A Comparison Of Microbiological Molecular Diagnostics In Cannabis Testing, With A Focus On *Aspergillus*.

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Over the last two decades, advances in DNA sequence analysis have facilitated the transition from traditional plate culturing to molecular/DNA-based methods that efficiently identify and quantify both culturable and non-culturable organisms in a sample. Molecular diagnostic platforms, utilizing technologies such as quantitative real time PCR (qPCR), DNA microarrays and in limited cases, Next Gen Sequencing (NGS), are quickly becoming the standard for microbial pathogen detection. Across food, agriculture, water testing and environmental screening, these methods ensure the highest quality and safest product to the market.

Similarly, with cannabis being consumed both through inhalation and ingestion, microbial testing is now a necessity, especially the testing of *Aspergillus* species (*A. niger, A. fumigatus, A. terreus, and A. flavus*) and other pathogenic organisms that are significant health risks to immune-compromised patients and consumers. There has been significant concern in the Cannabis industry with respect to varying test results from different methods.

PathogenDx initiated an independent evaluation and assessment of the accuracy of microbial methods currently used for compliance testing in the Cannabis sector. This evaluation was conducted and overseen by Dr. Reggie Gaudino and Anthony Torres from an independent cannabis testing lab. A side by side evaluation of plate culturing, qPCR, DNA microarray was conducted, with sequencing (NGS) used as a confirmation of the results in support of 4-species *Aspergillus* testing. In addition, detailed evaluation of the limits of sensitivity was undertaken for all the molecular methods to assess their capability in meeting state testing regulations. This paper illuminates the contrasting differences in the accuracy and detection limits of these methods, and the resulting implications on meeting testing standards that could potentially impact health and safety of consumers and patients.

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"In this study we evaluated the performance of plate culture, two qPCR methods as well as one DNA microarray method for the accuracy and detection of Aspergillus spp in cannabis flower samples."

Key Takeaways

- States that currently mandate *Aspergillus* spp testing as part of the microbial testing requirement require Presence/Absence or Detect/Non-Detect with a < 1 CFU/gram.
- DNA molecular methods should be the only way to detect and confirm the presence of *Aspergillus* spp. in cannabis and hemp products as traditional plate-based techniques are not sufficient for reliable detection of endophytes.
- Based on real-world cannabis samples and genomic DNA testing, the two qPCR methods evaluated in this study were not able to detect *Aspergillus* spp. at 1 CFU/gram.
    - qPCR method 1, using only a genus level primer reached a level of sensitivity of 50 CFU/gram. This method was more sensitive and accurate than qPCR method 2, which used a multiplex primer.
    - qPCR method 2, using a multiplex primer, reached a level of sensitivity of 100 CFU/gram.
    - However, qPCR 1 did not speciate and therefore neither method can comply with state standards.
- Utilizing real-world cannabis samples and genomic DNA testing, the microarray technology detected *Aspergillus* spp. down to 1 CFU/gram.
- The accuracy of all four methods tested was confirmed by a parallel analysis of prepared flower samples with and without naturally contaminated *Aspergillus* samples via next generation sequencing as well as Sanger sequencing.
- Both sequencing methods found that the DNA microarray test had the highest accuracy level of the four methods tested. Its accuracy level is suitable for *Aspergillus* testing pursuant to state regulatory standards.

Microbial Testing: A critical step in a dynamic industry.

Ensuring the safety of cannabis products promotes public safety and adds legitimacy to the cannabis industry. Cannabis-derived products are increasingly being used to treat various medical conditions. Thus, it is critical that these products are verified to be free of hazardous contaminants. The presence of pathogenic organisms can cause illness in healthy individuals, especially as a result of systemic infection, inflammation or allergic response, and thus poses a greater risk to immunocompromised and immunosuppressed patients (Yousef Gargani, 2011).

Regulations for evaluating the safety of cannabis products differ by state, including presence/absence and quantification of broad class indicators. Nonetheless, presence/absence detection of some organisms, such as *Salmonella* and pathogenic *Escherichia coli*, is consistent. Other organisms that some regulatory entities require to be tested include: *Aspergillus* species (*A. niger, A. fumigatus, A. terreus, and A. flavus*), *Clostridium botulinum, Staphylococcus aureus, and Listeria monocytogenes*.

Testing methods to detect specific microbes can be divided into two general categories: microbiological or traditional culture/chemical verification methods and molecular methods (Rajapaksa, 2018). Plate culturing is the predominant traditional verification method. Molecular methods include immunoassays, PCR, quantitative real time PCR (qPCR), DNA microarrays, and sequencing. Of the available molecular and traditional methods, PathogenDx chose to evaluate four of the most commonly used methods in the industry: plate culture, two qPCR methods, and one DNA microarray method. These methods were evaluated for the accuracy and limits of detection of *Aspergillus* spp in cannabis flower samples, and the results of all **four methods** were verified using Next Generation (NGS) and Sanger DNA sequencing.

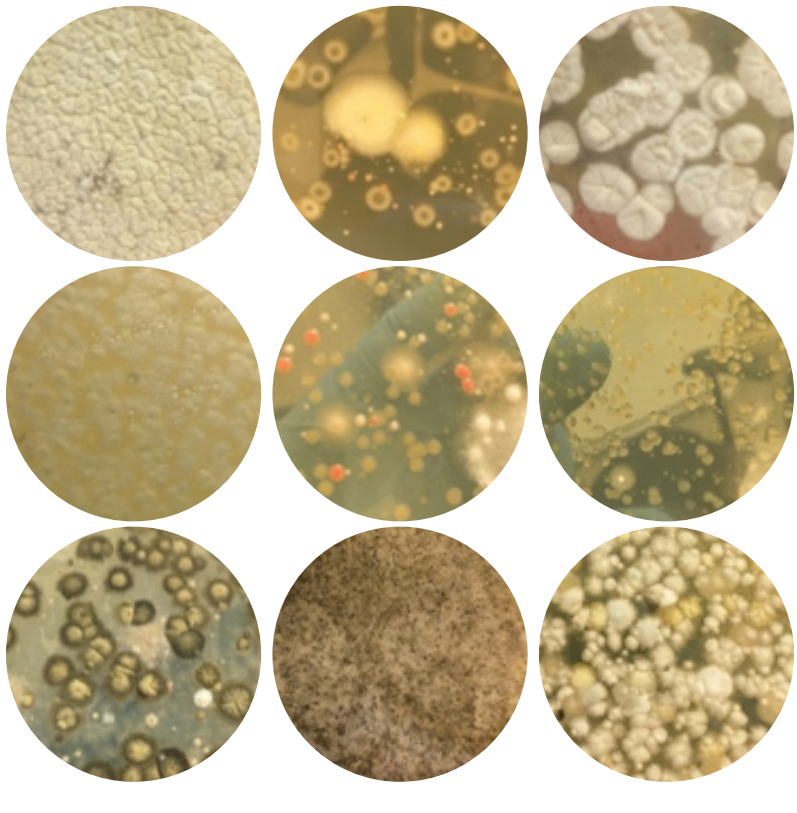


Figure 1: Plating results on Sabourad dextrose agar after 72 hours. Samples were diluted at 10 mL/gram analysis.

Experimental Conditions & Process

The third-party lab evaluated the same eighteen (18) 1-gram cannabis flower samples using the four methods described (plate culturing, two qPCR methods, and microarrays for the purpose of comparing and contrasting the relative accuracy of each method. The samples were subsequently sequenced using Sanger Sequencing to confirm the results (performed by the University of Arizona Genomic Core Facility). Serial dilutions with Genomic DNA also were performed to determine the sensitivity of these platforms.

The accuracy of all three methods tested were confirmed by parallel analysis of prepared flower samples with and without naturally contaminated Aspergillus samples via next generation sequencing as well as Sanger sequencing.

Traditional Methods versus DNA-Based Molecular Diagnostics

The traditional method of identifying microbials has been enrichment-based and involves culturing and plating on broad or selective agar media plates. Although commonplace in microbiology for well over a century, this methodology requires a high level of time, materials, and required expertise. For example, the time to get results for traditional or quick plating is a minimum of 24 hours and often closer to a week to two weeks for slower growing organisms, such as many fungi and including *Aspergillus* spp. The inefficiency of time-to-results can cause a loss of product or produce due to spoilage, costing millions of dollars. **In addition, traditional plating often suffers from false negatives, especially for *Aspergillus* spp, where faster and more prominent microorganisms such as *Penicillium* spp. outcompete for nutrients and mask its growth and identification due to enrichment bias** (Ku, 2017; Mahboob Nemati, 2016; James B Pettengill, 2012).

The results in Appendix A and Figure 1 (above) show significant differences in culture growth rate with no consistency in the specific organism being cultured. Therefore, identifying specific species through molecular methods is superior to using the culturing of potential microbial contaminants.

Molecular methods for microbial detection have proven to be more accurate and sensitive, and deliver more relevant and efficient results by dialing into the specific genome level of detection and providing results in 8-48 hours. (Souii, 2016; Hoorfar, 2011). Appendix E contains an overview of qPCR and DNA Microarray molecular methods (pages 24-26). In addition to eliminating the risk to lab personnel from the growth of hazardous organisms, DNA-based testing greatly reduces the time to detection and increases the accuracy of the detection of individual species, especially fungi, which are hard to speciate via culture methods in many cases.

Traditional plating often suffers from False negatives, especially for Aspergillus spp, where faster and more prominent microorganisms such as Penicillium spp. outcompete for nutrients and mask its growth and identification due to enrichment bias.

Microbial Detection by qPCR

Per manufacturer protocol, both qPCR techniques evaluated in this study require enrichment, DNA isolation, and downstream florescence detection using a qPCR probe. Prior to DNA isolation, an enrichment step for homogenous flower is performed with the samples in Tryptic Soy Broth to enable measurement of organisms. The experiments followed Original Equipment Manufacturers (OEM) instructions for two different qPCR microbial detection assays in this study. Both involved a 24-hour enrichment period, after which cells in solution were disrupted and purified using each assay manufacturer's protocol. Reactions were prepared similarly with water, qPCR master mix, and microorganism-specific primers and fluorescent probes. The reactions were carried out using the assay manufacturers suggestions, including recommended qPCR systems (specifically, method 1 required the Agilent AriaMx), and where OEM systems were not recommended, the manufacturers PCR cycling conditions were applied using industry standard commercially available qPCR systems (Quant Studio 5 (ThermoFisher), or Lightcycler 480 (Roche)), to measure either the appropriate fluorophore for target microorganism or reaction controls.

Appendix B contains results for each qPCR assay (pages 14-15). For identical samples tested on sequencing, *Aspergillus* was not detected equally by both commercially available qPCR assays, although controls were observed with results predicted by each manufacturer for both types of qPCR assays.

- **qPCR assay 1 (qPCR system and genus-level primer) detected the *Aspergillus* genus for 2 samples tested. Method 1 did not have species-specific assays available at the time of the test and could not be speciated.**
- **qPCR assay 2 (Agilent qPCR instrument using the multiplex genus and species-level primers) did not detect either the genus or species for the samples previously confirmed by DNA sequencing to contain the *Aspergillus* spp.**

The limit of detection was also measured for both qPCR assays.

Purified DNA (*A. niger* alone or both *A. niger* and *A. flavus*, depending on the assay) was titrated into PBS to generate a dilution series used as an input spanning from 10^4 genomic copies to 1 genomic copy of DNA per qPCR reaction. This input was used for either genus or species-level qPCR assays.

Appendix C contains the results for this evaluation (Pages 18 & 19). qPCR Method 1 detected *A. niger* DNA to 10^1 DNA copies per reaction, while qPCR Method 2 is more than 10x less sensitive; ~ 10^2 DNA copies per reaction. The detection limit seen for qPCR Method 1 is within expected values within the industry (Amin Forootan, 2017; Ricchi, 2017). Method 2 detected at a rate more than 10x lower than Method 1.

For identical samples tested on sequencing, Aspergillus was not detected equally by both commercially available qPCR assays: qPCR Method#1 matching 2/10 and qPCR Method#2 not matching any of positive sequencing results.

Microbial Detection by a DNA Microarray

To evaluate the DNA microarray platform, several homogenized and evenly distributed cannabis samples were assayed in this study by two separate laboratories using the same microarray procedure. These samples were analyzed to determine the presence or absence of *A. flavus* and *A. niger* in 1 gram of cannabis flower **(Table 1; Appendix D).**

Table 1: Results from the microarray assay. Red indicates a result of Negative. Yellow indicates a result of Positive.

	Lab 1		Lab 2	
Sample	*A niger*	*A flavus*	*A niger*	*A flavus*
1	Negative	Negative	Positive	Negative
2	Negative	Negative	Negative	Negative
3	Positive	Negative	Positive	Positive
4	Negative	Negative	Positive	Negative
5	Positive	Negative	Positive	Negative
6	Positive	Negative	Negative	Negative
7	Positive	Negative	Positive	Negative
8	Negative	Negative	Negative	Negative
9	Negative	Negative	Negative	Negative
10	Positive	Positive	Positive	Positive
11	Negative	Negative	Negative	Negative
12	Negative	Negative	Negative	Negative
13	Positive	Negative	Positive	Negative
14	Negative	Positive	Negative	Positive
15	Negative	Negative	Negative	Negative
16	Negative	Negative	Negative	Negative
17	Negative	Negative	Negative	Negative
18	Positive	Negative	Positive	Negative

Microbial Detection by Next Generation Sequencing/Sanger DNA Sequencing

To verify the presence of the different *Aspergillus* spp in the samples, Arizona State University performed Next Generation Sequencing (NGS) for broad identification (18S and ITS) of the microbial profile in the flower samples and Sanger DNA Sequencing to confirm the presence of *A. niger* and *A. flavus*. To confirm the presence of *A. niger* and *A. flavus*, a two-step nested PCR reaction, using species-specific primers followed by M13 primers for Sanger sequencing, was performed. The prepared DNA was sent to the University of Arizona Genomics Core Facility using species-specific primers for Sanger sequencing.

Table 2 contains the sequences utilized specific to each *Aspergillus* spp present in the cannabis samples tested. Using sequences amplified from the cannabis samples, the corresponding *Aspergillus* spp was confirmed in all cannabis flower samples determined to be positive in Table 1. Previously identified and confirmed species-specific SNPs are shown in Table 2. A representative sample of the results are provided in Table 3.

Due to the expensive nature of NGS and Sanger Sequencing, although highly sensitive and accurate when utilizing organism specific primers, the two sequencing methods are not economically practical as a microbial testing platform for cannabis at this time. As this study found, however, NGS and Sanger Sequencing is effective for additional confirmation and quality control.

Using sequences amplified from the cannabis samples, the corresponding Aspergillus spp was confirmed in Microarray testing for all cannabis flower samples determined to be positive.

Method Comparison

Microbial testing has largely utilized traditional methods of cell culture-based identification. Using molecular diagnostic tools, the speed at which these organisms can be detected has increased. Eliminating the culturing of pathogenic organisms makes the microbial testing laboratory safer and, with a reduced waste stream, less prone to systemic contamination due to accidental dissemination of cultured organisms as spores.

Reference Organism	Approximate Base Position											A. fla
	136	160	162	171	190	216	243	244	256	257	275 HV MOTIF	292
A. flavus (A) Control	A	T	G	-	C	A	C	C	C	C	CGCAAATCAATC	C
A. flavus (B) Control	A	T	G	-	C	A	C	C	C	C	CGCAAATCAATC	T
A. niger	-	T	G	-	G	A	A	T	T	T	GTTTTCCAACCA	N/A
A. brasiliensis	-	T	G	T	G	A	A	T	T	T	GTTTTCCAACCA	N/A
P. chrysogenum	-	C	C	G	G	G	C	C	C	C	CAACCCAAATTT	N/A
P. citreonigrum	-	C	C	T	G	G	C	C	C	C	ACATCAATCTTT	N/A
P. olsonii	-	C	C	T	G	G	C	C	C	T	CAACCAAATTT	N/A
P. brevicompactum	-	C	C	T	G	G	C	C	C	T	CAACCAAATTT	N/A
P. citrinum	-	C	C	C	G	G	C	C	C	C	CCCCCAACCTTT	N/A
P. copticola	-	C	C	C	G	G	C	C	C	C	AATCCCCCCCTC	N/A
P. paxilli	-	C	C	-	G	G	C	C	C	C	CCCCCCCTCAAT	N/A

Table 2: Reference sequences for *A. flavus*, *A. niger*, and *A. brasiliensis* as well as common *Penicillium* spp found in cannabis flower. The sequence alignments highlight the sequence differences used to distinguish *A. niger* and *A. flavus* from *A. brasiliensis* and *Penicillium* spp.

Sample #	Approximate Base Position											A. fla
	136	160	162	171	190	216	243	244	256	257	275 HV MOTIF	292
1 (*Niger* Specific Primers)	-	T	G	-	G	A	A	T	T	T	GTTTTCCAACCA	N/A
3 (*Niger* Specific Primers)	-	T	G	-	G	A	A	T	T	T	GTTTTCCAACCA	N/A
3 (*Flavus* Specific Primers)	A	T	G	-	C	A	C	C	C	C	CGCAAATCAATC	C
4 (*Niger* Specific Primers)	-	T	G	-	G	A	A	T	T	T	GTTTTCCAACCA	N/A
4 (*Flavus* Specific Primers)	A	T	G	-	C	A	C	C	C	C	CGCAAATCAATC	T
6 (*Niger* Specific Primers)	-	T	G	-	G	A	A	T	T	T	GTTTTCCAACCA	N/A
10 (*Niger* Specific Primers)	-	T	G	-	G	A	A	T	T	T	GTTTTCCAACCA	N/A
10 (*Flavus* Specific Primers)	A	T	G	-	C	A	C	C	C	C	CGCAAATCAATC	T
13 (*Niger* Specific Primers)	-	T	G	-	G	A	A	T	T	T	GTTTTCCAACCA	N/A

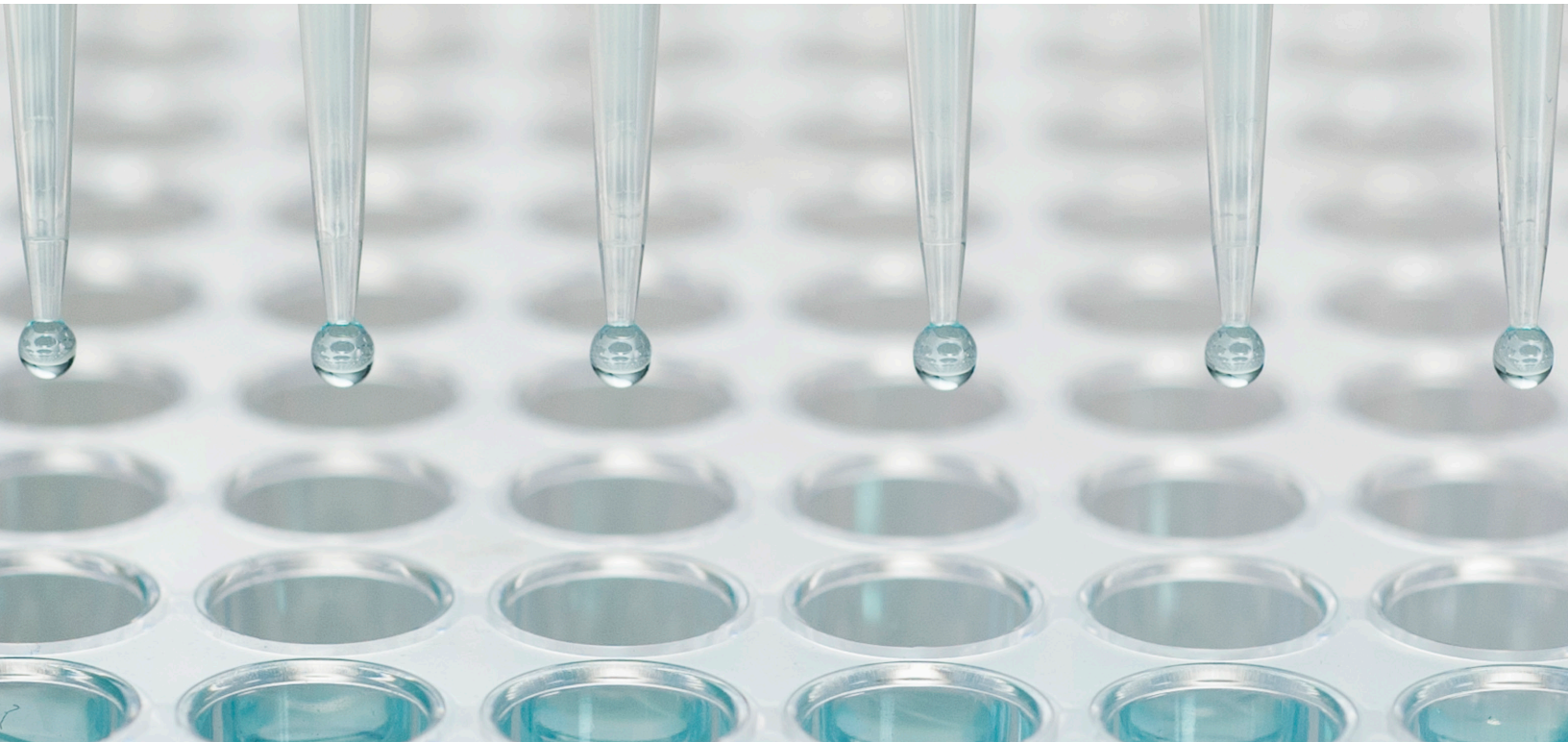
Table 3: Sanger sequencing of common fungal species. Purple indicates two variants of *A. flavus*. Green indicates *A. niger*. Yellow Indicates sequences of selected *Penicillum* spp. The single nucleotide polymorphisms are listed, if applicaple, at multiple sequence locations. HV - Hypervariable Region.

Further, eliminating culturing removes the possibility that species are lost because they are outcompeted or consumed by other organisms, causing an unpredictable bias for or against certain species (McKernan, 2019). In the set of cannabis flower samples described in this study, plating results identified a complex matrix of fungal organisms but did not identify the pathogenic *Aspergillus* spp.

The ability to detect the presence of the target organism differed between the two qPCR assays;

- qPCR Method 1 agreed with the DNA sequencing for two samples and displayed the level of sensitivity that is reported for qPCR platforms (Smith 2008).
- qPCR Method 2 test did not detect any of the target organisms at the levels present in the sample and did not meet the level of sensitivity of Method 1 (Tables 4 and 5).

The microarray assay proves to be the most accurate DNA-based method for testing Aspergillus species in cannabis with 8/10 at Lab #1 and 9/10 at Lab #2 matching positive Sequencing results.



Summary

The key findings of this study are summarized in Summary Table 4 below.

Table 4: Summary Table

Sample Number		DNA Microarray - Lab 1 Array	DNA Microarray - Lab 2 Array	Sequencing	qPCR Method #1	qPCR Method #2	Plate Culture
Conditions							
Sample Prep		Manufacture	Manufacture	Manufacture	Manufacture	Manufacture	
Enrichment		No	No	No	Yes	Yes	Yes
Sample Number	**Sample Type**	**Results**					
1	Flower	Negative	Pos (Niger)	Pos (Niger)	Pos (nonspecific)	Negative	Large Contamination - No Clear Aspergillus
2	Flower	Negative	Negative	Negative	Negative	Negative	Large Contamination - No Clear Aspergillus
3	Flower	Pos (Niger)	Pos (Niger/ Flavus)	Pos (Niger/ Flavus)	Pos (nonspecific)	Negative	Large Contamination - No Clear Aspergillus
4	Flower	Negative	Pos (Niger)	Pos (Niger/Flavus)	Negative	Negative	Large Contamination - No Clear Aspergillus
5	Flower	Pos (Niger)	Pos (Niger)	Pos (Niger)	Negative	Negative	Large Contamination - No Clear Aspergillus
6	Flower	Pos (Niger)	Negative	Pos (Niger)	Negative	Negative	Large Contamination - No Clear Aspergillus
7	Flower	Pos (Niger)	Pos (Niger)	Pos (Niger)	Negative	Negative	Large Contamination - No Clear Aspergillus
8	Flower	Negative	Negative	Negative	Negative	Negative	Large Contamination - No Clear Aspergillus
9	Flower	Negative	Negative	Negative	Negative	Negative	Large Contamination - No Clear Aspergillus
10	Flower	Pos (Niger/ Flavus)	Pos (Niger/ Flavus)	Pos (Niger/ Flavus)	Negative	Negative	Large Contamination - No Clear Aspergillus
11	Flower	Negative	Negative	Negative	Negative	Negative	Large Contamination - No Clear Aspergillus
12	Flower	Negative	Negative	Negative	Negative	Negative	Large Contamination - No Clear Aspergillus
13	Flower	Pos (Niger)	Pos (Niger)	Pos (Niger)	Negative	Negative	Large Contamination - No Clear Aspergillus
14	Flower	Pos (Flavus)	Pos (Flavus)	Pos (Flavus)	Negative	Negative	Large Contamination - No Clear Aspergillus
15	Flower	Negative	Negative	Negative	Negative	Negative	Large Contamination - No Clear Aspergillus
16	Flower	Negative	Negative	Negative	Negative	Negative	Large Contamination - No Clear Aspergillus
17	Flower	Negative	Negative	Negative	Negative	Negative	Large Contamination - No Clear Aspergillus
18	Flower	Pos (Niger)	Pos (Niger)	Pos (Niger)	Negative	Negative	Large Contamination - No Clear Aspergillus

Sample results from method **matching Positive Samples** from Sequencing	**8 out of 10**	**9 out of 10**	**2 out of 10**	**0 out of 10**	**0 out of 10**
Sample results from method **matching Negative Samples** from Sequencing	**8 out of 8**	**8 out of 8**	**8 out of 8**	**8 out of 8**	**8 out of 8**
Sample results from method that **does not match** Sequencing	**2 out of 18**	**1 out of 18**	**10 out of 18**	**8 out of 18**	**8 out of 18**

In Table 4, the results of the microbial analysis data obtained from the 18 representative cannabis flower samples is summarized (see Appendices for each method's detailed results).

The methods based on endpoint PCR (DNA Microarray & DNA Sequencing) are presented as the three left-most columns, whereas the real time qPCR-based methods are presented as column 4 and 5, with plate base culture presented to the right, as column 6.

The trends are instructive. As seen from plate culture, there is a substantial fungal load detected on all 18 of the flower samples. However, despite a massive level of microbial outgrowth, no explicit detection of *Aspergillus* is seen. It is possible that *Aspergillus* contamination could have been present, but obscured by plate overgrowth.

Table 5: Plate Culture Summary of Results

Method – Plate Culture	Plate Culture
Positives Samples Matching (%)	0%
Positives Samples Not Matching (%)	100%

Previous microbial analysis of cannabis has found that fungi, especially *Aspergillus*, culture poorly (Kevin McKernan, 2016). As a result, DNA sequencing methods have been found to be better suited to detect low levels of *Aspergillus* contamination, especially in the context of extensive additional microbial contamination on the same sample. For that reason, in this study, the same 18 samples have been subjected to fluid phase enrichment, followed by DNA purification endpoint PCR, and then Sanger DNA sequencing. Those data are presented in column 3. **From sequencing, 10 of the 18 samples display *Aspergillus* DNA (*niger* and/or *flavus*), and 8 of the 18 samples were negative. Based on these findings, the endpoint PCR-Sanger DNA sequencing data was a more accurate representation of the presence of *Aspergillus* contamination among the 18 cannabis flower samples.**

Neither qPCR method showed acceptable concordance to DNA sequencing (Method 1; 2/18 matches, Method 2; 0/18) with respect to detecting *Aspergillus* in the 18 samples at either the genus or species level.

Table 6: Summary of qPCR Results

Method – qPCR	Method #1	Method #2
Positives Samples Matching (%)	20%	0%
Positives Samples Not Matching (%)	80%	100%

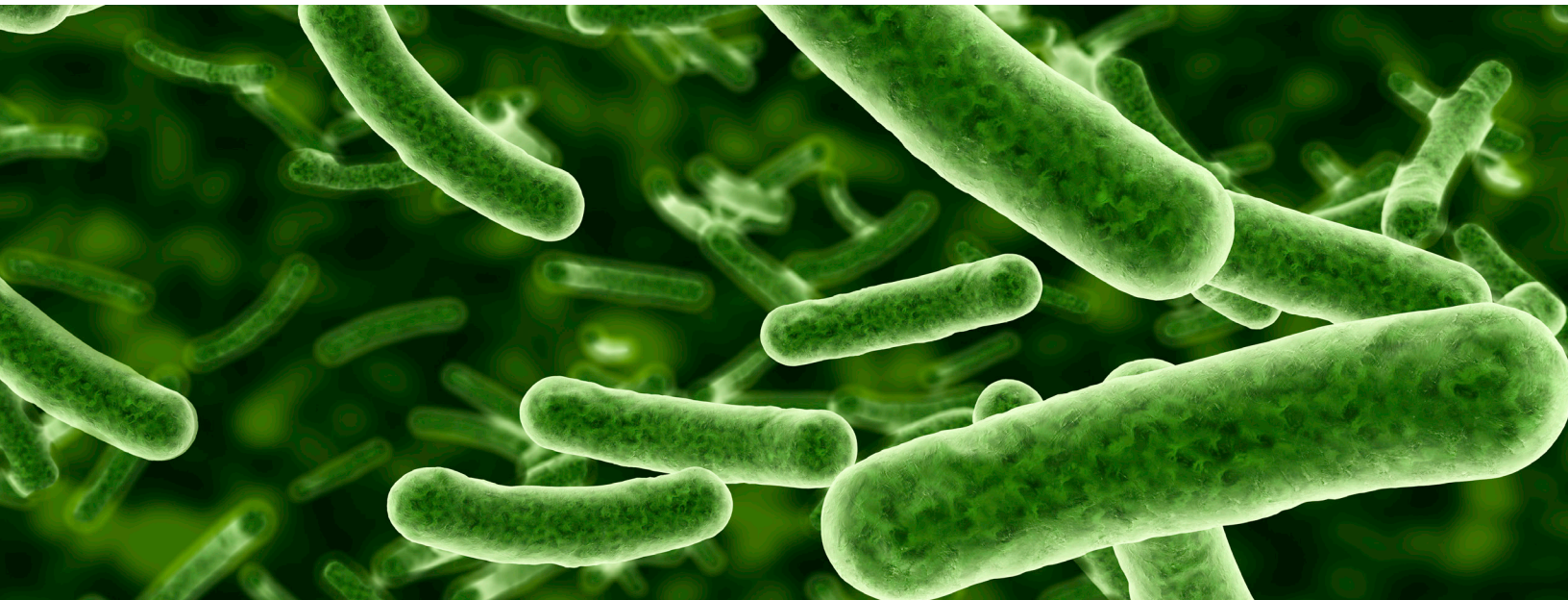
Both qPCR methods were observed to have a high error rate in detecting *Aspergillus* spp. The lack of agreement of Methods 1 and 2 with the microarray results and the ability of Method 1 to detect *Aspergillus* genus to a 10x lower CFU than Method 2 demonstrates differences in their relative sensitivity for the detection of low levels of *Aspergillus* contamination, as compared to each other and to the DNA microarray. The limit of detection (LOD) assessment conducted on these qPCR methods found that qPCR Method #1 had an LOD of $\mathbf{10^1}$ CFU range while qPCR Method #2 had an LOD of $\mathbf{10.^2}$

DNA microarray testing performed on the same samples in two different labs showed a higher level of concordance with the sequencing standard (16/18, 17/18 matches) than any of the other methods tested.

Table 7: Summary of Microarray Results

Method – DNA Microarray	Lab #1	Lab #2
Positives Samples Matching (%)	80%	90%
Positives Samples Not Matching (%)	20%	10%

The DNA-microarray assay used in this study is a type of endpoint PCR. PCR is better suited for detecting low DNA levels in the presence of a large DNA background than qPCR (Patrick Bastien, 2008). This study finds that the number of genomic copies detected by Microarray was significantly lower than with either Method 1 or Method 2.



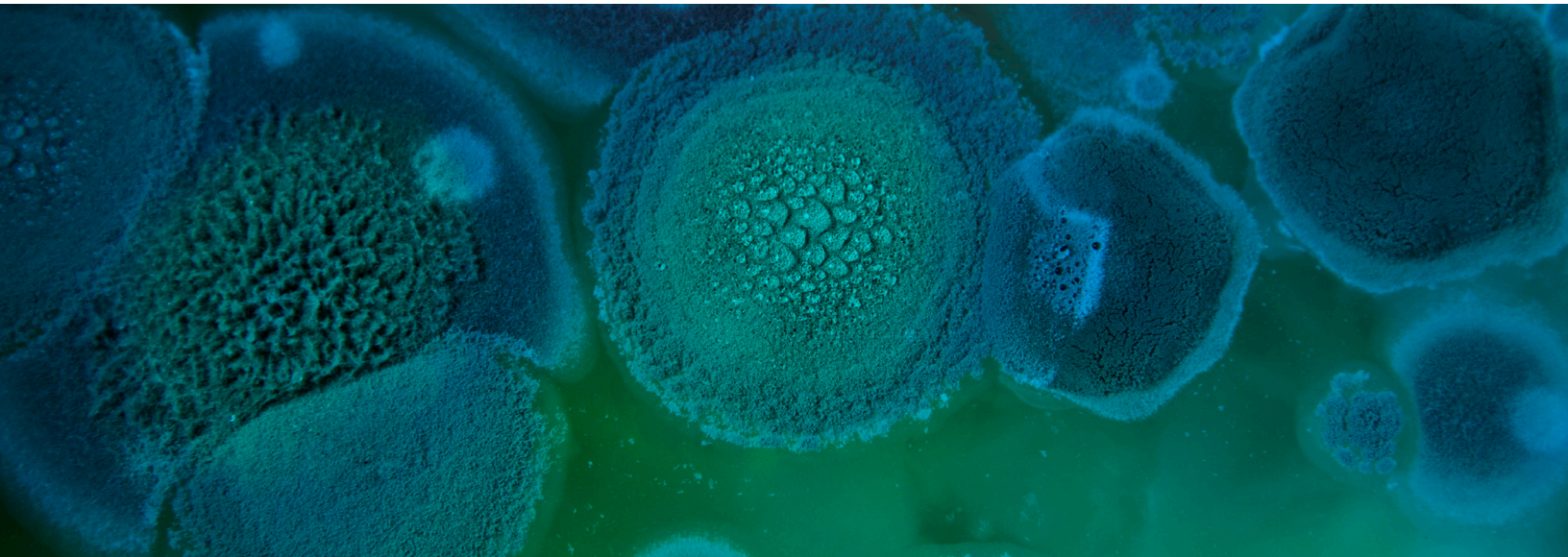
Conclusion

The results of this microbial method comparison challenge the conventional conclusion that plate culture is the "gold standard" of testing technologies, especially for microbial testing of *Aspergillus* species. In this study, sequencing results found that plate culture after enrichment does not reliably detect *Aspergillus* (See Table 8).

DNA methods are more sensitive and specific than plate culture for the detection of fungal contamination on cannabis such as *Aspergillus* (Kevin McKernan, 2016). However, the data suggest that the two qPCR methods tested appear to display significant levels of "false negative" outcomes. The differences could be due to inadequate sensitivity or loss/consumption of certain organisms during culture, when compared to DNA sequencing. DNA-microarray assay is similar to endpoint PCR, and the data suggest that the microarray method provides more accurate data as defined by the results obtained from DNA sequencing. Taken together, the DNA microarray assay was the most accurate DNA-based method for testing *Aspergillus* species in cannabis.

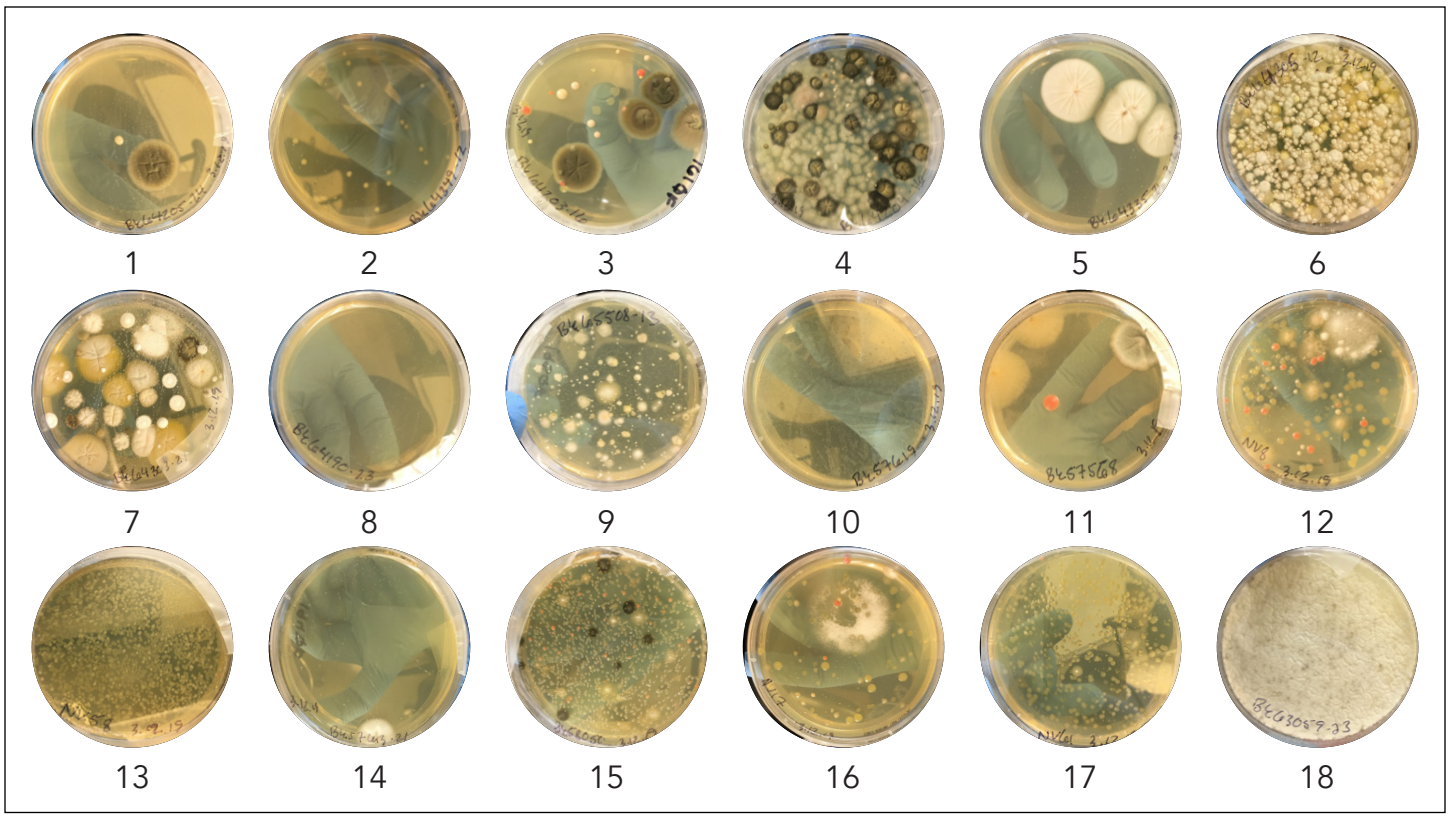
Table 8: Overall Summary of Results

	DNA Microarray - Lab #1 Array	**DNA Microarray - Lab #2 Array**	**qPCR Method #1**	**qPCR Method #2**	**Plate Culture**
Sample results from method **matching Positive Samples** from Sequencing	**8 out of 10**	**9 out of 10**	**2 out of 10**	**0 out of 10**	**0 out of 10**
Sample results from method **matching Negative Samples** from Sequencing	**8 out of 8**	**8 out of 8**	**8 out of 8**	**8 out of 8**	**8 out of 8**
Sample results from method that **does not match** Sequencing	**2 out of 18**	**1 out of 18**	**10 out of 18**	**8 out of 18**	**8 out of 18**



Appendix A
Plate Culture data

Appendix A – Representative fungal growth plates for each sample evaluated.



Appendix B

qPCR Method #1 and qPCR Method #2 Detection Results for samples confirmed positive by Sequencing for *Aspergillus* Genus and Species

qPCR Method #1: *Aspergillus* Genus Assay				
Channel	**Ct**	**Sample Number**	**PCR Result**	**Assay Result**
FAM	0	5	-	No *Aspergillus* Detected
VIC	23.008196	5	+	
FAM	35.751442	1	+	*Aspergillus* Detected ***(Questionable)***
VIC	22.857885	1	+	
FAM	0	4	-	No *Aspergillus* Detected
VIC	22.833048	4	+	
FAM	35.906944	3	+	*Aspergillus* Detected ***(Questionable)***
VIC	23.214401	3	+	
FAM	0	6	-	No *Aspergillus* Detected
VIC	23.00907	6	+	
FAM	0	7	-	No *Aspergillus* Detected
VIC	22.66361	7	+	
FAM	0	10	-	No *Aspergillus* Detected
VIC	22.845068	10	+	
FAM	0	13	-	No *Aspergillus* Detected
VIC	23.030277	13	+	
FAM	0	14	-	No *Aspergillus* Detected
VIC	23.010326	14	+	

Table 6. Results of qPCR method 1, evaluation of samples for *Aspergillus* spp.
Yellow Highlighted Ct values is considered to be **outside** of qPCR range for a positive result

qPCR Method #2: *Aspergillus* Genus Multiplex Assay				
Channel	**Ct**	**Sample Number**	**PCR Result**	**Assay Result**
FAM	0	1	-	No *Aspergillus* Detected
VIC	23.049427	1	+	
FAM	0	3	-	No *Aspergillus* Detected
VIC	25.728178	3	+	
FAM	0	4	-	No *Aspergillus* Detected
VIC	25.885878	4	+	
FAM	0	5	-	No *Aspergillus* Detected
VIC	24.710373	5	+	
FAM	0	6	-	No *Aspergillus* Detected
VIC	23.115564	6	+	
FAM	0	7	-	No *Aspergillus* Detected
VIC	19.660324	7	+	
FAM	0	10	-	No *Aspergillus* Detected
VIC	21.1156	10	+	
FAM	0	13	-	No *Aspergillus* Detected
VIC	22.056034	13	+	

qPCR method 2 - A. niger and flavus species specific primers test with tested samples.

qPCR Method #2: *Aspergillus niger* Species Assay				
Channel	**Ct**	**Sample Number**	**PCR Result**	**Assay Result**
FAM	0	1	-	No *Aspergillus* Detected
VIC	24.013842	1	+	
FAM	0	3	-	No *Aspergillus* Detected
VIC	25.728178	3	+	
FAM	0	4	-	No *Aspergillus* Detected
VIC	28.136799	4	+	
FAM	0	5	-	No *Aspergillus* Detected
VIC	26.06391	5	+	
FAM	0	6	-	No *Aspergillus* Detected
VIC	23.164606	6	+	
FAM	0	7	-	No *Aspergillus* Detected
VIC	24.710342	7	+	
FAM	0	10	-	No *Aspergillus* Detected
VIC	21.39899	10	+	
FAM	0	13	-	No *Aspergillus* Detected
VIC	21.764795	13	+	

qPCR Method #2: *Aspergillus flavus* Species Assay				
Channel	**Ct**	**Sample Number**	**PCR Result**	**Assay Result**
FAM	0	1	-	No *Aspergillus* Detected
VIC	22.661604	1	+	
FAM	0	3	-	No *Aspergillus* Detected
VIC	26.085764	3	+	
FAM	0	4	-	No *Aspergillus* Detected
VIC	26.095825	4	+	
FAM	0	5	-	No *Aspergillus* Detected
VIC	24.266834	5	+	
FAM	0	6	-	No *Aspergillus* Detected
VIC	21.732374	6	+	
FAM	0	7	-	No *Aspergillus* Detected
VIC	22.33345	7	+	
FAM	0	10	-	No *Aspergillus* Detected
VIC	19.659721	10	+	
FAM	0	13	-	No *Aspergillus* Detected
VIC	20.375305	13	+	
FAM	0	14	-	No *Aspergillus* Detected
VIC	21.845537	14	+	

Appendix C

Limits of Detection study of qPCR Method #1 and qPCR Method #2 by genomic DNA spiking study for *Aspergillus* Genus and Species

qPCR Method #1: *Aspergillus* Genus Assay				
Channel	**Ct**	**Sample Number**	**PCR Result**	**Assay Result**
FAM	16.08	*Aspergillus Niger* gDNA 625,000 copies	+	*Aspergillus* Detected
VIC	23.45	*Aspergillus Niger* gDNA 625,000 copies	+	
FAM	19.36	*Aspergillus Niger* gDNA 62,500 copies	+	*Aspergillus* Detected
VIC	23.04	*Aspergillus Niger* gDNA 62,500 copies	+	
FAM	23.69	*Aspergillus Niger* gDNA 6250 copies	+	*Aspergillus* Detected
VIC	22.96	*Aspergillus Niger* gDNA 6250 copies	+	
FAM	27.71	*Aspergillus Niger* gDNA 625 copies	+	*Aspergillus* Detected
VIC	22.80	*Aspergillus Niger* gDNA 625 copies	+	
FAM	31.38	*Aspergillus Niger* gDNA 62.5 copies	+	*Aspergillus* Detected
VIC	22.84	*Aspergillus Niger* gDNA 62.5 copies	+	
FAM	0.00	*Aspergillus Niger* gDNA 6.25 copies	-	No *Aspergillus* Detected
VIC	22.88	*Aspergillus Niger* gDNA 6.25 copies	+	
FAM	14.93	Positive Control	+	*Aspergillus* Detected
VIC	22.40	Positive Control	+	
FAM	0.00	Negative Control	-	No *Aspergillus* Detected
VIC	25.95	Negative Control	+	

Table 4. Results of qPCR method 1, genomic DNA evaluation of *Aspergillus* genus primers.
Green Highlighted Ct value is considered to be reasonable within qPCR range for positive results

qPCR Method #2: *Aspergillus* Genus Multiplex Assay				
Channel	**Ct**	**Sample Number**	**PCR Result**	**Assay Result**
FAM	19.59	*Aspergillus Niger* gDNA 625,000 copies	+	*Aspergillus* Detected
VIC	27.98	*Aspergillus Niger* gDNA 625,000 copies	+	
FAM	22.94	*Aspergillus Niger* gDNA 62,500 copies	+	*Aspergillus* Detected
VIC	27.93	*Aspergillus Niger* gDNA 62,500 copies	+	
FAM	26.63	*Aspergillus Niger* gDNA 6250 copies	+	*Aspergillus* Detected
VIC	28.02	*Aspergillus Niger* gDNA 6250 copies	+	
FAM	30.47	*Aspergillus Niger* gDNA 625 copies	+	*Aspergillus* Detected
VIC	27.76	*Aspergillus Niger* gDNA 625 copies	+	
FAM	40.32	*Aspergillus Niger* gDNA 62.5 copies	+	*Aspergillus* Detected ***(Questionable)***
VIC	27.74	*Aspergillus Niger* gDNA 62.5 copies	+	
FAM	48.01	*Aspergillus Niger* gDNA 6.25 copies	+	*Aspergillus* Detected ***(Questionable)***
VIC	30.62	*Aspergillus Niger* gDNA 6.25 copies	+	
FAM	11.66	Positive Control	+	*Aspergillus* Detected
VIC	31.02	Positive Control	+	
FAM	0.00	Negative Control	-	No *Aspergillus* Detected
VIC	0.00	Negative Control	-	

Table 5. Results of qPCR method #2, genomic DNA evaluation of *Aspergillus* genus primers.
Green Highlighted Ct value is considered to be reasonable within qPCR range for positive result
Yellow Highlighted Ct values is considered to be **outside** of qPCR range for a positive result

Appendix C: qPCR method #2: *A. niger* and flavus species specific primers test with genomic DNA.

qPCR Method #2: *Aspergillus niger* Species Assay				
Channel	**Ct**	**Sample Number**	**PCR Result**	**Assay Result**
FAM	17.34	*A. niger* gDNA 625,000 copies	+	*Aspergillus* Detected
VIC	29.69	*A. niger* gDNA 625,000 copies	+	
FAM	20.73	*A. niger* gDNA 62,500 copies	+	*Aspergillus* Detected
VIC	29.81	*A. niger* gDNA 62,500 copies	+	
FAM	23.98	*A. niger* gDNA 6250 copies	+	*Aspergillus* Detected
VIC	29.71	*A. niger* gDNA 6250 copies	+	
FAM	29.31	A. niger gDNA 625 copies	+	*Aspergillus* Detected
VIC	29.77	*A. niger* gDNA 625 copies	+	
FAM	35.99	*A. niger* gDNA 62.5 copies	+	*Aspergillus* Detected ***(Questionable)***
VIC	29.22	*A. niger* gDNA 62.5 copies	+	
FAM	0.00	*A. niger* gDNA 6.25 copies	-	No *Aspergillus* Detected
VIC	28.99	*A. niger* gDNA 6.25 copies	+	
FAM	13.70	Positive Control	+	Control Detected
VIC	29.10	Positive Control	+	
FAM	0.00	Negative Control	-	No Control Detected
VIC	0.00	Negative Control	-	

qPCR Method #2: *Aspergillus flavus* Species Assay				
Channel	**Ct**	**Sample Number**	**PCR Result**	**Assay Result**
FAM	14.085521	*A. flavus* gDNA 625,000 copies	+	*Aspergillus* Detected
VIC	32.116604	*A. flavus* gDNA 625,000 copies	+	
FAM	16.654589	*A. flavus* gDNA 62,500 copies	+	*Aspergillus* Detected
VIC	32.88441	*A. flavus* gDNA 62,500 copies	+	
FAM	20.274805	*A. flavus* gDNA 6250 copies	+	*Aspergillus* Detected
VIC	32.940475	*A. flavus* gDNA 6250 copies	+	
FAM	23.9684	*A. flavus* gDNA 625 copies	+	*Aspergillus* Detected
VIC	32.54495	*A. flavus* gDNA 625 copies	+	
FAM	27.943314	*A. flavus* gDNA 62.5 copies	+	*Aspergillus* Detected
VIC	31.935144	*A. flavus* gDNA 62.5 copies	+	
FAM	31.996887	*A. flavus* gDNA 6.25 copies	+	*Aspergillus* Detected ***(Questionable)***
VIC	32.047882	*A. flavus* gDNA 6.25 copies	+	
FAM	26.856306	Positive Control	+	Control Detected
VIC	37.56267	Positive Control	+	
FAM	0	Negative Control	-	No Control Detected
VIC	0	Negative Control	-	

Green Highlighted Ct value is considered to be reasonable within qPCR range for positive result
Yellow Highlighted Ct values is considered to be outside of qPCR range for a positive result

Appendix D

DNA Microarray Results – Independent Lab #1 & Lab #2 Results

Appendix D – Full table of DNA-Microarray Results

Lab #1: Slide 7017005007 RFU values. The bracketed number are the 1 CFU/gram cutoff values.

Slide	701700 5007	701700 5007	701700 5007	701700 5007	701700 5007	701700 5007	701700 5007	701700 5007	701700 5007	701700 5007	701700 5007	701700 5007
Sample ID Number	N/A	1	N/A	2	3	4	5	6	7	8	NTC	NTC
Well	5	6	8	5	6	4	4	1	2	2	10	6
Negative Control	1008 (2889)	759 (2889)	412 (2889)	715 (2889)	874 (2889)	912 (2889)	631 (2889)	1064 (2889)	608 (2889)	859 (2889)	632 (2889)	758 (2889)
PCR Positive Control - Fungi	63723 (2265)	64164 (2265)	56488 (2265)	63943 (2265)	63704 (2265)	63528 (2265)	63481 (2265)	63445 (2265)	63624 (2265)	63862 (2265)	63999 (2265)	63735 (2265)
Aspergillus flavus	940 (2939)	643 (2939)	546 (2939)	645 (2939)	1012 (2939)	711 (2939)	773 (2939)	952 (2939)	795 (2939)	877 (2939)	700 (2939)	808 (2939)
Aspergillus fumigatus	1321 (3163)	947 (3163)	736 (3163)	1116 (3163)	1083 (3163)	1088 (3163)	899 (3163)	1194 (3163)	841 (3163)	1268 (3163)	898 (3163)	1051 (3163)
Aspergillus niger	2000 (2729)	811 (2729)	671 (2729)	912 (2729)	42913 (2729)	1572 (2729)	28621 (2729)	4242 (2729)	4458 (2729)	1098 (2729)	1173 (2729)	934 (2729)
Aspergillus terreus	1105 (1400)	1010 (1400)	849 (1400)	982 (1400)	3428 (1400)	1044 (1400)	747 (1400)	908 (1400)	854 (1400)	871 (1400)	903 (1400)	585 (1400)
Negative Control	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected
PCR Positive Control - Fungi	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected
Aspergillus flavus	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected
Aspergillus fumigatus	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected
Aspergillus niger	Not Detected	Not Detected	Not Detected	Not Detected	Detected	Not Detected	Detected	Detected	Detected	Not Detected	Not Detected	Not Detected
Aspergillus terreus	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected

Lab #1: Slide 7017005042 RFU values. The bracketed number are the 1 CFU/gram cutoff values.

Slide	701700 5042	701700 5042	701700 5042	701700 5042	701700 5042	701700 5042	701700 5042	701700 5042	701700 5042	701700 5042	701700 5042	701700 5042
Sample ID Number	9	10	11	12	13	14	15	16	17	18	NTC	NTC
Well	5	3	4	1	3	3	1	8	2	6	12	12
Negative Control	789 (2889)	805 (2889)	908 (2889)	769 (2889)	1007 (2889)	935 (2889)	660 (2889)	731 (2889)	656 (2889)	1160 (2889)	872 (2889)	872 (2889)
PCR Positive Control - Fungi	63738 (2265)	63719 (2265)	63788 (2265)	63179 (2265)	63563 (2265)	64152 (2265)	63916 (2265)	63958 (2265)	56353 (2265)	63517 (2265)	63730 (2265)	63730 (2265)
Aspergillus flavus	1146 (2939)	20839 (2939)	944 (2939)	1079 (2939)	1204 (2939)	15280 (2939)	1107 (2939)	677 (2939)	854 (2939)	990 (2939)	925 (2939)	925 (2939)
Aspergillus fumigatus	910 (3163)	1070 (3163)	1024 (3163)	810 (3163)	1924 (3163)	1217 (3163)	1007 (3163)	958 (3163)	1145 (3163)	1317 (3163)	1348 (3163)	1348 (3163)
Aspergillus niger	1062 (2729)	62564 (2729)	1105 (2729)	727 (2729)	16764 (2729)	1084 (2729)	941 (2729)	822 (2729)	1097 (2729)	50140 (2729)	1112 (2729)	1112 (2729)
Aspergillus terreus	594 (1400)	12003 (1400)	683 (1400)	734 (1400)	1453 (1400)	1127 (1400)	822 (1400)	1100 (1400)	1191 (1400)	1166 (1400)	771 (1400)	771 (1400)
Negative Control	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected
PCR Positive Control - Fungi	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected
Aspergillus flavus	Not Detected	Detected	Not Detected	Not Detected	Not Detected	Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected
Aspergillus fumigatus	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected
Aspergillus niger	Not Detected	Detected	Not Detected	Not Detected	Detected	Not Detected	Not Detected	Not Detected	Not Detected	Detected	Not Detected	Not Detected
Aspergillus terreus	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected

Lab #2: Slide 7015006093 RFU values. The bracketed number are the 1 CFU/gram cutoff values.

Slide	70150 06093	70150 06093	70150 06093	70150 06093	70150 06093	70150 06093	70150 06093	70150 06093	70150 06093	70150 06093	70150 06093	701500 6093
Sample ID Number	N/A	1	N/A	2	3	4	5	6	7	8	N/A	Niger/T erreus Matrix Spike
Well	1	2	3	4	5	6	7	8	9	10	11	12
Negative Control	84 (2889)	223 (2889)	0 (2889)	39 (2889)	3 (2889)	0 (2889)	0 (2889)	51 (2889)	84 (2889)	128 (2889)	0 (2889)	0 (2889)
PCR Positive Control - Fungi	62705 (2265)	62855 (2265)	62843 (2265)	62769 (2265)	62963 (2265)	62973 (2265)	62818 (2265)	62846 (2265)	62819 (2265)	62464 (2265)	62513 (2265)	62372 (2265)
Aspergillus flavus	167 (2939)	305 (2939)	425 (2939)	0 (2939)	7800 (2939)	579 (2939)	0 (2939)	118 (2939)	166 (2939)	103 (2939)	0 (2939)	0 (2939)
Aspergillus fumigatus	1502 (3163)	485 (3163)	758 (3163)	565 (3163)	270 (3163)	129 (3163)	222 (3163)	1386 (3163)	288 (3163)	400 (3163)	243 (3163)	58 (3163)
Aspergillus niger	861 (2729)	38744 (2729)	0 (2729)	0 (2729)	37987 (2729)	27074 (2729)	29323 (2729)	1345 (2729)	3527 (2729)	207 (2729)	0 (2729)	52341 (2729)
Aspergillus terreus	373 (1400)	302 (1400)	186 (1400)	645 (1400)	174 (1400)	0 (1400)	453 (1400)	112 (1400)	183 (1400)	318 (1400)	201 (1400)	47586 (1400)
Negative Control	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected
PCR Positive Control - Fungi	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected
Aspergillus flavus	Not Detected	Not Detected	Not Detected	Not Detected	Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected
Aspergillus fumigatus	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected
Aspergillus niger	Not Detected	Detected	Not Detected	Not Detected	Detected	Detected	Detected	Not Detected	Detected	Not Detected	Not Detected	Detected
Aspergillus terreus	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Detected

Lab #2: Slide RFU values. The bracketed numbers are the 1 CFU/gram cutoff values.

Slide	70150 06094	70150 06094	70150 06094	70150 06094	70150 06094	70150 06094	70150 06094	70150 06094	70150 06094	70150 06094	70150 06094	7015006 094
Sample ID Number	9	10	11	12	13	14	15	16	17	18	N/A	Flavus/F umigatus Matrix Spike
Well	1	2	3	4	5	6	7	8	9	10	11	12
Negative Control	227 (2889)	118 (2889)	4 (2889)	61 (2889)	191 (2889)	0 (2889)	158 (2889)	22 (2889)	0 (2889)	0 (2889)	0 (2889)	0 (2889)
PCR Positive Control - Fungi	62705 (2265)	62774 (2265)	62785 (2265)	62454 (2265)	62628 (2265)	62529 (2265)	62688 (2265)	62751 (2265)	62688 (2265)	62625 (2265)	62679 (2265)	62498 (2265)
Aspergillus flavus	115 (2939)	10682 (2939)	0 (2939)	0 (2939)	617 (2939)	40479 (2939)	321 (2939)	0 (2939)	0 (2939)	0 (2939)	0 (2939)	62387 (2939)
Aspergillus fumigatus	567 (3163)	175 (3163)	312 (3163)	152 (3163)	1439 (3163)	704 (3163)	544 (3163)	438 (3163)	474 (3163)	485 (3163)	198 (3163)	45662 (3163)
Aspergillus niger	31 (2729)	43252 (2729)	0 (2729)	288 (2729)	5254 (2729)	0 (2729)	62 (2729)	0 (2729)	0 (2729)	37612 (2729)	0 (2729)	0 (2729)
Aspergillus terreus	317 (1400)	284 (1400)	233 (1400)	158 (1400)	294 (1400)	73 (1400)	345 (1400)	231 (1400)	1 (1400)	204 (1400)	226 (1400)	252 (1400)
Negative Control	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected
PCR Positive Control - Fungi	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected	Detected
Aspergillus flavus	Not Detected	Detected	Not Detected	Not Detected	Not Detected	Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Detected
Aspergillus fumigatus	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Detected
Aspergillus niger	Not Detected	Detected	Not Detected	Not Detected	Detected	Not Detected	Not Detected	Not Detected	Not Detected	Detected	Not Detected	Not Detected
Aspergillus terreus	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected	Not Detected

Appendix E

An Overview of DNA Molecular methods

Molecular diagnostic tools, particularly DNA microarray technology, have been used in human diagnostics for years, but are still relatively new to regulatory testing. Recently, they have been accepted and adopted by several regulatory agencies, including the USDA and FDA, due to the 2011 Food Safety Modernization Safety Act (Rasooly, 2008; McLoughlin, 2011). By employing the detection of DNA as opposed to the actual organisms, molecular diagnostics reduces the risk of systemic contamination of the laboratory with microorganisms, especially those that produce and disseminate airborne spores, such as *Aspergillus* species.

DNA-based microbial detection assays, and in particular DNA microarray microbial detection assays, have reduced the detection of certain organisms from days or weeks to hours, while delivering the flexibility in screening multiple organisms in triplicate, and sensitivity to comply with a diverse range of microbial testing regulations. This allows for increased efficiency in throughput at **a fraction of the cost** to other molecular methods.

Although most states currently mandate a short list of target organisms, the ability to test hundreds of different primers and probes simultaneously for the same organisms provides increased specificity that is not matched by single-plex or multiplex qPCR reactions. In addition, as the pathogen list increases over time and more microbial hazards are identified, the increase in content is **easily managed**.

All DNA-based microbial detection assays begin by extracting microbial organisms from pre-homogenized cannabis material. This is executed through vigorous surface washing in an aqueous medium (e.g. buffered peptone water, Butterfield's solution, tryptic soy broth, or phosphate buffered saline). It is here where the DNA-based methods diverge. For the qPCR methods currently in use in cannabis testing, there is a period of enrichment, typically overnight, causing a delay in turnaround time and bias in terms of the microbial content. In the past, enrichment was commonly used for culture-based methods and was later incorporated into a number of molecular tests. However, experimental evidence indicates that enrichment can have bias either for or against the microbes of interest, and thus could provide a significant frequency of false negative results (James B Pettengill, 2012; John Dunbar, 1997).

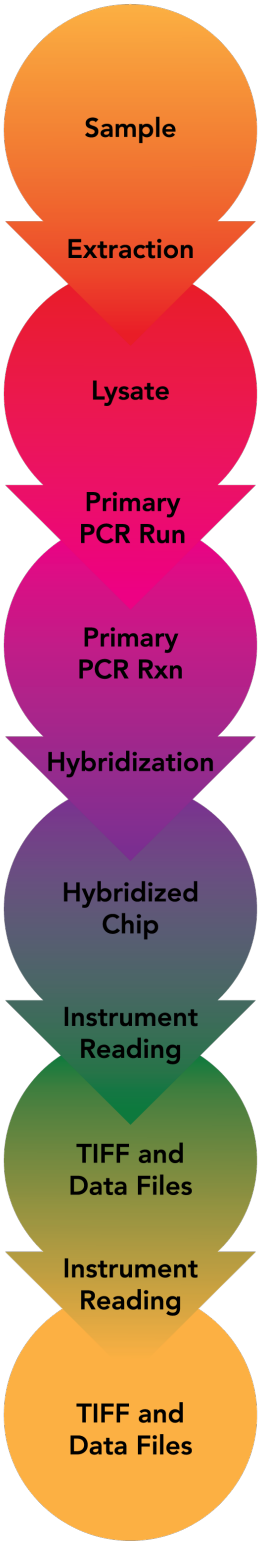


Figure 2: Process Pipeline for microbial testing using microarray

The DNA-based microarray methodology is designed to be performed without sample enrichment—thus eliminating enrichment bias. The sample (after surface washing only, or after washing and enrichment depending on state **regulations**) is then centrifuged to separate the cannabis material from the supernatant which is collected for DNA analysis.

For qPCR methods, genus-level detection for *Aspergillus* spp. is initially conducted and followed by separate reactions for detection of each species if the genus-level detection provides a positive result. This means that if any or all the samples show detection at the genus-level, each must then be rerun for species-level detection. The DNA microarray detection platform includes the required species level in each sample well; therefore, it reduces the time to meaningful results at the specific species level, so the test can be run on a first-pass. The results can be obtained more rapidly (in under 8 hours) using the DNA microarray approach. There is less opportunity for operator error, no need for sample retesting, and the approach is more conducive for a smoother high throughput flow. The microarray provides the necessary results during the first run, while the qPCR methods require that an additional qPCR run is performed for the species-specific reactions, which can interrupt the testing process flow for maximal throughput, and increase the cost per test.

The DNA-based microarray assays are configured to use a two-tiered PCR approach that allows for an initial loci enhancement PCR that targets a specific DNA region and a subsequent nested PCR reaction that introduces a fluorescence dye labeling of the resulting amplified DNA (Figure 2). The product of the two sequential PCR reactions is hybridized onto a matrix consisting of probes to specific organisms or genes without subsequent purification for microarray analysis. On a DNA microarray, the matrix is defined as series of spots consisting of a single type of DNA probe (synthetic single stranded DNA) that is complementary to the labeled PCR reaction product. Each matrix contains PCR positive controls, numerous negative controls, and the specific probes are printed in triplicate to provide greater confidence in the microarray results. In this microarray, 48 different nucleic acid probes, each printed in triplicate resulting in 144 probes per microarray, are printed as a 12 x 12 matrix—12 identical matrices per 1"x 3" glass slide—thus allowing 12 samples to be analyzed in parallel. The presence or absence of organisms is determined using relative fluorescence intensity (RFU) analysis of the pattern of spots formed as the unknown DNA binds to one or more of the 144 probes on the array, each being complementary to a microbial species.

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