PathoSEEK® Hop Latent Viroid Detection Assay Version 2

Method Developer Validation

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Introduction

Hop Latent Viroid (HLVd) is arguably the most destructive cannabis plant pathogen. One study estimates that as much as 90% of California cannabis was at one point infected with HLVd, costing nearly $4 billion in lost yields.

Once infected, plants can show a variety of symptoms, ranging from no obvious signs (asymptomatic) to a nearly 50% reduction in yield and potency. HLVd transmits from one plant to another via contact with infected plant sap. This most commonly occurs via tools, equipment, and workers who have come into contact with infected plant sap. Cultivators should always sterilize tools, equipment, and hands with a 10% bleach solution before starting work on a new plant. HLVd can also spread through cloning when cuttings are taken from an infected mother, shared water supplies, and leafhoppers.

The PathoSEEK® Hop Latent Viroid Detection Assay is a reverse-transcriptase quantitative PCR (RT-qPCR or QRT-PCR) assay that screens for the presence of HLVd. The PathoSEEK Hop Latent Viroid Detection Assay is designed to detect HLVd in cannabis leaves and roots, in a single RT-qPCR (Reverse Transcription Quantitative Polymerase Chain Reaction).

Version 2 of the HLVd Method includes the following updates:

- Optimized primer and probe concentrations
- Lyophilized RT-qPCR Master Mix reagent (RT-qPCR Master Kit v2)
- Two Options for sample lysis
    - Quick Lysis - Nucleic Acid Extraction Solution
    - Pure Prep - Nucleic Acid Purification Solution

Objective

To evaluate the PathoSEEK Hop Latent Viroid Detection Assay v2 using both the Quick Lysis method and Pure Prep method for the detection of Hop Latent Viroid in cannabis leaves and roots.

Materials

Assay Name: PathoSEEK® Hop Latent Viroid Detection Assay v2

Assay Components:

1. Sample Preparation Options:
    a. Quick Lysis - P/N 420240
    b. Pure Prep - P/N 420031
2. PathoSEEK® RT-qPCR Master Kit Version 2 - P/N 420207
3. PathoSEEK® Hop Latent Viroid Positive Control - P/N 420123
4. PathoSEEK® Hop Latent Viroid Detection Assay Version 2 - P/N 420135

Method Developer Validation Data

Limit of Detection

The Limit of Detection (LOD) is a measure of the qPCR assay's sensitivity. The LOD was tested by doing a 10-fold serial dilution using the HLVd positive control. The approximate copy number was determined using the provided OD260 and amplicon length. This experiment was performed on both the Biorad CFX96 qPCR Instrument and the Biomolecular Systems Mic qPCR Instrument along with the Myra Liquid Handling System.

These studies indicated that the assay can reliably detect down to 7 viroid copies, similar to other assays available on the market.

Table 1: LOD results using 20uL Reaction Volume in 96 well plate

Approximate Copy Number	Target (FAM)	Average Ct FAM	Average Ct HEX
660,000,000	HLVd	12.14	29.98
66,000,000	HLVd	15.11	23.08
6,600,000	HLVd	18.16	23.21
660,000	HLVd	20.75	23.56
66,000	HLVd	23.94	23.74
6,600	HLVd	27.11	23.85
660	HLVd	29.92	23.89
66	HLVd	32.79	23.89
7	HLVd	35.51	24.06
1	HLVd	ND	ND

Table 2: LOD results using 10uL Reaction Volume - Mic/Myra ONLY

Approximate Copy Number	Target	Average Ct FAM	Average Ct HEX
660,000,000	HLVd	12.88	34.16
66,000,000	HLVd	16.01	25.5
6,600,000	HLVd	19.24	23.75
660,000	HLVd	21.96	23.45
66,000	HLVd	24.92	23.39
6,600	HLVd	27.9	23.38
660	HLVd	30.84	23.33
66	HLVd	33.84	23.27
7	HLVd	37.38	23.35
1	HLVd	ND	ND

Circular RNA Limit of Detection

A circular RNA template provides a better analog for how the assay behaves in a real-world environment because it tests the RNA to DNA conversion step in RT-qPCR. Viroids are circular RNAs, which are very stable molecules and difficult to denature; therefore, we see different priming characteristics when compared to positive controls (e.g., linear DNA).

We performed a circular RNA LOD using a synthetic circular RNA genome. The approximate copy number was determined using the provided OD260 and amplicon length. A 10-fold serial dilution was performed down to 651 copies, after which the dilutions were halved down to 163 copies. This experiment was performed on both the Biorad CFX96 qPCR Instrument.

These studies indicated that the assay can reliably detect down to 163 circular RNA copies. At the time of publishing, Medicinal Genomics is the only assay provider to perform an LOD using circular RNA.

Table 3 - Circular RNA qPCR LOD

Approximate Copy Number	Target	Average Ct Fam	Average Ct Hex
6,508,000,000	HLVd	11.64	N/A
650,800,000	HLVd	14.59	N/A
65,080,000	HLVd	17.82	N/A
6,508,000	HLVd	21.06	N/A
650,800	HLVd	23.92	N/A
65,080	HLVd	27.00	N/A
6,508	HLVd	30.59	N/A
651	HLVd	33.42	N/A
325	HLVd	34.74	N/A
163	HLVd	35.49	N/A

Inclusivity

Predicted amplicons from our PathoSEEK® Hop Latent Viroid Detection Assay were extracted from HLVd genomic sequences deposited into the public NCBI sequence repository. Synthetic DNA representing 11 distinct HLVd amplicons termed *genomic blocks* were ordered from IDT and used to test and validate the PathoSEEK® Hop Latent Viroid Detection assay, and all 11 genomic blocks tested positive. These 11 amplicon sequences are found in 25 HLVd genomic sequences; 23 from NCBI and an additional two provided by Zamir Punja. A summary of these is presented in Table 4.

Table 4. List of 25 Inclusion Strains

NCBI Accession	Strain Name	Target	Result
MK795561.1	Hop latent viroid strain CV_42	HLVd	+
MK795569.1	Hop latent viroid strain CV_50	HLVd	+
MK795585.1	Hop latent viroid strain CV_66	HLVd	+
MK795587.1	Hop latent viroid strain CV_68	HLVd	+
MK795605.1	Hop latent viroid strain CV_86	HLVd	+
MK795619.1	Hop latent viroid strain CV_100	HLVd	+
MK795620.1	Hop latent viroid strain CV_101	HLVd	+
MK795623.1	Hop latent viroid strain CV_104	HLVd	+
MK795625.1	Hop latent viroid strain CV_106	HLVd	+
OK143457.1	Hop latent viroid isolate HLVd-BR	HLVd	+
NC_003611.1	HLVd RefSeq	HLVd	+
MK876285.1	Hop latent viroid isolate C1	HLVd	+
MK774671.1	Hop latent viroid isolate HpLVd001	HLVd	+
KT600318.1	Hop latent viroid isolate GVdC_HLVd01	HLVd	+
KT600317.1	Hop latent viroid isolate GVdC_HLVd02	HLVd	+
EF613191.1	Hop latent viroid isolate Y3	HLVd	+
EF613190.1	Hop latent viroid isolate T13	HLVd	+
EF613187.1	Hop latent viroid isolate S4	HLVd	+

EF613184.1	Hop latent viroid isolate H13	HLVd	+
EF613182.1	Hop latent viroid isolate C6	HLVd	+
MK876286.1	Hop latent viroid isolate C2	HLVd	+
EF613186.1	Hop latent viroid isolate K9	HLVd	+
EF613183.1	Hop latent viroid isolate H2	HLVd	+
NA	Mac_full_assembly_Contig_9-Punja_et_al	HLVd	+
NA	G54-2_Full_assembly_Contig_7-Punja_et_al	HLVd	+

Exclusivity

The PathoSEEK Hop Latent Viroid Detection Assay was tested on 32 live organisms and 21 purified DNA organisms from ATCC (American Type Culture Collection). The live target organisms were cultured under optimal conditions for the growth of the organism followed by DNA Purification. These 32 live organisms were isolated without RNAse such that the transcriptome and the genome were present. A synthetic cannabis DNA spike was included in the extraction of the live organisms to ensure that extraction was successful. Exclusivity results showed the PathoSEEK Hop Latent Viroid Detection Assay is highly specific in identifying Hop Latent Viroid, and the assays successfully excluded 53 non-target organisms. (See Table 5)

Table 5. List of 53 exclusion organisms.

Organism	Source	Origin	Target	Result
Alternaria alternata	ATCC 6663	Not Available	HLVd	-
Acinetobacter baumanii	ATCC 19606	Urine	HLVd	-
Aspergillus alabamensis	ATCC MYA-3633	Not Available	HLVd	-
Aspergillus caesiellus	ATCC 42693	Dried chilies, New Guinea	HLVd	-
Aspergillus carneus	ATCC 13549	Not Available	HLVd	-
Aspergillus deflectus	ATCC 62502	Wheat, China	HLVd	-
Aspergillus fijiensis	ATCC 20611	Not Available	HLVd	-
Aspergillus fischeri	ATCC 66641	Not Available	HLVd	-
Aspergillus flavus	ATCC MYA-3631	Human biopst, USA	HLVd	-
Aspergillus flavus	ATCC 200026	Peanut cotyledons, USA	HLVd	-
Aspergillus fumigatus	ATCC 13073	Human pulmonary lesion, Maryland	HLVd	-
Aspergillus japonicus	ATCC 16873	Soil, Panama	HLVd	-
Aspergillus nidulans	ATCC 38163	Not Available	HLVd	-
Aspergillus niger	ATCC 13496	Soil, Louisiana	HLVd	-
Aspergillus oryzae	ATCC 10124	Not Available	HLVd	-
Aspergillus parasiticus	ATCC 56775	Not Available	HLVd	-
Aspergillus pseudoterreus Peterson et al	ATCC 10020	Not Available	HLVd	-
Aspergillus terreus	ATCC 12238	Not Available	HLVd	-
Aspergillus tubingensis	ATCC 1004	Clinical Isolate, New York City, NY	HLVd	-
Aspergillus tubingensis	ATCC MYA 4996	Not Available	HLVd	-
Aspergillus ustus	ATCC 1041	Culture containment, USA	HLVd	-
Aspergillus versicolor	ATCC 11730	Cellophane gas mask, India	HLVd	-
Candida albicans	ATCC 10231	Man with bronchomycosis	HLVd	-
Cryptococcus neoformans	ATCC 208821	Patient with Hodgkin's disease, New York	HLVd	-
Fusarium proliferatum	ATCC 76097	Raw cotton, North Carolina	HLVd	-
Fusarium solani	ATCC 52628	Guatemala	HLVd	-
Mucor hiemalis	ATCC 28935	Soil in spruce forest, Germany	HLVd	-
Mucor luteus	ATCC 28932	Not Available	HLVd	-
Penicillium chrysogenum	ATCC 18476	Chesse?, USSR	HLVd	-
Penicillium venetum	ATCC 16025	Hyacinthus sp. Bulb, England	HLVd	-
Talaromyces marneffei	ATCC 18224	Not Available	HLVd	-

Yarrowia lipolytica	ATCC 20390	Nonsporulating diploid	HLVd	-
Vibrio cholerae Strain N16961	ATCC 39315D-5	Not Available	HLVd	-
Escherichia Coli (STEC)	ATCC BAA-2440D	Not Available	HLVd	-
Salmonella houtenae Strain MZ1443	ATCC BAA-1580D-5	Not Available	HLVd	-
Aspergillis clavatus	ATCC 1007D-2	Not Available	HLVd	-
Salmonella bongori	ATCC 43975D-5	Not Available	HLVd	-
Escherichia Coli Strain TY-2482	ATCC BAA-2326D9	Not Available	HLVd	-
Enterobacter aerogenes	ATCC 15038D-5	Not Available	HLVd	-
Listeria seeligeri Strain 1120	ATCC 35967D-5	Not Available	HLVd	-
Listeria welshimeri strain V8	ATCC 35897D-5	Not Available	HLVd	-
Salmonella typhimurium	ATCC 700720D-5	Not Available	HLVd	-
Salmonella Indicia Strain MZ1447	ATCC BAA-1578D-5	Not Available	HLVd	-
Pseudomonas aeruginosa	ATCC 47085 D-5	Not Available	HLVd	-
Staphylococcus aureus	ATCC 6538 D-5	Not Available	HLVd	-
Yersinia pestis	ATCC BAA-1511 D-5	Not Available	HLVd	-
Lactobacillus acidophilus	ATCC 4357 D-5	Not Available	HLVd	-
Klebsiella pneumoniae	ATCC 700721 D-5	Not Available	HLVd	-
Shigella flexneri	ATCC 29903 D-5	Not Available	HLVd	-
Pseudomonas aeruginosa	ATCC 9027 D-5	Not Available	HLVd	-
Pseudomonas syringae	ATCC BAA-871 D-5	Not Available	HLVd	-
Candida albicans	ATCC 10231 D-5	Not Available	HLVd	-
Aspergillus brasiliensis	ATCC 16404 D-2	Not Available	HLVd	-

Sample Type

Recent studies have suggested that roots produce the most robust signal in HLVd-infected plants. A laboratory partner in Florida collected root and leaf samples from 40 different plants that were suspected to have HLVd infection and processed them using the PathoSEEK Hop Latent Viroid Detection Assay with Leaf Punch Lysis Solution protocol. The results showed that 15 of the root samples tested positive for HLVd, while only 1 leaf sample tested positive. These results confirm that an infection of HLVd can be found in just the root and not the leaf of an infected plant.

Table 6. Results from suspected HLVd-infected plants.

Sample Name	HLVd		Cannabis DNA	
	Cq Leaf (FAM)	Cq Root (FAM)	Cq Leaf (HEX)	Cq Root (HEX)
Sample-01	ND	25.25	27.46	32.03
Sample-02	ND	ND	27.81	38.21
Sample-03	ND	24.65	27.73	31.80
Sample-04	ND	24.75	28.35	30.33
Sample-05	ND	25.62	27.60	31.23
Sample-06	ND	25.64	28.25	30.77
Sample-07	ND	23.23	27.91	29.41
Sample-08	ND	ND	26.39	30.99
Sample-09	ND	ND	27.29	31.86
Sample-10	ND	ND	25.19	31.88
Sample-11	ND	ND	28.75	33.28
Sample-12	ND	ND	27.66	29.91
Sample-13	ND	ND	27.57	33.70
Sample-14	ND	23.77	28.21	29.75
Sample-15	ND	24.73	27.00	31.87
Sample-16	ND	ND	27.60	31.32
Sample-17	ND	ND	28.74	32.25
Sample-18	ND	ND	27.71	34.75
Sample-19	ND	ND	28.22	32.86
Sample-20	ND	ND	28.89	30.83
Sample-21	ND	ND	27.94	29.80
Sample-22	ND	22.47	29.01	28.53
Sample-23	ND	ND	29.85	30.49

Sample-24	ND	ND	28.06	32.72
Sample-25	ND	ND	27.74	30.86
Sample-26	ND	ND	27.60	31.52
Sample-27	ND	ND	27.00	32.88
Sample-28	ND	23.52	29.35	30.61
Sample-29	ND	23.76	29.27	31.46
Sample-30	ND	28.20	27.43	31.50
Sample-31	ND	ND	28.27	30.36
Sample-32	ND	ND	28.83	29.78
Sample-33	ND	ND	27.60	32.03
Sample-34	ND	ND	27.34	31.76
Sample-35	ND	ND	28.53	28.94
Sample-36	ND	ND	27.54	32.12
Sample-37	ND	34.37	27.41	30.75
Sample-38	ND	ND	27.06	30.61
Sample-39	ND	24.50	28.00	29.33
Sample-40	24.30	20.59	29.68	26.50
Positive Control	13.17	15.54	No Cq	No Cq
NTC	No Cq	No Cq	39.24	No Cq

Lysis Method Comparison

We offer two different lysis methods that are compatible with PathoSEEK® Hop Latent Viroid Detection Assay v2.

- Quick Lysis is a simple boil-based prep, which requires no manual pipette steps.
- Pure Prep is a bead-based purification method that concentrates the DNA and RNA present in the sample and eliminates PCR inhibitors.

Internal experiments comparing Pure Prep to Quick Lysis showed that Pure Prep recovered 10 times more HLVd RNA from infected root samples than Quick Lysis.

Figure 1: Results from Lysis Buffer Comparison on Infected Roots

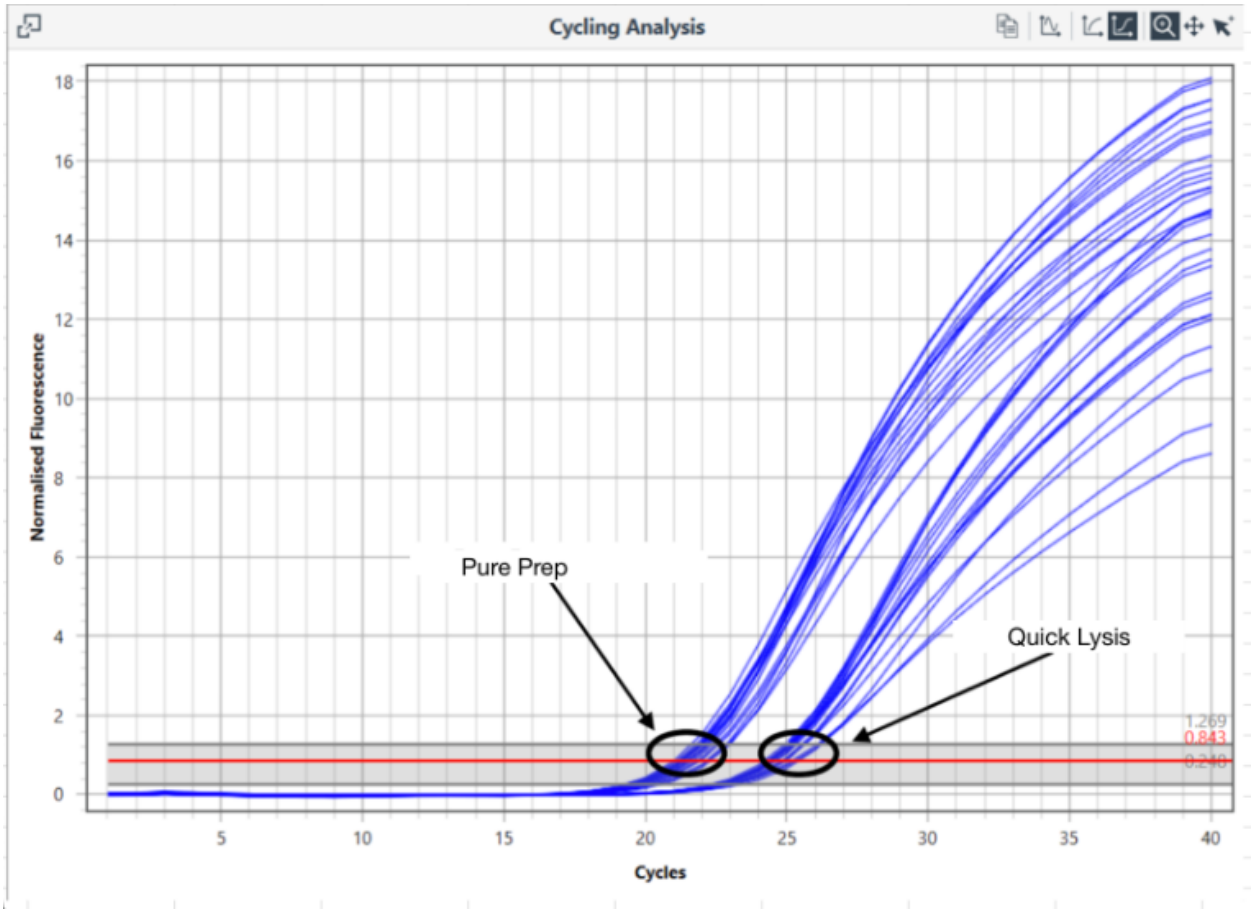

Table 7: Sample Prep Comparison on Infected Roots

Sample	Method	Cq Fam	Cq Hex
Infected Root	Pure Prep	20.99	21.16
Infected Root	Pure Prep	22.14	22.32
Infected Root	Pure Prep	21.22	22.23
Infected Root	Pure Prep	21.30	21.17
Infected Root	Pure Prep	21.53	22.53
Infected Root	Pure Prep	21.29	21.58
Infected Root	Pure Prep	22.16	22.04
Infected Root	Pure Prep	21.79	22.32
Infected Root	Pure Prep	21.17	21.32
Infected Root	Pure Prep	22.26	22.72
Infected Root	Pure Prep	21.24	21.88
Infected Root	Pure Prep	21.09	21.26
Infected Root	Pure Prep	21.64	22.65
Infected Root	Pure Prep	21.40	21.80
Infected Root	Pure Prep	21.90	21.87
Infected Root	Pure Prep	21.46	21.95
Infected Root	Quick Lysis	24.39	21.84
Infected Root	Quick Lysis	24.39	22.09
Infected Root	Quick Lysis	24.40	21.94
Infected Root	Quick Lysis	24.93	22.50
Infected Root	Quick Lysis	24.41	22.46
Infected Root	Quick Lysis	25.15	22.49
Infected Root	Quick Lysis	25.26	22.04
Infected Root	Quick Lysis	24.57	21.84
Infected Root	Quick Lysis	24.17	21.62
Infected Root	Quick Lysis	24.51	21.70
Infected Root	Quick Lysis	24.31	22.08
Infected Root	Quick Lysis	25.38	22.42
Infected Root	Quick Lysis	24.35	22.48
Infected Root	Quick Lysis	24.79	22.35
Infected Root	Quick Lysis	24.80	21.99
Infected Root	Quick Lysis	24.29	22.04
Positive Control		19.13	ND

Conclusion

The PathoSEEK Hop Latent Viroid Detection Assay v2 is a highly specific and sensitive method for the detection of Hop Latent Viroid in cannabis leaves and roots.

If the objective is to detect HLVd infection as early as possible, when the viroid load is very low, then the Pure Prep Nucleic Acid Purification method is the best option. Pure Prep recovers and purifies more genetic material from the sample and reduces PCR inhibitors resulting in higher sensitivity and higher probability of detecting a low level infection.

The Quick Lysis Nucleic Acid Extraction method is a straightforward boil-based prep, which requires no manual pipette steps, making it a scalable option that is perfect for customers who are screening a large number of plants.

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