MyFloraDNA

MFDetect™

Assay for HLVd

Abstract

Hop latent viroid (HLVd) poses a formidable challenge to cannabis growers worldwide, threatening crop health, productivity, and profitability. The asymptomatic nature of HLVd along with the lack of effective control and eradication techniques necessitates the development of robust and reliable early detection systems. In response to this critical need, MyFloraDNA's research team has developed MFDetect™, an innovative testing platform designed to facilitate rapid, sensitive, and precise diagnostics for HLVd in cannabis plants. This document details the Limit of Detection (LOD), or the lowest HLVd copy number that the MFDetect™ technology can detect in infected plant tissue. We additionally demonstrate the high degree of specificity of HLVd detection with MFDetect™. Combined, the data presented here demonstrates that the sensitivity and specificity of MFDetect™ is comparable to the standard TaqMan quantitative reverse transcriptase PCR (RT-qPCR), consistently detecting ~5 copies of HLVd with high statistical confidence.

(i) Introduction

Hop latent viroid (HLVd), a circular RNA molecule with no protein coat, presents a significant threat to the cannabis industry by causing stunted growth, reduced yields, and ultimately impacting cannabinoid and terpene production. Despite its detrimental effects, detecting HLVd infections in cannabis plants remains challenging. This, exacerbated by a lack of proper disease management and mitigation practices, has facilitated the rapid spread of disease. There is an urgent need for advanced diagnostic tools suitable for early and accurate detection to mitigate the spread of HLVd and protect cannabis crops.

(ii) The Need for Reliable Detection Methods

HLVd infections can remain asymptomatic in cannabis plants, making visual detection ineffective, particularly in the early stages of infection. Furthermore, the viroid's stability in plant tissues and on propagation tools complicates mitigation efforts, emphasizing the importance of early detection to prevent its spread. A highly sensitive, accurate and reliable diagnostic technique that can detect low viroid load in the early stages of growth in cannabis is much needed.

(iii) Introducing MFDetect™

MFDetect™ represents a cutting-edge solution to the challenges posed by HLVd in cannabis cultivation. Developed by the MyFloraDNA research team, this proprietary technology combines high-throughput handling procedures, RT-LAMP, and quantitative PCR to enable rapid, sensitive, and precise detection of HLVd in cannabis plants. Our talented R&D team has spent years of research developing a recipe that combines a unique nucleic acid extraction method, expertly designed high-temperature-resistant primers, and customized DNA-binding dyes to empower our clients to catch the viroid before it wreaks havoc on their crop. MFDetect™ offers unparalleled rapidity, sensitivity and specificity, allowing for the high throughput detection of low viroid titers in tissue samples at a relatively low cost to the client.

(iv) Limit of Detection (LOD)

Primer design, RNA extraction, gBlock and primer dilution, RNA amplification, data scoring and analysis were performed according to our previous publication (Fernandez i Marti et al 2023). A series of 15-fold dilutions was prepared ranging from 20479 to 0.0001 HLVD transcript copies/µL. Three uL of each dilution was added to the reaction mix, equating to 61437 to 0.0003 copies per 25 µL PCR reaction. In parallel, the same dilution series was also used to spike negative biological controls. No Template Control (NTC) samples containing water were also included in duplicates. The same dilution series was also tested using standard commercially available RT-qPCR. The lowest viroid load that consistently produced a clear and repeatedly positive result in the MFDetect™ prior to amplification of any negative controls was used to establish LOD for this assay. Our experiment revealed that our limit of detection for HLVD is 5 copies per microliter of sample (Figure 1, Table 1). To ensure robust test validation, these runs were performed in triplicates. The LOD observed with the MFDetect™ assay corresponded to an RT-qPCR CT value of less than 33, emphasizing the comparable sensitivity and accuracy of the two diagnostic methods in detecting HLVd infections.

Numerous studies have now demonstrated that 1–10 copies of viral RNA template per reaction were sufficient for successful detection of coronavirus using RT- LAMP amplification assay, which were ~ 100-fold more sensitive than conventional RT-PCR methods (Shirato et al 2018, Poon et al 2004). Xin et al. (2021) demonstrated that a multiplexed RT-LAMP assay could directly detect as low as 1.5 copies/µL of SARS-CoV-2 particles in saliva, without the need of RNA isolation. Zhao et al (2015) showed that RT-LAMP assay for Tomato Chlorosis Virus (ToCV) can detect viral dilutions up to 2.0×10(-7)ng, which is 100-times more sensitive than reverse transcription-polymerase chain reaction (RT-PCR).

Target Copies per uL	HLVd Detected	Time to Positive (mins)	Detected Using MFDetect
20,479	3/3	21	+
5,120	3/3	22	+
1,280	3/3	25	+
320	3/3	27	+
80	3/3	29	+
20	3/3	32	+
5	**3/3**	**33**	**+**
1.25	1/3	36	+/-
0.31	0/3	N/A	-
0	0/3	N/A	-

Table 1: Limit of Detection of MF Detect™: A series of dilutions of the synthetic DNA was run to determine the minimum copy number of detection. The results were reconfirmed using biological positive controls and experiments were performed in triplicates.

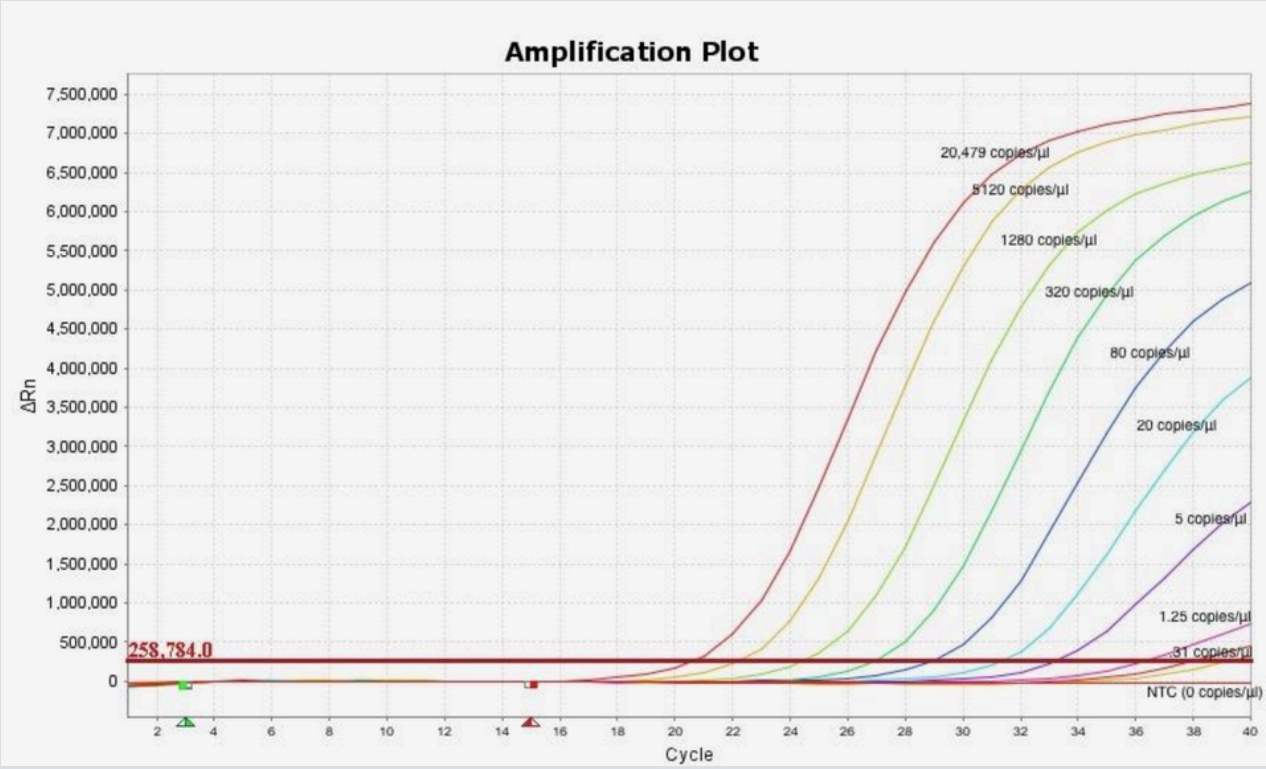

Figure 1: Real time amplification curve of the aforementioned serial dilution experiment.

(v) Specificity

MFDetect™ assay was tested against other pathogens common in cannabis i.e., Lettuce Chlorosis Virus (LCV), Alfalfa Mosaic Virus (AMV), Cannabis Cryptic Virus (CCV), Beet Curly Top Virus (BCTV) , Tobacco Mosaic Virus (TMV), Tomato Mosaic Virus (ToMV), Arabis Mosaic Virus (ArMV), Tomato Ringspot Virus (ToRSV), Fusarium botrytis, Fusarium oxysporum, Fusarium solani , Golovinomyces ambrosiae (Powdery Mildew), and Pythium myriotylum . No amplification was observed in no template controls, and no amplification was observed in all non-target pathogens, demonstrating high specificity. To date, MyFloraDNA has successfully completed HLVd testing in over >350,000 samples from >800 clients with each test plate harboring the positive and non-template controls. The large volume of negative controls run alongside these samples provides high quality data indicating the specificity of this assay. By targeting distinct regions of the HLVd genome and utilizing an annealing temperature of 67˚C, highly reliable and accurate diagnostic results have been achieved. Our findings align with Kokane et al. (2021), who found that the standardized RT-LAMP assay was highly specific and successfully detected all 35 Indian Citrus Ringspot Virus (ICRSV) isolates from Mandarin while avoiding all cross-reactivity with 17 isolates of five other citrus pathogens.

(vi) MFDetect and Taqman qRT-PCR comparison

Our results also revealed 99% agreement between MFDetect™ and TaqMan RT-qPCR for the detection of HLVD infected plants (Figure 2). Our findings aligned with Warghane et al. (2017), who found that the sensitivity of RT-LAMP for Citrus Tristeza Virus (CTV) was 100 times greater than conventional one step RT-PCR assays. They found a maximum detection limit as low as 0.0001ng RNA in individual reaction mixtures. Similarly, the study by Inaba et al. (2021) showed that the sensitivity and specificity of the RT-LAMP method is 100% comparable to RT-qPCR.

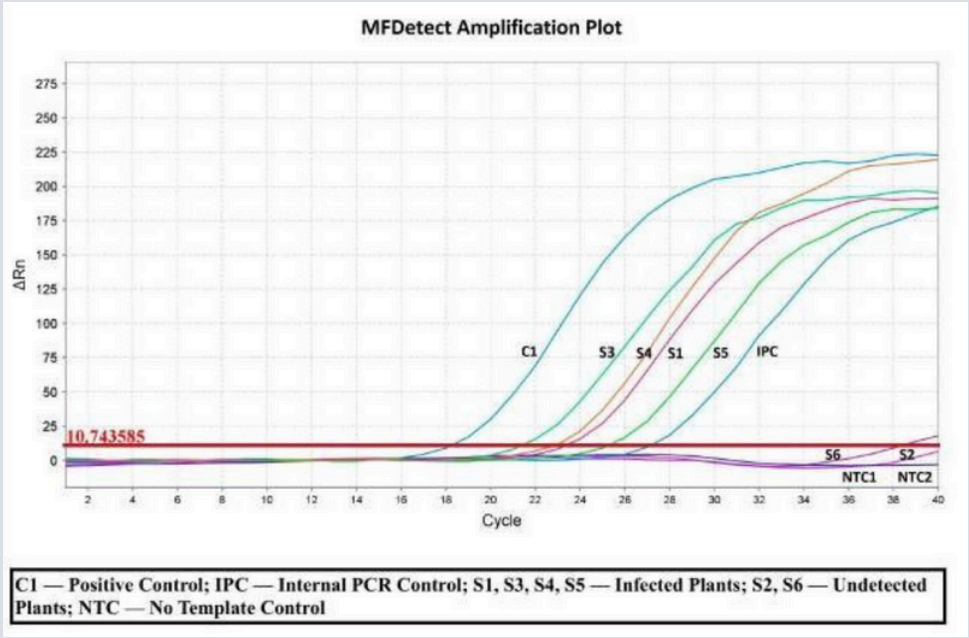

Figure 2: MFDetect™ real time fluorescence curves.

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(ii) Conclusion:

The development and validation of MFDetect™ represent a significant advancement in the field of cannabis pathogen detection. By offering rapid, sensitive, and precise diagnostics for HLVd infections, MFDetect™ equips cannabis growers with the tools necessary to protect their crops and preserve crop health, productivity, and profitability. As the cannabis industry continues to evolve, MFDetect™ stands poised to play a crucial role in ensuring the safety and sustainability of cannabis cultivation worldwide.

(viii) References:

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