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Real-time PCR detection of *Listeria monocytogenes* using an integrated microfluidics platform

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Abstract

A miniaturized, fully automated, PCR-based detection system has been developed for the rapid detection of bacterial pathogens. Monolithic DNA purification/real-time PCR silicon chips were fabricated and tested for their ability to purify and detect the pathogeneic bacterium *Listeria monocytogenes*. Using silica-coated microstructures, nucleic acids could be selectively bound, washed and eluted for subsequent real-time PCR. These microstructures were included in an integrated detection microchip containing two distinct regions, one for DNA purification and one for real-time PCR. Using an automated detection platform with integrated microprocessor, pumps, valves, thermocycler and fluorescence detection modules, microchips were used to purify and detect bacterial DNA by real-time PCR amplification using SYBR Green fluorescent dye. Between 10^4 and 10^7 *L. monocytogenes* cells could be detected using this system with an average turnaround time of 45 min.

Keywords: Biosensors; DNA; Purification; Detection; Real-time PCR; Microchip; Microfabrication

1. Introduction

In the past decade there has been an increased demand for rapid and accurate methods of detecting pathogenic bacteria, viruses and other disease-causing agents. In response to these demands, biosensors have been developed utilizing a variety of existing semiconductor processing strategies. The resulting devices, collectively known as lab-on-a-chip devices, incorporate multiple laboratory processes in a semi-automated, miniaturized format [14,16,17]. Many of these devices utilize the polymerase chain reaction (PCR) which is relatively robust, however, a variety of contaminants can inhibit amplification and diminish the success of such analytical instruments [29]. In order to circumvent this problem, DNA must be extracted and purified from a sample through a variety of lysis protocols and purification techniques [2,20,21,32]. In a previous study, our laboratory demonstrated effective bacterial cell lysis and DNA purification in a microfabricated silicon chip [4]. Using a guanidinium thiocyanate-based lysis buffer, DNA was extracted from bacteria and selectively bound to silica-coated microstructures. After a series of washing steps, the purified DNA was then eluted from the chip and utilized for PCR amplification.

In order to extend the utility of chip-based DNA purification, we have developed an integrated DNA purification and real-time PCR-based biosensor that is the subject of the research presented here. Successful chip-based DNA purification and PCR requires not only manufacturing of the detection microchips, but also development of a platform to perform the necessary thermal cycling, fluorescent measurement and fluid control systems. In previous studies, several strategies have been used to fulfill these requirements. For PCR thermal cycling, groups have employed multiple tech-

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niques, including infrared light [7], thermoelectric heater-coolers [15], and resistive electrodes [27]. In addition to changing the temperature of the entire reaction chamber, other methods have used so-called "flow-through" PCR in which the sample is passed through different thermal regions on the chip [27,28]. Moving fluids through micro analytical devices has also been a challenge. For bench-top applications, precise fluid control is often achieved with syringe pumps due to their high precision and ease of use. In addition to syringe pumps, multiple groups have reported on the use of electroosmotic pumps [13,17,22,26], miniaturized peristaltic pumps [30] and thermally driven pumps [35]. Electroosmotic pumps are intrinsically simple with few moving parts, but are highly dependent upon the geometry of the microchannels [22] and the chemical composition of the fluid to be pumped [3,19,30]. Both thermal and electroosmotic pumps are subject to bubble formation from thermal and electrolytic effects, respectively [30]. Bubbles scatter light and can reduce the sensitivity of an instrument relying on optical detection. Miniaturized peristaltic pumps offer an alternative pumping strategy, but require complicated gas control systems for actuating the microfluidic valves [30]. These systems can be overly cumbersome for integration into a portable detection system.

In the field of fluorescence detection, there have been relatively few reports of miniaturized excitation and emission sources for microchip devices. Most devices utilize bulky, bench-top excitation sources, including lasers and mercury lamps [9,14,16,31]. In addition, detection has commonly been accomplished with microscope-based CCD cameras or other large instruments that severely inhibit portability [9,14,16]. In contrast to these larger systems, light emitting diodes (LEDs) have been used as excitation sources, combined with miniaturized detectors such as photodiodes and miniaturized photomultiplier tubes [5,8]. Dasgupta et al. [5] reported on such an LED-based system for fluorescence excitation which has similarities to the detection system used in our device. Because of its low power requirements, LED-based excitation is highly useful for portable analytical devices.

We report here on the construction of a miniaturized lab-on-a-chip for the detection of the food pathogen *Listeria monocytogenes*. *L. monocytogenes* is a Gram positive bacterium that has been responsible for several food-related outbreaks in the past decade. Although *L. monocytogenes* is rarely lethal to healthy adults, it is highly virulent in the elderly, newborns, immunocompromised individuals and pregnant women [6]. Because this organism is a current threat to food safety, it is an ideal organism to use for model studies of the miniaturized detection system described here. Previous studies have demonstrated real-time PCR-based detection of *L. monocytogenes* using stationary laboratory equipment with high accuracy and sensitivity, providing detection limits as low as 50 cells [1,12,23–25]. We have incorporated these detection methods into a miniaturized device that is capable of both purifying DNA from intact *L. monocytogenes* cells and performing real-time PCR-based detection. This device is intended as a proof-of-concept system to demonstrate bacterial detection in a miniaturized format. The small size of the components, as well as its low power requirements make this system an ideal candidate for further miniaturization into a hand-held, point-of-care device.

2. Experimental

2.1. Reagents

Phosphate buffered saline (PBS), pH 7.4, guanidinium isothiocyanate (GuSCN), 70% ethanol (EtOH), ethylenediaminetetraacetic acid (EDTA), Sigmacote, Triton X-100, Tris (Trizma base), and SYBR Green JumpStart Taq ReadyMix, were obtained from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) 10 mg/ml and bacteriophage Lambda DNA (500 μg/ml) were obtained from New England Biolabs (Beverly, MA). SureStart Taq DNA polymerase (5U/μl) was obtained from Stratagene (La Jolla, CA). BioMix PCR master mix and Hyperladder I DNA ladder were obtained from Bioline (Randolph, MA). Sylgard 184 poly(dimethyl) siloxane elastomer kits were obtained from Ellsworth Adhesives (Germantown, WI). Tryptic soy broth, brain-heart infusion, and Bacto™ agar were obtained from BD Difco (Franklin Lakes, NJ).

2.2. Bacterial growth and preparation

Listeria monocytogenes and *Bacillus globigii* cultures were grown in brain-heart infusion (BHI) and tryptic soy broth (TSB), respectively at 37 °C for 12 h and were serially diluted in PBS. Enumeration of *L. monocytogenes* and *B. globigii* was performed by plating serially diluted cultures onto BHI and tryptic soy (TSA) agar plates and determining the number of colony forming units (CFU) after 12 h incubation at 37 °C. Total DNA was extracted from 10^9 cells (1 ml) using a Qiagen (Valencia, CA) DNeasy Kit with slightly modified procedures. The cells were resuspended in 1 ml of lysis buffer L5 (4.7M GuSCN, 1% v/v Triton X-100 in pH 7.0 TE buffer) and incubated at 25 °C for 10 min. This lysate was then applied to the DNeasy microspin columns and subsequent purification steps were followed according to the manufacturer's specifications. Purified DNA was then quantified using PicoGreen fluorescent dye (Molecular Probes, Eugene, OR) in a Tecan (Durham, NC) microplate fluorometer using bacteriophage Lambda DNA as a standard. For integrated DNA purification and PCR using microfluidic chips, *L. monocytogenes* cells were first diluted in PBS to achieve various cell concentrations. Cell lysis was then achieved by mixing 90 μl of lysis buffer L5 with 10 μl of cells and incubating at room temperature for 5 min. This lysate was then pumped into the chip using the integrated syringe pumps.

2.3. PCR amplification

PCR amplification of nucleic acid targets was carried out using standard protocols. A 544-bp fragment from the *Listeria monocytogenes hlyA* gene was amplified using primers HLYP8 and HLYP4R previously reported by Norton and Batt [23]. PCR reactions consisted of 25 μl SYBR Green JumpStart Taq Ready Mix (Sigma, St. Louis, MO), 50 nmol of each primer, 1 μl template DNA, in a total volume of 50 μl. Reactions were cycled in an MJ Research thermocycler (Waltham, MA) under the following conditions: 95 °C denaturation for 5 min, 40 cycles of 95 °C for 10 s, 57 °C for 15 s, 72 °C for 20 s, followed by a 5 min extension at 72 °C. DNA amplification was confirmed by gel electrophoresis. Real-time PCR was performed on an ABI Prism 7000 real-time thermocycler (Applied Biosystems, Foster City, CA). For these experiments, various amounts of template DNA were used in the same reaction conditions as described above. Microchip-based PCR amplification was performed using the same reaction conditions and fluorescence was monitored during the 72 °C extension step of each cycle. For optimized microchip PCR, SYBR Green JumpStart Ready Mix was mixed at 1.35 times the standard concentration for a 50 μl reaction: 25 μl Ready Mix, 50 nmol each primer, 2.5 units Stratagene Sure Start Taq polymerase (La Jolla, CA), and dH_2O to a final volume of 37.5 μl.

2.4. Microchip design and fabrication

The sensor chip described here incorporates a microfabricated DNA purification chamber with a second PCR amplification chamber, connected by microfluidic channels. The DNA purification section contains an array of 10 μm square pillars that were etched 50 μm deep in silicon to form a microfluidic channel. Construction of the DNA purification region was performed as previously described [4]. The PCR amplification chamber was constructed using soft lithography techniques for poly(dimethyl siloxane) (PDMS) and SU-8 photoresist (Microchem, Newton, MA) described by Xia and Whitesides [34]. Briefly, PDMS was cured in an SU-8 mold of the PCR chamber and then bonded to a 50 μm thick PDMS membrane. Bonding was achieved by exposing both PDMS substrates to an oxygen plasma for 20 s in a Harrick Model PDC-001 (Ossinning, NY) Plasma Cleaner/Sterilizer at 200–600 mTorr with 30 W DC power applied to the RF coil. The PDMS substrates were then pressed together and baked at 60 °C for 30 min to achieve maximum bonding strength. After bonding, the PDMS structures were peeled from the wafer and were bonded to the microfabricated Si chips to seal the chambers. For fluidic connections 30 ga stainless steel tubing was inserted into holes in the PDMS and was glued in place using Miller-Stephenson 907 Epoxy (Danbury, CT). Connections between the tubing and the syringe pump were made using 0.010 in. microbore tubing (Small Parts, Miami Lakes, FL). An assembled microchip is shown in Fig. 1. Microfabrication of this device was carried out at the Cornell Nanoscale Facility (Ithaca, NY).

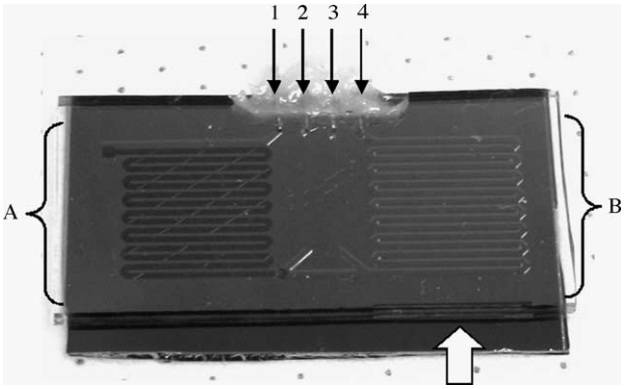

Fig. 1. An optical micrograph of the DNA purification/real-time PCR microchip is shown. The nucleic acid purification region is shown in (A) while the real-time PCR region is shown in (B). The fluid connections are (1) sample input, (2) waste outlet, (3) PCR reagent input, and (4) reaction outlet. The large white arrow denotes the lateral path for fluorescent excitation for real-time PCR.

2.5. Apparatus

A microcontroller-based instrument was built to automate fluid handling and control thermal cycling operation. The system was designed to require low power (20 W) and occupy a small footprint for future development of a portable, point-of-care device. Fig. 2A and B shows a schematic diagram of the instrument. The instrument has an electronics module consisting of a controller board and power amplifiers for driving an automatic syringe pump, a thermoelectric heater/cooler, a fluorescence excitation/emission module, and a pressure valve. During operation, the entire system is controlled by a Z-world microcontroller board (Rabbit, Davis, CA) and is programmed to carry out the amplification steps sequentially. In order to provide accurate fluid control and movement throughout the system, a multiple-channel syringe pump was designed to allow for parallel pumping of multiple fluids. A single Faulhaber AM1525-15A 102:1 HEAM152412 stepper motor (MicroMo, Clearwater, FL) actuates this pump that can drive individual syringes by selective engagement using PIC Design, Inc. RW1-333 electromagnetic clutches (Middlebury, CT). Fluid flow direction and chip pressurization are controlled by a Moog MicroValve (East Aurora, NY).

The temperature of the detection microchip is cycled by a Melcor HOT 2.1-31-F2A (Trenton, NJ) thermoelectric heater/cooler (TEC) that is, in turn, controlled by a Hytek (Carson City, NV) 5640 TEC control chip and Hytek 5670 control board. A 10 kΩ thermistor mounted on the chip measures the temperature and is used as the feedback element by the microcontroller to control cycling parameters. The Hytek 5670 control board was modified so that three separate temperature set-points could be achieved by switching between temperature set-point resistors with relay switches.

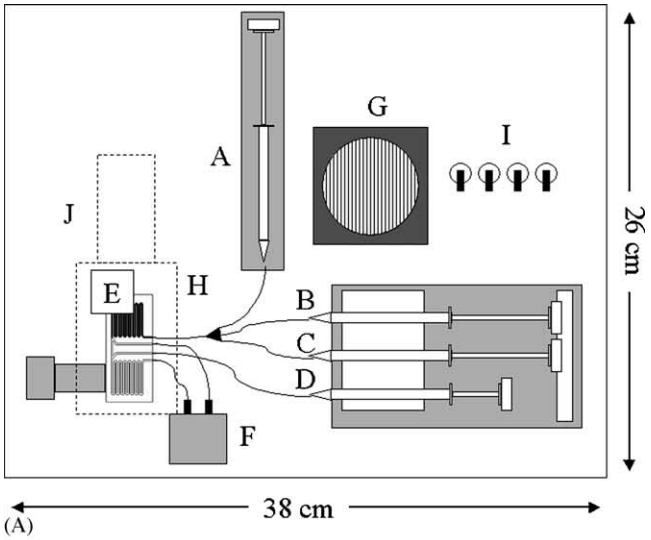

Fig. 2. A schematic of the assembled bacterial detection system is shown in A and a photographic image in B. The system includes integrated syringe pumps (A–D), Moog micro valve (F), cooling fan (G), LED-based fluorescence excitation/detection system (H – dotted outline) with PMT detector (J), power toggle switches (I). The microfluidic purification/detection chip (E) is inserted into the unit directly above the thermoelectric heater cooler. The syringes are connected to the chip via Tygon^{TM} tubing (black lines) and contain the sample lysate (A), ethanol wash buffer (B), dH_2O (C), and PCR master mix (D). The Moog micro valve (F) is also connected to the chip via tubing and controls pressurization and fluid flow through the chip outputs. The entire unit measures 36 cm × 28 cm × 15 cm.

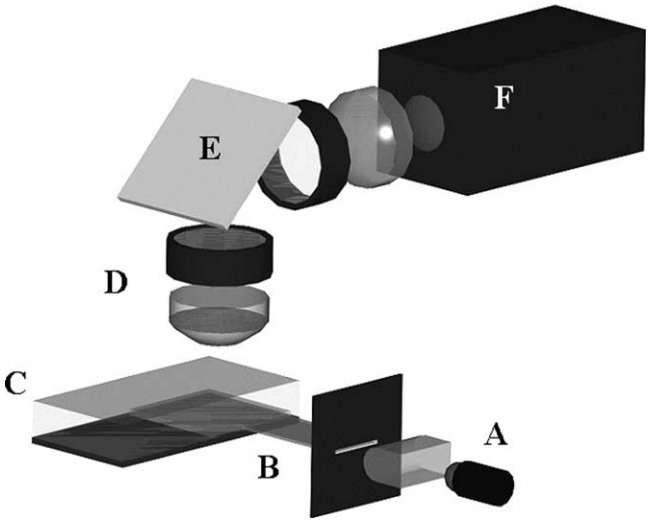

Fig. 3. The fluorescence excitation/detection system is shown. An 480 nm wavelength LED (A) is used to illuminate the PCR chamber of the microfluidic detection chip (C) through a chrome-plated glass waveguide (B). Upon fluorescence of the real-time PCR reaction mixture, the emitted wavelengths are passed through a plano-convex lens and filtered through a Chroma Inc. (Rockingham, VT) D535/40m band pass filter (D). The light is then reflected by a mirror (E) through a second D535/40 m filter and plano-convex lens and into a miniaturized Hammamatsu (Bridgewater, NJ) H5784-20 PMT (F) for quantification of fluorescence intensity.

Optical detection for real-time PCR was achieved using an LED-based fluorescence excitation source and miniaturized photomultiplier tube (PMT) for detection. The fluorescence detection system both excites and detects fluorescence in PCR microchips during amplification reactions and is similar to a system described by Dasgupta et al. [5]. The sample is excited by a 480 nm blue light emitting diode (LED) requiring 80 mW of power. The LED is filtered using a Chroma Inc. D480/30x excitation filter and laterally excites the detection microchip through a chrome-coated glass waveguide (Fig. 3). The resulting fluorescence is filtered by two Chroma Inc. (Rockingham, VT) D535/40 m emission filters and detected by a Hamamatsu (Bridgewater, NJ) H5784-20 photomultiplier tube (PMT) at 520 nm. The light from the LED uniformly illuminates the detection region on the chip while the PMT detects the fluorescent emission. Plano-convex lenses were used to focus emitted light from the detection microchip through the first emission filter, off of a 45° mirror, through a second emission filter and into the PMT (Fig. 3). The following specifications describe the optical parameters of the system. The clear aperture for imaging the reaction chamber is 6.46 mm in diameter which is 33% of the area of the 10 mm square chamber. This translates into a 6.46 mm spot size at the focal point. The numerical aperture (of the objective lens) is 0.41 and has a working F-number of 0.925. Using Eq. (1), the depth of focus (DOF) for the microfluidic channels was calculated to 574 μm (where A is the F-number, s_o the object distance, f the focal length, and d the image size). The microfluidic channels of the PCR chamber are 100 μm in height, well within the depth of focus. The image size on

the PMT is 4.92 mm in diameter and the image NA is 0.54. The magnification for the system is 0.75×.

$$\mathrm{DOF} = \frac{2Ads_o(s_o - f)f^2}{f^4 - A^2d^2{s_o}^2} \quad (1)$$

The entire system is mounted in a portable box enclosure (Fig. 2) that measures (36 cm × 28 cm × 15 cm) and has a total weight of 4 kg. During a typical detection protocol, a program is loaded into the Z-world controller's flash memory from a laptop computer through serial inputs. The program executes fluid pumping, chip pressurization, thermal cycling, and fluorescence detection sequentially. During the real-time PCR reaction, fluorescence data is collected during the 72 °C extension step and is either stored in the microcontroller's flash memory or is directly output to a laptop computer.

3. Results

3.1. Integrated detection platform performance

The integrated detection platform was designed to perform automated DNA purification and real-time PCR in a self-contained system. Individual components of the instrument were characterized separately. During testing, the pump was shown to be capable of pumping at flow rates from 1.7 to 50 μl/min. Fluid flow rates were determined by pumping fluids into 50 μl graduated glass microcapillaries at known motor stepping frequencies for a given length of time. After the flow rate calibration of the instrument, the on-board microprocessor was used to drive the pump at known frequencies and times making it possible to determine volumetric accuracy in the graduated microcapillary tubes. The accuracy of the pumping rate was measured to be ±0.1 μl/min. An electrically actuated microvalve from Moog (East Aurora, NY) was used to direct fluid flow and pressurize the system in preparation for thermal cycling. This is important for switching the direction of fluid flow between purification and PCR procedures on the chip and for preventing bubble formation during thermal cycling. Without pressurization, dissolved gasses and microscopic bubbles in the reaction mixture can increase in volume, especially during the 95 °C portion of PCR thermal cycling. This results in bubble formation, causing increased light scattering that degrades the fluorescent signal from the real-time PCR reaction. Pressurization above 1 atm reduces gaseous volume changes at high temperatures, preventing bubble formation. During testing of the fluidic system, the entire sample preparation procedure, including DNA purification, DNA elution and chip pressurization took approximately 15 min. The on-board TEC-based thermocycler was tested for its ability to rapidly and accurately cycle between the necessary temperatures for PCR (Fig. 4). The average heating and cooling rates for this thermocycler were both 3.1 °C/s. Using cycling parameters of 95 °C for 10 s, 57 °C for 15 s and 72 °C for 20 s, an entire 40 cycle reaction could be completed in 35 min. In comparison, the ABI Prism 7000 real-time thermocycler that was used for validation experiments required 1 h and 20 min while using the identical cycling parameters, nearly four times longer than our instrument. Combined with the 15 min needed for sample preparation, the entire process of preparation and detection took only 50 min with our system. A similar portable device reported by Liu et al. required 3.5 h for the detection of 10^3 *Escherichia coli* cells.

3.2. Real-time PCR

To initially test real-time PCR reactions we fabricated chips that only contained the PCR amplification chamber and did not contain a DNA purification region. Using purified *L. monocytogenes* DNA and Sigma JumpStart SYBR Green master mix, we performed real-time PCR in the ampli-

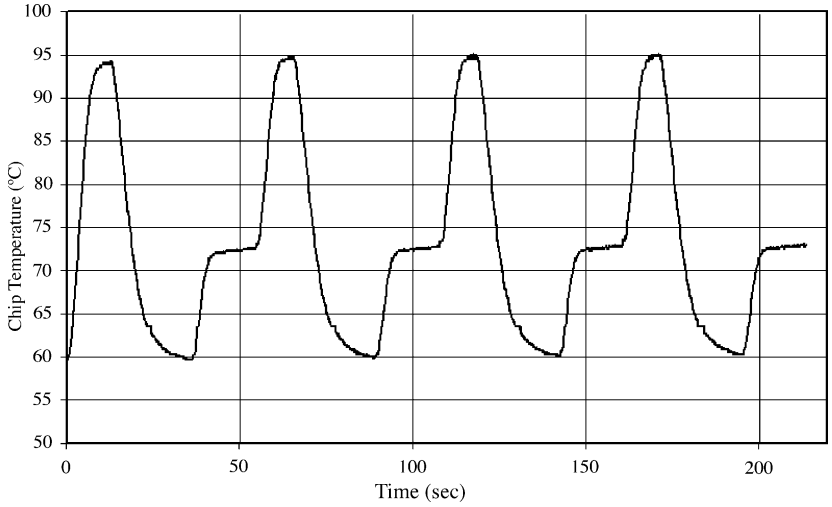

Fig. 4. The temperature profile of the detection microchip is shown for the standard cycling parameters used for real-time microchip PCR. The average cycling time is less than 60 s and typical 40 cycle PCR reactions could be performed in 35 min.

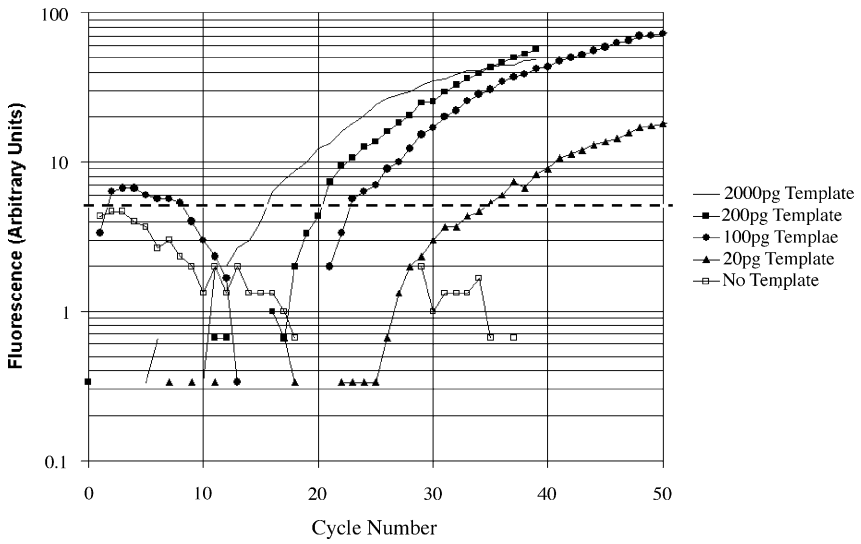

Fig. 5. Real-time PCR plots from purified *L. monocytogenes* DNA amplified on microchips. The amount of template DNA was quantified and added to each reaction. The dotted horizontal line shows the threshold fluorescence (5 units) used to calculate the threshold cycle (C_T) for each reaction.

fication chamber of our microchips. The on-chip PCR conditions were optimized by addition of 5 units of extra Taq DNA polymerase and pre-treatment of the PCR chamber with 50 μl of 10 mg/ml BSA (data not shown). After optimization of the on-chip PCR conditions, we performed real-time PCR reactions using varying starting concentrations of template DNA. Purified *L. monocytogenes* DNA was diluted to varying concentrations yielding 2000, 200, 100 and 20 pg of DNA per 5 μl of reaction mixture. Five microliters of this reaction mixture was pumped into the amplification chamber and was thermally cycled as described above. Fluorescence intensity of the reaction mixture was monitored during the 72 °C extension step of each cycle and completed reactions were analyzed by gel electrophoresis to confirm amplification (data not shown). The average background fluorescence was measured for the first 10 cycles of the reaction and was subtracted from each fluorescent reading, resulting in a standardized fluorescence curve for each reaction (Fig. 5). As can be seen, the initial fluorescence intensity of the reaction decreases during the first 5–10 cycles. This is likely due to SYBR Green dye interactions with the PDMS surfaces of the reaction chamber. Because a passive reference dye was not used for these tests, we cannot correct for interactions between dye molecules and the surfaces of the chamber. During these initial cycles, some dye likely binds to the PDMS surfaces, removing it from solution and decreasing overall fluorescence intensity. Using

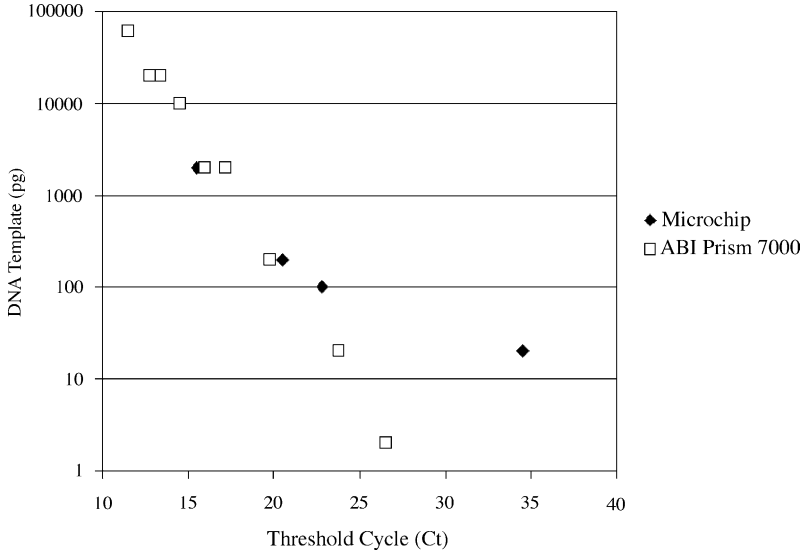

Fig. 6. Real-time PCR data from a microchip-based system and a conventional ABI Prism 7000 real-time thermocycler. The threshold cycle (C_T) for each reaction is shown as compared to the starting amount of template *L. monocytogenes* DNA. All PCR reactions were performed with Sigma JumpStart SYBR Green master mix.

these standardized curves, the threshold cycle (C_T) for each reaction was determined by measuring the cycle at which the fluorescence intensity increased above 5 units. This value was chosen because it was the intensity at which the signal increased above the initial noise level in the first 10 cycles. As seen in Fig. 5, the C_T of the PCR reaction varied inversely with the starting concentration of the *L. monocytogenes* DNA template. As the starting concentration of template was decreased 10-fold, the C_T increased by several cycles. With a threshold value of 5 fluorescence units, the C_T for 2000, 200, 100 and 20 pg of template were 15.5, 20.5, 23 and 34.5, respectively. A reaction performed without template showed no increase in fluorescence, providing evidence that the fluorescence increase is dependent on amplification of the *L. monocytogenes* DNA. As shown in Fig. 6, the starting amount of template DNA varies inversely with the C_T. This is typical of real-time PCR since greater amounts of template DNA provide more targets for amplification, resulting in faster increase above the detection threshold [10,11,25]. In addition to these data, real-time PCR amplification was also performed in a commercial real-time thermocycler (ABI Prism 7000) to compare our microchip-based system with standard 96-well tube-based systems. These data are also shown in Fig. 6, demonstrating correlation between the data collected with the microchip-based system.

3.3. Bacterial detection

In order to use our system for bacterial detection, we performed on-chip purification of *L. monocytogenes* DNA followed by on-chip real-time PCR. Although a DNA purification step is not always necessary for successful PCR amplification, it is often required when using environmental or otherwise complex samples. For the initial device development described here, we determined to use cultured cells as targets to better understand the maximum sensitivity of the system. Known concentrations of *L. monocytogenes* cells were added to lysis buffer L5 and allowed to incubate for 5 min. Following cell lysis, this mixture was pumped into the chip for DNA purification as described previously [4]. Following DNA binding and washing, dH_2O was pumped into the purification region to recover DNA for amplification in the PCR chamber. Simultaneous pumping of a concentrated PCR master mix through a second input port allowed for parallel flow of eluted DNA and master mix into the amplification chamber. By varying the pumping speeds of these two fluids, they could be pumped into the amplification chamber in a volumetric ratio that yielded the appropriate final concentration of the master mix. A variety of concentrations and pumping speeds were explored, yielding a final master mix concentration of 1.35 times the normal concentration (see Section 2) and a pumping speed ratio of 3:1, where master mix was pumped at 3 μl/min, while dH_2O was pumped at 1 μl/min.

To explore bacterial detection sensitivity in our device, we used decreasing numbers of *L. monocytogenes* cells for on-chip DNA purification and real-time PCR. Using the modified DNA elution and mixing method described above, *L. monocytogenes* lysate was pumped into microchips for DNA binding and washing with 70% EtOH, followed by elution into the PCR amplification chamber. During DNA elution with dH_2O, Sigma JumpStart master mix with *L. monocytogenes hlyA* primers was pumped into the amplification chamber in parallel and the entire system was pressurized to prevent bubble formation during thermal cycling. The microchips were then thermally cycled for 50 cycles using the same parameters described for purified DNA reactions. Fluorescence measurements were made during the amplification phase of each cycle and completed reactions were analyzed by gel electrophoresis to confirm amplification of the appropriately sized fragment (Fig. 7B). The fluorescence results were normalized as described above and a threshold of 5 fluorescence units was used to determine C_T values. As shown in Fig. 7A, we were able to purify DNA and detect with real-time PCR, between 10^7 and 10^4 *L. monocytogenes* cells. Attempts at detecting 10^3 and fewer cells were unsuccessful as determined by real-

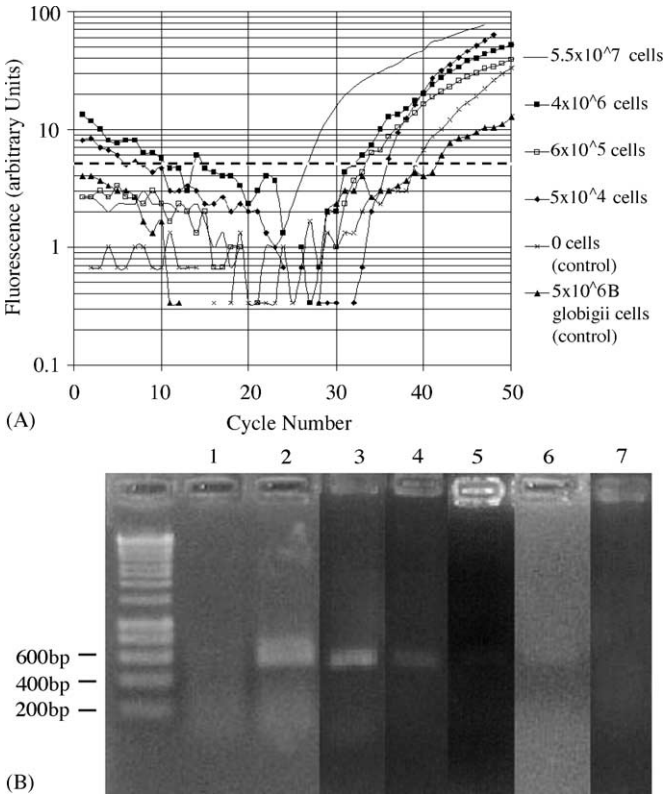

Fig. 7. (A) On-chip DNA purification and real-time PCR amplification of various numbers of *L. monocytogenes* cells. The dotted horizontal line shows the threshold fluorescence (5 units) used to calculate the threshold cycle (C_T) for each reaction. (B) Gel electrophoresis data for on-chip DNA purification and real-time PCR amplification of *L. monocytogenes*. Since electrophoresis of the on-chip reaction and controls was performed after each test, individual lanes from each on-chip test are shown compiled into a single figure. Lanes are as follows: (1) negative control; (2) positive control (cycled off-chip); (3) 5.5×10^7 cells; (4) 4×10^6 cells; (5) 6×10^5 cells; (6) 5×10^4 cells; (7) 5×10^6 *B. globigii* cells.

time fluorescence data and gel electrophoresis of completed reactions. Several control reactions were performed using *B. globigii* cells and *L. monocytogenes hlyA* PCR primers. A negative control using lysis buffer without cells was also performed. For these controls, the entire microchip purification and real-time PCR was performed for accurate comparison to the positive controls. These negative controls provide evidence that the threshold cycle for a positive result must be less than 40 cycles. Both the no-cell and *B. globigii* controls exhibited increases in fluorescence after 40 cycles (Fig. 7A). This is common for real-time PCR reactions using SYBR Green and is thought to be due to formation of primer-dimers and non-specific amplification of DNA [8]. This was confirmed by performing gel electrophoresis of the negative control samples in which streaks of both high and low molecular weight DNA were observed (data not shown). Because SYBR Green binds to any double stranded DNA, a non-specific increase in dsDNA can give rise to fluorescence and potential false-positive results. Therefore, the effective limits of detection for this system are limited to reactions that reach the threshold fluorescence level within 40 cycles.

4. Discussion

We have fabricated and tested an integrated microchip-based DNA purification and real-time PCR system for bacterial detection. Although current PCR-based methods can be used to identify *L. monocytogenes* and other bacterial pathogens, most systems require manual nucleic acid extraction and sample preparation that is time consuming and requires multiple laboratory instruments [10,18,23,33]. In an improvement over other systems, our device presents a fully automated method of purifying DNA from bacterial cells and preparing samples for PCR-based detection. This approach of integrated sample preparation and detection is imperative for environmental or otherwise complex samples since a variety of contaminants can inhibit PCR amplification. Although no mixed samples were tested for this study, the successful detection of cultured cells provides a benchmark for detection sensitivity with this instrument. As reported here, the current limits of detection for this system are approximately 10^4 *L. monocytogenes* cells. The average time required for DNA purification during these experiments was approximately 15 min, which combined with real-time PCR resulted in the detection of 10^4 *L. monocytogenes* cells in 45 min and 10^7 cells in only 37 min. This level of detection is higher than reported for another portable real-time PCR detector that could detect as few as 500 cells in approximately 7 min [8], however that instrument requires manual sample preparation. Manual purification could be more efficient and/or effective than our chip-based DNA purification, but is more time consuming and less portable than the automated system in our device. Another system, reported by Liu et al., was able to purify and detect 10^3 *E. coli* cells, but required 3.5 h for complete analysis. In comparison to both of these methods, conventional methods of *L. monocytogenes* detection, as outlined by the Bacteriological Analytical Manual, include cell culturing on microbiological media and require at least 24–48 h for detection. In relation to these detection methods, our device performs at lower sensitivity but is faster and incorporates on-board sample preparation. In addition to being fast, the instrument can be powered by a standard rechargeable laptop computer battery due to its low power consumption. Due to its small size and low power requirements, this system can be further developed as a truly portable, hand-held device. By optimizing the purification efficiency and the fluorescence detection system, it should be possible to increase sensitivity. The utility of this system can also be extended to other organisms and incorporate alternative fluorogenic PCR techniques, including the 5′ nuclease assay. Current work in our laboratory includes the integration of immunomagnetic sample preparation and concentration, as well as detection of alternative organisms such as *Salmonella typhimurium*, *Leishmania donovani*, and *Bacillus subtilis*. While there is ongoing work to improve the detection limit and effectiveness of this system, this work represents one of the first fully automated, miniaturized systems for integrated sample preparation and detection of bacterial pathogens.

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