# HLVd RT-qPCR Detection Test

Version: 1.0
Release: April 2025

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# OVERVIEW

The HLVd RT-qPCR Detection Test is a molecular test for **detecting Hop Latent Viroid (HLVd) infection** in *Cannabis* roots.

HLVd, short for Hop Latent viroid, is a single-stranded RNA pathogen known for its ability to infect a broad range of plant species, including *Cannabis*.

Key characteristics of HLVd include:

1. It causes "hop latent disease" or "dudding disease" in *Cannabis*
2. Infection symptoms include stunted growth, reduced yield, smaller flowers, and lower cannabinoid content
3. It's "latent" because infected plants may not show obvious symptoms for extended periods
4. It spreads primarily through vegetative propagation (cloning) and mechanical transmission during cultivation

HLVd has become a significant concern in commercial *Cannabis* cultivation as it can substantially reduce crop value while being difficult to detect visually in early stages.

**Testing and strict sanitation protocols are important for managing HLVd in cultivation facilities**

## MOLECULAR TEST DISCLAIMER

The results obtained from this molecular test are intended for the detection of plant pathogens and should be used as part of a comprehensive plant health management program. While the test is designed to be highly sensitive and specific, it is important to note that results may not always be definitive. False positive and false negative results can occur for a variety of reasons.

For optimal results, it is recommended that the test be performed following the provided guidelines and under controlled laboratory conditions.

**False Positives:** A positive result may occur in certain circumstances where the test detects non-target organisms or residual nucleic acid from prior infections. This could be caused by cross-contamination, improper sample handling, or the presence of similar genetic sequences in the plant sample.

**False Negatives:** A negative result does not guarantee that the plant is free of the pathogen. False negatives may arise due to factors such as low viral titer, suboptimal sampling procedures, or the presence of substances that affect the test's sensitivity such as soil residue and traces of fertilizers .

**Recommendations**

- When critical decisions depend on results confirm results by repeating the test
- Consider using an independent diagnostic methods for confirmation (e.g. sending the samples to an external lab)
- Follow all protocol instructions precisely

**This test is provided without warranties. Users assume responsibility for confirming results through additional methods when making management decisions.**

# HLVd RT-qPCR Detection Test

## PROTOCOL AT A GLANCE

| Step | | Time (approx.) |
|---|---|---|
| 1 | Sample collection | 3 min per sample |
| 2 | Lysis setup | 5 min |
| 3 | Sample preparation | 3 min per sample |
| 4 | Lysis | 100 min (hands-off) |
| 5 | RT-qPCR setup | 30 min |
| 6 | RT-qPCR | 100 min (hands off) |
| STOP | *Optional stopping point: once you complete the RT-qPCR run and save the file, you can proceed with data analysis at a later time* | |
| 7 | Result analysis | 15 min |

# KIT COMPONENTS

| Catalog number | Tests | Controls |
|---|---|---|
| DX-0421-02 | 42 | 6 |
| DX-0421-04 | 84 | 12 |

## SUPPLIED IN KIT

| Reagents and Supplies | DX-0421-02 | DX-0421-04 | Storage |
|---|---|---|---|
| Enzyme A1 | 1 tube | 2 tubes | Freezer |
| HLVd Probe Q1 | 1 tube | 1 tube | Freezer |
| HLVd Positive Control 1 | 1 tube | 1 tube | Freezer |
| DEB™ Extraction Buffer | 1 tube | 2 tubes | Freezer |
| Metal punches | 1 bag | 2 bags | Room temp. |
| PCR tubes | 1 bag | 2 bags | Room temp. |

## SUPPLIED BY USER

### Equipment

All items are available at https://dx.minipcr.com.

If you're starting your lab, we recommend the **qPCR Bundle (QP-2510-50)**.

| Item | Quantity | Recommended product | Cat. Number |
|---|---|---|---|
| **Real time thermal cycler** | 1 | q16 thermal cycler | QP-1100-01 |
| **Micropipettes** | 1 each | 1-10 µl H-style | QP-1001-05 |
| | | 2-20 µl H-style | QP-1001-01 |
| | | 100-1000 µl H-style | QP-1002-02 |
| | | 4 µl fixed volume minipette | QP-1003-01 |
| | | 20 µl fixed volume minipette | QP-1003-03 |
| **Microcentrifuge tube rack** | 1 | Microcentrifuge tube rack, 60-well | CM-1003-10 |
| **PCR tube rack** | 2 | 0.1 ml PCR tube rack - 96 wells | CM-1003-11 |
| **Microcentrifuge** | 1 | Gyro™ Microcentrifuge, fixed speed | QP-1800-01 |

## Consumables

All consumables are one-time use.

| Item | Quantity | Recommended product | Cat. Number |
|---|---|---|---|
| **Micropipette filter tips** | 2 boxes each (sufficient for processing up to 190 samples) | 1-10 µl range | 4AA75 |
| | | 2-20 µl range | 4AA76 |
| | | 20-200 µl range | 4AA77 |
| | | 100-1000 µl range | 4AA78 |
| **Microcentrifuge tubes** | 1 tube per run | 1.5 ml volume | 6AA02 |

## Other common supplies

- Disposable laboratory gloves
- Protective eyewear
- Lab coat
- 2 Bottles or sprayers with bleach solution. Prepare freshly by mixing 1 part household bleach + 19 parts water.
- 1 Bottle or sprayer with distilled water
- 2 Fine point permanent markers
- Flat end toothpicks
- 3 containers with lid to dispose of used tubes and tips
- Parchment paper or similar wax paper
- Tissue paper
- Labels to identify plants
- Zip-lock bags
- Disposable razor blades to cut particularly tough roots and a container for properly deposing them
- Disposable tweezers to help handle samples
- Scissors for collecting roots
- Tubes (1.5 ml or 5 ml) for cleaning the roots
- Vortex

# BEFORE YOU START

## LABORATORY GUIDELINES

**RT-qPCR is an extremely sensitive technique that can detect minute amounts of RNA. Always follow the practices outlined below to minimize the risk of contamination.**

- Set up your lab in an area that is removed from possible sources of plant RNA (*e.g.*, away from the greenhouse area).
- Keep each step of the process in three separate areas and avoid unnecessary trafficking between them.

| | **Reagents area** | **Sampling area** | **qPCR area** |
|---|---|---|---|
| **Purpose** | Preparing extraction buffer and master mix | Preparing plant samples.<br>Transferring lysates and positive control to tubes with the master mix | Running the lysis and the RT-qPCR test<br>Data analysis |
| **Precautions** | Plant material, material from the Sampling area or qPCR area should NOT be brought to this area. | Material from the qPCR area should NOT be brought to this area<br><br>Carefully clean surfaces and clean or change gloves in between samples when collecting roots. | Discard the tubes without opening them after the RT-qPCR step. |
| **Materials** | *Reagents*<br>DEB™ Extraction Buffer<br>Enzyme A1<br>HLVd Probe Q1<br><br>*Equipment*<br>20-200 µl and 100-1000 µl micropipettes<br>20 µl fixed volume minipette<br>Marker<br>Tube rack<br>Trash container<br>Bottle with bleach solution<br><br>*Consumables*<br>Tissue paper<br>Gloves<br>20 µl, 200 µl, and 1000 µl filter tips<br>PCR and 1.5 ml tubes | *Reagents*<br>HLVd Positive Control 1<br>Plant samples<br><br>*Equipment*<br>1-10 µl micropipette<br>4 µl fixed volume minipette<br>Marker<br>Tube rack<br>Trash container<br>Bottle with bleach solution<br>Bottle with distilled water<br>Microcentrifuge<br><br>*Consumables*<br>Tissue paper<br>Gloves<br>10 µl and 20 µl filter tips<br>Punches<br>Parchment paper<br>Razor blades and disposable tweezers (optional)<br>Tissue paper | *Reagents*<br>No reagents<br><br>*Equipment*<br>q16 thermal cycler<br>USB Drive<br>USB extension cable<br>Windows or Mac computer<br>Trash container<br><br>*Consumables*<br>13 PCR tubes<br>Gloves |

## BEST PRACTICES

- Clean your hands and wear a clean lab coat before running a test, especially after contact with any plants or plant parts.
- Maintain clean work areas. Spray freshly prepared bleach solution (1 part bleach + 19 parts water) on work surfaces before and after every use.
- Always use filter tips. Do not use the tips included with the minipettes as those don't have filters.
- Change gloves between samples, or spray them with the bleach solution.
- Wear a clean lab coat when preparing the reagents.
- Discard materials such as tips, tubes, and gloves in a container that can be closed. Empty it often and clean it with a bleach solution.
- Keep all tubes closed except for the one that you are actively using.

**Failure to follow good laboratory practices can result in contamination that will invalidate results. A contaminated laboratory is difficult to clean up.**

## CONTROLS

**Positive and negative controls are mandatory.**

To ensure accurate results and proper interpretation, each run **must include** both a negative and a positive control. Omitting these controls will hinder the software''s ability to automatically analyze the data.

### Negative control (mandatory)

The negative control will always be assigned to position 1 in the q16 thermal cycler. Follow manufacturer recommendations for other qPCR thermal cyclers.
Amplification in the negative control indicates contamination and invalidates the results of the test batch.

### Positive control (mandatory)

The positive control will always be assigned to position 2 in the q16 thermal cycler. Follow manufacturer recommendations for other qPCR thermal cyclers.
The positive control is a *non-infectious* synthetic DNA sequence.

**Handle the positive control with care to avoid contaminating other tubes, equipment, and surfaces**
**Make sure the liquid is thawed and at the bottom of the tube before opening the tube**
**Contamination with the positive control will result in false positive tests**

Amplification in the positive control indicates the test is working as expected but does not preclude the possibility of contamination.

# HLVd RT-qPCR Detection Test

## q16 THERMAL CYCLER

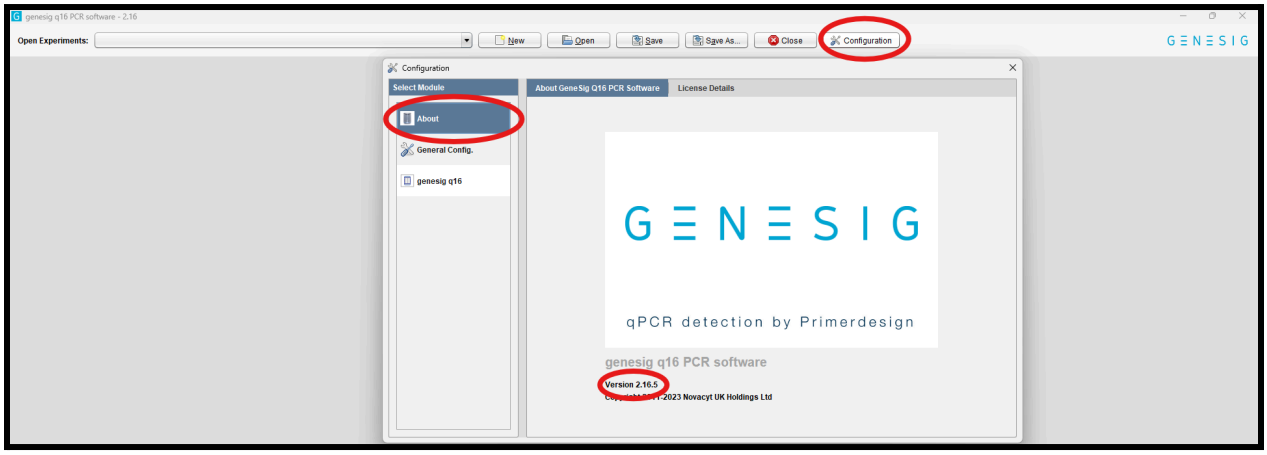
Read the q16 user's guide before proceeding. This is a quick guideline.

q16 software version 2.16.5 & q16 user's guide

### Install the software version 2.16.5

- **The software version 2.16.5 is available on our website (dx.minipcr.com)**
- **The USB Drive provided with your q16 thermal cycler contains software for Windows and MacOS operating systems**. Please open the software file matching your chosen operating system.
    - *Windows:* Double-click on the Windows installer and follow the on-screen instructions to install your genesig® q16 software on Windows.
    - *MacOS:* Double-click on the disk image and follow the on-screen instructions to install your genesig®q16 software on your Mac computer.

**IMPORTANT: make sure to install version 2.16.5 of the software. Previous versions are not compatible with this test. You can verify the installed version by clicking under Configuration/About.**



# PROCEDURE

**Please read these instructions completely before continuing**

## SETUP

Prepare sufficient reagents for screening all plants, plus two additional reactions for the negative control and positive controls.

Keep track of the plant and samples ID during the entire process. Refer to the tables below for recommended sample labeling and the necessary number of tubes.

### Suggested sample labeling

| Plant ID | Lysis Tube ID | RT-PCR Tube ID | Default name on software |
|---|---|---|---|
| Not Applicable | 1 | 1 | Negative control |
| Not Applicable | No lysis tube needed | 2 | Positive control |
| Plant A | 3 | 3 | Sample 1 |
| Plant B | 4 | 4 | Sample 2 |
| ..... | .... | ..... | ..... |
| Plant N | 16 | 16 | Sample 14 |

### Tubes needed for a full q16 run

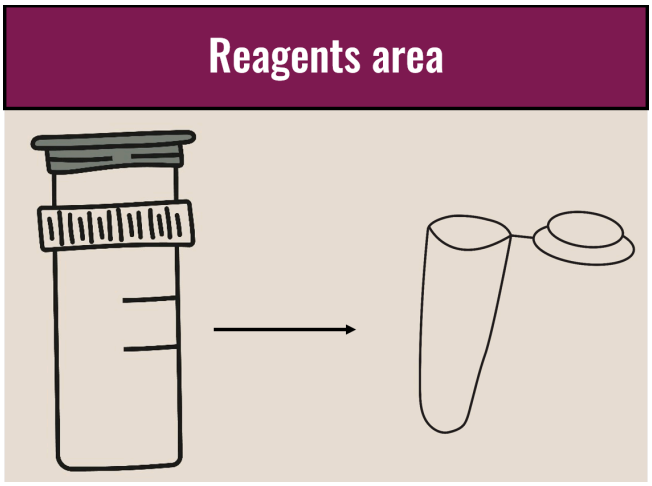
| Reactions | Tubes Needed | |
|---|---|---|
| 14 Tests<br>1 Negative control<br>1 Positive control | 15 for lysis | 16 for PCR |

## STEP 1: ROOT COLLECTION

**For optimal results, initiate testing promptly once all samples are collected.**

1. Prepare the necessary material to collect the samples:
    a. Gloves
    b. Marker
    c. Plant labels
    d. Zip-lock bags
    e. Disposable tweezers (optional)
2. Label the plant and an unused zip-lock bag with a unique ID.
3. Collect the root samples:
    a. Plants grown in soil:
        i. Lightly brush the topsoil of the plant to reveal the roots.
        ii. Pull gently on a root to break it away from the root mass.
        iii. Collect at least four roots per plant, each at least two inches long, evenly spaced around the base of the plant. Taking samples from different areas helps ensure a more accurate representation of the plant's infection status.
        iv. Gently remove the excess of soil by shaking the root sample and using a paper towel.
        v. Store samples in the labeled zip-lock bag.
    b. Plants grown in a hydroponic system:
        i. Use sterile scissors to cut small pieces of roots. Make sure the roots are white and do not show signs of rot. Collect at least four roots per plant, each at least two inches long, evenly spaced around the base of the plant. Taking samples from different areas helps ensure a more accurate representation of the plant's infection status.
        ii. Store samples in the labeled zip-lock bag.
4. Change gloves and scissors or clean them with bleach between samples.
5. Repeat steps 1-5 for all your samples.

## STEP 2. LYSIS SETUP




**Once all samples are collected, proceed with the test as soon as possible.**

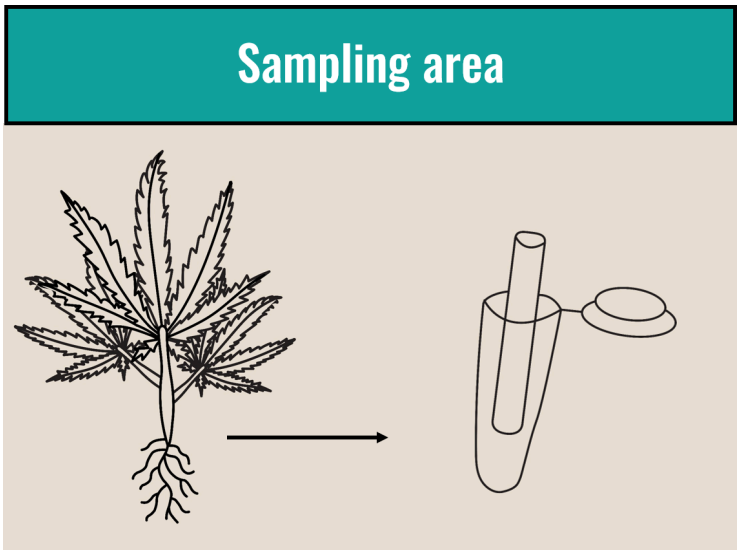
**Prepare the DEB™ Extraction Buffer in the reagents area, away from where plant samples are processed and the RT-qPCR is run to prevent contamination**

1. Wipe all surfaces with freshly prepared bleach solution.
2. Thaw the DEB™ Extraction Buffer at room temperature.
3. Add **100 µl of DEB™ Extraction Buffer to each PCR tube.** You will need one PCR tube per plant sample and one tube for the negative control.

| Sample | Lysis Tube ID |
|---|---|
| Negative control | 1 |
| Positive control | No lysis tube needed |
| Plant A | 3 |
| Plant B | 4 |
| ..... | .... |
| Plant N | 16 |

4. Close all caps.
5. Label the **caps** with the assigned ID.
6. Any remaining DEB™ Extraction Buffer can be stored at room temperature.

## STEP 3. SAMPLE PREPARATION




All sample preparation steps should be carried out in the dedicated sampling area.

**It is crucial to carefully clean work surfaces with the bleach solution before and after collecting the samples, and to clean or change gloves in between samples. This will prevent cross-contamination between samples.**

1. Prepare the materials to process the samples:
    a. Parchment paper
    b. Punches
    c. Toothpicks
    d. Disposable blades (optional)
    e. Tweezers (optional)
2. First, process the negative control tube.

**The negative control tube should always be the first tube to be handled and should be closed before any other sample is processed.**

3. Open tube labeled as “1” and insert a clean metal punch into the tube containing DEB buffer. Use a toothpick to collect the metal punch from inside the tube and discard both.
4. Close the tube. **Press strongly, the lid might be hard to close.** Make sure the cap is evenly seated on the rim of the tube.
5. Proceed to clean the roots and collect the samples.

**It is crucial to carefully clean the roots before running the test, especially if it is from a soil cultivated plant. Soil contaminants will likely result in false negative results due to failure of RNA extraction.**

## Hydroponic roots

**Open only one tube at a time to avoid contamination.**

How to collect root samples video tutorial

1. Take the root from the zip-lock bag and lay it on a piece of paper towel.
2. Gently rinse the root with distilled water.
3. Dry the root by gently tapping it with a dry paper towel.

**It is essential that the root is clean and shows no signs of rot. RNA extraction will not be successful from rotten roots.**

4. Move the root over to a piece of parchment paper.
5. Avoid touching the root directly with your gloves. Hold the root by flipping a corner of the parchment paper over it.
6. Press the smaller end of the punch on the roots and cut the root.
7. The root sample should cover approximately 90%-100% of the punch.

Example of an hydroponic root sample.
The tissue covers 90%-100% of the punch area.

8. Place the punch in the tube with DEB Extraction Buffer and make sure that the root is in contact with the liquid. You can use the toothpick to push the root out of the punch into the buffer. Discard both the toothpick and the punch.
9. Close the tube. **Press strongly, the lid might be hard to close!**
10. Clean the outside surface of the tube with the bleach solution.
11. Clean your gloves with the bleach solution or replace them.
12. Process the next samples by repeating steps 1-11.
13. **Check that all tubes are tightly closed.**

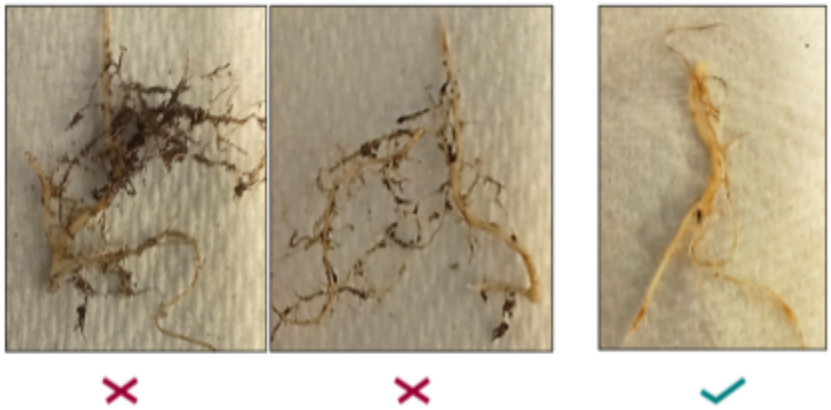
## Soil roots

**Open only one tube at a time to avoid contamination.**

1. Shake the root to remove as much soil debris as possible.
2. Gently clean the root to remove all soil debris.
    a. Wipe the roots with a distilled water-moisten paper towel. Repeat the procedure until the root is very clean without visible soil debris.

b. If the roots are very tender simply rinse it with distilled water using a wash bottle. Repeat the procedure until the root is very clean without visible soil debris.
c. Alternatively, move the root sample into a tube (1.5 ml tube or 5 ml canonical tube) with DI water. Vortex the tube to de-attach the soil debris from the root. If needed, discard the water and repeat the procedure till the roots look clean.

**The root must be very clean and free of any soil or debris. Repeat the cleaning procedure as many times as necessary, as soil residues will impair the performance of the RT-qPCR test**



Examples of soil root samples. The left and the center pictures show roots that have not been cleaned enough. The picture to the right shows an acceptable root.

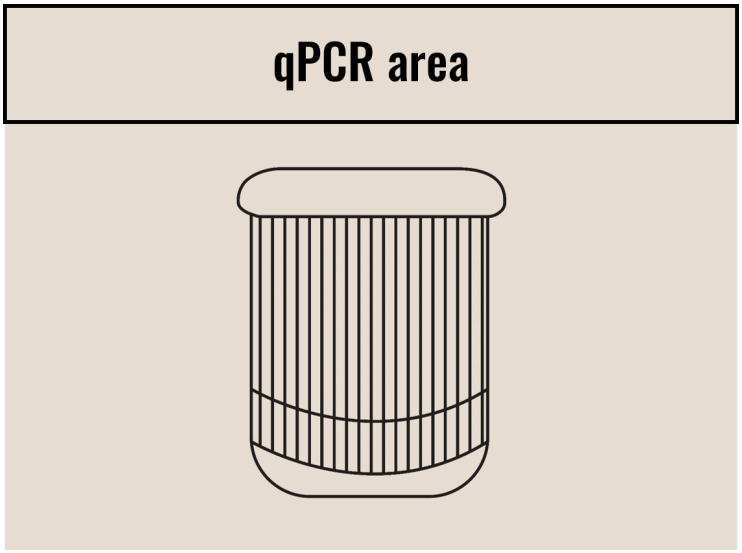
3. Dry the root by gently tapping it with a dry paper towel and move it over a piece of parchment paper.
4. Avoid touching the root directly with your gloves. Hold the root by flipping a corner of the parchment paper over it.

Example of a soil roots sample.
The tissue covers 90%-100% of the punch area.

5. Press the smaller end of the punch on the root and cut it. Use a disposable razor blade if the root is particularly tough.
6. The root sample should cover approximately 90%-100% of the punch.

7. Transfer the root in the tube with DEB Extraction Buffer with the punch or using disposable tweezers. Make sure that the root is in contact with the liquid. If needed, use a toothpick to push the root out of the punch into the buffer. Discard both the toothpick and the punch.
8. Close the tube. **Press strongly, the lid might be hard to close.**
9. Clean the surface of the tube with the bleach solution.
10. Clean your gloves with the bleach solution or replace them.
11. Process the next samples by repeating steps 1-10.
12. **Check that all tubes are tightly closed.**

## STEP 4. LYSIS

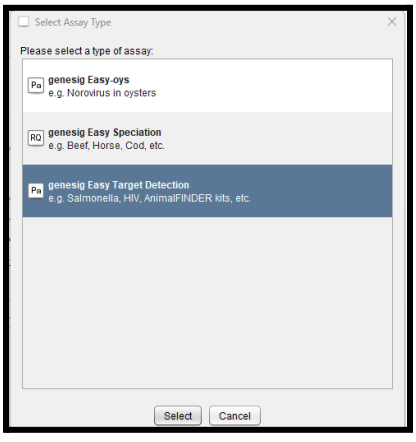



During this step you will extract RNA from the roots using the q16 thermal cycler.

**Please, read the genesig® q16 user's guide before operating the q16 thermal cycler.**

1. Connect the q16 thermal cycler to the power supply, then plug the power supply into an electrical outlet. The machine will make an audible announcement and the LED will display a blue static light to signal that the machine is on. There is no on/off switch.
2. Connect the USB adaptor cable to the bottom of the q16 thermal cycler.
3. Plug the USB Drive to your computer.
4. Open the q16 app on your computer.
5. Select "New".

6. Select "genesig Easy Target Detection".

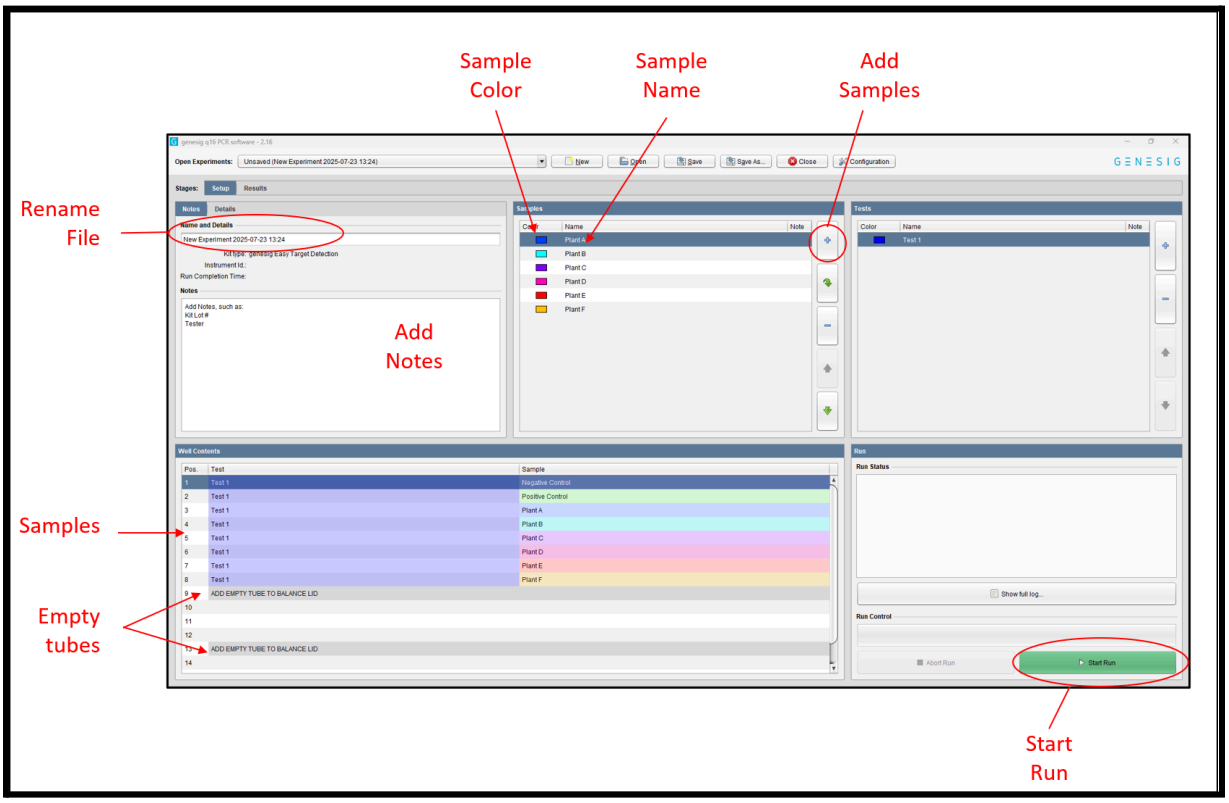


7. Rename the Experiment as you prefer.
8. Under “Samples” select “+” and add the number of samples based on your run.

**Notes:**
-The negative and positive control will be automatically assigned to position 1 and 2, respectively. **There is no need to put a tube in position 2 at this stage.**
-The software may highlight positions where empty tubes should be added to level to lid.

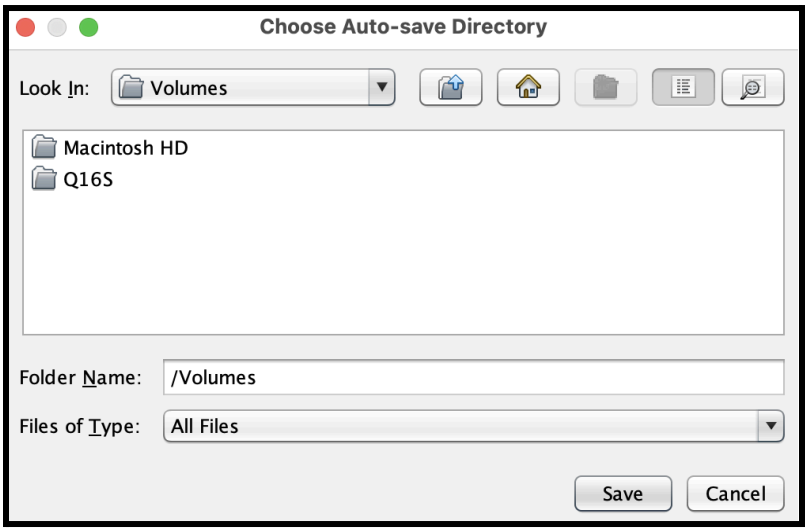
**Optional**: rename the samples and change color by selecting the sample and typing in the box and/or selecting the relative box color.

9. Click on “Start Run”. Note that the q16 thermal cycler will not yet start the program.

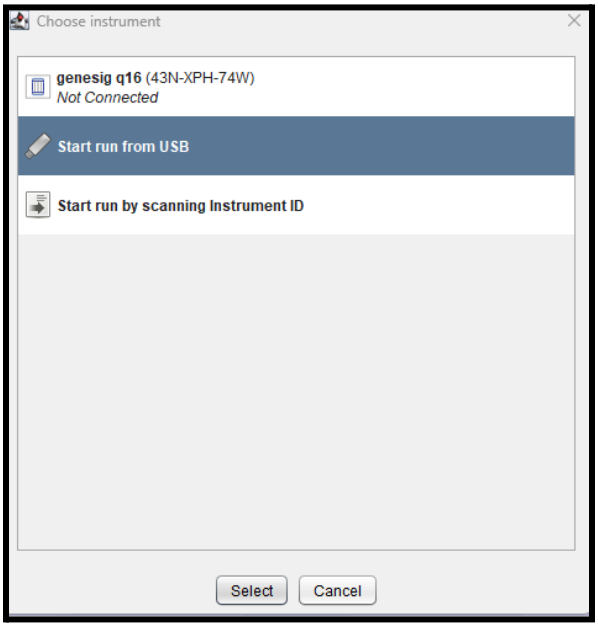



10. Select the *USB drive* as the directory for saving the data and click on “Ok”.

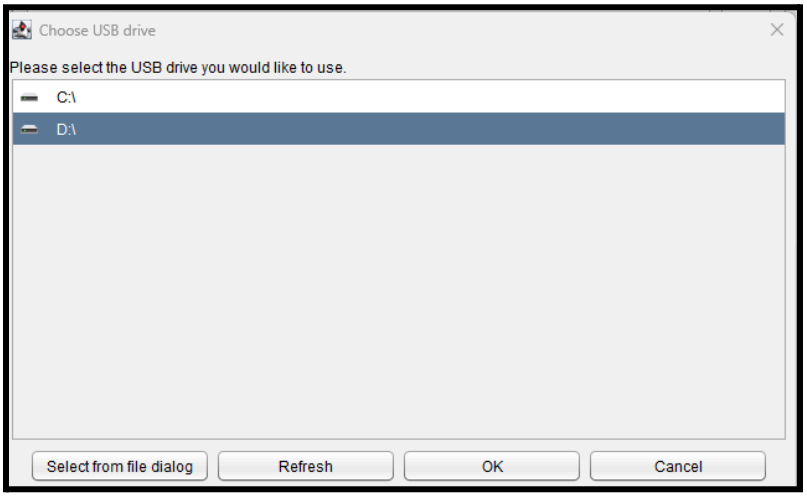
On a Mac, navigate to Volumes and select the name of the USB Drive.



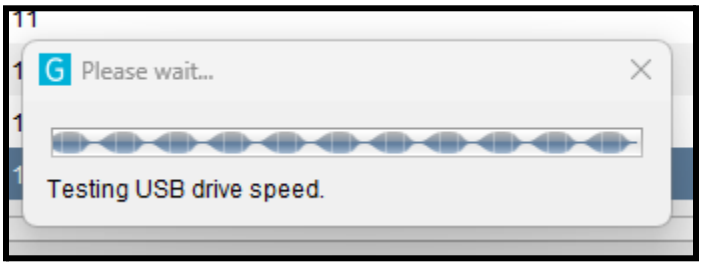

11. Select “Start run from USB” and click on “Select”.



12. Select the USB Drive and click “OK”.




13. The software will test the USB Drive. Once the test is correctly completed, remove the USB Drive from your computer.

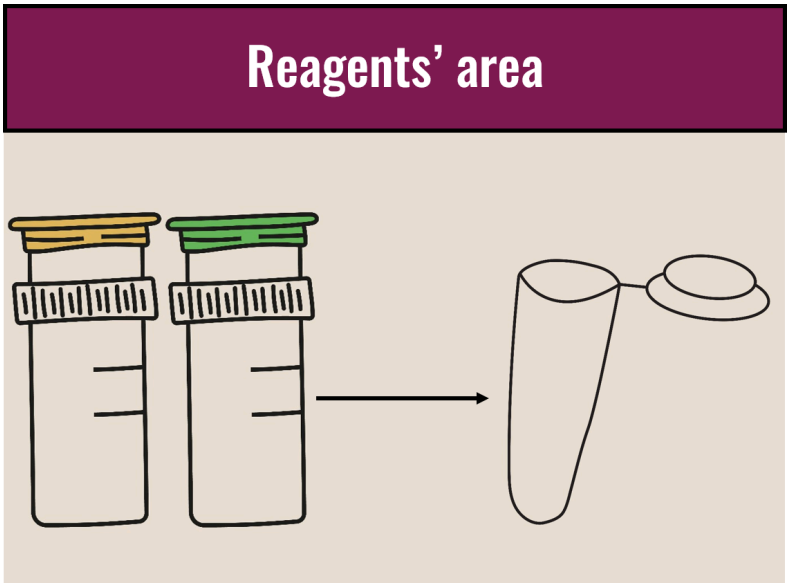



**Note:** If the software detected the USB Drive as too slow to load the program, try again repeating steps 10-13.

14. **To open the q16, push the lid down and twist a quarter turn anti-clockwise, then lift.**
15. **Load the samples in the assigned positions, making sure to add an empty tube in position 2 and to other positions assigned by the software, if needed. Close the lid.**
16. **Plug the USB Drive into the USB connector cable.**
17. When you start the run, the machine will make an audible announcement and the LED will turn green. The LED's color will change throughout the run, which takes approximately 1 hour and 40 minutes. A circling rainbow light pattern indicates that the program is completed. **We recommend preparing the RT-qPCR reactions (STEP 5. RT-qPCR SETUP) approximately 30 min before the run is completed.**

**Important note: No data will be analyzed from this Lysis step.**

## STEP 5. RT-qPCR SETUP




In this step, you will prepare the master mix in the reagents area. You will need the Enzyme A1, the HLVd Probe Q1, and the PCR tubes.

1. Wipe all surfaces with freshly prepared cleaning solution.
2. Thaw the provided reagents (Enzyme A1 and HLVd Probe Q1) at room temperature.
3. Prepare the master mix by following the instructions in the table below.
   -These calculations account for loss of volume during pipetting. You may have a small amount of liquid left in the tube.
   -The total number of reactions is the sum of the number of tests plus the negative and the positive controls.

| Reagent | Volume for 1 reaction (µl) | Volume for 8 reactions (µl) | Volume for 16 reactions (µl) |
|---|---|---|---|
| Enzyme A1 | 11 | 88 | 176 |
| HLVd Probe Q1 | 11 | 88 | 176 |
| **Total volume** | **22** | **176** | **352** |

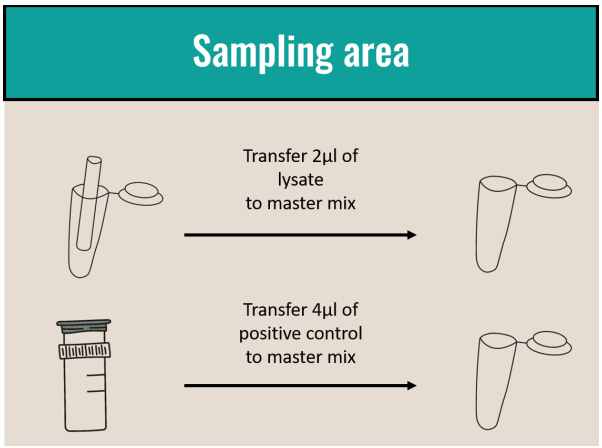
4. Add the reagents to a 1.5 ml microcentrifuge tube. Gently mix up and down until the solution is well mixed.
5. Put the Enzyme A1 and HLVd Probe Q1 back in the freezer.
6. Using the dedicated **20 µl fixed volume minipette** with **a 2-20 µl filter tip** to add 20 µl of the master mix to each PCR tube. There is no need to change tips at this time.
7. Close the tubes.

8. Label tubes with the proper ID on the top.

| Test | PCR Tube ID |
|---|---|
| **Negative control** | 1 |
| **Positive control** | 2 |
| **Sample 1** | 3 |
| **Sample 2** | 4 |
| **.....** | .... |
| **Sample 14** | 16 |

9. Move the tubes to the sampling area.

## STEP 6. RT-qPCR



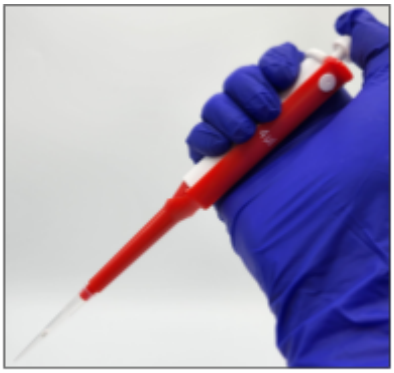

In this step, you will add the extracted RNA and the positive control to the master mix and run the RT-qPCR in the q16 thermal cycler.

### Transfer the lysates and the positive control

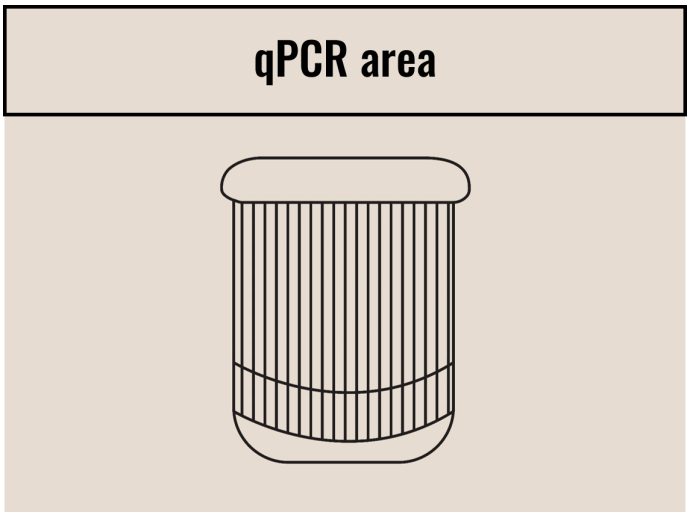
#### Open only one tube at a time to avoid contamination

1. Once the program in the genesig q16 thermal cycler is completed, gently open the lid and move the lysates to the **sampling area**.
2. First, process the negative control. Transfer 2 µl from the "lysate tube" to the PCR tube using the dedicated **H10 pipette** with a **1-10 µl filter tip**. Close both tubes tightly. Discard the tip.
3. Proceed with the root samples. Transfer 2 µl from the "lysate tube" to the PCR tube containing the master mix. Close both tubes tightly. Discard the tip.
4. Process all samples one at a time by repeating step 3.
5. Thaw the positive control.
6. Using the dedicated **4 µl fixed volume minipette** and a **2-20 µl filter tip**, transfer 4 µl to the tube labeled "2". Close both tubes.
7. **Store the positive control in the freezer.**
8. **Check that all the PCR tubes containing the master mix are closed tightly.**
9. **Briefly spin down the microcentrifuge to remove bubbles** (please, refer to the Appendix on how to operate a microcentrifuge). Bubbles interfere with optical readings. Eliminating bubbles ensures accurate interpretation of the results.

Example of a 1-10 µl micropipette set to 2 µl

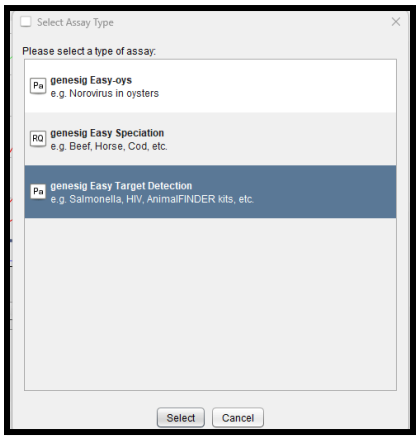

Example of 4 µl minipette with a 20 µl filter tip

## Start the RT-qPCR




1. Move to the qPCR area. Remove the USB Drive from the connector cable.
2. Connect the USB Drive to your computer.
3. Delete the lysis run file (PCR 1.USB) from the USB Drive.
4. Open the q16 app on your computer.
5. Select “New”.

6. Select “genesig Easy Target Detection”.



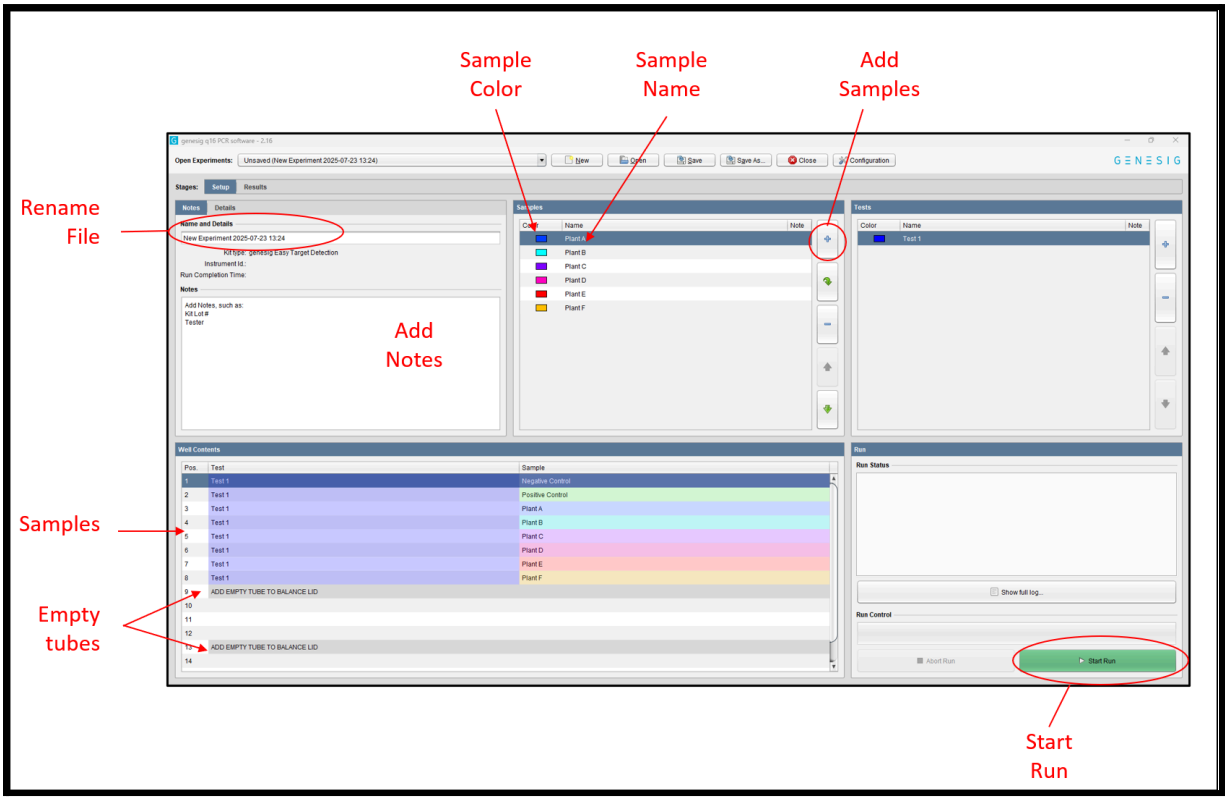

7. Rename the Experiment as you prefer.
8. Under “Samples” select “+” and add the number of samples based on your run.

**Notes:**
-The negative and positive control will be automatically assigned to position 1 and 2, respectively.

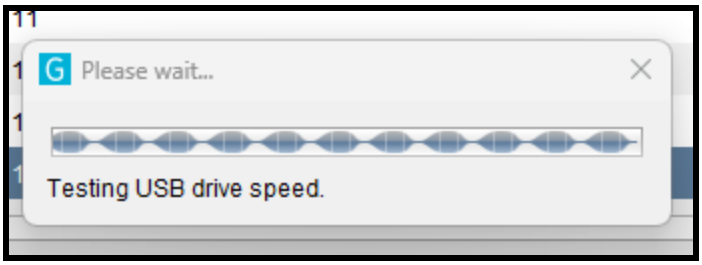
-The software will highlight where to add “empty” tubes, to make sure the lid will properly close.

**Optional**: rename the samples and change color by selecting the sample and typing in the box and/or selecting the relative box color.

9. Click on “Start Run”. Note that the q16 thermal cycler will not yet start the program.



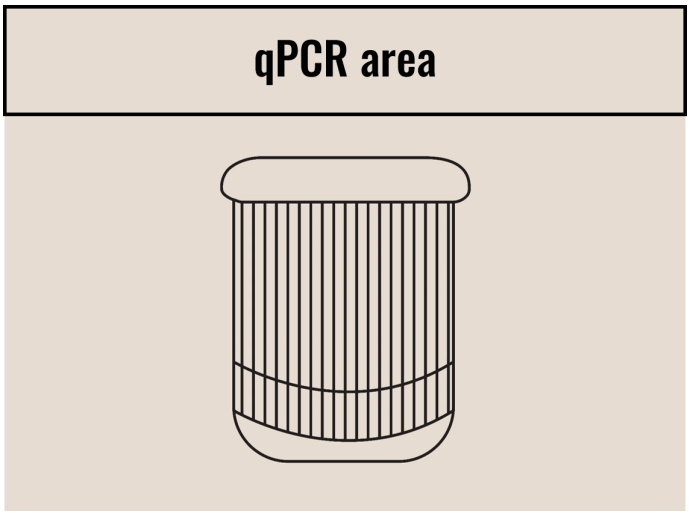

10. Select the USB Drive as the directory for saving the data and click on “Ok”.
11. Select “Start run from USB” and click on “Select”.
12. Select the USB Drive and click “OK”.
13. The software will test the USB Drive. Once the test is correctly completed, remove the USB Drive from your computer.




**Note:** If the software detected the USB as too slow to load the program, try again repeating steps 10-13.

14. **Load the samples in the assigned positions.**
    **Reminder: the negative control should be always in position "1", while the positive control should be always in position "2".**
15. Gently close the lid.
16. **Plug the USB Flash Drive into the USB connector cable.**
17. When you start the run, the machine will make an audible announcement and the LED will turn green. The LED's color will change throughout the run, which takes approximately 1 hour and 40 minutes. The program is complete when you see a circling rainbow light pattern. **Never open the tubes once the RT-qPCR step is completed!**
18. Once the run is completed, open the lid and discard the tubes.
19. Gently close the lid, unplug the machine, and proceed with data analysis.

## STEP 7. RESULT ANALYSIS




In this step you will analyze the results. Please, refer to the EXPECTED RESULTS section for more information.

**The analysis requires checking both the automated result table as well as the amplification curves.**

1. Remove the USB Drive from the USB connector cable and insert it in your PC. Transfer the run file (PCR 1. USB) into a directory where you store your results.
2. Open the q16 app and open the file you would like to analyze.
3. Click on the "Results" tab.
4. Click on "Details" close to the top "Summary" panel. Curves should appear.
5. Negative control is labeled as "PASS" in the result table.
6. Positive control is labeled as "PASS" in the result table.

**Note:** if either the Negative control or the Negative control failed, a warning message should appear in the Summary and the Notes panel.

7. **Manually check the amplification plot and Cq value for both the Internal Control and the Test channels for each sample**. **Refer to Result Interpretation, Expected Results, and Troubleshooting sections.**

    a. **Internal Control channel:** the sample shows a clear sigmoid curve and Cq < 36. Proceed with test channel analysis.

    **Lack of a clear sigmoid curve and/or Cq > 36 indicates sub-optimal RNA extraction and the test should be considered inconclusive.**

b. **Test channel:**

i. **Negative**: no sigmoid curve and no Cq detected (n/a). The software will label this as “NEGATIVE”.

ii. **Inconclusive**: the sample does not show a clear sigmoid curve and/or Cq > 36. The software might indicate this sample as “POSITIVE”. However, conclusions from samples showing sub-optimal amplification should be taken consciously.

**Check the troubleshooting section and repeat the test by collecting a new specimen from the same plant.**

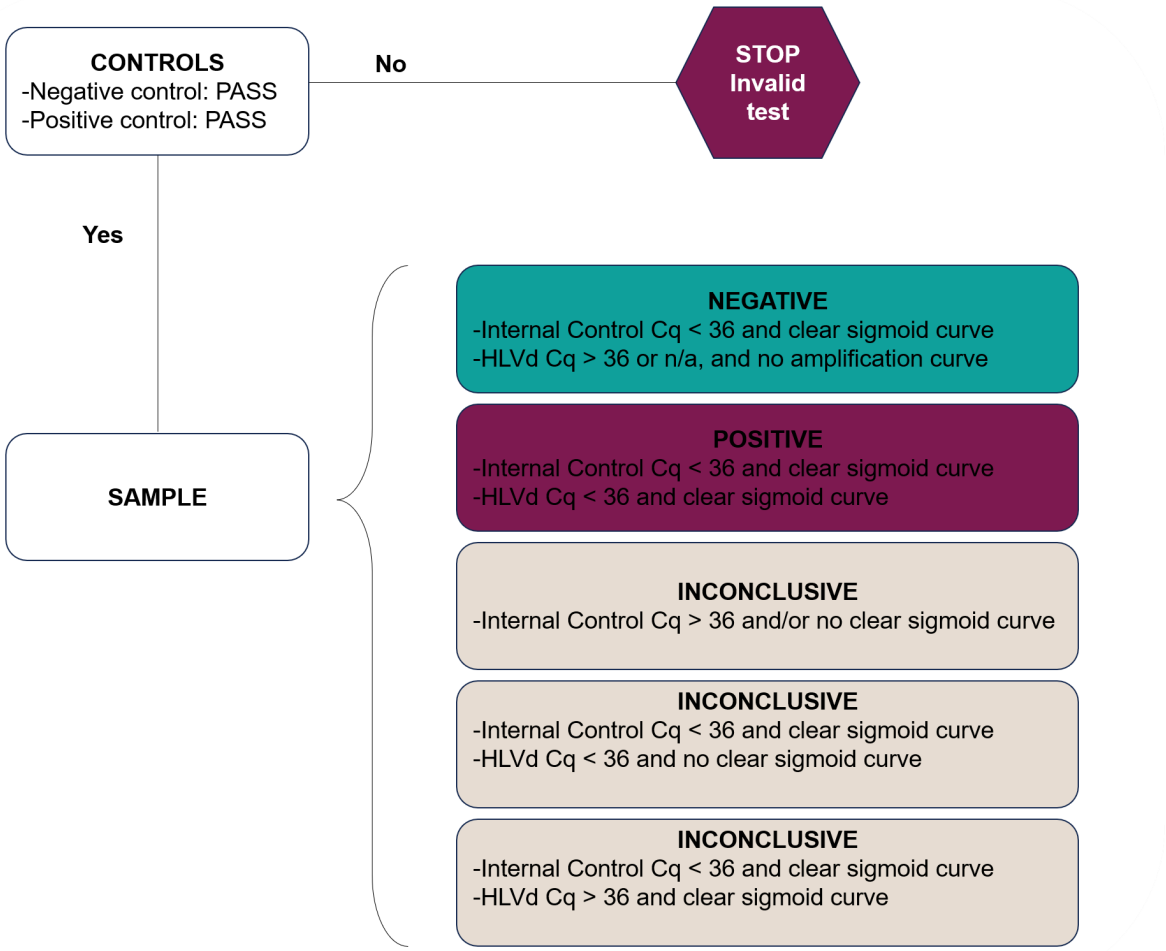
iii. **Positive**: clear sigmoid curve and Cq < 36. The software will label this as “POSITIVE”.

**We recommend repeating a test if a sample is determined positive. Repeat the test by collecting a new specimen from the same plant.**

**Important Note**: The copy number, which represents the number of viroid molecules present in the initial sample, provided by the software might not be accurate. This is due to several factors that can affect the results, including the efficiency of the RNA extraction and the specific part of the root that was sampled. Viroid concentration might vary in different areas of the plant, especially during the early stages of infection, and these variations can impact the final copy number.

8. Export the results for future record as .csv or .pdf (optional).
9. Delete the run file PCR 1. USB from the USB Drive.

# HLVd RT-qPCR Detection Test



| Sample | Software table | Curve Shape and Cq | Result Interpretation |
|---|---|---|---|
| Negative control | PASS | Both Internal control and Test show no clear sigmoid curve and/or Cq > 36. | Results as expected, the test is valid. |
| Negative control | FAILED | Internal Control and/or test show a clear sigmoid curve and/or Cq < 36. | Contamination.<br>Tests should be considered invalid and they will be labeled as " TEST CONTAMINATED".<br>Deeply clean the lab and repeat all the tests. |
| Positive control | PASS | Test: Clear sigmoid curve, 14 < Cq < 22. | Result as expected, the q16 thermal cycler correctly reads fluorescence. |
| Positive control | FAILED | Test: No clear sigmoid curve or Cq < 14 or Cq >22. | -Inaccurate pipetting when adding the positive control<br>If the positive control is detected as FAILED, the tests will be labeled as "TEST FAILED".<br>-If no signal is detected, this might indicate issues with the fluorescence reading. Contact support at dx@minipcr.com |
| Sample | NEGATIVE | Internal control: clear sigmoid curve and Ct < 36.<br>Test: no clear sigmoid curve, Cq not detected (n/a). | **The sample is HLVd negative.** |
| Sample | NEGATIVE | Internal control: no clear sigmoid curve and/or Cq > 36. | **Inconclusive.**<br>No clear sigmoid curve in the Internal Control indicates |

| | | | |
|---|---|---|---|
| | | | sub-optimal RNA extraction. Repeat the test. |
| Sample | POSITIVE | Internal control: clear sigmoid curve and Ct < 36.<br>Test: clear sigmoid curve and Cq < 36. | **The sample is HLVd positive.** |
| Sample | POSITIVE | Internal control: clear sigmoid curve and Ct < 36.<br>Test: not clear sigmoid curve and Cq < 36. | **Inconclusive.**<br>Repeat the test. |
| Sample | SAMPLE PREPARATION FAILED | Internal control: no clear sigmoid curve and/or Cq > 36. | **Inconclusive.**<br>No clear sigmoid curve in the Internal Control indicates sub-optimal RNA extraction. Repeat the test. |
| Sample | POSITIVE RESULT, POOR QUALITY SAMPLE | Internal control: no clear sigmoid curve and or Cq >36.<br>Test: Cq < 36, clear sigmoid curve. | **Inconclusive.**<br>No clear sigmoid curve in the Internal Control indicates sub-optimal RNA extraction. Repeat the test. |

# EXPECTED RESULTS

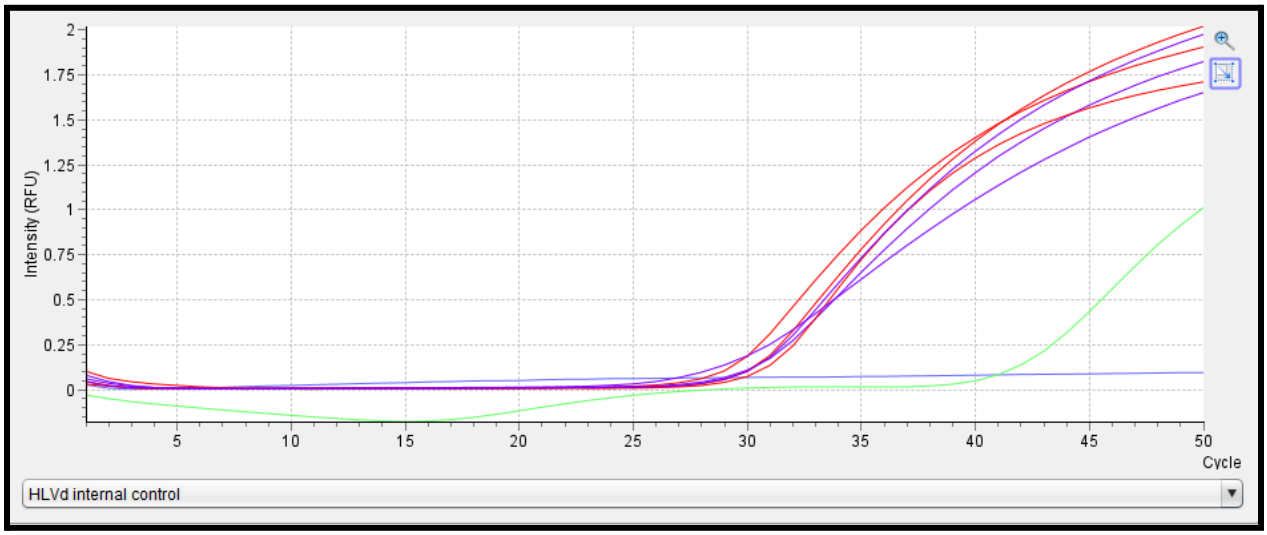
**The test result must be interpreted integrating the analysis of the amplification curve shape and the Cq.**

The Internal Control indicates whether RNA was extracted efficiently, and the Test is used to know if the sample is HLVd positive or negative.

### Internal Control channel

All samples except for the *negative control* (light blue, flat line) should show a clear sigmoid curve and Cq < 36.

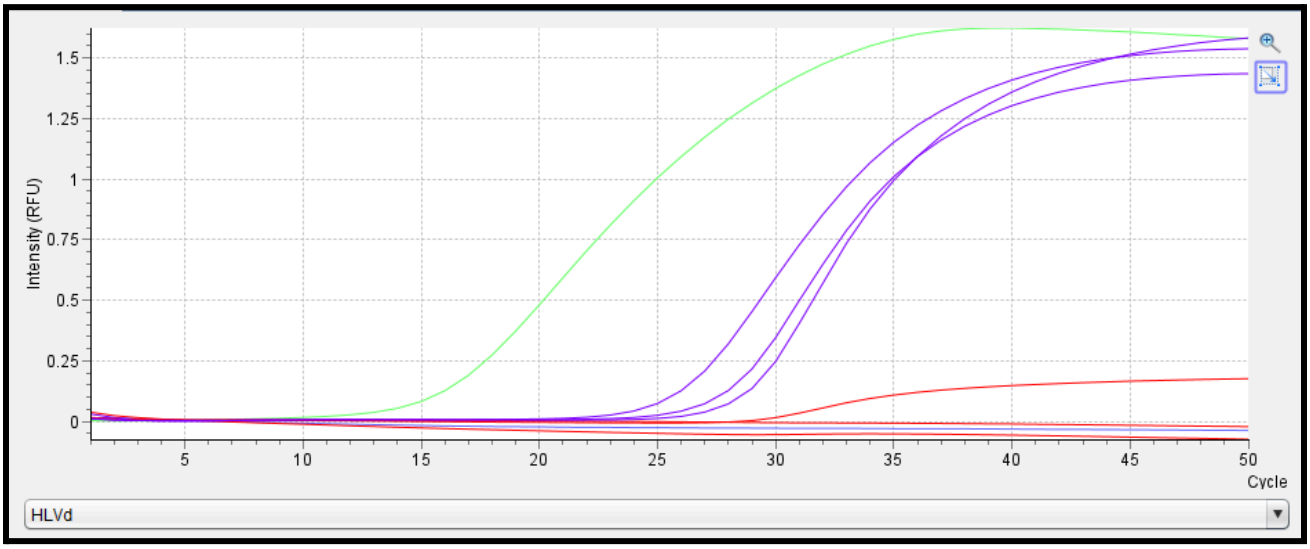
- HLVd Negative samples are in red and HLVd positive samples are in purple.



# HLVd RT-qPCR Detection Test

## Test channel

- The positive control (green) and the positive samples (purple) should show clear sigmoid curves and Cq < 36.
- The Negative control (light blue) and negative plant samples (red) show no amplification (flat lines).



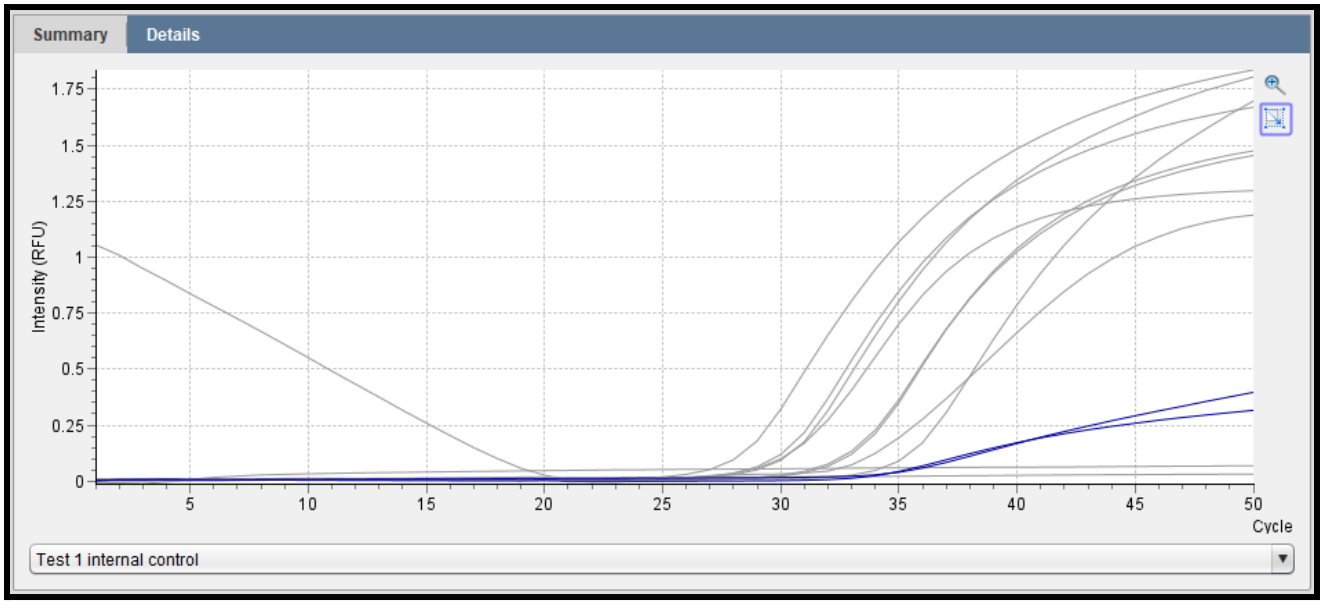

## Software table

| # | Test | Sample | Status | Copy Number | Cq (Test) | Cq (I.C.) |
|---|---|---|---|---|---|---|
| 1 | HLVd | Negative Control | PASS | n/a | n/a | n/a |
| 2 | HLVd | Positive Control | PASS | n/a | 15.38 | 41.10 |
| 3 | HLVd | Plant A (Negative) | NEGATIVE | n/a | n/a | 28.30 |
| 4 | HLVd | Plant B (Positive) | POSITIVE | 634 | 27.00 | 29.50 |
| 5 | HLVd | Plant C (Negative) | NEGATIVE | n/a | n/a | 29.81 |
| 6 | HLVd | Plant D (Negative) | NEGATIVE | n/a | n/a | 29.25 |
| 7 | HLVd | Plant E (Positive) | POSITIVE | 2,337 | 25.12 | 28.54 |
| 8 | HLVd | Plant F (Positive) | POSITIVE | 370 | 27.78 | 29.37 |

# TROUBLESHOOTING

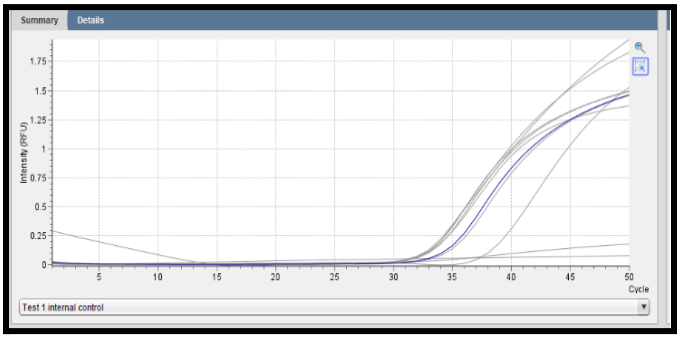
## Inconclusive result, example I

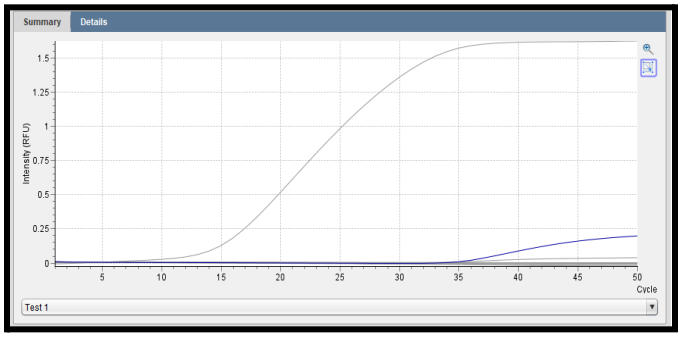
The samples in blue do not show the expected clear amplification curve (sigmoid shape) in the internal control channel. Indeed, the lines are diagonals. This indicates that the lysis was sub-optimal. We recommend repeating the test.




## Inconclusive result, example II

Sample 1 (blue) shows a clear amplification curve and Cq < 36 for the Internal Control, this indicates that the lysis process was successful (left picture and table below).
The test channel shows a Cq < 36 (34.22). However, there is not a clear sigmoid curve for the test channel (right picture). Therefore, the test should be considered inconclusive.



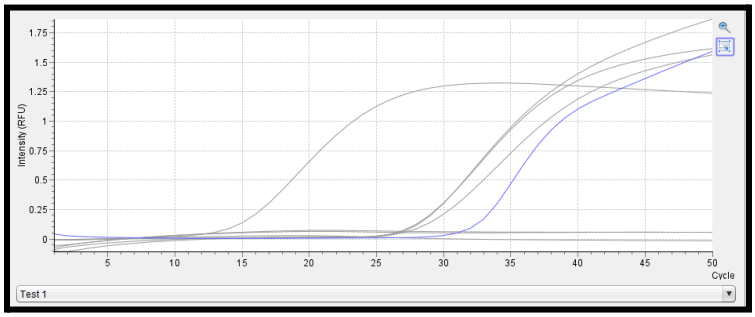




| # | Test | Sample | Status | Copy Number | Cq (Test) | Cq (I.C.) |
|---|---|---|---|---|---|---|
| 1 | Test 1 | Negative Control | PASS | n/a | n/a | n/a |
| 2 | Test 1 | Positive Control | PASS | n/a | 14.52 | 37.37 |
| 3 | Test 1 | Sample 1 | POSITIVE | 2 | 34.22 | 33.54 |

# HLVd RT-qPCR Detection Test

## Invalid test, contamination

The negative control (light blue curve) shows a clear sigmoid curve and Cq < 36 for the Test channel. This indicates contamination.
We recommend discarding already opened tip boxes and current reagents aliquots.
Deeply clean the lab with bleach and clean the lab coats.
Start with new reagents and new tip boxes.




| # | Test | Sample | Status | Copy Number | Cq(Test) | Cq(I.C.) |
|---|---|---|---|---|---|---|
| 1 | Test 1 | Negative Control | FAIL | n/a | 31.41 | 30.47 |

# APPENDIX

## TUBE TYPES

| Tube type | Picture | Description |
|---|---|---|
| Screw-cap reagent tube |  | Reagents for DNA and RNA extraction and RT-qPCR. |
| 1.5 ml microcentrifuge tubes |  | Used to prepare the master mix. Not included in the kit. Available at dx.minipcr.com |
| PCR tubes |  | Used for the lysis and PCR steps. Included in the kit. |

## MICROPIPETTING

Good micropipetting skills are essential to run the test successfully. Practice with water or colored liquids before using the reagents in this kit.

Micropipetting video tutorial

We recommend dedicated micropipettes and minipettes per area:
-Reagents area: one 100-1000 µl micropipette, one 20-200 µl micropipette, and one 20 µl fixed volume pipette.
-Sampling area: one 1-10 µl micropipette and one 4 µl fixed volume minipette.

**To reduce the contamination risk:**

- Dedicate specific micropipettes and filter tip boxes to each area
- Always use filter tips
- Use only the dedicated minipette for transferring the master mix and the positive control. In case of contamination, dispose of the minipette and use a new one.

Always select the right micropipette and tip based on the volume that you need to transfer according to the tables in the following page.

| Volume range | Micropipette | Tips |
|---|---|---|
| 1-10 µl |  H10 |  |
| 2-20 µl |  H20 |  |
| 20-200 µl |  H200 |  |
| 100-1000 µl |  H1000 |  |

| Volume | Minipette | Tips |
|---|---|---|
| 4 µl |  |  |
| 20 µl |  |  |

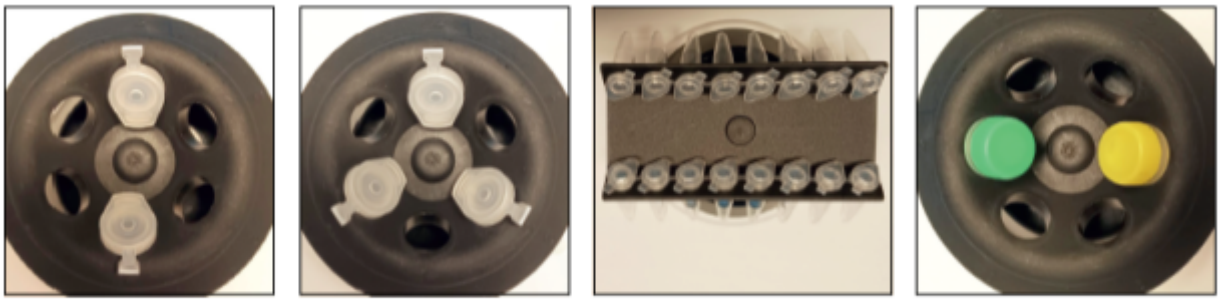
## LIQUID HANDLING

**Make sure that all liquid is collected at the bottom of the tubes before opening them.**

**Spin down the PCR tubes before the RT-PCR step. Bubbles can interfere with the RT-PCR process.**

Follow these steps to use the microcentrifuge:

1. Ensure that the contents of the tubes are fully defrosted.
2. Place the tubes in the rotor, making sure they are balanced as shown in the pictures. The tubes should have approximately the same amount of liquid in them. If needed, prepare a tube with water to balance the rotor. Refer to the manufacturer's instructions for details.
3. Spin for 5 seconds at 10,000 RPM.



Examples of balanced microcentrifuges. Tubes match in size and contain the same volumes, making their weights comparable

## SCIENTIFIC BASIS OF THE ASSAY

### HLVd is a viroid: a circular pathogenic RNA

Hop Latent Viroid (HLVd) poses a serious risk to *Cannabis* cultivation, warranting a comprehensive understanding of its characteristics, implications, and preventing measures to reduce the infections (Buirs & Punja, 2024).

HLVd is a viroid belonging to the family *Pospiviroidae*. It is a small, circular, single-stranded RNA pathogen known for its ability to infect a broad range of plant species, including *Cannabis* (Atallah et al., 2024).

In *Cannabis*, HLVd infections manifest through symptoms such as stunted growth, leaf distortion, chlorosis, reduced vigor, lower water intake, reduced flower mass and trichomes, and diminished cannabinoid production. Importantly, different *Cannabis* strains show different levels of subjectivity to HLVd infection and symptoms (Adkar-Purushothama et al., 2023; Buirs & Punja, 2024; Punja et al., 2024).

HLVd primarily spreads through vegetative propagation methods such as cloning and cuttings, where infected plant material serves as a reservoir for the viroid. Furthermore, mechanical transmission via contaminated tools or equipment further facilitates the rapid dissemination of HLVd within cultivation facilities (Buirs & Punja, 2024). Therefore, the first line of defense against HLVd spreading is implementation of good growing practices and monitoring through molecular testing. Besides, recent studies suggest that HLVd can also be transmitted by an infected plant to the seeds (Punja et al., 2024).

When spread through mechanical transmission, HLVd enters the plant's phloem at the point of infection, travels to the roots, where it is replicated, and then moves systematically throughout the entire plant over a period of approximately 6 weeks (Atallah et al., 2024).

Once inside the cell, the viroid RNA hijacks the host cellular machinery to replicate itself. Unlike viruses, viroids lack a protein coat and rely solely on host machinery for replication. The viroid RNA serves as a template for the plant RNA polymerases to produce more copies of the viroid. As the viroid replicates, it can interfere with the normal gene expression of the host plant. Although the molecular mechanism behind HLVd infection symptoms have not been elucidated yet, recent evidence suggests that viroid RNA molecules can interact with host proteins or RNA molecules, disrupting normal cellular processes such as transcription, translation, and RNA processing. This interference can lead to the dysregulation of important

plant genes, affecting growth, development, and defense mechanisms (Adkar-Purushothama et al., 2023; Atallah et al., 2024; McKernan et al., 2022).

### RT-qPCR allows detection of RNA

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) is a sensitive molecular technique for the detection and quantification of RNA molecules. First, the reverse transcriptase converts RNA molecules into complementary DNA (cDNA). Then, target cDNAs are amplified and quantified during the qPCR step, using specific primers and fluorescent probes. Indeed, during amplification, the probes bind to the target DNA sequence, and upon amplification, the fluorescent signal emitted and increases proportionally to the amount of amplified DNA, allowing for real-time quantification of the target sequences. Notably, using multiple primers and probes conjugated to different fluorophores allows simultaneous identification of multiple targets in the same reaction (multiplex assay). In other words, it is possible to check the presence of an Internal Control gene, assuring the efficiency of RNA extraction, as well as a specific target, such as the viroid sequences, in the same tube.

### How to read and interpret RT-qPCR data

A clear sigmoid curve in RT-qPCR is crucial as it demonstrates successful and reliable amplification of the target RNA. This characteristic S-shaped curve signifies the exponential phase of PCR, where the amount of amplified product doubles with each cycle. The curve's plateau indicates the reaction has reached its endpoint, where amplification has ceased.

The cycle threshold (Ct or Cq) value is the cycle number at which the fluorescence signal crosses a defined threshold, indicating detectable amplification. It is inversely proportional to the initial amount of target RNA; a lower Cq indicates a higher starting concentration. Cq values exceeding 36 are generally considered unreliable because at such high Cq values, the signal is often very close to background noise, making it difficult to distinguish true amplification from random fluctuations. Additionally, potential inhibitors or degradation of reagents become more impactful in later cycles, further compromising the reliability of the results.

# HLVd RT-qPCR Detection Test

## Test validation

HLVd RT-qPCR Detection Test is a multiplex assay that allows detection of HLVd and *Cannabis* RNA as internal control, using a simple RNA extraction from *Cannabis* roots. We evaluated our test taking into consideration inclusivity and specificity of the reagents targeting HLVd, specificity of the reagents for targeting the internal control.

### *HLVd Inclusivity*

In the context of primer design for a molecular diagnostic test, inclusivity refers to the extent that primers correctly detect and amplify the target nucleic acid sequences from all relevant variants of the organism or pathogen being tested. If primers are not inclusive, they might fail to detect certain strains or variants, leading to false negative results and misdiagnosis.

We performed two *in-silico* inclusivity analyses of the 90 HLVd variants publicly available on the NCBI database. These sequences are all reported in peer-reviewed published papers (Atallah et al., 2024) and are 256 nt long. We aligned our primer sequences with the selected HLVd genomes using Clustal-Omega MSA - EMBL. Our primers perfectly match 87 strains, and contain some mismatches to 3 strains that may reduce efficiency. The same results were obtained by performing the analysis using BLAST-NCBI.

### *HLVd Specificity*

Specificity in primer design refers to the extent that primers exclusively amplify the intended target DNA sequence. We evaluated the specificity of the primers with *in-silico* BLAST-NCBI analysis and Primer-BLAST. This analysis showed that our reagents target only HLVd and not other viruses or viroids.

### *Internal Control Specificity*

We designed mRNA specific primers to detect the exon-exon junction of a gene commonly expressed in *Cannabis* roots. Specificity of the primers have been evaluated with *in-silico* BLAST-NCBI analysis and Primer-BLAST. This analysis showed that our reagents target only one specific mRNA in *Cannabis*.

## Summary

To sum up, this RT-qPCR assay enables growers to accurately identify the presence of HLVd in plant samples with high specificity and sensitivity. Early detection through RT-qPCR empowers cultivators to enact timely management strategies, thereby curbing the spread of HLVd and mitigating crop losses (Adkar-Purushothama et al., 2023; Buirs & Punja, 2024).

## References

Adkar-Purushothama, C. R., Sano, T., & Perreault, J. P. (2023). Hop Latent Viroid: A Hidden Threat to the Cannabis Industry. In Viruses (Vol. 15, Issue 3). MDPI. https://doi.org/10.3390/v15030681

Atallah, O. O., Yassin, S. M., & Verchot, J. (2024). New Insights into Hop Latent Viroid Detection, Infectivity, Host Range, and Transmission. Viruses, 16(1). https://doi.org/10.3390/v16010030

Buirs, L., & Punja, Z. K. (2024). Integrated Management of Pathogens and Microbes in Cannabis sativa L. (Cannabis) under Greenhouse Conditions. Plants, 13(6), 786. https://doi.org/10.3390/plants13060786

McKernan, K., Kane, L., & McLaughlin, S. (2022). Hop Latent Viroid shares a 19 nucleotide sequence with Cannabis sativa COG7. https://doi.org/10.31219/osf.io/bwnmv

Punja, Z. K., Kahl, D., Reade, R., Xiang, Y., Munz, J., & Nachappa, P. (2024). Challenges to Cannabis sativa Production from Pathogens and Microbes—The Role of Molecular Diagnostics and Bioinformatics. International Journal of Molecular Sciences, 25(1). https://doi.org/10.3390/ijms25010014

**Questions? Email dx@minipcr.com or call +1-781-990-8727 for technical support**