

PathoSEEK[®] Virus/Viroid Detection Assays on the Chai Bio Open PCR

User Guide

**Quantitative Reverse Transcription PCR (RT-qPCR) Assays for the detection of
Virus/viroid in cannabis plants**

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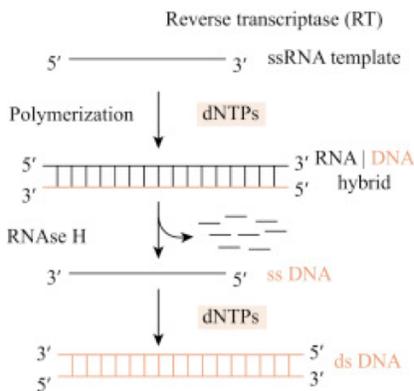
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Introduction

The PathoSEEK[®] Cannabis Virus Multiplex detection kit combines a reverse transcriptase with a multiplexed quantitative PCR assay designed to simultaneously detect Hop Latent Viroid (HLVd), Lettuce Chlorosis Virus and Cannabis Cryptic Virus in addition to an internal plant DNA reaction control to ensure accurate detection of plant pathogen contamination for every reaction. Unlike other techniques, this multiplexing strategy verifies the performance of the assay when detecting plant pathogens, resulting in the minimization of false negatives due to reaction set-up errors or failing experimental conditions. **The Open PCR is only equipped with 2 channels, therefore can only be used for the detection of HLVd.**

Process Overview

The process for determining the presence of a viral plant pathogen uses a reverse transcriptase enzyme to create complementary DNA (cDNA) from a messenger RNA (mRNA) template followed by quantitative PCR (qPCR) using a multiplex system of primers to detect both cannabis DNA and the targets of interest. Below is a simplified depiction of the reverse transcription followed by quantitative PCR whereas the cDNA becomes the template for qPCR.



Step 1:
Primers and probe
bind to target DNA.

Step 2:
PCR occurs, primers are
extended on forward and
reverse DNA strands.

Step 3:
Probe is degraded as a result
of polymerization and
fluorescent signal is
generated.

Step 4:
Target DNA is amplified and
fluorescent signal can be
measured and quantified.

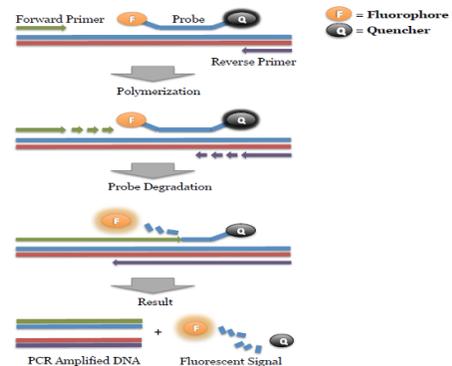


Figure 1: Overview Reverse Transcription Figure 2: Overview of qPCR

Materials and Methods

Test Kit Name: PathoSEEK[®] Virus Multiplex Detection Assay A

Test Kit Information

1. Leaf Punch Lysis Solution- P/N 420208
2. PathoSEEK[®] Virus Multiplex Detection Assay A - P/N 420121.
3. PathoSEEK[®] Virus Multiplex Detection Assay Positive Control - P/N 420126.
4. Medicinal Genomics RT - qPCR Master Kit - P/N 420204.

Test Kit Components

1. Leaf Punch Lysis Solution- P/N 420208
 - a. Lysis Solution — packaged in 12 x 0.2 mL PCR 8-tube strips with attached optical cap strip in a 96 well plate (Store at -15 to -20°C). Expires 1 Year from Date of Manufacture.
2. PathoSEEK[®] Virus Multiplex Detection Assay A - P/N 420121
 - a. Assay — 1 tube (Store kit at -15 to -20°C). Expires 2 Years from Date of Manufacture.
3. PathoSEEK[®] Virus Multiplex **HLVd**, CCV, LCV Positive Control - P/N 420126.
 - a. Control — 1 tube (Store at -15 to -20°C). Expires 2 Years from Date of Manufacture.
4. RT - qPCR Master Kit - P/N 420204

- a. RT/RNase Block — 1 tube (Store at -15 to -20°C). Expires 2 Years from Date of Manufacture.
- b. DTT — 1 tube (Store at -15 to -20°C). Expires 2 Years from Date of Manufacture.
- c. Nuclease Free Water —1 tube (Store at -15 to -20°C). Expires 2 Years from Date of Manufacture.
- d. RT - qPCR Master Mix — 1 tube (Store at -15 to -20°C). Expires 2 Years from Date of Manufacture.

Supplies, Reagents, and Equipment

1. Chai Bio Open PCR Dual Channel Catalog Number E013201
2. 0.1mL PCR 8Tube Strips, Optical Caps (USA Scientific, #1402-2300 or similar)
3. Multi-channel pipette P50 or P20 (optional)
4. Single channel pipette P10, P20 and P200
5. Filtered pipette tips for P10, P20, P50, and P200
6. Compact PCR Tube Rack (USA Scientific, #2300-9602 or similar)
7. 1.5mL tubes (USA Scientific #1415-2600)
8. Reservoirs (Fisher Scientific #14-222-398 or similar) (optional)
9. Crushed ice or cold racks (1.5ul Tube Bench-top Cryogenic Racks, VWR #89004-558 or similar)
10. Dual rotor personal microcentrifuge (USA Scientific #2641-0016)
11. Table top Vortex Genie (Scientific Industries #SI-0236 or Similar)
12. Refrigerator, 4°C

13. Freezer, -20°C
14. Laboratory Gloves (USA Scientific, # 4904-3300 or similar)
15. Permanent Marker (Sharpie)
16. Plant Stakes (Optional for labeling)
17. Toothpicks for punching out leaf punches (can use pipette tips as well)

Reagents

1. 10% bleach

Safety Precautions and Recommendations for Best Results

Environment

1. The quality of results depends on the strict compliance with Good Laboratory Practices (for example, the EN ISO 7218 standard), especially concerning PCR:
 - a. Never circulate lab equipment from one workstation to another.
 - b. Always use a positive and negative control for each series of amplification reactions.
 - c. Periodically verify the accuracy and precision of pipette, as well as correct functioning of the instruments.
 - d. Change gloves often, especially if you suspect contamination.
 - e. Clean workspaces periodically with 10% bleach and other decontaminating agents. Use powder-free gloves and avoid fingerprints and writing on tube caps because both can interfere with data acquisition.

Safety Precautions

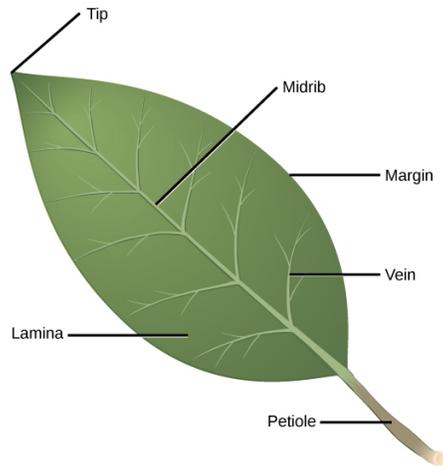
1. Assay users should observe good lab practices and safety precautions when performing this assay. Wear protective gloves, lab coats, eye/face protection as indicated by your quality system.
2. It is the responsibility of each laboratory to handle waste and effluents processed according to their nature and degree of hazardousness and to treat and dispose of them in accordance with applicable local, state, and federal regulations.

Intended User

The PathoSEEK[®] Virus Detection Assays and all components required to perform the method are intended for use by trained personnel familiar with laboratory techniques associated with viral detection.

Leaf Sampling:

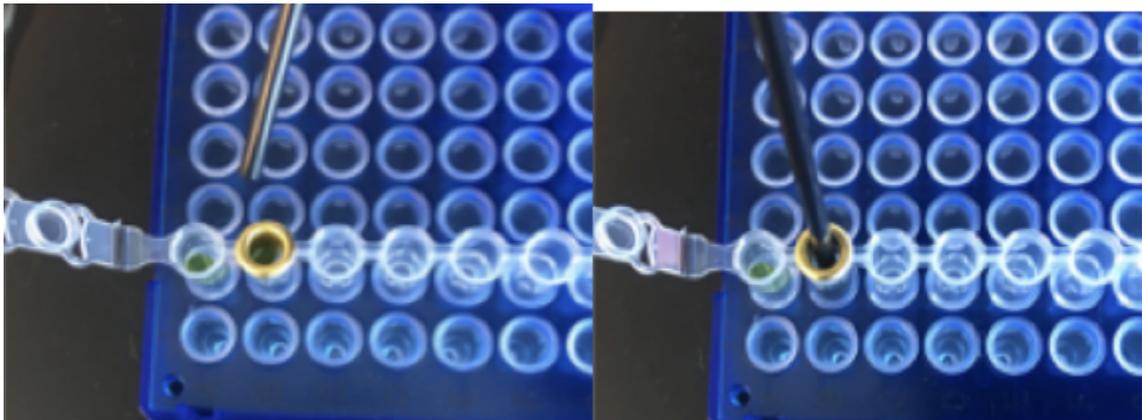
1. Put on a new pair of sterile gloves.
2. Remove strip tube(s) containing pre-aliquoted Leaf Punch Lysis Solution and allow them to thaw.
3. Once thawed, quick spin using a mini centrifuge with strip tube adapter to bring all contents of solution to the bottom of the tubes.
4. Label the wells of the Leaf Punch Lysis Solution strip tubes being used.
5. Obtain a disposable leaf punch ring. Press leaf punch down into leaf on sterile surface and rotate to remove a small section.
 - a. We recommend obtaining 1-3 punches from the midrib in the leaf, or stem. The older tissue of the midrib should have a higher viral titer. See picture below.
 - b. One to multiple punches can be transferred into one Leaf Punch Lysis tube. We have lysed from 1 to 10 punches at once. The amount of plant material necessary is dependent on the level of infection.
 - c. Determining number of leaf punches necessary will depend on:
 - i. Level of viral infection
 - ii. Where the leaf is sampled (broad part of leaf vs midrib)
 - iii. Age/thickness of leaf
 - iv. Too much leaf material can result in overloading the assay. A dilution of the lysed material may be necessary.



6. Remove cap from one strip tube and place leaf punch into the top of the strip tube.

Discharge leaf punch with toothpick or pipette tip. Use a fresh toothpick or pipette tip for every leaf punch.

7. Close cap of strip tube and discard leaf punch ring.



8. Repeat steps 5-7 for each sample being tested.

9. Change gloves in between each leaf sampling.

10. Once finished with leaf sampling, run a lysis program on the Bio-Rad Instrument.

Lysis Reaction Setup:

- Make sure all of the strip tube caps are firmly snapped into place on each tube.
- Briefly spin all strip tubes using mini-centrifuge with strip tube attachment to ensure that the Lysis Solution and leaf punch are in the bottom of the wells of the strip tubes. In some cases, the leaf punch may float onto top of the Lysis Solution. This is OK as long as it is in contact with the liquid.
- Place all of the sealed strip tubes onto the Open PCR.



- If using the Open PCR over a LAN/WiFi, sign on to the instrument's unique website by typing the IP address on the instrument screen into your browser address window and Log in. If logging in for the first time, create username and password.

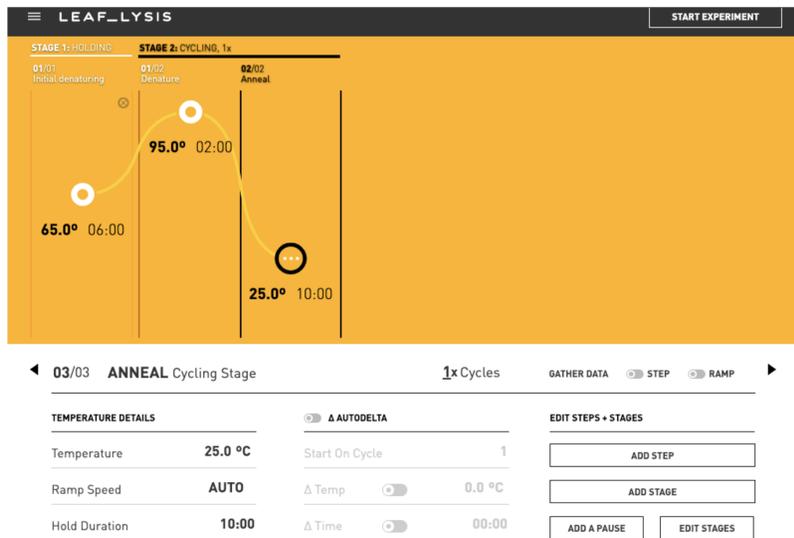


- Create a new experiment on the Open qPCR Instrument called “Leaf_Lysis”



- Program the proper Thermal Profile for the Leaf_Lysis method
 - a. The Temperature Parameters are as follows:
 - i. 6 minutes at 65°C
 - ii. 2 minutes at 95°C
 - iii. 10 minutes at 25°C

- b. Program the “Initial Denaturing” step to be 6 minutes at 65°C.
- c. Program the “Denature” Cycling Stage to be 2 minutes at 95°C. Change the Number of Cycles to be 1 cycle.
- d. Program the “Anneal” Cycling Stage to be 10 minutes at 25°C. Turn off the “Gather Data” option at this step, as there are no fluorophores included in the lysis step
- e. Close the lid and click “Start Experiment”.
- f. After approximately 18 minutes when the lysis program is complete, remove the strip tubes from the instrument and briefly spin in a mini-centrifuge with strip tube attachment to bring contents of each well to the bottom.
- g. If not already, allow the samples to come to room temperature for at least 5 minutes before continuing to qPCR detection assay setup.



RT- qPCR Protocol

1. Remove RT - qPCR Reagents, Viral Assay Probe Mix tube, and assay positive control from the -20°C freezer.
 - a. Allow all reagents to defrost at room temperature. Once defrosted, place tubes on ice.
2. Before preparing the master mix, invert or vortex (do not vortex the RT - qPCR Master Mix) and spin-down the reagents.
 - a. Assay Probe Mix tube – Vortex quickly followed by a pulse spin-down in a micro centrifuge.
 - b. Assay Positive Control tube – Vortex quickly followed by a pulse spin-down in a micro centrifuge.
 - c. DTT - Vortex quickly followed by a pulse spin-down in a micro centrifuge.
 - d. RT/RNase Block- Vortex quickly followed by a pulse spin-down in a micro centrifuge.
 - e. Water - Vortex quickly followed by a pulse spin-down in a micro centrifuge.
 - f. RT - qPCR Master Mix – Generously invert the bottle 5-10 times to mix followed by a wrist flick to bring contents to the bottom of the bottle.
 - g. Return all reagents to the ice.

Note: Do not vortex the RT - qPCR Master Mix at any point during the protocol.

3. Prepare master mix in a 1.5mL tube (the probe mix also contains the internal plant control, SCCG probe mix). Label tube with RT- qPCR MM. Always prepare enough master mix for 1 or 2 additional reactions over the total number of tests to account for pipetting and dead volumes. Be sure to include two extra reactions for the qPCR positive and negative controls. For example, if testing 10 plants, you would need to make enough master mix for 13 or 14 reactions, which would account for 1 or 2 excess.

Note: *It is best to add water first and master mix last.*

Reagents	1 Reaction	24 reactions (plus 1 excess rxn)	48 reactions (plus 2 excess rxn)
RT - qPCR Master Mix	10 μ L	250 μ L	500 μ L
Virus Assay Probe Mix	1 μ L	25 μ L	50 μ L
RT/RNAse Block	1 μ L	25 μ L	50 μ L
DTT(100m)	0.2 μ L	5 μ L	10 μ L
Water	5.8 μ L	145 μ L	290 μ L
Total	18 μ L	450 μ L	900 μ L

- a. Once combined, carefully tip mix or invert the tube 5 times to combine.
 - b. Pulse spin-down in micro centrifuge.
 - c. Place MM tube on ice until used in step 5.
4. Make positive control
 - a. For the positive control(s), transfer 1 μ L of multiplex control into 9 μ L of water

- b. For the negative control, use water (found in the kit).

Note: *It is best to add the largest volume reagent first, in this case H₂O.*

5. Transfer Master Mix to strip tubes
 - a. Add 18 µL of RT - qPCR MM to each well of strip tubes that will be used for reactions including sample wells, positive control well, and negative control well.
6. Transfer lysed samples, positive and negative controls to the corresponding reaction tubes
 - a. Open caps of tubes containing lysed leaf sample carefully (pay attention not to spill any liquid).
Transfer 2µL of each lysed sample into a separate well of the qPCR plate
 - b. Transfer 2 uL of the positive control into the designated positive control well and 2 µL of the negative control (water) into the designated negative control well.
 - c. NOTE: Be sure to use a new filtered tip for each transfer!
 - d. Mix each reaction well with a pipette set to approximately 15 µL to ensure sample and master mix are thoroughly mixed
7. Create a New Experiment on the Chai Bio website corresponding with the instrument.
8. **Thermal Profile:**
 - a. Under Setup>Thermal Profile, create the following PCR thermal profile.
 - i. RT reaction 50 °C for 10 minutes
 - ii. 95 °C for 3 minutes
 - iii. 40 cycles of 95°C for 5 seconds and 65°C for 30 seconds
 - iv. Data collection



9. Close the lid and click “Start Experiment”.
10. Save the experiment with the [User] and [date]
11. When the run is complete, immediately dispose of the tubes. Do not open the tube caps after the run to avoid contamination in the lab.

Detailed Assay Data Analysis

1. **Virus detection**
 - a. Select the Experiment once the Run is Complete
 - b. When the Data Analysis window opens, change the “Color By” option to “Channel” and the “Plot Type” to “Log” at the top of the page. Ensure “Baseline Subtraction” and both channels are selected.

Amplification Curve ▾

COLOR BY

WELL CHANNEL

PLOT TYPE

LOG LINEAR



BASELINE SUBTRACTION

AMPLIFICATION SETTINGS

CHOOSE CHANNEL:



- c. Highlight the wells of interest in the Well Map under the Graphical Display
 - i. Amplification plots will be available for viewing
 - ii. The Cq values will appear to the right in the table
- d. Controls
 - i. HLVd Positive Control, on the FAM fluorophore, has a Cq value < 35.
 - 1. Visually confirm with the curve on the graph.
- e. Unknown Samples (HLVd)
 - i. Internal Control, on the HEX fluorophore, has a Cq value < 35.
 - 1. Visually confirm with the curve on the graph
 - ii. A **virus positive** result for the unknown sample.
 - 1. Any Cq value for the FAM fluorophore < 35.
 - 2. Visually confirm with the curve on the graph. (It is very important to confirm with the amplification curve when a presence result occurred. Sometimes the background amplification will give a false positive reading, especially when Cq reading is less than 15. See troubleshooting guide below.)
 - iii. An “absence” result for the unknown sample.
 - 1. No Cq value for the FAM fluorophore or a Cq value >35.

Troubleshooting Guide

Symptom	Reason	Solution
Internal control (SCCG Primer) failure	Extraction Failure	Repeat SenSATIVAx™ and PathoSEEK™ by following the protocol.
	Residual ethanol in elution	Ethanol is an inhibitor to PCR. Return to the SenSATIVAx™ protocol and repeat all steps.
	Mix up in Reaction Setup	Repeat the qPCR by following the protocol.
	Missing Fluorophore on plate set up	In the Data Analysis window click on View/Edit Plate Setup from the Settings drop down. All wells should have both FAM and HEX. Once completed and the window is closed the analysis should automatically update.
Internal Control (SCCG) Positive result on positive or negative control samples or samples that do not contain plant DNA No Cq or a Cq of more than 35 is acceptable. Any Cq lower than 35 constitutes a rerun	Plant DNA contamination in a reagent	Troubleshoot which reagent was contaminated; use new reagents, thoroughly clean all pipettes and bench areas with 10% bleach solution.
	qPCR bench too close to extraction area	Designate separate benches, pipettes etc. for extractions and qPCR setup
Positive Negative Control	Small Cq value <15	Visually confirm that there is an amplification curve. If not, this is a low level background and is to be expected.
	Contamination	Repeat the qPCR by following the protocol.
	Insufficient pre-setup bleaching	Wipe down the lab workspace and all equipment with 10% Bleach. Repeat qPCR.
Negative Positive Control	Mix up in Reaction Setup	Repeat the qPCR by following the protocol.
Total run failure	Excessive vortex of the qPCR Master Mix	Repeat the qPCR by following the protocol.
Background Amplification	Unclear	This is usually seen with a very low Cq reading (<15), the curve is usually missing the exponential growth phase, but rather a gradual increase of fluorescence signal. This is usually a negative result, but should be repeated.

Glossary and Definitions

Deoxyribonucleic acid (DNA) is a molecule that encodes the genetic instructions used in the development and functioning of all known living organisms.

Reverse Transcription (RT): the process by which an enzyme, called reverse transcriptase, converts the viral RNA genome into a complementary DNA (cDNA) molecule.

Polymerase Chain Reaction (PCR) is a technology in molecular biology used to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

A **fluorophore** is a fluorescent chemical compound that can re-emit light upon light excitation.

The **Negative Controls** are the reactions where no Cq is expected. It helps to ensure that all Assay specific reactions are clean of contaminants.

The assay specific **Positive Controls** are the reactions where a Cq is expected. It helps ensure that all Assay specific reactions are working correctly. The Assay specific Positive Control is targeting the pathogen using the FAM, Texas Red and Cy5 Fluorophores.

Amplification of the **Internal Control** is expected in every reaction containing cannabis DNA. It ensures the DNA isolation procedure was successful. The internal control targets plant DNA, or more specifically, a Single Copy Control Gene (SCCG), using the HEX Fluorophore.

DISCLAIMER

This test was developed, and its performance characteristics determined by Medicinal Genomics Company, for laboratory use. Any deviations from this protocol are not supported by MGC

The results may vary based on laboratory conditions. Altitude and humidity are among factors known to affect the growth of bacterial and fungal species. All thresholds were determined based on the results using the BIO-RAD CFX96 Touch® Real-Time PCR Detection System. It is recommended that thresholds be calibrated for each specific laboratory setting.

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