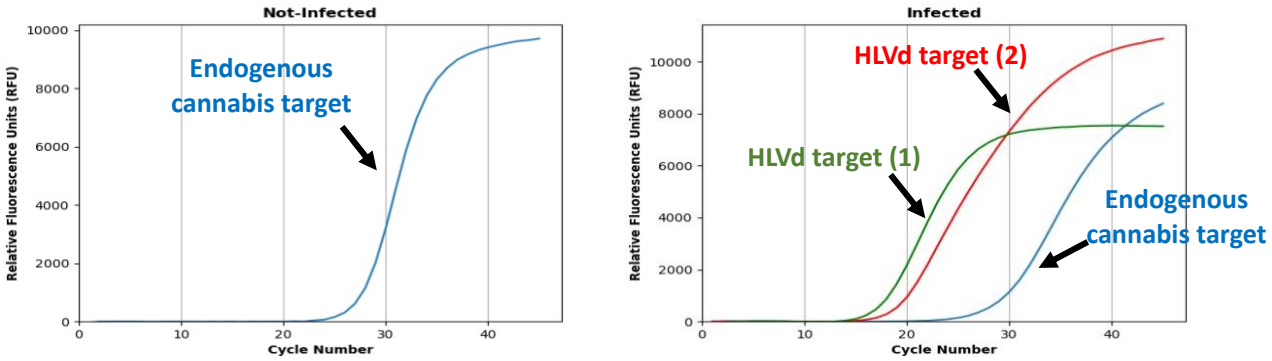
General information:

Hop Latent Viroid (HLVd) is a small, circular, infectious agent. As opposed to viruses and other pathogens, HLVd lacks an outside protein and is only composed of genetic material (RNA). Viroids generally spread via mechanical transmission on unsterilized cutting tools and equipment. HLVd has been detected in most geographical locations around the world and identified in cannabis plants throughout the United States. Common symptoms of HLVd include stunted growth, brittle stems, leaf malformation and reduced flower mass. However, plants may initially appear asymptomatic or with subtle symptoms making detection by eye difficult. The most reliable way to determine if HLVd is spreading in grow operation is by performing regular screening of plants using PCR, or other molecular (nucleic acid amplification) tests.

Description of Assay:

TUMI Genomics' Hop Latent Viroid (HLVd) assay is for the detection of HLVd infection from cannabis/hemp plant tissue. The assay uses a triplex, taqman qRT-PCR to detect the presence of HLVd RNA sequences. The primer/probe sets target two regions in the HLV genome as well as an endogenous plant transcript as a positive control. Because this assay detects two independent targets in the HLVd genome, the chance of false positive results is dramatically reduced. Additionally, each reaction includes a control amplicon from an endogenous cannabis target, allowing simultaneous confirmation of successful nucleic acid extraction and activity of reaction components. By using a proprietary method for nucleic acid extraction, TUMI Genomics laboratory is able to substantially reduce sample to result time allowing a 24-36 hour turnaround time on all samples. Representative results produced by the assay are shown below:



Representative results for samples not infected (left) and infected (right) with HLVd.

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.

To determine the limit of detection (LoD) of the TUMI Genomics HLVd qRT-PCR assay we spiked negative plant extract with decreasing amount of HLVd sequence for ten replicates of each tested concentration as shown in the table below. **These studies indicated that the assay can reliably detect down to 7.5 viroid copies per reaction.**

Table shows the results from studies performed to determine the LoD of TUMI Genomics HLVd qRT-PCR assay. The identified LoD is indicated in yellow.

Sample Concentration	Fraction Positive	Mean CT plant control	Standard Deviation	Mean CT HLVd (Target #1)	Standard Deviation	Mean CT HLVd (Target #2)	Standard Deviation
500 copies/reaction	10/10	28.72	+/- 1.04	26.81	+/- 0.13	27.68	+/- 0.32
250 copies/reaction	10/10	29.07	+/- 1.32	27.08	+/- 0.11	28.13	+/- 0.21
125 copies/reaction	10/10	29.56	+/-1.57	28.38	+/- 0.16	29.61	+/- 0.23
31 copies/reaction	10/10	30.24	+/- 0.14	30.44	+/- 0.87	31.05	+/- 0.15
15 copies/reaction	10/10	29.65	+/- 0.07	31.41	+/- 0.29	32.06	+/- 0.71
7.5 copies/reaction	10/10	29.42	+/- 0.16	32.01	+/- 0.122	33.03	+/- 0.45
3.8 copies/reaction	3/10	29.23	+/- 0.15	32.71	+/- 1.53	33.93	+/- 0.73
0 copies/reaction	0/30	28.67	+/- 1.16	NaN	NaN	NaN	NaN

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 of the TUMI Genomics' test 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 HLVd primer/probe set.

- To adjust for short input sequences and increase the number of detected sequences, the parameter word_size was set to 5.
- Match and mismatch scores were set to 1 and -3, respectively.
- The penalty to create and extend a gap in an alignment was set to 5 and 2, respectively.

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 100% with sequences from the Hop Latent Viroid genome. **No cross-reactivity above 80% was found with any other tested microorganism.**

The table shows the identity of each tested microorganism and the percent cross-reactivity with the primer/probe sets used to target the HLVd genome. Cross-reactivity is shown separately for each primer/probe set.

Organism	% Cross reactivity - HLVd target (1) primer/probe set	% Cross reactivity - HLVd target (2) primer/probe set
Hop latent viroid	**100.00%**	**100.00%**
Alfalfa mosaic virus	49.25%	50.00%
Alternaria alternata strain	71.64%	67.65%
Arabis mosaic virus	49.25%	51.47%
Aster yellows witches'-broom	61.19%	54.41%
Beet curly top	44.78%	50.00%
Berkeleyomyces basicola strain	74.63%	67.65%
Botrytis cinerea B05.10	65.67%	75.00%
Cannabis cryptic virus	43.28%	33.82%
Cercospora cf. flagellaris	74.63%	70.59%
Cladosporium cladosporioides strain	68.66%	66.18%
Colletotrichum fioriniae strain	77.61%	73.53%
Cucumber mosaic virus	44.78%	47.06%
Curvularia lunata strain	73.13%	76.47%
Fusarium oxysporum f.	71.64%	69.12%
Fusarium sambucinum strain	70.15%	66.18%
Fusarium solani strain	74.63%	67.65%
Hop mosaic virus	41.79%	48.53%
Hop stunt viroid	38.81%	41.18%
Macrophomina phaseolina isolate	77.61%	70.59%
Phoma sp. XZ068	70.15%	72.06%
Phytophthora citricola strain	71.64%	72.06%
Pseudomonas syringae strain	64.18%	61.76%
Pseudoperonospora humuli isolate	64.18%	66.18%
Pythium aphanidermatum DAOM	62.69%	66.18%
Pythium arrhenomanes ATCC	67.16%	57.35%
Pythium brassicum strain	65.67%	63.24%
Pythium guiyangense strain	68.66%	66.18%
Pythium insidiosum DNA,	70.15%	66.18%
Pythium oligandrum strain	76.12%	67.65%
Pythium periplocum strain	71.64%	67.65%
Rhizoctonia solani strain	76.12%	69.12%
Sclerotinia sclerotiorum 1980	76.12%	73.53%
Stemphylium lycopersici strain	71.64%	67.65%
Stemphylium vesicarium strain	74.63%	64.71%
Tetranychus urticae unplaced	73.13%	73.53%
Tobacco ringspot virus	47.76%	48.53%

Tobacco streak virus	44.78%	41.18%
Tomato mosaic virus	41.79%	47.06%
Tomato ringspot virus	41.79%	41.18%
Verticillium albo-atrum strain	71.64%	70.59%
Verticillium dahliae VdLs.17	68.66%	72.06%
Xanthomonas cannabis pv.	59.70%	60.29%

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.

An in-silico inclusivity analysis was performed by aligning all primer sequences from both targets in the hop latent viroid genome detected by TUMI Genomics RT-qPCR assay against hop latent viroid variants deposited within GenBank. A total of 155 genomes were in the repository. Of those, 91% (141) have one mismatch or less in the primers used to detect at least one of the two targets. These genomes would be readily detected by our assay. Of the 14 remaining genomes, the maximum number of mismatches for any single primer was two with the majority being in reverse primer sets (11/14). These types of mismatches are unlikely to impact amplification efficiency in one-step RT-PCRs reactions due to the increased hybridization efficiency of DNA/RNA hybrids during the reverse transcription step (Stadhouders et. al, 2010). The mismatches between our primers and the remaining 3 genomes are all > 5 basepairs from the 3' end of the primer sequence, indicating these mismatches are unlikely to inhibit amplification (Christopherson et. al, 1997), but could result in underestimation of viroid load.

Therefore, TUMI genomics assay reliability detects 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.

Clinical/comparative validation: The purpose of a clinical/comparative analysis is to determine the accuracy of a test when compared to another test available on the market. In clinical diagnostics, the comparison is generally made against a test that has already gained regulatory body approval (i.e. FDA or USDA). Because no regulatory body currently governs cannabis diagnostics, a fully correct accuracy analysis is not possible. However, it is possible to compare an assay to another lab offering cannabis pathogen diagnostics to determine how closely the two labs agree.

Positive predictive value (PPV): This value indicates the probability of the test finding a positive that was found by the comparison lab.

100% (24/24) samples identified by an independent reference laboratory as positive for HLVd also tested positive by TUMI Genomics HLVd qRT-PCR assay.

Negative predictive value (NPV): This value indicates the probability of the test finding a negative that was found by the comparison lab.

92% (82/89) samples identified as negative by an independent reference laboratory also tested negative by TUMI Genomics HLVd qRT-PCR assay.

Overall Agreement

Of the 113 samples compared between TUMI Genomics labs and the comparator lab, 104 samples agreed representing an overall agreement of 94% between both labs.

Table shows summary data for positive and negative predictive value of TUMI Genomics qRT-PCR test for HLVd compared to a reference lab.

		Reference Laboratory Results	
		Positive	**Negative**
TUMI Genomics qRT-PCR assay results	**Positive**	24	89
	Negative	0	82 *
	PPA	100% (87.9%-99%)	
	NPA	92% (84.4%96.4%)*	

*We noted that among the 7 negative samples that tested positive by TUMI Genomics HLVd qRT-PCR assay, the average viroid load per reaction was 7255 copies per reaction, whereas the average viroid load among samples testing positive in both assays was $1.74X10^7$ copies per reaction. These values suggest that the discrepancy between the reference lab results and TUMI Genomics HLVd qRT-PCR assay is likely due to increased sensitivity of the TUMI Genomics qRT-PCR assay compared to the comparator lab (see table below).

Table shows the CT values from all samples testing positive by TUMI Genomics qRT-PCR along with CT values associated with HLVd amplification and the reference lab results. Samples showing a discrepancy between TUMI Genomics qRT-PCR results and the reference lab are shown in red.

TUMI Genomics HLVd qRT-PCR result	CT value for HLVd (Target #1)	Estimated Viroid Load (copies per reaction)	Reference Lab Result
Positive	**26.18**	**2.77E+03**	**Negative**
Positive	**23.02**	**2.89E+04**	**Negative**
Positive	**31.69**	**4.62E+01**	**Negative**
Positive	**23.90**	**1.50E+04**	**Negative**
Positive	**28.64**	**4.45E+02**	**Negative**
Positive	**27.85**	**8.00E+02**	**Negative**
Positive	**26.18**	**2.77E+03**	**Negative**
Positive	26.45	2.26E+03	Positive
Positive	18.95	5.94E+05	Positive
Positive	22.05	5.94E+04	Positive
Positive	14.21	2.01E+07	Positive
Positive	15.89	5.77E+06	Positive
Positive	14.96	1.15E+07	Positive
Positive	13.77	2.79E+07	Positive
Positive	13.28	4.01E+07	Positive
Positive	13.64	3.07E+07	Positive
Positive	15.51	7.65E+06	Positive
Positive	15.96	5.48E+06	Positive
Positive	13.60	3.16E+07	Positive
Positive	12.19	9.01E+07	Positive
Positive	13.72	2.89E+07	Positive
Positive	14.13	2.13E+07	Positive
Positive	19.38	4.32E+05	Positive
Positive	14.01	2.33E+07	Positive

Positive	14.65	1.45E+07	Positive
Positive	13.10	4.58E+07	Positive
Positive	16.99	2.55E+06	Positive
Positive	15.37	8.49E+06	Positive
Positive	19.21	4.90E+05	Positive
Positive	18.18	1.05E+06	Positive
Positive	25.21	5.69E+03	Positive

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. Garrido-Lecca 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+garrido-lecca&sort=date

Erika Lasda, PhD: Dr. Lasda received her undergraduate degree in biology from the University of Richmond, Virginia and her graduate degree from the University of Colorado, Boulder in biochemistry and molecular genetics. Her graduate work focused on mechanisms of RNA splicing in *C. elegans* and her post-doctoral work focused on determining mechanisms of circular RNA translation, degradation, and excretion. Following her post-doctoral studies, Dr. Lasda worked as a research associate investigating the function of non-coding RNAs and protein/RNA interactions. She also worked with Dr. Saldi in the COVID-19 surveillance lab at CU, Boulder coordinating PCR diagnostics and result interpretation. She is a lead scientist and director of laboratory operations at TUMI Genomics.

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