Microbiological examination of nonsterile Cannabis products: Molecular Microbial Enumeration Tests and the limitation of Colony Forming Units.

Kevin McKernan, Yvonne Helbert, Heather Ebling, Adam Cox, Liam T. Kane, Lei Zhang
Medicinal Genomics, Woburn MA

Cannabis microbial testing presents unique challenges. Unlike food testing, cannabis testing has to consider various routes of administration beyond just oral administration. Cannabis flowers produce high concentrations of antimicrobial cannabinoids and terpenoids and thus represent a different matrix than traditional foods [1, 2]. In 2018, it is estimated that 50% of cannabis is consumed via vaporizing or smoking oils and flowers while the other half is consumed in Marijuana Infused Products or MIPs. There are also transdermal patches, salves and suppositories that all present different microbial considerations.

Several recent publications have surveyed cannabis flower microbiological communities[3-5]. These have detected several concerning genus and species such as *Aspergillus niger, Aspergillus fumigatus, Aspergillus flavus, Aspergillus terreus, Penicillium paxilli* and *Penicillium citrinum, Clostridium botulinum, Eschericia coli, Salmonella* and *Staphyloccus*. There are several documented cannabis complications and even fatalities due to Aspergillosis in immuno-compromised patients[6-18]. A recent paper even demonstrates a case of cannabis derived Aspergillosis in an immune competent patient[19].

It is unknown to what extent *Aspergillus* produces mycotoxins in cannabis and to what extent those toxins enrich in the cannabis extraction process. Llewellyn *et al* . published laboratory settings where to 8.7ug/g of aflatoxin could be produce with inoculated "marihuana" but the work was performed in 1977 and still leaves many questions regarding if this can occur in the wild [20]. It is also unknown if *Clostridium botulinum* produces botulinum toxin in cannabis oils. *E.coli* or *Salmonella* food poisoning events are likely to trigger febrile seizures in many epileptic patients relying on Cannabis oils[21]. While mycotoxin producing *P.citrinum* and *P.paxilli* DNA have been detected in cannabis flowers, it is unknown to what extent these nanomolar, lipid soluble compounds are even expressed on cannabis flowers. Cannabinoids are often consumed in micromolar concentrations implying even rare contaminants should be considered. Paxilline in particular is responsible for Ryegrass staggers in livestock and in theory may complicate many anti-epileptic properties of cannabidiol[22].

Grain silos of cannabis?

There is currently no published cannabis failure rate for mycotoxin testing presumably due to the fast turn over and short shelf duration of cannabis flowers. Mycotoxin production is usually a saprophytic state found in agricultural grain silos where longer term crop storage is more common place[23].

While long saprophytic storage conditions in cannabis are rare today, there is no reason to assume there will not be such things in the future given the crops utility and nutritional value. As production increases with continued cannabis normalization, we anticipate commercial agricultural practices will move into the market to leverage current infrastructure and economies of scale.

The recent popularity of vaporizing oils presents many unanswered questions regarding *Aspergillus* spore viability and mycotoxin concentration in current cannabinoid extraction systems[24, 25].

One must also recognize that strict and nonspecific microbial regulations can eliminate the use of beneficial microorganisms in agriculture and deliver unforeseen consequences in the marketplace. Mandated laboratory testing for non-specific total yeast and mold petri-dish assays (Total Yeast and Mold (TYM)) will fail commonly used chitinase (anti-fungal) producing microorganisms like *Trichoderma hazarium. Tricoderma hazarium* is known to reduce Aspergillus growth[26, 27]. These false TYM failures can induce growers to use less biological fungicides and more chemical fungicides. These chemical fungicides are known to concentrate preferentially over cannabinoids in some extraction methods[28]. Anti-fungal beneficial microbes that are harmless to human health are far easier to sterilize than small molecule fungicides like myclobutanil. Myclobutanil converts into hydrogen cyanide under many smoking and vaporization conditions and is now a common contaminant in cannabis[29].

To further complicate microbial testing, many growers are under the impression that cannabis extraction techniques redeem microbial contaminated cannabis products. There is no peer-reviewed literature on the survival of lipophilic *Clostridium* or *Aspergillus* spores and mycotoxins in cannabis extraction techniques. Many pesticides, insecticides and fungicides are known to concentrate with cannabis extraction[30]. Little is known if mycotoxins also concentrate in cannabis extraction. Due to these concerns the Denver Department of Environmental Health issued a *C.botulinum* warning in 2017[31].

Culture based methods versus molecular methods

While culture based methods have been in use for over 100 years, a century later publications continue to remind us that less than 5% of the microbial species are culturable[32, 33]. Molecular methods often leverage amplification of rDNA internal transcribed spacers or ITS regions[3, 4]. As a result, these PCR products can detect unculturable organisms and organisms that clump and distort CFU/g enumeration (Figure 1 and Figure 2). This clumping artifact is referred to as heterogeneous macrocolonies and is known to occur with Aspergillus[34]. This creates significant quantification issues for states requiring single CFU/g failure thresholds as the clumping creates sampling bias. There is little resolution between 0 and 1000 colonies with Aspergillus plated on SabDex , Potato Dextrose agar or 3M TYM films. In addition, Aspergillus are fairly ubiquitous in the environment and not all Aspergillus species are harmful. As a result, many states (CA, AK, NV) mandate *A. niger, A. flavus, A. fumigatus* and *A. terreus* testing. These species synthesize mycotoxins and pasteurization resistant pathogenic spores[35]. These can be difficult to discern with plating methods while molecular methods can easily itemize them. A recent case in Alaska had a lab using agar mistake benign *Aspergillus brasiliensis* with *Aspergilllus niger*[36]. The lab using culture ultimately closed its doors citing banking issues. *C.botulinum* also produces pasteurization resistant spores and is an anaerobe that is incompatible with aerobic plate counts[37].

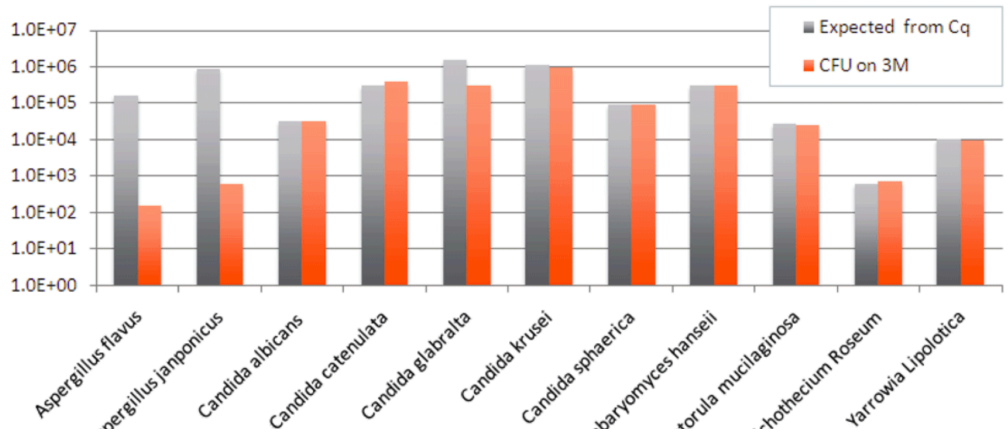


Figure 1. Reprinted from McKernan *et al*. CFU/g correlations with qPCR Cq is fairly consistent with ATCC fungi that do not form heterogeneous macro-colonies. *Aspergillus* clumps and creates sampling bias during

plating effectively limiting the dynamic range of the assay and complicating single CFU/g accuracy. Note the large discordance between estimated genomic copy numbers with *Aspergillus* and colonies detected with 3M.

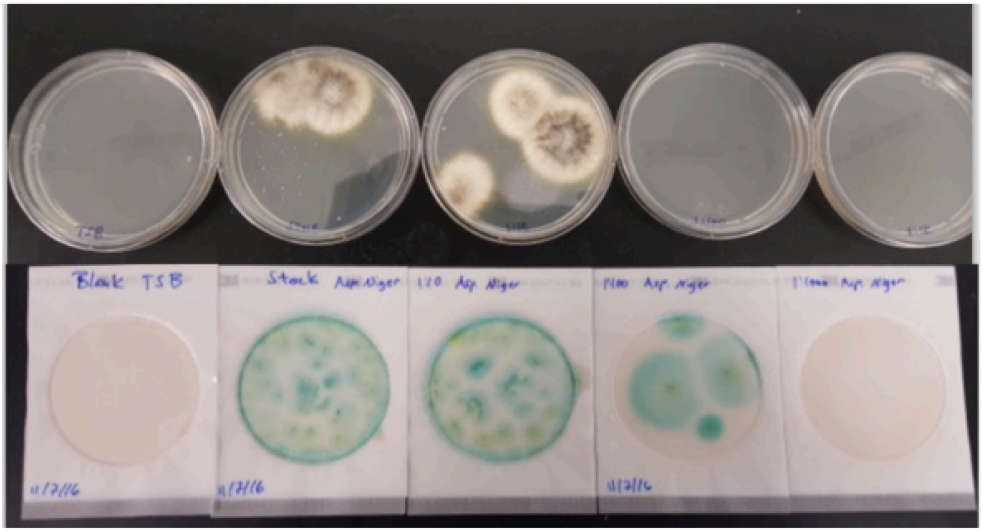


Figure 2. *Aspergillus niger* plated on 3M Petrifilm® and SabDex agar. Enlarged colonies are the result of 100-1000 conidia clumped into a heterogeneous macro-colony.

To properly calibrate single CFU/g Aspergillus sensitivity with qPCR, declumping of the conidia is required. This can be achieved by harvesting conidia in 1% Tween-80. Thorough vortexing and filtration through a 5um spin column delivers individual, declumped conidia (Figure 2B). While this filtration process is helpful for single CFU quantitation and calibration it is impractical to apply to real cannabis samples due to the significant loss in yield seen in the filtration process. This would fail to properly measure the full risk profile on cannabis. Nevertheless, the process does enable the validation of single CFU/g sensitivity.

Once spores are declumped and easy to count, it is important to demonstrate that the lysis conditions can in fact lyse the spores. One can quantitate this with a hemocytometer spore count coupled with qPCR of solutions that have spores with and without lysis buffer.

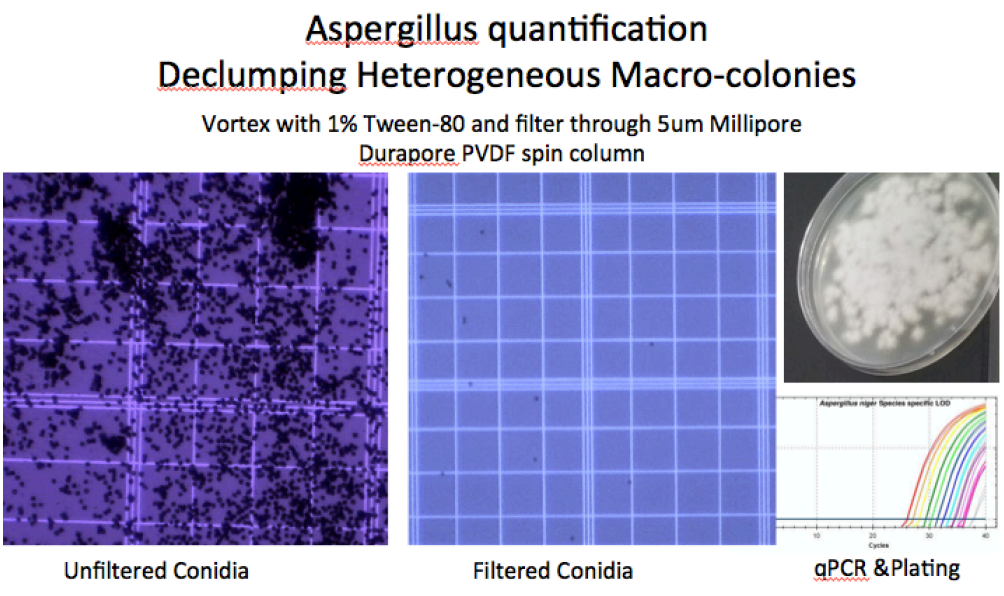


Figure 2B. *Aspergillus niger* Heterogenous macrocolonies can be declumped by vortexing in surfactants like Tween-80 and filtered for 5um single spores. This enables proper enumeration on plating to bring higher qPCR concordance with Aspergillus CFU/g. This is not advised for routine cannabis safety testing due to the loss in yield via

A commonly cited but incorrect limitation to molecular methods is that they cannot inform microbial viability or discern live versus dead DNA. As a result, there is misguided concern that molecular methods will penalize growers that properly sterilize their cannabis. There are several published methods describing viability PCR[38, 39] but the simplest

method suggested by the EPA is to simply retest (qPCR) all failed samples 24 hours later after incubation in a growth media[40]. Since this is limited to the samples that fail immediate testing, re-testing 24 hours later in TSB is restricted to fewer samples and not as cumbersome. Any sample that has viable cells or partially sterilized material will grow and demonstrate an increase in DNA concentration 24 hours later. It is important to underscore that this method is constrained to those organisms that *can culture* in TSB in a given time frame and temperature. Nevertheless, qPCR delivers higher genus and species specificity than culture-based methods.

In addition to being able to discern live from dead organisms, qPCR assays also have a lysis and DNA purification step. This step de-clumps macro-colonies and provides better sampling with *Aspergillus* while also removing any cannabinoids, terpenoids, sugars and other inhibitors from the assay that might interfere with plate count chemistry or polymerase chemistry. This is particularly important with the diverse matrices found in MIPs which have been shown to interfere with culture conditions[41].

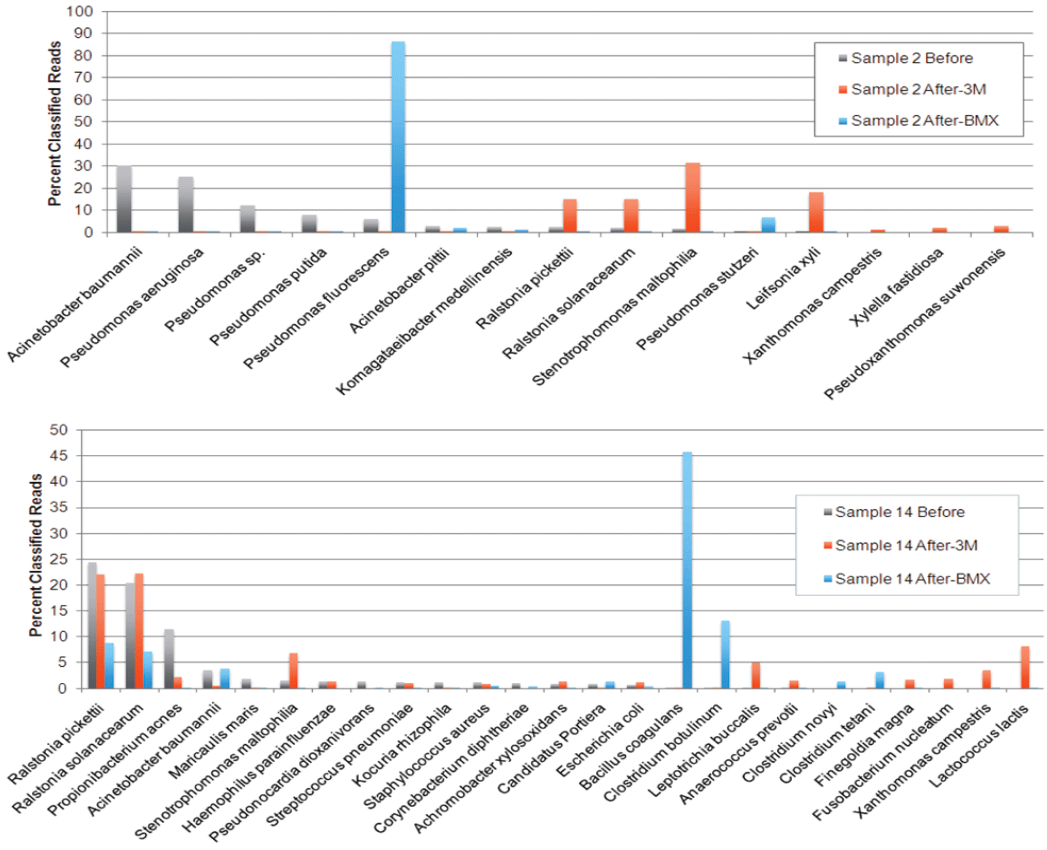


Figure 3A- Comparison of classified read percentages for bacterial 16S DNA on samples 2 and 14, before and after culturing on 3M and Biomerieux TEMPO® (BMX) media. The results represent all species observed down to 1% of classified reads. Large shifts in species prevalence are seen after growth on the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

Live versus Dead is not a binary debate

Beuchat *et al.* demonstrate the importance of varying nutrients, pH, and water activity required for resuscitation of stressed or partially viable cells[42]. Dried banana chips required 120 hours to fully resuscitate organisms in 5 different culture mediums. The dehydration is believed to leave the cells and spores in a partially viable state. Cannabis is also dried. 120 hours is much longer than most culture based techniques being utilized in the cannabis industry (48-60 hours) thus challenging the validity of any viability benefit from short duration plating. The five days required to capture these partially viable cells leaves one to question what will then be growing on the cannabis being tested that many days later. Measuring the entire DNA load on the plant can be done in under 5 hours. The

pursuit of viability has also lead to gentle homogenization techniques that likely fail to liberate endophytes. Fusarium is a pathogenic endophyte. Molecular methods tend to lyse cells and more aggressively liberate endophytes and declump filamentous fungi. Molecular techniques often utilize liberated cannabis DNA as an internal DNA purification control demonstrating that the lysis buffer is capable of dissolving plant walls and any respective endophytes. Thus the pursuit of viability not only limits the sensitivity of the assay but also provides a false sense of safety when it fails to address dried goods.

Clinical use of qPCR for Aspergillus in blood has been met with some resistance due to poor standardization.

White *et al*. describe the utilization of qPCR for Aspergillus detection in clinical blood isolates and how these techniques have failed to pass clinical milestones by the European Organization for Research and Treatment of Cancer and the Mycosis Study Group in 2006 (EORTC/MSG)[43]. Much of this resistance was related to the lack of commercially available kits with proper positive and internal controls. Many labs were home brewing their own LDT tests and most of the discordance was attributed to DNA extraction procedures eluting DNA in over 100ul, lysing less than 3ml of blood and the lack of internal controls to identify failed PCR reactions.

It is important to note that blood detection of Invasive Aspergillus (IA) has a Beta-D-Glucan assay that is currently FDA approved and thus the threshold for qPCR adoption is much higher[44]. As of 2015 these methods have reached equivalence and are being considered for inclusion into the EORTC/MSG[45]. It is not clear the blood detection debate is very informative to the cannabis industry. The gold standard Beta-D-Glucan (Fungitell®) assays used in blood are unlikely to be relevant in food due to high levels of naturally occurring Beta-D-Glucans in various non blood born and off target fungi and plants[46]. As a result these methods are not published for use in the food industry.

Microbiomes from before and after culturing are highly discordant

Cannabis ITS sequencing of microbes that existed before and after culturing on 3M Petrifilm® and Biomerieux TEMPO® culturing devices have demonstrated very low specificity and drastic differences in a species prevalence from before culture to after culture[3]. This implies the two different methods of culturing are differentially altering the initial risk profile while also suffering from specificity. This is not a surprise given the TEMPO® YM system uses chloramphenicol while the 3M system does not. As much as 60% of the DNA isolated from these TYM 3M Petrifilm® assays and the Biomerieux TEMPO® YM cartridges were bacteria. Many were known to be chloramphenicol resistant. Bacteria also tend to have faster doubling times (30minutes compared to 2 or more hours for yeast and molds) suggesting bacteria may form a saturated culture in the first 24 hours while the yeast continues to grow for 72 hours.

Sequencing of these off target bacteria often reveals *Bacillus* species know to produce lactic acid or Pseudomonas species known to produce salicylic acid. The degree to which these contaminating species alter the yeast and mold enumeration is unknown. The Biomerieux TEMPO® platform utilizes a pH sensitive fluorescent dye to monitor the growth curve. A decrease in pH will decrease fluorescence and infer higher yeast and mold counts. It is assumed that these contaminating acid producing bacteria are decreasing the pH or altering the fluorescence and falsely elevating the yeast and mold counts. *Bacillus* is commonly used in the cannabis industry as a foliar spray known as Serenade ®. Likewise, molds may produce acidic compounds at varying rates. *Penicillium citrinum*

produces ACC and Tanzawaic acids[47]. It is unclear to what extent this expression affects pH in culture and how specific pH is as a proxy for CFU/g in a mixed microbiome growth environment.

To test this theory we sent a chloramphenicol resistant, salicyclic acid producing *Pseudomonas aeruginosa* culture to two testing laboratories using the TEMPO® system and received failing TYM (9x10^4) counts and cloudy cartridge images with passing BTGN results. This implies chloramphenicol resistant bacteria that can alter the pH of the broth can create false positive Yeast and Mold tests with this platform. Pseudomonas should trigger a BTGN according to the USP.

Figure 3B- Bacterial growth in common TYM assays. *Pseudomonas aeruginosa* ATCC#10145 plated on 3M TYM Petri-film and in Biomerieux TEMPO YM Cartridge. *Pseudomonas aeruginosa* is chloramphenicol resistant and produces salicylic acid. TEMPO detection is based on a pH sensitive fluorescent dye.

Pseudomonas aeruginosa is responsible for 11-14% of hospital acquired infections and is particularly problematic with Cystic Fibrosis patients[48]. CF patients are experimenting with cannabinoid use for the reduction of fibrosis. Corbus Pharmaceuticals is pursuing modified cannabinoids for CF patients in FDA trials. 100,000 CFU/g of TAC are allowable but only 1,000 CFU/g are allowable for BTGN. Any lab using only Biomerieux will be vulnerable to passing samples with 1001- 99,999 CFU/g of Pseudomonas.

Certificate ID: 30518 Received: 5/4/2018
Client Sample ID: PA-250CAMP
Lot Number:
Matrix: Tincture - Microbe Liquid

Medicinal Genomics
12 Gill St., Suite 3700
Woburn, MA 01801
Attn: Yvonne Helbert

Authorization:	Signature:	Date:
Matthew Silva, Chemical Engineer		5/8/2018

PJLA Testing
Accreditation
80585

The data contained within this report was collected in accordance with the requirements of ISO/IEC17025:2005. I attest that the information contained within the report has been reviewed for accuracy and checked against the quality control requirements for each method. These results relate only to the test article listed in this report. Reports may not be reproduced except in their entirety.

MB1: Microbiological Contaminants [WI-10-09] *Analyst: MS* *Test Date: 5/4/2018*

This test method was performed in accordance with the requirements of ISO/IEC 17025. These results relate only to the test article listed in this report. Reports may not be reproduced except in their entirety.

30518-MB1

Symbol	Analysis	Results	Units	Limits*	Status
AC	Total Aerobic Bacterial Count	>490,000	CFU/g	100,000 CFU/g	FAIL
CC	Total Coliform Bacterial Count	<100	CFU/g	1,000 CFU/g	PASS
EB	Total Bile Tolerant Gram Negative Count	<100	CFU/g	1,000 CFU/g	PASS
YM	Total Yeast & Mold	=110,000	CFU/g	10,000 CFU/g	FAIL

Note: All recorded Microbiological tests are within the established limits.

Figure 3C. Pseudomonas aeruginosa grown in CAMP fails Biomerieux YM assay and AC assay. It does not trigger the EB failure. It is a Bile Tolerant Gram Negative bacteria and should fail that test. Hardy Diagnostic plates deliver different results. Both Pass Pseudomonas.

One must also be mindful that many foliar sprays used to combat yeast and molds utilize hydrogen peroxide or vinegar and may affect the pH of cannabis tested. It would be preferable to have a tool that measures the microbes more specifically than one that is exposed to the pH impact of the multitude of additives one might find on Cannabis.

Several studies have also found spices and other food products can alter the UV fluorescence chemistry of the TEMPO® YM platform and thus will require deeper dilutions to get accurate quantitation[49, 50]. Kunika demonstrates 5 out of 9 spiced foods created such interference on the TEMPO® YM platform (onion grits, cinnamon, yeast extract, ground caraway, beetroot juice concentrate) while Owen *et al.* demonstrate peppercorn interference. Onions, caraway, cinnamon and peppercorns all produce Beta Caryophyllene, a sesquiterpenoid also found in high concentrations in some cannabis cultivars. Cannabis is more likely to fall into the category of a spice given the potent expression of various acidic cannabinoids and terpenoids. Other molecular studies have also found that background off-target microbiota produce discordant results in culture based *E.coli* enumeration compared to qPCR[51].

	BMX	3M
18S Primers w/ 16S hits	520	334
18S Primers w/18S hits	194,986	174,320
% off target	0.266685813	0.191601652
16S Primers with 16S hits	2,900,912	606,942
16S Primers with 18S hits	114,646	204
% off target	3.952067488	0.033611119

Figure 4: BLAST analysis of the organisms found via sequencing the ITS PCR products obtained from Biomerieux TEMPO® YM cartridges (BMX) and 3M Petrifilm® YM plates. 18S primers deliver over 99.7% fungal sequences. 16S primers deliver over 96% bacterial sequences. Off target 16S sequences were predominantly 16S fungal mitochondrial sequence.

PCR based methods described by McKernan *et. al* demonstrated over 96% specificity with the limited discordance being derived from fungal mitochondrial 16S sequence being misclassified as bacterial DNA[3]. PCR also offers the capacity to survey the microbial community on the flower sample without exposing it to a new carbon source or selective bias. This capacity to

accurately quantitate *Aspergillus* and other non-culturable fungi is turning out to be imperative for the cannabis industry.

Given these published warnings, and observed concerns over contaminating species or compounds from spices, a robust validated study of cannabis in culture is required before general food guidelines are assumed to have adequate inclusion and exclusion criteria.

ITS amplification considerations

Many molds produce mycelium that have multiple nuclei per cell. While this may seem problematic for molecular counting, fragmented mycelia are culturable[52-54]. Given molds are generally a mixture of conidia and mycelium (and homogenization techniques partially fragment these structures), we believe it is best to measure the number of infective nuclei as opposed to number of cells in a given Yeast and Mold test. When counting nuclei, one must be aware of the approximate copy number of your DNA target per nuclei.

ITS copy number can vary between organisms. ITS regions can have 20-100 copies in fungi[55]. This can be both a benefit and a curse. While having more than one target per genome can enable sub-CFU sensitivity and reduce digital PCR artifacts, it can complicate CFU calculations. These ITS copy number variations are usually less than a log scale in variation[56]. The variation we see with single spore forming CFUs and spore clumping into heterogeneous macrocolonies can create 3 log scales of variance[3, 34, 57, 58]. Filamentous fungi clumping has created rigorous standards bodies to develop methods that ensure inter-laboratory consistency quantitating these organisms[59]. These methods are amenable to monocultures studying anti-fungal compounds but cannot be performed on wild microbiome samples due to the careful pipetting and centrifugation that is required to isolate homogenous spore suspensions[59]. ITS copy number is nevertheless important to keep in consideration as you compared total genomic copy numbers to ITS Cq and CFU.

Another benefit of ITS targets is that these are the first regions of the genome to replicate in viable cells and as a result have been demonstrated to assist in viability PCR[38, 39]. While single copy regions can be designed, these are harder to find conserved universally in all Fungi. Single copy targets also suffer from digital PCR artifacts at low dilutions.

Implications of endofungal toxigenic bacteria.

Many filamentous fungi are hosts for toxigenic bacteria. Ralstonia is a plant and human pathogenic bacteria found in cannabis and is known to infect many fungi found on Cannabis[60, 61]. Several clinical reports of Ralstonia infections in exist in Cancer patients, Cystic Fibrosis patients and other immune-compromised patients[62-64]. It is unlikely that bacteria internal to a fungi will produce colonies on bacterial petri dishes. Likewise, samples with low fungal counts on TYM petri dishes could still contain high levels of toxigenic bacteria. Ralstonia further complicates quantitating molds as it induces the formation of chlamydospores in many filamentous fungi[60]. Chlamydospores are multicellular, asexual spore clumps that are induced for environmental survival. These are difficult to homogenize and create similar quantitation problems described earlier with Aspergillus.

Rhizopus is an example of a mold known to be a host for the toxigenic endofungal symbiote *Burkholderia-Rhizopus*[65]. *Rhizopus microsporus* is known to be involved in fatal fungal infections affecting immune-compromised patients[66]. Rhizoxin is required for Rhizopus pathogenicity and can induce liver failure[67].

Powdery Mildew, Botrytis and sterilization.
Botrytis cinerea and powdery mildew are the two most common fungal plant pathogens found on Cannabis. Neither of these culture on 48-hour agar lawns or Petrifilms®. Culture conditions often require specialized media and 2-6 weeks of growth[68-71]. Some species of powdery mildew can be cultured on other plant tissue-based medias but this has not been validated on cannabis derived powdery mildew(CDPM) and the definition of a colony forming unit is in question. CDPM has recently been sequenced and inoculation on cannabis plant and is believed to be a Golovinomyces genus[72].

Powdery mildews are known to be obligate biotrophs that often speciate to grow on a single hosts species[73]. Culturing of CDPM may have to be performed on Cannabis leaf based medias making for a complicated product that cannot ship across state lines. There is little to no evidence in the clinical literature regarding human health risks with inhalation of powdery mildew, however litigation regarding an employee allergic reaction during prolonged cannabis trimming is ongoing in Massachusetts[74].

Botrytis is responsible for cannabis bud-rot. It is often used in wine production and known as Noble rot. This implies it is safe to consume orally however, *Botrytis cinerea* can also produce allergic reactions[75]. The CDC has published ITS sequencing work demonstrating high Botrytis spore exposure with cannabis trimming environments[76]. These common plant fungi are not being accurately quantified by the current CFU/g based regulations and we believe molecular methods will be the only mechanisms to do so.

To address this gap, Medicinal Genomics sequenced the genome of cannabis-derived powdery mildew. Its' ITS sequence is 98% identical to *P.macularis* and *Golovinomyces ambrosiae*. Pepin *et al*. have since published a *Golovinomyces chichoracearum* powdery mildew on Canadian cannabis[72]. With this information, we developed a PCR based assay for this species[77]. Validation of this assay via correlation to CFU/g metrics requires development of CDPM culturing conditions that produce enumerable colonies or calibration to conidia counts on a hemocytometer. Medicinal Genomics is also developing qPCR assays for *Botrytis cinerea.*

Cryptococcus
In 2018, Shapiro *et al.* published a case study of "a daily cannabis smoker without evidence of immunodeficiency presenting with confirmed *Cryptococcus neoformans* meningitis". Further ITS sequencing of the patients cannabis samples revealed several varieties of *Cryptococcus* species[78]. *Cryptococcus* is a slow growing mold that also requires specialized culture conditions[79]. There are benign forms of *Cryptococcus* so speciation is critical and difficult to achieve with culture alone. Pathogenic species include *C. neoformans, C.gatti*. Both species are addressable with ITS PCR. Peripheral Crytococcosis infections are usually treatable with fluconazole while CNS infections require intravenous amphotericin B.

Fusarium oxysporum and other organisms of concern
Many other organisms have been reported on Cannabis that present culturing problems. *Fusarium oxysporum* has been reported to cause fusariosis. Hundreds of cases of Fusariosis have been described in the literature[80-84]. Several cannabis derived cases are described[85]. *Fusarium* is also quite ubiquitous and mostly a risk for immuno-compromised patients but has a very high fatality rate

when acquired. This is of significant concern given *Fusarium oxysporum* is one of the more widely used biocontrol agents for cannabis eradication programs[86].

Staphylococcus aureus* and *Pseudomonas aeruginosa have also been listed as organisms of concern in some jurisdictions. The Pseudomonas genera is fairly ubiquitous and infects both humans and plants. Many species are benign and used as bio-control agents to limit harmful fungi. Speciation of harmful versus beneficial Pseudomonas is critical[87-90]. *Pseudomonas aeruginosa* is often a problem for burn victims and Cystic Fibrosis patients. Cannabinoids are popular amongst cystic fibrosis patients but it is unknown if such patients could or would want to resort to inhalation based delivery [91-93]. Corbus Pharmaceuticals has been advancing modified cannabinoids through the FDA to treat Cystic Fibrosis and other fibrotic diseases. Given the likely use of Cannabis by Cystic Fibrosis patients, *Pseudomonas aeruginosis* screening is a valid concern. Despite being a bacteria, Pseudomonas also grows on 3M TYM Petrifilm® and in Biomerieux TEMPO® Yeast and Mold cartridges. *Pseudomonas aeruginosa* is chloramphenicol resistant and known to produce Salicylic acid that can interfere with pH based fungal detection [94].

Staphylococcus aureus is always a health concern regarding antibiotic resistance and MRSA (methicillin resistant *Staphylococcus aureus).* Nevertheless, cannabinoids are proving to be some of the more promising anti-MRSA compounds[1, 95, 96]. Distinguishing MRSA from benign human skin-commensals like Coagulase-negative staphylococci (CoNS) is challenging without molecular techniques[97].

Other mycotoxin producing fungi have been recorded on Cannabis such as *P.citrinum* and *P.paxilli.* The mycotoxin production of these species on cannabis has not been quantitated to date but their potency is nanomolar compared to the micromolar usage of cannabinoids. Low level contamination of these lipid soluble mycotoxins is an unknown risk but published literature on their interference with cannabidiol exists in rats suggesting caution. In the most extreme cases fungal mycotoxins are classified as weapons[98].

Mycobacterium tuberculosis

An Australian study found 29 people infected by the same strain of *M.tuberculosis* believed to be spread from sharing the same bong[99]. Additional *M.tuberculosis* reports in Seattle WA were discovered in 2004[100]. While cannabis paraphernalia may be a vector for certain human pathogens, there is also some evidence that cannabis can be a host for mycobacterium[3, 101]. There are several forms of Non-Tuberculosis mycobacteria (NTM) that also present clinical risk[102]. More work is required to understand which species of Mycobacterium present risks to cannabis patients. Most of these studies rely on genotyping to speciate the Mycobacteria of clinical concern.

***Mucor* and mucormycosis**

Mucor is a fungal genus consisting of many pathogenic and benign species. *Mucor* infections can be life threatening in immunocompromised and diabetic patients. 90% of cases are caused by *Rhizopus oryzae* and PCR based tests usually target multiple species.*[103].* It is important to underscore that *Rhizopus oryzae* is used to ferment Tempe and the FDA categorizes it as GRAS (generally recognized as safe). *Rhizopus orzyae* has also been reported to reduce aflatoxin production of other species [104, 105]. Nevertheless, *Rhizopus orzyae* presents a very different risk profile to immunocompromised patients. It usually requires a laceration or ulceration to become infectious.

Mucor infections are usually found in sinuses, brain and lungs. Amphotericin B and Isavuconazole are often used to fight the infections [106]. Rhinocerebral mucormycosis is the most common manifestation of the infection and has 30-100% mortality depending on the patient pre-existing diagnosis. *Mucor* has been found on Cannabis flowers in two independent ITS sequencing studies[3, 5]. More work is required to speciate the genus detected on cannabis and if these species are the same species reported in hospital infections[107].

Listeria monocytogenes

Listeriosis is the 3rd most frequent form of food borne illness[108]. It is an intracellular gram-positive pathogen that can grow in refrigeration temperatures and is known to create biofilms[109]. Large doses of cannabinoids have been shown to weaken immune response to *Listeria* in mice models of disease[110]. The dosages studied in this work were several orders of magnitude higher (38-150mg/kg THC) than recreational cannabis dosages (5mg/75kg). *Listeria* has not yet been recorded on cannabis but it is important to realize the existing published sequencing surveys are still very preliminary with small sample sizes. *Listeria* is more likely relevant to cannabis edible manufacturing and clean kitchen methodologies. *Listeria* detection requires selective enrichment and speciation with colony morphology and sugar fermentation. More rapid PCR based tools have been published that can ascertain speciation and viability in under 48 hours[111]. No jurisdictions currently require *Listeria* testing for Cannabis but it is being reviewed as a concern in Colorado[112].

In summary, there is substantial evidence for culture based systems presenting significant vulnerabilities for the most severe microbial risks found on Cannabis and guidelines anchored in CFU/g will continue to inhibit proper quantification of these risks[113]. We believe cannabis microbial enumeration guidelines moving forward should consider molecular methods. Genomic techniques are gaining adoption at the FDA Genome Trakr network[114] and we believe this is related to the unreliability of culture based platforms to properly inform on microbial health hazards in a timely manner. The remainder of this document will focus on protocol and methods validation considerations used in validating a commercially available cannabis microbial detection platform known as PathoSEEK ®.

Methods Validation

There are currently only four peer-reviewed papers that describe Cannabis microbial methods and they all utilize ITS PCR[3-5, 115]. While there is a temptation to lift what is commonly used in the food industry and assume it will behave accordingly on cannabis, most regulators and certification agencies will not allow the superimposition of a method validation between different matrices. If you change the target matrix (milk to cannabis), you need to revalidate the assay on the new matrix. As of this writing, there are no peer-reviewed culture-based techniques that have performed a cannabis matrix validation in the literature. Likewise, no regulator will allow a manufacturers validation to transfer to another laboratory setting. Manufacturers validations need to be re-examined in the laboratories they are implemented in. This is also true with ISO certifications. Below are a few guidelines on how to perform such a validation.

Depending on the Country or State, some or all of the following live organisms and genomic DNA (Table 1) should ordered from ATCC or other tissue culture banks to serve as spike in controls for methods validation. A Bio Level 2 (BL2) Safety lab is required by law to handle human pathogens. Some human and plant pathogens like Fusarium may require USDA approval before shipment.

Spike-ins should be measured on both flowers and at least 3 different MIP matrices (Chocolate, gummy, shatter) at 3 or more different dilution levels. MIPs that are inhaled are often held to tighter regulatory standards than oral MIPs (California).

Table 1, Live Organisms Evaluated:

Live Organisms	Source
Aspergillus flavus	ATCC# 16870
Aspergillus fumigatus	ATCC# 16903
Aspergillus niger	ATCC# 15475
Aspergillus terreus	ATCC# 16793
Aspergillus janponicus	ATCC# 16873
Candida albicans	ATCC# 10231
Candida catenulate	ATCC# 10565
Candida glabralta	ATCC# 15545
Candida krusei	ATCC# 28870
Candida sphaerica	ATCC# 8565
Debaryomyces hanseii	ATCC# 10623
Rhodotorula mucilaginosa	ATCC# 4557
Trichothecium Roseum	ATCC# 90473
Yarrowia lipolytica	ATCC# 18944
Salmonella	ATCC# 700720
Saccharomyces cerevisiae	The Gold Pitch, Giga Yeast, Inc.
E.*coli*	DH10B (New England Biolabs, #C3020K)

Table 2, DNA Organisms Evaluated

DNA Organisms	Source
Aspergillus flavus	ATCC# 9643D-2
Aspergillus fumigatus	ATCC# 1022D-5
Aspergillus niger	ATCC# 1015D-2
Aspergillus terreus	ATCC# 20542D-2
E.coli	ATCC# 8739D-5
Penicillium chrysogenum	ATCC# 10106D-5
Klebsiella pneumoniae	ATCC# 700721D-5
STEC O111	ATCC# BAA-2440D-5
Salmonella enterica	ATCC# 700720D-5
Coliform/Entero Positive Control	MGC# 420314
Total Yeast and Mold Positive Control	MGC# 420303
Total Aerobic Count Positive Control	MGC # 420306

E. *coli*/Salmonella Positive Control	MGC# 420313

California regulations (2018) require inclusion and exclusion analysis be performed for STEC *E.coli, Salmonella, Aspergillus terreus, Aspegillus niger, Aspergillus flavus, Aspergillus fumigatus*. This requires additional ATCC strains to demonstrate differentiation from benign and ubiquitous *Aspergillus*. Molecular methods are required for this level of species specificity. For inclusion criteria for STEC *E.coli*, ATCC has published a very helpful guideline describing the "Big Six" *E.coli*. Table 3 has additional "Big Six" *E.coli* strains to demonstrate STEC assays are inclusive of these health hazards and that these primers don't amplify off target organisms (https://www.atcc.org/~/media/PDFs/Big%20Six.ashx)

Table 3, List of species tested with PathoSEEK and corresponding results:

Species	Type	ATCC #	Total Y&M	TAC	TC	TE	E. coli	Sal	STEC	A.fla	A.fum	A.nig	A. terr
Aeromonas hydrophilia	B	7965D		Pos (+)	Pos (+)	Pos (+)							
Aeromonas hydrophilia Strain CDC 359-60	B	7966D-5		Pos (+)	Pos (+)	Pos (+)							
Aspergillus aculeatus	M	24147	Pos (+)							Neg (-)	Neg (-)	Neg (-)	Neg (-)
Aspergillus brasiliensis WLRI 034 (120)	M	16404D-2	Pos (+)	Neg (-)			Neg (-)	Neg (-)		Neg (-)	Neg (-)	Neg (-)	Neg (-)
Aspergillus carbonarius	M	1025	Pos (+)							Neg (-)	Neg (-)	Neg (-)	Neg (-)
Aspergillus flavus	M	16870	Pos (+)					Neg (-)	Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)
Aspergillus flavus SN3	M	9643D-2	Pos (+)	Neg (-)				Neg (-)	Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)
Aspergillus fumigatus	M	16903	Pos (+)					Neg (-)	Neg (-)	Neg (-)	Pos (+)	Neg (-)	Neg (-)
Aspergillus fumigatus 118	M	1022D-2	Pos (+)					Neg (-)	Neg (-)	Neg (-)	Pos (+)	Neg (-)	Neg (-)
Aspergillus janponicus	M	16873	Pos (+)							Neg (-)	Neg (-)	Neg (-)	Neg (-)
Aspergillus niger	M	15475	Pos (+)					Neg (-)	Neg (-)	Neg (-)	Neg (-)	Pos (+)	Neg (-)
Aspergillus niger	M	1015D-2	Pos (+)	Neg (-)	Neg (-)	Neg (-)		Neg (-)	Neg (-)	Neg (-)	Neg (-)	Pos (+)	Neg (-)
Aspergillus oryzae	M	1010	Pos (+)										
Aspergillus terreus	M	16793	Pos (+)					Neg (-)	Neg (-)	Neg (-)	Neg (-)	Neg (-)	Pos(+)
Aspergillus terreus	M	20542D-2	Pos (+)	Neg (-)	Neg (-)	Neg (-)		Neg (-)	Neg (-)	Neg (-)	Neg (-)	Neg (-)	Pos(+)
Aspergillus ustus	M	1041	Pos (+)							Neg (-)	Neg (-)	Neg (-)	Neg (-)
Bacillus subtilis subsp. Spizizenii	B	6633D-5	Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)					
Bacillus subtilis subsp. Spizizenii	B	6633			Neg (-)	Neg (-)							
Candida albicans	Y	10231D-5	Pos (+)	Neg (-)			Neg (-)	Neg (-)		Neg (-)	Neg (-)	Neg (-)	
Candida albicans	Y	10231	Pos (+)		Neg (-)	Neg (-)							
Candida catenulata	Y	10565	Pos (+)										
Candida glabralta	Y	15545	Pos (+)										
Candida guilliermondii	Y	90197	Pos (+)										

Candida krusei	Y	28870	Pos (+)										
Candida lusitiniae	Y	344449	Pos (+)										
Candida sphaerica	Y	8565	Pos (+)										
Clostridium sporogenes	B	11437FD -5	Neg(-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)					
Clostridium sporogenes	B	11437			Neg (-)	Neg (-)							
Debaryomyces hanseii	Y	10623	Pos (+)										
E.coli DH10B™ Compentent Cells	B	NEB		Pos (+)	Pos (+)	Pos (+)							
Enterobacter aerogenes	B	15038D-5		Pos (+)	Pos (+)	Pos (+)							
Escherichia coli	B	8739D-5	Neg(-)	Pos (+)	Pos (+)	Pos (+)	Pos (+)	Neg (-)	Neg (-)				
Escherichia coli	B	8739					Pos (+)	Neg (-)					
Escherichia coli Strain 2000-3039	B	BAA-2193D-5						Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)
Escherichia coli Strain 2002-3211	B	BAA-2219D-5						Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)
Escherichia coli Strain 2003-3014	B	BAA-2196D-5						Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)
Escherichia coli Strain 2006-3008	B	BAA-2215D-5						Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)
Escherichia coli Strain 99-3311	B	BAA-2192D-5						Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)
Escherichia coli Strain O111	B	BAA-2440D-5		Pos (+)	Pos (+)	Pos (+)	Pos (+)	Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)
Geotrichum Candidum	M	74170	Pos (+)										
Geotrichum capitatum	M	28575	Pos (+)										
Klebsiella pneumoniae	B	700721D-5		Pos (+)	Pos (+)	Pos (+)							
Lactobacillus acidophilos	B	4357				Neg (-)							
Lactobacillus acidophilus	B	4357D-5		Pos (+)	Neg (-)	Neg (-)							
Listeria monocytogenes	B	19115D-5		Pos (+)	Neg (-)								
Mucor racemosus	M	22365	Pos (+)										
Penicillium chrysogenum	M	10106D-2	Pos (+)	Neg (-)		Neg (-)				Neg (-)	Neg (-)	Neg (-)	
Penicillium citrinum	M	36382	Pos (+)										
Penicillium paxilli	M	96516	Pos (+)										
Penicillum Citrinum	M	36382	Pos (+)										
Pseudomonas aeruginosa	B	9027D-5	Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)					
Pseudomonas aeruginosa	B	9027		Pos (+)									
Pseudomonas syringae pathovar tomato	B	BAA-871D-5		Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)					
Rhodotorula mucilaginosa	Y	4557	Pos (+)										
Saccharomyces cerevisiae	Y	-	Pos (+)										
Salmonella enterica serova Typhimurium LT2	B	700720D-5	Neg (-)	Pos (+)	Pos (+)	Pos (+)	Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)	Neg (-)
Salmonella enterica subsp.	B	700720					Neg (-)	Pos (+)					

aureus													
Salmonella enterica subsp. houtenae (IV)	B	BAA-1580D-5					Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)	Neg (-)
Salmonella enterica subsp. salamae (II)	B	BAA-1582D-5					Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)	Neg (-)
Salmonella enterica subsp. indica (VI)	B	BAA-1578D-5					Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)	Neg (-)
Staphylococcus aureus subsp. Aureus	B	6538D-5	Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)					
Staphylococcus aureus subsp. aureus	B	6538		Pos (+)	Neg (-)	Neg (-)							
Trichoderma virens	M	13213	Pos (+)										
Trichothecium Roseum	M	90473	Pos (+)										
Vibrio cholerae	B	39315D-5		Pos (+)	Neg (-)	Pos (+)							
Yarrowia lipolytica	M	18944	Pos (+)										
Yersinia pestis K25	B	BAA-1511D-5		Pos (+)	Neg (-)	Pos (+)							

Species	Type	ATCC #	Total Y&M	TAC	TC	TE	E. coli	Sal	STEC	A.fla	A.fum	A.nig	A. terr
Aeromonas hydrophilia	B	7965D		Pos (+)	Pos (+)	Pos (+)							
Aeromonas hydrophilia Strain CDC 359-60	B	7966D-5		Pos (+)	Pos (+)	Pos (+)							
Aspergillus aculeatus	M	24147	Pos (+)							Neg (-)	Neg (-)	Neg (-)	Neg (-)
Aspergillus brasiliensis WLRI 034 (120)	M	16404D-2	Pos (+)	Neg (-)			Neg (-)	Neg (-)		Neg (-)	Neg (-)	Neg (-)	Neg (-)
Aspergillus carbonarius	M	1025	Pos (+)							Neg (-)	Neg (-)	Neg (-)	Neg (-)
Aspergillus flavus	M	16870	Pos (+)					Neg (-)	Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)
Aspergillus flavus SN3	M	9643D-2	Pos (+)	Neg (-)				Neg (-)	Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)
Aspergillus fumigatus	M	16903	Pos (+)					Neg (-)	Neg (-)	Neg (-)	Pos (+)	Neg (-)	Neg (-)
Aspergillus fumigatus 118	M	1022D-2	Pos (+)					Neg (-)	Neg (-)	Neg (-)	Pos (+)	Neg (-)	Neg (-)
Aspergillus janponicus	M	16873	Pos (+)							Neg (-)	Neg (-)	Neg (-)	Neg (-)
Aspergillus niger	M	15475	Pos (+)					Neg (-)	Neg (-)	Neg (-)	Neg (-)	Pos (+)	Neg (-)
Aspergillus niger	M	1015D-2	Pos (+)	Neg (-)	Neg (-)	Neg (-)		Neg (-)	Neg (-)	Neg (-)	Neg (-)	Pos (+)	Neg (-)
Aspergillus oryzae	M	1010	Pos (+)										
Aspergillus terreus	M	16793	Pos (+)					Neg (-)	Neg (-)	Neg (-)	Neg (-)	Neg (-)	Pos(+)
Aspergillus terreus	M	20542D-2	Pos (+)	Neg (-)	Neg (-)	Neg (-)		Neg (-)	Neg (-)	Neg (-)	Neg (-)	Neg (-)	Pos(+)
Aspergillus ustus	M	1041	Pos (+)							Neg (-)	Neg (-)	Neg (-)	Neg (-)
Bacillus subtilis subsp. Spizizenii	B	6633D-5	Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)					
Bacillus subtilis subsp. Spizizenii	B	6633			Neg (-)	Neg (-)							
Candida albicans	Y	10231D-5	Pos (+)	Neg (-)			Neg (-)	Neg (-)		Neg (-)	Neg (-)	Neg (-)	
Candida albicans	Y	10231	Pos (+)		Neg (-)	Neg (-)							
Candida catenulata	Y	10565	Pos (+)										
Candida glabralta	Y	15545	Pos (+)										
Candida guilliermondii	Y	90197	Pos (+)										
Candida krusei	Y	28870	Pos (+)										
Candida lusitiniae	Y	344449	Pos (+)										
Candida sphaerica	Y	8565	Pos (+)										
Clostridium sporogenes	B	11437FD-5	Neg(-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)					
Clostridium sporogenes	B	11437			Neg (-)	Neg (-)							

Debaryomyces hanseii	Y	10623	Pos (+)										
E.coli DH10BTM Compentent Cells	B	NEB		Pos (+)	Pos (+)	Pos (+)							
Enterobacter aerogenes	B	15038D-5		Pos (+)	Pos (+)	Pos (+)							
Escherichia coli	B	8739D-5	Neg(-)	Pos (+)	Pos (+)	Pos (+)	Pos (+)	Neg (-)	Neg (-)				
Escherichia coli	B	8739					Pos (+)	Neg (-)					
Escherichia coli Strain 2000-3039	B	BAA-2193D-5						Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)
Escherichia coli Strain 2002-3211	B	BAA-2219D-5						Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)
Escherichia coli Strain 2003-3014	B	BAA-2196D-5						Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)
Escherichia coli Strain 2006-3008	B	BAA-2215D-5						Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)
Escherichia coli Strain 99-3311	B	BAA-2192D-5						Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)
Escherichia coli Strain O111	B	BAA-2440D-5		Pos (+)	Pos (+)	Pos (+)	Pos (+)	Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)
Geotrichum Candidum	M	74170	Pos (+)										
Geotrichum capitatum	M	28575	Pos (+)										
Klebsiella pneumoniae	B	700721D-5		Pos (+)	Pos (+)	Pos (+)							
Lactobacillus acidophilos	B	4357				Neg (-)							
Lactobacillus acidophilus	B	4357D-5		Pos (+)	Neg (-)	Neg (-)							
Listeria monocytogenes	B	19115D-5		Pos (+)	Neg (-)								
Mucor racemosus	M	22365	Pos (+)										
Penicillium chrysogenum	M	10106D-2	Pos (+)	Neg (-)		Neg (-)				Neg (-)	Neg (-)	Neg (-)	
Penicillium citrinum	M	36382	Pos (+)										
Penicillium paxilli	M	96516	Pos (+)										
Penicillum Citrinum	M	36382	Pos (+)										
Pseudomonas aeruginosa	B	9027D-5	Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)					
Pseudomonas aeruginosa	B	9027		Pos (+)									
Pseudomonas syringae pathovar tomato	B	BAA-871D-5		Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)					
Rhodotorula mucilaginosa	Y	4557	Pos (+)										
Saccharomyces cerevisiae	Y	-	Pos (+)										
Salmonella enterica serova Typhimurium LT2	B	700720D-5	Neg (-)	Pos (+)	Pos (+)	Pos (+)	Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)	Neg (-)
Salmonella enterica subsp. aureus	B	700720					Neg (-)	Pos (+)					
Salmonella enterica subsp. houtenae (IV)	B	BAA-1580D-5					Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)	Neg (-)
Salmonella enterica subsp. salamae (II)	B	BAA-1582D-5					Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)	Neg (-)
Salmonella enterica subsp. indica (VI)	B	BAA-1578D-5					Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)	Neg (-)
Staphylococcus aureus subsp. Aureus	B	6538D-5	Neg (-)	Pos (+)	Neg (-)	Neg (-)	Neg (-)	Neg (-)					
Staphylococcus aureus subsp. aureus	B	6538		Pos (+)	Neg (-)	Neg (-)							
Trichoderma virens	M	13213	Pos (+)										
Trichothecium Roseum	M	90473	Pos (+)										
Vibrio cholerae	B	39315D-5		Pos (+)	Neg (-)	Pos (+)							
Yarrowia lipolytica	M	18944	Pos (+)										
Yersinia pestis K25	B	BAA-1511D-5		Pos (+)	Neg (-)	Pos (+)							

Table 3 Legend:

B = bacteria, M = Mold, Y = yeast

Note: A blank cell means that the species was not tested with that assay

A filled well means that the species was tested with the assay and shows results of the test.

In-Silico Inclusion Analysis

Since *Aspergillus* species can be difficult to obtain and culture and those that can be purchased from ATCC do not always have genome sequencing available, we chose to also perform an *in-silico* analysis of Whole Genome Shotgun assemblies in NCBI.

Four different Whole Genome Shotgun assemblies for *Aspergillus flavus* were downloaded from NCBI. PathoSEEK ® positive control sequence was compared with BLAST to find 100% identical sequence to 3 of the strains. The single SNP found in one of the genome assemblies was NOT under any PathoSEEK ® primer or probe sequences suggesting all 4 of these subspecies will amplify with our primers. The variant strain was isolated from peanuts in Georgia but was a draft assembly at 26X coverage in over 6,423 contigs. It is very likely that this is a sequencing error in this reference due to low coverage. Other genomes were sequenced over 50X and in less than 900 contigs.

This analysis was repeated for *Aspergillus terreus, fumigatus and niger.* Only one *Aspergillus terreus* genome assembly showed polymorphisms in the positive control sequence (isolated from the international space station to assess microgravity mutation rates) and NONE of these polymorphisms were found in PathoSEEK ® primers and probe sequences implying identical amplification should occur.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Aspergillus flavus strain JAU2 S1_R1_(paired)_contig_64, whole genome shotgun sequence	444	444	100%	2.00E-124	100%	NKQP01000063.1
Aspergillus flavus strain NRRL 21882 S3_R1_(paired)_contig_33, whole genome shotgun sequence	444	444	100%	2.00E-124	100%	NKQQ01000032.1
Aspergillus flavus AF70 751, whole genome shotgun sequence	444	444	100%	1.00E-124	100%	JZDT01000286.1
Aspergillus flavus strain 3-2 contig0005419, whole genome shotgun sequence	433	433	100%	3.00E-121	99%	LOAQ01005419.1

Description	Max score	Total score	Query cover	E value	Ident	Accession
Aspergillus niger ATCC 1015, whole genome shotgun sequence	451	451	100%	8.00E-127	100%	ACJE01000004.1
Aspergillus niger CBS 513.88 supercontig An01	451	451	100%	8.00E-127	100%	NT_166518.1
Aspergillus niger strain ATCC 10864 scaffold65, whole genome shotgun sequence	451	451	100%	8.00E-127	100%	MCQH01000014.1
Aspergillus niger strain L2 unitig_2_quiver, whole genome shotgun sequence	451	451	100%	8.00E-127	100%	LKBF01000018.1

Description	Max score	Total score	Query cover	E value	Ident	Accession
Aspergillus fumigatus Af293 chromosome 4, whole genome shotgun sequence	442	442	100%	4.00E-124	100%	NC_007197.1
Aspergillus fumigatus A1163 scf_000011 genomic scaffold, whole genome shotgun sequence	442	442	100%	4.00E-124	100%	DS499604.1
Aspergillus fumigatus isolate HMR AF 706 scaffold_744, whole genome shotgun sequence	442	442	100%	4.00E-124	100%	NKHS01000743.1
Aspergillus fumigatus isolate HMR AF 270 scaffold_512, whole genome shotgun sequence	442	442	100%	4.00E-124	100%	NKHT01000511.1

Description	Max score	Total score	Query cover	E value	Ident	Accession
Aspergillus terreus NIH2624 scaffold_4 genomic scaffold, whole genome shotgun sequence	333	333	100%	2.00E-91	100%	NT_165927.1
Aspergillus terreus strain 45A scaffold_23, whole genome shotgun sequence	333	333	100%	2.00E-91	100%	LWBM01000023.1
Aspergillus terreus strain IMV 01167 jcf7180000027336, whole genome shotgun sequence	255	255	100%	4.00E-68	92%	MSJE01000028.1
Aspergillus terreus strain w25 NODE_5_length_746679_cov_26.5335, whole genome shotgun sequence	333	333	100%	2.00E-91	100%	PCZV01000005.1

Table 4. *In-silico* BLAST analysis of Whole Genome Shotgun datasets in NCBI.

A similar analysis was performed with our *Salmonella* Primers and Probe sequences. 146 *Salmonella* Subspecies were identified with 100% identical sequence. 34 are shown in the below table for reference.

Table 5. *in-silico* Salmonella BLAST analysis of target sequence

Description	Max score	Total score	Query cover	E value	Ident	Accession
Salmonella enterica subsp. enterica serovar Typhimurium strain BL10 chromosome, complete genome	281	281	100%	3.00E-72	100%	CP024619.1
Salmonella enterica subsp. enterica serovar Enteritidis strain 92-0392 chromosome, complete genome	281	281	100%	3.00E-72	100%	CP018657.1
Salmonella enterica subsp. enterica serovar Typhimurium isolate VNB151-sc-2315230 genome assembly, chromosome: 1	281	281	100%	3.00E-72	100%	LT795114.1
Salmonella enterica subsp. enterica strain RM11060 chromosome, complete genome	281	281	100%	3.00E-72	100%	CP022658.1
Salmonella enterica subsp. enterica strain RM11065 chromosome, complete genome	281	281	100%	3.00E-72	100%	CP022663.1
Salmonella enterica subsp. enterica serovar Paratyphi A strain FDAARGOS_368 chromosome, complete genome	281	281	100%	3.00E-72	100%	CP023508.1
Salmonella enterica subsp. enterica serovar Typhimurium strain WW012 chromosome, complete genome	281	281	100%	3.00E-72	100%	CP022168.1
Salmonella enterica subsp. enterica serovar Saintpaul strain SGB23 chromosome, complete genome	281	281	100%	3.00E-72	100%	CP023166.1
Salmonella enterica subsp. enterica strain 08-00436, complete genome	281	281	100%	3.00E-72	100%	CP020492.1
Salmonella enterica subsp. enterica serovar Manhattan strain SA20084699 chromosome, complete genome	281	281	100%	3.00E-72	100%	CP022497.1
Salmonella enterica subsp. enterica serovar Saintpaul strain SA20031783 chromosome, complete genome	281	281	100%	3.00E-72	100%	CP022491.1
Salmonella enterica subsp. enterica serovar Braenderup strain SA20026289 chromosome, complete genome	281	281	100%	3.00E-72	100%	CP022490.1
Salmonella enterica subsp. enterica serovar Typhimurium strain TW-Stm6 chromosome, complete genome	281	281	100%	3.00E-72	100%	CP019649.1
Salmonella enterica subsp. enterica serovar Typhimurium strain FDAARGOS_321, complete genome	281	281	100%	3.00E-72	100%	CP022070.1
Salmonella enterica strain FDAARGOS_313, complete genome	281	281	100%	3.00E-72	100%	CP022069.1
Salmonella enterica strain FDAARGOS_312, complete genome	281	281	100%	3.00E-72	100%	CP022062.1
Salmonella enterica subsp. enterica serovar India str. SA20085604, complete genome	281	281	100%	3.00E-72	100%	CP022015.1
Salmonella enterica subsp. enterica strain ST1120, complete genome	281	281	100%	3.00E-72	100%	CP021909.1
Salmonella enterica subsp. enterica serovar Minnesota strain CFSAN017963, complete genome	281	281	100%	3.00E-72	100%	CP017720.1
Salmonella enterica subsp. enterica strain 16A242, complete genome	281	281	100%	3.00E-72	100%	CP020922.1
Salmonella enterica subsp. enterica strain 2012K-0678, genome	281	281	100%	3.00E-72	100%	CP020718.1
Salmonella enterica strain FORC_030, complete genome	281	281	100%	3.00E-72	100%	CP015598.1
Salmonella enterica subsp. enterica serovar Typhimurium strain RM10607, complete genome	281	281	100%	3.00E-72	100%	CP013720.1
Salmonella enterica subsp. enterica serovar Typhimurium str. USDA-ARS-USMARC-1899, complete genome	281	281	100%	3.00E-72	100%	CP007235.2
Salmonella enterica subsp. enterica serovar Typhimurium str. USDA-ARS-USMARC-1898, complete genome	281	281	100%	3.00E-72	100%	CP014971.2
Salmonella enterica subsp. enterica serovar Typhimurium str. CDC H2662, complete genome	281	281	100%	3.00E-72	100%	CP014979.2
Salmonella enterica subsp. enterica serovar Typhimurium str. USDA-ARS-USMARC-1810, complete genome	281	281	100%	3.00E-72	100%	CP014982.2
Salmonella enterica subsp. enterica serovar Typhimurium strain 81741, complete genome	281	281	100%	3.00E-72	100%	CP019442.1
Salmonella enterica subsp. enterica serovar Yovokome str. S-1850, complete genome	281	281	100%	3.00E-72	100%	CP019418.1
Salmonella enterica subsp. enterica serovar Wandsworth str. SA20092095, complete genome	281	281	100%	3.00E-72	100%	CP019417.1
Salmonella enterica subsp. enterica serovar Nitra strain S-1687, complete genome	281	281	100%	3.00E-72	100%	CP019416.1
Salmonella enterica subsp. enterica serovar Manchester str. ST278, complete genome	281	281	100%	3.00E-72	100%	CP019414.1
Salmonella enterica subsp. enterica serovar Krefeld str. SA20030536, complete genome	281	281	100%	3.00E-72	100%	CP019413.1

Sample Homogenization

250mg to 1g of homogenization is required in most states. Homogenization of 250mg/1g of flower in 3.55ml/14.2ml Tryptic Soy Broth (TSB) can be performed in a WhirlPak bag. These bags have a 300um mesh filter in them to eliminate large debris from the homogenization process. This filter is too large to prevent trichomes (50-150um) from filtering through and these often require centrifugation to fully eliminate. Lysis buffer should be added before any centrifugation steps or bacterial and mold cells will pellet with the trichomes and residual plant debris.

Homogenization techniques need to be carefully scrutinized. Some homogenization protocols used for cannabinoid and terpenoid testing may grind the plant matter and microbes into an unviable state in methanol or other organic solvents. Liquid nitrogen or other forms of aggressive homogenization can alter the viability of the microbes and present false negative tests for culture based techniques. Extreme homogenization can also create fine powders that obstruct or saturate various solid phase particles or surfaces often used for DNA purification. Filter pore sizes need to be scrutinized to ensure homogenization debris is properly filtered while bacterial and mold cells are not omitted from the sample.

Commonly used phosphate rich buffers and broths should be avoided for homogenization and growth if your microbial quantification method requires DNA purification. Most DNA isolation techniques use solid phases that are attracted to the phosphodiester bond in DNA. Many of these DNA isolation techniques actually use phosphates to elute their DNA from the solid phase. As a result, the use of phosphate rich homogenization buffers can saturate the downstream DNA preps' solid phase with phosphates such that little to no DNA is captured.

Internal DNA purification controls are advised so one can differentiate a DNA purification failure from an amplification failure. Some kits (PathoSEEK ®) target amplicons unique to the cannabis plant as an internal purification control. If this signal does not appear, the homogenization and DNA purification should be scrutinized and repeated.

Sampling Bias

Whether using culture or molecular methods such as PCR, neither approach affords assaying the entire 3.55ml or 14.2ml homogenate. Usually a small subsampling of this total homogenate is plated or placed into qPCR. This subsampling needs to be accounted for or single CFU/g measurements cannot be made regarding the entire cannabis sample. For example, if only 1/10th of the original sample is plated or placed into PCR and the original sample was at 1 CFU/g, 90% of the time you pipette that subsampling you will get negative results and only one time will you get a positive hit.

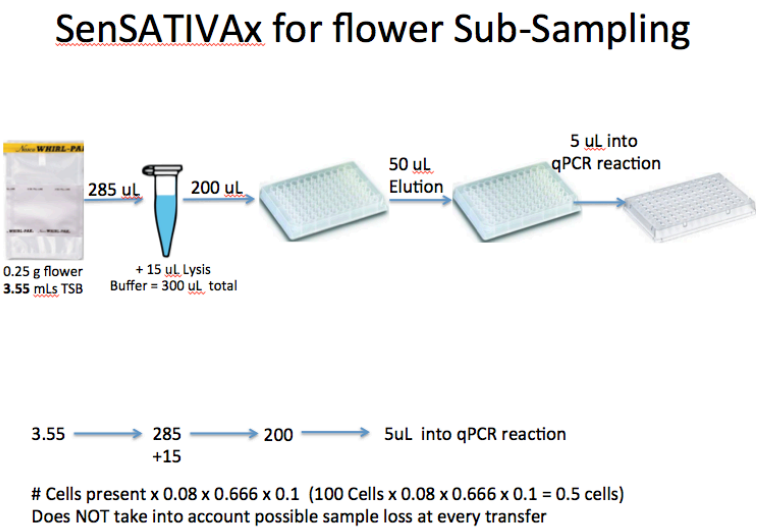


Figure 5- Diagram of subsampling for SenSATIVAx DNA purification from Flower.

For this reason most single CFU tests that subsample, use an enrichment step where they allow the E.coli to incubate for a set number of doubling times to compensate for the subsampling. Assuming E.coli doubles every half hour at 37C in static growth with TSB, 8 doublings (2^8 = 256) will compensate for 100 fold subsampling. This enrichment step will also ensure that molecular methods are only measuring viable cells. Where possible, growth rates should be measured in the presence of a cannabis matrix and the broth selected due to the inhibitory nature of certain cannabis matrix components (Figure 6).

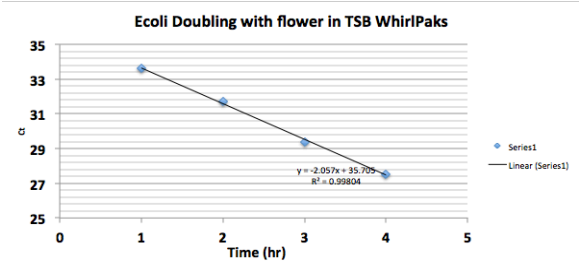


Figure 6- E.coli doubling rate in TSB Whirlpak bags with cannabis matrix present.

PCR Efficiency and Limit of Detection (LOD)

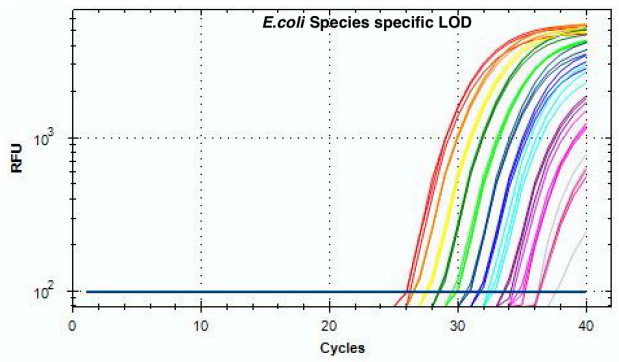
In order to achieve concordance with CFU/g one needs to measure PCR efficiency and the limits of detection (LOD) across a broad dynamic range. To achieve this, 2 fold serial dilutions across 12 data dilution points are performed in triplicate. %RSD, PCR efficiency and R squared can be calculated from the replicated dilutions. AOAC guidelines should be followed for proper concordance with limited dynamic range techniques like plating (http://multimedia.3m.com/mws/media/241188O/3m-petrifilm-plate-certificates-recognitions-validations.pdf).

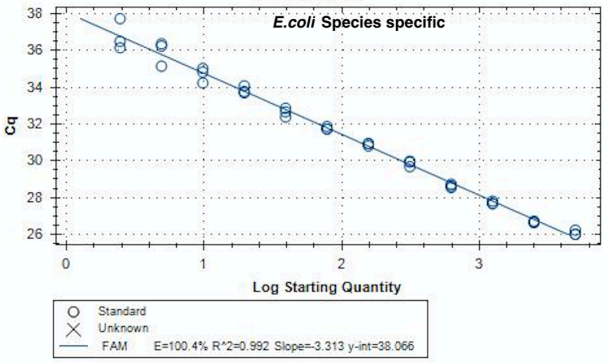
Table 6.

Assay	Copies of STEC O111 DNA	Cq Value (FAM)	%RSD
E.*coli* Specific	5,000	26.23	0.50
E.*coli* Specific	5,000	26.00	
E.*coli* Specific	5,000	26.01	
E.*coli* Specific	2,500	26.68	0.16

E.*coli* Specific	2,500	26.63	
E.*coli* Specific	2,500	26.72	
E.*coli* Specific	1,250	27.69	0.28
E.*coli* Specific	1,250	27.66	
E.*coli* Specific	1,250	27.81	
E.*coli* Specific	625	28.55	0.32
E.*coli* Specific	625	28.73	
E.*coli* Specific	625	28.63	
E.*coli* Specific	313	29.68	0.54
E.*coli* Specific	313	29.99	
E.*coli* Specific	313	29.93	
E.*coli* Specific	156	30.93	0.25
E.*coli* Specific	156	30.81	
E.*coli* Specific	156	30.94	
E.*coli* Specific	78	31.87	0.26
E.*coli* Specific	78	31.74	
E.*coli* Specific	78	31.72	
E.*coli* Specific	39	32.67	0.75
E.*coli* Specific	39	32.38	
E.*coli* Specific	39	32.87	
E.*coli* Specific	20	33.70	0.60
E.*coli* Specific	20	33.78	
E.*coli* Specific	20	34.08	
E.*coli* Specific	10	34.24	1.19
E.*coli* Specific	10	34.83	
E.*coli* Specific	10	35.03	
E.*coli* Specific	5	36.37	1.86
E.*coli* Specific	5	35.15	
E.*coli* Specific	5	36.24	
E.*coli* Specific	2	36.15	2.26
E.*coli* Specific	2	36.50	
E.*coli* Specific	2	37.74	
E.*coli* Specific	0	Not Detected	Not Applicable
E.*coli* Specific	0	Not Detected	
E.*coli* Specific	0	Not Detected	

Figure 7: E.*coli* qPCR Dilution Curves and qPCR Efficiency (E)





Correlation of these results with plating live species will result in an equation that enables conversion of Cq/g to CFU/g. Each assay should have its own equation and the equation may differ based on the use of extract versus flower due to the impact of the matrix as a carbon source for growth on plates. The broader spectrum assays like TAC and TYM will have lower concordance than the more specific assays since 95-99% of microbes don't culture and many molds don't form colonies[116]. Thus the broader the scope of the test, the higher likelihood unculturable organisms will trigger qPCR signals and the higher likelihood some organisms will grow that do not PCR with ITS or 16S primer sequences. While the unculturable organisms will never be known with plating, DNA sequencing can provide a list of thousands of organisms your primers are known capture. This cannot be underscored enough as each organism ordered from ATCC for spike in assessment on cannabis is $300 making the acquisition of a list of spike in organisms that can culture in the present of cannabis matrix very expensive. As a result there is currently no peer reviewed published validation data regarding what organisms derived from cannabis can grow on plates or 3M Petrifilm®. There are multiple studies using PCR and DNA sequencing to acquire this information with and without culture.

Since many regulators are looking for strong correlations with Cq->CFU, the only way to achieve this is to design primers that do not amplify unculturable molds. Commonly found non-culturable but harmless molds like *Botrytis cinerea* and Cannabis derived powdery mildew (CDPM) can be designed to not amplify with certain primer sets. It is recommended that species specific assays exist for these common molds in the event regulatory bodies decide their allergenic potential needs to be monitored in the future.

Table 7, Cq to CFU/g Equations for Flower, MIP/Extract and Gummy

Matrix	Microbial Test	Cq to CFU/g Conversion Equation
Plant	Total Yeast and Mold	$CFU/g = 10^{[(36.671 - Cq\ Value)/3.1194]}$
Plant	Total Aerobic Count	$CFU/g = 10^{[(35.111 - Cq\ Value)/2.8883]}$
Plant	Total Coliform	$CFU/g = 10^{[(40.073 - Cq\ Value)/3.3417]}$
Plant	Total Enterobacteriaceae	$CFU/g = 10^{[(41.218 - Cq\ Value)/4.3708]}$
MIP/Extract	Total Yeast and Mold	$CFU/g = 10^{[(54.972 - Cq\ Value)/5.8485]}$
MIP/Extract	Total Aerobic Count	$CFU/g = 10^{[(38.076 - Cq\ Value)/3.2249]}$
MIP/Extract	Total Coliform	$CFU/g = 10^{[(41.935 - Cq\ Value)/3.6274]}$

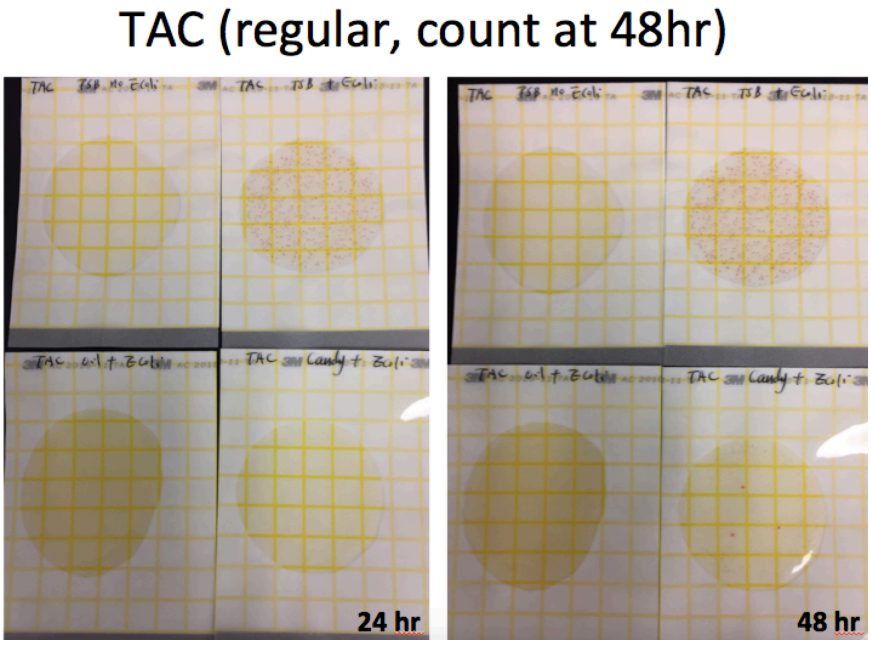
MIP/Extract	Total Enterobacteriaceae	CFU/g = $10^{[(38.407 - \text{Cq Value})/3.3041]}$
Gummy	Total Yeast and Mold	CFU/g = $10^{[(52.989 - \text{Cq Value})/4.9718]}$
Gummy	Total Aerobic Count	CFU/g = $10^{[(37.235 - \text{Cq Value})/2.356]}$
Gummy	Total Coliform	CFU/g = $10^{[(52.888 - \text{Cq Value})/5.9643]}$
Gummy	Total Enterobacteriaceae	CFU/g = $10^{[(44.81 - \text{Cq Value})/4.9665]}$

One will notice significantly altered Cq->CFU conversions with various Extracts or MIPs. To better understand this we performed spike in platings with various MIP matrices to better understand the impact of these carbon sources on culturing conditions.

Marijuana Infused Products (MIP) interference and impact on validation

MIPs are a very diverse class of matrices that behave very differently than cannabis flowers. Gummy bears, chocolates, oils and tinctures all present different challenges to culture based techniques as the sugars and carbohydrates can radically alter the carbon sources available for growth. Some oil loving microbes like *Clostridium botulinum* have been found in cannabis and these require anaerobic conditions to culture. These varying matrices also present challenges to homogenization and DNA purification techniques. To address this, it is important to understand the various inhibitors to your plating and PCR technologies. 3M Coliform plates list citrate as an inhibitor to their reporter assay. Citrate is commonly used in cannabis cultivation in the form of Cal-Mag or Calcium and Magnesium citrate. Citric acid is commonly used as a foliar spray to thwart off plant pathogens. Likewise, certain terpenoids have been listed as polymerase inhibitors suggesting direct PCR without DNA purification may present variable results with different cannabis chemotypes[117]. The list of potential additives, chemotypes and their impact on culture is a near infinite list. An ethanol based DNA purification step simplifies this inhibitory complexity greatly.

Figure 8- MIP inhibition of Petrifilm®



To assess the impact of MIPs on CFU/g enumeration, we spiked in live *E.coli* cells into various MIPs to measure the qPCR signal and compared these to the colony counts with and without MIPs (Figure 8). To our surprise *E.coli* cells spiked into many of the MIPs failed to grow despite growth in the TSB controls. This implies the MIPs are interfering with the reporter assay on the films or that the MIPs are antiseptic in nature. Many MIPs use citric acid as a sour flavoring ingredient and can cause interference with 3M reporter chemistry. qPCR signal was constant implying there is DNA on the films but the colony formation or reporting is inhibited. This underscores the importance of

revalidation with novel matrices. More can be found on this topic at https://www.medicinalgenomics.com/mips-and-extracts-negative-impact-on-plating/.

Precision

Precision contains two sub-tasks: **repeatability** and **robustness**. Repeatability is defined as the precision under the same operating conditions (intra-assay precision). Robustness is defined as the degree of precision of test results obtained by the analysis of the same samples under a variety of typical test conditions, such as different days and analysts. With this in mind, it was necessary for us to display the repeatability and robustness of the SenSATIVAx™ Flower/Leaf DNA Extraction Kit and the SenSATIVAx™ MIP/Extract DNA Extraction Kit and the PathoSEEK® qPCR assays.

Precision of *Aspergillus* species-specific and multiplex assays

The *Aspergillus* multiplex assay combines the *Aspergillus A. flavus, A. fumigatus, A. niger* and *A. terreus* detection assays together in one reaction. The detection probe for each species is on the FAM channel; therefore, if a positive result were to occur, the only way to determine which species was present would be to run all four individual *Aspergillus* assays (presumably on many fewer samples as Aspergillus failure rates are usually under 3% (personal communication with Cannabis testing laboratories).

Live *Aspergillus flavus*, *fumigatus*, *niger* and *terreus* organisms were grown to saturation in TSB. The species-specific assay vs. multiplex assay was tested by adding 100 µL of the species-specific culture into 3.45 mL of TSB with 0.25g of flower present. The multiplex assay was also tested by adding 100 µL of each species culture to 3.15 mL of TSB with 0.25g of flower present. For each matrix tested, a non-spiked control was included to show that the product being tested was clean and the qPCR signal was from the spiked live organism. This was followed by DNA extraction using the SenSATIVAx™ Flower/Leaf DNA Extraction Kit. The extracted DNA was tested using the PathoSEEK™ *Aspergillus flavus, A. fumigatus, A. niger*, A. *terreus* and or *Aspergillus* multiplex assay in replicates of six for the single species spike-ins and in triplicate for the multiple species spike.

The data presented in Table 26 shows that *Aspergillus flavus, A. fumigatus, A. niger* and *A. terreus* can be detected at similar Ct values when either the *Aspergillus* species-specific assay or the *Aspergillus* multiplex assay is used. The table also presents the repeatability of the extracted DNA when tested using the *Aspergillus flavus, A. fumigatus, A. niger*, *A. terreus* and or *Aspergillus* multiplex assays.

Table 8, Precision of *Aspergillus* Assays

Sample	Organism Spiked	qPCR Assay	Cq	%RSD
Flower	*Aspergillus flavus*	*Aspergillus flavus*	25.20	0.70
Flower	*Aspergillus flavus*	*Aspergillus flavus*	25.06	
Flower	*Aspergillus flavus*	*Aspergillus flavus*	24.94	
Flower	*Aspergillus flavus*	*Aspergillus flavus*	24.88	
Flower	*Aspergillus flavus*	*Aspergillus flavus*	24.76	
Flower	*Aspergillus flavus*	*Aspergillus flavus*	25.19	
Flower	*Aspergillus flavus*	*Aspergillus* Multiplex	24.57	0.90
Flower	*Aspergillus flavus*	*Aspergillus* Multiplex	24.40	
Flower	*Aspergillus flavus*	*Aspergillus* Multiplex	24.47	
Flower	*Aspergillus flavus*	*Aspergillus* Multiplex	24.68	
Flower	*Aspergillus flavus*	*Aspergillus* Multiplex	25.03	
Flower	*Aspergillus flavus*	*Aspergillus* Multiplex	24.58	
Flower	*A. flavus, fumigatus, niger, terreus*	*Aspergillus flavus*	25.43	1.91

Flower	*A. flavus, fumigatus, niger, terreus*	*Aspergillus flavus*	24.48	
Flower	*A. flavus, fumigatus, niger, terreus*	*Aspergillus flavus*	25.01	
Flower	None	*Aspergillus flavus*	Not Detected	Not Applicable
Flower	None	*Aspergillus flavus*	Not Detected	
Flower	None	*Aspergillus flavus*	Not Detected	
Flower	*Aspergillus fumigatus*	*Aspergillus fumigatus*	18.60	0.82
Flower	*Aspergillus fumigatus*	*Aspergillus fumigatus*	18.60	
Flower	*Aspergillus fumigatus*	*Aspergillus fumigatus*	18.72	
Flower	*Aspergillus fumigatus*	*Aspergillus fumigatus*	18.47	
Flower	*Aspergillus fumigatus*	*Aspergillus fumigatus*	18.53	
Flower	*Aspergillus fumigatus*	*Aspergillus fumigatus*	18.90	
Flower	*Aspergillus fumigatus*	*Aspergillus* Multiplex	20.36	3.84
Flower	*Aspergillus fumigatus*	*Aspergillus* Multiplex	21.04	
Flower	*Aspergillus fumigatus*	*Aspergillus* Multiplex	20.32	
Flower	*Aspergillus fumigatus*	*Aspergillus* Multiplex	19.59	
Flower	*Aspergillus fumigatus*	*Aspergillus* Multiplex	18.82	
Flower	*Aspergillus fumigatus*	*Aspergillus* Multiplex	20.39	
Flower	*A. flavus, fumigatus, niger, terreus*	*Aspergillus fumigatus*	21.55	0.15
Flower	*A. flavus, fumigatus, niger, terreus*	*Aspergillus fumigatus*	21.54	
Flower	*A. flavus, fumigatus, niger, terreus*	*Aspergillus fumigatus*	21.49	
Flower	None	*Aspergillus fumigatus*	Not Detected	Not Applicable
Flower	None	*Aspergillus fumigatus*	Not Detected	
Flower	None	*Aspergillus fumigatus*	Not Detected	
Flower	*Aspergillus niger*	*Aspergillus niger*	26.66	0.29
Flower	*Aspergillus niger*	*Aspergillus niger*	26.56	
Flower	*Aspergillus niger*	*Aspergillus niger*	26.47	
Flower	*Aspergillus niger*	*Aspergillus niger*	26.65	
Flower	*Aspergillus niger*	*Aspergillus niger*	26.52	
Flower	*Aspergillus niger*	*Aspergillus niger*	26.52	
Flower	*Aspergillus niger*	*Aspergillus* Multiplex	26.97	0.33
Flower	*Aspergillus niger*	*Aspergillus* Multiplex	26.92	
Flower	*Aspergillus niger*	*Aspergillus* Multiplex	26.95	
Flower	*Aspergillus niger*	*Aspergillus* Multiplex	27.15	
Flower	*Aspergillus niger*	*Aspergillus* Multiplex	26.98	
Flower	*Aspergillus niger*	*Aspergillus* Multiplex	27.09	
Flower	*A. flavus, fumigatus, niger, terreus*	*Aspergillus niger*	32.06	0.53
Flower	*A. flavus, fumigatus, niger, terreus*	*Aspergillus niger*	32.14	
Flower	*A. flavus, fumigatus, niger, terreus*	*Aspergillus niger*	32.39	
Flower	None	*Aspergillus niger*	Not Detected	Not Applicable
Flower	None	*Aspergillus niger*	Not Detected	
Flower	None	*Aspergillus niger*	Not Detected	
Flower	*Aspergillus terreus*	*Aspergillus terreus*	25.43	0.98
Flower	*Aspergillus terreus*	*Aspergillus terreus*	25.35	
Flower	*Aspergillus terreus*	*Aspergillus terreus*	24.88	
Flower	*Aspergillus terreus*	*Aspergillus terreus*	24.91	
Flower	*Aspergillus terreus*	*Aspergillus terreus*	24.88	
Flower	*Aspergillus terreus*	*Aspergillus terreus*	25.07	
Flower	*Aspergillus terreus*	*Aspergillus* Multiplex	27.34	3.65
Flower	*Aspergillus terreus*	*Aspergillus* Multiplex	25.03	
Flower	*Aspergillus terreus*	*Aspergillus* Multiplex	25.21	
Flower	*Aspergillus terreus*	*Aspergillus* Multiplex	25.01	
Flower	*Aspergillus terreus*	*Aspergillus* Multiplex	25.26	
Flower	*Aspergillus terreus*	*Aspergillus* Multiplex	26.30	
Flower	*A. flavus, fumigatus, niger, terreus*	*Aspergillus terreus*	26.62	0.94
Flower	*A. flavus, fumigatus, niger, terreus*	*Aspergillus terreus*	26.81	
Flower	*A. flavus, fumigatus, niger, terreus*	*Aspergillus terreus*	27.12	

Flower	None	*Aspergillus terreus*	Not Detected	Not Applicable
Flower	None	*Aspergillus terreus*	Not Detected	
Flower	None	*Aspergillus terreus*	Not Detected	
Flower	*A. flavus, fumigatus, niger, terreus*	*Aspergillus* Multiplex	24.04	5.73
Flower	*A. flavus, fumigatus, niger, terreus*	*Aspergillus* Multiplex	23.68	
Flower	*A. flavus, fumigatus, niger, terreus*	*Aspergillus* Multiplex	21.59	

Robustness

Robustness should measure day to day variance and lab operator to lab operator variance for both Flower and MIPs and preferably itemize homogenization and DNA purification related variances from qPCR variance with spike in controls. A few examples are provided in Table 9,10,11.

Table 9, Sampling Repeatability:

Sample	qPCR Assay	Cq
Extract Sample 2	Total Coliform	21.07
Extract Sample 2	Total Coliform	20.93
Extract Sample 2	Total Coliform	20.80
Extract Sample 2	Total Coliform	20.99
Extract Sample 2	Total Coliform	21.07

Cq Percent RSD	**0.534**

Table 10, Sample Robustness:

Sample & Well	qPCR Assay	Cq
Extract Sample 1	Total Coliform	20.58
Extract Sample 2	Total Coliform	20.47
Extract Sample 3	Total Coliform	20.56
Extract Sample 4	Total Coliform	20.62
Extract Sample 5	Total Coliform	21.05
Extract Sample 6	Total Coliform	20.81

Cq Percent RSD	**1.034**

Table 11, User-to-User Robustness:

Sample	User	qPCR Assay	Cq
Extract 1	User 1	Total Yeast & Mold	23.95
Extract 1	User 2	Total Yeast & Mold	22.14
Extract 1	User 3	Total Yeast & Mold	21.90
Extract 2	User 1	Total Yeast & Mold	24.45
Extract 2	User 2	Total Yeast & Mold	24.59
Extract 2	User 3	Total Yeast & Mold	25.21
Extract 3	User 1	Total Yeast & Mold	32.13
Extract 3	User 2	Total Yeast & Mold	32.18
Extract 3	User 3	Total Yeast & Mold	33.70

Extract 1 (3 users)

Cq Percent RSD	4.94

Extract 2 (3 users)

Cq Percent RSD	1.63

Extract 3 (3 users)

Cq Percent RSD	2.73

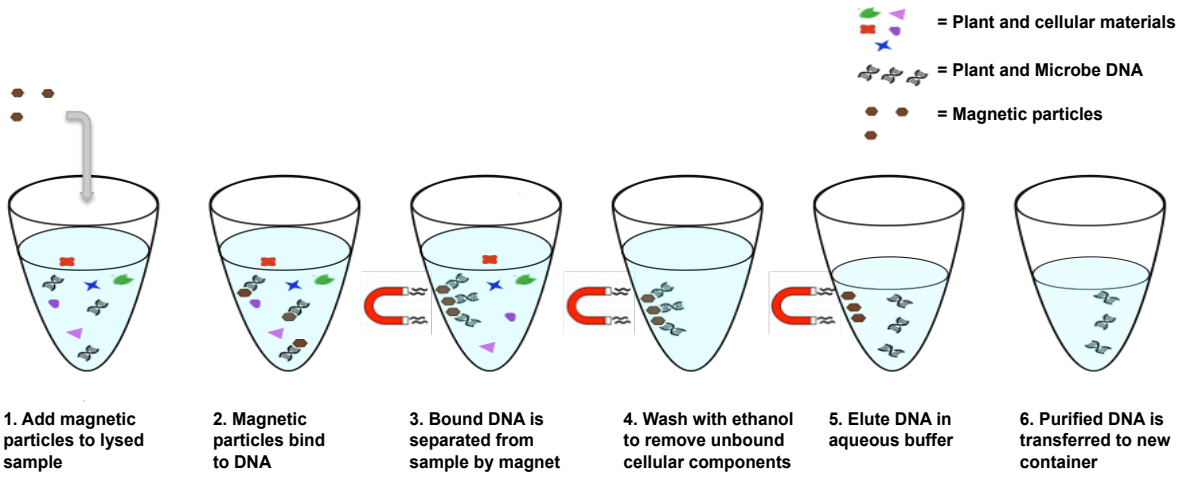
SenSATIVAX condensed Protocol

Introduction

SenSATIVAx™ is a proprietary DNA isolation process that uses magnetic particles to isolate and purify both plant and microbial DNA from a raw, homogenized plant sample. This approach is designed for ease of use and minimal requirement of laboratory equipment. Large centrifuges have been replaced with lightweight mini-fuges, magnetic particles, and magnets. The use of magnetic particles affords 8 tip or 96 tip automation, enabling both minimal entry costs and high throughput applications. DNA can be isolated from a single sample or a large batch in under 1 hour. Hands-on time is less than 45 minutes.

To enable minimal laboratory overhead, all organic solvents have been replaced with non-caustic reagents and 70% EtOH. Magnet plates are available for purchase from Medicinal Genomics (part #420202).

Process Overview



Kit Specifications

The SenSATIVAx™ Plant/Microbial DNA Purification Kit contains 200 reactions (Medicinal Genomics #420001) or 1000 reactions (Medicinal Genomics #420206) worth of reagents.

Materials Supplied in the Kit

- MGC Lysis Buffer (Store at Room Temperature, 20°C to 28°C)
- MGC Binding Buffer (Store at 2-8°C)
- MGC Elution Buffer (Store at Room Temperature, 20°C to 28°C)

Materials Supplied by the User:
Consumables & Hardware:

- Whirl-Pak bags (Nasco #B01385WA)
- Solo Cups or Beaker (optional)
- MGC Enrichment Broth, store at 2°C-8°C (Medicinal Genomics #420205)
- 1.5 mL Eppendorf tubes (Multiple Suppliers)
- 96 well plate magnet (Medicinal Genomics #420202)
- 96 well extraction plate (Perkin Elmer #6008290)
- Adhesive optical seal for qPCR plates (Bio-Rad Microseal® # MSB-1001 or USA Scientific TempPlate® RT Optical Film # 2978-2100)
- Multi channel pipettes P20 and P300, or P50 and P1000 (optional)
- Single channel pipettes P20, P200, & P1000
- Filtered pipette tips for P20, P50, P200, & P1000
- Eppendorf tube rack
- Scientific scale (milligram)
- Incubator, that can reach 37°C (VWR® Personal Size Incubator # 97025-630, or similar)

- Table top mini tube centrifuge (VWR® Mini Centrifuge #10067-588 or 6-place personal microcentrifuge for 1.5/2.0 ml tubes # 2631-0006, or similar)

- Table top Vortex Genie (Scientific Industries #SI-0236 or Similar)

Reagents:

- 10% Bleach
- 70% Ethanol (EtOH) (American Bioanalytical product # AB00844-01000)

Extraction #1 Protocol (CFU Threshold Assays):

1. Begin with a 10% bleach wipe down of the workspace, including the bench top and all equipment being used.

2. Remove the MCG Binding Buffer and the MGC Enrichment Broth from the 2-8°C refrigerator (it should come to room temperature before use).

3. Before weighing out the sample to be tested, make sure that the entire sample is broken up and thoroughly homogenized. A well-homogenized sample will ensure more accurate testing.

4. Label a new Whirl-Pak bag with the "[sample name] [date]". After homogenization, weigh out **0.22-0.28g,** and put into the labeled Whirl-Pak bag. Make sure to add all of the sample material to one side of the mesh layer inside the Whirl-Pak bag. If processing multiple plant samples, be sure to change gloves between each, to ensure there is no cross contamination of flowers during the weighing process.
    a. Add **3.55mL** of MGC Enrichment Broth to Whirl-Pak bag.
    Note: MGC Enrichment Broth is a growth medium and the perfect condition for microbes to grow. Due to this, it is best to pour the approximate amount of MGC Enrichment Broth into another sterile tube or container as to not contaminate the whole bottle. Nothing should go into this bottle. Return it to the 2-8°C refrigerator immediately after use.
        i. Close the Whirl-Pak bag by folding the top over three times.
    b. Mix the homogenized plant material in MGC Enrichment Broth for at least **1 minute** with your fingers, one sample at a time.

5. Prepare and label a 1.5mL tube with the "[sample name] [date]". Aspirate **285 µL** from the side of the filter bag, free of plant debris, and dispense into the 1.5mL tube.

a. If using a presence/absence test (E.coli, Salmonella or Aspergillus), save and incubate the Whirl-Pak bag at 37°C for **16-24 hours**, and proceed to the Extraction #2 set up. Incubate a full 24 hours for Aspergillus testing.

6. Add **15µL** of MGC Lysis buffer to the 285µL sample.
   a. Vortex for 5 seconds, and incubate on the bench for **2 minutes.**

7. After 2 minute incubation, spin for at least **30 seconds** in a bench top mini centrifuge.

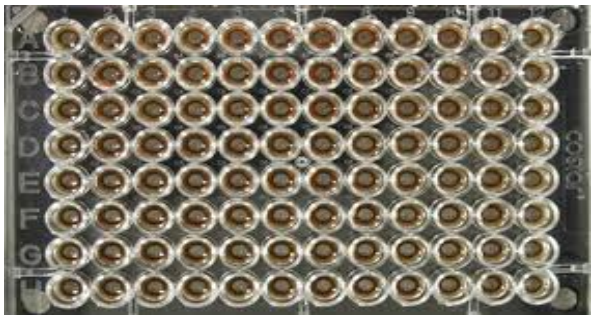
8. Remove the **200**µL of supernatant from the 1.5ml tube containing the centrifuged sample, being careful not to disturb the pellet at the bottom of the tube. Place the 200µL in a labeled 96 well extraction plate labeled with "Extraction Plate Day1 [date]" or "Extraction Plate Day2 [date]".
   Note: Pellet size will vary depending on trichome density.

9. Vortex MGC Binding Buffer thoroughly before use, be sure that the magnetic particles are completely
   re-suspended in buffer at least 30 seconds.

10. Add **200µL** of MGC Binding Buffer (this liquid is very viscous) to the 200µL sample, and pipette tip mix 15 times.
    a. Incubate the plate on the bench for at least **5 minutes**.
    Note: Be careful to avoid adding too many bubbles by pipetting gently when tip mixing. This is extremely important as to not contaminate the wells in proximity.

11. Place the extraction plate onto the 96 well plate magnet plate for at least **5 minutes**.

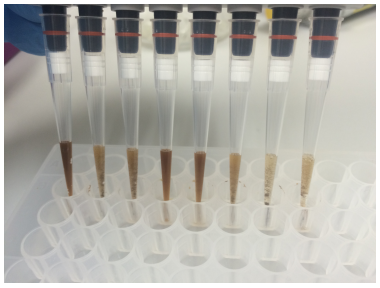
12. After 5 min incubation, remove as much of the 400ul of the supernatant as possible. Be careful not to disturb or aspirate the beads.
    a. Add **400µL** of 70% ethanol (EtOH) with the extraction plate still on the magnet plate.
    b. Wait at least **30 seconds,** and remove all the EtOH.
    Note: Take the pipet tip to the bottom center of the well to remove liquid.



13. Again, add **400µL** of 70% EtOH with the extraction plate still on the magnet plate. Wait at least **30 seconds** and remove all the EtOH.
    Note: If EtOH still remains in the wells, go back in with a smaller pipet tip to remove the excess.

14. After all the EtOH has been removed let the beads dry at room temperature on the magnet plate for at least **15 minutes**. Be sure to remove all EtOH, as any leftover can inhibit qPCR results.

15. Remove the extraction plate from the magnet plate, and add **50µL** of MGC Elution Buffer.
    a. Tip mix approximately 15 times or until the beads are completely re-suspended.
    Note: The re-suspensions may appear varied in their appearance, but the result will be the same.



    b. Incubate the plate for at least **1 minute** on the bench before returning the plate to the magnet plate.
    c. Let the plate sit on the magnet for at least **1 minute** before transferring the eluent to a new extraction plate labeled with "Final Extract Day 1 [date]" or "Final Extract Day 2 [date]".
    Note: To save space and consumables, both day 1 and day 2 extracts can be stored in separate wells on the same extraction plate.

16. Seal the plate with the adhesive seal, making sure to completely seal the plate wells using a pen or flat object to slide back and forth along the seal. Store at -20°C until ready to perform qPCR protocol.

<u>Extraction #2 Protocol (Presence/Absence)</u>

After an **16-24 hour** incubation at 37°C has occurred remove **285µL** from the side of the filter bag free of plant debris, and put into a 1.5mL tube and repeat steps 5-16.

Note: If using the Aspergillus Specific Detection Assays, you must incubate for the full 24 hours to insure proper growth.

PCR Conditions

This PCR method utilizes a published decontamination protocol known as D.R.E.AM PCR [118, 119]. DREAM PCR utilizes a low concentration of 5-hydroxy methyl cytosine in PCR. This base can be targeted by 5-hydroxy methyl cytosine specific endonucleases like AbaSI to specifically digest only PCR amplified DNA and thus decontaminate a laboratory of post PCR products. The method is analogous to the use of uracil in PCR but more compatible for sequencing with uracil-illiterate next generation sequencers.

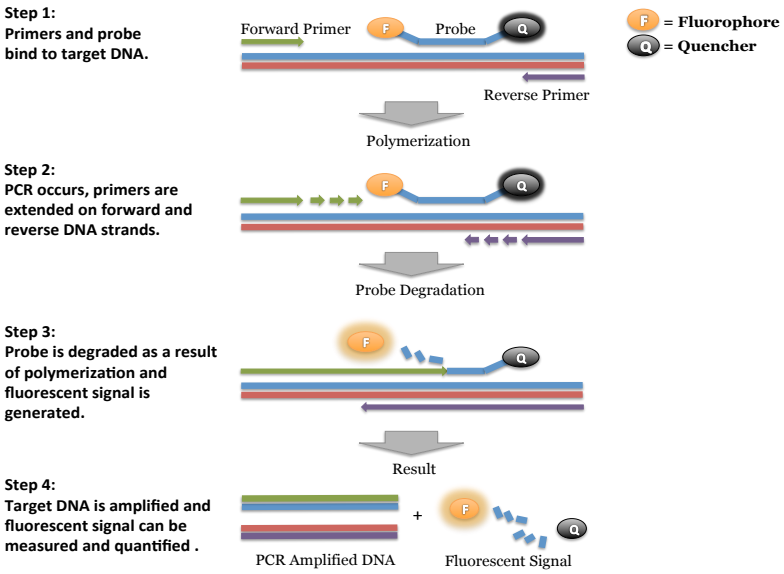
PathoSEEK™ Microbial Safety Testing Platform utilizes a novel, contamination-free, PCR-based assay and provides an internal plant DNA control for every reaction. It is a simple two-step protocol, which is flexible and automation compatible.

PathoSEEK™ microbial detection assays use a multiplexing strategy with an internal plant DNA reaction control to ensure accurate detection of microbial species for every reaction. Unlike other

techniques, this multiplexing strategy verifies the performance of the assay when detecting pathogens, resulting in the minimization of false negative results due to reaction set-up errors or failing experimental conditions.

Process Overview

The PathoSEEK™ process includes Real-time quantitative PCR assays using a multiplex system of primers to detect potential pathogens within the plant, extract or MIP (Marijuana Infused Product) sample. Below is a simplified depiction of the qPCR assays. The forward and reverse primers have universal primer tails to enable potential Next Generation Sequencing of resulting products.



Kit Specifications

The qPCR Master Kit contains 125 reactions (Medicinal Genomics # 420002). Each PathoSEEK™ Detection Assay Probe Mix contains 200 reactions. Each PathoSEEK™ Positive Control contains 60 reactions.

The Master Mix is manufactured in a GMP facility by New England Biolabs as a 5X solution.

1X Solution

60mM Tris-SO4
20mM (NH4)2SO4
5mM MgSO4
3% Glycerol
0.6mM dATP
0.6mM dTTP
0.6mM dCTP
0.6mM dGTP
0.8mM Hydroxymethyl dCTP
125 units/mL LongAmp Hot Start Taq DNA Polymerase (pH 9.1 @25C)

Materials Supplied in the Kit

qPCR Master Kit, store at -15 to -20°C upon arrival [Medicinal Genomics #420002].

- Reaction Buffer (10x)
- Decontamination Enzyme (10 Units/µL) – (Not used in this protocol)
- qPCR Master Mix (5x)
- Nuclease Free Water

PathoSEEK™ Detection Assays and Positive Controls, ordered separately, store at -15°C to -20°C upon arrival.

- Salmonella and *E.coli* Multiplex Detection Assay (Medicinal Genomics #420113)
  - Salmonella and *E.coli* Positive Control (Medicinal Genomics #420313)
- Salmonella and Shiga Toxin producing *E.coli* (STEC) Detection Assay (Medicinal Genomics #420122)
  - Salmonella and Shiga Toxin producing *E.coli* (STEC) Positive Control (Medicinal Genomics #420322)
- Yeast & Mold Detection Assay (Medicinal Genomics #420103)
  - Yeast & Mold Positive Control (Medicinal Genomics #420303)
- Total Aerobic Count (TAC) Detection Assay (Medicinal Genomics #420106)
  - Total Aerobic Count (TAC) Positive Control (Medicinal Genomics #420306)
- Total Enterobacteriaceae and Coliform Detection Assay (Medicinal Genomics #420114)
  - Total Enterobacteriaceae and Coliform Positive Control (Medicinal Genomics #420314)
- *Aspergillus niger* Detection Assay (Medicinal Genomics #420109)
  - *Aspergillus niger* Positive Control (Medicinal Genomics #420309)
- *Aspergillus flavus* Detection Assay (Medicinal Genomics #420111)
  - *Aspergillus flavus* Positive Control (Medicinal Genomics #420311)

- *Aspergillus fumigatus* Detection Assay (Medicinal Genomics #420110)
    - *Aspergillus fumigatus* Positive Control (Medicinal Genomics #420310)
- *Aspergillus terreus* Detection Assay (Medicinal Genomics #420129)
    - *Aspergillus terreus* Positive Control (Medicinal Genomics #420329)
- *Aspergillus* Multiplex Detection Assay (Medicinal Genomics #420004)
    - Assay Control: Use any of the four available Aspergillus Controls
- Single Copy Control Gene (SCCG) Positive Control (Medicinal Genomics #420326, for use with Medicinal Genomics SenSATIVAx for MIP/Extract DNA Extraction Kit)

Materials Supplied by the User

Consumables & Hardware

- Agilent AriaMx Real-Time PCR System G8830A Option 010 FAM, ROX, and HEX (Contact Agilent)
- Agilent HP 650 Notebook PC option 650 (Contact Agilent)
- 96 well optical qPCR plates (Agilent AriaMx 96 well plates, Agilent # 401490, 401491, or 401494 or Fisher Scientific 96-Well Armadillo PCR Plate, Fisher # AB2396)
- Adhesive optical seal for qPCR plates (Agilent adhesive plate seals, Agilent # 401492 or USA Scientific TempPlate® RT Optical Film # 2978-2100)
- Multi-channel pipette P50 or P20 (optional)
- Single channel pipette P10, P20 and P200
- Filtered pipette tips for P10, P20, P50, and P200
- Crushed ice or cold racks (96 well PCR Cryogenic Rack, VWR #89004-570 and 1.5µL Tube Benchtop Cryogenic Racks, VWR #89004-558 or similar)
- Freezer, -20°C
- Table top mini plate centrifuge (Fisher Scientific #14-100-143 or similar)

- Table top mini tube centrifuge (VWR® Mini Centrifuge #10067-588 or 6-place personal microcentrifuge for 1.5/2.0 ml tubes # 2631-0006, or similar)

- Table top Vortex Genie (Scientific Industries #SI-0236 or Similar)

Reagents

- 10% bleach

Real-Time Quantitative PCR (qPCR) Protocol

1. **Using the 10% bleach solution, wipe down the workspace, including the bench top and all equipment being used (except the Agilent AriaMX Instrument).**
2. **Remove qPCR reagents including qPCR Master Mix, water, reaction buffer and assay probe mixes to be used from the -20°C freezer. Place qPCR master mix on ice. Allow remaining tubes to thaw at room temperature. Once thawed, immediately place tubes on ice**
3. **Remove positive control tubes needed from -20°C freezer. Allow tubes to thaw at room temperature. Once thawed, place tubes on ice.**

4. **Before preparing the reaction, invert or vortex and spin-down the reagents.**
   - **4.1. Assay probe mix tubes, reaction buffer, positive controls and water – Vortex quickly followed by a pulse spin-down in a microcentrifuge.**
   - **4.2. qPCR Master Mix – Invert the tube 5 times (do not vortex), followed by a pulse spin-down in a microcentrifuge.**
   - **4.3. Return all reagents to the ice.**

     Note: Do not vortex the qPCR Master Mix at any point during the protocol.

5. **Make a separate master mix in a 1.5mL tube for each assay type being run. All probe mixes contain the internal plant control, SCCG probe mix, and the probe for the microbial targets. Label each tube with [Assay Name] MM. Always prepare enough master mix for 1 or 2 additional reactions over the total number of tests to account for pipetting and dead volumes.**

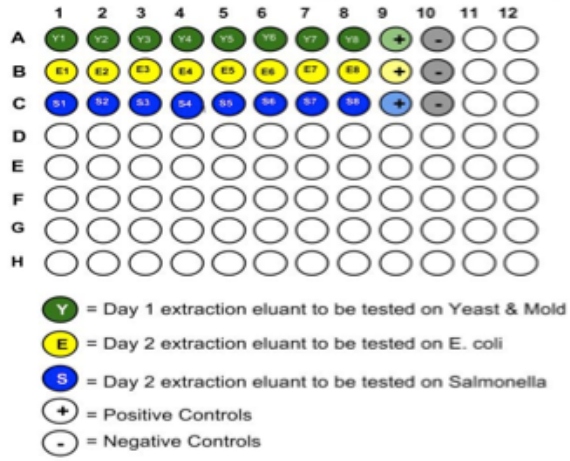
   Note: *It is best to add the largest volume reagent first, in this case water.*

Reagents	1 Reaction	24 reactions (plus 1 excess rxn)	48 reactions (plus 2 excess rxn)
qPCR Master Mix	3.75µL	93.75µL	187.5µL
Assay Probe Mix (Assay Specific)	1µL	25µL	50µL
Reaction Buffer	0.8µL	20µl	40µl
Water	8.2	205µL	410µL
Total	13.75µL	343.75µL	687.5µL

   - **5.1. Once combined gently tip mix or invert the tube 5 times to combine the assay master mix.**
     - 5.1.1. **Pulse spin-down tube in microcentrifuge.**
     - 5.1.2. **Place MM tubes on ice until used.**
     - 5.1.3. **For the positive control(s), make a 1:10 dilution of each assay being run**
       - **5.1.3.1. 1µL of Positive Control dilute with 9µL of water (found in the kit)**
       - **5.1.3.2. For the negative control, use water (found in the kit).**

     Note: It is best to add the largest volume reagent first, in this case the 9 µL water then the 1 µL of positive control, pipette mix well to ensure control DNA is in solution

Below is an example plate setup. This will vary depending on which assays are being tested.



6. **Place the Extraction Plate on the magnet. This is to ensure no magnetic beads are transferred into the qPCR reactions if there are some left over from the extraction elution process.**
7. **Use a new 96-well optical qPCR plate and label the plate "qPCR Plate_[date]".**
8. **Carefully remove the seal from the Extraction Plate and transfer 5µL of each sample into the corresponding well on the qPCR plate. Keep the extraction plate on the magnet when aspirating the 5µL.**
   - **8.1. Add 5µL of the diluted Positive Controls to their corresponding wells. Then add 5µL of water to the corresponding Negative Control wells.**

   Note: ALWAYS use a fresh tip for every liquid transfer into the qPCR plate

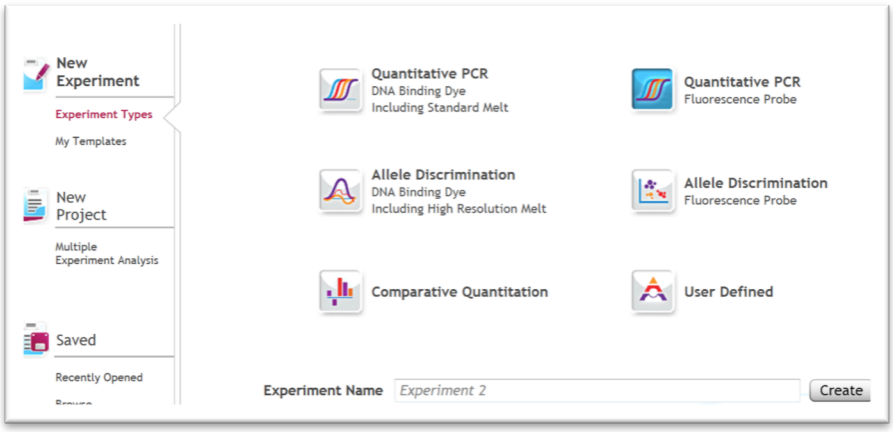
9. **Add 13.75µL of specific Assay Probe MM to each corresponding sample well, positive control well, and negative control well in the qPCR plate. Gently tip mix a few times after each addition of qPCR master mix. Be careful to not introduce bubbles during this mix.**

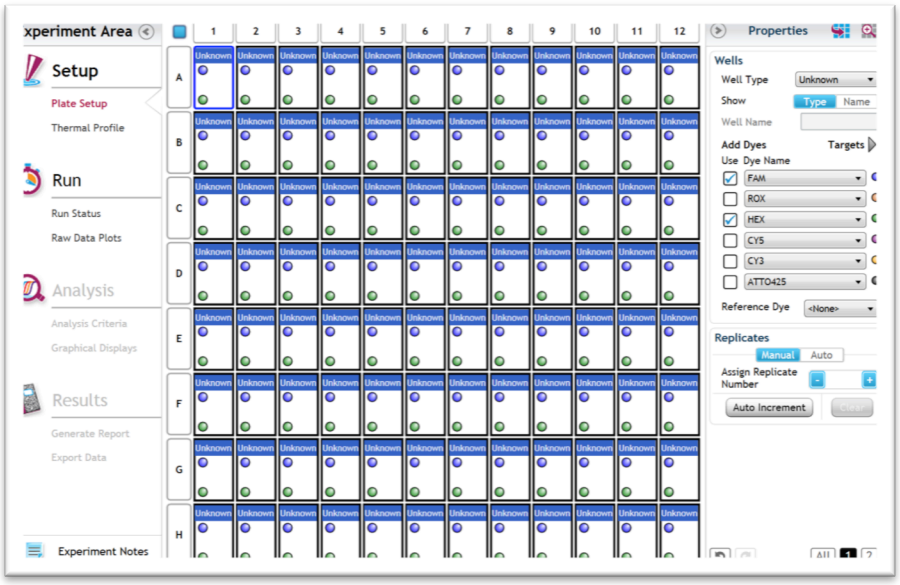
   Note: *It may be helpful to label each of the corresponding column wells to accurately dispense the correct samples*

   - **9.1. Seal the plate with the adhesive seal, making sure to completely seal the plate wells using a pen or flat object to slide back and forth along the seal.**
10. **Spin-down for at least 1 minute in plate microcentrifuge.**

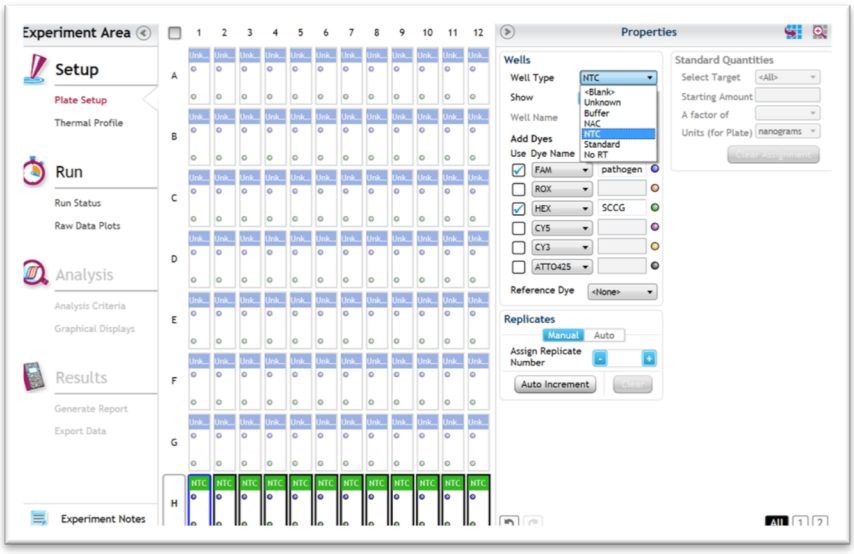
    Note: *Check for bubbles at the bottom of the wells (minimal bubbles on the surface of the liquid is acceptable). If bubbles remain in the bottom of the wells, spin-down for another minute.*

11. **Place the sealed plate onto the Agilent AriaMX instrument, positioning the A1 well in the top left corner**
12. **Create a New Experiment on the Agilent qPCR instrument.**
    - 12.1.1. **Select "Quantitative PCR" from Experiment Types. Under Setup>Plate Setup, select FAM, HEX and ROX channel collection. ROX is only necessary if running multiplexed assays, *E. coli*/Salmonella or Coliform/Entero.**



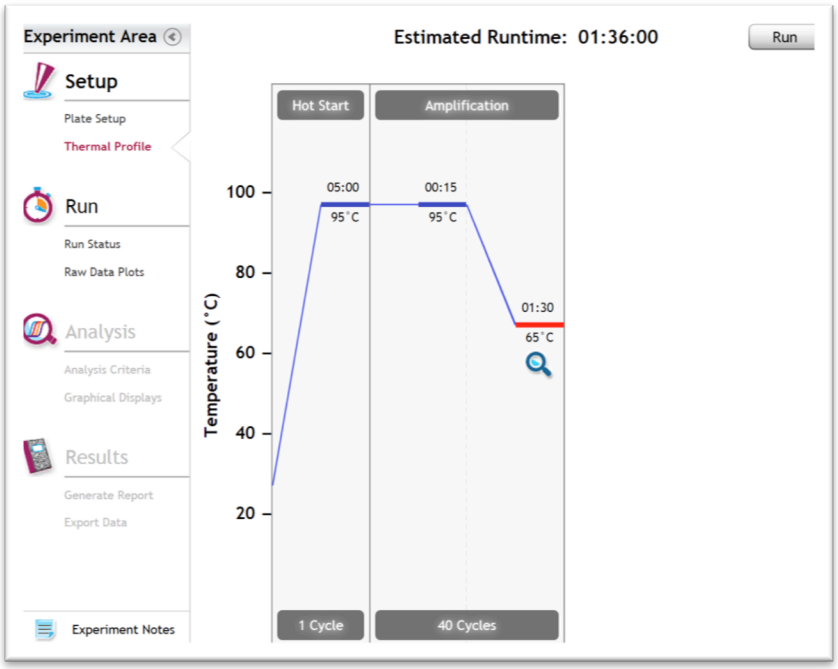


12.2. Change the well types to reflect your plate set up. Add Target names to include "pathogen name" for FAM or ROX and SCCG (single copy control gene) for HEX.



12.3. Under Setup>Thermal Profile, create the following PCR thermal profile.

- Hot start at 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds and 65°C for 90 seconds.



12.4. Close the lid and click "Start Run".
12.5. Save the experiment with the [User] and [date]
12.6. When run is complete, immediately dispose of the plate. Do not open the plate seal after the run to avoid contamination in the lab.

Glossary and Definitions

Deoxyribonucleic acid (DNA) is a molecule that encodes the genetic instructions used in the development and functioning of all known living organisms.

Polymerase Chain Reaction (PCR) is a technology in molecular biology used to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

A **fluorophore** is a fluorescent chemical compound that can re-emit light upon light excitation.

The **Negative Controls** are the reactions where no Cq is expected. It helps to ensure that all Assay specific reactions are clean of contaminates.

The assay specific **Positive Controls** are the reactions where a Cq is expected. It helps ensure that all Assay specific reactions are working correctly. The Assay specific Positive Control is targeting the pathogen using the FAM flourophore.

The **Internal Control** is added to every sample reaction where a Cq is expected. It ensures the effectiveness and efficiency of each reaction. The internal control is targeting a Single Copy Control Gene or SCCG, using the HEX flourophore.

Troubleshooting Guide:

Symptom	Reason	Solution
Clumpy/Grainy Beads	Over-manipulation of plant with MGC Enrichment Broth	Over manipulation of the plant can cause the release of extra cellular debris therefore clogging the beads with extra material. To ensure this does not occur, only manipulate the plant material for 1 minute.
	Too many tricomes and/or insufficient spinning	Some plants produce more tricomes than others resulting in carry-over into extraction plate. To ensure this doesn't happen, it may be necessary to spin the tube for longer than the recommended 30 seconds. Also, be sure not to disturb the pellet. If the pellet is disturbed or tricomes are still visible, re-centrifuge the tube and try again.
Bead Loss	Insufficient time on the magnet	Make sure the supernatant has fully cleared before removing. Failure to do so will result in bead loss, which will result in DNA loss.
	Insufficient pipetting	Make sure no beads are aspirated during supernatant removal; dispense back supernatant, and attempt again with a smaller volume after beads have re-settled..
Extra elution volume	Insufficient removal of Ethanol	Make sure ALL ethanol is removed before drying. This may require a second or third aspiration. Carry-over ethanol can cause inhibition in qPCR.

PathoSEEK condensed Protocol

PathoSEEK™ Analysis Quick Reference Tables:

FLOWER

Table 1: Flower Samples ONLY – No Decontamination Step

PathoSEEK™ Assay	Cq Value (High CFU count)	Fluor	Negative Control (Cq)	CFU threshold (CFU/g)
Aspergillus	≤ 40	FAM	> 40	Presence/Absence
E. coli	≤ 40	FAM	> 40	Presence/Absence
STEC *E. coli*	≤ 40	FAM	> 40	Presence/Absence
Salmonella	≤ 40	TXR	> 40	Presence/Absence
Total Aerobic Count	≤ 20.7	FAM	> 30	100,000 (10^5)
Total Coliform	≤ 30.5	FAM	> 40	1,000 (10^3)
Total Enterobacteriaceae	≤ 28.1	TXR	> 40	1,000 (10^3)
Total Yeast and Mold	≤ 24.2	FAM	> 40	10,000 (10^4)
Internal Control*	≤35	HEX	*Internal control verifies the presence or absence of plant DNA	
Assay Positive Controls	≤35	FAM/TXR		

NON – FLOWER Matrices

Table 2: All Concentrates, MIP Samples – No Decontamination Step
(Except gummy, see table 3)

PathoSEEK™ Assay	Cq Value (High CFU count)	Fluor	Negative Control (Cq)	CFU threshold (CFU/g)
Aspergillus	≤ 40	FAM	> 40	Presence/Absence
E. coli	≤ 40	FAM	> 40	Presence/Absence
STEC *E. coli*	≤ 40	FAM	> 40	Presence/Absence
Salmonella	≤ 40	TXR	> 40	Presence/Absence
Total Aerobic Count	≤ 25.2	FAM	> 35	10,000 (10^4)
Total Coliform	≤ 34.7	FAM	> 40	100 (10^2)
Total Enterobacteriaceae	≤ 31.8	TXR	> 40	100 (10^2)
Total Yeast and Mold	≤ 37.4	FAM	> 40	1000 (10^3)

Internal Control*	≤40	HEX	*Internal control verifies the presence or absence of spiked plant positive control (SCCG)
Assay Positive Controls	≤35	FAM/TXR	

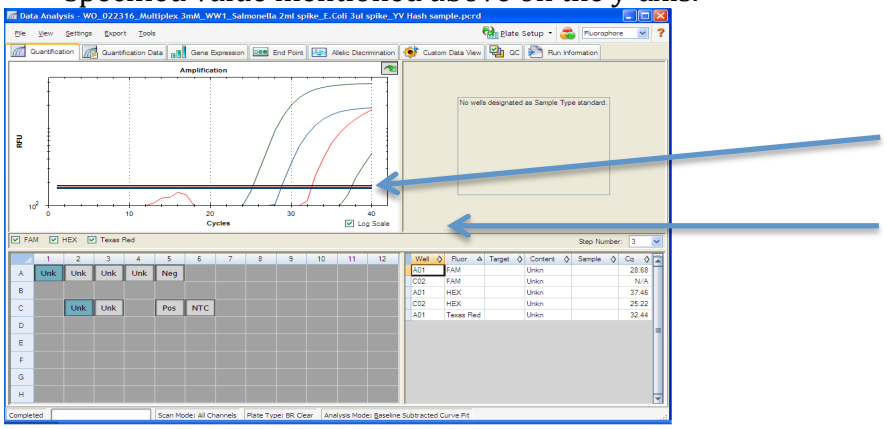
GUMMY

Table 3: Gummy – No Decontamination Step

PathoSEEK™ Assay	Cq Value (High CFU count)	Fluor	Negative Control (Cq)	CFU threshold (CFU/g)
Aspergillus	≤ 40	FAM	> 40	Presence/Absence
E. coli	≤ 40	FAM	> 40	Presence/Absence
STEC *E. coli*	≤ 40	FAM	> 40	Presence/Absence
Salmonella	≤ 40	TXR	> 40	Presence/Absence
Total Aerobic Count	≤ 27.8	FAM	> 35	10,000 (10^4)
Total Coliform	≤ 40	FAM	>40	100 (10^2)
Total Enterobacteriaceae	≤ 34.9	TXR	> 40	100 (10^2)
Total Yeast and Mold	≤ 38.1	FAM	> 40	1000 (10^3)
Internal Control*	≤35	HEX	*Internal control verifies the presence or absence of spiked plant positive control (SCCG)	
Assay Positive Controls	≤35	FAM/TXR		

Detailed Assay Data Analysis

1. **Presence / Absence Multiplex Assay: *E. coli* & Salmonella**
   - **1.1.** The Data Analysis window will open automatically when the run is complete.
   - **1.2.** Highlight well(s) of interest.
     - The graph will appear above.
     - The Cq values will appear to the right.
   - **1.3.** To analyze the results
     - Start by turning the graph to Log Scale and manually moving the threshold lines to 10^2 for the FAM and TEXAS RED fluorophores. Also manually move the threshold line for the HEX fluorophore half way between 10^2 and 10^3.
       - To turn the graph to Log Scale, click on the box at the bottom right of the graph.
       - To adjust the threshold, click on the horizontal lines, and move them to the specified value mentioned above on the y-axis.



     - Controls
       - Assay-specific Positive Controls, on the FAM and TEXAS RED fluorophores, has a Cq value ≤ 35.
         - Visually confirm with the curve on the graph.
       - Assay-specific Negative Control, on the FAM and TEXAS RED fluorophores, has no Cq value.
         - Visually confirm with the curve on the graph.
     - Unknown *E. coli* Target **(FAM fluorophore detects *E. coli*)**
       - Internal Control, on the HEX fluorophore, has a Cq value ≤ 35 for flower samples, ≤ 40 for all other matrices.
         - Visually confirm with the curve on the graph
       - A "presence" result for the unknown *E. coli* target.
         - Any Cq value for the FAM fluorophore ≤ 40.
         - Visually confirm with the curve on the graph.
           - It is very important to confirm with the amplification curve when a presence result occurred. Sometimes the background amplification will give a false positive reading, especially when Cq reading is less than 15. (See troubleshooting guide below for more details.)
       - An "absence" result for the unknown *E. coli* target.
         - No Cq value for the FAM fluorophore.
         - Visually confirm no curve on the graph.

- Unknown Salmonella Target **(TEXAS RED fluorophore detects Salmonella)**
    - Internal Control, on the HEX fluorophore, has a Cq value ≤ 35 for flower samples, ≤ 40 for all other matrices.
        - Visually confirm with the curve on the graph
    - A "presence" or failing result for the unknown Salmonella target.
        - Any Cq value for the TEXAS RED fluorophore ≤ 40.
        - Visually confirm with the curve on the graph.
            - It is very important to confirm with the amplification curve when a presence result occurred. Sometimes the background amplification will give a false positive reading, especially when Cq reading is less than 15. (See troubleshooting guide below for more details.)
    - An "absence" or passing result for the unknown Salmonella target.
        - No Cq value for the TEXAS RED fluorophore.
        - Visually confirm no curve on the graph.

2. **Presence / Absence Singleplex and Multiplex Assay: Aspergillus (all species specific and multiplex assays)**

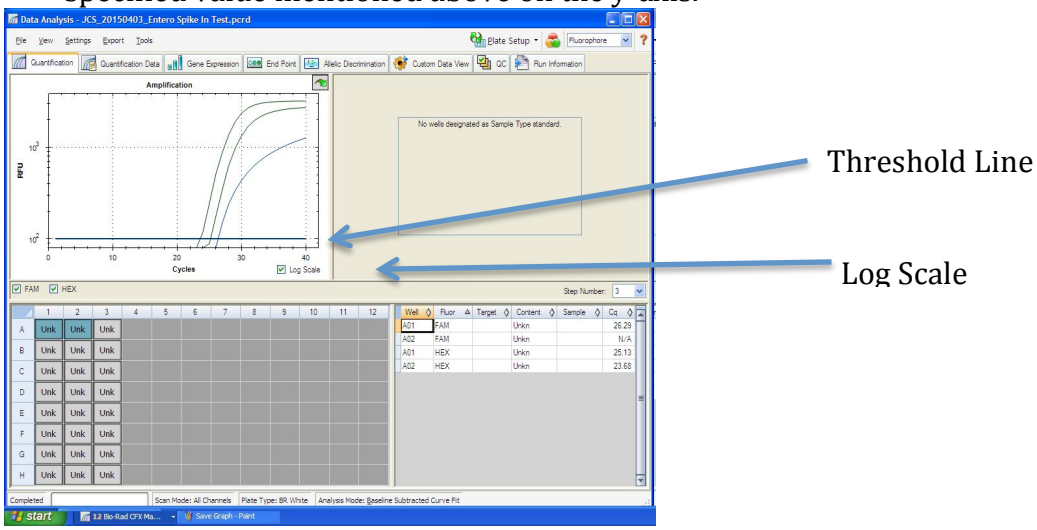
2.1. The Data Analysis window will open automatically when the run is complete.

2.2. Highlight the well of interest.

- The graph will appear above.
- The Cq values will appear to the right.

2.3. To analyze the results

- Start by turning the graph to Log Scale and manually moving the threshold to 10^2 for the FAM fluorophore. Also manually move the threshold for the HEX fluorophore half way between 10^2 and 10^3.
    - To turn the graph to Log Scale, click on the box at the bottom right of the graph.
    - To adjust the threshold, click on the horizontal lines, and move them to the specified value mentioned above on the y-axis.



- Controls
    - Assay-specific Positive Control, on the FAM fluorophore, has a Cq value ≤ 35.
        - Visually confirm with the curve on the graph.
    - Assay-specific Negative Control, on the FAM fluorophore, has no Cq value.

        - Visually confirm with the curve on the graph.
- Unknown Aspergillus Target **(FAM fluorophore detects all 4 Aspergillus species in multiplex assay)**
    - Internal Control, on the HEX fluorophore, has a Cq value ≤ 35 for flower samples, ≤ 40 for all other matrices.
        - Visually confirm with the curve on the graph
    - A "presence" or failing result for the unknown Aspergillus target.
        - Any Cq value for the FAM fluorophore ≤ 40.
        - Visually confirm with the curve on the graph.
            - It is very important to confirm with the amplification curve when a presence result occurred. Sometimes the background amplification will give a false positive reading, especially when Cq reading is less than 15. (See troubleshooting guide below for more details.)
    - An "absence" or passing result for the unknown Aspergillus target.
        - No Cq value for the FAM fluorophore.
        - Visually confirm no curve on the graph.

3. CFU Threshold Assay: Total Aerobic Count

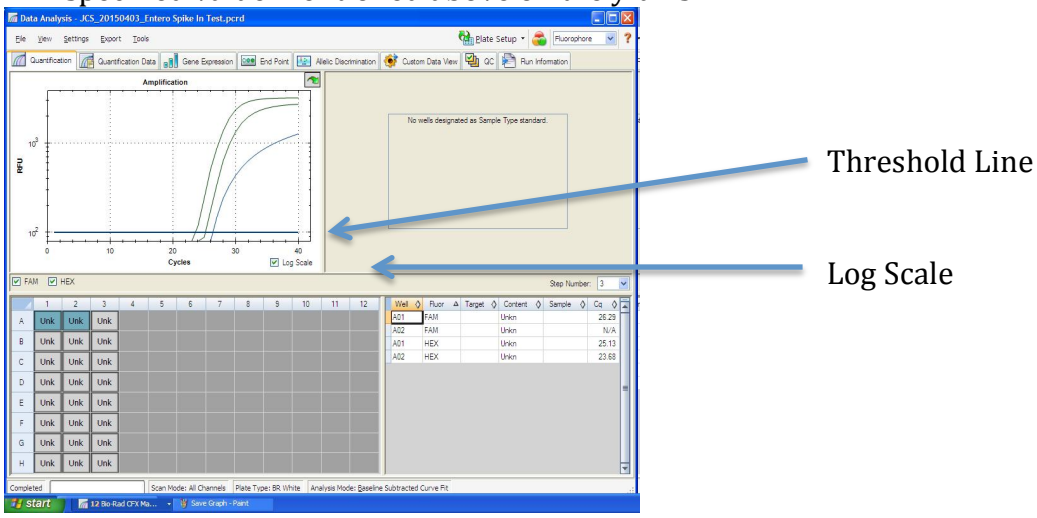
3.1. The Data Analysis window will open automatically when the run is complete.

3.2. Highlight the well of interest.

- The graph will appear above.
- The Cq values will appear to the right.

3.3. To analyze the results.

- Start by turning the graph to Log Scale and manually moving the threshold to 10^2 for the FAM fluorophore. Also manually move the threshold for the HEX fluorophore half way between 10^2 and 10^3.
    - To turn the graph to Log Scale, click on the box to the bottom right of the graph.
    - To adjust the threshold, click on the horizontal lines, and move them to the specified value mentioned above on the y-axis.



- Controls
    - Assay-specific Positive Control, on the FAM fluorophore, has a Cq value ≤ 35
        - Visually confirm with the curve on the graph.

- Assay-specific Negative Control, on the FAM fluorophore, has a Cq value of > 30 or no Cq value.
    - Visually confirm with the curve on the graph.
- Unknown Aerobic Count Target **(FAM fluorophore detects Total Aerobic Count Bacteria)**
    - Internal Control, on the HEX fluorophore, has a Cq value ≤ 35 for flower samples, ≤ 40 for all other matrices.
        - Visually confirm with the curve on the graph.
    - A high CFU count result for the unknown TAC target.
        - **Passing Sample Result:** Check Cq Value on the FAM Fluorophore. See Tables 1-3 for Cq cutoff value depending on matrix being tested.
        - Visually confirm with the curve on the graph
            - It is very important to confirm with the amplification curve when a high CFU count occurred. Sometimes the background amplification will give a false positive reading, especially when Cq reading is less than 15. (See troubleshooting guide below for more details.)
    - A low CFU count result for the unknown TAC target.
        - **Failing Sample Result:** Check Cq Value on the FAM Fluorophore. See Tables 1-3 for Cq cutoff value depending on matrix being tested.
        - Visually confirm with the curve on the graph.

4. CFU Threshold Assay: Total Yeast & Mold

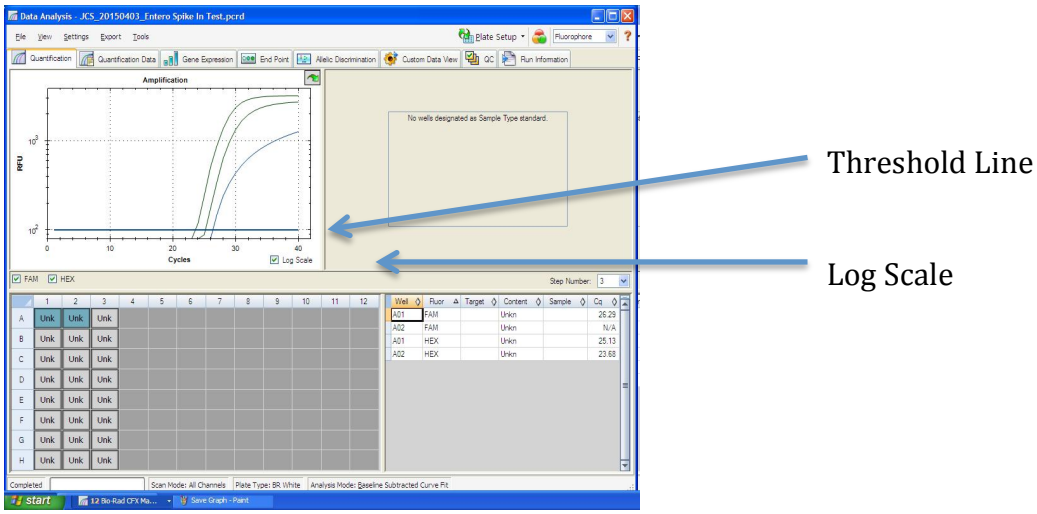
4.1. The Data Analysis window will open automatically when the run is complete.

4.2. Highlight the well of interest.

- The graph will appear above.
- The Cq values will appear to the right.

4.3. To analyze the results.

- Start by turning the graph to Log Scale and manually moving the threshold to 10^2 for the FAM fluorophore. Also manually move the threshold for the HEX fluorophore half way between 10^2 and 10^3.
    - To turn the graph to Log Scale, click on the box to the bottom right of the graph.
    - To adjust the threshold, click on the horizontal lines, and move them to the specified value mentioned above on the y-axis.



- Controls
  - Assay-specific Positive Control, on the FAM fluorophore, has a Cq value ≤ 35
    - Visually confirm with the curve on the graph.
  - Assay-specific Negative Control, on the FAM fluorophore, has no Cq value.
    - Visually confirm with the curve on the graph.
- Unknown Yeast and Mold Target **(FAM fluorophore detects Total Yeast & Mold)**
  - Internal Control, on the HEX fluorophore, has a Cq value ≤ 35 for flower samples, ≤ 40 for all other matrices.
    - Visually confirm with the curve on the graph.
  - A high CFU count result for the unknown Y&M target.
    - **Passing Sample Result:** Check Cq value on the FAM Fluorophore. See Tables 1-3 for Cq cutoff value depending on matrix being tested.
    - Visually confirm with the curve on the graph
      - It is very important to confirm with the amplification curve when a high CFU count occurred. Sometimes the background amplification will give a false positive reading, especially when Cq reading is less than 15. (See troubleshooting guide below for more details.)
  - A low CFU count result for the unknown Y&M target.
    - **Failing Sample Result:** Check Cq value on the FAM Fluorophore. See Tables 1-3 for Cq cutoff value depending on matrix being tested.
    - Visually confirm with the curve on the graph.

5. CFU Threshold Multiplex Assay: Total Coliform and Enterobacteriaceae

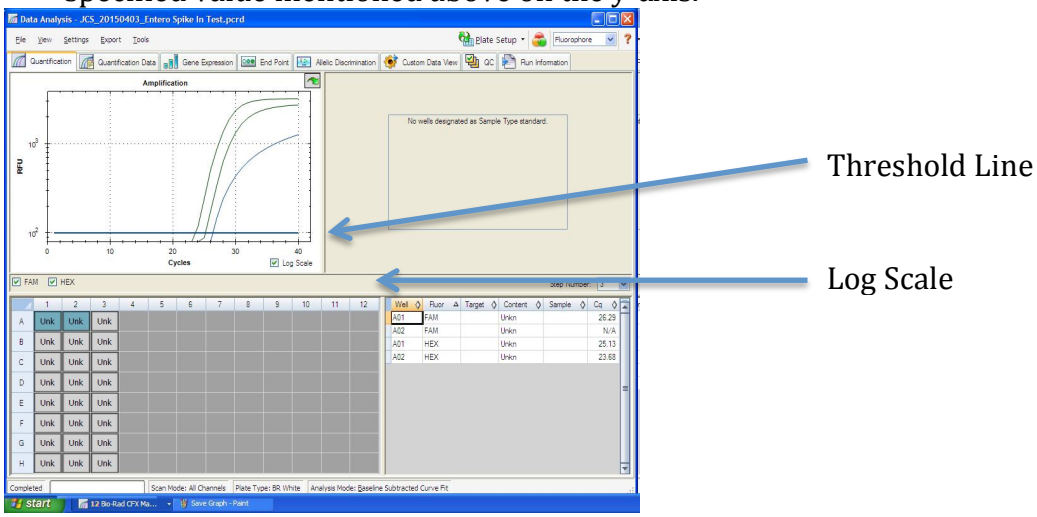
5.1. The Data Analysis window will open automatically when the run is complete.

5.2. Highlight the well of interest.

- The graph will appear above.
- The Cq values will appear to the right.

5.3. To analyze the results.

- Start by turning the graph to Log Scale and manually moving the threshold to 10^2 for the FAM and TEXAS RED fluorophore. Also manually move the threshold for the HEX fluorophore half way between 10^2 and 10^3.
    - To turn the graph to Log Scale, click on the box to the bottom right of the graph.
    - To adjust the threshold, click on the horizontal lines, and move them to the specified value mentioned above on the y-axis.



- Controls
    - Assay-specific Positive Control, on the FAM fluorophore, has a Cq value ≤ 35
        - Visually confirm with the curve on the graph.
    - Assay-specific Negative Control, on the FAM fluorophore, has no Cq value.
        - Visually confirm with the curve on the graph.
- Unknown Coliform Target (**FAM fluorophore detects Total Coliform)**
    - Internal Control, on the HEX fluorophore, has a Cq value ≤ 35 for flower samples, ≤ 40 for all other matrices.
        - Visually confirm with the curve on the graph.
    - A high CFU count result for the unknown coliform target.
        - **Passing Sample Result:** Check Cq Value on the FAM Fluorophore. See Tables 1-3 for Cq cutoff value depending on matrix being tested.
        - Visually confirm with the curve on the graph
            - It is very important to confirm with the amplification curve when a high CFU count occurred. Sometimes the background amplification will give a false positive reading, especially when Cq reading is less than 15. (See troubleshooting guide below for more detail.)
    - A low CFU count result for the unknown coliform target.
        - **Failing Sample Result:** Check Cq value on the FAM Fluorophore. See Tables 1-3 for Cq cutoff value depending on matrix being tested.
        - Visually confirm with the curve on the graph.

- Unknown Entero Target (**TEXAS RED fluorophore detects Entero)**

- Internal Control, on the HEX fluorophore, has a Cq value ≤ 35 for flower samples, ≤ 40 for non flower matrices.
    - Visually confirm with the curve on the graph.
- A high CFU count result for the unknown Entero target.
    - **Passing Sample Result:** Check Cq Value on the TEXAS RED Fluorophore. See Tables 1-3 for Cq cutoff value depending on matrix being tested.
    - Visually confirm with the curve on the graph
        - It is very important to confirm with the amplification curve when a high CFU count occurred. Sometimes the background amplification will give a false positive reading, especially when Cq reading is less than 15. (See troubleshooting guide below for more detail.)
- A low CFU count result for the unknown Entero target.
    - **Failing Sample Result:** Check Cq value on the TEXAS RED Fluorophore. See Tables 1-3 for Cq cutoff value depending on matrix being tested.
    - Visually confirm with the curve on the graph.

Table 4, Cq to CFU Conversion Equation Table (No Decontamination Step)

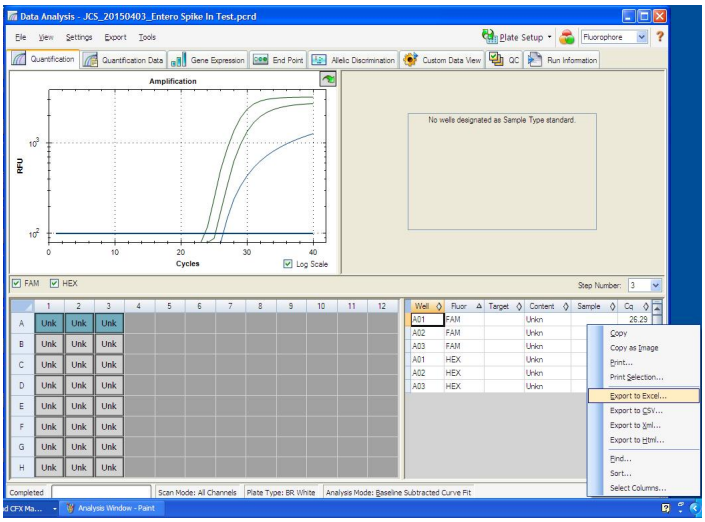
Matrix	Microbial Test	Cq to CFU/g Conversion Equation
Flower	Total Yeast and Mold	$CFU/g = 10^{[(36.671 - Cq\ Value)/3.1194]}$
Flower	Total Aerobic Count	$CFU/g = 10^{[(35.111 - Cq\ Value)/2.8883]}$
Flower	Total Coliform	$CFU/g = 10^{[(40.073 - Cq\ Value)/3.3417]}$
Flower	Total Enterobacteriaceae	$CFU/g = 10^{[(41.218 - Cq\ Value)/4.3708]}$
MIP/Extract	Total Yeast and Mold	$CFU/g = 10^{[(54.972 - Cq\ Value)/5.8485]}$
MIP/Extract	Total Aerobic Count	$CFU/g = 10^{[(38.076 - Cq\ Value)/3.2249]}$
MIP/Extract	Total Coliform	$CFU/g = 10^{[(41.935 - Cq\ Value)/3.6274]}$
MIP/Extract	Total Enterobacteriaceae	$CFU/g = 10^{[(38.407 - Cq\ Value)/3.3041]}$
Gummy	Total Yeast and Mold	$CFU/g = 10^{[(52.989 - Cq\ Value)/4.9718]}$
Gummy	Total Aerobic Count	$CFU/g = 10^{[(37.235 - Cq\ Value)/2.356]}$
Gummy	Total Coliform	$CFU/g = 10^{[(52.888 - Cq\ Value)/5.9643]}$
Gummy	Total Enterobacteriaceae	$CFU/g = 10^{[(44.81 - Cq\ Value)/4.9665]}$

Please Contact support@medicinalgenomics.com for an easy to use conversion spreadsheet

6. Export the Data

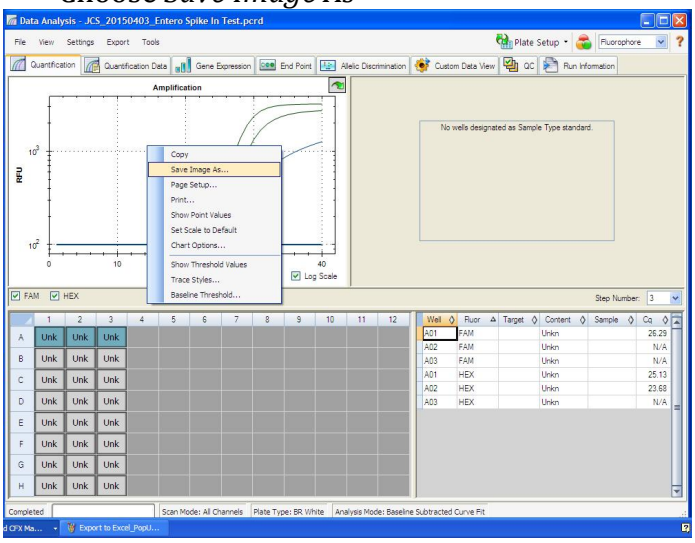
6.1. Exporting the Cq values into an Excel spreadsheet.

- To export the Cq values to an Excel spreadsheet, right-click on the chart on the bottom right of the screen.
- Choose *Export To Excel…*



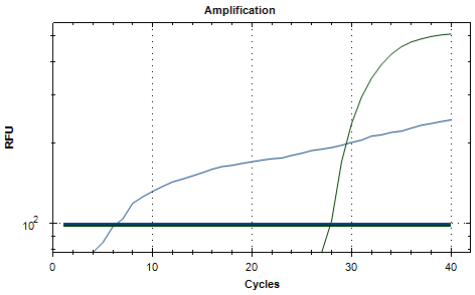
6.2. Saving a visual of the graph

- To save a picture of the graph, right-click the graph on the top left of the screen.
- Choose *Save Image As*



Troubleshooting Guide

Symptom	Reason	Solution
Internal control (SCCG Primer) failure	Extraction Failure	Repeat SenSATIVAx™ and PathoSEEK™ by following the protocol.
	Residual ethanol in elution	Ethanol is an inhibitor to PCR. Return to the SenSATIVAx® protocol and repeat all steps.

Symptom	Reason	Solution
	Mix-up in Reaction Setup	Repeat the qPCR by following the protocol.
	Missing Fluorophore on plate set up	In the Data Analysis window click "View/Edit Plate Setup" from the Settings drop down. All wells should have both FAM and HEX, Multiplex Samples should also have Texas Red. Once completed and the window is closed, the analysis should automatically update.
Internal Control (SCCG) Positive result on positive or negative control samples or samples that do not contain plant DNA	Plant DNA contamination in a reagent	Troubleshoot which reagent was contaminated; use new reagents, thoroughly clean all pipettes and bench areas with 10% bleach solution.
	qPCR bench too close to extraction area	Designate separate benches, pipettes etc. for extractions and qPCR setup
Positive Negative Control	Small Cq value <15	Visually confirm that there is an amplification curve. If not, this is low level background and is to be expected.
	Carry over	Repeat the qPCR by following the protocol.
	Insufficient pre-setup bleaching	Wipe down the lab workspace and all equipment with 10% Bleach. Repeat qPCR.
Negative Positive Control	Mix-up in Reaction Setup	Repeat the qPCR by following the protocol.
Total run failure	Excessive vortex of the qPCR Master Mix	Repeat the qPCR by following the protocol.
Background Amplification 	Unclear	This is usually seen with a very low Cq reading (<15). The curve is usually missing an exponential growth phase, but rather appears as a gradual increase of fluorescent signal. Visual analysis of the graph is necessary to determine if signal is real or background. This is usually a negative result. If still unclear after visual analysis, it is suggested to re-run the assay.

State to state differences

Each state relies on a patchwork of different suggested regulations from AHP, USP, AOAC, AOCS or others. AOAC recommendations for other foods require many replicates and dilutions to manage the limited dynamic range of a petri-dish. Most labs in operation today, do not have BL2 laboratories and are assuming the manufacturers validation is valid in a novel matrix. This assumption of a historical gold standard is dangerous and spike-in experiments with these organisms onto cannabis matrices and MIP matrices is required for accurate risk assessments.

Summary

There are an estimated 42 million cannabis users in the USA. This number is predicted to rise with further legalization. As cannabis legalization continues, and medical use expands, accurate and validated methods are required to assess exposure and risk across diverse route of administration and matrices. Molecular methods are required to meet many of these demands.

1. Appendino G, Gibbons S, Giana A, Pagani A, Grassi G, Stavri M, et al. Antibacterial cannabinoids from Cannabis sativa: a structure-activity study. Journal of natural products. 2008 Aug;71(8):1427-30. PubMed PMID: 18681481.
2. Van Klingeren B, Ten Ham M. Antibacterial activity of delta9-tetrahydrocannabinol and cannabidiol. Antonie van Leeuwenhoek. 1976;42(1-2):9-12. PubMed PMID: 1085130.
3. McKernan K, Spangler J, Helbert Y, Lynch RC, Devitt-Lee A, Zhang L, et al. Metagenomic analysis of medicinal Cannabis samples; pathogenic bacteria, toxigenic fungi, and beneficial microbes grow in culture-based yeast and mold tests. F1000Research. 2016;5:2471. PubMed PMID: 27853518. Pubmed Central PMCID: 5089129.
4. McKernan K, Spangler J, Zhang L, Tadigotla V, Helbert Y, Foss T, et al. Cannabis microbiome sequencing reveals several mycotoxic fungi native to dispensary grade Cannabis flowers. F1000Research. 2015;4:1422. PubMed PMID: 27303623. Pubmed Central PMCID: 4897766.
5. Thompson GR, 3rd, Tuscano JM, Dennis M, Singapuri A, Libertini S, Gaudino R, et al. A microbiome assessment of medical marijuana. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2017 Apr;23(4):269-70. PubMed PMID: 27956269.
6. Sutton S, Lum BL, Torti FM. Possible risk of invasive pulmonary aspergillosis with marijuana use during chemotherapy for small cell lung cancer. Drug intelligence & clinical pharmacy. 1986 Apr;20(4):289-91. PubMed PMID: 3009125.
7. Verweij PE, Kerremans JJ, Voss A, Meis JF. Fungal contamination of tobacco and marijuana. Jama. 2000 Dec 13;284(22):2875. PubMed PMID: 11147983.
8. Ruchlemer R, Amit-Kohn M, Raveh D, Hanus L. Inhaled medicinal cannabis and the immunocompromised patient. Supportive care in cancer : official journal of the Multinational Association of Supportive Care in Cancer. 2015 Mar;23(3):819-22. PubMed PMID: 25216851.
9. Gargani Y, Bishop P, Denning DW. Too many mouldy joints - marijuana and chronic pulmonary aspergillosis. Mediterranean journal of hematology and infectious diseases. 2011;3(1):e2011005. PubMed PMID: 21625309. Pubmed Central PMCID: 3103256.

10. Bal A, Agarwal AN, Das A, Suri V, Varma SC. Chronic necrotising pulmonary aspergillosis in a marijuana addict: a new cause of amyloidosis. Pathology. 2010 Feb;42(2):197-200. PubMed PMID: 20085530.
11. Szyper-Kravitz M, Lang R, Manor Y, Lahav M. Early invasive pulmonary aspergillosis in a leukemia patient linked to aspergillus contaminated marijuana smoking. Leukemia & lymphoma. 2001 Nov-Dec;42(6):1433-7. PubMed PMID: 11911432.
12. Marks WH, Florence L, Lieberman J, Chapman P, Howard D, Roberts P, et al. Successfully treated invasive pulmonary aspergillosis associated with smoking marijuana in a renal transplant recipient. Transplantation. 1996 Jun 27;61(12):1771-4. PubMed PMID: 8685958.
13. Llamas R, Hart DR, Schneider NS. Allergic bronchopulmonary aspergillosis associated with smoking moldy marihuana. Chest. 1978 Jun;73(6):871-2. PubMed PMID: 657864.
14. Hamadeh R, Ardehali A, Locksley RM, York MK. Fatal aspergillosis associated with smoking contaminated marijuana, in a marrow transplant recipient. Chest. 1988 Aug;94(2):432-3. PubMed PMID: 3293934.
15. Chusid MJ, Gelfand JA, Nutter C, Fauci AS. Letter: Pulmonary aspergillosis, inhalation of contaminated marijuana smoke, chronic granulomatous disease. Annals of internal medicine. 1975 May;82(5):682-3. PubMed PMID: 1094876.
16. Cescon DW, Page AV, Richardson S, Moore MJ, Boerner S, Gold WL. Invasive pulmonary aspergillosis associated with marijuana use in a man with colorectal cancer. Journal of clinical oncology : official journal of the American Society of Clinical Oncology. 2008 May 1;26(13):2214-5. PubMed PMID: 18445848.
17. Remington TL, Fuller J, Chiu I. Chronic necrotizing pulmonary aspergillosis in a patient with diabetes and marijuana use. CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne. 2015 Nov 17;187(17):1305-8. PubMed PMID: 26100839. Pubmed Central PMCID: 4646751.
18. Kagen SL. Aspergillus: an inhalable contaminant of marihuana. The New England journal of medicine. 1981 Feb 19;304(8):483-4. PubMed PMID: 7453776.
19. Babu TM GM, Urban MA, et al. Aspergillosis Presenting as Multiple Pulmonary Nodules in an Immunocompetent Cannabis User. Journal of Toxicology and Pharmacology. 2017;1:004.
20. Llewellyn GC, O'Rear CE. Examination of fungal growth and aflatoxin production on marihuana. Mycopathologia. 1977 Dec 16;62(2):109-12. PubMed PMID: 414138.
21. Taylor DN, Wachsmuth IK, Shangkuan YH, Schmidt EV, Barrett TJ, Schrader JS, et al. Salmonellosis associated with marijuana: a multistate outbreak traced by plasmid fingerprinting. The New England journal of medicine. 1982 May 27;306(21):1249-53. PubMed PMID: 7070444.
22. Shirazi-zand Z, Ahmad-Molaei L, Motamedi F, Naderi N. The role of potassium BK channels in anticonvulsant effect of cannabidiol in pentylenetetrazole and maximal electroshock models of seizure in mice. Epilepsy & behavior : E&B. 2013 Jul;28(1):1-7. PubMed PMID: 23644464.
23. Tekaia F, Latge JP. Aspergillus fumigatus: saprophyte or pathogen? Current opinion in microbiology. 2005 Aug;8(4):385-92. PubMed PMID: 16019255.
24. Russo EB. Current Therapeutic Cannabis Controversies and Clinical Trial Design Issues. Frontiers in pharmacology. 2016;7:309. PubMed PMID: 27683558. Pubmed Central PMCID: 5022003.
25. Sullivan N, Elzinga S, Raber JC. Determination of pesticide residues in cannabis smoke. Journal of toxicology. 2013;2013:378168. PubMed PMID: 23737769. Pubmed Central PMCID: 3666265.
26. Abou-Zeid AM, Altalhi AD, Abd El-Fattah RI. Fungal control of pathogenic fungi isolated from wild plants in Taif Governorate, Saudia Arabia. Roumanian archives of microbiology and immunology. 2007 Jul-Dec;66(3-4):90-6. PubMed PMID: 18928069.

27. Hasan MM, Rahman SM, Kim GH, Abdallah E, Oh DH. Antagonistic potentiality of Trichoderma harzianum towards seed-borne fungal pathogens of winter wheat cv. Protiva in vitro and in vivo. Journal of microbiology and biotechnology. 2012 May;22(5):585-91. PubMed PMID: 22561850.
28. Subritzky T, Pettigrew S, Lenton S. Into the void: Regulating pesticide use in Colorado's commercial cannabis markets. The International journal on drug policy. 2017 Apr;42:86-96. PubMed PMID: 28173984.
29. Wurzer. Safety Testing of Medical Cannabis. CannMed. 2016;https://courtagen.wistia.com/medias/dh2l944tqe#.
30. Raber JC, Elzinga S, Kaplan C. Understanding dabs: contamination concerns of cannabis concentrates and cannabinoid transfer during the act of dabbing. The Journal of toxicological sciences. 2015 Dec;40(6):797-803. PubMed PMID: 26558460.
31. Health DDoE. https://www.medicinalgenomics.com/wp-content/uploads/2016/08/Special-concerns-with-marijuana-extractions-and-infusions_Denver_Botulism_160824.pdf.
32. Kalyuzhnaya MG, Lapidus A, Ivanova N, Copeland AC, McHardy AC, Szeto E, et al. High-resolution metagenomics targets specific functional types in complex microbial communities. Nature biotechnology. 2008 Sep;26(9):1029-34. PubMed PMID: 18711340.
33. Tringe SG, Rubin EM. Metagenomics: DNA sequencing of environmental samples. Nature reviews Genetics. 2005 Nov;6(11):805-14. PubMed PMID: 16304596.
34. de Bekker C, van Veluw GJ, Vinck A, Wiebenga LA, Wosten HA. 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.
35. 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.
36. Maguire S. http://www.ktuu.com/content/news/Auditing-marijuana-labs--472254833.html. KTUU. 2018.
37. Zeiller M, Rothballer M, Iwobi AN, Bohnel H, Gessler F, Hartmann A, et al. Systemic colonization of clover (Trifolium repens) by Clostridium botulinum strain 2301. Frontiers in microbiology. 2015;6:1207. PubMed PMID: 26583010. Pubmed Central PMCID: 4628109.
38. Delgado-Viscogliosi P, Solignac L, Delattre JM. Viability PCR, a culture-independent method for rapid and selective quantification of viable Legionella pneumophila cells in environmental water samples. Applied and environmental microbiology. 2009 Jun;75(11):3502-12. PubMed PMID: 19363080. Pubmed Central PMCID: 2687295.
39. Cangelosi GA, Meschke JS. Dead or alive: molecular assessment of microbial viability. Applied and environmental microbiology. 2014 Oct;80(19):5884-91. PubMed PMID: 25038100. Pubmed Central PMCID: 4178667.
40. EPA. https://www.medicinalgenomics.com/wp-content/uploads/2014/12/final_microbiology_method_guidance_110409.pdf.
41. Genomics M. MIPs interfere with 3M petri-film chemistry. https://wwwmedicinalgenomicscom/mips-and-extracts-negative-impact-on-plating/. 2017.
42. Beuchat LR, Mann DA. Comparison of New and Traditional Culture-Dependent Media for Enumerating Foodborne Yeasts and Molds. Journal of food protection. 2016 Jan;79(1):95-111. PubMed PMID: 26735035.
43. White PL, Bretagne S, Klingspor L, Melchers WJ, McCulloch E, Schulz B, et al. Aspergillus PCR: one step closer to standardization. Journal of clinical microbiology. 2010 Apr;48(4):1231-40. PubMed PMID: 20147637. Pubmed Central PMCID: 2849576.

44. Powers-Fletcher MV, Hanson KE. Molecular Diagnostic Testing for Aspergillus. Journal of clinical microbiology. 2016 Nov;54(11):2655-60. PubMed PMID: 27487954. Pubmed Central PMCID: 5078539.
45. White PL, Barnes RA, Springer J, Klingspor L, Cuenca-Estrella M, Morton CO, et al. Clinical Performance of Aspergillus PCR for Testing Serum and Plasma: a Study by the European Aspergillus PCR Initiative. Journal of clinical microbiology. 2015 Sep;53(9):2832-7. PubMed PMID: 26085618. Pubmed Central PMCID: 4540904.
46. Burton RA, Fincher GB. (1,3;1,4)-beta-D-glucans in cell walls of the poaceae, lower plants, and fungi: a tale of two linkages. Molecular plant. 2009 Sep;2(5):873-82. PubMed PMID: 19825664.
47. El-Neketi M, Ebrahim W, Lin W, Gedara S, Badria F, Saad HE, et al. Alkaloids and polyketides from Penicillium citrinum, an endophyte isolated from the Moroccan plant Ceratonia siliqua. Journal of natural products. 2013 Jun 28;76(6):1099-104. PubMed PMID: 23713692.
48. LaBauve AE, Wargo MJ. Growth and laboratory maintenance of Pseudomonas aeruginosa. Current protocols in microbiology. 2012 May;Chapter 6:Unit 6E 1. PubMed PMID: 22549165. Pubmed Central PMCID: 4296558.
49. Kunicka A. TEMPO® SYSTEM IN MICROBIOLOGICAL ANALYSIS OF SPICES AND FOOD ADDITIVES. Polish Journal of Food and Nutrition Sciences. 2007;57(3):67-9.
50. Owen M, Willis C, Lamph D. Evaluation of the TEMPO((R)) most probable number technique for the enumeration of Enterobacteriaceae in food and dairy products. Journal of applied microbiology. 2010 Nov;109(5):1810-6. PubMed PMID: 20649835.
51. Delbeke S, Ceuppens S, Holvoet K, Samuels E, Sampers I, Uyttendaele M. Multiplex real-time PCR and culture methods for detection of Shiga toxin-producing Escherichia coli and Salmonella Thompson in strawberries, a lettuce mix and basil. International journal of food microbiology. 2015 Jan 16;193:1-7. PubMed PMID: 25462916.
52. Granade TC, Artis WM. Antimycotic susceptibility testing of dermatophytes in microcultures with a standardized fragmented mycelial inoculum. Antimicrobial agents and chemotherapy. 1980 Apr;17(4):725-9. PubMed PMID: 7396462. Pubmed Central PMCID: 283859.
53. Dorrell WW, Page RM. The Use of Fragmented Mycelial Inoculum in the Culture of Fungi. Journal of bacteriology. 1947 Mar;53(3):360-1. PubMed PMID: 16561281. Pubmed Central PMCID: 518317.
54. Granade TC, Hehmann MF, Artis WM. Monitoring of filamentous fungal growth by in situ microspectrophotometry, fragmented mycelium absorbance density, and 14C incorporation: alternatives to mycelial dry weight. Applied and environmental microbiology. 1985 Jan;49(1):101-8. PubMed PMID: 3883895. Pubmed Central PMCID: 238352.
55. Herrera ML, Vallor AC, Gelfond JA, Patterson TF, Wickes BL. Strain-dependent variation in 18S ribosomal DNA Copy numbers in Aspergillus fumigatus. Journal of clinical microbiology. 2009 May;47(5):1325-32. PubMed PMID: 19261786. Pubmed Central PMCID: 2681831.
56. Maleszka R, Clark-Walker GD. Yeasts have a four-fold variation in ribosomal DNA copy number. Yeast. 1993 Jan;9(1):53-8. PubMed PMID: 8442387.
57. Perez AA, Sanchez CC, Patino JM, Rubiolo AC, Santiago LG. Surface adsorption behaviour of milk whey protein and pectin mixtures under conditions of air-water interface saturation. Colloids and surfaces B, Biointerfaces. 2011 Jul 1;85(2):306-15. PubMed PMID: 21440425.
58. Espinel-Ingroff A, Kerkering TM. Spectrophotometric method of inoculum preparation for the in vitro susceptibility testing of filamentous fungi. Journal of clinical microbiology. 1991 Feb;29(2):393-4. PubMed PMID: 2007647. Pubmed Central PMCID: 269774.
59. Espinel-Ingroff A, Bartlett M, Bowden R, Chin NX, Cooper C, Jr., Fothergill A, et al. Multicenter evaluation of proposed standardized procedure for antifungal susceptibility testing of filamentous

fungi. Journal of clinical microbiology. 1997 Jan;35(1):139-43. PubMed PMID: 8968895. Pubmed Central PMCID: 229526.
60. Spraker JE, Sanchez LM, Lowe TM, Dorrestein PC, Keller NP. Ralstonia solanacearum lipopeptide induces chlamydospore development in fungi and facilitates bacterial entry into fungal tissues. The ISME journal. 2016 Sep;10(9):2317-30. PubMed PMID: 26943626. Pubmed Central PMCID: 4989320.
61. Spraker JE, Jewell K, Roze LV, Scherf J, Ndagano D, Beaudry R, et al. A volatile relationship: profiling an inter-kingdom dialogue between two plant pathogens, Ralstonia Solanacearum and Aspergillus Flavus. Journal of chemical ecology. 2014 May;40(5):502-13. PubMed PMID: 24801606.
62. Lucarelli C, Di Domenico EG, Toma L, Bracco D, Prignano G, Fortunati M, et al. Ralstonia mannitolilytica infections in an oncologic day ward: description of a cluster among high-risk patients. Antimicrobial resistance and infection control. 2017;6:20. PubMed PMID: 28191308. Pubmed Central PMCID: 5297155.
63. Waugh JB, Granger WM, Gaggar A. Incidence, relevance and response for Ralsfonia respiratory infections. Clinical laboratory science : journal of the American Society for Medical Technology. 2010 Spring;23(2):99-106. PubMed PMID: 20499534. Pubmed Central PMCID: 4086841.
64. Coenye T, Vandamme P, LiPuma JJ. Infection by Ralstonia species in cystic fibrosis patients: identification of R. pickettii and R. mannitolilytica by polymerase chain reaction. Emerging infectious diseases. 2002 Jul;8(7):692-6. PubMed PMID: 12095436. Pubmed Central PMCID: 2730328.
65. Scherlach K, Partida-Martinez LP, Dahse HM, Hertweck C. Antimitotic rhizoxin derivatives from a cultured bacterial endosymbiont of the rice pathogenic fungus Rhizopus microsporus. Journal of the American Chemical Society. 2006 Sep 6;128(35):11529-36. PubMed PMID: 16939276.
66. Wilson T, Rabie CJ, Fincham JE, Steyn PS, Schipper MA. Toxicity of rhizonin A, isolated from Rhizopus microsporus, in laboratory animals. Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association. 1984 Apr;22(4):275-81. PubMed PMID: 6539275.
67. Lackner G, Hertweck C. Impact of endofungal bacteria on infection biology, food safety, and drug development. PLoS pathogens. 2011 Jun;7(6):e1002096. PubMed PMID: 21738468. Pubmed Central PMCID: 3128126.
68. nyi S. Paecilomyces farinosus destroys powdery mildew colonies in detached leaf cultures but not on whole plants. European Journal of Plant Pathology. 2006;115:351–356.
69. Diguta CF, Rousseaux S, Weidmann S, Bretin N, Vincent B, Guilloux-Benatier M, et al. Development of a qPCR assay for specific quantification of Botrytis cinerea on grapes. FEMS microbiology letters. 2010 Dec;313(1):81-7. PubMed PMID: 20946385.
70. Edwards SG, Seddon B. Selective media for the specific isolation and enumeration of Botrytis cinerea conidia. Letters in applied microbiology. 2001 Feb;32(2):63-6. PubMed PMID: 11169044.
71. Kiss L. Advances in the identification of emerging powdery mildew fungi using morphological and molecular data. Acta microbiologica et immunologica Hungarica. 2002;49(2-3):245-8. PubMed PMID: 12109154.
72. Pepin. Occurrence of Powdery Mildew Caused by Golovinomyces cichoracearum sensu lato on Cannabis sativa in Canada. Plant Disease. 2018.
73. Suda W, Nagasaki A, Shishido M. Powdery mildew-infection changes bacterial community composition in the phyllosphere. Microbes and environments. 2009;24(3):217-23. PubMed PMID: 21566376.
74. Crawford. IS THERE MOLDY CANNABIS IN MASS? https://digbostoncom/is-there-moldy-cannabis-in-mass/.

75. Jurgensen CW, Madsen A. Exposure to the airborne mould Botrytis and its health effects. Annals of agricultural and environmental medicine : AAEM. 2009;16(2):183-96. PubMed PMID: 20047250.
76. Green BJ, Couch JR, Lemons AR, Burton NC, Victory KR, Nayak AP, et al. Microbial hazards during harvesting and processing at an outdoor United States cannabis farm. Journal of occupational and environmental hygiene. 2018 Jan 25:0. PubMed PMID: 29370578.
77. McKernan K. WHOLE GENOME SEQUENCING OF SEVERAL CANNABIS DERIVED POWDERY MILDEW SAMPLES AND THE DEVELOPMENT OF FIELD PORTABLE COLORIMETRIC DETECTION TOOLS FOR INFECTING FUNGI. ICRS 2017. 2017;http://www.icrs.co/SYMPOSIUM.2017/ICRS2017.FINAL.PROGRAMME.pdf.
78. Shapiro B. Cryptococcal meningitis in a daily cannabis smoker without evidence of immunodeficiency. BMJ Case Reports. 2018.
79. Castro e Silva Dde M, Santos DC, Pukinskas SR, Oshida JT, Oliveira L, Carvallho AF, et al. A new culture medium for recovering the agents of Cryptococcosis from environmental sources. Brazilian journal of microbiology : [publication of the Brazilian Society for Microbiology]. 2015 Jun;46(2):355-8. PubMed PMID: 26273249. Pubmed Central PMCID: 4507526.
80. Nucci M, Anaissie E. Fusarium infections in immunocompromised patients. Clinical microbiology reviews. 2007 Oct;20(4):695-704. PubMed PMID: 17934079. Pubmed Central PMCID: 2176050.
81. Nucci M, Marr KA, Queiroz-Telles F, Martins CA, Trabasso P, Costa S, et al. Fusarium infection in hematopoietic stem cell transplant recipients. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2004 May 1;38(9):1237-42. PubMed PMID: 15127334.
82. Nucci M. Emerging moulds: Fusarium, Scedosporium and Zygomycetes in transplant recipients. Current opinion in infectious diseases. 2003 Dec;16(6):607-12. PubMed PMID: 14624113.
83. Nucci M, Anaissie EJ, Queiroz-Telles F, Martins CA, Trabasso P, Solza C, et al. Outcome predictors of 84 patients with hematologic malignancies and Fusarium infection. Cancer. 2003 Jul 15;98(2):315-9. PubMed PMID: 12872351.
84. Nucci M, Anaissie E. Cutaneous infection by Fusarium species in healthy and immunocompromised hosts: implications for diagnosis and management. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2002 Oct 15;35(8):909-20. PubMed PMID: 12355377.
85. Khwaja S, Yacoub A, Cheema A, Rihana N, Russo R, Velez AP, et al. Marijuana Smoking in Patients With Leukemia. Cancer control : journal of the Moffitt Cancer Center. 2016 Jul;23(3):278-83. PubMed PMID: 27556668.
86. K. S. Tiourebaev GVS, M. Dolgovskaya , M. K. McCarthy , T. W. Anderson , L. D. Carsten , A. L. Pilgeram & D. C. Sands. Biological Control of Infestations of Ditchweed (Cannabis sativa) with Fusarium oxysporum f.sp. Cannabis in Kazakhstan. Biocontrol Science and Technology 2011;11(4).
87. Validov S, Kamilova F, Qi S, Stephan D, Wang JJ, Makarova N, et al. Selection of bacteria able to control Fusarium oxysporum f. sp. radicis-lycopersici in stonewool substrate. Journal of applied microbiology. 2007 Feb;102(2):461-71. PubMed PMID: 17241352.
88. Bolwerk A, Lagopodi AL, Wijfjes AH, Lamers GE, Chin AWTF, Lugtenberg BJ, et al. Interactions in the tomato rhizosphere of two Pseudomonas biocontrol strains with the phytopathogenic fungus Fusarium oxysporum f. sp. radicis-lycopersici. Molecular plant-microbe interactions : MPMI. 2003 Nov;16(11):983-93. PubMed PMID: 14601666.
89. Chin AWTF, Bloemberg GV, Mulders IH, Dekkers LC, Lugtenberg BJ. Root colonization by phenazine-1-carboxamide-producing bacterium Pseudomonas chlororaphis PCL1391 is essential for

biocontrol of tomato foot and root rot. Molecular plant-microbe interactions : MPMI. 2000 Dec;13(12):1340-5. PubMed PMID: 11106026.
90. Dekkers LC, Mulders IH, Phoelich CC, Chin AWTF, Wijfjes AH, Lugtenberg BJ. The sss colonization gene of the tomato-Fusarium oxysporum f. sp. radicis-lycopersici biocontrol strain Pseudomonas fluorescens WCS365 can improve root colonization of other wild-type pseudomonas spp.bacteria. Molecular plant-microbe interactions : MPMI. 2000 Nov;13(11):1177-83. PubMed PMID: 11059484.
91. Fride E. Cannabinoids and Cystic Fibrosis. Journal of Cannabis Therapeutics. 2008;2(1):59-71.
92. Fride E. The endocannabinoid-CB(1) receptor system in pre- and postnatal life. European journal of pharmacology. 2004 Oct 1;500(1-3):289-97. PubMed PMID: 15464041.
93. Bregman T, Fride E. Behavioral alterations in cystic fibrosis mice are prevented by cannabinoid treatment in infancy. Journal of basic and clinical physiology and pharmacology. 2011 Jun 17;22(1-2):33-6. PubMed PMID: 22865361.
94. De Meyer G, Hofte M. Salicylic Acid Produced by the Rhizobacterium Pseudomonas aeruginosa 7NSK2 Induces Resistance to Leaf Infection by Botrytis cinerea on Bean. Phytopathology. 1997 Jun;87(6):588-93. PubMed PMID: 18945074.
95. Russo EB. Taming THC: potential cannabis synergy and phytocannabinoid-terpenoid entourage effects. British journal of pharmacology. 2011 Aug;163(7):1344-64. PubMed PMID: 21749363. Pubmed Central PMCID: 3165946.
96. Radwan MM, Elsohly MA, Slade D, Ahmed SA, Khan IA, Ross SA. Biologically active cannabinoids from high-potency Cannabis sativa. Journal of natural products. 2009 May 22;72(5):906-11. PubMed PMID: 19344127. Pubmed Central PMCID: 4886613.
97. Zhang K, Sparling J, Chow BL, Elsayed S, Hussain Z, Church DL, et al. New quadriplex PCR assay for detection of methicillin and mupirocin resistance and simultaneous discrimination of Staphylococcus aureus from coagulase-negative staphylococci. Journal of clinical microbiology. 2004 Nov;42(11):4947-55. PubMed PMID: 15528678. Pubmed Central PMCID: 525205.
98. Paterson RR. Fungi and fungal toxins as weapons. Mycological research. 2006 Sep;110(Pt 9):1003-10. PubMed PMID: 16908123.
99. Thu K, Hayes M, Miles S, Tierney L, Foy A. Marijuana 'bong' smoking and tuberculosis. Internal medicine journal. 2013 Apr;43(4):456-8. PubMed PMID: 23551310.
100. Oeltmann JE, Oren E, Haddad MB, Lake L, Harrington TA, Ijaz K, et al. Tuberculosis outbreak in marijuana users, Seattle, Washington, 2004. Emerging infectious diseases. 2006 Jul;12(7):1156-9. PubMed PMID: 16836841. Pubmed Central PMCID: 3291049.
101. Kusari P, Kusari S, Lamshoft M, Sezgin S, Spiteller M, Kayser O. Quorum quenching is an antivirulence strategy employed by endophytic bacteria. Applied microbiology and biotechnology. 2014 Aug;98(16):7173-83. PubMed PMID: 24846733.
102. Uchiya KI, Tomida S, Nakagawa T, Asahi S, Nikai T, Ogawa K. Comparative genome analyses of Mycobacterium avium reveal genomic features of its subspecies and strains that cause progression of pulmonary disease. Scientific reports. 2017 Jan 3;7:39750. PubMed PMID: 28045086. Pubmed Central PMCID: 5206733.
103. Lengerova M, Racil Z, Hrncirova K, Kocmanova I, Volfova P, Ricna D, et al. Rapid detection and identification of mucormycetes in bronchoalveolar lavage samples from immunocompromised patients with pulmonary infiltrates by use of high-resolution melt analysis. Journal of clinical microbiology. 2014 Aug;52(8):2824-8. PubMed PMID: 24850354. Pubmed Central PMCID: 4136189.
104. Winkler S, Susani S, Willinger B, Apsner R, Rosenkranz AR, Potzi R, et al. Gastric mucormycosis due to Rhizopus oryzae in a renal transplant recipient. Journal of clinical microbiology. 1996 Oct;34(10):2585-7. PubMed PMID: 8880524. Pubmed Central PMCID: 229322.

105. Hackbart HC, Machado AR, Christ-Ribeiro A, Prietto L, Badiale-Furlong E. Reduction of aflatoxins by Rhizopus oryzae and Trichoderma reesei. Mycotoxin research. 2014 Aug;30(3):141-9. PubMed PMID: 24925827.
106. Spellberg B, Edwards J, Jr., Ibrahim A. Novel perspectives on mucormycosis: pathophysiology, presentation, and management. Clinical microbiology reviews. 2005 Jul;18(3):556-69. PubMed PMID: 16020690. Pubmed Central PMCID: 1195964.
107. Millon L, Larosa F, Lepiller Q, Legrand F, Rocchi S, Daguindau E, et al. Quantitative polymerase chain reaction detection of circulating DNA in serum for early diagnosis of mucormycosis in immunocompromised patients. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2013 May;56(10):e95-101. PubMed PMID: 23420816.
108. Ramaswamy V, Cresence VM, Rejitha JS, Lekshmi MU, Dharsana KS, Prasad SP, et al. Listeria--review of epidemiology and pathogenesis. Journal of microbiology, immunology, and infection = Wei mian yu gan ran za zhi. 2007 Feb;40(1):4-13. PubMed PMID: 17332901.
109. Arizcun C, Vasseur C, Labadie JC. Effect of several decontamination procedures on Listeria monocytogenes growing in biofilms. Journal of food protection. 1998 Jun;61(6):731-4. PubMed PMID: 9709259.
110. Morahan PS, Klykken PC, Smith SH, Harris LS, Munson AE. Effects of cannabinoids on host resistance to Listeria monocytogenes and herpes simplex virus. Infection and immunity. 1979 Mar;23(3):670-4. PubMed PMID: 313368. Pubmed Central PMCID: 414217.
111. Gasanov U, Hughes D, Hansbro PM. Methods for the isolation and identification of Listeria spp. and Listeria monocytogenes: a review. FEMS microbiology reviews. 2005 Nov;29(5):851-75. PubMed PMID: 16219509.
112. Ward M. Microbial Testing in Cannabis: Challenges and Best Practices. Emerald Conference. 2018;https://www.emeraldcommunity.net/trLuyiuvieBoewO/Emerald-Conference-0108-Matthew-Ward.pdf.
113. Cundell. The limitations of the colony-forming unit in microbiology. The Eurpean Pharmaceutical Review. 2015;20(6).
114. GenomeTrakr. https://www.fda.gov/Food/FoodScienceResearch/WholeGenomeSequencingProgramWGS/ucm363134.htm.
115. Shapiro B. Cryptococcal meningitis in a daily cannabis smoker without evidence of immunodeficiency. BMJ Case Reports. 2018.
116. Stewart EJ. Growing unculturable bacteria. Journal of bacteriology. 2012 Aug;194(16):4151-60. PubMed PMID: 22661685. Pubmed Central PMCID: 3416243.
117. Astani A, Reichling J, Schnitzler P. Comparative study on the antiviral activity of selected monoterpenes derived from essential oils. Phytotherapy research : PTR. 2010 May;24(5):673-9. PubMed PMID: 19653195.
118. McKernan KJ, Spangler J, Zhang L, Tadigotla V, McLaughlin S, Warner J, et al. Expanded genetic codes in next generation sequencing enable decontamination and mitochondrial enrichment. PloS one. 2014;9(5):e96492. PubMed PMID: 24788618. Pubmed Central PMCID: 4008621.
119. McKernan KJ, Spangler J, Helbert Y, Zhang L, Tadigotla V. DREAMing of a patent-free human genome for clinical sequencing. Nature biotechnology. 2013 Oct;31(10):884-7. PubMed PMID: 24104751.