Information pest: *Hop stunt viroid*

Hop stunt viroid (*HSVd*) is a covalently closed, single-stranded RNA molecule of 297 nucleotides and belongs to the genus *Hostuviroid* in the *Pospiviroidae* family. *HSVd* was named due to the first identification of the pathogen on hop plants originating from Japan. *HSVd* is responsible of the cachexia disease of Citrus, characterized by symptoms of "stem-pitting" and production of gum in the bark. In a wide range of host species, infection by *HSVd* appears to be latent, whereas in hop, Citrus and Prunus species, it causes specific disorders and economic damage. *HSVd* is a widespread viroid in various producing areas.

Introduction

HSVd has a rod-like conformation with five domains, a central conserved region and a terminal conserved region. The SYBR *Hop stunt viroid* set has been developed by Qualiplante based on the amplification of a target sequence located on the V domain where the pathogenicity domain is located.
This product should be used only for research purposes.

Intended use

The qPCR set is validated for the detection of *Hop stunt viroid* in One-Step Real-Time RT-PCR (SYBR-Green® technology). The SYBR-Green® technology allows to confirm that a sample generating an amplification signal is produced only by nucleic acids of the pathogen of interest, by interpretating the melting peak.
This set offers a sensitive diagnostic method to detect *HSVd* and permits to avoid amplicon contamination in routine diagnosis. Suitable tissues are plant leaves. The performance of this set has been demonstrated using several nucleic acid extraction procedures and different viroid-infected host species (Etrog citron, sour orange, Orlando tangelo, Washington navel sweet orange and Verna lemon).

Set format and content

Two sets are available for 24 and 96 tests.

Article N°	Product name
7HSVd-S2	SYBR *Hop stunt viroid* (*HSVd*) - set 24
7HSVd-S9	SYBR *Hop stunt viroid* (*HSVd*) - set 96

Content	set 24	set 96
Direct Master Mix	24 tests 7HSVd-S2-DM-	2x48 tests 7HSVd-S9-DM-
RT-Enzyme	24 tests 7HSVd-S2-RT-	96 tests 7HSVd-S9-RT-
Positive Control	3 tests 7HSVd-S2-PC-	8 tests 7HSVd-S9-PC-
Negative Control	3 tests 7HSVd-S2-NC-	8 tests 7CEVd-S9-NC-

Storage conditions

This set can be shipped at room temperature but upon receipt it should be stored immediately at the recommended storage temperature: **from -30 ° C to -10 ° C**.
Avoid prolonged exposure to light and repeated freeze and thaw cycles.

Shelf life

If the set is correctly stored, at constant-temperature freezer, its performance is guaranteed until the expiration date indicated on the tubes label.

Materials and equipment (not provided)

- RNA extraction tools and reagents
- Nuclease-free filter tips and micropipettes
- Optical grade nuclease-free tubes/plate
- Disposable latex or vinyl gloves
- Thermal cycler for Real-Time PCR with filters calibrated for SYBR-Green®

Nucleic acids extraction

Extract RNA from samples according to your usual protocol. Upon request, Qualiplante can recommend you an extraction method.

Preparation of the *HSVd* 1-Step master mix

a) Slowly thaw Direct Master Mix and RT-Enzyme by placing it on ice or at 4°C.
b) Shake briefly Direct Master Mix and RT-Enzyme and spin down the liquid.
c) In a new tube called ***HSVd* 1-Step master mix**, mix 17,6 µl of Direct Master Mix and 0,4 µl of RT-Enzyme per reaction. Do not forget to count the Positive Control and the Negative Control in the number of reactions to prepare.

Example:	1 rxn	10 rxns
Direct Master Mix	17,6 µl	176,0 µl
RT-enzyme	0,4 µl	4,0 µl

d) Store the ***HSVd* 1-Step master mix** by placing it on ice or at 4°C.

Reaction set-up

a) Shake briefly ***HSVd* 1-Step master mix** and spin down the liquid.
b) Add 18 µl of ***HSVd* 1-Step master mix** (without RNA template) to each PCR tubes or wells of an optical-grade PCR plate.
c) Add 2 µl of RNA template to the **HSVd 1-Step master mix**. Do not forget to prepare a PCR tube or well of an optical-grade PCR plate for the Positive Control and the Negative Control.

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Components	Volume/PCR tube or well
RNA template or Positive control or Negative control	2 µl
***HSVD* 1-Step master mix**	18 µl
Total Volume / PCR tube or well	20 µl

In order to confirm the absence of any reagent's contamination, we strongly recommend including a no-template control (e.g. DEPC water) in the assay.

Run and thermal cycling

a) Seal carefully the PCR tubes or PCR plate. Centrifuge briefly to collect components at the bottom of the PCR tubes or wells of the plate. Protect from light before thermocycling.
b) Load the PCR tubes or plate into the thermal-cycler and follow the thermal cycling below:

Steps	Temp (°C)	Time	Cycle(s)
Reverse transcription	40°C	15 min	1
Enzyme activation	95°C	10 min	1
Denaturation	95°C	15 sec	
Annealing	60°C	30 sec	40
Elongation	72°C	30 sec	
Melt temperature	Follow the instructions of your thermal cycler		

Results analysis

The reaction for *HSVd* will generate a specific SYBR®-labeled amplification curve and a specific melting-curve.

Fig.1: Example of amplification curves relative to a *HSVd* positive sample and negative sample.

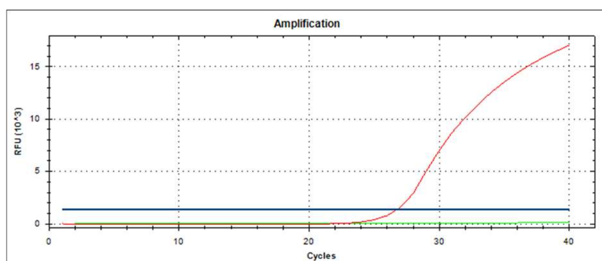

fig.1 shows the amplification curves associated to a *HSVd* infected sample or Positive Control (red curve) and to a healthy sample or Negative Control (green curve).

Fig.2: Example of melting curves relative to a *HSVd* positive sample and negative sample

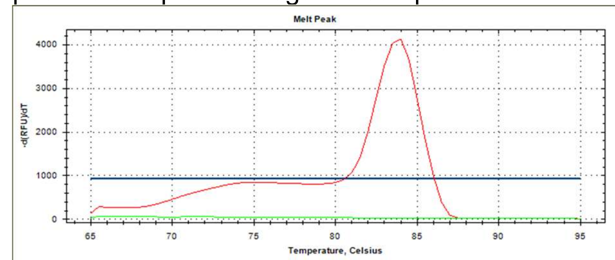

fig.2 shows the melting curve associated to a *HSVd* infected sample or Positive Control (red curve - Tm=84,0°C) and to a healthy sample or Negative Control (green curve) on Biorad CFX96 machine.

ANALYSIS VALIDATION AND RESULTS INTERPRETATION

For a correct interpretation of results, always:

- check if results of Positive Control and Negative Control pass,
- combine amplification curves analysis with melting curves analysis,
- confirm that the samples melting temperatures match the Positive Control melting temperature.

Step 1: Check the Ct values of Positive Control and Negative Control

Well	Ct	Interpretation
Positive Control	30 or less	Go to step 2 and 3
	Above 30 or no Ct	Fail*
Negative Control	No Ct	Go to step 2 and 3
	Less than 30	Fail**

* Repeat the assay, ensuring that steps of the user guide are carefully performed
** The mix or the Negative Control was contaminated with *HSVd* nucleic acids. Repeat the assays after identifying and removing the potential source of contamination

Step 2: Check Ct value in the samples well

Well	Ct	Interpretation
Sample	35 or less	Go to step 3
	More than 35 or no Ct	Negative

Step 3: See melting temperature

Well	Tm	Interpretation
Sample	Tm differs no more than ± 1°C from Tm of Positive Control	Positive
	Tm differs more than ± 1°C from Tm of Positive Control	Negative

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Special handling instructions

This set was designed to be used by laboratory staff trained to follow the usual molecular biology precautions. Always perform the tests in a nuclease-free work environment. Always wear gloves when handling samples containing DNA/RNA and the components of the set. Do not touch any set components with an ungloved hand. Use appropriate laboratory disposable parts. Use nuclease-free tubes and filter tips to avoid degradation and cross-contamination. Do not use components from sets with different batch numbers in the same test procedure. Do not interchange reagents with other sets. To avoid cross-contamination, use separate rooms for (a) nucleic acids extraction, (b) preparation of the Master Mix and (c) amplification. 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. Do not use reagents after their expiration date.

Troubleshooting

Post-PCR data analysis shows no amplification, or amplification plots look grossly abnormal:

Possible causes	Corrective actions
Evaporation of the sample due to inadequate sealing of the plate	Repeat the test using the appropriate tools to seal correctly the plate
Consumables are not appropriate for the method	Repeat the test using consumables recommended by the thermal cycler supplier
The quality of nucleic acid extracted is low	Repeat the extraction step. Ensure that the method of extraction has been performed correctly. In any doubt, contact us
Abnormal amplification	Centrifuge the plate briefly to spin down the contents and eliminate any air bubbles

No amplification reaction is observed in the positive control well, while other samples are positive:

Possible causes	Corrective actions
The positive control provided with the set was not added into the reaction well	Repeat the test. If the problem persists, contact us

An amplification plot is observed in the negative control well:

Possible causes	Corrective actions
Contamination of the negative control or the Master Mix with target-positive nucleic acid	Repeat the test by applying appropriate quality procedures to prevent contamination. Seal the plate correctly

Warranty and Responsibilities

Qualiplante SAS guarantees the buyer exclusively concerning the quality of reagents and of the components used to produce the Sets. Any product not fulfilling the specifications included in the product sheet will be replaced. This warranty limits Qualiplante SAS responsibility to the replacement of the product. No other warranties, of any kind, express or implied-are provided by Qualiplante SAS.
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Sets components are intended, developed, designed, and sold for Research Purpose Only. Product claims are subject to change.

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