

# Fluorometric Paper-Based, Loop-Mediated Isothermal Amplification Devices for Quantitative Point-of-Care Detection of Methicillin-Resistant *Staphylococcus aureus* (MRSA)

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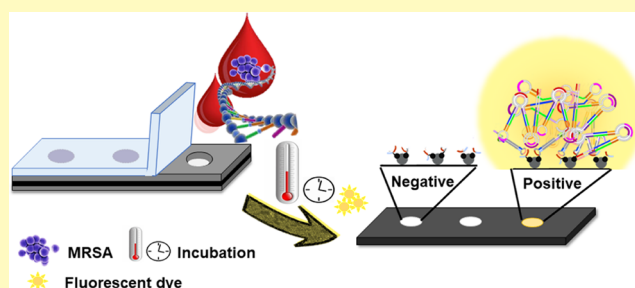
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**ABSTRACT:** Loop-mediated isothermal amplification (LAMP) has been widely used to detect many infectious diseases. However, minor inconveniences during the steps of adding reaction ingredients and lack of simple color results hinder point-of-care detection. We therefore invented a fluorometric paper-based LAMP by incorporating LAMP reagents, including a biotinylated primer, onto a cellulose membrane paper, with a simple DNA fluorescent dye incubation that demonstrated rapid and accurate results parallel to quantitative polymerase chain reaction (qPCR) methods. This technology allows for instant paper strip detection of methicillin-resistant *Staphylococcus aureus* (MRSA) in the laboratory and clinical samples. MRSA represents a major public health problem as it can cause infections in different parts of the human body and yet is resistant to commonly used antibiotics. In this study, we optimized LAMP reaction ingredients and incubation conditions following a central composite design (CCD) that yielded the shortest reaction time with high sensitivity. These CCD components and conditions were used to construct the paper-based LAMP reaction by immobilizing the biotinylated primer and the rest of the LAMP reagents to produce the ready-to-use MRSA diagnostic device. Our paper-based LAMP device could detect as low as 10 ag (equivalent to 1 copy) of the MRSA gene *mecA* within 36–43 min, was evaluated using both laboratory (individual cultures of MRSA and non-MRSA bacteria) and clinical blood samples to be 100% specific and sensitive compared to qPCR results, and had 35 day stability under 25 °C storage. Furthermore, the color readout allows for quantitation of MRSA copies. Hence, this device is applicable for point-of-care MRSA detection.

**KEYWORDS:** molecular diagnostic, DNA detection, loop-mediated isothermal amplification (LAMP), methicillin-resistant *Staphylococcus aureus* (MRSA), paper-based analytical device, point-of-care, quantitative detection



Methicillin-resistant *Staphylococcus aureus* (MRSA) is ubiquitous in hospitals and community settings and represents one major bacterial pathogen worldwide. MRSA can cause infections in different parts of the human body, from skin and cellulitis to invasive bacteremia, endocarditis, and medical device-associated infections. These diseases can be fatal especially in immunocompromised individuals.<sup>1,2</sup> MRSA is of healthcare concern because the bacteria resist all  $\beta$ -lactam antibiotics, e.g., penicillin, methicillin, and amoxicillin, and are highly transmissible through contagious contact with infected persons or contaminated objects.<sup>3,4</sup> The resistance to  $\beta$ -lactam antibiotics in MRSA is mediated by a mobile genetic element, which harbors a *mecA* gene that encodes penicillin-binding protein2a (PBP2a). While  $\beta$ -lactam antibiotics can inhibit cell wall synthesis resulting in cell death of general bacteria, the PBP2a in MRSA does not bind the  $\beta$ -lactam antibiotics and the bacterial cell wall synthesis remains functional.<sup>5,6</sup> Furthermore,

aerial dispersal of MRSA had been reported, and when compared with methicillin-susceptible *S. aureus* (MSSA), MRSA exhibited a greater mortality rate.<sup>7,8</sup> Therefore, early diagnosis of MRSA, including routine screening for MRSA carriers, is critically important to prevent the outbreak in hospitals and communities.<sup>9</sup>

Traditional detection methods of MRSA in clinics rely on *S. aureus* culture followed by antibiotic susceptibility testing such as antibiotic disc diffusion. These methods are labor-intensive, high priced, and require 2–3 days for culture and antibiotic

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Table 1. Primers for MRSA LAMP and PCR<sup>a</sup>

reactions	primer names	oligonucleotide sequences (5'-3')	T <sub>m</sub> (°C)
PCR	MRSA_F	AGATTGGGATCATAGCGTCAT	62.7
	MRSA_R	TTGAGGGTGGATAGCAGTACC	63.2
LAMP	MRSA_FIP	ACCTAATAGATGTGAAGTCGCTTTTTCATCTTACAATAATGAAACAGAA	76.0
	MRSA_BIP	TATGTTGGTCCCATTAACTCTGAAGTTCCTTTTACCAATAACTGCA	79.7
	MRSA_F3	GATGAATATTTAAGWGATTCGC	58.7
	MRSA_B3	TGGAGCTTTTATCGTAAAGTT	59.0
	MRSA_LF	TTCTAGAGGATAGTTACGACT	52.1
	MRSA_LB	CAAAAAGAATATAAAGGCTATAA	53.8
	MRSA_F	AGATTGGGATCATAGCGTCAT	62.7
PCR	MRSA_R	TTGAGGGTGGATAGCAGTACC	63.2
	MRSA_FIP	ACCTAATAGATGTGAAGTCGCTTTTTCATCTTACAATAATGAAACAGAA	76.0
LAMP	MRSA_BIP	TATGTTGGTCCCATTAACTCTGAAGTTCCTTTTACCAATAACTGCA	79.7
	MRSA_F3	GATGAATATTTAAGWGATTCGC	58.7
	MRSA_B3	TGGAGCTTTTATCGTAAAGTT	59.0
	MRSA_LF	TTCTAGAGGATAGTTACGACT	52.1
	MRSA_LB	CAAAAAGAATATAAAGGCTATAA	53.8

<sup>a</sup>The underlined letter in MRSA\_FIP represents a biotinylated base.

susceptibility tests.<sup>10</sup> The methods are also not as sensitive and precise as a conventional molecular diagnostic technique such as polymerase chain reaction (PCR) that relies on target gene *mecA* amplification and detection. Nevertheless, PCR requires special instruments including an expensive thermal cycler and an electrophoresis apparatus, which local clinics generally do not have, and the methods are rather complicated for nonscientist users. Recently, a loop-mediated isothermal amplification (LAMP) technique has been developed that is compatible with PCR but does not require a thermal cycler or an electrophoresis apparatus.

Today, LAMP represents one of the most widely used isothermal nucleic amplification techniques for point-of-care applications. LAMP comprises 4–6 target-specific primers and *Bst* DNA polymerase as a strand displacement enzyme to allow specific and sensitive intercalating amplification of the targeted gene at a single temperature within a rapid time frame (15–60 min). Unlike PCR, neither an additional 90–95 °C denaturation of dsDNA to a ssDNA template nor a 72 °C nucleotide extension is required. LAMP thereby needs only a simple heating pad (or a heat block) (~55–65 °C). In addition, the enzyme *Bst* DNA polymerase that is used in LAMP possesses greater efficiency in the presence of an inhibitor in the template (i.e., high or low pH, or salt, in clinical and food samples) than the *Taq* DNA polymerase that is typically used in PCR. Further, a simple 5 min boiling for crude DNA lysis has been reported to be efficient for subsequent LAMP.<sup>11–13</sup> However, the steps required to add LAMP reaction ingredients and the lack of simple color results hinder equipment-free and point-of-care detection.

Recently, a paper-based device has become promising for diagnostic applications at the point-of-care and for resource-constrained testings.<sup>14–16</sup> The paper-based platform is user-friendly and low-cost. However, the paper-based LAMP technique remains limited as most LAMP reactions are performed in a tube or all LAMP reagents are mixed prior to applying and analyzing the results on a membrane paper.<sup>17–20</sup> Some recent studies developed dried LAMP reagents as an all-in-one step onto a glass fiber<sup>21</sup> or a poly(ethylene terephthalate) (PET) film.<sup>22</sup> The prior prototype consisted of sample extraction and amplification parts connecting via automatic flow control that showed significant variation following a

position of the reaction pad owing to an unequal lateral flow from a loading pad to an absorbent pad, and its incubation step requires moisture (an extra need compared with our prototype). This prototype also lacked a stability (shelf life) report.<sup>21</sup> The latter prototype consisted of sample extraction and amplification parts connecting via a microfluid channel. The highlight is that this prototype contains a circuit that allows heating during a reaction incubation step to be performed via connection to a mobile phone. However, its limit of detection was relatively poor (100 copies) and the prototype's performance reduced very much in sensitivity after 21 days of ambient temperature storage. Additionally, the prototype construction of both designs are complicated and required some special instruments, such as a photolithograph and a plastic molder (CO<sub>2</sub> laser),<sup>21,22</sup> while we aimed to construct a prototype that could be made locally without any special instrument. For disposal recycling, the polymers glass fiber and PET are also known to induce carcinogenesis, so the waste requires special management.<sup>23,24</sup> We herein developed a ready-to-use and carcinogenic free (human health safe), cellulose paper-based LAMP device by utilizing the interaction between a biotin-tagged primer and a streptavidin central composite design (CCD) for optimizing LAMP reagents and conditions, coupled with a DNA fluorescent dye (e.g., SYBR Green I) for rapid point-of-care identification of MRSA. The sensitivity and specificity, as well as the stability of the paper-based LAMP activity and assay efficiency, were determined using laboratory strains and clinical blood samples.

## EXPERIMENTAL SECTION

**Bacterial Strains and Crude DNA Extraction.** MRSA strains used in this study included USA300 from the Department of Health Sciences and Technology, ETH Zürich, Switzerland, and six clinical isolates (clin1–6) from Phramongkutkdao Hospital, Bangkok, Thailand. The following non-MRSA strains were used as negative controls: *S. aureus* Newman (MSSA), *Staphylococcus epidermidis* MP04 (methicillin-sensitive CoNS), *Escherichia coli* JM109, *Enterococcus faecalis* ATCC 19433, and *Listeria monocytogenes* Scott A. The bacterial DNA was extracted by boiling in TE buffer (10 mM Tris–HCl, 1 mM disodium EDTA, pH 8.0) (40 µL of microbes/20 µL of TE buffer) for 5 min, immediately placed on ice, centrifuged at 13 000g for 10 min, and the supernatant was served as the template DNA.<sup>11,13</sup> The concentration and purity of the crude extracted DNA were determined

by a nanodrop spectrophotometer (Thermo Scientific, NY, U.K.) at wavelengths A260 and A260/A280, respectively.

**LAMP Primer Design.** The *mecA* gene sequences of MRSA retrieved from GenBank (<http://www.ncbi.nlm.nih.gov/genbank/>) (Table S1) were aligned to design six LAMP primers specific to *mecA* sequences using software Primer Explorer Ver. 5 (<http://primerexplorer.jp/elamp5.0.0/index.html>) and manual curation. The specificity of each primer was checked by BLASTN against the nonredundant GenBank database. Table 1 lists the MRSA LAMP primers, provided that a primer MRSA\_FIP was designed to tag biotin at the middle base (T) between regions F1 and F2 that resulted in the MRSA\_FIP primer sequence. This causes the MRSA\_FIP to append to the membrane paper via the biotin and streptavidin interaction; meanwhile, this position least interrupts the loop amplification process in LAMP.<sup>25</sup>

**Optimization of MRSA LAMP Reaction.** LAMP reaction (15  $\mu$ L tube) comprised 1.6  $\mu$ M each of MRSA\_FIP and MRSA\_BIP, 1.4  $\mu$ M each of MRSA\_LF and MRSA\_LB, 0.2  $\mu$ M each of MRSA\_F3 and MRSA\_B3, 1.4 mM dNTPs (Promega, Wisconsin), 1 M betaine (USB Corporation, Ohio), MgSO<sub>4</sub> (New England Biolabs, Massachusetts), 1 $\times$  Thermopol buffer (New England Biolabs), *Bst* DNA polymerase large fragment (New England Biolabs), 0.08 $\times$  SYBR Green I (Sigma, New York), and DNA template (10 ag–1 fg). To determine an optimal LAMP reaction, three variable composites of the LAMP reaction including (X1) temperature, (X2) MgSO<sub>4</sub>, and (X3) *Bst* DNA polymerase were optimized following central composite design (CCD) analysis.<sup>26–28</sup> Note that the parameters of X1 ( $^{\circ}$ C) and quantities of X2 (mM) and X3 (units, U) were from previously established works.<sup>11,12,29</sup> The number of experiments required for the CCD was computed by  $2^n + 2n + 6$  (where  $n$  is the number of independent composites; and six replications), equal to 20 experiments (Table 2A). The real-time LAMP reaction kinetic was analyzed by QuantStudio 3 Real-Time PCR (Kyoto, Japan), while the predicted reaction time ( $Y$ ) was computed from a CCD equation  $Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$ , where  $\beta_0$  is the constant;  $\beta_1$ ,  $\beta_2$ , and  $\beta_3$  are the linear coefficients;  $\beta_{11}$ ,  $\beta_{12}$ ,  $\beta_{13}$ , and  $\beta_{23}$  are the cross-product coefficients; and  $\beta_{11}$ ,  $\beta_{22}$ , and  $\beta_{33}$  are the quadratic coefficients, respectively (Table 2B). Corresponding graphs were plotted to determine a relationship among the variable 2–3 independent composites and the LAMP reaction time, and the combination composites that yielded the least reaction time analysis were considered the optimal parameters for X1, X2, and X3. These parameters were used to construct a paper-based MRSA LAMP reaction.

**Design and Fabrication of Paper-Based LAMP Devices.** A paper-based device consisted of a sandwichlike bottom base, a reaction pad in the center, and a clear top seal (Figure 1A). Two pieces of a double-sided adhesive tape (each 2 mm thickness), of 20 mm  $\times$  20 mm squares, were made as the bottom base, with the upper double-sided adhesive tape punched to have a 6 mm diameter and 2 mm deep hole by a puncher. Between the double-sided tapes was placed a wax-coated paper (the reaction layer for the reaction pad to be placed). This hole represents a cell where the LAMP reaction takes place. A wax-coated paper is a cellulose membrane paper that was wax-printed over the entire surface using a ColorQube 8570 printer (Xerox Corporation, Connecticut)<sup>30</sup> and then cut into a 20 mm  $\times$  20 mm square.

To construct the reaction pad, initially, a mixture of 0.08 g of (2,2,6,6-tetramethylpiperidin-1-yl)oxidanyl (TEMPO), 0.5 g of NaBr, and 100 mL of Na<sub>2</sub>CO<sub>3</sub>/NaHCO<sub>3</sub> buffer (pH 10.8) was placed onto a 5 g cellulose membrane paper (Whatman No. 1). The pH was adjusted to 10–11 using NaOH (1 N), and the mixture and the paper were incubated at room temperature for 1–3 h. Then, the paper was washed with ddH<sub>2</sub>O, followed by 95% ethanol, and dried in the drying oven at 40  $^{\circ}$ C for 12–24 h to produce the TEMPO-oxidized cellulose paper. Following, 50 mL of NaCl (15% w/v) was added to the TEMPO-oxidized cellulose paper, incubated at room temperature for 1 h, washed with ddH<sub>2</sub>O, and dried in a drying oven at 40  $^{\circ}$ C for 12–24 h. Next, 5 mmole *N*-(3-dimethylamino propyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) and 5 mmole *N*-hydroxysuccinimide (NHS) in 20 mL of ddH<sub>2</sub>O were added, and the TEMPO-oxidized cellulose paper

**Table 2.** (A) Experimental parameters for CCD analysis based on three variable composites (X1, temperature; X2, MgSO<sub>4</sub>; and X3, *Bst* DNA polymerase) and (B) reaction time (min) from actual experiment and theoretical prediction<sup>a</sup>

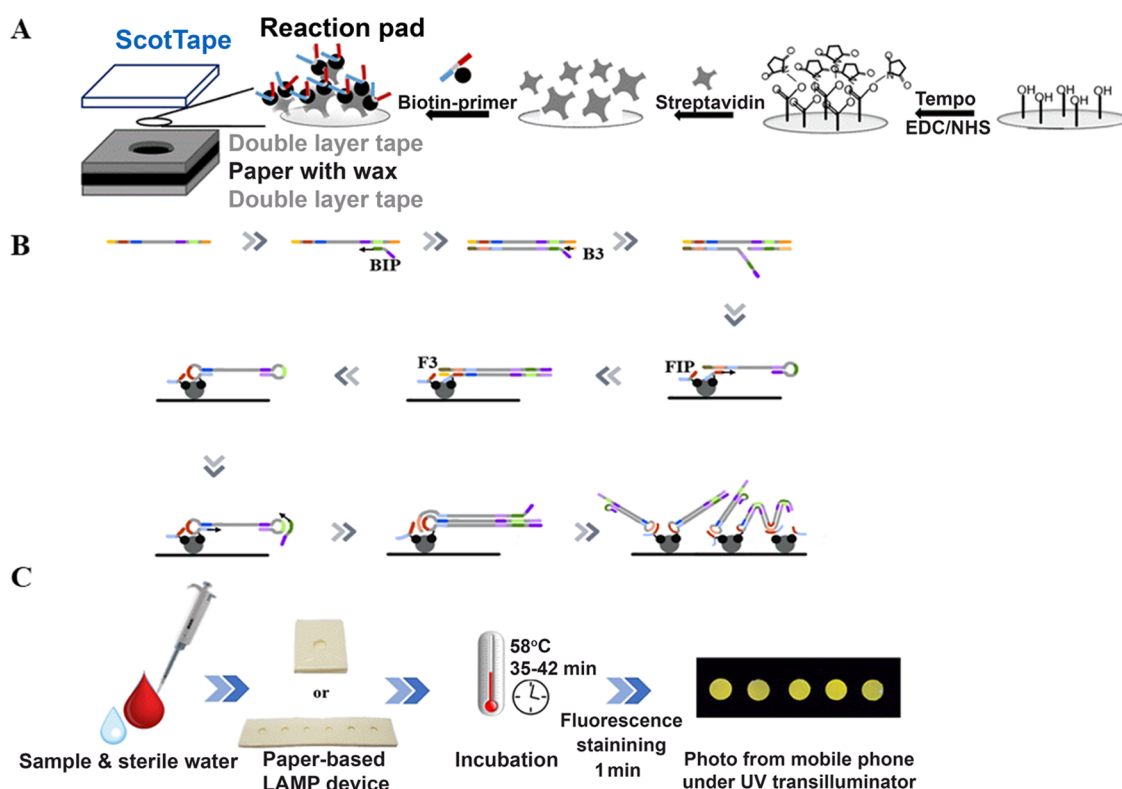
A.			
experiments	X1 (temperature, $^{\circ}$ C)	X2 (MgSO <sub>4</sub> , mM)	X3 ( <i>Bst</i> DNA polymerase, U)
1	60 (+1)	8 (+1)	8 (+1)
2	60 (+1)	8 (+1)	4 (−1)
3	60 (+1)	4 (−1)	8 (+1)
4	60 (+1)	4 (−1)	4 (−1)
5	56 (−1)	8 (+1)	8 (+1)
6	56 (−1)	8 (+1)	4 (−1)
7	56 (−1)	4 (−1)	8 (+1)
8	56 (−1)	4 (−1)	4 (−1)
9	61.36 (+1)	6 (0)	6 (0)
10	54.64 (−1)	6 (0)	6 (0)
11	58 (0)	9.36 (+1)	6 (0)
12	58 (0)	2.64 (−1)	6 (0)
13	58 (0)	6 (0)	9.36 (+1)
14	58 (0)	6 (0)	2.64 (−1)
15–20	58 (0)	6 (0)	6 (0)
B.			
experiments	actual time (min)	predicted time (min)	
1	16.33	17.94	
2	29.67	31.54	
3	22.73	22.56	
4	45.17	42.56	
5	29.50	32.48	
6	34.57	35.11	
7	18.63	17.13	
8	27.40	26.16	
9	30.33	30.10	
10	28.83	28.54	
11	36.43	32.44	
12	25.33	28.80	
13	15.17	13.61	
14	31.60	32.63	
15	19.40	19.77	
16	20.23	19.77	
17	19.40	19.77	
18	20.00	19.77	
19	20.33	19.77	
20	19.17	19.77	

<sup>a</sup>The values in parentheses (+1, 0, or −1) represent the higher, intermediate, or lower values, respectively, for each variable composite, and CCD experimental parameters must contain five values each of (+1), (0), and (−1) for each variable composite.

was incubated at room temperature for 3 h and washed with ddH<sub>2</sub>O. After this treatment, the TEMPO-oxidized cellulose membrane paper was ready to be punched by a hole puncher to make 6 mm diameter discs (the reaction pad), and streptavidin (1  $\mu$ g/disc) (Himedia, Mumbai, India) was dropped onto the reaction pad and dried at room temperature for 24 h. The redundant streptavidin was discarded, and 1.6  $\mu$ M MRSA\_FIP per reaction pad was dropped onto the top face of the reaction pad.<sup>20,31,32</sup> These reaction pads can be stored for a long term at 4  $^{\circ}$ C.

Prior to use, and for the stability (shelf life) test of the paper-based LAMP device, the LAMP reaction mixtures (for 15  $\mu$ L reaction) including the rest of LAMP primers (Table 1), MgSO<sub>4</sub>, *Bst* DNA polymerase large fragment, dNTPs, betaine, 1 $\times$  Thermopol buffer, (optional) 0.08  $\times$  SYBR Green I, and 3% poly(vinyl alcohol) (PVA)





**Figure 1.** Schematic of a paper-based LAMP device. (A) Design of the device and MRSA\_FIP (biotinylated) immobilization, (B) LAMP reaction steps on the paper, and (C) overview steps for paper-based LAMP device usage.

(Sigma-Aldrich, Missouri) were dropped onto the reaction pad, dried in a sterile air flow at 25 °C 1–2 h, and stored at 4 °C (or 25 °C for the stability test). To use our paper-based MRSA LAMP device, the reaction pad was placed on the reaction layer of the sandwichlike bottom base, 1  $\mu$ L of DNA sample (i.e., crude DNA extraction) and 14  $\mu$ L of sterile water were pipetted onto the reaction pad, covered by the clear top seal to prevent evaporation during the LAMP incubation (15–40 min), and the result was analyzed by fluorescent color (Figure 1A,C). Figure 1B describes the LAMP reaction steps when the MRSA\_FIP was attached to the paper.

**Fluorescence Monitoring and Analysis.** Either SYBR Green I was added together with the LAMP reagents or to the paper-based LAMP product;<sup>33,34</sup> the product visualization was allowed under an ultraviolet (UV) transilluminator (Labnet International Inc., New Jersey) at 302 nm wavelength and 15 min in a dark room. Real-time analysis of kinetic fluorescence intensity through time (min) of LAMP products was performed using QuantStudio 3 Real-Time PCR. For on-site and equipment-free quantitation of MRSA (*mecA*) copies, the result could be read by a portable LED transilluminator (BluView Transilluminator, MajorScience, California) or photographed by an auto mode camera of a typical smartphone (e.g., Samsung Note 5, aperture *f*/1.9, exposure time 0.02–0.03 s, ISO 500), the photograph was then converted into grayscale and the intensity value was measured by ImageJ software (<https://imagej.nih.gov/ij/download.html>). Blank, or negative control (no DNA template in the LAMP reaction), was set as the background intensity value. The product intensity values from tenfold serial dilution LAMP products provided a regressive equation to quantitate the MRSA copies.

**Scanning Electron Microscopy (SEM) Analysis of MRSA\_FIP Immobilized Papers.** The surface topographies of the TEMPO-oxidized cellulose membrane papers, before and after streptavidin immobilization, were analyzed at the Scientific and Technological Research Equipment Centre, Chulalongkorn University, Thailand. SEM was performed using a JSM-IT500HR scanning electron microscope with an energy-dispersive X-ray spectrometer (JOEL, Massachusetts).

**Specificity and Detection Limit of Paper-Based LAMP Devices.** The specificity of the paper-based LAMP device was determined using MRSA USA300 and five non-MRSA strains aforementioned in the bacterial strain and crude DNA extraction. The detection limit was determined from tenfold serial dilutions of MRSA USA300 genomic DNA. The LAMP products were stained by a DNA fluorescent dye; we used SYBR Green I or a Diamond nucleic acid dye (Promega, Wisconsin). A photograph was taken, the intensity value was analyzed by ImageJ software, and a quantitative regression equation for MRSA copies was derived. Of all experiments, a minimum of three independent replicates were performed. The detection limit is the lowest copy number that our paper-based LAMP reaction gave fluorescence intensity.

**Evaluation of Clinical Samples and Stability of Paper-Based LAMP Devices.** Clinical blood samples from three healthy volunteers were collected by clinicians at King Chulalongkorn Memorial Hospital, Thailand. The protocols were approved by the Ethical Committee of the Faculty of Medicine, Chulalongkorn University (E.C. No. 654/60) and conducted based on the guidelines by the Declaration of Helsinki. The clinical blood samples were tested MRSA-negative, so 0.15 pg of MRSA USA300 genome (equivalent 50 *mecA* copies) and 10 ng of each of *S. aureus* or *E. coli* genomes were added to the clinical blood samples for the evaluation of the clinical samples and stability of the paper-based LAMP device. For stability evaluation of the paper-based LAMP device, the device was stored at 25 °C for a specified number of days before being tested to detect against positive and negative laboratory and clinical blood samples. The percent efficiency of the LAMP reaction was the fluorescence intensity results against the referencing 0 day results, and a minimum of five independent replicates were performed to obtain avg.  $\pm$  S.D. values.

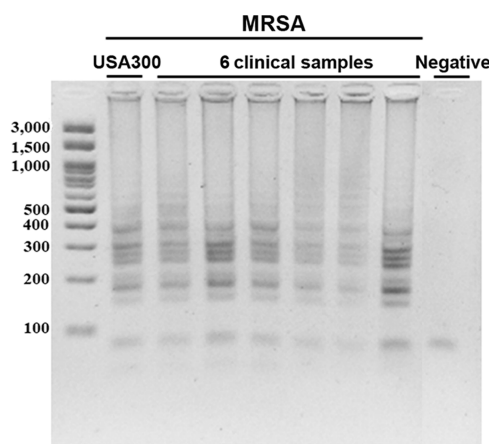
## RESULTS AND DISCUSSION

**Optimization of MRSA LAMP Reaction.** The CCD analysis was performed to determine the optimal LAMP reaction from three essential LAMP parameters (i.e., incubation temperature,  $\text{MgSO}_4$  concentration, and *Bst* DNA polymerase

units). The combinations of varying ranges of each composite (Table 2A:  $X_1$ , 54.64–61.36 °C;  $X_2$ , 2.64–9.36 mM; and  $X_3$ , 2.64–9.36 U)<sup>12,29</sup> were analyzed to identify the shortest incubation time to a positive LAMP result. Experiment 13 ( $X_1$ , 58 °C;  $X_2$ , 6 mM; and  $X_3$ , 9.36 U) showed the relatively shortest incubation time (Table 2B). Mathematical computation gave a theoretical  $Y$  equation as  $19.77 - 0.46X_1 + 1.08X_2 - 5.66X_3 + 3.38X_1^2 + 3.84X_2^2 + 1.18X_3^2 - 4.99X_1X_2 - 2.74X_1X_3 + 1.6X_2X_3$ . Note that the theoretical prediction times ( $Y$ ) were close to the actual reported times (Table 2B), given that the statistic Pearson correlation between the actual and predicted data was positively correlated ( $R^2 = 0.974$ ,  $P < 0.001$ ). Further, the response graphs were plotted for two-variable composites, while one composite was kept constant at the predetermined optimum.

Figure S1 demonstrates the correlations between variable composites: in Figure S1A, the higher concentration of  $MgSO_4$  was associated with the higher incubation temperature (the relatively shortest reaction time 19.5 min at 57.4 °C and 5.4 mM). Too much  $MgSO_4$  (salt) affects the ability of primers to anneal and *Bst* DNA polymerase activity.<sup>35,36</sup> Thus, a higher incubation temperature (or a longer incubation time) is required. Figure S1B demonstrates the small positive correlation between the *Bst* DNA polymerase activity and the incubation temperature. Although the *Bst* DNA polymerase could remain active in the temperature range of 55–65 °C,<sup>37,38</sup> the relatively shortest reaction time of 12.34 min was found to be at 59.2 °C with ~9–9.36 U of *Bst* DNA polymerase. Our testing ranges of incubation temperature provided a better effect than the enzyme as optimal melting temperatures for the four LAMP primers (oligonucleotide sequences) allowed annealing to the DNA template for nucleic acids amplification (Figure 1B).<sup>11</sup> For example, our MRSA\_FIP and MRSA\_BIP primers had melting (annealing) temperatures of 58.9 °C and 60.8 °C, respectively. Between the *Bst* DNA polymerase and  $MgSO_4$ , the relatively shortest reaction time of 12.69 min was found at 9.36 U and around 4.6–5.4 mM, respectively (Figure S1C). This low concentration of  $MgSO_4$  was consistent with the fact that high salt concentrations can precipitate DNA and primer oligonucleotides that make both unavailable for the annealing step of LAMP.<sup>39,40</sup> Subsequently, the CCD, following manual computation curation using  $Y = 19.77 - 0.46X_1 + 1.08X_2 - 5.66X_3 + 3.38X_1^2 + 3.84X_2^2 + 1.18X_3^2 - 4.99X_1X_2 - 2.74X_1X_3 + 1.6X_2X_3$  to find the optimal combination values for the least LAMP reaction time for  $X_1$ ,  $X_2$ , and  $X_3$  from the preferential ranges of each variable composite retrieved from the LAMP response graph in Figure S1, yielded the optimized MRSA LAMP reaction consisting of 58 °C incubation temperature, 5 mM  $MgSO_4$ , and 9 U *Bst* DNA polymerase. The incubation time was 18 min. Additionally, the specificity of the MRSA LAMP was verified against different MRSA strains, including USA300 that is a virulent and the most prevalent community-associated strain in the United States (Figure 2). The LAMP products appear as ladderlike bands on agarose gel electrophoresis (Figure 2).<sup>11,12,29</sup>

Furthermore, we compared the LAMP reaction efficiencies between the MRSA\_FIP with and without tagged biotin using real-time fluorescence intensity monitoring (QuantStudio 3 Real-Time PCR). The LAMP reaction that utilized MRSA\_FIP with tagged biotin started amplification at 18 min, slower than using MRSA\_FIP without tagged biotin by 5 min (Figure S2). A LAMP reaction, using MRSA\_FIP without tagged biotin but with added free biotin particles of equal amount to that being



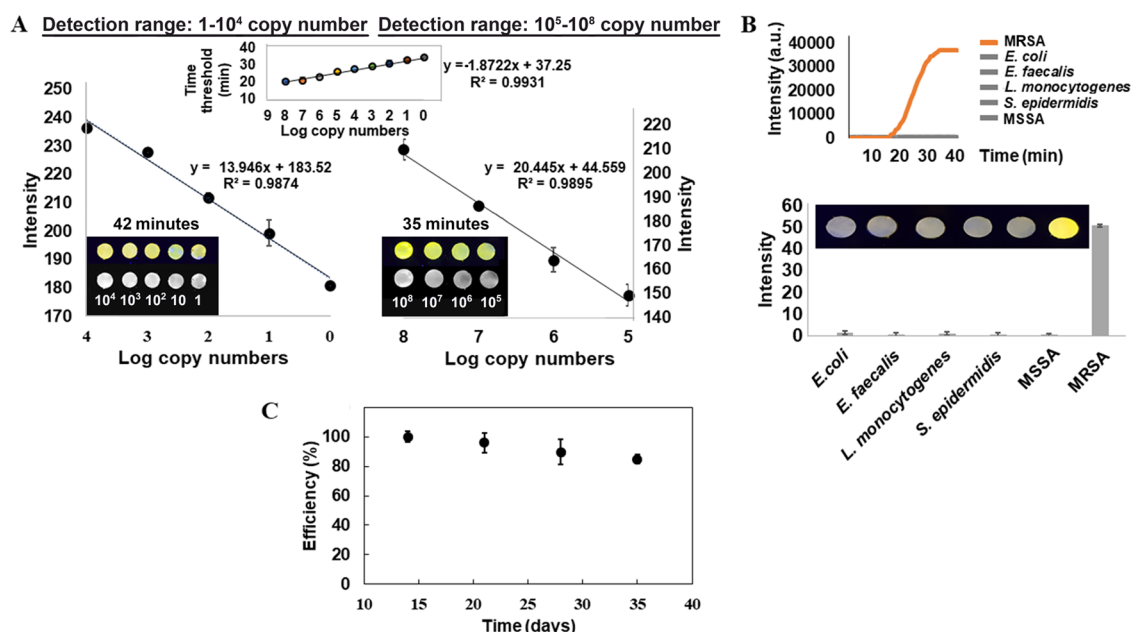
**Figure 2.** Specificity of MRSA LAMP reactions in tubes. Amplified products were verified by agarose gel electrophoresis.

used with the MRSA\_FIP with tagged biotin, was included to demonstrate a slight inhibitory effect of biotin. Biotin affects the steric hindrance and reduces the annealing kinetics between the primers and the DNA sequences.<sup>41</sup> As our MRSA\_FIP had biotin appended to the sequence, the poorer LAMP efficiency was observed.

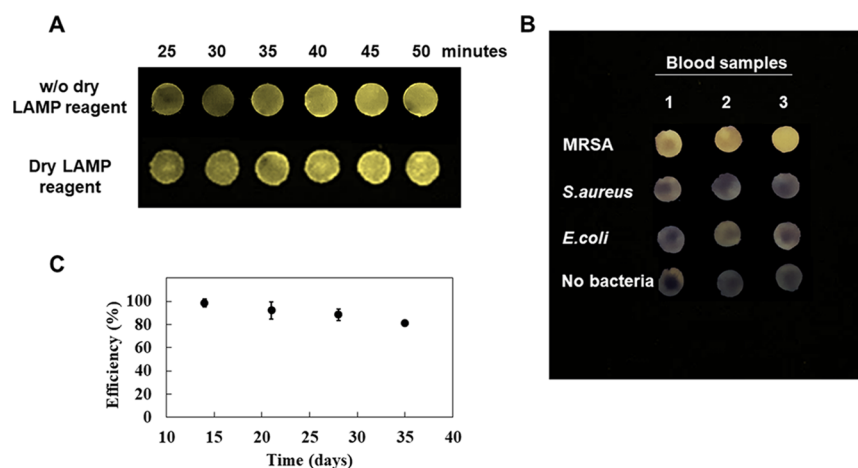
**Fabrication of Paper-Based LAMP Devices.** Oxidization of a reaction pad using TEMPO and EDC/NHS allowed binding of streptavidin (Figure S3).<sup>31,42–44</sup> Following the addition of MRSA\_FIP, where the tagged biotin bound streptavidin, and the rest of the LAMP ingredients and appropriate incubation condition (temperature and time), the LAMP process occurred, as described in Figure 1B. To replace inconvenient and time-consuming methods such as agarose gel electrophoresis, analysis of the LAMP DNA products on the paper-based device simply required a 1 min incubation with a DNA fluorescent dye (i.e., SYBR Green I) (Figure 1C).

We determined whether the LAMP reaction steps (Figure 1B) and hence the LAMP products occurred in the reaction pad or a liquid phase. After the paper-based LAMP reaction was completed, we transferred the liquid phase to a sterile tube, stained with SYBR Green I, and found no to very faint fluorescence intensity. In contrast, when we removed the liquid phase and performed the SYBR Green I staining on the reaction pad, there was a clear fluorescent signal (Figure 1C). In brief, the targeted DNA template was hybridized with the B2 region of MRSA\_BIP and initiated a complementary DNA strand synthesis with 3' loop (named "BIP loop strand"). The outer primer MRSA\_B3 hybridized to the outer region of B2 and synthesized another complementary DNA strand, displacing the BIP strand detached. This BIP strand then hybridized to the primer MRSA\_FIP on the reaction pad to initiate another complementary DNA strand with 5' loop to the BIP strand (named "BIP loop, FIP loop strand"). This dumbbell-like BIP loop, the FIP loop strand, is the initiator for continuous syntheses of multiloop amplicons. The final LAMP products therefore appeared as cauliflowers in structures (Figure 1B) or ladderlike bands in agarose gel electrophoresis (Figure 2).<sup>11</sup>

The immobilization of MRSA-FIP to the reaction pad is essential to allow the LAMP product to append on top of the reaction pad for the optimally detected fluorescent signal. Without this immobilization (e.g., all reagents dried inside the paper reaction well and the fluorescence detected from the supernatant), the LAMP product might be in the supernatant,



**Figure 3.** Detection limit and specificity of quantitative paper-based MRSA LAMP devices. (A) Quantitative regression analyses derived from fluorescence intensity readings of paper-based LAMP devices using tenfold dilutions of MRSA USA300 DNA (10 ag to 1 ng) as a template; (B) specificity analyses of paper-based LAMP devices against (top inlet) real-time LAMP as a reference assay, using 1 ng of each of *E. coli*, *E. faecalis*, *L. monocytogenes*, *S. epidermidis*, and *S. aureus* Newman (MSSA) as negative controls and MRSA USA300 as a positive control; and (C) stability of paper-based LAMP in detecting 0.15 pg of MRSA USA300 DNA for up to 35 days. In (A), the photographs are fluorescence photographs (top row), followed by the fluorescence converted to grayscale photographs (second row), and the third row below the fluorescence and the grayscale photographs represents a copy number (e.g., 10 and 1 are 10 and 1 copy numbers, in order). Following the real-time quantitative regression analyses that identified appropriate incubation time for log copy numbers, we used 42 min as the incubation time for  $1\text{--}10^4$  copy numbers and 35 min as the incubation time for  $10^5\text{--}10^8$  copy numbers. In (B), only a fluorescence photograph is shown.



**Figure 4.** Performance and shelf life of the paper-based MRSA LAMP device in clinical blood sample detection. (A) Comparative LAMP reaction performances between dried LAMP reagents on the paper versus no dried LAMP reagents (except MRSA\_FIP) on the paper but adding liquid reagents onto the reaction pad of a device upon usage, (B) clinical blood samples tests without and with 50 copies of bacteria, and (C) efficiency of detecting clinical blood samples with 50 copies MRSA after days of 25 °C storage. The quantitative colorimetric data (a.u.) for (A) and (B) are given in Table S2. Note that the paper-based MRSA LAMP reactions in Figure 4 were incubated for 40 min, while those in Figure 3A were for 42 and 35 min, so their quantitative colorimetric data were not directly correlated.

immersed in or under the reaction pad. As cellulose membrane paper is an absorbent material, the LAMP product of a nonimmobilized primer might be absorbed in the cellulose paper, resulting in the poorer efficacy to detect the signal by the DNA fluorescence staining and thus offering the much lower detection limit and sensitivity compared with the mobilized one (data not shown).

**Sensitivity and Specificity of Quantitative Paper-Based LAMP Devices.** The detection limit of the paper-

based LAMP device was determined using tenfold serial dilutions of MRSA USA300 DNA, from approximately  $1 \times 10^0$  to  $1 \times 10^8$  *mecA* copies (equivalent 10 ag to 1 ng). The fluorescence intensity correlated with the amplifying LAMP product,<sup>45,46</sup> and the minimum amount of DNA template that resulted in a sufficient amount of LAMP product that could be detected by the DNA fluorescent dye defined the sensitivity of the device. Figure 3A demonstrates that our paper-based MRSA LAMP device could detect as low as 1 *mecA* copy (10 ag) if the



incubation time was increased to 42 min. For  $\geq 1 \times 10^5$  *mecA* copies (1 pg), the incubation time was 35 min. Linear regression analyses of both DNA template ranges, one at  $1 \times 10^0$  to  $1 \times 10^4$  and the other at  $1 \times 10^5$  to  $1 \times 10^8$  *mecA* copies, yielded statistically reliable equations for quantitation of the DNA template (log copy number) from the fluorescent signal (intensity, a.u.) ( $R^2 = 0.9874$ – $0.9895$ ). Note that the longer incubation time in the paper-based LAMP compared with the liquid LAMP (in the tube) was consistent with previous reports, owing to the reaction on the paper (molecular mobility) being not as efficient as that in the liquid.<sup>41</sup> Moreover, we analyzed the real-time fluorescence intensity using QuantStudio 3 Real-Time PCR of the LAMP reactions in tubes to plot the minimum fluorescent detection time at each log copy number of the DNA template and confirmed the linear relationship between the fluorescent detection time and the log copy number,<sup>47,48</sup>  $y = 1.8722x + 37.25$  ( $R^2 = 0.9931$ ) (Figure 3A, top inset graph). The shorter fluorescent detection time for liquid LAMP in the tube compared to the paper-based LAMP corresponded to the shorter incubation time for the liquid LAMP in the tube.

For the specificity of the paper-based MRSA LAMP device, in addition to the fact that all LAMP primers were checked for specificity to MRSA *mecA* sequences in the GenBank database and that the reactions had been tested in tubes (Figure 2), different genera of bacteria and MSSA were tested. Proper positive and negative fluorescent signals were determined, as shown in Figure 3B. We additionally performed the control (absence of target DNA) experiment. The control showed no fluorescence as observed by the naked eye under a UV transilluminator ( $93.28 \pm 0.94$  a.u.), similar to those in non-*mecA*-carrying bacteria experiments (*E. coli*, *E. faecalis*, *L. monocytogenes*, *S. epidermidis*, and MSSA). The fluorescence intensities of these negative samples were all lower than 100 a.u. (Figure 3B).

**Stability of Our Paper-Based LAMP Device and Clinical Sample Detection.** To use our paper-based MRSA LAMP device as a point-of-care detection test, the stability (shelf life) of the device was determined. First, we analyzed the effect of dried reagents and PVA, a well-known preservative.<sup>47,49–51</sup> We found an equal minimum LAMP incubation time for fluorescent signal detection between the LAMP reaction using dried LAMP reagents on the paper, in which we included PVA, and the LAMP reaction with no dried LAMP reagent (except MRSA\_FIP) on the paper but adding liquid reagents onto the reaction pad of the device upon use (Figure 4A, 40 min). The dry LAMP reagent platform showed an intensity of  $151.52 \pm 1.19$  a.u. at a 40 min incubation period (Table S2). This finding suggested no effect of the dried reagents and PVA and supported the user-friendly platform of the device since all of the LAMP ingredients were dried on the reaction pad and the users would only add 14  $\mu$ L of sterile water and 1  $\mu$ L of DNA sample upon use (Figure 1C, DNA sample from 5 min boiling). Using a cellulose membrane paper with simple fabrication offers our paper-based device to be able to be constructed at local settings, the low production cost, and safe health and environment compared to those that were constructed from glass fiber and plastic materials.<sup>23,24,52</sup> Next, we evaluated the shelf life of the paper-based LAMP device following different days of room temperature (25 °C) storage. Testing on crude DNA from individual bacterial species culture, the percent efficiency of the LAMP reaction (determined from five repetitive experiments) remained at  $85 \pm 2.98\%$  for 35 days (Figure 3C).

To validate the practicability of our paper-based MRSA LAMP diagnostic device in real life point-of-care uses, the devices were used to detect three clinical blood samples from healthy volunteers, without and with added specific bacteria. Figure 4B shows that the devices could properly detect MRSA from the blood DNA. Additionally, the fluorescence intensities of the positive MRSA reactions (Table S2,  $205.98 \pm 4.31$  a.u.) were quantitated using the linearized graph derived in Figure 3A, and the computed copy number (avg. 53.02 copies) was close to the actual inoculation (50 copies). The shelf life of the devices was also tested against these clinical blood samples with MRSA for five minimum repetitive experiments, and their percent efficiencies of the LAMP reaction remained at 81.25–100% for 35 days of the device storage at room temperature (Figure 4C). The stability of the devices may be much extended if the devices could be stored at lower temperatures, e.g., in a refrigerator or a freezer.<sup>53</sup> Further, as a LAMP reaction could contain 2–3 target gene primers, our paper-based LAMP diagnostic platform could be future developed to detect multiple genes (or species) simultaneously, which represents an advantage over a current CRISPR/Cas system that requires a complicated system for multiplex detection (i.e., separate specific Cas/crRNA complexes for each target gene (or species) via channel networks, like wells or arrays).<sup>54</sup>

## CONCLUSIONS

We first invented the paper-based LAMP device (Thailand patent no. 2001005176) that are appropriate for point-of-care and local testing, where the LAMP reaction and the fluorescent color readout were performed on a single paper device platform, and validated the devices and their shelf life on clinical blood samples detection. As MRSA is a prevalent drug-resistant pathogen in hospitals and community settings, the sensitivity and practicability of the paper-based MRSA device for point-of-care detection are essential. The user protocols are simple, the result is rapid (<1 h), and the detection limit is ultrasensitive (as low as 1 copy *mecA* or equivalent to 10 ag MRSA genome). After 5 min boiling for DNA sample preparation, the only requirement for the user is a typical heating pad (or a heat block or a water bath). Moreover, this paper-based MRSA LAMP device had a shelf life at room temperature for 35 days or even longer under storage at <25 °C. Conversion of a fluorescent image to intensity data also allowed the copy number of the MRSA to be quantitated. Together, we demonstrated our paper-based MRSA LAMP device prototype to be a powerful tool that can be used for MRSA screening and monitoring of treatment at the point-of-care.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acssensors.0c01405>.

Analyses of LAMP response graphs for reaction time against each two-variable composite, real-time LAMP analyses showing minor LAMP reaction efficiencies in the presence of free biotin particles and MRSA\_FIP (with tagged biotin), SEM micrographs of TEMPO-oxidized cellulose membrane paper, without and with streptavidin, list of MRSA strains for LAMP primers design, and quantitative colorimetric data of Figure 4A,B (PDF)

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## Author Contributions

I.C. performed experiments, analyzed data, and drafted the manuscript. A.S. helped perform experiments. Y.T., D.W., and D.D. helped draft and revise the manuscript. P.D.H., M.S., A.J.M., and N.S. advised experiments. A.L. provided clinical samples. A.J.M., S.T., and N.S. provided bacterial strains. N.S. initiated and conceived the study, coordinated experiments and data analyses, and drafted and revised the manuscript. All authors have given approval to the final version of the manuscript.

## Notes

The authors declare no competing financial interest.

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