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Method Article

Optimization of Loop-Mediated Isothermal Amplification (LAMP) reaction mixture for biosensor applications

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A B S T R A C T

Genetically Modified (GM) foods are becoming the future of agriculture on surviving global natural disasters and climate change by their enhanced production efficiency and improved functional properties. On the other hand, their adverse health and environmental effects, ample evidence on transgene leakage of Genetically Modified Organisms (GMOs) to crops have raised questions on their benefits and risks. Consequently, low-cost, reliable, rapid, and practical detection of GMOs have been important. GMO-detection platforms should be capable of stably storing detection reagents for long-delivery distances with varying ambient temperatures. In this study, we developed an event-specific, closed tube colorimetric GMO detection method based on Loop-Mediated Isothermal Amplification (LAMP) technique which can be integrated into GMO-detection platforms. The entire detection process optimized to 30 min and isothermally at 65 °C. The durability of the LAMP mixture in the test tubes showed that the LAMP reaction mixture, in which *Bst* polymerase and DNA sample was later included, yielded DNA amplicons for 3 days at room temperature, and for 6 days at 4 °C.

- Simple, stable, and cheap storage method of LAMP reaction mixture for GMO-detection technologies.
- GMO-detection platforms can stably store detection reagents for long-delivery distances with varying ambient temperatures.
- Any DNA sample can be used in the field or resource-limited setting by untrained personnel.

A R T I C L E I N F O

Method name: Loop-Mediated Isothermal Amplification (LAMP) for genetically modified food detection
Keywords: Loop-Mediated Isothermal Amplification (LAMP), Genetically Modified Foods (GM foods), Resource-limited settings, Storage
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More specific subject area:	Storage of reaction reagents in biosensors
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Introduction

Genetically Modified Organisms (GMOs) products have been widespread and the increase in their production will undoubtably continue with the discussion on the benefits and risks of Genetically Modified (GM) foods. Particularly, a variety of hypothesized potential adverse health and environmental effects of GM foods have made their detection methods essential. Along this line, GMO-detection platforms should be affordable, hand-held, easy-to-use, field-deployed, and their test results should be real-time [13]. Still, most of the detection methods for GM products relies on well-established laboratory techniques those require trained personnel, expensive laboratory settings and interpretation of obtained results to conclude the tests. Polymerase Chain Reaction (PCR), Real-Time PCR (RT-PCR), or digital PCR (dPCR) are among the broadly performed nucleic acid amplification-based detection tests in the laboratories [15,19,26]. Although they have high sensitivity, they demand expensive and specialized equipment that can be run by a specialist. PCR-based tests require longer test run time [15,19,26]. Moreover, their readout cannot be visualized by naked eye in real time.

In the past two decades, limitations of PCR have been overcome by isothermal nucleic acid amplification techniques [3,11,28]. These methods do not require cyclic temperature changes, instead it can be conducted at a constant temperature using simple instruments – e.g., a conventional water bath or heat block – which drastically reduces equipment costs [4,5,11]. Hence, fast and inexpensive detection methods can be used in comparison to PCR methods. Several isothermal exponential amplification methods have been developed, among them Loop-Mediated Isothermal Amplification (LAMP) exponentially amplifies nucleic acid template using 4–6 primers and DNA polymerase and provides 10^9 copies of target DNA at 60–65°C in less than an hour [20]. LAMP provides high selectivity, specificity, efficiency and rapidity; therefore, it becomes an adequate DNA amplification method for GMO-detection platforms [4,18,21,22,24,30]. DNA-detection technologies mostly focused on providing rapid, sensitive, and accurate detection in cheap, reusable, portable devices those Rese [12]–[14].

However, when they include reaction mixtures, their storage and delivery, which directly contributes their performance and affordability, are also become an important technical challenge to solve. Since, these novel DNA-detection platforms use thermally unstable reagents for the enzymatic reactions to amplify nucleic acids, they must be stored and transported in refrigerated conditions [6,9,16,17,27]. This common limitation of DNA-detection platforms restricts their usage in resource limited settings, where reliable cold-chain equipment is not available [2,6,23]. Therefore, the reagents, those will be used in the DNA-detection platforms, should be carefully considered. Moreover, stability of the reagents inside the DNA-detection biosensors might contribute to determine their shelf life.

In this study, we exhibited the durability of the LAMP reaction mixture in the test tubes, excluded from *Bst* polymerase enzyme and DNA sample, for 3 days at room temperature (RT) and 6 days at 4 °C. Our results might enable rapid detection of nucleic acids in resource-limited fields where transportation and storage conditions are not favorable. Herein, we showed the readout of the most convenient LAMP reaction mixture and its storage conditions using the lectin gene of soybean (Glycine max). Lectin gene has been widely used as a species control for all soybean samples. The P35S primer pair is used to detect transition site from the Cauliflower Mosaic Virus 35S promoter sequence in Roundup Ready Soybean (RRS, containing 10% gts40–3–2/RRS). We used RRS with P35S primer set to present specificity of the LAMP reactions.

Experimental section

DNA extraction and primer sequences

Soybean, MON89788 (100% GMO) is certified reference material (CRM), was obtained from the American Oil Chemists' Society (Boulder, Urbana, USA). Certified reference materials for Roundup Ready Soybean (RRS, gts40–3–2, 10% GMO) was obtained from Sigma Aldrich (St. Louis, MO, USA). Overall DNA extraction completed from 200 mg of soybean sample using the Macherey-Nagel NucleoSpin Plant Purification II kit according to the manufacturers' instructions. At the final step, 40 ml Elution Buffer was used 2 times to recover DNA for obtaining the highest concentration. DNA yield and purity were evaluated by UV Spectrophotometry at 230, 260 and 280 nm using a NanoDrop 2000c instrument (Thermo Scientific, Wilmington, DE, USA). DNA integrity was detected by agarose gel electrophoresis, in which 400–1200 ng/25 µl DNA samples were separated on 1% agarose gels containing GelRed nucleic acid stain (Biotium, Hayward, CA, USA) in 0.5 Tris/Borate/EDTA (TBE) buffer. Primer sequences were reported in our previous work. The oligonucleotides were obtained from Oligomer (Ankara, TURKEY) [10,13].

LAMP reaction

Each LAMP reaction was performed in a final volume of 25 µl containing 8 U *Bst* DNA polymerase (large fragment; New England Biolabs) in 1X ThermoPol Reaction buffer (20 mM Tris–HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$ and 0.1% Triton X-100; New England Biolabs) supplemented with 6 mM $MgSO_4$, 1 M betaine and 1.4 mM of each dNTP, 2 µL DNA template, 6.5 µl double-distilled water (ddH2O), and 1.6 µl, 1X primer mix. Each reaction was incubated at 65 °C for 30 min in a heat block. LAMP results were determined according to color change provided by Hydroxy Naphthol Blue (HNB), which was added in 120 µM to each test reaction before the incubation.

Shelf life of LAMP reaction reagents

The preparation of the reactive mixtures was kept both at room temperature (25°C) and in the refrigerator at 4 °C for 7 days to test their shelf life. LAMP reagents were mixed in a PCR tube and stored wrapped in aluminum foil for preventing light transmittance. Next, efficiency of the LAMP reaction was tested by introducing the sample DNA and *Bst* polymerase enzyme at 65 °C for 30 min.

Detection and quantification of the LAMP product

When the reaction incubation completed, the color of each reaction was assessed by naked eye where negative results were differentiated from positive results by a color change from violet to sky blue. LAMP reaction products were also resolved electrophoretically (1% agarose in 0.5X TBE buffer) and visualized using BioRad nucleic acid stain in BioRad Gel Doc EZ System (Berkeley, CA, USA).

Data quantification was performed using ImageJ software (Version 2.0 National Institutes of Health, Rockville, MD, USA). Intensity of the bands in the images was acquired from the agarose gel images by removing the backgrounds of the DNA bands and defining a rectangular region of interest. Then, lane profile plots were drawn based on the measured areas (Analyze-Gels-Plot Lanes). The obtained data was analyzed and presented using GraphPad Prism software (Version 5). Student's *t*-test was used to determine statistical significance of changes in band intensities. Figures show the data as mean ± standard deviation. The experiments were performed in triplicate.

Results and discussion

Currently, widespread application of molecular biology techniques in research, diagnosis, and education is costly and often unaffordable for many laboratories and schools in resource-poor countries [2]. Particularly, in case of colorimetric tests, it is important to know the stability of the indicator to facilitate the use of detection platforms in the field [1]. In this study, we reported a LAMP

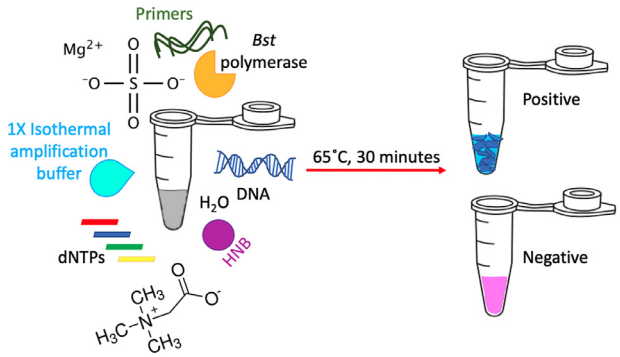

Fig. 1. Schematic representation of LAMP assay. Reaction mixture consists of *Bst* polymerase, DNA, HNB, dNTPs, 10x isothermal amplification buffer, primers, betaine, Mg^{2+} ions and water. LAMP reaction occurs at 65°C for 30 min. Positive and negative results are distinguished according to color change.

assay for detection of GM soybean sample that addressed stability of the reaction mixture without *Bst* polymerase enzyme and DNA sample. These results might contribute to development of DNA-detection platforms, which includes reaction mixtures, those can be stored or delivered [7,22,29,31]. Thanks to insensitivity of LAMP method to DNA-impurities, its short reaction time and a constant temperature requirement for DNA amplification, it has become more convenient method for resource-limited settings. Furthermore, the inclusion of HNB in the LAMP master mix allows visualization of the LAMP results under indoor light by naked eye and eliminates the need to open the reaction vials. Hence, it simplifies the workflow and minimizes the risks of contamination. Fig. 1 shows the schematic of the LAMP reaction. The LAMP reaction is quantified by measuring the Mg^{2+} ion concentration levels. During the LAMP reaction large amounts of pyrophosphate ions are generated, they react with Mg^{2+} ions and therefore, the Mg^{2+} ion concentration decreases as the LAMP reaction progresses [31]. In 2009, Goto and his co-workers used HNB as an indicator for the LAMP reaction that monitors the change of the Mg^{2+} ion concentration in the reaction at the first time [8]. Here, the LAMP reaction was performed with HNB to obtain visible results without additional detection steps [25].

In this study, LAMP reactions were optimized for detection of GM soybean samples in the tubes according to color change by HNB, Fig. 2. Table 2 presents the tested conditions for optimization of the LAMP reactions. The reaction mixture (RM) 5 with 6 mM $MgSO_4$, 120 µM HNB and 7.5 µl ddH2O presented the most ideal reagent concentrations where the color change can be easily distinguished by naked eye. Therefore, RM5 was used to test the shelf life of LAMP reaction mixtures.

Fig. 2 shows the specificity of the LAMP reactions using lectin and P35S primer sets with Roundup Ready Soybean (RRS, containing 10% gts40–3–2/RRS) and MON89788 (100% GMO) soybean samples. Fig. 2a shows the colorimetric readouts of the LAMP reactions with the order of lectin primers with 10% RSS, lectin primers with MON89788, P35 primers with 10% RRS, and P35 primers with MON89788 samples. Positive results can be distinguished by sky blue color. The only negative result, visualized in violet color, was obtained from the LAMP reaction using the P35 primers with the MON89788 samples. Fig. 2b demonstrates the DNA bands on agarose gel electrophoresis using the reaction tubes from Fig. 2a. Fig. 2c exhibits DNA quantification by agarose gel electrophoresis using Fig. 2b. LAMP reactions were performed as three replicates using a heat block. In this experiment, lectin gene is used since it is a housekeeping gene that can be used as a molecular marker for soybean. Fig. 2 shows that lectin gene can be expressed in both MON89788 and RSS soybean samples. The P35S primer pair is used to detect transition site from the Cauliflower Mosaic Virus 35S promoter sequence in RRS. When LAMP reactions were performed using P35S primer set, P35S gene was only detected in 10% RSS sample. MON89788 soybean does not contain the Cauliflower Mosaic Virus 35S promoter sequence.

Storage of the reaction mixture in detection platforms might contribute to increase usage of colorimetric assays in a more reliable and easy way. While shelf life of master mixes relies on dry

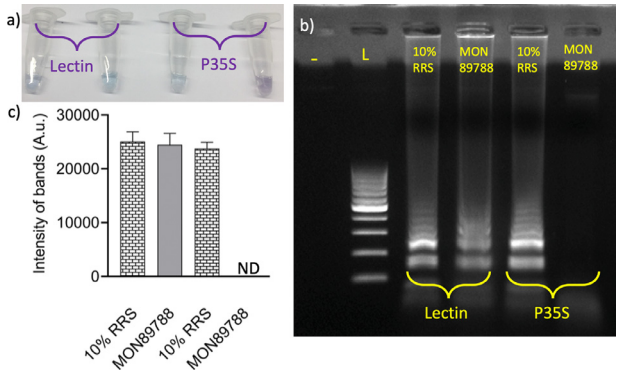

Fig. 2. Specificity of LAMP-based detection. LAMP reactions using lectin and P35S primer sets with RSS (10% gts40–3–2/RRS soybean) and MON89788 (100% GMO) template DNA. (**a)** Colorimetric results. (**b)** Gel electrophoresis results with the order of the samples shown in a. 1-kbp DNA ladder is used. (**c)** Intensity measurements of the amplified DNA fragments following the order of the samples shown in b (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.). Intensity measurements of the DNA bands show mean ± standard deviation of three independent experiments. ND: Not detected.

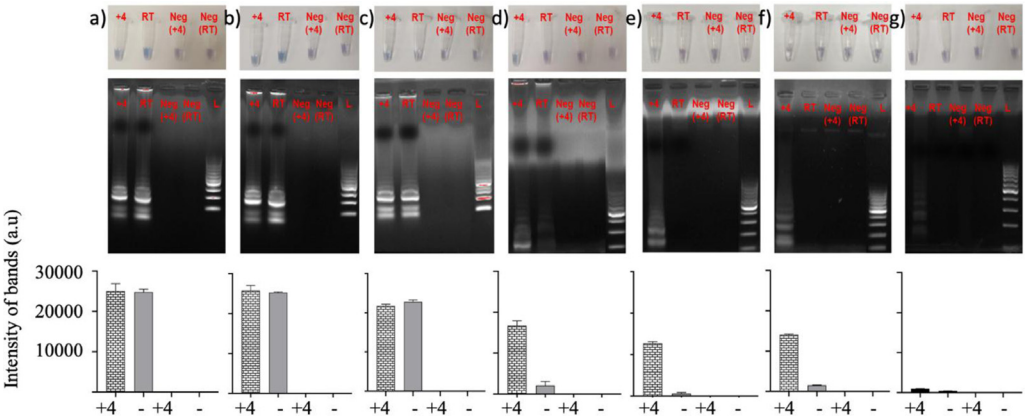

Fig. 3. Testing stability of the LAMP reaction mixtures (RM) at RT (25°C) and at 4°C for 7 days. LAMP reactions using the lectin primer set with 100 ng of MON89788 template DNA were incubated for (**a)** 1, (**b)** 2, (**c)** 3, (**d)** 4, (**e)** 5, (**f)** 6, (**g)** 7 days either at RT or 4°C fridge. Neg: Negative control, LAMP reaction without DNA template. L: 1-kbp DNA ladder. Results were presented by gel electrophoresis (1% agarose gel), colorimetric images (images of the reaction tubes) and the intensity measurements of the DNA bands.

storage conditions, their repeated freezing and thawing can lead to loss of reactivity in their long-term storage. For a regular LAMP assay, DNA polymerase, primers, and dNTPs need to be stored at −20 °C. Herein, we verified that LAMP reaction mixture without DNA template and polymerase enzyme, can be stored at 4 °C for 6 days and at RT for 3 days. The results were verified by DNA amplicons on agarose gel electrophoresis, Fig. 3.

In this study, we showed the readout of the most convenient LAMP reaction mixture and its storage conditions using the lectin gene of soybean. Lectin gene has been widely used as a housekeeping gene to confirm soybean species. The storage of the reaction mixture is not target gene or species specific. When different genes from different species are identified using LAMP protocol, storage of their reaction mixture will remain the same. Our results suggest that LAMP reaction mixture (RM 5) without DNA template and polymerase enzyme can be delivered in a biosensor or a PCR test tube at 4 °C for 6 days and at RT for 3 days. The end-user can only add the enzyme and

Table 1
Lectin and P35S primer sets used in this study [10].

Lectin-F3: ACAACTTTTGAAAAGTACCCAAT
Lectin-B3: GAACTTGTTCCAGCTGAAAG
Lectin-FIP: CATTGCTTTGCTTCAGCTAAATTGCTGCTAGTATAAATAGGGGCATG
Lectin-BIP: AACCCAGAATGTGGTTGTATCTCTCAGTTTCCGCTGAGTTTGC
P35S_LampF: GTCTTCAAAGCAAGTGGTTTTGGATAGTGGGATTGTGCG
P35S_LampR: TTCCACGATGCTCCTCGTTTTCCTCTGCCGACAGTGG
P35S_LoopF: TCCACTGACGTAAGGG
P35S_LoopR: GGGGTCCATCTTTGGG
P35S_DisplF: AGGAAGGGTCTTGCG
P35S_DisplR: ATAAAGGAAAGGCCATCG

Table 2
The tested concentrations of reagents for optimization of LAMP readout.

Reagents	RM1	RM2	RM3	RM4	RM5
1X Isothermal Amplification Buffer (µl)	2.5	2.5	2.5	2.5	2.5
$MgSO_4$ (mM)	4	8	6	8	6
10x Primers (µl)	2.5	2.5	2.5	2.5	2.5
10 mM dNTPs (µl)	3.5	3.5	3.5	3.5	3.5
Betaine (µl)	5	5	5	5	5
HNB (µM)	120	120	240	240	120
Bst 2.0 (µl)	1	1	1	1	1
Template DNA (ML)	2	2	2	2	2
Double Distilled Water (µl)	7.5	6.5	6.5	6.5	7.5
Total volume (µl)	**25**	**25**	**25**	**25**	**25**

sample DNA into the reaction which can be practical for an untrained person. Besides, LAMP assays require 60–65°C temperature by a heating block or water bath, which is mostly available in many resource-limited settings and does not require expertise for their operation. Therefore, our results might provide a solution for LAMP-based colorimetric DNA-detection biosensors containing premixed reaction reagents.

Conclusion

Stability of reaction mixtures is an important parameter for the newly designed DNA detection platforms which includes premixed reaction reagents. In this study, a LAMP based colorimetric GMO detection method was optimized for testing the durability of LAMP reaction mixtures for 7 days both at RT and 4°C. First, LAMP reaction mixture was prepared without DNA template and *Bst* polymerase and stored either at RT or 4°C. Next, DNA template and *Bst* polymerase was added into the reaction mixture and the LAMP reaction was performed at 65 °C for 30 min. Afterwards, color change was visualized, the blue color indicated the existence of GMO, positive reactions. Finally, verification of the results was performed by gel electrophoresis. Our method presents that LAMP reaction can be performed when the reaction mixture without polymerase enzyme and DNA template is stored for 3 days at RT and 6 days at 4°C.

Table 1

Declaration of Competing Interest

The authors declare no competing interests.

Acknowledgments

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