

## Integrated Microcapillary for Sample-to-Answer Nucleic Acid Pretreatment, Amplification, and Detection

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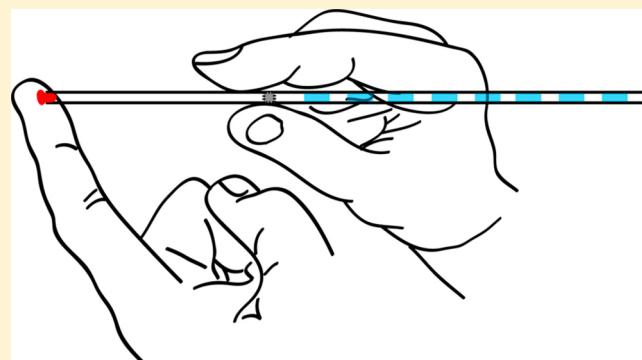
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W Web-Enhanced Feature S Supporting Information

**ABSTRACT:** This work develops an integrated microcapillary-based loop-mediated isothermal amplification (icLAMP) containing preloaded reagents and DNA extraction card, allowing for sample-to-answer screening of single nucleotide polymorphisms (SNPs) typing of the CYP2C19 gene from untreated blood samples with minimal user operation. With all reagents and the DNA extraction card preloaded inside the capillary, this icLAMP system can achieve on-site pretreatment, extraction, amplification, and detection of nucleic acids within 150 min, without the requirement for advanced instruments. As icLAMP technology carries many advantages such as disposability, easy operation, low cost, and reduced cross contamination and biohazard risks, we expect this system to have a great impact on point-of-care (POC) nucleic acid detection.



Point-of-care (POC) molecular diagnostic testing with sample-to-answer capability has great promise for early diagnostics of diseases and assessment of genetic disorders.<sup>1–4</sup> To increase the detection sensitivity and specificity, nucleic acid amplification is always required.<sup>5,6</sup> Compared to other nucleic acid amplification methods, loop-mediated isothermal amplification (LAMP) is isothermal and highly sensitive and does not require the extracted DNA to be highly purified or denaturized, suggesting its potential applicability to the POC format.<sup>7–9</sup> To accompany the goal of POC diagnostics, the whole nucleic acid detection procedure, including sample pretreatment, extraction, separation, amplification, and readout, should be integrated in a small, low cost, disposable device with user-friendly operation.<sup>10–15</sup> Although some microfluidic devices for nucleic acid analysis are small in size, they normally require bulky and complicated external supplies for liquid manipulation, thermal control, and data analysis.<sup>16–21</sup>

We previously miniaturized the conventional LAMP into a multichannel microfluidic system ( $\mu$ LAMP) for parallel detection of pseudorabies virus (PRV) with enhanced sensitivity and specificity.<sup>22</sup> The optical fibers were also embedded into the  $\mu$ LAMP to monitor the amplification in real time instead of using an expensive turbidimeter.<sup>23</sup> To further integrate the procedures of sample pretreatment, a

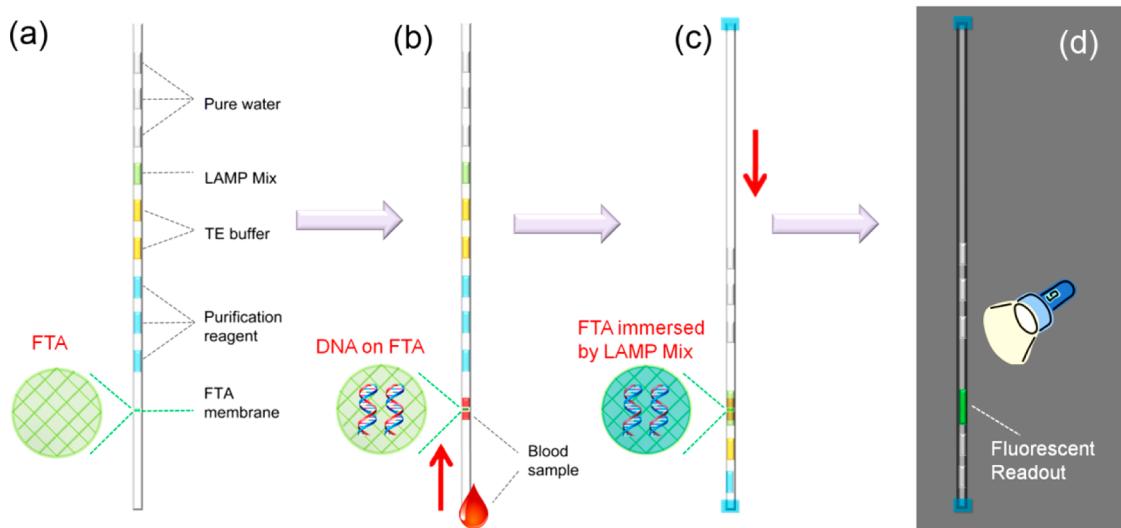
thermal lysis chamber was designed for the LAMP device, which was separated from the reaction chamber by a screw valve.<sup>24</sup> This system initially realized the sample-to-answer identification of bacteria. Other researchers designed a microfluidic LAMP cassette equipped with an integrated Flinders Technology Associates (FTA) membrane for on-chip capture and concentration of nucleic acids, which were directly used as templates for real-time LAMP monitored by a fluorescent reader.<sup>25</sup> To avoid contamination during nucleic acid amplification in microfluidic chips, we recently developed a straightforward and multiplexed microcapillary-based LAMP system (cLAMP) for simultaneous detection of two targets of human immunodeficiency virus (HIV).<sup>26</sup> However, this cLAMP lacks function of sample pretreatment and requires manually pipetting multiple reagents into the microcapillary.<sup>27</sup>

In this paper, we improve upon the advantages of cLAMP, such as having rapid detection and being free of contamination/bulky equipment, to endow the microcapillary-based LAMP with new functions including sample pretreatment, stable storage of preloaded reagents, and easy operation. This truly

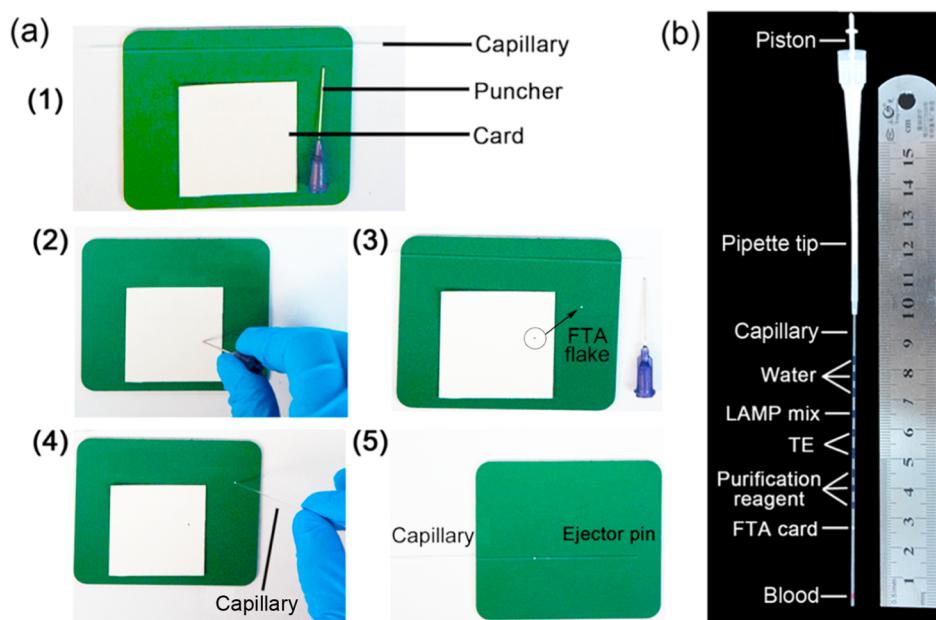
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**Figure 1.** (a) Integrated capillary LAMP (icLAMP) with preloaded reagents. A membrane used to extract DNA from blood samples was inserted into the microcapillary before fluidic operations. (b) 200 nL of blood sample was introduced into the microcapillary and contacted with the FTA card with the aid of a pipet tip and a piston. DNA from blood sample was extracted and trapped onto the FTA card. (c) After sequential washing of the FTA card with segments of purification reagent and TE (derived from Tris and EDTA) buffer, the FTA card was immersed in a LAMP mix. The ends of the microcapillary were sealed by reagent/water droplets, air, and epoxy glue to avoid contamination during amplification. (d) A visual readout of the fluorescent signal after the LAMP reaction with a hand-held flashlight.



**Figure 2.** (a) The assembly of icLAMP system: (1) components for preparing icLAMP: a puncher (500  $\mu\text{m}$  ID), a microcapillary (500  $\mu\text{m}$  ID), a piece of FTA card, and a cutting mat; (2) cutting the FTA card with the punch; (3) the FTA flake with a diameter of 500  $\mu\text{m}$ ; (4) the FTA flake was picked up by the microcapillary; (5) pushing the FTA flake into the microcapillary using the ejector pin of 375  $\mu\text{m}$  OD. (b) The microcapillary with preloaded reagents was connected to a pipet tip with a piston to provide positive/negative pressure for fluid manipulation.

integrated sample-to-answer microcapillary-based LAMP system (icLAMP) is used for detection of the *CYP2C19* gene from an untreated, freshly drawn blood sample by simply using a positive-displacement pipet tip and a hand-held UV-flashlight. As the icLAMP only requires 200 nL of whole blood, we can use a finger-pricking device to obtain finger blood and introduce it into the capillary. We also demonstrate the applicability of this icLAMP for collection, amplification, and detection of nucleic acids from bacteria.

## EXPERIMENTAL SECTION

**Materials.** The human whole blood samples were collected from patients before receiving treatment with Plavix (clopidogrel), an antiplatelet agent used to inhibit blood clots, at Beijing Tiantan Hospital or Peking Union Medical College Hospital. All blood specimens were treated by the anticoagulant EDTA immediately after collection. The primers for single nucleotide polymorphisms (SNPs) typing of the *CYP2C19* gene of humans are shown in the Supporting Information, Table S1.<sup>28</sup> The *CYP2C19* genotyping kit was from BaiO (Shanghai). A finger-pricking device (ACCU-CHEK Softclix,

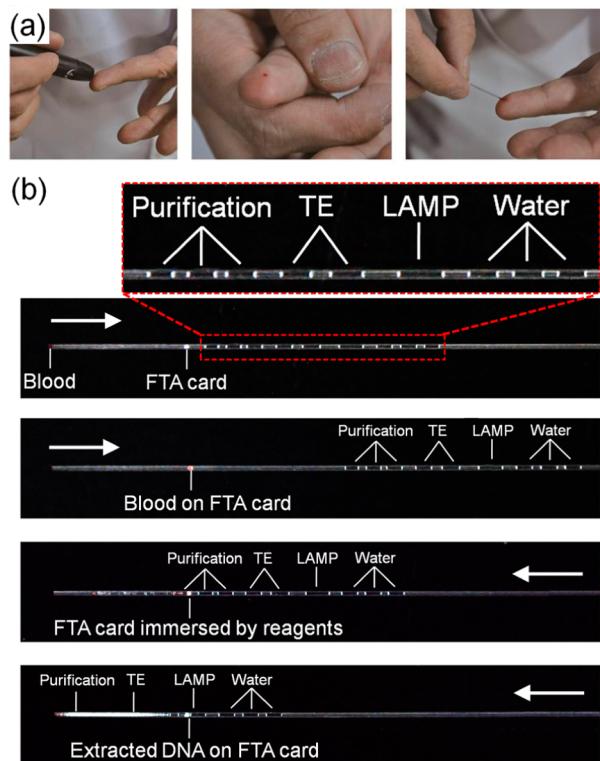
FP001, Roche) was used to obtain blood samples from the finger.

The LAMP mix for the CYP2C19 gene contains 1× ThermoPol Buffer (containing 2.0 mM MgSO<sub>4</sub>), in total 8.0 mM MgSO<sub>4</sub>, 0.5 mM MnCl<sub>2</sub>, 25.0 μM calcein, 0.5 mM dNTPs, 0.2 μM each of the outer primer (F3 and B3), 1.6 μM each of inner primer (FIP and BIP), 0.8 μM each of the loop primer (LF and LB), 1.0 M betaine, and 0.32 U/μL *Bst* polymerase. Glass microcapillaries with inner diameter (ID) of 500 μm (or 1000 μm), outer diameter (OD) of 1000 μm (or 1500 μm), and 100 mm in length were purchased from ChuanHuada (Chengdu, China). Epoxy glue with quick-curing properties used for sealing the ends of microcapillaries was from 3M (Scotch-Weld Epoxy Adhesive DP-105, USA). The Whatman FTA card for extracting and storing genomic DNA from blood samples was from GE healthcare (USA). Positive-displacement pipet tips with pistons to manipulate the droplets inside the microcapillaries were obtained from Gilson Medical Electronics (Microman CP-2S, USA). A hand-held UV-flashlight (ZY-S12, Small Sun) for the LAMP readout was purchased from an online store ([www.taobao.com](http://www.taobao.com)). The Heraeus Multifuge X1R centrifugal machine (Thermo) was used to centrifuge the microcapillaries of different diameters.

**Assembly of icLAMP System.** For the sample-to-answer icLAMP system, we inserted a trimmed piece of FTA card into the microcapillary before we introduced samples, wash solutions, amplification reagents, and water segments (Figure 1). In accordance with the diameter of the microcapillary, we prepared the membrane flake with an appropriate size by punching the card with a homemade sharp flat-tip needle. In this case, the inner diameter of the microcapillary is 500 μm, so we punched the card with the tip needle with a diameter of 500 μm and inserted the membrane flake into the microcapillary with the aid of thinner tubing with an outer diameter of 375 μm (Figure 2 and Movie 1).

After insertion of the FTA card inside the microcapillary, we preloaded three segments of purification reagent (600 nL × 3), two segments of TE buffer (derived from Tris and EDTA, composed of 10 mM Tris, 0.1 mM EDTA, pH 8.0, 600 nL × 2), one segment of LAMP reaction mix (1 μL), and three segments of water droplets (600 nL × 3) into the microcapillary in sequence using negative-pressure introduction (Figure 2). After loading the reagents and water droplets, we used two customized polydimethylsiloxane (PDMS) plugs to protect the ends of the microcapillary. As each reagent/water droplet inside the microcapillary was quarantined by adjacent air segments, the surface tension at the interface could prevent the movement of lipid droplets inside the microcapillary under harsh conditions such as violent shaking (Movie 2).

**Operation of icLAMP System.** We first connected the microcapillary containing preloaded multiple reagents and an FTA card with a positive-displacement pipet tip. 200 nL of blood sample was then introduced into the microcapillary from one end by pulling the piston inside the pipet tip to produce a negative pressure from the other end (Figure 3). The blood sample was immersed with the FTA card for at least 1 h, allowing the DNA to be extracted fully. Three segments of preloaded purification reagent and two segments of TE buffer passed through the FTA card in a sequence by pushing the piston. Each segment stayed on the FTA card for 5 min, which was equivalent to the necessary washing and purification for the pretreatment of the blood sample (Figure 3). The segment of LAMP mix finally covered the whole FTA area, and the ends of



**Figure 3.** Working procedure of an FTA-integrated microcapillary for blood analysis. (a) Blood collection using a finger-pricking device. (b) Fluid handling inside the microcapillary. The white arrow indicates the direction of the fluids.

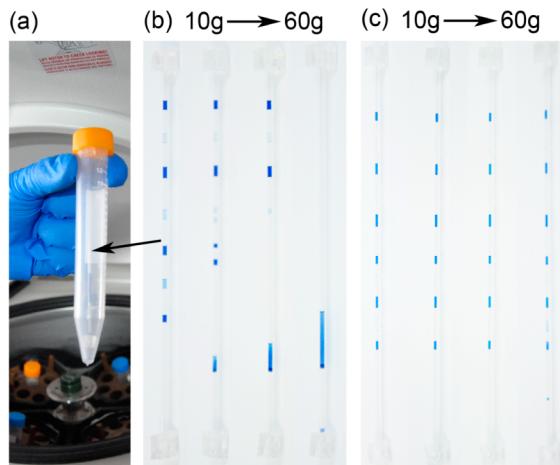
microcapillary were sealed with glue. In this manner, the LAMP reaction area was protected by reagent/water droplets, air, and epoxy glue to avoid contamination. We carried out thermal reaction for the SNPs typing for 1 h at 65 °C by placing the capillary into an oven. The result could be directly read out with the naked eye when using a hand-held UV-flashlight to excite the green fluorescence after amplification.

## RESULTS AND DISCUSSION

**icLAMP System.** In this study, we insert an FTA flake into the microcapillary and stop the sample solution in the position where the flake is located. The volume of the blood sample injected into the microcapillary is enough to completely immerse the cell-lysis membrane. After washing with buffers and reacting with LAMP mix, the extracted DNA on the FTA flake could be amplified at 65 °C. The total assay time is 150 min including 60 min of lysing blood cells and extracting DNA by the FTA card, 25 min of washing and purifying trapped DNA on the FTA card, 60 min of amplification of DNA, and 5 min of fluid manipulation. To verify that no liquid evaporation occurred, the main cause of contamination during the LAMP reaction, we introduced several segments of dyes into the microcapillary, sealed the two ends with glue, and marked the positions of dyes by a red pen before heat treatment. After heating the microcapillary inside an oven at 65 °C for 90 min, we examined the positions and lengths of dye segments, and no reduction or evaporation of liquid was observed (Figure S1, Supporting Information).

We also used centrifugation to produce large forces on droplets inside the capillary, to demonstrate the reliability and robustness of the icLAMP for storing reagents. Microcapillaries

of 500 or 1000  $\mu\text{m}$  ID were preloaded with an FTA card, and dye/water droplets were separated from each other by air spacers and sealed by two-removable PDMS plugs. The microcapillaries were centrifuged inside a Heraeus Multifuge X1R (Thermo) at 10g, 20g, 40g, and 60g for 1 min (Figure 4a).



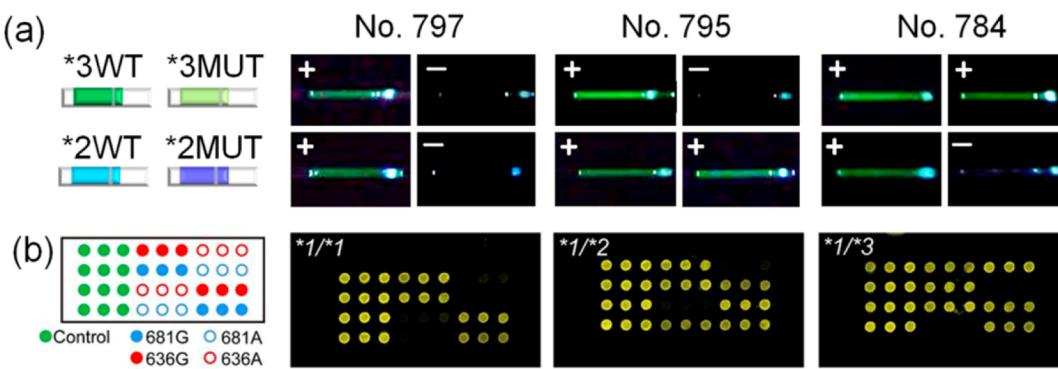
**Figure 4.** (a) Microcapillaries of 500 or 1000  $\mu\text{m}$  ID with a preloaded FTA card and dye/water droplets were centrifuged at different rotating speeds to demonstrate the reliability of icLAMP for storing reagents. (b) Droplets inside the capillary of 1000  $\mu\text{m}$  ID moved after centrifugation at 10–60g. (c) Droplets inside the capillary of 500  $\mu\text{m}$  ID had no observable change in positions after centrifugation at 10–40g, while a small movement of droplets was observed at 60g. The rotating speed from left to right is 10g, 20g, 40g, and 60g.

After centrifugation, we compared the positions of liquid droplets inside capillaries with different IDs (Figure 4b,c). There was no observable change in positions of droplets inside the capillary of 500  $\mu\text{m}$  ID after centrifugation at 10–40g, due to the large surface tension of droplets inside the small capillary.<sup>29</sup> If the rotating speed increased to 60g, the centrifugal force exceeds the surface tension, resulting in a small movement of droplets inside the capillary. In comparison, the surface tension acting on droplets inside the capillary of 1000  $\mu\text{m}$  ID could not compete with the droplet gravity, and thus, a significant movement of droplets occurred even at 10g. This experiment suggests that a reagents-preloaded microcapillary of

500  $\mu\text{m}$  ID is suited for storage and transportation with sufficient robustness.

A similar approach of utilizing a cartridge prefilled by sequentially injecting plugs of reagents was previously adopted in heterogeneous immunoassays.<sup>30,31</sup> This technique allows the end-users to accomplish the entire assay by simply driving the liquid flow in one direction, which saves the trouble of repeated pipetting. This strategy has been successfully utilized in the underdeveloped regions that previously have no access to advanced diagnostic tools.<sup>32</sup> In order to ensure specificity and sensitivity, most immunoassays are carried out in the heterogeneous reaction system that performs multistep immobilization and washing on a solid surface which adsorb protein molecules as well as immunocomplexes.<sup>33</sup> Unlike immunoassays, however, nucleic acid detection based on amplification techniques is usually carried out in a homogeneous system, making the aforementioned approach originally unavailable in the field of nucleic acid detection.<sup>22,23</sup> The emergence of the cell-lysis card makes the homogeneous amplification a quasi-heterogeneous system, which allows the nucleic acids to be adsorbed on the solid surface of the card. This is the essential reason for the availability of the prefilling approach, and the final goal is to facilitate the end-user as well.

**SNPs Typing.** We applied the sample-to-answer icLAMP system to a clinical SNPs typing of the human CYP2C19 gene. CYP2C19 is a member of the cytochrome P450 gene family, which is related to the metabolic pathway of many drugs in humans. For example, clopidogrel can inhibit platelet aggregation and has been approved for prevention and treatment of coronary artery disease and peripheral vascular disease, the activation of which requires the CYP2C19 liver enzyme. Individuals with a lower level of CYP2C19 liver enzyme may not get the full effect. Therefore, rapid, accurate, and personalized genetic testing of CYP2C19 could provide the direct guidance as to the choice of drug dosage.<sup>34</sup> There are several SNP sites in different positions of the CYP2C19 gene. Carriers of loss-of-function CYP2C19\*2 allele (position: 681G > A, an SNP on exon 5) and CYP2C19\*3 allele (position: 636G > A, an SNP on exon 4) show poorer ability to metabolize the antiplatelet drugs compared to the wild-type, namely, CYP2C19\*1.



**Figure 5.** (a) CYP2C19 SNPs typing using allele-specific icLAMP. For testing one blood sample, four microcapillaries were required with each capillary corresponding to one type of primer. Two mutation sites (\*2 and \*3) in three different whole blood samples (No. 797, 795, and 784) were tested. The genotype for each sample was determined by the presence (+) or absence (−) of the fluorescence signal after amplification (WT = wild-type; MUT = mutant). (b) CYP2C19 genotyping for blood samples (No. 797, 795, and 784) using a commercial kit (BaiO, Shanghai). The schematic shows the arrangement of target probes for different SNP sites, and the other images showed the experimental results. Six parallel tests were performed for each probe. The fluorescent image determined the genotype of each sample, as indicated in the upper-left corner of each image.

We carried out allele-specific LAMP by using four groups of primers (Table S1, Supporting Information)<sup>28</sup> to distinguish the alleles for each blood sample (Figure 5). We first introduced 200 nL of whole blood equivalent to  $\sim 10^3$  copies of genome DNA into the FTA flake inside the microcapillary. FTA technology is universally applicable for nucleic acid extraction/storage at room temperature and is much milder than other traditional methods such as thermal lysis. Some researchers have combined the FTA card with microfluidic devices, but they still lysed cells by additional reagents while the card itself contains chemicals for cell lysis.<sup>25</sup> In our icLAMP system, the extracted DNA was stored on the FTA card. During isothermal amplification, the DNA was immersed by the LAMP mix containing the fluorescence indicator, calcein, the fluorescence of which was initially quenched by Mn<sup>2+</sup>. As the reaction proceeded, Mn<sup>2+</sup> was precipitated with the pyrophosphoric acid produced by the reaction. Thus, the calcein was separated from Mn<sup>2+</sup> and combined with Mg<sup>2+</sup> in the LAMP mix, resulting in a detectable fluorescence under UV light.<sup>35</sup>

We determined that sample No. 797 was homozygous wild-type (so-called extensive metabolizer), because it can only be amplified by the wild-type allele-specific primer. Similarly, sample No. 795 was determined to be heterozygous \*2 (so-called intermediate metabolizer), and sample No. 784 was determined to be heterozygous \*3 (so-called intermediate metabolizer as well), respectively (Figure 5). The commercial CYP2C19 genotyping kit combining PCR amplification and DNA microarray analysis (BaiO, China) yielded the same results (Figure 5). Compared to icLAMP for SNPs analysis with sample-to-answer capability, the CYP2C19 genotyping kit required complicated procedures such as (1) extraction of nucleic acids from blood samples; (2) amplification of nucleic acids by PCR; (3) detection of PCR products by DNA hybridization reaction and chromogenic reaction at 42 °C with several rounds of adding and removing different reagents; (4) signal readout using a specific instrument. The comparison between icLAMP and the commercial kit was presented in Table 1.

**Table 1. Comparison between icLAMP and the CYP2C19 Genotyping Kit**

properties	icLAMP	commercial kit
function integration	high	low
sample	blood	extracted nucleic acid
sample volume	0.2 μL	5 μL
total time	150 min	>530 min
total cost	< \$0.2	> \$15
procedures	simple	complex

In addition, The FTA technology could be applied for collection and isolation of nucleic acids from different samples including blood, bacteria, viruses, and plants. To extend the applicability of the icLAMP system to bacteria, we inserted a trimmed piece of CloneSaver Card (GE healthcare, which also utilizes the FTA technology but specifically designed to fit applications in bacterial specimens) into the microcapillary and preloaded TE buffer (600 nL × 2), LAMP reaction mix (1 μL), and water droplets (600 nL × 3) into the microcapillary in sequence before introduction of *E. coli* into the microcapillary (Figure S2a, Supporting Information). The components of LAMP reaction mix for *E. coli* carrying the conserved fragment of H1N1 could be found in our previous publication.<sup>26</sup> We

demonstrated that immersing the card membrane in the sample solution for 1 h could extract DNA from *E. coli* that are protected by a cell wall (Figure S2b, Supporting Information). If we did not wait long enough (the instructions of the CloneSaver card states that at least 1 h is required), the DNA could not be efficiently extracted from the bacteria (Figure S2c, Supporting Information).

## CONCLUSION

The developed icLAMP system could perform simultaneous genetic testing starting at crude specimens for multiple samples and/or multiple mutation sites and get the results within  $\sim 150$  min, with low cost, low sample/reagent consumption, and operational convenience. This icLAMP technology may expand the application range of the microcapillary-based analytical system toward POC testing.

## ASSOCIATED CONTENT

### S Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

### W Web-Enhanced Features

Two movies, one showing the assembly of the icLAMP system and another showing the microcapillary being shaken, are available in the HTML version of the paper.

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### Notes

The authors declare no competing financial interest.

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