

Optimized Cannabis Microbial Testing: Combined Use of Medicinal Genomics Extraction Methods With the AriaMx qPCR Instrument

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Introduction

Recent legislation legalizing the medicinal or recreational use of cannabis or cannabinoid products in the United States and Canada has led to tremendous growth in this industry. Providing consumers access to safe cannabis products is a priority, and in these jurisdictions, laws mandate testing for certain microbes.

The presence of bacteria and fungi in cannabis poses a potential threat to consumers if those microbes include pathogenic or toxigenic species. The current industry standard for detecting harmful microbes on cannabis flower is culture-based testing. However, the culture-based methods used in testing labs have never been validated for use on the cannabis plant. In fact, culture-based yeast and mold tests have shown false positives due to off-target bacterial species growth. Most alarming, *aspergillus*, the only microbe to ever be associated with harming consumers concerning cannabis, grows poorly in culture mediums and is severely underreported by current culture-based platforms. *Aspergillus*, *fusarium*, and many pathogenic bacteria are plant endophytes, and can only be thoroughly surveyed by lysing open plant cell walls. Culture-based assays demand intact and living organisms for the test to run properly, and cannot be used to survey the endophytes of the plant. This results in inaccuracy or failure to detect these pathogens through culture-based testing.

This application note highlights the shortcomings of culture-based methods borrowed from the food industry and the advantages of using quantitative polymerase chain reaction (qPCR) detection. qPCR is capable of accurately and rapidly identifying a wide spectrum of micro-organisms present on cannabis samples based on those micro-organisms' unique genetic signature or DNA sequence. Unlike food testing, cannabis testing has to consider various routes of administration beyond just oral administration. A successful testing method needs to detect micro-organisms in many different matrices, and be compatible with more than just cannabis flower.

What is qPCR?

Polymerase chain reaction (PCR) is a common medical research technique used to amplify a single copy or copies of a segment of DNA. Kerry Mullis invented PCR in 1988, for which he and his colleagues won the Nobel Prize for chemistry in 1993. PCR is extremely sensitive, requiring only a few DNA molecules in a single reaction for amplification across several orders of magnitude of detection. qPCR made its debut in 1993, and uses the linearity of DNA amplification to determine quantities of a known unique sequence in a DNA sample. Using a fluorescent probe reporter, it is possible to measure the amplification of a targeted DNA molecule during the PCR and see the amplification occurring in real time. For review, see Reference 1 and Figure 1 for one cycle of qPCR. <https://www.ncbi.nlm.nih.gov/probe/docs/techqpcr/>

Figure 2 describes how this increase in fluorescence signal translates into quantification. In brief, if a targeted DNA molecule is present, fluorescence will accumulate in the reaction tube until the signal reaches and exceeds a predetermined value in that tube. If more target DNA molecule is present, that signal accumulates and becomes visible before samples containing less of the target DNA. Figure 2 shows this in a plot of two different samples of high and low target levels (blue versus yellow line). The output value from this analysis is the fractional cycle number (C_q), at which the signal curve exceeds the predetermined value, known as the threshold (red line). Using this C_q relationship, we can estimate or, if compared to the result from a sample of known quantity, accurately determine absolute numbers of that target DNA. This QPCR result can then be converted to common microbial terms such as colony forming unit (CFU).

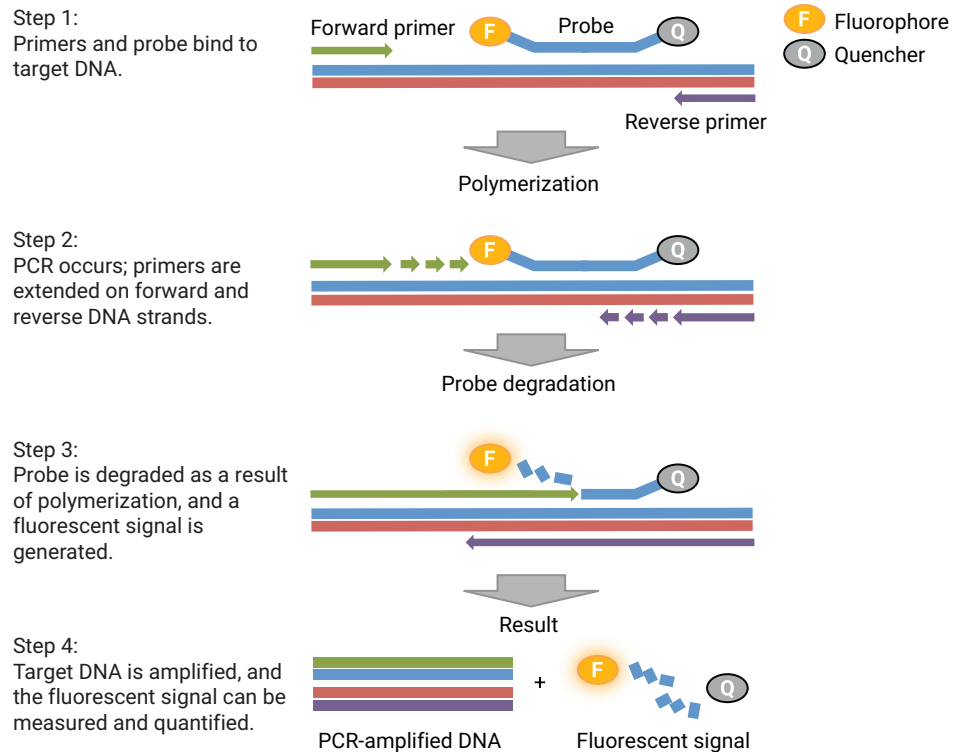


Figure 1. One cycle of qPCR.

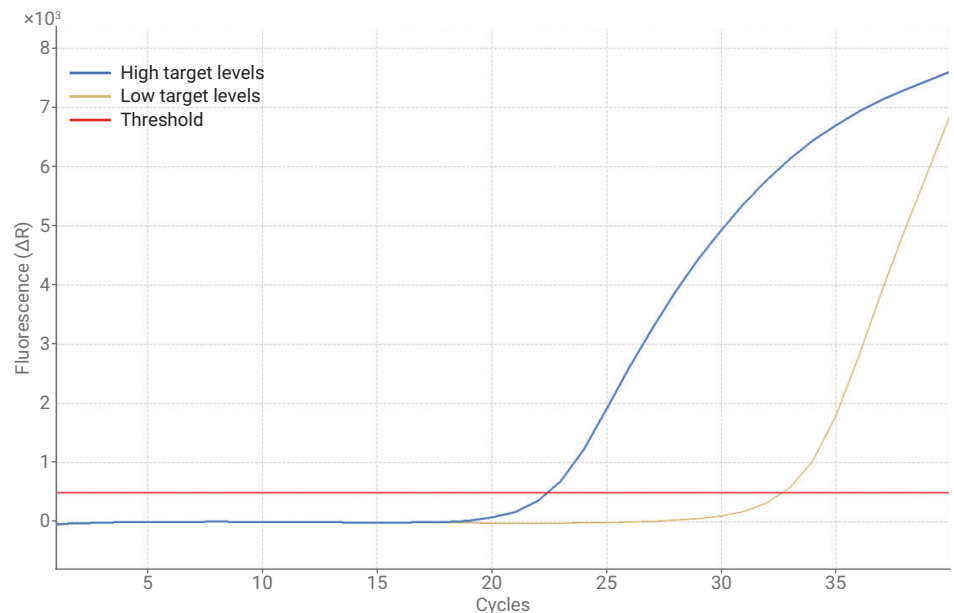


Figure 2. qPCR Amplification plots for high versus low target DNA levels. This plot shows the accumulation of fluorescence across 40 cycles of qPCR for a low titer micro-organism sample (yellow) and a higher titer sample (blue). The greater amount of micro-organism DNA in the blue sample will generate fluorescent signal earlier with fewer cycles, and achieves a pre-established value (red line) earlier. The point at which the sample signal crosses this threshold is the output for that sample, or quantitative cycle or C_q.

Of particular importance to PCR analysis is the design of primers and probes, the short DNA sequences that determine what part of the target DNA sequence will be amplified. Primers are designed to bind adjacently to the target sequence, and are specific to the target DNA such that a single DNA base difference can determine binding or not. This specificity is what makes qPCR such a useful tool for the detection of pathogens in cannabis since it can select for pathogenic strains and detect them, but ignores closely related strains². This reduces the frequency of false positives in pathogen detection, a frequent problem with current culture-based cannabis testing.



PCR requires that heat be applied to the tube containing the DNA, and the addition of amplification enzymes. Detection and quantification requires the PCR system to include a light source and a fluorescence detector. Agilent Technologies manufactures qPCR detection systems that have these capabilities. The Agilent AriaMx qPCR system accurately heats samples in a 96-well plate format, and scans for fluorescent signals using a specific light emitting diode (LED) and photodiode detection system.

Development and validation of reagents for the qPCR detection of common pathogens found on cannabis has been performed by Medicinal Genomics, Woburn Massachusetts. The complete platform for DNA extraction and detection, PathoSEEK, is fully validated on cannabis and related matrices. To ensure accurate setup and reduce

reagent contamination, each assay includes internal controls for the presence or absence of cannabis DNA, and positive and negative controls for each assay. As part of the protocol, a valuable PCR decontamination step ensures clean results with fewer false positives, and requires less dedicated lab space.

Table 1. Performance specifications of the Agilent Aria MX system.

Feature	Description
Excitation source	Eight dye-specific LEDs per optical module
Detection sources	Eight silicon photo-detectors per optical module
Probe dyes/LEDs available	SYBR/FAM 462.5–516.0 nm detectable Cq for 0.001 ng DNA per reaction HEX 535.0–555.0 nm detectable Cq for 0.001 ng DNA per reaction ROX 585.0–610.0 nm detectable Cq for 0.001 ng DNA per reaction CY3 542.0–568.5 nm detectable Cq for 0.001 ng DNA per reaction CY5 635.0–665.0 nm detectable Cq for 0.001 ng DNA per reaction ATTO425 435.0–475.0 nm detectable Cq for 0.001 ng DNA per reaction
Reaction volume	10 µL to 30 µL
System temperature range	25.0–99.9 °C Heating: 6 °C/sec Cooling: 2.5 °C/sec Accuracy: ±0.2 °C
Dynamic range	Nine orders of magnitude
Multiplex	Five channels
Temperature uniformity	±0.4 °C
Data acquisition time	Three-second scanning time for data acquisition in all six channels
Electrical power (input)	100–240 VAC, 50/60Hz, 1,100 A
Operating environment	20 to 30 °C, 20 to 80 % noncondensing humidity, 7,500 feet max altitude
Weight	50 lbs (23 kg)
Dimensions	19.7" W × 18.1" D × 16.5" H (50 cm × 46 cm × 42 cm)
Sample containers	0.2 mL tubes, 96-well plates, strip tubes

Assay workflow

The Medicinal Genomics PathoSEEK series of assays detects all of the pathogenic organisms required by many state cannabis agencies and Canada. DNA must first be extracted from plant cells and microbial cells that may be present on the cannabis being tested. To simplify this process, Medicinal Genomics developed a magnetic bead-based extraction kit named SenSATIVax. In brief, the cannabis flower, leaf, or marijuana-infused product is homogenized and, if necessary, allowed to culture in a growth medium, which will generate more pathogens, indicating the presence of live bacteria on the cannabis product. This culture medium is then subjected to a DNA extraction, followed by an optional decontamination reaction, which will rid the sample of any previously amplified DNA. This sample is then used as a template for the PathoSEEK assay. Detection of many of these pathogens is done in multiplex. This means that in a single reaction, two to four microbes are targeted. The presence of cannabis DNA and microbial contamination is based on the sample amplification curve achieving an assay-specific fluorescent value within a predetermined number of PCR cycles. Additionally, assay positive and negative controls show evidence and absence of amplification, respectively.

Experimental comparison of methods

A set of 15 medicinal cannabis samples was analyzed using PathoSEEK qPCR and two commercially available culture-based methods. To enumerate the bacteria and fungi present before and after growth on culture-based media, all samples were then subjected to next-generation sequencing and metagenomics analysis.

While culture-based methods have been in use for over 100 years, publications continue to remind us that less than 5 % of the microbial species are culturable^{4,5}. Molecular methods often leverage amplification of ribosomal DNA, internal transcribed spacers, or ITS regions^{3,6}. As a result, these PCR products can detect unculturable organisms and organisms that clump and distort CFU/g enumeration such as *aspergillus* species (Figure 5).

Aspergillus demonstrates log scales lower growth at room temperature than most other yeast. The *Expected* value from Figure 5 is the inferred CFU count converted from the Cq measurement using the formula:

$$\text{CFU/g} = 10^{[(42.185 - \text{Cq Value})/3.691]}$$

Using this conversion to CFU from a qPCR-derived Cq value, we show the discrepancy and underreporting of the *aspergillus* by culture-plating methods.

Results and discussion

Metagenomic sequencing data were collected on 15 samples, directly from plant material and after culture on both the 3M Petrifilm and Biomérieux platforms³. The sequencing results demonstrate substantial shifts in the presence and abundance of bacterial and fungal species after growth on the two platforms. Thus, both culture-based platforms are detecting and enumerating only a subset of the species present, and the final composition of microbes after growth is markedly different from the starting sample. Most concerning is the frequent identification of bacterial species in systems designed for the exclusive quantification of yeast and mold, as quantified by elevated total aerobic count (TAC) Cq values after culture in the BMX total yeast and mold (TYM) medium. These observations call into question the specificity claims of these culture-based testing platforms. The presence of bacterial colonies on TYM growth plates or cards may falsely increase the rejection rate of cannabis samples for fungal contamination.

Perhaps the most concerning observation is that one of the most dangerous fungal pathogens that may be found in cannabis products, *aspergillus*, grows poorly, and is severely underreported by current culture-based platforms. The differential growth of other toxigenic fungi, depending on the companion species present, further influences the results. Bacterial pathogens are not uncommon, and beneficial bacteria are also capable of influencing the growth or inhibition of other flora.



Figure 3. Assay workflow. DNA decontamination means use of a restriction enzyme to digest the potential contaminant amplicon DNA from a previous qPCR. For more detail on this method see <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4008621/>.

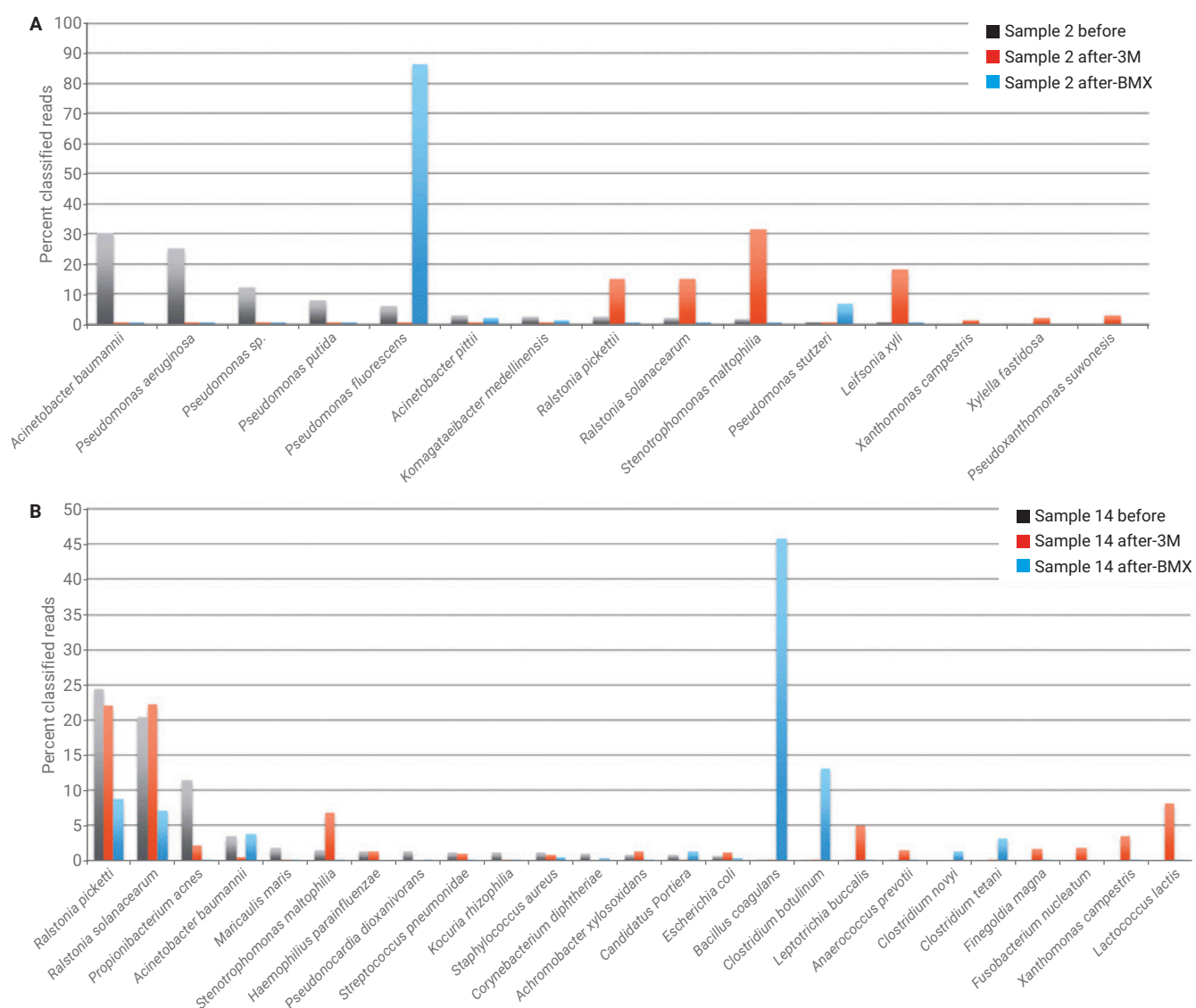


Figure 4. Genomic profiles of before and after culturing. Comparison of classified read percentages for bacterial 16S DNA on samples 2 and 14, before and after culturing on 3M and BMX media. The results represent all species observed down to 1 % of classified reads. Large shifts in species prevalence are seen after growth on the two culture-based platforms.

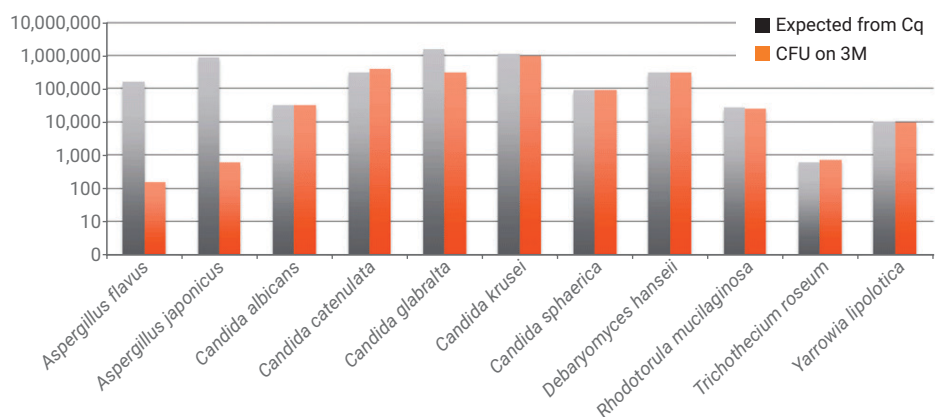


Figure 5. Comparative growth of *aspergillus* species and other fungi on 3M Petrifilm.

Conclusion

The AriaMX real-time qPCR instrument with Medicinal Genomics SenSATIVax and PathogINDICATOR extraction and qPCR reagent kits provide an optimal assay for fast, accurate, and scalable microbial testing on cannabis flower or cannabinoid products.

References

1. For PCR and Quantitative PCR review, <https://www.ncbi.nlm.nih.gov/probe/docs/techqpcr/>
2. <https://www.medicinalgenomics.com/dna-based-testing-can-distinguish-aspergillus-brasilensis-aspergillus-niger/>
3. McKernan, K.; *et al.* Metagenomic analysis of medicinal Cannabis samples; pathogenic bacteria, toxigenic fungi, and beneficial microbes grow in culture-based yeast and mold tests, *F1000 Research*.
4. de Bekker, C.; *et al.* Heterogeneity of *Aspergillus niger* microcolonies in liquid shaken cultures. *Applied and Environmental Microbiology* **2011** Feb, 77(4), 1263-7. PubMed PMID: 21169437. Pubmed Central PMCID: 3067247.
5. Fujikawa, H.; Itoh, T. Tailing of thermal inactivation curve of *Aspergillus niger* spores. *Applied and Environmental Microbiology* **1996** Oct, 62(10), 3745-9. PubMed PMID: 8837430. Pubmed Central PMCID: 168182
6. McKernan, K.; *et al.* Cannabis microbiome sequencing reveals several mycotoxic fungi native to dispensary grade Cannabis flowers. *F1000 Research*. **2015**, 4, 1422. PubMed PMID: 27303623. Pubmed Central PMCID: 4897766.

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