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Allele-Specific Isothermal Amplification Method Using Unmodified Self-Stabilizing Competitive Primers

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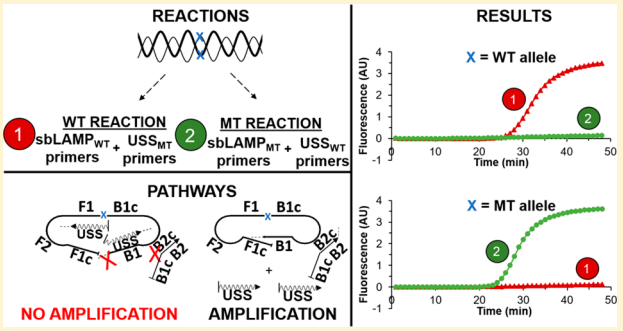
Supporting Information

ABSTRACT: Rapid and specific detection of single nucleotide polymorphisms (SNPs) related to drug resistance in infectious diseases is crucial for accurate prognostics, therapeutics and disease management at point-of-care. Here, we present a novel amplification method and provide universal guidelines for the detection of SNPs at isothermal conditions. This method, called USS-sbLAMP, consists of SNP-based loop-mediated isothermal amplification (sbLAMP) primers and unmodified self-stabilizing (USS) competitive primers that robustly delay or prevent unspecific amplification. Both sets of primers are incorporated into the same reaction mixture, but always targeting different alleles; one set specific to the wild type allele and the other to the mutant allele. The mechanism of action relies on thermodynamically favored hybridization of totally complementary primers, enabling allele-specific amplification. We successfully validate our method by detecting SNPs, *C580Y* and *Y493H*, in the *Plasmodium falciparum kelch 13* gene that are responsible for resistance to artemisinin-based combination therapies currently used globally in the treatment of malaria. USS-sbLAMP primers can efficiently discriminate between SNPs with high sensitivity (limit of detection of 5×10^1 copies per reaction), efficiency, specificity and rapidness (<35 min) with the capability of quantitative measurements for point-of-care diagnosis, treatment guidance, and epidemiological reporting of drug-resistance.



The emergence of drug resistance is a constant threat to global public health, limiting the ability to treat infectious diseases effectively and compromising medical procedures.[1] Rapid detection of single nucleotide polymorphisms (SNPs) that are linked with resistance phenotypes in infectious pathogens is, therefore, key to improving treatment efficacy and guiding clinical usage of drugs toward development of personalized medicine. However, the detection of genetic markers is still a major challenge for molecular-based diagnostic technologies, especially for those aiming to be deployed at point-of-care (PoC).

Currently, high-throughput methods such as Next Generation Sequencing or Sanger sequencing are considered the gold standard for low frequency allele detection. Despite their advancement in the past decade, their high cost and time to report results limit their use outside specialized laboratories.[2,3] A variety of PCR-based methods have been developed to enhance detection of genetic markers. These allele-specific PCR (AS-PCR) techniques can be classified into two main groups: major allele suppression methods and minor allele enrichment methods. Within the first group, blocking primers have been designed to suppress the amplification of the major allele, enabling minor allele amplification by allele-specific primers. Different approaches have been reported such as (i) PCR blocking primers with a poly-A tail of four nucleotides[4] or with 3′ end modifications (ddNTPs, carbon-spacer, or inverted DNA nucleotides),[5−7] (ii) PCR clamping[6] based on the incorporation of peptide nucleic acids,[8] bridged nucleic acids,[9] or locked nucleic acids,[10] or (iii) PCR amplification of previously treated DNA with lambda exonuclease.[11] Within the second group, AS-PCR techniques have focused on enhancing the amplification of the minor allele[12] by the incorporation of (i) primers specific to the SNP at their 3′ end,[13] (ii) primers with mismatches within the four bases from the 3′ end,[14,15] or (iii) primers of different lengths for melting temperature (T_m) analysis.[16] However, the need of thermal cycling, gel electrophoresis,[17] and modified oligonucleotides for validation

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elevate the time and cost per sample, preventing these PCR-based methods from being widespread to decentralized areas.

An alternative to PCR is isothermal DNA amplification techniques, which offer nucleic acid synthesis at constant temperature using simpler and less complex equipment better suited to applications at PoC. Several isothermal techniques have been reported, each one with its own innovative characteristics and temperature requirements in the range of 30−65 °C, depending on the enzymes used in the reaction. The most popular techniques are (i) recombinase polymerase amplification,[18] (ii) nucleic acid sequence-based amplification,[19] (iii) loop-mediated isothermal amplification (LAMP),[20] and (iv) helicase-dependent amplification.[21] Among them, LAMP has become popular due to advantages such as its high efficiency, high amplification yield, high specificity due to four to six primers in the reaction, strand displacement DNA synthesis between 60 and 65 °C, and the capability of visual detection of products due to precipitation of magnesium pyrophosphate.[22,23] Allele-specific LAMP (AS-LAMP) has been previously described for minor allele amplification. Several approaches have been reported such as (i) placing the SNP at the 5′ end of overlapped FIP and BIP primers,[24,25] (ii) placing the SNP at the 3′ end of B2 or LB primer,[26] or (iii) introducing additional mismatches.[27,28] These methods either delay the onset of the unspecific reaction, or the time-to-positive (TTP) of the specific reaction is too late such that their applicability is limited to test high concentrated samples. Each design is unique and target dependent, preventing their standardization across other relevant SNPs. The addition of universal QProbe[29] or the utilization of the *Taq Mut* enzyme[30] have addressed this to an extent, however, the need of melting analysis, complex primer design, or low specificity prevent the use of these methods at the PoC.

Here, we describe a new method and propose universal primer design guidelines for enhanced SNP detection at isothermal conditions based on (i) SNP-based loop-mediated isothermal amplification (sbLAMP) primers, which consist of six primers targeting eight different regions, with two of them responsible for AS-LAMP amplification, and (ii) the novel unmodified self-stabilizing (USS) competitive primers, complementary to the SNP at their 5′ end, which are responsible for robustly delaying or preventing unspecific sbLAMP amplification. In contrast to conventional blocking PCR primers, which present 3′ end modifications, the proposed USS primers are chemically unmodified with capability to extend (higher stability) and compete with other primers and intermediate amplified structures in the reaction. We demonstrate successful application of our method, defining universal primer design guidelines based on the detection of the SNP *C580Y* located in the gene *kelch 13* (*K13*) of the AT-rich genome of *P. falciparum* responsible for resistance to artemisinin-based drug treatment of malaria.[31,32] As validation, the same principles were used to develop USS-sbLAMP primers for detection of another antimalarial-resistant *K13* SNP, *Y493H*. The proposed method has shown high sensitivity, efficiency, specificity and rapid TTP (<35 min) for SNP detection, and it is also applicable to mixed populations. The primer design guidelines we provide at the end of each section aim to give a foundation for the design of new USS-sbLAMP primer sets for the detection of other relevant targets. Diagnostic tests incorporating the proposed isothermal chemistry will greatly expand the capability of rapid SNP screening, including in limited-resource settings (LRS) where rapid, sensitive, and specific diagnostics of infectious diseases are of vital importance.

EXPERIMENTAL SECTION

Primer Design of USS-sbLAMP Specific to SNP *C580Y* and SNP *Y493H*. The USS-sbLAMP method consists of a total of eight primers targeting ten distinct regions of the DNA template. The sbLAMP primer set is composed of two outer primers (F3 and B3), two loop primers (LF and LB) and two inner primers (sbFIP and sbBIP), where F1c and B1c locate the SNP at their 5′ end. The USS primer set consists of a forward blocking competitive primer (FB) and a backward blocking competitive primer (BB). The USS-sbLAMP primer sets for the specific detection of *C580Y* and *Y493H* were designed based on the gene *K13* of *P. falciparum*. Consensus reference genomic sequences from all human-infective *Plasmodium* species (*PF3D7_1343700.1*, *PFIT_1342900*, *PKNH_1257700*, *PKNOH_S09541100*, *PVP01_1211100*, *PVX_083080*, *PmUG01_12021200*, *POcGH01_12019400*) were retrieved from Plasmodium Genomic Resource (PlasmoDB)[33] and aligned using MUSCLE algorithm[34] in Geneious 10.0.5 software.[35] The sbLAMP primer set was designed using Primer Explorer V5 (Eiken Chemical Co. Ltd., Tokyo, Japan; http://primerexplorer.jp/lampv5e/index.html) and optimized manually to locate the SNP at the 5′ end of F1c and B1c, following the method described by Eiken Chemical Co., Ltd.,[24] with the novelty of considering the local GC% composition for the design of FIP and BIP primers, named as sbFIP and sbBIP. Different lengths of F1c and B1c were designed for the SNP *C580Y* (17 bp, 19 bp, 21 bp, and 25 bp for F1c, and 21 bp, 25 bp, 27 bp, and 29 bp for B1c) and for the SNP *Y493H* (21 bp for F1c and 17 bp for B1c). The USS primer sets were designed based on the most suitable pair of F1c and B1c for allele-specific amplification based on experimental data (see Results and Discussion). FB and BB primers of different lengths were manually designed, always equal or longer than the selected F1c and B1c. They were between 0 and 6 bp longer at the 3′ end (additional 0 to −3.93 kcal/mol^{-1}) and locate the SNP within the 3 bp (up to −1.31 kcal/mol^{-1}) closer to their 5′ end (FB0, FB1, FB2 and BB0, BB1 and BB2), where the number indicates the position of the SNP from the 5′ end. To investigate the performance of the competitive primers, reverse-complementary primers to FB/BB, named FA/BA, were designed, and 3′ end modifications (/3AmMo/) were added to each primer set, named FA*/BA* and FB*/BB*. All primers used in this paper can be found in Table S1 and final USS-sbLAMP primer sets in Table S2.

USS-sbLAMP Mechanism. The incorporation of USS primers in the sbLAMP reaction enhances allele discrimination by preventing unspecific sbLAMP amplification (caused by unspecific sbLAMP primers) through thermodynamically favored hybridization to the template due to total complementarity and self-stabilizing capability. USS primers and sbFIP/sbBIP primers (within sbLAMP) are in the same reaction mixture but always targeting different alleles, one set specific to the wild type (WT) allele and the other to the mutant (MT) allele. Allelic discrimination is possible by comparing the outcome of two independent reactions: WT reaction ($sbLAMP_{WT}$ and USS_{MT} primers) and MT reaction ($sbLAMP_{MT}$ and USS_{WT} primers), as shown in Scheme 1. sbLAMP follows the LAMP method described by Notomi et al.[20] It is initiated by the binding of two inner primers sbFIP (F1c-F2) and sbBIP (B1c-B2), which bind to F2c and B2c

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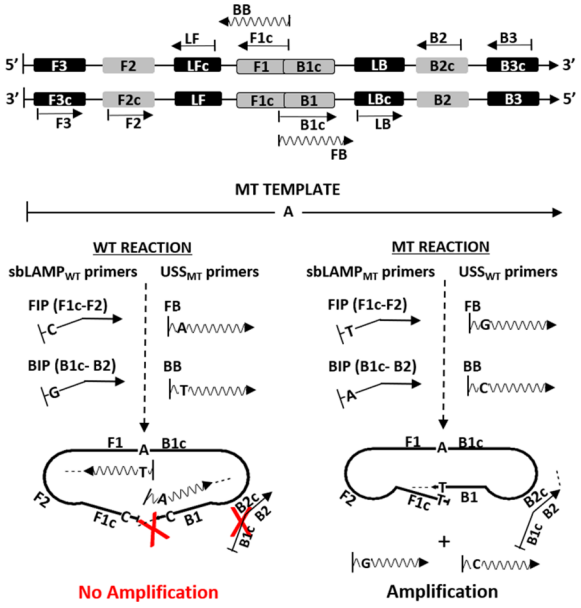
Scheme 1. Mechanism of USS-sbLAMP in the Presence of Mutant DNA[a]



[a]As an example, mutant (MT) DNA presents allele A and it is uniquely amplified within the MT reaction (sbLAMP$_{MT}$ and USS$_{WT}$ primers). Amplification of the MT DNA within the wild type (WT) reaction (sbLAMP$_{WT}$ and USS$_{MT}$ primers) is significantly delayed or prevented by the annealing of complementary USS$_{MT}$ primers to the template.

regions, respectively, leaving F1c and B1c free. Two outer primers F3 and B3 displace the strands, releasing single-stranded DNA (ssDNA). The free F1c and B1c form a dumbbell-like structure by annealing to their complementary sequences F1 and B1, respectively. It is rapidly linearized from its 3′ end, and the binding of sbFIP and sbBIP initiate the cyclic amplification step. During this step, LF and LB primers bind their complementary sequences, which are in between B1c and B2c and F1 and F2, further accelerating the reaction.[36]

The complementarity of the USS primers to the target template (with SNP specificity) suggests the possibility of hybridization during the initiation stage of sbLAMP, causing a general delay in specific and unspecific reactions until USS primers are displaced by F3/B3 and sbFIP/sbBIP (F2 and B2, specifically). At the end of the initiation stage, the annealing of the USS primers to the template and the formation of the dumbbell-like structure with the binding of sbFIP and sbBIP cannot occur simultaneously. Consequently, there will be a competition and the most energetically favorable reaction, the specific reaction, will occur at a first stage. In case that the DNA template is not specific to the sequence of the USS primers, they may anneal, but the 5′ end will be unbound leaving a toehold for loop formation and subsequent amplification by specific sbLAMP primers. If the DNA template is specific to the sequence of the USS primers, a second stage will take place. The association between the USS primers and the template is not transient and a highly stable primer-template complex is formed as nucleotides are incorporated by the DNA polymerase. FB will anneal to the B1 region of the dumbbell-like structure preventing the annealing between B1 and B1c regions of this structure (this 3′ end cannot act as a primer) and the annealing of B2 of the BIP primer to the B2c region of the dumbbell-like structure; BB will anneal to the F1 region of the dumbbell-like structure preventing the annealing between F1 and F1c regions of this structure (this 3′ end cannot act as a primer) and the annealing of F2 of the FIP primer to the F2c region of the dumbbell-like structure. This self-stabilizing behavior will create byproducts that do not prime amplification and, therefore, will inhibit the formation of dumbbell-like structures preventing unspecific amplification.

The hybridization energies of specific FB/BB must be favorable with respect to the energies of unspecific F1c/B1c, $\Delta G^{\circ}_{(F1c/B1c)} > \Delta G^{\circ}_{(FB/BB)}$. To achieve that, the design of USS primers followed two principles: primer elongation along the 3′ end and displacement of the position of the SNP from their 5′ end. Regarding the first principle, FB/BB primers should be equal or longer than F1c/B1c. Overlapping with the 5′ end of LF and LB must be minimum (1−2 bp) and preferably avoided, although it might be tolerated since only one of the USS primers will be slightly compromised (some mismatches with the template, although it might be able to bind because the region is free) when preventing the formation of the dumbbell structure. Since USS primers should not delay the TTP, their free energy values should not exceed in more than 10 kcal mol^{-1} the free energy values of F1c/B1c. Regarding the second principle, placing the SNP slightly away from the 5′ end of USS primers might enhance their specificity due to the fact that a local region around the SNP will be opened or closed based on their complementarity at the SNP position. Depending on the sequence of the target, placing the SNP at their 5′ end could be the most optimum configuration.

Thermodynamic Calculations of USS-sbLAMP Primers. Possible secondary structures, primer dimer formation, and hybridization stability were checked using NUPACK[37] and NetPrimer (Premier Biosoft, NetPrimer; https://www.premierbiosoft.com/netprimer/). Equations[38−40] can be found in Table S3 and obtained ΔG° values in Tables S4, S7, S9, and S11.

Samples and DNA Extraction Methods. Two gBlock Gene fragments of 607 bp were purchased from Integrated DNA Technologies (Table S2) and resuspended in TE buffer to 5 ng/μL stock solutions (stored at −20 °C). The WT synthetic DNA template (named here as WT template) contained SNPs *580C* and *493Y*; the MT synthetic DNA template (named here as MT template) contained the corresponding drug-resistant mutations, *580Y* and *493H*. *P. falciparum* genomic DNA (gDNA) was isolated using the PureLink Genomic DNA Mini Kit (ThermoFisher Scientific) from Cambodian and Thai culture adapted asexual parasites harboring the WT *K13* allele (ANL1 and ARN1G, respectively), a Thai isolate harboring *K13 539T* mutation (APS2G), a Cambodian isolate containing *K13 493H* mutation (ANL8G), and a Cambodian isolate with *K13 580Y* mutation (ANL5G). *P. ovale curtisi*, *P. ovale wallikeri*, *P. vivax*, *P. malariae*, and *P. knowlesi* clinical isolates (gDNA) were kindly provided by Prof. Colin Sutherland. The samples were stored at −20 °C until experiments were performed.

USS-sbLAMP Reaction Conditions. Two independent reactions, WT reaction and MT reaction, were performed with each target. Each reaction mixture contained the following: 1.5 μL of 10× isothermal buffer, 0.9 μL of $MgSO_4$ (100 mM stock), 2.1 μL of dNTPs (10 mM stock), 0.375 μL of BSA (20 mg/mL stock), 2.4 μL of Betaine (5 M stock), 0.375 μL of SYTO 9 Green (20 μM stock), 0.6 μL of *Bst* 2.0 DNA polymerase (8,000 U/mL stock), 3 μL of different

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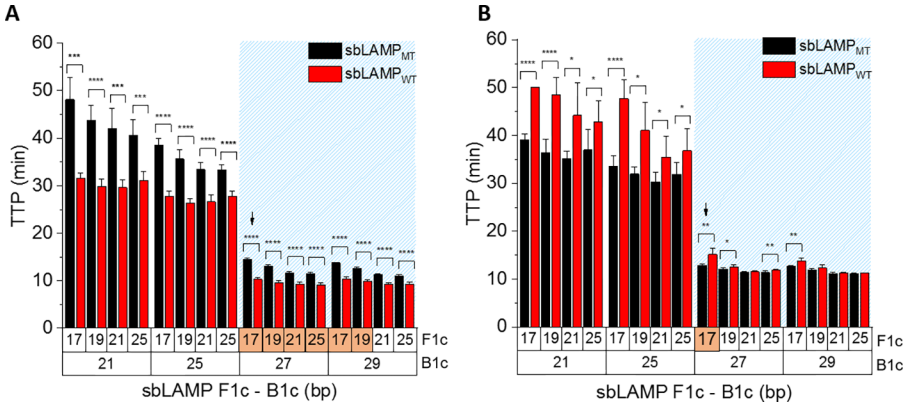

Allele	F1c \ B1c	17 bp	19 bp	21 bp	25 bp
WT 580C	21 bp	16.5	14.0	12.4	9.6
	25 bp	10.7	9.3	6.8	5.5
	27 bp	4.2	3.4	2.4	2.3
	29 bp	3.3	2.8	1.9	1.7
MT 580Y	21 bp	10.9	12.2	9.1	5.9
	25 bp	14.2	9.1	5.1	5.0
	27 bp	2.3	0.4	0.1	0.5
	29 bp	1.1	0.4	0.1	0.1

Figure 1. Results of sbLAMP using different F1c-B1c primer lengths. (A) Comparison of WT template (*580C*) amplification by WT specific (sbLAMP$_{WT}$) and MT specific (sbLAMP$_{MT}$) primer sets with different lengths of F1c and B1c. (B) Comparison of MT template (*580Y*) amplification by WT specific (sbLAMP$_{WT}$) and MT specific (sbLAMP$_{MT}$) primer sets with different lengths of F1c and B1c. (C) Table showing the ΔTTP values between specific and unspecific primer sets. Average TTP values of specific reactions below 20 min are blue shadowed. ΔTTP values above 2 min are orange colored. Average of two experiments performed in triplicates using 5 × 10^4 copies/reaction of synthetic DNA. Selected sbLAMP primer sets are indicated with arrows in the plots and dark blue cells in the table.

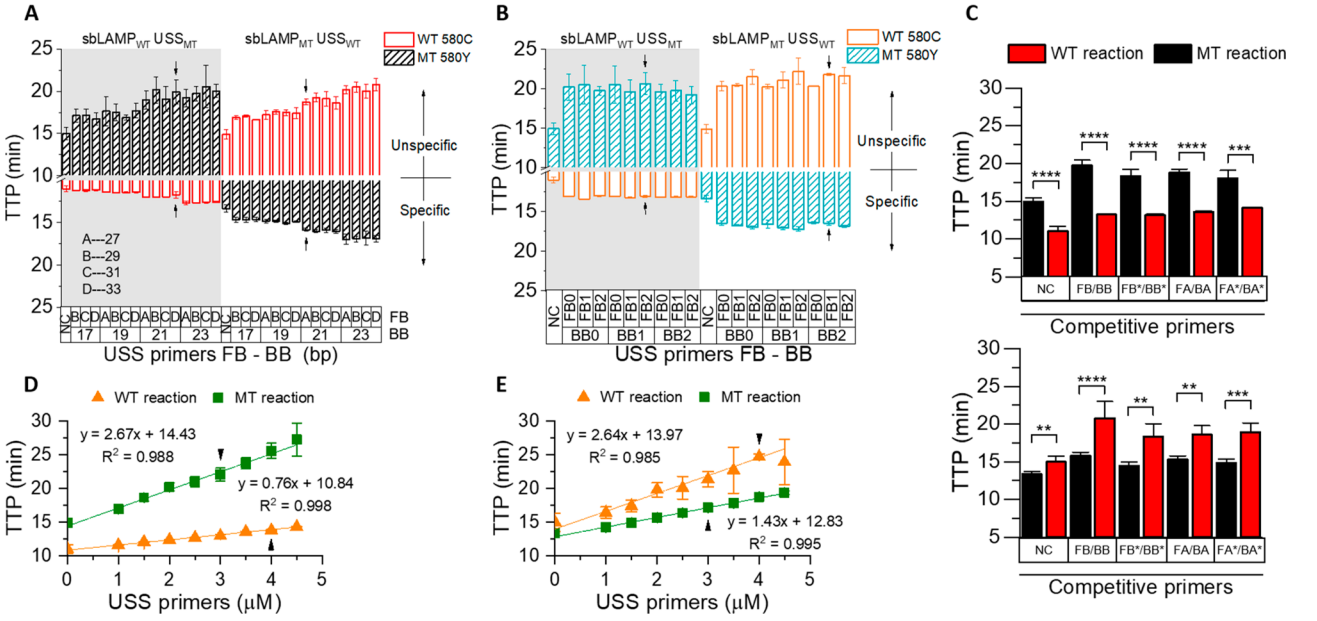

Figure 2. Incorporation of USS primer for enhancement of sbLAMP. (A) Performance comparison of USS primers with different lengths added to sbLAMP reaction at 2 μM/reaction for detecting SNP *C580Y*. A total of 15 different combinations were tested. Selected USS primers are indicated with arrows (****p*-value). (B) Performance comparison of redesigned USS primers added to sbLAMP reaction at 2 μM/reaction for detecting SNP *C580Y*. A total of 9 different combinations were tested. Selected USS primers are indicated by arrows (****p*-value). (C) Comparison of modified, unmodified and reverse competitive primers for allele-specific detection (WT template in the upper section, and MT template in the lower section). (D, E) Optimisation of the concentration of USS primers (FB/BB). WT template (*580C*) in (D) and MT template (*580Y*) in (E). Selected concentrations for the detection of SNP *C580Y* are labeled by arrows, USS$_{MT}$ at 4 μM and USS$_{WT}$ at 3 μM. Average of two experiments performed in triplicates using 5 × 10^4 copies/reaction of synthetic DNA. Negative control (NC), no addition of USS primers.

concentrations of synthetic DNA or gDNA, 1.5 μL of 10× sbLAMP primer mixture (20 μM sbBIP/sbFIP, 10 μM LF/LB, and 2.5 μM B3/F3), and enough nuclease-free water (ThermoFisher Scientific) to bring the volume to 15 μL. All reagents were purchased to New England BioLabs and all synthetic DNA to Integrated DNA Technologies. Reactions were performed at 63 °C for 50 min for screening purposes and 30−35 min for final assays. For high resolution melting, SYTO 9 Green was replaced by EvaGreen (Biotium, California) at a final concentration of 0.6 μM and one cycle was performed at 95 °C for 60 s, 40 °C for 60 s, 65 °C for 1 s, and 97 °C for 1 s (Figure S1). Experiments were performed twice, and each condition was run in triplicates (5 μL each reaction) loading the reactions into LightCycler 480 Multiwell Plates 96 (Roche Diagnostics) utilizing a LightCycler 96 Real-Time PCR System (Roche Diagnostics). Competitive primers (FA/BA, FA*/BA*, FB/BB, or FB*/BB*) were incorporated into the sbLAMP 10× primer mixture at different final concentrations: 1, 1.5, 2, 2.5, 3, 3.5, 4, and 4.5 μM. Nuclease-free water was adjusted to bring the volume to 15 μL. sbLAMP and USS primers were purchased from Integrated DNA Technologies and resuspended in nuclease-free water to 100 and 400 μM stock solutions, respectively. The solutions were stored at 4 °C.

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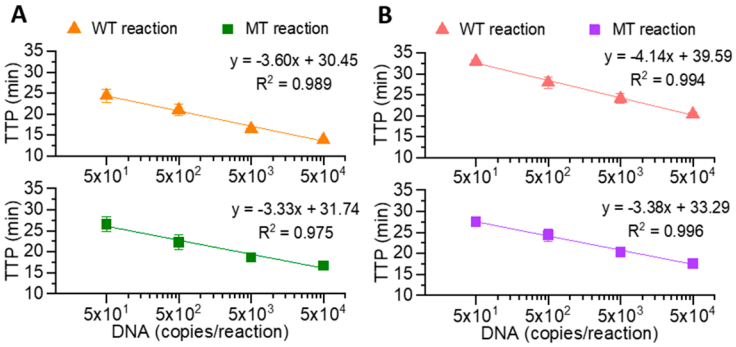

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SNP	DNA (copies/reaction)	WT template TTP ± SD (min)		MT template TTP ± SD (min)	
		USS_{MT} $sbLAMP_{WT}$	USS_{WT} $sbLAMP_{MT}$	USS_{MT} $sbLAMP_{WT}$	USS_{WT} $sbLAMP_{MT}$
SNP C580Y	5 x 10^4	13.9 ± 0.2	22.8 ± 1.3	25.2 ± 1.1	16.7 ± 0.7
	5 x 10^3	16.5 ± 0.3	26.2 ± 2.1	NEG	18.7 ± 0.6
	5 x 10^2	21.0 ± 1.4	NEG	NEG	22.3 ± 1.8
	5 x 10^1	24.4 ± 1.6	NEG	NEG	26.6 ± 1.8
SNP Y493H	5 x 10^4	20.4 ± 0.4	29.0 ± 1.2	NEG	17.6 ± 0.4
	5 x 10^3	24.1 ± 1.2	NEG	NEG	20.4 ± 0.4
	5 x 10^2	27.9 ± 1.4	NEG	NEG	24.3 ± 1.5
	5 x 10^1	32.9 ± 0.4	NEG	NEG	27.6 ± 0.5

Figure 3. Standard curves of specific USS-sbLAMP reactions for detection of SNP *C580Y* and SNP *Y493H*. (A) USS-sbLAMP amplification of serially diluted WT template (*580C*) and MT template (*580Y*), in the upper and lower section, respectively. (B) USS-sbLAMP amplification of serially diluted WT template (*493Y*) and MT template (*493H*), in the upper and lower section, respectively. (C) Table showing the TTP values of WT and MT reactions with WT or MT template independently. Reactions were considered negative above 30 min for *C580Y* and above 35 min for *Y493H*. Average of two experiments performed in triplicates.

Sensitivity of USS-sbLAMP Method. Sensitivity was evaluated using 10-fold serial dilutions of synthetic DNA: 5 × 10^4, 5 × 10^3, 5 × 10^2, and 5 × 10^1 copies/reaction. Standard curves were generated by plotting the TTP against copies/reaction with errors at one standard deviation. Sensitivity of mixed populations was evaluated by spiking synthetic DNA harboring the WT allele and the MT allele at different ratios (100/0, 80/20, 50/50. 20/80, and 0/100, in percentages) from stock solution at 2.5 × 10^3 copies/reaction.

Cross-Reactivity of USS-sbLAMP Method and Detection of Clinical Isolates. Ten clinical isolates (gDNA) were used to evaluate the feasibility of USS-sbLAMP and to prove the absence of cross-reactivity with any other human-infective *Plasmodium* species. Samples included *P. ovale curtisi*, *P. ovale wallikeri*, *P. vivax*, *P. malariae*, *P. knowlesi* (2 samples), *P. falciparum* harboring *WT K13* alleles (2 samples), *P. falciparum* harboring the MT allele *580Y*, *493H*, and *539T* allele (3 different samples). Experiments were performed as described above.

Statistical Analysis. Data is presented as mean TTP ± standard deviation; *p*-values were calculated by Student's heteroscedastic t-test, with a two-sided distribution. Statistically significant difference was considered as **p*-value < 0.05, ***p*-value < 0.01, ****p*-value < 0.001, *****p*-value < 0.0001; k-means cluster analysis and ANOVA test were performed in Origin software (OriginLab, Northampton, MA).

RESULTS AND DISCUSSION

sbLAMP Method. Different lengths of F1c and B1c were designed in order to study the impact of local GC% content, T_m and primer length in (i) allele-specificity and (ii) TTP. The design of overlapping primers for allele detection is not trivial, and the dissimilarity of the sequence in terms of ACTG composition upstream and downstream the position of the SNP should be taken into account. In general, primers are equal in length (23−24 bp)[24] and are considered specific and stable as long as they present optimal GC% content and T_m to anneal to the DNA template.[41] Due to the fact that the GC% content and T_m are different at local regions with respect to the position of the SNP, allele-specific primers sbFIP and sbBIP might be of different lengths. F1c and B1c of four different lengths each (Table S1) were designed with hybridization energies considered between −10.00 and −23.00 kcal mol^{-1} (Table S4), increasing proportionally to the length of the primers and the GC%. Real-time amplification experiments of

Table 1. Study of the USS-sbLAMP Method in Mixed Populations Harbouring SNPs *C580Y* and *Y493H*[a]

spiked DNA WT/MT (%)	WT reaction *580C* TTP ± SD (min)	MT reaction *580Y* TTP ± SD (min)
100/0	15.6 ± 0.6	NEG
80/20	15.9 ± 0.7	19.5 ± 0.9
50/50	16.2 ± 0.7	19.1 ± 0.2
20/80	16.7 ± 0.9	18.6 ± 0.4
0/100	NEG	18.1 ± 0.4
spiked DNA WT/MT (%)	WT reaction *493Y* TTP ± SD (min)	MT reaction *493H* TTP ± SD (min)
100/0	23.0 ± 1.9	NEG
80/20	23.7 ± 1.4	22.8 ± 1.3
50/50	24.4 ± 1.9	21.1 ± 1.8
20/80	25.9 ± 1.9	20.4 ± 1.8
0/100	NEG	20.0 ± 1.5

[a]Spiked mixed populations at different ratios (100/0, 80/20, 50/50, 20/80, and 0/100, in percentages) from stock at 2.5 × 10^3 copies/reaction. Average of two experiments performed in triplicates.

WT (Figure 1A) and MT (Figure 1B) templates were performed independently. Two independent reactions, WT specific ($sbLAMP_{WT}$) and MT specific ($sbLAMP_{MT}$), were tested with each template. A total of 16 combinations of F1c-B1c were performed; F1c of 17, 19, 21, and 25 bp and B1c of 21, 25, 27, and 29 bp. For both DNA templates, the TTP of specific and unspecific reactions was reduced as B1c was elongated from 21 to 29 bp, and allele-specific detection was enhanced as F1c was shortened from 25 to 17 bp.

The amplification time difference (ΔTTP) between specific and unspecific reactions with each template is presented in Figure 1C. Detailed TTP values of all reactions are reported in Table S5. The results in Figure 1 showed the existence of a critical length for B1c (>27 bp) at which the TTP was significantly reduced (highlighted in blue). Two highly significant clusters were obtained by performing k-means cluster analysis and ANOVA test of sbLAMP results of WT and MT templates ($P > F = 4.44 \times 10^{-15}$ and $P > F = 1.53 \times 10^{-14}$, respectively). The selected sbLAMP primer sets according to the highest ΔTTP were $F1c_{17}$-$B1c_{27}$ for both alleles (indicated by arrows in Figure 1A,B, and dark blue cells in Figure 1C).

Guideline 1. Allele-specificity and TTP were significantly enhanced by modifying the length of F1c and B1c with respect to the standard sizes. It is recommended to design allele-

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Table 2. Cross-Validation of the USS-sbLAMP Method for Detection of SNP *C580Y* and SNP *Y493*[a]

sample	WT reaction *580C* TTP ± SD (min)	MT reaction *580Y* TTP ± SD (min)	WT reaction *493Y* TTP ± SD (min)	MT reaction *493H* TTP ± SD (min)	Pan-P TTP ± SD (min)
580C[1]	17.8 ± 0.2	NEG	26.3 ± 1.4	NEG	10.5 ± 0.7
580C[2]	16.7 ± 0.1	NEG	25.0 ± 1.6	NEG	9.2 ± 0.4
580Y	NEG	18.5 ± 0.6	25.9 ± 1.8	NEG	8.1 ± 0.1
493H	13.8 ± 0.3	21.8 ± 0.9	NEG	18.5 ± 0.2	7.2 ± 0.1
539T	16.8 ± 0.4	NEG	26.1 ± 1.8	NEG	8.9 ± 0.2
Poc	NEG	NEG	NEG	NEG	9.2 ± 0.7
Pow	NEG	NEG	NEG	NEG	20.3 ± 9.7
Pv	NEG	NEG	NEG	NEG	9.3 ± 0.4
Pm	NEG	NEG	NEG	NEG	9.5 ± 0.7
Pk[1]	NEG	NEG	NEG	NEG	5.5 ± 0.1
Pk[2]	NEG	NEG	NEG	NEG	6.3 ± 0.3
NTC	NEG	NEG	NEG	NEG	NEG

[a]No cross-reactivity with any human-infective *Plasmodium*. Published Pan-Plasmodium primer set[50] was used as positive control. Samples tested were: *580C*[1] (*P. falciparum* WT *K13* sample 1), *580C*[2] (*P. falciparum* WT *K13* sample 2), *580Y* (*P. falciparum K13 580Y*), *493H* (*P. falciparum K13 493H*), *539T* (*P. falciparum K13 539T*), Poc (*P. ovale curtisi*), Pow (*P. ovale wallikeri*), Pv (*P. vivax*), Pm (*P. malariae*), Pk[1] (*P. knowlesi* sample 1), Pk[2] (*P. knowlesi* sample 2), and NTC (non-template control). Experiment was performed in triplicates.

specific primers of different lengths depending on the GC% content at the local region. Shortening the primer that sits at the richer GC% region proved to enhance allele-specificity (preferably not less than 13 bp, i.e. −8.52 kcal mol^{-1}). Elongating the primer that sits at the richer AT% region with respect to the other primer equalized their free energy values and T_m (even favoring the primer that sits at the richer AT% region) such that both primers performed properly (early TTP). For optimal performance (allele-specificity and early TTP) more than 15 bp difference (−9.83 kcal mol^{-1}) between F1c and B1c should be avoided.

Incorporation of USS Primers for Enhancement of sbLAMP. USS primers were incorporated into the sbLAMP reaction at 2 μM to prevent unspecific sbLAMP amplification. FB and BB of different lengths (FB of 27, 29, 31, and 33 bp and BB of 17, 19, 21, and 23 bp) were designed (Table S1) and a total of 15 different combinations were tested. Real-time amplification experiments of WT and MT templates were performed independently. Two independent reactions, WT reaction ($sbLAMP_{WT}$ and USS_{MT}), and MT reaction ($sbLAMP_{MT}$ and USS_{WT}), were tested with each template. The TTP of specific reactions are shown in the lower section of Figure 2A and the TTP of unspecific reactions in the upper section. Detailed TTP values can be found in Table S6. As the length of USS primers is increased unspecific reactions are significantly delayed, while specific reactions are slightly delayed. Consequently, there is a trade-off between allele-specificity and sensitivity. The length of BB had a higher impact on the delay of both reactions than the length of FB. This behavior might be attributed to the richer GC% content of the BB region and hence, the free energy hybridization values (Table S7). In general, USS primers must be within an optimal length range, and results of both templates, WT and MT, must be interpreted as a whole to select the most suitable USS primer set. The criterion for the selection of the most optimal size of FB/BB was based on (i) minimizing the TTP of the specific reactions and (ii) maximizing the TTP of the unspecific reactions. Because both criteria cannot be fulfilled by any USS primer set, a balance is considered as the optimal. The selected lengths were USS_{WT} FB_{27}/BB_{21} and USS_{MT} FB_{33}/BB_{21}. The ΔTTP between specific and unspecific reactions was statistically significant (****p-value) for both templates, WT and MT.

To enhance allele-specificity, the selected FB/BB primers were redesigned to locate the SNP at three different positions from their 5′ end (5′X···3′ named as FB0/BB0, 5′NX···3′ named as FB1/BB1, and 5′NNX···3′ named as FB2/BB2 where X denoted the SNP) by adding nucleotides totally complementary to the DNA template (Table S1). A total of nine different combinations were tested. Detailed TTP values can be found in Table S8. According to the estimated free energy values presented in Table S9, as far as the SNP is displaced away from the 5′ end, the specific hybridization is favored due to enhanced primer stability (additional −0.66 to −1.31 kcal mol^{-1}). However, the risk of creating an internal bulge between unspecific USS primers and the DNA template prevents to locate the SNP far from the third position (i.e., FB2/BB2) from the 5′ end of FB/BB primers. TTP of specific reactions were not significantly affected by the incorporation of the redesigned USS primers. However, unspecific reactions were further delayed, contributing to allele-specific detection. Based on the experimental results obtained in Figure 2B the selected redesigned primers were USS_{WT} $FB1_{27}/BB2_{21}$ and USS_{MT} $FB2_{33}/BB1_{21}$. The ΔTTP between specific and unspecific reactions was statistically significant (****p-value) for both templates, WT and MT.

Guideline 2. The nucleotide sequence of FB/BB primers is restricted by the template, and their length is limited by the 5′ end of LF and LB primers. It is recommended that USS primers are between 0 to 8 bp longer than F1c/B1c primers (additional 0 to −5.24 kcal mol^{-1}) with GC clamps at their 3′ end, if possible, to favor their stability upon hybridization. Locating the SNP within the three base pairs closer to their 5′ end (preferably FB1/BB1 or FB2/BB2, meaning an additional −0.66 to −1.31 kcal mol^{-1}) favor specific hybridization to their target template. Placing the SNP more than 4 bp (additional −2.62 kcal mol^{-1}) away the 5′ end should be avoided due to the high risk of internal bulges formation and unspecific amplification.

Comparison of Modified, Unmodified, and Reverse Competitive Primers. Based on the selected USS_{WT} $FB1_{27}/BB2_{21}$ and USS_{MT} $FB2_{33}/BB1_{21}$ primers, their reverse-complement and the addition of 3′ end modifications were studied.

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Reverse-complement primers to FB/BB, named FA/BA, located the SNP at the 3′ end in contrast to FB/BB which located the SNP at the 5′ end. The 3′ end modified versions of FB/BB and FA/BA, named FB*/BB* and FA*/BA*, included an amino modification which prevented the elongation of the primers by the DNA polymerase. The results presented in Figure 2C showed the similar behavior of the four primers in combination with sbLAMP. This data proved for the first time that unmodified primers (FB/BB and FA/BA) can perform similarly or better for allele-specific detection than modified primers, based on statistical analysis. *t* test was performed to compare the ΔTTP values obtained using modified and unmodified primers with each template. In the case of the WT template, FB/BB and FA/BA performed better than their modified versions (*p*-value **** for both of them). In the case of the MT template, FB/BB outperformed FB*/BB* (*p*-value *), but no statistical significance was observed for FA/BA and FA*/BA*. Regarding the two sets of unmodified primers, FB/BB presented higher ΔTTP values than FA/BA. Compared to other strategies based on 3′ end chemical modifications,[14,15,42] and the addition of mismatches at 3′ or 5′ end of FIP[28] or BIP,[27] our methodology relies on total complementary unmodified primers with extension capability. The easier design and the lack of chemical modifications reduce the cost of the assay and production time, positioning this method as a promising molecular-based technique for SNP discrimination to be used at PoC.

Guideline 3. Unmodified primers performed similarly or better than 3′ end chemically modified primers to prevent unspecific amplification by sbLAMP, based on statistical analysis. The use of FB/BB (SNP placed closed to the 5′ end) is recommended due to higher ΔTTP between specific and unspecific reactions, easier design and lower cost and production time.

Study of Different Concentrations of USS Primers. The selected USS_{WT} $FB1_{27}/BB2_{21}$ and USS_{MT} $FB2_{33}/BB1_{21}$ primers were added to the sbLAMP reaction mix at different concentrations: 1, 1.5, 2, 2.5, 3, 3.5, 4, and 4.5 μM. Real-time amplification experiments of WT and MT templates were performed independently. For each template, WT and MT reactions were tested (Figure 2D,E). Specific reactions were slightly delayed as the concentration of the USS primers was increased. The TTP values followed a linear fit with slopes of 0.76 and 1.48 for the WT and the MT template, respectively. Although the unspecific reactions also followed a linear fit, their slopes were sharper, denoting the significance of the concentration of FB/BB on delaying unspecific amplification. The TTP of specific and unspecific reactions was evaluated to discern the optimum concentration of USS primers for allele discrimination. Analyzing all the combinations of different concentrations, the one presenting the highest ΔTTP for both templates without compromising the other was selected as the optimum (Table S10). Selected concentrations were USS_{WT} at 3 μM and USS_{MT} at 4 μM.

Guideline 4. The concentration of USS primers in the reaction mixture should be higher (preferably between 1.5× to 2.5×) than the concentrations of sbFIP and sbBIP, to not compromise the limit of the detection of sbLAMP.

Sensitivity of USS-sbLAMP Method. Sensitivity was tested using 10-fold serial dilutions (5×10^4, 5×10^3, 5×10^2, and 5×10^1 copies/reaction) of WT and MT templates independently (Figure 3A,C) for detection of SNP *C580Y*. Amplification curves are presented in Figure S2. For both alleles, the detection limit was 5×10^1 copies/reaction within 30 min. Standard curves were generated with R^2 values of 0.989 and 0.975, for WT and MT template, respectively, denoting the ability of the assay to robustly quantify samples. Pure WT and MT templates at concentrations below 5×10^3 and 5×10^4 copies/reaction, respectively, were uniquely amplified by their corresponding specific reactions providing "yes/no" results. Sensitivity of sbLAMP was not disrupted by the incorporation of the USS primers, and amplification of the nontarget template was successfully delayed or inhibited. Two reactions are always assessed (WT and MT reactions) for each sample, providing two TTP values being one of them negative at certain sample concentration (samples can be diluted if needed). Consequently, having always two reactions ensures the high specificity of the assay. Amplification was performed in less than 30 min at low DNA copies/reaction indicating the rapidness achieved utilizing the USS-sbLAMP method compared to other techniques based on PCR thermal cycling such as molecular beacons, TaqMan or FRET,[43,44] which require complex designs, more than 1 h to finish and usually postamplification analysis. Other reported isothermal assays usually need between 20 to 75 min to finish,[45,46] do not report quantitative data,[47] rely on the addition of probes (Au NPs) to enhance specificity,[48] lack amplification data at low concentrations,[26] or require sequencing for product specificity.[49]

Validation of the USS-sbLAMP Method with the Artemisinin-Resistant SNP *Y493H*. Following the above created guidelines for the design of USS-sbLAMP primers, a specific USS-sbLAMP primer set was developed to detect a second *K13* SNP, *Y439H* (Table S2). USS_{MT}-$sbLAMP_{WT}$ primer set consisted of $F1c_{21}$-$B1c_{17}$ + $FB2_{24}/BB1_{26}$ at 4.5 μM and USS_{WT}-$sbLAMP_{MT}$ primer set consisted of $F1c_{21}$-$B1c_{17}$ + $FB1_{24}/BB0_{26}$ at 4.5 μM. Primer properties and hybridization energies are provided in Table S11. Sensitivity was tested using 10-fold serial dilutions (5×10^4, 5×10^3, 5×10^2, and 5×10^1 copies/reaction) of WT and MT templates independently (Figure 3B,C). Amplification curves are presented in Figure S3. For both alleles, the limit of detection was 5×10^1 copies/reaction within 35 min. Standard curves were generated with R^2 values of 0.994 and 0.996, for WT and MT template. Pure WT and MT templates at concentrations below 5×10^4 copies/reaction and 5×10^5 copies/reaction, respectively, were uniquely amplified by their corresponding specific reactions.

Study of the USS-sbLAMP Method in Mixed Populations Harboring SNPs *C580Y* and *Y493H*. USS-sbLAMP primer sets for allele-specific detection of SNP *C580Y* and SNP *Y493H* were evaluated with spiked mixed populations at different ratios (100/0, 80/20, 50/50, 20/80, and 0/100, in percentages). The four alleles were clearly discriminated within their respective specific reactions, as shown in Table 1. Unspecific reactions did not amplify, demonstrating the specificity of the method considering mixed populations at 80/20% as the limit for this experiment.

Cross-Validation of USS-sbLAMP Method for Detection of SNP *C580Y* and SNP*Y493H* Using Clinical Isolates. The specificity of USS-sbLAMP for detecting SNP *C580Y* and *Y493H* was tested using gDNA samples from all human-infective *Plasmodium* species: *P. falciparum*, *P. ovale curtisi*, *P. ovale wallikeri*, *P. vivax*, *P. malariae*, and *P. knowlesi*, to prove the absence of cross-reactivity with any of them. In addition, three different *P. falciparum* samples harboring the mutations *580Y*, *493H*, and *539T* were tested. There was no

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cross-reactivity with any of the human-infective *Plasmodium* species (Table 2). The sample harboring the *539R* mutation was uniquely amplified by the WT reactions *580C* and *493Y*, denoting the high specificity achieved by the MT reactions *580Y* and *493H*, which only amplified the samples harboring their respective mutations.

CONCLUSION

The proposed USS-sbLAMP method achieves high sensitivity, efficiency, specificity, and rapidness (TTP < 35 min) for the detection of SNPs at isothermal conditions suitable for PoC applications. For the first time here, judiciously designed sbLAMP primers for allele-specific amplification are combined with novel unmodified self-stabilizing (USS) competitive primers specific to the SNP at their 5′ end which robustly delay or prevent unspecific sbLAMP amplification. The special design, based on the local GC% content at the SNP position enhanced allele specificity, and the superior concentration of USS primers suppressed significantly unspecific amplification offering an excellent linear working range with a limit of detection of 5×10^1 copies/reaction for the detection of two of the most important artemisinin-resistant SNPs *C580Y* and *Y493H* within 30 and 35 min, respectively. This universal isothermal method uses chemically unmodified primers totally complementary to the target template except at the SNP position (either wild-type or mutant allele), which significantly reduces the cost of reagents and equipment avoiding the need of thermal cycling and electrophoresis for product validation. The guidelines we provide aim to enable others to develop their own USS-sbLAMP primer sets to detect any kind of SNP, which can relate to drug resistance, disease susceptibility or cancer development. Diagnostic platforms that use CMOS-based ISFET electrochemical biosensors,[51,52] which identify targets using pH-based nucleic acid amplification, will perfectly couple with the USS-sbLAMP method described here, with efforts currently underway to integrate these. Any diagnostic device that can incorporate the proposed method will greatly expand the capability of rapid SNP screening at PoC, including LRS where infectious disease diagnosis and rapid drug resistance screening are urgently needed.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b02416.

Primer sequences, detailed TTP values, thermodynamic equations and values, high resolution melting, and standard amplification curves (PDF)

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Notes

The authors declare no competing financial interest.

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