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Supporting Information

Enzymatic Incorporation of Multiple Dyes for Increased Sensitivity in QD-FRET Sensing for DNA Methylation Detection

Vasudev J. Bailey,[a] Brian P. Keeley,[b] Yi Zhang,[a] Yi-Ping Ho,[b] Hariharan Easwaran,[c] Malcolm V. Brock,[c] Kristen L. Pelosky,[c] Hetty E. Carraway,[c] Stephen B. Baylin,[c] James G. Herman,[c] and Tza-Huei Wang*[a, b]

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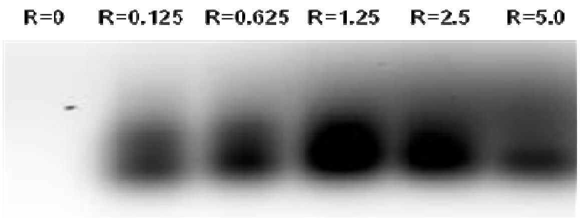
Table S1: Table of primers.

Gene	**Unmethylated Forward**	**Unmethylated Reverse**	Product Size (bp)
$p15^{INK4b}$	5'-GGTTGGTTTTTTATTTTGTTAGAGTGAGGT-3'	5'-AACCACTCTAACCACAAAATACAAACACA-3'	80
RASSF1A	5'-TGTGTGGTTTTTTTTAGTTTTTTTTTGTTG-3’	5’-CCCAACATAACCCAATTAAACCA-3’	81
CDH13	5′-TTGTGGGGTTTGTTTTTTGT-3′	5′-AACATTTTCATTCATACACACA-3′	120
ASC/TMS1	5'-GAAGGTGGGGAGTTTAGGTTTTGTTTT-3'	5'-AAATTCTCCAACACATCCAAAATAACAT-3'	140
$p16^{INK4a}$	5'-GGTTGGTTTTTTATTTTGTTAGAGTGAGGT-3'	5'-AACCACTCTAACCACAAAATACAAACACA-3'	151
Gene	**Methylated Forward**	**Methylated Reverse**	
$p15^{INK4b}$	5'-GGTTTTTTATTTTGTTAGAGCGAGGC-3'	5'-TAACCGCAAAATACGAACGCG-3'	68
RASSF1A	5’-CGGTTTTTTTTAGTTTTTTTTCGTCG-3’	5’-TAACTTTAAAGGCTAACAAACGCGAA-3’	76
CDH13	5′-TCGCGGGGTTCGTTTTTCGC-3′	5′-GACGTTTTCATTCATACACGCG-3′	112
ASC/TMS1	5'-GCGGGGAGTTTAGGTTTCGTTTC-3'	5'-CCAACGCATCCAAAATAACGTCG-3'	130
$p16^{INK4a}$	5'-TTATTAGAGGGTGGGGCGGATCGC-3'	5'-TAACCGCAAAATACGAACGCG-3'	150

Methods

Optimization of Cy5-dCTP Protocol for *$p16^{INK4a}$*.

Cy5-dCTP/ dCTP (R)	0	0.5	0.875	1.25	1.875
Relative PCR Yield (%)	100	82±4.2	74±6.7	61±3.3	47±12.1
Cy5 dye/ DNA	0	3.28±0.3	4.52±0.56	5.8±0.73	6.6±0.71



PCR with Cy5-dCTP

For methylated target DNA, PCR efficiency decreases with increase in Cy5-dCTP due to the close proximity of multiple cytosines. Utilizing only Cy5-dCTP without dCTP can cause premature termination of PCR product due to steric hindrance of incorporated Cy5 that prevents any further nucleotide incorporation. This demonstrates that incorporation efficiency is a result of the ratio of Cy5-dCTP/ dCTP. In order to determine the optimum amount of dNTP: Cy5-dCTP/dCTP ratios, several titrations were performed using varying ratios and the final amplified labeled product was run through a gel electrophoresis. DNA product concentration was quantified using the NanoDrop 1000 (NanoDrop Technologies, Inc). Additionally, we also quantified Cy5 incorporation by measuring total fluorescence using a Typhoon™ Scanner (GE Healthcare). Upon exciting the PCR product with a 525 nm light source, the incorporated Cy5 in the product is directly excited and visualized. Based on the number of Cy5 incorporated, the intensity observed varies (lower panel gel). We found that the amount of Cy5 incorporated into the 150 bp product was greatest when 1.5mM dATP, 1.5mM dGTP, 1.5 mM dTTP (the combination that is hereby referred to as dNTP minus dCTP), and 1 mM Cy5-dCTP and 1.25 mM Cy5-dCTP was used. This ensured that the Cy5-dCTP/ dCTP ratio was 1.25:1. Further, when this was increased to 2.5 to 1 and 5:1, there was a considerable decrease in the concentration of product. Total PCR yield was reduced two fold from maximum levels when the ratio of Cy5-dCTP/ dCTP was at 1.875. Thus, the ratio of 1.25 was selected for the experiments, as it allowed for enough dye incorporation without substantially compromising DNA yield. Similar optimization was performed with all genes in this paper.

DNA Isolation and Methylation Specific Modification

Genomic DNA was isolated from whole blood and serum mediums. Peripheral blood lymphocytes (NL) were obtained from normal volunteers, while sputum and serum samples were received from patients with a known smoking history. All patient samples were obtained after adequate informed consent and.IRB approval for clinical studies. Leukocyte DNA treated with SSSI methyltransferase produced fully methylated DNA (IVD) of *in vitro* sequences. Genomic DNA was isolated by standard phenol-chloroform extraction, and bisulfite conversion for methylation specific modification was performed as previously described.

Clinical Sample Collection

The samples used for this study were obtained from the non-small cell lung cancer (NSCLC) spore bank. Samples had been collected to investigate epigenetic characteristics of various types of collected biological samples (tissue, blood, sputum).While the goal is to collect early stage lung cancers, the bank consisted of samples from all stages of cancer due to pathological outcomes at the time of surgery. From this bank, there were eight patient samples which had previously been analyzed using a nested MSP approach, and were thus chosen to demonstrate the Cy5-dCTP QD-FRET analysis.

Primers

Primer sequences have been previously validated (Table 1). The primers were sequenced with an addition of 5' labeling of the forward primer with biotin (Integrated DNA Technologies (IDT)).

PCR Amplification for FRET Analysis

PCR Buffer was modified with Cy5-dCTP. The optimized PCR reaction consisted of 2 μL of target DNA and 23 μL of reaction buffer. The reaction buffer included 10x PCR buffer (16.6 mM ammonium sulfate/ 67 mM Tris; pH 8.8/ 6.7 mM $MgCl_2$/ 10 nM 2-mercaptoethanol), dATPs (Continental Lab Products, 1.25 mM), dGTPs (Continental Lab Products, 1.25 mM), dTTPs (Continental Lab Products, 1.25 mM), dCTPs (Continental Lab Products, 2.5 mM), Cy5-dCTP (GE Amersham, 2.5 mM), MSP gene dependent primers (300 ng of each), and HotStart Taq polymerase (Qiagen Corporation). Thermocycling conditions for $p16^{INK4a}$ were conducted as follows: 95 °C for 15 min, followed by a repetition of 35 cycles incorporating 95 °C for 30 s, 64 °C (annealing temperature) for 30 s, 72 °C for 30 s, and finally completed with a 5 min extension cycle at 72 °C. $p15^{INK4b}$, *TMS1*, *RASSF1A*, *CDH13* utilize annealing temperatures of 58, 58, 62 and 60°C respectively, however, all other thermocycling conditions were similarly performed as previously stated. Controls for unmethylated (NL), fully methylated (IVD), and the absence of template (water control) were utilized. For standard MSP results, 5 μL of PCR product were loaded onto a 2%, GelStar (Lonza) stained agarose gel and directly visualized under UV illumination. Qualitative results were also be obtained by the detection of fluorescent gel bands with the Typhoon™ scanner (GE Healthcare) using 630 nm excitation and 670BP30 filter with a PMT voltage of 400-500V. DNA concentration measurements were made using the NanoDrop 1000 (NanoDrop Technologies, Inc).

Fluorescence Detection and FRET Analysis

Modified MSP with biotin labeled primers and Cy5-dCTP was performed as previously described. Products were subsequently subjected to PCR purification (QIAquick PCR Purification Kit, Qiagen Corporation), and eluted with dH_2O in order to isolate PCR product in proper solution for QD fluorescence detection. For DNA conjugation to QD (Qdot® 605 ITK™ Streptavidin Conjugate Kit, Invitrogen Corporation), 1 μL of 100 mM NaCL was mixed with 7 μL of purified PCR product. 2 μL of 2 nM QD was then added to the mixture and left undisturbed for 15 min. Fluorescence of this nanocomplex was measured using the NanoDrop 3300 Fluorospectrometer (NanoDrop Technolgoies, Inc). After QD excitation with UV light/ blue laser, fluorescence peaks were measured at 605 nm and 670 nm due to QD and Cy5 photoluminescence respectively. FRET efficiency (E), was calculated using the proximity ratio formalism: $E = I_A / (I_A + I_D)$ (I_A and I_D referring to the acceptor and donor intensity respectfully). Additionally, normalization of the calculated E permitted quantitative analysis of each methylated DNA sequence

Enzymatic Extension Using Cy5-dCTP

In order to analyze incorporation as well as observe increasing signal with increasing amplification, 150 ng of 150 bp synthesized oligonucleotide (IDTDNA) was used with *p16*^{INK4a} methylation specific primers (5'-GGTTG GTTTT TTATT TTGT TAGAG TGAGG T-3' 5'-Biotin-AACCA CTCTA ACCAC AAAAT ACAAA CACA-3'). 10 µL of Cy5-dCTP is added to the reaction buffer included 10x PCR buffer (16.6 mM ammonium sulfate/ 67 mM Tris; pH 8.8/ 6.7 mM $MgCl_2$/ 10 nM 2-mercaptoethanol), dATPs (Continental Lab Products, 1.25 mM), dGTPs (Continental Lab Products, 1.25 mM), dTTPs (Continental Lab Products, 1.25 mM), dCTPs (Continental Lab Products, 2.5 mM), Cy5-dCTP (GE Amersham, 2.5 mM), MSP gene dependent primers (300 ng of each), and HotStart Taq polymerase (Qiagen Corporation). Thermocycling conditions for *p16*^{INK4a} were conducted as follows: 95 °C for 15 minutes, followed by a repetition of either 0, 1, 2, 3 or 4 cycles of 95 °C for 30 seconds, 64 °C (annealing temperature) for 30 seconds, 72 °C for 30 seconds, and a final extension for 5 minutes at 72 °C. The resulting product was purified and conjugated with QD605 and subsequently analyzed. Normalized FRET efficiency is computed by subtracting the FRET efficiency of water control for each replication from the target reaction and then assigning 0 cycles (background noise) of PCR a normalized value of 0.

Titration Analysis for Varying Acceptor to Donor Ratios for Single and Multi-labeled Products

PCR was performed using conditions that have previously been described. One set of reactions consisted labeling the PCR product with a single Cy5 fluorophore while another set included using Cy5-dCTP. Concentrations of the product were measured using the Nanodrop 1000 (NanoDrop Technologies, Inc). Dilutions of the product were made to equalize concentrations and fix acceptor:donor ratios. Diluted product was conjugated with QD605 and FRET measurements were made.

Analysis in Patient Serum Samples

Bisulfite treated serum samples were subject to PCR with methylated *p16*^{INK4a} primers with conditions that have been described previously. One set of reactions were carried out to label the product with a single fluorophore while another set was with the optimized Cy5-dCTP protocol. Fluorescence measurements were then carried out after the respective products were conjugated with QD605. If signal was below the water control, the samples were scored as unmethylated. The results were tabulated for both the single and multi-labeled approach.

Single Molecule Analysis with Genomic DNA

Cy5-dCTP was enzymatically incorporated into 8 µg of genomic cell line (RKO) DNA that has been bisulfite treated and *in vitro* methylated (IVD) and amplified with *ASC/TMS1* primers (5'-GCGGG GAGTT TAGGT TTCGT TTC-3' and 5'-Biotin-AAAT TCTCC AACA CATCC AAAAT AACAT-3'). A separate reaction with similar reagents but no DNA was run as a water control. Single-molecule measurements were taken using Confocor 2 (Ziess, Inc) that has an avalanche photodetector (APD). Photon counts for multi-labeled product as well as water control were measured for a total of 1000 seconds in order to computer single molecule photon counts.

Mixing Experiment

Mixing experiments involved combining methylated control DNA (IVD) with unmethylated control (NL) in ratios such that the total input DNA still remained 150 ng. Percent methylation described is defined as the ratio of methylated DNA to unmethylated DNA and was varied ranging from 100%, 75%, 50%, 25%, 1%, and 0%. This mixture served as template for 16 cycles of PCR amplification with Cy5-dCTP. Having purified the product, DNA concentration was reduced for 100% template and the same dilution factor was correspondingly used for all other percent methylation.

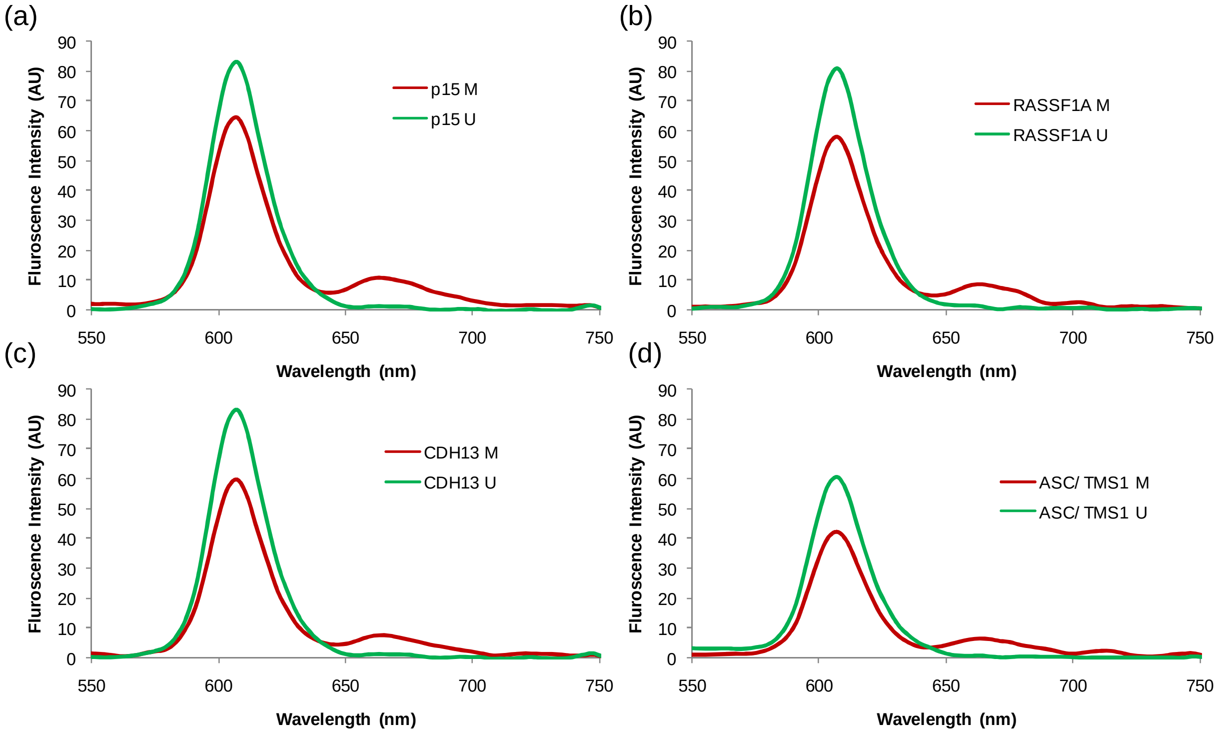


Figure S1: Fluorescence spectra for 4 genes (*p15*^{INK4b}, *RASSF1A, CDH13, ASC/ TMS1*) to validate that Cy5-dCTP can be extended to other genes irrespective of base pair length and sequence. 150 ng of IVD methylated control is amplified with methylated and unmethylated primers for the 4 genes. Product obtained is quantified for DNA concentration using the Nanodrop 1000. Equal DNA concentrations are used to quantify FRET for all four products in order to correct for varying PCR efficiencies. Panels (a)-(d) all show quenching of QD (605 nm) and emission of Cy5 (670 nm) when amplified with methylated primers. When amplified with unmethylated primers, no QD quenching or Cy5 emission is observed. M=methylated, U=unmethylated, IVD=*in vitro* DNA.

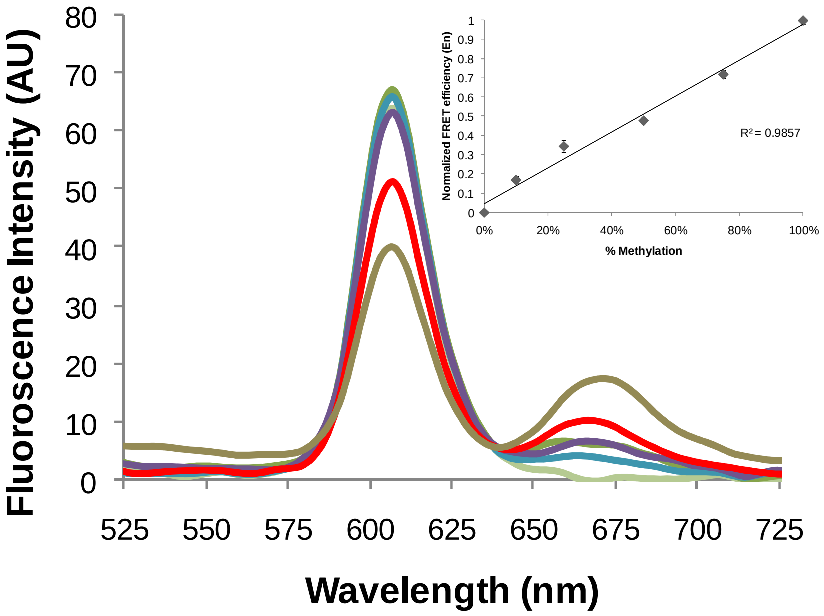


Figure S2: Quantification of methylation. Dilution experiment of *in vitro* methylated DNA (IVD) and normal lymphocytes (NL) amplified with $p16^{INK4a}$ primers and Cy5-dCTP. Mixtures of defined methylation levels range from 100%, 75%, 50%, 25%, and 1% of the total 150 ng input DNA by varying quantities of IVD methylated control diluted in a background of unmethylated NL. The mixture was used as input template for 16 cycles PCR reaction with labeled $p16^{INK4a}$ primers. As seen in the inset, normalized FRET efficiency indicates a linear correlation of measured methylation to the input methylation.