Electronic Supplementary Material (ESI) for ChemComm.

Electronic Supplementary Information (ESI)

for

eMethylsorb: Electrochemical biosensing for the quantification of DNA methylation at CpG resolution using DNA-gold affinity interactions

Abu Ali Ibn Sina, Sidney Howell, Laura G. Carrascosa, Sakandar Rauf, Muhammad J. A. Shiddiky* and Matt Trau**

Methods

Material. All the reagents used for the experiments were of analytical grade and purchased from Sigma Aldrich (Australia). UltraPure™ DNase/RNase-free distilled water (Invitrogen, Australia) was used to carry out the experiments. Synthetic DNA oligonucleotides were purchased from IBA (Germany). MCF7, BT474 and MDA-MB-231 breast cancer cells lines were purchased from ATCC (USA).

Preparation of DNA samples. DNeasy blood and tissue kit (QIAGEN Pty. Ltd., Venlo, Netherlands) was used to extract the Genomic DNAs from 10^5 cells plates of MCF7, MDA-MB-231 and BT 474 cells according to manufacturer's instructions. Briefly, the cells were suspended in lysis buffer to lyse and release the nucleic acids and proteins into the solution. The digestion of protein and RNA was done by using proteinase and RNase enzymes. Subsequent centrifugation step removed the digested protein and RNA. An elution buffer was used to elute the purified DNA. A REPLI-g whole genome amplification kit (QIAGEN Pty. Ltd., Venlo, Netherlands) was used to prepare the whole genomic amplified (WGA) DNA by amplifying 50 ng of the human genomic DNA (Roche, Germany) according to manufacturer's instructions. The purified DNAs were stored at -20ºC.

Bisulfite treatment. Bisulfite treatment of purified DNA was performed by using a MethylEasyXceed kit (Human Genetic Signatures Pty. Ltd., NSW, Australia) according to manufacturer's instructions. Briefly, 4 µg of DNA were incubated with 150 mM NaOH solution at 37 °C for 15 minutes followed by treatment with sodium bisulfite at 80 °C for 45 minutes. The DNA solution containing sodium bisulfite was then mixed with 800 µL of water, 2 µL of glycogen (20 mg/mL, Fermentas, USA) and

1 mL of iso-propanol. The mixture was incubated on ice for 30 minutes followed by centrifugation at 14000 g for 10 minutes. The supernatant was removed and washed with 70% ethanol to precipitate the DNA pellet. The washing step was repeated twice to completely remove residual sodium bisulfite salts from the precipitated DNA pellet. The pellet was then resuspended in elution buffer and desulphonicated at 95 °C for 20 minutes.

Quantification of DNA. To normalize the DNA copy number from each DNA source, the relative amount of *EN1* genes in bisulfite treated cell and WGA DNA samples were determined by real-time amplification of the housekeeping Col2A1 gene using a Rotor-Gene 6000 thermomixer (Corbett Research, Mortlake, Australia) and performing comparative analysis of Ct (i.e. the fractional PCR cycle number at which the reporter fluorescence is greater than the threshold). Each 20 µL of the PCR mixture contains 1 unit Taq DNA polymerase (AmpliTaq DNA Polymerase, Applied Biosystems, Australia), 2 mM $MgCl_2$ 1X PCR buffer (AmpliTaq 10X PCR buffer) 0.2 mM each dNTP, 1% triton X-100, 10 µM SYTO 9 dye and 250 nM of each Col2A1 forward and reverse primer (Supplementary Table 1). Equal volume of the target cell sample and WGA DNA were added as template to the PCR reaction. Thermal cycling was carried out in a Rotor-Gene 6000 thermomixer (Corbett Research, Mortlake, Australia) using the following conditions: denaturation at 94 ºC for 10 minutes followed by 40 cycles of 94 ºC for 30 sec, 55 ºC for 30 sec and 72 ºC for 30 sec.

Asymmetric PCR. Asymmetric PCR was performed to obtain ss-stranded DNA amplicons containing the target sequence. Briefly, bisulfite treated samples were amplified in a 60 µL reaction containing 1.5 unit Taq DNA polymerase (AmpliTaq DNA Polymerase, Applied Biosystems, Australia), 0.7X PCR buffer (AmpliTaq 10X PCR buffer) 0.2 mM each dNTP, 0.1% tween, 125 nM of forward primer and 375 nM reverse primer (Supplementary table 1). Cycling was carried out in a Bio-Rad thermo cycler (MJ Mini Personal Thermal Cycler) using the following conditions: denaturation at 94 ºC for 10 minutes followed by 49 cycles of 94 ºC for 30 sec, 61 ºC for 45 sec and 72 ºC for 30 sec.

Electrochemical detection of DNA methylation. All electrochemical experiments were carried out using a CH1040C potentiostat (CH Instruments) with a three-

electrode system consisting of a gold working electrode (2 mm in diameter), Pt counter electrode, and Ag/AgCl reference electrode (all electrodes are from CH Instruments, USA). Differential pulse voltammetric (DPV) experiments were conducted in 10 mM PBS solution containing 2.5mM [$K_3Fe(CN)_6$] and 2.5mM [$K_4Fe(CN)_6$] electrolyte solution. DPV signals were obtained with a potential step of 5 mV, pulse amplitude of 50 mV, pulse width of 50 ms, and pulse period of 100 ms. For DNA methylation detection, the gold electrodes were initially cleaned by polishing with Alumina polishing powder (CH Instruments) followed by ultra-sonication with acetone and deionised water for 5 minutes and then dried under the flow of nitrogen. DPV signals of clean electrodes were measured in electrolyte solution to get the baseline current. The electrodes were then incubated in 250 µL of synthetic DNA samples (for genomic DNA, 20 µL of amplified DNA spiked in 250 µL of SSC5X buffer). The electrodes were then washed three times with PBS for 5 minutes. The relative DPV currents (i.e., $\%i_{r,}$ percent difference of the DPV signals generated for DNA sample (i_{sample}) with respect to the baseline current ($i_{baseline}$)) due to the adsorption of DNA samples were then measured by using equation 1. The difference in relative DPV signals between unmethylated and methylated DNA was calculated by using equation 2.

$$\text{Relative DPV signals } (\%i_r) = [(i_{baseline} - i_{sample})/i_{baseline}] \times 100 \quad \ldots \quad \ldots \quad (1)$$

$$\text{Current difference } (\Delta i_r) = \%i_{r,unmethylated} - \%i_{r,methylated} \quad \ldots \quad \ldots \quad (2)$$

where $\%i_{r,methylated}$ and $\%i_{r,unmethylated}$ are the relative DPV signals for the methylated, and unmethylated samples, respectively.

Table S1. List of the DNA sequences used in this study. The CpG sites in the *EN1* region of interest are highlighted in red colour. The G/A base changes in synthetic DNA representing methylated and unmethylated samples that would be generated after bisulphite treatment and asymmetric PCR of *EN1* gene are highlighted with green and orange colour.

Description	Sequence
Region of interest from *EN1* gene with eight potentially methylate CpG sites (chr2:119604022-119604075)	5'-TTGGTGCCCTGCGCTCCGGGGCTCCCCGCG CCGCCTCCACTGCCGCCGCCACCG-3'
Forward primer for asymmetric PCR of *EN1* region	5'-ATTCAGTCCACAACAAYGTTGGTTGAG TTTATAAGTAGGATAGT-3'
Reverse primer for asymmetric PCR of *EN1* region	5'-ACRACCRCAACAACCAAACCCT-3'
Forward primer for quantifying DNA using the Col2A1 gene	5'-GTAATGTTAGGAGTATTTTGTGGGTA-3'
Reverse primer for quantifying DNA using the Col2A1 gene	5'-CTACCCCAAAAAAACCCAATCCTA-3'
Forward primer used for sequencing of *EN1* region	5'-GAGGTTGTTGTGAGTTAGTTTTGATT-3'
Reverse primer used for sequencing of *EN1* region	5'-ACRACCRCAACAACCAAACCCT-3'
Synthetic methylated DNA mimicking the bisulphite treated and asymmetric PCR processed *En1* region with 8 methylated CpG sites	5'-GATAACGACGACAATAAAAACGACGCGAA AAACCCCGAAACGCAAAACACCAA-3'
Synthetic methylated DNA mimicking the bisulphite treated and asymmetric PCR processed *En1* region with 4 methylated CpG sites	5'-GATAACAACGACAATAAAAACGACACGAA AAACCCCAAAACACAAAACACCAA-3'
Synthetic methylated DNA mimicking the bisulphite treated and asymmetric PCR processed *En1* region with 1 methylated CpG sites	5'-GATAACAACAACAATAAAAACAACACAAAA AACCCCAAAACACAAAACACCAA-3'
Synthetic unmethylated DNA mimicking the bisulphite treated and asymmetric PCR processed *En1* region with 0 methylated CpG sites	5'-AATAACAACAACAATAAAAACAACACAAAA AACCCCAAAACACAAAACACCAA-3'