

Journal Pre-proof

Shunting microfluidic PCR device for rapid bacterial detection

Abbas Salman, Helen Carney, Simon Bateson, Zulfiquar Ali



PII: S0039-9140(19)30936-1

DOI: <https://doi.org/10.1016/j.talanta.2019.120303>

Reference: TAL 120303

To appear in: *Talanta*

Received Date: 3 May 2019

Revised Date: 25 August 2019

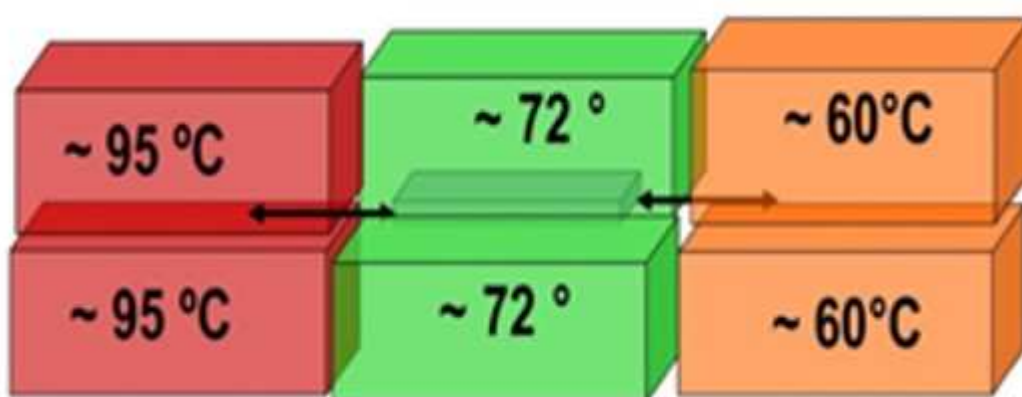
Accepted Date: 27 August 2019

Please cite this article as: A. Salman, H. Carney, S. Bateson, Z. Ali, Shunting microfluidic PCR device for rapid bacterial detection, *Talanta* (2019), doi: <https://doi.org/10.1016/j.talanta.2019.120303>.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2019 Published by Elsevier B.V.

Graphical Abstract



Journal Pre-proof

Shunting microfluidic PCR device for rapid bacterial detection

Abbas Salman¹, Helen Carney¹, Simon Bateson² and Zulfiquir Ali^{2†}

¹School of Science, Engineering and Design, Teesside University, Middlesbrough, TS1 3BX, United Kingdom

²Healthcare Innovation Centre, Teesside University, Middlesbrough TS1 3BX, United Kingdom

[†]Corresponding author at: Healthcare Innovation Centre, Teesside University,

Middlesbrough, TS1 3BX, United Kingdom. Tel.: +44 1642 342469;; E-mail:

z.ali@tees.ac.uk

Abstract

Polymerase chain reaction (PCR) is commonly used for the analysis of nucleic acids in a variety of applications including clinical. There is, however, a need for a low cost portable PCR device that allows rapid identification of pathogenic bacteria. We report a shunting PCR microfluidic device comprising: polycarbonate microfluidic PCR chip; shunting thermal cycler and fluorescence detector. The microfluidic PCR chip – fabricated using micro-milling and thermal fusion bonding for sealing of the cover – was shunted between three double side temperature zones for thermal cycling. Rapid amplification was observed with heating and cooling rates of 1.8 °C/s and 2 °C/s respectively. Lock-in photodetector for fluorescence detection of the microfluidic PCR chip achieved at 95% confidence an LOD of 75pM FITC and 0.7 ng μl^{-1} of dsDNA using a QuantiFluor assay kit. The device was validated using universal primers - based on chromosomal DNA extracted from non-pathogenic K-12 subtype of *Escherichia coli* (*E. coli*) – for amplification of fragments of 250, 552 and 1500 bp. PCR amplification was demonstrated, with annealing temperatures ranging between 54°C to 68°C, and confirmed using gel electrophoresis. The developed shunting PCR microfluidic device will allow for low cost and portable nucleic acid amplification for the detection of infectious diseases.

Key words: Shunting PCR, DNA analysis, fluorescence, microfluidic, point-of-care

1.0 Introduction

PCR has revolutionised the analysis of nucleic acids. There is an increasing trend in the use of microfluidic devices for point-of-care testing[1]. Robust portable microfluidic PCR systems for pathogen detection for point-of-care (PoC), food control, clinical or environmental applications could supplement or replace expensive bench top laboratory apparatus [2]. Microfluidic based PCR facilitate faster DNA amplification because of smaller thermal capacity and a larger heat transfer rate between the PCR sample and heater element. These systems consist of three components: reaction chamber; thermal cycler; and PCR product detector. PCR microfluidic devices have been made using various materials such as silicon [3][4][5], glass [6], polycarbonate [7], polyamide [8] and PMMA [9].

There are three main types of microfluidic PCR devices, namely: chamber-based stationary PCR; continuous-flow PCR; and shunting PCR. In all of these types the primary objective is to achieve faster thermal cycling and use of reduced volumes of expensive sample/reagents [10]. A number of chamber-based stationary PCR [3][5][6][7] [8][11] and continuous-flow PCR microfluidic devices [12][13][14][15][16][17][18] have been reported. Continuous-flow PCR has the advantage over chamber-based stationary PCR of faster heating and cooling times and consequently reduced total time of the PCR reaction. A drawback for continuous-flow PCR is the difficulty in collection of PCR products for further analysis, such as gel-electrophoresis, for determination of amplicon sizes and for real-time PCR. Shunting PCR offers possible advantages of both stationary-based chamber and continuous-flow PCR modules with faster heating and cooling times and ability to extract PCR products for further analysis. Although shunting PCR has

the potential for faster heating and cooling cycling than stationary-based PCR this is potentially limited in terms of speed of the PCR process when compared with continuous-flow PCR. The number of cycles required for best possible amplification often depends on either the initial concentration of the PCR constituents and the efficiency of each amplification step. Typically the most effective amplification takes place around 40 cycles with often no amplified products in fewer than 20 cycles and potentially a higher level of amplification of nonspecific products between 50 or 60 cycles [19].

Both single-well and multiple-well PCR microfluidic devices have been reported [3][4]. Heating is typically achieved using either contact or non-contact heating [8][20]. Detection of PCR products has been carried out using: gel electrophoresis [5][9][13]; fluorescence end-point [4][7]; as well as real time fluorescence intensity detection by either fluorescence microscope [3][9] or by an in-house fluorescence detection module [21].

We describe here a shunting PCR approach where the sample chamber of the microfluidic device is moved between different temperature zones and the sample settles at the desired temperatures for known residence times. This approach has the advantage of fast cycling times because of the thin fluidic layer of the microfluidic chip and the large static heating system with high thermal inertia elements to induce rapid temperature change, in both heating and cooling phases. We also incorporate a highly sensitive lock-in photodetector for fluorescence detection to monitor the PCR progress by detection of intercalating dye which incorporates within the PCR products. The developed system was demonstrated for the amplification and

detection of three primer sizes of a model non-pathogenic subtype of *Escherichia coli* (*E. coli*). The simplicity of the approach allows for use within the clinical, environmental and food sectors including within low to medium income countries. Moreover, this approach could potentially be extended for use within real-time PCR.

2.0 Materials and methods

2.1 Reagents and Materials

Three sets of primers were designed to produce fragments of differing lengths ranging from 250 to 1500 base pairs. Each primer pair was tested in a conventional GeneAmp 9700 PCR instrument (Applied Biosystems, UK) and in the developed shunting microfluidic PCR device followed by agarose gel electrophoresis to analyse the size of the DNA amplicons. A summary of the nucleotide sequences of the oligonucleotides used and their target genes presented in Table 1. Q-PCR Master Mix (Promega UK Cat. no. A6001) was used for the PCR protocol. This included buffer, Taq enzyme for amplification and SYBR Green 1 dye, which fluoresces on binding to dsDNA components. Bovine serum albumin (BioLabs, UK) was used as part of the PCR reaction and pre-coating the microfluidic device to avoid contamination. A Quant-iT dsDNA High-Sensitivity Assay Kit (Thermo Fisher Cat. no. Q33120), with a fluorescent DNA-binding dye, was used for determination of the concentration of the extracted dsDNA. Comparison of sensitivity of the developed lock-in fluorescence detection with commercial systems for measurement of DNA concentration used a QuantiFluor assay kit (Promega, UK, Cat. no. E2670).

2.2 PCR amplification

Escherichia coli (K-12) bacterial culture was incubated in nutrient broth (Oxide, UK) for 24 hours at 37 °C and 120 rpm to a final optical density (OD) of 2 at 600nm. Cells were harvested from 1.5mL culture by centrifugation at 13000g for 10min. The cell pellet was re-suspended in 500 µl lysis buffer (0.5 % SDS, 20 µg/ml proteinase k) and incubated at 55 °C for 30 min. Pre-warmed 5M Na Cl (100 µl) and CTAB (Cetyl Trimethyl Ammonium Bromide) solutions (80 µl) were added and the sample incubated at 65 °C for 10 min for genomic DNA extraction [22], further information is provided within the SI. Isoamyl alcohol: chloroform (1: 24; 680 µl) was added, followed by centrifugation for 5min at 13000g. The top aqueous layer of the sample was removed to a new tube and 360µl isopropanol added. Samples were incubated at room temperature for 30min to precipitate the DNA, followed by centrifugation for 10 min at 13000 g. The supernatant was removed and the DNA pellet washed with 300µl pre-chilled 70% ethanol and centrifuged for a further 10min at 13000 g. The supernatant was removed and the pellet allowed to air dry prior to re-suspension in 100 µl Tris-EDTA solution at pH 8. DNA concentration was measured using a Quant-iT dsDNA High-Sensitivity Assay Kit (Thermo Fisher Catalog no. Q33120) and the average of two sample measurements gave a DNA concentration of 245 ng µl⁻¹, see further details in the SI. The A268/280 ratio was found to be 1.8 which indicates that the DNA yield is pure, free from RNA and proteins. This DNA batch was used to carry out all the PCR tests either in a commercial thermo cycler or the developed shunting microfluidic PCR device.

A 50 µl volume PCR reagent mix was prepared containing: Promega Q-PCR Master Mix (0.025 U/µl Taq DNA polymerase, 1.5 mM MgCl₂, 200 mM dNTPs in reaction buffer pH8.5); forward and reverse primers (1 µM); bovine serum albumin (0.4 µg/µl);

and template DNA 24 (ng/ μ l). The 50 μ l PCR reagent mix was divided into two 25 μ l portions, with the two aliquots being amplified using either the commercial thermal cycler and the developed shunting microfluidic PCR device.

The PCR microfluidic chip was rinsed with sterile distilled water three times and once with analytical grade isopropanol (BDH, UK) and then oven dried at 100°C for 30 minutes to clean both the chip from any cross contaminants. Bovine serum albumin (25 μ l, 1 μ g/ml; BioLabs, UK) diluted with buffer - 20mM KPO₄ (pH 7.0 at 25°C), 50 mM NaCl, 0.1 mM EDTA and 5% glycerol (New England Biolabs NEB #B9001, UK) - was inserted into the reservoir chamber with a micro syringe. The PCR microfluidic device was placed in a centrifuge (Sigma 1-14K, Germany) by positioning the injection port towards the central rotor and the sample chamber at the outer side. The device was fixed by a small masking tape and the centrifuge lid was then closed and the centrifuge turned on. The PCR microfluidic chip was centrifuged for 5 secs at 14000 rpm to transfer the BSA to the sample chamber and incubated at 4 °C for 18 hours to coat the microfluidic chip with BSA and avoid inhibitors. The chip was then centrifuged at 14000 rpm to remove BSA and rinsed three times with PCR grade water. In order to reduce or eliminate any cross contamination that may cause inhibition of DNA amplification, the chambers were sterilised before use by exposure to 30 min Ultra Violet (UV) light (UV Sterilisation cabinet, Scie-Plas Ltd., UK).

PCR reagent mix (25 μ l) and mineral oil (20 μ l) (Fisher Bio-reagent, UK) were loaded sequentially into the reservoir chamber of the PCR microfluidic chip, followed by centrifugation for 5 secs at 14000 rpm to displace the PCR reagent mixture into the sample chamber. Mineral oil was added to avoid the formation of air bubbles during the process and prevent reagents from evaporating (McPherson and Moller, 2006).

The thermal cycling conditions for the GeneAmp 9700 (Applied Biosystems) commercial thermal cycler and the developed shunting microfluidic PCR device are provided in SI Table 6. PCR products amplified from both devices were loaded onto a 1.5% (w/v) agarose gel stained with 1 mg/l SYBR safe (Invitrogen, UK). The gels were run using horizontal electrophoresis at 90V for 80 min. Hyper-Ladder 1 (Bioline, UK) was used as a marker for size determination of the products.

3.0 Results and discussion

3.1 Shunting thermal cycler

The thermal cycler was made using three heater plates – each plate having dimensions of 85mm L x 50.8mm W - modified to attain the desired temperature level appropriate for each zone. The heater plate for extension and denaturation were respectively 72⁰C and 95⁰C and with the annealing heater plate being allowed to vary depending on the amplicon that was used (Figure 1 B). The heater plates were placed in series from high to low temperature going from the denaturation to extension to annealing. The PCR microfluidic chip was passed for specified periods of time between the three temperature zones using a rotational servo (HS-5755MG, digital quarter scale, from Hitec, UK). The servo was controlled by data acquisition software programmed using C# to trigger the position of the chip in the thermal cycler for a defined period. Temperature was monitored by inserting the thermistor within the inlet port after the reagents had been included in the microfluidic device and used to ensure that the PCR microfluidic chip is at the correct temperature. Temperature profiles for the developed shunting thermal cycler with a PCR microfluidic chip were compared with the commercial GeneAmp (model No. 9700) and Rotor Gene thermo cyclers (Model No. 3000). This was done by placing a micro

thermistor (Farnell-UK) in the centre of the water filled sample chamber of the PCR microfluidic chip and with the data collected using a data-logger (Pico Technology, device type is ADC-24). For the commercial thermal cyclers the micro thermistor was secured in the centre of water filled Eppendorf tube.

The developed shunting thermal cycler is intended for fast heating and cooling cycles with accurate temperature profile whilst minimizing the power requirements. The heaters in the three temperature zones were set to specific temperatures, which avoided overshooting of the temperature set points and consequently provided more optimal amplification, with shorter cycle times and faster amplification. It follows then that this should result in higher quality of products being produced since overshooting of the desired temperatures would result in lower enzyme activity.

The developed shunting thermal cycler for the microfluidic PCR chip provided rapid and predictable temperature cycling. **Figure 2** shows the temperature profile as a function of time for thermal cycling of our developed shunting thermal cycler compared with the commercial Rotor Gene and GeneAmp thermal cyclers. The GeneAmp cycler with slower heating and cooling cycles will slow the reaction process whilst the faster ramping rates of the Rotorgene tended to produce overshooting. Our developed shunting thermal cyclers had faster amplification than the GeneAmp which reduces the amplification time and avoids overshooting of the temperature set points as observed by the Rotor Gene which will affect the quality of amplification products produced..

The shunting microfluidic PCR device has the advantage of high thermal capacity for the thermal cycler with a low thermal capacity for the microfluidic PCR chip this allows faster cycle times and reduces the total time required for PCR amplification.

The temperature within the microfluidic PCR chip increased from $\sim 47\text{ }^{\circ}\text{C}$ to $\sim 92\text{ }^{\circ}\text{C}$ at a rate of $1.0\text{ degree per second}$ and decreased from $\sim 92\text{ }^{\circ}\text{C}$ to $60\text{ }^{\circ}\text{C}$ at a rate of $\sim 1.8\text{ degree per second}$ ($^{\circ}\text{C/s}$). The temperature rose from $60\text{ }^{\circ}\text{C}$ to $72\text{ }^{\circ}\text{C}$ and from $72\text{ }^{\circ}\text{C}$ (elongation step) to $92\text{ }^{\circ}\text{C}$ (denaturation step) at a rate of $2.0\text{ }^{\circ}\text{C/s}$. The initial denaturation stage requires two minutes to ensure complete denaturation of dsDNA. There was a need to add an additional 10 seconds to allow the PCR mixture to attain the desired temperature from the elongation to denaturation steps so that the total time required for the denaturation step was two minutes and 10 seconds. It is particularly important that the time taken to reach the desired temperatures for the denaturation and annealing steps is minimized to reduce nonspecific amplification. Moreover overshooting beyond the desired temperature for any of the steps may lead to polymerase enzyme inactivation. Our shunting thermal cycler achieves fast DNA amplification through improving the ramping rate of heating and cooling for the PCR mixture.

3.2 Microfluidic PCR Chip

A polycarbonate slide ($34\times 20\times 1.0\text{ mm}$) was used to fabricate microfluidic PCR chip comprising three layers, a polycarbonate (PC) fluidic layer (thickness, $1000\mu\text{m}$) with fluidic architecture sandwiched between two sealing polycarbonate films (thickness $127\text{ }\mu\text{m}$). The microfluidic PCR chip comprises an injection port ($5\times 1\text{mm}$) leading to reservoir chamber, which is subsequently connected by a channel ($20\times 1\text{mm}$) to a sample chamber (6mm diameter). The PC parts were well cleaned prior to use by soaking them with decon 90 and rinsed with distilled water before being sonicated in distilled water for 10 minutes to remove any decon 90 residues, which inhibits bonding. The PC parts are placed in container of isopropan-2-ol (Analar grade, BDH)

for a final rinse then and placed in an oven at 105 °C for at least 2 hours to dry. The three layers were aligned and then placed on top of each other along with a weight of 12.5kg (245 Pascal) in an oven at 165 °C - above the PC transition temperature (148°C) - for two hours to bind the different layers. After two hours, the temperature was decreased by 10 °C every 15 minutes towards room temperature. The temperature and pressure conditions used were sufficient to achieve high bond strength and conserve the fluidic structure of the PCR microfluidic chip.

The microfluidic PCR chip was developed as a simple and disposable element that could be used for rapid DNA amplification. The complexity of the device is reduced to a minimum to allow low cost fabrication. The chip comprises reservoir and sample chambers, which are connected by a channel (Figure 1A). The reservoir chamber provides the opening for fluids and works as a supplier cavity to the sample chamber. The channel serves as a two-way pressure control valve, which connects the reservoir with the sample chamber.

The material used for fabrication of the microfluidic PCR chips needs to take into account the chemical, mechanical, optical and thermal properties that are best suited for the PCR reaction and the thermal cycler design. Most of the early microfluidic PCR chips were fabricated in silicon or glass. Silicon has the advantage that it can be closely integrate the fluidics, transducers and associated electronics elements using well-established microfabrication technologies. Glass has attractive optical properties. However, glass and silicon are known to be a source of PCR inhibition. Polymeric materials have been explored as more flexible alternatives for the fabrication of microfluidic PCR chambers. Polymers have a number of advantages including low cost, biocompatibility and integration of pneumatically actuated fluidic valves for sophisticated fluidic manipulation[23]. A drawback to the use of polymers

is the relatively low thermal diffusivities which can limit heat transfer and DNA amplification in the PCR chamber [24]. PCR chambers have also been produced using PET film [8],[10]. We have adopted to use polycarbonate since this is already widely used for PCR within conventional Eppendorf tubes. Our approach has the advantage of use of a thin polymer film with improved heat transfer characteristics.

3.3 Lock-in fluorescence detection

Fluorescence detection was carried out using a lock-in photodetector system comprising: an analogue to digital converter (ADC) DDC112 (Texas Instruments Inc., Dallas TX, USA); photodiode detector (ODD-525W (OPTO Diode Corp., California, USA) and blue LED as a fluorescence excitation source (DS23) from (Lumileds Lighting, U.S.A). The ADC has a current integrating input capable of single bit resolution as low as (94 fA) (which is ideal for sampling low level photodetector outputs) and automatically provides the accumulation process. It also has a 20-bit resolution providing a wide dynamic range useful for sampling low-level optical signals in the presence of relatively high ambient light levels. Charge integration is continuous as each input uses two integrators; while one is being digitised, the other is integrating. For each of its two inputs, the DDC112 combines current-to voltage conversion, continuous integration, programmable full-scale range, A/D conversion, and digital filtering to provide digital results with a high level of precision and wide dynamic range. In addition to the internal programmable full-scale ranges, external integrating capacitors allow an additional user-settable full-scale range of up to 1000 pC (data sheet, Texas Instruments). In this device, a selective photodiode detector and a dichroic filter are used to detect only the desired wavelength of the light.

The photodetector has a universal serial bus (USB) interface, which can be connected to any personal computer to control all the photodetector functions. The software programmed used was C# language. This software is programmed to control the photodetector device functions. The device controller was also established to power the LED lamp, which could be switched on only if the personal computer was powered. Furthermore, the excitation light integration time is fully controlled by the software programme. The microprocessor in the detector functions as a digital lock-in amplifier so that the detection of noise signals in the system and its surroundings is minimised [25].

The shunting thermal cycler system was combined with the fluorescence detection module to allow both DNA amplification and end point detection. Fluorescence was measured at the start and end point of the amplification process. The LED uniformly illuminates the detection region from the side of the microfluidic PCR chip while the photodetector detects the fluorescent emission at 90 degree. The photodiode detector therefore collected the fluorescent emission at (520nm) and was geometrically separated from the excitation signal at (480nm). In practice, scattering of the excitation light led to a requirement for additional wavelength filtering to achieve good fluorescence sensitivity. Fluorescence light was transferred by means of a 45 centimetres long and 600 μm diameter fibre optic (MM Patch Cables, Thorlab, USA) through an emission filter that is above the reaction chamber (5mm) of the microfluidic PCR chip.

Comparisons of fluorescence detection in the microfluidic PCR chip using both the developed lock-in photodetector system and a commercial HR4000 spectrometer (Ocean optics, USA) was made using FITC and ds DNA QuantiFluor kit assay

(Promega Ca. no. E2670,) . Double stranded DNA detection using the Quantifluor kit assay was also made in a standard 96 well plate with a Synergy HT Multi-Mode Microplate Reader (BioTek UK). Comparison of the lock-in fluorescence detection with the HR4000 spectrometer (Ocean optics, USA) used the same LED excitation source and fibre optic collection of the fluorescence emission. An LOD of 75pM was found for FITC in the microfluidic PCR chip using the developed lock-in fluorescence detection (Figure 3 A) against an LOD of 92pM for the HR4000 spectrometer (Ocean Optics) with both at 95% confidence. Comparison of ds DNA calibration assay using QuantiFluor kit in the microfluidic PCR chip gave at 95% confidence an LOD for DNA of $0.7 \text{ ng } \mu\text{l}^{-1}$ for the developed lock-in fluorescence detection (Figure 3 B) and an LOD of $4.0 \text{ ng } \mu\text{l}^{-1}$ for the HR4000 spectrometer (Ocean Optics). The LOD for the dsDNA using the QuantiFluor in a standard 96 well plate with a Synergy microplate reader was $0.2 \text{ ng } \mu\text{l}^{-1}$.

A number of different approaches have been described for the integration of fluorescence detection with microfluidic chips. The use of polyfluorine based (polymer LED) as light source and avalanche photodiode as a detector has been demonstrated with limit of detection (LOD) of $1 \text{ } \mu\text{M}$ for fluorescein dye[26]. A microfluidic device with an integrated OLED excitation source and a PMT as detector for on-chip fluorescence detection using a pinhole and interference filter for masking the excitation light has also been developed [27] with an LOD of $3 \text{ } \mu\text{M}$ for Alexa 532 dye. Moreover, a compact device using an OLED a slight source and an integrated p-i-n photodiode as a detector, with interference filter to mask the excitation light provided an LOD of $10 \text{ } \mu\text{M}$ of TAMRA dye [28]. Rhodamine 6G has been detected with an LOD of 10 nM with a thin microfluidic chamber that used organic devices

both as optical sources (organic light emitting device) and detectors (organic photodiode) [29]. A cross-polarization scheme, placed on the both sides of the chamber, was used to mask the pump light from the signal detector.

3.4 PCR amplification validation

Amplification conditions were first optimised for time and temperature of annealing, denaturation and extension for three different sized amplicons (250 bp, 552 bp and 1500 bp) using a standard 9700 GeneAmp thermal cycler (Applied Science, UK). The 250 bp is a universal primer set, which is easy to optimise, and can be used to test different PCR systems. The primer set designed to produce DNA fragments of 552bp was selected to target a fragment of putative electron transfer flavoprotein FixA in *Escherichia coli* str. K-12 substr DH10B. The target locus is predicted to be the gene responsible for the synthesis of flavoprotein FixA in *Escherichia coli* str. K-12, which is an electron transfer flavoprotein subunit, required for anaerobic carnitine reduction [30]. The third primer set tested was near complete 16S rRNA [31] and was used to evaluate the ability of the prototype thermal cycler to amplify a large size PCR product. Amplification of such a large PCR amplicon is a challenge for PCR since it is difficult to optimise conditions.

The amplification conditions developed within the standard GeneAmp thermal cycler were then further optimised for the microfluidic PCR device and are given in S1 Tables six to nine. The amplification within the microfluidic PCR device was determined by fluorescence measurements before and after completing the amplification reactions. The extent of amplification is indicated by the fluorescence signal from SYBR Green I dye which binds to the PCR products. During the denaturation step, all SYBR Green molecules intercalated between the two strands

are released and used again in the next amplification cycle. As the size of the amplicon increases then the amount of SYBR Green I that can intercalate between the two DNA strands increases, yielding higher fluorescence intensity and sensitivity. It should, however, be noted that SYBR Green I will bind to any double stranded DNA, so the fluorescence signal can also arise from non-specific dsDNA and potentially lead to false positive results. The amount of non-specific dsDNA present in the reaction will, however, be relatively small within 35 cycles. The effective limits of detection for this system are therefore limited to reactions that reach the threshold fluorescence level within these number of cycles.

Figure 4A-C shows the fluorescence emission spectrum from the microfluidic PCR before and after amplification using an Ocean Optics spectrometer. Fluorescence measurements within the microfluidic PCR chip were also made at 520nm using the lock-in fluorescence detector are shown in Figure 4 D. These results reveal that there is a significant fluorescence signal produced after amplification of the samples for each of the three amplicons using the microfluidic PCR device which suggests that the target PCR amplicon was correctly amplified. Confirmation of amplification of the appropriately sized fragment was carried out by analysis using gel electrophoresis. Samples were taken out from both the microfluidic PCR chip and a 9700 GeneAmp thermal cycler (Applied Science, UK) after amplification and applied on the agarose gel. Figure 5 A–C shows that the same amplicons are identically amplified in both systems and Figure 5D shows confirmation of differences in sizes for the three amplicons on a single gel using the standard 9700 GeneAmp thermal cycler. Fluorescence detection has the advantage over agarose gel electrophoresis since it is difficult to visualise amplicons below 100 bp using gel electrophoresis but these can be relatively easily detected by fluorescence dye. Moreover, the

fluorescence intensity and sensitivity will increase in proportion to the size of the amplicons [32].

The shunting microfluidic PCR described here is intended to be inexpensive, portable and easy to operate with fast amplification rates. The microfluidic PCR device has a simple design so that it can be manufactured in high volume as a low cost disposable polymer device which avoids having to reuse the device and reduce the possibility of contamination. The most expensive element of the cost will be the assay reagents and we estimate that the overall cost per assay could be under £10, reducing cost with a higher volume of devices. The power consumption of the thermal cycler is small so that this can be developed as a battery supplied system for portable pathogen diagnostics. The repeatability and reproducibility of our developed system was demonstrated against a standard thermal cycler using gel electrophoresis assay to confirm the primers sizes. The utility of our system is that it can also be extended to other organisms and incorporate alternative fluorophores.

4.0 Conclusion

We have described a shunting microfluidic PCR device for DNA amplification with fluorescence detection and have demonstrated this for three different primer sets targeting the non-pathogenic K-12 subtype of *Escherichia coli* (*E. coli*). The system was capable of performing PCR amplification with different annealing temperatures ranging from 54 to 68 °C and amplifying three different sizes of PCR products of 250, 552 and 1500 bp. A low cost lock-in photodetector for fluorescence detection was found to have an LOD of 75pM FITC and 0.7 ng μl^{-1} of dsDNA at 95% confidence. The system was used for measuring the intensity of the fluorescence signal resulting from the interaction between the fluorescent dyes/probes and an increasing amount

of dsDNAs. The developed approach has high sensitivity and has the potential to quantify PCR products in real-time without performing slab gel or capillary electrophoresis. The shunting microfluidic PCR device allows for the development of low cost, easy to use and portable instrument for bacterial detection.

REFERENCES

- [1] S. O'Sullivan, Z. Ali, X. Jiang, R. Abdolvand, M.S. Ünlü, H.P. Da Silva, J.T. Baca, B. Kim, S. Scott, M.I. Sajid, S. Moradian, H. Mansoorzare, A. Holzinger, Developments in transduction, connectivity and AI/machine learning for point-of-care testing, *Sensors* (Switzerland). 19 (2019) 1–31. doi:10.3390/s19081917.
- [2] C. Zhang, D. Xing, Miniaturized PCR chips for nucleic acid amplification and analysis: latest advances and future trends, *Nucleic Acids Res.* 35 (2007) 4223–4237. doi:10.1093/nar/gkm389.
- [3] H. Nagai, Y. Murakami, Y. Morita, K. Yokoyama, E. Tamiya, Development of A Microchamber Array for Picoliter PCR, *Anal. Chem.* 73 (2001) 1043–1047. doi:10.1021/ac000648u.
- [4] Y. Matsubara, K. Kerman, M. Kobayashi, S. Yamamura, Y. Morita, E. Tamiya, Microchamber array based DNA quantification and specific sequence detection from a single copy via PCR in nanoliter volumes, *Biosens. Bioelectron.* 20 (2005) 1482–1490. doi:10.1016/j.bios.2004.07.002.
- [5] Y.-C. Lin, M.-Y. Huang, K.-C. Young, T.-T. Chang, C.-Y. Wu, A rapid micro-polymerase chain reaction system for hepatitis C virus amplification, *Sensors Actuators B Chem.* 71 (2000) 2–8. doi:10.1016/S0925-4005(00)00558-X.
- [6] E.T. Lagally, I. Medintz, R.A. Mathies, Single-Molecule DNA Amplification and

- Analysis in an Integrated Microfluidic Device, *Anal. Chem.* 73 (2001) 565–570.
doi:10.1021/ac001026b.
- [7] J. Yang, Y. Liu, C.B. Rauch, R.L. Stevens, R.H. Liu, R. Lenigk, P. Grodzinski, High sensitivity PCR assay in plastic micro reactors, *Lab Chip.* 2 (2002) 179.
doi:10.1039/b208405h.
- [8] B.C. Giordano, J. Ferrance, S. Swedberg, A.F.R. Hühmer, J.P. Landers, Polymerase Chain Reaction in Polymeric Microchips: DNA Amplification in Less Than 240 Seconds, *Anal. Biochem.* 291 (2001) 124–132.
doi:10.1006/abio.2000.4974.
- [9] D.-S. Lee, S.H. Park, H. Yang, K.-H. Chung, T.H. Yoon, S.-J. Kim, K. Kim, Y.T. Kim, Bulk-micromachined submicroliter-volume PCR chip with very rapid thermal response and low power consumption Electronic supplementary information (ESI) available: details of numerical simulation. See <http://www.rsc.org/suppdata/lc/b3/b313547k/>, *Lab Chip.* 4 (2004) 401.
doi:10.1039/b313547k.
- [10] Q. Zou, Y. Miao, Y. Chen, U. Sridhar, C.S. Chong, T. Chai, Y. Tie, C.H.L. Teh, T.M. Lim, C. Heng, Micro-assembled multi-chamber thermal cycler for low-cost reaction chip thermal multiplexing, *Sensors Actuators A Phys.* 102 (2002) 114–121. doi:10.1016/S0924-4247(02)00384-9.
- [11] T.M.-H. Lee, M.C. Carles, I.-M. Hsing, Microfabricated PCR-electrochemical device for simultaneous DNA amplification and detection, *Lab Chip.* 3 (2003) 100. doi:10.1039/b300799e.
- [12] M.U. Kopp, Chemical Amplification: Continuous-Flow PCR on a Chip, *Science* (80-.). 280 (1998) 1046–1048. doi:10.1126/science.280.5366.1046.
- [13] P.J. Obeid, T.K. Christopoulos, H.J. Crabtree, C.J. Backhouse, Microfabricated

- Device for DNA and RNA Amplification by Continuous-Flow Polymerase Chain Reaction and Reverse Transcription-Polymerase Chain Reaction with Cycle Number Selection, *Anal. Chem.* 75 (2003) 288–295. doi:10.1021/ac0260239.
- [14] M. Hashimoto, P.-C. Chen, M.W. Mitchell, D.E. Nikitopoulos, S.A. Soper, M.C. Murphy, Rapid PCR in a continuous flow device, *Lab Chip.* 4 (2004) 638. doi:10.1039/b406860b.
- [15] J. Liu, M. Enzelberger, S. Quake, A nanoliter rotary device for polymerase chain reaction, *Electrophoresis.* 23 (2002) 1531. doi:10.1002/1522-2683(200205)23:10<1531::AID-ELPS1531>3.0.CO;2-D.
- [16] X. Jiang, N. Shao, W. Jing, S. Tao, S. Liu, G. Sui, Microfluidic chip integrating high throughput continuous-flow PCR and DNA hybridization for bacteria analysis, *Talanta.* 122 (2014) 246–250. doi:10.1016/j.talanta.2014.01.053.
- [17] H. Tachibana, M. Saito, K. Tsuji, K. Yamanaka, L.Q. Hoa, E. Tamiya, Self-propelled continuous-flow PCR in capillary-driven microfluidic device: Microfluidic behavior and DNA amplification, *Sensors Actuators B Chem.* 206 (2015) 303–310. doi:10.1016/j.snb.2014.09.004.
- [18] M. Guarnaccia, R. Iemmolo, S. Petralia, S. Conoci, S. Cavallaro, Miniaturized Real-Time PCR on a Q3 System for Rapid KRAS Genotyping, *Sensors.* 17 (2017) 831. doi:10.3390/s17040831.
- [19] S. Harris, D.B. Jones, Optimisation of the polymerase chain reaction, *Br. J. Biomed. Sci.* 54 (1997) 166–173. <https://www.scopus.com/inward/record.uri?eid=2-s2.0-0030839566&partnerID=40&md5=f82e837ca9d6f8ff9feabb3a6018a465>.
- [20] G. Hu, Q. Xiang, R. Fu, B. Xu, R. Venditti, D. Li, Electrokinetically controlled real-time polymerase chain reaction in microchannel using Joule heating

- effect, *Anal. Chim. Acta.* 557 (2006) 146–151. doi:10.1016/j.aca.2005.10.021.
- [21] N.C. Cady, S. Stelick, M. V Kunnnavakkam, C.A. Batt, Real-time PCR detection of *Listeria monocytogenes* using an integrated microfluidics platform, *Sensors Actuators, B Chem.* 107 (2005) 332–341. doi:10.1016/j.snb.2004.10.022.
- [22] M.G. Murray, W.F. Thompson, Rapid isolation of high molecular weight plant DNA, *Nucleic Acids Res.* 8 (1980) 4321–4326. doi:10.1093/nar/8.19.4321.
- [23] G. Pasirayi, S.M. Scott, M. Islam, L. O'Hare, S. Bateson, Z. Ali, Low cost microfluidic cell culture array using normally closed valves for cytotoxicity assay, *Talanta.* 129 (2014) 491–498. doi:10.1016/j.talanta.2014.06.020.
- [24] H. Becker, C. Gärtner, Polymer microfabrication technologies for microfluidic systems, *Anal. Bioanal. Chem.* 390 (2008) 89–111. doi:10.1007/s00216-007-1692-2.
- [25] S. Bateson, A USB high resolution lock-in photometer, *AIMS Electron. Electr. Eng.* 3 (2019) 1–15. doi:10.3934/ElectrEng.2019.1.1.
- [26] J.B. Edel, N.P. Beard, O. Hofmann, J.C. DeMello, D.D.C. Bradley, A.J. DeMello, Thin-film polymer light emitting diodes as integrated excitation sources for microscale capillary electrophoresis, *Lab Chip.* 4 (2004) 136. doi:10.1039/b313503a.
- [27] B. Yao, G. Luo, L. Wang, Y. Gao, G. Lei, K. Ren, L. Chen, Y. Wang, Y. Hu, Y. Qiu, A microfluidic device using a green organic light emitting diode as an integrated excitation source, *Lab Chip.* 5 (2005) 1041. doi:10.1039/b504959h.
- [28] Y.-H. Kim, K.-S. Shin, J.-Y. Kang, E.-G. Yang, K.-K. Paek, D.-S. Seo, B.-K. Ju, Poly(dimethylsiloxane)-Based Packaging Technique for Microchip Fluorescence Detection System Applications, *J. Microelectromechanical Syst.* 15 (2006) 1152–1158. doi:10.1109/JMEMS.2006.880355.

- [29] A. Banerjee, Y. Shuai, R. Dixit, I. Papautsky, D. Klotzkin, Concentration dependence of fluorescence signal in a microfluidic fluorescence detector, *J. Lumin.* 130 (2010) 1095–1100. doi:10.1016/j.jlumin.2010.02.002.
- [30] M.H. Tsai, M.H. Saier, Phylogenetic characterization of the ubiquitous electron transfer flavoprotein families ETF- α and ETF- β , *Res. Microbiol.* 146 (1995) 397–404. doi:10.1016/0923-2508(96)80285-3.
- [31] A. Felske, A. Wolterink, R. Van Lis, A.D. Akkermans, Phylogeny of the main bacterial 16S rRNA sequences in Drentse A grassland soils (The Netherlands), *Appl. Environ. Microbiol.* 64 (1998) 871–9. <http://www.ncbi.nlm.nih.gov/pubmed/9501427>.
- [32] F. Debode, A. Marien, É. Janssen, C. Bragard, G. Berben, The influence of amplicon length on real-time PCR results , *Biotechnol. Agron. Soc. Environ.* 21 (2017) 3–11. <https://www.scopus.com/inward/record.uri?eid=2-s2.0-85015873617&partnerID=40&md5=68b659d0f5ade34cd1ea2048748e0eef>.

Table 1 A summary of the nucleotide sequences of the oligonucleotides used and their target genes.

Primer used	Nucleotide sequence	Amplicon size (bp)	Target gene
Primer set 1:Forward	5`-CGCCCGCCGCGCCCCCGCCCGG CCCGCCGCCCCCGCCCACTCCTACG GGA GGCAGC-3`	250	16S rRNA gene V3 region
Reverse	5`-GTATTACCGCGGCTGCTG-3`		
Primer set 2:Forward	5`-AGCAACAGGCAGCAGAGGCG-3`	552	flavoprotein FixA in (<i>E Coli</i>)
Reverse	5`-GACGTTTCGCGCTGTTTCGGC-3`		
Primer set 3:Forward	5`GTTTGATCCTGGCTCAG-3`	1500	Near complete 16S rRNA. Gene
Reverse	5`GGTTACCTTGTTACGACTT-3`		

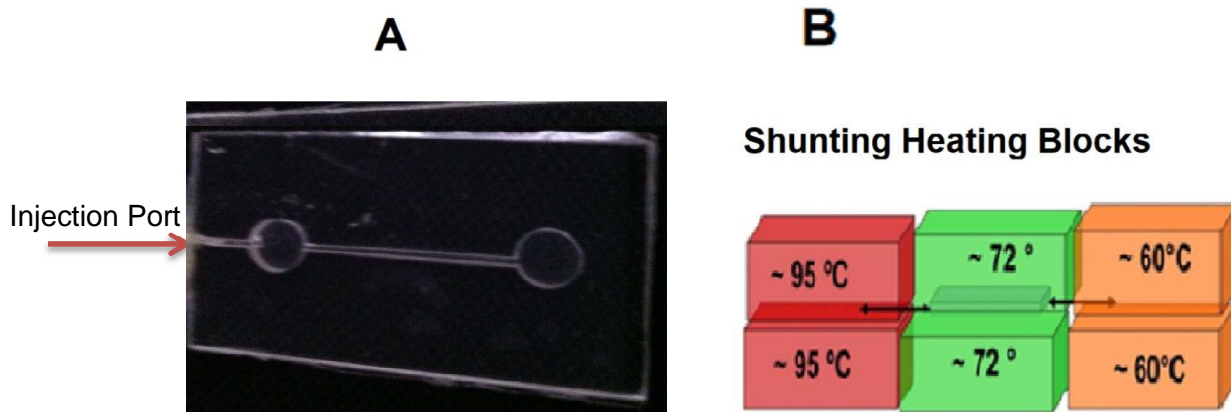


Figure 1 **A:** PCR microfluidic device. **B:** Schematic showing PCR microfluidic device, with PCR reaction mix, shuttling between three constantly heated regions.

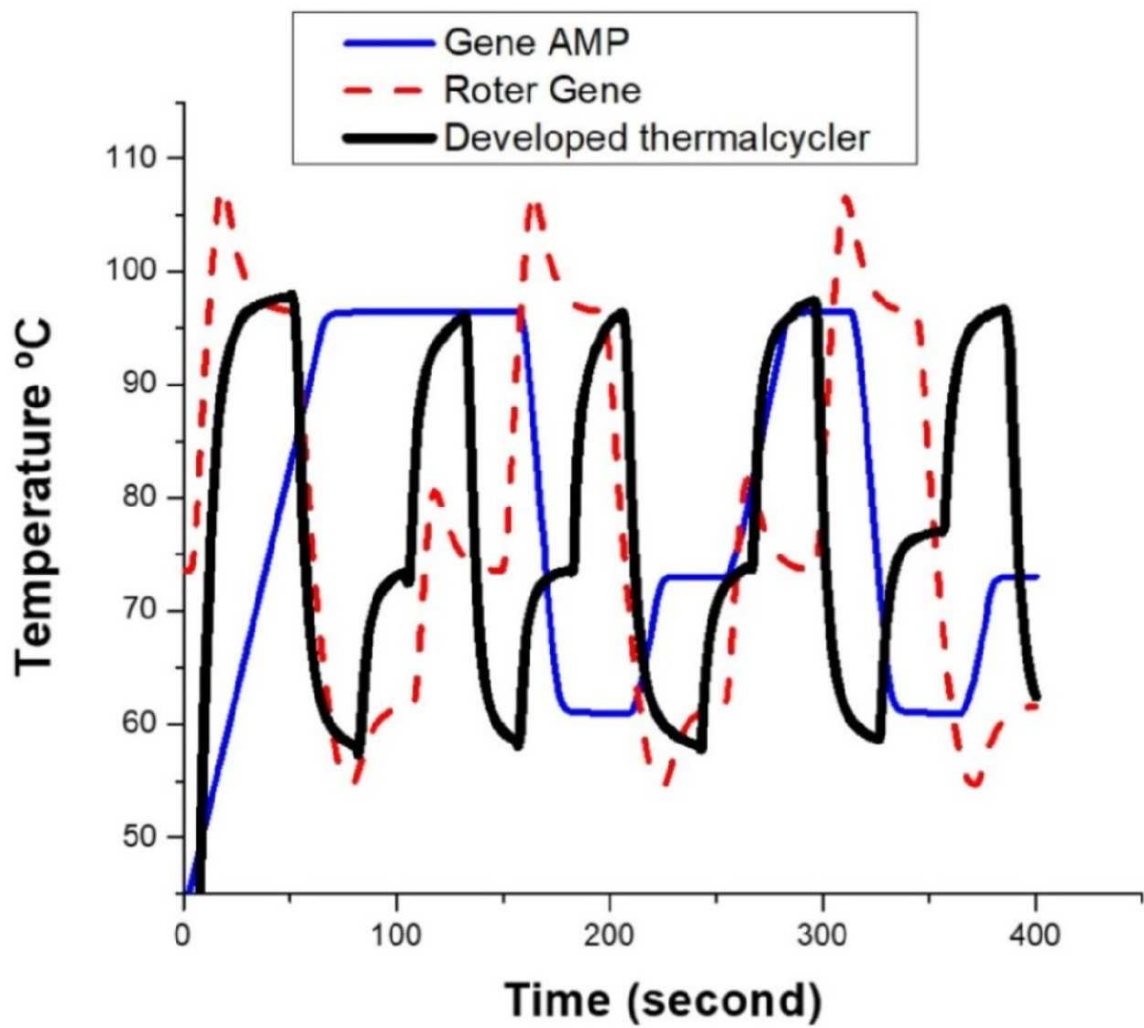


Figure 2 Comparison of heating profiles of GeneAmp and Roter Gene with the developed thermal cycler device, where each cycle of PCR in the can be completed in 60 second (10 sec denature, 20 sec anneal, 30 sec extend).

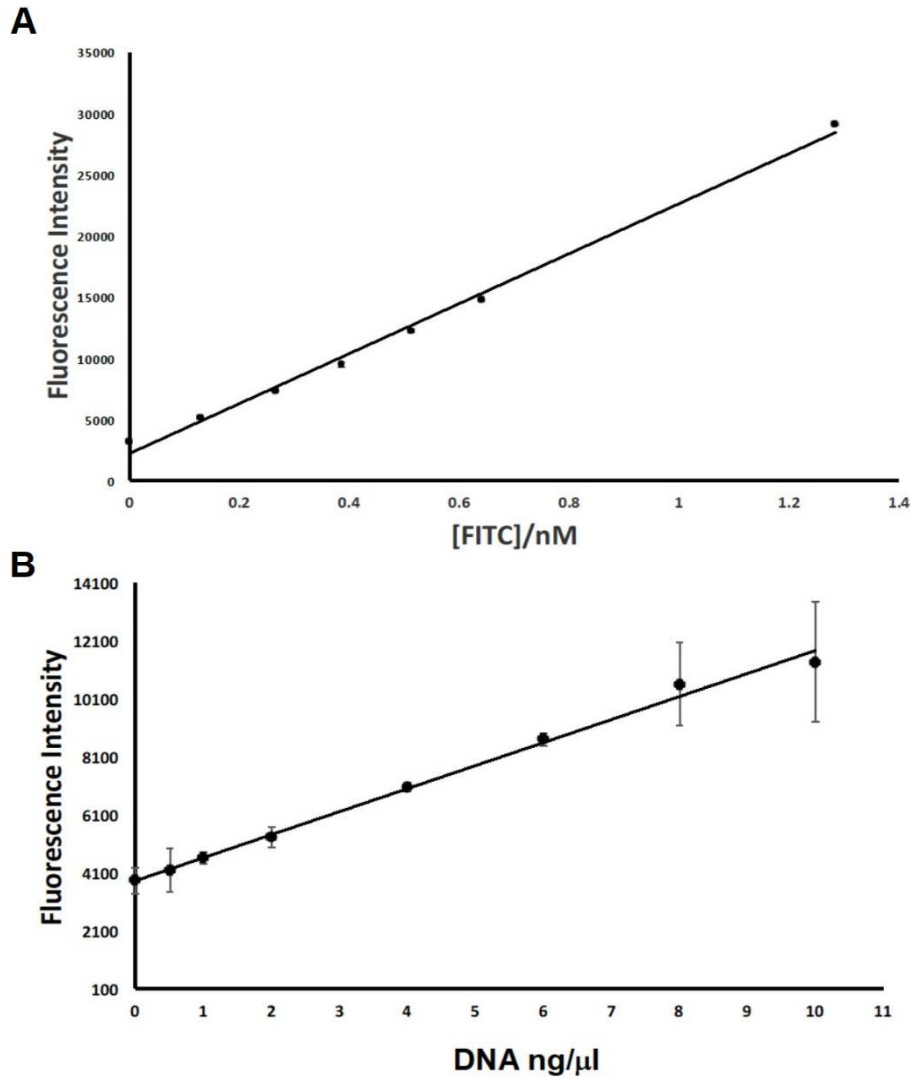


Figure 3 Fluorescence intensity measurements within the microfluidic PCR device with the developed lock-in photodetector system. **A:** Varying concentrations of FITC giving an LOD of 75pM for FITC and an equation of line of $y = 20409x + 2223$ and $R^2 = 0.995$ **B:** Varying dsDNA using QuantiFlour with an LOD of $0.7 \text{ ng } \mu\text{l}^{-1}$ of dsDNA and an equation of line of $y = 793x + 3821$ and $R^2 = 0.9942$.

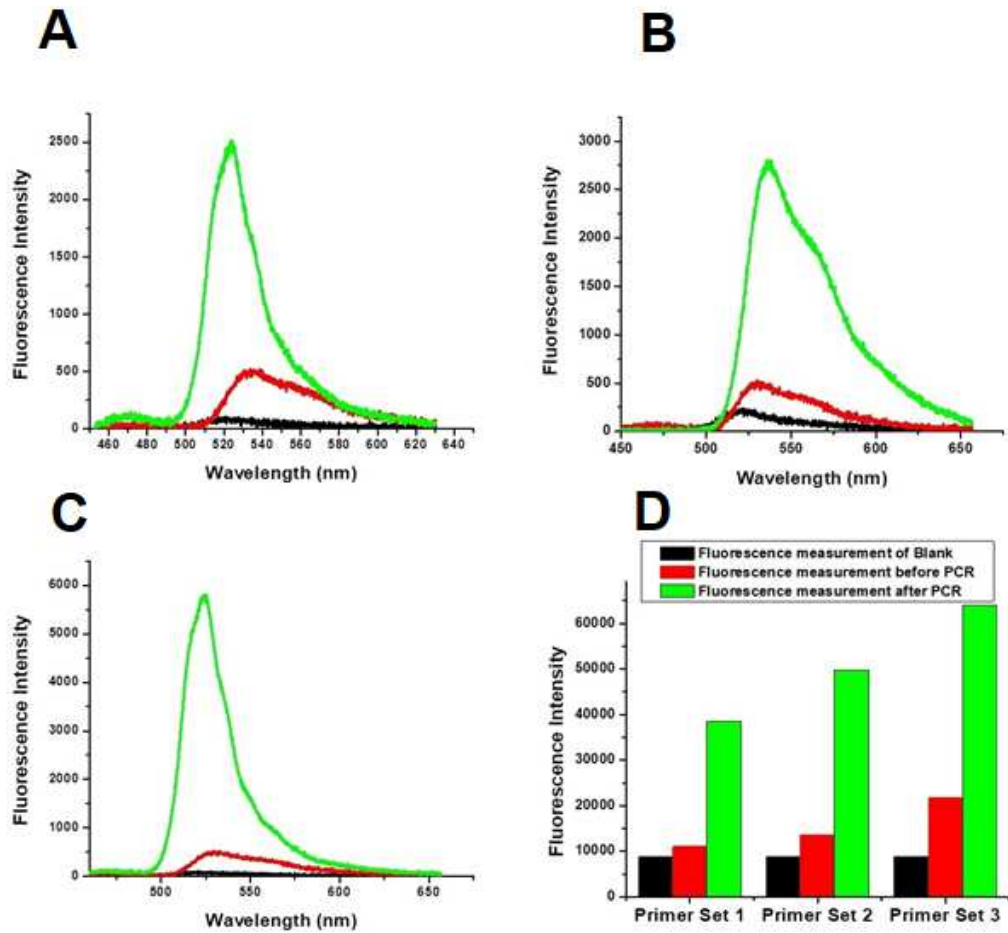


Figure 4 Fluorescence measurements from microfluidic PCR device using blue LED and HR4000 spectrometer with 100 μ m fibre optic of PCR mix along with SYBR Green after sample amplification for **(A)** 250bp amplicon **(B)** 552 bp amplicon **(C)** 1500 bp amplicon and for **(D)** primer sets 1,2 and 3 as in Table 1.

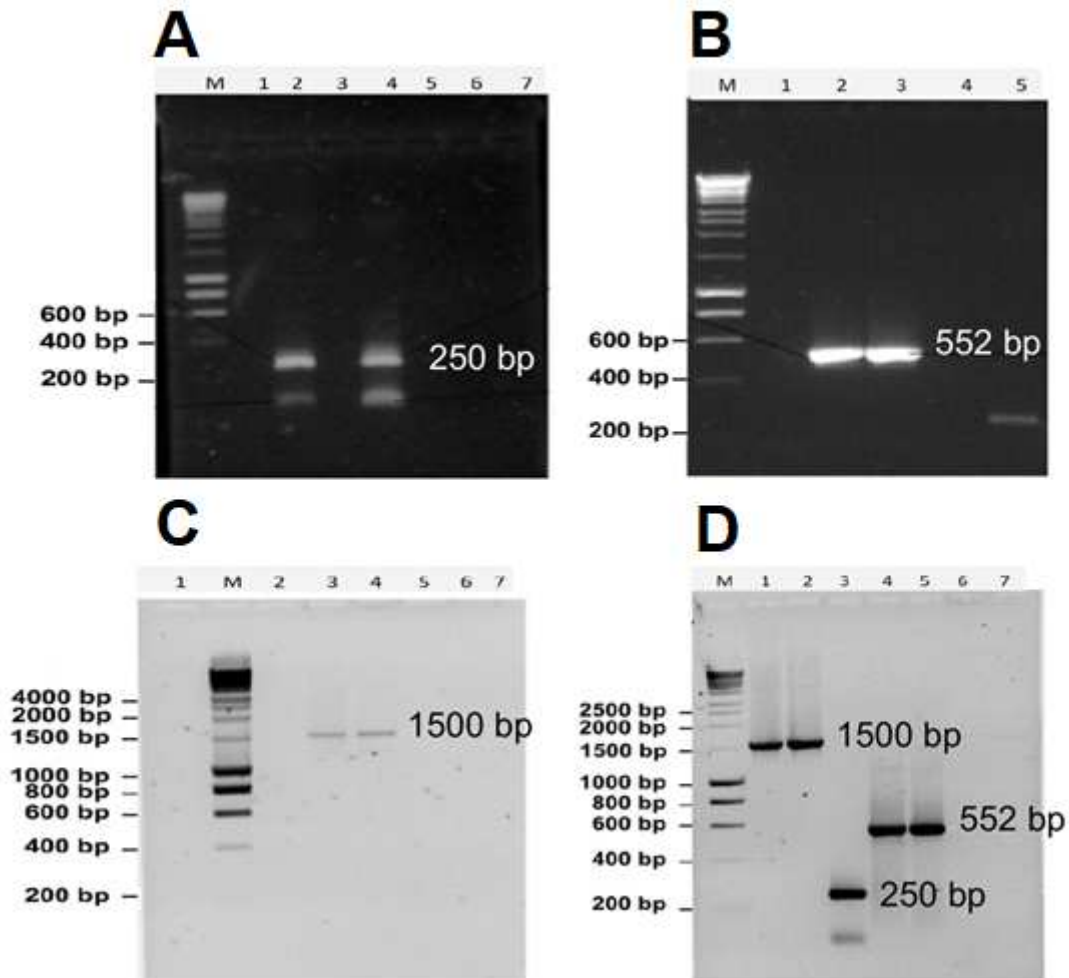


Figure 5 Sample resolutions on 1.5 % (w/v) of agarose gel electrophoresis at 90V for 80 minutes 12.5 μ l of PCR products were placed in each lane. The Lane M in **A-D** contains Hyperladder 1 (Bioline, UK). For **A-C** comparison is made of standard thermal cycler (GeneAmp 9700, Applied Science, UK) and the developed shunting PCR device. **A**: amplified amplicon size 250 bp with Lane 2 for standard thermal cycler and Lane 4 for the developed shunting PCR device **B**: amplified amplicon size is 552 bp with Lane 2 for the standard thermal cycler and Lane 3 for developed shunting PCR device **C**: amplified amplicon size 1500 bp where Lane 3 uses the standard thermal cycler and Lane 4 for the developed shunting PCR device. **(D)** Lane 1 and 2: amplicon size 1500 bp, lane 3: amplicon size 250 bp, lane 4 and 5: amplicon size 552 bp of DNA lane 6 and 7 Negative controls, all amplified using standard thermal cycler.

Highlights

- ❖ Novel PCR device comprising: double side shunting thermal cycler; polycarbonate microfluidic PCR chip and lock-in photodetector for fluorescence detection.
- ❖ Rapid amplification was observed with heating and cooling rates of 1.8 °C/s and 2 °C/s respectively.
- ❖ Lock-in photodetector with an LOD of 75 pM for FITC and 0.7 ng μl^{-1} of dsDNA using Quan-iT dsDNA high sensitivity kit assay.
- ❖ PCR device was validated for amplification of 250, 552 and 1500 bp fragments using universal primers based on chromosomal DNA extracted from non-pathogenic K-12 subtype of *Escherichia coli* (E.coli).