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DNA METHYLATION

METHYLATION ANALYSIS

# Novel Multiplex MethyLight Protocol for Detection of DNA Methylation in Patient Tissues and Bodily Fluids

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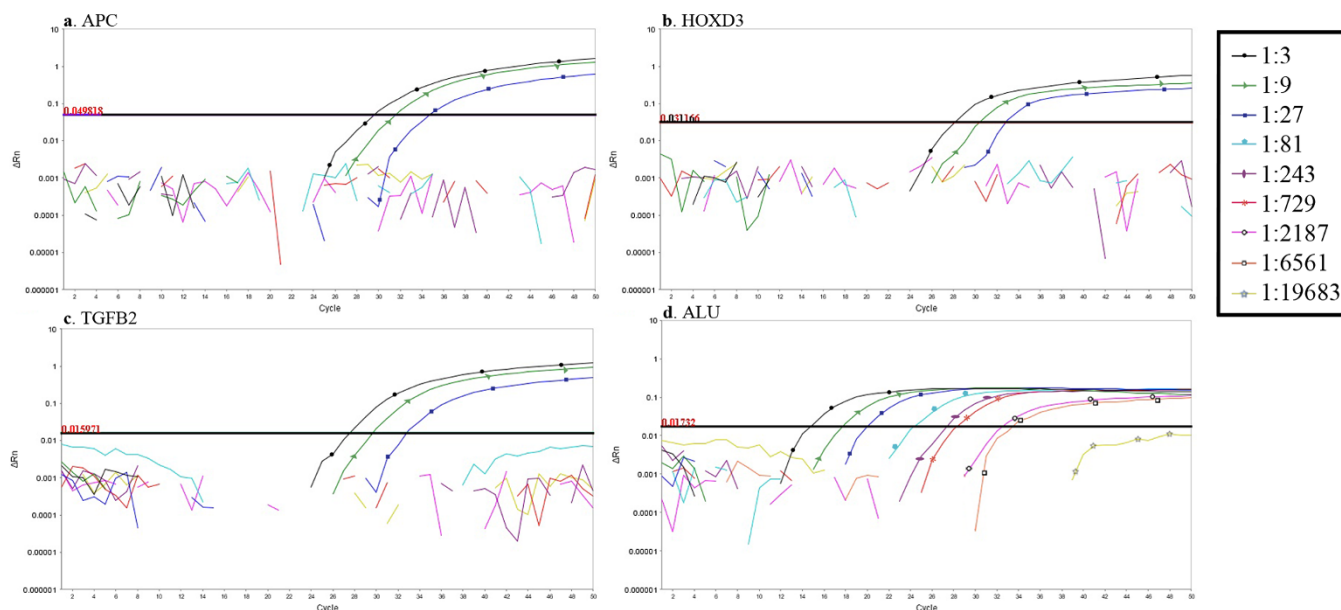
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Aberrant DNA methylation is a hallmark of cancer and is an important potential biomarker. Particularly, combined analysis of a panel of hypermethylated genes shows the most promising clinical performance. Herein, we developed, optimized and standardized a multiplex MethyLight assay to simultaneously detect hypermethylation of *APC*, *HOXD3* and *TGFB2* in DNA extracted from prostate cancer (PCa) cell lines, archival tissue specimens, and urine samples. We established that the assay is capable of discriminating between fully methylated and unmethylated alleles with 100% specificity and demonstrated the assay as highly accurate and reproducible as the singleplex approach. For proof of principle, we analyzed the methylation status of these genes in tissue and urine samples of PCa patients as well as PCa-free controls. These data show that the multiplex MethyLight assay offers a significant advantage when working with limited quantities of DNA and has potential applications in research and clinical settings.

**D**NA Methylation plays a key role in regulating a diverse array of functions in normal and disease states. Alterations in DNA methylation are a hallmark of human cancers and have been shown to contribute to disease initiation and progression<sup>1,2</sup>. Assessment of tumor-specific DNA methylation changes has a broad range of applications from providing insights into disease pathogenesis to biomarker discovery. Tumor cells are characterized by a methylome distinct from that of normal cells, consisting of global hypomethylation, contributing to genomic instability and activation of silenced oncogenes<sup>3</sup>. Additionally, site-specific hypermethylation contributing to silencing of tumor-suppressor genes and microRNAs has been identified to play a crucial role in tumorigenesis<sup>4</sup>. Further, DNA methylation is heritable yet also reversible, making it an attractive therapeutic target<sup>5</sup>.

Differential methylation patterns of selected candidate genes have been shown to serve as promising biomarkers for early diagnosis, prognosis, disease monitoring, prediction of response to therapy, and assessment of risk of recurrence<sup>6–11</sup>. Importantly, it has been found that assessment of a panel of such biomarkers dramatically improves the sensitivity and specificity compared to any single marker<sup>12</sup>. Therefore, analysis of multiple DNA methylation-based biomarkers is becoming increasingly important in translational research. DNA methylation events have several advantages with respect to their use as cancer biomarkers since they can be tissue- and tumor-type specific<sup>13</sup>. DNA is stable and easy to isolate from various biological sources and methylation analysis can be performed on a diverse array of specimens ranging from fresh, frozen or formalin fixed tissues, to different types of biofluids including plasma, serum, urine or saliva samples<sup>14</sup>. However, despite their promise, DNA methylation measurements have not come into widespread use in the clinic<sup>15</sup>. There are a number of potential reasons for this. A significant technological challenge is the sensitive detection of specific DNA methylation patterns occurring at low abundance and/or availability of limited quantities of clinical samples.

Methylation specific PCR-based techniques are among the easiest and quickest methods for DNA methylation analysis that have the potential to be used in everyday laboratory practice for screening of a large number of samples<sup>16</sup>. One such technique, MethyLight, is based on sensitive and quantitative, methylation-specific, fluorescence based real-time PCR assay<sup>17,18</sup>. MethyLight assays quantify DNA methylation at a particular locus by using DNA oligonucleotides that anneal differentially to bisulfite-converted DNA according to their methylation status in the original genomic DNA. MethyLight is broadly applicable for analysis of large series of samples.



**Figure 1** | Analysis of the sensitivity of the multiplex MethyLight technique for (A) *APC*, (B) *HOXD3*, (C) *TGFB2*. CpGenome™ universal methylated DNA was serially diluted in 3-fold increments from 1 : 3 to 1 : 19683 and subsequent multiplex analysis was performed. The resulting relative fluorescence ( $\Delta R_n$ ) was plotted as a function of cycle number. Increasing dilutions are indicated by different colors and shapes, as shown on the right. (D) *ALU* control reaction was included to determine the detection of input DNA in each dilution. The black horizontal line indicates the threshold used for calculating the amount of template DNA. A positive signal was noted for a specific dilution when the corresponding amplification curve has passed the threshold line.

Although highly sensitive for the detection of methylation signal, conventional MethyLight assay can only analyze one gene at a time, which can be potentially limiting when attempting to analyze multiple markers using particularly small quantities of DNA obtained from sources such as needle biopsy, saliva, serum, fine needle aspirates or urine.

In this manuscript, we describe the development of a multiplex MethyLight assay that allows for the co-amplification of multiple genes in one reaction mixture. We selected a panel of three well-studied methylation gene markers in prostate cancer (PCa), namely, *APC*, *HOXD3* and *TGFB2*<sup>19–21</sup>. We first established the limits of the technique by measuring its sensitivity, specificity, accuracy and reproducibility of the detection of methylated versus unmethylated DNA. Subsequently, we analyzed the methylation status of these genes in PCa cell lines, archival formalin-fixed paraffin-embedded (FFPE) PCa tissue, fresh frozen benign prostatic tissue, and urine samples from PCa patients as well as PCa-free controls, in order to test the feasibility and applicability of this technique.

## Results

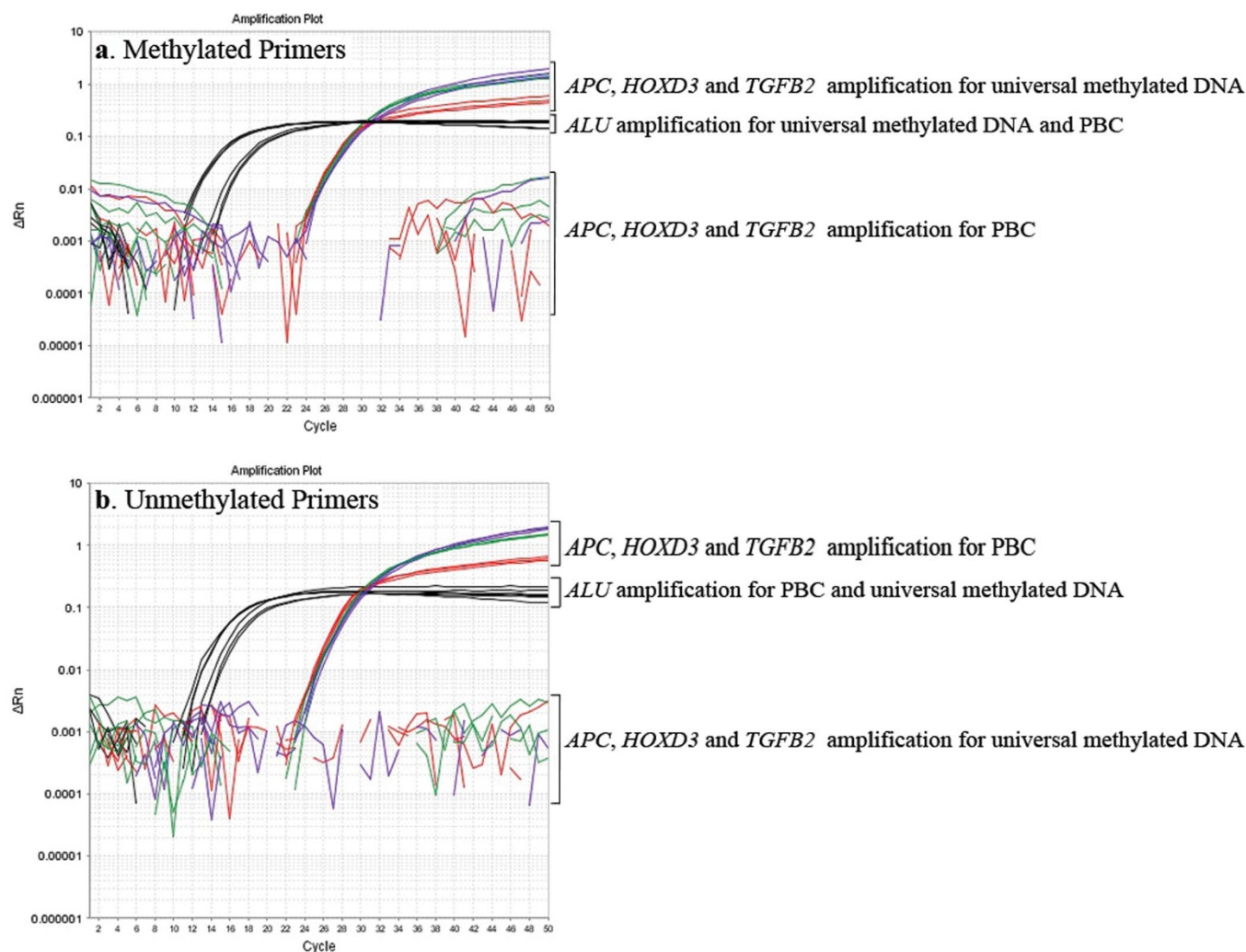
### Parameters important for multiplex MethyLight assay design.

Important parameters for the successful development of multiplex MethyLight are primer design, fluorescent dye selection and optimization of reaction conditions. For the initial development of a multiplex MethyLight assay, we chose MethyLight primers and probe sets used for DNA methylation analysis of *APC*, *HOXD3* and *TGFB2* genes as described in our previous studies<sup>19–21</sup>. For methylation-independent MethyLight control reaction, we selected *ALU* sequences to measure the amount of input DNA. *ALU* repetitive elements represent a significant portion of the human genome hence they are less susceptible to normalization errors caused by cancer-associated aneuploidy and copy number changes<sup>22</sup>. *In silico* analysis of these primer and probe sequences revealed minimal unfavorable interactions, defined as minimal propensity to form dimers based on sequence homology and delta Gibbs free energy, allowing them to be multiplexed<sup>23</sup>. The ABI 7500 Real-Time PCR System, used in this

study, has five channels for target detection; one of these is required (the ROX dye, emission maximum at 605 nm) in our master mix to serve as a passive reference, thus we selected four fluorescent dyes to label four different gene probes which have sufficient spectral separation to avoid overlap between signals of different targets: HEX (554 nm), CY5 (650 nm), TAMRA (583 nm), and FAM (520 nm). Next, we optimized the multiplex MethyLight reaction conditions by running a set of PCR reactions with a range of concentrations of dNTPs (200–600  $\mu$ M),  $MgCl_2$  (3.5–10.5 mM), *Taq* polymerase enzyme (0.5–1.0 units), primers (0.5–8.0  $\mu$ M) and probes (0.1–4.0  $\mu$ M). Thus, we have established that the optimal multiplex MethyLight reaction conditions are 400  $\mu$ M dNTPs, 10.5 mM  $MgCl_2$ , 1.0 units of *Taq* polymerase enzyme, primer concentrations of 8.0  $\mu$ M for *APC*, *HOXD3* and *TGFB2*, and 1.0  $\mu$ M for *ALU*, probe concentrations of 2.66  $\mu$ M for *APC* and *TGFB2*, 2.0  $\mu$ M for *HOXD3* and 0.1  $\mu$ M for *ALU* (data not shown).

### Sensitivity and specificity of the multiplex MethyLight assay for discrimination of methylated versus unmethylated DNA.

We performed a serial dilution experiment to determine the sensitivity of detection of *APC*, *HOXD3* and *TGFB2* methylation in the multiplex MethyLight assay. Seventy nanograms of bisulfite modified CpGenome™ universal methylated DNA was serially diluted in 3-fold increments from 1 : 3 up to 1 : 19683. The *APC*, *HOXD3* and *TGFB2* methylated reactions were then used to track the decreasing amount of methylated DNA in the serial dilution series. The amplification plots resulting from this experiment are shown in Figure 1. The *APC*, *HOXD3* and *TGFB2* reactions show a decreasing detection of methylated alleles in each subsequent dilution, as indicated by the increasing cycle number at which each reaction signal crosses the detection threshold. We have determined that in a multiplex MethyLight setting, the sensitivity limit of *APC*, *HOXD3* and *TGFB2* methylation is the 1 : 27 dilution (estimated to contain 0.37 ng of methylated DNA). Further, we established that the *ALU* control reaction sensitivity of detection is the 1 : 6561 dilution (0.0016 ng). Similarly, bisulfite modified CpGenome™ universal methylated DNA was serially diluted in the same manner



**Figure 2 | Analysis of the specificity of *APC*, *HOXD3* and *TGFB2* multiplex MethyLight assay.** We performed separate reactions using either the methylated or unmethylated primers on both human peripheral blood cell (PBC) DNA and CpGenome™ universal methylated DNA. The resulting relative reaction fluorescence ( $\Delta R_n$ ) was plotted as a function of cycle number. The (A) methylated or (B) unmethylated reactions were 100% specific because the methylated primers did not cross-react with PBC DNA (0% methylated) and the methylated primers did not cross-react with CpGenome™ universal methylated DNA (100% methylated). Both CpGenome™ universal methylated DNA and PBC were positive for their *ALU* reactions, indicating that there was sufficient input DNA in each sample.

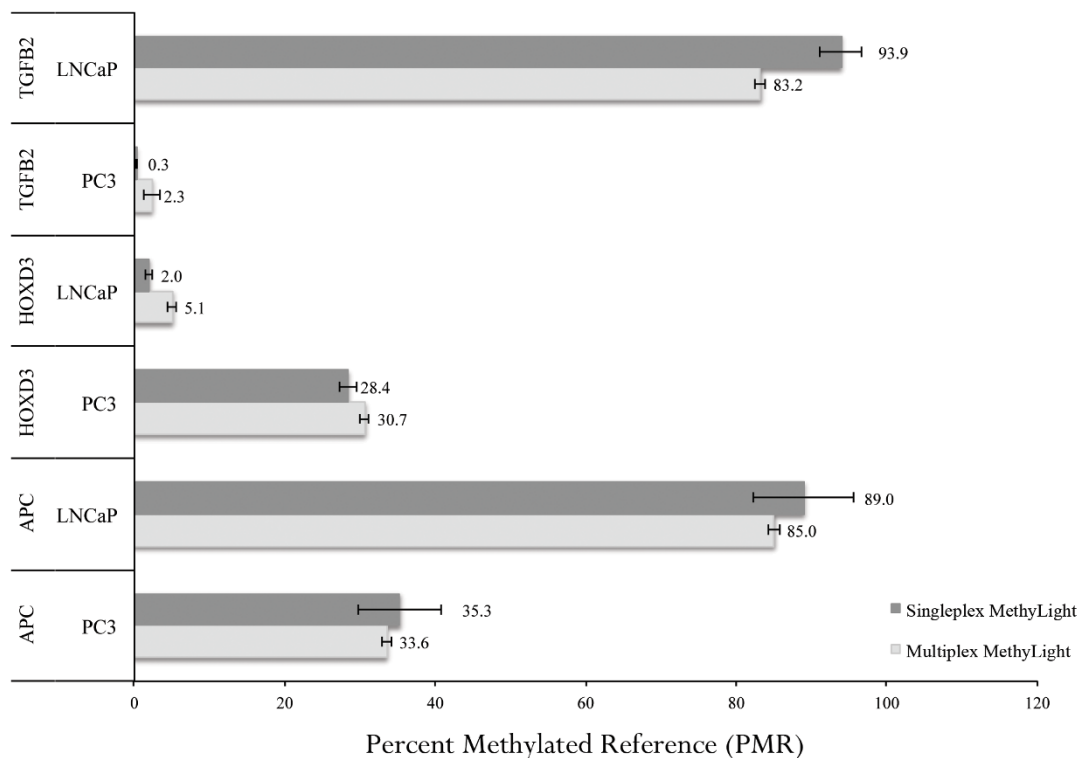
in human peripheral blood cell (PBC) DNA which is expected to harbor minimal methylation of the selected genes. The PBC DNA and all of the dilution reactions had overlapping *ALU* amplification curves, indicating they contained equal amounts of DNA (10 ng). PBC DNA displayed minimal methylation of *TGFB2* (<1 PMR). In this setting, the sensitivity limit of *APC*, *HOXD3* and *TGFB2* methylation is also the 1:27 dilution (estimated to contain 0.37 ng of methylated DNA and 9.63 ng of PBC DNA, respectively).

Next, to assess the specificity of detection of the *APC*, *HOXD3* and *TGFB2* multiplex MethyLight assay, we performed separate reactions using either the methylated or unmethylated primer-probe sets of each gene, on both human peripheral blood cell (PBC) DNA (expected to harbor minimal methylation of the selected genes) and CpGenome™ universal methylated DNA (expected to show robust methylation). The resulting amplification plots of *APC*, *HOXD3* and *TGFB2* are shown in Figure 2. Consistent with its unmethylated status, PBC DNA yielded an amplification signal for multiplex MethyLight reactions with the unmethylated primers, while there was no detectable signal for the methylated reaction. In contrast, CpGenome™ universal methylated DNA yielded a robust amplification signal in the methylated reactions, but not in the unmethylated reactions for all 3 genes. Both the PBC and

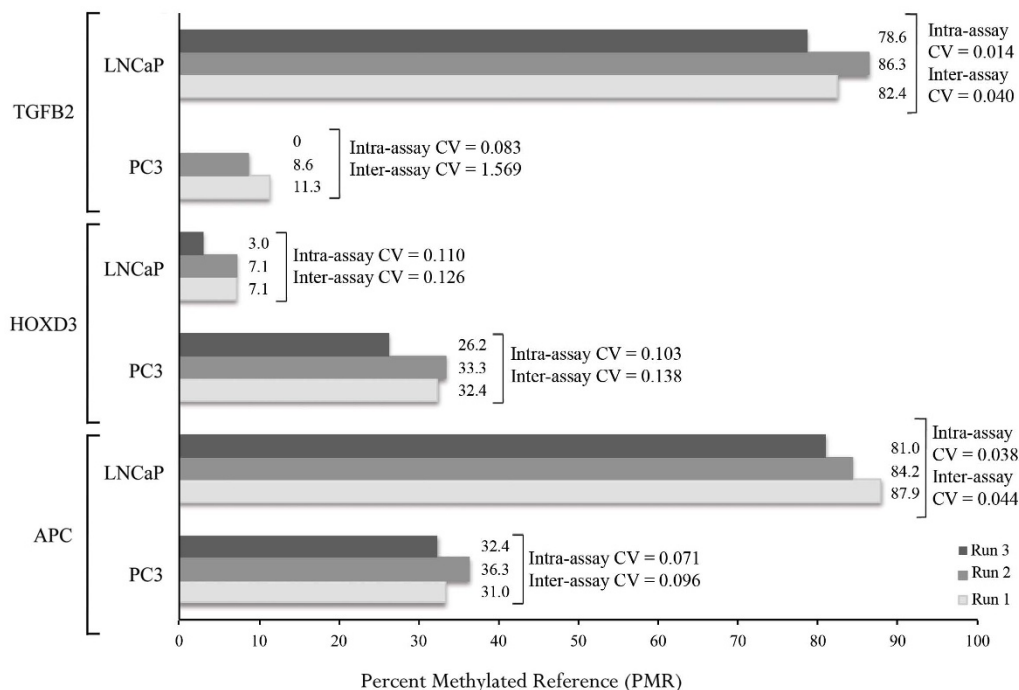
CpGenome™ universal methylated DNA were positive for their *ALU* reactions, indicating that the minimal required amount of input DNA was present in the multiplex assay. Therefore, multiplex MethyLight technology can efficiently discriminate between fully methylated versus unmethylated alleles with 100% specificity.

#### Accuracy and reproducibility of the multiplex MethyLight assay.

To test the accuracy of the *APC*, *HOXD3* and *TGFB2* reactions in the multiplex MethyLight setting, we first performed a series of conventional singleplex MethyLight assays to quantify methylation levels of *APC*, *HOXD3* and *TGFB2* each separately in the human prostate cancer (PCa) derived cell lines LNCaP and PC3. We then performed multiplex MethyLight assays to analyze the methylation levels of these genes in the same cell lines. As shown in Figure 3, there were no significant differences in percent methylated reference (PMR) values between singleplex and multiplex MethyLight assays (paired t-test  $P$ -Value > 0.05) for each of the three genes in either of the two cell lines. Additionally, to test the reproducibility of the multiplex MethyLight assay we performed 2–6 additional independent runs of the assay in the same PCa cell lines. The mean PMR values and/or inter-assay and intra-assay coefficient of variation (CV) are shown in Figure 4. The inter-assay and intra-assay CV



**Figure 3** | Representative analysis of the accuracy of multiplex MethyLight through the comparison of PMR values for *APC*, *HOXD3* and *TGFB2* genes in DNA from human prostate cancer cell lines LNCaP and PC3 to singleplex MethyLight PMR values. The PMR for each gene was calculated from the average of 3–7 independent assays with duplicate reactions per gene in each assay. Paired t-test analysis was performed to assess differences in PMR values between singleplex and multiplex MethyLight assays for each of the three genes in either cell line (paired t-test *P*-Value > 0.05).



**Figure 4** | Analysis of the reproducibility of the multiplex MethyLight reactions for *APC*, *HOXD3* and *TGFB2*. Three independent multiplex MethyLight assays were performed in duplicate reactions for the prostate cancer cell lines LNCaP and PC3. The intra-assay coefficient of variation (CV) was calculated as the average for the duplicate reactions in each independent multiplex assay. The inter-assay CV was calculated between the 3–7 independent multiplex assays. Although *TGFB2* methylation in PC3 cell line shows high CV value, it is not statistically significant between singleplex and multiplex assays by Student's t-test.



values ranged from 0.044 to 0.138, and 0.014 to 0.110, respectively, indicative of only a modest discordance in PMR values between independent multiplex MethyLight runs, suggesting that the technique generates highly reproducible results. Only in PC3 cells, CV value of 1.569 is observed for *TGFB2* due to high variance in the methylation of this gene in this cell line. Although *TGFB2* methylation in PC3 cell line shows high variance and CV value, it is not statistically significant between singleplex and multiplex assays by Student's t-test.

**Application of the multiplex MethyLight assay to measure DNA methylation in patient samples.** The main potential application of the multiplex MethyLight technology is aimed at rapid DNA methylation analysis of multiple loci in clinical samples with limited DNA quantities. A number of studies have shown that *APC*, *HOXD3* and *TGFB2* are aberrantly methylated in PCa and each is associated with disease progression and biochemical recurrence<sup>19,20</sup>. Further, in previous studies, we have shown that when combined, the methylation status of these genes outperformed any single marker for the prediction of disease progression and biochemical recurrence<sup>21</sup>. Therefore, as a proof of principle experiment, we tested our multiplex MethyLight method by measuring the methylation levels of *APC*, *HOXD3* and *TGFB2* in 10 FFPE PCa tissues, 5 fresh frozen benign prostatic tissues, 10 urine samples obtained from unrelated PCa patients, and 5 urine samples from PCa-free controls. As seen in Table 1, we detected PMR values above zero for at least one gene in all but one of the tested tissues and all urine samples from PCa patients. Two of the urine samples from PCa-free controls had PMR values above zero for the *APC* gene (PMR = 0.2 and 0.3 for samples 4 and 5, respectively). All 30 DNA samples gave rise to a positive signal in their *ALU* reactions (Ct < 19), confirming that the minimal required amount of input DNA was present in each reaction to perform the multiplex assay.

## Discussion

This study reports the development of a multiplex MethyLight assay for the simultaneous analysis of *APC*, *HOXD3*, *TGFB2* methylation markers and the reference marker *ALU*. We have shown that this method is highly specific, accurate and reproducible for the quantitative detection of methylated versus unmethylated alleles. Further, we have demonstrated that this approach can be used to rapidly detect DNA methylation in patients' fresh frozen and FFPE tissues as well as urine samples. However, this study was not aimed at analyzing the clinical sensitivity and specificity of the multiplex methylation markers in PCa detection. The clinical utility of *APC*, *HOXD3* and *TGFB2* multiplex methylation assay will be investigated in future studies.

Incorporating a multiplex protocol for MethyLight assay makes the technique better suited for use with precious clinical samples, is more economical and enhances throughput, thereby increasing overall efficiency of the methylation detection experiments. Multiplex MethyLight requires smaller amounts of template DNA because it can be used to analyze multiple loci concomitantly in one assay, compared to the singleplex MethyLight assay. This is especially beneficial for analyzing clinical samples with limited DNA quantities such as biopsy material. The Multiplex protocol is also more economical, as it allows for smaller amounts of reagents (i.e. oligonucleotides, MgCl<sub>2</sub>, enzyme) to be used in each multiplex assay when compared to the singleplex approach. A standard multiplex run in this study required only one standard curve for quantification of four genes. This decreases the amount of CpGenome™ universal methylated DNA used and allows for the simultaneous analysis of three DNA methylation markers in up to 41 samples in duplicate in a 96-well plate format in each assay, thus saving time and increasing efficiency.

Some limitations of our assay should be noted. The multiplex MethyLight assay optimized in this study was performed on ABI 7500 Real-Time PCR system, which supports multiplexing of up to

**Table 1 | Multiplex MethyLight data (PMR) for *APC*, *HOXD3* and *TGFB2* in FFPE prostate cancer tissues, post-DRE urine samples of PCa patients as well as fresh frozen tissues and urine samples from PCa-free controls**

	Average PMR		
	APC	HOXD3	TGFB2
<b>Prostate Cancer Tissues</b>			
1	39.7	4.1	10.0
2	0.0	0.0	1.1
3	63.4	29.1	22.1
4	44.0	15.9	10.8
5	35.0	7.8	13.1
6	11.9	9.8	3.3
7	34.3	13.3	13.9
8	42.3	1.8	14.0
9	13.2	10.7	12.7
10	16.7	16.9	3.4
<b>Benign Prostatic Tissues</b>			
1	0.0	0.0	0.0
2	0.2	7.9	2.5
3	0.6	17.0	1.2
4	5.2	12.2	2.4
5	0.9	12.7	1.3
<b>Prostate Cancer Patients Urine Samples</b>			
1	4.8	1.6	0.0
2	0.0	12.9	0.0
3	1.6	7.6	0.8
4	0.0	3.9	0.0
5	0.6	4.5	0.0
6	0.8	0.2	0.0
7	0.0	1.3	0.0
8	0.0	0.8	0.6
9	0.0	0.4	0.0
10	0.1	0.3	0.1
<b>Prostate Cancer-free Controls Urine Samples</b>			
1	0.0	0.0	0.0
2	0.0	0.0	0.0
3	0.0	0.0	0.0
4	0.3	0.0	0.0
5	0.2	0.0	0.0

five fluorescent dyes. A greater number of methylation marks could be potentially incorporated into the multiplex MethyLight assay when used with other platform such as the QuantStudio™ 12 K Flex Real-Time PCR System (Applied Biosystems), which supports multiplexing of up to six fluorogenic probes. Another factor that may limit the number of markers that can be multiplexed in a MethyLight assay is primers and probe design. MethyLight technology is based on the detection of methylation-dependent sequence differences in DNA following bisulfite modification, which leads to a significant reduction in DNA complexity. This makes the design of highly specific primers and probes challenging and requires special attention to be paid to parameters such as homology of primers and probes with their target sequences, oligonucleotides length, CG content, and concentration. Lastly, the quantitative sensitivity of the multiplex MethyLight assay developed in this study is limited, yet sufficient for the detection of *APC*, *HOXD3* and *TGFB2* methylation in FFPE tissue and urine samples. Further, we have shown that in a multiplex setting the methylation of *APC*, *HOXD3* and *TGFB2* could be detected in the presence of large excess of unmethylated DNA. This further suggests that this approach can be used to detect aberrant methylation patterns in heterogeneous clinical samples with substantial contamination of unmethylated DNA. It is important to note that the presence of sufficient DNA amounts in this study was evaluated by the *ALU* reactions. However, this only indicates that the minimal required amount of input DNA was present in the



multiplex assay, not that sufficient cancer DNA is present in the sample. The sensitivity of detection for this assay could be potentially further improved by incorporating Locked Nucleic Acids (LNA) in primers and/or probes<sup>24,25</sup>. LNA probes and primers are nucleic acid analogues that contain a bicyclic furanose ring in the ribose sugar which is chemically locked in an RNA-mimicking conformation. This chemistry allows for increased specificity and improved detection limit for real-time PCR assays.

Different multiplex approaches have been developed to evaluate DNA methylation in various sources of patient DNA samples. Some of these include multiplex ligation-dependent probe amplification (MLPA), bisulfite assisted genomic sequencing PCR (BSP), loop mediated isothermal amplification (MS-LAMP), nested MSP, fluorescence resonance energy transfer (FRET), padlock probes (PP), and multi-component nucleic acid enzymes (MNAzymes)<sup>26–36</sup>. However, all these multiplex DNA methylation analysis methods have several disadvantages compared to MethyLight technology. First, approaches such as BSP, PP and nested MSP, are based on gel electrophoresis, which is generally less sensitive than real-time PCR. These approaches also require relatively greater quantities of template DNA. Second, probe development for approaches such as MLPA and MS-LAMP is much more complicated, costly and time consuming. Third, most of the above-mentioned approaches are multi-step procedures that cannot be performed in a single tube increasing the risk of contamination. Lastly, novel approaches such as FRET and MNAzymes are based on chemistries that are not as well established and need to be further validated. Other approaches based on methylation specific fluorescent probe detection by real-time PCR have been previously reported<sup>37–40</sup>. However, the control reactions utilized in these approaches were *ACTB* and not *ALU* based, which is a less stable and/or less reproducible measure of bisulfite-converted input DNA, especially when analyzing tumor samples with local amplifications or deletions of single genes. Multiplex digital MethyLight has also been utilized for DNA methylation analysis<sup>41</sup>. Digital MethyLight is shown to have greater sensitivity and accuracy than the singleplex MethyLight assay in detecting individual methylated DNA molecules in clinical samples. Therefore, Digital MethyLight technology can potentially be applied to the *APC*, *HOXD3*, *TGFB2* multiplex MethyLight assay in the future.

This is the first study to develop a multiplex MethyLight assay for the analysis of the three-gene panel consisting of *APC*, *HOXD3* and *TGFB2* genes, which have previously shown promise as prognostic biomarkers for PCa. Our multiplex MethyLight assay was able to successfully determine methylation levels of these three genes in fresh frozen and FFPE tissue as well as urine samples obtained from PCa patients and PCa-free individuals. This proof of principal cohort included 20 PCa samples and 10 healthy controls, thus is only adequate to demonstrate the feasibility of the multiplex assay. To ensure high specificity of this multiplex assay in future studies, an individual PMR threshold value will need to be set for each individual gene that will allow for clear distinction between PCa and normal as has been done for other multiplex methylation assays<sup>38</sup>. Taking an approach similar to that used in this study, multiplex MethyLight assays can be further developed for other promising DNA methylation biomarker panels. One such example is the *APC*, *MGMT*, *RASSF2A* and *Wif-1* biomarker panel, which have shown high diagnostic sensitivity and specificity in colon cancer<sup>42,43</sup>.

Future studies based on this multiplex MethyLight assay will focus on analyzing the clinical utility of *APC*, *HOXD3* and *TGFB2* as methylation biomarkers, as well as expanding the number of candidate markers and/or different combinations of methylation gene markers that can be multiplexed in the MethyLight assay, and increasing the number of samples that can be analyzed employing a single standard curve by using 384- or 1536- well plates. Additionally, different types of clinical samples such as needle biopsies, oral lavage, fine needle aspirates and serum samples will be tested.

In summary, multiplex MethyLight represents a powerful method for measuring DNA methylation of a gene panel, with the potential to improve research, encourage innovation and improved translation of biomarkers to the clinic.

## Methods

**Patient samples and cell lines.** Radical prostatectomy (RP) FFPE tissue samples used in this study were a subgroup of 10 samples obtained from patients diagnosed with PCa and who underwent RP at the University Health Network (UHN) in Toronto between 2007–2011<sup>44</sup>. The 10 samples were selected based on high *KLK10* methylation status and were expected to harbor methylation of other genes thus serving as good test samples for our proof-of-principle experiment. Another series of five cystoprostatectomy (CP) fresh frozen prostatic tissue samples was collected from UHN, reviewed by a pathologist (T.vdK.) and confirmed as benign. Additionally, post digital rectal exam (DRE) urine specimens of 10 unrelated patients diagnosed with organ-confined, intermediate risk PCa at UHN and examined by the attending urooncologist (N.F.) as well as five voided urine samples from PCa-free controls were obtained from the UHN Genito-Urinary Prostate Clinic. Patient consent was obtained for accrual of urine and surgically excised tissue following RP or CP at the UHN tissue bank, and according to the approved protocols of the Research Ethics Board (REB) at UHN and Mount Sinai Hospital in Toronto. The human prostate cancer cell lines LNCaP (ATCC # CRL-1740) and PC-3 (ATCC # 59500) were obtained from Drs. R. Bristow, and E. Diamandis at the University of Toronto.

**DNA extraction and sodium bisulfite modification.** Cell pellets were isolated from 35–70 mL of whole urine by centrifugation (2000 g, 10 minutes) and DNA was extracted using QiagenQIAampDNA Microkit (Qiagen Inc.) according to the manufacturer's protocol. DNA extracted from cell lines, FFPE tissue and urine samples (100–400 ng genomic DNA for each) was bisulfite modified using the Zymogen EZ DNA Methylation Gold Kit (Zymo Research) as per manufacturer's protocol and was eluted into a final concentration ranging from 10 ng/μL–20 ng/μL. For experiments involving serial dilutions, the concentration of CpGenome™ universal methylated (Chemicon, Millipore) and PBC DNA was measured by NanoDrop 1000 (Thermo Scientific) prior to bisulfite modification and diluted to final concentration of 5 ng/μL. Following bisulfite conversion, the DNA concentration was again verified by NanoDrop. Next, based on these measurements, 70 ng of bisulfite modified CpGenome™ universal methylated DNA and 140 ng bisulfite modified PBC DNA were mixed at a concentration of 5 ng/μL and serially diluted in 3-fold increments from 1 : 3 (1.67 ng/μL) up to 1 : 19683 (0.0003 ng/μL). In each PCR reaction 2 ul of each dilution were ran in triplicate.

**MethyLight assays.** Singleplex MethyLight assays for each gene were performed by incubating the bisulfite converted DNA in one PCR reaction well with 200 μM dNTPs, 0.3 μM forward and reverse primers, 0.1 μM probe, 3.5 mM MgCl<sub>2</sub>, 0.01% Tween-20, 0.05% gelatin and 0.5 units of *Taq* polymerase in a 30 ul reaction volume on a 96-well plate (Applied Biosystems). For multiplex MethyLight assay, bisulfite converted DNA was incubated with 400 μM dNTPs, 10.5 mM MgCl<sub>2</sub>, 0.01% Tween-20, 0.05% gelatin, 1.0 units of *Taq* polymerase and varied concentrations of each primer and probe set as follows: 8.0 μM *APC*, *HOXD3* and *TGFB2* forward and reverse primers each, 1.0 μM *ALU* forward and reverse primers each, 2.66 μM *APC*

**Table 2 | Primer-probe sequences of the genes analyzed by multiplex MethyLight**

Gene name	Primer/probe sequence
<b>APC</b>	
Forward primer	5'-GAACCAAACGCTCCCCAT-3'
Reverse primer	5'-TTATATGTCGGTACGTGCGTTTATAT-3'
Probe	5'-/HEX/CCCGTCGAAAACCCGCCGATTA/BHQ1/-3'
<b>HOXD3</b>	
Forward primer	5'-TAAAAGTTTATGGTTGCGC-3'
Reverse primer	5'-TTACGAACACTAAACTACACCCG-3'
Probe	5'-/CY5/ACAAAACGTTCCCGACGCTTCTAAAA/BHQ1/-3'
<b>TGFB2</b>	
Forward primer	5'-TTTTAGGAGAAGGCGAGTCG-3'
Reverse primer	5'-CTCCTTAACGTAATACTCTTCGTCG-3'
Probe	5'-/TAMRA/TCTCGCGCTCGCAAACGACC/BHQ1/-3'
<b>ALU</b>	
Forward primer	5'-GGTTAGGTATAGTGTTTATATTGTAATTTAGTA-3'
Reverse primer	5'-ATTAACATAACTAATCTTAAACTCCTAACCTCA-3'
Probe	5'-/6-FAM/CCTACCTTAACCTCCC/MBNFQ1/-3'



and *TGFβ2*, 2.0 μM *HOXD3* and 0.1 μM *ALU* probe each, in a 30 μl reaction volume on a 96-well plate. The ratio between the values of *APC*, *HOXD3* or *TGFβ2* and *ALU* was used as a measure of the percent methylation ratio (PMR) according to the formula of Eads et al.<sup>18</sup> as follows:

$$\left[ \frac{(\text{gene}/ALU)_{\text{sample}}}{(\text{gene}/ALU)_{\text{CpGenome universal methylated DNA}}} \right] \times 100\%$$

Primers and probe sequences used for Multiplex MethyLight are listed in Table 2. Oligonucleotides for unmethylated reactions were identical, except CG was replaced with TG and CA in the forward and reverse sequences, respectively. All oligonucleotide sequences were ordered from Integrated DNA Technologies and analyzed *in silico* for potential non-specific interactions using the IDT heterodimer oligoanalyzer algorithm: <http://www.idtdna.com/analyzer/applications/oligoanalyzer>. All PCR reactions were performed on an Applied Biosystems 7500 Real-Time PCR system (Applied Biosystems) with an initial denaturation at 95 °C for 10 minutes, followed by 50 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Data acquisition and analysis was performed on the Sequence Detection Software v2.0.6. In each run, CpGenome™ universal methylated DNA (Chemicon, Millipore) was integrated as a positive control as well as standard curve material. Multiplex *ALU* reactions were considered positive when sufficient input DNA was present, which as defined as *ALU* Ct value ≤ 19. Above this cutoff, not detecting methylated DNA of either assay could be either due to a lack of methylation or due to insufficient quality/quantity of template DNA. The selection of this Ct cutoff was based on technical parameters determined during multiplex MethyLight assay optimization and standardization. Briefly, *ALU* Ct value of 19 corresponded to the lowest DNA concentration on the standard curve that reliably detected *APC*, *HOXD3* and *TGFβ2* methylation in the same reaction. Intra-assay and inter-assay coefficient of variation (CV) and differences between singleplex and multiplex MethyLight PMR values were determined using paired t-test on Microsoft® Office Excel for Mac 2011 (Microsoft).

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## Author contributions

E.O.-M., D.Z., K.K. and B.B. contributed to experimental design, analysis and interpretation of data. E.O.-M. and D.Z. performed all singleplex and multiplex MethyLight experiments.



T.vdK. reviewed the complete set of H&E stained slides from each RP. N.F. identified and recruited suitable patients who provided urine samples for the study. B.B. supervised the project. All authors discussed the results, critically reviewed the manuscript, read and approved the final version.

### Additional information

**Competing financial interests:** The authors declare no competing financial interests.

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