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Fully 3D Printed Integrated Reactor Array for Point-of-Care Molecular Diagnostics

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Abstract

Molecular diagnostics that involve nucleic acid amplification tests (NAATs) are crucial for prevention and treatment of infectious diseases. In this study, we developed a simple, inexpensive, disposable, fully 3D printed microfluidic reactor array that is capable of carrying out extraction, concentration and isothermal amplification of nucleic acids in variety of body fluids. The method allows rapid molecular diagnostic tests for infectious diseases at point of care. A simple leak-proof polymerization strategy was developed to integrate flow-through nucleic acid isolation membranes into microfluidic devices, yielding a multifunctional diagnostic platform. Static coating technology was adopted to improve the biocompatibility of our 3D printed device. We demonstrated the

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Conflict of interest

The authors declare no competing financial interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version.

suitability of our device for both end-point colorimetric qualitative detection and real-time fluorescence quantitative detection. We applied our diagnostic device to detection of *Plasmodium falciparum* in plasma samples and *Neisseria meningitidis* in cerebrospinal fluid (CSF) samples by loop-mediated, isothermal amplification (LAMP) within 50 minutes. The detection limits were 100 fg for *P. falciparum* and 50 colony-forming unit (CFU) for *N. meningitidis* per reaction, which are comparable to that of benchtop instruments. This rapid and inexpensive 3D printed device has great potential for point-of-care molecular diagnosis of infectious disease in resource-limited settings.

Graphical abstract

Keywords

3D Printing; Microfluidic Chip; Nucleic acid isolation; Fluorescence and Colorimetry; Isothermal Amplification; Point of Care Diagnostics

1. Introduction

Nucleic acid amplification tests (NAATs), often referred to as molecular diagnostics, are based on the detection of DNA or RNA (nucleic acid; NA) from pathogens which inform diagnosis of the presence, progression, and host response to infectious agents (Gubala et al., 2012). NAATs can provide highly sensitive and selective detection of pathogens due to the sequence-specific enzymatic nucleic acid amplification, which enables detection of minute quantities of viral, bacterial or parasite NA (Innis et al., 1988; Yang et al., 2004). Polymerase chain reaction (PCR) is considered the gold standard in molecular diagnostics due to its high sensitivity and specificity, but PCR is burdened by complex thermal cycling mostly confining PCR assays to centralized laboratories (Espy et al., 2006). As an alternative to PCR technology, new generation of isothermal amplification methods like loop mediated isothermal amplification (LAMP) (Njiru 2012), recombinase polymerase amplification (RPA) (Euler et al., 2013) etc., are currently being implemented in clinical and rapid onsite diagnostics due to their simplicity, robustness and capability to alleviate the use of complex thermal cycling operations (Li et al., 2015; Notomi et al., 2000). Recently, there has been considerable interest in combining such simple isothermal amplification methods with microfluidics technology for point-of-care (POC) applications, providing *in vitro* diagnostic capability outside of clinical laboratories (Zhao et al., 2015; Giuffrida et al., 2017).

Conventional microfluidic fabrication methods such as soft lithography, micromachining, embossing and injection molding have proved successful and reliable for molecular diagnostics applications, but they are challenged by rapid prototyping, bulky instrumentation, need for expertise and high cost (Ho et al., 2015; McDonald et al., 2000; Abgrall et al., 2007). The recent emergence of additive manufacturing (i.e., 3D printing) has provided an attractive alternative for rapidly producing complex microfluidic devices for bioanalytical applications (Bishop et al., 2016; Gross et al., 2014). Advancements and accessibility of inexpensive desktop 3D printers that can reach printing resolutions as low as the 25 μm range attracted researchers in many fields like prosthetics, biomedical scaffold printing, biochemical analysis, energy storage devices etc (Bonyar et al., 2010; Schubert et al., 2014). For instance, 3D printing technology has been successfully used to make microfluidic devices for chemical and nanoparticle synthesis (Bishop et al., 2015; Kitson et al., 2013), pathogen bacteria detection (Lee et al., 2015), protein diagnostics (Kadimisetty et al., 2016), blood separation and component evaluation (Chen et al., 2014; Liu et al., 2016), DNA toxicity testing (Kadimisetty et al., 2017), electrochemical sensors (Erkal et al., 2014), cell growth monitoring (Anderson et al., 2013) etc.

Plasmodium falciparum (*P. falciparum*) is a protozoan parasite that can cause severe malaria, one of the main worldwide health threats causing significant mortality and socioeconomic impact (Snow et al., 2005). Malaria is associated with an estimated 429,000 deaths each year and widely distributed in over 109 countries (World Malaria Report 2016, World Health Organization, 2016). Bacterial meningitis caused by *Neisseria meningitides* (*N. meningitidis*) is another deadly infectious disease (Heyderman et al., 2004) with a high mortality rate, causing an estimated 500,000 cases and 50,000 deaths each year (Harrison et al., 2009). Rapid and sensitive diagnostic detection of *P. falciparum* specific gDNA in blood plasma, and *N. meningitidis* bacterial DNA in cerebrospinal fluid (CSF) could markedly facilitate diagnosis and effective treatment, significantly control the mortality rates, and enhance disease surveillance activities (Lee et al., 2015; Guerin et al., 2002).

In this study, we report a miniaturized, inexpensive and multifunctional 3D printed POC diagnostic device that can rapidly extract, pre-concentrate, purify, amplify and detect nucleic acids from complex biological samples such as plasma and CSF. A leak proof polymerization method was developed to embed flow-through isolation membranes into microfluidic devices for nucleic acid extraction and concentration. Static coating technology was used to enhance the biocompatibility of our 3D printed device for nucleic acid-based amplification testing. Use of our 3D printed reactor array has been demonstrated for both end-point colorimetric qualitative detection and real-time fluorescence quantitative detection. Using this device, we detected *P. falciparum* and *N. meningitidis* bacteria from plasma and CSF, respectively, using LAMP assays within 50 minutes.

2. Experimental Section

2.1 Chemicals and Materials

Bovine serum albumin (BSA), Poly (ethylene glycol) 8000 (PEG), poly(vinyl alcohol) (PVA) are from Sigma-Aldrich, RT-PCR grade water from Ambion, Inc., intercalating Eva Green fluorescent dye from Biotium, Isothermal Master Mix used in LAMP reaction buffer

from ISO-001nd, OptiGene, Horsham, UK, WarmStart® colorimetric LAMP 2X master mix from New England Biolabs Inc. *N. meningitidis* (ATCC 13098) from American Type Culture Collection (ATCC, Rockville, MD). *N. meningitidis* was grown in Tryptic Soy Broth (BD, Sparks, MD) and counted on Trypticase™ Soy Agar with 5% Sheep Blood (BD, Sparks, MD).

2.2 3D printed reactor array design and fabrication

We use an inexpensive, high definition Stereolithographic (SLA) laser based Form 2, 3D printer (Form labs) to fabricate our multifunctional molecular diagnostic device. Clear methacrylate based resin (Forms Labs, FLGPCL02) was used for prototyping. Initially a computer aided design (CAD) was created using Autodesk 123D design (.stl format). The files were uploaded to the printer with print resolution of 50 μm and printed. Then, printed parts were removed from the build platform of the printer and immersed into isopropanol (IPA) for 10 min followed by 5-min ultra-sonication to remove any uncured resin in the microfluidic channels. The printed objects were then thoroughly washed with water, and air dried. To improve the transparency of the 3D printed device for optical detection, the surfaces of the devices were coated with heat resistant acrylic spray (Rust-Oleum Clear Gloss Enamel spray, 257884) (Fig. S1).

The 3D printed arrays consist of two part bondable structures (Part I and Part II) that allow integration of flow-through isolation membranes for nucleic acid extraction and purification (Fig. 1 and Fig. S1). Part I and II are designed to fit together *via* simple align guide structures. Our 3D printed array has four separate amplification reactors on a single chip to enable multiple replicate or multiple analyte analysis. If desired, more reactors can be fabricated on a single chip. Each amplification reactor was designed with a pocket that can incorporate Qiagen™ DNeasy blood and tissue (Qiagen 69504) silica membranes (Song et al., 2016). The silica material have been demonstrated to bind nucleic acids at low pH in the presence of high concentrations of chaotropic salts present in the lysis buffer (Boom et al., 1990). Thus, the prepared membrane enclosed part I was aligned, stamped with photopolymer (3D printing resin) and attached with Part II. This integrated microfluidic array was exposed to UV lamp at 410 nm for 5 to 10 min to irreversibly bond and make a robust unibody multifunctional reactor array. 3D printed array in Fig.2A is 24 mm (L) $\times$ 12 mm (W) $\times$ 3.5 mm (H) in dimensions and the internal feature dimensions are 1.5 mm (diameter) $\times$ 1.5 mm (depth) for silica membrane pocket, 1.0 mm in diameter for microchannels and amplification reactor is 9.5 mm (L) $\times$ 1.5 mm (W) and 0.8 mm in height with reactor volume of $16 \pm 1 \mu\text{L}$ (n=3). Inlet and outlet port diameters are 1.2 mm that fit tightly to a 100 μL pipette tip (Sharp barrier tips, Denville scientific Inc.) for performing extraction and purification.

2.3 Surface modification of 3D printed reactor array

Previously NA amplification efficiencies have been shown to be enhanced when inner surfaces of the microfluidic chip were exposed to either static or dynamic coating with PEG (Panaro et al., 2004), polyvinylpyrrolidone (PVP), PVA (Lou et al., 2004), BSA (Erill et al., 2004), or deposition of oxide layers (Zanoli et al., 2012). Static coating on the microfluidic walls of the amplification reactor in Part I was done by adding 2% aqueous solutions of

BSA, PEG and PVA at room temperature. The coating solution was allowed to incubate for 30 min. The coated arrays were dried at 60°C for 5 min after removing the excess solution. Thus, coated Part I of the arrays were incorporated with NA isolation membranes and bonded to Part II *via* photopolymerization for LAMP assays.

2.4 Sample preparation and chip-based assay

Plasma samples were obtained by centrifugation of fresh whole blood followed by spiking with known concentration of *P. falciparum* target genomic DNA (gDNA). The lysate plasma samples were prepared by mixing 20 µL plasma, 20 µL of Qiagen AL lysis buffer and 20 µL ethanol. CSF samples were spiked with known concentration of *Neisseria meningitidis* bacteria or gDNA as required. CSF lysate samples were prepared by mixing, 100 µL of CSF, 20 µL of Proteinase K and 100 µL of AL lysis buffer and incubated at 56°C for 15 min. The resulting lysate was treated with 100 µL of ethanol and loaded into the reactor array for nucleic acid extraction and concentration.

Isolation of nucleic acids inside the 3D printed reactor array was performed by pipetting the sample into the inlet port or using a small suction pump at the outlet port that allows samples to pass through a silica membrane (Fig. 2A), binding the NAs, while the lysed cellular debris flows through. Nucleic acids were isolated and enriched at room temperature in real time while passed through the silica membrane. Binding of NA was followed by two wash steps, ethanol based wash buffers 75 µL of AW1 and 150 µL of AW2 from Qiagen kits to remove any remaining contaminants. The 3D printed reactor arrays were then left to dry for 30 seconds to evaporate residual ethanol by applying a suction pump for 30 seconds.

After the NA isolation and purification was done, the amplification reactor was directly filled with 17 µL of LAMP reaction buffer that contains 5.4 µL PCR grade water, 0.6 µL EvaGreen® fluorescent dye, 0.9 µL target specific LAMP primer mix (See SI, Table S1), 10.2 µL of Optigene® LAMP master mixture. Both inlet and outlet ports were then sealed using PCR Sealers™ tape to avoid evaporation and contamination during the isothermal amplification. Then the 3D printed array was placed on the heated platform of the battery operated portable heater to initiate LAMP amplification (Fig. 2B). For the combination assay (both colorimetric detection and real-time fluorescence analysis), the reaction buffer contained 8.5 µL WarmStart® LAMP master mix, 0.6 µL EvaGreen® dye, 0.9 µL primer mix and 7.1 µL of RT-PCR grade water.

2.5 Detection and data analysis

Isothermal NA amplification and real-time fluorescence detection was performed using our portable homemade processor which combines a battery-operated heater module and a USB fluorescence microscope (AM4113T-GFBW Dino-Lite Premier, AnMo Electronics, Taipei, Taiwan) as previously described (Liu et al., 2014) (Fig. 2C). The heater module is equipped with a polyamide based thin-film heater and precisely maintain a temperature of 65°C for performing LAMP. LAMP amplification was initiated by placing the 3D printed reactor array on the heated platform and fluorescence was detected via USB microscope that is positioned right above the reactor array (Fig. 2B).

Colorimetric detection was scored after 50 min of reaction time when the 3D printed reactor array was taken outside of the heater. Simultaneously the fluorescence signal of the reactor array was real-time monitored by the USB fluorescence microscope. Using MatLab®, the fluorescence intensities were integrated, yielding normalized average fluorescence intensities from all the 4 amplification reactors at every specified time interval (1 minute intervals for 50 min). Normalized fluorescent intensities were plotted against time to obtain real time amplification curves.

3. Results and Discussion

3.1 Rapid prototyping of the 3D printed reactor array

Rapid prototyping of microfluidic devices poses a number of challenges. Traditional fabrication techniques such as microfabrication, injection molding and milling are slow, expensive and labintensive. Here, simple stereolithographic (SLA) based 3D printing technology was used to fabricate microfluidic devices due to its high resolution, low roughness and availability of optically transparent acrylate-based resin. To improve the transparency of our 3D printed reactor array for optical detection, the device's surface was coated with a thin heat resistant acrylic film by spraying (Fig. S1). To incorporate NA isolation membranes into microfluidic devices, polymerization was used to bond the 3D printed devices using a UV-curable 3D printing resin. The nucleic acid yield of the isolation membrane was 62 ± 24 % (n=3), which was comparable to our previous result (Chen et al., 2010). The nucleic acids captured by the membrane were used directly as templates for amplification without elution.

The cost for printing 3D arrays is ~ 0.15 \$, nucleic acid extraction reagents for four amplification reactors cost ~ 0.65\$, assay reagents ~ 0.35\$, leading to total cost per assay of ~ 1.15 \$. Low volume, rapid prototyping and mass production using a desktop printer that can print up to 48 devices simultaneously (5.6 min per array) within 4.5 hours enables cost effective scalability for developing world settings. Multiple amplification reactors fabricated by 3D printing technology enables future adaptation of the platforms for detection of multiple targets concurrently. These inexpensive 3D printed microfluidic devices are designed for single use, due to possible contamination. A detailed comparison of current method with that of other LAMP based nucleic acid detection methods is given in SI, Table S2.

3.2 Biocompatibility of 3D printed device and its surface modification

In general, the biocompatibility of 3D printed devices for biomedical applications, including *in vitro* diagnostic devices, is still not widely established (Macdonald et al., 2016; Lee et al., 2016). Thus, it is crucial to optimize and assess surface modification of our 3D printed device to achieve high-efficiency enzymatic amplification of nucleic acid. We evaluated the performance of the 3D printed array without surface modification in the real-time LAMP assay, plotting normalized fluorescent intensities as a function of time. Threshold time (T_h), time taken to reach 50% saturation value is used to quantify the amplification process. Fig. 3A shows a LAMP experiment with *P. falciparum* gDNA in Phosphate Buffer Saline (PBS) in a 3D printed array without any surface modifications. We tested different concentrations

of *P. falciparum* gDNA spiked in PBS samples ranging from 1000 pg to 0.01 pg per reaction. Results obtained indicate rapid amplification for 1000 to 1 pg per reaction samples within 20 min, with lowest detectable concentration of 1 pg. Although the 3D printed array without any surface coating showed biocompatibility with NA amplification, the sensitivity and detection limits could be improved with surface modification.

Surface modification *via* physical adsorption (static coating) has been explored for improving amplification efficiency and stability by preventing non-specific adsorption of polymerase (Wong et al., 2009; Lou et al., 2004; Erill et al., 2003). We employed static coating of BSA, PEG, PVA to the amplification reactors of the 3D printed array to evaluate their applicability in enhancing the efficiency of the LAMP assay. Fig. 3B (BSA), 3C (PEG) and 3D (PVA) shows amplification results in coated arrays for *P. falciparum* gDNA spiked PBS samples ranging from 1000 pg to 0.01 pg as a function of time. Results suggest that BSA-coated arrays showed similar amplification efficiency and sensitivity compared to non-coated arrays with dynamic ranges extending up to 1 pg. Both PEG-coated and PVA-coated arrays show enhanced sensitivity and efficiency (shorter threshold times) compared to BSA and non-coated arrays with dynamic ranges extending up to 0.1 pg. Decreased adsorption of polymerases and LAMP inhibitors in the presence of PVA/PEG enhanced the efficiency and sensitivity. Our polymer coated 3D arrays showed extremely good reproducibility (n=3) with RSD's ranging from 2 to 8 %. PEG coating was used for the rest of the experiments due to its slightly better efficiency compared to PVA (Fig. 3C and D). Besides improving amplification efficiency and sensitivity, we noticed that static coating improved reproducibility which is essential for reliable molecular diagnostics.

3.3 Colorimetric and fluorescence based detection in the 3D printed reactor array

Excellent optical clarity (Fig. 2A and Fig. S1) and low auto fluorescence (Fig. 4A) of our 3D printed material, made it possible for both colorimetric and fluorescence based detection. To test these methods, we used WarmStart® LAMP master mix, which was designed with a visible pH indicator that turns from pink to yellow color in the presence of amplification products. During NA amplification, DNA polymerase activity results in production of protons, thus lowering the pH allowing colorimetric detection (www.neb.com/warmstart; Toumazou et al., 2013). Four amplification reactors of the 3D printed microfluidic array were loaded with different concentration of either *P.falciparum* or *N. meningitidis* gDNA, reactor A (Negative control), reactors B-D with different concentrations of gDNA. A representative colorimetric and fluorescence based signal for *N. meningitidis* gDNA is as shown in Fig. 4A, no color change and increase in fluorescence intensity were observed for reactor A (Negative control) from 0 minutes to 50 minutes, whereas reactors spiked with gDNA showed color change to yellow and increase in fluorescence intensity within 50 min, confirming the presence of amplicons and successful isothermal amplification.

Real-time amplification curves showing fluorescence intensity increase as a function of time are in Fig. 4B (*P. Falciparum*) and 4C (*N. meningitidis*). In the presence of our combination reaction mix, the fluorescence intensity gradually increased with increase in time for concentrations ranging from 0.1 pg to 1000 pg for *P. falciparum* and 50 to 5000 CFU for *N. meningitidis* gDNA per amplification reaction. Fluorescence saturation levels were reached

faster for higher concentrations of gDNA, Fig. 4B and D, indicated by a decrease in threshold time with increase in concentration. Quantification of the amplification process was summarized in Fig. 4C and E as calibration curves showing log concentration of gDNA added per reaction vs threshold time (T_h) in minutes. Threshold time decreased linearly as a function of increased gDNA added per reaction with good RSD's ranging from 2 to 5% ($n=3$). No false positives were observed in the negative controls. Our results from combination assay in 3D printed device were compared with conventional benchtop LAMP assays and we obtained a similar detection limit of 50 CFU for *N. meningitidis* gDNA (SI, Fig. S2B).

Optigene® LAMP Isothermal Master Mix was used for real time fluorescent analysis for biocompatibility tests, Fig. 3, and WarmStart® master mix with intercalating fluorescent dye was used for the combination assay, Fig. 4, in the amplification reaction buffer. Both buffers have similar sensitivity but amplification efficiency was higher for Optigene® LAMP buffer due to shorter amplification time. Saturation of fluorescence signal for 100 fg per reaction in Optigene® reaction buffer was within 36 min whereas for the WarmStart® master mix is within 45 min, which contributes to the presence of pyrophosphatase in Optigene® LAMP buffer that can improve amplification efficiency and reduce reaction times (Miyamoto et al., 2015). Our benchtop LAMP experiments revealed similar results, SI, Fig. S2.

3.4 *P. falciparum* gDNA detection in plasma and *N. meningitidis* bacteria detection in CSF Samples

To test the suitability of our 3D printed integrated reactor array for point-of-care molecular diagnostics, we detected *P. falciparum* gDNA detection in plasma and *N. meningitidis* bacteria in CSF samples. Plasma was spiked with *P. falciparum* gDNA at 0.5 pg, 5 pg and 50 pg per reaction. As shown in Fig. 5A and B, amplification curves showed specific amplification up to 0.5 pg reaction, reaching a saturation point around 45 min. The higher the target nucleic acid concentration was, the earlier the real-time fluorescence curve increased above the baseline. The calibration curves show typical linear decrease with increase in target concentration with good RSD's ranging from 2 to 12 % ($n=3$). Amplification and calibration curves obtained from combination assays of *N. meningitidis* bacteria in CSF samples at concentration of 50, 500 and 5000 CFU/reaction are as shown in Fig. 5C and D. The detection limit of 50 CFU of *N. meningitidis* from 100 μ L CSF sample with saturation was reached within 40 min with good reproducibility, RSD's ranging from 8 to 12% ($n=3$). Using similar methodology (Song et al., 2016), we performed POC testing in Botswana to evaluate *N. meningitidis* in CSF of 6 patient samples. Out of the evaluated samples 4 were found to be negative and 2 samples were tested positive. These results were confirmed by the standard culture method.

4. Conclusion and outlook

We demonstrated a simple, inexpensive microfluidic molecular diagnostic device fabricated with 3D Printing technology. A photopolymerization approach was developed to hermetically bond the 3D printed device to integrate the NA isolation membranes into reactors and yield a multifunctional diagnostic device. To improve the biocompatibility of

the 3D printed device, we assessed different surface coatings and showed that PEG-coating produced the best results for nucleic acid-based molecular diagnostics.

Our multifunctional 3D printed reactor array has been demonstrated to be suitable for both end-point colorimetric detection (for yes/no detection) and real-time fluorescence detection (for quantitative detection) in complex biological samples by using colorimetric pH indicator and intercalating fluorescent dye, respectively. The low-cost 3D printed reactor array can consistently detect 100 fg/reaction of *P. falciparum* gDNA and 50 CFU/reaction of *N. meningitidis* bacteria in body fluids, respectively, achieving performance on par with benchtop equipment. This methodology can be easily expanded to other biological matrices like saliva, urine, etc. Such simple, inexpensive 3D printed microfluidic devices provide a new avenue for molecular detection at the point of care in resource-limited settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- The first low cost 3D-printed reactor array for nucleic acid amplification tests (NAATs) at the point of care
- A simple leak-proof polymerization method was developed to integrate flow-through nucleic acid isolation membranes into 3D printed devices
- Static coating technology was adopted to improve the biocompatibility of the 3D printed devices
- 3D-printed devices have been demonstrated to be suitable for both end-point colorimetric qualitative detection and real-time fluorescence quantitative detection
- Detection limits were 100 fg for *P. falciparum* in plasma samples and 50 colony-forming unit (CFU) for *N. meningitidis* in cerebrospinal fluid (CSF) samples

Fig. 1. Schematic illustration of assembly of multifunctional 3D printed reactor array for NAATs with leak proof bonding. 3D printed parts are packed with silica membranes followed by stamping of photo polymer (3D printing resin) that acts as bonding agent. The 3D printed parts are aligned and attached together and photo polymerized under UV light to form a unibody 3D printed reactor array.

Fig. 2. Portable heater module with USB microscope and heat platform for 3D printed array to process LAMP amplification. A) Finished 3D printed array integrated NA isolation silica membrane. B) 3D printed array on a thin film heater inside the heater module. C) Portable heating and imaging platform for real-time fluorescence analysis.

Fig.3.

LAMP amplification curves of serial dilutions of *P. falciparum* gDNA in PBS samples with or without static coating in the 3D printed amplification reactors. A) no coating, B) BSA coating, C) PEG coating and D) PVA coating (n=3). Optigene® LAMP Isothermal Master Mix was used. Note: rxn = reaction

Fig. 4. Colorimetric and fluorescence based detection for NAATs in 3D printed reactor array. A) Representative photographs of colorimetric LAMP assay for detection of *N. meningitidis* with 0, 50, 500 and 5000 CFU/reaction on the same chip, alongside LAMP fluorescence based image at given time interval. B) LAMP amplification curves for *P. falciparum* with 0, 0.1, 1, 10, 100, 1000 pg per reaction. C) Calibration curve for *P. falciparum* as function of log target concentration, $n=3$. D) LAMP amplification curves for *N. meningitidis* with 0, 50,

500, 5000 CFU per reaction. E) Calibration curve for *N. meningitidis* as function of log target concentration, n=3. WarmStart® LAMP master mix was used.

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Fig. 5.

A and B) LAMP amplification curves and calibration curve for quantitative detection of *P. falciparum* gDNA in plasma samples, spiked concentrations of 0, 0.5, 5 and 50 pg/reaction. C and D) LAMP amplification curves and calibration curve for quantitative detection of *N. meningitidis* bacteria in CSF samples at concentration of 50, 500 and 5000 CFU/reaction, $n=3$. WarmStart® LAMP master mix was used.