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Incorporating asymmetric PCR and microarray hybridization protocols onto an integrated microfluidic device, screening for the *Escherichia coli* ssrA gene

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ABSTRACT

We present a microfluidic cartridge system capable of implementing Nucleic Acid (NA) *in-vitro* amplification followed by microarray fluorescence detection. System functionality was verified by implementing Polymerase Chain Reaction (PCR), followed by hybridization, targeting the *Escherichia coli* ssrA gene. The denaturation temperature was reduced from 95 °C to 85 °C and incorporated a modified primer ratio (10:1,forward:reverse). This two-step asymmetric PCR protocol addressed microfluidic evaporation, bubble formation and amplicon re-annealing prior to hybridisation. The PCR extension step was removed, shortening the overall amplification time. The cartridge control system implemented sample heating, fluorescence detection and fluidic actuation. The microarray incorporated silver nanoparticles for enhanced fluorescence detection by localised surface plasmon resonance (LSPR), demonstrating a 0.2 nM target DNA detection limit. The cartridge manufacture process incorporated elastomeric "pinch" valves eliminating the need for flexible membrane layers. The valves were robust to thermo-pneumatic pressure generated during thermocycling, with a leak pressure of 340 kPa.

1. Introduction

Advances in microfluidics, biochemistry and nanotechnologies have facilitated diagnostic test transfer from the laboratory to clinical settings [1] (e.g.BiofireDX,Alere-I). Microfluidics facilitates biochemical tests using microliter volumes, while nanoparticles enhance detection and emerging biochemistries (e.g. NASBA–Nucleic Acid Specific Base Amplification) are fluidic friendly, achieving rapid low temperature amplification [2]. However, isothermal amplification techniques (e.g. NASBA) have complex enzymatic steps [2], requiring additional microfluidic chambers and valves. Quantitative PCR (qPCR) is a popular detection approach with point of care (POC) molecular diagnostic systems. PCR biochemistry is well established and extensively applied to genomic [3–5] and disease monitoring [6–8] applications. The challenge for POC systems is implementing full "sample in answer out", protocols on a single microfluidic device. Systems have been reported using individual devices for sample preparation [9–11] and NA detection [12–15]. A proof of principle instrument [16,17], demonstrated RNA extraction followed by NASBA amplification on separate microfluidic modules. While buccal swab sample lysis followed by qPCR was also demonstrated on separate devices [18]. A complete "sample in answer out" fluidic device, generated genetic profiles from whole blood samples [19], incorporating lysis, PCR amplification and capillary electrophoresis (CE). Multiple amplification chambers [16,17] are required for multiplex qPCR detection, while microfluidic hybridisation [20,21] offers increased multiplex capacity without increased fluidic complexity. With real-time assays, limited label combinations are available for spectral multiplexing due to filter combinations and fluorophore crosstalk.

A challenge for realtime amplification assays is multiplex capacity, conventionally for infectious disease screening, primer design targets pathogen strains of interest, indicating a positive/negative result. However to identify numerous pathogens & strains in a single test, multiple multiplex amplification reactions are required, a limitation for point of care tests due to increased fluidic complexity. With inherited disease screening, mutations caused by numer-

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ous combinations of single nucleotide polymorphisms, require 100′s or 1000′s of targets to be identified. In point of care testing, incorporating microarray detection enhances multiplex capacity, reduces microfluidic complexity (e.g. sample splitting/aliquoting) while delivering rapid results. Assay controls are also readily incorporated into microarrays, with additional oligonucleotide probes targeting specific housekeeping genes. With realtime amplification, additional primers/beacons are incorporated into each tube requiring at least duplex reactions. From an instrumentation perspective limitations around fluorophore combinations due to filters and dye crosstalk, restricts spectral multiplex capacity and requires spatial multiplexing. The possibility to incorporate high density microarray detection with target amplification, is an interesting alternative to realtime amplification point of care tests.

In this work, a two-step asymmetric PCR protocol, followed by hybridisation was implemented within a microfluidic cartridge. The PCR protocol implemented denaturation and annealing with the former temperature lowered from 95 °C to 85 °C, reducing bubble formation and evaporation. Shortened denaturation and annealing times (30 s) reduced amplification time below fifty minutes. Asymmetric PCR (10:1,forward:reverse primers), addressed amplicon re-annealing prior to hybridisation, removing the need for sample fragmentation. Low temperature PCR has previously been reported [22], to generate high fidelity DNA (Human Papillomavirus) using 85 °C denaturation. While aptamer production was implemented with single stranded DNA (ssDNA) generated by asymmetric PCR [23]. However, combining low temperature denaturation with asymmetric PCR in a microfluidic device, hasn't previously been reported. Few examples of microfluidic asymmetric PCR are reported, electrochemical microarray detection was used to distinguish between *E.coli* and *Bacillus subtilis* [24,25], where asymmetric PCR product was more efficiently modified for electrochemical labelling [26]. In this work, to achieve improved fluorescence signal, the microarray was printed on a silver nanoparticle (160 nm) substrate, delivering enhanced sensitivity through LSPR [27–30]. Where microarray surface to dye molecule separation is <20 nm, optimised resonance energy transfer occurs from metal to fluorophore, minimising background interference [28]. Metal nanoparticles have previously been incorporated into microfluidic devices enhancing fluorescence detection [31–33].

Typically integrated microfluidic valves are deflected membrane [34,35] or phase change configurations [18,36,37], requiring additional complexity in microfluidic design, manufacture and instrumentation. Soft lithography has improved multilayer membrane valve manufacturability [38], with specific application demonstrated for DNA synthesis [39]. While integrated torque valves, were demonstrated on a device screening nasal samples for respiratory viruses [40]. Multilayer soft lithography has been used to define pinch valve structures within pneumatically activated valve networks [41]. Where individual valve channels are manufactured in PDMS, then aligned and sealed to fluidic channels. Commercially available "duckbill" check valves were incorporated into a microfluidic device for rapid DNA forensic analysis [42], for sample loading and directional flow control, while paraffin phase change valves were used to seal the PCR chamber for thermocycling. However these approaches require multiple layers incorporated during cartridge manufacture. We adapt a valve approach using integrated elastomeric pinch valves. These are formed after fluidic cartridge assembly, realising a flexible round channel profile for optimal sealing compared to conventional square channels. This eliminated multi-layer fluidic structures defining valves, channels and interconnects allowing the fluidic design to be manufactured in a single layer. Multiple valve actuations (>100 times) demonstrated reliable performance and were robust to thermo-pneumatic pressures generated during PCR thermocycling.

2. Materials and methods

2.1. Microfluidic device fabrication

The microfluidic cartridge was injection moulded using Polypropylene (GoodFellows UK, Ltd.) on a Babyplast 6/10P machine (Babyplast; Molteno, LC, Italy), pellets were dried for sixty minutes at 55 °C. The mould system parameters were set for polypropylene: Injection nozzle 195 °C, injection chamber 210 °C, plastic melt chamber 220 °C, with three second injection at 60 bar. The manufactured components were cleaned as follows: (i) forty minutes at 50 °C in an ultrasonic bath (10% methanol) (ii) forty minutes at 50 °C in an ultrasonic bath (0.1% TWEEN 20), (iii) forty minutes at 20 °C in an ultrasonic bath (de-ionised water) and (iv) ten minute ozone clean (ProCleaner™ BioForce Nanoscience, UT). The microfluidic component was designed using computer-aided design (CAD) software (Solid Edge; Siemens, Plano, TX) and the injection mould was milled in brass using a Computer Numerical Control (CNC) milling machine (Bridgeport GX 480 VMC;Elmira,NY). The chamber dimensions were 500 μm deep, 4 mm wide and 15 mm long. The design tapered from 4 mm to 500 μm wide at the inlet/outlet channels. The pinch valves were located upstream and downstream of each chamber (PCR and hybridization). To ameliorate DNA or enzyme adsorption, fluidic channels and chambers were coated with Polyethylene glycol (PEG) (Sigma Aldrich) (0.5% PEG in methanol). The chambers were filled

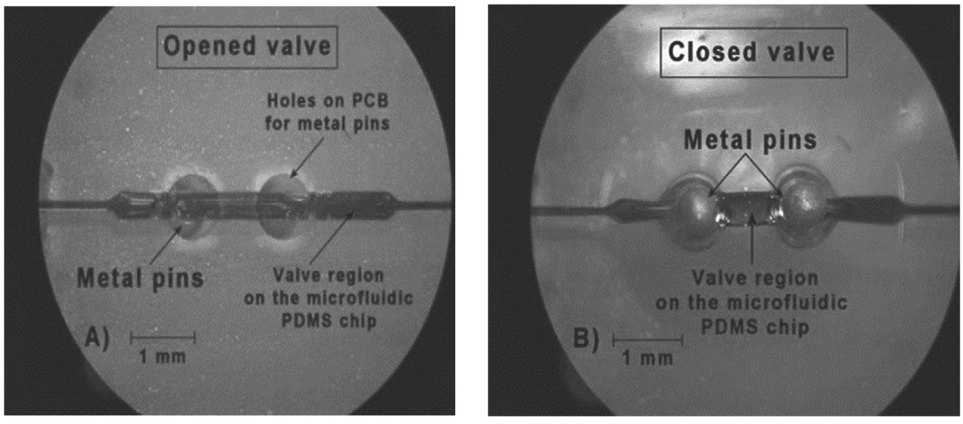

Fig. 1. The pinch valve structure is formed by curing a silicone plug around a capillary, forming the fluidic pinch valve. The open valve (A) is achieved by solenoid/pin release. The valve is closed (B) by compressing the valve with solenoid activated metal pins.