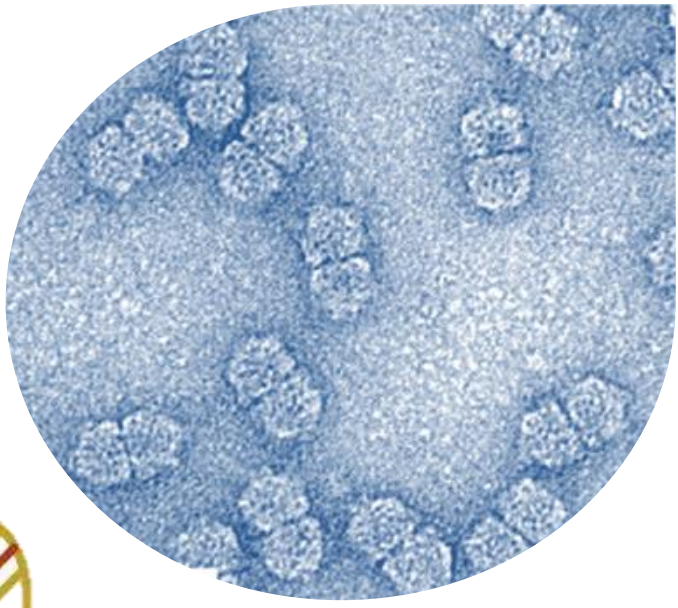


Training Workshop on Diagnostics of Begomovirus and Development of LAMP-PCR

(Training Manual and Laboratory Protocol)

This training workshop is supported by the Government of Japan through the Japan – ASEAN Integration Fund (JAIF)

Collaboration between the following institutions:

Authors

Dr. Marita S. Pinili
Dr. Sri Hendrastuti Hidayat
Dr. Masashi Ugaki
Ms. Norhayati Madiha
Mr. Freddiewebb B. Signabon

2022 JAIF-APHCN

TABLE OF CONTENTS

I. Composition and Preparation of Buffers and Other Reagents for DAS-ELISA (BIOREBA AG, Switzerland)

1. Extraction buffer (General)

(pH 7.4; for 1000 ml)
In distilled water (pH adjusted with HCl)

Compound	Concentration (1x)	
TRIS	20 mM	2.40g
NaCl	137 mM	8.00g
PVP K25 (MW 24000)	2% (w/v)	20.00g
Tween 20	0.05 % (w/v)	0.50g
KCl	2.7 mM	0.20g
NaN_3	0.02 % (w/v)	0.20g

Extraction buffer (General) available as 10x concentrate)

2. Coating buffer

(pH 9.6; for 1000 ml)
In distilled water;

Compound	
Na_2CO_3	1.59g
$NaHCO_3$	2.93g
NaN_3	0.20g

Coating buffer available as tablets for 100ml each

3. Conjugate buffer

(pH 7.4; for 1000ml)
In distilled water (pH adjusted with HCl)

Compound	Concentration (1x)	
TRIS	20 mM	2.40g
NaCl	137 mM	8.00g
PVP K25 (MW24000)	2 % (w/v)	20.00g
Tween 20	0.05% (w/v)	0.50g
BSA (bovine serum albumin)	0.2 % (w/v)	2.00g
$MgCl_2 .6H_2O$	1 mM	0.20g
KCl	2.7 mM	0.20g
NaN_3	0.02 % (w/v)	0.20g

Conjugate buffer available as 10x concentrate

4. Substrate buffer

(pH 9.8; for 1000ml)
In distilled water (pH adjusted with HCl)

Compound	Concentration (1x)	
Diethanolamine	1 M	97.00ml
NaN_3	0.02 % (w/v)	0.20g

Substrate buffer available as 5x concentrate

5. Washing buffer PBS-Tween

(pH 7.4; for 1000ml)
In distilled water;

Compound	Concentration (1x)	
NaCl	137 mM	8.00g

KH_2PO_4	1.5 mM	0.20g
Na_2HPO_4	8.1 mM	1.15g
KCl	2.7 mM	0.20g
Tween 20	0.05 % (w/v)	0.50g

Contains no preservatives. Use within 2 days or add 0.2g/LNaN_3. Washing buffer available as tablets or 1000ml each, or as pre-mixed powder for larger amounts.

6. pNPP substrate tablets

Compound	Weight per Tablet
Para Nitrophenylphosphate	20 mg
Total tablet weight	200 mg +/- 2%

Note: All buffers should be stored at 4°C. They are stable for 1 month after dilution/preparation. Use the prepared buffers at room temperature (20-25°C).

II. DOUBLE ANTIBODY SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY (DAS-ELISA)

(BIOREBA AG, Switzerland)

Procedures;

1. Coating: binding specific antibodies

    1.1. Dilute the IgG 1:1000 in coating buffer (e.g., 20 µl IgG in 20 ml coating buffer)
    1.2. Pipette 100 µl diluted IgG to each well.
    1.3. Cover the plate (e.g., Parafilm or plate cover).
    1.4. Incubate the plate at 30°C for 4 h or 4°C overnight.
    1.5. Wash the plate 3 x with washing buffer per well. (see point 5.)

2. Antigen: incubation with plant extract

    2.1. Homogenize the samples in extraction buffer.
    2.2. Add 100 µl of the extract to each well.
    2.3. Cover the plates with Parafilm or a plate cover and incubate at 4°C overnight.
    2.4. Wash the plate 3 x with 200 µl washing buffer per well. (see point 5.)

3. Conjugate: incubate with an enzyme-labelled antibody

    3.1. Dilute the conjugate 1:1000 in conjugate buffer (e.g., 20 µl conjugate in 20 ml conjugate buffer).
    3.2. Add 100 µl to each well.
    3.3. Cover the plate with Parafilm or cover plate and incubate at 30°C for 5 h.
    3.4. Wash the plate 3 x with 200 µl washing buffer per well. (see point 5.)

4. Substrate: the color reaction indicates positive samples

    4.1. Dissolve the substrate pNPP (para-nitrophenylphosphate) in substrate buffer to give a final concentration of 1 mg/ml.
    4.2. Add 100 µl of the substrate solution to each well.
    4.3. Incubate the plate at room temperature (20-25°C in the dark).
    4.4. Monitor the color development visually and/or photometrically at 405 nm (for ELISA readers with individual filters) or at 405/492 nm (for ELISA Readers with dual filters). Measure after 30-120 min.

5. Washing steps (washing the plate)

    5.1. For the washing procedure empty the content of the plate by tapping it dry. Ensure that there is nothing left in the well to avoid contamination from one well to the other. Another method is to use an automated ELISA washer.

5.2. Wash each well 3 times with 200 µl washing buffer. After each washing empty the content of the plate by tapping it dry.

5.3. After the last washing remove the remaining liquid by tapping it onto a clean paper towel.

Note: Specificity and sampling: *The coating reagent contains polyclonal antibodies; the conjugate consists of monoclonal antibodies, developed against a TYLCV isolate from Jemen. This broad-spectrum reagent combination detects all isolates of TYLCV but also other begomoviruses such as Tomato mottle virus (ToMoV), Bean golden mosaic virus (BGMV), Squash leaf curl virus (SLCV), and African cassava mosaic virus (ACMV). For increased test security, several samples of young leaves from different parts of the plant should be taken and extracted at 1:20 in extraction buffer.*

6. Alternative incubation conditions

6.1. Coating step: A shorter incubation time such as 2.5 h at 37°C gives similar results. Alternatively, an overnight incubation in the refrigerator at 4°C is possible. Coated plates can be kept in the refrigerator at 4°C for a maximum of one week (plates covered, preserved such as NaN_3 in the buffer).

6.2. Conjugate step: A shorter incubation time at 37°C for 2 h gives similar results if the substrate incubation is prolonged to 1.5 h as compared to 1 h as standard.

III. PLANT DNA EXTRACTION

1. Materials and Reagents

Preparation for Plant DNA extraction

Below is a list of the stock solutions needed for DNA extraction and PCR at *Centre for marker Discovery and Validation* (*CMDV*).

1.1 Stock Solution

Buffer preparation

Extraction Buffer:	**Final Conc**	**100 ml**	**200 ml**	**300 ml**	**400 ml**	**500 ml**	**600 ml**	**700 ml**	**800 ml**
PVP	2%	2 g	4 g	6 g	8 g	10 g	12 g	14 g	16 g
Sodium Sarkosyl	1%	1 g	2 g	3 g	4 g	5 g	6 g	7 g	8 g
DIECA	4 mM	90 mg	180 mg	270 mg	360 mg	450 mg	540 mg	630 mg	720 mg
Ascorbic Acid	5 mM	88 mg	176 mg	264 mg	352 mg	440 mg	528 mg	616 mg	704 mg
5M NaCl	1.4M	28 ml	56 ml	84 ml	112 ml	140 ml	168 ml	196 ml	224 ml
1M Tris-HCl (pH8.0)	100mM	10 ml	20 ml	30 ml	40 ml	50 ml	60 ml	70 ml	80 ml
0.5M EDTA	20mM	4 ml	8 ml	12 ml	16 ml	20 ml	24 ml	28 ml	32 ml

5M NaCl

NaCl	292.2g
ddH_2O	1000 ml
Total volume	1000 ml

Autoclave to sterilize

0.5M EDTA (pH 8)

EDTA	186.1g
ddH_2O	1000 ml
Total volume	1000 ml

pH to 8 with ~20g of NaOH pellets and autoclave to sterilize

1M Tris-HCl (pH 8)

Tris	121.1g
ddH_2O	1000 ml
Total volume	1000 ml

pH to 8 with HCl and autoclave to sterilize

2. Extraction Procedure

Prior DNA extraction, preheat the water bath at 65°C.

2.1 Before beginning DNA extraction, prepare Extraction buffer according to sample number (1 sample used 600 µl Extraction buffer).

2.2 Use puncher to punch 3 leaf disks and put in 2 ml tube and add one stainless ball and incubate the tube in -80 °C (preferably overnight / minimum 2 h).

2.3 Remove the tube (s) from –80°C and grind in Mixer Mill for 1 min at 30 Hz.

2.4 Centrifuge at 5500 RPM for 1 min at 25°C in Beckman centrifuge.

2.5 Add 600 µl of extraction buffer to tube and invert the tube several times to re-suspend the ground tissue in the extraction buffer.

2.6 Incubate at 65°C for 1 h. Shake the tube every 15 min.

2.7 Remove tube from water bath and place at 4°C for 30 min.

2.8 Centrifuge at 5500 RPM for 15 min at 25°C in Beckman centrifuge.

2.9 Use pipet to transfer 500 µl of the supernatant to a new 2 ml tube and add 350µl cold isopropanol.

2.10 Let sit for 15 min on bench at room temperature.

2.11 Centrifuge at 5500 RPM for 15 min at 25°C.

2.12 Discard the supernatant by inverting the tube over the Waste Isopropanol /Ethanol. Waste bottle under the fume hood.

2.13 Rinse DNA pellets with 300 µl of 70% ethanol using pipette.

2.14 Discard the supernatant by inverting the tube over the Waste Isopropanol / Ethanol Waste bottle under the fume hood.

2.15 Invert the tube on a Kimwipe to remove as much excess ethanol as possible.

2.16 Let pellets dry under the fume hood for 2 h or at 37°C for 30 min.

2.17 Re-suspend the DNA in 50 µl of TE/RNAse.

2.18 Place the tube at 4°C fridge overnight.

2.19 Shake the tube for 1 min at 500 RPM prior centrifuge for 1 min at 5500 RPM.

2.20 Transfer the DNA into 96-well Greiner ELISA round-bottom plate using pipette.

2.21 Check the DNA quality by loading 2 µl of each sample with 3 µl of 1X loading dye on a 0.8% agarose gel. Once the gel electrophoresis is complete, check the gel photos using gel Imager.

2.22 Measure the DNA concentration using Epoch Biotech.

2.23 Store the plate(s) in the 4°C fridge.

3. PCR Conditions

PCR cocktail

No.	Composition	Volume (µl)
1	PCR Buffer (10X)	1.00
2	MgCl2 (50 mM)	0.50
3	dNTP (2 mM)	1.00
4	Primer F* (10 µM)	0.06
5	Primer R (10 µM)	0.06
6	Taq Invitrogen (5U/mL)	0.10
7	DNA (40 ng/ µL)	1.00
8	H2O	6.28
	Final volume:	**10.00**

PCR Primers for *Tomato yellow leaf curl virus* (TYLCV)

Primer Name	Nucleotide sequence (5' – 3')	Expected band size (bp)
TYLCV-F	ACGCATGCCTCTAATCCAGTGTA	543
TYLCV - R	CAAATAAGGCGTGTAGAC	

PCR Cycle

Steps	PCR conditions
1	94°C – 3 min
2	94°C – 30 s
3	**56°C – 30 s**
4	72°C – 30 s
5	Cycle to steps 2-4 for more 29 times
6	72°C – 10 min
7	10°C - forever

4. Agarose gel

4.1. Dissolve 0.8 g gel agarose in 100 ml of 1X TAE.
4.2. Microwave until agarose gel dissolve completely.
4.3. Let cold down to 65°C and add Ethidium bromide and pour to casting mold.

50X TAE (Tris-acetic acid-EDTA) (pH 8)

Tris	242.0 g
0.5M EDTA pH 8	100 ml
Glacial acetic acid	57.1 g
ddH_2O	1000 ml
Total volume	1000 ml

10X Loading dye for Agarose Gel

Glycerol	20 ml
Bromophenol blue	0.06 g
ddH_2O	40 ml
Total volume	40 ml

Note: place in a sterile falcon tube

IV. AMPLIFICATION OF TARGET BEGOMOVIRUS DNA BY POLYMERASE CHAIN REACTION

For *Pepper yellow leaf curl Indonesia virus* (PepYLCIV)

Dr. Sri Hendrastuti Hidayat
srihendrastuti@apps.ipb.ac.id

1.1. Prepare the PCR reaction mixture.

The amplification reaction consists of the following;

Composition	Concentration	
DNA template		1 µl
Primer Forward	1 µM	1 µl
Primer Reverse	1 µM	1 µl
Reagent Mixture (DreamTaq Green amplification kit, Thermo Scientific, US) or Accu Power PCR Master Mix (BioNeer, Korea)		10 µl
Distilled water		7 µl
Final reaction volume		20 µl

1.2. PCR conditions

Table 1. Primers used to amplify Begomovirus

Primer code	Nucleotide sequence (5' – 3')	PCR product (bp)	Source*
Universal primer			Li *et al*. (2004)
SPG1	CCCCKGTGCGWRAATCCAT	912	
SPG2	ATCCVAAYWTYCAGGGAGCT		
Specific DNA-A			Koeda *et al*. (2017)
PepYLCIV A-F	ACAGCAACTATCAAGAACGATC	468	
PepYLCIV A-R	ATCTGGACTCGTTTACGTCCTC		
Specific DNA-B			Koeda *et al*. (2017)
PepYLCIV B-F	TGTCCTCATCGTAGTCACACA	385	
PepYLCIV B-R	GAAGATAGTCTGTACCGTCAT		

*Li R, Sarbagh S, Hurtt S. 2004. Detection of *Begomoviruses* in sweetpotato by polymerase chain reaction. *Plant Dis*. 88:1347-1351.

Koeda S, Homma K, Tanaka Y, Onizaki D, Kesumawati E, Zakaria S, Kanzaki S. 2017. Inoculation of Capsicums with *Pepper Yellow Leaf Curl Indonesia Virus* by Combining Agroinoculation and Grafting. *J Horticulture Science*.

Universal Primer SPG1/SPG2

Steps	PCR Conditions
1	94°C – 5 min
2	94°C – 1min
3	**50°C – 1 min**
4	72°C – 1 min
5	Cycle to step 2-4 for more 34 times
6	72°C – 1 min
7	10°C - forever

Specific DNA-A PepYLCIV A-F/PepYLCIV A-R

Steps	PCR Conditions
1	94°C – 5 min
2	94°C – 1min
3	**63°C – 1 min**
4	72°C – 1 min
5	Cycle to step 2-4 for more 34 times
6	72°C – 1 min
7	10°C - forever

Specific DNA-B PepYLCIV-B F/PepYLCIV B-R

Steps	
1	94°C – 5 min
2	94°C – 1min
3	**58°C – 1 min**
4	72°C – 1 min
5	Cycle to step 2-4 for more 34 times
6	72°C – 1 min
7	10°C - forever

1.3. Visualize the amplified DNA product using electrophoresis on 1% agarose gel.

V. DETECTION OF BEGOMOVIRUS FROM WHITEFLY, *BEMISIA TABACI*

Hi-Yield Genomics DNA Mini Kit (Tissue)

1. Tissue Dissociation

1.1. Anaesthetize 10 whiteflies using 70% Ethanol and blot dry in tissue paper.
1.2. Transfer the whiteflies to a 1.5 ml microcentrifuge tube.
1.3. Use the provided micropestle to grind the tissue to a pulp.
1.4. Add 200 µl of GT BUFFER to the tube and continue to homogenize the sample tissue by grinding.

2. Lysis

2.1. Add 20 µl of Proteinase K to the sample mixture and mix vortex.
2.2. Incubate at 60°C for 30 min to lyse the sample. During incubation, invert the tube every 5 min.
2.3. Add 200 µl of GBT BUFFER and mix by vortex for 5 sec.
2.4. Incubate 70°C for 20 min or until the sample lysate is clear.

(During incubation, invert the tube every 5 min. this time, preheat the required Elution Buffer (100 µl per sample) to 70°C (for step 5 DNA elution)

2.5. Centrifuge for 2 min at full speed and transfer the supernatant to new 1.5 ml microcentrifuge tube.

3. DNA Binding

3.1. Add 200 µl of absolute ethanol to the sample lysate and vortex immediately for 10 sec. If precipitate appears, break it up by pipetting.
3.2. Place a GT Columm in a 2 ml Collection Tube.
3.3. Transfer all of the mixture (including any precipitate) to the GT Column.
3.4. Centrifuge at full speed for 2 min.
3.5. Discard the 2 ml Collection Tube containing the flow-through and transfer the GT Column to a new 2ml Collection Tube.

4. Wash

4.1. Add 400 µl of W1 Buffer to the GT Column.
4.2. Centrifuge at the full speed for 30 sec.
4.3. Discard the flow-through and place the GT Column back in the 2 ml Collection Tube.
4.4. Add 600 µl of Wash Buffer to the GT Column.
4.5. Centrifuge at the full speed for 30 sec.
4.6. Discard the flow-through and place the GT Column back in the 2 ml Collection Tube.

4.7. Centrifuge again for 3 min at full speed to dry the column matrix.

5. DNA Elution

5.1. Transfer the dried GT Column to a clean 1.5 ml microcentrifuge tube.
5.2. Add 100 µl of preheated Elution Buffer to the center of the center of column matrix.
5.3. Let stand for 5 min or until the Elution Buffer is absorbed by the matrix.
5.4. Centrifuge a full speed for 30 sec to elute the purified DNA.

Proceed to DNA amplification of the target virus genome using PCR assay protocol.

VI. BEGOMOVIRUS TRANSMISSION USING INSECT VECTOR

Begomovirus transmission using whiteflies (Polston et al., 2013)

1. Whitefly colony maintenance

1.1 *Environmental condition*. Whiteflies should be maintained in controlled growth room for optimal colony growth. A temperature of 28ºC, 30-50% RH, and 14 hr photoperoid will yield a colony that develops from egg to adult emergence in 18 days. RH should be kept below 70% to discourage insect and fungal growth. Fertilizer rates and watering must also be reduced to discouraged fungal growth and salt accumulation. Light intensity for the colony should be fairly high. Cleanliness is essential to maintain optimal rearing conditions.

1.2. *Colony cages.* Whiteflies must be maintained on plants in cages, rather than free in the growth room. Cages can be constructed using different materials but must allow for ventilation, ease of access, ability to prevent whitefly escape/infiltration, and of sufficient size to maintain enough plants to generate the whitefly population needed.

1.3. *Colony plant preparation.* The species of plant selected should be able to support a high whitefly population without collapsing. Plant chosen for non-viruliferous colony should be non-host for the virus of interest while a host of the virus should be selected in maintaining a viruliferous colony. Selected plants should grow fairly quickly to support emergence of new adults within 18 days of being introduced to the colony but not so quickly as to outgrow the cages within four weeks. Dwarf, bush, or patio-type plant cultivars are recommended as they often produce a similar leaf area but not without stem elongation.

1.4. It is very important to rear plants in cages that exclude whiteflies and other insects. Infested plants with whiteflies, thrips, or mites before they enter the colony can cause the colony to collapse and have the potential to be infected with insect borne viruses which will interfere with virus transmission studies.

2. Whitefly colony establishment

2.2. Start the colony for the first time using clean whiteflies (free of plant viruses, other insects, and insect pathogens). These can be obtained from collaborators or from the field. If field collected, whiteflies should be reared for at least 8 weeks on non-host plants of the plant virus, and check for absence of plant symptoms to be sure they are free of plant viruses.

2.3. Week 1. Introduce whiteflies to the first cage of plants by aspiration of known numbers or by gently shaking whiteflies from another source plant

depending on the demands on the colony. During week 1, whiteflies will lay eggs on the underside of plant leaves. An approximate population size can be predicted for each emergence based on the number of adults used to lay eggs and the average number of eggs laid by the female whitefly on the host plant.

2.4. Week 2. Begin another cage by introducing whiteflies to new host plant to lay eggs. The plants in the cage started in week 1 will have eggs, and some immature whiteflies covering the undersides of the leaves. Some adult whiteflies introduced during week 1 can still be alive.

2.5. Week 3. Begin a third cage by introducing whiteflies to new plant to lay eggs. The plants in the cage started in week 1 will have immature as well as many new adult whiteflies emerging and there should be a very noticeable increase in the adult whitefly population. The plants in the cage started in week 2 will have many eggs and immature whiteflies covering the underside of the leaves.

2.6. Week 4. Begin a fourth cage by introducing whiteflies to new plants to lay eggs. The plants in the cage started in week 1 will have many adult whiteflies that are approximately one -week post emergence. The plants in the cage started at week 2 will have many new adult whiteflies emerging and there should be very noticeable increase in whitefly population. The plants in the cage started in week 3 will have many eggs and immature whiteflies covering the underside of the leaves.

2.7. Week after the first 4 weeks: Each week, start a new cage by introducing the adult whiteflies from the fourth oldest cage (the cage started in week 1) onto new plants in a new, clean cage. The old cage should be removed, and its plant discarded. These whiteflies are approximately 1 week – post emergence. In the case of viruliferous colony, these whiteflies will transmit virus to the new plants as well as lay eggs for the next generation of whiteflies.

2.8. Plan transmission experiments to use the whiteflies that will emerge in the third week's cage. If more whiteflies are need, plant numbers can be increased, and more whiteflies can be added to the week 1 cage to increased whitefly population.

3. Method for Inoculation of test plants

3.2. To ensure high transmission rates:

3.2.1. Whiteflies must be handled as gently as possible to prevent damage to the insect which will reduce transmission rates.

3.2.2. There must be adequate leaf area available for whiteflies to either probe or feed. An increase in acquisition time or increase in the number of acquisition host plants may increase low rates of infectivity in test plants due to crowding in either the acquisition or inoculation access period.

3.3. Collection of whiteflies. To move large number of whiteflies, as is needed for resistance screening, it is only necessary to gently shake the whitefly infested plants over the test plants. Be sure to shake the plants over many locations to minimize aggregation effects.

3.4. Newly emerged adult whiteflies (1-3 days post emergence) are highly active and feed often so tend to give the highest transmission rates. Older whiteflies still transmit but at a lower frequency. Multiple whiteflies per test plant (15-40 per plant) should be used for high rates of transmission since a ratio of 1 whitefly per plant often results in unacceptably low transmission rates. The number needed depend upon the virus and species of the acquisition and test plants.

3.5. Assemble aspiration devices and collection vials.

3.6. With one hand, hold a yellow plastic card inside the colony cage that contains the whiteflies to be collected. Gently tap the plants to encourage the adult whiteflies to fly. Whiteflies will be attracted to the yellow card and will fly from the plant to the card where they can be collected using an aspirator and a very gentle breath. Never aspirate whiteflies feeding on plants as their stylets are still embedded while feeding that may result in the breakage of their stylet rendering them unable to acquire or transmit the virus.

3.7. Collect about 20 adult whiteflies into a single collection vial to minimize physical damage to the insects from repeated aspirations.

3.8. To change the collection vial, gently tap the vial on hard surface and cap with parafilm while the whiteflies are disoriented from the tapping.

3.9. Put a new collect vial on the aspirator and repeat until the number of whiteflies needed are collected. Whiteflies can remain in the collection vials at room temperature for several hours.

3.10. *Acquisition.* Whiteflies are placed on a virus infected plant and allowed to feed on the infected plants for 48 – 72 hr. Acquisition periods longer 72 hr generally do not increase transmission rates. For those viruses transmitted in a non-persistent or semipersistent manner, 1 hr and several hours, respectively, are sufficient acquisition access periods. Best results are obtained when whiteflies are given free access to plants in cages that confine the insects to a single plant or multiple plants.

3.11. *Inoculation*. Prepare a cage appropriate for the size of the inoculation and place the test plants inside. If only one plant is to be inoculated, consider a single plant cage. If more than one, consider a small PVC frame bag cage or aluminum cage. The size of the cage used for inoculation should be slightly larger than the size of the plant (s) to be inoculated. Higher transmission rates are obtained when whiteflies are kept close to the plant canopy and not given a lot of free space.

3.12. Collection of whiteflies from acquisition hosts depends on the needs of the experiment. If small numbers are needed, aspirate whiteflies as described above. Place the collection vials of whiteflies inside the cage near the plants and remove the lid. Open the vial and release the whiteflies or invert and gently tap the vial to release the whiteflies. If large numbers are needed, simply move the acquisition plants into the cages and gently shake the whiteflies off the acquisition host plant. For both types on introduction, be sure to distribute the whiteflies across the plants to minimize aggregation and ensure uniform inoculation.

3.13. Allow the plants to probe or feed for the appropriate amount of time.

3.14. Check whiteflies at least once during inoculation access period to ensure that whiteflies are probing or feeding by opening the cage and turning over some leaves of each plant.

3.15. For longer inoculation access periods, disturb the plants gently (bamboo stick or equivalent) to brush the top of the plants and encourage the whiteflies to redistribute on the plants. This helps to ensure higher transmission rate by countering the natural tendency of whiteflies to aggregate.

3.16. Termination. Inoculation access period is ended by killing the whiteflies with approved chemicals. Apply two insecticides one after the other. A contact insecticide to quickly terminate adult whiteflies and a systemic insecticide to terminate any whiteflies that develop in the following weeks and those missed by the contact insecticide.

Reference:
Polston JE, Capobianco H. 2013. Transmitting plant viruses using whiteflies. J. Vis. Exp. (81), e4332, doi:10.3791/4332.

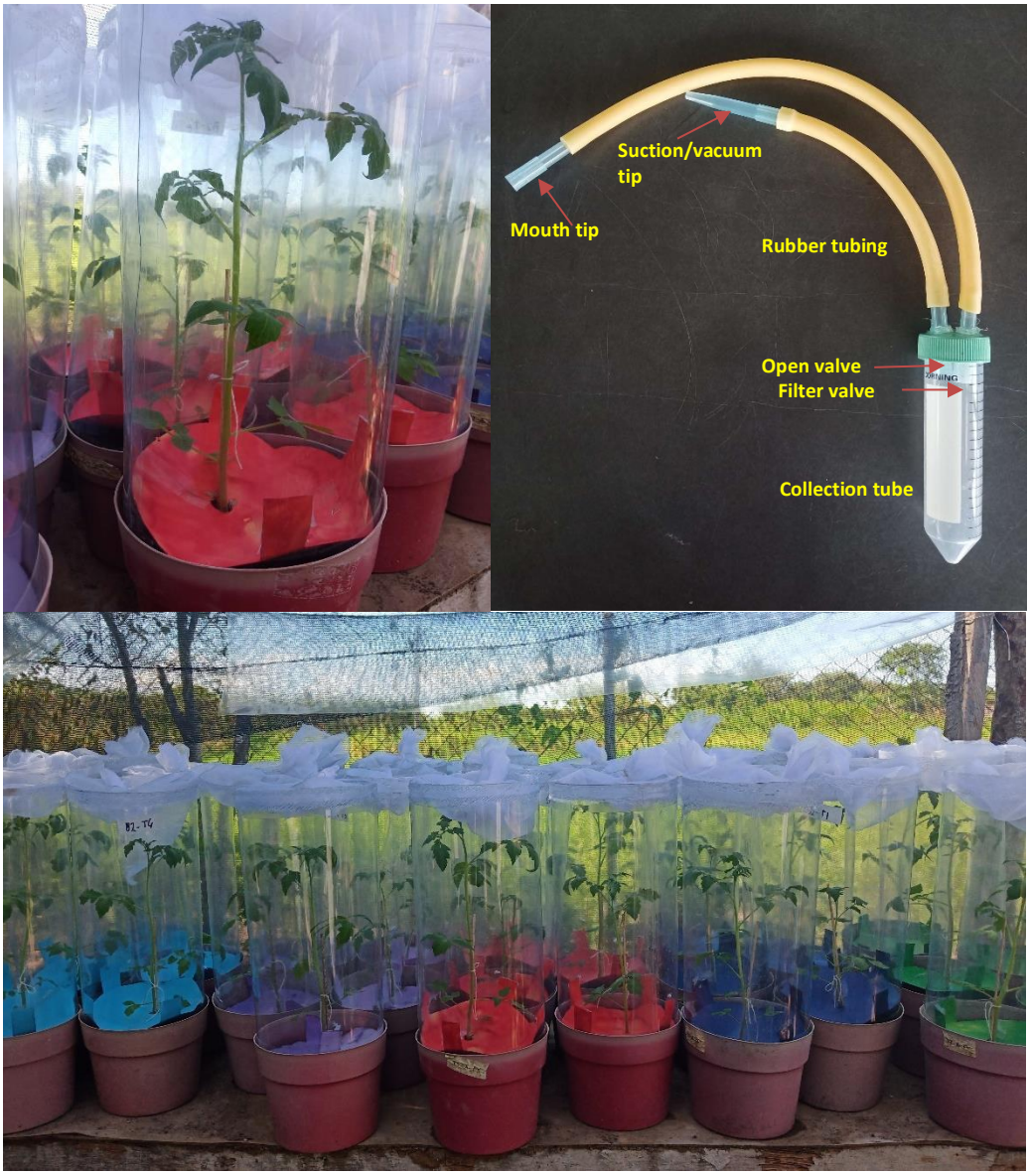
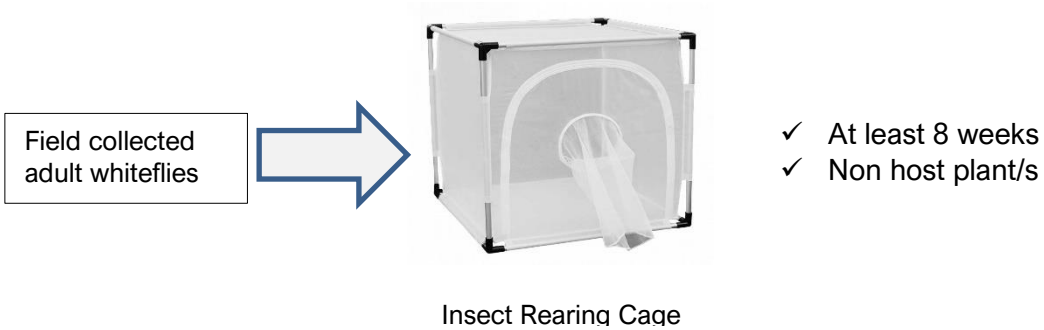
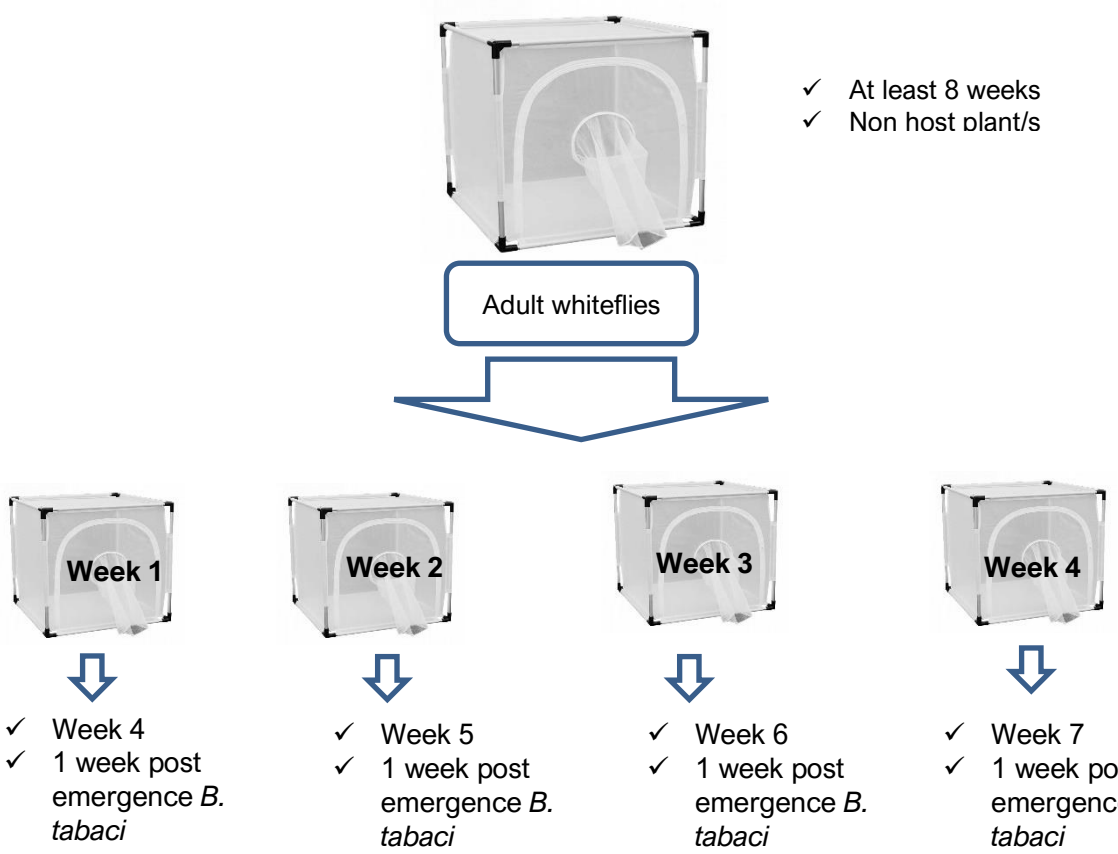


Figure 1. Tomato seedling with colored paper on the base (upper left) inside a Mylar cage to serve as host for whitefly population. Improvised suction device for collecting and inoculating whiteflies (upper right). Treated tomato seedlings under screenhouse condition (lower).

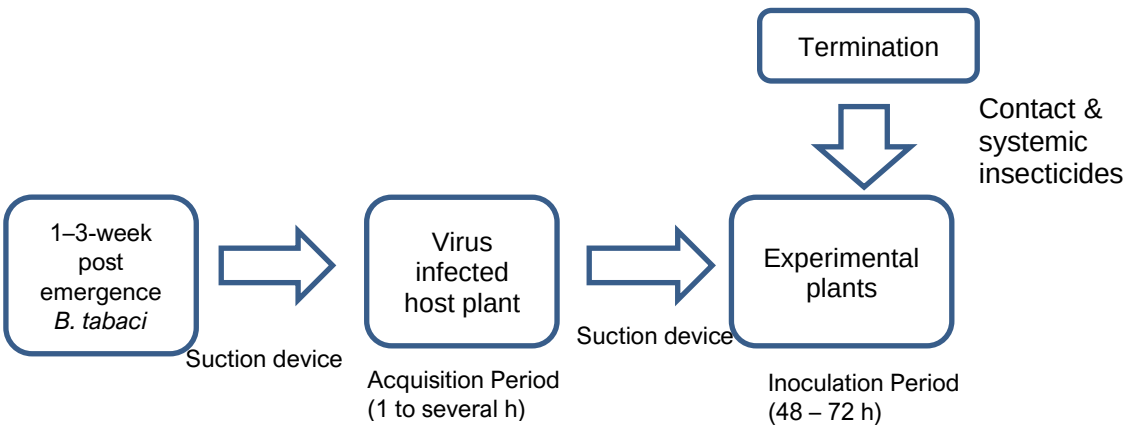
B. tabaci Maintenance



B. tabaci colony establishment



Method of inoculation



VII. LAMP – MEDIATED DETECTION OF GEMINIVIRUSES

Masashi Ugaki, The University of Tokyo
ugaki@k.u-tokyo.ac.jp

These protocols were presented at the "Training Workshop on Begomovirus: Detection, Identification and Etiology and Development of Loop-Mediated Isothermal Amplification (LAMP) Kit as a Diagnostic Tool" held from September 18 to 30, 2022.

<Preface>

The rapid, sensitive, and reliable diagnosis of plant pathogens is crucial in controlling plant diseases. Owing to their ability to amplify signals, nucleic acid amplification techniques, including polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP), are generally more sensitive than serological techniques in detecting pathogens. In addition, nucleic acid amplification techniques may identify pathogen species using species-specific primer sets.

The most widely used of these techniques is the PCR, which exponentially amplifies a defined segment of target nucleic acids using a set of two primers and heat-stable DNA polymerase. However, the PCR has some limitations, in that its reaction temperatures should be repeatedly shifted, and thus, it requires an (expensive) thermal cycler and takes a long reaction time. Another technique that overcomes this weakness is LAMP, in which the reaction temperature is constant (isothermal) at 60°C–65°C. LAMP can be performed in an (inexpensive) heating block, an air incubator, or even in hot water in a thermos flask. In this workshop, we will perform LAMP reactions by mixing commercially available reagents – the most inexpensive approach to LAMP. This procedure is given in "PROTOCOL 1" below.

This protocol is efficient, provided we perform LAMP in the laboratory. However, when we want to perform the reaction outdoors in agricultural fields, the protocol has one serious drawback: most reagents used, especially DNA polymerase, should be kept at −20°C until use. However, we cannot carry a freezer to the field. To overcome this problem, we will perform another LAMP procedure in this workshop, where a commercial "dried LAMP kit" is used. The kit requires only pure water and primer solution, and all other reagents are premixed and dried beforehand. The kit can be stored or carried at room temperature for months, and no freezer is necessary. The protocol is also given below as "PROTOCOL 2."

Finally, the only remaining problem is that the commercial dried LAMP kit used in the protocol above is (very) expensive. Although this commercial kit's composition and preparation procedure are not publicized, we, the research group at the University of Tokyo, have tried to construct our own homemade "dried" LAMP kit. The current best protocol is given in "PROTOCOL 3" below, which allows making of far less expensive kit. We will NOT construct this dried kit during this workshop since it requires more reagents, high-performance machines (e.g., freeze dryer), and a long preparation time.

Anybody can use this protocol without prior permission or restrictions, so please try it later if you like.

<Notes>

- The protocols given in this workshop can be used by anyone without prior permission or restrictions. However, when you construct LAMP “kits,” you cannot “sell” them since the LAMP technique itself is subject to patents issued for Eiken Chemical Company, Japan.

- Our activity for construction of homemade dried LAMP kit has been supported by an international collaborative program Science and Technology Research Partnership for Sustainable Development (SATREPS) sponsored by the Japanese Government, under the project titled “Development and Dissemination of Sustainable Production System Based on Invasive Pest Management of Cassava in Vietnam, Cambodia, and Thailand” (2017–2022).

- For any questions or comments, please get in touch with Masashi Ugaki (ugaki@k.u-tokyo.ac.jp). If you find improvements to these protocols, we will be happy to hear them.

<Technical Comments>

(i) These protocols use six LAMP primers designed to detect Sri Lankan cassava mosaic virus (SLCMV), a begomovirus, which causes cassava mosaic diseases. To detect other pathogens, specific LAMP primers should be designed using one of the following websites:

PrimerExplorer
https://primerexplorer.jp/e/index.html

NEB LAMP Primer Design Tool
https://lamp.neb.com/#!/

(ii) LAMP use six primers (Fig. 1). Four of them, FIP, BIP, F3 and B3 primers are essential and called “core” primers. Whereas the other two, LF and LB are “loop” primers that accelerate LAMP amplification.

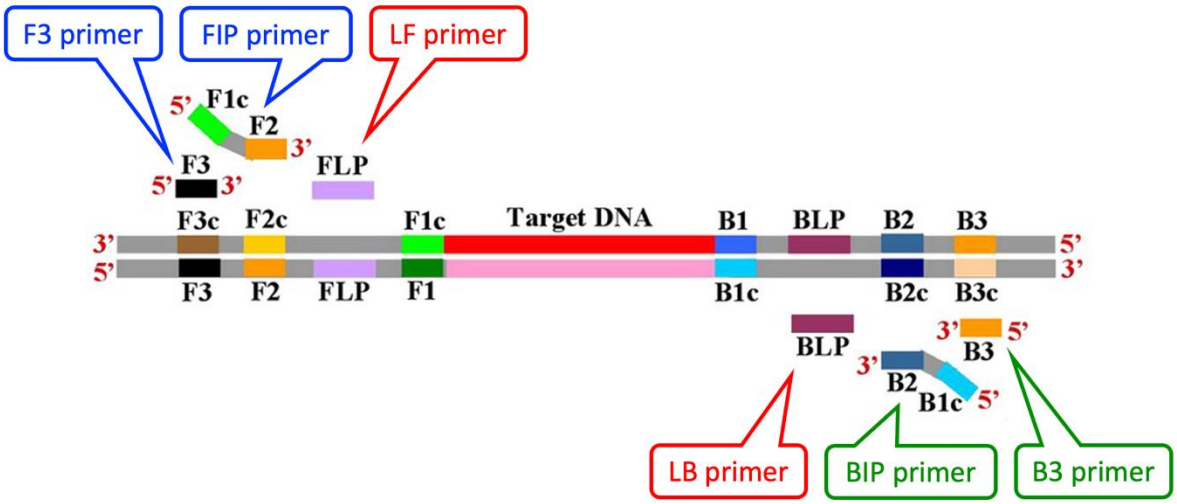


Figure 1. Six primers used for LAMP amplification. The region between 5´ end of F2 and 3´ end of B2c will be amplified.

Most protocols utilize pre-mixture of all six primers, but the following protocols utilize two separate pre-mixtures of four (core primers) and two (loop primers). This enables us to fine-tune amplification efficiency. At the first trial, add the same volume of core primer mix and loop primer mix. If the amplification is too much, reduce the volume of loop mixture mix; if the amplification is not enough, increase the volume of loop primer mix.

(iii) LAMP is more sensitive than the PCR. Accordingly, “false positives” or positive results from negative samples without any target DNA can easily occur without sufficient attention to detail. The following countermeasures should be taken to avoid the occurrence of false positives:

- Always include at least two negative control templates, specifically pure water as a template and a healthy plant sample, to detect false positives.

- Adding too much DNA template into LAMP reactions can result in the nonspecific annealing of primers to nontarget sequences, resulting in false–positive amplification. This also suggests that only an extremely small amount of DNA is sufficient for LAMP; sufficient DNA can be isolated by breaking symptomatic plant tissues with the tip of a wooden toothpick and washing the tip in the LAMP reaction mixture.

- Wear gloves while handling reagents.

- Use “filtered” pipette tips whenever possible. The most common cause of false positives is the contamination of “the inside of the pipettes” with the target DNA. When a target-positive liquid sample is aspirated using a pipette, negative air pressure is created between the pipette piston and the liquid sample. This pressure creates “aerosols” or tiny invisible drops of liquid from the liquid surface. The aerosols containing the target DNA are aspirated into the pipette shaft, which will contaminate it. The filtered tips effectively prevent this type of contamination.

- I recommend not to open reaction tubes after completion, since pipetting the LAMP product is the most common source of pipette contamination. If you include a chromogenic or fluorogenic dye in the LAMP reaction, positive or negative results after the reaction can be judged by the naked eye under white light or UV light without opening the tube.

- When you want to double-check your positive or negative results, you can judge the results also by running the LAMP product in agarose gel electrophoresis for observing the ladder-like multimeric amplification products that appear when positive.

- If you want to prepare homemade LAMP kits for your constant use, they should be prepared in a separate room from the room where positive controls are made, plant samples are handled, and the kit is used for detection. Separate sets of pipettes should be used in these two rooms.

< PROTOCOL 1 – Standard (wet) LAMP (for SLCMV Detection) >

This protocol is supposed to be followed in laboratories.

Organize the following materials:
Capped microcentrifuge tubes (0.2 mL)
Pipettes
Pipette tips (preferably "filtered" ones for they effectively prevent false-positive amplifications in LAMP)
Microcentrifuge
Thermal cycler, heating block, air incubator, or heating water bath

Prepare the following reagents:
Pure water
TE buffer (10 mM tris-HCl, pH 8, 1 mM EDTA)
Bst 2.0 DNA polymerase (New England Biolabs; or similar DNA polymerase with strand-displacement activity, stored at −20°C)
10× Isothermal Amplification Buffer (New England Biolabs; this will be included with the enzyme, stored at −20°C)
100 mM $MgSO_4$ (this might be included with the enzyme; stored at −20°C)
10 mM dNTP mix (it is better to use a 10 mM stock instead of a 2.5 mM stock, stored at −20°C)
25 mM $MnSO_4$ (stored at −20°C)
1.25 mM calcein (Sigma C0875 or equivalent; stored at −20°C)
100 µM (100 pmol/µL) of the following six primers (for SLCMV detection; stored at −20°C):

Primer name:	Sequence
SLCMV-F3:	5´-CCGCG TCACA AAAGA CAAG-3´
SLCMV-B3:	5´-TCTTA CCCAC GCGAT GAGT-3´
SLCMV-FIP:	5´-TCCCC TAGGA ACATCT GGGCT TGGAC AAACA GGCCC ATGAA-3´
SLCMV-BIP:	5´-GAGTC CAGAC ACGAT GTGGT CCGCC CAACT CCACG AGTGA-3´
SLCMV-LF:	5´-GTACC ACCTG GGCTT CCGA-3´
SLCMV-LB:	5´-ATAGG TAAGG TCATG TGCAT CTCT-3

Positive control DNA samples (for SLCMV detection)
either:
SLCMV-infected plant leaves,
3-10 pg/µL total DNA extracted from SLCMV-infected plant leaves, or
0.1 pg/µL of an infectious clone of SLCMV DNA-A
Negative control DNA samples
(i) either:
healthy plant leaves, or
DNA extracted from healthy plant leaves
(ii) either pure water or TE without any DNA

Procedure:

1. Prepare 50 µL (for 50 reactions) of 25× LAMP core primer mix by mixing the reagents as follows:
   5 µL of pure water
   20 µL of 100 µM primer SLCMV-FIP (= 40 µM; final concentration 1.6 µM)
   20 µL of 100 µM primer SLCMV-BIP (= 40 µM; final concentration 1.6 µM)
   2.5 µL of 100 µM primer SLCMV-F3 (= 5 µM; final concentration 0.2 µM)
   2.5 µL of 100 µM primer SLCMV-B3 (= 5 µM; final concentration 0.2 µM)

2. Prepare 50 µL (for 50 reactions) of 25× LAMP loop primer mix by mixing the reagents as follows:
   40 µL of pure water
   5 µL of 100 µM primer SLCMV-LF (= 10 µM; final concentration 0.4 µM)
   5 µL of 100 µM primer SLCMV-LB (= 10 µM; final concentration 0.4 µM)

3. Add the following in a 0.2 mL tube in this order:

Pure water	10.5 µL
10× Isothermal amplification buffer	2.5 µL (final 1×)
100 mM $MgSO_4$	1.5 µL (final 6 mM)
25 mM $MnSO_4$	1.0 µL (final 1 mM)
1.25 mM calcein	1.0 µL (final 0.05 mM)
10 mM dNTP mix	3.5 µL (final 1.4 mM)
25× LAMP core primer mix	1.0 µL (final 1×)
25× LAMP loop primer mix	1.0 µL (final 1×)

   Mix well by tapping the tube and centrifuge it briefly.

4. Add 1 µL of 8 units/µL Bst 2.0 DNA polymerase (final 8 units per reaction).
   Mix well by tapping the tube and centrifuge it briefly.

5. Add positive or negative DNA controls as follows.

5-1 (Use of plant materials)
   Insert the tip of a wooden toothpick into the vein of a diseased (positive) or a healthy (negative) plant slightly breaking the vein tissue but not passing through the leaf. Wash the toothpick in the reaction mixture.

5-2 (Use of DNA extracted from plants)
   Add 2 µL of TE (total 25 µL) containing 3–10 pg of plant total DNA extracted from a diseased (positive) or a healthy (negative) plant.

5-3 (Use of infectious clone of SLCMV DNA-A)
   Add 2 µL of 0.1 pg/µL of an infectious clone of SLCMV DNA-A (final concentration, 0.2 pg/reaction) (positive) or 2 µL of pure water or TE (negative).
   Mix well by tapping the tube and centrifuge it briefly.

6. Incubate the tube at 65°C for 45–60 min.

7. Determine whether the target sequence was amplified (positive for the target) or not (negative for the target).

7-1 Under white light, a negative sample is transparent and slightly orange (Fig. 2, upper right), and a positive sample is turbid and white green (Fig. 2, upper left).

More clearly, only a positive sample gives fluorescence under UV light (365 nm) (Fig. 2, lower left).
The tube should be observed immediately and photographed for record. Otherwise, the negative reactions in the tubes will slowly turn positive, as the DNA polymerase is still active. To maintain the results for a longer time, the enzyme should be inactivated by heating the tubes at 80°C for 5 min or 95°C for 2 min.

7-2 Amplification products can be observed also by running the reaction mixture in an agarose gel electrophoresis. Unlike PCR which produces a single-sized band at the presence of the target, LAMP gives multiple-sized, ladder-like bands (Fig. 3). However, it is generally recommended not to analyze the results by electrophoresis, since pipetting the products can be a source of contamination of the pipette.

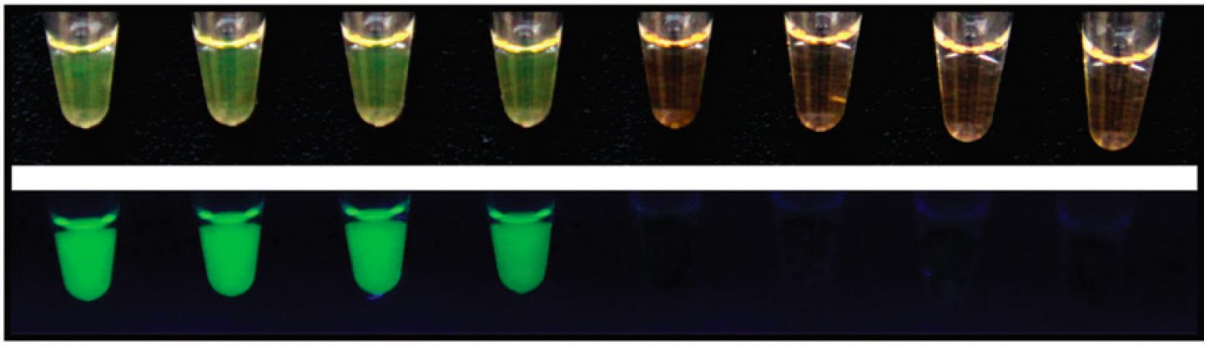


Figure 2. LAMP reaction products analyzed by the naked eye.
Upper panel; Under white light. Left four tubes, positive: a bit turbid and white green. Right four tubes, negative: transparent and slightly orange. Lower panel; Under UV light (365 nm). Left four tubes, positive: with green fluorescence. Right four, negative: without fluorescence.

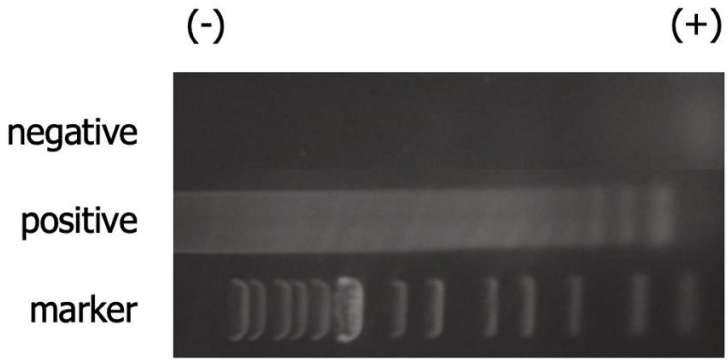


Figure 3. LAMP reaction products analyzed by agarose gel electrophoresis.
Upper lane; Negative for the target with no amplification product. Middle lane; Positive for the target with ladder-like amplification products. Lower lane; Molecular size markers.

< PROTOCOL 2 – LAMP (for SLCMV Detection) Using Commercial Dried LAMP Kit >

This protocol is supposed to be followed outdoors in agricultural fields.

Bring the following materials to the field:
Disposable plastic gloves
Pipettes
Pipette tips (preferably "filtered" ones for they effectively prevent false-positive amplifications in LAMP)
Portable microcentrifuge with an 8-microtube rotor (battery-driven one such as "Multi Spin," TOMY Co., Japan, Fig. 4A)
Wooden toothpicks (sterilized beforehand at 160°C for 2 hr)
Hot water (around 95°C at the departure) kept hot in a thermos bottle
Bottled water
Thermometer
Thermos food flask with a wide opening (Fig. 4B left)
Hand-made styrofoam "float" to hold eight-connected microtubes (Fig. 4B right)
Portable UV lamp (365 nm wavelength)

Bring the following reagents to the field:
Dried LAMP Kit (Loopamp RNA/DNA Amplification Reagent D, Cat No. LMP247, Eiken Chemical Co., Japan) stored at room temperature. This kit contains all reagents required for LAMP except for primers and templates. The reagents are dried and attached inside of the cap (lid) of the reaction tube.
Pure water
Primer mixture (for SLCMV detection) prepared by mixing the following three reagents. Bring the mixture to the field while chilled with ice.
(i) pure water: 13 volume
(ii) 25× core primer mixture: one volume

Concentration and primer name:	Sequence
5 μM SLCMV-F3:	5′-CCGCG TCACA AAAGA CAAG-3′
5 μM SLCMV-B3:	5′-TCTTA CCCAC GCGAT GAGT-3′
40 μM SLCMV-FIP:	5′-TCCCC TAGGA ACATCT GGGCT TGGAC AAACA GGCCC ATGAA-3′
40 μM SLCMV-BIP:	5′-GAGTC CAGAC ACGAT GTGGT CCGCC CAACT CCACG AGTGA-3′

(iii) 25× loop primer mixture: one volume (total 15 vol)

Concentration and primer name:	Sequence
10 μM SLCMV-LF:	5′-GTACC ACCTG GGCTT CCGA-3′
10 μM SLCMV-LB:	5′-ATAGG TAAGG TCATG TGCAT CTCT-3′

Positive control DNA samples (for SLCMV detection)
either:
SLCMV-infected plant leaves,
3–10 pg/μL total DNA extracted from SLCMV-infected plant leaves, or
0.1 pg/μL of an infectious clone of SLCMV DNA-A
Negative control DNA samples
either:

healthy plant leaves, or
3–10 pg/μL total DNA extracted from healthy plant leaves
Mineral oil

Procedure:

1. Ware gloves.

2. Take a necessary number (total number of samples and controls) of the connected tubes of the dried LAMP kit. To cut a bridge between the tubes, use scissors to avoid any impact on the dried reagents that are attached inside of the cap. (Do not tear the tubes.) Restore the remaining reaction tubes to the original aluminum pack and seal it for storage.

3. Dispense 15.0 μL of the primer mixture to the "bottom" of each reaction tube.

4. Add 10.0 μL of pure water to the inside of the "cap (lid)" of the reaction tube where there are dried reagents attached. Slowly pipette the water up and down several times to dissolved the dried reagents, and transfer them all to the bottom of the tube.

6. Add one drop of mineral oil to the first negative control (no template control) tube, and close the cap so as to avoid any subsequent contamination. Keep other caps open.

7. Insert the tip of a toothpick into the vein of a healthy plant, slightly breaking the vein tissue but not passing through the leaf (Fig. 5), and wash the tip in the primer mixture in the bottom of the second negative control (healthy plant control) tube. Add one drop of mineral oil and close the cap of the tube. Change gloves.

8. Similarly insert the tip of toothpicks into the veins of the sample (symptomatic) plants as described above. This is because begomoviruses reside mainly in the phloem tissue. Wash the tip in the reaction mixture of a new tube, add one drop of mineral oil, and close the cap of the tube. Change gloves for each sampling.

9. Lastly add 2 μL of the positive control reagents to the positive control tube, gently tap the tube to mix, add one drop of mineral oil, and close the cap of the tube.

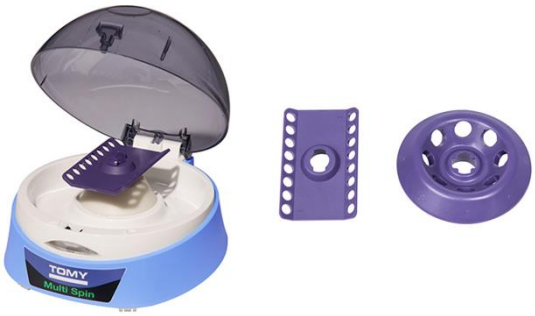
10. Mix hot water and bottled water in the thermos food flask, and adjust the temperature to 65°C–67°C.

11. Insert the tubes into a float and put them into the hot water in the thermos food flask. Close the lid and leave them for 45 min–60 min. LAMP reaction can proceed at 65°C–60°C.

12. Observe the sample (Fig. 6). Under white light, a negative sample is transparent and slightly orange, and a positive sample is turbid and white green. More clearly, only a positive sample gives fluorescence under UV light (365 nm). When in the field, a portable UV lamp may be used. The tube should be observed immediately

and photographed for record. Otherwise, the negative reactions in the tubes will slowly turn positive, as the DNA polymerase is still active.

A



B

Figure 4. Some useful equipment in an open field.
A. An example of a portable, battery-driven microcentrifuge and its rotors; Multi Spin, TOMY Co., Japan. B. An example of a double-skin, vacuum thermos food flask with a wide opening (left) and a handmade polystyrene foam float holding 8-connected microtubes (right).

Figure 5. Sampling of begomoviruses from a vein of a healthy control (left) or a diseased symptomatic (right) cassava leaves by using the tip of a sterilized wooden toothpick.

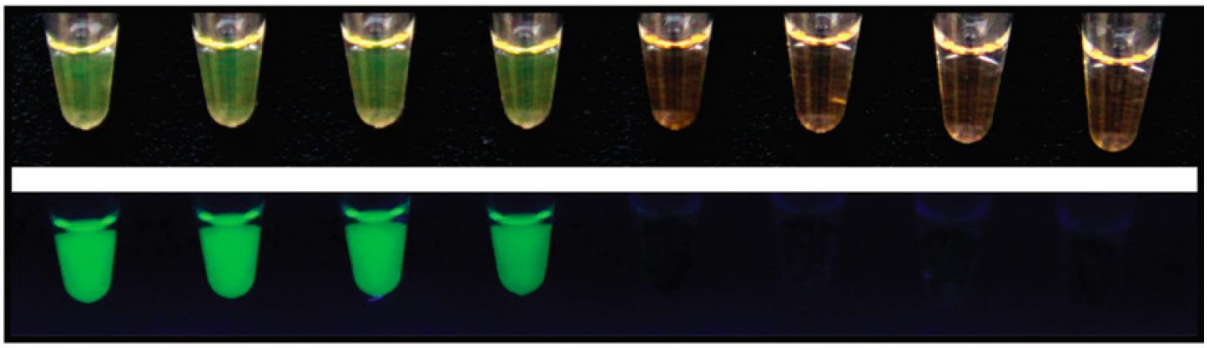


Figure 6 (same as Figure 2). LAMP reaction products analyzed by naked eyes.
(Upper panel) Under white light. Left four tubes, positive: a bit turbid and white green. Right four tubes, negative: transparent and slightly orange.
(Lower panel) Under UV light (365 nm). Left four tubes, positive: with green fluorescence. Right four, negative: without fluorescence.

<The Modified Version of Protocol 2 that we followed in the "Training Workshop on Begomovirus" held from September 18 to 30, 2022>

Protocol 2 above using the commercial dried LAMP kit is intended to be followed in the field, but in the "Workshop" we performed LAMP in a laboratory. Therefore there were some modifications. To avoid confusion, I would like to list the differences between Protocol 2 and what we did in the "Workshop" using the dried LAMP kit.

- Primers
(Protocol 2)
Adding 15 µL of premixed "Primer mixture" prepared by mixing the following three reagents.
(i) pure water: 13 volume
(ii) 25× core primer mixture: one volume
(iii) 25× loop primer mixture: one volume

(Workshop)
Adding 13 µL of pure water
Adding 1 µL of 25× core primer mixture
Adding 1 µL of 25× loop primer mixture

*Concentrations were the same.

- Mineral oil
(Protocol 2)
Use mineral oil to avoid evaporation.

(Workshop)
We did not use mineral oil, since we used a thermal cycler which prevents evaporation.

-Detection
(Protocol 2)
Detection by naked eyes for turbidity and by UV for fluorescence.

(Workshop)
Detection by naked eyed for turbidity, by UV for fluorescence and by electrophoresis for typical amplification products.

<PROTOCOL 3 - LAMP (for SLCMV Detection) Using Homemade Dried LAMP Kit>

This protocol covers construction of "homemade" dried LAMP kit in a laboratory and use of it outdoors in agricultural fields.
Before constructing the dried LAMP kit, you should run normal (wet) LAMP by using only positive and negative controls as described in PROTOCOL 1, so that you can check your reagents and primers are fine.

<<Construction of homemade dried LAMP kit>>

Prepare the following apparatuses:
Capped microcentrifuge tubes (1.5 mL and 8-connected 0.2 mL tubes)
Pipettes
Tips (preferably "filtered" ones for they effectively prevent false-positive amplifications in LAMP)
Microcentrifuge
Freeze-drying apparatus (Centrifugal vacuum concentrator equipped with a cold trap and driven by a vacuum pump, Fig. 6)
Sealable, moisture-free, light-shielded aluminum bag

Prepare the following reagents:
Pure water
TE buffer (10 mM tris-HCl, pH 8, 1 mM EDTA)
Bst 2.0 DNA polymerase (New England Biolabs; or similar DNA polymerase with strand-displacement activity, stored at −20°C)
10× Isothermal Amplification Buffer (New England Biolabs; this will be included with the enzyme, stored at −20°C)
100 mM $MgSO_4$ (this might be included with the enzyme, stored at −20°C)
10 mM dNTP mix (it is better to use a 10 mM stock instead of a 2.5 mM stock, stored at −20°C)
25 mM $MnSO_4$ (stored at −20°C)
1.25 mM calcein (Sigma C0875 or equivalent; stored at −20°C)
100 µM (100 pmol/µL) of the following six primers (for SLCMV detection; stored at −20°C):

Primer name:	Sequence
SLCMV-F3:	5′-CCGCG TCACA AAAGA CAAG-3′
SLCMV-B3:	5′-TCTTA CCCAC GCGAT GAGT-3′
SLCMV-FIP:	5′-TCCCC TAGGA ACATCT GGGCT TGGAC AAACA GGCCC ATGAA-3′
SLCMV-BIP:	5′-GAGTC CAGAC ACGAT GTGGT CCGCC CAACT CCACG AGTGA-3′
SLCMV-LF:	5′-GTACC ACCTG GGCTT CCGA-3′
SLCMV-LB:	5′-ATAGG TAAGG TCATG TGCAT CTCT-3′

Positive control DNA samples (for SLCMV detection)
either:
SLCMV-infected plant leaves,
3-10 pg/µL DNA extracted from SLCMV-infected plant leaves, or

0.1 pg/μL of an infectious clone of SLCMV DNA-A
Negative control DNA samples
(i) either:
healthy plant leaves, or
DNA extracted from healthy plant leaves
(ii) either pure water or TE without any DNA
500 mg/mL trehalose dihydrate
Liquid nitrogen

Procedure:

1. Prepare 50 μL (for 50 reactions) of 25× LAMP core primer mix by mixing the reagents as follows:
   5 μL of pure water
   20 μL of 100 μM primer SLCMV-FIP (= 40 μM; final concentration 1.6 μM)
   20 μL of 100 μM primer SLCMV-BIP (= 40 μM; final concentration 1.6 μM)
   2.5 μL of 100 μM primer SLCMV-F3 (= 5 μM; final concentration 0.2 μM)
   2.5 μL of 100 μM primer SLCMV-B3 (= 5 μM; final concentration 0.2 μM)

2. Prepare 50 μL (for 50 reactions) of 25× LAMP loop primer mix by mixing the reagents as follows:
   40 μL of pure water
   5 μL of 100 μM primer SLCMV-LF (= 10 μM; final concentration 0.4 μM)
   5 μL of 100 μM primer SLCMV-LB (= 10 μM; final concentration 0.4 μM)

3. Add the following (for eight tubes) in a 1.5 mL tube in this order:

Pure water	60 μL
10× Isothermal amplification buffer	20 μL (final 1×)
100 mM $MgSO_4$	12 μL (final 6 mM)
25 mM $MnSO_4$	8 μl (final 1 mM)
1.25 mM calcein	8 μL (final 0.05 mM)
10 mM dNTP mix	28 μL (final 1.4 mM)
25× LAMP core primer mix	8 μL (final 1×)
25× LAMP loop primer mix	8 μL (final 1×)

   Mix well by tapping the tube and centrifuge it briefly.

4. Add 8 μL of 8 units/μL Bst 2.0 DNA polymerase (final 8 units per reaction).
   Mix well by tapping (not by vortexing for the enzyme is fragile) the tube and centrifuge it briefly.

5. Add 40 μL of 500 mg/mL trehalose dihydrate (final 100 mg/mL or 10% (w/v); total, 200 μL).
   Mix well by tapping (not by vortexing) the tube and centrifuge it briefly.

6. Aliquot into eight-connected 0.2-mL tubes by 25 μL. (I often do it by 24.5 μL, for the last tube often becomes short by Murphy's law :-)

7. Quickly freeze the tubes by dipping it in liquid nitrogen (−196°C) for 10 s or more. It is important to freeze the tube very quickly.

8. The frozen tubes should be quickly placed into a precooled freeze-drying apparatus, and the reaction should be freeze-dried under a vacuum until it is completely dry. We spin the tube at a low speed (100 rpm) but not at a high speed, because we do not want to “pack” the precipitate. The time required for complete drying depends on the freeze-drying apparatus, but it may take many hours.

9. After complete drying, the cap should be closed and the tube should be placed in a sealable, moisture-free, light-shielded aluminum bag, heat-sealed, and kept at room temperature. The dried kit can be stored at room temperature or at 4°C for several months or longer. After using some of the reaction tubes, make sure to heat-seal the bag again, for humidity may affect activity of dried DNA polymerase.

Figure 7. An example of centrifugal vacuum concentrator (left) equipped with a cold trap (middle) and driven by a vacuum pump (right).

Notes and comments:

- Difference from the commercial kit:
Please note there are some differences between “commercial” dried LAMP kit in the PROTOCOL 2 and “homemade” one in this protocol.
1) The reagents used, their concentration, or manufacturing procedure should be different between the two, for those of the commercial kit have not been publicized.
2) Commercial kit does not contain primers, and this homemade one does (for easier use).
3) Dried reagents are attached inside of the “cap (lid)” of the commercial kit tube, and they are attached in the “bottom” of this homemade kit tube.

- Why trehalose is added:
In the course of making this dried LAMP protocol, we first “simply dried up” LAMP reagent mixture described in the PROTOCOL 1, then added water to it, and subjected

it to LAMP reaction. But no LAMP amplification occurred. It is generally known that, in the aqueous solution, proteins keep their specific tertiary structure (necessary for their activity) by interaction between their hydrophilic amino acid residues and surrounding water molecules. We therefore believed that the drying of the LAMP mixture removed water molecules associated with the DNA polymerase, a protein, and denatured the protein's tertiary structure irreversibly. Based on this hypothesis, we speculated that adding a “hydroxyl group-rich substance” in the mixture may keep the protein structure intact after drying, since the -OH groups may interact with hydrophilic amino acids in place of the leaving water molecules (Fig. 7). We therefore added various kinds of hydroxy group-rich substances in the LAMP mixture before drying. At long last we finally found that high concentrations of a few non-reducing sugars are effective in keeping the polymerase's activity upon drying and rehydration. Among them, trehalose worked best and thus this protocol includes addition of trehalose.

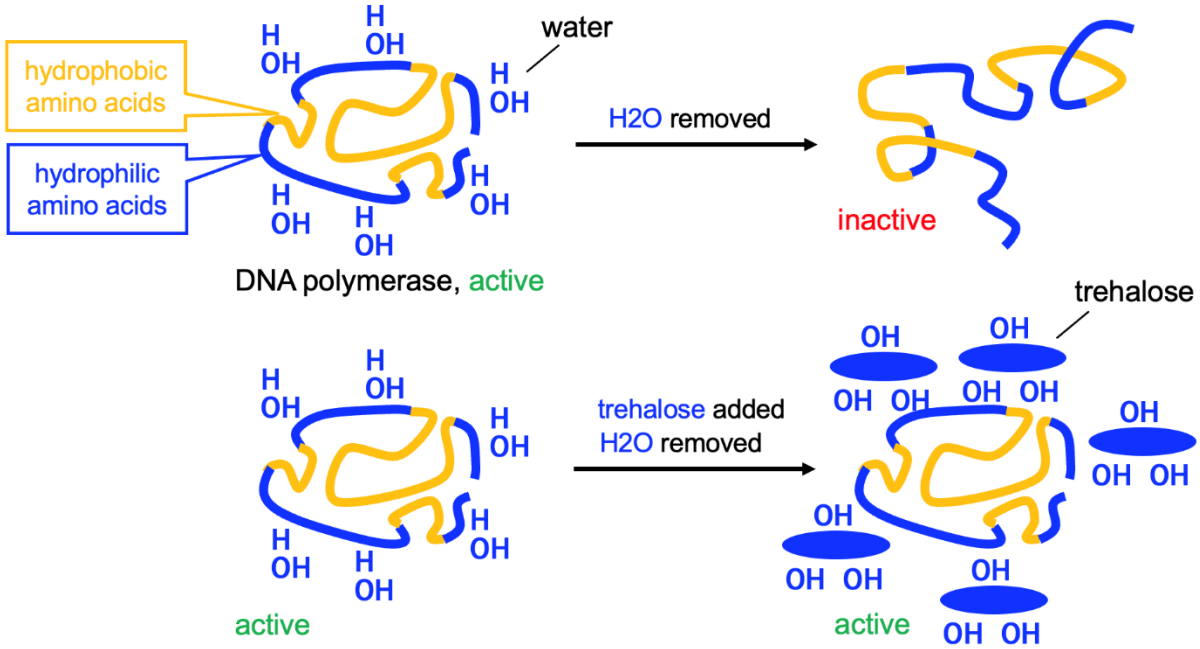


Figure 8. A putative model why trehalose prevents DNA polymerase from inactivation upon drying. The enzyme keeps its specific tertiary structure (and thus its activity) because of the interaction between its hydrophilic amino acids and surrounding water molecules (upper left). When water molecules are removed by drying, the enzyme loses its proper structure (denatures) irreversibly and thus is inactivated (upper right). When trehalose (or certain hydroxy group-rich substances) is added and then water is removed, the OH-groups of trehalose should interact with the enzyme's hydrophilic amino acids (in place of water molecules) and will keep its structure (and activity) intact (lower right).

-Why quick freezing and freeze-drying under vacuum:
In the course of optimizing the protocol, we tried various ways of drying LAMP mixture, and rapid freezing by liquid nitrogen and subsequent freeze-drying under vacuum worked best. When the LAMP mixture was not frozen before drying or it was dried under atmospheric air pressure (not under vacuum), water was lost by way of

“vaporization” and it took a long time later to dissolve the dried mixture with water again. When the mixture was frozen and dried under vacuum, water was lost by way of “sublimation” and it was easy to dissolve the mixture. When the mixture was frozen slowly by placing the tube in a −80°C freezer for 30 min, the resultant kit still worked, but the kit’s sensitivity decreased to some extent.

<PROTOCOL 3 (continued)>

<<Use of homemade dried LAMP kit>>

This part of the protocol is supposed to be followed outdoors in agricultural fields. This looks similar to PROTOCOL 2 above (dried LAMP using a commercial dried LAMP kit), but is slightly simpler compared to PROTOCOL 2.

Bring the following materials to the field:
Disposable plastic gloves
Pipettes
Pipette tips (preferably "filtered" ones for they effectively prevent false-positive amplifications in LAMP)
Portable microcentrifuge with an 8-microtube rotor (battery-driven one such as "Multi Spin," TOMY Co., Japan, Fig. 9A)
Wooden toothpicks (sterilized beforehand at 160°C for 2 hr)
Hot water (around 95°C at the departure) kept hot in a thermos bottle
Bottled water
Thermometer
Thermos food flask with a wide opening (Fig. 9B left)
Hand-made styrofoam "float" to hold eight-connected microtubes (Fig. 9B right)
Portable UV lamp (365 nm wavelength)

Bring the following reagents to the field:
homemade Dried LAMP Kit constructed as above
Pure water
Positive control DNA samples (for SLCMV detection)
  either:
  SLCMV-infected plant leaves,
  3–10 pg/μL total DNA extracted from SLCMV-infected plant leaves, or
  0.1 pg/μL of an infectious clone of SLCMV DNA-A
Negative control DNA samples
  either:
  healthy plant leaves, or
  3–10 pg/μL total DNA extracted from healthy plant leaves

Procedure:
1. Ware gloves.

2. Take a necessary number (total number of samples and controls) of the connected tubes of the dried LAMP kit. To cut a bridge between the tubes, use scissors and do not tear. Restore the remaining reaction tubes to the original aluminum pack and seal it for storage.

3. Dispense 25.0 μL of pure water to each LAMP reaction tube. At this point, do not try to dissolve the dried reagents by tapping the tubes. You will have to do so later. Keep caps open.

4. Close the cap of the first negative control (no template control) tube, so as to avoid any subsequent contamination. Keep other caps open.

5. Insert the tip of a toothpick into the vein of a healthy plant, slightly breaking the vein tissue but not passing through the leaf (Fig. 9), and wash the tip in the primer mixture in the bottom of the second negative control (healthy plant control) tube. Close the cap of the tube. Change gloves.

6. Similarly insert the tip of toothpicks into the veins of the sample (symptomatic) plants as described above. This is because begomoviruses reside mainly in the phloem tissue. Wash the tip in the primer mixture and close the cap of the tube. Change gloves for each sampling.

7. Lastly add 2 µL of the positive control reagents to the positive control tube, and close the cap of the tube.

8. Mix hot water and bottled water in the thermos food flask, and adjust the temperature to 65°C–67°C.

9. Tap the reaction tubes to completely dissolve the dried LAMP reagents at the bottom of the tubes.

10. Spin down the reaction mixture using a portable 8-microtube centrifuge.

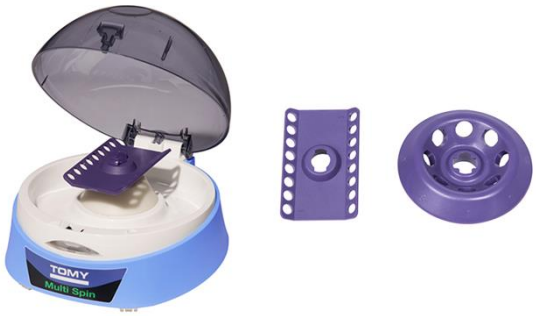
11. Insert the tubes into a float and put them into the hot water in the thermos food flask. Close the lid and leave them for 30 min–60 min. LAMP reaction can proceed at 65°C–60°C.

12. Observe the sample (Fig. 10). Under white light, a negative sample is transparent and slightly orange, and a positive sample is turbid and white green. More clearly, only a positive sample gives fluorescence under UV light (365 nm). When in the field, a portable UV lump may be used. The tube should be observed immediately and photographed for record. Otherwise, the negative reactions in the tubes will slowly turn positive, as the DNA polymerase is still active.

13. We do not recommend electrophoresis confirmation of positive results where positive sample gives ladder-like multimeric bands (Fig. 12), because handling of positive LAMP mixture is the serious source of contamination of pipettes and eventually the whole laboratory. However, if there is a strong need to bring the reaction tubes from the field to the laboratory, you may try the following:

- Make boiled water (95°C) by a water heater driven by some electric sources such as the car battery. Dip the tube into the hot water for 2 min to kill the DNA polymerase. Now you can bring them at room temperature.

A



B

Figure 9 (same as Figure 4). Some useful equipment in an open field.
A. An example of a portable, battery-driven microcentrifuge and its rotors; Multi Spin, TOMY Co., Japan. B. An example of a double-skin, vacuum thermos food flask with a wide opening (left) and a handmade polystyrene foam float holding 8-connected microtubes (right).

Figure 10 (same as Figure 5). Sampling of begomoviruses from a vein of a healthy control (left) or a diseased symptomatic (right) cassava leaves by using the tip of a sterilized wooden toothpick.

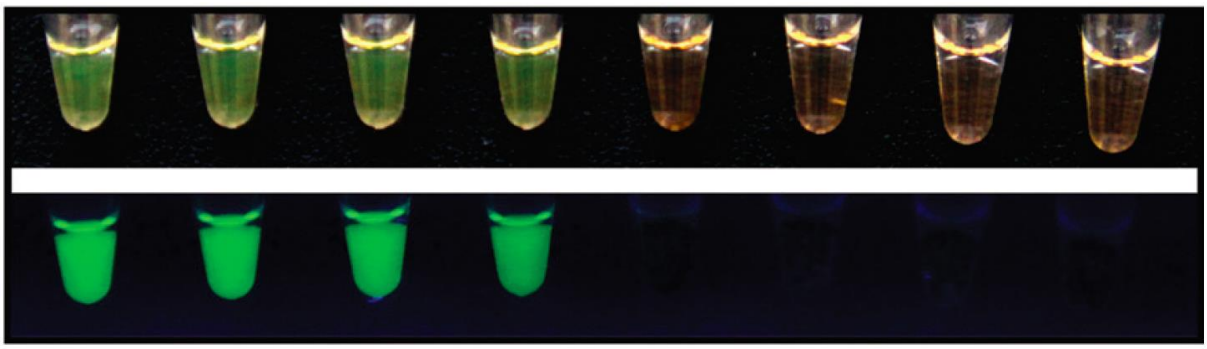


Figure 11 (same as Figure 2). LAMP reaction products analyzed by naked eyes.
(Upper panel) Under white light. Left four tubes, positive: a bit turbid and white green. Right four tubes, negative: transparent and slightly orange.
(Lower panel) Under UV light (365 nm). Left four tubes, positive: with green fluorescence. Right four, negative: without fluorescence.

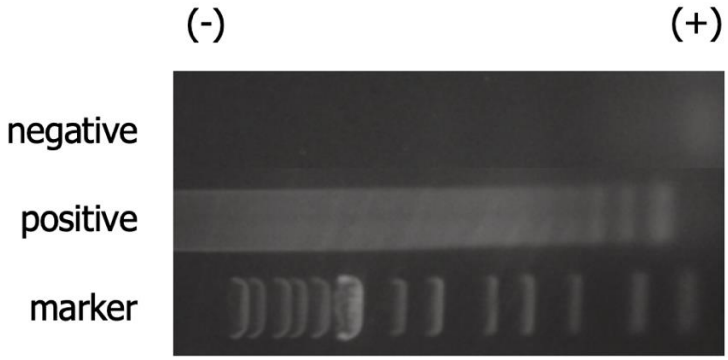


Figure 12 (same as Fig. 3). LAMP reaction products analyzed by agarose gel electrophoresis.
Upper lane; Negative for the target with no amplification product. Middle lane; Positive for the target with ladder-like amplification products. Lower lane; Molecular size markers.