Journal Name

COMMUNICATION

A multiplex microplatform for the detection of multiple DNA methylation events using gold-DNA affinity

Received 00th January 20xx,
Accepted 00th January 20xx

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DOI: 10.1039/x0xx00000x

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We report a new multiplexed strategy for the electrochemical detection of regional DNA methylation across multiple regions. Using the sequence dependent affinity of bisulfite treated DNA towards gold surfaces, the method integrates the high sensitivity of micro-fabricated multiplex device comprising microarray of gold electrodes, with the powerful multiplexing capability of multiplex-PCR. The synergy of this combination enables the monitoring of the methylation changes across several genomic regions simultaneously from as low as 500pg/μl of DNA with no sequencing requirement.

Introduction

The pervasive epigenetic alteration in the levels and distribution of DNA methylcytosines across the genomes has been considered as a prominent hallmark of cancer.¹ More specifically, during cancer, many regulatory regions undergo substantial hypermethylation at sites that are enriched in CpG dinucleotides- the so called CpG islands.¹⁻³ Accurate detection of these regions can provide a clear understanding of the complex relationships between epigenetic alterations and gene expression in normal and cancer cells. This knowledge is also opening the ways to develop new epigenetics-based strategies for cancer diagnosis and treatment. However, with few exceptions, most cancer types require the simultaneous analysis of many genomic regions from an epigenetics-based perspective. There is also a thirst for simple and rapid methods that can readily provide actionable clinical information. Currently, most multiplexed conventional methods for DNA methylation analysis require sophisticated and expensive instrumentation which are not suitable for clinical application.^{4, 5} Thus a simple and inexpensive multiplex technique can possibly drive out this limitation and provide a better alternative for the detection of cancer in clinic.

Recently, we reported a methodology for DNA methylation detection, coined as “Methylsorb”⁶ or “eMethylsorb”,⁷⁻⁹ which showed great promise for clinical applications due to the simplicity of the detection approach. The methods were based on the different adsorption ability of bisulphite treated (followed by asymmetric PCR) ssDNA amplicons (generated from methylated and unmethylated DNA) onto the bare gold surface. The bisulfite treatment changes unmethylated cytosines into uracils, which are copied to adenine in the complementary strand during the asymmetric PCR process. In contrast, methylated cytosines remain unchanged and are copied as guanines during the same process. Since the ssDNA follows the A>G>C>T adsorption trend,^{10, 11} the DNA amplicon enriched with adenines, which was generated from unmethylated templates adsorbed relatively strongly in compare to the guanine enriched amplicon derived from the methylated templates. This simple adsorption process therefore enabled clear and fast determination of the average methylation status of a given genomic region. Despite this approach has single CpG site resolution and circumvent the need for DNA sequencing, simultaneous analysis of multiple regions remain challenging due to the use of asymmetric PCR in generating ssDNA amplicons. In addition, performing individual PCR reactions for each region translates into a need for larger amounts of DNA input, which are often limited in the clinic.

To circumvent this drawback, herein, we report a new multiplex microplatform that synergistically combines the multiplex PCR with the multiplex microdevice and significantly improves the eMethylsorb approach. With this approach, the multiplex PCR can amplify several gene regions in a single step from minute quantities of DNA input, while the micro-device can perform multiple electrochemical measurements with high sensitivity within a single platform. The combination of these multiplex platforms therefore addresses the current demand for a facile, rapid and multiplexed methodology for DNA methylation in a simple, yet robust manner. The underlying principle of this approach is presented in scheme 1. Briefly, the genomic DNA extracted from cells undergoes bisulfite

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Scheme 1. Methodological approach for the multiplex detection of DNA methylation using DNA-gold affinity interaction in a multiplex micro-device.

treatment and multiplex PCR using biotinylated forward primers. This generates a pool of multiple ds-DNA amplicons corresponding to the targeted regions, which are biotinylated in the forward strand. Since our strategy only reads ssDNA, the amplicons are next dehybridized and all forward (5'-3') strands are removed from the amplicon pool by streptavidin coated magnetic beads. This thus generates a new pool of ssDNA amplicons comprising only the reverse (3'-5') ssDNA sequences. Then, each individual ssDNA amplicon is specifically isolated from the pool via hybridization with a complementary DNA molecule previously loaded onto the surface of magnetic beads. Finally, each captured amplicon is eluted using NaOH solution and separately dropped onto the surface of the gold microelectrodes within the multiplex device. This multiplexed device was previously reported by our group¹² and contains 16 micro-wells, with each well comprehending four

microelectrodes (Scheme 1). This configuration thus enables the possibility of obtaining 64 measurements from a single device. As reported in our previous studies, the level of DNA adsorption is quantified by measuring the Faradaic current generated by the $[\text{Fe}(\text{CN})_6]^{3-/4-}$ system in the electrolyte solution.^{7, 8} In this system, the generated current is the function of the adsorbed DNA (i.e. the more adsorbed DNA, the less current) which is presumably due to the stronger columbic repulsion between $[\text{Fe}(\text{CN})_6]^{3-/4-}$ and negatively charged adsorbed DNA.

Experimental

Materials

All the reagents used for the experiments were of analytical grade. UltraPure™ DNase/RNase-free distilled water (Invitrogen, Australia) was used to carry out the experiments. Synthetic DNA oligonucleotides were purchased from IBA (Germany). Breast cancer cells lines were purchased from ATCC (USA).

Preparation of DNA samples

Genomic DNAs were extracted from 10^5 breast cancer cells pellets by using DNeasy blood and tissue kit (QIAGEN Pty. Ltd., Venlo, Netherlands) according to manufacturer's instructions. Briefly, the cells were lysed by using lysis buffer to release the nucleic acids and proteins into the solution. The proteins and RNAs were digested using proteinase and RNase enzymes. Subsequently, the solution was centrifuged to remove the digested protein and RNA. The purified DNA was eluted in low TE buffer. 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 -20°C.

Bisulfite treatment

Bisulfite treatment was performed using a MethylEasyXceed kit (Human Genetic Signatures Pty. Ltd., NSW, Australia) according to manufacturer's instructions. Briefly, 4 µg of purified 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 isopropanol. 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 re-suspended in elution buffer and desulphonated at 95 °C for 20 minutes.

Asymmetric PCR

Asymmetric PCR was performed to obtain ss-stranded DNA amplicons of individual regions. The input bisulfite treated DNA sample) was amplified in a 20 μ L reaction containing 0.1 unit Taq DNA polymerase (GoTaq Hot Start DNA Polymerase, Promega, Australia), 5X PCR buffer (GoTaq 5X Reaction buffer) 0.2 mM each dNTP, CES, 125 nM of forward primer and 375 nM reverse primer (ESI table 3) The PCR was performed in a Bio-Rad thermo cycler (MJ Mini Personal Thermal Cycler) using the following conditions: denaturation at 95 $^{\circ}$ C for 10 minutes followed by 35 cycles of 95 $^{\circ}$ C for 30 sec, 55 $^{\circ}$ C for 45 sec and 72 $^{\circ}$ C for 30 sec.

Multiplex PCR

Multiplex PCR was performed to generate a pool of ds-DNA amplicons representing multiple target gene regions. Briefly, bisulfite treated samples were amplified in a 20 μ L reaction containing 0.1 unit Taq DNA polymerase (GoTaq Hot Start DNA Polymerase, Promega, Australia), 5X PCR buffer (GoTaq 5X Reaction buffer) 0.2 mM each dNTP, CES, 250nM biotinylated forward primer and 250 nM reverse primer for each gene region. The PCR reaction was performed in a Bio-Rad thermo cycler (MJ Mini Personal Thermal Cycler) using the following conditions: denaturation at 95 $^{\circ}$ C for 10 minutes followed by 35 cycles of 95 $^{\circ}$ C for 30 sec, 55 $^{\circ}$ C for 45 sec and 72 $^{\circ}$ C for 30 sec.

Magnetic Separation of forward Strand

Streptavidin coated magnetic beads (Dynabeads[™] MyOne[™] Streptavidin C1) are obtained from Thermo fisher Scientific. Desired amount of beads were washed and re-suspended in SSC5X buffer. Since biotinylated forward primers were used for multiplex PCR, it generated ds-DNA amplicons containing biotinylated forward strands. To remove the biotinylated forward strands, PCR amplified ds-DNA solution was first denatured by adding a NaOH solution. This mixture was subsequently added to the designated amount of streptavidin coated magnetic bead solution. The ratio of bead and ds-DNA was calculated by the binding ratio provided by the manufacturer. To ensure maximum capture of the forward strand, we used 1.5 times higher amount of beads than the amount suggested by manufacturer. The solution was well-mixed with the pipette, incubated with gentle shaking and then placed in a magnetic stand to separate the magnetic beads bound with the biotinylated forward strands. To achieve maximum denaturation and recovery of reverse strand, we optimised the incubation time and concentration of NaOH solution. As shown in figure S1, 0.3M NaOH solution with 8 minutes incubation at room temperature with gentle shaking provided us the maximum recovery of the reverse strands. The solution was neutralized by adding 0.3M Tris-HCl solution and the supernatant containing ss-DNA pool of multiple gene regions was then separated for further analysis.

Functionalization of magnetic beads with complementary sequences to target regions

Desired amount of beads were washed and re-suspended in SSC5X buffer. Designated amount of biotinylated synthetic oligonucleotides for the target region (e.g., oligo 1 complementary to the reverse strand of region1) were mixed with the beads as per manufacturer instruction. The ratio of bead and biotinylated synthetic oligonucleotides was calculated as per manufacturer instruction.

Isolation of antisense strand of individual gene

Oligo-functionalized beads were sequentially mixed with the ss-DNA PCR pool containing reverse strand of multiple gene regions. To achieve maximum binding of reverse stand with the synthetic complementary oligo sequence for region 1, the mixture was shaken and incubated for 15 min. The beads containing reverse strands for region 1 were then separated by using magnetic stand. The supernatant containing reverse strands of other regions (i.e. region 2 and 3) was reused again to separate them individually according to the same procedure. To denature the reverse stand of region 1 from the bead bound complementary oligo sequence, the beads were re-suspended in NaOH solution and incubated according to the optimised condition mentioned for 1st pull down. The solution was neutralized by adding 0.3M Tris-HCl solution and the beads were separated using magnetic stand. The supernatant containing ss-DNA of region 1 was then quantified and used for electrochemical analysis. The total bead isolation protocol is depicted in ESI scheme S1 for further clarification. These steps were repeated for isolating the other target regions in a sequential manner.

Device Fabrication and Electrochemical detection

Microdevices were fabricated at Australian National Fabrication Facility, Queensland node as reported previously (See details on the ESI of ref S1). All electrochemical experiments were carried out using a CH1040C potentiostat (CH Instruments) with a three-electrode system consisting of gold microelectrodes as working electrode (100 μ m in diameter), Pt counter electrode, and Ag quasi reference electrode. Differential pulse voltammetric (DPV) experiments were conducted in 10 mM PBS solution containing 2.5mM [K₃Fe(CN)₆] and 2.5mM [K₄Fe(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, DPV signals of microelectrodes were measured in electrolyte solution to get the baseline current. An aliquot of 10 μ l of DNA was then adsorbed into each well of the device under optimised conditions. The electrodes were then washed three times with PBS. The relative adsorption currents (i.e., %*i_r*, % difference of the DPV signal 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 adsorption currents between unmethylated and methylated DNA was calculated by using equation 2.

Adsorption current(%*i_r*) = [(*i_{baseline}* - *i_{sample}*)/*i_{baseline}*] $\times$ 100...(1)

Relative Current difference(Δi_r) = %*i_{r,unmethylated}* - %*i_{r,methylated}*...(2)

where $%i_{r, \text{methylated}}$ and $%i_{r, \text{unmethylated}}$ are the relative adsorption currents for the methylated, and unmethylated samples, respectively.

Results and Discussion

The use of microelectrodes for DNA detection provides special advantages, particularly, in terms of sensitivity in comparison to the conventional macroelectrodes. This is mainly due to their small surface area (i.e. diameter = 100 μm vs 2mm in macro-electrodes) which significantly enhances the detection sensitivity due to the high signal to noise ratio.¹² We thus sought to compare the performance of the microdevice with macroelectrodes used in our previous (i.e. eMethylsorb) approach to determine its analytical performance. First, we compared the sensitivity of the device by analysing a synthetic ssDNA fragment mimicking the magnetically isolated reverse amplicon generated from the methylated and unmethylated region contained in the engrailed homeobox1 (EN1) gene. The methylation analysis of this region was previously reported by eMethylsorb approach.⁷ This region contains eight potentially methylated CpG sites located within a span of 53 bases and has been regarded as a potential methylation biomarker in different cancer types.¹³ Figure 1A shows that the new assay can clearly differentiate the methylation status of ssDNA sequences at 500pM concentration, which is 20 times more sensitive than our previously reported eMethylsorb using macroelectrodes (e.g. LOD 10nM vs 500pM for the macro and microelectrodes respectively). To determine the analytical performance in DNA samples derived from real genomic DNA, we extended the analysis of this EN1 region for the DNA extracted from MCF7 breast cancer cell line. The unmethylated control was generated from a genomic DNA template which has previously undergone whole genome amplification (WGA) — a process that removes all the methylation marks from DNA. For these samples, the ssDNA amplicons were obtained as per our previous eMethylsorb standard protocol (i.e., via bisulfite treatment and asymmetric PCR amplification). Using these sequences, we optimized key operating parameters such as assay time and sample concentration. Figure 1C indicates that our assay can differentiate methylated and unmethylated DNA sequence from as low as 500pg/ μL of PCR amplified DNA (i.e. $\Delta i_r = 11.85 \pm 3.13$, see Experimental for detail calculation of $%i_r$ and Δi_r). However, the maximum difference was observed for the 5ng/ μL concentration of the DNAs (i.e. Figure 1C, 1B, $\Delta i_r = 41.18 \pm 1.86$). We also found that 15 min adsorption time provides us the maximum difference (i.e. $\Delta i_r = 41.18 \pm 1.86$) between the two sequences. Beyond 15 minutes adsorption, the difference (i.e. Δi_r) decreased presumably due to the electrode surface starting to saturate in both, methylated and unmethylated, derived DNA cases.

The ability of our assay in detecting heterogeneous methylation was further assessed by using a solution containing designated proportions of methylated and unmethylated DNA derived from EN1 region of MCF7 breast cancer cell line and WGA DNA respectively (i.e., Meth/Unmeth at 0%/100%, 10%/90%, 25%/75%, 50%/50%, 75%/25%, and 100%/0%). Quantifying the heterogeneous methylation in

Figure 1. Optimization of the operating parameters and the sensitivity of the assay. (A,B): Mean values of relative current ($%i_r$) obtained for the adsorption of synthetic(A) and cell derived(B) methylated and unmethylated EN1 gene region respectively (C,D): Mean values of relative current difference (Δi_r) obtained for the adsorption of cell derived methylated and unmethylated DNA at different concentration(C) and time(D). (E): DPV signals for the designated mixture of cell derived methylated and unmethylated samples and corresponding calibration plot. Each bar represents the average of three separate trials ($n=3$). Error bars represent the standard deviation of measurements (relative standard deviation (%RSD) was found to be < 5% for $n= 3$)

cancer is very important because patient tissue samples often contain a mixture of cancer and healthy cells providing a heterogeneous methylation profile.¹⁴ In addition, some regions in the genome could be differentially methylated in different stage of cancer in different patients. As shown in Figure 1E, the DPV signals increased with the increase of methylation levels indicating the lower adsorption of the DNA with higher methylation. This suggests that that our method is capable to identify differentially methylated regions. Furthermore, we achieved high sensitivity in analysing heterogeneous samples with low methylation percentage (i.e. 10% Methylation).

Finally, to demonstrate the multiplexing capability of our assay, we performed detection using the full protocol as described in Scheme 1, which integrates the multiplex PCR system with our multiplex micro device. We designed primers to target 3 regions located within the chromosomes 6 (region 1), 16 (region 2) and 19 (region 3) of the human genome (See ESI for details), and performed multiplex PCR for these 3 regions on three separate genomic DNA templates extracted from 3 different breast cancer cell lines (i.e., BT474, MCF7,

Figure 2. DPV signals for the adsorption of methylated regions (region1 (black dash line), region2 (blue dash line) and region3 (red dash line) derived from A) MCF7, B) BT474 and C) T47D cell line and unmethylated regions ((region1 (black), region2 (blue) and region3 (red)) derived from WGA DNA. Right panel: bar graph for the corresponding relative current mean values. Each data point represents the average of three separate trials, and error bars represent the standard deviation of measurements (%RSD $\leq$ 5% for $n=3$).

47D). These three regions have recently been found to be highly methylated in breast cancer.¹⁵ To avoid the dimer formation during the PCR reaction, the primers were carefully designed using "Primer Suite" which is a dedicated primer design software for multiplex-PCR on bisulfite treated DNA and provides excellent performance in reducing the likelihood of primer dimer formation.¹⁶ The formation of a single PCR amplification band for all amplicons was confirmed by gel electrophoresis (See ESI figure S2 for gel picture of individual PCR). Following multiplex PCR reaction, we used NaOH to dehybridize all dsDNA amplicons and subsequently removed the forward strand from the PCR pool using streptavidin coated magnetic beads. In this step, we optimized

dehybridization conditions (e.g. NaOH concentration and incubation time) to avoid the coincidental pull-down of reverse strand along with the forward. We found that 0.3M NaOH with 8 minutes incubation time provided us the best dehybridization conditions in our assay (see Experimental section for detail optimisation experiments). In addition, we found that a slight excess of streptavidin coated magnetic beads than suggested by manufacturer ensured maximum capture of forward strands from the PCR pool. Next, we performed the second pull-down using magnetic beads loaded with synthetic ss-DNA sequences complementary to the selected target. Captured DNA amplicons were then individually eluted from the beads with incubation with 0.3M NaOH for another 8 minutes. This step facilitates the sequential isolation of individual regions from the same reaction mixture providing more luxury to work with small volume of sample.

To test the specificity of the pull-down steps, we performed control experiments using beads modified with non-complementary sequences. We also used unmodified beads to test the nonspecific interaction of beads with DNA (See Experimental for details). The data in ESI Table S1 shows that both the experiments provided negligible concentration of the eluted ss-DNA amplicons which is below the limit of our detection (LOD = 500pg/ μ l, Figure 1C). This data confirm the high specificity of our bead based separation protocol. Under these optimised conditions, we performed the adsorption experiments for the three amplicons derived from the multiplex PCR and pull-down isolation of the three selected cell lines. As shown in Figure 3, relative DPV current (i.e. % I_r) for the three regions derived from cancer cell lines provided significantly lower relative current than the same region derived from an unmethylated genomic DNA (WGA). The relative DPV current difference (i.e. ΔI_r) was found to be 31–41% (See ESI table 2 for details) depending on the region, which could be due to the different number of CpG sites present in each region. These values nevertheless suggest that these regions are highly methylated. Further analysis with next generation sequencing confirmed the methylation status of these regions (data not shown). This confirms the high potential of this approach to be used as a platform for methylation analysis. Although techniques like microarrays and sequencing are highly sensitive and multiplexable, future improvements with an integrated micro-fabricated device to perform the PCR and pull-down in the same platform could enhance the sensitivity, simplicity and multiplexing capabilities of this approach.

Conclusions

In conclusion, we developed a multiplex platform for the simultaneous detection of multiple DNA methylation targets using gold-DNA affinity interaction. The developed method provides critical improvements over current methylation approach including i) 16plex micro-device which can analyse up to 16 samples in replicates (i.e., 64 measurements at the same time), ii) integrated with multiplex PCR to analyse several

amplified target regions, iii) inexpensive platform and high detection sensitivity. We believe that this micro-fabricated multiplex platform could have broad applications in cancer research and potentially find its relevance as simple diagnostic tool.

Acknowledgements

This work was supported by the UQ Postdoctoral Research Fellowship (2012001456), ARCDP (DP120102503) and National Breast Cancer Foundation of Australia (CG-12-07). These grants have significantly contributed to the environment to stimulate the research described here. Device fabrication was performed at the Australian National Fabrication Facility, Queensland node (ANFF-Q).

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Electronic Supplementary Information (ESI)

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A multiplex microplatform for the detection of multiple DNA methylation events using gold-DNA affinity

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Scheme S1: Magnetic bead isolation protocol: Isolation of individual gene from multiplex PCR pool containing ds-DNA of multiple genes.

Figure S1: Gel picture for the individual PCR reaction with primer designed for region 1, 2 and 3 from MCF7, BT474, T47D cell line and WGA DNA

Figure S2: Optimisation of the parameters for denaturation and elution of ss-DNA. (A) Mean values of the ss-DNA concentration (after the removal of forward strand) at PCR pool after eluting with different concentration of NaOH solution (B) Mean values of the ss-DNA concentration at PCR pool after eluting with NaOH solution at different incubation time. Each bar represents the average of three separate trials (n=3). Error bars represent the standard deviation of measurements (relative standard deviation (%RSD) was found to be < 5% for n= 3).

Table S1: Concentration of ss-DNA sample after the bead isolation using specific sequence, nonspecific sequence and unmodified beads.

DNA		Concentration after the isolation using magnetic beads modified with specific sequence (ng/μl)	Concentration after the isolation using magnetic beads modified with nonspecific sequence(ng/μl)	Concentration after the isolation using unmodified magnetic beads(ng/μl)
WGA	Region1(Chr6)	5.82±0.27	0.19±0.04	0.28±0.06
	Region2(Chr16)	5.09±0.21	0.12±0.05	0.21±0.08
	Region3(Chr19)	4.81±0.64	0.16±0.07	0.18±0.09
MCF7	Region 1(Chr6)	5.73±0.53	0.10±0.09	0.14±0.08
	Region2(Chr16)	5.47±0.65	0.09±0.05	0.11±0.04
	Region3(Chr19)	5.86±0.42	0.10±0.06	0.13±0.06
BT474	Region 1(Chr6)	5.39±0.57	0.06±0.04	0.14±0.06
	Region2(Chr16)	5.55±0.48	0.22±0.06	0.17±0.06
	Region3(Chr19)	4.98±0.49	0.25±0.08	0.15±0.08
T47D	Region 1(Chr6)	5.54±0.47	0.19±0.08	0.13±0.09
	Region2(Chr16)	5.58±0.63	0.25±0.06	0.16±0.06
	Region3(Chr19)	5.49±0.39	0.15±0.08	0.13±0.06

Table S2: Relative Current difference (Δi_r) between methylated gene regions from (MCF7, BT474 & T47D) and unmethylated gene regions from WGA.

Gene region	Δi_r for MCF7	Δi_r for BT474	Δi_r for T47D
Region1 (Chromosome 6)	39.85±2.91	38.02±2.85	38.50±3.11
Region2 (Chromosome 16)	38.72±2.46	39.93±2.52	41.21±2.37
Region3 (Chromosome 19)	36.21±2.81	33.05±2.23	31.84±2.12

Table S3. List of the DNA sequences used in this study.

Description	Sequence
Synthetic methylated DNA mimicking the bisulphite treated and asymmetric PCR processed <i>En1</i> region with 8 methylated CpG sites	5'-GATAACGACGACAATAAAAAACGACGGAAAAACCCCG AAACGCAAAACACCAA-3'
Synthetic methylated DNA mimicking the bisulphite treated and asymmetric PCR processed <i>En1</i> region with 8 methylated CpG sites	5'-AATAACACAACAATAAAAAACAACAAAAAACCCCA AAACACAAAACACCAA-3'
<i>EN1</i> gene with eight potentially methylate CpG sites (chr2:119604022-119604075)	5'-TTGGTGCCCTGCGCTCCGGGGCTCCC CGCG CCGCCTCCACTGCGCGCCACCG-3'
Region1: A region of 7 potentially methylated CpG sites chr6:26745623-26745741	5'-CACTCCTTTCTACTTTGCTTCCGTAGCTTCTGAGCAACC CGTGAAGAAATTGACGGAACCGGAAGGAATTACCGCCAGTTTC TTCCA CGGGCCGTCAAGGCCAATATAAGTCCCCAC-3'
Region 2: A region of RBFOX1 gene with 9 potentially methylate CpG sites chr16:6069891-6070005	5'-CTCCAGCTTATGGTGAGTGTGGCTGGGGGTGCAGAGAG CGCACGGGAATTGGGGGTCTGGGGCCGAGAACGTGACCGCAGC CGGGCTCGCGGGAGTTCTAGGTAAGTCCAGGC-3'
Region 3: A region of ZNF729 gene regions with 5 potentially methylated CpG sites chr19:22469491-22469594	5'-CCTCAGTCTCCTTCAGCCATAAGATGGCGAATGCGCTGA CTGCGGGACCCTGGGCGACCTGTCTCCTCCATGCGCTGTGACTGT GCCCTGGCCTGGAGCCCTC-3'

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