Multiplex qPCR and Cannabis Microbiome Sequencing Reveals Several Bacteria and Fungi Native to Cannabis Flowers

Kevin McKernan, Jessica Spangler, Lei Zhang, Vasisht Tadigotla, Yvonne Helbert, Kellie Dodd, Douglas Smith
Medicinal Genomics Corporation

Introduction

The Center for Disease Control estimates 128,000 people in the U.S. are hospitalized annually due to food-borne illnesses. As a result, the detection of mold and bacteria on agricultural products has become an important safety consideration. This risk extends itself to medical Cannabis and is of particular concern with inhaled, vaporized and even concentrated Cannabis products. As a result, third-party microbial testing has become a regulatory requirement in the medical and recreational Cannabis markets. Medicinal Genomics has developed a novel PCR-based assay for the detection of pathogenic microbes in Cannabis materials. This process consists of a proprietary system for DNA extraction called SenSATIVAx™ and a novel PCR assay called PathogINDICAtor™.

PathogINDICAtor™ utilizes a novel qPCR-based assay that is contamination-free and provides an internal plant DNA control for every reaction. DNA detection is based on a 5' nuclease assay that directly measures the amount of plant and microbe DNA in a given sample. This technique provides robust sensitivity, specificity, and multiplexing capability. Positive results can then be confirmed through microbiome sequencing. PathogINDICAtor™ is able to provide a contamination-free technique through a proprietary process known as DREAM PCR[1, 2], which uses methylation-specific restriction enzymes to prevent the carryover of amplified products from one reaction to the next. We present favorable comparisons to conventional techniques.

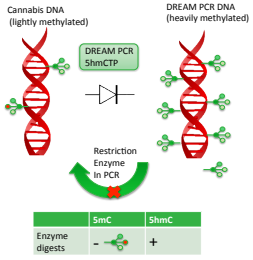
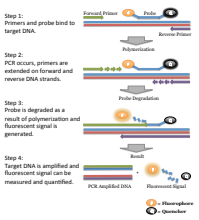


Figure 1 (above): DREAM PCR generates heavily methylated DNA that cannot survive digestion.

Figure 2 (below): Process of a typical 5' nuclease fluorescent assay. PathogINDICAtor™ includes 5' universal tails and ShmCTP in PCR.



MGC Offerings

Our current assay offerings for use with SenSATIVAx™ and PathogINDICAtor™ are listed below.

Presence/Absence Tests
- ***E.coli***
- ***E.coli* STEC only**
- **Salmonella**
- **Aspergillus** (*A.niger, A.fumigatus, & A.flavus* species specific assays coming soon)

Threshold Tests
- **Total Yeast & Mold (18S)**
- **Total Aerobic Bacteria (16S)**
- **Total Enterobacteriaceae (16S)**
- **Total Coliform**

Examples of PathogINDICAtor™ Data

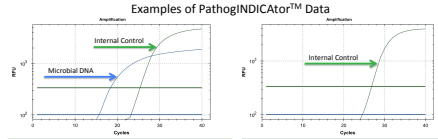
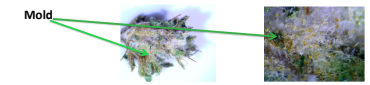


Figure 3: Sample containing plant and microbe DNA. (Plant DNA = Green, Microbial DNA = blue). X-axis is cycles (every 1.5 minutes). Y-axis is relative fluorescence units in a log 10 scale.

Figure 4: Sample with no microbe DNA present. (Plant DNA = Green, Microbial DNA detection = Blue)

Example 1: Comparison of a Visually-Contaminated Sample



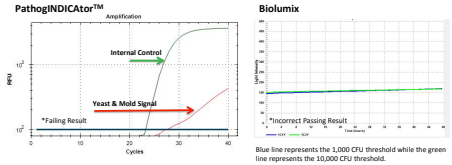


Figure 5: With only 1% of microbes able to grow into colonies[3], there is the potential for false negative results with conventional culture-based methods. The cannabis sample has visible mold growing, however, the culture-based method does not detect the microbes. PathogINDICAtor™ detects and fails the sample.

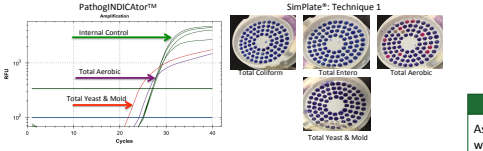
Example 2: Comparison of Multiple Methods

Total Coliform (1,000 CFU/g)			
SimPlate®(CFU/g)	3M™Petrifilm™ (CFU/g)	Biolumix	PathogINDICAtor™ (Cq)
0	0	pass	Cq>40

Total Enero (1,000 CFU/g)			
SimPlate®(CFU/g)	3M™Petrifilm™ (CFU/g)	Biolumix	PathogINDICAtor™ (Cq)
0	0	pass	Cq>40

Total Aerobic (100,000 CFU/g)			
SimPlate®(CFU/g)	3M™Petrifilm™ (CFU/g)	Biolumix	PathogINDICAtor™ (Cq)
24000 (pass)	24000 (pass)	pass	24.03 (pass)

Yeast/Mold (10,000 CFU/g)			
SimPlate®(CFU/g)	3M™Petrifilm™ (CFU/g)	Biolumix	PathogINDICAtor™ (Cq)
0	0	pass	21.71 (fail)



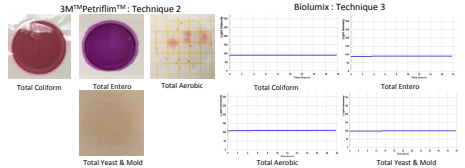


Figure 6: To aid in validation of SenSATIVAx™ and PathogINDICAtor™, we utilized three alternative culture-based assays (3M™Petrifilm™, SimPlate®, and Biolumix). When a sample does not correlate we reflex to next-generation sequencing of that sample to confirm our assay is correct.

The Microbiome Profile of the Sequenced Positive Result

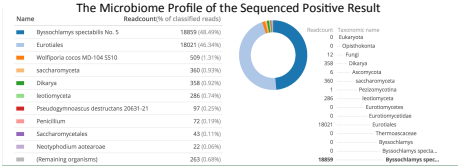


Figure 7: After the positive result using PathogINDICAtor™, the product was next-generation sequenced on and Illumina MiSeq with our Yeast & Mold Detection Assay to reveal the microbes contained in the sample. Using OneCodex to analyze the data, this sample is primarily Bysochlamys, which produces mycotoxin and should be detected for safety. (Sample 3 in Figure 8 is also a representation of this sample)
In general, this data can be used for many purposes, most notably to determine the possible contaminates in a greenhouse or grow room prior to wider infestation.

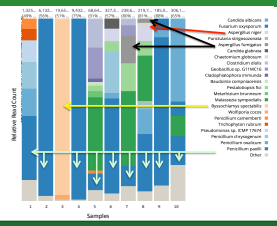


Figure 8: A look at multiple sequenced PathogINDICAtor™ positive cannabis samples and the microbiomes found on each. This data can help in determining trends within multiple cannabis samples. Notice that 9 of 10 have Penicillium as a part of the microbiome, which is a known endophyte[4].
Sample 1 is the Australian Bastard sample referenced in the presentation titled "Genomic, Terpene, and Cannabinoid Profiles of a Putatively Novel Cannabis Species".

Discussion

As many regulations are beginning to mandate "heat killing" of microbial content, we must remain aware of how these drying techniques often confound culture-based methods used to monitor colony forming units (CFU). Even though this "heat kill" process may be effective at sterilizing some of the culturable microbial content it does not eliminate various pathogenic toxins like Aflatoxin or the DNA that encodes the Aflatoxin gene. This is of particular importance as Aflatoxin is a carcinogen. The clearance of Aflatoxin requires the liver enzyme CYP3A4 and this liver enzyme is potently inhibited by Cannabinoids[5-7]. With the publication of the Cannabis genome and many pathogenic microbial genomes, we designed several multiplexed quantitative PCR (qPCR) assays to detect pathogenic DNA in a background of host Cannabis DNA.

One of these qPCR assays is designed to detect total yeast and mold and thus targets the 18S rDNA ITS (Internal Transcribed Spacer) regions. ITS regions are routinely used to sequence the microbiomes of samples and identify and itemize the collection of microbial communities present in a given sample. The addition of next-generation sequencing primer tails to this yeast and mold qPCR assay enables reflexive sequencing of samples testing positive for yeast and mold. Next-generation sequencing thus reveals a digital record of the microbiological community on a given Cannabis sample. Cannabis samples that tested positive for yeast and mold with qPCR and culture-based techniques were sequenced to precisely identify the collection of microbes present in the failing qPCR samples. Microbes harmful to both the plant and humans were identified (Botrytis, Magneporthe, Fusarium and Aspergillus) while over 180 different microbes including endophytes were also present. This qPCR assay presents a real-time tool for both quality testing and geospatial monitoring of microbial communities in cannabis production facilities. This highly sensitive assay allows for the detection of very low levels of contamination. This can be used to proactively monitor for outbreaks before they can spread.

Research and Development Contact
Jessica Spangler
Medicinal Genomics Corporation
Email: Jessica.Spangler@medicinalgenomics.com

Business Development Contact
Kellie Dodd
Medicinal Genomics Corporation
Email: Kellie.Dodd@medicinalgenomics.com

Sales Contact
John Geanacopoulos
Medicinal Genomics Corporation
Email: jg@medicinalgenomics.com

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