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LAMP

Using SuperScript IV RT-LAMP Master Mix to detect DNA from fungal and fungus-like plant pathogens

Introduction

Food and economic losses due to crop infections caused by fungi and fungus-like pathogens (e.g., oomycetes) are persistent issues impacting agriculture around the world. In order to reduce disease damage to crops and sustain a productive agricultural sector, cost-effective, sensitive, and accurate disease detection methods are required [1]. Loop-mediated isothermal amplification (LAMP) is considered an easy, highly efficient, and fast diagnostic tool for early diagnosis of various human, animal, and plant pathogens. A typical LAMP reaction employs a set of 4–6 specially designed oligonucleotide primers recognizing 6–8 distinct regions of a target gene, and a thermophilic DNA polymerase with a strong strand displacement activity, such as *Bst* DNA polymerase, for DNA amplification. A primary advantage of LAMP is that it does not require specialized equipment such as a thermal cycler and can be performed simply with a thermal block and/or a water bath maintaining a constant temperature (65°C) for incubation of reactions.

Here we demonstrate the use of Invitrogen™ SuperScript™ IV RT-LAMP Master Mix for rapid isothermal amplification of genomic DNA (gDNA) from the widespread fungi *Botrytis cinerea* and *Fusarium graminearum* and oomycetes *Phytophthora cactorum* and *Phytophthora plurivora*. Although this master mix is formulated for RT-LAMP, containing Invitrogen™ SuperScript™ IV Reverse Transcriptase, it can be conveniently used to amplify from DNA templates.

Cleaning instructions

Before setting up LAMP reactions, the laboratory workspace should be properly cleaned and disinfected to reduce the risk of environmental and random template contamination. Clean the laboratory workspace and all equipment thoroughly using the following reagents in the following order:

1. 70% ethanol
2. Invitrogen™ DNA*Zap*™ PCR DNA Degradation Solutions (Cat. No. AM9890)
3. Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water (Cat. No. 10977049)
4. 70% ethanol

Important notes

LAMP is a highly sensitive detection method, and precautions should be taken to prevent any carryover contamination of new reactions with amplification products from previous reactions.

- Use separate workspaces as well as specially dedicated equipment and supplies for reaction setup and amplification.
- Use only dedicated equipment and supplies for real-time LAMP. Use of pipette tips with aerosol filters is strongly recommended for preparing DNA samples and setting up LAMP reactions.
- We recommend setting up LAMP mixtures in a laminar flow cabinet equipped with a UV lamp.
- Change gloves frequently—put on fresh gloves after cleaning the workspace and equipment, before DNA sample preparation, and after LAMP mixture setup.
- Always include negative control reactions (e.g., no-template control, or NTC) to check for background amplification.
- Close tubes or wells with NTC reactions before adding DNA template to other reactions, to reduce the risk of sample cross-contamination.
- Prior to each real-time LAMP experiment, prepare a fresh 50 µM solution of Invitrogen™ SYTO™ 9 Green Fluorescent Nucleic Acid Stain by diluting the 5 mM SYTO 9 stain stock solution 1:100 in nuclease-free water.

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Materials

Reagents

- Invitrogen™ SuperScript™ IV RT-LAMP Master Mix (2X) (Cat. No. A51801)
- Invitrogen™ SYTO™ 9 Green Fluorescent Nucleic Acid Stain (Cat. No. S34854)
- Thermo Scientific™ Water, nuclease-free (Cat. No. R0581)
- 70% ethanol
- Invitrogen™ DNA*Zap*™ PCR DNA Degradation Solutions (Cat. No. AM9890)
- Invitrogen™ UltraPure™ DNase/RNase-Free Distilled Water (Cat. No. 10977049)
- Invitrogen™ SYBR™ Green I Nucleic Acid Gel Stain (Cat. No. S7567)
- Invitrogen™ E-Gel™ EX Double Comb Agarose Gels, 2% with SYBR™ Gold DNA stain (Cat. No. A42346)
- Invitrogen™ E-Gel™ 48 Agarose Gels, 2% with SYBR™ Safe DNA stain (Cat. No. G800802)
- Invitrogen™ E-Gel™ 1 Kb Plus Express DNA Ladder (Cat. No. 10488091)

LAMP targets and primer sets

Target regions and primer sets used to detect fungal and oomycete plant pathogens are described in Table 1.

Table 1. Targets and primer sets.

Pathogen	Target	Primer set
Botrytis cinerea	Intergenic spacer (IGS) of the nuclear ribosomal DNA (rDNA) sequence	See ref. 2
Fusarium graminearum	Region within chromosome 1	Custom-designed at Thermo Fisher Scientific
Phytophthora cactorum	Translation elongation factor 1-α (*EF1a*) gene	Custom-designed at Thermo Fisher Scientific
Phytophthora plurivora		

Methods

The reaction mixes were prepared on ice following the setup shown in Table 2. Each LAMP reaction was carried out in a total volume of 25 µL. Nuclease-free, nonstick Eppendorf™ DNA LoBind™ 1.5 mL tubes, along with sterile filtered pipette tips, were used to prevent aerosol contamination and maximize sample recovery.

Table 2 shows components of the reaction mixture required to perform the LAMP assay for the detection of four different plant pathogens individually. LAMP reactions must be prepared in separate reaction tubes or wells for each pathogen or each target sequence.

Table 2. Reaction setup for *B. cinerea*, *F. graminearum*, *P. cactorum*, and *P. plurivora* gDNA detection.

Component of the reaction mix	Detection of target DNA	NTC	Final concentration
SuperScript IV RT-LAMP Master Mix (2X)	12.5 µL	12.5 µL	1X
100 µM FIP/BIP primer	0.4 µL each	0.4 µL each	1.6 µM
100 µM F3/B3 primer	0.05 µL each	0.05 µL each	0.2 µM
100 µM LoopF/LoopB primer	0.1 µL each	0.1 µL each	0.4 µM
50 µM fresh solution of SYTO 9 Green Fluorescent Nucleic Acid Stain (optional)*	2.5 µL	2.5 µL	5 µM
Nuclease-free water	8.4 µL	9.4 µL	
NTC tubes or wells were closed prior to adding target DNA			
Genomic DNA template	1 µL		
Total volume	**25 µL**	**25 µL**	

* For real-time fluorescence detection only.

Real-time fluorometric LAMP

LAMP reactions were performed using the Applied Biosystems™ QuantStudio™ 6 Flex and 7 Flex Real-Time PCR Systems, with isothermal amplification conditions (Table 3). Specificity of the LAMP amplification products and reaction kinetics were analyzed using Applied Biosystems™ QuantStudio™ Real-Time PCR Software for QuantStudio 6 Flex and 7 Flex Systems. A melt curve analysis was performed to verify the specificity of the amplified products. The real-time LAMP reactions were performed in quadruplicate, and negative (no-template) controls were included.

Table 3. Isothermal amplification protocol.

Number of cycles	Step	Temperature	Time
60	Amplification	65°C	30 seconds
1	Inactivation	95°C	2 minutes
—	Melt curve	60–95°C	—

Colorimetric endpoint detection

Naked-eye visualization and gel analysis of LAMP products were performed using the same reaction setup as described in Table 2, except that SYTO 9 stain was replaced with nuclease-free water. Colorimetric LAMP reactions were performed on the preheated thermal block at 65°C for different incubation times (15–30 min), depending on the pathogen being analyzed. Reactions were terminated by inactivating the polymerase in SuperScript IV RT-LAMP Master Mix at 95°C for 2 minutes. Along with each reaction, an NTC reaction was also run.

Colorimetric naked-eye detection

The reaction tubes were removed after termination of the reaction, and 1 µL of 10-fold diluted SYBR Green I stain was added to each reaction tube for color change evaluation by naked eye. To avoid contamination with amplicons from the positive reaction tubes, negative control reactions were stained first. For the same reason, only one tube at a time was opened, and closed quickly after adding the SYBR Green I stain. Positive results were determined by a color change from orange (negative) to yellow-green (positive).

Agarose gel electrophoresis

The results of the colorimetric naked-eye visualization were further verified by agarose gel electrophoresis using either E-Gel EX Double Comb Agarose Gels, 2% with SYBR Gold DNA stain, or E-Gel *48* Agarose Gels, 2% with SYBR Safe DNA stain, on the Invitrogen™ E-Gel™ Power Snap Electrophoresis System. For the LAMP reaction samples initially stained with SYBR Green I stain, a 10-fold dilution was used. E-Gel 1 Kb Plus Express DNA Ladder was diluted according to the product manual. The prepared ladder and samples were transferred into wells of an E-Gel agarose gel, starting with the negative control reactions. The lanes for NTC samples were distanced from positive test samples to minimize the risk of contamination.

Results and discussion

Real-time fluorometric LAMP detection

B. cinerea: A real-time LAMP reaction using SuperScript IV RT-LAMP Master Mix was run to detect the intergenic spacer (IGS) sequence in the *B. cinerea* nuclear ribosomal DNA (rDNA). The results presented in Figure 1A show that the amplification signals of the target region were detected in less than 10 minutes, regardless of gDNA input. *B. cinerea* gDNA at 500, 1,000, and 5,000 copies per reaction were consistently amplified in all 4 replicates. No amplification signals were observed in the NTC replicates in the 60-cycle reaction.

P. cactorum **and** ***P. plurivora:*** LAMP primers targeting the translation elongation factor 1-α (*EF1a*) gene were designed to detect both *P. cactorum* and *P. plurivora*. SuperScript IV RT-LAMP Master Mix amplified *P. cactorum* gDNA in less than 10 minutes (Figure 1B), whereas the amplification signal from *P. plurivora* gDNA was detected in more than 10 but less than 15 minutes (Figure 1C). As expected, specific amplification signals of the target *EF1a* gene were detected earlier when the template input was higher. All reactions were repeated 4 times with consistent results. Negative results were obtained for all 8 NTC replicates in 30-minute reactions.

F. graminearum: The sequence selected for designing LAMP primers was a region within chromosome 1, unique to *F. graminearum*. Results presented in Figure 1D show that the reaction replicates containing 500 copies of the gDNA template were highly variable in reaching detectable signals. This suggests that primers could be further optimized to achieve higher specificity and sensitivity, and overall better performance. Nevertheless, overall across the range of template copy numbers, the amplifications with these primers and SuperScript IV RT-LAMP Master Mix were detectable between 10 and 18 minutes, on average. No amplification was evident in NTC repeats in 30-minute reactions.

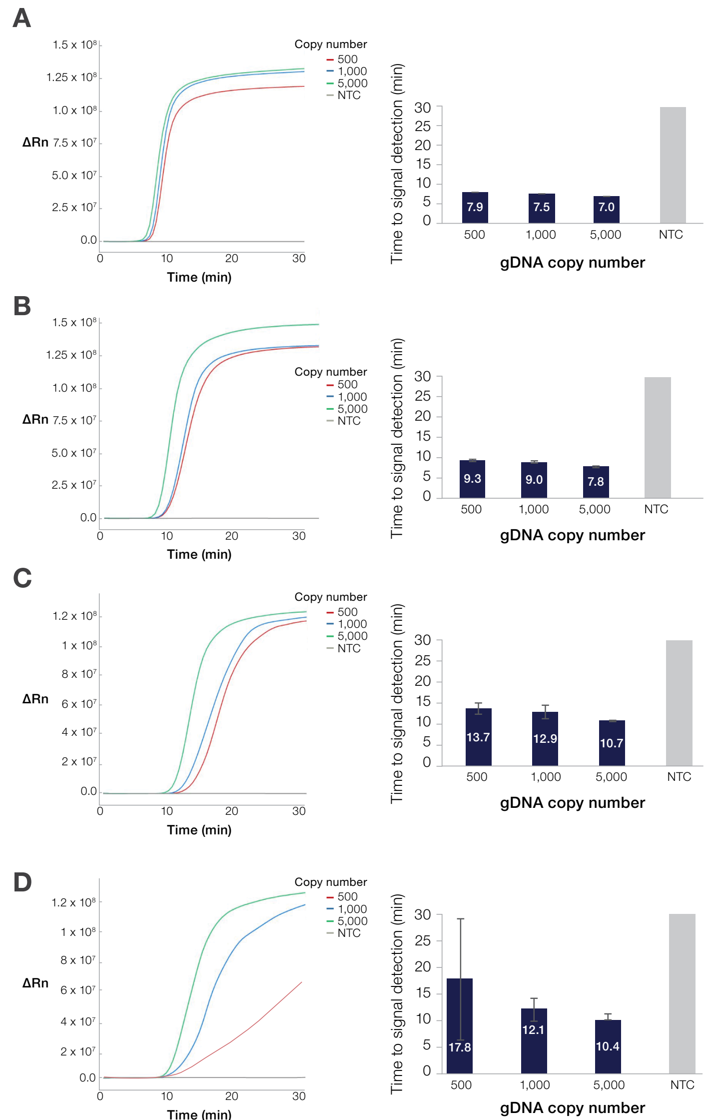


Figure 1. Average amplification of four different fungal and oomycete gDNA targets via real-time LAMP using SuperScript IV RT-LAMP Master Mix. (A) *B. cinerea*, **(B)** *P. cactorum*, **(C)** *P. plurivora*, and **(D)** *F. graminearum*. Amplification kinetics were demonstrated using different gDNA input amounts: 500, 1,000, and 5,000 copies of gDNA template. Error bars represent standard deviation of reaction speed (time to signal detection), calculated from multiple technical replicates (n = 4) for each tested sample.

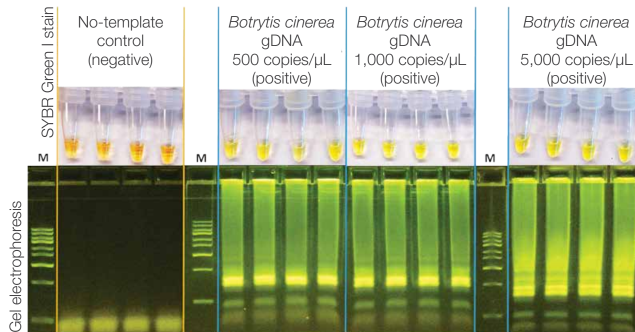
Endpoint colorimetric LAMP detection

The outcome of LAMP reactions was also assessed by visual inspection of DNA accumulation in the reaction tubes by fluorescence visible to the naked eye (Figure 2). The specificity and sensitivity of the visual LAMP assay was evaluated by utilizing serially diluted gDNA at concentrations of 500–5,000 or 1,000–5,000 copies per reaction along with NTCs. The change in the color of the solution was observed directly by naked eye following the addition of SYBR Green I stain. An orange color indicated no amplification, whereas a change from orange to yellow-green demonstrated the presence of LAMP amplicons. The same amplification products were confirmed by agarose gel electrophoresis. Reaction tubes showing a positive reaction exhibited a ladder-like pattern that indicates stem loops with inverted repeats. NTCs did not give any visible fluorescence or ladder-like pattern, indicating the absence of LAMP amplicons.

Solution color indicates:

Gel electrophoresis pattern indicates:

A



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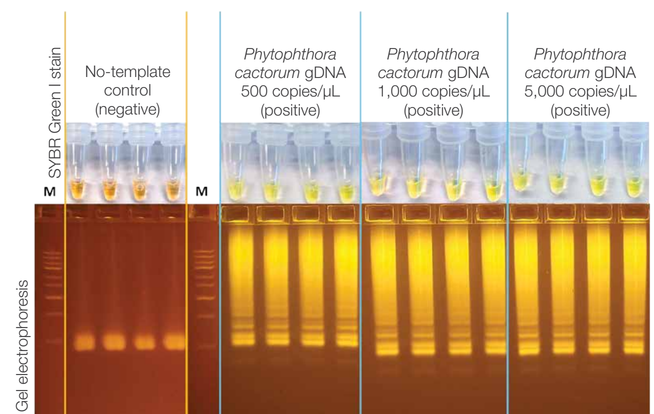

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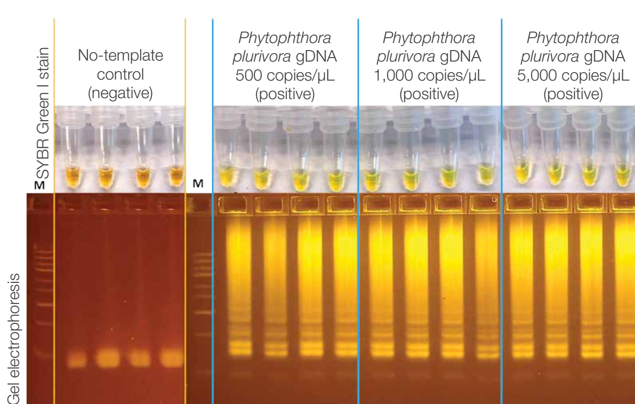

D

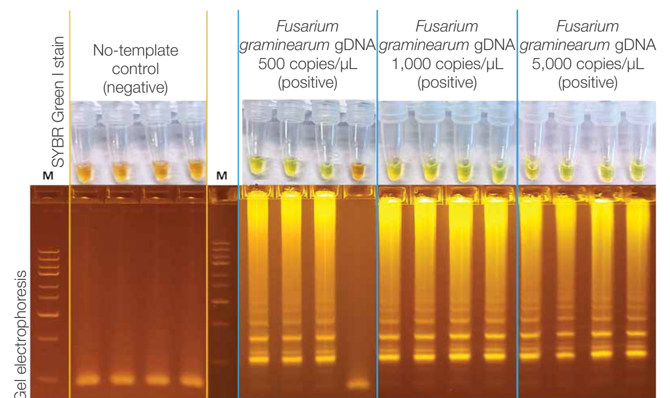

Figure 2. Endpoint analysis of LAMP results using SYBR Green I Nucleic Acid Gel Stain for the naked-eye detection and characteristic ladder-like pattern visualization on agarose gels with the E-Gel Power Snap Electrophoresis System. Purified gDNA from four different plant pathogens, **(A)** *B. cinerea*, **(B)** *P. cactorum*, **(C)** *P. plurivora*, and **(D)** *F. graminearum*, were used along with NTCs. LAMP reactions containing *B. cinerea*, *P. cactorum*, and *P. plurivora* templates were incubated for 15 minutes. LAMP reactions with *F. graminearum* were performed for 30 minutes. Upper panels: Color changes induced by SYBR Green I stain after addition of 1 μL of the diluted stain into each reaction tube (bright yellow-green color: LAMP product; orange color: no amplification). Lower panels: Successful LAMP reaction verified by visualization of a ladder-like pattern in the E-Gel Double Comb Agarose Gel (2%) with SYBR Gold DNA stain and E-Gel 48 Agarose Gels (2%) with SYBR Safe DNA stain. Lane M contains the E-Gel 1 Kb Plus Express DNA Ladder.

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Conclusions

SuperScript IV RT-LAMP Master Mix enables fast reaction preparation, outstanding amplification speed, and ease of use for the detection of gDNA from plant pathogens. Real-time fluorometric and endpoint colorimetric LAMP reactions with SuperScript IV RT-LAMP Master Mix detected gDNA from tested plant pathogens in less than 18 and 30 minutes, respectively. These results demonstrate that SuperScript IV RT-LAMP Master Mix can be used in the development of highly specific, rapid, and sensitive LAMP assays for the detection of plant diseases caused by fungi and oomycetes.

Acknowledgments

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References

1. Fang Y, Ramasamy R (2015) Current and prospective methods for plant disease detection. *Biosensors* 5(3):537–561.
2. Tomlinson J, Dickinson M, Boonham N (2010) Detection of *Botrytis cinerea* by loop-mediated isothermal amplification. *Lett Appl Microbiol* 51(6):650–657.

Ordering information

Description	Cat. No.
SuperScript IV RT-LAMP Master Mix (2X)	A51801
SYTO 9 Green Fluorescent Nucleic Acid Stain	S34854
Water, nuclease-free	R0581
DNA*Zap* PCR DNA Degradation Solutions	AM9890
UltraPure DNase/RNase-Free Distilled Water	10977049
SYBR Green I Nucleic Acid Gel Stain	S7567
E-Gel EX Double Comb Agarose Gels, 2% with SYBR Gold DNA stain	A42346
E-Gel 48 Agarose Gels, 2% with SYBR Safe DNA stain	G800802
E-Gel 1 Kb Plus Express DNA Ladder	10488091

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