Advances in Mutation Detection using Loop-Mediated Isothermal Amplification

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Abstract

Detection of mutations and single-nucleotide polymorphisms is highly important for diagnostic applications. Loop-mediated isothermal amplification (LAMP) is a powerful technique for the rapid and sensitive detection of nucleic acids. However, LAMP traditionally does not possess the ability to resolve single-nucleotide differences within the target sequence. Due to its speed and isothermal nature, LAMP is ideally suited for point-of-care applications in resource limited settings. Recently, different approaches have been developed and applied to enable single-nucleotide differentiation within target sequences. This mini-review highlights advancements in mutation detection using LAMP. Methods involving primer design and modification to enable sequence differentiation are discussed. In addition, the development of probe-based detection methods for mutation detection are also covered.

Introduction

Nucleic acids (NAs) play a prominent role as biomarkers for a wide range of diseases. Since its development, polymerase chain reaction (PCR) has been broadly applied for NA diagnostics and has become the gold standard for many applications. PCR leverages the unique Watson-Crick base pairing of NAs to amplify specific sequences through multiple heating and cooling cycles. To further increase PCR specificity, fluorescent probes including TaqMan and molecular beacons are often used to discriminate single nucleotide differences within an amplified sequence. This has allowed for the development of assays capable of discriminating single-nucleotide polymorphisms (SNPs) for certain diagnostics such as cancer[1] and multi-drug resistant tuberculosis (MDR-TB).[2]

While PCR is considered the gold standard for many diagnostic tests, there remain formidable drawbacks that prevent its use in resource-limited settings and peripheral laboratories. Significant hinderances include the need for sophisticated thermal-cycling equipment and imaging modules for real-time detection. In addition, some PCR methods require extended incubation periods (<1 h) and can suffer inhibition from molecules present in biological matrices.[3] These limitations increase the difficulty of developing PCR-based diagnostics that can be performed in non-laboratory settings.

Isothermal amplification techniques have been developed and applied for NA detection in order to circumvent the aforementioned drawbacks. These techniques include, but are not limited to, recombinase polymerase amplification (RPA),[4] rolling circle-amplification (RCA),[5] and loop-mediated isothermal amplification (LAMP).[6] The isothermal nature of these methods eliminate the need for complex thermal cycling equipment and high temperatures. In addition, several colorimetric detection methods have been developed to allow for easy visualization and identification of positive samples.[7,8]

LAMP has been the most popular and widely implemented isothermal amplification technique. It relies on 4-6 primers and a DNA polymerase possessing strong strand displacement activity to amplify the target NA sequence. A general amplification schematic is shown in figure 1A. These characteristics have allowed LAMP to achieve equal or lower detection limits to PCR for certain targets.[9] However, due to the length of the primers and the concentration that is often required, LAMP suffers from non-specific amplification arising from the formation of primer-dimers.[10]

Traditional detection methods for LAMP include turbidimetry and the use of dyes to identify when amplification has occurred. Popular dyes include metal indicators such as hydroxy naphthol blue (HNB)[7] and calcein,[11] which change color as the Mg^{2+} concentration decreases during the progression of the amplification reaction. Other dyes, such as SYBR Green I,[12] exhibit increased fluorescence in the presence of double-stranded DNA that is generated during amplification. These traditional methods are unable to differentiate between amplification of the desired sequence and spurious amplification occurring from primer-dimers. Careful primer design and reaction optimization can be used to minimize non-specific amplification and avoid false positive reactions. However, these strategies are unable to provide sufficient specificity when attempting to achieve single-nucleotide differentiation in sequences. Differentiating these types of sequences is highly desirable as they can be an indicator of disease. Therefore, tools capable of rapidly and accurately identifying these sequences are highly desired.

LAMP provides a platform for the rapid identification of sequences that can be deployed in resource-limited settings. Becherer et al. recently published a comprehensive review which discusses advancements made for sequence-specific detection of LAMP.[13] Herein, we discuss recent advances in the development of sequence-specific LAMP detection methods with particular emphasis on the differentiation of mutations/single-nucleotide differences. Several different strategies have been developed and can be broadly categorized into primer- and probe-based approaches. Primer-based methods rely on primer design and modification to achieve differentiation of similar sequences. Probe-based approaches typically involve the modification of LAMP primers with fluorophore/quencher pairs to provide a sequence-specific signal.

SNP detection using primer-based approaches

Single Enzyme Methods

Ding et al. reported a strategy for SNP detection called probe-enhanced loop-mediated isothermal amplification (PE-LAMP).[14] This strategy relies on designing LAMP primers to contain the SNP within the region of one of the loop-primers. Differentiation between the wild-type and SNP sequence was achieved due to the difference in amplification speed. When the loop-primer that was a perfect complement to the sequence was present, the LAMP reaction was significantly accelerated compared to when the mismatch was in the reaction. One important parameter that was identified and optimized was the length of the loop-primer. It was found that the shortest (11 nucleotide (nt)) loop-primer tested yielded an amplification time difference of greater than 40 min between the perfect complement and SNP. This was likely due to the decreased melting temperature of the loop-primer and subsequent reduced stability when a mismatch was present. The method was paired with neutral-red dye for colorimetric detection allowing the SNP to be detected when it comprised only 0.1% of the total NA concentration in the sample.

A similar strategy was developed by Itonaga et al. and involved the incorporation of a peptide-nucleic acid (PNA) and locked nucleic acid (LNA) for the detection of KRAS mutations.[15] This gene codes for a protein within the RAS/MAPK pathway which is responsible for relaying signals from the exterior of the cell to the nucleus. This assay involved the addition of a PNA sequence that was complementary to the wild-type allele. When the wild-type sequence was present, the PNA was able to bind resulting in a significant slowing of the reaction. In addition, a LNA that was complementary to the mutant sequence was included and this allowed for rapid amplification to occur only when the mutant allele was present. Calcein was used as the dye for detection which enabled the use of a real-time qPCR instrument as well as viewing the reaction containers with the naked eye. The assay was applied for the mutation detection of four distinct cell lines. Amplification was unable to be detected from two wild-type cell lines while positive reactions were observed for the two mutant cell lines tested. Moreover, several different ratios of mutant:wild type DNA were tested to identify the limit of detection of the method. It was found that the mutant could still be detected down to a 0.1% mutant to wild-type ratio.

An allele-specific LAMP (AS-LAMP) method was developed by Carlos et al. that utilized gold nanoparticles functionalized with single-stranded DNA (ssDNA) for the detection of a SNP responsible for lactose intolerance.[16] This method consisted of performing two parallel LAMP reactions, which contained a F3 primer that was either complementary to the wild type or the mutant sequence. The SNP was placed in the 3' end to prevent amplification from occurring if a mismatched sequence was present. Gold nanoparticles served as the detection platform as their visual appearance changed based on the presence of the amplified target due to aggregation when the target was absent. A blue color indicated a negative reaction while a pink appearance revealed the presence of the target. The capability of the method to detect the mutation was tested using 6 biological samples of each genotype. This approach allowed for discrimination based on the presence of the correct F3 primer as well as the addition of the ssDNA functionalized nanoparticles. The ssDNA sequence was complementary to a portion of the LAMP amplicon facilitating aggregation if the desired sequence was amplified, in addition to enabling differentiation between spurious and specific amplification.

In another study, Malpartida-Cardenas et al. developed an AS-LAMP method for SNP detection.[17] Similar to the previous study, this work utilized two separate, parallel reactions to independently identify the mutant and wild-type allele. The strategy involved the incorporation of two extra primers, termed unmodified self-stabilizing primers (USSP), to delay amplification of the mismatched sequence. These primers were designed to possess the SNP at the 5' end and target the F1 and B1 regions of the LAMP target. In the mutant reaction, USSPs complementary to the wild-type sequence were added while the reverse was done for the wild-type reaction. These primers competed with the FIP and BIP primers during the loop-formation of the amplification process, thereby delaying amplification when the complementary sequence was present. A general overview of the method is represented in Figure 2. A wide range of parameters were optimized and tested during the study to create universal-primer design guidelines. One interesting result was a comparison of modified and unmodified USSPs. The modified primers contained a blocking group on the 3' end and prevented extension from occurring. Surprisingly, it was found that the modified primers performed comparably to their unmodified counterparts in some cases and worse in others. This approach was successfully applied for the detection of two SNPs responsible for resistance to artemisinin-based combination therapies in malaria treatment and *PIK3CA* p.H1047R breast cancer mutation.

. Several other AS-LAMP assays have been developed following a similar strategy by placing the SNP on the 5' end of both FIP and BIP primers to delay amplification of the untargeted sequence. Zhang et al. developed an assay for the detection of CYP2C19 polymorphisms from clinical samples.[18] Tamura et al. developed and applied an AS-LAMP assay for the detection of N526K *ftsI* mutation of β-lactamase-negative ampicillin-resistant *Haemophilus influenzae.*[19] This strategy was also applied for the detection and differentiation of wild-type and vaccine strains of Mink Enteritis Virus.[20] Differentiation and genotyping of ABO blood types was also achieved with an AS-LAMP method.[21]

An approach was developed by Yongkiettrakul et al. for SNP detection of antifoulant resistant *Plasmodium falciparum* which employed a lateral-flow dipstick for detection.[22] Primer design was performed manually and contained the SNP location on the 5' end of the FIP and BIP primers. A 5' modified fluorescein isothiocyanate (FITC) primer was used to enable detection on the lateral flow devices. It was unclear which primer was modified with biotin to enable the detection.

Multiple enzyme methods

In addition to utilizing primer-design to detect SNPs, several studies have developed methods that incorporate additional enzymes in the reaction system. Du et al. developed a strategy for the detection of the most common KRAS mutation (codon 12, G>T).[23] The assay involved the RNase H2 enzyme and a modified BIP primer containing an RNA base at the SNP location and a 3' blocker to prevent extension. The RNase H2 enzyme binds to RNA-DNA duplexes and cleaves the RNA strand, enabling extension of the primer if the perfect complement is present. If the mismatched sequence is present, the amplification reaction is significantly delayed. Initial experiments to demonstrate the specificity of the enzyme for the desired target were performed by incubating a short oligonucleotide probe containing an internal FAM fluorophore, a 5' quencher, and an RNA base in between the fluorophore-quencher pair. This probe was incubated with either an oligonucleotide that was complementary to the probe or contained the SNP and the RNase H2 enzyme. Fluorescence was measured and observed to increase exponentially when the perfect complement was present while increasing linearly when the mismatch was added. A mutation abundance as low as 0.01% could be detected with this technique. The method was expanded and shown to successfully discriminate between complement and mismatch when any RNA base was used (e.g., rA, rU, rC, rG).

A different approach by Fu et al. incorporated a ligase to initiate the LAMP reaction and differentiate between mismatches.[24] The assay consisted of two ligation substrates with each forming a part of the stem-loop dumbbell structure required for LAMP. One substrate was complementary to both the mutant and wild-type sequence and the other substrate contained the SNP on the 3' end as well as another mismatch 2 bases away to further destabilize binding of non-complementary sequences. When the mutant sequence was present, hybridization occurred which allowed for the ligation reaction to occur and subsequent generation of the dumbbell structure. The generated dumbbell was then transferred to a separate reaction container where LAMP was performed with additional BIP and FIP primers. A few parameters tested and optimized included the addition of a second mismatch in one of the ligation substrates. Several different conditions were tested which placed the second mismatch 1,2,3,4, and 5 nucleotides away from the 3' SNP determining mismatch. It was found that the largest difference in amplification time between the mutant and wild-type sequences was achieved when the second mismatch was placed 2 bases away. However, a drawback to this method was the need for an independent ligase reaction that required a temperature program (30 heat and cool cycles), which increased the sample-handling steps, total time, and complexity of instrumentation required.

Probe-based approaches for SNP detection

Higgins et al. developed a method which utilized a modified loop-primer probe and endonuclease IV for SNP detection.[25] The probe consisted of the following three components: a 5' quencher, an abasic site, and an internal fluorophore. This approach exploits the enhanced endonuclease activity when a double-stranded abasic site is present. When the mutant sequence was present, the endonuclease cleaved the abasic site and allowed for the fluorophore to be displaced by the polymerase, leading to an increase in observed fluorescence. If the wild-type sequence was present, endonuclease activity was significantly reduced and little to no fluorescence signal could be detected. The approach was successfully applied for the single-plex and multiplex detection of different targets. Multiplexed detection could occur by choosing different fluorophores in the modified loop-primer. This approach improves upon TEC-LAMP which utilizes *Tth* endonuclease IV as the enzyme, as it was unable to differentiate between SNPs using similar probes.

Another probe-based approach that has been developed involves the use of strand-displacement probes to achieve discrimination between perfectly matched sequences and SNPs.[26] These probes were designed to target the single-stranded loop regions between the F1/F2 and B1/B2 regions of the LAMP target. A representative schematic of the approach is shown in Figure 3. Strand-displacement probes were composed of two components: reporter F and reporter Q. Reporter F was complementary to the target and is 3' or 5' modified with a fluorophore. Reporter Q hybridizes with reporter F, contains a corresponding quencher, and is generally shorter than reporter F by 10 nucleotides. In the presence of the target sequence, reporter F will bind to the target leading to the displacement of reporter Q, resulting in an increase in the fluorescence detected. The length of reporter F is critical in allowing rapid strand-exchange to occur under LAMP conditions. The SNP-detection capabilities of the method was tested with BRAF V600E, a mutation present in 90% of melanomas. Significantly higher fluorescence could be observed when reporter F was complementary to the target sequence. Discrimination was most successful at a temperature of 60 °C, as higher temperatures potentially reduced the stability of the reporter F and Q duplex. The method was shown to successfully detect down to 5% of the mutant allele in the presence of 95% wild-type sequence.

The aforementioned approach was subsequently modified by Du et al. for the detection of the same target using low cost, commercially-available lateral-flow immunoassay strips (pregnancy tests).[27] A modified reporter was conjugated with human chorionic gonadotropin (hCG), which could be detected by the pregnancy strips. Two different approaches were employed, as shown in Figure 4. It was found that the large LAMP amplicons were unable to migrate through the lateral flow device. Therefore, in one approach, a LAMP positive reaction was indicated by a negative test strip as the hCG modified reporter was bound to the amplicon and unable to migrate through the lateral-flow device. Conversely, amplification of the mismatched sequence yielded a positive signal in the test-band, as the reporter was not incorporated into the amplicon. Another approach was developed to yield a positive test strip result in the presence of the target nucleic acid and relied on a three-way junction reporter that contained a sequence complementary to the target bound to a magnetic bead and a hCG modified sequence. In the presence of the complementary sequence, the magnetic bead-labeled primer hybridized with the target and enabled release of the hCG-labeled sequence. Upon magnetic separation, the hCG-labeled sequence could be detected on the lateral-flow device. One disadvantage of strand-displacement probes is the required pre-annealing step to make the probe. This adds an additional step to the process, which increases assay complexity.

Recently, molecular beacons (MBs) have been demonstrated to impart sequence-specificity to LAMP detection.[28] MBs are dually-labeled with a fluorophore-quencher pair that possess a hairpin structure that remains closed until a target sequence is present. Upon hybridization with the target, the probe ‘opens up’ and leads to an increase in fluorescence. In absence of the target, the hairpin structure remains closed resulting in minimal fluorescence. These probes have previously been shown to successfully detect SNPs using qPCR and show great promise in LAMP assays.

Varona et al. demonstrated the successful visual detection of a SNP by the combined use of a MB and HNB, a traditional non-specific indicator of amplification.[29] The strategy involved the use of a transilluminator for the visual identification of positive reactions. Figure 5 shows a representative image of the results. When a negative reaction occurred, strong red fluorescence could be observed due to the presence of HNB. Strong green fluorescence was observed in the presence of the perfect complement while the SNP sequence resulted in significantly decreased green fluorescence. Several parameters were optimized in order to achieve the greatest visual differentiation. The reaction temperature was found to be important to consider as it allows for greater destabilization between the MB and the mismatched sequence. HNB concentration was also varied in order to achieve a clear and distinct signal from the negative samples. A 1% mutation abundance could be visually differentiated with this method.

In a subsequent study, an assay was designed for the detection of BRAF V600E with MB-LAMP.[30] Two distinct MBs possessing two fluorophores (FAM and HEX) that were complementary to either the wild-type (FAM) or mutant (HEX) alleles were designed. Endpoint detection was performed with a plate reader, negating the need for real-time fluorescence measurements and allowing for detection of 5% mutation abundance using this assay. In addition, the method could be coupled with polymeric ionic liquid-based solid-phase microextraction for the isolation of DNA from human plasma in clinically-relevant concentrations, demonstrating its potential in clinical applications. The biggest challenge associated with MB-LAMP assay implementation is the MB design. Loop-primers must be carefully chosen to maximize the stability of the MB-to the target while destabilizing the mismatched sequence. In addition, careful optimization of the stem must be made to achieve optimal results.

Ding et al. developed an interesting strategy with a MB-like probe containing an RNA nucleotide in the SNP location as well as incorporating the use of RNase H2.[31] In this approach, the hairpin structure of the MB was eliminated and the probe became linearized during the amplification process (61 °C). If the complementary (mutant) sequence was present, the RNase H2 cleaves the RNA nucleotide, allowing for separation of the fluorophore-quencher pair and accelerated amplification. Upon completion of the reaction and cooling to room temperature, high fluorescence could be observed due to cleaving of the MB by the RNase. However, when the mismatch (wild-type) was present and the RNase was unable to hydrolyze the probe, the MB remained intact and formed the hairpin structure upon cooling. A significant decrease in the observed fluorescence can be observed when reactions containing the wild-type sequence were performed. A schematic of the method and visual appearance of the reaction containers is shown in Figure 6. The developed approach was applied for the detection of a KRAS mutation with successful detection being achieved utilizing real-time fluorescence detection and visually with a transilluminator. Ten (10) copies per reaction could be positively identified and a mutation abundance as low as 0.01% was successfully detected with both methods. Detection was achieved from pure plasmid DNA as well as genomic DNA from KRAS mutant type cells (LS 174T cell).

Tani et al. previously developed a universal probe called the QProbe for the detection of SNPs following PCR amplification using melt-curve analysis.[32] This method was later applied for SNP detection in LAMP by Ayukawa et al.[33] The QProbe is a short, 3' fluorophore-labeled LNA oligonucleotide. The complementary sequence of the QProbe is added onto a short sequence that is complementary to the desired target. A key aspect of the target sequence is the need for a guanine base at the 5' end. When these conditions are satisfied, the target and its complement hybridize, which brings the QProbe sequence in close proximity to the guanine base, leading to a quenching of fluorescence. After amplification, a melt-curve is performed and the derivative of the fluorescence calculated. Mismatches in the target sequence will lead to decreased duplex stability and lower melting temperatures. A significant drawback to this method using LAMP is the need for real-time fluorescence monitoring as well as precise thermal cycling equipment.

A similar strategy employed by Komura et al. also relied on differential annealing curves to differentiate between SNPs.[34] In this approach, two separate probes were designed and each contained either a fluorophore or quencher of a quencher pair. These probes were designed to bind in close proximity to each other within the amplicon, allowing quenching when both probes bound their respective targets. The quencher probe was designed to contain the SNP region. Following LAMP, the annealing temperature could be determined by monitoring the fluorescence; as the temperature decreased, the quencher probe was able to bind its complementary target at higher temperatures than the mismatch, resulting in significant difference in the annealing temperature and allowing for the differentiation of SNPs.

Conclusions and Outlook

LAMP is a powerful tool for the rapid amplification of NA sequences and holds great promise for use in diagnostic applications. In particular, SNP detection remains a highly relevant and significant field of research. LAMP has incredible potential for use in SNP detection due to its speed and low detection limits. This is particularly the case for point-of-care applications or in resource limited settings where affordable, rapid, and specific methods are highly desired or absolutely required. This mini-review highlights various methodologies that have been developed to enable fast and accurate SNP identification. The development of a robust, universal technique for SNP detection could allow for LAMP-based systems to be more widely accepted in the diagnostic industry. In addition, the development of digital LAMP assays capable of SNP differentiation would be beneficial in order to provide highly quantitative information from what is typically a qualitative technique.

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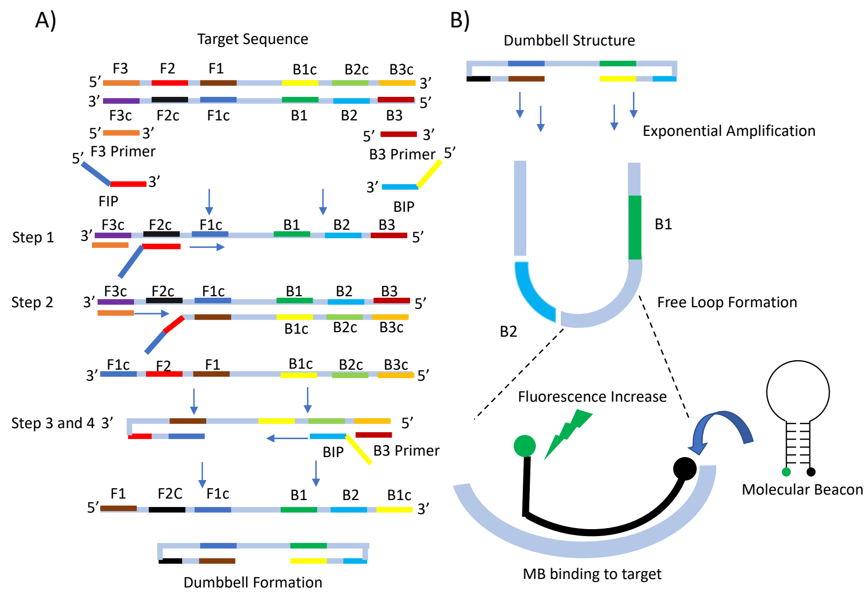

Figure 1. A) General schematic describing LAMP. B) Illustration of mechanism for MB-based specific detection of LAMP. Adapted with permission from ref. 29. Copyright 2019 American Chemical Society.

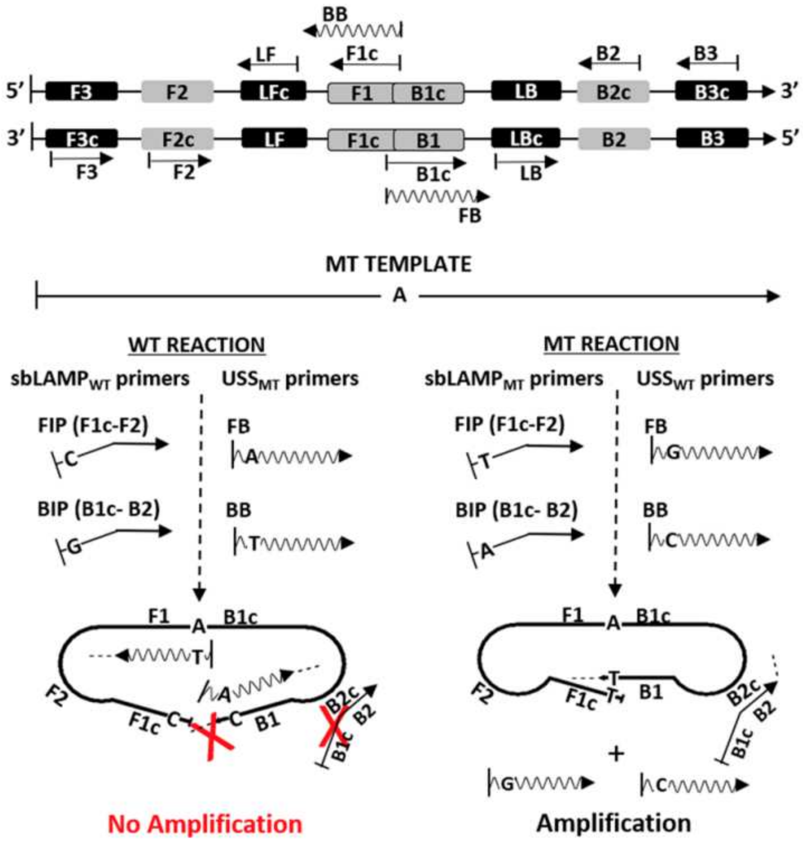

Figure 2. Allele-specific LAMP using unmodified, self-stabilizing primers for SNP detection. Adapted with permission from ref. 17. Copyright 2018 American Chemical Society.

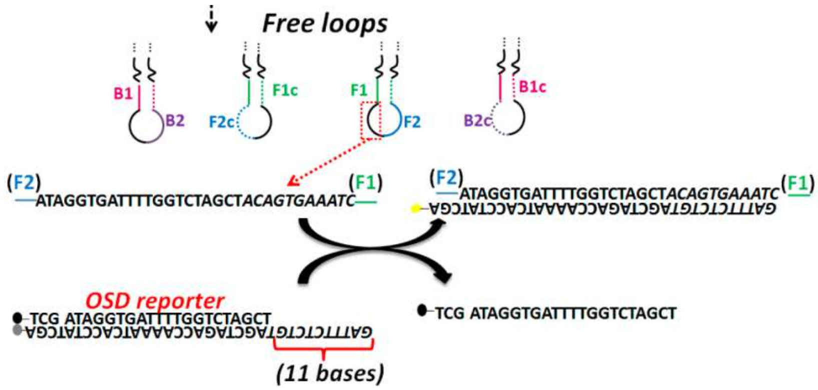

Figure 3. Schematic representing the specific detection of LAMP using OSD reporter probes. Adapted with permission from ref. 26. Copyright 2015 American Chemical Society.

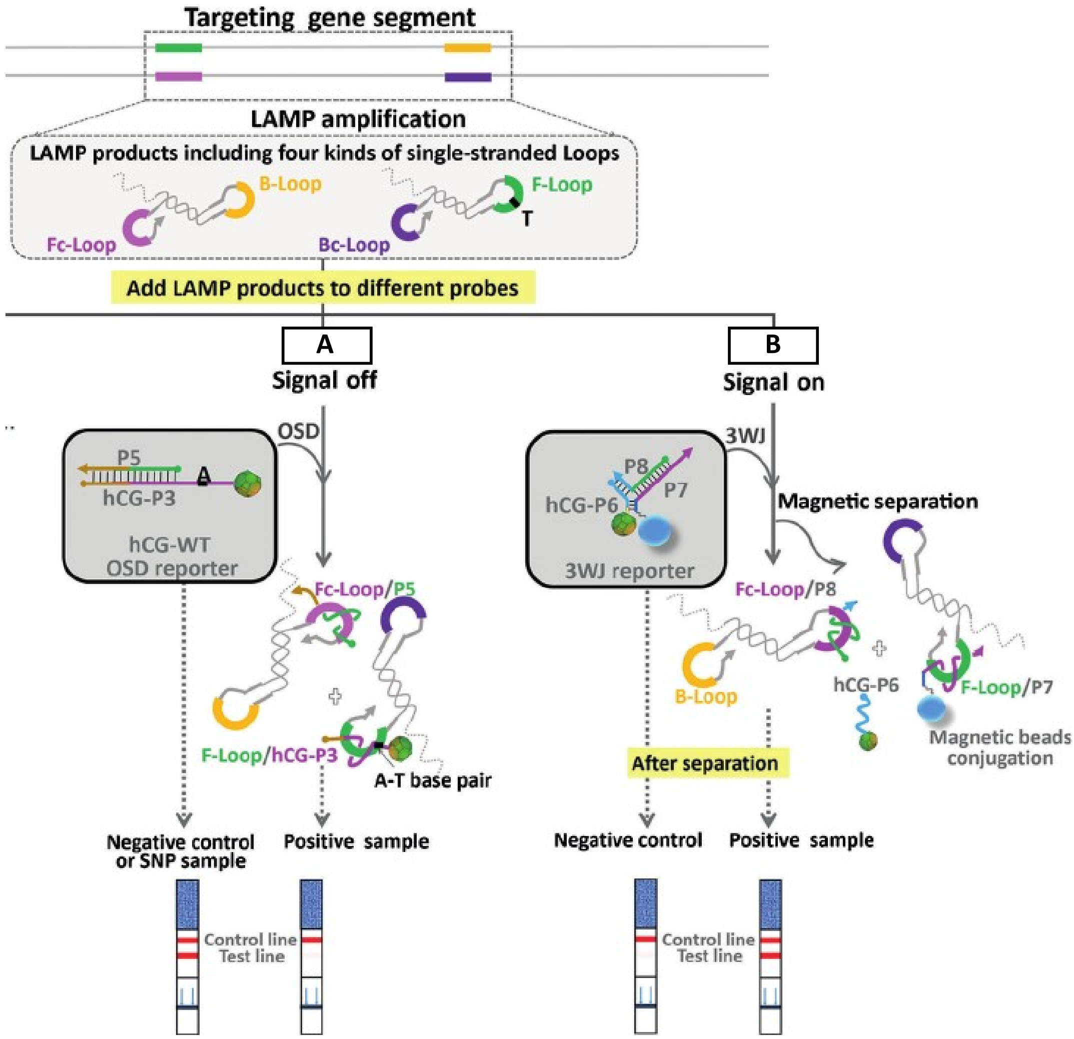

Figure 4. Overview of strand-displacement approaches to achieve detection of BRAF V600E on commercially-available pregnancy test strips. A) hCG-modified probe binds to the LAMP amplicon leading to a negative signal on the test strip. B) A three way junction is used that displaces an hCG modified oligonucleotide during LAMP to yield a positive test strip when the target is detected. Adapted with permission from ref. 27. Copyright 2016 Wiley-VCH.

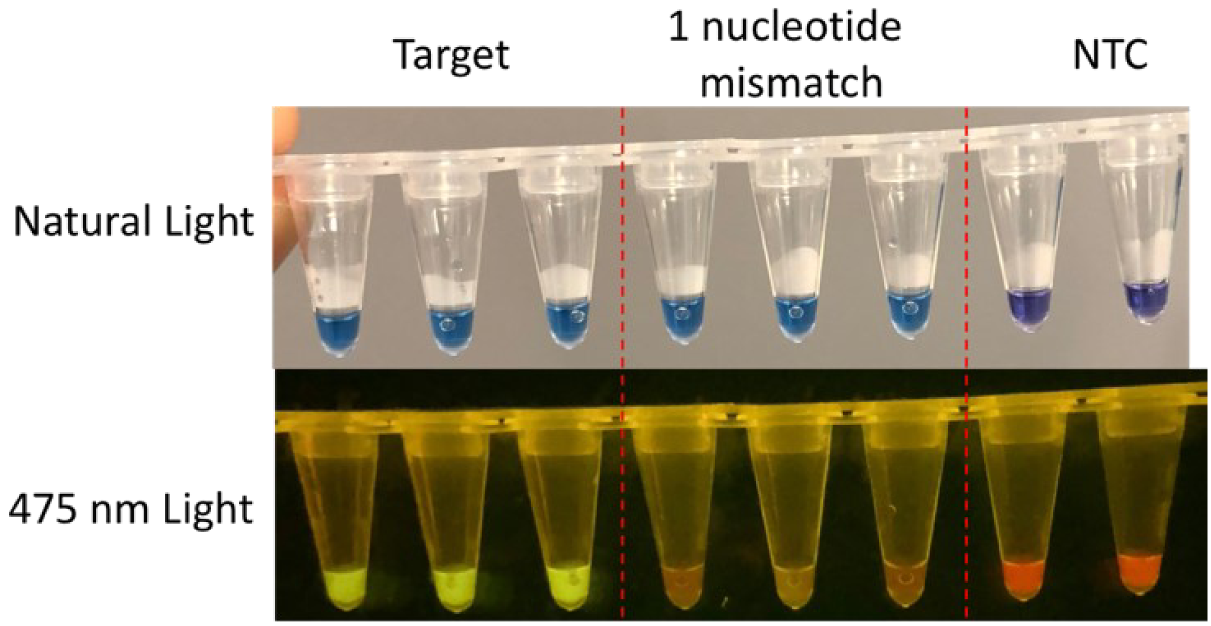

Figure 5. MB-LAMP reactions containing HNB for the target and single-nucleotide mismatch, as viewed by natural light and under 475 nm irradiation. Adapted with permission from ref. 29. Copyright 2019 American Chemical Society.

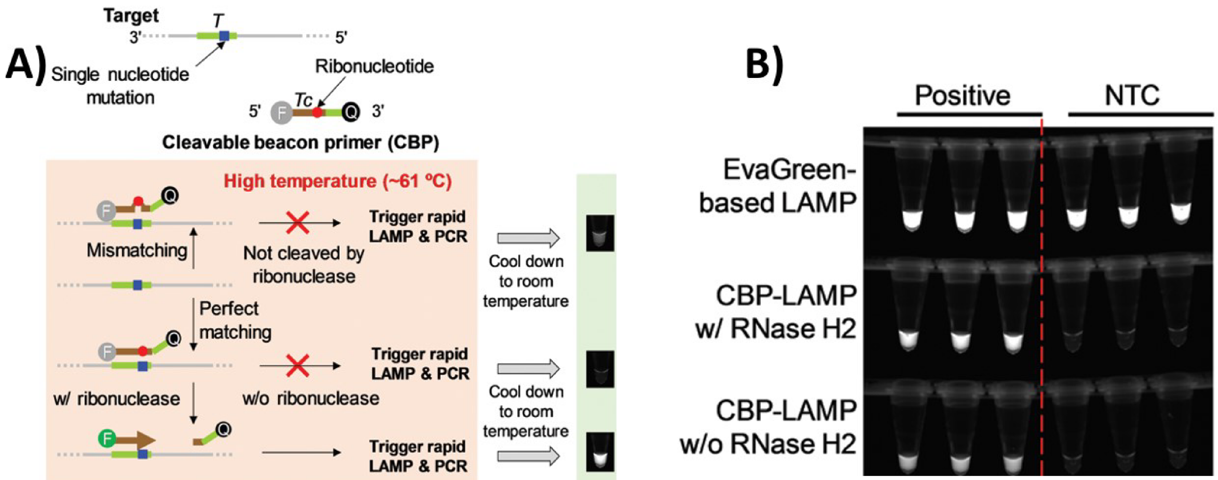

Figure 6. A) Schematic describing the utilization of cleavable beacon primer (CBP) and a ribonuclease for SNP detection. B) Images comparing the fluorescence from reaction containers containing EvaGreen or the CBP with or without RNase H2. Adapted with permission from ref. 31. Copyright 2019 Royal Society of Chemistry.

References

(1) Pratt, E. D.; Cowan, R. W.; Manning, S. L.; Qiao, E.; Cameron, H.; Schradle, K.; Simeone, D. M.; Zhen, D. B. Multiplex Enrichment and Detection of Rare KRAS Mutations in Liquid Biopsy Samples Using Digital Droplet Pre-Amplification. *Anal. Chem.* **2019**, *91* (12), 7516–7523.

(2) Pandey, P.; Pant, N. D.; Rijal, K. R.; Shrestha, B.; Kattel, S.; Banjara, M. R.; Maharjan, B.; Rajendra, K. C. Diagnostic Accuracy of GeneXpert MTB/RIF Assay in Comparison to Conventional Drug Susceptibility Testing Method for the Diagnosis of Multidrug-Resistant Tuberculosis. *PLoS One* **2017**, *12* (1).

(3) Demeke, T.; Jenkins, G. R. Influence of DNA Extraction Methods, PCR Inhibitors and Quantification Methods on Real-Time PCR Assay of Biotechnology-Derived Traits. *Anal. Bioanal. Chem.* **2010**, *396* (6), 1977–1990.

(4) Piepenburg, O.; Williams, C. H.; Stemple, D. L.; Armes, N. A. DNA Detection Using Recombination Proteins. *PLoS Biol.* **2006**, *4* (7), e204.

(5) Ali, M. M.; Li, F.; Zhang, Z.; Zhang, K.; Kang, D.-K.; Ankrum, J. A.; Le, X. C.; Zhao, W. Rolling Circle Amplification: A Versatile Tool for Chemical Biology, Materials Science and Medicine. *Chem. Soc. Rev.* **2014**, *43* (10), 3324–3341.

(6) Notomi, T.; Okayama, H.; Masubuchi, H.; Yonekawa, T.; Watanabe, K.; Amino, N.; Hase, T. Loop-Mediated Isothermal Amplification of DNA. *Nucleic Acids Res.* **2000**, *28* (12), E63.

(7) Goto, M.; Honda, E.; Ogura, A.; Nomoto, A.; Hanaki, K. I. Colorimetric Detection of Loop-Mediated Isothermal Amplification Reaction by Using Hydroxy Naphthol Blue. *Biotechniques* **2009**, *46* (3), 167–172.

(8) Ding, X.; Wu, W.; Zhu, Q.; Zhang, T.; Jin, W.; Mu, Y. Mixed-Dye-Based Label-Free and Sensitive Dual Fluorescence for the Product Detection of Nucleic Acid Isothermal Multiple-Self-Matching-Initiated Amplification. *Anal. Chem.* **2015**, *87* (20), 10306–10314.

(9) Khan, M.; Wang, R.; Li, B.; Liu, P.; Weng, Q.; Chen, Q. Comparative Evaluation of the LAMP Assay and PCR-Based Assays for the Rapid Detection of Alternaria Solani. *Front. Microbiol.* **2018**, *9* (SEP), 2089.

(10) Rolando, J. C.; Jue, E.; Barlow, J. T.; Ismagilov, R. F. Real-Time Kinetics and High-Resolution Melt Curves in Single-Molecule Digital LAMP to Differentiate and Study Specific and Non-Specific Amplification. *Nucleic Acids Res.* **2020**, *48* (7), e42–e42.

(11) Xu, G.; Zhao, H.; Cooper, J. M.; Reboud, J. A Capillary-Based Multiplexed Isothermal Nucleic Acid-Based Test for Sexually Transmitted Diseases in Patients. *Chem. Commun.* **2016**, *52* (82), 12187–12190.

(12) Njiru, Z. K.; Mikosza, A. S. J.; Armstrong, T.; Enyaru, J. C.; Ndung'u, J. M.; Thompson, A. R. C. Loop-Mediated Isothermal Amplification (LAMP) Method for Rapid Detection of Trypanosoma Brucei Rhodesiense. *PLoS Negl. Trop. Dis.* **2008**, *2* (2), e147.

(13) Becherer, L.; Borst, N.; Bakheit, M.; Frischmann, S.; Zengerle, R.; von Stetten, F. Loop-Mediated Isothermal Amplification (LAMP)–Review and Classification of Methods for Sequence-Specific Detection. *Anal. Methods* **2020**, *12* (6), 717–746.

(14) Ding, S.; Chen, R.; Chen, G.; Li, M.; Wang, J.; Zou, J.; Du, F.; Dong, J.; Cui, X.; Huang, X. One-Step Colorimetric Genotyping of Single Nucleotide Polymorphism Using Probe-Enhanced Loop-Mediated Isothermal Amplification (PE-LAMP). *Theranostics* **2019**, *9* (13), 3723.

(15) Itonaga, M.; Matsuzaki, I.; Warigaya, K.; Tamura, T.; Shimizu, Y.; Fujimoto, M.; Kojima, F.; Ichinose, M.; Murata, S. Novel Methodology for Rapid Detection of KRAS Mutation Using PNA-LNA Mediated Loop-Mediated Isothermal Amplification. *PLoS One* **2016**, *11* (3), e0151654.

(16) Carlos, F. F.; Veigas, B.; Matias, A. S.; Doria, G.; Flores, O.; Baptista, P. V. Allele Specific LAMP-Gold Nanoparticle for Characterization of Single Nucleotide Polymorphisms. *Biotechnol. Reports* **2017**, *16*, 21–25.

(17) Malpartida-Cardenas, K.; Rodriguez-Manzano, J.; Yu, L. S.; Delves, M. J.; Nguon, C.; Chotivanich, K.; Baum, J.; Georgiou, P. Allele-Specific Isothermal Amplification Method Using Unmodified Self-Stabilizing Competitive Primers. *Anal. Chem.* **2018**, *90* (20), 11972–11980.

(18) Zhang, C.; Yao, Y.; Zhu, J.-L.; Zhang, S.-N.; Zhang, S.-S.; Wei, H.; Hui, W.-L.; Cui, Y.-L. Establishment and Application of a Real-Time Loop-Mediated Isothermal Amplification System for the Detection of CYP2C19 Polymorphisms. *Sci. Rep.* **2016**, *6* (1), 1–7.

(19) Tamura, S.; Maeda, T.; Misawa, K.; Osa, M.; Hamamoto, T.; Yuki, A.; Imai, K.; Mikita, K.; Morichika, K.; Kawana, A. Development of a Highly Resolved Loop-Mediated Isothermal Amplification Method to Detect the N526K FtsI Mutation of β-Lactamase-Negative Ampicillin-Resistant Haemophilus Influenzae. *J. Microbiol. Methods* **2017**, *141*, 108–114.

(20) Lin, P.; Wang, H.; Cheng, Y.; Song, S.; Sun, Y.; Zhang, M.; Guo, L.; Yi, L.; Tong, M.; Cao, Z. Loop-Mediated Isothermal Amplification-Single Nucleotide Polymorphism Analysis for Detection and Differentiation of Wild-Type and Vaccine Strains of Mink Enteritis Virus. *Sci. Rep.* **2018**, *8* (1), 1–8.

(21) Zhang, C.; Zhu, J.; Yang, J.; Wan, Y.; Ma, T.; Cui, Y. Determination of ABO Blood Group Genotypes Using the Real-time Loop-mediated Isothermal Amplification Method. *Mol. Med. Rep.* **2015**, *12* (4), 5963–5966.

(22) Yongkiettrakul, S.; Kampeera, J.; Chareanchim, W.; Rattanajak, R.; Pornthanakasem, W.; Kiatpathomchai, W.; Kongkasuriyachai, D. Simple Detection of Single Nucleotide Polymorphism in Plasmodium Falciparum by SNP-LAMP Assay Combined with Lateral Flow Dipstick. *Parasitol. Int.* **2017**, *66* (1), 964–971.

(23) Du, W.-F.; Ge, J.-H.; Li, J.-J.; Tang, L.-J.; Yu, R.-Q.; Jiang, J.-H. Single-Step, High-Specificity Detection of Single Nucleotide Mutation by Primer-Activatable Loop-Mediated Isothermal Amplification (PA-LAMP). *Anal. Chim. Acta* **2019**, *1050*, 132–138.

(24) Fu, Y.; Duan, X.; Huang, J.; Huang, L.; Zhang, L.; Cheng, W.; Ding, S.; Min, X. Detection of KRAS Mutation via Ligation-Initiated LAMP Reaction. *Sci. Rep.* **2019**, *9* (1), 1–7.

(25) Higgins, O.; Smith, T. J. Loop-Primer Endonuclease Cleavage–Loop-Mediated Isothermal Amplification Technology for Multiplex Pathogen Detection and Single-Nucleotide Polymorphism Identification. *J. Mol. Diagnostics* **2020**, *22* (5), 640–651.

(26) Jiang, Y. S.; Bhadra, S.; Li, B.; Wu, Y. R.; Milligan, J. N.; Ellington, A. D. Robust Strand Exchange Reactions for the Sequence-Specific, Real-Time Detection of Nucleic Acid Amplicons. *Anal. Chem.* **2015**, *87* (6), 3314–3320.

(27) Du, Y.; Pothukuchy, A.; Gollihar, J. D.; Nourani, A.; Li, B.; Ellington, A. D. Coupling Sensitive Nucleic Acid Amplification with Commercial Pregnancy Test Strips. *Angew. Chemie - Int. Ed.* **2017**, *56* (4), 992–996.

(28) Liu, W.; Huang, S.; Liu, N.; Dong, D.; Yang, Z.; Tang, Y.; Ma, W.; He, X.; Ao, D.; Xu, Y.; Zou, D.; Huang, L. Establishment of an Accurate and Fast Detection Method Using Molecular Beacons in Loop-Mediated Isothermal Amplification Assay. *Sci. Rep.* **2017**, *7* (1), 40125.

(29) Varona, M.; Anderson, J. L. Visual Detection of Single-Nucleotide Polymorphisms Using Molecular Beacon Loop-Mediated Isothermal Amplification with Centrifuge-Free DNA Extraction. *Anal. Chem.* **2019**, *91* (11), 6991–6995.

(30) Varona, M.; Eitzmann, D. R.; Pagariya, D.; Anand, R. K.; Anderson, J. L. Solid-Phase Microextraction Enables Isolation of BRAF V600E Circulating Tumor DNA from Human Plasma for Detection with a Molecular Beacon Loop-Mediated Isothermal Amplification Assay. *Anal. Chem.* **2020**, *92* (4), 3346–3353.

(31) Ding, X.; Yin, K.; Chen, J.; Wang, K.; Liu, C. A Ribonuclease-Dependent Cleavable Beacon Primer Triggering DNA Amplification for Single Nucleotide Mutation Detection with Ultrahigh Sensitivity and Selectivity. *Chem. Commun.* **2019**, *55* (84), 12623–12626.

(32) Tani, H.; Miyata, R.; Ichikawa, K.; Morishita, S.; Kurata, S.; Nakamura, K.; Tsuneda, S.; Sekiguchi, Y.; Noda, N. Universal Quenching Probe System: Flexible, Specific, and Cost-Effective Real-Time Polymerase Chain Reaction Method. *Anal. Chem.* **2009**, *81* (14), 5678–5685.

(33) Ayukawa, Y.; Hanyuda, S.; Fujita, N.; Komatsu, K.; Arie, T. Novel Loop-Mediated Isothermal Amplification (LAMP) Assay with a Universal QProbe Can Detect SNPs Determining Races in Plant Pathogenic Fungi. *Sci. Rep.* **2017**, *7* (1), 1–9.

(34) Komura, R.; Kawakami, T.; Nakajima, K.; Suzuki, H.; Nakashima, C. Simultaneous Detection of Benzimidazole-Resistant Strains of Fusarium Head Blight Using the Loop-Mediated Isothermal Amplification-Fluorescent Loop Primer Method. *J. Gen. Plant Pathol.* **2018**, *84* (4), 247–253.

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