Loop-mediated isothermal amplification (LAMP) Assay for detection of grapevine red blotch virus (GRBV)

This is a protocol providing guidance for conducting a rapid, colorimetric assay for the detection of grapevine red blotch virus (GRBV). Results are viewed as a color change of a solution in a tube (indicative of a pH change). Conducting the assay requires a clean space, a limited amount of equipment and chemical reagents and takes an experience user about one hour (or more, depending on the number of samples). When first learning the technique, considerably more time will be required. Details of this assay were published in 2019.

> Romero, J.L.R., Carver, G.D., Johnson, P.A., Perry, K.L., Thompson, J.R., 2019. A rapid, sensitive and inexpensive method for detection of grapevine red blotch virus without tissue extraction using loop-mediated isothermal amplification. Arch. Virol. 164, 1453–1457. https://doi.org/10.1007/s00705-019-04207-y

Note that the tissue sampling/extraction described in this publication as been updated, as described below. For questions on methods and reagents, or a copy of the above publication, contact: Dr. Keith Perry, Cornell University, KLP3@cornell.edu

Reagents required

-Distilled water to prepare the LAMP reaction mix wherein 45 uL volumes are required. DI water will also be required in small volumes (10 uL) for preparation of each plant sample in the 5 ml tubes. Prepare a set of 500 uL aliquots in 1.5 ml microfuge tubes for single use.

-DNA oligonucleotide primer set (aliquoted into tubes in as 12.5 µl volumes, enough for "10" assays in theory, 8 assays in practice; one does not want to end up not having sufficient volume; details below).

-WarmStart® Colorimetric LAMP 2X Master Mix, New England Biolabs, catalog # M1800S or M1800L, aliquoted into tubes in as 62.5 µl volumes, enough for "10" assays in theory, 8 assays in practice (one does not want to end up not having sufficient volume).

Equipment required

-Incubator or water bath for 65˚C / 149˚F incubation.
-Thermometers (two), previously checked for consistency.
-Pipet tips for 0.5 to 2 (or 10 µl) volumes ('smallest pipet tip')
-Pipet tips for 10 to 20 µl volumes ('small pipet tip')
-Pipet tips for maximum 200 µl volumes ('mid-size pipet tip')
-Plastic bags to collect samples
-Marker to label bags
-Cooler or container to cool down samples or avoid their overheating in a vehicle.
-Razor blades for collecting samples from canes, disposable (can be re-used if washed and baked to remove contaminating DNA)
-Paper towels or butcher paper on which to work with each sample

-1.5 ml centrifuge tubes, capped, polypropylene
-5 ml centrifuge tubes, capped, polypropylene
-200 µl, thin-walled polypropylene microfuge tubes ('PCR' tubes'), preferably in strips of 8 tubes for easy handling.
-'P-20' pipettor with a capacity to transfer from (0.5) 1 to 20 µl volumes
-'P-200' pipettor with a capacity to transfer from 20 to 200 µl volumes
-(optional) 2 µl capacity pipettor ('P-2') with a capacity to transfer from 0.5 to 2.0 µl volume or a 'P-10' pipettor with a capacity to transfer from 0.5 to 10 µl volume. This size allows one to more accurately transfer the required 0.5 µl sample volume. Alternatively use the P-20 pipettor set at 0.5 µl.
-(optional) small microfuge that can accommodate strips of eight 200 µl, thin-walled polypropylene microfuge tubes ('PCR' tubes')
-camera (mobile phone) to document test results.

Protocol

1. Field sampling: petioles and canes

The easiest, most practical plant material for reason of ease of handling is the leaf petiole. Collect six older, larger leaves with intact petioles; only three will be used for a first assay. The additional three leaves can be used if re-testing is required. When sampling, avoid touching the wet end of the detached petiole so as to avoid cross contamination. It is recommended that samples be taken from 3 different canes of a single vine. It is important to select the oldest (lower) leaves on a canes. Sampling mid to late in the season is preferable to early in the season.

The most reliable plant material for testing is the woody cane for bark scrapings. Collect three woody canes from different areas of a single vine. One can use the same clippers to obtain canes from different vines without fear of cross contamination, since the cut ends will not be sampled.

Place the petiole or cane sample in a labeled plastic bag; transfer and store in a cooler or refrigerator (4˚C / 39˚F) as soon as possible. Label the bag with sufficient information to know the vineyard and preferable the vine from which the sample was taken. It is helpful to mark the vine with flagging in case re-sampling is required.

2.. **Preparations of reagents and materials**

-All reagents should be stored frozen (standard freezer temperature, -20˚C / -4˚F). Before use, allow components to warm to room temperature.

-Note: reagents are prepared as frozen aliquots sufficient to perform at least 8 tests at a time (corresponding to strips of 8 tubes); volumes are nominally for 10 reactions.

-Pre-heat an incubator or water bath to 65˚C / 149˚F

-5 ml centrifuge tubes, capped, polypropylene; this allows a pipet tip with the plant sample to be placed inside it and the cap closed to limit cross contamination. Alternatively, a 1.5 ml polypropylene microfuge tube can be used, but this cannot be closed so care must be taken that the tip does not fall out.

3. Prepare tubes for tissue samples

Label the 5 ml centrifuge tubes, one for each plant sample to be tested. Into the bottom of each tube, transfer 10 µl of distilled water using a p-20 pipettor with a small pipet tip (with 10-20 µl capacity)

4a. Pipette tip sampling of petiole

To perform the assay in the clean work space, it is best to use gloved hands for each sample; alternatively, wash hands with soap in between each sample preparation. Set up a paper towel or piece of paper on a table to cover the space where you will work. Insert the pointed end of a small pipet tip, into the broken or cut end of a petiole. The tissue should be spongy and one can feel the tip penetrating the tissue; if in doubt repeat puncturing of the tissue; if it still doesn't feel like the tip is entering tissue, break/cut off a small piece of the end of the petiole (~5 mm) and repeat the puncturing of the tissue. Look closely at the end of the tip to see if you can see any tissue or liquid. You may not be able to see anything and this is acceptable; more is not better and you do not want to see a piece of tissue attached to the tip, as this is too much and may interfere with the assay.

Using the same pipet tip, sample (puncture) three different petioles. After sampling, ideally you should be able to see a miniscule amount of sap (or tissue) in the tip; again, you do not want any obvious pieces of tissue on the outside of the tip. If during the process, at any point the tip bends, start over with a new pipet tip.

Place the tip into the 10 µl droplet of water in a 5 ml centrifuge tube and close the cap. Holding the top of the tube with two fingers, flick the tube with a finger so that the pipet tip spins in order to mix. Shake the tube downward to bring down any droplets on the side of the tube. This sample should be stable sitting on a table top at room temperature while you work.

Clean up all the plant material, placing the leaves and petioles back in the original sample bag. Discard the paper on top of which you were working. Wipe down the table surface if tissue contaminated this surface.

4b. Pipette tip sampling of cane (dormant or not)

Using a single razor blade, scrape aware the outer bark tissue to expose the (green-) white cambial tissue. Do this for each of the three canes, only exposing a small area, perhaps 1 cm / ½ inch in length. Using a small pipet tip, scrape tissue from all three canes (described below) and place the pipet tip into the 10 µl droplet of water in a 5 ml centrifuge tube and close the cap. Holding the top of the tube with two fingers, flick the tube with a finger so that the pipet tip spins in order to mix. Shake the tube downward to bring down any droplets on the side of the tube. This sample should be stable sitting on a table top at room temperature while you work.

To scrape tissue, hold a small pipet tip with thumb and finger close to the end for strength and stability, and angle the pipet tip on the exposed cambial tissue such that it is almost parallel.

Push the tip along the tissue to scrape some off into the tip. The tip needs to be nearly parallel to the surface, because if you try to poke the tip into the tissue at an oblique angle, it will bend the tip. If during the process, at any point the tip bends, start over with a new pipet tip. Look closely at the end of the tip to see if you can see any tissue or liquid. Ideally you should be able to see something or to have felt the tip removing tissue. Unlike with petioles, it may be difficult to know if tissue or sap has been punctured / removed. More is not better and you do not want to see an abundance of tissue attached to the tip, as this would be too much and may interfere with the assay.

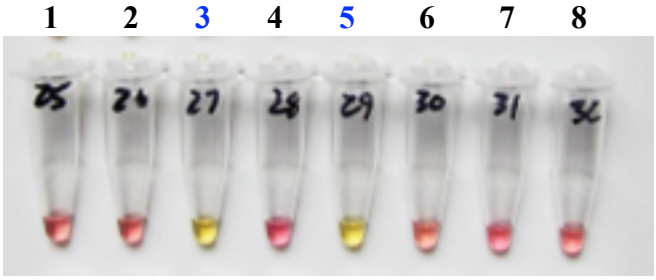
5. Experimental design

Note: Samples are tested using strips of eight tubes. The first strip should have four important controls and can thus accommodate four plant samples. Subsequent strips only need one control and can accommodate seven plant samples. When first setting up and learning the assay, it is recommended to start small and only do one strip of tubes (four plant samples) so as to become familiar with material handling and all steps.

First strip of tubes:

1. Plant sample #1, 0.5 µl
2. Plant sample #2, 0.5 µl
3. Plant sample #3, 0.5 µl
4. Plant sample #4, 0.5 µl
5. Positive control plant sample, 0.5 µl
6. Negative control plant sample, 0.5 µl
7. Negative control 0.5 µl of water
8. Negative control no addition of 0.5 µl volume

Example of first strip LAMP assay results – pink is a negative result (no virus detected) yellow is a positive result (virus detected). The two positive results are for plant sample #3 and the positive control sample



Second strip of tubes:

1. Plant sample #5, 0.5 µl
2. Plant sample #6, 0.5 µl
3. Plant sample #7, 0.5 µl
4. Plant sample #8, 0.5 µl
5. Plant sample #9, 0.5 µl
6. Plant sample #10, 0.5 µl
7. Plant sample #11, 0.5 µl
8. Negative control no addition of 0.5 µl volume

6. Set up for the LAMP assay

Set out:

-all samples to be tested (in 5 ml capped centrifuge tubes)
-aliquots of water, a positive control, and a negative control
-small volume pipettor (P-2, P-10, or P-20)
-smallest size pipet tips for 0.5 to 2 (or 10 µl) volumes to be use to transfer the 0.5 µl samples
-strip of eight 200 µl capacity 'PCR tubes', each with 12 µl volume of the prepared LAMP reagents (step 7 below)

Note: If not well-experienced, practice pipetting manipulations. After pipetting up a small volume, hold the pipet tip up to the light to confirm you can see a very small volume in the tip. Familiarize yourself with what different volumes look like, e.g. 12 µl, and especially the 0.5 µl volume which is difficult to see. You must be able to view and confirm the presence of the desired volumes in the pipet tip at each step in the process.

7. Combining the LAMP reagents to prepare the LAMP reaction mix

This is the last step before starting the LAMP assay. Thaw microfuge tubes with frozen aliquots of the following:

-Distilled water (45 µl will be required)
-DNA oligonucleotide primer set (12.5 µl)
-WarmStart® Colorimetric LAMP 2X Master Mix (62.5 µl)
Total volume = 120 µl

- Shake down or microfuge the tubes to make sure the liquid is all at the bottom of the tube. It is normal to see some precipitate in the colorimetric LAMP Master Mix after thawing the aliquot. Make sure to adequately thaw and then tap tube until mixed.

- Using a P-20 pipettor with a 20 µl capacity tip, transfer 12.5 µl of the primer mix into the tube with the LAMP 2X master mix; gently pipet up and down once rinse the tip.

- Using a 200 µl capacity pipet tip and a P-200 pipettor, transfer 45 µl of water into the tube with the LAMP 2X master mix; gently pipet up and down once rinse the tip.
- Cap the microfuge tube with combined reagents. Holding the top of the tube with two fingers, flick the tube with a finger two or three times in order to mix. Shake the tube downward to bring down any droplets on the side of the tube.

- Using a P-20 pipettor with a 20 µl capacity tip, transfer 12.0 µl of the LAMP reaction mix into the bottom of each of eight 200 µl capacity 'PCR tubes'. Loosely close the caps of all the tubes. Tightly close the cap of tube #8 (the negative control with no further addition). Set aside and ultimately discard the remaining 24 µl (theoretically) LAMP reaction mix.

8. Starting the LAMP assay

Use freshly gloved or washed hands. Pick up the 5 ml centrifuge tube with plant sample #1, gently flick the tube to mix, shake the solution down to the bottom of the tube, and visually inspect the tube to confirm you can see the droplet of water at the bottom (there is still a pipet tip in the tube). Open the tube with care to avoid your finger touching the rim of the opened tube, in which case you can cross-contaminate samples. If you do contact the rim, change gloves or wash hands before proceeding to the next plant sample.

Pick up the smallest volume pipettor (e.g. a P-2, P-10, or P-20 pipettor) with the smallest pipet tip available (e.g. tips for 0.5 to 2 (or 10 µl) volumes) and set at 0.5 µl. Holding the 5ml tube up to the light so that you can see the water droplet at the bottom, insert the pipet tip into the droplet and remove 0.5 µl. Remove the pipet tip from the tube and hold it up to the light to confirm you can see a very small volume in the tip. Open one of the eight 200 µl capacity 'PCR tubes' with the 12 µl of LAMP reaction mix and transfer the 0.5 µl sample into the reaction mix. To do this, hold the 200 µl capacity 'PCR tube' up to the light and observe the pipet tip enter into the 12 µl droplet of reagents. Pipet up and down once to make sure the 0.5 µl sample has been introduced into the solution. Close the cap tightly.

Repeat the above process for the six remaining samples/controls; this would correspond to tubes #2 through #7 in the first strip of tubes above. Every time you do an addition, you must hold the tubes up to the light and visually confirm the transfer of volumes.

Double check the caps of the eight tubes are tightly secured; this can most easily be done if the tubes are set in a rack and one can press down on the tube. Holding one end of the strip of tubes flick them a few times. Flip the strip so that you are holding the other end of the strip of tubes and repeat the flicking. Shake the strip of tubes downward to ensure all the red colored reagent is at the bottom of the tube. This can also more easily be done with a pulse of a small microfuge, a piece of equipment one will want to have if performing large numbers of assays.

Take note of the red/pink color of the reagent in each tube. Note if any of the samples start off with a more orange or other color; this would be problematic, as it indicates the pH has been changed simply by the addition of plant material.

Check the temperature of the incubator or water bath to be used for the reactions (65˚C / 149˚F). Transfer the strip of eight tubes into the incubater/water bath and set timer for 35 minutes. Remove the strip of eight tubes, dry off any moisture on the outside, shake the liquid to

the bottom of the tube, place on a white paper background, and take a photograph to document the test results.

9. Interpreting results of the LAMP assay

Referencing the first strip of tubes (above 5. Experimental design), the most important tubes are the last four control tubes.

Negative controls - Working backwards, tube #8 is expected to have retained the original pink/red color, showing that the reagents are stable and what is to be expected for a negative test result. Tube #7 provides the same information, and if still pink/red shows that there has not been any unexpected contamination during the sample manipulations. Tube #6 is a negative plant control and if still pink/red shows what is to be expected for a negative test result and that there has not been any unexpected contamination. All of these controls should be included in at least one strip of tubes every time an assay is performed; they are essential in order to have confidence in interpreting the results. One of the biggest challenges in performing LAMP assays is that of cross-contamination resulting in 'false positives'. If the negative controls all remain pink/red, one has confidence the likelihood of obtaining false positives is very low.

Positive controls - Tube #5 is the positive control. If the color has changed to yellow, it shows that all of your reagents are working as expected and that the assay has been performed properly.

Tubes #1 to #4 are the experimental plant samples. Yellow indicates the virus is present in the tested plant; red/pink indicates the virus has not been detected. If all the controls are working as expected AND there has not been any cross-contamination, one can have high confidence that any tested plant showing yellow is infected with GRBV.

Although a red/pink color indicates GRBV has not been detected, it does not necessarily mean the plant is not infected. There are many reasons why a plant might test negative; the sample may not have been representative of the whole plant or the virus may not be equally distributed in the plant and not have been present in the selected petioles or cane.

10. Reconfirming results of the LAMP assay

Do the test results obtained make sense? Are they what you might have expected based on symptoms, the history of a planting material, or past test results? What are the consequences of a positive test result or a 'false positive', i.e. a positive test result when the plant is actually <u>not</u> infected. What are the consequences of the opposite scenario, a negative test result when the vine is actually infected with GRBV (a 'false negative').

One has the option to re-test and reconfirm an initial result with the LAMP assay. This can be done with three different leaves (or canes) from the original sample. Or even better, an independent re-sampling of the plant can be made. Re-testing and reconfirmation can also be done at a different time of year. One can also send material to a commercial testing service, but if you do so, make sure to submit an identical sample to what has been LAMP tested, i.e. the same leaves/petioles or canes.

All diagnostic tests have their limitations; none are perfect. The reliability of results will always be a function of how representative a sample was submitted and tested. The LAMP assay

is more sensitive than a PCR assays. This higher sensitivity can be an advantage but also poses a greater risk of cross-contamination and 'false positives'.

11. Experimental controls

Positive and negative controls are essential. To ensure reproducibility, controls should be prepared in a larger volume and aliquots prepared and frozen at -20˚C / -4˚F. Aliquots of the same tube can be used every time an assay is performed, so you know what to expect if reagents and methods are working well.

One could request from a cooperating research laboratory that purified nucleic acid extracts from GRBV-infected and uninfected plants be provided to the individual performing LAMP assays. Once received, the user should make multiple aliquots in 1.5 ml microfuge tubes (or other sizes) before using it. This will ensure reliable, non-contaminated stocks are always available to fall back upon should any problems arise.

Alternatively, one could prepare aqueous extracts from known GRBV-infected and uninfected plants; this plant tissue (cane or petiole) could be requested from a cooperating research laboratory. Alternatively, an individual may have access to previously tested plants. Aliquots of the extracts would be prepared, stored frozen and the performance of these extracts could then be verified. A suggested starting point is a given weight of petiole tissue ground in water and diluted to a final ratio of x grams tissue : 100x grams water.

Though trivial, aliquots of distilled water need to be available for one of the negative controls (above in section 5. Experimental design, first strip of tubes, tube #7, 0.5 µl of water addition) and for use in other parts of the assay.

12. Key reagents - DNA oligonucleotide primer set

The primer set will need to be prepared in a laboratory by someone familiar with ordering and manipulating synthetic DNAs. The per assay cost is negligible, but the one-time preparation requires an initial investment of money and especially time. In short, one orders the six DNA oligonucleotides, resuspends them in buffer, mixes them in a volume of water to the specified concentrations, and then prepares single use 12.5 µl volume aliquots.

Specific information on the mix is as follows:

LAMP Set I				10X LAMP primer mix			
			Final Concentration (µM)	Volume (µl) of 1 mM stock	Volume (µl) of 1 mM stock	Volume (µl) of 1 mM stock	Volume (µl) of 1 mM stock
				To make 200 µl	To make 200 µl	To make 1000 µl	To make 5000 µl
p1825	LAMP.168-F3	GAATCGTTTGAATCGTAAGAGA	0.2	0.4	1	2	10
p1826	LAMP.168-B3	CAGACAAATAAATACGATTCCTTTC	0.2	0.4	1	2	10
p1827	LAMP.168-FIP	AATGACTCCTGCGGCTTCTTTCGTATTTTGGGTTCGAAGA	1.6	3.2	8	16	80
p1828	LAMP.168-BIP	TCAAAGACGTCGTCTGGTTGTCATCATTACGTCCTCCACC	1.6	3.2	8	16	80
p1842	LAMP.168-LB	GCTTTTAAAAACGACGTGT	0.4	0.8	2	4	20
p1857	LAMP.168-LF	TTCACGCCAACAACAAGT	0.4	0.8	2	4	20
			Total	8.8	22	44	220
			Water to 200 µl	191.2			
			Water to 500 µl		478		
			Water to 1000 µl			956	
			Water to 5000 µl				4780

13. Key reagents - -WarmStart® Colorimetric LAMP 2X Master Mix, New England Biolabs, catalog # M1800S or M1800L, aliquoted into tubes in as 62.5 µl volumes, enough for "10" assays in theory, 8 assays in practice (one does not want to end up not having sufficient volume).

Before using in assays, this reagent is aliquoted into tubes in 62.5 µl volumes, enough for "10" assays in theory, 8 assays in practice (one does not want to end up not having sufficient volume). The preparation of single use aliquots is done to minimize any cross-contamination between experiments. Although not recommended, this reagent can be thawed and refrozen. A smaller volume of the reagent can be used and the remainder saved, although this is designed to be single use aliquots.

14. Notes on setting up for larger numbers of reactions

Setting up one reaction at a time is not efficient and subject to greater error. In practice, we set up the reagents for 10 reactions; we set up for 1 or 2 more reactions than intended to avoid running short on the mix. Thus, a mix for 10 reactions is prepared to perform 8 assays at a time (including controls). 12 µl of the mix is aliquoted into each of the eight tubes (one complete strip of 8 tubes); the remainder of the mix is discarded. The compositions for doing this would be:

	For 5 reactions	10 reactions	20 reactions
Warm start 2x reagent	31.25	62.5	125
Primer mix	6.25	12.5	25
Water	22.5	45	90
Total volume	**60**	**120**	**240**

Since the 2X Master Mix reagent is aliquoted for use for 10 reactions, reagents are prepared for multiples of 10 reagents. For instance, if one had 10 plant samples + 5 controls, use two aliquots of the 2x reagent, add the primer and water to each, then combine into a single tube with 240 µl total volume. There will be waste and the cost of the 2X Master Mix is significant, but the most costly aspect of the procedure is the time and labor.