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Supporting Information

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Rapid, Sensitive, and Quantitative Detection of Pathogenic DNA at the Point of Care through Microfluidic Electrochemical Quantitative Loop-Mediated Isothermal Amplification**

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Supporting Information

Materials and Reagents. Reagent-grade chemicals, including 6-mercapto-1-hexanol (6-MCH), sulfuric acid (H_2SO_4), methylene blue (MB), and betaine were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. LAMP DNA primers were ordered from IDT (Coralville, IA). Bst DNA polymerase (large fragment), 10× ThermoPol reaction buffer, dNTPs, and low molecular weight DNA ladder were obtained from New England Biolabs (Ipswich, MA). Nuclease-free water (not DEPC-treated), TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0), 4-20% gradient polyacrylamide TBE gels, 10× BlueJuice gel loading buffer, and SYBR Gold nucleic acid stain were purchased from Life Technologies (Carlsbad, CA). Purified genomic DNA of *Salmonella entrica entrica* Typhimurium, *Escherichia coli*, and *Shigella flexneri* were acquired from ATCC (Manassas, VA). Genomic DNA was reconstituted in TE buffer at 20 ng/µL. A custom high-salt phosphate buffer containing 100 mM sodium phosphate, 1 M sodium chloride (NaCl) and 1 mM magnesium chloride ($MgCl_2$) was prepared to dilute 6-MCH for passivation of the gold working electrode.

Fabrication of MEQ-LAMP Chips. MEQ-LAMP chips were assembled from three modular, separately fabricated layers – the electrode substrate, the chamber layer, and the fluidic via substrate – in a manner similar to previously described methods.[1] For the electrode substrate, platinum reference and counter electrodes and gold working electrodes were sequentially microfabricated on a 4-inch-diameter, 500-µm-thick Borofloat glass wafer (Mark Optics, Santa Ana, CA) through successive standard lift-off processes. The lift-off consisted of transparency mask (Grayphics, Santa Barbara, CA) based contact photolithography and electron-beam evaporation-based metal deposition (180 nm of platinum or gold on 20 nm titanium for adhesion) in a VES 2550 evaporation chamber (Temescal, Livermore, CA), followed by immersion and gentle sonication in acetone. The chamber layer was formed from a 250-µm-thick PDMS sheet (BISCO Silicones, Rogers Corporation, Carol Stream, IL) with the channel design cut using a programmable sign-cutting tool (CE5000-60, Graphtec, Santa Ana, CA). For the fluidic via substrate, eyelet holes were drilled through a second Borofloat glass wafer with a 1.1-mm-diameter diamond drill bit (Triple Ripple, Abrasive Technology, Lewis Center, OH) using a programmable CNC milling machine (Flashcut CNC, San Carlos, CA). The electrode wafer and fluidic via substrate were diced (DAD-2H/6, Disco, Tokyo, Japan) into individual chips prior to assembly. The three modular layers were manually assembled in a fume hood. During the assembly, one side of the PDMS layer was corona treated with a high-frequency emitter (BD-20AC, Electro-Technic Products, Chicago, IL) for approximately 5 seconds before being bonded to the fluidic via substrate. This assembly was allowed to rest for several minutes to ensure strong bonding between PDMS and glass. Subsequently, the other side of the PDMS was corona treated and bonded to the electrode substrate to finish device assembly. The complete chip measured 30 mm × 7.5 mm, with a microfluidic chamber volume of approximately 20 µL.

We cleaned each chip's gold working electrode with 50 mM H_2SO_4 via cyclic voltammetry, with twelve potential sweeps ranging from -0.8 to 1.0 V (with respect to the platinum reference electrode) applied at 0.1 V s^{-1} with a sample interval of 0.001 V and 10 µA sensitivity, readying it for surface passivation. After a brief rinse with deionized (DI) water, the gold electrodes were passivated with 10 mM 6-MCH in high salt phosphate buffer for 2 h. The device was placed on a hot plate at 65 °C for 20 minutes to thermally remove defects in the self-assembled 6-MCH monolayer. After this thermal treatment, the device was immediately rinsed with DI water before loading the LAMP reaction mix into the device.

LAMP Reaction Assembly. The reaction was assembled within a laminar flow hood in a separate laboratory to prevent carry-over contamination. Typical LAMP reaction mix (25 µL) contained the following: 20 mM Tris buffer, 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.8 M betaine, 1.4 mM dNTP, 0.64 U/µL *Bst* DNA polymerase, 0.1% BSA, 10 µM MB, 3.2 µM of each FIP and BIP primers, 0.4 µM of each F3 and B3 primers, 0.8 µM of each LF and LB primers, and 1 µL genomic target DNA diluted from the stock with TE buffer to obtain the desired concentration. During the reaction assembly, the reagents were kept cold with a 96-well PCR cold block (Eppendorf, Hauppauge, NY). The primer sequences are as follows:

FIP: 5' – GACGACTGGTACTGATCGATAGTTTTTCAACGTTTCCTGCGG – 3'
BIP: 5' – CCGGTGAAATTATCGCCACACAAAACCCACCGCCAGG – 3'
F3: 5' – GGCGATATTGGTGTTTATGGGG – 3'
B3: 5' – AACGATAAACTGGACCACGG – 3'
LF: 5' – GACGAAAGAGCGTGGTAATTAAC – 3'
LB: 5' – GGGCAATTCGTTATTGGCGATAG – 3'

Experimental Procedures of MEQ-LAMP Reactions. Assembled LAMP reaction mixture was pipette-loaded into 6-MCH-passivated and rinsed MEQ-LAMP chips. The device eyelets were sealed with PCR film (Microseal 'B' Adhesive Seals, Bio-

Rad, Hercules, CA) to prevent reaction contamination and reagent evaporation. The chips were connected to a potentiostat (CHI 660D, CH Instruments, Austin, TX) for electrochemical measurements via custom card-edge connectors. After three room-temperature pre-LAMP measurements (1-minute intervals for 3 minutes), the chips were mounted onto a digital block heater (VWR International, Radnor, PA) maintained at 65 °C to initiate the reaction. The thermal contact between the chips and the heater was established by a thermal compound (Arctic Alumina, Arctic Silver, Inc., Visalia, CA), which also served as an adhesive to keep chips attached to the heater during the reaction. Up to three chips could be run in a single experiment. We simultaneously initiated a series of time-course electrochemical measurements, measuring the redox current in each chip every minute for 60 minutes. In parallel, bench-top, PCR-tube control reactions were performed in a thermocycler (DYAD 220 Peltier Thermal Cycler, MJ Research, Inc., Waltham, MA), also at 65 °C for 60 minutes. At the end of the LAMP reaction, the chips were removed from the heater, and the currents were measured every minute for 10 minutes as the chips cooled down to room temperature. The amplified LAMP reaction products were pipette extracted from the chips and analyzed via gel electrophoresis. Unreacted LAMP mixes containing exactly the same reagents were subsequently loaded into the chips, and the redox current was measured every minute for 3 minutes at room temperature, similar to the pre-LAMP measurements. The pre-LAMP, post-LAMP, and unreacted LAMP current measurements were used to monitor the changes at the working electrode surface during the course of the reaction.

Electrochemical Measurements. Square wave voltammetry (SWV) was selected for monitoring the MB redox current during LAMP reactions due to its fast measurement time (~4 seconds per measurement). The SWV parameters were as followed: 0.2 V initial voltage, -0.4 V final voltage, 0.004 V increment, 0.025 V amplitude, 100 Hz frequency, 1 second quiet time and 1 μA sensitivity. A custom potentiostat macro command was written to enable the simultaneous monitoring of up to three chips.

Gel Electrophoresis. Both on-chip and bench-top amplified LAMP products were analyzed via gel electrophoresis using a 4-20% gradient polyacrylamide TBE gel. Each sample to be analyzed contains 1 μL LAMP reaction product, 1 μL loading dye, and 8 μL 0.5× TBE. In parallel, each lane of ladder contains 0.5 μL low molecular weight DNA ladder, 1 μL loading dye, and 8.5 μL 0.5× TBE. Electrophoresis was performed with an XCell *SureLock* Mini-Cell (Life Technologies) system in 0.5× TBE buffer at 200 V for 60 min. The gel was stained with 1× SYBR Gold nucleic acid stain for approximately 5 to 10 minutes. Gel images were taken with a Kodak Gel Logic 200 imaging system equipped with Kodak molecular imaging software (Carestream Health, Inc., Rochester, NY).

Electrochemical Data Processing. The time-course current measurements were normalized to the first measurement. The normalized current trace was plotted and further processed with Origin 7.5 data analysis and graphing software (OriginLab, Northampton, MA). The normalized current trace was smoothed by the Origin built-in five-point adjacent averaging function. The derivative curve was subsequently calculated from the smoothed current trace using the Origin built-in derivative function. The derivative trace facilitated the reliable identification of the signal threshold – designated as the local minimum of the current derivative trace – and the corresponding time-to-threshold – defined as the reaction time required for a particular sample reaction to reach the signal threshold.

Specificity of LAMP Reaction. We tested the specificity of our LAMP amplification by performing MB-supplemented LAMP reactions using our *InvA* gene primer set in Eppendorf PCR tubes and bench-top thermocycler against *Salmonella* Typhimurium, *Shigella flexneri*, or *Escherichia coli* genomic DNA (2×10^4 copies per 25-μL reaction) as the target and analyzing the results with gel electrophoresis. Both *S. flexneri* and *E. coli* are enteric bacteria that are closely related to *Salmonella*, and thus provide a stringent evaluation for the reaction specificity. We observed strong bands of amplified DNA from the *S.* Typhimurium sample (Figure S1, lane 1), but no product from the *S. flexneri* or *E. coli* samples (Figure S1, lanes 2 and 3), thus demonstrating the high specificity of our LAMP reaction.

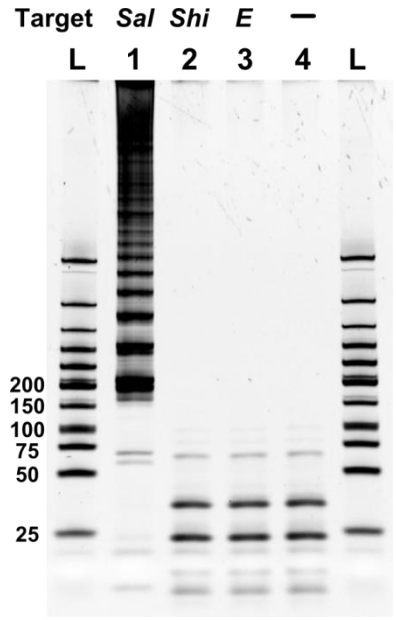

Figure S1. Specificity of LAMP Reaction. We observed strong bands of amplified DNA from the *S.* Typhimurium sample (lane 1), but no product from either the *S. flexneri* or the *E. coli* sample (lanes 2 and 3), thus demonstrating the high specificity of our LAMP reaction. Lane 4 represents the no-target negative control. Lanes L in this figure and subsequent figures indicate the low molecular weight DNA ladder.

Fidelity of Microfluidic MB-LAMP Reaction. We subsequently demonstrated successful microfluidic, on-chip MB-LAMP reactions with high amplification efficiency. To do so, we performed standard, MB-free and MB-supplemented LAMP reactions in PCR tubes and a bench-top thermocycler, while also conducting a MB-supplemented microfluidic MEQ-LAMP reaction, all using the *InvA* primers and purified *S.* Typhimurium genomic DNA (approximately 2×10^4 copies per 25-µL reaction) as the target. We analyzed the amplified product from each reaction with gel electrophoresis, and observed similar distribution of amplicon bands and band intensities between the three reactions (Figure S2, lanes 1, 3, and 5), indicating the success of the microfluidic LAMP reaction. These results also show that the addition of MB does not adversely affect the LAMP reaction and that there is a negligible penalty for conducting the reaction on-chip.

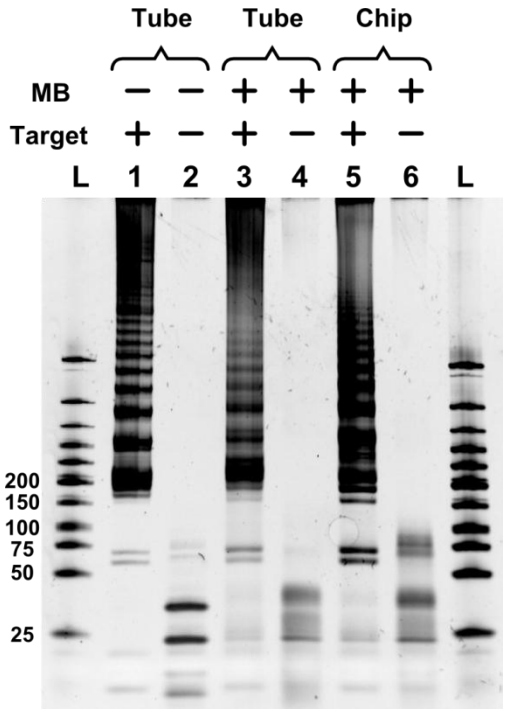

Figure S2. Fidelity and Efficiency of Microfluidic MB-LAMP Reaction. We successfully demonstrated that the microfluidic, on-chip MB-LAMP reaction (lane 5) displayed amplification efficiency equivalent to a standard MB-free LAMP reaction (lane 1) or MB-LAMP reaction (lane 3) performed with a bench-top thermocycler. All three reactions contained approximately 2×10^4 copies of purified *S.* Typhimurium genomic DNA per 25-µL reaction, and yielded similar amplicon band sizes and intensities, indicating similar amplification performances. Lanes 2, 4, and 6 represent no-target negative controls.

Thermal-Induced MB Dissociation from Alkanethiol Monolayer. Previous research has suggested that MB gets incorporated into self-assembled alkanethiol monolayers.[2, 3] We thus hypothesized that the current decrease observed in Region 1 ($t < 10$ minutes) of our negative control (no-target) MEQ-LAMP reactions arises because heating the device from room temperature (~22 °C) to 65 °C forces MB molecules initially entrapped within the alkanethiol passivation monolayer on the working electrode to dissociate into solution. This dissociation lowers the local MB concentration close to the electrode surface, thus reducing the current. To test our model, we performed one reaction at room temperature (~22 °C) and one reaction at the typical LAMP reaction temperature of 65 °C and compared their current traces in Region 1. We employed simplified, non-amplifiable MEQ-LAMP reactions lacking both target DNA and primers, so that we could decouple the current changes from the LAMP reaction itself. We observed typical signal decrease in Region 1 with the device incubated at 65 °C (Figure S3, red). However, the decrease in Region 1 was negligible for the reaction conducted at 22 °C (Figure S3, black) supporting our model.

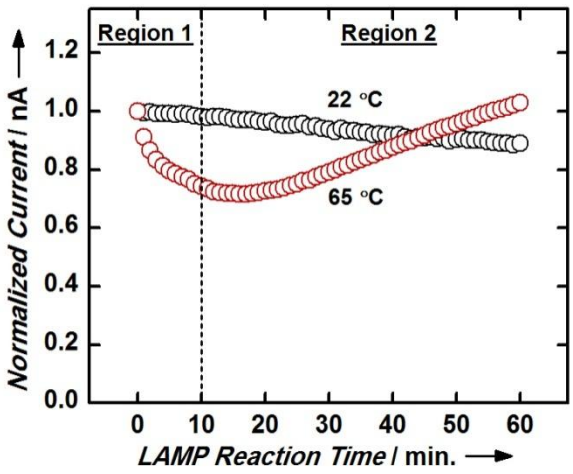

Figure S3. Comparison of MEQ-LAMP Current Traces under Various Temperature Conditions. We performed two non-amplifiable MEQ-LAMP reactions, one at room temperature (~22 °C) and another at the typical LAMP reaction temperature (65 °C), and compared their current traces. The 65 °C reaction trace shows the typical two-region curve: an initial decrease in Region 1 followed by a steady increase in Region 2. In comparison, the current remained near-constant at 22 °C, suggesting that this temperature was too low to free entrapped MB from the alkanethiol monolayer at early time-points or to induce degradation of the alkanethiol monolayer at later time-points.

Heat-Induced Degradation of Alkanethiol Monolayer. At time-points in Region 2 (t > 10 minutes), we observed a steady increase in the redox peak current as a function of time, which we attribute to the breaking of the gold-thiol bond that attaches the alkanethiol passivation monolayer to the electrode surface.[4-6] As this monolayer dissociates, a greater number of MB molecules can approach the electrode surface unimpeded, causing the redox current to rise. To test this hypothesis, we first compared the current traces from the experiment described above (Figure S3). It was clear that the current increase observed in Region 2 occurred exclusively at 65 °C, indicating that it was directly associated with the higher reaction temperature (Figure S3, Region 2).

To provide additional evidence that the alkanethiol monolayer degraded during the high-temperature reaction, we performed another experiment in which we measured the current obtained with the non-amplifiable MEQ-LAMP reaction solution at 22 °C before and after maintaining the device at either 22 °C or 65 °C for one hour. Importantly, in both cases we emptied the chip and re-loaded fresh reaction mix after the one-hour incubation. Since the reaction mixes were identical, any change in the current trace must originate from the temperature-induced changes at the electrode surface. For the room temperature treatment, the current did not change, indicating no changes to the electrode surface (Figure S4A). On the other hand, pre-incubating the chip at 65 °C caused a notable current increase, suggesting that the passivation layer had degraded as a result of the higher reaction temperature (Figure S4B), thus allowing more MB to approach the electrode surface unimpeded and causing the redox current to rise.

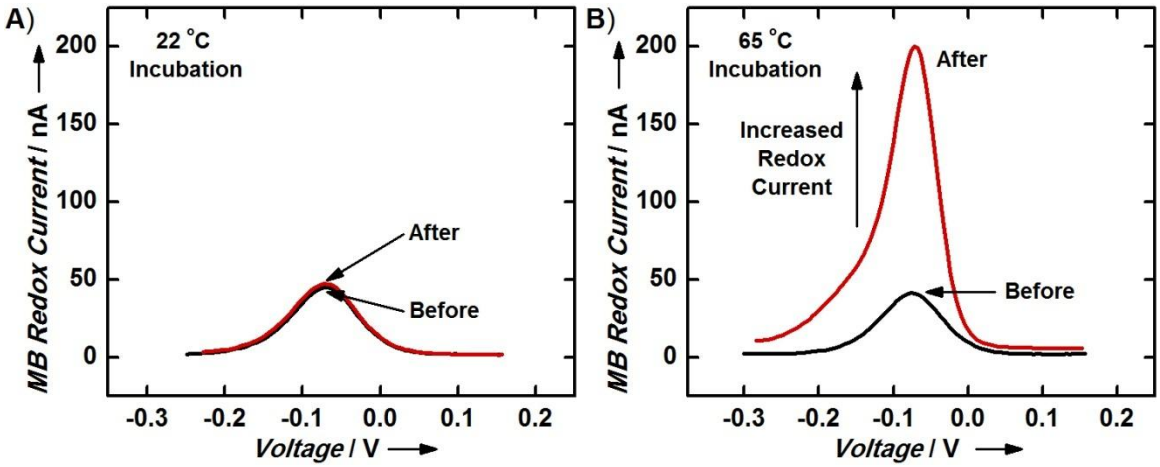

Figure S4. Validation of Thermally-Induced Degradation of Alkanethiol Monolayer. We compared the currents measured at room temperature before and after a one-hour reaction at either (A) 22 °C or (B) 65 °C. At 22 °C, the current did not change, indicating no effect on the electrode surface. On the other hand, one hour at 65 °C caused a large current increase, suggesting that the passivation layer had degraded as a result of the high reaction temperature.

Verification of MEQ-LAMP Time-to-Threshold Measurements. To verify the time-to-threshold (t_{TH}) observed in the various MEQ-LAMP reactions, we performed bench-top MB-LAMP reactions using different initial target copy numbers for varying lengths of reaction time and analyzed the results via gel electrophoresis. Specifically, we serially diluted (10-fold) the initial target dose from 2×10^4 down to 2×10^1 genome copies per 25-µL reaction and increased the reaction time with a 10-minute increment from 15 minutes (2×10^4 copies) up to 45 minutes (2×10^1 copies) – good approximations of the t_{TH} measured at each target dose. We analyzed the amplified product from each reaction with gel electrophoresis, and observed similar distribution of amplicon bands and band intensities between the four reactions (Figure S5, lanes 1 to 4), indicating the similar reaction statuses for these samples at their perspective reaction time points. This experiment thus verified the t_{TH} observed from the MEQ-LAMP reactions.

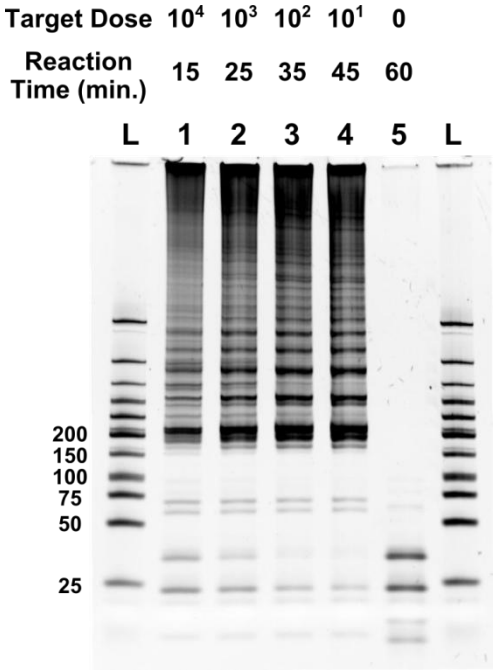

Figure S5. Verification of Time-to-Threshold Measurements from MEQ-LAMP Reactions. We performed bench-top MB-LAMP reactions by serially diluting (10-fold) the initial target dose from 2×10^4 down to 2×10^1 copies genome copies per 25-µL reaction and increasing the reaction time with a 10-minute increment from 15 minutes (2×10^4 copies) up to 45 minutes (2×10^1 copies) – good approximations of the time-to-threshold (t_{TH}) observed at each target dose. Analyzing the amplified product from each sample with gel electrophoresis, we observed similar distribution of amplicon bands and band intensities between the four reactions (lanes 1 to 4), thus validating the t_{TH} values measured from the MEQ-LAMP reactions. Lane 5 represents the no-target negative control.

References

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