**SMALL VOLUME HYPERMETHYLATED DNA ENRICHMENT FOR EPIGENETICS**

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**ABSTRACT**

We present a small volume microfluidic chip for low concentration (≤1 ng/ml) capture and elution of hypermethylated DNA (hmDNA) using solid-phase extraction. All steps of the assay have been qualitatively characterized using a real-time surface plasmon resonance biosensor. A capture/elution efficiency of 84% from the assay has been quantitatively characterized using calibrated fluorescence spectroscopy.

**KEYWORDS**

Hypermethylated DNA, Methyl-binding domain protein (MBD), solid-phase extraction

**INTRODUCTION**

Hypermethylated CpG islands at the promoter region of genomic DNA are an accepted epigenetic cancer marker for many types of cancer [1]. For early warning diagnostics it is imperative that a small number of hmDNA copies are detected, which typically requires concentration enhancement. A small volume solid-phase hmDNA extraction chip has been realized in a microfabricated lab-on-chip platform using a methyl binding domain protein (MBD2, Life Technologies) [2] conjugated directly to the large area chip surfaces. The MBD2 protein coated surfaces are used to specifically capture hmDNA, which is then subsequently eluted. The focus of this article is to demonstrate the capture and elution of hmDNA and to improve the efficiency of the chip based capture and elution for low concentration hmDNA samples. This type of lab-on-chip platform is necessary to realize fully integrated microscale analysis systems. For epigenetics, small volume solid-phase hypermethylated DNA capture and elution systems are becoming increasingly important as opposed to conventional large volume magnetic bead based solution assay products.

**EXPERIMENTAL**

We have designed and implemented parallel channel and parallel pillar array chips [3,4] as they provide large surface area (Figure 1a-1b). The pillar channels are 50 µm in height and the pillars have a square geometry (10 µm on a side) rotated 45 degrees with respect to the inlet flow stream in order to maximize the flow velocity for efficient sample elution (Figure 1e). The chips were manufactured by anodically bonding an oxidized silicon wafer that contains the microfluidic channels to a borofloat glass wafer that contains the inlet and outlet holes. The microfluidic channels were formed by deep reactive ion etching of the silicon wafer and followed by the growth of a 300 nm thick silicon dioxide layer.

![Figure 1. Chip designs and simulation. (a) straight parallel channels (b) bifurcated parallel channels (c) SEM of pillar array (d) SEM of parallel channels (e) simulated flow velocity of the pillar array.](image)

The inlet and outlet holes were created in the glass wafer using powder blasting with alumina particles. External fluidic connections were made using polyetheretherketone tubing (1574 PEEK™ tubing, Upchurch Scientific) with interface fittings (Nanoport, Upchurch Scientific) attached to the glass layer. The reagents and samples were delivered using pressure driven flow.

The MBD2 surface functionalization scheme is shown in Figure 2. An amine functional group is first covalently attached to the silicon dioxide surface using 3-amino-propyl-trimethoxy alkyl silane (APTES). Following immersion in a 2% APTES solution, the glass-silicon chip was washed with ethanol and heated at 120°C for 15 min. prior to further coupling steps inside the chip. A biotinylated surface is formed using a 10 mM biotin-N-hydroxysuccinimide ester in phosphate buffered saline (PBS) buffer. The biotinylated surface was exposed to a 1µM streptavidin (SA) solution. The biotinylated MBD2 protein is then attached to available sites on the SA covered surface. The MBD2
protein has a mass of 29 kDa.

![Figure 2. Surface functionalization scheme for hmDNA capture using MBD2 proteins.](image)

The DNA used for capture is 80 bp dsDNA with eight symmetric 5-methyl-CpG islands and molecular weight 49537 g/mol (~50 kDa) (Figure 3). A surface plasmon resonance (SPR) biosensor was used to characterize the MBD protein based hmDNA capture/elution assay.

![Figure 3. 80 bp dsDNA with eight symmetric 5-mCpG islands used for hmDNA capture experiments.](image)

Following the preparation of the silicon-glass chip with the MBD2 capture proteins, the chip was used to capture hmDNA. Briefly the MBD2 chip was loaded with hmDNA in incubation/wash buffer consisting of 160 mM NaCl for 1 hour by flowing the 1 ml load volume through the chip. A wash step with 100 µl buffer is followed by an elution step with 1 ml high ionic strength elution buffer consisting of 2M NaCl. The incubation buffers, MBD2 proteins and elution buffers were used from the Methyl Miner Kit, (Life Technologies) [2]. The microfluidic chips have been used for the capture and elution of hmDNA at various concentrations. The eluted DNA is de-salted with ethanol precipitation and re-suspended in 1 ml wash buffer and the concentration measured with pico-green intercalating dye (Quant-IT, Invitrogen) fluorescence assay.

RESULTS and DISCUSSION

The fabricated parallel channel and pillar chips with MBD2 capture surface were used for the capture/elution experiments of hmDNA (Figure 1c and 1d). A flow velocity of ~0.7 mm/s has been estimated numerically using finite element simulations (Comsol) (Figure 1e), which enhances hmDNA elution from the surfaces. Fluorescence spectroscopy using SA-Alexafluor488, which binds to available MBD2 biotin sites as in Figures 3(a), 3(b) and 3(c), show attachment of the MBD2 protein to the biotinylated surface. The SA only control surface, without the MBD2 protein, followed by SA-488 introduction, shows minimal fluorescence (Figure 3d), which is most likely due to nearly complete biotin binding to the APTES from the high SA concentration (1µM).

![Figure 3. AlexaFluor488-SA used for surface imaging (a) and (b) MBD attachment to oxide in parallel channels (c) MBD attachment to oxide pillar array (d) control (no-MBD).](image)

The MBD2 proteins can distinguish between hmDNA and non-hmDNA as demonstrated with real-time SPR experiments (Figure 4). The large SPR signals are caused by the injection of different buffer solutions required for each assay step. A control biotinylated gold surface did not bind to the MBD protein and hmDNA.
Table 1 lists capture/elution results quantified using the complete assay protocol and fluorescence spectroscopy. Calibration plots of fluorescence intensity variations as a function of hmDNA concentrations were measured and used for all quantification experiments. For input concentrations ~1 ng/ml, and lower, the capture/elution assay functions with very high efficiency ~84%, which can be further improved by experimental refinement. The assay appears to be well saturated with ~4 ng of DNA at higher concentrations ~100 ng/ml. From these measurements and considering that the total chip area for the combined design of parallel channels with pillars is 1.7 cm², the protein attachment density is ~3×10¹⁰ MBD2-molecules/cm² (assuming 100% capture efficiency), which is reasonable considering the mass of the MBD2 protein. Our present method uses large elution volume post processing with ethanol precipitation, which can also add to loss of the recovered DNA. We are currently optimizing the protocol to reduce the elution volume by at least 100×, so that captured hmDNA can be eluted in small volume providing highly concentrated and purified hmDNA samples for further analysis.

Table 1. Measured results from full capture/elution hmDNA assay using fluorescence quantification.

<table>
<thead>
<tr>
<th>Input concentration (ng/ml)</th>
<th>Output concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.924</td>
</tr>
<tr>
<td>123</td>
<td>4</td>
</tr>
<tr>
<td>200</td>
<td>3.8</td>
</tr>
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CONCLUSION
We have presented a small volume microfluidic chip that is capable of capturing low concentrations of hypermethylated DNA (≤1 ng/ml) with a capture/elution efficiency of 84%. All steps of the assay have been qualitatively characterized using a real-time surface plasmon resonance biosensor. The assay has been quantitatively characterized using calibrated fluorescence spectroscopy.

REFERENCES

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