SAMPLE-TO-ANSWER MOBILE MALARIA MOLECULAR DIAGNOSITSTIC SYSTEM FOR RESOURCE-LIMITING AREAS

Gihoon Choi[1], Daniel Song[2], Sony Shrestha[3], Jun Miao[3], Liwang Cui[3], and Weihua Guan[1,2]
[1]The Department of Electrical Engineering, Pennsylvania State University, University Park, USA
[2]The Department of Biomedical Engineering, Pennsylvania State University, University Park, USA
[3]The Department of Entomology, Pennsylvania State University, University Park, USA

ABSTRACT

This work reports a field-deployable "sample-to-answer" molecular diagnostic system (AnyMDx) for species-specific malaria detection at the point of need. The portable nucleic acid diagnostic system uses a disposable microfluidic disc, which incorporates integrated sample preparation steps of DNA extraction, purification, elution, and amplification. Built on our previous success with highly sensitive singleplex *P. falciparum* detection (low detection limit of ~0.6 parasites/μl) [1], here we report the specificity performances of AnyMDx for distinguishing two of the most common endemic malaria species (*P. falciparum* and *P. vivax*). The AnyMDx system is fully automated and delivers real-time multiplexed species-specific molecular answers directly from the raw blood samples within 40 minutes without any requirement of laboratory infrastructures.

INTRODUCTION

Despite global malaria control efforts, malaria still causes ~214 million cases worldwide per year and remains a major public health issue. For effective malaria eradication campaign, a rapid, accurate, and sensitive diagnosis is highly required. Currently, field detection of malaria relies heavily on microscopy or immunoassay-based RDT with a detection limit of ~50-100 parasites/μl, which is incapable of detecting low parasite densities in asymptomatic parasite carriers [2]. In addition, specificity test to identify the fatal malaria species is challenging for microscopy and RDT [3, 4]. Failure to recognize the different species of human malaria parasites can lead to overuse of antimalarial drugs, which can cause the development of resistance to antimalarial drugs. Therefore, highly sensitive and species-specific malaria diagnostic tools are urgently needed in malaria endemic settings.

Modern nucleic acid testing (NAT) methods have been employed as a malaria diagnostic tool due to its significantly improved sensitivity with low detection limits (~1 parasite/μl) [5]. Moreover, NATs have been shown higher specificity against the various malaria species than microscopic examination and RDTs [2, 4]. This level of sensitivity and specificity enables clinicians to detect sub-patent asymptomatic carriers of malaria parasites with accurate identification of the most critical human malaria parasite species (*P. falciparum and P. vivax*) [4, 5]. Hence, the NAT based detection facilitate targeted control and is critical for accurate chemotherapies in high malaria prevalence region.

Among the various NAT technologies, loop-mediated isothermal nucleic acid amplification (LAMP) attracts significant attention for potential integration in the mobile molecular diagnostic system for field use because of its simplicity, rapidness, sensitivity, and specificity [6]. We have previously shown a "sample-to-answer" nucleic acid testing system for highly sensitive singleplex *P. falciparum* detection (low detection limit of ~0.6 parasites/μl) [1].

In this work, we present the specificity performances of AnyMDx for distinguishing two of the most common endemic malaria (*P. falciparum* and *P. vivax*). As a mobile malaria molecular diagnostic system, AnyMDx has the following attributes: i) standalone; ii) seamlessly integrated and automated DNA sample preparation; iii) real-time fluorescence detection; iv) rapid and robust detection; and v) sensitive and species-specific diagnosis. Within 40 minutes, AnyMDx can specifically recognize the two most common malaria species (*P. falciparum* and *P. vivax)* and deliver sensitive molecular answers from raw blood samples without any requirement of specialist and laboratory infrastructures.

MATERIALS AND METHODS

Instrumentation

Figure 1 shows the exploded structure of AnyMDx analyzer. We used a commercial 3D printer for quick prototyping of the AnyMDx platform. The platform has a small footprint (12×13×13 cm) and powered by a rechargeable lithium-ion battery. This portable battery allows 14 hours of operation before recharging, thus it is highly suited to the resources-limited environment. The platform consists of four functional parts: mechanical subsystem (servo motor/spindle platter/compact disc), optical subsystem (LED/optical sensor), and thermal subsystem (Peltier heater and thermal sensors). A programmable microcontroller unit (MCU) interfaces with each subsystem through a customized PCB board to process automated sample preparation, nucleic acid amplification, and real-time detection. For the real-time optical detection, a LED light (λ= 488 nm) is guided towards the reaction chamber through polymer optical fibers. The optical fiber and LED light source are coupled by a 3D printed adapter. The incidence of excitation LED light is perpendicular to the optical sensor. Hence, the optimal signal-to-noise ratio is achieved by reducing the total amount of excitation LED light into the optical sensor. For the thermal subsystem, a temperature of Peltier heater is continuously monitored micro-thermistor. A preset reaction temperature is maintained by the feedback control for DNA amplification process. An LCD provides a user-friendly interface for easy operation and data display. In addition, low power Bluetooth module is incorporated to deliver the diagnostic results to the patients.

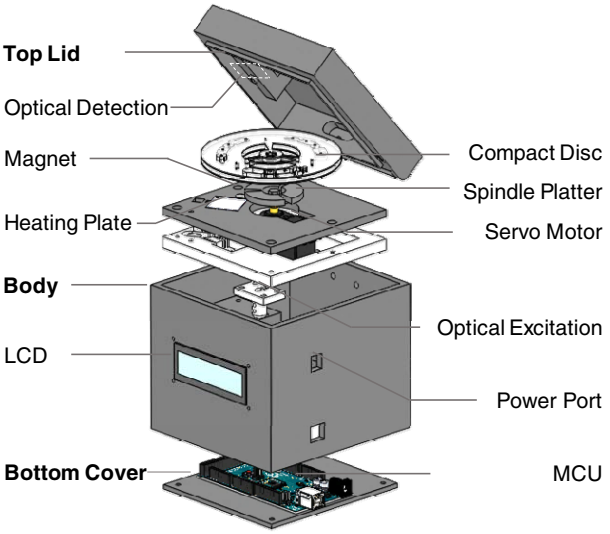

Figure 1: Exploded structure of the standalone and mobile nucleic acid testing system (AnyMDx).

Reagent compact disc

The 3.2 mm thick of microfluidic compact disc consists of top, spacer, and bottom poly(methyl methacrylate) (PMMA) layers. Each layer is patterned using (Epilog Helix Laser System) and laminated with adhesive solvent (Figure 2). Three spatially separated testing units were designed to perform *P. falciparum*-specific, *P. vivax*-specific and no template control (NTC). Each testing unit comprises a LAMP reaction chamber, a washing chamber, and a binding chamber, and two valving chambers. A sharp bending angle of the teeth-shaped valves maximizes the structural pinning effect to prevent the reagent mixing under agitation. The valving chamber adjacent to the reaction chamber was filled with FC-40 oil to prevent evaporation while thermal process.

Loop-mediated isothermal nucleic acid amplification

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), species-specific primer set, $MgSO_4$, calcein, $MnCl_2$, deoxyribonucleotide triphosphates (dNTPs), *Bst* 2.0 DNA Polymerase, Betaine, DNA template, and PCR grade H_2O. The LAMP assay is performed at a constant temperature (65°C). Two primer sets were synthesized through IDT to specifically amplify the 213-bp region of the *P. falciparum* DNA and the 127-bp region of the *P. vivax* DNA [7, 8].

Automated sample preparation

In order to extract malaria genomic DNA (gDNA) from whole blood lysates, commercial nucleic acid purification kit (ChargeSwitch forensic DNA purification kit, Invitrogen) is used in the reagent compact disc. Due to the negative surface charge polarity of DNA, pH dependent magnetic beads can attract or repel the nucleic acid by the different pH values of the preloaded reagent (Figure 3). The whole blood lysate is prepared from 10 µl of malaria parasite-infected whole blood samples by adding 1000 µl of lysis buffer and 10 µl of proteinase K. Only 180 µl of the lysate is introduced to the binding

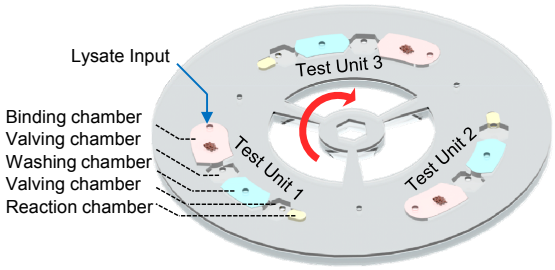

Figure 2: Schematics of the reagent compact disc, showing the three independent testing units. All reagents were premixed and preloaded in the ready-to-use fashion.

chamber through the inlet on reagent compact disc. After the lysate loading, the automated sample preparation is sequentially performed for DNA binding, purification, and elution. The 10 µl of preloaded magnetic beads are actuated by rotating the compact disc against a stationary magnet (Figure 3). The DNA-carrying beads are transferred from chamber to chamber until the purified DNAs reach the reaction chamber. The whole DNA sample preparation from the human whole blood can be automated in less than 10 minutes.

Data analysis

The relative fluorescence unit (RFU) is real-time measured every 2.5 seconds during the amplification process. The RFU values are smoothed by averaging a fixed number of consecutive data points. The positive reaction can be determined by the radical increase in measured RFU level. We defined a detection threshold time (T_t) when the slope of measured RFU that reaches the peak.

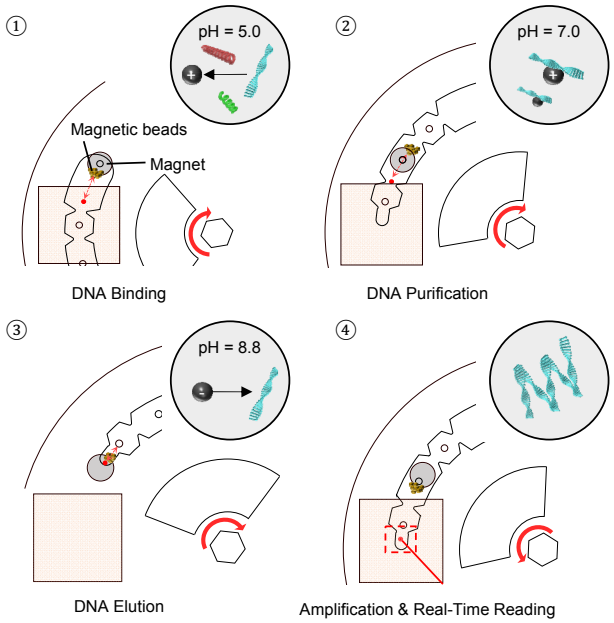

Figure 3: Illustration of automated sample preparation and DNA amplification steps on the reagent compact disc. By rotating the disc against a stationary magnet, the pH dependent charge-switchable magnetic beads were directed from binding chamber to reaction chamber.

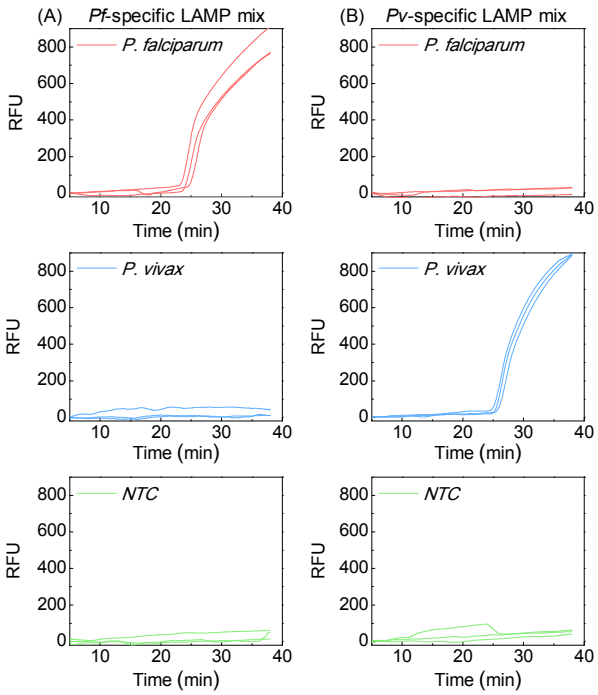

Figure 4: Specificity test for detecting the P. falciparum (Pf) and P. vivax (Pv) gDNA sample. The species-specific amplification results were obtained using AnyMDx analyzer. (NTC - no template control)

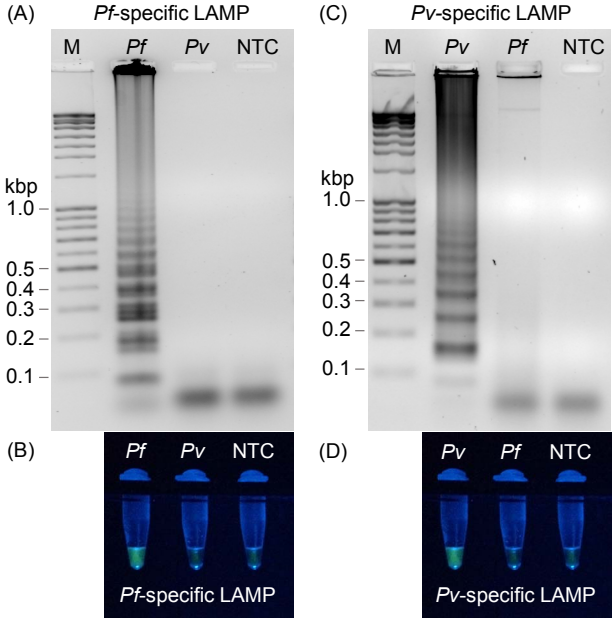

Figure 5: Verification of LAMP amplicons from Pf-specific LAMP (A, B) and Pv-specific LAMP (C, D) using gel electrophoresis analysis and green fluorescence emission under the blue LED illumination (Pf: P. falciparum, Pv: P. vivax, NTC: no template control)

RESULTS AND DISCUSSION

Specificity Test

To demonstrate the precise identification of the human malaria species, two species-specific LAMP master mixes, which are specific for *P. falciparum* (*Pf*-specific LAMP) and *P. vivax (Pv*-specific LAMP), were used. gDNA of *P. falciparum* and *P. vivax* parasites were bench extracted and purified for assay validation. 1 µl of prepared gDNA samples were amplified on the reagent compact disc and real-time monitored using the AnyMDx instrument. For each species-specific LAMP master mix, the gDNA of *P. falciparum, P. vivax* parasites, and no template control (NTC) were tested (Figure 4).

As shown in Figure 4, a sharp increase in RFU level was observed from *P. falciparum* sample against the *Pf*-specific LAMP and *P. vivax* gDNA against the *Pv*-specific LAMP. Whereas, *P. falciparum* gDNA against *Pv*-specific LAMP and *P. vivax* gDNA against the *pf*-specific LAMP showed no increase of RFU values. The results clearly imply that the AnyMDx system can specifically distinguish two different malaria species within 30 minutes.

To confirm if the LAMP amplicons were indeed from the designed target sequence, the amplicons were loaded onto the 2% agarose gel for electrophoresis analysis. In general, positive LAMP amplicons shows a ladder-like pattern with multiple bands due to its inverted-repeat structures [9]. As shown in Figures 5A and 5B, ~200 bp of *P. falciparum* amplicon and ~100 bp of the *P. vivax* amplicon were in accordance with the length of the designed target sequence (213 bp and 127 bp respectively). In addition, we performed the blue LED illumination test in the darkroom to confirm the emission of strong green fluorescence (implying the presence of amplicons) by the naked eyes. As a result, we observed the clear green emission of *P. falciparum* and *P. vivax* amplicons in PCR tube (Figure 5B and 5D).

Sensitivity Test

In order to quantitatively assess the relationship between threshold time and gDNA concentration, we tested purified *P. falciparum* gDNA, which was 10-fold serially diluted with Tris-EDTA buffer. As shown in Figure 6A, the detection threshold times were observed with different concentrations of gDNA. We were able to conclude that the threshold time is inversely proportional to gDNA concentrations (Figure 6A-inset).

After the quantitative validation of the relationship between threshold time and gDNA concentration, we evaluated diagnostic sensitivity of the AnyMDx system for analyzing infected whole blood samples with varying parasitemias of *P. falciparum*. Whole blood, containing different parasitemias of *P. falciparum* (from 2% to 0.00002%, and 2% parasitemia corresponds to ~60,000 parasites/µl), were prepared by diluting fresh human RBCs at 45% hematocrit. Healthy RBCs (hRBC) were used as the negative control. Clear exponential increases were observed in the amplification curves of the *P. falciparum*-infected RBC, whereas the negative controls (hRBC) exhibited no amplification (Figure 6B). The AnyMDx system successfully detected and quantified the *P. falciparum*-infected blood sample with low-level parasitemia (e.g., 0.00002%), which corresponds to a detection limit of ~0.6 parasites/µl. This level of sensitivity is essential for detecting early-stage asymptomatic parasite carriers of low parasite densities (~1 parasite/µl) [5]. In addition, the results can be obtained in less than 40 minutes, including the automated sample preparation steps and nucleic acid amplification process.

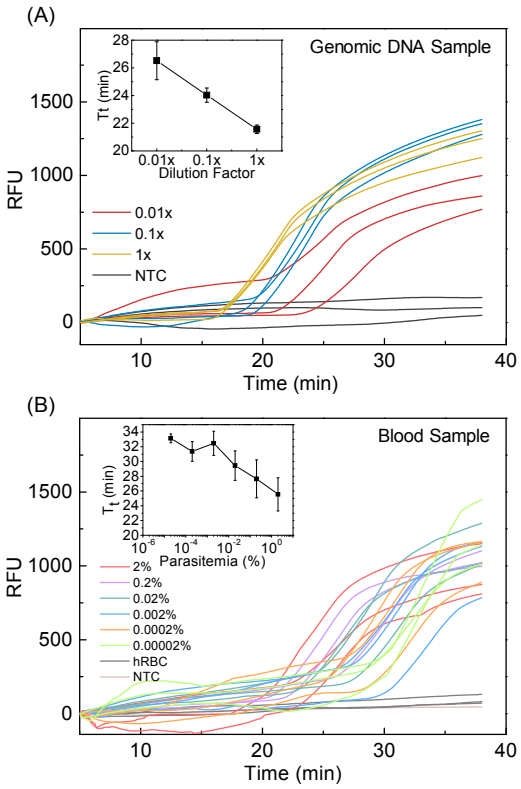

Figure 6: (A) Quantitative validation of the relationship between threshold time (T_t) and P. falciparum gDNA, (B) Sensitivity test for detecting P. falciparum-infected whole blood sample. (NTC: no template control)

We conclude that the AnyMDx system can deliver ultrasensitive and quantitative molecular answers for malaria infections in remote settings without supporting infrastructure.

CONCLUSION

We developed a highly sensitive and specific mobile molecular diagnostic system for rapid and accurate diagnosis of malaria infection in resource-limited areas at the point of need. The standalone and user-friendly system works in an "insert-and-test" fashion and can distinguish two most common malaria species (*P. falciparum* and *P. vivax*) in under 40 minutes. We believe that the AnyMDx system represents a new paradigm for malaria diagnosis in the field settings.

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CONTACT

*W. Guan, tel: +1-814-8675748; wzg111@psu.edu