PathoSEEK® Total Yeast and Mold Count Assay with SenSATIVAx® TLP DNA Purification & Grim Reefer® Free DNA Removal

Manufacturer's Validation v2

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Abstract

Background

Yeasts and molds have been known to cause deterioration and decomposition of cannabis. Certain species of yeast and mold, such as *Aspergillus fumigatus*, can produce toxins and infect immuno-compromised patients with fatal Aspergillosis. The PathoSEEK® Total Yeast and Mold Count (TYM) Detection Assay with SenSATIVAx® Thaumatin Like Protein (TLP) Enzyme Extraction and Grim Reefer® Free DNA Removal Protocol (the MGC TYM Method) is a DNA purification and qPCR assay method for the rapid enumeration of yeasts and molds in cannabis flower. The Thaumatin Like Protein (TLP) is a beta glucanase that digests the glucan cell wall of some yeasts that are difficult to lyse. The Grim Reefer® Free DNA Removal kit eliminates free or extracellular DNA from samples prior to qPCR analysis.

Objective

To evaluate the MGC TYM Method for the enumeration of total yeast and mold in cannabis flower (delta 9-tetrahydrocannabinol >0.3%).

Results

The MGC TYM Method is a rapid, alternative method to traditional plating procedures for the enumeration of yeast and molds in cannabis flower. Inclusivity and exclusivity results show the MGC TYM method is highly specific in discriminating target organisms found in cannabis flowers from non-target organisms. The MGC TYM Method eliminates subjectivity with colony interpretation, offering a rapid alternative to traditional plating procedures.

Conclusions

The MGC TYM Method is a rapid, alternative method to traditional plating procedures for the enumeration of yeast and molds in cannabis flower.

Materials and Methods

Test Kit Information

PathoSEEK® Total Yeast and Mold Detection Assay with SenSATIVAx® TLP Enzyme Extraction Protocol and Grim Reefer Free DNA Removal

Test Kit Components:

1. SenSATIVAx® Flower Purification / Leaf DNA Purification Kit – MGC P/N 420001 (200 extractions)
    a. Binding Buffer - 1 bottle (store at 2-8°C)
    b. Elution Buffer - 1 bottle (store at 20-28°C)
    c. Lysis Solution - 1 bottle (store at 20-28°C)
2. SenSATIVAx® TLP Extraction Enzyme - MGC P/N 420206 (50 extractions). Store at -15°C to -20°C.
    a. 2.0mL clear top - 1 vial
3. PathoSEEK® PathoSEEK® Total Yeast & Mold Detection Assay —MGC P/N 420103 (200 tests). Store kit at -15 to -20°C.
    a. 1.5mL clear top – 1 vial
4. Grim Reefer Free DNA Removal Kit - MGC P/N 420145 - Store at -15°C to -20°C.
    a. Grim Reefer Buffer - 1 vial (P/N 420142)
    b. Grim Reefer Enzyme 1 vial (P/N 420141)

5. Grim Reefer Assay -MGC P/N 420143 - Store at -15°C to -20°C.
6. Grim Reefer Control -MGC P/N 420144 Store at -15°C to -20°C.
7. qPCR Master Kit v3 – MGC P/N 420201 (200 tests). Store kit at -15 to -20°C.
    a. Reaction Buffer (10x).— 1 vial.
    b. qPCR Master Mix (5x).—1 vial.
    c. Nuclease Free Water.—2 vials.

Method Developer Validation

For the inclusivity evaluation, Fifty-five (55) strains of yeast and mold, as identified by the AOAC, were evaluated. Target strains were cultured in TSB for 48 hours at room temperature (20-28 °C). After incubation, cultures were diluted in TSB to levels of 100-1,000 CFU/mL. Thirty-one (31) exclusivity organisms were cultured onto non-selective agar under optimal conditions for growth. Exclusivity cultures were analyzed undiluted. Inclusivity and exclusivity cultures were randomized, blind coded, and analyzed by the MGC TYM Method.

Results

Of the 55 inclusivity strains tested, 51 were correctly detected by the PathoSEEK® TYM Method. Two inclusivity isolates, *Arthrinium arundinis* and *Phytophthora infestans* were not detected by the MGC TYM Method or PDA agar. The other two isolates not detected by the MGC TYM Method were *Botrytis cinerea* and *Scopulariopsis acremonium* but were able to be recovered on PDA agar. Of the 31 exclusivity strains tested, all 31 were correctly excluded. Tables 1 and 2 present a summary of the results.

Table 1: Inclusivity Results, PathoSEEK® Total Yeast and Mold Count Assay

#	Species	Strain	Source	PathoSEEK® TYM Result
1	*Alternaria alternata*	6663	Not Available	Detected
2	*Arthrinium arundinis*	96021	Bing cherry fruit, Prunus avium cv. Bing, Wenatchee, WA	Not Detected
3	*Aspergillus aculeatus*	24147	Fragaria sp., Brazil	Detected
4	*Aspergillus brasiliensis*	9642	Wireless Radio Equipment	Detected
5	*Aspergillus brasiliensis*	16404	Blueberry	Detected
6	*Aspergillus carbonarius*	MYA-4641	Grape berry, Brindis, Apulia, Italy	Detected
7	*Aspergillus caesiellus*	42693	Dried chillies, New Guinea	Detected
8	*Aspergillus carneus*	13549	France	Detected
9	*Aspergillus clavatus*	1007	Not Available	Detected
10	*Aspergillus deflectus*	62502	Wheat, China	Detected
11	*Aspergillus flavus*	9643	Shoe Sole, New Guinea	Detected
12	*Aspergillus fijiensis Varga et al*	20611	Not Available	Detected
13	*Aspergillus fumigatus*	204305	Human sputum, Virginia	Detected
14	*Aspergillus japonicus*	16873	Soil, Panama	Detected
15	*Aspergillus nidulans*	38163	Not Available	Detected
16	*Aspergillus niger*	16888	Not Available	Detected
17	*Aspergillus niveus glaucus*	10075	Not Available	Detected
18	*Aspergillus ochraceus*	18500	Rubber Sheet	Detected
19	*Aspergillus oryzae*	42149	Cereal	Detected
20	*Aspergillus parasiticus*	56775	Not Available	Detected
21	*Aspergillus tamarii*	1005	Tomato	Detected
22	*Aspergillus terreus*	1012	Soil, Connecticut	Detected
23	*Aspergillus tubingensis*	1004	Not Available	Detected
24	*Aspergillus ustus*	1041	Culture Contaminant, USA	Detected
25	*Aspergillus versicolor*	11730	Cellophane Gas Mask, India	Detected
26	*Aureobasidium species*	62921	Not Available	Detected
27	*Beauveria bassiana*	44860	Soil, Georgia	Detected
28	*Botrytis cinerea*	11542	Azalea Flowers, Washington, DC	Not Detected
29	*Candida albicans*	10231	Man with bronchomycosis	Detected
30	*Candida tropicalis*	13803	Not Available	Detected
31	*Cladosporium sphaerospermum*	11288	Human Nails	Detected
32	*Cryptococcus laurentii*	18803	Palm Wine, Malaffou, Congo	Detected

33	*Cryptococcus neoformans*	208821	Patient with Hodgkin's Disease, New York	Detected
34	*Fusarium proliferatum*	76097	Raw Cotton, North Carolina	Detected
35	*Fusarium oxysporum*	62506	Celery, Apium graveolens var. dulce, California, USA	Detected
36	*Fusarium solani*	52628	Cardamom fruit pod, Elettaria cardamomum, Guatemala	Detected
37	*Fusarium sporotrichioides*	24631	Corn, USA	Detected
38	*Fusarium verticillioides*	MYA4922	Maize, Visalia, CA, USA	Detected
39	*Mucor circinelloides*	38592	Not Available	Detected
40	*Mucor hiemalis*	28935	Soil in Spruce Forest, Germany	Detected
41	*Paecilomyces species*	13435	Soil, Japan	Detected
42	*Penicillium brevicompactum*	9056	Not Available	Detected
43	*Penicillium citrinum*	10105	Egypt	Detected
44	*Penicillium chrysogenum*	18476	Cheese, USSR	Detected
45	*Penicillium expansum*	28885	Grape Berry, California	Detected
46	*Penicillium rubens*	11709	Selected from Wis. 48-701, ATCC 11707, after N-mustard exposure	Detected
47	*Penicillium simplicissimum*	48706	Not Available	Detected
48	*Penicillium venetum*	16025	Acidic Soil, England	Detected
49	*Phytophthora infestans*	MYA1113	Hyacinthus sp. bulb, England	Not Detected
50	*Purpureocillium lilacinum*	10114	Potato tuber, Solanum tuberosum, Glasston, MN	Detected
51	*Rhizopus oryzae*	52748	Soil, Ithaca, NY	Detected
52	*Rhizopus stolonifer*	14037	Not Available	Detected
53	*Scopulariopsis acremonium*	58636	Not Available	Not Detected
54	*Yarrowia lipolytica*	20390	Chicken House Soil, Alberta, Canada	Detected
55	*Talaromyces pinophilus*	11797	Non Sporulating Diploid	Detected

Table 2: Exclusivity Results, PathoSEEK® Total Yeast and Mold Count Assay

#	Species	Strain	Source	PathoSEEK® TYM Result
1	*Acinetobacter baumannii*	19606	Urine	Not Detected
2	*Aeromonas hydrophila*	7966	From a tin of milk with fishy odor	Not Detected
3	*Burkholderia multivorans*	17616	Soil enriched with anthranilate at 41C, Berkeley, CA	Not Detected
4	*Bacillus subtilis*	11774	No Source Listed	Not Detected
5	*Citrobacter braakii*	3037	No Source Listed	Not Detected
6	*Citrobacter koseri*	25408	Throat	Not Detected
7	*Edwardsiella tarda*	23672	No Source Listed	Not Detected

8	*Enterobacter aerogenes*	13048	Sputum, South Carolina Dept. of Health and Environmental Control	Not Detected
9	*Enterobacter cloacae*	13047	Spinal Fluid	Not Detected
10	*Erwinia rhapontici*	29290	English pink wheat grains, England	Not Detected
11	*Escherichia coli*	25922	Clinical Isolate	Not Detected
12	*Escherichia coli O157:H7*	35150	Human Feces	Not Detected
13	*Escherichia hermannii*	700368	No Source Listed	Not Detected
14	*Escherichia vulneris*	33821	Human wound, Bethesda MD	Not Detected
15	*Hafnia alvei*	51873	Human feces, The Netherlands	Not Detected
16	*Klebsiella oxytoca*	51983	Human blood, Albany NY, USA	Not Detected
17	*Klebsiella pneumonia*	BAA-2146	Human urine	Not Detected
18	*Listeria monocytogenes*	7647	Bovine	Not Detected
19	*Morganella morganii*	25829	Stool of infant (Providence City Hospital)	Not Detected
20	*Pantoea agglomerans*	43348	Gypsophila paniculata galls, California	Not Detected
21	*Proteus mirabilis*	43071	Rectum, Georgia	Not Detected
22	*Pseudomonas aeruginosa*	15442	Animal room water bottle	Not Detected
23	*Pseudomonas aeruginosa*	35554	No Source Listed	Not Detected
24	*Pseudomonas fluorescens*	13525	Pre-filter tanks, England	Not Detected
25	*Pseudomonas putida*	47054	No source listed	Not Detected
26	*Ralstonia insidiosa*	49129	Clinical isolate	Not Detected
27	*Rahnella species*	33991	Soil	Not Detected
28	*Salmonella enterica*	13311	Feces, Human, 1911	Not Detected
29	*Stenotrophomonas maltophilia*	13637	Oropharyngeal region of patient with mouth cancer	Not Detected
30	*Staphylococcus aureus*	12600	Pleural Fluid	Not Detected
31	*Serratia marcescens*	27137	Human isolate	Not Detected

Generation of Cq to CFU Conversion Equation for flower samples

The Cq to CFU/g equation was generated by running ten known Yeast and Mold cultures on MGC TYM Method compared against plating on DRBC plates. The MGC TYM Method was done in replicates of five 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 = -0.1267x + 6.6781 where y = the log10 of the plating data on DRBC and x = the Cq on qPCR. (Figure 1)

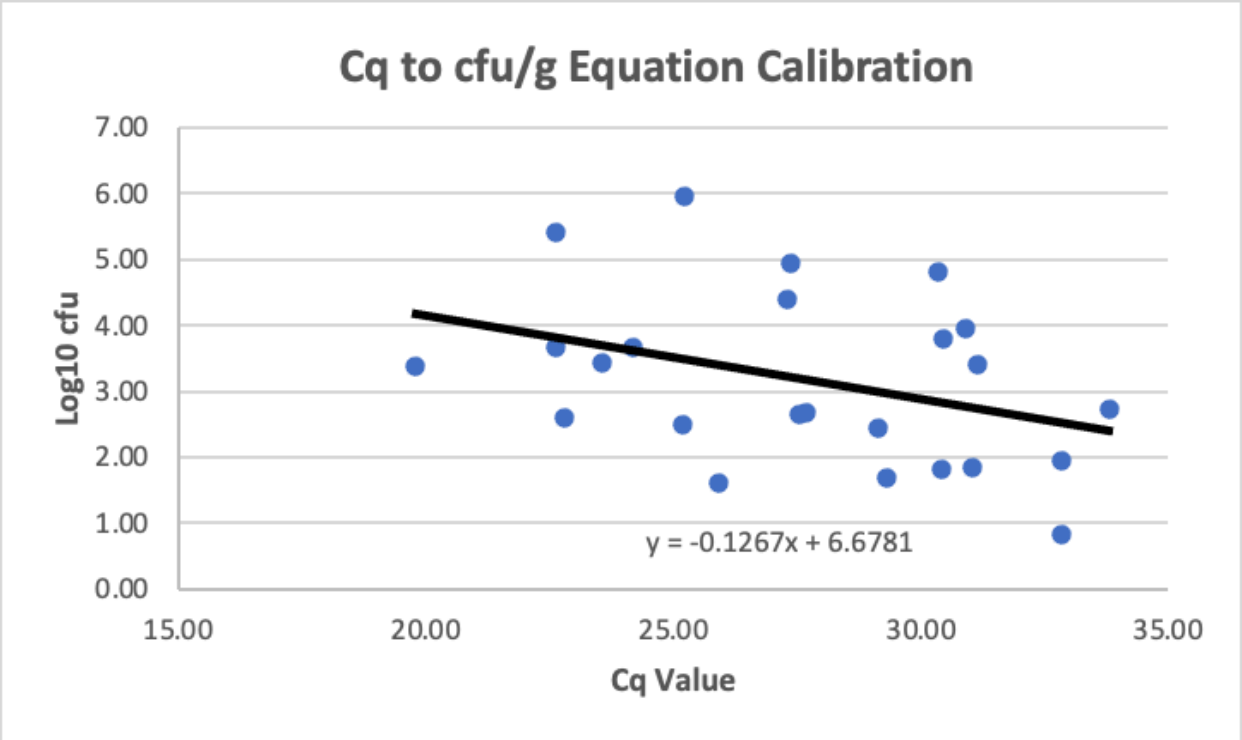


Figure 1 - Cq to CFU/g Equation

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

$$Y = -0.1267X + 6.6781$$

Perform an inverse logarithmic transformation of Y to and multiply the result by 20 to obtain CFU/g.

Table 3: Cq to CFU Conversion Equations

Matrix	Microbial Test	Cq to CFU Conversion Equation
Flower	Total Yeast and Mold Count	CFU/g = 10^((-0.1267*Cq)+6.6781) Multiply resulting CFU x 20 to account for upfront dilution factor
MIP	Total Yeast and Mold Count	IF Cq<40, Plate confirm for enumeration

Limit of Detection

The limit of detection (LOD) is used to describe the smallest concentration of a species that can be reliably measured by the Medicinal Genomics (MGC) PathoSEEK® qPCR 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:

$$\text{number of copies} = X \text{ ng} * 6.0221 \times 10^{23} \text{ molecules/mole} / (N \times 650 \text{ g/mole}) * 1 \times 10^{9} \text{ ng/g}.$$

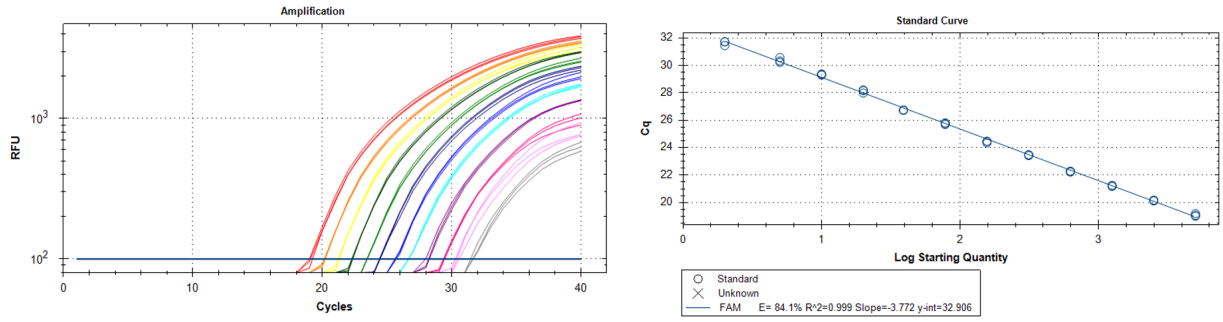
The following data demonstrates the experiments used to calculate the limit of detection when using the MGC TYM Method.

Table 4: LOD, Total Yeast and Mold Assay

Assay	DNA Copies (A. niger)	Cq Value	%RSD
TYM	5000	19.06	0.09
TYM	5000	19.01	
TYM	5000	19.19	
TYM	2500	20.14	0.03
TYM	2500	20.18	
TYM	2500	20.12	
TYM	1250	21.17	0.04
TYM	1250	21.25	
TYM	1250	21.24	
TYM	625	22.29	0.03
TYM	625	22.23	
TYM	625	22.26	
TYM	313	23.49	0.03
TYM	313	23.47	
TYM	313	23.43	
TYM	156	24.47	0.06
TYM	156	24.46	
TYM	156	24.36	
TYM	78	25.81	0.06
TYM	78	25.69	
TYM	78	25.76	
TYM	39	26.73	0.01
TYM	39	26.74	
TYM	39	26.75	
TYM	20	28.17	0.12
TYM	20	27.99	
TYM	20	28.22	
TYM	10	29.38	0.04
TYM	10	29.36	
TYM	10	29.31	
TYM	5	30.23	0.18
TYM	5	30.59	
TYM	5	30.33	
TYM	2	31.46	0.16
TYM	2	31.73	
TYM	2	31.74	

TYM	No Spike	ND	Not Detected
TYM	No Spike	ND	
TYM	No Spike	ND	

Figure 2: Total Yeast and Mold qPCR Dilution Curves and qPCR Efficiency (E)



Action Limit Study

Flower Matrix

Fifty Naturally contaminated Cannabis flower samples with varying levels of yeast and mold contamination were tested via Potato Dextrose Agar (PDA) with TA and Dichloran Rose Bengal Chloramphenicol (DRBC) vs qPCR. The DNA purifications and qPCR analysis were performed according to the PathoSEEK® Total Yeast and Mold Count Assay with SenSATIVAx® TLP DNA Purification & Grim Reefer® Free DNA Removal User Guide v2. See Table 5 below for resulting data.

Table 5: Flower Matrix qPCR Results compared to PDA and DRBC Agar Plates

Contamination Level	qPCR Cq Value	qPCR cq to cfu/g result	Average cfu Result PDA w/ TA (cfu/g)	Average cfu results DRBC (cfu/g)
Low	ND	ND	140	490
Low	ND	ND	120	290
Low	ND	ND	130	500
Low	ND	ND	130	450
Low	ND	ND	150	590
Low/Medium	ND	ND	430	870
Low/Medium	36.64	2,171	1,510	1,810
Low/Medium	37.13	1,881	1,750	2,090
Low/Medium	35.61	2,932	1,680	1,900
Low/Medium	ND	ND	250	710
Low/Medium	38.20	1,377	2,630	2,150
Low/Medium	36.53	2,244	2,290	2,300
Low/Medium	38.41	1,295	1,820	1,890
Low/Medium	38.17	1,390	1,760	1,570
Low/Medium	38.68	1,198	1,840	1,350
Low/Medium	38.83	1,145	1,360	1,570
Low/Medium	ND	ND	270	520
Low/Medium	38.14	1,403	1,640	2,840
Low/Medium	ND	ND	440	850
Low/Medium	ND	ND	820	930
Low/Medium	39.00	1,090	1,940	2,140
Low/Medium	38.30	1,340	1,770	1,880
Low/Medium	36.83	2,058	2,180	2,200
Low/Medium	37.52	1,682	1,840	2,290
Low/Medium	38.15	1,400	1,220	1,720
Medium/High	36.50	2,265	3,130	8,100
Medium/High	30.51	12,994	17,540	17,760
Medium/High	30.31	13,788	17,360	17,980
Medium/High	32.35	7,599	5,510	6,060
Medium/High	32.00	8,404	7,740	7,580
Medium/High	31.70	9,173	6,670	6,410
Medium/High	30.01	15,010	17,160	17,320
Medium/High	34.04	4,638	6,070	7,630
Medium/High	33.53	5,383	6,580	7,540
Medium/High	30.79	11,963	18,240	17,550
Medium/High	30.01	15,038	17,560	17,540
Medium/High	29.61	16,891	17,700	17,840
Medium/High	31.83	8,831	9,850	9,820
Medium/High	30.54	12,876	10,370	10,170
Medium/High	29.45	17,718	19,450	19,200
Medium/High	29.10	19,587	20,470	18,990
Medium/High	28.02	26,877	19,280	18,820
Medium/High	30.65	12,473	11,340	11,060
Medium/High	29.10	19,581	19,140	19,320
Medium/High	30.20	14,233	18,370	14,720
High	26.49	41,926	39,000	38,500
High	26.32	44,155	67,500	39,000
High	26.32	44,109	47,500	42,500
High	27.00	36,122	61,500	46,000
High	27.30	33,139	50,000	41,500

MIP Matrix

We recommend enriching non-flower (MIP) samples in TSB and processing as pass/fail as described in the PathoSEEK® Total Yeast and Mold Count Assay with SenSATIVAx® TLP DNA Purification & Grim Reefer® Free DNA Removal User Guide v2. Refer to data in Table 7 below.

Proficiency Testing/Certified Reference Material Results

Flower Matrix

Material Used - NSI Part Number CMCQ-085, Quantitative Yeast and Mold in HEMP

Testing was performed using MGC's Total Yeast and Mold Assay with TLP in addition to plating on DRBC agar plates. A 1:20 upfront dilution in nuclease free water was used.

Note: For more information on using NSI CRMs or PTs with PathoSEEK please contact support@medicinalgenomics.com

Table 6: NSI Hemp CRM Results

NSI Acceptance Limits = 3.88E+04 - 1.55E+05 (38,800 - 155,000 cfu/g)

Sample	Extraction	Dilution	Target	Ct (FAM)	Average	Ct(HEX)	Avg CFU/g qPCR	Avg CFU/g DRBC
TYM in Hemp	1	1:20	TYM	26.32	26.21	26.65	45,566	90,000
TYM in Hemp	1	1:20	TYM	26.61		26.65		
TYM in Hemp	2	1:20	TYM	25.60		26.01		
TYM in Hemp	2	1:20	TYM	26.30		26.55		
Positive Control				11.91				
NTC				No Cq				

MIP Matrix

Material Used - NSI 51-00054, C. albicans CRM for USP 51

CRM is provided as a lyophilized pellet. The pellet was serially diluted in nuclease free water and spiked into the presence of a 2.4 mL TSB and 1g hemp oil matrix. The samples were then enriched overnight at 37° C for 18 hours. After enrichment, the samples were processed with the MGC TYM Method. Results are below in Table 7.

Table 7: Results of Hemp Oil samples spiked with NSI C. albicans CRM

Sample	Spike Level (CFU)	Fam (TYM) Cq	HEX (IC) Cq
Hemp Oil	243	No Cq	25.87
Hemp Oil	243	No Cq	26.17
Hemp Oil	696	37.09	26.26
Hemp Oil	696	35.21	25.81
Positive Control	N/A	12.61	No Cq
NTC	N/A	No Cq	No Cq

Conclusions

The data derived from the use of commercially available Proficiency Tests/CRMs for total yeast and mold indicate that the MGC TYM Method falls within range compared to enumeration data provided by the NSI CoA for yeasts and molds in cannabis flower. For MIPs, detection of ≤1000 CFU/g C. albicans is achieved with enrichment. For MIP samples, if detection is observed via qPCR on a sample post enrichment, enumeration and viability should be determined with the use of the culture plate method of choice. We recommend DRBC as that is the AOAC preferred plating method.