BIOREBA

qPCR Hop latent viroid (HLVd) set

User Guide

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1. Introduction

1.1 General

The qPCR HLVd set enables the detection of Hop latent viroid (HLVd) with a duplex real-time RT-PCR. Viroid RNA of HLVd, extracted from hop or hemp samples, is amplified with a specific primer pair in a one-step RT-PCR reaction. The amplification of the cDNA can be monitored in real time, because the specific probes are labeled with fluorophores (HLVd: FAM). An internal positive control (IPC) is designed to detect a cytochrome oxidase (COX) gene sequence with a fluorophore-labeled (IC: ROX) specific probe. The control shows whether the extraction of samples, the reverse transcription and the amplification of the RNA worked as intended.
The qPCR HLVd set was developed and validated by BIOREBA. Suitable tissue sources of viroid RNA are hop/hemp leaves. For the extraction of RNA, it is recommended to use standard RNA extraction protocols established for plant material. Upon request, BIOREBA can recommend an optimal extraction method.

Two sets are available for 96 or 192 reactions (see page 4 for details).

1.2 Information: HLVd

Hop latent viroid (HLVd) was mentioned the first time in 1988 and was found in commercial hop varieties (*Humulus lupulus*) in Spain (1). Since this viroid did not induce any visible disease symptoms in hops, it was named "Hop latent viroid" (2). Worldwide surveys revealed the presence of HLVd in most of the tested hop cultivars (2). Although HLVd-infected hop plants are symptomless, infection significantly reduces yield as well as bitter acids and terpene content of hop cones, which drastically increases the economic impact of the viroid (3).
In hemp (*Cannabis sativa*), HLVd has been first observed in California (USA) in 2017 and was found to be the causative agent of the formerly called "duds" or "dudding disease". This disease has become the most devastating cannabis disease in cannabis-growing areas. Observed symptoms are shorter internodal spacing, smaller leaves, stunting, malformation, chlorosis, brittle stems, reduced vigor, lower water intake, reduced flower mass and trichomes (3). These symptoms are reflected in yield and loss of quality with up to 50% reduction of cannabinoid and terpene production (3).
HLVd belongs to the genus of *Pospiviroidae*, one out of the two currently known viroid families. Viroids are very small, protein-free and plant pathogenic RNA molecules, that are predominantly known to cause diseases in plants. HLVd has a covalently closed structure with circular RNA of 256 nucleotides (4).
HLVd can be transmitted over long distances and can be introduced into hop and hemp by infected propagative materials and transmits mechanically, by grafting or by vegetative propagation (5). To date, HLVd transmission by vectors is not known, transmission by pollen or seeds has been reported as being low or even non-existing (4).

1.3 Special handling instructions

Perform the tests in an RNase-free work environment. Always wear gloves when handling samples containing RNA and the components of the set. Do not touch any set components with an ungloved hand. Keep all components tightly sealed when not in use. Use appropriate laboratory disposable parts. In particular, use nuclease-free tubes and filter tips to avoid RNA degradation and cross-contamination.
Do not use components from sets with different lot numbers in the same test procedure. In order to avoid cross-contamination and obtain reliable results, it is essential to strictly follow the protocol in this manual. Avoid unnecessary freeze-thaw cycles of the set components.

1.4 Warranty and liability

BIOREBA products are guaranteed to meet the specifications described on the product certificate of analysis and in the user guide, which is included with every shipment. No further warranties are given. If you have any questions about specifications or performance, please contact our administrative office (admin@bioreba.ch).

Our products are for laboratory use only and are not intended for human or animal applications. Should a product fail for reasons other than inappropriate handling or misuse, BIOREBA will replace the product free of charge or refund the purchase price.

BIOREBA shall not be liable for any direct or indirect, special or consequential damage of any kind resulting from the use of our products.

2. Intended use

The qPCR HLVd set is validated for the simultaneous detection (multiplex) of Hop latent viroid (HLVd) and a cytochrome oxidase (COX) gene sequence which serves as internal positive control (IPC) by one-step real- time RT-PCR. Suitable tissues are hop/hemp leave samples. Samples of up to 25 leaves can be pooled for RNA extraction and analyzed according to this qPCR protocol.

3. Format, content, storage conditions and quality

3.1 Set format and contents

		Components		
Sales Part No.	**Product name**	**Colour of screw cap**	**Name**	**Volume**
899600	qPCR HLVd set 96	Blue	Taq Master Mix (2x) Art. No. 831412	1.2 ml
		Yellow	RT Master Mix (50x) Art. No. 830414	0.05 ml
		Clear	Primers/Probes Mix_HLVd/COX (10x) Art. No. 890100	0.2 ml
		–	Nuclease-free water Art. No. T143.4	1 ml
		–	HLVd positive control (PC) Art. No. 890053	30 µl
		–	Plant RNA negative control (NC) Art. No. 830043	30 µl
899200	qPCR HLVd set 192	Blue	Taq Master Mix (2x) Art. No. 831412	2 x 1.2 ml
		Yellow	RT Master Mix (50x) Art. No. 830414	2 x 0.05 ml
		Clear	Primers/Probes Mix_HLVd/COX (10x) Art. No. 890100	2 x 0.2 ml
		–	Nuclease-free water Art. No. T143.4	2 x 1 ml
		–	HLVd positive control (PC) Art. No. 890053	30 µl
		–	Plant RNA negative control (NC) Art. No. 830043	30 µl

3.2 Storage conditions

Store all qPCR components (Taq Master Mix, RT Master Mix, Primers/Probes Mix_HLVd/COX, Nuclease-free Water) at -30 °C to -10 °C.

3.3 Lot-to-Lot consistency

Quality control of the qPCR set is performed based on predetermined specifications to ensure consistent product quality. See lot-dependent certificate of analysis included with the shipment.

4. Specificity and Sensitivity information

4.1 Specificity

The primer pair used in this product specifically amplify a 113 basepair (bp) fragment of the HLVd circular RNA.

The qPCR HLVd set is suitable for detecting HLVd in hop/hemp leaves.

All isolates of HLVd tested so far can be detected (isolates from locations in Switzerland, Czech Republic and Slowenia).

No cross reactivity was observed with the following viruses (AMV, ArMV, BYDV, CMV, ErLV, GLRaV-4, GLRaV-6, PLRV, RBDV, SLRSV, TMV and TRSV) and no negative effect was observed from host matrix plant species (hop and hemp).

4.2 Sensitivity

The sensitivity of this product is high and was tested with leave samples from hop and hemp. The limit of detection for all tested samples and isolates ranged from 10^{-3} to 10^{-4}.

For leaves screening or routine testing in greenhouses, we mixed leaves of one HLVd-infected plant with leaves from different numbers of healthy hop/hemp plants. The sensitivity of the qPCR HLVd set for the distinct mixing ratios was as follows:

- Infected plant: Ct 23.95
- Mixed sample 3 plants (1 infected and 2 healthy): Ct 24.02
- Mixed sample 5 plants (1 infected and 4 healthy): Ct 24.33
- Mixed sample 10 plants (1 infected and 9 healthy): Ct 25.95
- Mixed sample 25 plants (1 infected and 24 healthy): Ct 27.03

Full validation data is available on request.

5. Materials and equipment required (not provided)

- RNase-free filter tips and micropipettes
- Optical grade RNase-free tubes/plate
- Disposable latex or vinyl gloves
- Thermal cycler for real-time PCR

6. Protocol

Please pay attention to the following points:

- The protocol in this manual must be followed.
- Create an RNase-free environment by cleaning the bench with 1 % bleach followed by 70 % ethanol.
- Gloves must be worn at all times.
- Use nuclease-free tubes and filter tips.
- Use appropriate eye protection and wear protective clothing.
- To avoid cross-contamination, use separate rooms for
  a) nucleic acid extraction,
  b) preparation of the Master Mix and
  c) amplification.
- Avoid unnecessary freeze-thaw cycles of the qPCR components.

6.1 RNA extraction

For the extraction of RNA it is recommended to use standard RNA extraction protocols established for plant material. Upon request, BIOREBA can recommend an optimal extraction method.

6.2 Preparation of the qPCR samples

1. Slowly thaw the set components (with the exception of the RT Master Mix!) on ice or at 4 °C. Thereafter, the components should always be kept on ice. It is not necessary to thaw the RT Master Mix (the enzyme is in glycerol.) The RT Master Mix should always be kept at -20 °C.

2. Shake the tubes briefly, and spin down the liquid.

3. To prepare the reaction mix, first determine the number of reactions and then increase the number by 1 or 2 to have enough volume for all reactions.

4. Prepare the reaction mix (without RNA template) by combining the components of the set to reach a final volume of 20 µl per reaction (see Table 1).

Component	Volume
Taq Master Mix (2x) Blue	10 µl
RT Master Mix (50x) Yellow	0.4 µl
Primers/Probes Mix_HLVd/COX (10x) Clear	2 µl
RNA Template	2 – 5 µl
Nuclease-free water	to reach a final reaction volume of 20 µl

Table 1: Preparation of reaction mix

5. Add the reaction mix (without RNA template) to each PCR tube or well of an optical-grade PCR plate.

6. Add 2-5 µl RNA template to the reaction mix.

7. Seal the PCR tubes or PCR plates, centrifuge briefly to collect components at the bottom of the PCR tubes or wells. Protect from light before thermocycling.

6.3 Thermal cycling

Place the PCR tubes or PCR plate in a thermocycler. Start cycling according to the program below (Table 2).

Step	Cycles	Temperature	Time
Reverse Transcription	1	50 °C	20 min
RT inactivation	1	95 °C	5 min
Denaturation Annealing/Extension	40	95 °C 60 °C	15 sec 30 sec

Table 2: PCR cycling conditions

6.4 Monitoring the PCR amplification

To monitor PCR amplification in real-time, an appropriate thermocycler is required, which can measure the fluorescence of the following fluorophores:

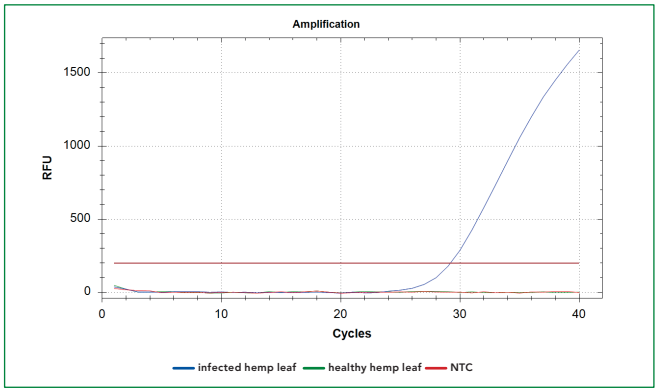
Dye	Virus/Control	Max. EX (nm)	Max. EM (nm)
FAM	HLVd	495	520
ROX	COX (IPC)	576	601

Table 3: Fluorophores overview

Please refer to the manufacturer's manual for information on programming the thermocycler, monitoring and evaluation.

6.5 Amplification of typical samples

The graph below shows the amplification curve of a HLVd-infected sample. No amplification was detected in the healthy control sample and the "no template control" (NTC).



Evaluation criteria
In order to distinguish positive from negative samples we recommend taking the following criteria into account:

A) The Ct value
B) The PCR efficiency
C) The delta RFU
(the difference between baseline and final RFU)

The range of values for each of the above can be determined for every channel by means of a dilution series of a known sample.

7. References

(1) Pallas, V., Navarro, A., & Flores, R. (1987). Isolation of a viroid-like RNA from hop different from hop stunt viroid. Journal of General Virology, 68(12), 3201-3205.

(2) Puchta, H., Ramm, K., & Sänger, H. L. (1988). The molecular structure of hop latent viroid (HLV), a new viroid occurring worldwide in hops. Nucleic acids research, 16(10), 4197-4216.

(3) Barbara, D. J., Morton, A., Adams, A. N., & P. GREEN, C. (1990). Some effects of hop latent viroid on two cultivars of hop (Humulus lupulus) in the UK. Annals of applied biology, 117(2), 359-366.

(4) Adkar-Purushothama, C. R., Sano, T., & Perreault, J. P. (2023). Hop Latent Viroid: A Hidden Threat to the Cannabis Industry. Viruses, 15(3), 681.

(5) Lavagi, I., Matoušek, J., & Vidalakis, G. (2017). Other cocadviroids. In Viroids and satellites (pp. 275-287). Academic Press.

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