PathoSEEK® Bile Tolerant Gram Negative (BTGN) Detection Assay v3 with SenSATIVAx® for BTGN Detection in Cannabis Flower and MIP Matrices

Method Developer Validation

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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 v3 is a qPCR detection assay for the rapid detection and enumeration of BTGN in cannabis flower matrices, and presence absence testing in cannabis infused products.

Objective:

To evaluate the PathoSEEK® Bile Tolerant Gram Negative Detection Assay v3, using the SenSATIVAx® flower extraction protocol for the detection and enumeration of BTGN in cannabis flower (delta 9-tetrahydrocannabinol >0.3%; 1g).

To evaluate the PathoSEEK® Bile Tolerant Gram Negative Detection Assay v3 as a screen for presence or absence of BTGN in infused products.

Results:

Inclusivity and exclusivity results showed the PathoSEEK® Bile Tolerant Gram Negative Detection 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® Bile Tolerant Gram Negative Detection Assay v3 were validated through the development of an enumeration curve using ten distinct bacterial species, with subsequent plating on 3M™ Petrifilm™ Enterobacteriaceae (EB) Count Plates. Following curve establishment, a certified reference material (CRM) for Quantitative EB in Hemp was procured from NSI and analyzed employing the SenSATIVAx flower extraction kit and PathoSEEK® Bile Tolerant Gram Negative Detection Assay v3. The resultant qPCR Cq values were converted to colony forming units per gram (CFU/g) utilizing a conversion equation and compared with data obtained via 3M™ Petrifilm™ Enterobacteriaceae (EB) Plates, as well as the NSI value provided on the CRM Certificate of Analysis. The results demonstrated comparability with those from 3M and alignment with the specifications detailed 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 (2-4 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 (2-4 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® Bile Tolerant Gram Negative Detection Assay v3 Kit - **P/N 420539**

Component Name	Qty Provided	Storage Conditions
PathoSEEK® Amplification Mix *Includes 2 tubes nuclease free water for resuspension*	4 Vials (50 rxns/each)	RT (20-28 ºC)/ -15 to -20 ºC*
PathoSEEK® Bile Tolerant Gram Negative Detection Assay v3	1 Tube (200 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

Inclusivity and Exclusivity

Methodology

For the inclusivity evaluation, 34 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® BTGN v3 method.

Results

Of the 34 Inclusivity strains tested, 34 were correctly detected by the PathoSEEK® BTGN v3 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® BTGN v3

Species	ATCC#	Pathoseek BTGN v3 Result
Aeromonas hydrophila	7965 DNA	Detected
Aeromonas hydrophila	7966	Detected
Citrobacter braakii	3037	Detected
Citrobacter freundii	8090	Detected
Citrobacter koseri	25408	Detected

Cronobacter sakazakii	BAA-894	Detected
Enterobacter aerogenes	15038 DNA	Detected
Escherichia hermannii	700368	Detected
Escherichia coli Strain 2005-3287 O145	BAA-2223	Detected
Escherichia coli Strain 2000-3039 O45:H2	BAA-2193 DNA	Detected
Escherichia coli Strain 2002-3211 O121-H19	BAA-2219 DNA	Detected
Escherichia coli Strain 2003-3014 O26:H11	BAA 2196 DNA	Detected
Escherichia coli Strain 2006-3008 O103:H11	BAA 2215 DNA	Detected
Escherichia coli Strain 99-3311 O145	BAA 2192 DNA	Detected
Escherichia coli Strain O111	BAA 2440 DNA	Detected
Hafnia alvei	51873	Detected
Klebsiella pneumonia	200721 DNA	Detected
Klebsiella oxytoca	51983	Detected
Morganella morganii	25829	Detected
Pantoea agglomerans	43348	Detected
Proteus mirabilis	43071	Detected
Proteus vulgaris	8427	Detected
Rahnella aquatilis	33991	Detected
Salmonella bongori	43975D-5	Detected
Escherichia hermannii	6962	Detected
Salmonella enterica subsp. arizonae	BAA-731D-5	Detected
Salmonella enterica subsp. diarizonae	BAA-1579D-5	Detected
Salmonella enterica subsp. houtene	BAA-1580D-5	Detected
Salmonella enterica subsp. indica	BAA-15780D-5	Detected
Salmonella enterica subsp. Salamae	BAA-1582D-5	Detected
Shigella flexneri	29903D-5	Detected
Vibrio cholerae	39315D-5	Detected
Yersinia enterocolitica	9610	Detected

Pseudomonas aeruginosa	9027	Detected

Table 2: Results for Exclusivity of the PathoSEEK® BTGNv3

Species	ATCC#	Pathoseek BTGN v3 Result
Aspergillus flavus	9643	Not Detected
Aspergillus niger	1015	Not Detected
Aspergillus terreus	20542	Not Detected
Bacillus subtilis	11774	Not Detected
Candida albicans	10231	Not Detected
Clostridium sporogenes	11437	Not Detected
Lactobacillus acidophilus	4357	Not Detected
Listeria monocytogenes	19115D-5	Not Detected
Listeria seeligeri	35967D-5	Not Detected
Listeria wilshire	35897D-5	Not Detected
Penicillium chrysogenum	10160 DNA	Not Detected
Penicillium rubens	11709	Not Detected
Staphylococcus aureus	6538	Not Detected

Note: Sanger sequencing of the assay amplicon confirmed cross-reactivity with *Pseudomonas rhizosphaerae*, an off-target species. This organism was not included in the original primer exclusion criteria, as it is not represented in common microbial reference collections such as ATCC. Because *P. rhizosphaerae* is not commercially available for purchase or propagation, it cannot be incorporated into formal exclusivity testing. To date, no clinical evidence has linked *P. rhizosphaerae* to human disease, although its presence in inhaled agricultural products warrants continued evaluation. Because *P. rhizosphaerae* is not commercially available from ATCC, its colony-forming characteristics cannot be readily assessed. As a result, the degree to which this species may contribute to inflated CFU/g estimates in the P. aeruginosa assay remains undetermined.

Cq to CFU Conversion 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 = -0.2627x + 11.716,** 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 = -0.2627x + 11.716.
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 in TSB to determine the final CFU (x 20).

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 Material Results

To verify the Cq to CFU/g conversion equation, two different CRMs supplied by NSI, Quantitative EB in hemp (Cat # FM-730) and Quantitative Coliform/E. coli in hemp (Cat # FM-727) were assessed. The reference materials were prepared according to the PathoSEEK® Bile Tolerant Gram Negative Detection Assay v3 User Guide (with Grim Reefer) in triplicate, using the above equation to convert Cq to CFU/g. The results fell within the acceptance limits of the CofA provided for the CRMs.

Table 3: CFU results of the CRMs

Sample	Assay	Cq FAM	Cq to CFU
Quant. Coliform/E. coli in Hemp	BTGN v3	31.77	*46,902*
Quant. Coliform/E. coli in Hemp	BTGN v3	32.21	*35,835*
Quant. Coliform/E. coli in Hemp	BTGN v3	31.76	*47,240*

Quant. EB in Hemp	BTGN v3	31.33	*61,029*
Quant. EB in Hemp	BTGN v3	31.30	*62,435*
Quant. EB in Hemp	BTGN v3	31.75	*47,354*

Marijuana Infused Product (MIP) Testing Confirmation

To validate the functionality of PathoSEEK® BTGN v3 with Marijuana Infused Products (MIPs), chocolate and oil samples were analyzed. When used with non-flower matrices (such as MIPs), the assay is designed for presence/absence screening. The method is not designed to report a CFU/g result in non-flower matrices.

Chocolate and oil samples were enriched with a live Salmonella culture for 24 hours and subsequently processed using the SenSATIVAx MIP protocol. Growth of the Salmonella culture was concurrently confirmed through plating on 3M RAC plates. The results may be found in Table 4.

Table 4: Results of the Infused Product (MIP) testing

Sample	Assay	Cq FAM	Cq Hex
Salmonella in Chocolate	BTGN v3	(+)	(+)
Salmonella in Chocolate	BTGN v3	(+)	(+)
Salmonella in Chocolate	BTGN v3	(+)	(+)
Salmonella in Oil	BTGN v3	(+)	(+)
Salmonella in Oil	BTGN v3	(+)	(+)
Salmonella in Oil	BTGN v3	(+)	(+)
Positive Control	BTGN v3	(+)	(-)
NTC	BTGN v3	(-)	(-)

Conclusions

The PathoSEEK® BTGN v3 qPCR Assay with SenSATIVAx® DNA Purification 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 method produced comparable results to 3M Petrifilm EB and CC plates for the enumeration of Bile Tolerant Gram Negative bacteria in cannabis flower.

REVISION HISTORY

Version	Date	Description
v1	June 2025	Validation data generated with the use of Amplification Mix and v3 Assay design

DISCLAIMER

This test was developed and its performance characteristics determined by Medicinal Genomics Corporation, 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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