Electronic Supplementary Information (ESI)

for

Molecular inversion probe-based SPR biosensing for specific, label-free and real-time detection of regional DNA methylation

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Methods

1. DNA extraction and bisulfite treatment

Detection of regional DNA methylation in our study was performed using DNA extracted from MCF7 cells and a control whole genome amplified (WGA) DNA that is unmethylated. MCF7 genomic DNA was extracted from 10^5 cells using the DNeasy blood and tissue kit (QIAGEN Pty. Ltd., Venlo, Netherlands) according to manufacturer's instructions. Briefly, the cells were suspended in lysis buffer to release the nucleic acids and proteins into the solution. Protein and RNA were digested with proteinase and RNase enzymes respectively and then removed by centrifuging the solution in a spin column. The purified DNA was eluted from the column in 100 µL of elution buffer and stored at -20 °C. Subsequently, the purified DNA was bisulfite treated using a MethylEasy Xceed kit (Human Genetic Signatures Pty. Ltd., New South Wales, Australia) according to the manufacturer's instructions. Briefly, 4 µg of DNA were incubated with 150 mM sodium hydroxide solution at 37 °C for 15 min followed by treatment with sodium bisulfite at 80 °C for 45 min. After treatment, the DNA solution containing sodium bisulfite was mixed with 800 µL of water, 2 µL of glycogen (20 mg/mL, Fermentas, USA) and 1000 µL of iso-propanol. The mixture was incubated on ice for 30 min followed by centrifugation at 14000 g for 10 min. 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 DNA precipitate. The DNA precipitate pellet was then resuspended in elution buffer and desulphonicated at 95 °C for 20 min. The control WGA DNA was, prepared by amplifying 50 ng of the human genomic DNA (Roche, Germany) using REPLI-g whole genome amplification kit (QIAGEN

Pty. Ltd., Venlo, Netherlands) according to manufacturer's instructions and then bisulfite treated using the similar protocol as described previously. Finally, to normalize the DNA copy number from each source, the relative amount of *En1* gene in bisulfite treated MCF7 and WGA DNA was determined by performing comparative Ct analysis using a Rotor-Gene 6000 thermomixer (Corbett Research, Mortlake, Australia). Each 20 µL PCR reaction contained 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 (Table S1). Equal volume of each MCF7 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 min, followed by 40 cycles of 94 ºC for 30 s, 58 ºC for 30 s and 72 ºC for 30 s.

2. Regional DNA methylation interrogation by MIPs and generation of single stranded DNA amplicons using asymmetric PCR

Following DNA extraction, the *En1* region of interest contained in the DNA template was interrogated with a unique MIP using a fill-in approach. In this approach 1 pg (22 aM) of molecular inversion probes (MIPs) (Table S1) were mixed with 50 ng of bisulfite treated genomic DNA in a 10 µL reaction containing 1X ligase buffer (200 mM Tris-HCl (pH 8.3), 250 mM KCl, 100 mM $MgCl_2$, 5 mM NAD, and 0.1% Triton X-100). The reaction was denatured at 95 °C for 6 min followed by 85 °C for 10 min. The temperature was then gradually decreased from 70 °C to 56 °C at the rate of 1 °C/30 s followed by 3 h incubation at 56 °C in a thermocycler. In these conditions, binding of the MIP to the genomic DNA was achieved generating a broad gap of 53 bases containing the methylated region to be interrogated. The filling of this gap was performed by adding 5 µL of the fill in mixture containing 1 unit ampligase enzyme (Ampligase Thermostable DNA Ligase, Epicentre Biotechnologies, Wisconsin, USA), 1 unit DNA stoffel fragment polymerase (Applied biosystems, USA), 125 nM of each dNTP (Roche diagnostics, Manheim, Germany) in 1X ligase buffer (200 mM Tris-HCl (pH 8.3), 250 mM KCl, 100 mM MgCl2, 5 mM NAD, and 0.1% Triton X-100). The reaction mixture was incubated at 56 °C for 2 h followed by thermal cycling for four reaction cycles on a Bio-Rad thermo cycler (MJ Mini Personal Thermal Cycler) using the following conditions, initial denaturation at 95 °C for 6 min.

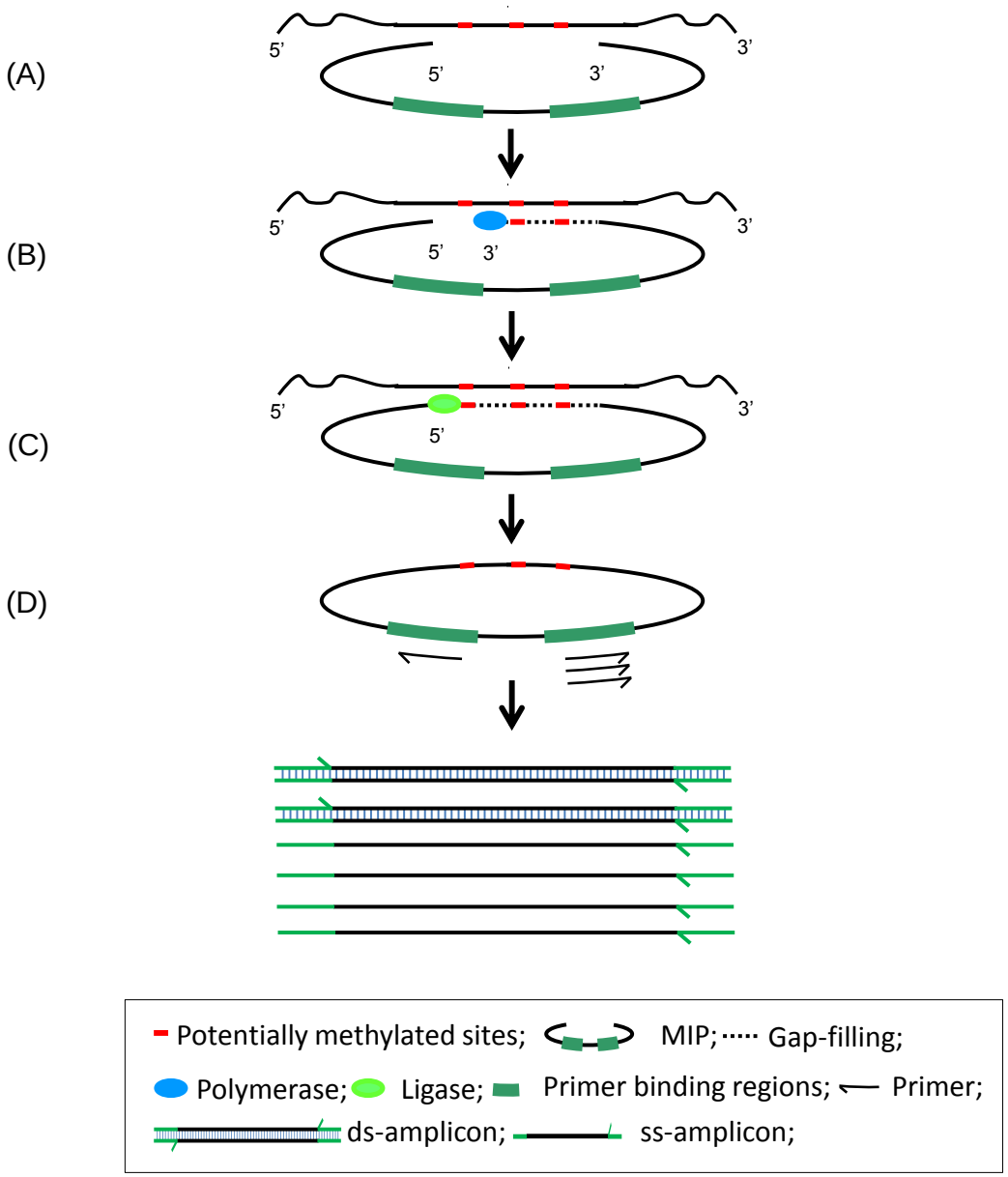

Fig. S1. A) *MIP designed to detect methylation pattern of CpG dinucleotides in an epiallele was mixed with bisulfite treated DNA and allowed to hybridise.* ***(B)*** *The gap between the recognition ends of the MIP was filled by a polymerase (blue).* ***(C)*** *A DNA ligase (green) was then used to circularise the MIP by covalently coupling the 5' end of the recognition ends with the 3' end of the newly synthesised DNA strand.* ***(D)*** *Universal forward and reverse primers in different molar ratios were used to amplify the circularised MIP and generate single stranded DNA amplicons.*

followed by 85 °C for 10 min. The temperature was gradually decreased from 70 °C to 56 °C at the rate of 1 °C/30 s followed by 4 h incubation at 56 °C. In order to digest all non-circularized MIPs, the entire volume of the ligation reaction was incubated with 10 units of exonuclease I (NEB, Ipswich, MA) and 50 units of exonuclease III (NEB, Ipswich, MA) at 37 °C for 30 min. The enzymes were heat deactivated by incubating at 80 °C for 20 min. Subsequently, the entire volume of the reaction mix containing the circularised MIPs was asymmetrically amplified by PCR 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 each universal forward primer and 375 nM universal reverse primer (Table S1). Thermal 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 min followed by 50 cycles of 94 ºC for 30 s, 65 ºC for 45 s and 72 ºC for 30 s.

3. Sequence and structure analysis of MIP's derived amplicons from MCF7 cells and WGA DNA.

To investigate the methylation status of the interrogation region of *En1* gene in MCF7 cells we employed bisulfite based sequencing. Briefly, the resulting PCR amplicon was electrophoresed through an agarose gel and purified using a QIAquick Gel Extraction Kit (QIAGEN Pty. Ltd., Venlo, Netherlands) according to manufacturer's instructions. The agarose gel containing the PCR amplicon was melted in a dissolving buffer (Buffer QG) and centrifuged through a spin column to wash away impurities. The purified DNA was eluted in 40 μL of DNase free water and quantified using a ND-1000 Nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA). The purified DNA was then mixed with the primer (20 ng DNA + 0.5 μL of 10 μM primer in 12 μL H_2O) and sequenced using Sanger sequencing protocol by the Australian Genome Research Facility (AGRF, Brisbane). 3D amplicon structures were then modelled and predicted using Mfold software.[1] Figure S2A depicts the sequencing output showing the methylated status of MCF7 cells. Figure S2B shows the 3D structures of methylated and unmethylated amplicons after bisulfite treatment. The regions matched by the oligo helpers employed during our study to unstructure these amplicons are also highlighted.

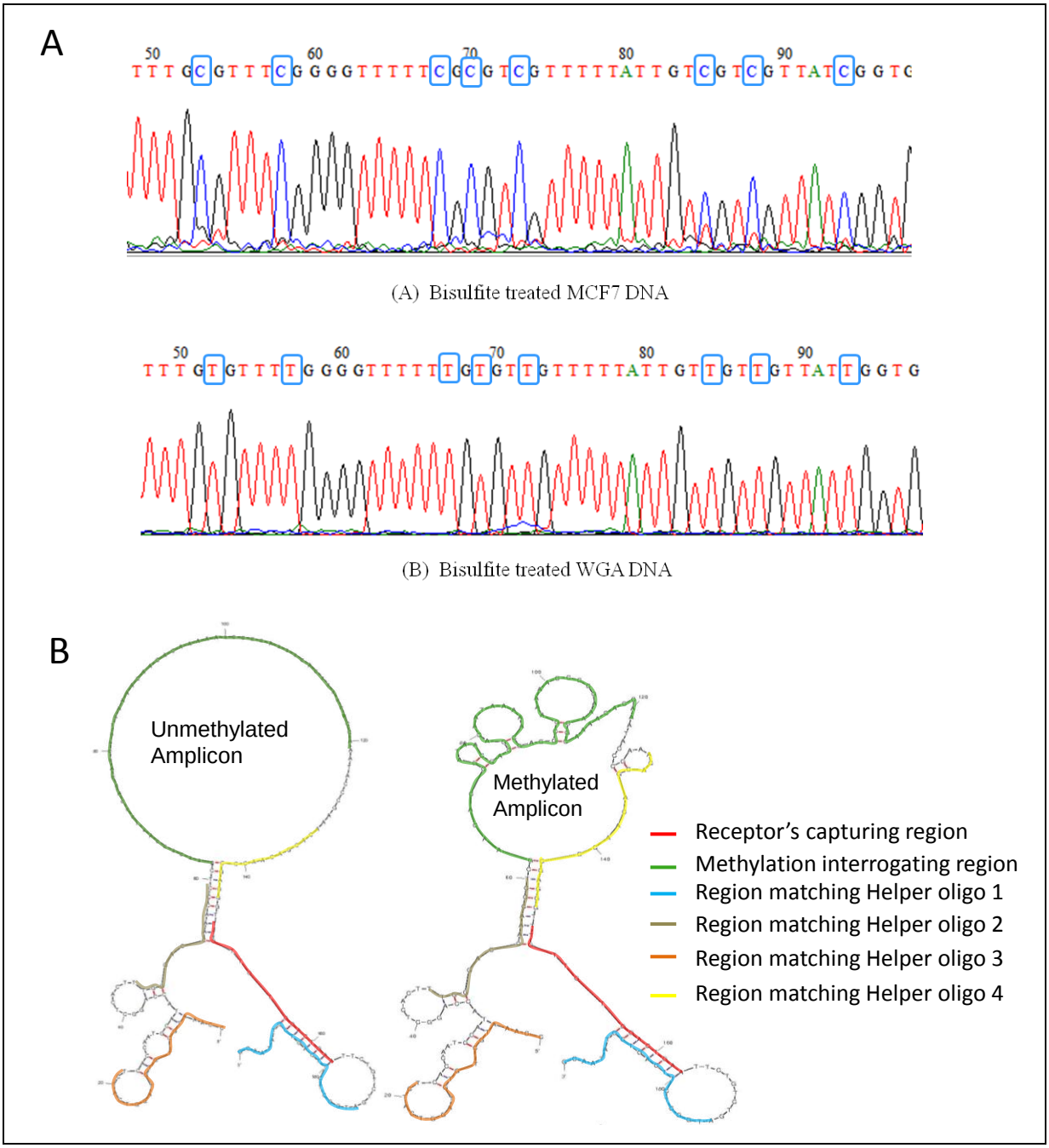

Fig. S2. A) *Sanger sequencing of En1 gene in Bisulfite treated MCF7 DNA (up) and WGA DNA (down). PCR primers were designed to amplify the interrogation region containing the eight CpG sites in the En1 gene.* ***B)*** *MIP derived amplicon's structure provided by Mfold software. Interrogating and receptor matching regions are highlighted in green and red respectively. Regions matching the helpers oligos are also indicated.*

4. Description of the SPR device

We employed a home-made SPR sensor of reduced size with a single microfluidic channel and using wavelength interrogation at a fixed 68º angle of incidence (Figure S3). Halogen light source (Oceans Optics, HL-2000-HP) was fiber-coupled to a multi-mode fiber with a small core (Thorlabs, ϕf = 50 μm, M14L01) to decrease the size of the resulting light spot. Light was subsequently collimated with a collimation lens (Thorlabs, C330TME-B) mounted inside a lens tube which also consisted of a platform that allows for tilt-correction. Finally, the collimated light beam was TM polarized with a UV-VIS polarizer (Thorlabs, LPVIS050). The reflected light was collected by a fiber-coupled (Thorlabs, f = 1 mm, M35L01) light

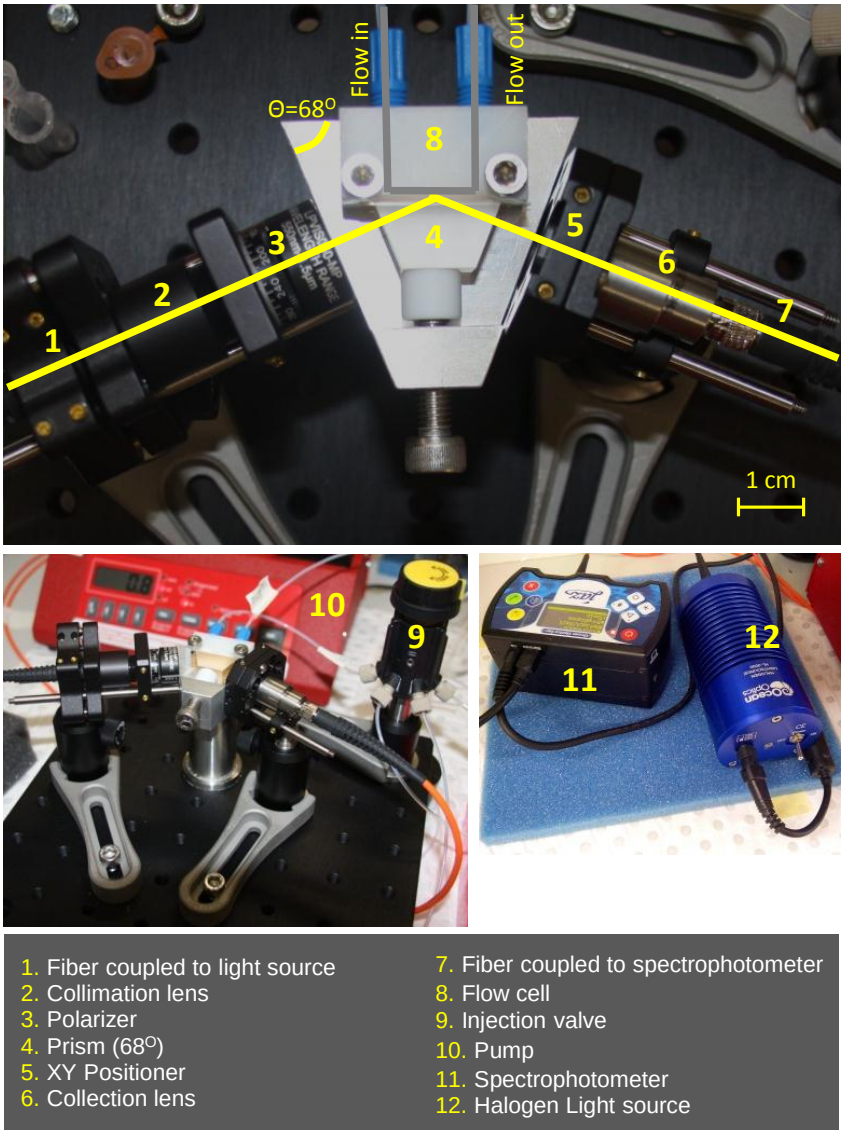

Fig. S3. *Image of the SPR biosensor employed in this study and its components.*

collection lens (Thorlabs, F230SMA-B), that was attached to a XY translator mount (Thorlabs, SSP05) in order to allow for the manual optimization of the collected light signal. Spectral analysis was carried out with a low-cost, CCD spectrometer (Ocean Optics, Spectrasuite Jaz Module). The gold coated (50 nm) SPR chip 24.5 x 0.3 mm in size (SSens) was clamped between the prism and the flow cell. To prevent any leakage, the flow cell excluding the flow channel was sealed with a thin parafilm layer. The liquid was pumped using a syringe pump (Chromolytic, NE-1000) at the designated flow rate. The flow cell and the microfluidics network were connected using two M6 screw flow connectors that assured proper in- and outflow of liquid in the system.

5. Detection of regional methylation using SPR biosensor

Initially, the SPR sensor chips were cleaned by rinsing the chips with acetone, ethanol and dH_2O previously heated up to their boiling point and dried under nitrogen flux. Subsequently, the chips were dipped in piranha solution (70% H_2SO_4–30% H_2O_2) for few seconds, rinsed with water and dried under nitrogen flux. The sensor chips were biofunctionalized by incubating with a solution of 1 µM DNA receptors and 100 nM of Mercaptohexanol (MCH) in PB buffer (50 mM Phosphate buffer- 0.75 mM NaCl; pH 7.0) for 3h in a humid chamber. Following this, the chips were rinsed with water, dried under nitrogen flux and placed in the SPR platform. To prevent nonspecific adsorption of DNA on the sensor surface, the chips were again blocked using an aqueous solution of 1 mM MCH. The generated PCR amplicons and/or designated concentrations of S-MIPs were hybridized *in situ* with the receptor probe. Prior to this hybridization step, PCR amplicons and/or S-MIPs were mixed with 200 nM of helpers in 200 µL Sodium Saline Citrate (SSC) 5X buffer (0.75 M in NaCl, 0.075 M in sodium citrate; pH 7.0) followed by heating to 90 ºC and slowly cooling to RT. For experiments using higher concentrations of S-MIPs (> 200 nM) the concentration of helpers were increased accordingly. To interrogate the region of methylation present in the PCR amplicons a 300 nM short reading oligo (Table S1) in 200 µL SSC 5X buffer was hybridized to the PCR amplicons. The SPR signals were monitored using home-made labview software. The MCH and all other subsequent samples were injected into the SPR platform using a syringe pump operating at a flow rate of 0.8 ml/h.

Table S1. Summary of DNA sequences employed in this study.

SAMPLE AMPLIFICATION		
Short name	**Description**	**Sequence**
MIP	Molecular inversion probe (I, inosine)	*5'P-ATACCCIAAACACIACAACCCIAGGGTTTGTTG TGGACTGAATTCTGTCTGATGGCTCTTCAGTCCT ATAACGUUUCCAAATGCTGTGTAGGTCATCTCAC CAATGCATACCAGGCTCACTTTGGGACAACAACC AAACCCTCIA*
Universal Fw-primer	Forward primer for amplifying MIP	*CGTTATAGGACTGAAGAGCCAT*
Universal Rv-primer	Reverse primer for amplifying MIP	*CCAAATGCTGTGTAGGTCATCT*
En1 Fw-primer	Forward primer for direct PCR	GAGGTTGTTGTGAGTTAGTTTTGATT
En1 Rv-primer	Reverse primer for direct PCR	AC(G/A)ACC(G/A)CAACAACCAAACCCT
Col2A1 Fw-primer	Forward primer for quantifying DNA	*GTAATGTTAGGAGTATTTTGTGGGTA*
Col2A1 Rv-Primer	Reverse primer for quantifying DNA	*CTACCCCAAAAAAACCCAATCCTA*

SPR BIOSENSING		
Short name	**Description**	**Sequence**
DNA-Receptor	DNA sequence for amplicon capture: the amplicon matching sequence is underlined	5- SH-TTTTTTTTTTTTTTTATTCAGTCCACAACAA-3
S-MIP$_{Meth}$	Synthetic amplicon homologue to the MIP employed for interrogating EN1 promoter region on MCF7 cells (Methylated): the receptor matching sequence is underlined; bases representing methylated sites are bolded; region matching the reading-oligo is highlighted in grey	5'-CAAACCCTCGAACACC**G**ATAAC**G**AC**G**ACAATAA AAAC**G**AC**G**C**G**AAAAACCCC**G**AAAC**G**CAAAACAC CAAATACCCGAAACACGACAACCCGAGGGTTTGTT GTGGACTGAATTCTGTCTG-3'
S-MIP$_{Unmeth}$	Synthetic amplicon homologue to the MIP employed for interrogating EN1 promoter region on WGA DNA (unmethylated): the receptor matching sequence is underlined; bases representing unmethylated sites are bolded; region matching the reading-oligo is highlighted in grey.	5'-CCAAACCCTCGAACACC**A**ATAAC**A**AC**A**ACAATA AAAAC**A**AC**A**C**A**AAAAACCCC**A**AAAC**A**CAAAACAC CAAATACCCGAAACACGACAACCCGAGGGTTTGTT GTGGACTGAATTCTGTCTG-3'
Reading-Oligo	Oligo perfectly matching the fully interrogated sequence	5- GTTTTGCGTTTCGGGGTTTTTCGCGTCGTTTTTA TTGTCGTCGTTATCGGTGTTC-3

	from the methylated MIP amplicon	
Oligo-helper01	Oligo matching folded regions within the MIP's amplicon	5'-CGTTATAGGACTGAAGAGCCAT-3'
Oligo-helper02	Oligo matching folded regions within the MIP's amplicon	5'-AGGGTTTGGTCCCAA-3'
Oligo-helper03	Oligo matching folded regions within the MIP's amplicon	5'- AGATGACCTACACAGCATTTGG-3'
Oligo-helper04	Oligo matching folded regions within the MIP's amplicon	5'- CCTCGGGTTGTCGTGT-3'

REFERENCES:

S1. University of Albany, *http://mfold.rna.albany.edu/*.