

Detection of cassava mosaic geminivirus using loop-mediated isothermal amplification

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# Detection of cassava mosaic geminivirus using loop-mediated isothermal amplification

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## **Abstract**

A real-time loop mediated isothermal amplification (LAMP) is described to diagnose cassava mosaic disease (CMD). The disease continues to cause great damage in Sub-Saharan African countries threatening food security. Early detection of the pathogen is paramount to control the spread of the disease and promote the safe exchange of clean planting material. Real-time LAMP in combination with a crude but rapid method of DNA extraction can be used to detect the two most widely distributed species of cassava mosaic geminivirus in Sub-Saharan Africa, African cassava mosaic virus (ACMV) and the East African cassava mosaic virus (EACMV), in less than 30 min at a single operation temperature (65 °C) without the need for any expensive instrumentation. Further, this protocol avoids the laborious extraction of high-quality DNA and making it suitable to be used in poorly resourced laboratories and in field settings.

**Key words:** Begomovirus, ACMV, EACMV, LAMP, on-site detection, virus diagnostics

## 1. Introduction

Cassava mosaic disease (CMD) is a viral disease that causes substantial yield losses in Sub-Saharan Africa and more recently in Southeast Asia, endangering food security in these regions [1, 2]. CMD is caused by 11 known cassava mosaic geminivirus species all belonging to the genus *Begomovirus* in the family *Geminiviridae* [3]. In Sub-Saharan Africa the two most widely distributed species of cassava mosaic geminivirus are the African cassava mosaic virus (ACMV) and the East African cassava mosaic virus (EACMV) [4]. Coinfection of two or more virus species is a common feature in the etiology of the diseases that usually leads to increased symptom severity. Cassava mosaic geminiviruses are known to be transmitted either by infected stem cuttings or by members of the cryptic whitefly species complex *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) [5].

Early detection of CMD is critical to achieve effective control, both from a biosecurity as well as an endemic pest perspective [6, 7]. The use of virus-free planting materials remains one of the most effective methods to control the spread of the disease. The development and implementation of specific, sensitive and rapid diagnostic methods is paramount in seed systems to achieve this goal.

Several serological and PCR-based methods have been developed and applied for the diagnosis of CMD [4, 5]. Different factors must be taken into consideration when selecting the most appropriate detection method, including sensitivity, specificity, cost, and the time required to obtain results. PCR-based assays are frequently favoured due to their high sensitivity and specificity [4], however they require trained personnel and sophisticated equipment that might be difficult to implement in poorly equipped laboratories or in field settings. Furthermore, these methods usually need the extraction of high-quality DNA from the sample material, which is time-consuming and frequently involves hazardous chemicals.

Isothermal amplification methods, like the Loop mediated isothermal AMPlification (LAMP) [8] overcome the disadvantages of the PCR-based methods, offering rapid detection systems with specificity and sensitivity similar to PCR assays [9]. LAMP is a rapid amplification method that uses two pairs of primers and a *Bst* DNA polymerase with strand displacing activity to generate amplification products containing stem-loop structures. Amplification can be accelerated by the addition of loop primers [10] which bind to the looped amplicons. Whilst loop primers are not essential for the amplification mechanism to proceed, assays with two loop primers typically amplify target DNA in a shorter period. The *Bst* DNA polymerase used in LAMP assays is more robust and less affected by proteins, polysaccharides or polyphenolic compounds present in plant material that can inhibit PCR assays. As a result, there is no need to use high-quality DNA and simple crude tissue lysis methods can be used to generate extracts for LAMP assays [11, 12, 13, 14]. LAMP products can be visualized by colour change reactions (Fig.1) [15] although weak positive results can be difficult to interpret. LAMP amplification results can also be visualised through agarose gel electrophoresis, lateral flow devices (LFDs) [16] or in real-time using a fluorescent intercalating dye of probe. Real-time detection is preferred as the close tube reaction greatly reduces risk of post-amplification contamination. Portable fluorescent devices like the Genie II (Fig. 2; Optisense, Ltd.) have been developed for real-time isothermal amplification. These devices are particularly suitable for use in a diagnostic lab or in remote locations due to its small size and portability, and its ability to run on battery power for several hours.

In this chapter, we describe an alkaline polyethylene glycol protocol for preparation of fast, crude extracts that can be used with LAMP for the detection of ACMV and EACMV.

## 2. Materials

### 2.1 Crude extract preparation

1. Cassava leaf samples (*see Note 1*).
2. Alkaline PEG buffer. Add 20 mM KOH (or NaOH), pH 13.3-13.5 to 6% Polyethylene glycol (PEG) 200 (Sigma).
3. Sterile nuclease-free water.
4. Screw cap tubes.
5. Stainless steel ball bearings (8-10mm diameter).
6. Ice bucket and ice.
7. Vortex.

### 2.2 Real-time LAMP

1. LAMP mastermix (e.g., GspSSD2.0 Isothermal Mastermix (ISO-004), OptiGene) (*see Note 2*).
2. ACMV and EACMV LAMP primers (Table 1).
3. Microcentrifuge.
4. Fluorometer/real-time PCR instrument (e.g., Genie II (Optigene)) (*see Note 3*)
5. A set of pipettes and corresponding sterile/filter tips.
6. Sterile 1.5 ml microfuge tubes and a microfuge rack.
7. Genie® Strips tubes (Optigene) (*see Note 4*).

### 3. Methods

#### 3.1 Alkaline PEG DNA extraction

1. Add 1ml Alkaline PEG buffer and a stainless-steel ball-bearing to a screw cap tube.
2. Place a small piece (1-2cm<sup>2</sup>) of leaf material to be tested into the tube.
3. Replace cap and shake bottle vigorously for 1 – 2 minutes, or until the sample is a mid-green colour (*see Note 5*).
4. Dilute the PEG extract by a factor of one in ten by transferring 10 µl PEG extract into a tube containing 90 µl nuclease-free water and shake or vortex to mix. The DNA sample is now ready to be used for LAMP amplification. (*see Note 6*).

#### 3.2 Preparation of primer mix

1. Prepare the primer mix according to Table 1. Prepare one primer mix for each target virus.

#### 3.3 Real-time LAMP amplification

1. For each target virus, set up the LAMP reaction mixture (total reaction volume 25 µl) as follows: 15 µl of LAMP master mix, 2.5 µl of primer mixture (see Table 1) and 5.5 µl of nuclease-free water (*see Note 7*).
2. Pipette 23 µl of LAMP master mix into the Genie tube-strips.
3. Keep strip tubes on ice until ready to use. Reactions can be stored on ice for several hours prior to use.
4. Add 2 µl of diluted plant extract to each tube and close the lids firmly (*see Note 8*). Use a fresh tip for each sample.
5. Spin the 8-well strip briefly.

6. Place the 8-well strip into the Genie II machine.
7. Run the LAMP for 30min at 65°C to allow real-time data capture followed a melting process from 98 °C to 80 °C with a ramp rate of  $-0.05$  °C/s (*see Note 9*).
8. The results can be observed as amplification plots along with the annealing profile of the amplified DNA (*see Note 10*).

#### 4. Notes

1. It is important to collect both symptomatic and asymptomatic cassava leaf samples as some cassava varieties might be infected with viruses but not showing disease symptoms.
2. Several commercial isothermal master mixes are available to purchase. The New England Biolabs mastermix, WarmStart® LAMP kit, is a good alternative to the Optigene mastermix used in this study. These master mixes contain the necessary reagents including DNA polymerase and fluorescence dye to perform reactions in real-time.
3. Real-time LAMP can be run in any fluorescent reading device including real-time PCR instruments. In this protocol the Genie II (Optisense, Ltd.) instrument was used.
4. The Genie Strips tubes are specifically designed to fit the Genie II machine used in this protocol. If using another fluorometer/real-time PCR instrument other 0.2 ml tubes or 8-well strips can be used.
5. If the extract is too clear it might not contain enough DNA in solution due to poor cell disruption. Avoid dark green extracts as these might contain higher levels of plant contaminants that can interfere with LAMP reaction that could result in false-negative results.
6. Ideally crude extracts would be prepared just prior to amplification. When handling several samples it is recommended to put extracts on ice until use in LAMP reactions.
7. As a guide, multiply the volume of each component by the numbers of samples to be tested +3. One extra sample will be the negative control (where DNA is replaced with molecular biology grade water), one the positive control and one to accommodate pipetting errors. To minimise pipetting errors and reduce the risk of losing small volumes of liquid, add the components in a particular order, starting with larger volumes first.
8. The tubes are designed to be closed once and have a locking mechanism preventing the tubes from being re-opened after amplification.

9. Do not open the tubes after the completion of the amplification reaction, as this carries the risk of contamination with amplification product.
10. The amplification plot (Fig. 3) shows, on the y-axis the fluorescence values and on the x-axis the time in minutes. The threshold reflects a significant increase of fluorescence over the baseline signal. It is set to distinguish relevant amplification signal from the background. Any reaction with an amplification curve that goes above the threshold is considered a positive reaction. Any straight line, that stays below the threshold is considered a negative reaction. The negative control or no-template control (NTC) should appear as a straight line. The threshold line can be automatically defined by the software or manually adjusted by the user. The time of amplification can also be an indication of the amount of target we have in the samples. The more diluted the sample is, or the less amount of target in the sample, more time is needed to detect it.

## 5. References

1. Carvajal-Yepes M, Cardwell K, Nelson A, et al (2019). A global surveillance system for crop diseases. *Science* 364: 1237–1239. doi:10.1126/science.aaw1572.
2. Kreuze J, Cuellar W, Kumar L, et al (2023). New technologies provide innovative opportunities to enhance understanding of major virus diseases threatening global food security. *Phytopathol.* doi:10.1094/PHYTO-12-22-0457-V.
3. Rey C, Vanderschuren H (2017). Cassava Mosaic and Brown Streak Diseases: Current Perspectives and Beyond. *Annu Rev Virol* 4: 429-452. doi:10.1146/annurev-virology-101416-041913.
4. Abarshi M M, Mohammed I U, Jeremiah S C, et al (2012). Multiplex RT-PCR assays for the simultaneous detection of both RNA and DNA viruses infecting cassava and the common occurrence of mixed infections by two cassava brown streak viruses in East Africa. *J Virol Methods* 179: 176–184. doi:10.1016/j.jviromet.2011.10.020.
5. Legg J P, Kumar P L, Makesh Kumar T, et al (2015). Cassava virus diseases: biology, epidemiology, and management. *Adv Virus Res* 91: 85–142. doi:10.1016/bs.aivir.2014.10.001.
6. Mumford RA, Macarthur R, Boonham N (2016). The role and challenges of new diagnostic technology in plant biosecurity. *Food Secur.* 8: 103–109. doi:10.1007/s12571-015-0533-y.
7. Silva G, Tomlinson J, Onkokesung N, et al (2021). Plant pest surveillance: from satellites to molecules. *Emerg. Top. Life Sci.* 5: 275–287. doi:10.1042/ETLS20200300.
8. Notomi T, Okayama H, Masubuchi H, et al (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res* 28, e63. doi:10.1093/nar/28.12.e63.

9. Boonham N, Kreuze J, Winter S, et al. (2014). Methods in virus diagnostics: from ELISA to next generation sequencing. *Virus Res.* 186: 20–31. doi:10.1016/j.virusres.2013.12.007.
10. Nagamine K, Hase T, Notomi T (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Mol Cell Probes* 16: 223-229. doi:10.1006/mcpr.2002.0415.
11. Tomlinson JA, Boonham N, Hughes K J D, et al (2005). On-site DNA extraction and real-time PCR for detection of *Phytophthora ramorum* in the field. *Appl. Environ. Microbiol.* 71: 6702–6710. doi:10.1128/AEM.71.11.6702-6710.2005.
12. Okereke N, Tomlinson J, Adams I et al (2017). Protocol for cost effective detection of cassava mosaic virus. *Nig Agr J* 48(1), pp.96-102.
13. Nkere C K, Oyekanmi J O, Silva G, et al (2018). Chromogenic detection of yam mosaic virus by closed-tube reverse transcription loop-mediated isothermal amplification (CT-RT-LAMP). *Arch. Virol.* 163: 1057–1061. doi:10.1007/s00705-018-3706-0.
14. Festus R O, Seal S E, Prempeh R, et al (2022). Improved Reverse Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) for the Rapid and Sensitive Detection of Yam mosaic virus. *Viruses* 1592. doi:10.3390/v15071592.
15. Tomlinson JA, Boonham N, Dickinson M (2010a). Development and evaluation of a one-hour DNA extraction and loop-mediated isothermal amplification assay for rapid detection of phytoplasmas. *Plant Pathol.* 59: 465-471. doi:10.1111/j.1365-3059.2009.02233.x.
16. Tomlinson J A, Dickinson MJ, Boonham N (2010b). Rapid detection of *Phytophthora ramorum* and *P. kernoviae* by two-minute DNA extraction followed by isothermal amplification and amplicon detection by generic lateral flow device. *Phytopathol.* 100: 143–149. doi:10.1094/PHYTO-100-2-0143.

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**Table 1. LAMP primer sequences and volume of each primer needed to prepare the primer mix**

Primer	Sequence 5' – 3'	Volume in microlitres (µl) (concentration of starting solution)	Reference
ACMV_F3	GCTCGTAATTATGTCGAAGCG	0.5 (10 µM)	
ACMV_B3	TAGGTATGTCTGGGCTTCTGTA	0.5 (10 µM)	
ACMV_FIP_dg	CTGTATGGGCTGTCGAAGTTCAGACCAGGAGATATCATCATTTMCA	0.5 (100 µM)	[12]
ACMV_BIP_dg	GAACCKTGCTACTGCCCCACTTTCTGTACATGGGYCTGTT	0.5 (100 µM)	
ACMV_FL_dg	ACGAACCTTGGAKYCTGG	0.25 (100 µM)	
ACMV_BL	TCCACGTCACAAATCGAAAACG	0.25 (100 µM)	
EACMV_F3dg	CTGTTACATYTKGAGCAGGAATA	0.5 (10 µM)	
EACMV_B3dg	GGTGACGCGGACAGTRGG	0.5 (10 µM)	
EACMV_FIPdg	TCTCCTGGTCGCTTCGACATAAGTACTGTTGGKCKGAGT	0.5 (100 µM)	
EACMV_BIPdg	CCCKTATCCAAGGTGCGGACAACAACACGGTTCKTGTATG	0.5 (100 µM)	
EACMV_FLdg	TTACACCGTAGMACMCCTAT	0.25 (100 µM)	
EACMV_BLDg	AGGCTGAACTTCGACASYC	0.25 (100 µM)	

ACMV: African cassava mosaic virus; EACMV: East African cassava mosaic virus; B & F: Backward and Forward; FIP & BIP: Forward and Backward Inner Primer; FL & BL: Forward and Backward Loops; dg: indicates presence of degeneracies in the primer sequence; µM: micro molar

## Figure Legends

Figure 1. Example of LAMP products visualized by colour change reactions. Inclusion of SYBR Green in the reaction creates a colour change at the end of LAMP reactions. The green and orange solutions in the tubes represent positive and negative reactions, respectively.

Figure 2. Image of a Genie II portable fluorescent devices (© Optisense Ltd.).

Figure 3. Real-time loop mediated isothermal amplification for detection of ACMV and EACMV. NTC: no-template control.

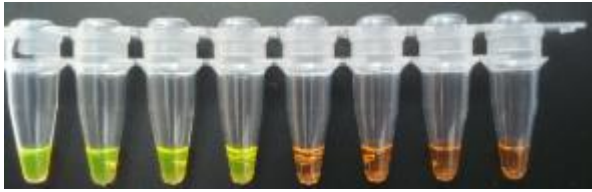


Figure 1.

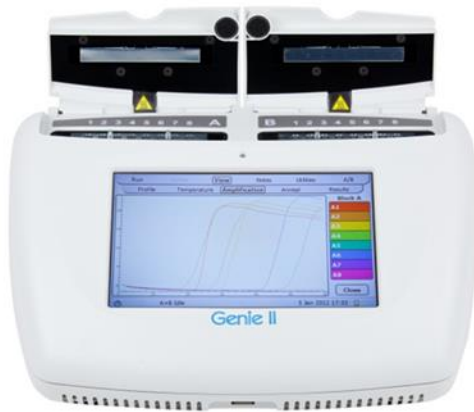


Figure 2.

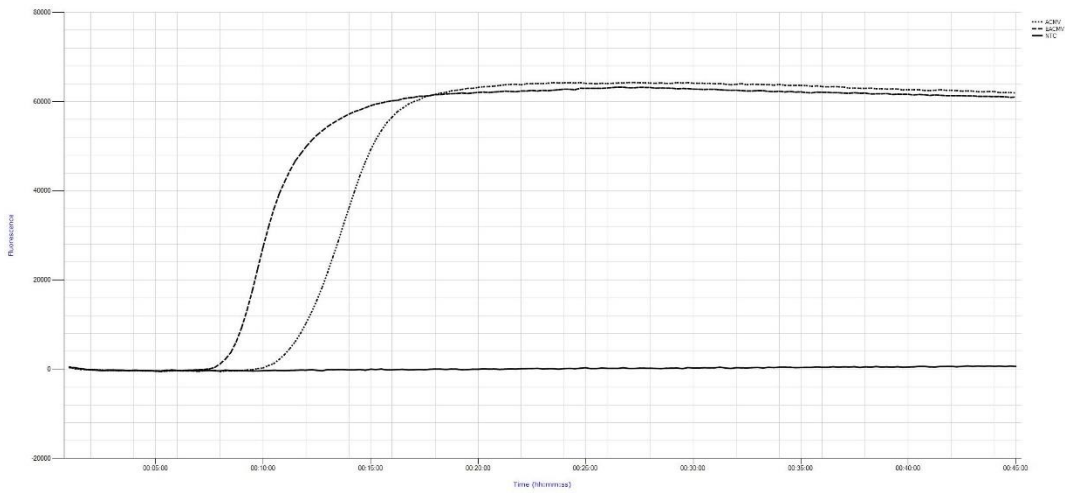


Figure 3.