DNA Extraction, Purification and Quantification Using Micromachined Microfluidic Chip

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ABSTRACT

This paper introduces a new DNA extraction and purification method from whole blood. Mixing and separation steps are performed using functionalised superparamagnetic beads suspended in cell lysis buffer in a circular chamber that is sandwiched between two electromagnets. Non-uniform nature of magnetic field causes temporal and spatial distribution of beads within the chamber. This process efficiently mixes the lysis buffer and whole blood in order to extract DNA from white blood cells. Functionalized surface of the magnetic beads then attract the exposed DNA molecules. Finally, DNA-attached magnetic beads are attracted to the bottom of the chamber by activating the bottom electrode. DNA molecules are extracted from magnetic beads by washing and re-suspension processes. In this work a 25µl PMMA chamber, 500µm in depth and 8mm in diameter was fabricated to purify DNA from spiked bacteria cell culture into the whole blood sample using Promega Magazorb DNA extraction kit. Spiked bacterial cell culture was prepared from a Gram positive; *B. Subtilis* and a Gram negative, *E. coli* cell cultures with 100,000 copy levels. The lysis was performed in 5min incubation at 56°C followed by 5min incubation for binding process. Thermal stability was generated and controlled by the same external coils which were used for continuous mixing and on-chip clamping of the magnetic beads in the chamber. The yield/purity and recovery levels of the extracted DNA were evaluated using quantitative UV spectrophotometer and real-time PCR assay respectively. The preliminary result indicated successful lysis of *E. coli* and *B. subtilis* using modified extraction protocol. Excellent DNA recovery levels were obtained using chip-based extraction process with a good quality index.

Keywords: DNA extraction, Magnetic mixer, Magnetic bead.

1 INTRODUCTION

A magnetic bead-based DNA extraction and purification method was previously introduced using two different extraction devices [1, 2], static extraction using a square-shaped microchamber (integrated key-type electrodes) and continuous extraction using a microchannel (external electromagnets). Superparamagnetic beads were used to perform the mixing of the blood sample and lysis buffer. Non-uniform magnetic field was generated to create temporal and spatial distribution of beads within the extraction device. This process efficiently mixed the lysis buffer and blood in order to extract DNA from lysed cells. Released DNA molecules were then captured by functionalised surface of the magnetic beads. Finally, DNA-attached beads were clamped using DC magnetic field and DNA molecules were purified by washing and resuspension processes.

A problem with the previously reported extraction device was the low extracted DNA recovery levels, which was only sufficient to be detected using thermal cycling PCR assay. In this paper, the efficiency of the static extraction approach (using microchamber) was further evaluated using modified chamber (circular geometry) and new improved external magnetic coils. This has consequently improved the efficiency of the mixing and extraction to achieve a very high DNA recovery levels, comparable to the standard bench-top extraction process.

2 MICROFLUIDIC CHIP FABRICATION

A schematic diagram of the microchamber is depicted in Figure 1. The chamber was made form PMMA as core material, which possess high mechanical rigidity and is well known material used in microfluidic chips. PDMS, which is another well known bio-compatible material, was used to fabricate the internal surfaces of the microchamber. The chamber comprises three layers structure.

The middle part, which is in contact with bio-fluid, was made of PDMS that is sandwiched between two PMMA outer layers. PMMA was die-cut to accommodate the chamber and port vias. Since it is important to accommodate the external coils at the closest possible distance from the chamber, a 1mm sheet of PMMA was used for fabrication of outer layers. A recess was hot-embossed into the PMMA to provide an end-wall of approximately 250 µm thickness, and to form a cylindrical wall some 9 mm in diameter to accommodate the winded coils.

After embossing the two PMMA layers were micromilled (#73 drill) to create the porting. Four ports were initially designed for inlets and outlets. One port was designed for air vent, two ports for inlets (reagent and sample) and the last port for outlet. Two inlet and outlet

port pairs were positioned at 180° angle around the circumference and were symmetrically positioned in a vertical line to create a T-shape junction.

A 12 mm diameter pitch circle was used for drilling the ports. A 500∓15 µm thick cast sheet of PDMS was then die-cut to fabricate the middle chamber layer. The necessary vias were made to link to the outer PMMA layers. It was initially intended to pressure-assemble and seal three layers by using two 1.6 mm diameter screws. This method failed to securely seal the device. Therefore, a thin layer of PMDS was coated on PMMA to act as a bonding agent.

The bonding of PDMS thin layer was made with an elastomer mix of 5:1 base/curing agent ratio, rather than usual 10:1 ratio. This new mixture ratio has lower viscosity and reduced cure time and most importantly improved bonding to PMMA. This PDMS mixture was left at room temperature for 1 hour before assembling the chip. After sealing, the sandwiched layers were placed between two aluminium blocks at 60°C temperature for 1½ hour to cure the PDMS. Port-way feeds were made from 6mm length stainless steel tubing set into 2.4mm×1mm polystyrene collars and drilled (#73) to form ferrules. The ferrules were then secured into the PMMA ports using epoxy resin. Finally, 100mm length, 250µm bore medical-grade PVC tubing was used to provide the microfluidic networks at inlets and outlets. A photograph of microfabricated microfluidic four-port chip is shown in Figure 2 with attached feed-line tubing.

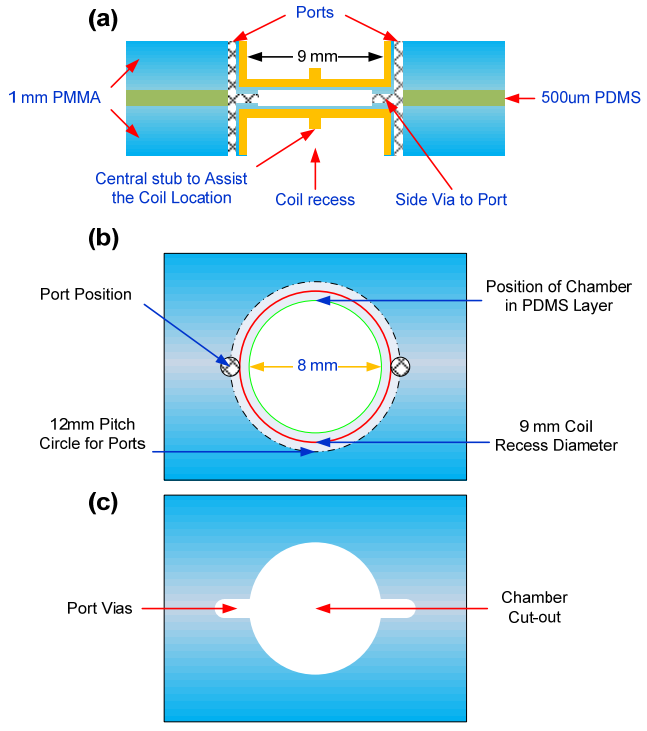

Figure 1: Schematic diagram of the microfabricated microchamber. a) Cross-sectional view. b) Plane view. c) Chamber cut-out and position of vias.

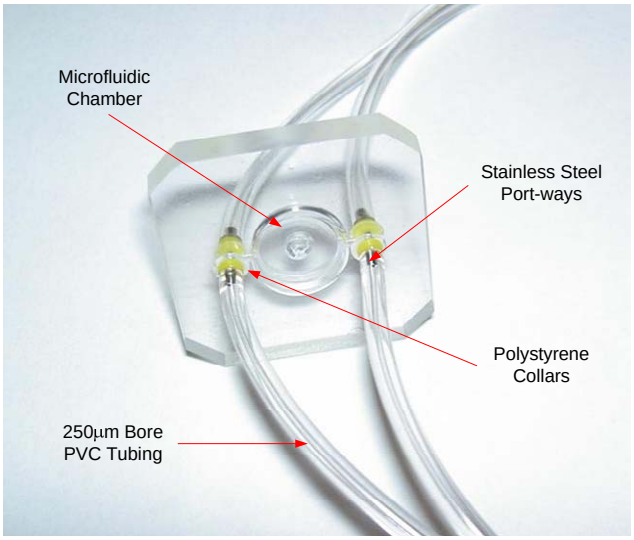

Figure 2: A photograph of microfabricated 15µl chamber with PVC tubing.

Two magnetic coils were made using aluminium bobbins with 2mm winding width, 4mm winding depth and 8mm coil overall outer diameter. The thickness of bobbin's cheek on the chamber side was narrowed down to 0.25mm to maximise the strength of magnetic field. Bobbins were wound using copper wire, gauge 42.

3 EXPERIMENTAL SETUP

The experiment was performed using low-volume disposable chips (25µl, 8 mm in diameters and 500µm chamber depth). MagaZorb DNA Mini-prep Kit (Promega, Ltd.) was used to perform the extraction process and all reagents were scaled down relatively. Two different Bacterial cell cultures (*B. subtilis* and *E. coli*) were spiked into the blood at approximately 100,000 copy levels. Prior to the spiking process, a real-time PCR assay was developed in order to calculate the concentration of the cell cultures. Fresh blood was used as the negative extraction control in addition to the standard negative control (water sample). Spiked blood experiment was carried out in five replicates to reduce the variability and a parallel manual bench-top extraction was also performed using the same reagents and samples for comparison purpose. Two-step protocol (combined proteinase k/lysis step, then binding step) was used for extraction and the enzymatic incubation steps were performed in two separate steps, 5 minutes lysis step at 56°C and 5 minutes binding step at room temperature. The required temperature was generated and maintained using the external magnetic coils and attached temperature sensor. Chip-based washing and elution steps were performed inside the chip.

The whole chip-based extraction process is summarised as follows:

- Introduction of the spiked blood sample and lysis buffer into the chip using a 25µl Hamilton syringes.
- Five minutes incubation of blood/lysis buffer at 56°C.
- Introduction of magnetic beads suspended in binding buffer to the lysed blood sample inside the chip using a 25µl Hamilton syringes.
- Five minutes incubation of the lysed blood/beads/binding buffer with active magnetic mixing. The mixing was performed using two external heatsink coils at switching frequency of f=1 Hz and coil current of 200 mA.
- Performing the washing process with on-chip clamping of the magnetic beads using the bottom coil. The clamping current was increased to 250 mA and the washing buffer was introduced to the chip using a 100µl Hamilton syringe. A total of 200µl washing buffer was dispensed in two steps.
- Washing buffer was removed using a 25µl Hamilton syringe, while magnetic beads remained inside the chip under magnetic clamping force.
- 25µl elution buffer was introduced into the chip using a 25µl Hamilton syringe and switching magnetic field was used to mix the DNA-attached beads with the elution buffer. This process was performed in 10 minutes.
- Elution buffer (contained suspended extracted DNA molecules) was removed from the chip using a 25µl Hamilton syringe, while the magnetic beads remained clamped inside the chip.
- This experiment was repeated another time on different day with similar extraction conditions.

4 RESULTS AND DISCUSSION

A successful temperature control was obtained using the bottom coil, which resulted in a very fast and complete lysis process. As a result of efficient magnetic mixing, a very homogeneous mixture of the binding buffer and lysed blood was also observed. A very efficient chip clamping process was achieved during both washing process and removing the sample after elution step. However, a percentage of the beads were lost (on average <10%) during the clamping and washing process. Figure 3 illustrates a series of screen-shots captured from different steps during extraction process describing lysis progress, magnetic mixing progress, beads clamping/washing process and elution step.

4.1 UV Spectrophotometry

Extracted DNA sample was analysed using standard DNA quantitation technique (Nanodrop ND1000 spectrophotometer) to evaluate the total DNA yields and purity factors. The averaged results of Nanodrop analysis are shown in Table 1 for both manual and chip-based extractions (data is given for two different extractions). These measurements were performed in three replicates for each sample and the device was blanked with the elution buffer. Both extractions produced high quality DNA, as shown by A260/A280 quality index. However, DNA quality in manual extraction is higher than the chip-based extraction. It was observed that the device is highly sensitive to the residual washing buffer inside the sample during extraction process. This sensitivity can affect the DNA yield measurements significantly (as shown in DNA yield of chip extraction control). Therefore the measurements were corrected based on the extraction controls. Another problem with this quantitation technique was the sensitivity of the device to the presence of magnetic beads inside the sample (it can be seen as high DNA yield in chip/blood (1) sample in Table 1). Nevertheless, very high DNA yields were obtained using both extraction methods, which describe the extraction efficiency using two-step protocol.

4.2 Real-time PCR Assay

In addition to the standard DNA quantitation technique (UV spectrophotometer), two different quantitative real-time PCR assays were developed to determine bacterial DNA extraction recovery levels using Applied Biosystems 7900HT Fast Real-Time PCR system. Determination analysis was performed in separate experiments using two 384-well plates for two microorganisms, *B. subtilis* and *E. coli*. Standard curves were generated using ⟦10⟧^6-⟦10⟧^1 gDNA copies and were used to calculate approximate concentration of the recovered bacterial DNA within the extracted DNA samples (using 2µl sample volumes).

The PCR plates were setup using 3x replicates of each extracted sample and the standard DNA samples. The regular 1:100 dilution ratios of the original cell cultures (used in the previous qPCR experiments) were replaced with 1:1000 dilution ratios to decrease the effect of PCR inhibitors. In addition, replicates of the cell cultures (1:1000 dilutions) were increased to 9x and pooled averaged results were used to determine the extracted DNA recovery levels of *B. subtilis* and *E. coli* microorganisms. This process can increase the accuracy of DNA recovery calculations. In a different approach, DNA recovery levels were calculated based on the extracted DNA samples from the original cell cultures used for spiking experiments. The extraction process was performed using the QIAamp DNA mini kit. Pooled averaged results of 9x replicates were used to estimate the extracted DNA recovery levels.

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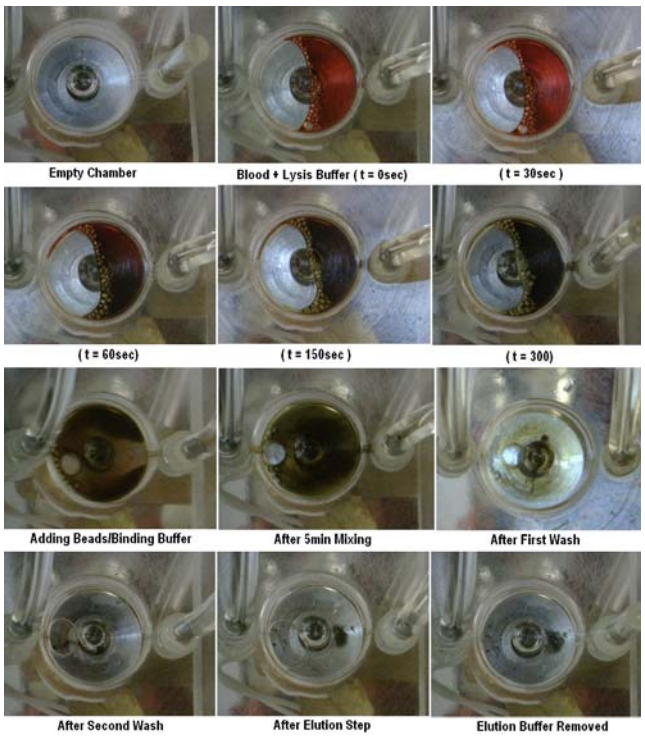

Figure 3: A series of screen-shots captured from different steps during chip-based extraction process (describing: lysis progress, magnetic mixing progress, beads clamping/washing process and elution step).

	Average A260/A280	Average DNA Yield (ng/µl)	Corrected Average Yield (ng/µl)	Standard Deviation
Manual Cells/Blood (1)	2.13	15.63	12.42	6.55
Manual Blood (1)	1.96	8.43	5.23	0.10
Manual Cells/Blood (2)	2.35	8.24	5.03	1.23
Manual Blood (2)	2.47	6.46	3.25	0.12
Manual Control	2.8	3.20	0.00	0.00
Chip Cells/Blood (1)	1.61	31.14	8.55	6.74
Chip Blood (1)	1.51	88.16	65.57	41.43
Chip Cells/Blood (2)	1.68	29.27	6.68	6.44
Chip Blood (2)	2.10	24.39	1.80	0.44
Chip Control	1.69	22.59	0.00	0.00

Table 1: Nanodrop quantitation results.

Spiked Blood Sample	% Recovery (DNA-based)	% Recovery (Cell-based)
B. subtilis (Chip)	58.5	20.2
B. subtilis (Manual)	59.5	21.5
E. coli (Chip)	43	62
E. coli (Manual)	52	79

Table 2: Real-time PCR quantitation results.

The PCR plates were setup using 3x replicates of each extracted sample and the standard DNA samples. The regular 1:100 dilution ratios of the original cell cultures (used in the previous qPCR experiments) were replaced with 1:1000 dilution ratios to decrease the effect of PCR inhibitors. In addition, replicates of the cell cultures (1:1000 dilutions) were increased to 9x and pooled averaged results were used to determine the extracted DNA recovery levels of *B. subtilis* and *E. coli* microorganisms. This process can increase the accuracy of DNA recovery calculations. In a different approach, DNA recovery levels were calculated based on the extracted DNA samples from the original cell cultures used for spiking experiments. The extraction process was performed using the QIAamp DNA mini kit. Pooled averaged results of 9x replicates were used to estimate the extracted DNA recovery levels.

Presence of *B. subtilis* and *E. coli* was not observed in non-spiked blood samples and negative controls. The approximate genomic equivalent of spiked bacterial cell concentration was calculated using these two above approaches (using original cells culture and extracted DNA from cell cultures). Estimated recovery levels for the spiked *B. subtilis* bacterial cells were calculated for chip-based and manual extraction process based on the above two approaches and are given in Table 2.

Excellent DNA recovery levels were obtained with both chip-based and manual extraction process. Similar DNA recovery levels were obtained with both manual and chip-based extractions, which indicate comparable extraction efficiencies in both techniques. These results indicate significant improvement in extraction efficiency using two-step protocol.

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