Sequence-Specific Detection of ORF1a, BRAF, and ompW DNA Sequences with Loop Mediated Isothermal Amplification on Lateral Flow Immunoassay Strips Enabled by Molecular Beacons

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Abstract

Loop-mediated isothermal amplification (LAMP) holds great potential for point-of-care (POC) diagnostics due to its speed and sensitivity. However, differentiation between spurious amplification and amplification of the target sequence is a challenge. Herein, we develop the use of molecular beacon (MB) probes for the sequence-specific detection of LAMP on commercially-available lateral flow immunoassay (LFIA) strips. The detection of three unique DNA sequences, including ORF1a from SARS-CoV-2, is demonstrated. In addition, the method is capable of detecting clinically-relevant single-nucleotide polymorphisms (BRAF V600E). For all sequences tested, the LFIA method offers similar sensitivity to fluorescence detection using a qPCR instrument. We also demonstrate the coupling of the method with solid-phase microextraction to enable isolation and detection of the target sequences from human plasma, pond water, and artificial saliva. Lastly, a 3D printed device is designed and implemented to prevent contamination caused by opening the reaction containers after LAMP.

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Introduction

The SARS-CoV-2 pandemic has revealed the dire need for robust and accurate diagnostic platforms that facilitate rapid detection of pathogens. In particular, diagnostic tools that can provide rapid and accurate information with limited laboratory equipment are highly desirable. These tools shorten the time between receiving a clinical sample and obtaining a result, significantly increasing sample throughput and allowing prompt administration of treatment. In addition, easy-to-use diagnostics allow for minimally trained individuals to perform analyses and interpret the results, allowing for the facile establishment of testing centers and enabling in-home testing.

Loop-mediated isothermal amplification (LAMP) is a technique that offers distinct advantages over polymerase chain reaction (PCR) including shorter run-times while maintaining lower or equal detection limits.[1,2,3] In addition, LAMP offers increased robustness and resistance to inhibitors compared to PCR.[4] LAMP also possesses high potential for use in point-of-care (POC) diagnostics due to its simplicity of use and compatibility with colorimetric detection methods. Colorimetric LAMP assays have previously been developed for the detection of various targets including *M. tuberculosis*,[5] *E. coli*,[6] and SARS-COV-2.[7–11]

Traditional detection methods for LAMP include turbidimetry, the use of metal indicators such as calcein and hydroxynapthol blue (HNB)[12] and double-stranded DNA binding dyes such as SYBR Green I.[13] These non-specific methods become a hinderance when designing LAMP diagnostics, as false positives become indistinguishable from actual positive samples. Several attempts have been made to develop probe-based systems for the sequence-specific detection of LAMP. These include, but are not limited to, the use of strand displacement probes,[14–16] detection of amplification by release of quenching,[17] fluorogenic bidirectional displacement probes,[18] and more recently molecular beacons (MBs).[19] We have recently exploited MBs in LAMP for the development of POC assays.[20,21] However, these studies required a dedicated instrument for the detection step. To further simplify detection, a system that does not necessitate additional equipment would be highly beneficial. Phillips et al. developed an instrument-free method which used strand displacement probes to enable specific detection on commercially-available lateral flow immunoassay (LFIA) strips.[16] However, strand displacement probes require a pre-annealing step, thereby

increasing the number of overall steps and total assay time. Moreover, these probes, unlike TaqMan and MBs, are not commonly used for PCR-based diagnostics.

In this study, we the leverage the specificity of MBs to enable detection of LAMP on commercially-available LFIA strips. This strategy is implemented for the qualitative detection of three unique, clinically-relevant sequences. Successful detection of the ompW gene of *Vibrio cholera*, BRAF V600E SNP, and ORF1a gene from SARS-CoV-2 is demonstrated. In addition, this detection scheme yields identical sensitivity to real-time fluorescence detection for all studied sequences. In addition to challenges posed by the specific detection of LAMP, the rapid isolation of sufficiently pure nucleic acid (NA) from complex biological matrixes remains a limiting factor in NA-based diagnostics. To address this challenge, we demonstrate the compatibility of this detection method with solid-phase microextraction (SPME) to enable rapid NA isolation and detection from pond water, human plasma, and artificial saliva.

Experimental

SPME Extraction Procedure

A schematic of the SPME procedure and chemical composition of the sorbent can be found in Figure S1. A 1.0 mL volume of either pond water, plasma, or artificial saliva was placed in a 1.5 mL DNA LoBind tube (Eppendorf, Hamburg, Germany) and spiked with DNA. The SPME sorbent was inserted through the cap of the tube, immersed in the extraction solution, and agitated with a Fisher-Brand digital vortex mixer (Fisher Scientific, Hampton, NH) for 2 min at 2500 rpm. The fiber was then washed in deionized water and transferred into 10 µL of 1 M NaCl desorption solution for 30 min. A 1 µL aliquot of the desorption solution was then subjected to MB-LAMP with lateral-flow detection.

SPME Compatible LAMP Buffer

Reaction mixtures (10 µL) were prepared with the following components: 1.4 mM of each dNTP, 50 mM Tris-HCl pH: 8.8, 8 mM $MgSO_4$, 1.6 µM FIP and BIP primers, 0.2 µM F3 and B3 primers, 0.5 µM of MB, 0.4 µM of biotinylated loop backward primer, 3.2 U Bst 2.0 WarmStart DNA polymerase, and 1 µL of template DNA solution. A 1 µL volume from the SPME desorption solution composed of 1 M NaCl was

added into the reaction mixture. Amplification was performed by heating at 60.5 °C for 1 h on a CFX96 Touch real-time PCR detection system with fluorescence measurements being recorded every 30 seconds. All primers and DNA sequences are shown in Table S1. Detailed information regarding the reagents and standard LAMP reactions are provided in the Supporting Information

3D Printing Device

The 3D printed device was modeled using Autodesk Inventor Professional 2020. An Ultimaker S3 (Utrecht, Netherlands) was used with Ultimaker ABS filament (2.85 mm). A layer height of 0.2 mm, infill density of 100%, and extruder temperature of 220 °C was used. Additionally, the bed temperature was set to 85 °C.

Lateral-Flow Immunoassay Assays

A HybriDetect Universal Lateral Flow Assay Kit was obtained from Milenia Biotec (Giessen, Germany) and used for all lateral-flow experiments. Assays were performed according to the manufacturer's instructions. Additional details can be found in the supporting information.

Results and Discussion

MB-LAMP-LFIA Assay Design and Implementation

The general MB-LAMP-LFIA design is shown in Figure 1A. Briefly, one of the loop primers is converted to a MB labeled with a FAM fluorophore while the other loop primer is biotinylated on the 5' end. This enables incorporation of the biotinylated primer into the amplicon. The MB imparts specificity by hybridizing to the opposite loop, allowing for detection on the LFIA strip only when the biotinylated primer is incorporated into the amplicon and the MB is hybridized to its complementary loop sequence. MBs are dual-labeled oligonucleotide probes possessing a stem-loop structure.[22] Figure 1B shows a representative depiction of the visual appearance that would be expected on a positive and negative LFIA strip.

In a proof-of-concept experiment, LFIA detection was first attempted with the ompW and BRAF sequences. Initial experiments demonstrated clear, specific detection of amplicons on the LFIA strips, as evidenced by the dark test band that appeared when the target sequence was present. Figure S2 demonstrates the sensitivity of the method for each of the chosen sequences. For the ompW sequence, 226

copies per reaction could be observed on the LFIA strips based on triplicate reactions. In addition, two-thirds of the reactions containing 22.6 copies yielded positive results. This sensitivity is equal to that obtained using real-time detection (Figure S3), indicating no decrease in performance when the MB-LAMP-LFIA method was employed. It should also be noted that the intensity of the test bands remained virtually identical when examining different template concentrations. This can be observed by both visual examination of the LFIA strips (Figure 2 A,C) and by ImageJ analysis (Figure 2 B,D). The ability to obtain high test band intensities even when low concentrations of target are amplified is highly beneficial for the unambiguous determination of positive samples. Similarly, as few as 22.4 copies per reaction of the BRAF sequence could be detected in triplicate. These results were also consistent with real-time sensitivity values (Figure S4).

The developed method was then compared with a previous strategy for LAMP detection on LFIA strips that relied on the labeling of the loop primers with either a biotin or FAM moiety.[23,24] As demonstrated in Figures S5 and S6, the labeled primer strategy yielded similar sensitivity to the MB-LAMP method for both the ompW and BRAF sequences. However, light test bands could be observed for the NTCs and the 10^0 copies per reaction of the ompW sequence, indicating lack of specificity of the traditional method (Figure S5 A,B) .

Single-Nucleotide Polymorphism (SNP) Detection

To determine whether MB-LAMP could enable SNP detection on a LFIA strip, triplicate MB-LAMP reactions for a wild-type BRAF sequence and the BRAF V600E variant were performed with a MB complementary to the wild-type BRAF. Figure 2 shows the resulting images and ImageJ analysis following MB-LAMP with LFIA analysis of both wild-type and mutant sequences. Visually, the wild-type can be easily differentiated from the mutant sequence. While a faint test band does appear when the mutant is amplified, the resulting intensity from the wild-type sequence is over 2-times greater, as shown in Figure 2B. As shown in Figure S7, there is no difference in intensity, either visually or with ImageJ analysis, between the wild-type and mutant sequences when the traditional strategy is employed. This result highlights the specificity imparted by the MB when used in LAMP for LFIA detection.

SNPs often represent a small percentage of the total NA population within a sample. Therefore, detecting the target sequence in the presence of interfering sequence is highly desirable. The ability of the MB-LAMP-LFIA method to detect the wild-type sequence in the presence of various amounts of mutant DNA was investigated. It can be observed in Figure 2C that the method is able to detect the wild-type sequence in a 1:1 copy number ratio with the mutant sequence. However, when higher amounts of the mutant sequence are present, the method is unable to detect the wild-type sequence. Future work will focus on developing strategies to enable detection of lower target:mutant ratios.

Assay design for ORF1a detection

Enabling the rapid and simple detection of the SARS-CoV-2 virus is of high worldwide significance. Traditional reverse transcription (RT)-PCR methods can detect extremely low quantities of the viral target (10^1-10^2 copies per reaction)[25]; however, centralized laboratories are required to perform the analysis due to the specialized instruments required and need for highly trained personnel. Enzyme linked immunosorbent assays are an alternative to RT-PCR and require less equipment. However, these tests have higher rates of false negatives compared to RT-PCR tests which can lead to an underestimation of total positive cases.[26]The development of an MB-LAMP method compatible with the LFIA detection scheme would be highly beneficial. Such a method would provide high accuracy, due to the specificity imparted by the MB probe, and facilitate easy interpretation of results. Several LAMP methods have been developed for SARS-CoV-2 detection. However, the majority of these methods rely on non-specific, colorimetric indicators for positive sample identification. Very recently, a preprint by Sherrill-Mix et al. demonstrated the use of molecular beacons containing locked nucleic acid bases in LAMP for the fluorescence detection of SARS-CoV-2.[27]

A recently developed LAMP primer set by Zhang et al. targeting the ORF1a gene of the SARS-CoV-2 virus was used for the assay in this study, with some modifications.[10] The forward loop primer was chosen to be converted into a MB, while the backward loop primer was biotinylated at the 5’ end. MB stem length plays an important role in determining the MB’s annealing temperature to its desired target. Two MBs with stems consisting of 5 and 6 nucleotides (nt) were designed and used for LAMP

analysis with real-time fluorescence and LFIA detection. Figure 3A shows the LFIA strips following amplification with each MB. Visually, the test band is significantly darker when the 5-nt stem MB is used. ImageJ analysis, shown in Figure 3B, reveals that the 5-nt stem MB yields an approximate two-fold increase in band intensity compared to the 6-nt stem MB. In both cases, the NTCs remained clear without a test-band. Annealing profiles were performed with both MBs to determine whether the annealing temperature to the target was dependent on stem length and if these results correlated to the observed band intensities. Fluorescence plots in Figure S8 show an apparent decrease in the melting temperature of the MB to its target when the stem length is increased from 5 to 6 nucleotides.

MB concentration could play a significant role in the perceived intensity of the test band, and therefore in assay efficacy. Initially, several concentrations of MB ranging from 0.1-0.6 μM were tested using real-time fluorescence monitoring and LFIA analysis. Results in Figure S9 (A-B) show that test-band intensity remains fairly constant across the concentrations tested, with minimal drop-off between the highest (0.6 μM) and lowest (0.1 μM) concentrations. Interestingly, when compared with the real-time fluorescence results, the decrease in the endpoint fluorescence with lower MB concentrations following amplification is significant (Figure S9 C,D). Several lower concentrations of MB (0.05 μM-0.005 μM) were also tested to identify where a decrease in the test-band intensity may be observed. Results shown in Figure S10 illustrate a perceived decrease in the test band intensity for the concentrations tested. In particular, 0.025 μM and 0.005 μM have significantly different appearance and intensities when compared with 0.05 μM. When compared to the real-time results, the 0.025 μM and 0.005 μM samples were unable to be detected. These results suggest that LFIA detection is more tolerant to changes in MB concentration than real-time fluorescence detection. The sensitivity was then determined for LFIA detection using the 5-nt stem MB. Results in Figure 3 C and D show the method is capable of detecting as few as 2.3×10^2 copies per reaction. These results are consistent with the real-time data obtained in our laboratory as well as previously demonstrated colorimetric and real-time results.[11]

Isolation of DNA from Complex Matrices using SPME

NA isolation from complex samples is a critical step in order to minimize the introduction of inhibitors into the enzymatic reaction and increase DNA concentration for optimal detection. SPME is a sample preparation technique that circumvents some of the drawbacks of traditional methods. Recently, polymeric ionic liquids (PIL) have been shown as SPME sorbent phases for the isolation of NAs from complex samples.[28,29] During the desorption step, 1 M NaCl is used to desorb DNA from the PIL-based sorbent phase. However, high salt concentration is known to inhibit and delay LAMP reactions.[28] To circumvent this, a custom-buffer was developed that used NaCl from the desorption solution to avoid dilution.[28] Figure S11 illustrates a comparison between LFIA following MB-LAMP of pure ORF1a sequence in either NEB Isothermal Buffer or the custom-buffer. As shown, the resulting test-band intensities are similar between the two buffers, indicating similar performance on the LFIA strips. Triplicate extractions were performed from human plasma spiked with the wild-type BRAF sequence at clinically-relevant concentrations (10^7 copies mL^{-1}).[30] Successful detection was achieved on the LFIA strips in all extractions performed. Table S2 contains a summary of the MB-LAMP-LFIA results after SPME from all matrices tested. Detection was also achieved for ompW and ORF1a in pond water and artificial saliva, respectively. A recent study suggested that saliva may yield better sensitivity for SARS-CoV-2 virus detection compared to nasopharyngeal swabs,[31] which would allow for easier sample collection and lower detection limits.

Integration of 3D Printed Device to Minimize Contamination

A significant disadvantage of LFIA is the need to open the reaction containers following amplification to add the assay diluent and the strip. Opening the reaction container can lead to widespread contamination of the environment with the amplicon, leading to positive NTCs. A closed system where the amplification and subsequent detection step can be performed is highly desirable. Commercially-available systems that can enclose reaction systems post-amplification to enable detection on LFIA do exist. However, this system increases the cost and complexity of analysis. A closed system where the amplification step and LFIA detection can be performed all-in-one would be highly beneficial.

3D printing represents a promising alternative for the creation of cheap, simple, and easy-to-use diagnostic devices. In this study, fused-deposition modeling is used to create a device that can mitigate contamination by enclosing amplification and LFIA detection. Figure S12 shows a model and image of the device. The device is composed of two "trap doors" and one inlet to enable the addition of the LFIA buffer. During amplification, the device is placed over a conventional polypropylene PCR tube and a 3D printed covering is placed through slit #1 and slit #2 (Figure S12). The LFIA strip is placed in the device and rests on slit #2 while amplification progresses. After amplification, the covering on slit #1 can be removed and the assay buffer inserted through the diluent port. Once the reaction mixture and assay buffer are mixed, the covering from slit #2 can be removed to allow the LFIA strip to reach the resulting mixture and enable detection through the LFIA test viewing window. This device is also compatible with PCR, recombinase polymerase amplification or any other amplification-based technique coupled with LFIA detection.

Conclusions

In this study, the instrument-free, specific detection of LAMP enabled by molecular beacons was demonstrated on commercially-available LFIA strips. The versatility of the method was demonstrated by the successful detection of three unique, clinically relevant sequences (ompW, BRAF, and ORF1a). High specificity was also demonstrated through the detection and differentiation of a clinically-relevant SNP (BRAF V600E). In addition, SPME is shown to be compatible with the developed MB-LAMP-LFIA method to enable detection of nucleic acid sequences from pond water, human plasma, and artificial saliva. The developed method has potential for use in low-resource settings and peripheral laboratories to enable sensitive and specific detection of diseases.

Supporting Information: DNA primers and sequences used, PIL structure, SPME extraction results, sensitivity for LAMP assays, annealing profiles, MB concentration optimization, 3D printed device diagram and image, reagents, and LAMP conditions.

Acknowledgements: J.L.A. acknowledges funding from the Chemical Measurement and Imaging Program at the National Science Foundation (Grant No. CHE-1709372). J.L.A. and D.R.E. thank the Alice Hudson Professorship for support.

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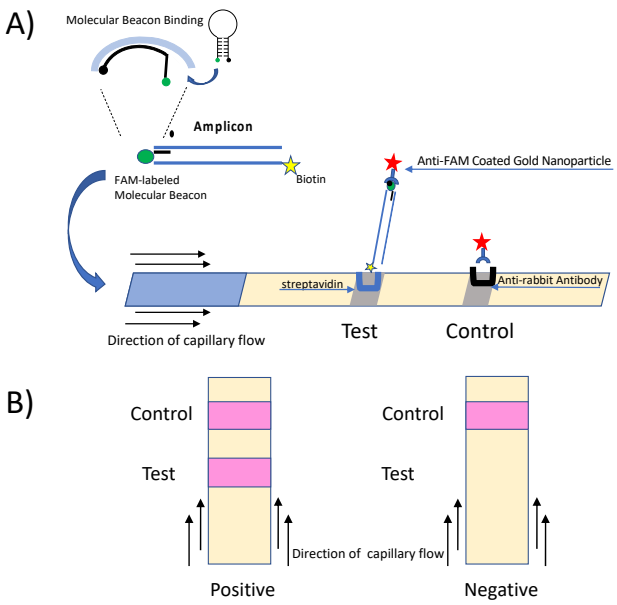


Figure 1. A) Schematic representation of MB-LAMP detection on a commercially-available LFIA strip. B) Representative illustration of LFIA strips indicating positive or negative samples.

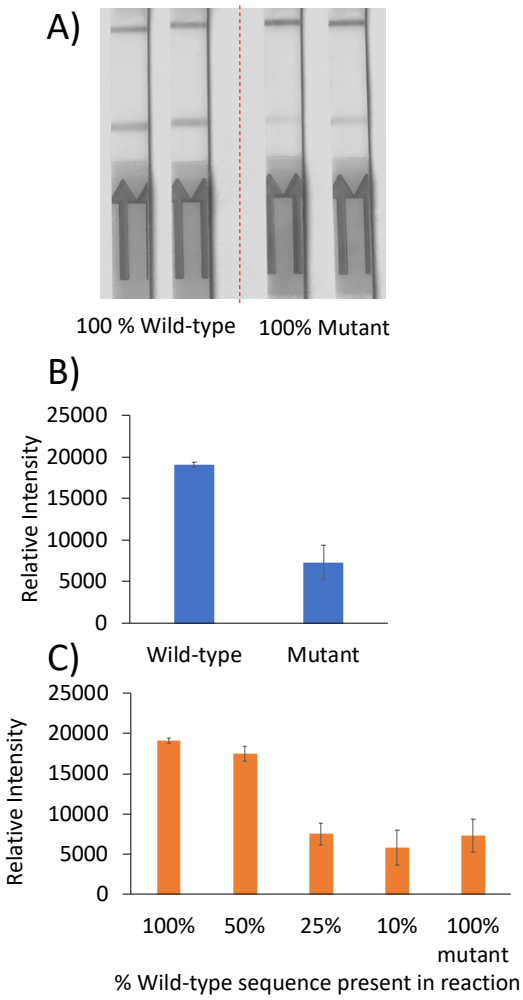


Figure 2. A) MB-LAMP-LFIA strips of the wild-type or mutant BRAF sequences using a FAM-labeled MB complementary to the wild-type sequence. B) Intensity of the test bands following ImageJ analysis of 100% wild-type and 100% mutant BRAF sequences. C) Resulting intensities of the test-bands following ImageJ analysis of various ratios of wild-type:mutant sequence. Triplicate reactions were used for analyses performed on ImageJ.

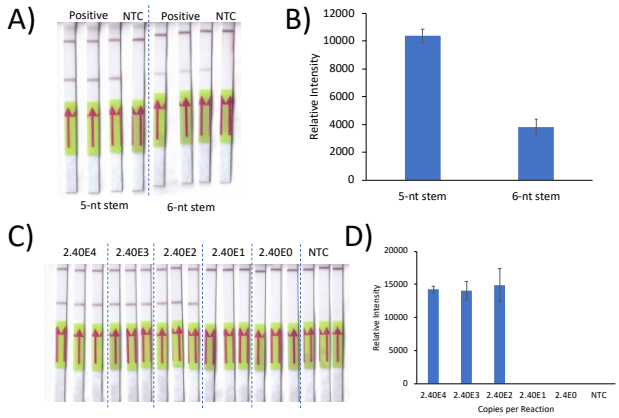
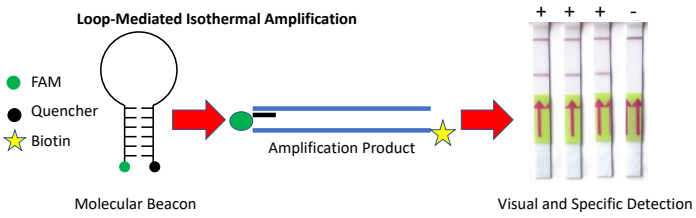


Figure 3. A) MB-LAMP-LFIA analysis of the ORF1a gene from SARS-CoV-2 using either a 5-nt stem or 6-nt stem MB for analysis. B) ImageJ analysis of the band intensities obtained from the 5-nt stem and 6-nt stem molecular beacons following MB-LAMP. C) Image of sensitivity results using the 5-nt stem MB and D) ImageJ analysis of the test-bands. Triplicate reactions were performed for all ImageJ analyses.

TOC Image


Loop-Mediated Isothermal Amplification
FAM
Quencher
Biotin
Amplification Product
+ + + -
Molecular Beacon
Visual and Specific Detection