Miniaturized Devices for Isothermal Amplification and Photometric Quantification of Pseudomonas Aeruginosa

Ramya Priya, Satish Kumar Dubey, and Sanket Goel

Abstract*—*Goal: **This study introduced a proof-of-concept prototype for isothermal recombinase polymerase amplification (RPA) with miniaturized photometric detection, enabling rapid P. aeruginosa detection. *Methods:* The researchers conducted the amplification process within a microchamber with a diameter of 10 mm, utilizing a standalone Thermostat driven thermal management setup. RPA, an amplification technique was employed, which required a lower operating temperature of 37 °C–40 °C to complete the reaction. The amplified amplicon was labeled with a fluorophore reporter, stimulated by an LED light source, and detected in real-time using a photodiode. *Results:* The developed prototype successfully demonstrated the rapid detection of P. aeruginosa using the RPA assay. The process only required the utilization of 0.04 ng of working concentration of DNA. The entire process, from amplification to detection, could be completed in over 15 minutes. The platform showed enhanced sensitivity and specificity, providing a cost-effective and accurate solution for on-site detection/quantification of pathogens. *Conclusions:* The integration of isothermal RPA with the miniaturized photometric detection platform proved successful in achieving the goal of rapid and specific pathogen detection. This study proved the benefits of Isothermal Nucleic Acid Amplification Technology (INAAT), emphasizing its potential as an accessible, user-friendly point-of-care technology for resource-constrained institutions. The RPA-based prototype demonstrated capability without requiring costly laboratory equipment or expertise. The developed platform, when combined with Internet of Things (IoT) enabled cloud platform, also allowed remote monitoring of data. Overall, the methodology presented in this study offered a cost-effective, accurate, and convenient solution for on-site testing in resource-limited settings.**

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***Index Terms*—Internet-of-things (IoT), isothermal nucleic acid amplification, led-photodiode, microcontroller, pseudomonas aeruginosa, recombinase polymerase amplification.**

***Impact Statement*—An RPA based isothermal amplification device with integrated optoelectronics for quantitative detection of target nucleic acid of sample in 15 minutes.**

I. Introduction

THROUGHOUT history, diseases have predominantly been attributed to various pathogens, including Protozoa, Fungi, Bacteria, Parasitic worms, Viruses, and Prions. The establishment and dissemination of dangerous bacterial infections within healthcare settings, including hospitals and other medical facilities, is a matter of substantial global concern [1]. In comparison to outbreaks caused by viruses and bacteria, it is evident that bacterial outbreaks have a higher incidence of hospitalizations and fatalities on a global scale [2], [3]. A typical opportunistic human pathogen bacterium called Pseudomonas Aeruginosa (P. aeruginosa) poses a serious threat to human health. Specifically, this Gram-negative bacterium is associated with the occurrence of serious infections that manifest as lung disease, cystic fibrosis, neutropenia, hospital-acquired pneumonia, urinary tract infections, and various other conditions [4]. The presence of P. aeruginosa can be observed ubiquitously in natural environment, encompassing many sources such as food, water, and soil, hence posing a substantial risk to the promotion of well-being. In response to concerns regarding nosocomial infection, food safety, and environmental safety, there has been a development of rapid, precise, targeted, and ultrasensitive technologies for the early detection of essential pathogens. The key to disease control is in the development of improved, more accurate, and timelier methods for identifying harmful diseases [5].

Finding effective techniques for P. aeruginosa detection has received a lot of attention. The two primary traditional techniques for identifying P. aeruginosa are immune-based techniques and bacterial culture. But these approaches have certain drawbacks, like being laborious or imprecise [6].

Since its invention by Mullis [7], For amplifying nucleic acids, using Polymerase Chain Reaction has been the method of choice. However, PCR has several cons: its high equipment cost, skilled technicians, susceptibility to group of inhibitors and contaminants, the necessity of thermal cycling, and so on. These

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limitations resulted in the headway of alternative methods such as Recombinase Polymerase Amplification (RPA) [8], Nucleic Acid Sequence-Based Amplification (NASBA) [9], Rolling Circle Amplification (RCA) [10], Loop-Mediated Isothermal Amplification (LAMP) [11] and Strand Displacement Amplification (SDA) [12], etc. Most of this alternative DNA amplification is isothermal, which need not to demand a thermal cycler [13]. These alternative methods offer advantages over PCR regarding speed, cost, scale, or portability.

Consequently, research has centered on developing detection methods for P. aeruginosa based on molecular diagnostic techniques, with the goal of reducing the time required for detection and streamlining the process itself. Numerous assays have been developed for P. aeruginosa determination, like biosensors [14], magnetic relaxation switch aptasensors [15], lateral flow tests [16], and microfluidic platforms [17], [18]. Recently, FRW Schmitz et al. [19] reported that P. aeruginosa can be detected by colorimetry using aptamers that have been conjugated with gold nanoparticles. The main drawback of this technique is it takes 2 hrs. - 5 hrs. for the required detection output. Gholami et al. reported the detection of P. aeruginosa from ocular samples validating of oprL gene, targeting P. aeruginosa using multiplexed Real-Time PCR process [20]. Another research group [21] identified the presence of Pyocyanin caused by the P. aeruginosa in the sputum sample using paper-based electrodes. In recent study Dong Kui, et al. reported a method for the quick and selective detection of P. aeruginosa that combines the loop-mediated isothermal amplification (LAMP) with the nanoparticles-lateral flow biosensor (LFB). Despite the facts these methods are accurate and sensitive, they are labor-intensive and take a lot of time, utilize potentially hazardous solvents, require specialized facilities, and involve tedious complicated operating procedures. As a result, their applicability in practical detections is limited. Literature says that RPA is a straightforward, quick, and sensitive IA technique. It enlarges a target DNA sequence by employing a recombinase, a single-stranded DNA-binding protein (SSB), and a strand-displacing DNA polymerase [22], it has been used successfully for different target organisms, such as viruses, bacteria, protozoa, and fungi, with varying types of samples [23]. There is much scope and interest in developing smaller bioanalytical devices that can replace the heavy, bulky equipment used for point-of-care diagnostics.

As a result, miniaturized, added with microfluidic devices, gives a new cutting-edge technology in point-of-care testing in diagnostic applications [24], [25]. These devices have sparked much attention due to their numerous advantages, including minimal reagent volume, short reaction time, low power consumption, lightning-fast analysis, and excellent value for the money. Using this implementation strategy, the number of biological experiments can be cut down by a significant amount, which is effective in doing so. A lot of focus has been employed on creating microfluidic devices over the past ten years that can effectively carry out a variety of formats and applications within this sector, including paper-based microfluidics [26], wearable microfluidics [27], and even digital microfluidics [28]. This is because microfluidics offers a wide variety of benefits, the majority of which are derived from its cut-down size. These advantages result in capabilities such as portability, accessibility, automation, and parallel processing, which are valued in a variety of molecular diagnostic applications.

To meet POCT standards, mobility is frequently given priority in the designs of isothermal amplification devices, which results in reduced efficiency and simpler functionality. To overcome such errors many IoT/AI convergence models are being developed to solve these challenges. [29], [30]. One such system is developed by Lui et al., [31] where IoT enabled isothermal amplification paper based microfluidic device is used to detect pathogens. However, paper-based devices have its limitations, one such is cross contamination which eventually restrict the use as POCT, particularly when conducting on-site testing.

Hence, there is an immediate requirement for a detection method that is on-site, rapid, equipment-independent, equipment-specific, and sensitive for P. aeruginosa. To this end, RPA on a polymer-based reaction on-chip (RoC) device for point-of-care (PoC) with IoT enabled prescreen diagnostics device has been incorporated in this work.

The present study focuses on the investigation of rapid and accurate identification of the P. aeruginosa strain that was isolated from patients with eye disease who were admitted to a specialized eye hospital in Hyderabad. The experiments were conducted and analyzed to compare conventional methods, which are commonly regarded as the gold standard (shown in supplementary data), with the proposed RPA-based isothermal amplification method employing a standalone temperature management unit. The primary method of detection involves the utilization of photometric detection with the application of SYBR Green dye I within a microreactor chip that has been fabricated.

II. Materials and Methods

A. Chemical and Instrumentation

TwistAmp Basic kit (TwistDx, Cambridge, U.K.) for Isothermal Amplification, and 1 Kb Plus Invitrogen DNA marker were procured from Thermo fisher Scientific. QIAamp DNA mini (QIAamp DNA mini kit; Qiagen, Hilden, Germany) for isolation of nucleic acids: QIAquick PCR purification kit (Qiagen, Hilden, Germany). The required bio reagents such as Acetic acid, Tris buffer, Agarose salts, Ethanol and Isopropyl alcohol (IPA) were procured (SR Life Sciences Pvt Ltd, India). Visualizing and fluorescence dyes like Ethidium bromide (EtBr), SYBR Green-I dye were purchased (Sigma-Aldrich, USA). 96-Well Thermal Cycler Veriti^{TM} was installed from S.R Life Science Solutions, and Gel-electrophoresis and Gel-doc systems were purchased from BioBee Tech Solutions (Bangalore, India). Glass slides (75 (L) $\times$ 25 mm (B)) Corning Borosil was purchased from Prabha Trade Impex Pvt. Ltd (Secunderabad, India), A custom made cartridge heater was procured from Ragatiya Heaters (Mumbai, India), Electronics components such as LED, Operational Amplifier (LM358P), Microcontrollers, Thermostats were Procured from Online platform Element 14, Bangalore Karnataka, Photodiode (S1087) was procured (Hamamatsu Photonics, Japan), and Oxygen Plasma Instrument CUTE-1MPR

TABLE I
LIST OF THE PRIMER SET USED TO TARGET THE SPECIFIC PATHOGEN

Target Gene	Primer sequence	Reference
Oprl F	ATGGAAATGCTGAAATTCGGC	[33]
Oprl *R*	CTTCTTCAGCTCGACGCGACG	[33]

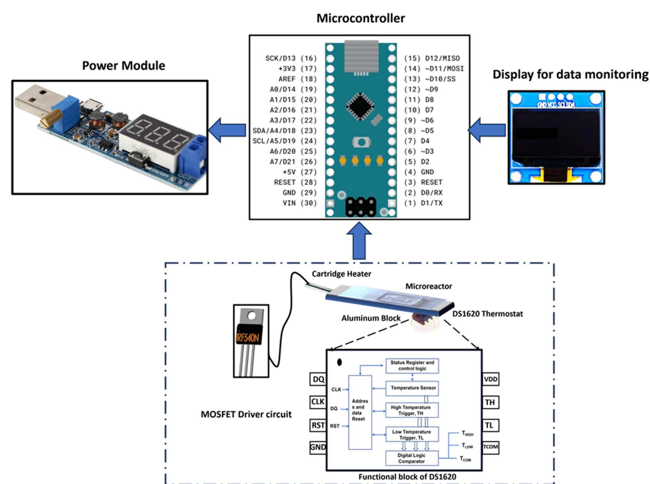

Fig. 1. Block diagram representation of the Thermal management setup. The power module is used to power up the microcontroller and the heater. Display which is connected to microcontroller to display the obtained temperature. The heater mosfet and DIP thermostat is controlled and monitored by the microcontroller.

was installed from Femto Science Inc. Korea. The Primer sequence shown in Table I is procured from (SIGMA Genosys, US). The sample preparation and pipettes used were sterile and autoclaved for every test. The tips which were used are sterile and filtered from ThermoFisher Scientific.

B. Primer Design

The primers used in this study is summarized in Table I. oprL was used as the target gene for the detection of P. aeruginosa. oprL and oprI lipoproteins which are outer membrane proteins are responsible for the resistance to antibiotics and antiseptics. L lipoprotein (oprL) is the main choice of consideration which helps in the detection of species of P. aeruginosa strain. Furthermore, an evaluation was conducted to evaluate its performance with phenotypical and regular biochemical identification methods.

III. RESULTS

A. Heater Sensor Circuit

The microcontroller-operated thermal management system is developed using D1620 Dual in-line Package (DIP) thermostat sensor. A MOSFET-based driver circuit is used to control the temperature level shown in Fig. 1. A custom-made cartridge heater manufactured with stainless steel with a dimension of 25 mm × 3 mm (Length × Diameter) is used for heating the microreactor. The cartridge heater, temperature sensor, and

Fig. 2. 3D package of the thermal management device with a microreactor placed on it.

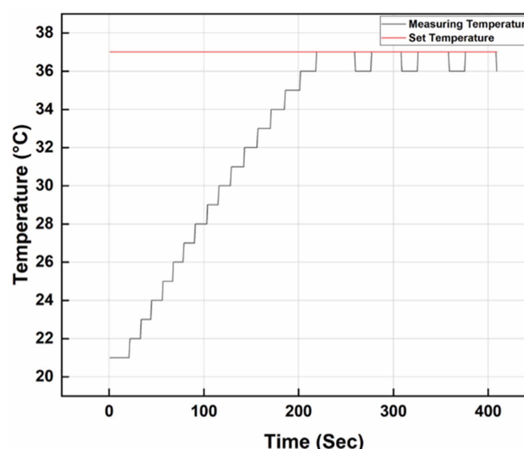

Fig. 3. Graphical representation of temperature acquiring to the set value using the thermal management set-up. Data was taken by the microcontroller using Arduino IDE.

driver circuit are all controlled by an Arduino nano, which acts as the command house of the circuitry shown in Fig. 2. This driver circuit is used in controlling the excess current not to flow to the heater. The temperature sensing and the controlling of the required temperature are done using the single-chip thermostat (DS1620). The max temperature is set as 37 °C. Once the heater attains the maximum temperature, the sensor senses the value and sends the signal to the microcontroller to turn off the supply to the MOSFET connected to the thermostat. At about 3.5 minutes of observation, the temperature of the block will reach the set temperature, as demonstrated in Fig. 3.

B. Detection / Quantification Unit

The detection / quantification unit is used in replacing spectrophotometer study. It consists of a monochromatic source from an LED and a detecting element as a photodiode. The specifications of the LED and detecting photodiode should be chosen in such a way that the peak emission of the LED and the peak sensitivity of the photodiode are approximately at the same wavelength of the fluorophore. The entire circuity data is operated with the help of the Node MCU microcontroller shown in Fig. 4. The data is observed through a cloud platform called Thingspeak. The current output reading from the photodiode is converted into voltage using a conventional trans-impedance circuit. A circuitry low pass 1st ordered filter is used to avoid

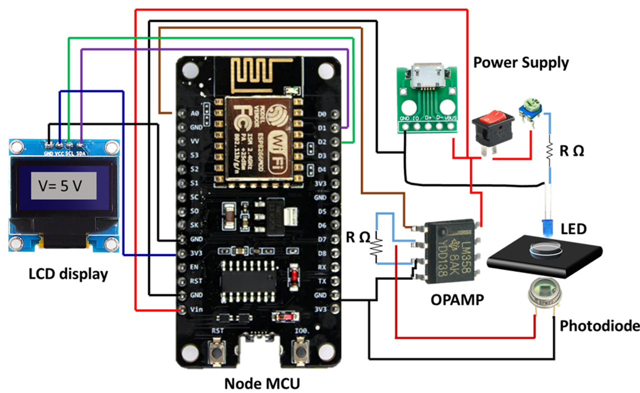

Fig. 4. A pictographic representation showing the circuit used in the detection unit integrated with the microreactor. The variation in the photodiode current is to read and amplified by the transimpedance amplifier arrangement of the opamp circuit. A customizable either battery (not shown) or 5V USB power module is used to energize the entire circuit.

noise and to ensure repeatability of the test results. Fig. 7(b) shows the actual image of the circuit where all the components were incorporated.

IV. Discussion

A. Establishment of PCR Reaction Condition

The templated reaction is carried out in a 96-well thermal cycler, well programmed to maintain 3 different temperature zones for denaturation, annealing, and extension. The performance is analyzed using three different PCR master mix, and their results were compared with RPA. For a total of 25 μL samples selected DreamTaq Green PCR Master Mix (2X), PCR Master Mix, SYBR Green Master Mix 2X DyNAmo Color Flash, DNA quantity was determined on a micro volume Spectrophotometer.

Taq polymer premix with SYBR green Enzyme and Plane Taq polymer and 2X Taq polymer of 12.5 μL were taken in separate 0.2 mL Eppendorf tube oprL specific forward and reverse primer of 1 μL each of 10 μM concentration is used. 1 μL DNA of P. aeruginosa specific template is used, finally nuclease-free water is used to make up the final volume. The reaction conditions of PCR were used as the denaturation stage of 95 °C for 30 sec of 30 cycles. Annealing of 45 sec at 62 °C and 72 °C at 1 min is followed. Melting Temperature (Tm) is taken into consideration according to the manufacturer. After amplification, the results were examined using electrophoresis by loading the sample in 0.7 % agarose gel using 100 V for 30 min. Fig. 5. shows the amplification of PA, and the bands were shown at 504 bp line.

B. Design and Validation of RPA Assay

The Recombinase Polymerase Amplification, also known as RPA, was carried out in accordance with the protocols outlined in the TwistAmp Basic kit (TwistDx, U.K.). To get a total of 50 μL worth of reaction samples from each optimised reaction, the following components were combined: 29.5 μL of primer-free Rehydration buffer, 12.2 μL nuclease-free water, 2.4 μL forward primer [10 μM], and 2.4 μL reverse primer [10 μM]. An amplicon with a length of 504 base pairs was produced by using

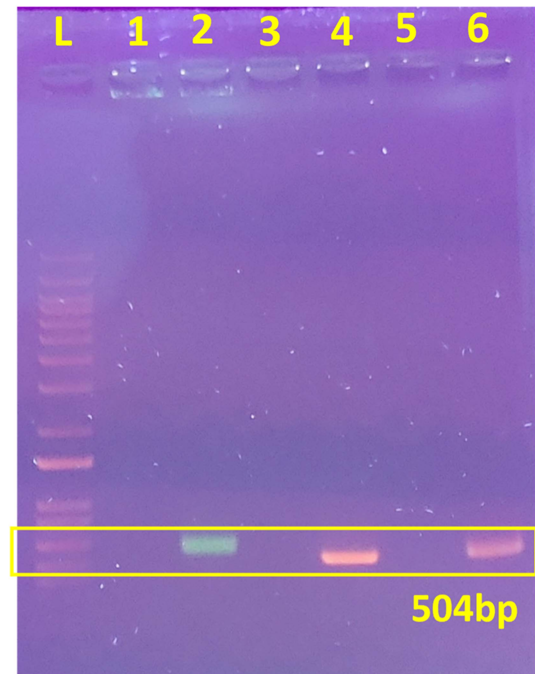

Fig. 5. Shows the PCR amplification of Pseudomonas genus-specific primers (oprL gene) using different Master mix, L = represents the marker (1 kb Marker), 2,4,6 are the clinical isolates of the genus, 1, 3, 5 are the no template (DNA) contains primer sets.

both forward and reverse primers. Finally, 2ng/μL (LoD of 0.04 ng) of genomic DNA is added and to initiate the reaction, 2.5 μL of a magnesium acetate (MgOAc) solution containing 280 mM was dispensed into the cap of each tube, and the tubes were thoroughly mixed to start the reaction. The reaction tubes were recapped, centrifuged and vortexed twice for a few seconds. Then these samples were shifted into the microreactor chamber of 60 μL of volume and placed on the heat block, where the heat is controlled by a microcontroller using a driver circuit. The well-maintained temperature of 37 °C using a DS1620 sensor and incubated for a period of 15–20 mins. After incubation, the samples undergo purification (Qiagen 28104, QIAquick PCR Cleanup) and are electrophoresed on a 0.7% agarose gel to analyze the purity, quality, appropriate size, and quantity of the product. At last, the Visualization is done upon ethidium bromide staining under the del doc system. Lane L in Fig. 6 represents the 1 kb ladder used as the marker. Lane 1 is the negative control, and Lane 2 is the positive amplicon implied using RPA on the device, whereas Lane 3,4 is the negative control and 5,6 are the positive control of the same amplicon using PCR samples on a conventional thermal cycler.

C. Detection/Quantification Principle

A through hole photodiode (S1087, Hamamasthu) with high sensitivity in the necessary spectral wavelength range served as the detection setup with a blue LED of 470 nm of peak wavelength and luminal intensity of 55 mcd (Element 14, Bangalore) measuring 3.3 mm. The photodiode was chosen so that the photocurrent was found to vary in a linear fashion with irradiance. An opamp is used to measure the voltage of the

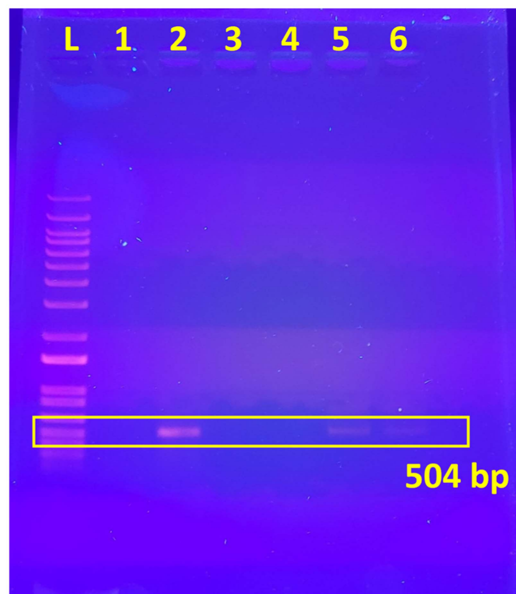

Fig. 6. Electrophoretic analysis of bacterial genomic DNA extracted from the clinical sample and amplified with P. aeruginosa primer set using the proposed thermal management system. Lane L, 1Kb DNA marker, Lane 1(Negative control), 2(Positive control), RPA amplified amplicon with oprL primer set (504 bp) performed in microreactor chamber, Lane 3,4 no DNA template (Negative control) with each primer set, lane 5,6 contain DNA template (Positive control) PCR- generated amplicon with oprL (504 bp) primer set performed in a thermal cycler.

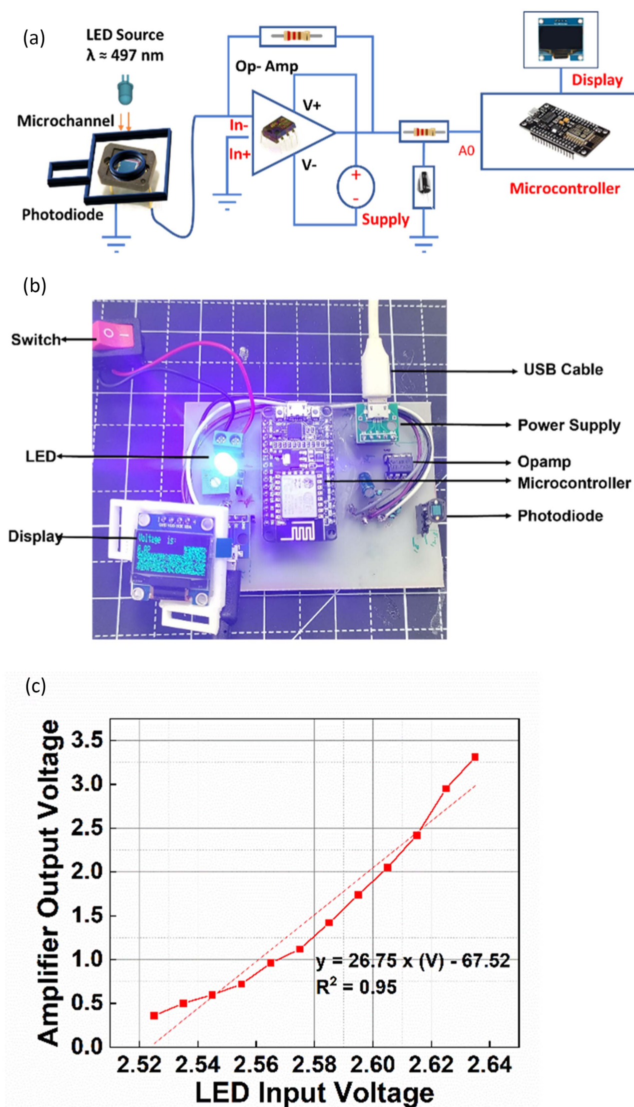

Fig. 7. (a) The schematic representation of the trans-impedance circuit used for the detection setup. (b) The realistic view of the components placed on the PCB design. (c) Amplifier output voltage (Vout) Vs. LED input voltage (Vin).

intensity to ensure high repeatability, low noise, and linear gain, transimpedance amplifier circuit (Fig. 7(a)) was created using a low noise operational amplifier (OPA Series, manufactured in the United States by Texas Instruments). To obtain the desired output voltage in the range of 0.1 to 4.9V from an opamp. The feedback resistor was calculated and chosen based on the reverse light current specification and the surface area of the photodiode. Suitable capacitors and resistors were chosen to create a bias at the non-inverting input and prevent noise from affecting the output. To maintain the intensity of the LED, a 10 Kilo Ohm potentiometer was used. The voltage at the input of the LED and the voltage at the output of the amplification circuit are shown in Fig. 7(c). The repeatability and linearity of the amplifier's output were impressive when compared to the input voltage of the LED with a correlation coefficient $R^2 = 0.95$.

The photometric-based sensing is realized using a LED and photodiode combination Fig. 7(b) is the representation of the components on the printed circuit board of the detection system, wherein the PMMA-based micro reservoir of dimension 4 millimeters (ϕ) and a thickness of 2 millimeters was placed in between LED and photodiode. The RPA amplified sample with different concentrations of DNA mixed with SYBR Green I dye is pipetted into the well of the reservoir, which helps in analyzing the absorbance of the sample.

The concentration of DNA in the range of 25–125 ng/μL while maintaining a fixed concentration of SYBR Green I of

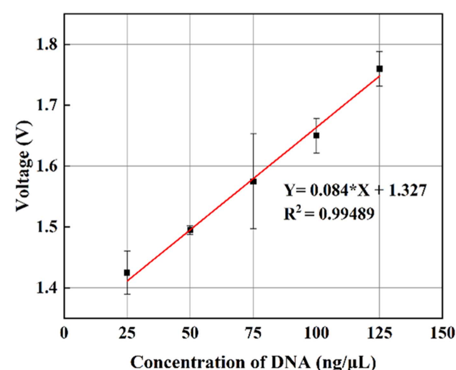

Fig. 8. Calibration plot for concentration of DNA versus Voltage.

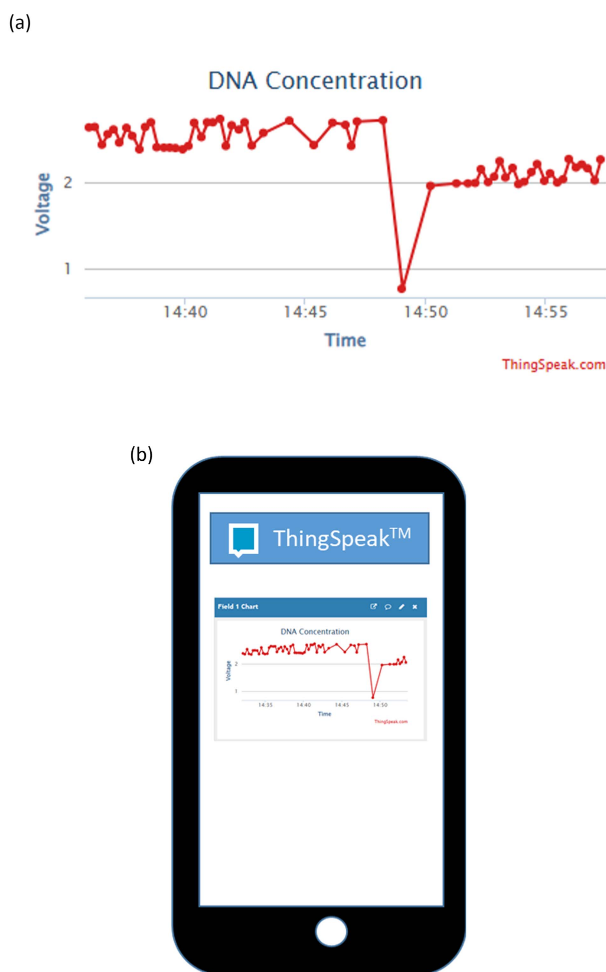

Fig. 9. Graphical data monitoring (a) real time data monitoring via *thingspeak* server, while change in voltage represents the change in the concentration of DNA. (b) Cloud data is accessed through mobile phone.

100x. The response to these variable concentrations was then analyzed using the prototyped device. The resultant DNA-dye complex exhibited absorption of blue light at 497 nm (max absorption at 497 nm) and subsequent emission of green light at 520 nm. The prototyped device response is designed in such a way that the photodiode with peak sensitivity at 520 nm is selected and functionalized using transimpedance circuitry. The voltage output from the transimpedance amplifier circuit is directed towards the analog inputs of the microcontroller, which can be further exhibited on the display module connected to the device and the cloud platform. The relationship between the change in the voltage and the change in the concentration of the DNA has been studied and represented in Fig. 8.

D. IoT Enabling for Real Time Remote Monitoring

A cloud-based platform service called *ThingSpeak* for IoT analytics enables users to compile, display, and examine real-time data streams as shown in Fig. 9. With the help of the IoT cloud platform *ThingSpeak*, remote monitoring of the pathogenic diseases and its outspread can be identified.

V. Conclusion

The work presents the design and implementation of a cost-effective, user-friendly, and portable device for Recombinase Polymerase Amplification (RPA). The device encompasses a microfluidic device, a microcontroller-operated thermal management system, and a miniaturized photometric detection unit. The operational control of the circuitry data of the device is facilitated through the utilization of the Node MCU microcontroller, cloud platform, and smartphone interface. Further, the applicability of the developed device is demonstrated through the rapid detection of P. aeruginosa using RPA assay. The methodology employed by the RPA is effective for the on-field detection of DNA. The developed device was amenable to detecting for a working concentration of genomic DNA as low as 0.04 ng in a sample of 50 μL in less than 15 mins. This device exhibited the capacity to provide affordable diagnostic capabilities even in resource-limited settings and helps in data storage and monitoring using *ThingSpeak* Cloud platform. Additionally, the RPA reagent can be further modified for a variety of applications by adjusting its composition on the paper-based lateral flow strip. Some of these applications include the detection of viruses and viral nucleic acids, the monitoring of water quality, and the testing of food.

Supplementary Material

Supplementary Material provides a background overview of RPA technique along with the chip design and the scheme of the work.

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