Validation of Pan-Fusarium Duplex-TaqMan qPCR Assay

Background

Fusarium is a root-infecting fungal pathogen. Fusarium infection can result in both root rot and damping-off (i.e. stunted growth with dark green or yellow curled leaves and root lesions, especially at root tips). In an indoor growing environment, introduction of diseased plant material such as cuttings, stock plants or seeds can result in inoculation and eventual spread of the fungus. Infected growing medium that has not been adequately sterilized can also be a source of infection.

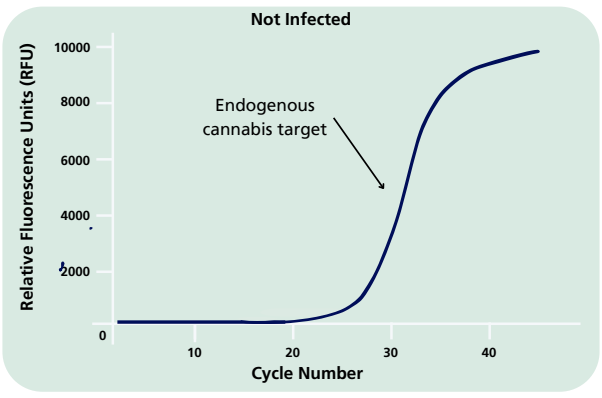
Once established, Fusarium can spread via unfiltered, recirculated water, in air, on tools/equipment and potentially by workers. There is no effective and safe cure for Fusarium in cannabis. Prevention by careful sterilization protocols and destruction of diseased plant material are the most effective methods to prevent/limit disease spread. Identification of infected plants and effective management through a preventative testing program can help limit introduction and spread of Fusarium and safeguard against catastrophic economic loss.

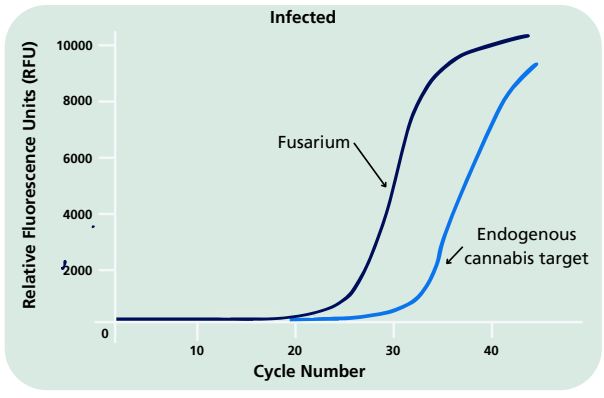
Multiple species of Fusarium have been characterized to infect cannabis. TUMI Genomics' Pan-Fusarium assay detects common species known to cause serious disease in cannabis including:

Fusarium oxysporum, Fusarium solani, Fusarium proliferatum, and Fusarium brachygibbosum.

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 Fusarium detected by this assay) was determined by testing serial dilutions (500 copies – 4 copies) of Fusarium genomic material spiked into negative cannabis plant extract and tested with ten replicates per concentration.

The smallest amount of Fusarium reliably detected by the assay was determined to be 390 femtograms of genetic material, which represents ~ 8 cells of Fusarium 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 Fusarium as well.

Sample Concentration	Fraction Positive	Mean CT Plant Control	Standard Deviation	Mean CT Pan Fusarium	Standard Deviations
500 copies/reaction	10/10	27.25	+/- 0.11	27.88	+/- 0.09
60 copies/reaction	10/10	27.27	+/- 0.07	27.83	+/- 0.09
30 copies/reaction	10/10	27.26	+/- 0.09	28.79	+/- 0.05
8 copies/reaction	10/10	27.23	+/- 0.07	30.94	+/- 0.22
4 copies/reaction	10/10	28.01	+/- 0.03	32.70	+/- 0.97

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 *Fusarium oxysporum* 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 Fusarium 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 Fusarium genome.

Organism	% Cross Reactivity
Fusarium oxysporum	100.00%
Fusarium solani	100.00%
Fusarium proliferatum	100.00%
Fusarium brachygibbosum	100.00%
Cladosporium cladosporioides strain	72.31%
Macrophomina phaseolina	72.31%
Botrytis cinerea B05.10	70.77%
Cercospora cf. flagellaris strain	69.23%
Verticillium albo-atrum strain	69.23%
Verticillium dahliae	69.23%
Berkeleyomyces basicola strain	67.69%
Curvularia lunata strain	67.69%
Pythium oligandrum strain	67.69%
Phoma sp. XZ068	66.15%
Sclerotinia sclerotiorum	66.15%
Colletotrichum fioriniae strain	64.62%
Pseudomonas syringae strain	64.62%
Rhizoctonia solani	63.08%
Alternaria alternata strain	61.54%
Fusarium sambucinum	60.00%
Tobacco streak virus	56.92%
Aster yellows witches'-broom	53.85%
Cucumber mosaic virus strain	53.85%
Pythium guiyangense strain	52.31%
Tetranychus urticae	50.77%
Phytophthora citricola strain	47.69%
Arabis mosaic virus	46.15%

Organism	% Cross Reactivity
Pythium insidiosum	46.15%
Stemphylium vesicarium strain	46.15%
Alfalfa mosaic virus	44.62%
Hop mosaic virus	44.62%
Stemphylium lycopersici strain	44.62%
Tomato mosaic virus	44.62%
Cannabis cryptic virus	43.08%
Pythium periplocum 43.08%	43.08%
Pythium aphanidermatum	41.54%
Hop latent viroid	40.00%
Tomato ringspot virus	40.00%
Beet curly top virus 35.38%	35.38%
Hop stunt viroid	33.85%
Phomopsis ganjea	30.77%
Pythium arrhenomanes	29.23%
Pythium brassicum strain	27.69%
Pseudoperonospora humuli isolate	26.15%
Xanthomonas cannabis pv.	21.54%

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