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Visual Detection of Single-Nucleotide Polymorphisms Using Molecular Beacon Loop-Mediated Isothermal Amplification with Centrifuge-Free DNA Extraction

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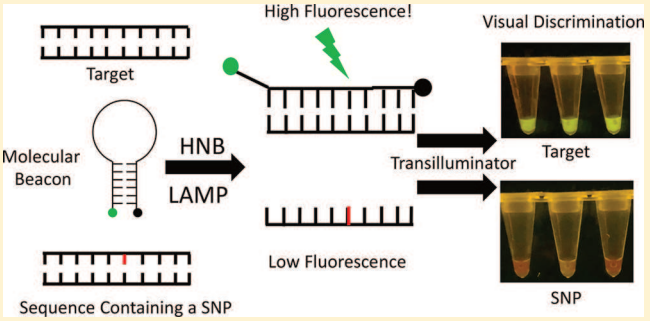
Supporting Information

ABSTRACT: Loop-mediated isothermal amplification (LAMP) is a powerful nucleic acid amplification technique due to its rapid and sensitive nature. These characteristics, in addition to low-cost and robustness, make LAMP an attractive alternative to polymerase chain reaction for point-of-care applications. However, sequence-specific detection remains a formidable challenge, particularly when single-nucleotide resolution is required. In this study, a LAMP method is developed for facile visual detection of single-nucleotide polymorphisms (SNPs) using molecular beacons (MBs) by exploiting the dual-fluorescence of fluorescein (6-FAM) and hydoxynaphthol blue (HNB). The method is coupled with solid-phase microextraction (SPME) to facilitate rapid extraction and detection of the target sequence. This work expands the use of MBs in LAMP for the visual detection of SNPs and facilitates the development of future LAMP assays for a wide-range of targets.



Nucleic acid amplification techniques are some of the most useful and widely employed in the biological and biomedical fields. Quantitative polymerase chain reaction (qPCR) is an established amplification method that has been extensively used for pathogen identification,[1] gene expression analysis,[2] and genotyping.[3] A qPCR instrument is composed of a thermal cycler, which accurately controls the temperature of the reaction containers and an optical module to measure fluorescence. However, these requirements preclude the facile implementation of this technique in resource-limited settings for point-of-care (POC) applications.[4]

Isothermal nucleic acid amplification (INAA) techniques have been developed to overcome some of the limitations posed by traditional methods.[5] Loop-mediated isothermal amplification (LAMP) is a popular INAA technique that relies on 4−6 primers for the amplification of a target sequence.[6] Since it is performed at a constant temperature and can be easily coupled with colorimetric detection, it is ideally suited for use at the POC.[7] One significant drawback to traditional visual detection methods for LAMP is the inability to detect specific amplicons. SYBR Green I is a DNA binding dye that fluoresces strongly in the presence of any double-stranded DNA that is produced, meaning spurious amplification is indistinguishable from specific amplification.[8] Hydroxynapthol blue (HNB)[9] and Calcein[10] have also been used to visually distinguish between negative and positive reactions. More specifically, when HNB is present in the reaction solution prior to amplification, the dye chelates excess Mg^{2+} in the solution and produces a violet color change. During the amplification process, insoluble magnesium pyrophosphate is formed,[11] which removes free Mg^{2+} from solution and changes the appearance of HNB in solution from violet to sky-blue.[9] However, these metal indicators only monitor the presence of Mg^{2+} in the reaction system and cannot provide direct evidence of specific amplification. Furthermore, certain clinically relevant applications require single-nucleotide resolution for accurate identification of drug resistant microorganisms[12] and cancer diagnoses.[13] While careful primer design can be used to mitigate nonspecific amplification, it does not negate the indirect nature of traditional colorimetric methods.

Strand displacement probes have been successfully used for sequence-specific detection of LAMP amplicons and single-nucleotide polymorphisms (SNPs) using real-time detection.[14,15] Similarly, molecular beacon (MB) probes were recently shown to be effective in the real-time detection of LAMP amplification;[16] however, their ability to discriminate SNPs has not been demonstrated. While the aforementioned methods have been shown with real-time detection, they require further study and modification for use at the POC. The ideal LAMP detection method for POC applications should be capable of detecting specific amplification sequences, possess single-nucleotide resolution, and produce results that can be easily visualized and interpreted. Herein, we demonstrate a

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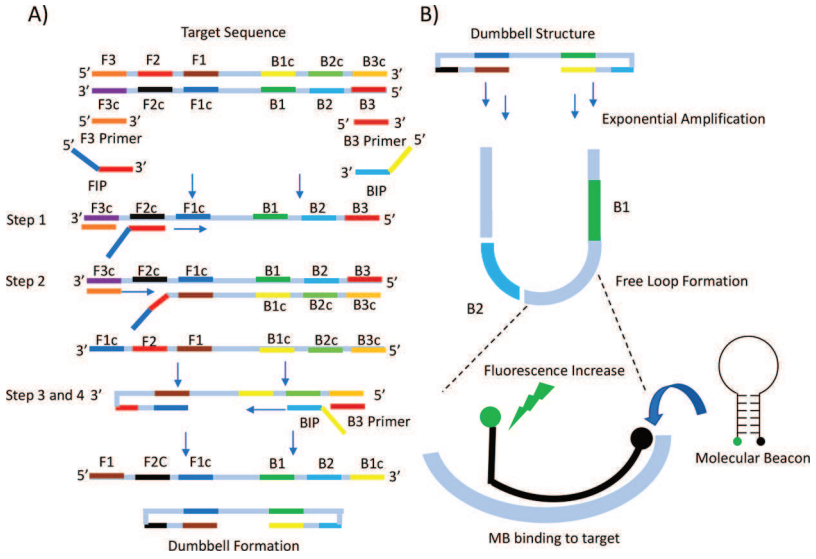

Figure 1. (A) General loop-mediated isothermal amplification (LAMP) schematic of the amplification process up to the formation of the self-matching dumbbell structure as well as illustration of primers and primer-binding sites. (B) Schematic demonstrating the binding of the molecular beacon to the loop region between the B1 and B2 regions of the target.

MB-LAMP method that exploits the fluorescence of HNB and fluorescein (FAM) to achieve single-nucleotide differentiation that can be identified visually with a transilluminator. Furthermore, we demonstrate the use of a SPME method for rapid, centrifuge-free isolation of DNA in less than 5 min and subsequent visual detection.

Figure 1A,B shows a general schematic of the LAMP amplification process and the detection using a MB probe, respectively. During MB-LAMP, single-stranded loops are generated between the B1 and B2 regions of the target. A real-time calibration curve was performed with the MB-LAMP method previously developed by Liu et al. and used as a comparison for subsequent experiments.[16] Figure S1 shows the method can detect down to 2.38×10^2 copies of the target, which is consistent with previous results.

To develop a visual detection method compatible with MB-LAMP, the addition of HNB was investigated. HNB has previously been coupled with SYBR Green I[17] and FAM[18] to develop a system that exploits the fluorescence of both dyes for easy visual identification of amplified targets. Since the MB used in this study utilizes FAM as the fluorophore, it was hypothesized that a similar system could be developed. Several samples with HNB concentrations ranging from 120 to 360 μM were tested and the amplification measured in real-time. Figure S2A shows a decrease in the threshold times when HNB is added to the reaction. Threshold times (T_t) are the real-time LAMP equivalents of quantification cycle (C_q) values in qPCR and indicate when the observed fluorescence is appreciably higher than the background. They are useful for assessing effects of changing reaction conditions on the amplification of the target. Increasing threshold times are an indication of inhibition of the reaction system.[19] The obtained maximum relative fluorescence units (RFUs) decreased significantly with the addition of more HNB, with a 4-fold difference in RFUs is observed from 0 μM to 360 μM (Figure S2B). The effect of HNB concentration on the visual identification of the reaction products was subsequently studied. Three positive reactions and three no template controls (NTC) were incubated for 1 h at 63 °C, and the reaction containers were subsequently visualized with a transilluminator. As shown in Figure S3, as the concentration of HNB is increased, the appearance of green fluorescence in the positive reaction containers was reduced. Conversely, red fluorescence derived from the NTC appeared to increase with the addition of more HNB.

To investigate the source for the change in visual appearance, fluorescence spectra of the reactions were obtained by exciting at 470 nm and recording fluorescence using a 10 nm step size, as shown in Figure S4. Maximum fluorescence was observed at 520 nm for the positive reactions, while the negative reactions exhibited a broad peak starting at 600 nm. Endpoint measurements were subsequently taken by recording emission spectra at 520 and 660 nm with excitation at 470 nm. As shown in Figure S5, the fluorescence observed at 520 nm remains constant for both the positive and negative reactions. However, the fluorescence obtained at 660 nm from the negative reaction increased noticeably as higher concentrations of HNB were used. This indicates that the concentration of HNB directly influenced the intensity of the red-light emission derived from the NTC, as determined by fluorescence measurements. On the other hand, the visual appearance of the positive reactions appear to change with increasing HNB concentration. However, the fluorescence at 520 nm remains constant regardless of how much HNB was added. Therefore, 240 μM was chosen for subsequent experiments as it provided the most clear and visually apparent fluorescence for both the positive control and NTC. A calibration curve using the method with 240 μM HNB was performed and is shown in Figure S6. The detection limit of this system (2.38×10^2) is identical to the reaction system without HNB.

Molecular beacons are able to detect single-nucleotide mismatches due to their unique structure.[20] The various conformations of a MB are illustrated in Figure S7A. When a target is present, the molecular beacon will unfold from its hairpin structure to bind the target, resulting in separation of the fluorophore−quencher pair and an increase in fluorescence. If mismatches are present in the sequence, binding will not occur until the temperature is sufficiently reduced, allowing differentiation of mismatched sequences if the appropriate

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conditions are chosen. Since MBs have not been previously employed in LAMP assays for SNP detection, it was necessary to investigate the effect of the SNP location on MB binding. As illustrated in Figure S7A, the 3′ end of the MB stem used in this study binds to the target. Therefore, it was necessary to ascertain whether the MB could discriminate single-nucleotide differences occurring in the stem and/or loop binding region. Annealing profiles were performed to determine the stability of MB binding to different mismatched sequences. The sequences studied are shown in Table S1. In this approach, the temperature is slowly reduced at a rate of 1 °C min^{-1} and the fluorescence recorded after each minute.[20,21] Two different mismatched sequences were tested, and a schematic representation of the location of these can be seen in Figure S7B. From the results shown in Figure 2, it was observed that

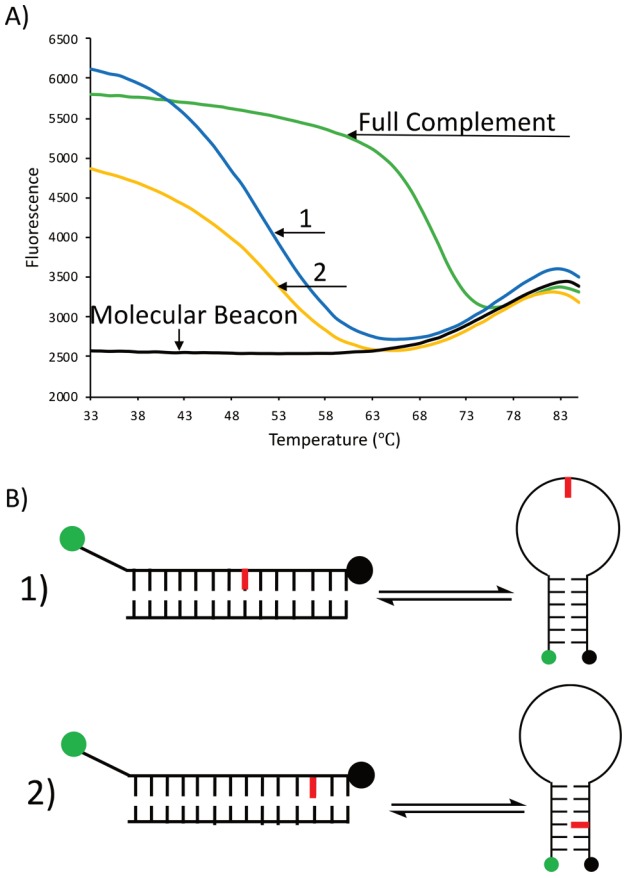

Figure 2. (A) Real-time results of the performed molecular beacon annealing profiles. (B) Representative illustration of the single nucleotide polymorphism (SNP) location within the sequences tested. The green circle represents the fluorophore while the black circle represents the quencher.

the MB did not produce a stable duplex with sequences containing a mismatch in the loop or stem structure. In contrast, the stability of the MB−target duplex was much higher. The mismatched structures are not stable at elevated temperatures; therefore, a fluorescence increase cannot be observed until the temperature is below 58 °C. Conversely, the MB binds with a higher stability to its complementary sequence enabling fluorescence at higher temperatures (65 °C).

To determine whether or not discrimination of mismatched sequences was possible using MB-LAMP, amplification was performed with a sequence containing a single-nucleotide difference in the loop binding region of the MB. The mismatched sequence can be found in Table S1. The SNP was placed in the same location as the mismatch used for the annealing profile experiments described previously. Figure S8 shows the real-time results indicating a significantly reduced fluorescence signal from the SNP-containing target. An 850 RFU difference is observed in the qPCR plots between the maximum fluorescence from the target and the SNP. When the reaction containers were visualized with a transilluminator, there was a clear and unambiguous difference in the appearance of the target and mismatched sequence, as shown in Figure 3. Furthermore, due to HNB in the reaction, a color-

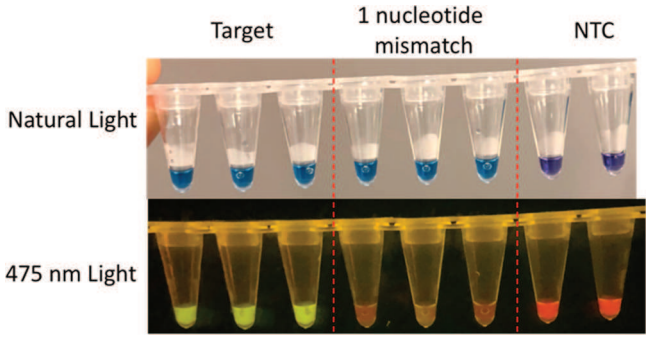

Figure 3. MB-LAMP reaction containers following amplification for 60 min at 63 °C as visualized under natural light or 475 nm light. For the assays containing the target and mismatched sequence, 1.0 pg of DNA was used.

change from purple to light blue using natural light could be observed between the positive and negative reactions (Figure 3). This has potential to be exploited in the future development of a visual, multiplexed detection system by employing natural light and a transilluminator to differentiate between amplification products.

In order to improve discrimination of the target from the mismatched products, the effect of different reaction temperatures on the resulting fluorescence was examined. Six reactions containing the mismatched sequences were carried out at 63 °C, 64 °C, and 65 °C, along with three positive reactions containing the target. After incubating for 1 h at the aforementioned temperatures, the fluorescence at 520 nm was recorded. Results shown in Figure S9 indicate a slight decrease in the fluorescence of the positive reaction at a reaction temperature of 65 °C. A more noticeable difference was observed in the reactions containing the SNP, as there was a significant change in the fluorescence signal as the temperature is increased. This was highlighted by nearly a 2-fold decrease in the fluorescence intensity observed from 63 to 65 °C. The difference between the two temperatures can be attributed to the destabilization of the MB-mismatch complex, as increased temperature reduces nonspecific binding, leading to a total decrease in the fluorescence output.[22]

Certain applications requiring single-nucleotide resolution involve the detection of low quantities of the desired sequence within a large excess of the wild type sequence. To test the ability of the MB-LAMP method in the detection of the target sequence in the presence of interfering DNA, a series of reactions were performed in which the ratio of target DNA to nontarget DNA containing the SNP was varied. Reactions were performed at 65 °C, and the fluorescence was monitored in

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real-time. Furthermore, the reaction containers were visualized after amplification under a transilluminator to verify that results could be visually interpreted. Results shown in Figure S8 demonstrate that the method is capable of detecting the target when it comprises 1% of the total mass of nucleic acid present, where 99% is the mismatched sequence.

In addition to the detection method, POC applications also require robust sample preparation techniques for the isolation of nucleic acids from complex samples. We have previously developed a rapid SPME method using polymeric ionic liquid sorbents for the extraction of DNA from complex samples.[23] An overview of the method can be seen in Figure 4A. One drawback to this method was the long desorption time (30 min) compared to the 2 min extraction time. To improve the overall speed of the method, different desorption containers were explored. By using a small vial featuring an insert, desorption into 50 μL of NaCl could be achieved in just 2 min by using vortex agitation. Results shown in Figure 4B demonstrate that comparable recoveries of the target DNA are observed when using 2 min of vortexing for the recovery in comparison to a 30 min static desorption step. Conversely, when a 2 min static desorption is performed, nearly half as much DNA is recovered versus using vortex agitation for 2 min (0.51 pg and 0.93 pg).

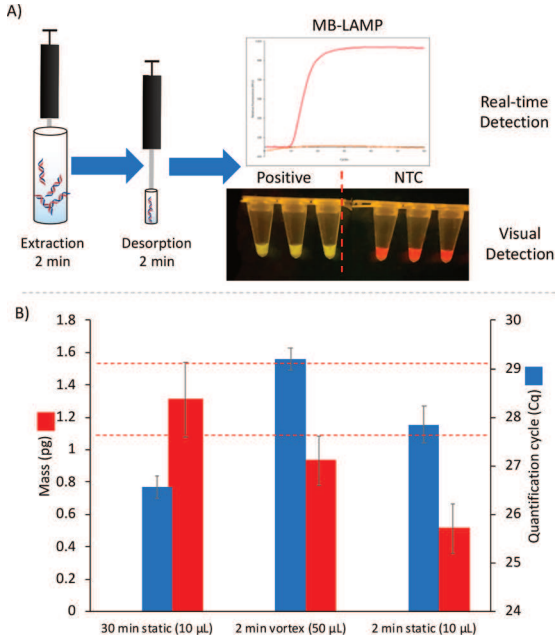

Figure 4. (A) Representative schematic of a SPME-based extraction from a cell lysate with real-time and visual MB-LAMP detection. (B) Comparison of desorption conditions following a 2 min SPME-DNA extraction. All extractions were performed in triplicate from a 84 pg mL^{-1} solution of DNA: total volume, 1.5 mL; 2 mM TE buffer pH 8; extraction time, 2 min with vortex (2500 rpm); desorption solvent, 1 M NaCl.

The compatibility of the extraction method to MB-LAMP was subsequently explored. As illustrated in Figure S10, the method was not tolerant to high concentrations of the desorption solution as evidenced by the increase in threshold times when more NaCl is added to the LAMP reaction. This would require a 10-fold dilution of the desorption solution. To circumvent this, a previously optimized isothermal reaction buffer compatible with 1 M NaCl was tested.[23] Inhibition caused by the NaCl was negated using this buffer allowing for direct interfacing of the SPME desorption solution to the isothermal reaction, thereby eliminating the need for dilution. In order to demonstrate the practical applicability of the method, extractions were performed from a cell lysate prepared from *Escherichia coli* K−12 cells containing a plasmid with the sequence of interest. Detailed culture, lysis, and extraction conditions can be found in the Supporting Information. After performing extractions from cellular suspensions of 10^6 colony forming units mL^{-1}, sufficient DNA was recovered for easy visualization with MB-LAMP. Triplicate extractions were performed to verify the reproducibility of the method. A representative image of the results from one extraction can be seen in Figure 4A. Three positive reactions and 3 NTCs were performed for each extraction.

To determine whether or not SNP discrimination could be possible following SPME-based extraction, annealing profiles were performed in the custom buffer and compared to the annealing profiles previously performed in the traditional Isothermal Buffer. As observed in Figure S11, the annealing profiles are virtually identical when performed in either buffers. This indicates that the specificity of the MB-LAMP method remains when coupled to SPME-based extraction.

The developed method provides a foundation for further development of MB-LAMP for POC applications. Through coupling MB-LAMP to SPME-based sample preparation, a rapid and "user-friendly" method for visually identifying specific amplification products was established. This MB-LAMP method can be readily applied in applications requiring single-nucleotide specificity for diagnostic applications. Future work will focus on expanding the use of SPME as a sample preparation tool with MB-LAMP analysis for cancer diagnostics and drug resistance in pathogens. We also aim to develop sequence-selective extraction methods compatible with MB-LAMP for more sensitive analysis of low-abundance sequences.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.9b01762.

Reagents and instrumentation, cell culture conditions, cell lysate extraction conditions, all oligonucleotide and target sequences used, LAMP calibration curves, HNB optimization including real-time plots, fluorescent measurements and emission spectra, molecular beacon conformations, target/mismatch representative real-time plots, temperature optimization, NaCl effects on LAMP, and annealing profiles in custom and Isothermal Buffer (PDF)

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Notes

The authors declare no competing financial interest.

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