Mobile All-In-One Malaria Molecular Diagnosis for Field Deployment in Resource-Limited Areas

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Abstract— **This paper presents a "sample-in-answer-out" molecular diagnostic system (AnyMDx) for blood-born malaria detection at the point of care, especially in resource-limiting settings. AnyMDx consists of a microfluidic reagent compact disk incorporating seamlessly integrated steps of DNA extraction, purification, elution, and amplification, as well as a mobile battery-powered analyzer. Low power thermal module and novel fluorescence sensing module are integrated into the analyzer for real-time monitoring of loop-mediated isothermal nucleic acid amplification (LAMP). The AnyMDx has an assay detection limit of ~0.6 parasites/μL against blood *P. falciparum* parasites, much lower than conventional microscope-based method (~50 parasites/μL) and immunoassay based RTD (~100 parasites/μL). AnyMDx is fully automated and thus is easily accessible to non-specialist with minimal molecular training. The sample-to-answer turnaround time for AnyMDx is less than 40 minutes.**

I. Introduction

Despite extensive international efforts, malaria still causes a staggering 243 million cases annually worldwide with half of the world's population remaining at risk of malaria [1, 2]. Malaria diagnosis - detection of malaria parasites in the peripheral blood of patients is required for proper treatments of the infections. Current malaria diagnosis in epidemic field settings relies exclusively on microscopy and RDTs [2-5]. However, both methods have a detection limit of ~50-100 parasites/μL, thus patients presenting with lower parasitemias are often missed [3-5]. So far, satisfactory performance of RDTs has only been achieved for diagnosing *P. falciparum* malaria. Furthermore, most RDTs for *P. falciparum* malaria are based on detection of the histidine-rich protein 2 (HRP2) of the parasite. Hence, the deletion of the hrp-2 gene in some *P. falciparum* populations resulted in a substantial proportion of the malaria cases misdiagnosed as false negatives.

Molecular methods based on nucleic acid amplification are much more sensitive with detection limits approaching ~1 parasite/μL. This level of sensitivity enables healthcare workers to identify asymptomatic malaria carriers of malaria parasites, who may serve as reservoirs for continued transmission. Asymptomatic infections often persist at very low parasite densities, below the threshold of detection by microscopy or RDTs. The detection of malaria parasites in this subpatent population enables targeted control and is essential for the malaria elimination campaigns currently unfolded in many malaria endemic countries.

Among various amplification assays, loop-mediated isothermal DNA amplification (LAMP) has emerged as a promising technology due to its rapidness, sensitivity and specificity [7-9]. However, LAMP based method still relies on laboratory infrastructures for DNA extraction, purification, elution, and amplification [1, 3, 9, 10]. A true "sample-in-answer-out" genetic testing is still challenging for practical setting [5, 9, 10, 13-15].

Herein, we demonstrate a field-deployable malaria genetic testing platform (AnyMDx). The AnyMDx has the following attributes: i) standalone, ii) sample-in-answer-out, iii) fully integrated and automated DNA sample preparation, iv) real-time fluorescence detection, and v) rapid and sensitive diagnosis. In addition, AnyMDx has smartphone connectivity for data logging and result reporting. Charge switchable magnetic beads were adopted in the microfluidic compact disk to streamline the DNA sample preparation steps (extraction-purification-elution). Sensitive and specific LAMP assay was performed on the same compact disk in a sealed manner (thus reducing the chance of cross-contamination). All required reagents from sample preparation to DNA amplification were preloaded into the microfluidic reagent compact disk in a ready-to-use fashion. With minimal hands-on work, the AnyMDx can deliver sensitive molecular answers directly from raw blood samples within 40 minutes without any requirement of laboratory infrastructures.

II. Materials and Instrumentation

A. AnyMDx Instrumentation

Figure 1 shows the exploded view of AnyMDx. The platform is of a small footprint (12×13×13 cm) and consists of four functional parts: mechanical components (servo motor/spindle platter/compact disk), optical components (LED/optical sensor), thermal modules (peltier heater and thermal sensors), and data connectivity components (GPS/Bluetooth). All components were controlled by a microcontroller unit (MCU) through a customized PCB board. We used a 3D printer for quick prototyping of the AnyMDx platform. For the real-time optical detection, we used an LED light source (λ= 488 nm) which is guided towards the reaction chamber through plastic optical fibers. The incidence of excitation LED light was perpendicular to

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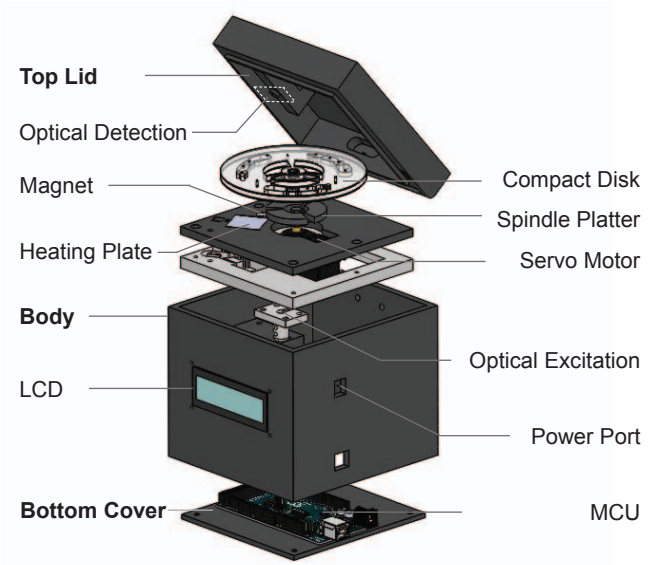

Figure 1. Schematic of functional parts in AnyMDx. The whole instrument has a footprint of 12×13×13 cm.

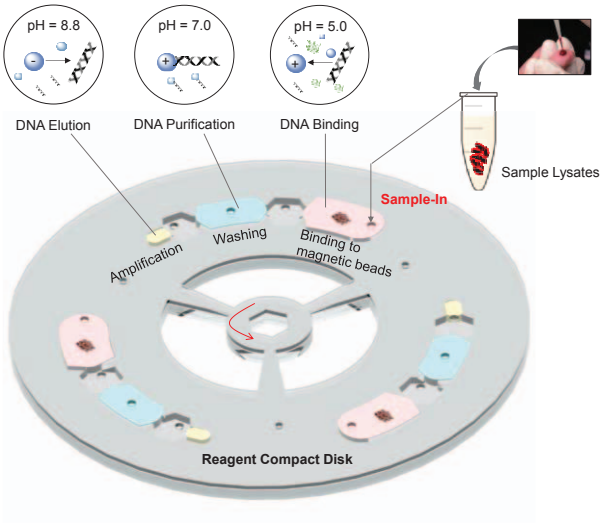

Figure 2. Schematic of reagent disk. The 4.8 mm-thick microfluidic reagent compact disk consists of three patterned PMMA layers. Three functional chambers are isolated with two teeth-structured valves. All reagents are premixed and loaded in the ready-to-use format. Sample lysate was prepared on the tube and loaded to the binding chamber. pH sensitive charge switchable magnetic beads binds to the DNA and carry over DNA towards the reaction chamber through washing chamber for purification and amplification.

the optical sensor. This configuration allows for an increased signal-to-noise ratio by minimizing the total amount of excitation light into the optical sensor.

An LCD provides a user-friendly interface for operating and data display. In addition, Low power Bluetooth module is incorporated for easy data connectivity. A GPS module also is embedded in the AnyMDx instrument to acquire geographical information of epidemic malaria infections. The whole system is Lithium-ion battery powered and requires no electricity on-site.

B. Reagent Compact Disk Fabrication

Figure 2 shows the microfluidic reagent compact disk for sample preparation and LAMP DNA amplification. Three microfluidic chambers, a 25 µL LAMP reaction chamber (yellow), a 150 µL washing chamber (sky blue), and a 210 µL DNA binding chamber (pink), were separated by the two 80 µL valve-spaces filled with FC-40 oil and air. The FC-40 oil completely sealed the LAMP reaction chamber to prevent evaporation while thermal process. Air-filled valve was surface treated with water-oil repellent (Daikin Unidyne TG-5601), creating the barrier for holding amphiphilic lysis buffer. Teeth-structures prevents the reagent from mixing by increasing the surface tension. The reagent compact disk is manufactured by laminating top, spacer, and bottom layers through the adhesive solvent. Each layer was designed and fabricated by laser cutting (Epilog Helix 24 Laser System).

C. Automated Sample Preparation

Commercially available DNA purification kit (Invitrogen ChargeSwitch forensic DNA purification kit) was adopted in the reagent compact disk for automated DNA sample preparation. The surface charge polarity of the magnetic beads is pH dependent, while the surface charge polarity of DNA is negative for a wide range of pH values. As a result, the nucleic acids can be attracted onto or repelled from the magnetic beads by adjusting pH values of the buffer solution (Figure 2). Lysate was prepared from 10 µL of human blood samples by adding 1000 µL of lysis buffer and 10 µL proteinase K in 1.5 mL microcentrifuge tube for 2 minutes at room temperature. Only 180 µL of the lysate was dispensed into the bead-binding chamber on the compact disk. After that, the automated sample preparation was executed via following three sequential steps: i) magnetic beads binding, ii) washing, and iii) elution (Figure 2). Beads were actuated by rotating the compact disk against a stationary magnet. Through well-designed control sequence, the DNA-carrying beads can be directed into different reagent chambers for sample preparation. The fully automated sample preparation process can seamlessly extract and purify the DNA sample from the human blood in less than 10 minutes.

D. LAMP Assay

The LAMP assay consists of isothermal buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 50 mM KCl, 2 mM $MgSO_4$, 0.1% Tween 20, pH 8.8), *P. falciparum*-specific primer set, $MgSO_4$, calcein, $MnCl_2$, deoxyribonucleotide triphosphates (dNTPs), *Bst* 2.0 DNA Polymerase, DNA template, and PCR grade H_2O. The LAMP assay is performed at a constant temperature (65°C). Primer set targeting mitochondrial gene was synthesized through IDT to specifically amplify the 213-bp region of the *P. falciparum* DNA [16]. Calcein was used for fluorescence signal development. The positive LAMP reaction can be determined by the dramatic increase in fluorescence reading in less than 30 minutes.

III. Results and Discussion

A. Validation of the LAMP assays on AnyMDx

The goal of the experiment was to validate the LAMP assay, and to confirm the fluorescence sensing mechanism on

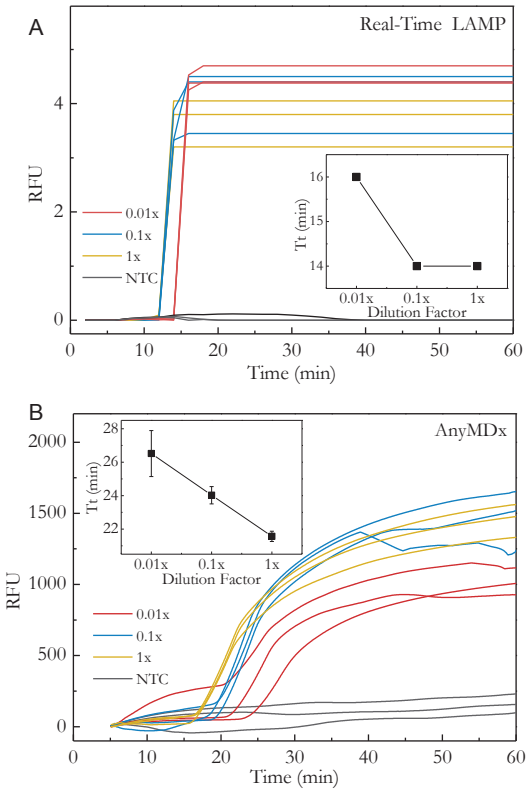

Figure 3. Validation of the LAMP assay using laboratory purified DNA. The DNA amplification results obtained from a real-time PCR machine (A), and the AnyMDx instrument (B).

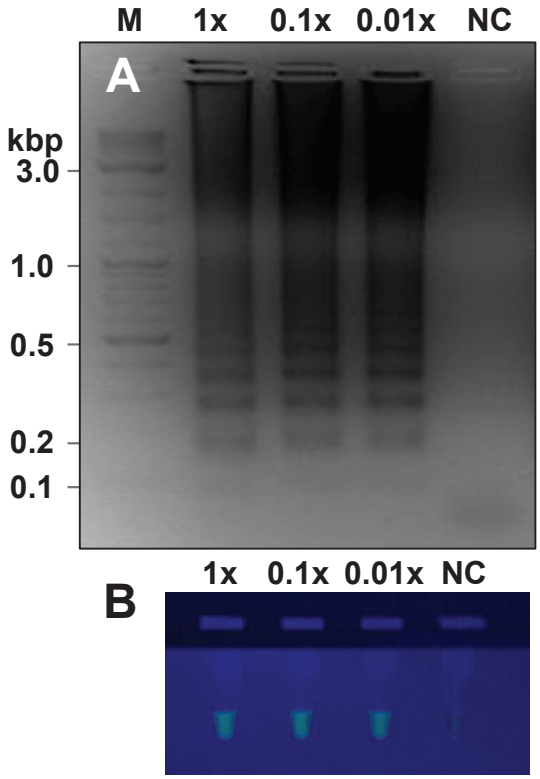

Figure 4. (A) Gel image and (B) Green emission under the blue LED illumination. 1x, 0.1x, and 0.01x denote the dilution factors of the *P. falciparum* DNA samples. 2% agarose gel was used (213-bp bands were expected).

AnyMDx. To evaluate the performance, the comparison was made between the conventional real-time PCR machine and AnyMDx instrument, using laboratory purified *P. falciparum* genomic DNA (gDNA).

10-fold serially diluted gDNA (yellow- 1:1 dilution, blue- 1:100 dilution, and red- 1:1000 dilution) with Tris-EDTA buffer were prepared as a positive control (Figure 3). LAMP cocktail without DNA template (black- NTC) was used as negative control.

As shown in the Figure 3A, DNA amplification was found only in the positive sample. The positive samples show a fluorescence signal 5 times higher than the negative control sample. After the amplification, the amplicons were loaded onto the 2% agarose gel for electrophoresis analysis. A clear ladder-like pattern with multiple bands of different molecular sizes was observed due to its inverted-repeat structures, implying the successful nucleic acid amplification (Figure 4A). More specifically, the bands of ~200 bp were in accordance with the length of the specifically selected target sequence (213 bp) (Figure 4A). In addition, amplification results could be clearly distinguished in the PCR tube under the blue LED illumination (Figure 4B). Strong green emission (implying positive reaction) was easily recognized by the naked eye, implying positive reaction. Figure 3B demonstrates the quantitative ability of fluorescence detection with the minimized and integrated optical sensor. We defined a detection threshold time (Tt) at the time when the slope of measured RFU reached the peak. The mean and standard deviation of threshold time were calculated from the triplicates and plotted on a semi-log scale. The experimental variations of threshold time for 1:100, 1:10, and 1:1 dilution samples were appeared to be 5.18%, 2.15%, and 1.42% respectively.

B. Sensitivity Test

Figure 5 showed the sensitivity of the AnyMDx instrument. *P. falciparum* parasites were cultured in fresh human red blood cells (RBCs). From the ring stage parasites, parasitemia was set up at 2% (~64,000 parasites/μL), then 10-fold serial dilution was made with healthy human blood at

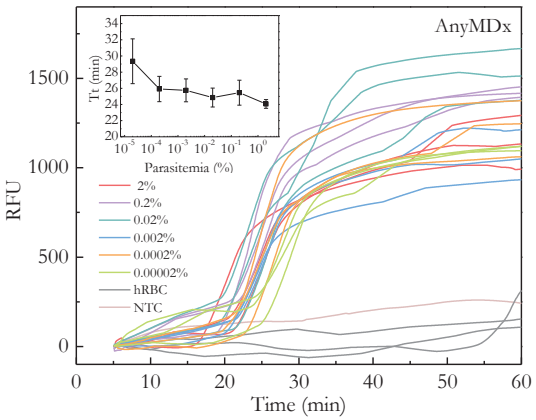

Figure 5. Sensitivity test of AnyMDx using cultured malaria in human blood. Six serially diluted infected RBC samples with healthy blood at 45% hematocrit were evaluated with AnyMDx. The % value represents the parasitemia of the infected RBC.

45% hematocrit to prepare six different parasitemia samples (from 2% to 0.00002%, and 2% parasitemia corresponds to ~60,000 parasites/μL) for quantitative analysis. All LAMP master mix without the template DNA was aliquoted from the same mother solution and loaded into the reagent compact disk. An amplification curve of the no template control (NTC) assured that the experimental environment was not contaminated by template DNA or LAMP amplicons. Triplicates for each concentration were performed to evaluate batch-to-batch variations. Most amplification events occurred between 24 to 30 minutes based on the parasitemia. The parasitemia of 0.00002% sample was successfully amplified, showing the detection limit is ~0.6 parasites/μL. Note that the parasite numbers in each LAMP reaction were expected to be one-fifth of the lysed parasite numbers because the only 180 μL of lysate was introduced from 1020 μL of lysate solution. This showed that the lower detection limit can be achieved by AnyMDx compared to conventional LAMP assay (less than 2 parasites/μL) [17-19]. The threshold time versus the parasitemia was plotted (Figure 5 inset). As expected, the amplification threshold time was inversely proportional to the parasitemia. The variations of threshold time for six 10-fold diluted parasitemia samples were calculated to be 11.5%, 6.1%, 5.7%, 4.5%, 5.9%, 1.8% respectively. It is anticipated that the variation can be improved with more sophisticated sample preparation process by the precise magnetic beads control in the reagent compact disk.

IV. Conclusion

We demonstrated a field-deployable molecular diagnostic system for quickly and accurately diagnose malaria infection in resource-limiting areas at the point of care. The standalone and mobile instrument works in a "sample-in-answer-out" fashion and can detect specific malaria species within 40 minutes from raw sample to answer. The detection limit is ~0.6 parasites/μL, much more sensitive than that of the microscopy and immunoassay based RDT method. We believe AnyMDx will create a new paradigm of malaria detection in resource-limiting regions.

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