

DNA methylation analysis by digital bisulfite genomic sequencing and digital MethyLight

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ABSTRACT

Alterations in cytosine-5 DNA methylation are frequently observed in most types of human cancer. Although assays utilizing PCR amplification of bisulfite-converted DNA are widely employed to analyze these DNA methylation alterations, they are generally limited in throughput capacity, detection sensitivity, and or resolution. Digital PCR, in which a DNA sample is analyzed in distributive fashion over multiple reaction chambers, allows for enumeration of discrete template DNA molecules, as well as sequestration of non-specific primer annealing templates into negative chambers, thereby increasing the signal-to-noise ratio in positive chambers. Here, we have applied digital PCR technology to bisulfite-converted DNA for single-molecule high-resolution DNA methylation analysis and for increased sensitivity DNA methylation detection. We developed digital bisulfite genomic DNA sequencing to efficiently determine single-basepair DNA methylation patterns on single-molecule DNA templates without an interim cloning step. We also developed digital MethyLight, which surpasses traditional MethyLight in detection sensitivity and quantitative accuracy for low quantities of DNA. Using digital MethyLight, we identified single-molecule, cancer-specific DNA hypermethylation events in the CpG islands of *RUNX3*, *CLDN5* and *FOXE1* present in plasma samples from breast cancer patients.

INTRODUCTION

Alterations of CpG island DNA hypermethylation and chromatin modification have been widely documented in human cancers (1,2). DNA methylation changes are not only detectable in tumors, but also in blood, as tumor-derived DNA is released into the bloodstream due to tumor necrosis and apoptosis (3,4). Cancer-specific DNA methylation alterations present in cancer tissues and blood of cancer patients can serve as diagnostic markers for risk assessment, progression, early detection, treatment prediction and monitoring (5).

The sensitive detection of specific DNA methylation patterns occurring at very low abundance presents technological challenges that are distinct from the challenges of determining the sequence of consecutive methylation states at single base-pair resolution in individual DNA molecules. The former requires high signal-to-noise ratio, and generally relies on methylation-specific PCR priming (MSP) (6), with optional further enhancement by methylation-specific probing (MethyLight) (7), whereas high-resolution sequencing requires low-sensitivity methylation-independent priming, combined with separation of PCR products for sequence analysis. This separation has traditionally been accomplished by a plasmid cloning step in *Escherichia coli* (*E. coli*) prior to sequencing (8). We show here that digital PCR can benefit both DNA methylation analysis strategies.

Digital PCR (9) was originally described as a tool for the amplification of individual molecules for purposes of identifying and counting individual DNA molecule sequence alterations, and now is well-established in determining

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The authors wish it to be known that, in their opinion, the first three authors should be regarded as joint First Authors

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coding mutations, loss of heterozygosity, allelic imbalance and SNP polymorphisms (10). By distributing a sample over multiple PCR reaction wells to a mean concentration well below one template molecule per well, amplification of single template molecules is achieved in a minority of the wells. This provides a digital readout of the original number of template molecules in the distributed sample. We have applied this principle to bisulfite genomic sequencing. By omitting the time- and labor-intensive cloning step in *E. coli* (8), digital bisulfite genomic sequencing greatly increases the efficiency of single-molecule DNA methylation analysis, and results in a significant cost reduction. PCR wells with positive amplification can be recognized by the use of SYBR Green, and sequencing can be performed directly on the PCR products following clean up. Thus, digital PCR not only provides information on the number of discrete templates, but can also be used to separate heterogeneous templates into separate amplifications for subsequent sequencing. A less well-recognized benefit of digital PCR is the sequestration of competing background molecules into negative wells that do not participate in the PCR amplification. As a consequence, the ratio of template-to-background improves in the positive wells. Competition for primer annealing by background DNA is a major problem in the detection of low-abundance methylation variants by MSP and MethyLight. This problem is particularly acute for these bisulfite-based detection methods, since sequence redundancy is increased in bisulfite-converted DNA, which contains only three bases outside of sites of DNA methylation (11).

In this study, we have applied digital PCR technology to two bisulfite-DNA based DNA methylation assays, digital bisulfite genomic DNA sequencing and digital MethyLight, to obtain DNA methylation information at high resolution or with high sensitivity, respectively. Both digital bisulfite genomic DNA sequencing and digital MethyLight are novel, fast, reliable and cost effective measures for determining DNA methylation information of individual DNA molecules, and are easily customizable to the analysis of any gene region and sample type.

MATERIALS AND METHODS

M.SssI and whole genome amplification treatments

Human peripheral blood leukocyte (PBL) DNA was purchased from Promega Corporation (Madison, WI) and 75 μ g DNA was treated with 75 units *M.SssI* methylase (New England Biolabs, Ipswich, MA) together with 0.16 mM *S*-adenosylmethionine (SAM) at 37°C for 16 h. Additional SAM and 25 units of *M.SssI* methylase were added and incubated overnight at 37°C. The enzyme was inactivated and directly used for bisulfite conversion. The *M.SssI*-DNA sample serves as a positive control for DNA methylation in bisulfite sequencing and MethyLight assays. Multiple *M.SssI* treatments are beneficial for completely methylating a DNA sample.

We used whole genome amplification (WGA) to generate unmethylated DNA for methylation analyses. In brief, 5 ng genomic PBL DNA was used as input for amplification. WGA-DNA was purified by phenol-chloroform

extraction, precipitated with ethanol, and re-suspended in 50 μ l water. We recovered 10–20 μ g WGA-DNA after purification. The WGA sample essentially contains background levels of DNA methylation, with less than 0.1% of methylated cytosines remaining in the WGA-DNA sample. Whole genome amplification using the REPLi-G kit (Sigma, Valencia, CA) was performed as described by the manufacturer.

Bisulfite conversion and recovery

DNA samples (1 μ g genomic DNA for each) were treated with bisulfite as previously described (11). The purified bisulfite-converted samples were eluted in 120 μ l volume, and in order to remove traces of ethanol-based PCR inhibitors, we then incubated the samples at 80°C for 20 min, and then stored the samples at –30°C until needed.

Digital bisulfite genomic DNA sequencing

Fresh-frozen tumor DNA (1 μ g) from two colorectal cancer patients (Laird IDs 6317 and 6363) was bisulfite converted and recovered as described above. For the conventional, cloning-based bisulfite DNA sequencing approach, we amplified a portion of the *MLH1* CpG island using forward and reverse primers listed in Table 1. The PCR was performed on a Robocycler (Stratagene, La Jolla, CA) containing 200 μ M dNTPs, 2 mM MgCl₂, 0.3 μ M forward and reverse primers and 0.5 units of *Taq* polymerase (Invitrogen, Carlsbad, CA). The PCR conditions are as follows: 95°C for 3 min, then 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for one min. A final incubation at 72°C for 15 min concluded the PCR. PCR products were verified by gel electrophoresis, and a small aliquot of the PCR reaction was used with the TOPO-TA cloning system (Invitrogen) as suggested by the manufacturer. Clones were picked from LB-Amp cultures, and then were screened and amplified using M13 primers. Positive clones were then sequenced using the following sequencing primer: 5'-GGT ATT TAA GTT GTT TAA TTA ATA GTT GT -3' by the USC/Norris Cancer Center DNA Sequencing Core Facility.

For the digital bisulfite genomic DNA sequencing assay, we first established the amount of bisulfite converted DNA to load on the 96-well PCR assay in order to avoid over- or under-loading the template DNA. To accomplish this, we determined the relative amounts of each sample through the use of a TaqMan PCR reaction (C-LESS-C1), which recognizes a DNA strand that does not contain cytosines, and hence will be able to amplify the total amount of DNA (bisulfite-converted or unconverted) in a PCR reaction well. The C-LESS forward sequence is provided in Table 1 and was purchased from Applied Biosystems. An unconverted DNA sample of known concentration was serially diluted and used as a standard curve. Since the C-LESS reaction amplifies both template strands of unconverted genomic DNA but only one strand of bisulfite-converted DNA, we expect that bisulfite-converted DNA will amplify one PCR cycle later than that of unconverted DNA. To correct for this, we multiplied the amount of bisulfite-converted DNA for each sample quantitated from the C-LESS standard curve

Table 1. PCR primer and probe DNA sequences

Reaction ID	HB-number	Forward primer sequence (5' to 3')	Reverse primer sequence (5' to 3')	Probe sequence (5' to 3')
MLH1-C2	HB-665	GAT TGG TAT TTA AGT TGT TTA ATT AAT AG	CAA TCA TCT CTT TAA TAA CAT TAA CTA A	
PITX2-M2	HB-235	AGT TCG GTT GCG CGG TT	TAC TTC CCT CCC CTA CCT CGT T	6FAM-CGA CGC TCG CCC GAA CGC TA-BHQ-1 ^a
C-LESS-C1	HB-344	TTG TAT GTA TGT GAG TGT GGG AGA GA	TTT CTT CCA CCC CTT CTC TTC C	6FAM-CTC CCC CTC TAA CTC TAT-MGBNFQ ^b
FOXE1-M1	HB-417	GGG TTA GTT CGC GAC GAT TTT	CGA ACC TAA CGT CCC CGA	6FAM-CGA ACG CTC GAC CCT TCT ACG AAA AAC T-BHQ-1
CLDN5-M1	HB-415	TGA GGG CGC GGG ATC	CCT AAA CCA ACC CAA AAT ACG CT	6FAM-CGA CCG CGA CTA AAA CAA CGA CGA ATA A-BHQ-1

^aBHQ-1 refers to a Black Hole Quencher at the 3' terminus of the probe.

^bMGBNFQ refers to a Minor Groove Binder non-fluorescent quencher in the 3' terminus of the probe.

by the PCR efficiency to determine the total amount of bisulfite-converted DNA for use in digital bisulfite sequencing assays.

We next performed digital PCR for the *MLH1* locus using bisulfite-specific forward and reverse primers (described in Table 1). For each assay, we generated PCR products from 15–20 molecules after loading 300–400 pg of bisulfite-converted DNA in each PCR plate. Assuming each haploid genome contains 3.3 pg DNA, we loaded 90–120 copies of bisulfite-converted DNA into each 96-well plate. In comparing the number of amplified DNA molecules to the estimate of genome equivalents loaded into the assay, we found an approximate 15–20% sensitivity of amplifying individual *MLH1* DNA molecules using digital bisulfite genomic DNA sequencing. In order to minimize the occurrence of two or more PCR templates in a single well, we limited each assay to a maximum of 20–30 amplifiable molecules per PCR plate.

Each PCR reaction used the iQ SYBR Green Supermix (Bio-Rad, Hercules, CA) and 0.3 μ M forward and reverse primers in a 1.44 ml total volume. This volume was dispersed in 15 μ l aliquots over an entire 96-well plate, and the PCR was performed using an Opticon real-time thermal cycler (Bio-Rad) using the PCR program of 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 55°C for 1 min. Using a melting curve analysis, we identified the melting curve of the PCR product in each well. Primer dimers melted at approximately 70°C, while PCR products derived from single template DNA molecules melted between 77–85°C.

We randomly chose true PCR products for sequencing. We removed 10 μ l from each well, and removed unused dNTPs and primers using the ExoSAP-IT kit (USB Corporation, Cleveland, Ohio) according to the manufacturer's specifications. The *MLH1* sequencing primer was added to each treated sample, and each sample was subsequently sequenced by the USC/Norris Comprehensive Cancer Center DNA Sequencing Core Facility. Interestingly, we noticed that the PCR products with high melting temperatures as reported by the melting curve analyses were CpG-methylation rich, while the PCR products with lower melting temperatures were generally unmethylated. We used this correlation as a quality control after obtaining

the bisulfite sequencing information of each single-molecule derived PCR product.

Digital MethyLight evaluation experiments

Each MethyLight reaction used in this report has been thoroughly and extensively evaluated for specificity to methylated DNA only. We routinely evaluate each MethyLight reaction on white blood cell DNA: \pm M.SssI treatment, \pm bisulfite conversion and \pm whole genome amplification. All MethyLight reactions used were positive in M.SssI-treated, bisulfite-converted DNA and negative in samples without M.SssI-treatment, without bisulfite conversion and with whole genome amplification. In addition, we have also tested each digital MethyLight assay for the presence of any false-positive signals in 20 PCR plates that do not contain template DNA. We failed to detect any amplification signals on any of these assays. Therefore, we feel confident that the signals we detect in this report are indeed amplifications of single methylated DNA molecules.

Each bisulfite-converted DNA sample was mixed with 200 μ M dNTPs, 0.3 μ M forward and reverse PCR primers, 0.1 μ M probe, 3.5 mM MgCl₂, 0.01% Tween-20, 0.05% gelatin and 50 units of *Taq* polymerase (Applied Biosystems, Foster City, CA) in a 2.85 ml total volume. The *PITX2* MethyLight primers and probe were obtained from BioSearch Technologies and are listed in Table 1. The entire reaction mixture was aliquotted over a 96-well plate at 30 μ l per PCR reaction well, and the PCR program used was 95°C for 10 min, followed by 50 cycles of 95°C for 15 s then 60°C for 1 min. The number of methylated DNA molecules was scored as the number of quality real-time PCR fluorescence curves over the entire PCR plate.

For the M.SssI-DNA dilution series (Figure 3B), bisulfite-converted, M.SssI-DNA was diluted in serial 1:3 dilutions, and the MethyLight PCR reaction mixture was the same as above, except we used 16.67 units of *Taq* polymerase in a 0.96 ml total volume, which was distributed in 10 μ l aliquots over 96 reaction wells. We used 184 ng of bisulfite-converted DNA for the most concentrated M.SssI-treated DNA sample and 3-fold dilutions of that sample in the remaining dilutions. The digital MethyLight PCR reactions were performed as above. The reactions were analyzed on an Opticon DNA

Engine Continuous Fluorescence Detector (Bio-Rad) and the number of positive amplifications for each sample was scored.

The approximate number of bisulfite-converted DNA molecules in the most concentrated M.SssI-DNA sample was determined using the C-LESS-C1 reaction. An unconverted DNA sample of known concentration was serially diluted and used as a standard curve, and the DNA concentration in the M.SssI-DNA sample was then determined in a similar manner to the digital bisulfite sequencing protocol. Since the C-LESS amplification of bisulfite-converted DNA will be delayed by one cycle compared to unconverted DNA, we multiplied this concentration by the PCR efficiency (1.83 in this experiment) of the C-LESS reaction as a correction factor. With this final concentration value, we determined the number of molecules present in the assayed DNA sample volume, and then extrapolated the number of DNA molecules for the remaining M.SssI-DNA dilution series. Based on these calculations, we detected approximately 25% of the available methylated *PITX2* DNA molecules in the digital MethyLight assay.

Comparison of digital and classic MethyLight assay sensitivities

M.SssI-DNA and WGA-DNA samples (1 µg each) were individually treated with bisulfite as described above. A mixture of 25 pg bisulfite-converted M.SssI-DNA and 50 ng of bisulfite-converted WGA-DNA was analyzed for *PITX2* methylation with the mixture analyzed in one well (classic MethyLight assay) and the remaining 95 wells of a PCR plate (digital MethyLight assay). The Classic MethyLight assay was performed by incubating the bisulfite-converted M.SssI- and WGA-DNA samples in one PCR reaction well with 200 µM dNTPs, 0.3 µM forward and reverse PCR primers, 0.1 µM probe, 3.5 mM MgCl₂, 0.01% Tween-20, 0.05% gelatin and 0.5 units of *Taq* polymerase in a 30 µl reaction volume. For the digital MethyLight assay, the bisulfite-converted M.SssI- and WGA-DNA samples were mixed with 200 µM dNTPs, 0.3 µM forward and reverse PCR primers, 0.1 µM probe, 3.5 mM MgCl₂, 0.01% Tween-20, 0.05% gelatin and 50 units of *Taq* polymerase in a 2.85 ml total volume. This reaction mixture was aliquotted over 95 PCR reaction wells with 30 µl per well. This comparison was analyzed 20 times for each assay.

Analysis of DNA methylation in plasma using digital MethyLight

Plasma from breast cancer patients and controls was obtained from the University of Texas M.D. Anderson Cancer Center (Houston, TX). DNA was purified from 500 µl plasma using the Qiagen Blood DNA kit (Qiagen, Valencia, CA) and converted with bisulfite using the Zymo EZ DNA methylation kit (Zymo, Orange, CA) according to manufacturer's specifications. For each sample, an amount of bisulfite-converted DNA equivalent to 100 µl of plasma was mixed with MethyLight reactions specific for *RUNX3* (RUNX3-M1, HB-181), *FOXE1* (FOXE1-M1, HB-417) or *CLDN5* (CLDN5-M1, HB-415). These

MethyLight primers and probes were obtained from Biosearch Technologies. Each digital MethyLight reaction was prepared with 200 µM dNTPs, 0.3 µM forward and reverse PCR primers, 0.1 µM probe, 3.5 mM MgCl₂, 0.01% Tween-20, 0.05% gelatin and 50 units of *Taq* polymerase in a 2.85 ml total volume. This volume was dispersed in 30 µl aliquots over an entire 96-well PCR reaction plate. For the multiplexed digital MethyLight assay, an amount of bisulfite-converted DNA present in 100 µl of each plasma sample was prepared the same as above, except each MethyLight reaction was present at a concentration of 0.1 µM forward and reverse PCR primers and 0.1 µM probe. Each digital MethyLight assay was performed on an Opticon Real-time PCR system, and the PCR program is 95°C for 10 min, followed by 50 cycles of 95°C for 15 s then 60°C for 1 min. The number of methylated DNA molecules was scored as the number of quality real-time PCR fluorescence curves over the entire PCR plate. The MethyLight primers for RUNX3-M1 have been previously described (12). The MethyLight primers probe sequences for CLDN5-M1 and FOXE1-M1 are listed in Table 1.

Microfluidic digital MethyLight

Bisulfite-converted M.SssI-treated DNA (1 µg) in a 110 µl volume was concentrated to a final volume of 30 µl by speed-vac evaporation. This sample was then serially diluted 1:5 and 3.76 µl of each dilution was used for microfluidic digital MethyLight analysis. We determined the amount of bisulfite-converted DNA from the C-LESS reaction as described above. We loaded 19.20 ng, 3.84 ng, 0.77 ng, 0.15 ng and 0.03 ng of bisulfite-converted DNA into separate channels of the microfluidic array. A mastermix for the *PITX2* MethyLight assay was prepared in a 8.24 µl total volume consisting of 200 µM dNTPs, 0.3 µM forward and reverse PCR primers, 0.1 µM probe, 3.5 mM MgCl₂, 0.05% Tween-20, 0.05% gelatin, 0.5 units of *Taq* polymerase. The 11 µl total reaction volume for each serial dilution was loaded onto a Fluidigm BioMark Digital Array according to manufacturer's specifications. Each reaction was subdivided into 1104 chambers, such that each chamber contained a 10 nl PCR reaction. The PCR program is the same as with the 96-well based digital MethyLight assay for 50 cycles. PCR products were visualized by fluorescence emission and detection by a CCD camera contained within the BioMark platform. Images were taken at nearly every cycle throughout the PCR program, and screening the TaqMan fluorescence curves for each chamber via BioMark software eliminated false positives.

RESULTS

Digital bisulfite genomic DNA sequencing

The human genome contains an abundance of DNA methylation information, and cancer-specific methylated DNA sequences are a powerful biomarker of disease, tumor recurrence and clinical outcome. Obtaining high-resolution continuous DNA methylation information for longer stretches of DNA is possible via bisulfite genomic

DNA sequencing, however, this assay is quite laborious and time inefficient with the required subcloning steps in order to isolate individual DNA molecules. Since digital PCR was shown to compartmentalize the individual template DNA molecules into separate PCR reaction wells, we applied this technology to bisulfite DNA sequencing with the hopes of quickly amplifying bisulfite-converted DNA of a specific locus for the purposes of obtaining high resolution DNA methylation sequence information.

Our approach, as described in Figure 1, was to compartmentalize and amplify individual bisulfite-converted DNA molecules in a 96-well PCR reaction plate with primers specific for bisulfite-converted DNA. PCR products derived from single template DNA molecules are then identified, purified and sequenced directly without a subcloning step. To test this, we designed a PCR reaction specific for bisulfite-converted DNA sequence within the *MLH1* CpG island (MLH1-C2) that can be used to compare both the conventional and digital bisulfite sequencing assays. The MLH1-C2 PCR primers are specific for bisulfite-converted DNA but are methylation-independent, such that all possible DNA methylation patterns can be amplified prior to sequencing. Fresh-frozen tumor DNA samples from two colorectal cancer patients were used, both shown to harbor *MLH1* DNA methylation by MethyLight analysis (12). Using the conventional bisulfite DNA sequencing approach first, we PCR amplified the *MLH1* locus for each bisulfite-converted sample, and then ligated each PCR product into a TOPO-TA vector. These were subsequently transformed into *E. coli* and subclones composed of individual DNA molecules were isolated and sequenced. One DNA sample (6363) showed extensive methylation of the *MLH1* CpG island, while individual clones of the other DNA sample (6317) showed fewer methylated CpG dinucleotides (Figure 2A).

We next performed digital PCR on the bisulfite-converted DNA samples using the same MLH1-C2 primers. In order to minimize the occurrence of two or more PCR templates in a single well, we targeted a maximum of 20–30 molecules per PCR plate. After PCR, wells containing valid amplified products were identified using a SYBR green melting curve analysis. An aliquot of the PCR reaction containing amplified DNA from single molecules was then purified using Exonuclease I and Shrimp Alkaline Phosphatase (Exo-SAP-IT) to remove primer and dNTPs, and was then subjected to DNA sequencing. The individual bisulfite-converted DNA molecules showed an *MLH1* DNA methylation profile comparable to those derived from TOPO-TA cloning-based DNA sequencing for each sample (Figure 2B). However, we did detect two instances in which both methylated and unmethylated signals for the same CpG (highlighted by the asterisk) were observed, suggesting that this may be the result of two DNA molecules present in one PCR reaction well prior to amplification or an error in the DNA sequence analysis for this CpG dinucleotide.

Digital MethyLight

DNA methylation alterations are abundant in human cancers, and one approach to early detection of cancer has

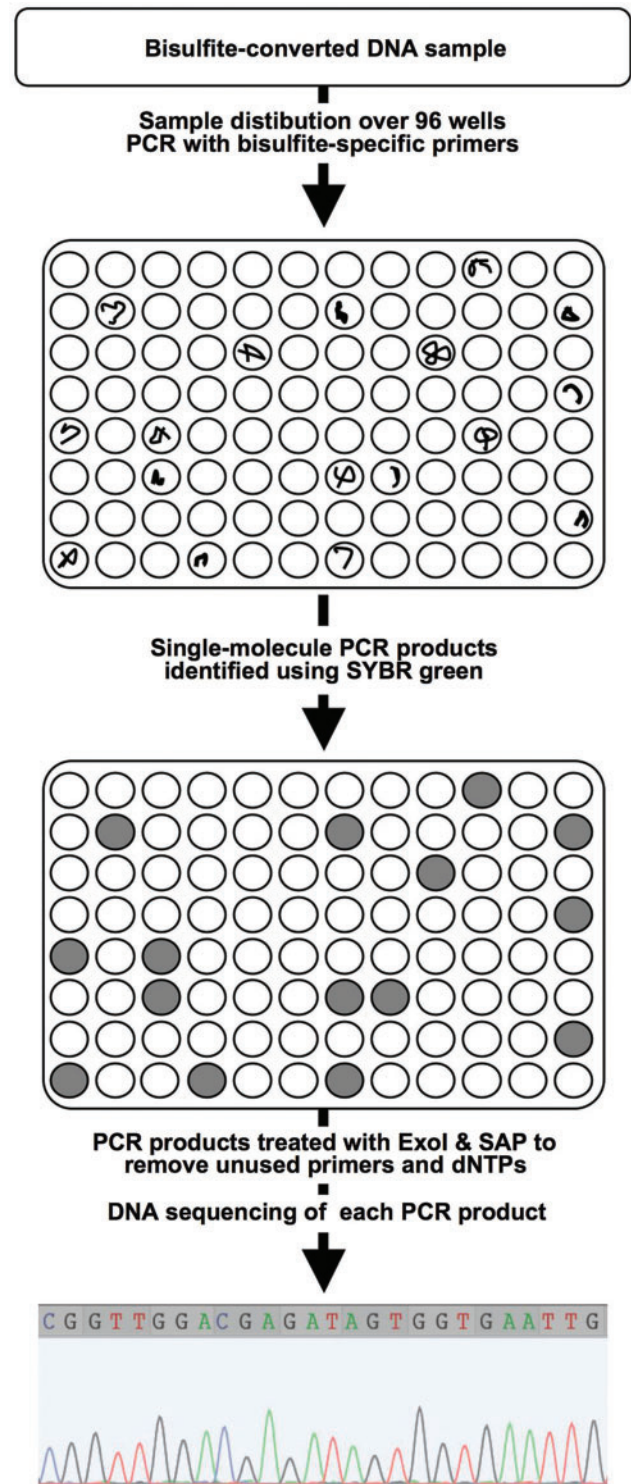


Figure 1. Digital bisulfite genomic DNA sequencing overview. An amount of bisulfite-converted DNA was loaded such that individual template DNA molecules were amplified on a 96-well PCR plate. We targeted a maximum of 20–30 molecules to be sequenced in each PCR plate in order to minimize the occurrence of two or more PCR template molecules per reaction well. Positive amplifications are evaluated by SYBR green melting curve analyses, and PCR products from these wells are removed and purified with Exonuclease I and Shrimp Alkaline Phosphatase (Exo-SAP-IT) to remove unused PCR primers and dNTPs. PCR products representing individual DNA molecules are then subject to DNA sequencing.

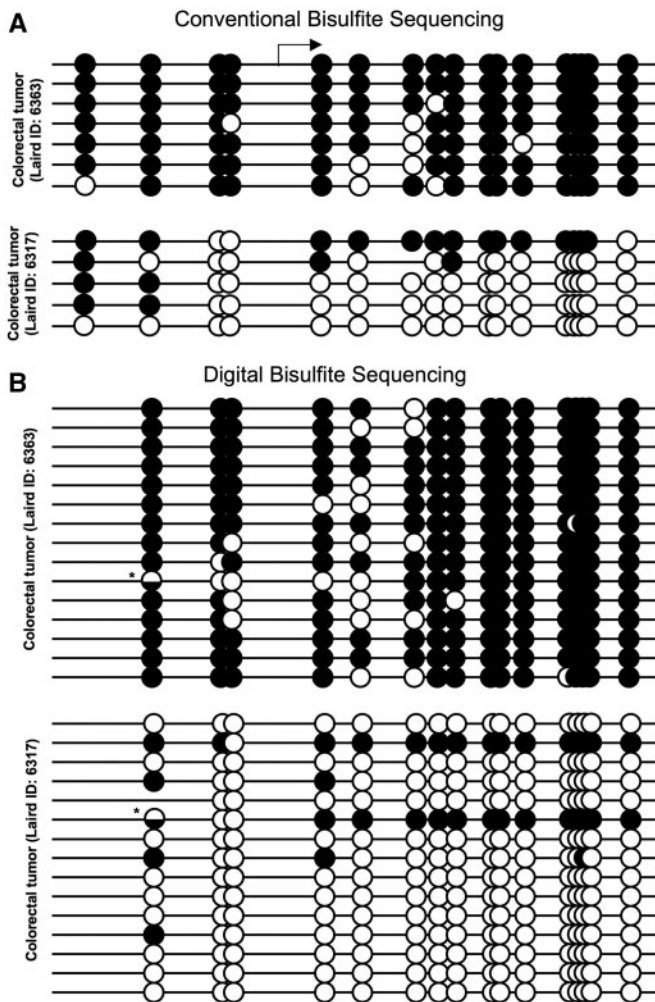


Figure 2. Comparison between conventional and digital bisulfite genomic DNA sequencing of *MLH1* CpG island. Bisulfite-converted tumor DNA from two colorectal cancer patients was amplified at the *MLH1* locus and subject to (A) TOPO-TA cloning followed by DNA sequencing, and (B) Digital bisulfite genomic DNA sequencing. Each horizontal line represents an individual DNA molecule, and the circles represent CpG dinucleotides. Filled in circles are methylated CpGs while open circles are unmethylated CpGs. The asterisk (*) represents DNA sequencing reads that gave signals for both methylation and the absence of methylation for a specific CpG dinucleotide, and may be indicative of two DNA molecules amplified in the PCR reaction well.

been to identify tumor-derived methylated DNA in cancer patient blood. However, this strategy has been hampered by relatively low sensitivity (5). This low sensitivity stems in part from the low absolute concentration of circulating tumor-derived DNA in some patients, combined with a large excess of PCR inhibitory contaminants and competing background DNA. Digital PCR was developed as a compartmentalized PCR reaction to allow for detection and counting of discrete template molecules (9). We theorized that digital PCR technology would have an additional benefit of sequestering background DNA and contaminants into wells that do not contain amplifiable templates, thereby increasing the signal-to-noise ratio of the positive wells. We tested this concept in a new application, termed digital MethyLight, which utilizes

MethyLight to interrogate a bisulfite-converted DNA sample distributed over multiple independent chambers. In the first implementation, we tested this principle in a 96-well plate format (Figure 3A).

We applied digital MethyLight to serial dilutions of *M.SssI*-treated DNA using a MethyLight reaction for methylated *PITX2* (Figure 3B). As the sample is diluted, the cycle threshold (Ct) values increase. However, as the number of available templates becomes limiting, the assay transitions from a quantitative measurement to a dichotomous measurement of stochastically distributed individual molecules. At this point, the mean Ct value no longer increases with further dilution, as one would expect for the detection of a single, discrete molecule, as demonstrated for digital bisulfite genomic sequencing. For *PITX2*, this occurs at approximately cycle 40 (Figure 3B).

We used a TaqMan PCR reaction (C-LESS-C1), which is derived from a unique DNA sequence near the *SLC24A3* gene that does not contain cytosines on one DNA strand to determine DNA quantities. This reaction can detect unconverted as well as bisulfite-converted DNA, and hence will be able to quantitatively measure the total amount of DNA independent of bisulfite-conversion. In comparing the number of methylated *PITX2* DNA molecules to the estimate of genome equivalents in the reaction (Figure 1B), we found an approximate 25% sensitivity of detecting and amplifying individual methylated *PITX2* DNA molecules using digital MethyLight (Figure 3B).

We compared the sensitivity of digital MethyLight and classic MethyLight assays under challenging conditions of a large excess of unmethylated DNA. We mixed 25 pg of *M.SssI*-treated, bisulfite-converted DNA (equivalent of approximately three to four cells) with a 2000-fold molar excess of genomic DNA devoid of DNA methylation by whole genome amplification. This mixture was analyzed 20 times for *PITX2* methylation, by both classic and digital MethyLight assays on 96-well PCR reaction plates, with one PCR reaction well of each plate dedicated to the Classic MethyLight assay and all remaining 95 wells of each plate constituting a single digital MethyLight assay (Figure 3C). By using mixtures of 25 pg *M.SssI*-DNA (methylated DNA) + 50 ng WGA-DNA (unmethylated DNA) in each assay, both classic and digital MethyLight assays interrogated equal quantities of methylated and unmethylated DNA, and therefore accurately compared assay sensitivities. Only 4 of the 20 classic assays (20%) detected *PITX2* methylation. However, 17 of 20 digital MethyLight assays (85%) were able to detect *PITX2* methylation, with many digital assays detecting multiple methylated *PITX2* loci, suggesting that digital MethyLight can detect methylated DNA molecules with an increased sensitivity compared to classic MethyLight. We determined that digital MethyLight technology was a statistically significant improvement over classic MethyLight through the use of a paired two-tailed *t*-test ($P < 0.0001$).

Microfluidic digital MethyLight

Even though digital MethyLight can detect single methylated DNA molecules, each 96-well assay is reagent intensive. Therefore, we tested digital MethyLight for its ability

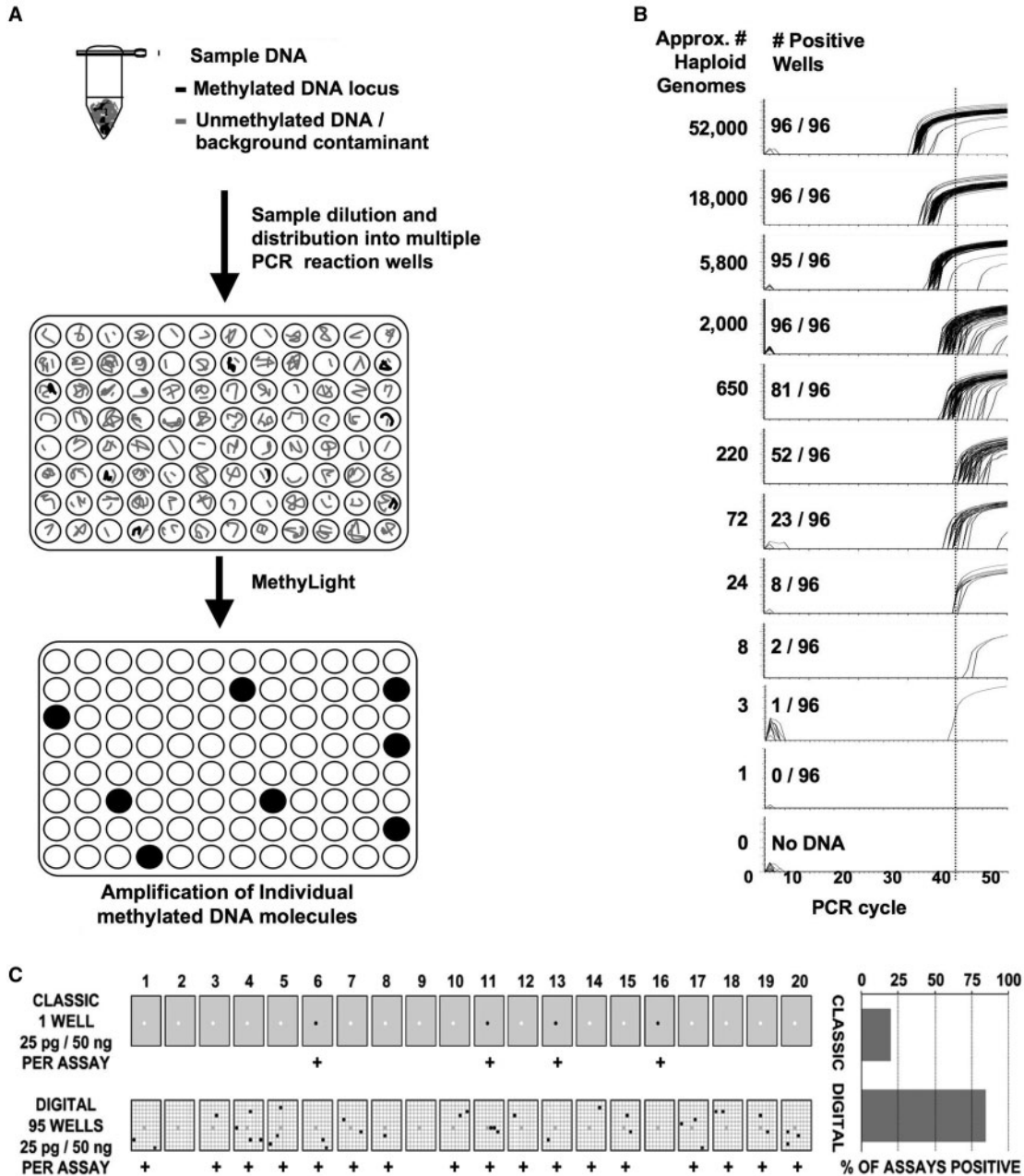


Figure 3. Digital MethyLight-based real-time PCR amplification. (A) Principle of amplifying individual methylated DNA molecules using digital MethyLight. A bisulfite-converted DNA sample is diluted and divided into multiple PCR reaction wells such that the target methylated DNA molecules are less than one molecule per reaction well. (B) Digital MethyLight was applied to serial dilutions of *in vitro* methylated DNA. The *PITX2* MethyLight reaction for each serial dilution was spread over 96 PCR reaction wells, the fluorescence signals for each dilution were plotted against the PCR cycle number and the number of positives were counted. The approximate number of haploid genomes was also evaluated for each dilution. The dashed vertical line represents the mean cycle threshold (Ct) value of single methylated *PITX2* DNA molecules. (C) Comparison of digital and classic MethyLight assay sensitivities. Two identical mixtures of 25 pg *M.SssI*-DNA plus 50 ng of unmethylated WGA-DNA were each analyzed for *PITX2* methylation with one mixture analyzed in one well (classic) and the second analyzed over the remaining 95 wells of a PCR plate (digital). This experiment was analyzed 20 times for each assay. The positive methylated *PITX2* molecules are indicated by the black wells and the + symbol indicates a positive signal for each assay. The percentage of assays positive for *PITX2* methylation is plotted for both classic and digital MethyLight assays.

to detect single methylated *PITX2* molecules on the Fluidigm microfluidic platform (13,14) in which 12 DNA samples can be assayed simultaneously. Each PCR reaction is compartmentalized into 1104 individual 10 nl reaction chambers, enabling the detection of single methylated DNA molecules in an 11 µl total reaction volume. Individually amplified methylated DNA molecules were

then visualized via the MethyLight probe fluorescence signals using a high-resolution CCD camera. We applied microfluidic MethyLight technology to serial dilutions of *M.SssI*-treated DNA (Figure 4). Using the microfluidic platform, we were also able to amplify single methylated *PITX2* molecules. This high-throughput digital MethyLight approach can successfully and sensitively

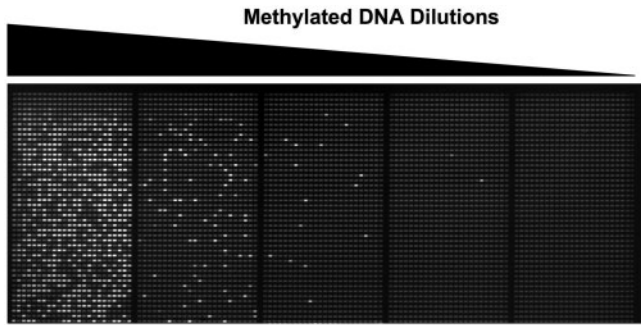


Figure 4. Detection of single methylated *PITX2* molecules serial dilutions of bisulfite-converted *M.SssI*-DNA using microfluidic digital MethyLight (Fluidigm). Each DNA sample was compartmentalized into 1104 reaction chambers of 10 nL each and amplifications were visualized by fluorescence emission in each positive chamber.

detect single molecule DNA methylation events in small PCR reaction volumes.

Detection of methylated DNA in breast cancer patient plasma using digital MethyLight

We tested the digital MethyLight technology on biological samples for the detection of tumor-derived, methylated DNA in the bloodstream. We applied digital MethyLight to DNA isolated from plasma of 44 breast cancer patients of different stages of disease and 13 apparently normal individuals. MethyLight reactions specific for methylated CpG islands located in the promoter regions of *FOXE1*, *CLDN5* and *RUNX3* were selected for this analysis. Using classic MethyLight, these reactions showed high cancer specificity in breast cancer tumor samples, and did not detect methylation in a test panel of plasma and white blood cells (WBC) from age-matched healthy control individuals (data not shown). As a result, these reactions would generate a low background signal from lysed WBCs and other free DNAs present in the breast cancer patient plasma samples.

We tested each of the three MethyLight reactions separately on bisulfite-converted DNA isolated from 100 μ L plasma using digital MethyLight, and we detected methylated DNA molecules in one stage II and several stage IV breast cancer patients, with the most abundant methylation seen in Stage IV patients (Figure 5). Methylated *FOXE1* and *RUNX3* molecules were more abundant than methylated *CLDN5* DNA, especially in the stage IV cases. To increase the sensitivity of methylated DNA detection, we multiplexed all three MethyLight reactions into one assay for each plasma sample. As expected, we detected an approximately cumulative number of DNA hypermethylation events using the multiplexed assay, thereby increasing sensitivity. One of the stage IV cases with background methylation levels of the individual markers became more evident after multiplexing, rising slightly above background levels (Figure 5). We found that although there were plasma samples with substantial amounts of free DNA, this did not correlate with the number of methylated DNA molecules in patient or control plasma based on an assessment of DNA quantities

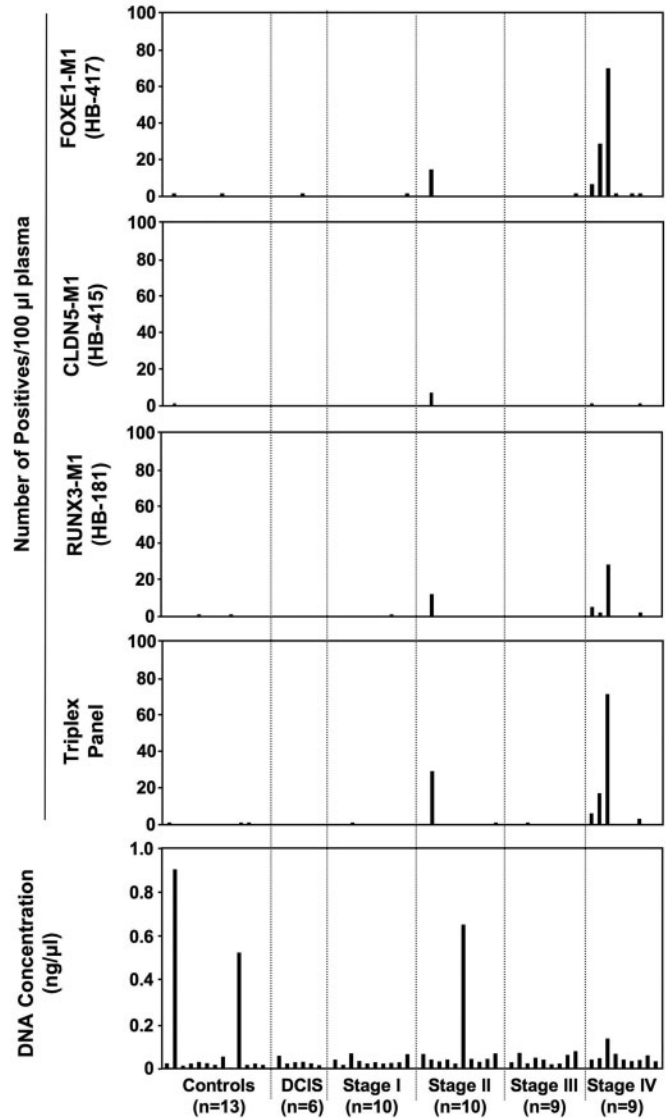


Figure 5. Digital MethyLight on plasma samples from 44 breast cancer patients and 13 apparently healthy controls. DNA from 500 μ L plasma was purified, bisulfite converted and a DNA amount from 100 μ L plasma was subject to digital MethyLight. Each sample was analyzed individually for *FOXE1*, *CLDN5* or *RUNX3* methylation, as well as with all three reactions multiplexed. The results are presented as the number of methylated molecules per 100 μ L plasma for each sample. The DNA concentration in each plasma sample was estimated based on a TaqMan reaction specific for *ALU* repetitive elements.

using a TaqMan PCR reaction specific for *ALU* repeats (Figure 5). We conclude that the careful selection of MethyLight reactions effectively avoids detecting DNA methylation from lysed white blood cells or background DNA methylation in plasma.

DISCUSSION

In this study, we have developed two novel DNA methylation analysis tools utilizing digital PCR technology. Digital MethyLight allows for detection of individually methylated DNA molecules in a large background of

unmethylated DNA, while digital bisulfite genomic DNA sequencing generates high-resolution DNA methylation information without the need for a subcloning step. Both assays are efficient and effective methods of obtaining DNA methylation information for samples with small amounts of DNA. Single-molecule analysis is possible by compartmentalizing the template across multiple PCR reaction wells. Not only are single molecules isolated, the background and other PCR contaminants are also diluted, and the ratio of primer to methylated template DNA is kept high.

Digital MethyLight was shown to be more sensitive than classic MethyLight in detecting a small number of methylated molecules in a large background of unmethylated DNA. Digital MethyLight, in compartmentalizing the methylated DNA molecules over multiple PCR wells, also reduces the background and contaminant levels, thereby reducing their PCR inhibitory effects and increasing methylated DNA detection sensitivity. This strategy allowed us to detect and quantify the number of individual methylated DNA molecules in plasma samples of breast cancer patients. Digital MethyLight is the most sensitive assay described to date for detecting methylated DNA in biological fluids.

The additional refinement of multiplexing digital MethyLight assays increased the sensitivity of detecting methylated DNA loci in plasma samples. Although the multiplexed assays detected DNA hypermethylation mostly in plasma from Stage IV breast cancer patients, we did detect DNA methylation in one stage II patient. This emphasizes the need to further improve the detection sensitivity so that earlier stages of disease can be detected. This may be achieved by using an increased number of multiplexed MethyLight markers in each digital MethyLight assay. Nonetheless, the CpG islands located in *RUNX3*, *FOXE1*, and *CLDN5* are promising DNA methylation markers for breast cancer patients. *RUNX3* DNA methylation was previously shown in breast cancer patients (15), while *FOXE1* and *CLDN5* methylation in breast cancer has not been described previously.

We used an amount of DNA present in a small volume (100 μ l) of serum for digital MethyLight-based detection. A recent study (16) identified DNA methylation of *SEPT9* in 69% of colorectal cancer plasma samples from measurements of milliliter volumes of patient plasma. While the amount of cancer patient plasma or serum is usually limiting for laboratory use, larger volumes of plasma or serum in digital MethyLight assays may increase the detection sensitivity of individual methylated DNA molecules. Nonetheless, the early detection of methylated DNA in biological fluids using digital MethyLight has great promise in cancer detection and surveillance.

We also described digital bisulfite DNA sequencing as a powerful method of amplifying individual bisulfite-converted DNA molecules for DNA sequencing. DNA methylation patterns of individual gene loci can be heterogeneous and an understanding of the DNA methylation patterns of individual molecules may be helpful to determine the role of DNA methylation in gene regulation and the mechanism of DNA methylation at specific gene loci. Digital bisulfite genomic DNA sequencing is a quick and efficient assay in which individual template DNA

molecules can be amplified, screened, purified and sequenced in the same day. This assay is time and labor effective in comparison to subcloning techniques to isolate individual bisulfite-converted DNA molecules.

Two recent reports also highlight the application of digital PCR to bisulfite-converted DNA. Chhibber and Schroeder (17) performed DNA sequencing after serial dilution of bisulfite-converted template DNA and showed that single-molecule PCR eliminated PCR and cloning biases. Snell and colleagues (18) reported the use of digital methylation-sensitive high-resolution melting (HS-HRM), followed by bisulfite genomic sequencing, to measure DNA methylation of the *BRCA1* promoter CpG island in breast cancer patients. Our digital bisulfite genomic DNA sequencing protocol also reduces PCR bias, circumvents cloning strategies and increases the signal-to-noise in detecting and amplifying individual methylated DNA molecules. We correlated melting temperature differences with differences in DNA methylation profiles of PCR products, as methylated bisulfite-converted DNA is more G:C rich and therefore melts at a higher temperature than unmethylated DNA.

A study from Taylor *et al.* (19) used 454 next-generation sequencing technology to identify individual molecule CpG methylation patterns in lymphoma and leukemia primary cells. While this assay is robust and powerful in generating large amounts of bisulfite sequencing data, there are substantial equipment and informatics requirements for 454 and other next-generation DNA sequencing platforms. Although digital bisulfite genomic DNA sequencing does not generate the amount of sequence data compared to 454 Sequencing, only a real-time PCR machine is required and approximately 20–30 individual molecules can be quickly assayed and sequenced. High-resolution sequence information of 20–30 DNA molecules can provide a detailed understanding of DNA methylation events at candidate gene loci. Digital bisulfite genomic DNA sequencing is an advantageous and flexible technology for determining single-molecule DNA methylation patterns of a wide range of DNA samples and gene loci.

In conclusion, the work presented here describes novel advancements in single-molecule DNA methylation detection and bisulfite sequencing. Digital MethyLight and digital bisulfite genomic DNA sequencing are cost and time effective methods in which a wide range of samples and loci can be assayed.

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Conflict of interest statement. P.W.L. is a shareholder, consultant and scientific advisory board member of

Epigenomics, AG, which has a commercial interest in DNA methylation markers. This work was not supported by Epigenomics, AG. Other authors have none.

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