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Robust Strand Exchange Reactions for the Sequence-Specific, Real-Time Detection of Nucleic Acid Amplicons

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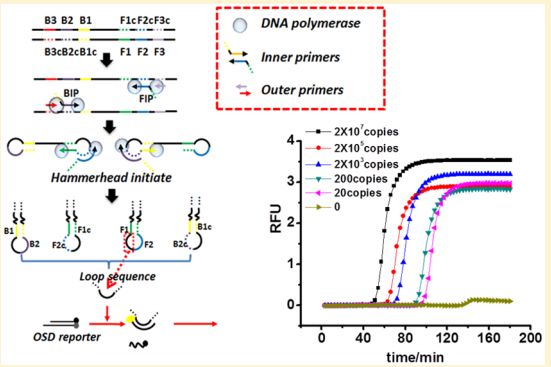
Supporting Information

ABSTRACT: Loop-mediated isothermal amplification (LAMP) of DNA is a powerful isothermal nucleic acid amplification method that can generate upward of 10^9 copies from less than 100 copies of template DNA within an hour. Unfortunately, although the amplification reactions are extremely powerful, real-time and specific detection of LAMP products remains analytically challenging. In order to both improve the specificity of LAMP detection and to make readout simpler and more reliable, we have replaced the intercalating dye typically used for monitoring in real-time fluorescence with a toehold-mediated strand exchange reaction termed one-step strand displacement (OSD). Due to the inherent sequence specificity of toehold-mediated strand exchange, the OSD reporter could successfully distinguish side products from true amplicons arising from templates corresponding to the biomedically relevant *M. tuberculosis* RNA polymerase (*rpoB*) and the melanoma-related biomarker BRAF. OSD allowed the Yes/No detection of *rpoB* in a complex mixture such as synthetic sputum and also demonstrated single nucleotide specificity in Yes/No detection of a mutant BRAF allele (V600E) in the presence of 20-fold more of the wild-type gene. Real-time detection of different genes in multiplex LAMP reactions also proved possible. The development of simple, readily designed, modular equivalents of TaqMan probes for isothermal amplification reactions should generally improve the applicability of these reactions and may eventually assist with the development of point-of-care tests.



Molecular diagnostics that can specifically detect sequences in real-time should be particularly valuable for point-of-need detection and point-of-care monitoring of infectious diseases.[1−3] Sequence amplification methods such as the polymerase chain reaction (PCR) have been widely used in clinical diagnostics,[4] but they have infrastructure requirements that make them less useful for point-of-care applications. In contrast, a series of powerful isothermal nucleic acid amplification (IsoT) techniques have been developed that have shown promising applications in research, diagnostics, forensics, medicine, and agriculture.[5,6] These techniques include self-sustained sequence replication (3SR[7]), nucleic acid sequence-based amplification (NASBA[8]), signal-mediated amplification of RNA technology (SMART[9]), strand displacement amplification (SDA[10]), isothermal multiple displacement amplification (IMDA[11]), helicase-dependent amplification (HDA[12]), single primer isothermal amplification (SPIA[13]), and loop-mediated isothermal amplification of DNA (LAMP[14]).

In general, however, isothermal amplification methods are plagued by the accumulation of spurious amplicons that occur during continuous amplification reactions and a corresponding loss in specificity. The lack of specificity inherent in robust isothermal amplification reactions such as LAMP are compounded during real-time detection because many detection methods rely on nonspecific signal transduction schemes, such as the intercalation of dyes into any double-stranded DNA amplicons that may arise,[15,16] increases in solution turbidity due to the release of pyrophosphates during polymerization.[17−19] Although nonspecific products can be distinguished by additional characterization steps, such as agarose gel electrophoresis,[20] such steps increase the complexity of the workflow. In contrast, our real-time sequence-specific detection method is overall faster and simpler.

There is thus a pressing need for a real-time, sequence-specific, and robust method for monitoring LAMP and other isothermal amplification reactions that can readily separate true signal from nonspecific noise. To overcome this challenge, we have relied on highly programmable, enzyme-free, strand-exchange nucleic acid circuits and computational devices for transducing amplicons produced during isothermal amplification into readily apprehended signals.[5,21−24]

Moreover, accurate SNP analysis is often crucial for diagnoses, such as the detection of drug resistant pathogens and cancer mutations.[25−28] SNP-specific LAMP primers and existing

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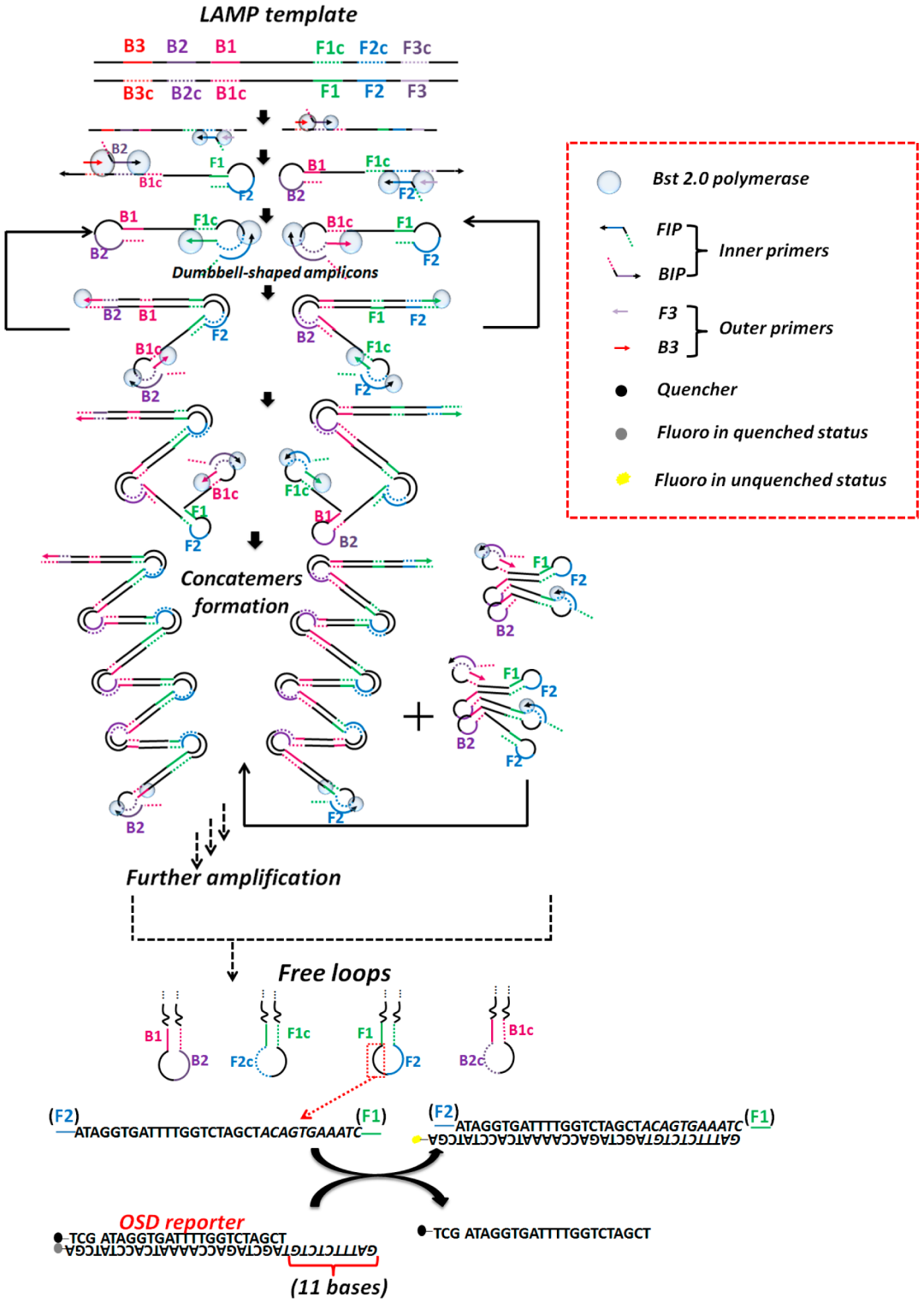

Figure 1. Schematic depicting the generation of LAMP amplicons and their detection by OSD reporters. Colored regions and labels correspond to the different primers; "c" denotes complementary sequences. At the bottom, the interactions between the loop sequence between F1 and F2 of the wild-type BRAF LAMP amplicon and the OSD probe are shown. The toehold regions are italicized; the length of the toehold is indicated below in parentheses. The dark circles on the OSD reporter are the fluorophore(fluoro) and quencher, and upon strand displacement, the fluorophore can be excited and is indicated as a yellow circle.

sequence-probing methodologies such as pyrosequencing[29] and high-resolution melting curve analyses[30] are cumbersome and often inefficient at SNP detection. However, nucleic acid circuits have also been shown to discriminate even slight defects in DNA structure[31] and single nucleotide polymorphisms (SNPs) in genes.[23] We have now developed thermostable, toehold-mediated, strand exchange devices called OSD (short for one-step strand displacement) probes that not only allow real-time sequence detection but also accurately distinguish single

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Anal. Chem. XXXX, XXX, XXX−XXX

nucleotide polymorphisms (SNPs) in LAMP amplicons with high signal-to-noise ratios.

EXPERIMENTAL SECTION

Chemicals and Oligonucleotides. All chemicals were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.) unless otherwise indicated. All enzymes and related buffers were purchased from New England Biolabs (NEB, Ipswich, MA, U.S.A.). All oligonucleotides were ordered from Integrated DNA Technology (IDT, Coralville, IA, U.S.A.). Oligonucleotide sequences are summarized in Table S1.

Standard LAMP Reactions and Electrophoretic Characterizations. Reaction mixtures containing template, 0.8 μM each **BIP** and **FIP** primers, 0.2 μM each **B3** and **F3** primers, 1 M betaine, and 0.4 mM dNTPs in a total volume of 24 μL of 1× Isothermal Buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8) were heated to 95 °C for 5 to 10 min. The solutions were chilled on ice for 2 min, and then 1 μL (8 U) of *Bst* polymerase 2.0 was added to initiate LAMP reactions. The reactions (with a final volume of 25 μL) were incubated at 65 or 60 °C for 3 h. Afterward, the samples were kept at 4 °C until they were analyzed by electrophoresis on a 1% SeaKem LE agarose gel. A DNA ladder (either from NEB, Ipswich, MA, U.S.A. or Life Technologies, Grand Island, NY, U.S.A.) was used as a size standard. Each well was loaded with 10 μL of sample and an additional 2 μL of 6× Orange Loading Dye (40% glycerol, 0.25% Orange G). The electrophoresis was developed at 90 to 110 V for 30 min.

Real-Time LAMP Detection with Either OSD or Evagreen. An OSD stock solution was prepared by annealing 10 μM **Reporter F** with 50 μM **Reporter Q** in Isothermal Buffer. The solution was incubated at 95 °C for 5 min followed by slow cooling to room temperature at a rate of 0.1 °C/s. LAMP-OSD reactions were prepared in almost the same manner as standard LAMP reactions, with the exception that the OSD reporter (at a final concentration of 60 nM **Reporter F**) or 1× Evagreen dye (Biotium, Hayward, CA, U.S.A.) was added to the reactions following primer annealing but prior to the addition of *Bst* 2.0 DNA polymerase. Subsequently, 20 μL of the LAMP-OSD solutions were transferred into a 96-well plate, which was maintained at 60 or 65 °C for 2 to 3 h. Fluorescence signals were recorded every 3 min with a LightCycler 96 (Roche, NC, U.S.A.).

Real-Time Multiplex LAMP Detection with OSD. Multiplex LAMP reactions were assembled by mixing 20 μL of HSV1 LAMP reactions and 15 μL of *cyt*B LAMP reactions, and seeding with different amounts of DNA templates (relative volumes were chosen following optimization experiments). The effective concentrations of reagents in the final multiplex LAMP reaction were the following: 0.46 μM each of HSV1 **FIP** and **BIP**, 0.11 μM each of HSV1 **F3** and **B3**, 0.34 μM each of *cyt*B **FIP** and **BIP**, 0.086 μM each of *cyt*B **F3** and **B3**, 0.4 mM dNTPs, 1 M betaine, 1× Isothermal Buffer, 2 mM $MgCl_2$, 28 nM HSV1 TYE665-labeled probe (preannealed with a 5-fold excess of the quencher strand), 21 nM *cyt*B TYE615-labeled probe (preannealed with a 5-fold excess of the quencher strand), and 8 U of *Bst* polymerase 2.0. LAMP reactions were amplified at 65 °C in the LightCycler 96 and fluorescence was measured every 3 min in the Texas Red (for TYE615) and Cy5 (for TYE665) channels.

All LAMP-OSD fluorescence data obtained from LightCycler 96 were analyzed using the "Absolute quantification" calculation available via the LightCycler 96 software and plotted against amplification time.

RESULTS AND DISCUSSION

OSD Design. OSD probes are essentially hemiduplex DNAs composed of hybridized oligonucleotides in which a target-binding strand contains a fluorophore at either its 5′ or 3′-end (**Reporter F**), and the opposing strand contains a corresponding quencher (**Reporter Q**). In general, **Reporter F** should be 10 or 11 bases longer than **Reporter Q**[32] in order to ensure a relatively fast toehold-mediated strand exchange reaction at 60 °C. Target-binding to the toehold will initiate a strand-exchange reaction that should lead to displacement of **Reporter F** from **Reporter Q** and thus to turning on the fluorophore. As an example, Figure S1 shows fluorescence changes when 60 nM BRAF OSD reporter was incubated with various concentrations of its cognate target and with negative controls. The OSD reporter responds in a template-specific manner, and will not signal the presence of background, nonspecific amplicons.

Real-Time Gene Detection with LAMP and OSDs. We have demonstrated the efficacy of OSDs by detecting SNPs of the melanoma biomarker gene BRAF. There are four primers in a typical LAMP reaction (Figure 1): two inner primers (**FIP** and **BIP**) and two outer primers (**F3** and **B3**). These primers are specific to six consecutive blocks of target sequences designated in their order of appearance from the 5′-end as **B3**, **B2**, **B1**, **F1c**, **F2c** and **F3c**. **FIP** (**F1c-F2**) is composed of the sequence **F2** that initiates the first stage of amplification and the sequence **F1c** that self-primes subsequent stages of amplification. Similarly, **BIP** is composed of sequences **B2** and **B1c** (**B1c-B2**). **FIP** and **BIP** hybridize via their respective **F2** and **B2** sequences to the complementary template sequences **F2c** and **B2c** to initiate complementary DNA synthesis by Bst DNA polymerase. Due to their lower concentration and binding efficiency, the outer primers **F3** and **B3** hybridize to their complementary target sequences at a slower rate and initiate DNA synthesis that displaces the preceding inner primer-initiated strands. These newly released strands serve as templates for complementary inner and outer primer-initiated strand displacement DNA synthesis resulting in two single-stranded, dumbbell-shaped amplicons whose 3′-ends are extended by Bst polymerase to form stem-loop hairpins. Inner primers then hybridize to the single-stranded loops and initiate another round of strand displacement synthesis that opens the original hairpin stem to form a concatemerized amplicon containing a self-priming 3′-end hairpin. The ensuing exponential amplification from continuous strand displacement DNA synthesis (initiated both by hybridization of inner primers to newly generated single-stranded loops and by self-priming) generates increasingly complex, double-stranded concatameric amplicons containing self-priming hairpins and single-stranded loops. The final LAMP product comprises large molecular weight concatemers that contain free loops between the **F1** and **F2**, **B1** and **B2**, **F1c** and **F2c**, and **B1c** and **B2c** regions (Figure 1). These free loop regions originate from target sequences that are absent in the primers. Hence, OSD probes designed to detect these target-specific loops can discriminate true-positive amplicons from primer amplification artifacts generated in the absence of specific targets.

The utility of sequence-specific probes is manifested, because LAMP is notorious for generating spurious amplicons. As with PCR, these are likely due to the formation and extension of primer dimers. In addition, though, because spurious LAMP products are often composed of a continuum of both short and very large amplicons (as shown in the negative control of Figure

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Anal. Chem. XXXX, XXX, XXX−XXX

2B), it seems likely that the production of these parasitic amplicons is exacerbated by the basic replication mechanisms

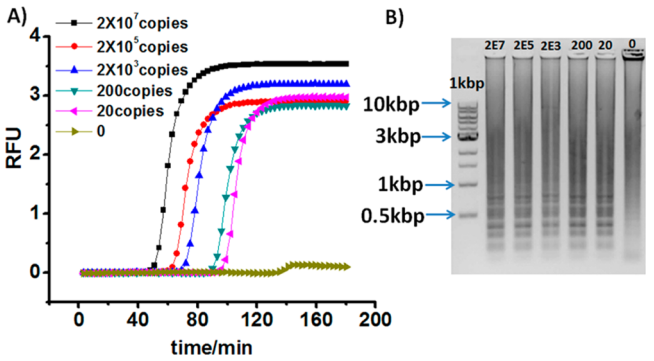

Figure 2. Performance of LAMP-OSD. (A) LAMP-OSD is shown as a function of time with varying amounts of the BRAF template. (B) The same samples from (A) are separated on a 1% agarose gel with 0.5 μg of 1 kb ladder on the left and stained with ethidium bromide.

inherent in the primer sequences, because these lead to the formation of concatamers that can provide additional binding sites for primer-binding and extension, either specific or nonspecific. Opportunities for generating background are further complicated by the fact that Bst DNA polymerase Bst DNA polymerase has an intrinsic nucleotidyl transferase activity that enables it to generate 3′ overhangs by nontemplated incorporation of random nucleotides,[33] and this polymerase has also been reported to multimerize both circular and linear DNAs.[34] Nonetheless, because our template-loop specific OSD reporter lacks complementarity to any primer-derived sequences, it should be activated only by true LAMP amplicons generated by target-specific replication and not by any primer-generated artifacts.

LAMP-OSD with different amounts of a *rpoB* (β subunit of bacterial RNA polymerase) plasmid template showed a fluorescence response that was quite similar to LAMP where detection was with a less specific intercalating dye (Figure S2). However, LAMP-OSD was able to clearly distinguish between template and a no-template control, whereas the reactions with the intercalating dye could not (Figure S2). Because we have largely eliminated background detection, the detection limit for LAMP-OSD for the wild-type BRAF gene was as low as 20 copies (Figure 2). However, Figure 2 also shows that while signal responses over time are roughly dependent on the input concentrations of template, this dependence is not strictly proportional to the concentration. However, our method is very useful as a sensitive Yes/No diagnostic.

Real-Time SNP Discrimination with LAMP and OSDs. Having shown that we could readily discriminate template from background, we next attempted to carry out real-time, single-nucleotide distinctions. In order to realize BRAF V600E SNP detection, the program ***PrimerExplorerV4*** was called upon to generate a set of primers that had the V600E mutation localized to the loop region between **F1** and **F2**. An OSD probe was designed to target the V600E mutation, and this should in turn lead to a low fluorescent signal with the wild-type BRAF gene.

In order to better match the LAMP with OSD, we carried out the joint reaction at several temperatures (60, 63, and 65 °C; Figure S3). The discrimination factor Q (ΔRFU_{WT} /ΔRFU_{SNP}) was greatest (8.1) at 60 °C. It is possible that higher temperatures

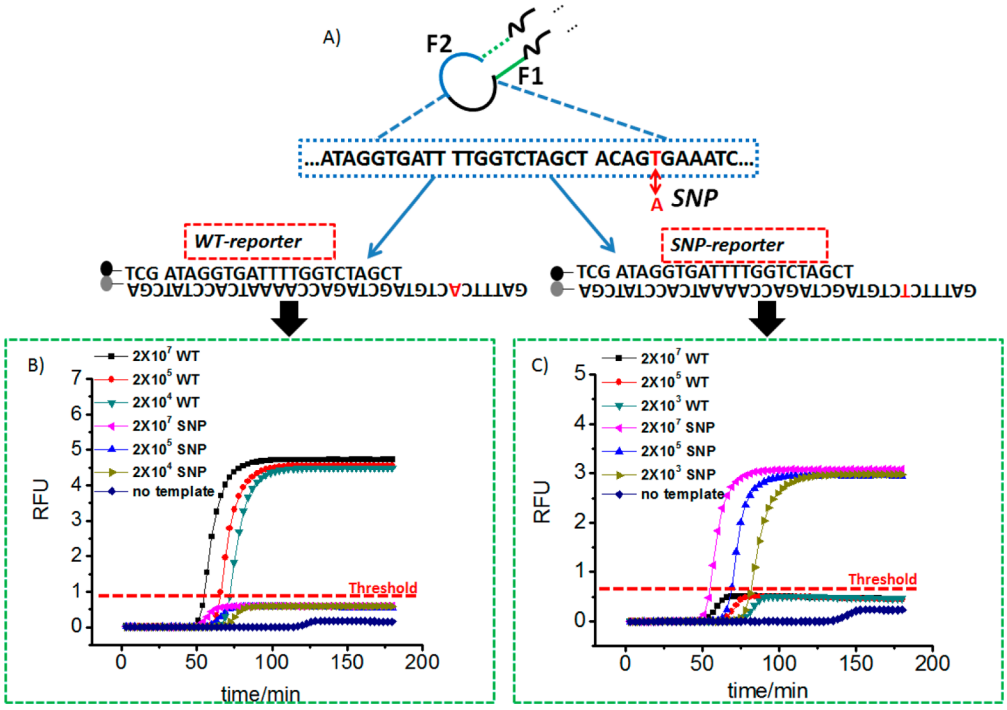

Figure 3. LAMP-OSD can distinguish single nucleotide polymorphisms. (A) Sequence interactions between LAMP amplicons that represent either the wild-type (WT) BRAF gene or the allele V600E (which contains a single nucleotide difference) and the OSD probes. The discriminating nucleotide position is shown in red in both amplicons and the OSD probes (where it is found in the toehold region). LAMP-OSD is shown as a function of time with varying amounts of either the wild-type (WT) BRAF gene or the allele V600E (labeled "SNP"), which contains a single nucleotide difference. Detection is with either a probe for the wild-type gene (B) or the V600E SNP (C). The red dashed lines in (B) and (C) show the RFU threshold for discriminating between signals from fully matched and mismatched alleles.

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Anal. Chem. XXXX, XXX, XXX−XXX

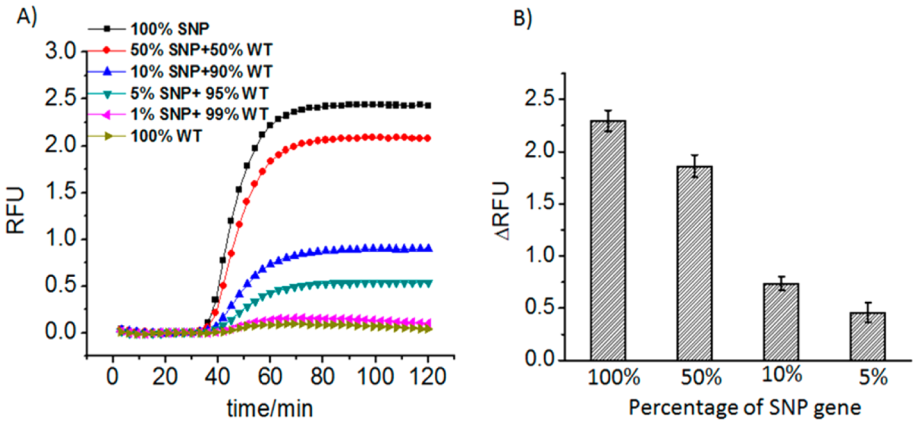

Figure 4. LAMP-OSD can detect different ratios of alleles. (A) Real-time fluorescence curves of LAMP reactions coupled with the V600E BRAF OSD probe for samples containing 100%SNP (V600E BRAF), 50%SNP + 50%WT, 10%SNP + 90%WT, 5%SNP + 95%WT, 1%SNP + 99%WT, 100%WT, respectively. (B) The relative plateau value of the fluorescence curves of (A) averaged over six experiments. The ΔRFU was determined by taking the average raw plateau value for amplicons arising from the SNP from 80 to 120 min, then subtracting background (the raw plateau value for WT alone; 100% WT).

allow the ends of the OSD reporter to breathe, effectively lengthening the toehold and leading to more strand displacement by otherwise mismatched amplicons. All further reactions were carried out at 60 or 55 °C.

LAMP-OSD was carried out with varying amounts (from 2×10^4 to 2×10^7 copies) of both the wild-type BRAF gene and its V600E mutant SNP (a T to A transversion). When the wild-type OSD probe was used, there was a concentration-dependent increase in signal for the wild-type templates, with little or no background from the mutant templates (Figure 3B). Conversely, when an OSD probe specific for the V600E allele was used, the mutant gene could be detected, and the wild-type gave little background (Figure 3C). The much lower fluorescence plateaus in the absence of a cognate template were consistent across samples. We therefore drew RFU threshold lines (Figure 3B,C) to discriminate between signals from fully matched and mismatched alleles. The discrimination between perfectly matched and mismatched LAMP amplicons is due in part to the fact that toehold binding is likely inefficient at the high temperatures at which LAMP is carried out, as well as because there may be an internal competition between OSD probes and complementary loop sequences (for example, the loop between **F1** and **F2** is complementary to the loop between **F1c** and **F2c**).

In qPCR, SNP discrimination is based on when wild-type and SNP signals "break through" the basal level of fluorescence necessary for a positive signal (i.e., differences in Ct values). However, with isothermal amplification, the common primers for the wild-type and SNP amplicons lead to the same degree of amplification, preventing discrimination based on Ct. Instead, the different loop sequences activate OSD to different degrees, resulting in different levels of fluorescence being produced by a perfectly matched versus a mismatched amplicon. Thus, the appropriate discrimination for isothermal amplification may not be Ct but rather the overall fluorescence produced. We mixed the mutant and wild-type templates such that the mutant template was present at 0%, 1%, 5%, 10%, 50%, or 100% of the population and carried out LAMP-OSD at 55 °C (Figure 4A). An increasing SNP-specific fluorescence signal was observed. A ΔRFU value was obtained by subtracting a given fluorescence signal for the mutant allele from the fluorescence signal when 100% of the population was wild-type. In 6 replicates, our method could specifically detect the V600E mutant template even when it was present in as little as 5% of the population (ΔRFU for 5% V600E − wild type = 0.46 ± 0.093, Figure 4B).

The BRAF V600E is typically detected by sequencing,[35,36] and it requires some 20 to 125 ng of extracted genomic DNA (about 5000 to 30 000 copies of human genomic DNA). Given that our method has a detection limit of as low as 20 copies of the BRAF gene and can discriminate between alleles based on single mismatches, our results are clinically relevant.

Multiplex, Real-Time Detection with LAMP and OSDs. Sequence-specific OSD probes are ideally suited for the deconvolution of multiplex LAMP reactions, because each probe can identify a different sequence and report a different color. As a demonstration, we coamplified cloned fragments of the *Plasmodium falciparum cyt*B gene and the herpes simplex virus 1 (HSV1) US4 genomic locus using LAMP. The *cyt*B-specific OSD probes were labeled with the fluorophore TYE615, whereas the HSV1-specific OSD probes were labeled with the fluorophore TYE665. As depicted in Figure 5, these different OSD probes were able to separately detect their cognate LAMP

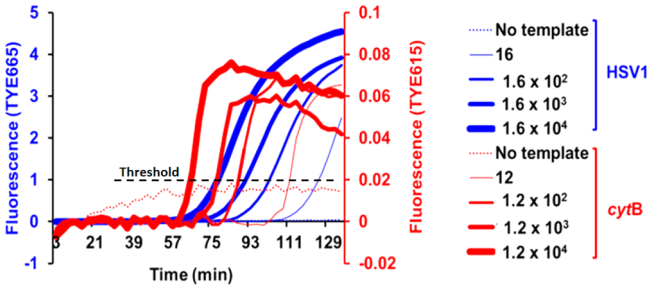

Figure 5. Real-time, sequence-specific detection by OSD probes of two templates in a multiplex LAMP reaction. HSV1 and *cyt*B templates were amplified with two sets of LAMP primers in parallel. OSD probes specific for HSV1 (TYE665-labeled; blue *Y*-axis) and *cyt*B (TYE615-labeled; red *Y*-axis) were used for detecting the two different amplicons. Blue and red traces show the real-time accumulation of amplicons from HSV1 and *cyt*B, respectively. The different line thicknesses correspond to different template mixtures. Five separate multiplex reactions are shown; for example, the thinnest line is 16 copies of HSV1 and 12 copies of *cyt*B, and the thickest line is 1.6×10^4 copies of HSV1 and 1.2×10^4 copies of *cyt*B. The black dashed line shows the RFU threshold for discriminating *cyt*B positive signals from fluorescence background.

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Anal. Chem. XXXX, XXX, XXX−XXX

amplicons, including at the limits of detection (less than 20 copies of the *cyt*B and HSV1 synthetic targets). It is noteworthy that the "no template" sample of *cyt*B also apparently gave a fluorescence increase. However, this increase is consistent with the background seen with other samples and is only seen here because the positive signal from *cyt*B, an extremely A:T rich amplicon, is lower than for other templates (compare y axes in Figure 5 for HSV1 and *cyt*B).

LAMP-OSD-Mediated Detection of *Mycobacterium tuberculosis* (MTB) Spiked into Synthetic Sputum. For any diagnostic method, the complexity of the sample in which an amplicon is found, such as saliva, sputum, or other clinical materials, will increase the risk of generating side-products or false negative results. Conversely, the robustness of a detection system to contaminants and interferents will determine whether it can be applied in clinical practice.[37] As a test of the robustness of LAMP-OSD system, we attempted to detect *Mycobacterium tuberculosis* spiked into synthetic sputum that contained human genomic DNA, salmon sperm DNA, mucin, and other components (provided by the Program for Appropriate Technology in Health, PATH, Seattle, WA).[38]

We first developed a LAMP-OSD system that could detect the *rpoB* (β subunit of bacterial RNA polymerase) gene of MTB, a protein that frequently is associated with mutations that lead to drug resistance. As with other amplicons, it proved possible to quickly identify a primer set and amplification protocol that yielded a detection limit of ~200 copies (Figure S4A). It is likely that this detection limit is higher than that seen with other genes because the G:C-rich sequence is known to be hard to be optimized for amplification assay.[39]

When similar assays were attempted with MTB spiked into synthetic sputum, we found that the Bst 2.0 polymerase was greatly inhibited by mucin. Therefore, we liquefied the mucin with 2% *N*-acetyl-L-cysteine and 1% sodium hydroxide[40] and lysed the bacteria via 5 rounds of freezing and thawing (see Supporting Information for more details). LAMP-OSD consistently detected the MTB *rpoB* gene at either 1000 or 100 genomic copies (Figure S4B and C). However, only 5 out of 8 samples showed a positive LAMP-OSD signal when only 10 genomic copies were present. Remarkably, these results are consistent with the detection limit observed with the *rpoB* plasmid in water and with other LAMP analyses carried out in pure solutions (Figure S4A).

CONCLUSION

LAMP is an ultrasensitive nucleic acid amplification method that can detect even a few copies of a DNA or RNA template within hours. However, the very power of the method frequently results in nonspecific amplification of background templates. In order to distinguish between true and false amplicons, we have developed a simple and robust oligonucleotide strand-displacement probe (OSD). This method can specifically detect as few as 20 copies of the BRAF, *cyt*B, and HSV1 templates, and 200 copies of the *rpoB* template. Transduction by an OSD probe could also be used to detect a mutant allele (V600E) in the presence of 20-fold more wild-type gene. The pathogen MTB could be detected in a complex background, synthetic sputum, at as low as 10 bacteria/μL.

These results are only possible because OSD probes are sequence-specific for the amplicon, acting in essence as the equivalent Taqman probes in qPCR. Because of this, OSD can be used to detect individual amplicons in multiplex reactions by the simple expedient of using sequence-specific probes that contain different dyes. In contrast, competing methods that rely on as fluorescence activation via intercalating dyes[41] are not sequence-specific and thus will label any amplicon, including nonspecific amplification products. Similarly, methods that rely upon quenching[42] or activation (via strand displacement; the DARQ system)[43] of fluorescently labeled primers cannot validate the sequence of the correct amplicon relative to nonspecific amplicons.

OSD probes can work in real-time, which offer several advantages relative to end-point analysis. Avoiding postprocessing of LAMP reactions (for example, by agarose gel electrophoretic separation,[20] pyrosequencing,[44] or multiplex detection with cationic polymers[45]) saves time and effort and reduces the chances of cross-contaminating reactions due to aerosolization of templates. Compared to other sequence-specific methods, such as the AB-Q probe,[46] the OSD probe is simpler in design and requires no auxiliary competitor in the system that could potentially reduce the sensitivity of LAMP assays.

However, because of the very robust amplification that occurs with LAMP, different amounts of template give nearly indistinguishable Ct shifts. It is therefore difficult to use LAMP for conventional quantitative analyses. That said, the very low limits of detection make LAMP-OSD ideal for Yes/No diagnostic applications or for quantitation via digital methods.[47] Although so far LAMP-OSD has only been used in a lab setting, additional optimizations may eventually make it possible to adapt this molecular signal amplifier and transducer to point-of-care devices. For example, improved primer designs may lead to speed increases in the overall LAMP reaction,[48] whereas changes in the toehold length may improve the kinetics of OSD.[49]

Beyond the intrinsic analytical advantages of using OSD probes, strand exchange methods are in general highly programmable and thus facilitate the ready adoption of new configurations of reactions.[50−52] New probes can be simply designed by knowing the sequences of new targets. Strand exchange probes can potentially feed into nucleic acid logic circuits,[49,53,54] allowing multi-input decision making in diagnostics. For example, multiple OSD probes that detected different regions of the same target could be used as inputs for an AND gate that would decrease false positive detection.[4] The outputs of such logical circuits could be the input for further signal processing or transduction into different downstream analytical devices.[24,55]

ASSOCIATED CONTENT

Supporting Information

Additional information including plasmids preparation, OSD design, and pretreatment of synthetic sputum. This material is available free of charge via Internet at t http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Hartman, M. R.; Ruiz, R. C. H.; Hamada, S.; Xu, C.; Yancey, K. G.; Yu, Y.; Han, W.; Luo, D. *Nanoscale* **2013**, *5*, 10141−10154.
(2) Griffith, D. E.; Aksamit, T.; Brown-Elliott, B. A.; Catanzaro, A.; Daley, C.; Gordin, F.; Holland, S. M.; Horsburgh, R.; Huitt, G.; Iademarco, M. F.; Iseman, M.; Olivier, K.; Ruoss, S.; von Reyn, C. F.; Wallace, R. J.; Winthrop, K.; Subcommittee, A. T. S. M. D. *Am. J. Respir. Crit. Care Med.* **2007**, *175*, 367−416.
(3) Dunlap, N. E.; Bass, J.; Fujiwara, P.; Hopewell, P.; Horsburgh, C. R.; Salfinger, M.; Simone, P. M.; Sci Assembly Microbiology, T. *Am. J. Respir. Crit. Care Med.* **2000**, *161*, 1376−1395.
(4) Saiki, R. K.; Bugawan, T. L.; Horn, G. T.; Mullis, K. B.; Erlich, H. A. *Nature* **1986**, *324*, 163−166.
(5) Li, B.; Chen, X.; Ellington, A. D. *Anal. Chem.* **2012**, *84*, 8371−8377.
(6) Asiello, P. J.; Baeumner, A. J. *Lab Chip* **2011**, *11*, 1420−1430.
(7) Guatelli, J. C.; Whitfield, K. M.; Kwoh, D. Y.; Barringer, K. J.; Richman, D. D.; Gingeras, T. R. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 1874−1878.
(8) Compton, J. *Nature* **1991**, *350*, 91−92.
(9) Wharam, S. D.; Marsh, P.; Lloyd, J. S.; Ray, T. D.; Mock, G. A.; Assenberg, R.; McPhee, J. E.; Brown, P.; Weston, A.; Cardy, D. L. *Nucleic Acids Res.* **2001**, *29*, E54−4.
(10) Walker, G. T.; Fraiser, M. S.; Schram, J. L.; Little, M. C.; Nadeau, J. G.; Malinowski, D. P. *Nucleic Acids Res.* **1992**, *20*, 1691−1696.
(11) Dean, F. B.; Hosono, S.; Fang, L. H.; Wu, X. H.; Faruqi, A. F.; Bray-Ward, P.; Sun, Z. Y.; Zong, Q. L.; Du, Y. F.; Du, J.; Driscoll, M.; Song, W. M.; Kingsmore, S. F.; Egholm, M.; Lasken, R. S. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 5261−5266.
(12) Vincent, M.; Xu, Y.; Kong, H. M. *EMBO Rep.* **2004**, *5*, 795−800.
(13) Kurn, N.; Chen, P. C.; Heath, J. D.; Kopf-Sill, A.; Stephens, K. M.; Wang, S. L. *Clin. Chem.* **2005**, *51*, 1973−1981.
(14) Notomi, T.; Okayama, H.; Masubuchi, H.; Yonekawa, T.; Watanabe, K.; Amino, N.; Hase, T. *Nucleic Acids Res.* **2000**, *28*, e63.
(15) Njiru, Z. K.; Mikosza, A. S. J.; Armstrong, T.; Enyaru, J. C.; Ndung'u, J. M.; Thompson, A. R. C. *PLoS Negl. Trop. Dis.* **2008**, *2*, e147.
(16) Iwamoto, T.; Sonobe, T.; Hayashi, K. *J. Clin. Microbiol.* **2003**, *41*, 2616−2622.
(17) Tomita, N.; Mori, Y.; Kanda, H.; Notomi, T. *Nat. Protoc.* **2008**, *3*, 877−882.
(18) Boehme, C. C.; Nabeta, P.; Henostroza, G.; Raqib, R.; Rahim, Z.; Gerhardt, M.; Sanga, E.; Hoelscher, M.; Notomi, T.; Hase, T.; Perkins, M. D. *J. Clin. Microbiol.* **2007**, *45*, 1936−1940.
(19) Pandey, B. D.; Poudel, A.; Yoda, T.; Tamaru, A.; Oda, N.; Fukushima, Y.; Lekhak, B.; Risal, B.; Acharya, B.; Sapkota, B.; Nakajima, C.; Taniguchi, T.; Phetsuksiri, B.; Suzuki, Y. *J. Med. Microbiol.* **2008**, *57*, 439−443.
(20) Aonuma, H.; Yoshimura, A.; Kobayashi, T.; Okado, K.; Badolo, A.; Nelson, B.; Kanuka, H.; Fukumoto, S. *Exp. Parasitol.* **2010**, *125*, 179−183.
(21) Benenson, Y.; Adar, R.; Paz-Elizur, T.; Livneh, Z.; Shapiro, E. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 2191−2196.
(22) Yin, P.; Choi, H. M. T.; Calvert, C. R.; Pierce, N. A. *Nature* **2008**, *451*, 318−322.
(23) Niu, S.; Jiang, Y.; Zhang, S. *Chem. Commun.* **2010**, *46*, 3089−3091.
(24) Li, B.; Ellington, A. D.; Chen, X. *Nucleic Acids Res.* **2011**, *39*, e110.
(25) Kiyohara, C.; Takayama, K.; Nakanishi, Y. *Lung Cancer* **2006**, *54*, 267.
(26) Yang, H.; Dinney, C. P.; Ye, Y.; Zhu, Y.; Grossman, H. B.; Wu, X. *Cancer Res.* **2008**, *68*, 2530−2537.
(27) Isla, D.; Sarries, C.; Rosell, R.; Alonso, G.; Domine, M.; Taron, M.; Lopez-Vivanco, G.; Camps, C.; Botia, M.; Nunez, L.; Sanchez-Ronco, M.; Sanchez, J. J.; Lopez-Brea, M.; Barneto, I.; Paredes, A.; Medina, B.; Artal, A.; Lianes, P.; Spanish Lung Canc, G. *Ann. Oncol.* **2004**, *15*, 1194−1203.
(28) Li, Q.; Luan, G.; Guo, Q.; Liang, J. *Nucleic Acids Res.* **2002**, *30*, e5.
(29) Spuesens, E. B. M.; Hoogenboezem, T.; Sluijter, M.; Hartwig, N. G.; van Rossum, A. M. C.; Vink, C. *J. Microbiol. Methods* **2010**, *82*, 214−222.
(30) Peuchant, O.; Menard, A.; Renaudin, H.; Morozumi, M.; Ubukata, K.; Bebear, C. M.; Pereyre, S. *J. Med. Microbiol.* **2009**, *64*, 52−58.
(31) Li, B.; Jiang, Y.; Chen, X.; Ellington, A. D. *J. Am. Chem. Soc.* **2012**, *134*, 13918−13921.
(32) Jiang, Y.; Li, B.; Milligan, J. N.; Bhadra, S.; Ellington, A. D. *J. Am. Chem. Soc.* **2013**, *135*, 7430−7433.
(33) Zyrina, N. V.; Zheleznaya, L. A.; Dvoretsky, E. V.; Vasiliev, V. D.; Chernov, A.; Matvienko, N. I. *Biol. Chem.* **2007**, *388*, 367−372.
(34) Hafner, G. J.; Yang, I. C.; Wolter, L. C.; Stafford, M. R.; Giffard, P. M. *Biotechniques* **2001**, *30*, 852−867.
(35) Qu, K.; Pan, Q.; Zhang, X.; Rodriguez, L.; Zhang, K.; Li, H.; Ho, A.; Sanders, H.; Sferruzza, A.; Cheng, S. M.; Nguyen, D.; Jones, D.; Waldman, F. *J. Mol. Diagn.* **2013**, *15*, 790−795.
(36) Spittle, C.; Ward, M. R.; Nathanson, K. L.; Gimotty, P. A.; Rappaport, E.; Brose, M. S.; Medina, A.; Letrero, R.; Herlyn, M.; Edwards, R. H. *J. Mol. Diagn.* **2007**, *9*, 464−471.
(37) Kohan, L.; Shahhosseiny, M. H.; Razavi, M. R.; Parivar, K.; Moslemi, E.; Werngren, J. *Afr. J. Biotechnol.* **2011**, *10*, 5096−5101.
(38) Sanders, N. N.; Van Rompaey, E.; De Smedt, S. C.; Demeester, J. *Am. J. Respir. Crit. Care Med.* **2001**, *164*, 486−493.
(39) Henke, W.; Herdel, K.; Jung, K.; Schnorr, D.; Loening, S. A. *Nucleic Acids Res.* **1997**, *25*, 3957−3958.
(40) Verma, S.; Dhole, T.; Kumar, M. *J. Clin. Microbiol.* **2013**, *51*, 3597−3601.
(41) Kouguchi, Y.; Fujiwara, T.; Teramoto, M.; Kuramoto, M. *Mol. Cell Probe* **2010**, *24*, 190−195.
(42) Zerilli, F.; Bonanno, C.; Shehi, E.; Amicarelli, G.; Adlerstein, D.; Makrigiorgos, G. M. *Clin. Chem.* **2010**, *56*, 1287−1296.
(43) Tanner, N. A.; Zhang, Y.; Evans, T. C., Jr. *BioTechniques* **2012**, *53*, 81−89.
(44) Liang, C.; Chu, Y.; Cheng, S.; Wu, H.; Kajiyama, T.; Kambara, H.; Zhou, G. *Anal. Chem.* **2012**, *84*, 3758−3763.
(45) Mori, Y.; Hirano, T.; Notomi, T. *BMC Biotechnol.* **2006**, *6*, 3.
(46) Tani, T.; Teramura, T.; Adachi, K.; Tsuneda, S.; Nakamura, K.; Kanagawa, T.; Nada, N. *Anal. Chem.* **2007**, *29*, 5608−5613.
(47) Vogelstein, B.; Kinzler, W. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 9236−9241.
(48) Bagmine, K.; Hase, T.; Notomi, T. *Mol. Cell. Probes* **2002**, *16*, 223−229.
(49) Zhang, D. Y.; Winfree, E. *J. Am. Chem. Soc.* **2009**, *131*, 17303−17314.
(50) Li, F.; Zhang, H.; Wang, Z.; Li, X.; Li, X. F.; Le, X. C. *J. Am. Chem. Soc.* **2013**, *135*, 2443−2446.
(51) Liu, M.; Fu, J.; Hejesen, C.; Yang, Y.; Woodbury, N. W.; Gothelf, K.; Liu, Y.; Yan, H. *Nat. Commun.* **2013**, *4*, 2127.
(52) Chen, S. X.; Zhang, D. Y.; Seelig, G. *Nat. Chem.* **2013**, *5*, 782−789.
(53) Seeling, G.; Soloveichik, D.; Zhang, D. Y.; Winfree, E. *Science* **2006**, *314*, 1585−1588.
(54) Zhu, J.; Zhang, L.; Li, T.; Dong, S.; Wang, E. *Adv. Mater.* **2013**, *25*, 2440−2444.
(55) Allen, P. B.; Arshad, S. A.; Li, B.; Chen, X.; Ellington, A. D. *Lab Chip* **2012**, *12*, 2951−2958.

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