

A Point-of-Care Instrument for Rapid Multiplexed Pathogen Genotyping

Frank B. Myers, Richard H. Henrikson, Liyi Xu, and Luke P. Lee

Abstract—We are leveraging recent advances in rapid nucleic acid amplification chemistries, self-powered microfluidics, and low-cost optoelectronics to develop instrumentation for pathogen genotyping in the developing world. A growing number of correlations are emerging between genetic mutations in pathogens and their infectivity, origin, and drug resistance. Particularly for diseases like tuberculosis, where multi-drug resistance is a growing concern, a rapid diagnostic which could inform prescription decisions for newly diagnosed patients would not only save lives and reduce prolonged sickness but would help slow the emergence of more virulent strains. Additionally, for pathogens such as HIV, there is a need for new assay formats which can inexpensively and quantitatively monitor pathogen load. We have developed a portable instrument which uses disposable microfluidic assay cartridges pre-loaded with lyophilized reagents for genetic amplification of multiple markers. The cartridges can be adapted for a variety of sample types (blood, sputum, saliva). The instrument controls assay temperature and quantitatively monitors real-time fluorescence signals from 96 individual reaction chambers. The platform can be tailored for different economic situations—from a quantitative electronic readout to a simple binary readout with the naked eye.

Keywords—point-of-care diagnostics, nucleic acids, genotyping, polymerase chain reaction, loop-mediated isothermal amplification, fluorescence, microfluidics, phototransistors, microcontroller

I. INTRODUCTION

THERE is great interest in developing rapid, inexpensive devices for infectious disease diagnosis in remote and resource-limited settings [1]. Beyond their value for clinical discovery and management, diagnostics serve an important role in disease surveillance and intervention [2].

In particular, tuberculosis (TB) has been identified as one of the most cost-effective and high-impact diseases for targeted intervention [3]. One third of the world's population has latent TB, with 8.8 million new cases of the disease and 2 million deaths annually [4]. These numbers are dramatically skewed towards the developing world, with TB now accounting for the majority of HIV-related deaths. It is thus extremely important to avoid wasting precious resources and medicines on misdiagnosis and retreatment,

yet sputum smear microscopy (the most common diagnostic technique) achieves just 40-60% sensitivity in field conditions, with this value dropping to 20% in cases of co-infection with HIV [4]. Furthermore, an assay capable of genotyping TB cases for drug resistance classification would have a significant impact on clinical outcomes overall [5].

Although developed economies have access to more accurate molecular diagnostics, including polymerase chain reaction (PCR) tests, they have yet to be translated into diagnostics that match the specific needs and requirements of point-of-care testing. Lateral flow immunoassays have made a significant impact in the diagnosis of diseases like HIV, but it is difficult to adapt this assay format to situations where a quantitative readout or molecular amplification is required. Recent efforts have focused on automating

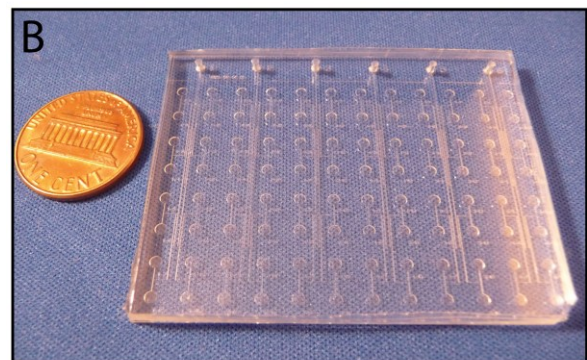
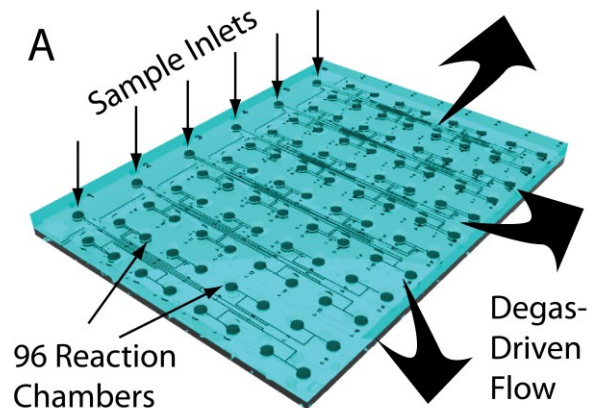


Fig. 1. Self-powered disposable microfluidic chip which contains preloaded lyophilized reagents for nucleic acid amplification. The chip is packaged in a vacuum sealed bag and once this bag is opened, the residual vacuum in the PDMS draws sample fluid into all of the reaction chambers and hydrates the reaction mix.

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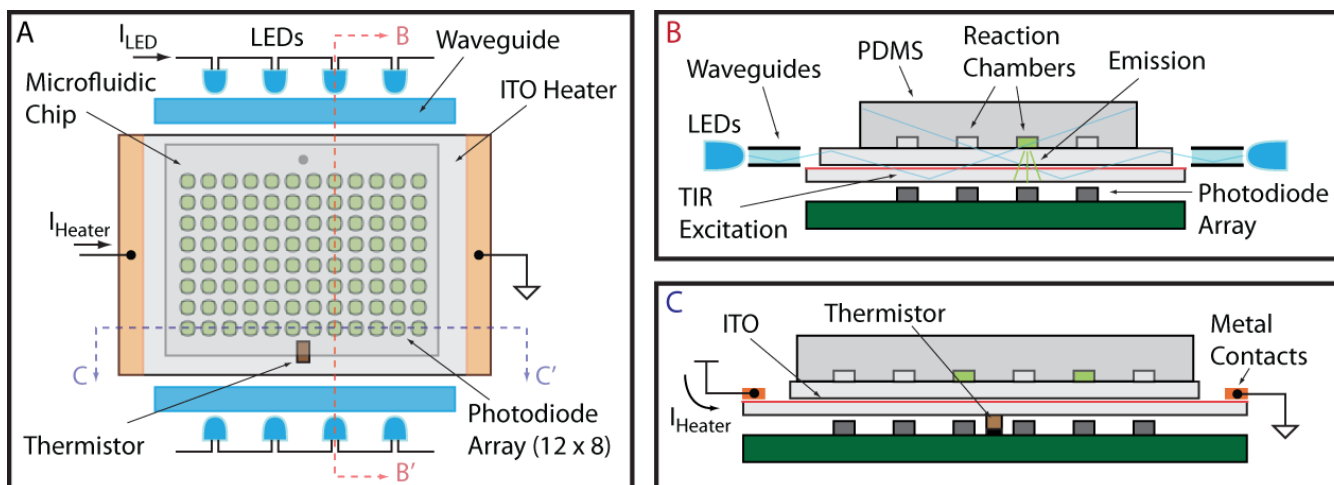


Fig. 2. (A) Top view of chip on top of instrument. Two rows of blue LEDs illuminate the chip from the side through a rectangular waveguide which ensures total internal reflection within the chip. The chip sits on top of a transparent heating element consisting of an ITO coated glass slide whose temperature is regulated with a thermistor epoxied underneath. Cross-sectioning lines indicate the views shown in (B) and (C). (B) Illumination and fluorescence detection scheme. (C) Temperature control scheme.

standard sample preparation and PCR techniques; however the significant assay cost and relatively low throughput remain prohibitive barriers for wide-scale adoption [6].

Recently, several methods have been developed to enable isothermal amplification, facilitating reduced power consumption and expanded options for material and chemical selection [7]. Furthermore, these methods offer improved performance over standard PCR, achieving highly sensitive and specific results in less than 45 minutes.

Loop-mediated isothermal amplification (LAMP) has been adapted for a range of assays, including infectious pathogen detection [8] and chronic disease typing [9]. There has recently been interest in leveraging this assay in a miniaturized format, however a complete integrated system for multiplexed sample analysis has yet to be adopted.

Fluorescent analysis offers a number of advantages over competing optical or electrochemical detection methods, however implementing sensitive fluorescence detection inexpensively remains a challenge [10].

II. DISPOSABLE MICROFLUIDIC DEVICE

We have developed a disposable microfluidic device featuring passive fluid actuation and sample preparation based on standard soft lithography techniques [11]. The chip measures 55 x 42 x 5 mm and consists of 96 parallel reaction chambers serviced by 6 separate sample inlets for multiplexed analysis. Chamber dimensions have been designed to meet sensitivity requirements for standard clinical assays.

The chambers are cast in polydimethylsiloxane (PDMS), and subsequently bonded to a glass slide after spotting and lyophilizing customized reaction mixes. The device is exposed to a vacuum of ~300 mTorr for >1 hour, and then vacuum sealed using a commercial food sealer. Due to the elastomeric pore structure of the PDMS, the chip will begin drawing in sample introduced into the inlets immediately upon exposure to atmospheric pressure. Samples can be completely loaded in less than 10 minutes.

Each chamber is preloaded with a lyophilized reaction mix containing custom reagents and primers optimized for both visual readout and optoelectronic quantification. Although we have used the LAMP method in the design of these primer sets, this chip is compatible with PCR, as well as other isothermal amplification techniques such as nucleic acid sequence-based amplification (NASBA) and recombinase polymerase amplification (RPA).

We have adapted the calcein metal indicator fluorophore as a tool for both real-time fluorescence measurement and naked-eye readout of signal based on initial target presence. Briefly, this method involves the use of a calcein dye that is initially quenched by manganese ions. As a byproduct of nucleic acid amplification, pyrophosphate groups are produced in abundance and readily precipitate out of solution as manganese pyrophosphate, removing the quencher from calcein and yielding a bright fluorescent signal [12].

III. INSTRUMENTATION

For quantitative readout of the assay, the chip is inserted into a battery-powered instrument which maintains assay temperature, illuminates the chip, and detects fluorescence emission from the reaction chambers using an array of phototransistors. It does this without the use of costly optical components, and without the need for alignment or focusing. The instrument is automated with an Arduino microcontroller board based on the Atmel Atmega2560 microcontroller. The instrument features a USB interface, a Secure Digital (SD) Flash memory card reader for storing assay parameters and results, and a 4.3 inch color touchscreen user interface (Amulet Technologies).

To run an assay, the microfluidic chip is inserted directly on top of a 47 x 67 mm indium tin oxide (ITO) coated glass slide which heats the chip to 60° C. The ITO is connected to a TPS61085 boost converter which steps up the voltage of the batteries to 12V. The boost circuit is capable of delivering over 1 A through the slide, and within one minute the slide reaches its set point temperature. The temperature is maintained via pulse-width modulation (PWM) of the boost

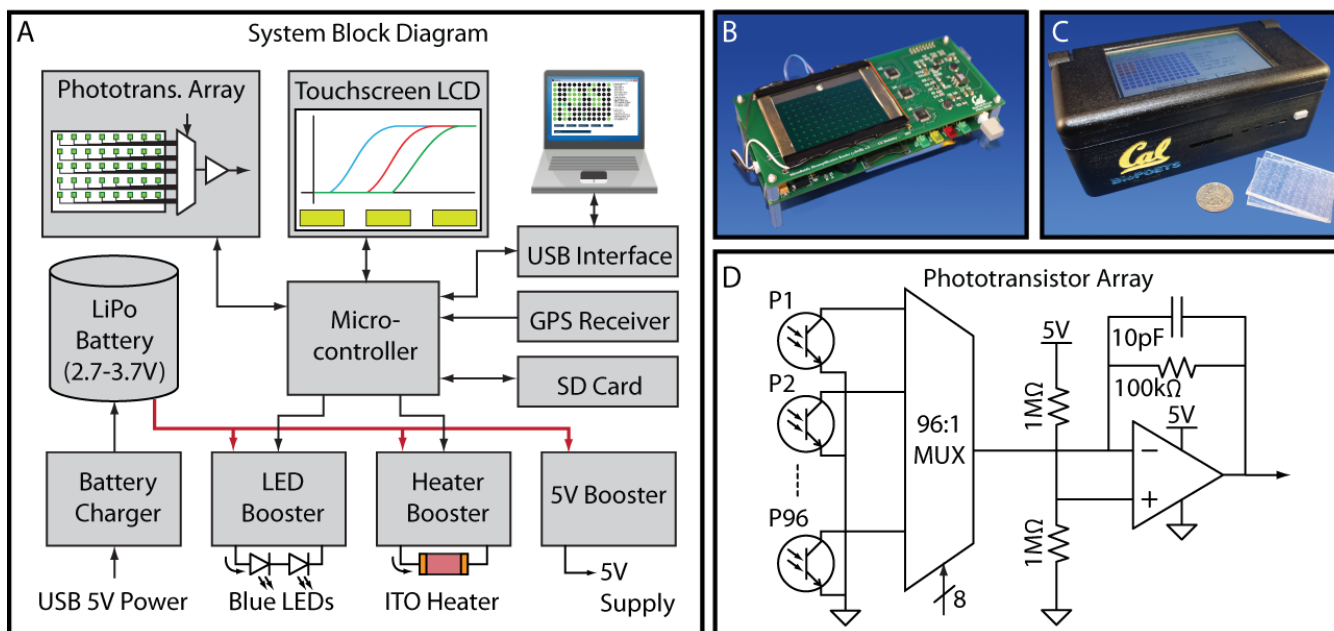


Fig. 3. (A) Block diagram showing all system components. The device is battery powered with a lithium polymer battery and a microcontroller automates LED/heater control and phototransistor measurements. In applications where it is cost effective, a touchscreen LCD can be included for assay setup and readout. Additionally, an optional GPS module geotags assay results, which can provide useful epidemiological information for field-based diagnostics. A USB interface allows both charging of the battery and device control with a PC. Assay results are stored on an SD card. (B) Prototype system consisting of two stacked PCBs with the microcontroller, SD card reader, USB interface, phototransistor array, LED/heater driver, and transimpedance amplifier circuitry, and lithium polymer battery. (C) Completed prototype in 3D printed enclosure with a hinged lid shown here along with two disposable assay cartridges and a US quarter for size comparison. An LCD module in the lid allows assay operation without a PC. (D) Multiplexed transimpedance photocurrent amplifier.

circuit. A thermistor in a half-bridge circuit configuration monitors assay temperature. For LAMP and other isothermal reactions, the temperature is held constant, whereas for PCR it can be cycled between two set points.

Six blue InGaN LEDs (peak = 472 nm) illuminate the chip from its sides through glass waveguides clad with black paint to minimize stray light. The waveguides promote total internal reflection (TIR) of excitation light within the chip. The refractive index of glass, PDMS, and water are similar enough that reflections of excitation light off of internal surfaces of the chip do not significantly contribute to background signal on the phototransistors. The LEDs are driven by a second boost circuit based on the MIC3289 which delivers a controlled current to the LEDs which does not vary with battery charge state. This chip provides 16 logarithmically-spaced intensity levels which are digitally selected.

The LAMP reaction emits green fluorescence (peak excitation = 480 nm, peak emission = 515 nm). This fluorescence is detected with a phototransistor located directly underneath each chamber. Importantly, there is a small air gap between the phototransistor housings and the ITO heater, which ensures TIR and prevents feedthrough of the excitation light into the phototransistors. Each one of the 96 phototransistor is wired to one of three 32:1 analog multiplexers (AD732). The microcontroller uses these multiplexers to raster through the phototransistor array, selecting one at a time for interrogation. The entire array is sampled at a specified interval (10 seconds, typically). The outputs of the multiplexers are connected to a transimpedance amplifier with an input biased to 2.5V. When a phototransistor is selected, this bias voltage allows collector

current to flow in proportion to the illuminance on the phototransistor's surface. Phototransistors, rather than photodiodes, were chosen because the leakage current of CMOS analog multiplexers is significant compared to photodiode currents, so crosstalk is an issue. Phototransistors provide significantly more current for the same illuminance and in this configuration they are biased through the multiplexer which eliminates the possibility of crosstalk.

The instrument is powered by a 3.7 V, 2000 mAh lithium polymer battery. A third boost converter (LTC3525) delivers

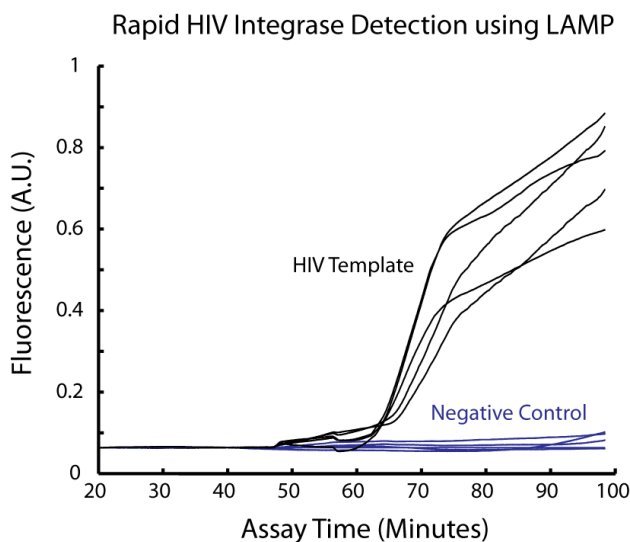


Fig. 4. Results of LAMP reaction detecting the HIV integrase gene using our microfluidic chip and portable instrument. A positive result is seen after 70 minutes. 5 positive (template present) and 5 negative assays (DI water) are shown.

a 5V supply to the digital electronics, LCD display, and transimpedance amplifier. A typical assay run lasts approximately 1.5 hours and consumes one amp-hour. The heater dominates power consumption. Future designs will incorporate an enclosure featuring better thermal insulation which will greatly reduce this power consumption and extend the life of the battery. Our ultimate goal is to create a fully-integrated, portable instrument which addresses the needs of remote/resource poor settings. The specific components included can be tailored for the situation. At its simplest, this instrument would be a handheld heater and LED illuminator which would allow qualitative naked eye readout.

IV. REAL-TIME LAMP GENOTYPING RESULTS

Figure 4 shows the results of a loop-mediated isothermal amplification (LAMP) assay for the HIV integrase gene using our microfluidic chip and electronic reader. The amplification mix has been lyophilized on the chip. Two independent samples are shown, one containing a plasmid with the HIV integrase gene at a concentration of 10^3 copies/ μL , and the other with only deionized water. Within 60 minutes, the sample containing the genetic target for our primers exhibits readily detectable signals above the negative template control background. Our results suggest that a microfluidic LAMP diagnostic would be a powerful tool for rapid detection and subtyping of a variety of pathogens.

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