

# High sensitivity PCR assay in plastic micro reactors

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Small volume operation and rapid thermal cycling have been subjects of numerous reports in micro reactor chip development. Sensitivity aspects of the micro PCR reactor have not been studied in detail, however, despite the fact that detection of rare targets or trace genomic material from clinical and/or environmental samples has been a great challenge for microfluidic devices. In this study, a serpentine shaped thin (0.75 mm) polycarbonate plastic PCR micro reactor was designed, constructed, and tested for not only its rapid operation and efficiency, but also its detection sensitivity and specificity, in amplification of *Escherichia coli* (*E. coli*) K12-specific gene fragment. At a template concentration as low as 10 *E. coli* cells (equivalent to 50 fg genomic DNA), a K12-specific gene product (221 bp) was adequately amplified with a total of 30 cycles in 30 min. Sensitivity of the PCR micro reactor was demonstrated with its ability to amplify K12-specific gene from 10 cells in the presence of 2% blood. Specificity of the polycarbonate PCR micro reactor was also proven through multiplex PCR and/or amplification of different pathogen-specific genes. This is, to our knowledge, the first systematic study of assay sensitivity and specificity performed in plastic, disposable micro PCR devices.

## Introduction

Lab-on-a-chip technology has been advancing rapidly in the last decade. The resulting on-chip operation has the potential to greatly improve assay sensitivity and ease of operation over currently used technologies. Multi-functional chips for sample pre-concentration, micro PCR amplification, and microchip electrophoretic separation have been demonstrated in several laboratories.<sup>1–3</sup> Currently, integration of sample processing steps into one system, to provide a complete sample-to-answer assay on the chip, is becoming the focus of development work.

Polymerase Chain Reaction (PCR) amplification is essential to all genetic analysis applications of integrated microchips. Rapid operation, minute sample volume (compatible with small volume requirements of downstream analysis in separation channels, for example), ease of sample transport to the analytical stage, and parallel amplification in many wells are among the advantages of amplification in the microchip environment. At the same time, there are numerous challenges to the microchip amplification approach, including: loss of sample to the chamber walls due to the dramatic increase in surface-to-volume ratio, evaporation in the small volume regime, and effective heat dissipation. Micro PCR devices have been successfully fabricated from glass,<sup>4–6</sup> silicon<sup>7–9</sup> and plastic.<sup>10–16</sup> Silicon, due to its superior thermal conductivity, allows for very fast temperature ramp times and results in very short on-chip protocols.<sup>8</sup> Similarly, successful and fast amplification assays have been demonstrated by many groups in glass.<sup>5,6,17,18</sup> Recently with increasing emphasis on disposable devices, the use of plastic and plastic fabrication methods have become very popular<sup>10–14</sup> in micro reactor development. Plastic chips are disposable, inexpensive, optically transparent and biocompatible.<sup>3,19</sup> These properties make them excellent candidates for mass produced microfluidic devices. Microfluidic devices have been demonstrated using several plastic materials, including *e.g.* polyimide,<sup>20</sup> polymethylmethacrylate (PMMA),<sup>21</sup> poly(dimethyl siloxane) (PDMS),<sup>22</sup> parylene<sup>23</sup> and polycarbonate.<sup>3</sup> Despite all the advantages, plastic presents a

major challenge to designers of PCR micro reactors due to its poor thermal conductivity,<sup>24</sup> which means that it is difficult to achieve rapid thermal cycling. Consequently, not many demonstrations of micro PCR assays in the plastic chips exist to date.

Low volume and rapid thermal cycling have been early motivations for on-chip PCR development. Landers' group demonstrated IR heated PCR devices containing a 1.7  $\mu$ l micro chamber; amplification was achieved by 15 thermal cycles in 4 min.<sup>20</sup> In an integrated monolithic silicon-glass device, sub-microliter (280 nl) DNA amplification was thermally cycled as fast as 30 s cycle<sup>-1</sup>.<sup>17</sup> The low volume limit for micro PCR was also investigated by Nagai and colleagues.<sup>25</sup> They demonstrated successful amplification in picoliter micro chamber array. Using a real-time PCR device, Belgrader *et al.* reported PCR detection of *Erwinia*, a vegetative bacterium, in 7 min.<sup>8</sup> An integrated rapid PCR-detection system was presented by Khandurina *et al.*<sup>26</sup> and Lagally *et al.*,<sup>18</sup> where efficient DNA amplification, electrophoretic sizing, and detection were achieved in 10–20 min.

Among all these demonstrations, the sensitivity aspect of micro PCR chip assay has not been well addressed. The initial template concentration used to achieve fast and small volume amplification was high, ranging from 0.1 ng phage DNA<sup>20</sup> to 100 ng human genomic DNA.<sup>27,28</sup> Few authors evaluated the sensitivity issue in microfluidic PCR reactors. The most sensitive micro PCR assay demonstrated, so far, was by Lagally *et al.*<sup>17</sup> A single molecule DNA template could be amplified in a glass integrated microfluidic device. Also, in an integrated glass sandwich structure, Mathies and coworkers<sup>18</sup> reported that amplification with a starting template concentration as low as 5–6 copies was achieved in their nanoliter-volume glass microchambers. For the 'real' sample (containing target cells, rather than purified DNA) analysis, the most sensitive silicon microstructure, which could perform rapid real time PCR analysis from a sample containing low concentration of target (*Erwinia*) cells, was reported by Belgrader and colleagues.<sup>8</sup> A positive amplicon signal was detected in less than 35 cycles (17 s per cycle time) with a starting template concentration as low

as 5 cells. In a flat polypropylene tube, Northrup and his group<sup>29</sup> demonstrated a real-time PCR analysis of 1000 bacillus spores.

Integration of the sample preparation module on-chip and amplification of target DNA from a 'real sample' (blood sample containing target cells) are currently among the most challenging steps in the development of lab-on-a-chip technology. Based on their previous work,<sup>9</sup> Yuen *et al.* designed and constructed a Plexiglas microchip module for blood sample preparation and nucleic acid amplification.<sup>30</sup> Human white blood cells (WBC) were isolated in an 8–9  $\mu\text{l}$  dual-purpose (cells isolation and PCR) glass-silicon microchip through a series of 3.5  $\mu\text{m}$  filters. The human coagulation factor V gene was subsequently amplified in the same microchip from DNA released from the human WBCs captured in the filter. Therefore, the initial template of human WBC DNA concentration from 3  $\mu\text{l}$  of human whole blood was > 10000 cells (> 10 ng DNA). An automated and integrated system for DNA genotyping directly from blood was reported by Zhang *et al.*<sup>31</sup> With addition of formamide, three short-tandem-repeat loci were amplified in fused-silica capillaries, directly from about 1% blood sample providing ng range of genomic DNA as the starting material.

*Escherichia coli* (*E. coli*) is one of the widely known pathogens causing intestinal and systemic illnesses in humans and animals through contaminated food, water, and soil. Environmental scientists have suggested that specific monitoring and analyzing of *E. coli* may be a good indicator of water, soil, sewage and food pollution.<sup>32–34</sup> Traditionally, analyses of *E. coli* and other pathogens have relied on culture techniques, which require selective-differential media, specific conditions, and at least a 16 h culture period. Although these approaches are reliable, they are time-consuming, and have low specificity for cross-reaction. Development of DNA techniques consisting of Polymerase Chain Reaction (PCR) and/or hybridization probes increased the precision and speed of microbial detection and identification while reducing labor intensity. The PCR approach has been widely applied to detect trace genetic material from medical, biological, and agriculture samples. In a clinical or research lab, PCR is still processed with sequential steps of nucleic acids extraction, purification and then amplification. PCR in microchip format provides a reduction in PCR cycling time, a reduction of sample volume, and a path to integration with other sample preparation modules of the microchip. In the effort to develop a highly sensitive and integratable PCR microchip, we have developed and constructed a PCR micro reactor for pathogen detections using plastic material, rather than silicon or glass.

Highly sensitive micro PCR operation is crucial to diagnostic applications for rare target analysis. Many biological target cells are normally present at very low concentrations in a biological sample. It has been known that starting with a reduced sample volume would be problematic for detecting rare cells or microorganisms from real medical samples,<sup>35,36</sup> since the reduced volume may not statistically contain enough of the target cells in which we are interested. Therefore, sample pre-concentration and a high sensitivity PCR micro reactor become more important for clinical applications. In order to perform rare target cell analysis in microfluidic devices, the sensitivity of a PCR micro reactor becomes very critical. In this study, we address three issues relevant to the further progress of on-chip PCR operation: (1) device design in a plastic platform, enabling rapid, reproducible assay; (2) the use of a 'real' sample, such as bacterial cells in blood or biological buffer, to directly amplify target-specific genes; (3) assay sensitivity at low target concentration.

In this paper, simple, low cost, integratable, and disposable polycarbonate PCR micro devices were fabricated using CO<sub>2</sub> laser direct writing. Care was taken to build PCR reactors with thin walls in order to enable sufficient heat transfer despite poor

the thermal conductivity of the plastic material. High sensitivity of micro PCR analysis was demonstrated using real bacteria, *Escherichia coli* (*E. coli*) cells. The K12 strain of *E. coli* bacterial cells was used as a pathogen model in this study. K12 cells were directly used in PCR reaction following thermal lysis performed in the same reactor, without any additional isolation and purification of genomic DNA. Successful amplification was achieved in this polycarbonate PCR micro reactor with a starting template concentration as low as ten K12 cells and as fast as 30 min. Ten *E. coli* cells could be adequately amplified directly from the sample containing 2% blood. Multiplex PCR was also demonstrated by amplifying *E. coli* K12, *E. faecalis*, and *S. salivarius* gene fragments, simultaneously.

## Materials and methods

### Device fabrication

The device fabrication procedure was similar to that described previously.<sup>3</sup> Briefly, the PCR micro reactor consists of two pieces of clear polycarbonate (PC) wafer (top and bottom) and one piece of black PC polycarbonate wafer (Piper Plastic, AZ), which are 250  $\mu\text{m}$  thick each, thus forming a stack of 750  $\mu\text{m}$ . The fluidic path design was first generated with CAD software on a computer and then the PC wafer work pieces were laser cut using a CO<sub>2</sub> laser engraving system (Universal Laser System Inc., Scottsdale, AZ). A planar serpentine PCR channel design was selected due to ease of fluidic transfer with minimal dead volume and bubble trapping (Fig. 1). In addition, the serpentine channel design enabled the micro reactor to have high holding volume in a given heating surface area with minimized chip size, compared to a straight channel. The thin PC wafer (250  $\mu\text{m}$ ) was selected for the improvement of heat transfer of the plastic device. A black PC wafer was used to fabricate the middle pieces containing all the fluidic structures and clear PC wafers were used as covers to enclose the fluidic structure. First, the serpentine channel was CO<sub>2</sub> laser machined out of black PC wafer, then the black PC piece was sandwiched between two clear PC pieces. The serpentine channel is 1.5 mm in width, 0.25 mm in height and holds 40  $\mu\text{l}$  of sample volume (Fig. 1A). At the end of the channel were inlet and outlet holes of 0.7 mm in diameter. The enclosed fluidic reactor is formed using thermal bonding (Carver, Inc. Wabash, IN) of three PC pieces at 133 °C and with 1400 psi pressure for 2 h. Since no photo mask is needed, this fabrication technique is extremely fast for building prototype microchip devices. Six or more micro PCR devices could be bonded simultaneously. Both inlet and outlet holes were sealed with double-sided adhesive tape ('9490LE', 3M, St. Paul, MN) following PCR reaction solution loading. To ensure that no leaking occurred during thermal cycling, a paraffin layer, which was put on the top side of the double-sided tape, was pressed onto the tape covered with a small piece of polycarbonate (0.25 mm) and compressed with a small hand-press. This leads to the paraffin pushing the double-sided tape into the cavity, thereby creating a leak-proof seal. PCR compatibility assays were performed for all the materials used for the device including both clear/black PC and double-sided tape.

### Instrumentation

The thermal cycler for the micro PCR device was designed and built in-house using two Peltier thermoelectric devices (1 inch  $\times$  1 inch, output power of 18.1 W). Aluminium cooling fins are attached to the backside of the Peltier devices as heat sinks. A small cooling fan was also attached to each of the two aluminium blocks for convective cooling. During PCR thermal cycling, the micro PCR device was sandwiched between the two

Peltier elements. The device temperature and Peltier surface temperature were monitored using thermo-couples (Fig. 2A and B). LabView™ software (National Instruments, Austin, TX) was used for thermal cycle temperature control.

### PCR amplification and product analysis

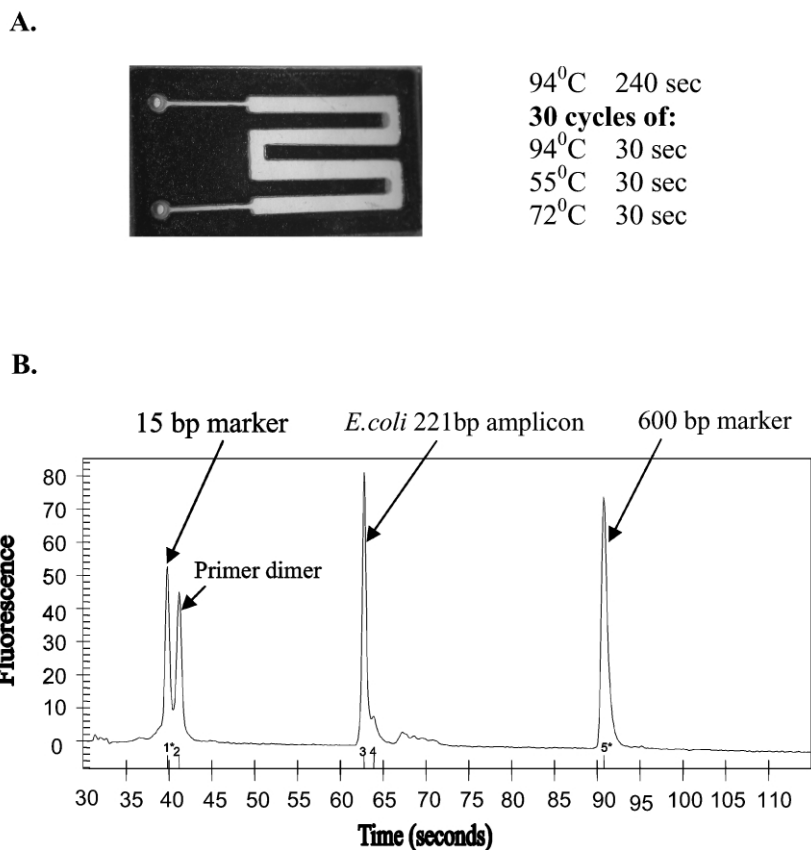
*E. coli* K12 cells were used as model organisms for pathogen detection assays. Overnight culture of *E. coli* K12 cells was quantified using a spectrophotometer (SPECTRONIC 20D+, ThermoSpectronic, Rochester, NY). Different concentrations of *E. coli* cell stock were achieved by series dilutions. Cell concentrations were verified prior to each experiment by plating 10  $\mu$ l of different stocks on LB plates and counting the colony forming units (cfu) following overnight culture. A pair of *E. coli* K12-specific primers were used to amplify a 221 bp *E. coli* K12-specific (MG1655) gene fragment (Fig. 1), with forward primer D3490-1: 5'-GGC-GTT-ATC-CCC-AGT-TTT-TAG-TGA-3' and reverse primer D3491-1: 5'-AAC-GGC-CAT-CAA-CAT-CGA-ATA-CAT-3'. The standard PCR reaction mixture was modified to accommodate the large surface to volume ratio ( $S/V = 9.33$ ) in micro PCR devices. Each PCR reaction mix (40  $\mu$ l) includes Tris-HCl (pH 8.3) 10 mM, KCl 50 mM, MgCl<sub>2</sub> 2 mM, gelatin 0.001%, dNTPs (deoxynucleotide triphosphates) 0.4 mM each, bovine serum albumin (BSA) 250  $\mu$ g ml<sup>-1</sup>, forward and reverse primers 0.5  $\mu$ M each, polyethylene glycol 8000 (PEG 8000, Sigma) 0.75%, and AmpliTaq DNA polymerase (Applied Biosystems) 5 units. *E. coli* K12 cells were used to perform PCR directly, instead of using purified *E. coli* DNAs. *E. coli* cells were lysed during the initial denature step at 94 °C, 4 min. Following the PCR reaction mixture loading, the inlet and outlet holes of the reactor were sealed using double-sided tape and paraffin by pressing to ensure no leakage. The PCR micro device was then placed into the Peltier thermal cyclers. The PCR thermal cycling was

controlled by LabView™ computer software. A standard PCR protocol was performed by an initial denature step at 94 °C for 4 min followed by 30 cycles of 94 °C 30 s, 55 °C 30 s and 72 °C 30 s, with a final extension at 72 °C for 3 min. During PCR assay optimization, the dwell time at each temperature step was shortened to 20 s or 15 s as indicated in the corresponding figure legend. The amplified PCR products were electrophoretically analyzed in an Agilent 2100 Bioanalyzer (Palo Alto, CA) using DNA 500 assay (Fig. 1B). All control PCR reactions (40  $\mu$ l) were performed in parallel, using 0.5 ml thin-wall PCR tubes (Molecular BioProducts, San Diego, CA) in a conventional DNA Engine™ Thermal Cycler (MJ Research Inc. South San Francisco, CA) under the same conditions (same volume, same reaction mix, same PCR cycle numbers and same reaction time). Dose-response curves were obtained from experiments using different number of input *E. coli* cells in PCR mix, namely: 10 cells, 100 cells, 10<sup>3</sup> cells, 10<sup>4</sup> cells, 10<sup>5</sup> cells, and 10<sup>6</sup> cells.

Amplification of *E. coli* K12-specific gene in the presence of 2% blood was achieved by adding 0.8  $\mu$ l of sheep blood (Colorado Serum Company, Denver, CO) into 40  $\mu$ l of PCR reaction mix which contained 3 mM of MgCl<sub>2</sub>, and was supplemented with 1X Q buffer (QIAGEN) in which the component could block potential inhibiting factors provided from the whole blood; AmpliTaq DNA polymerase was replaced with the Hot Start Taq DNA polymerase (QIAGEN, Valencia, CA) which requires a 15 min initial denature period.

### Multiplex amplification and analysis

In order to demonstrate multiplex PCR in the plastic micro reactor, three different bacteria cells were used in the same PCR reaction mix. The other surrogates for pathogenic microbes were *Enterococcus faecalis* (*E. fae.*), *Staphylococcus epidermi-*



**Fig. 1** A photograph of the polycarbonate PCR micro reactor (A). A symmetric PCR was performed and *E. coli* K12-specific gene (221 bp fragment) was amplified and analyzed using Agilent BioAnalyzer (B) DNA 500.

*tis* (*S. epid.*) and *Streptococcus salivarius* (*S. sal.*) cells. *E. coli* K12-specific primers (D3490-1 and D3491-1) amplified a 221 bp MG1655 gene fragment. The primers used to amplify *E. fae.* DNAE gene were: 5'-GCC-AGA-TTT-TTC-GTT-CGC-TCA-T-3' (forward) and 5'-AAA-TCG-GCA-ACT-TCT-CGC-TCA-G-3' (reverse); they were designed to amplify a 195 bp fragment. The primers used to amplify *S. sal.* DAL gene were: 5'-ATG-GGG-TCA-TCT-GTT-GGG-ATT-T-3' (forward) and 5'-GCC-GCA-TAG-ACA-CGC-ATT-TG-3' (reverse); they were designed to amplify a 293 bp fragment. The primers used to amplify *S. epid.* ARGC gene were 5'-TGC-GAT-ATT-GAT-GAG-TGG-TTC-C-3' (forward) and 5'-ATG-AAA-TGC-GTA-AGT-TCC-GAC-AT-3' (reverse); they were designed to amplify a 371 bp fragment. The primers ratio used in the multiplex PCR mix was *E. fae.*:*E. coli*:*S. sal.* = 1:1:2. All PCR primers were ordered from Operon Technology Inc. (Alameda, CA). Post amplification PCR products were analyzed by the Agilent Bioanalyzer using DNA 500 assay. The temperature profiles used for multiplex PCR were the same as the on-chip single template amplification, with an extra 6 PCR cycles added (36 cycles of 94 °C 30 s, 55 °C 30 s and 72 °C 30 s).

## Results

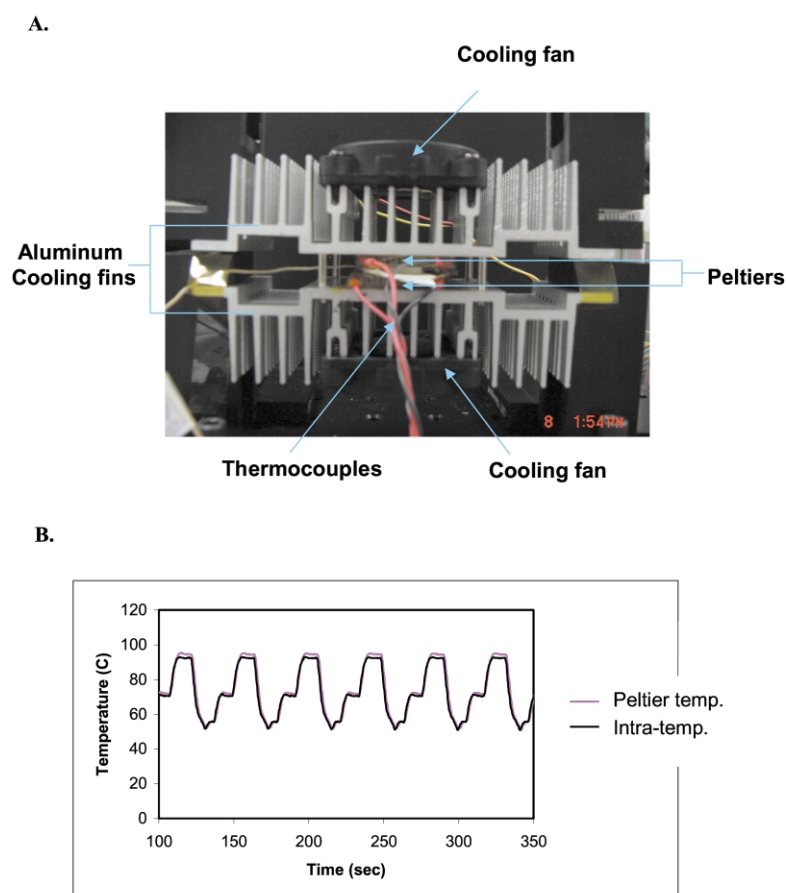
### PCR amplification

Polycarbonate (PC) has a poorer thermal conductivity than silicon or glass. The thickness of the PC reactor was kept thin (0.75 mm) in order to achieve a reasonable thermal cycle time. We have conducted a series of thermal transfer experiments using different thicknesses of polycarbonate wafer (*e.g.* 0.7 mm, 0.5 mm, 0.25 mm) and found that the intra-device temperature

would fit the set point temperature better by using thinner polycarbonate wafer (data not shown). The temperature cycling performance of the PCR micro reactor in a dual Peltier assembly is represented by trace curves in Fig. 2B (three cycles collected using LabView™ software). The Peltier surface temperature and intra-device temperature were measured by thermocouples. We have built a calibration chip which contained an embedded thermocouple to assess the intra-device temperature and compare it with the temperature of the Peltier surface. These data were used to the design thermal cycle profile used in amplification experiments. The chips used in the experiments did not have the embedded thermocouple. The calibration was performed before each experiment set. The polycarbonate wafers were tested for PCR compatibility (data not shown). Cycle times were initiated according to the intra-device temperature. The temperature profile (Fig. 2B) showed that the intra-device temperature (black line) followed the Peltier set temperature (purple line) well. In this configuration, heating from room temperature (25 °C) to denature temperature (94 °C) takes about 9 s, corresponding to a heating rate of 7–8 °C s<sup>-1</sup>. The cooling rate is slower, about 5–6 °C s<sup>-1</sup>. Both heating and cooling rates are significantly faster than those of conventional thermal cyclers (1 to 2 °C s<sup>-1</sup>). The time required for one complete cycle of denature, annealing and extension is about 18–19 s without counting the dwell time (15–30 s) at each temperature.

### PCR optimization

Our polycarbonate PCR micro reactors have a larger surface to volume ratio ( $S/V = 9.33$ ) than conventional PCR tubes. In order to improve the yield of PCR assay in the micro PCR device, the conventional PCR reaction mix was modified to accommodate a larger surface to volume ratio of the PCR micro

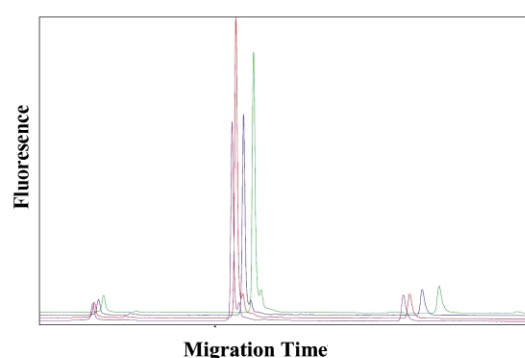


**Fig. 2** Peltier thermocycler for the PCR micro reactor (A). Peltier surface and intra-chamber temperatures are transduced by thermocouples. Cycle times are initiated according to the intra-chamber temperature. LabView™ controlled PCR cycles run automatically (B).

reactor as compared to regular PCR tubes. Polyethylene glycol (PEG, Sigma) 8000 is a molecule with large molecular weight. It has been shown that addition of PEG 8000 as a PCR buffer additive improved the PCR yield dramatically in polyimide microchips.<sup>20</sup> We have tested the effects of addition of PEG into our PCR mix with our PC plastic PCR chip. Different concentrations of PEG 8000 (0.5%, 0.75%, 1%) and/or PEG with different molecular weights (*e.g.* PEG 400, PEG 1000, PEG 8000 *etc.*) were tested in the PC micro reactor (data not shown). The best result was achieved by addition of 0.75% PEG 8000. With a given template concentration of  $10^6$  *E. coli* cells (about 5 ng DNA), the yield of 30 cycles PCR was  $13.3 \text{ ng } \mu\text{l}^{-1}$  for the PCR micro reactor as quantified by Agilent BioSizing analysis, which was about 82% of conventional MJ PCR machine yield ( $16.3 \text{ ng } \mu\text{l}^{-1}$ ) (Fig. 3). By adding 0.75% (w/v) PEG 8000, the PCR yield from the micro PCR device was improved to  $16.5 \text{ ng } \mu\text{l}^{-1}$  (from  $13.3 \text{ ng } \mu\text{l}^{-1}$ ), the same as or even higher than conventional MJ PCR yield. Another attempt was to test the devices pretreated with 5% polyvinyl pyrrolidone (PVP, Sigma), which was used for coating capillary walls<sup>31</sup> in capillary electrophoresis. 5% PVP pretreatment of the channels did not have a significant effect (Fig. 3) on amplification in the polycarbonate PCR micro reactor. Since BSA is routinely used in on-chip PCR assays, several concentrations of BSA in the PCR mix were examined in the PC micro reactor. In combination with 0.75% PEG, the addition of BSA at  $250 \text{ } \mu\text{g ml}^{-1}$  resulted in the best amplification yield in the PC micro reactor. Thereafter, 0.75% PEG and  $250 \text{ } \mu\text{g ml}^{-1}$  BSA were routinely included in the PCR mixture for on-chip assays. The possible mechanism may be due to the non-specific blocking of any potential sticking of nucleic acids and reagents to the plastic channel surface.<sup>3</sup>

### Sensitivity of the polycarbonate PCR micro reactor

Many of the to-date demonstrations of micro PCR chip assays resulted in rapid operation and small sample volume.<sup>5,17,18,20,25,27</sup> However, important concerns for practical application of these devices are their sensitivity and specificity. These factors, with few exemptions,<sup>8,17,18,29</sup> have not obtained significant attention thus far. To evaluate the sensitivity of the polycarbonate PCR micro devices, we performed PCR on



PCR Conditions	Amplicon Concentration
MJ-PCR: $10^6$ cells	16.3 ng/ $\mu\text{l}$
$\mu\text{PCR}$ : $10^6$ cells	13.3 ng/ $\mu\text{l}$
$\mu\text{PCR}$ : $10^6$ cells + 0.75% PEG	16.5 ng/ $\mu\text{l}$
$\mu\text{PCR}$ : $10^6$ cells +5% PVP Pre-treatment	12.3 ng/ $\mu\text{l}$

**Fig. 3** On-chip PCR optimization. *E. coli* K12 cells were amplified in either an MJ PCR thermocycler (MJ-PCR; green line) or a plastic PCR micro reactor ( $\mu\text{PCR}$ ) without (blue line) or with 0.75% polyethylene glycol 8000 (PEG 8000; red line) or 5% polyvinyl pyrrolidone (PVP; purple line). Improvement of amplification efficiency in the polycarbonate PCR micro reactor by addition of 0.75% PEG in the PCR mix was achieved. Pretreatment of the channel with 5% PVP had no effect.

samples containing different numbers of *E. coli* cells. The optimized PCR conditions were used. The templates were *E. coli* K12 cells with various cell numbers ranging from 10 cells to 1 million ( $10^6$ ) cells. Fig. 4A is the Agilent Bioanalyzer result showing that the K12-specific 221 bp amplicon peak could be detected from as low as 10 *E. coli* cells (50 fg genomic DNA) in the polycarbonate PCR micro reactors, with an amplification efficiency equal to 36% of the MJ conventional PCR thermal cycler. The estimated amount of genomic DNA is about 5 fg in one single *E. coli* cell. There are only 7 copies of the K12-specific 221 bp gene segment per cell (Fig. 4B). With an increase in the initial template concentration ( $10^6$  *E. coli* cells, about 5 ng DNA), the PCR yield from the PCR micro reactor was comparable to that of a conventional PCR thermal cycler (90% of MJ thermal cycler). Quantitative data are given in Fig. 4B. This dose-response curve shows that the PCR yield is a function of initial target cell concentration. As the amount of starting genomic material increased, the final PCR amplicon yield increased.

### Rapid operation of the polycarbonate PCR micro reactors

In the attempt to make the micro PCR operation fast, we tried to optimize the ramp time for each PCR cycle or to reduce the total number of PCR cycles while keeping ramp times the same (Fig. 5). In the presence of the same number of *E. coli* cells (*e.g.*  $10^6$  K12 cells) and the same cycle time ( $30 \text{ s step}^{-1}$ ), the yield from the micro PCR device was 46% of the conventional PCR machine yield in 20 cycles of PCR (data not shown) and the total duration time of the assay was 40 min. However, when the total PCR cycle number (30 cycles) was kept constant while the set time for each temperature steps was varied (30 s, 20 s, or 15 s), the PCR yield was very comparable between the conventional MJ machine and the micro PCR device, while the total PCR time was shortened from 50 min ( $90 \text{ s cycle}^{-1}$ ) to 30 min ( $45 \text{ s cycle}^{-1}$ ) (Fig. 5).

### Multiplex PCR assay in the polycarbonate PCR micro reactor

To further evaluate the specificity and functionality of the PCR micro reactor, additional bacterial cells, other than *E. coli* K12 cells, were used as DNA templates to amplify variable gene fragments. The surrogates of three bacteria strains were *Enterococcus faecalis* (*E. fae.*), *Staphylococcus epidermitis* (*S. epid.*), and *Streptococcus salivarius* (*S. sal.*). Fig. 6 demonstrates the amplification of four different gene products from four different bacterial cells: DNAE gene (195 bp) of *E. fae.*; K12-specific MG1655 gene (221 bp) of *E. coli* K12; DAL gene (293 bp) of *S. sal.* and ARGC gene (371 bp) of *S. epid.* 1000 *E. coli* K12 and *E. fae.* cells were used to perform PCR, which was equivalent to 5 pg DNA template. Compared to *E. coli* and *E. fae.*, *S. epidermitis* and *S. salivarius* genes were not easy to amplify directly from samples containing intact cells. Therefore, higher template concentration ( $10^4$  cells) was used for *S. epid.* and *S. sal.*, respectively. Following successful amplification of different gene products from various bacterial cell types, multiplex PCR was successfully performed in the PCR micro reactor as well. Results of cell lysis and multiplex PCR in a single plastic micro reactor are shown in Fig. 7, where 1000 cells of each *E. fae.*, *E. coli* K12, and *S. sal.* were present in the same PCR reaction mix. Whole bacterial cells were thermally lysed in PCR mixture during the initial four minutes denature (same as the conventional PCR machine). The ratio for the three sets of primers (six primers) used were *E. fae.* : *E. coli* : *S. sal.* = 1 : 1 : 2. Using the Agilent Bioanalyzer to size the products, the three peaks (195 bp; 221 bp; 293 bp) shown in Fig. 7 correspond to *E. faecalis*, *E. coli* K12, and *S. salivarius*, respectively. It was observed that with the starting material of 1000 cells or higher,



*E. fae.* gene amplification in chip PCR was similar to or higher than that in MJ PCR. At this point, the exact mechanism is not clear. PCR calibration curves are under construction for different microorganisms, especially at low template concentrations. Our multiplex experiments aimed at demonstration of the capacity, rather than systematic study to amplify all species under consideration. At the concentration of 1000 cells, successful amplification of *S. epid.* in a multiplex test was not yet achieved. It is possible that alternative primer design may improve the PCR yield of *S. epid.* in multiplex testing.

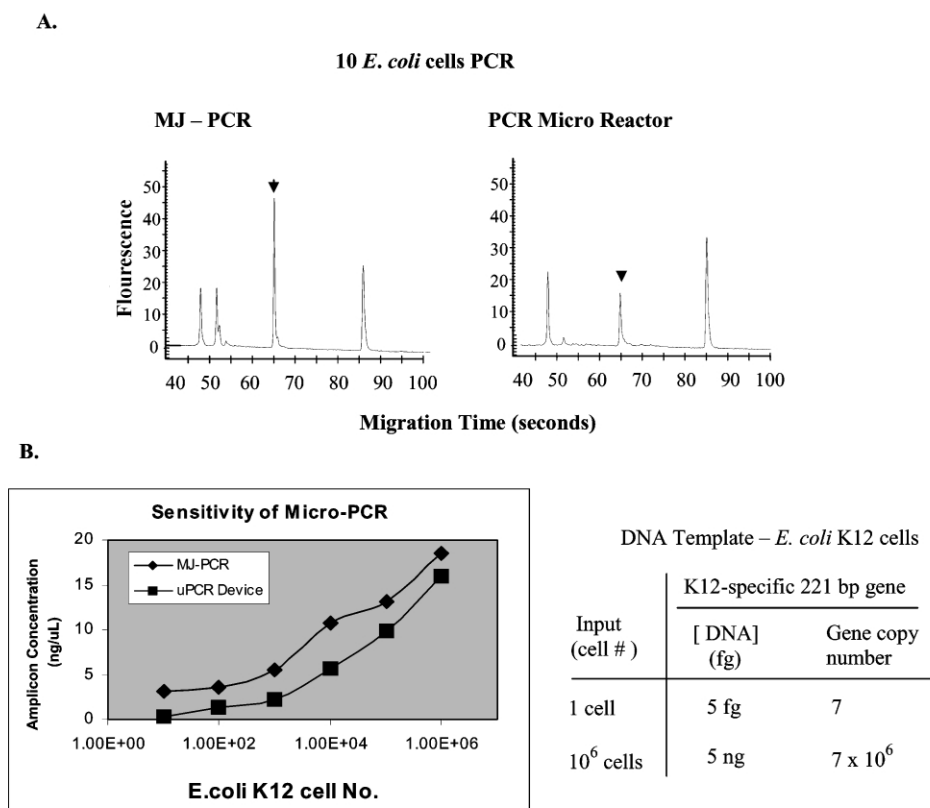
### Amplification of *E. coli* genes from 2% blood

Sensitivity and specificity of the PCR micro reactors were further evaluated by amplification of an *E. coli* K12-specific gene (221 bp) fragment in the presence of 2% blood. By adding 0.8  $\mu$ l of blood to the PCR mix,  $10^4$  nucleated white blood cells,

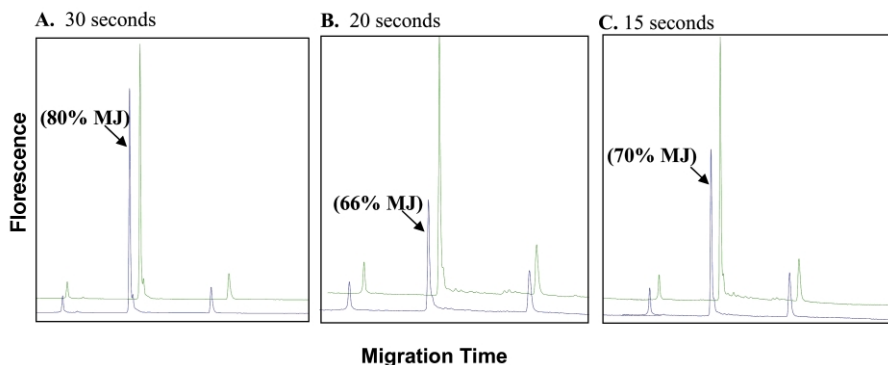
$10^6$  red blood cells,  $10^5$  platelets, and other components of the plasma were added into the PCR reaction mixture. *E. coli* K12-specific gene (221 bp) fragment was amplified adequately in the plastic PCR micro reactor (Fig. 8) from an initial template concentration of 10 cells (50 fg DNA). The amplification efficiency for the polycarbonate PCR micro reactor was 48% that of the conventional MJ PCR thermocycler. These data indicated a high sensitivity and specificity of the polycarbonate PCR micro reactor.

### Discussion

The use of micro fabricated devices in sample mixing, gene amplification and signal detection has been growing rapidly. One of the many application schemes for these devices is rapid and accurate pathogen detection.<sup>8</sup> In the current study, we systematically investigated the sensitivity and specificity of a



**Fig. 4** Sensitivity of polycarbonate PCR micro reactor. PCR was performed in either a conventional MJ PCR thermocycler or the plastic PCR micro reactor, in the presence of 10 *E. coli* cells (A). The concentrations of PCR product (a 221 bp amplicon) were 3.3  $\text{ng } \mu\text{l}^{-1}$  and 1.2  $\text{ng } \mu\text{l}^{-1}$  for the MJ thermocycler and the plastic micro reactor, respectively. A quantitative dose-response curve for amplification of *E. coli* cells, input *E. coli* K12 cell numbers versus amplicon concentration (221 bp) concentration ( $\text{ng } \mu\text{l}^{-1}$ ) following 30 cycles of PCR, is shown in panel B, diamonds represent the MJ PCR and squares represent the micro reactor PCR. The table insert shows a reference of how much genomic material and gene (221 bp) copies were added as starting template corresponding to the input of *E. coli* cell numbers.



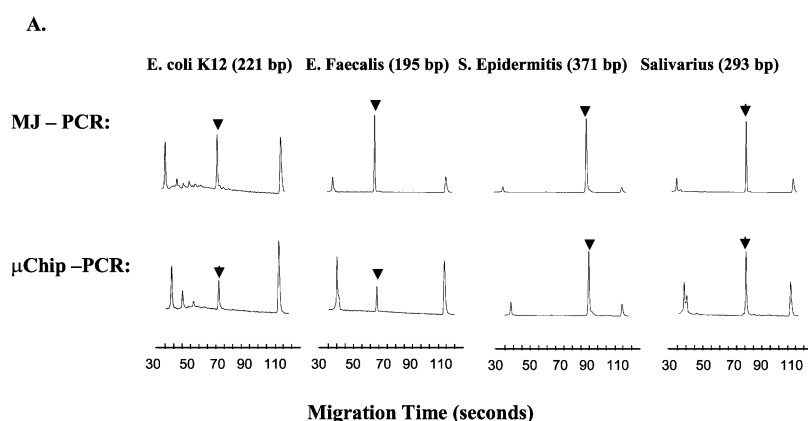
**Fig. 5** PCR cycles optimization. The time (seconds) at each temperature step was modified to 30 s (A), 20 s (B) or 15 s (C). The total time for amplifying  $10^6$  *E. coli* cells with 30 cycles of PCR was 50 min, 40 min, and 30 min, respectively. Green lines represent PCR performed in MJ thermocycler. Blue lines represent PCR performed in the plastic PCR micro reactor.

polycarbonate PCR micro reactor. Due to the biological compatibility,<sup>3,19</sup> abundant choices of material selection, low-cost manufacturing techniques, and device disposability, plastic material is becoming the platform of choice for future chip fabrication. The systematic studies of micro PCR operation in plastic micro reactors are few. In this study, we have undertaken such a study and successfully demonstrated the specific amplification of *E. coli* K12 gene in a PC plastic thin PCR micro reactor at very low cell (template) concentrations, *i.e.* we have addressed the sensitivity aspect of the plastic PCR micro reactors. In most medical and clinical situations, rare target cells<sup>35</sup> in unprocessed host blood samples, such as tumor cells during metastasis, bacterial cells during sepsis, and fetal cells in maternal blood<sup>36</sup> are typically present at very low concentrations (*i.e.* in the range of 10–1000 cells ml<sup>-1</sup>). The Cepheid real time PCR tube device was fabricated from plastic material (polypropylene, PP) with a total volume of 25–100  $\mu$ l. With their flat PP tube device, Northrup and his colleagues<sup>29</sup> could complete 45 cycles of amplification using 1000 cells (cfu) of *Bacillus subtilis* or *thuringensis* spores in <15 min. The difference in operation time between our PC micro reactor and Cepheid's PP tube micro device results not only from the use of plastic material, but also from the device configuration and thermal cycling methods. The most sensitive microstructure which could perform rapid, real time PCR analysis from a sample containing a low concentration of target (*Erwinia*) cells was made of silicon.<sup>8</sup> A positive amplicon signal could be detected in their study, in less than 35 cycles (17 s per cycle time) with the starting template concentration as low as 5 cells. There are 7 copies of the K12-specific gene fragment (221 bp) in each single *E. coli* cell. The template concentration of the genetic material from 10 *E. coli* cells in our study was about 70 copies. Therefore, the sensitivity obtained in our PC plastic PCR micro reactor was similar to that of the silicon chip reported by Belgrader *et al.*<sup>8</sup> in terms of initial template concentration, *i.e.* 5 cells in 25  $\mu$ l *vs.* 10 cells in 40  $\mu$ l, respectively. This detection sensitivity is considered to be relevant to clinical and environmental applications. A high assay sensitivity was also demonstrated in an integrated glass sandwich micro structure, where Lagally *et al.*<sup>18</sup> showed amplification and detection of as few as 5–6 copies of M13/pUC

gene in 10 min, with a micro chamber volume of 280 nl. Considering that M13/pUC is a well established cloning vector and the assay was carried out in a buffer environment, the prospective inhibition from a matrix of 'real' sample (blood sample and/or with whole target cells) was not present. The PC plastic serpentine channel PCR micro reactor discussed in this study held a maximum volume of 40  $\mu$ l and demonstrated a high sensitivity of amplifying 10 *E. coli* cells (50 fg DNA) from 2% blood sample, as compared to on-chip (glass-silicon) amplification of ng amounts of WBC DNA obtained by filtration from a sample containing 3  $\mu$ l of blood (30) and/or PCR from a 1% blood<sup>31</sup> sample in fused-silica capillaries. Although 40  $\mu$ l is not commonly considered a very small volume, this plastic PCR micro reactor was designed and fabricated with the intent of total on-chip integration.<sup>3</sup> Considering the need to integrate with the upstream sample preparation module which possesses a capacity to pre-concentrate potential rare target cells from a large volume (at least one milliliter) of raw sample, 40  $\mu$ l was considered a relatively small volume.

Only a few demonstrations have shown multiplex PCR on a microchip device.<sup>6,28,29</sup> Multiplex PCR using whole *E. coli* cells has been demonstrated in a glass microchip,<sup>6</sup> where different regions of *E. coli* genomic and plasmid DNAs were amplified simultaneously. Dilutions of *E. coli* colonies were used as DNA template, where cell lysis, multiplex PCR and electrophoretic analysis were executed sequentially on a monolithic microchip device, although the exact cell concentrations were not clearly reported. Cheng *et al.*<sup>28</sup> successfully demonstrated a locus-specific multiplex PCR for the human dystrophin gene using degenerate oligonucleotide primed-PCR (DOP-PCR) in a silicon-glass microchip. Although the data convincingly showed the feasibility of performing complex PCR assays in their microfabricated silicon-glass device, the amount of starting template concentration used was in the 100 ng– $\mu$ g range of human genomic DNA in their multiplex amplification which was performed over an hour period. In our PC plastic PCR micro reactor, 1000 cells (about 5 pg DNA) of three different pathogens, *E. coli* K12, *E. fae.*, and *S. sal.*, were amplified simultaneously and adequately.

Amplification from whole blood requires optimization of salt (K<sup>+</sup> and Mg<sup>2+</sup>) concentration according to sample volume and

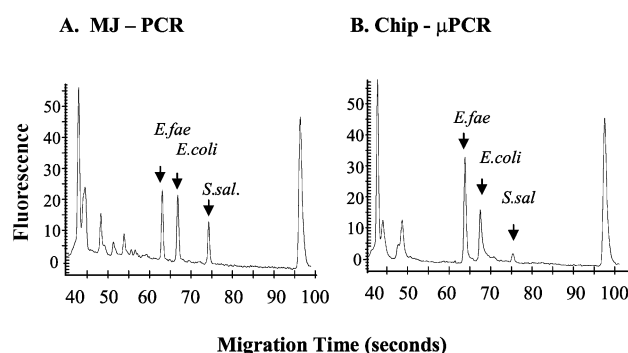


**B.**

Different Pathogen Cells	Amplicon Concentration (ng/ $\mu$ l)		
	MJ – PCR	$\mu$ PCR	% of MJ
<i>E. coli</i> K12 cells	1.9	0.83	44
<i>E. faecalis</i> cells	8.6	0.93	11
<i>S. epidermitis</i> cells	25.9	10.4	40
<i>S. salivarius</i> cells	7.4	3.1	42

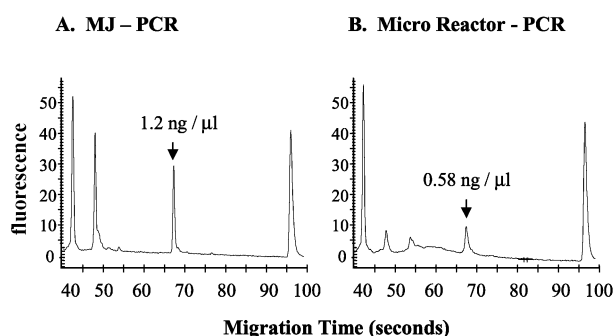
**Fig. 6** Microchip PCR amplification of different bacterial genes. 10<sup>3</sup> cells of *E. coli* K12 and *E. faecalis* and 10<sup>4</sup> cells of *S. epidermitis* and *S. salivarius* were used as templates to amplify *E. coli* K12-, *E. faecalis*-, *S. epidermitis*-, and *S. salivarius*-specific genes (221 bp; 195 bp; 371 bp; 293 bp), respectively (A). The quantitative data are shown in panel B, comparing MJ-PCR and the micro reactor PCR.

type of anticoagulant used<sup>37</sup> or addition of formamide in the PCR mixture.<sup>38</sup> Successful PCR amplification from unpurified blood samples has been reported in tubes using a conventional PCR thermocycler.<sup>37,38</sup> A high sensitivity amplification from a blood sample in a plastic micro PCR reactor or chip had not been demonstrated to date. An automated and integrated system for high-throughput DNA genotyping directly from blood was reported by Zhang *et al.*<sup>31</sup> Using a hot air thermocycler, samples containing whole blood were directly amplified in a fused-silica capillary and only one concentration (1%) of blood sample PCR was demonstrated, providing ng amounts of DNA as the starting genomic material. In another microchip module for blood sample preparation and amplification, Wilding's group demonstrated direct capture of WBC cells in a glass-silicon microchip with a series of micro filters, from 3  $\mu$ l of whole human blood, and subsequent amplification of human coagulation factor V from WBC DNAs in the same microchip,<sup>30</sup> in which > 10 ng WBC genomic DNA was used as initial template. For on-chip PCR from a blood sample in our PC plastic PCR micro reactor, the initial denature time was prolonged to 15 min, using the Hot Start Taq DNA polymerase (QIAGEN Valencia, CA), and also supplemented with Q buffer (QIAGEN) which is designed to accommodate the blood components which could inhibit PCR. Ten whole *E. coli* cells (50 fg *E. coli* genomic DNA) were adequately amplified from ng amounts of heterogeneous



	Amplicon concentration (ng / $\mu$ l)		
	<i>E. Fae</i>	<i>E. coli</i>	<i>S. sal</i>
MJ - PCR	0.73	0.7	0.4
Micro reactor PCR	1.2	0.6	<0.1

**Fig. 7** Multiplex amplification of three different bacteria-specific genes in the plastic PCR micro reactor. 1000 of each *E. faecalis*, *E. coli*, and *S. salivarius* cells were amplified simultaneously in either the conventional MJ thermocycler (A) or the plastic PCR micro reactor (B). Three amplicons of 195 bp, 221 bp, and 293 bp fragments were analyzed by Agilent Bioanalyzer as marked with arrows (upper panel). Quantitative comparison data are shown in the lower panel.



**Fig. 8** Amplification of *E. coli* K12-specific gene (221 bp) fragment from 10 *E. coli* cells in the presence of 2% blood. A 221 bp amplicon (arrows) was detected by Agilent Bioanalyzer. The amplicon concentration from the plastic PCR micro reactor (B) was 48% of that from MJ thermocycler (A).

mammalian genetic DNA pool (2% unpurified sheep blood) in our polycarbonate plastic PCR micro reactor (Fig. 8). This result further demonstrates the high sensitivity and specificity of our plastic PCR micro reactor.

In order to capture and detect the rare targets from a relatively large volume of clinical and environmental samples, the device sensitivity or detection limit takes priority over the time of operation. The PCR micro reactors demonstrated here meet the requirements of fairly fast operation (30 min for 30 cycles) along with the low manufacturing cost (plastic material instead of silicon or glass), and foremost, high sensitivity and specificity. These reactors permitted detection of as little as ten *E. coli* cells (50 fg DNA and 70 copies of the gene). The specificity of these polycarbonate PCR micro reactors was clearly demonstrated by their ability to amplify different pathogen-specific genes separately and multiplex amplification of three different bacteria specific genes simultaneously.

In addition, this highly sensitive PC plastic PCR micro reactor can be readily integrated<sup>3</sup> with upstream sample preparation and downstream detection. On-chip capture of rare target cells using immunomagnetic bead separation has been developed and demonstrated in our laboratory<sup>39</sup> and integration of DNA amplification and detection in the same PC plastic monolithic device has also been reported<sup>3</sup> previously. Similarly, it is very critical for a plastic PCR micro reactor to possess the ability to selectively amplify rare target genes from a heterogeneous, larger genetic background. The cellular components in 1  $\mu$ l of whole blood include about 10000 white blood cells, > 1 million of red blood cells and 100000 platelets, in addition to all other protein and lipid components. Operation of our plastic micro PCR reactor has shown that 10 *E. coli* cells could be amplified in the presence of 2% blood, which suggests that the PC PCR micro reactor could amplify a specific gene from a heterogeneous background, indicating its specificity and sensitivity.

In summary, the polycarbonate plastic PCR micro reactors reported here are easy to fabricate and capable of full assay functionality with high assay sensitivity and specificity. These plastic micro PCR devices will be useful for future clinical applications due to their ease of fabrication, low cost, high assay sensitivity, and capability for further integration with other portions of a sample-to-answer chip system.

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