Solar-thermal complex sample processing for nucleic acid based diagnostics in limited resource settings

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Abstract: The use of point-of-care (POC) devices in limited resource settings where access to commonly used infrastructure, such as water and electricity, can be restricted represents simultaneously one of the best application fits for POC systems as well as one of the most challenging places to deploy them. Of the many challenges involved in these systems, the preparation and processing of complex samples like stool, vomit, and biopsies are particularly difficult due to the high number and varied nature of mechanical and chemical interferents present in the sample. Previously we have demonstrated the ability to use solar-thermal energy to perform PCR based nucleic acid amplifications. In this work demonstrate how the technique, using similar infrastructure, can also be used to perform solar-thermal based sample processing system for extracting and isolating Vibrio Cholerae nucleic acids from fecal samples. The use of opto-thermal energy enables the use of sunlight to drive thermal lysing reactions in large volumes without the need for external electrical power. Using the system demonstrate the ability to reach a 95°C threshold in less than 5 minutes and maintain a stable sample temperature of +/- 2°C following the ramp up. The system is demonstrated to provide linear results between 10^4 and 10^8 CFU/mL when the released nucleic acids were quantified via traditional means. Additionally, we couple the sample processing unit with our previously demonstrated solar-thermal PCR and tablet based detection system to demonstrate very low power sample-in-answer-out detection.

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1. Introduction

Point-of-care (POC) or point-of-need devices provide the ability to carry out personalized, typically molecular, diagnostics at a site that is more convenient for the patient than a traditional clinical setting. Beyond being able to provide diagnostic accuracy that is at least comparable to traditional laboratory tests, the added constraints on POC tests is that they must be: packaged in such a way that they are self-contained, able to return results in a time period consistent with that which the patient is willing to wait, and operable with as little reliance on external infrastructure as possible [1–3]. The latter of these is particularly important in limited resource settings where reliable electricity, clean water, and refrigeration may not be available. Nucleic acid based detection methods, such as polymerase chain reaction (PCR), have been extensively developed for the detection of genetic markers of diseases with high sensitivity and specificity [4, 5]. This accuracy along with progress in the miniaturization and integration of the sample processing, amplification, and detection into a single package makes PCR based systems attractive platforms for POC testing [6, 7].

In all cases POC nucleic acid based testing requires upstream sample processing and usually purification. This is considered one of the most critical steps in successful system level development. This is largely due to high concentrations of interfering species (e.g. proteins, enzymes) which can be a problem both during nucleic acid amplification and/or at the detection step. This is particularly problematic for complex sample media, such as stool, vomit, or human biopsies, which have a very wide variety of chemical and mechanical interferents [8]. While laboratory procedures for processing these samples are well established, integrating the steps into a relatively simple package can be difficult, particularly with the additional constraints of limited resource settings. As such, most methods still rely on some level of sample preparation steps consisting of centrifugation and reagent refrigeration [9, 10]. Significant works have been done for the integration of all the analytical steps such as lysis, DNA extraction and purification on a single device [11–15], but sample preparation in the field from complex samples such as stool has yet to be investigated sufficiently.

*Vibrio Cholerae* is a comma-shaped, gram negative bacterium which is the cause of an acute diarrheal disease in humans commonly referred to as cholera [16–18]. Infection can be caused by ingestion of food or water contaminated with the cholera bacterium, and if left untreated may cause death through extreme dehydration and electrolyte imbalance. There are an estimated 3-5 million cholera cases worldwide every year and 100,000-120,000 result in deaths [19]. Cholera has a short incubation period of two hours to five days, sometimes causing rapid outbreaks of the disease [19] and increases the need for a rapid diagnostic for Cholera. Even though up to 80% of the cases can be successfully treated with oral rehydration salts [19], the high death rates indicate that early and rapid detection of the cholera is necessary to prevent spread of disease and to decrease the intensity of epidemics. Traditional methods to identify *V. Cholerae* involving culture, biochemical, and immunological assays are time-consuming and laborious [16, 20]. There are commercially available rapid detection tests such as the SMART™ test [21–24] and the Crystal VC® dipstick test [24–27]. These tests however have been reported to have sub-optimal field performance (as opposed to in-lab testing) resulting from: relatively low clinical sensitivity and specificity, high number of indeterminates, and variations in performance depending on the skill level of the user [24, 25].

In this paper, we present a solar-thermal sample processing system useful for processing stool samples at the point-of-need and demonstrate its usefulness in the nucleic acid based detection of *Vibrio Cholerae* nucleic acids. Previously, we have demonstrated the ability to use a simple lens and shadow mask to perform nucleic acid amplification via PCR [28]. This represented a low infrastructure and low energy method for performing molecularly specific
detection. Our goal here is to show that the same infrastructure can also be used to perform the upstream sample processing. As shown in Fig. 1, the sample processing system comprises of a solar-thermal DNA extraction method using a solar-incubator to thermally lyse the bacteria and to extract the nucleic acids. We have also integrated ChargeSwitch® magnetic microparticle-based technology to efficiently isolate the DNA following extraction. While our focus here is on demonstrating the sample processing methodology, to demonstrate complete sample-in-answer-out compatibility we have also integrated the system with our previously published solar-thermal PCR [28] and tablet based detection system enabling end-to-end diagnostics with extremely low energy usage.

Fig. 1. Solar-thermal sample processing system, components and operation. (a) Packaged solar-thermal incubator b) Polycarbonate block mounted with micro-centrifuge tube c) Solar-incubator cartridge that fits into the interchangeable cartridge area of the system. d) Overview of the system components.

2. Experimental

2.1 Solar-incubator design and fabrication

The solar-incubator was manufactured at Cornell University’s Laboratory of Atomic and Solid State Physics (LASSP). Black polycarbonate thermoplastic material was used to fabricate the solar-incubator as it has strong broadband optical absorptivity and a high melting temperature (155 °C). A 6 mm hole was drilled at the top of the solar incubator in order to mount a thermocouple which enabled tracking of the incubator’s temperature. The temperature distribution of the solar incubator was simulated using a three dimensional heat transfer model in COMSOL Multiphysics. The heat transfer model includes both the black polycarbonate absorption layer, and polypropylene microcentrifuge tube with water inside. Conductive heat transfer equations were used across all domains with appropriate conductive/convective boundary conditions. Characterization experiments were conducted with a solar-simulating apparatus consisting of a 100 W white light emitting diode (LED) lamp and lens, a cooling fan, an aluminum heat sink and 2 extra light collimation lenses which we have previously validated in Jiang et al. [28]

2.2 Solar thermal PCR and smartphone fluorescent detection

Detailed procedures for the solar-thermal PCR were previously reported in Jiang et al. [28] Briefly, a 75 mm diameter glass lens focuses light to the solar-PCR chip and the light is converted into heat by an absorption layer consisting of carbon black and polydimethylsiloxane (PDMS). Through a ring-shaped mask, three temperature zones at 95 °C (denaturation), 72 °C (extension) and 60 °C (annealing) are created along the radius of the
chip. A micropump is used to carry the sample through microfluidic channels that loop through these three temperature zones for 35 cycles at a rate of 1 µL/s. Three thermocouples are inserted into the solar-PCR chips to measure the on-chip temperatures throughout the test by a custom smartphone app. By changing the distance between lens and microfluidic chip, the desired temperature profile can be maintained, even when encountering differing levels of cloud cover and ambient temperature.

Fluorescence detection was conducted using a separate PDMS chip containing 4 chambers that each hold 20 µL volumes of sample which was illuminated using a 3.4 W blue LED (Sparkfun Electronics) further filtered through a blue filter (Thor Labs) to reduce lights at undesired wavelengths (see Jiang et al. [28] for further details). Each sample includes SYBR Green dye, which bind to double stranded DNA and emits in the green light range. An image of the excited samples is taken in the dark through the green filter using smartphone (Samsung, Galaxy Note 3) which was then analyzed using a smartphone app to plot the fluorescent signal intensity across each sample. Peaks in the fluorescent intensity correspond to amounts of cholera toxin DNA (ctxB) in the sample, enabling POC detection using a smartphone or tablet camera.

2.3 Stool sample collection and spiked stool sample preparation

Fresh canine stool samples were collected from College of Veterinary Medicine, Cornell University, and stored at 4 °C when not in use in order to minimize bacterial growth. After diluting the stool samples four times with 1X phosphate buffered saline (PBS) solution (Sigma Aldrich), they were spiked with fresh Escherichia coli (E. coli) HM109 bacterial culture which were previously transformed with pIDTSMART-AMP:ctxB plasmid (low copy number plasmid with complete ctxB gene, Table 1). Bacteria numbers were determined with a spectrophotometer (Spectramax 384, Molecular Devices LLC). The spiked stool samples were then prepared by adding known concentrations of ctxB transformed E. coli bacteria to the original stool samples. Spiked stool samples were used for the experiments directly.

| Name | Sequence |
|------|----------|
| ctxB probe 1 (24 bp) | 5’-CTGATTTTGTGCAGAATACCACA-3’ |
| ctxB probe 2 (24 bp) | 5’- CGCATGAGGGCTTTTATTATCCAA-3’ |
| ctxB target (269 bp) | CTGATTTTGTGCAGAATACCACAACACACAATACATACACGC TAAATGATAAGATATTTTTGCTACAGAATCTCTAGCTGAA AAAAGAGATGGGCTTATTATTACGTAAATGATAAGTCACA AAAAGGCTTAGAAGGTAGGAGGATACCTGAGGATTGC TATCTTTAAGGCTAAGGCTCGAAAATTTATGTGTAGGAAT AATAAAAAGGCTCATGCG |

2.4 Solar-thermal lysing of bacteria and DNA isolation using magnetic microbeads

To achieve bacterial lysing, 150 µL of spiked stool sample was transferred into 0.2 mL microcentrifuge tube and thermally lysed for 10 minutes at around 95 °C using the solar-incubator. Bacterial DNA was then isolated using the magnetic microbeads of the ChargeSwitch® gDNA Mini Bacteria Kit which uses magnetic microbead-based technology to provide rapid and efficient isolation of DNA. Magnetic microbeads are coated with an ionizable functional group whose affinity for nucleic acids is pH dependent, helping to facilitate nucleic acid isolation without the need for hazardous chemicals, centrifugation, or vacuum manifolds. Lysed stool samples are mixed with 15 µL magnetic microbeads with binding solution (pH < 6.0) to bind the DNA to magnetic microbeads. After incubating at room temperature for 1 min, beads were collected with a small magnet for 1 min until the beads formed a tight pellet. After removing the supernatant, 200 µL of wash buffer was added, (pH = 7.0) and mixed gently. Beads were again collected with the magnet for 1 minute, and the supernatant was removed. To be able to elute the DNA from beads, 80 µL of eluting buffer (pH = 8.5) was added and mixed gently. To increase the elution efficiency, the
solution was incubated at 60 °C for 5 minutes using solar-incubator. Then beads were collected with the magnet and supernatant containing DNA was transferred to a clean microcentrifuge tube for PCR amplification reactions. For characterization experiments, a standard gel electrophoresis system (Flash Gel System, Lonza) was used to analyze the amplified DNA.

2.5 ctxB target selection and primer design

Within the cholera toxin genome, part of the DNA that codes for ctxB was chosen as our target DNA. Specific primers for this sequence were designed using BLAST Primer Design (results shown in Table 1), and ordered from Integrated DNA Technologies Inc. (Coralville, IA).

2.6 PCR sample preparation and amplification

23 µL volume of DI water containing 4.3% w/v polyvinylpyrrolidone (PVP) (Sigma-Aldrich) is mixed with illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare) which is provided as room temperature stable dried beads that contain all the necessary reagents, except primers, for performing PCR analysis. When characterizing the DNA extraction and isolation performance of the solar incubator system exclusively, a standard thermal cycler was used.

Real-time PCR reactions were performed using QuantiNova SYBR Green PCR Kit (QIAGEN, Valencia, CA). All PCR reactions were performed in duplicate in a volume of 20 µl containing SYBR Green master mix, primers ctxB-F and ctxB-R and 1 µl of DNA template. Reaction condition was as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 58°C for 30 s. Fluorescence was measured at each 58°C step. PCR cycling was performed on an ABI Prism Sequence Detection System 7000 (Applied Biosystems, Foster City, CA, USA). Data were analyzed with the Sequence Detection Software 1.2.1 (Applied Biosystems, Foster City, CA, USA).

3. Results and discussion

3.1 Thermal characterization of solar-incubator

Sunlight has the opportunity to be used in novel optofluidic applications ranging from energy [29] and global health [30] largely due to its distributed nature as a ubiquitous power source and ease with which it can be converted to arbitrary spatial heat or illumination patterns. As noted above, the aim of this work has been to develop a system which uses solar-thermal heating during the sample preparation step to extract and isolate nucleic acids from fecal samples. Figure 1 shows an overview of the integrated system including details of the packing and system operation. Screenshots of the smartphone app which is used to operate and monitor the system during heating is presented in Fig. 2(a)-2(b) and a general overview of the operational procedure is provided in Fig. 2(c). Briefly, light is focused through the lens onto the polycarbonate holder which serves as the target optical absorber converting the incident light into heat. Optimization of the temperature is done manually by focusing or defocusing the upper lenses through adjusting the distance between it and the target. Mounts and cartridges were developed for both 0.2 mL or 0.5 mL microcentrifuge tubes (See Fig. 1(b)-1(c)) and are inserted into the incubator as shown in Fig. 1(d).
Fig. 2. (a,b) Screenshots from mobile app used to monitor system temperature and other operational controls. Connectivity to packaged system is via Bluetooth. (c) Overview of experimental and operational stages.

As shown in Fig. 3, the temperature of the liquid inside the tube generally reaches 95 °C within about 5 minutes for both 0.2mL, Fig. 3(a), and 0.5mL, Fig. 3(b), tubes. All experiments were conducted as outlined in section 2.1. As expected the polycarbonate material heating was faster than that of the liquid as a result of the time required for the heat to diffuse from the absorber to the tube. Generally, after a few minutes the system came to a stable system temperature enabling us to correlate the temperature of the incubator to that in the tube.

Finite element simulations were conducted using COMSOL Multiphysics in order to ensure that the thermal conditions within the sample tube were sufficiently uniform and that temperature variations within the sample were minimized. Standard steady-state diffusive heat transfer equations and boundary conditions were used within the computational domain which comprised of three different material regions. Inside the tube, it was assumed that the fluid was pure water (as the sample itself is relatively dilute within the buffer and does not have appreciably different heat transfer characteristics than water). The tube itself and the holder were simulated as polypropylene and polycarbonate respectively (matching the actual construction materials). The holder dimensions were 19 mm in length and width and 6.1mm in depth, with a fitting where half of the tube could fit. The tube was assumed to have a diameter of 6.5 mm at the top with a wall thickness of 0.30 mm. The tube was cylindrical at the top with a height of 8.5 mm, and then cylindrical at the bottom with a height of 11.5 mm that narrowed into 3mm diameter edge. We assumed that there was an intensity of 1038 W/m² shining from the bottom layer which was completely absorbed by the poly-carbonate within a layer thickness of 100 μm. As can be seen in Fig. 4 the temperature variation within the tube itself was less than ± 2°C. The variation along the depth of the system could be more significant, but still sufficiently consistent to enable correlation between the thermocouple measurement point and the tube.
Fig. 3. Experimental thermal characterization of the solar-incubator. Water temperature in the micro-centrifuge tube closely follows that of the polycarbonate substrate temperature, reaching near equivalence after about 5 minutes. Temperature ramp-up time and steady state value can be adjusted by changing lens distance. (a) 0.2 mL tube system (b) 0.5 mL tube system.

Fig. 4. Finite element simulations of solar-incubator elements. Polycarbonate material distributes and delivers the heat absorbed from the light and allows the liquid inside the micro-centrifuge tube to heat up to desired lysing temperatures Temperature distribution (a) with tube and (b) without tube. Temperature profiles at (c) horizontal and (d) vertical cross sections.

3.2 DNA extraction and isolation using solar-incubator and magnetic microparticles

Bacterial lysing is the first step for DNA analysis and involves releasing genomic materials and other cellular content by disassembly of the cellular membrane. A number of different lysing methods have been successfully demonstrated for POC applications such as chemical lysis, thermal lysis, and lysing by mechanical forces or electrical pulses [31]. The advantage of thermal lysing is that no extra lysing reagents are needed that may interfere with downstream reactions or require refrigeration prior to use. Thermal lysing has been previously been incorporated into microfluidic devices to perform on-chip lysis [32, 33].
Other works have also demonstrated follow on nucleic acid extraction capability which helps to isolate the nucleic acid from other cell components [34, 35].

Isolation of nucleic acids is an important step since non-isolated samples may contain inhibitor substances that can degrade DNA and inhibit downstream enzymatic reactions such as PCR or other target amplification processes [36]. Isolation can also help with target enrichment and increase the nucleic acid concentrations for sensitive detection applications [7]. DNA extraction and isolation in the same device for complex samples like stool remains a challenge for POC devices. Recently researchers have investigated minimal preparation needs for stool samples and their effect on cholera toxin detection results using microfluidic immunosensor [10]. However, sample preparation consisted of centrifugation or vacuum manifolds would not be appropriate for POC diagnostic applications where laboratory-based equipment is not available. Paper-based microfluidic devices have also been receiving increased attention for their ability to extract nucleic acids from clinical samples for sample-to-answer molecular diagnostic platforms for POC diagnostics [15, 37].

Our complete system has four main components: First, a solar-incubator for lysing the bacteria thermally by using sunlight and extracting DNA out of the cell for further genetic analysis; second, a magnetic microparticle-based DNA isolation which uses ChargeSwitch® technology to provide rapid and efficient purification of DNA; third, solar-PCR based DNA amplification which uses sunlight to realize PCR reaction; and finally, smartphone integration to the system for temperature measurements of the solar-incubator and solar-PCR devices, pump controls for the microfluidics required in the solar-PCR steps, and for result analysis when DNA concentration is determined by green fluorescence intensity.

Figure 5 shows the results of testing on different variations of the sample processing steps described above (steps 1 and 2) for ctxB amplification from stool samples. All results were validated using standard gel electrophoresis as described in the methods section of this paper. As can be seen, the combination of thermal lysing for 10 minutes at 95°C combined with magnetic bead based DNA extraction at 60°C for 5 minutes yielded successful amplification at both concentration levels. In all cases no amplification was possible when either the thermal lysing or extraction or isolation steps was not used, showing that these steps are essential to remove inhibitor substances found in the complex matrix of stool samples.

Fig. 5. ctxB PCR amplification from stool samples. No amplification occurred when either lysing or isolation steps were not used, indicating the importance of both steps in removing remove inhibitor substances found in the complex matrix of stool samples.
Fig. 6. Sensitivity of the system. Different concentrations of ctxB are extracted and isolated from spiked stool samples in the range of 10^4 to 10^8 CFU/mL. (a) Gel electrophoresis results and (b) Threshold cycle real-time PCR results showing quantification.

Figure 6(a) shows the variation in output fluorescence intensity following solar lysing and extraction steps as a function of the original concentration of organisms in the stool samples. As can be seen, good amplification was obtained over the range of tested concentration 10^4 to 10^8 CFU/mL with the amplification signal decreasing with concentration as expected. Real-time PCR was used for the quantification of ctxB positive E. coli that had been introduced into fecal samples. To do this fecal samples were spiked with 10^8, 10^7, 10^6, 10^5, 10^4 pure cultured cells. Collection of bacteria, lysis of the cells and isolation of DNA were performed as described in the methods section. Figure 6(b) shows a dissociation curve analysis that was performed at the end of the qPCR cycle. The linearity of the result shows the quantitative capability of the system within the range tested. We limited our low end concentration experiments here to 10^4 CFU/mL however we believe that both the linearity of the response and detection limit could be extend well beyond this range.

3.3 Solar thermal PCR and fluorescent measurements with smartphone app

As a final demonstration of the sample-in-answer-out capabilities of the system, we integrated the solar incubator technology presented here with our solar thermal PCR system which we previously demonstrated for the detection of nucleic acids associated with Kaposi Sarcoma [28]. As shown in Fig. 1(d), the solar incubator is largely interchangeable with the solar-PCR system requiring only the use of a different cartridge at the heating point and removal of the shadow mask. For this experiment, three samples were run through the combined system, inserted into the reader chip, and read vial fluorescence signal. Figure 7(a) shows an image of the experimental setup and detection chip. Readers are referred to the experimental section for further details. The three samples were positive (10^8 CFU/mL) and negative ctxB samples extracted from stool and a traditional PCR using negative control. As can be seen in Fig. 7(b) the smartphone app analyses the fluorescence image and compares the results against a previously determined threshold (the dashed line in Fig. 7(b)). This threshold was determined.
in Jiang et al. [28] and was based on obtaining optimal discrimination between positive and negative cases. The system predicted the correct result in all three cases.

![Fluorescent measurement setup](image)

**Fig. 7.** (a) Fluorescent measurement setup. Samples excited with a blue light and image is taken in the dark through the green filter using smartphone. (b) Smartphone app analyzes the fluorescent signal intensity across each sample where peaks correspond to the amount of ctxB in the sample. The system predicted the correct result in all three cases where positive (108 CFU/mL) (+) and negative (-) ctxB samples extracted from stool and a traditional PCR using negative control (NC). Inset shows the visual image of the resulted picture where distribution of the color in the image was digitally enhanced.

4. Conclusions and perspectives

In this work, we have developed a solar-thermal sample preparation system for point-of-care diagnostics and demonstrated its usefulness by detecting nucleic acid from *Vibrio Cholerae* that had been previously transformed into *E. coli* cells from raw stool samples. We demonstrated the ability to use light energy to perform thermal lysing of the bacteria, requiring only a few minutes to heat the system up to 95 °C, and the use of magnetic microbeads to isolate and remove the extracted nucleic acids. In addition to quantifying and validating the sample processing technique via traditional PCR means, we also demonstrated the compatibility of the system with our previously published solar-PCR system and amplification analysis using our smartphone application. When combined, these systems give us the ability to do rapid sample-in-answer-out in-field molecular diagnosis of cholera without the need for extensive laboratory equipment, chemicals or a dedicated power supply.

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