Supplementary Material (ESI) for Analyst
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Methylation detection without bisulphite or methylation-specific PCR

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Experimental Details

DNA Sample Preparations

The model system oligonucleotides (Table 1) were purchased from Geneworks Australia (SA, Australia). Serum samples were collected as described in Hoque et al. (11) and 14 normal samples and 14 breast cancer samples were chosen for analysis. DNA from 100 μL serum samples was extracted using a guanidinium isothiocyanate (GuSCN) protocol based on the method of Boom et al 1990. Briefly, the sample was mixed in a 1:1 ratio with lysis buffer ((a) 120g GuSCN dissolved in 100 mL 0.1M Tris-HCl pH 6.4; (b) add 15g Urea, 22 mL 0.2M EDTA pH 8.0, 2.6g Triton-X-100; NOTE: significant volume change with addition of GuSCN) including 100 μg proteinase K and 5 μg silica microparticle suspension (Sigma) and incubated for 15 minutes at 60C. The silica particles were then pelleted by centrifugation and the supernatant discarded. The pellet was washed three times in 500 μL wash buffer (120g GuSCN, 100mL 0.1M Tris-HCl pH 6.4, 25% ethanol) and twice in 70 % ethanol. After the removal of ethanol the pellets were heated for 60s at 56C to evaporate any remaining solvent. Finally, 100 μL ultra-pure water was added and the suspension heated at 40C for 15 minutes in a water bath. The supernatant was removed and stored frozen at -20C.

Microparticle functionalization

Particles were synthesized from a 3-mercaptopropyl trimethoxysilane (MPS) monomer in an emulsion/condensation process described in detail elsewhere (18). Amine functionality was introduced via incorporation of 3-aminopropyltrimethoxysilane (APS), carboxyl groups were provided by grafting a polyethylene glycol (PEG) linker and DNA probes were attached via standard EDC chemistry as described previously (18).

GST-MBD2b Protein Production

The GST-MBD2b fusion protein was synthesized based on the method of Pfeiffer et al 2005 (9). Briefly, the plasmid containing the human GST-MBD2b DNA sequence (donated by Dr Pfeiffer under a material transfer agreement) was transformed into BL21DE3 cells (Invitrogen, CA, USA). After streaking the transformed cells onto agar (supplemented with carbenicillin), two colonies were expanded in 5 mL LB medium overnight (37C, 250 rpm) and then diluted into 200 mL LB and grown under the same conditions for 2 hours. Induction of GST-MBD2b was initiated by the addition of 100 mM IPTG and after four hours the cells were pelleted and stored frozen for up to several weeks. Bacterial cell lysis was performed in 5mL STE buffer (0.01M Tris-HCl, 0.15M NaCl, 0.001M EDTA) containing 1 μL/mL protease inhibitor, 0.5mg lysozyme (Sigma, pre-warmed to room temperature) and 0.5 mL 15% sarcosyl solution. After careful vortexing to break the cell pellet, the mixture was sonicated for 4 × 30s intervals. GST-MBD2b protein was purified onto glutathione-agarose resin (Sigma) and eluted in PBS buffer (pH carefully controlled at pH = 8.0, very important) containing 100 mM reduced glutathione (Sigma). The concentration of eluted protein was determined by Biorad assay

and dialysis was performed using a Mini Dialysis Kit (8 kDa cutoff, GE Healthcare Life Sciences) into a PBS buffer. Storage was at -80 C for up to 12 months with 1% triton-X-100 (Sigma) added after dialysis.

DNA Hybridization and MBP Binding Assays

For DNA hybridization reactions, approximately 3000 probe-functionalized microparticles were combined with the target DNA (1 μM for model system, determined by Nanodrop measurement or 10μL purified serum sample) and 6X SSPE buffer (0.9 M NaCl, 60 mM sodium phosphate, 6 mM EDTA) supplemented with 0.05% triton-X-100 surfactant in a total volume of 20 μL. Reaction mix was heated in a PCR machine at 95C for 10 minutes followed by 55C for 30 minutes or 12 hours for clinical samples. After the reaction the particles were transferred to a Qiagen LiquiChip filter plate and washed three times in blocking buffer (PBS, 0.2% BSA, 0.05% triton-X-100) and incubated for 30 minutes. All samples were washed three times in incubation buffer (PBS, 0.1% BSA, 0.05% triton-X-100) and then incubated in a 1μg/mL solution of either GST-MBD2b or 5MeC-antibody (Aviva Systems Biology, San Diego, CA, US, cat no: AMM99021) diluted in IB. The same washing and incubation procedure was repeated for the FITC-labelled detection antibodies (Abcam, Cambridge, MA, USA) before a final washing step for flow analysis.

Assays for the model system were analysed on the FC500 (Beckman Coulter, Fullerton CA, USA) whilst serum assays were analysed using a BDLSRII (Becton Dickinson, Franklin Lakes NJ, USA). All assays were performed in triplicate and data is presented as average plus/minus the standard deviation as described in previous work.[18] The Cy5 fluorescent label on the target DNA sequences was not routinely used for quantitation – we observed large variations in the Cy5:DNA ratio (up to 30%), therefore the input DNA concentration was carefully monitored using the Nanodrop spectrophotometer. For experiments involving significantly different probe lengths, the Cy5 signal was used to normalise methylation data (Supplementary Figure 2). Data analysis was performed in GraphPad PRISM software (GraphPad Prism 5.02, GraphPad Software, Inc.) using t-tests for linear regressions for the model system and Mann-Whitney tests for clinical samples.

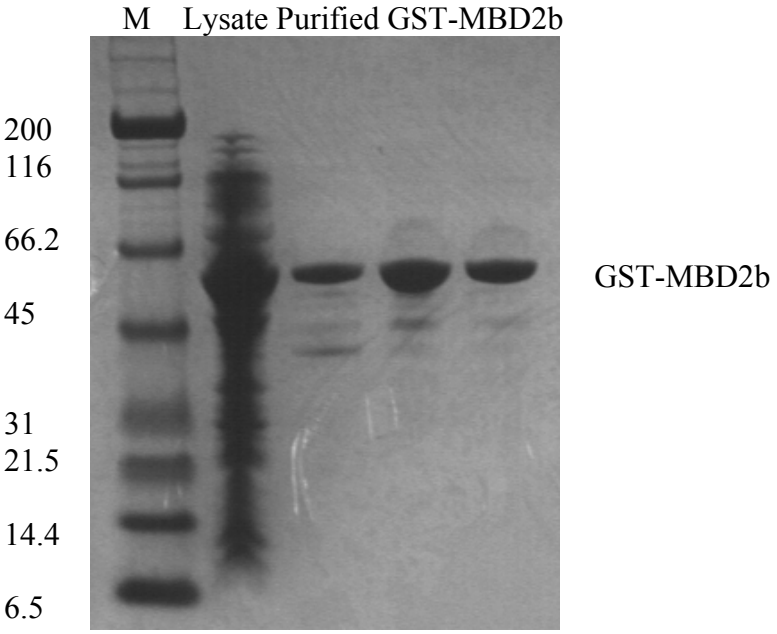
Quantitative Real Time PCR (QPCR)

QPCR was used to determine the DNA concentration in extracted serum samples. Real-time PCR (ABI7900, Applied Biosystems) for ALU and LINE-1 quantitation was performed using Quantace Sensimix (5 μL 2X PCR buffer, 0.067 μL of 30 μM primer mix (Table 1), 4.8 μL template). The cycling conditions were: 95C for 10 minutes, 40 cycles of 95C for 10s, 60C for 1 min and a final extension step at 72C for 5 minutes. Raw Ct values in triplicate were averaged and converted to relative amount, which was used to normalize the methylation data from flow cytometry for serum sample analysis. Methylation score as used in Fig. 4 is thus defined as the ratio of background-subtracted fluorescence intensity value from flow cytometry to relative DNA amount as calculated from qPCR. In Fig. 4 the average values are indicated by the line inside the box, the borders of the box represent the 25^{th} and 75^{th} quartiles and the whiskers indicate the highest and lowest value in each dataset.

Supplementary Table 1. Oligonucleotide probes and targets used in this study. Model system consists of a probe sequence (suffix "NH2" = 5'-amine modification for microparticle attachment) and complementary target (suffix "Cy5" = 5' Cy5 modification for detection by flow cytometry). The DOM-0 probe/target contains no methylated bases at all, however the DOM-x- probe/target contains x methylated CpG bases.

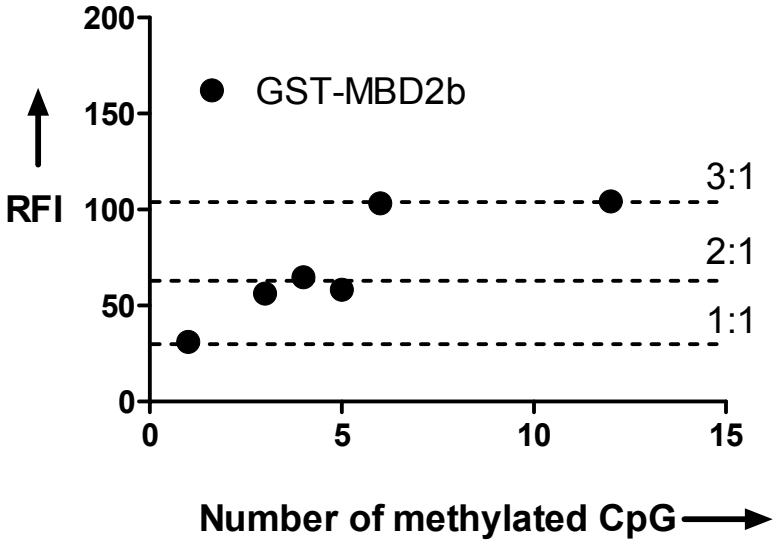
Name	Sequence[a]
DOM-0-NH_2	5'-GATCCGACGACGACGACGACGACGACGACGACGATC-3'
DOM-0-Cy5	3'-CTAGGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTAG-5
DOM-1-NH_2	5'-GATCCGACGACGACGACG**C**GACGACGACGACGACGATC-3'
DOM-1-Cy5	3'-CTAGGCTGCTGCTGCTGCTG**C**TGCTGCTGCTGCTGCTAG-5
DOM-3-NH_2	5'-GATCCGACGACGA**C**GA**C**GA**C**GACGACGACGACGACGATC-3'
DOM-3-Cy5	3'-CTAGGCTGCTGCTG**C**TG**C**TG**C**TGCTGCTGCTGCTGCTAG-5
DOM-4-NH_2	5'-GATCCGACGACGA**C**GA**C**GA**C**GA**C**GACGACGACGACGATC-3'
DOM-4-Cy5	3'-CTAGGCTGCTGCTG**C**TG**C**TG**C**TG**C**TGCTGCTGCTGCTAG-5
DOM-5-NH_2	5'-GATCCGACGA**C**GA**C**GA**C**GA**C**GA**C**GACGACGACGACGATC-3'
DOM-5-Cy5	3'-CTAGGCTGCTG**C**TG**C**TG**C**TG**C**TG**C**TGCTGCTGCTGCTAG-5
DOM-6-NH_2	5'-GATCCGACGA**C**GA**C**GA**C**GA**C**GA**C**GA**C**GACGACGACGACGATC-3'
DOM-6-Cy5	3'-CTAGGCTGCTG**C**TG**C**TG**C**TG**C**TG**C**TG**C**TGCTGCTGCTGCTAG-5
DOM-12-NH_2	5'-GATC**C**GA**C**GA**C**GA**C**GA**C**GA**C**GA**C**GA**C**GA**C**GA**C**GA**C**GATC-3'
DOM-12-Cy5	3'-CTAGG**C**TG**C**TG**C**TG**C**TG**C**TG**C**TG**C**TG**C**TG**C**TG**C**TG**C**TAG-5
ALU Probes	1) CTGGGATTACAGGAGTAAGCCACCGCGCCCGGCCGACTGAAGGGT 2) CGCGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAGG 3) GCACGATCTCGGCTCACCACAACTTCCACCTCCCAGGTTCAAGCGATTCTC
LINE-1 Probes	1) CCTGGAAAATCGGGTCTCTCCCACCCGAATATTGCGCTTTCGGACCGGCTT 2) CAGGGCGAGGCATTGTCTCACTTGGGAAGCGCAAGGGGTCAGGGAGTT 3) AGGTACTGGGTTCATCTCACTAGGGAGTGCCAGACAGTGGGCGCAGGTCAGTGGG
ALU PCR primers	For: CGGTGGCTCACGCCTGTA; Rev:GAGTGCAGTGGCGCGATC
LINE-1 PCR primers	For: TCTCACTAGGGAGTGCCAGACAG; Rev: CTGAGCCAGGTGTGGGATATAGTC
Long probe	5'GTGTCACCCGTGGTGGTGGCCTCGTCGCTAGGGCTCGTGTCGCTGGGCGGCAAGGCGAC CACGGCGTCGC-3'
Short probe	5'-TGGAGAACATCCTGGTGTCACCCGTGGTGGTGGCCTCGTC-3'
Long target	3'CACAGTGG**GC**ACCACCACCGGA**GC**A**GC**GATCCCGA**GC**ACA**GC**GACCC**GC**CGTTCC **GC**TG GT**GC**C**GC**A**GC**G-5'

[a] Bold **C** indicates 5-methyl-cytosine base in sequence



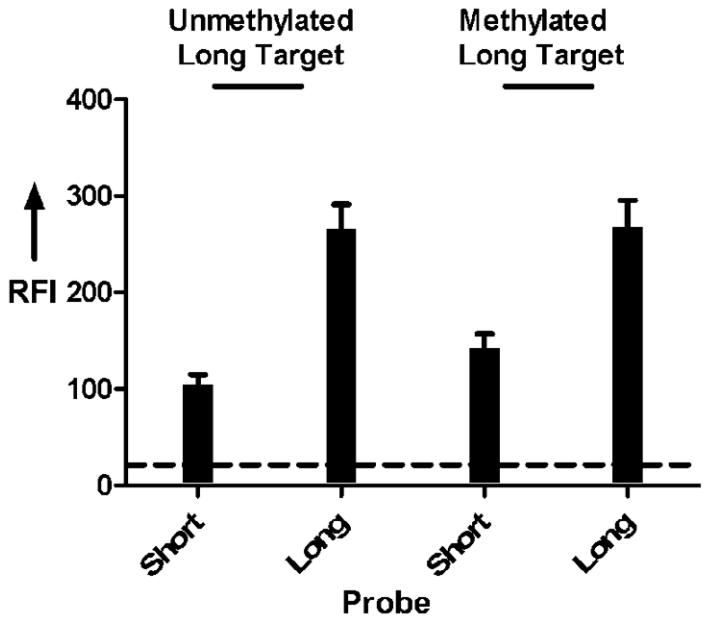
Supplementary Figure 1: SDS-PAGE gel showing the purification of the GST-MBD2b protein from bacterial lysate using GSH-agarose. The GST-MBD2b protein is approximately 50-60kDa in accordance with Rauch and Pfeiffer, *Laboratory Investigation* (2005) 85, 1172–1180.

GST-MBD2b plasmid was kindly provided under an MTA from Dr Gerd Pfeiffer and the resultant protein was produced in bacterial culture as described above. Supplementary Figure 3 shows the qualitative result of purification using glutathione-agarose resin.



Supplementary Figure 2: Flow cytometry data showing the increase in FITC intensity as a function of the number of symmetrically methylated 5MeC pairs in a duplex containing the DOM-12-NH_2 and –Cy5 sequences. Note that each data point represents the average of triplicate samples (standard deviations are also plotted here).

To validate the assay, we compared the microparticle results in Supplementary Figure 1 with previously reported data using the same model system of DNA sequences. Nan et al 1993 (16) formed symmetrically methylated DNA duplexes (identical DNA sequences to the probes/targets used in the current study) in solution and incubated each sample with the methylation binding domain of MeCP2 (similar to that used in the current study – GST-MBD2b was originally from MeCP1) and performed electrophoretic mobility shift assays (EMSA) to determine the MBP: DNA ratio. As shown in Supplementary Figure 1, GST-MBD2b specifically detected a single methylated CpG dinucleotide with respect to background, as reported also in Nan's study. As the DOM was increased, both assays registered increases in MBP binding with DOM in a "step-change" fashion, indicating a minimum number of symmetrically methylated CpG dinucleotide pairs required to accommodate more protein binding. In Nan's study, increases in GST-MBD2b binding occurred between DOM-0/1, DOM-3/4 and DOM-6/12, indicating a binding footprint of 12 bases centered on each methylated CpG dinucleotide. The microparticle assay showed similar step changes between DOM-0/1, DOM-1/3 and DOM-5/6, resulting in a binding footprint of 9-10 bases centered on each methylated CpG-dinucleotide. These results confirm the validity of the microparticle assay in comparison to an EMSA study using the same DNA sequences and a similar MBP.



Supplementary Figure 3: DNA hybridization results for long/short probes, measured by fluorescence intensity from Cy5-labelled target sequences via flow cytometry.

While the total target concentration was the assays in Figure 3 were kept constant (Nanodrop), we consistently observed a probe length-dependent difference in Cy5 fluorescence intensity upon hybridization (prior to, or after, MBD and FITC-Ab addition). This is expected because long probe/target sequences form more stable duplexes (e.g. cDNA arrays) in comparison to those involving short probes. We therefore

normalized the methylation data from these experiments to take into account changes in DNA concentration.

Supplementary Table 2. Amount of ALU/LINE-1 sequences in serum samples measured by qPCR

		Median	Min (ng)	Max (ng)
Cancer	ALU	0.183	0.004	11.0
Normal	ALU	0.033	0.001	10.4
Cancer	LINE-1	0.010	9.2×10^{-5}	0.683
Normal	LINE-1	3.0×10^{-4}	2.2×10^{-5}	0.233