Validation of Pan-Pythium Duplex-TaqMan qPCR Assay

Background

Pythium belongs to a distinct class of fungus-like, parasitic microbes called oomycetes. Primarily a root pathogen, Pythium is a common cause of root rot and damping off in cannabis plants and has major economic impacts in the cannabis industry. While there are more than 300 difference types of pathogenic Pythium, the subspecies most detected in diseased cannabis plants and identified by this assay are:

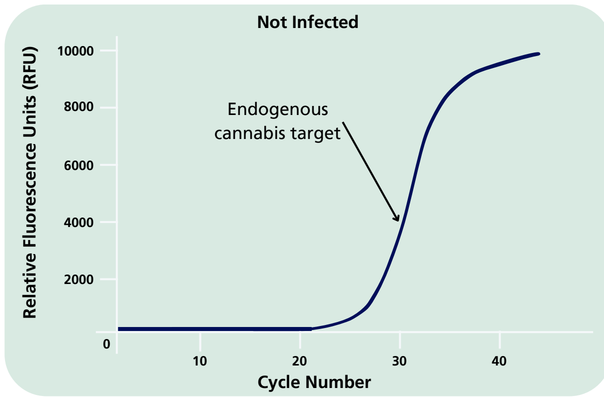
Pythium aphanidermatum
Pythium arrhenomanes
Pythium dissotocum
Pythium guiyangense
Pythium myriotylum
Pythium oligandrum
Pythium Ultimum
Pythium brassicae
Pythium insidiosum
Pythium periplocum

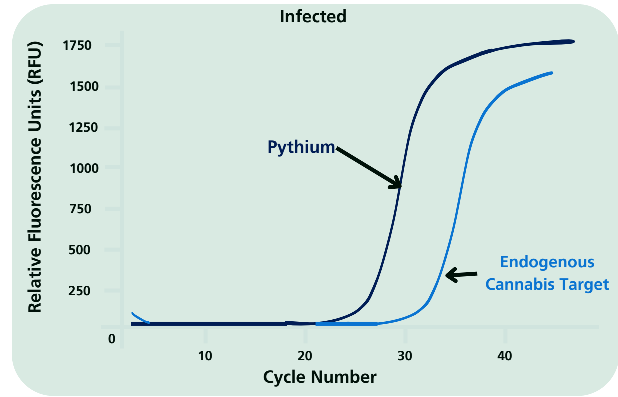
Pythium is generally considered a water mold and can exist and multiply in standing water, ditches, water tanks, and hydroponic systems. Pythium strains are opportunistic plant pathogens that can cause severe damage whenever plants are stressed (i.e. clone cuttings), at vulnerable stages (i.e. seedlings) or under high wet/humid environments. Most Pythium varieties are necrotrophs, meaning that infection results in necrotic tissue and can lead to plant death.

Pythium infections can manifest in seeds, roots, or crown and are major causes of damping-off and germination failure. In older plants, root and crown rot are often observed that can lead to slow growth and plant death. Pythium attacks feeder roots reducing the ability of plants to absorb water and salts from the soil, leading to weak plants with low production. If plants are showing poor yield, root rot, or yellowing leaves, Pythium should be considered as a potential cause.

General Information

TUMI Genomics' Fusarium diagnostic assay is for the detection of infection from cannabis and hemp plant tissue. The assay uses a duplex TaqMan qPCR to detect the presence of Fusarium DNA sequences. The primer/probe sets target the Fusarium genome as well as an endogenous plant transcript as a positive control. Because this assay detects a target that is in all cannabis tissues, it provides confirmation of sample integrity, successful nucleic acid extraction, and activity of reaction components in every sample. By using a proprietary method for nucleic acid extraction, TUMI Genomics laboratory can substantially reduce sample to result time allowing a 24-48-hour turnaround time on all samples. Representative results produced by the assay are shown below:





Test Validation

Technical validation: Technical validation consists of experiments performed by TUMI Genomics to determine the technical (in lab) limits and characteristics of the assay.

Limit of Detection (LoD): A limit of detection study determines the sensitivity of a test. The limit of detection is defined as the lowest concentration of pathogen where the test can still detect 95% of the true positive samples. A limit of detection can help you understand how well a test can find low-level/early infections.

The LoD (lowest concentration of Pythium detected by this assay) was determined by testing serial dilutions (63 copies – 4 copies) of Pythium genomic material spiked into negative cannabis plant extract and tested with ten replicates per concentration.

The smallest amount of Pythium reliably detected by the assay was determined to be ~ 8 cells of Pythium per reaction.

The table below shows the results of tests to determine the limit of detection (i.e. the smallest amount of pathogen this assay can identify). The limit of detection is highlighted in green. The limit of detection was confirmed for each individual species of Pythium as well.

Sample Concentration	Fraction Positive	Mean CT Plant Control	Standard Deviation	Mean CT Pan Pythium	Standard Deviations
62.5 copies/reaction	10/10	28.02	+/- 0.06	31.47	+/- 0.18
31.2 copies/reaction	10/10	27.81	+/- 0.12	32.47	+/- 0.16
15.6 copies/reaction	10/10	27.79	+/- 0.09	33.38	+/- 0.51
8 copies/reaction	10/10	27.76	+/- 0.12	34.19	+/- 0.48
4 copies/reaction	10/10	26.99	+/- 0.09	37.57	+/- 1.37

Cross Reactivity: A cross reactivity analysis determines the specificity of a test. Specificity means whether the test detects only the target pathogen versus giving a non-specific signal or throwing a positive result due to the presence of an unrelated pathogen. A specific test should be 100% specific for the target pathogen (i.e. hop latent viroid) and less than 80% specific for potential contaminating pathogens (like other viruses).

Cross-reactivity was evaluated using in silico analysis of primer sequences compared to genome sequences from microorganisms that are commonly found in the roots, stems, and leaves of Cannabis, hops, and hemp plants. Genomic sequences for the microorganisms were acquired from the National Center for Biotechnology Information database (NCBI) and the alignments were performed with the Basic local alignment search tool (BLAST). For each organism, percent cross-reactivity was determined by dividing the number of nucleotide sequences that matched with the organism by the total number of nucleotides in the Pythium primer/probe set.

Cross-reactivity is defined as greater than 80% similarity between the primer/probes set and any sequence present in the targeted microorganism. As expected, primer probe sequences matched sequences from the Pythium genome 100%. No cross-reactivity above 80% was found with any other tested microorganism.

Table shows the identity of each tested microorganism and the percent cross-reactivity with the primer/probe sets used to target the Pythium genome. Pathogens in red are predicted to be identified with 100% specificity. Pathogens in purple are identified with less certainty, but are very likely to be detected in this assay.

Organism	% Cross Reactivity
Pythium aphanidermatum	100.00%
Pythium arrhenomanes	100.00%
Pythium dissotocum	100.00%
Pythium guiyangense	100.00%
Pythium myriotylum	100.00%
Pythium oligandrum	100.00%
Pythium ultimum	100.00%
Pythium brassicae	98.53%
Pythium insidiosum	98.53%
Pythium periplocum	97.06%
Phytophthora citricola	95.59%

Organism	% Cross Reactivity
Pseudoperonospora cannabina	95.59%
Aster yellows witches'-broom phytoplasma	54.41%
Fusarium solani	54.41%
Fusarium brachygibbosum	52.94%
Macrophomina phaseolina	51.47%
Cucumber mosaic virus	50.00%
Tetranychus urticae	50.00%
Fusarium oxysporum	48.53%
Fusarium proliferatum	48.53%
Hop mosaic virus	48.53%
Sclerotinia sclerotiorum	48.53%

Organism	% Cross Reactivity
Verticillium dahliae	48.53%
Cercospora cf. flagellaris	45.59%
Rhizoctonia solani	45.59%
Cannabis cryptic virus	44.12%
Tobacco streak virus	44.12%
Alternaria alternata	42.65%
Berkeleyomyces basicola	42.65%
Alfalfa mosaic virus	41.18%
Arabis mosaic virus	41.18%
Tobacco ringspot virus	41.18%
Tomato mosaic virus	41.18%
Hop latent viroid	39.71%
Pseudomonas syringae	39.71%
Tomato ringspot virus	39.71%
Beet curly top virus	38.24%
Hop stunt viroid	38.24%
Botrytis cinerea	35.29%
Cladosporium cladosporioides	35.29%
Curvularia lunata	35.29%
Fusarium sambucinum	35.29%
Phoma	35.29%
Stemphylium lycopersici	35.29%
Stemphylium vesicarium	35.29%
Verticillium albo-atrum	35.29%
Colletotrichum fioriniae	30.88%
Xanthomonas cannabis	23.53%
Stemphylium vesicarium	35.29%

Inclusivity: An inclusivity analysis determines how well a test can detect different known sequence variants of a given pathogen. An acceptable inclusivity analysis should show that an assay has been designed in a way that can reasonability detect all, or the vast majority, of known variants of the target pathogen.

This analysis indicated that the primers and probes used in this assay reliability detect 98% of known hop latent viroid variants (152/155) with little to no reduction in amplification efficiency and will identify 100% of all known hop latent viroid variants in a yes/no diagnostic assay.

To ensure inclusivity of the assay, all Fusarium primer and probe sequences were designed specifically against reference genomes identified from infections in cannabis (reviewed in Gwinn et. al, 2022). All accessions identified from pathogenic cannabis tissue can be amplified using the primer sequences.

*Gwinn, Kimberly et. al, Diseases of Cannabis sativa Caused by Diverse Fusarium Species. Frontiers in Agronomy, Jan 18, 2022. https://doi.org/10.3389/fagro.2021.796062

TUMI Genomics Lead Scientists

Tassa Saldi, PhD: Dr. Saldi received her undergraduate and graduate degrees in molecular biology from the University of Colorado in Boulder and completed her post-doctoral studies at the Health Sciences Center, University of Colorado, Denver. Her graduate work explored the molecular mechanism underpinning Amyotrophic Lateral Sclerosis (ALS) and the role of double-stranded RNA accumulation and heterochromatin in pathogenesis.

Continuing her work on structured RNA during post-doctoral work, Dr. Saldi investigated the role of genome-wide nascent RNA secondary structure in co-transcriptional splicing, A-to-I RNA editing and transcription termination. Her work was supported by fellowships from the American Cancer Society and the RNA Biosciences Intuitive (RBI). Following her postdoc, Dr. Saldi directed the COVID-19 surveillance lab at CU, Boulder where she supervised a team of 8 scientists and designed and validated multiple PCR assays to detect SARS-CoV-2 in human saliva. She is a lead scientist and CSO of TUMI Genomics.

Her publications can be found here:
https://pubmed.ncbi.nlm.nih.gov/term=Tassa+Saldi&sort=date

Alfonso Garrido-Lecca, PhD: Dr. Garrido-Lecca received an undergraduate degree in biology with a minor in chemistry from Texas A&M University. He pursued his PhD at the University of Colorado, Boulder in molecular biology. His graduate work focused on using the unique genetic organization of C. elegans to understand how genes are expressed and RNA transcripts processed. His postdoctoral work focused on the regulation of microRNAs in leukemia and was supported by a fellowship from the Linda Crnic Institute for Down Syndrome and the National Institute of Health T32 training grant. Dr. GarridoLecca is a lead scientist at TUMI Genomics and head of Research and Development.

His publications can be found here:
https://pubmed.ncbi.nlm.nih.gov/term=alfonso+garridolecca&sort=date