MethylScreen: DNA methylation density monitoring using quantitative PCR

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Aberrant gene silencing of genes through cytosine methylation has been demonstrated during the development of many types of cancers including prostate cancer. Several genes including GSTP1 *have been shown to be methylated in prostate cancer leading to the suggestion and demonstration that methylation status of such genes could be used as cancer diagnosis markers alone or in support of histology. We developed a bisulfite-free alternative, MethylScreen technology, an assay for DNA methylation detection utilizing combined restriction from both methylation-sensitive restriction enzymes (MSRE) and methylation-dependent restriction enzymes (MDRE). MethylScreen was used to analyze the 5′ region of* GSTP1 *in cell lines, in vitro methylated DNA populations, and flash-frozen tissue samples in an effort to characterize the output and analytical performance characteristics of the assay. The output from the quantitative PCR assay suggested that it could not only detect fully methylated molecules in a mixed population below the 1% level, but it could also quantify the abundance of intermediately methylated molecules. Interestingly, the interpreted output from the four quantitative PCRs closely resembled the molecular population as described by clone-based bisulfite genomic sequencing.*

INTRODUCTION

The association between DNA methylation and diseases such as cancer opens the door to the development of new clinical testing technology, applicable in both diagnostic and prognostic settings. Methylated DNA possesses all of the desired hallmarks of an optimal biomarker amenable to testing in a clinical setting. Compared with protein and mRNA, methylated DNA is chemically stable and less subject to transient alterations due to biological variability. In many cancers, cell-free, tumor-derived DNA is shed and can be detected in a patient's serum (1,2). Therefore, detection of occult malignant cells may be achievable without direct tumor sampling (see Reference 3 for a review). Some of the better studied examples of genes whose transcription is silenced by methylation in cancer include p16[INK4a], hMLH1, and *GSTP1*, the most frequently hypermethylated gene in prostate cancer (4).

Although a small set of genes have been characterized that are affected by methylation of specific residues within their upstream regions (5,6), for most genes the overall density of methylation surrounding the transcriptional start sites correlates best with gene expression (7). While methylation of specific sites may have an important impact upon expression of the gene, cells rarely modify only those sites (5), suggesting that regional methylation may be the basis of the mechanism of DNA methylation-associated transcriptional inactivation rather than methylation of specific residues. Therefore, a regional perspective seems warranted when determining target DNA methylation status. It would seem that most biologically (and potentially clinically) relevant information should ideally reveal not only whether a DNA target contains methylation, but also the extent of methylation occupancy within the target region and the abundance or load of those aberrantly methylated molecules.

Previously developed methods for methylation detection rely on digestion of target DNA with methylation-sensitive restriction enzymes (MSREs) for which restriction is blocked by methylation. Digestion is followed by qualitative measurements such as Southern blot analysis, or semiquantitative measurements of restriction-refractory template in end point PCR (8), or by quantitative PCR (9). Alternative methods employ bisulfite conversion of unmethylated cytosine residues, followed by direct DNA sequencing (10) or amplification selecting for specific methylated base configurations [i.e., methylation-specific PCR (MSP)] (11) alone or in combination with quantitative PCR [(qMSP) (12) or MethyLight (13)]. Each of these approaches has associated limitations. MSRE-based methods are prone to false-positive results due to incomplete restriction, while bisulfite conversion techniques are laborious in terms of time and reagent consumption (14). In addition, amplification primers and detection probes used in all MSP-based methods by necessity encompass several CpG dinucleotides in the sequences, and only DNA that has the complementary methylation state for all cytosines covered by the primers/probes will be recognized in the assay. Given the large number of differing

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methylation configurations that may be associated with gene silencing at a given locus, these assays can only detect a tiny fraction of the total number of erroneously methylated molecules present in a sample.

To overcome many of the limitations associated with existing methylation detection technologies and to develop an assay that is particularly suited for the clinical laboratory, we have developed a novel method, MethylScreen, which when coupled with quantitative PCR permits sensitive detection and quantification of cytosine methylation in genomic loci (15). The MethylScreen technology is based upon the combination of methylation-sensitive and methylation-dependent restriction enzymes in single- and double-digests. The inclusion of a methylation-dependent restriction enzyme (MDRE) in the assay design not only eliminates false-positive reporting but also provides additional valuable information about the methylation density present in the region studied. The method is fast and efficient, and amenable to high-throughput clinical formats. Bisulfite conversion of DNA is not required, allowing the assay to be performed on nanogram quantities of starting template. In this report, we present an analysis of the performance characteristics of the MethylScreen assay, along with the methods necessary to interpret results from the assay. Importantly, in this study the MethylScreen measurements obtained were validated using bisulfite genomic sequencing. Our results suggest there may be an inherent benefit of application of the MethylScreen approach to biomarker-based cancer detection in tissue samples.

MATERIALS AND METHODS

Nucleic Acid Isolation

Genomic DNA was isolated from the LNCaP cell line (ATCC, Manassas, VA, USA; accession no. CRL-1740), DU145 cell line (ATCC; accession no. HTB-81), and CUGI RP11-715f10 bacterial artificial chromosome (BAC) clone (Invitrogen, Carlsbad, CA, USA) using MasterPure Complete DNA and RNA Purification kit and purified by phenol-chloroform extraction. Purified human
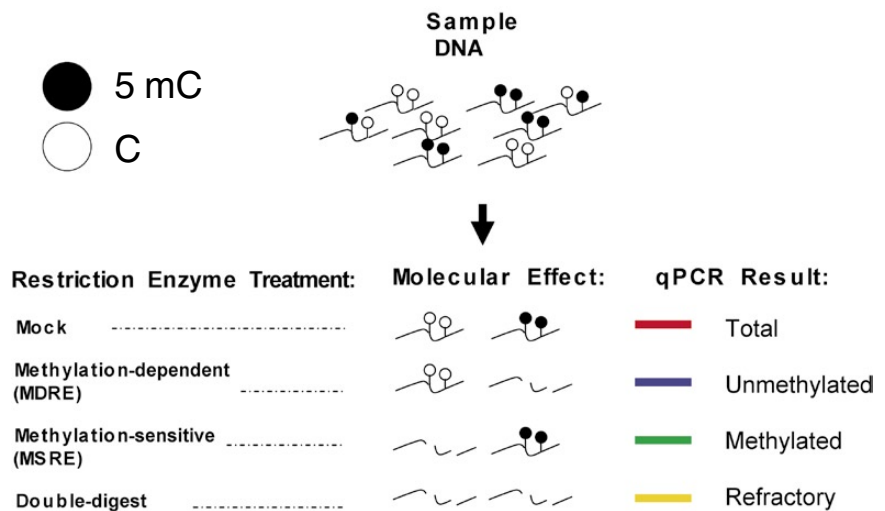

Figure 1. MethylScreen technology procedure overview. Depicted here are simulated results obtained from each of the four treatments in a MethylScreen assay. The genomic DNA obtained from a sample is depicted as wavy molecules. The cytosine bases methylation occupancy on each molecule is reflected by the shading of the circles on each molecule. Open is unmethylated, shaded is methylated. The results of each treatment upon the simulated population are depicted through the selective destruction of particular molecules (dashed wavy molecules). The SYBR Green kinetic reaction profiles are presented as follows: red represents the mock-treated reaction, quantifying the total molecular population under study; blue reflects the methylation-dependent restriction enzyme (MDRE) reactions, quantifying the unmethylated molecular population; green represents the methylation-sensitive restriction enzyme (MSRE)-treated portion and allows quantification of the densely methylated fraction. The fraction of the population not amenable to analysis results from using the double-digest as template (gold).

male blood genomic DNA for wild-type control was obtained from Novagen (Madison, WI, USA), and purified genomic DNA from the LNCaP cell line was obtained from ATCC (accession no. CRL-1740D). Human placental DNA was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Growth of Cell Lines and 715f10 Clone

LNCaP and DU145 cell lines were grown, maintained, and subcultured in isolation following ATCC guidelines. Invitrogen's *Escherichia coli* culturing recommendations were followed to grow and maintain the 715f10 clone.

Methylation Reactions

Male blood genomic DNA and BAC DNA were treated with one of two DNA modifying enzymes. *Hha*I methylase and SssI methylase were used to impart different methylation patterns on the *GSTP1* region of interest. The genomic DNA was then incubated with either of the two methylases for

1 h at 37°C, then incubated at 65°C to halt methylase activity. Each 1000-µL reaction contained an equivalent amount of male blood genomic DNA (20 µg), 1× NEB buffer 2 [10 mM Tris-HCl, 55 mM NaCl, 10 mM MgCl$_2$, 1mM dithiothreitol (DTT)], and glycerol. The *Hha*I methylase reaction consisted of 160 µM S-adenosyl-methionine (SAM) and 250 U *Hha*I methylase. The SssI methylase reaction consisted of 320 µM SAM and 40 U SssI methylase.

Digestion of Nucleic Acids

Genomic DNA was digested using four restriction enzyme-reagent containing reactions. Each reaction consisted of equivalent amounts of 1× NEB buffer 2 (10 mM Tris-Hcl, 55 mM NaCl, 10 mM MgCl$_2$, 1 mM DTT), 1 µg/mL bovine serum albumin (BSA), 2 mM guanosine-5′-triphosphate (GTP), 3% glycerol, and water. McrBC alone, *Hha*I alone, McrBC and *Hha*I combined, or 50% glycerol was then added to the reactions and combined with sample DNA. In the mock-treated reactions, restriction enzyme was replaced with

Table 1. Summary of Inter- and Intra-Assay Variation Study

A

DU145	Dense (%)	Sparse (%)	Int (%)	Refractory (%)
Lo	0.2	6.8	51.9	0.0
Hi	9.9	38.5	92.0	0.8
Range	9.7	31.7	40.0	0.8
Mean	3.6	19.2	77.2	0.2
SD	2.0	6.9	8.5	0.2
CV	56.1	35.9	11.0	120.1
Total ($n = 60^a$)	59	59	59	59
Phenotype				
Met	10			
Unmet		0		
Int			49	
Total ($n = 60^a$)	10		49	

B

Tumor	Dense (%)	Sparse (%)	Int (%)	Refractory (%)
Lo	12.9	21.7	30.9	0.5
Hi	25.8	47.1	62.3	1.1
Range	12.9	25.4	31.4	0.7
Mean	20.5	31.5	31.4	0.7
SD	3.9	7.8	10.5	0.2
CV	19.3	24.7	21.5	31.5
Total ($n = 10$)	10	10	10	10
Phenotype				
Met	10			
Unmet		0		
Int			0	
Total ($n = 10$)	10			

Panel A contains the data from three different analysis days, during which 20 independent MethylScreen GSTP1 assays were performed upon the DU145 cell line. There was no significant difference between the daily results [i.e., overlapping sem error bars (data not shown)]. Panel B contains the data from 10 independent analysis results from a pool of 10 prostate tumor samples. Phenotypes Met (methylated), Unmet (unmethylated), and Int (intermediately methylated) are phenotype calls. Met calls were made using a >5.6% cutoff for %Met. Int calls were made using a percent Met <5.6 and a percent Int >5.6. 5.6% is the mean dense DU145 + the SD Range = largest C_t - smallest C_t (Hi-Lo). Mean is arithmetic average. SD, standard deviation; CV, coefficient of variation of the population fractions = SD/mean × 100; total run, the number of independent assays in consideration.
^{a}One assay failed, so total attempts was 60 but total successes was 59. %methylated, %unmethylated, %intermediate, and %refractory were calculated following conversion back to copies (see Materials and Methods).

50% glycerol in order to maintain homogeneity of the restriction digest cocktail. Each digestion contained 1 µg sample genomic DNA in a volume of 100 µL. McrBC digestions consisted of 30 U McrBC, and *Hha*I digestions consisted of 60 U *Hha*I. Digestions were incubated for 6 h at 37°C to ensure complete digestion and then incubated at 65°C to halt enzyme activity. Overnight digestion has been employed without deleterious consequence (data not shown).

MethylScreen Reactions

Following enzyme digestion, samples were analyzed by fluorescence-based, quantitative PCR using the DNA Engine Opticon 2 (Bio-Rad Laboratories, Hercules, CA, USA). Detection of amplified products was enabled by SYBR Green fluorescent dye. Using locus-specific PCR primers, intact genomic DNA was able to be amplified, while genomic DNA that had been cleaved by one of the two restriction endonucleases (either within the locus or within the priming region) remained unamplified. A change in available loci for amplification is detected in the fluorescence cycle threshold among the separate digests. Standard curve data was obtained from 10-fold serial dilutions of blood genomic DNA. The threshold was set by maximizing the regression fit to the standard curve. To ensure specificity, a no-template control was included. The PCR amplification was performed in a 96-well optical tray with a 50-µL reaction volume. The tray was sealed with a Microseal 'B' plate seal and compressed against the detection system by a 96-well optical compression pad. Each reaction consisted of 75.2 nM each primer, 4 µL digested template DNA, 25 µL FailSafe 'G' Real-Time PCR buffer, and FailSafe Real-Time PCR enzyme at the manufacturer's recommended reaction conditions. PCR was performed at the following conditions: 95°C for 3 min, followed by 50 cycles at 95°C for 1 min; 69°C for 15 s; and 68°C for 30 s, followed by a plate read. A high-temperature plate read of 87°C for 5 s was performed after each amplification cycle to prevent the accumulation of unwanted products from interfering with the cycle threshold detection. To ensure amplification of desired products, melting curve analysis was performed following the real-time reaction. The melting curve range was 65°–95°C, holding for 1 s at increments of 0.2°C for product detection.

MethylScreen Primer Sequences

A single set of PCR primers was designed for the amplification of genomic DNA at the 5′ end of the promoter region of the *GSTP1* gene. The PCR product was 404 bp in length and contained five *Hha*I restriction sites and >50 CpG sites. The forward primer sequence was 5′-GCGCGGCGACTCCGGGGACT CCA-3′, and the reverse primer sequence was 5′-CGAGGCGCTCCGGGCCCC CTGAA-3′. The PCR product coordinates on chromosome 11 were Start-67107674, End-67108077, NCBI v35.

DNA Methylation Occupancy Calculations

DNA methylation occupancy calculations were performed as previously described (16).

Bisulfite Mutagenesis

A

B

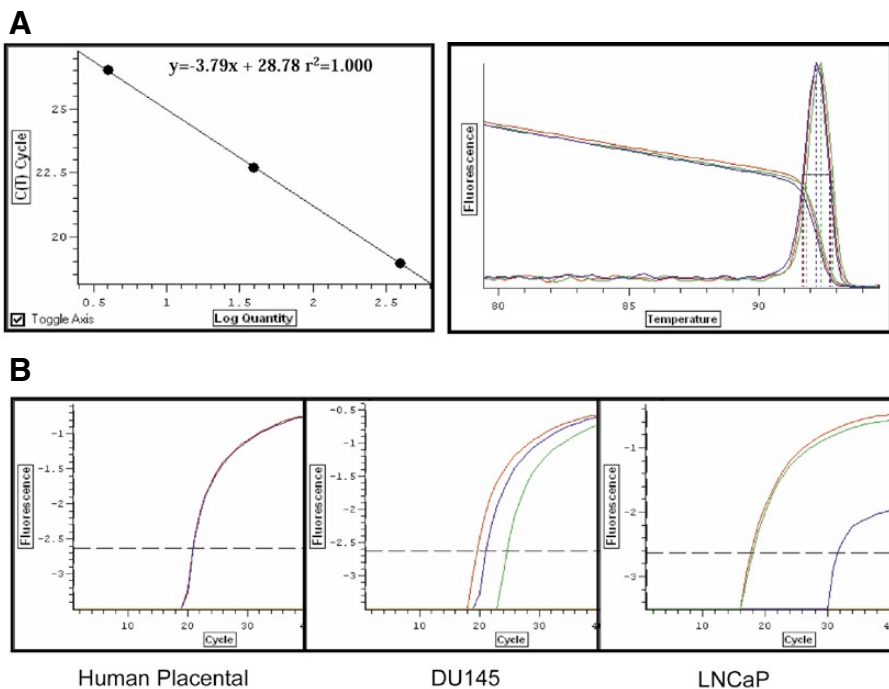Human Placental   DU145   LNCaP

Figure 2. MethylScreen analysis reveals that DU145 contains partially cytosine-methylated *GSTP1* copies. (A) Representative SYBR Green quantitative PCR results and the resultant melt curve analysis derived from the *GSTP1* primers across a 10-fold standard dilution curve using human placental genomic DNA as the template. Each point in the template dilution curve has two experimental PCR replicates. The melt curve demonstrates the PCR product's melting signature across all six PCRs. (B) Representative MethylScreen *GSTP1* analysis results obtained from three control genomes. Human placental is the negative control; DU145 is the intermediate control; and LNCaP is the positive control. The SYBR Green kinetic reaction profiles are presented as follows: red represents the mock-treated reaction, quantifying the total molecular population under study; blue reflects the methylation-dependent restriction enzyme (MDRE) reactions, quantifying the unmethylated molecular population; green represents the methylation-sensitive restriction enzyme (MSRE)-treated portion and allows quantification of the densely methylated fraction. The fraction of the population not amenable to analysis results from using the double-digest as template (gold).

Template for the amplification of bisulfite sequences was prepared by bisulfite mutagenesis using the EZ DNA Methylation kit (Zymo Research, Orange, CA, USA). Each mutagenesis contained 1 µg genomic DNA, and conversions were performed according to the manufacturer's recommendations.

Amplification of Bisulfite-Mutagenized DNA

PCR products to be cloned for bisulfite sequencing were prepared following two rounds of amplification. Data were obtained by analysis of a 900-bp region of the *GSTP1* gene.

First-round amplification was performed in a 50-µL volume containing 6 ng each round one primer, 25 µL FailSafe 'G' PCR buffer, 2.5 U *Taq* DNA polymerase (Invitrogen), and 0.5 µL converted DNA. Round-one PCR conditions were as follows: 95°C for 2 min, followed by 5 cycles at 95°C for 30 s; 45°C for 30 s; and 68°C for 1 min, then 30 cycles at 95°C for 30 s; 50°C for 30 s, 68°C for 1 min; followed by 68°C for 2 min and a 10°C hold. Second-round amplification was performed in a 50-µL volume containing 6 ng each round two primer, 25 µL FailSafe 'G' PCR buffer, 2.5 U *Taq* DNA polymerase (Invitrogen), and 0.5 µL Round-one

amplified product. Round-two PCR conditions were as follows: 95°C for 2 min, followed by 30 cycles at 95°C for 30 s; 50°C for 30 s; 68°C for 1 min; followed by 68°C for 2 min and a 10°C hold.

Primer Sequences

All PCR products were tested for amplification bias by evaluating their ability to reconstruct a 50:50 mixture of LNCaP and blood genomic DNA, or a 50:50 mixture of blood DNA that had been in vitro methylated with M.*Hha*I. The 900-bp PCR product displayed a modest representation bias of methylated gene copies with the LNCaP DNA, and neither displayed bias with the sparsely in vitro methylated control (data not shown). With both of the 900-bp cases, the bias resulted in recovery of fewer methylated gene copies than expected at random, however the effect was modest; such an effect has been observed before (17).

Nine Hundred-Base Pair PCR Product

Round-one and -two primer pairs were used as described in Millar et al. (18). Round-one forward primer sequence was 5′-TTTGTTGTTTGTTTATTT

TTTAGGTTT-3′. Round-one reverse primer sequence: 5′-AACCTAATAC TACCTTAACCCCAT-3′. Round-two forward primer sequence: 5′-GGG ATTTGGGAAAGAGGGAAA-GGTTT-3′. Round-two reverse primer sequence: 5′-ACTAAAAACT CTAAACCCCATCCC-3′.

Cloning of Bisulfite-Mutagenized PCR-Amplified Template Molecules

Round-two PCR products were cloned using the topoisomerase-mediated method (TOPO pCR2.1) using 4 µL PCR product, 1 µL vector, and 1 µL diluted salt solution. Ligation reaction occurred at room temperature for 20 min. Two microliters of the ligation were then electroporated into 50 µL TOP10 cells, and clones were selected according to the manufacturer's instruction (Invitrogen).

Bisulfite Genomic Sequencing Analysis

DNA was isolated using Perfectprep Plasmid 96, Spin Direct Bind kit, and forward and reverse DNA sequences were obtained using M13 sequencing primers. Sequence data was generated by the 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).
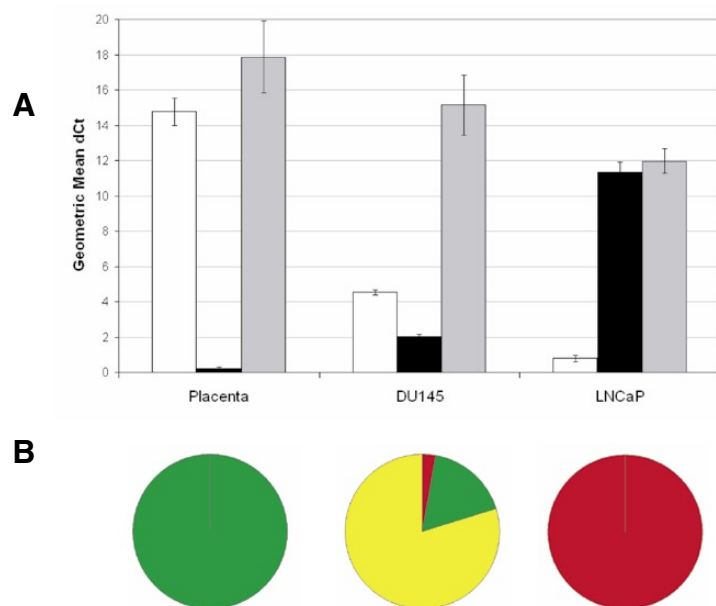
Figure 3. Reproducibility of results from control genome analysis and data interpretation. (A) The results from MethylScreen *GSTP1* quantitative PCR analysis as a column chart of the average change in cycle threshold (C_t) conditioned by the treatments in three control genomes. For each genome (placental, DU145 cell line, and LNCaP cell line); the white columns reflect the average change in C_t between the MSRE (*Hha*I) and the mock digest; the black columns reflect the average change in C_t between the MDRE (McrBC) and the mock digest; and the gray columns reflect the average analytical window (e.g., the change in C_t between the double-digest and the mock digest). (B) A pie chart representation of the differently methylated populations in each sample. The ΔC_t values in panel A were converted back to copies and are expressed as a fractional proportion of the total number of copies present (see Materials and Methods). The green wedge reflects the %sparsely methylated, yellow reflects the %intermediately methylated, and red reflects the %densely methylated.

The data were analyzed using Orion's MethylMapper technique (19).

RESULTS

Principles of the MethylScreen Technology

The MethylScreen protocol is pictorially represented in Figure 1. Briefly, methylation-sensitive and methylation-dependent restriction enzyme treatments selectively digest the unmethylated or the methylated DNA, respectively, and a treatment with both enzymes serves as a control for how much DNA is participating in the assay. DNA remaining after each treatment is quantified by real-time PCR using primers that flank the region of interest, and the amount of DNA remaining is compared with pre-treatment DNA concentrations.

The procedure involves only two main steps. First, the DNA is separated into four equal aliquots. The first aliquot is treated with a methylation-sensitive restriction enzyme (MSRE) that cuts within the region of interest. For analysis of the *GSTP1* promoter sequence, we chose to use *Hha*I. However, we have also employed either *Aci*I or *Hpy*CH4IV with similar results (data not shown). The second aliquot is treated with a methylation-dependent restriction enzyme (MDRE).

While the procedure could work for quantification of adenine methylation (6mA), most utility will likely come from analysis of cytosine methylation. When quantifying cytosine methylation, McrBC is employed; McrBC is an MDRE that recognizes a pair of methylated cytosine residues in the context 5′-Pu^mC ($N_{40-2000}$) Pu^mC-3′ and cleaves within approximately 30 bp from one of the methylated residues (20). The third DNA aliquot is mock-treated, wherein digestion buffer cocktail is added but no enzymes are present. The mock treatment determines the total amount of DNA put into the assay. The fourth aliquot is treated with both the

MSRE and the MDRE, resulting in a double-digest. The double-digest is used to assess the amount of DNA available to participate in both single treatments. Careful selection of enzymatic activities should even allow for the analysis of hemi-methylated DNA through the use of additional single- and double-digestion treatments (Reference 21 and rebase.neb.com).

In the second step of MethylScreen, DNA from all four aliquots is used as template in quantitative PCRs using primers that amplify the region of interest. We have successfully employed probe-based (i.e., TaqMan, or molecular beacons) technologies, intercalating dyes (i.e., SYBR Green), and fluorogenic self-quenching primers (i.e., LUX primers) for amplification detection (data not shown).

GSTP1 Assay

Representative MethylScreen assay results demonstrating *GSTP1* methylation detection are depicted in Figure 2. Panel A depicts a standard template dilution and the product's signature melting curve. Assay performance can be judged by correct discrimination of three differentially methylated control templates (Figure 2B). For all three of the samples presented, the red profile represents the mock digest, the blue represents the McrBC, the green the *Hha*I, and the yellow is the double-digest. The prostate cancer cell line LNCaP has been previously reported to contain only heavily methylated and silent *GSTP1* copies. The DU145 prostate tumor cell line also contains methylated *GSTP1* copies, but the level of methylation is lower per copy and the cell line expresses *GSTP1* (22). Finally, human placenta genomic DNA was the source of unmethylated *GSTP1* copies. As expected, the McrBC predominates relative to the mock digests in the LNCaP sample is intermediate in DU145 and has little activity in the placental genome (blue versus red in each), while the *Hha*I exhibited the reciprocal activity (green versus red in each). The double-digests did not cross the threshold (yellow versus red). Variance was assessed by change in cycle threshold (C_t) as a result of the treatments (Figure 3). Panel A in Figure 3 depicts the average change in C_t (±SEM) for templates treated with

the sensitive enzyme (white boxes), the dependent (black boxes), or the double-digest (gray boxes) relative to the mock-treated portion of each genomic DNA. The number of copies in each population was calculated, and the size of each population was expressed as a percentage of the total (Figure 3B). In these DNA samples (placental, DU145, and LNCaP), nearly all of the DNA was amenable to analysis since the ΔC_t between the mock and the double-digest was >10 cycles (Figure 3A). As predicted, the LNCaP genome consisted of nearly all densely methylated *GSTP1* copies. There appeared to be a small amount of molecular hetero-geneity in LNCaP *GSTP1* promoter methylation since the C_t conditioned by McrBC alone was less than the C_t attained by the double-digest (i.e., some *Hha*I sites were unoccupied on a small fraction of molecules), indicating a small intermediately methylated population exists. In contrast with LNCaP and placental DNA, DU145 displayed a large population of intermediately methylated molecules. The placental genome contained very little 5 mC per *GSTP1* copy. These results were verified by bisulfite genomic sequencing by our group (data not shown) and have been previously published by other groups (22).

McrBC and Density

McrBC behaves differently from many other restriction enzymes in that it has loose site specificity. Target cleavage does not occur directly at the Pu^mC site. Rather, current models posit that the enzyme binds at this site and then translocates along the DNA. Restriction occurs at random sites in the vicinity of the Pu^mC after collision of two separate McrBC complexes (23). It therefore follows that cleavage will occur more frequently in regions where there are many Pu^mC sites present. These results suggest that McrBC may be capable of responding not only to the presence of Pu^mC sites, which are available in methylated templates, but also to the density of Pu^mC sites per DNA fragment, when the ratio of enzyme to target is held constant. One could expect that using McrBC in the four condition MethylScreen assay may lead to distinctly different amplification
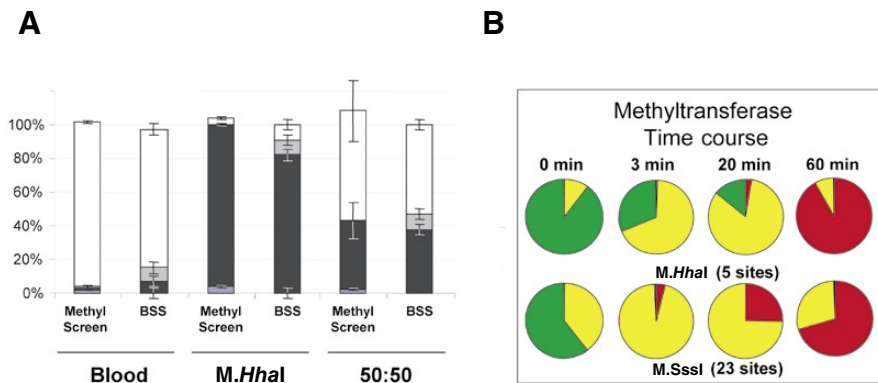
Figure 4. MethylScreen technology resolves complex mixtures of heterogeneously methylated molecules. (A) The MethylScreen *GSTP1* results obtained from unmethylated (blood), M.*Hha*I-methylated blood and 50:50 mixtures of M.*Hha*I methylated:unmethylated DNA, along with the results obtained from clone-based bisulfite-based genomic sequencing on the same molecular populations (BSS). White reflects the unmethylated molecular population for both technologies, while black represents the proportion uniformly methylated by M.*Hha*I. The gray boxes reflect the portion of molecules with some but not total M.*Hha*I methylation. The blue-gray boxes reflect the fraction of the population refractory to MethylScreen analysis. The error bars reflect the SEM for each population. Panel B depicts the interpreted molecular populations following MethylScreen *GSTP1* analysis of a time course panel of blood genomic DNA methylated with either M.*Hha*I or M.SssI. The M.SssI time course was carried out under conditions that favor non-processive methylation. The green wedge reflects the %sparsely methylated, yellow reflects the %intermediately methylated, and red reflects the %densely methylated.

profiles, based upon whether the input DNA is sparsely, intermediately, or densely methylated, and based upon the load of these methylated molecules in the population.

Indirect confirmation of this hypothesis came through analysis of the MethylScreen results obtained from DU145 and LNCaP in comparison to human placental DNA. MethylScreen results in Figure 2 show significant differences between the three genomic DNA samples. DU145's ΔC_t values, in contrast, for McrBC-Mock and *Hha*I-Mock were intermediate to those obtained from LNCaP and placenta (Figure 2 and Figure 3). The simplest explanation for this observation suggested that the simultaneous monitoring of multiple MSRE and MDRE restriction sites within a target provided the apparent density monitoring capability, and thus provided additional detail as to a sample population's true methylation state.

Direct confirmation of this hypothesis was achieved through in vitro manipulation of genomic methylation levels through time course analysis upon human blood genomic DNA using either M.*Hha*I, or M.SssI (Figure 4). First, homogenously methylated control populations were generated using M.*Hha*I and human placental

DNA as the template, along with a mass equivalent mixture. Each of these templates was then interrogated with MethylScreen and subjected to clone-based bisulfite sequencing for confirmation. We obtained double-stranded sequence from more than 30 clones per library using a four-primer strategy on the approximate 900-bp inserts (see Materials and Methods section). The results are depicted in Figure 4A. The MethylScreen results closely matched the bisulfite-sequencing results and were therefore considered validated.

Subsequently, the *GSTP1* MethylScreen assay was then performed upon the time course from each partially methylated template using *Hha*I. These three treatments generated partial methylation reactions with up to 5 or 23 monitorable McrBC half-sites per target *GSTP1* promoter region product, respectively. The proportion of intermediately and densely methylated templates increased across each time point in the methylase reaction (proportion of yellow and red, respectively, in Figure 4B). Of particular interest was the observation that the reaction conditions employed were able to distinguish the M.*Hha*I, from the M.SssI reaction at the 20 min time points, in a manner that correlated with the number of available half-sites (Figure 4B).
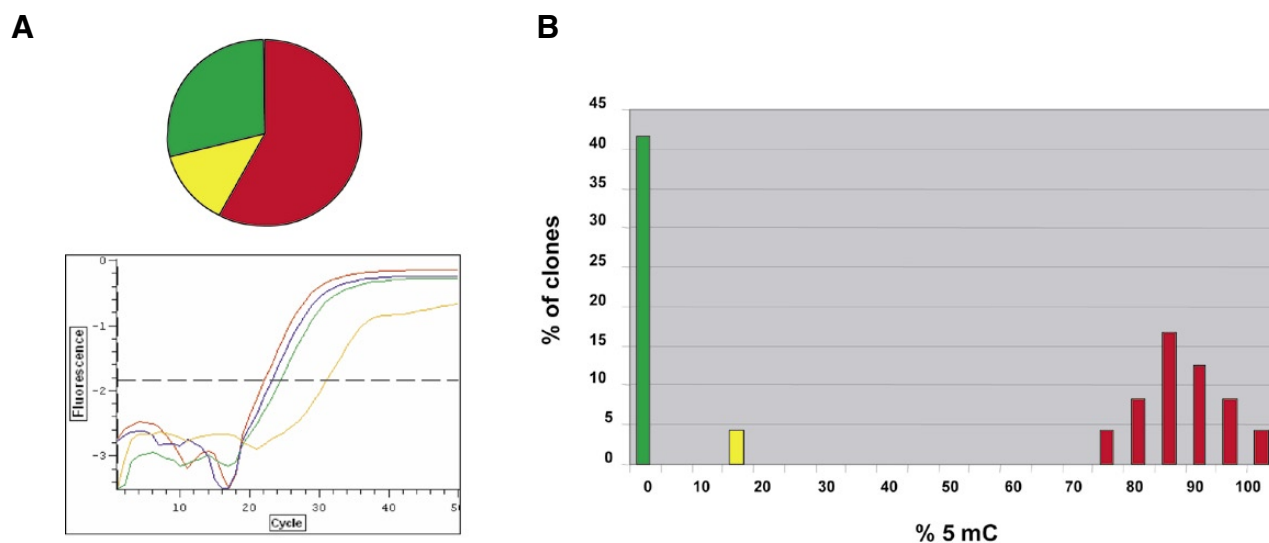
Figure 5. Bisulfite genomic sequencing results from a prostate tumor sample closely match the MethylScreen molecular population predictions. (A) The kinetic reaction profiles obtained from the four treatments in a MethylScreen *GSTP1* analysis of a flash-frozen prostate cancer sample along with the pie chart depiction of the interpreted molecular populations. The kinetic profile is presented as follows: red represents the mock-treated reaction, quantifying the total molecular population under study; blue reflects the methylation-dependent restriction enzyme (MDRE) reactions, quantifying the unmethylated molecular population; green represents the methylation-sensitive restriction enzyme (MSRE)-treated portion and allows quantification of the densely methylated fraction. The fraction of the population not amenable to analysis results from using the double-digest as template (gold). For the pie chart, green wedge reflects the %sparsely methylated, yellow reflects the %intermediately methylated, and red reflects the %densely methylated. The tumor sample had a neoplastic cellularity of >80% and was sum Gleason score >7. The results from clone-based bisulfite genomic sequencing of the *GSTP1* locus are depicted as a bar chart. The y-axis reflects the fraction of the 25 clones in the population as a percent of total clones. The x-axis expresses the average 5 mC per molecule considering the 53 CG sites in the region sequenced as a percent occupancy. The bars on the chart are colored to correlate with the MethylScreen pie chart based upon whether the methylation detected blocked the five analytical *Hha*I sites.

Unfortunately, we were not able to generate a homogeneously methylated template with M.SssI. Repeated attempts to gain a completely M.SssI-methylated template were unsuccessful when employing either genomic DNA or a BAC spanning the region. A further complication was the finding that the amount of topoisomerase activity with the M.SssI methylase in reactions longer than 45 min substantially reduced the effective concentration of DNA in that sample. However, we feel that the LNCaP genome behaved substantially similar to what a nearly homogeneously methylated population would and should have resembled. Comparison of the time course at the 60 min time with LNCaP substituted for the M.SssI suggests that the assay's output is responsive to the number of target half-sites occupied per priming region (compare Figure 4B with Figure 2B).

Density Monitoring Ability Affords Molecular Resolution Among Different Types of Populations

A population of DNA that is 50% methylated could exist in a number of different ways. One-half of the DNA population could be 100% methylated, with the other half being almost entirely unmethylated, as in the case of imprinted loci (i.e., hemizygous methylation). Alternatively, the entire population of DNA could be 50% methylated. Serendipitously, we tested the assay's ability to discriminate between these two alternatives directly (Figure 4).

The 50:50 hemizygous mixture gave very different results from any of the time points in the partial methylation reactions. This finding confirmed the ability to resolve a mixed fully methylated and unmethylated population, as well as appeared to indicate the assay had the capability to resolve highly heterogeneously methylated populations. This capacity has been observed before (16). Because early (24) and subsequent published reports (18) have suggested that tumor samples contain highly complex mixtures of molecules with different methylation patterns in different abundances, we reasoned that direct confirmation of the density monitoring hypothesis could be achieved through the analysis of DNA methylation patterns from tumor samples. A final direct test of the assay was chosen to be a population of molecules biologically produced by a tumor followed with bisulfite-sequencing validation of the result.

Figure 5 depicts representative results obtained from the MethylScreen analysis of *GSTP1* promoter methylation present within a commercially obtained prostate tumor sample along with a pie chart representing the interpreted result (panel A). The color of the pie chart is meant to correspond to the dense (red), intermediate (yellow), and sparsely methylated populations identified by bisulfite genomic sequencing (panel B). The tumor specimen displayed >80% neoplastic cellularity (Asterand, plc,

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Detroit, MI, USA). The MethylScreen results appeared to suggest that the sample was a mixture of nearly equal portions of unmethylated gene copies and nearly fully methylated gene copies (i.e., the methylated promoter elements) had most of the monitorable CpGs methylated. That is, the ΔC_t of green-*Hha*I and blue-McrBC was each about one cycle relative to the red-mock digest. In order to verify that an approximate 50:50 mixture of heavily methylated and unmethylated DNA was present in the tumor, bisulfite sequence analysis of the DNA was carried out on 20 clones. The sequence data confirmed that there were two distinct molecular populations present, representing both heavily methylated and mostly unmethylated DNA (Figure 5B). We obtained a similar MethylScreen result from a pool of 10 prostate tumor samples (see next section and Table 1B).

MethylScreen Assay Reproducibility

One criticism of restriction enzyme mediated cytosine methylation detection is that the enzyme performance is variable and not reproducible, leading to false-methylated sequence detection (see Reference 25 for a review). We have not observed this. Table 1A summarizes the data obtained from 20 independent assays performed on DU145 genomic DNA on each of three different days (3 × 20 = 60 assays). DU145 was selected as a source for the assay since both the MSRE and MDRE's activity could be assessed. Table 1B summarizes the results obtained from analysis of 10 independent assays of flash-frozen tumor DNA. Since we did not possess enough tumor DNA from one sample to support 10 independent assays, and our purpose was to assess uniformity of the results, we used pooled DNA from 10 prostate tumors. Each member sample in the pool contained a neoplastic cellularity >60% with a sum Gleason score of 7. There were 59 successful assays in the DU145 analysis; one experimental replicate was excluded due to PCR failure. The standard deviations (SD) and coefficients of variation (CV) calculated suggest that the variance of quantitative PCR is much larger than the variance of the restriction enzyme digestions, since the mock-treated portion behaved the same as each single treatment (calcula-

tions in Table 1). In the primary tumor pool, the refractory population (%Ref) was very small; on average only 0.7% of the population did not participate in the restriction analysis (Table 1B and Materials and Methods section). As expected with relatively inefficient primers (Figure 2A), variation observed in the analytical-window calculation [ΔC_t DD-Mock (where DD is the double-digest; see Materials and Methods section)] were consistent with a Monte-Carlo effect being observed at the later cycles (26). There was no difference in performance across the 3 days (data not shown). A cutoff for phenotypically classifying DU145 was set by considering the %dense calculation and its SD (5.6%). DU145 was consistently classified to be substantially methylated (Met = 11 of 59, where %dense > 5.6%) or intermediately methylated (Int = 48 of 59; %dense < 5.6% but %intermediate > 5.6%); it was never classified as unmethylated (%dense + %intermediate < 5.6%). The pooled DNA from the primary tumors behaved even more uniformly than the cell line DNA (Table 1B). All 10 of the assays categorized the sample as substantially methylated (Met in Table 1B).

MethylScreen Assay Sensitivity

The sensitivity of the MethylScreen assay is of course established by the restrictability of the sample. The size of the restriction-refractory population establishes the number of cycles in the analytical window (i.e., ΔC_t between the DD and the mock). The analytical windows obtained from the cell line assays, including the sensitivity study, were larger than those obtained from the primary tumors (%Ref, Table 1A versus Table 1B). The commercially obtained placental genomic DNA yielded the largest analytical window achieved to date (>12 cycles; Figure 2B and 3A). Tumor samples routinely yield smaller windows (approximately six to seven cycles; Figure 5, Table 1B, and data not shown). With windows >10 cycles, the assay is tremendously sensitive; we have repeatedly detected methylated molecules at a dilution factor of 4000 (or 0.025%) using just 40 ng DNA/PCR (data not shown). However, given that most tumor samples examined have exhibited windows between six and

seven cycles, a more routine sensitivity may be expected to be in the range of one methylated molecule in the presence of 64 to 128 unmethylated (or 1.5%–0.7%) (Table 1B and Figure 5).

DISCUSSION

Principles of the MethylScreen Technology

In the MethylScreen protocol, methylation-sensitive and methylation-dependent restriction enzyme treatments selectively digest the unmethylated or the methylated DNA, respectively, and a treatment with both enzymes serves as a control for how much DNA is participating in the assay. DNA remaining after each treatment is quantified by real-time PCR using primers that flank the region of interest, and the amount of DNA remaining is compared with pretreatment DNA concentrations.

The procedure represents an improvement over approaches first described by Raleigh and colleagues (27) and refinements over the method published by Yamada et al. (8). Largely this is because of the analytical and calculation control provided by the double-digest, the utilization of quantitative PCR, and the use of the notion that intermediately methylated molecules may be subject to the action of both enzymes such that accounting for them balances the equation $I =$ Total-Sparse-Dense-Refractory from a molecular perspective. This idea is the key to resolution of the constituents of each molecular population in a way that matches the results obtained by bisulfite genomic sequencing.

The inclusion of both a methylation-sensitive restriction enzyme (MSRE) and a methylation-dependent restriction enzyme (MDRE) yields additional information and protects against false-positives stemming from incomplete digestion, since restriction failure by both activities yields a very different measurement than a methylated target call. Failure of restriction by both of the enzymatic activities collapses the assay's analytic window, which is the number of cycles between the mock digested sample and the double-digest (i.e., a small ΔC_t value indicates a large refractory population). By its very

construction, MethylScreen should demonstrate a low false-positive rate. The false-negative rate will be governed by the sensitivity of the assay to detect alterations of the normal background methylation pattern in the sample of interest. Establishing a meaningful background methylation level will likely be very important and can only result from further study. Clearly, optimizing sample restrictability to be more like the placental sample here would be a key first step to establishing a low background noise for any assay.

A criticism of MSRE-based methylation detection is that not enough of the genome can be covered due to the site specificity requirements of the enzymes. No currently known enzyme is capable of detecting cytosine methylation better than McrBC. This is because McrBC effectively has a 3-bp recognition site (R^{m}CG). Our computational assessment of >24,000 transcriptional start-site flanking regions suggests that a six MSRE collection would be necessary so that a >97% chance exists that a test can be built for any locus (R.W.C., J.A.B., and J.A.J., unpublished observation). The methylation-sensitive enzymes *Hha*I, *Hpa*II, *Aci*I, and *Hpy*CH4IV provide the best genomic coverage in the human genome, and all are compatible with McrBC digestion conditions (H.H. and J.A.J., unpublished observation). For most regions of interest (i.e., CpG islands) there are multiple *Aci*I or *Hha*I sites in the majority 300–400-bp intervals. Such site density (2–5 sites per PCR product) makes the methylation density monitoring capability of MethylScreen possible.

Assay Sensitivity

Undoubtedly assay sensitivity is a direct consequence of the unmethylated genomic background's overall restrictability. The primary tissue samples exhibited smaller analytical windows than the placental DNA suggesting this test example may represent an idealized situation. That being said, the routinely achieved window of six to seven cycles observed with human tumor samples makes the assay as sensitive as qMSP, and other bisulfite-requiring PCR-based assays (28).

The amount of sample restricted does not impact assay sensitivity. Formally, a large amount of sample is necessary to achieve sensitivities of detection >1 in 1000 templates, this is a consequence of PCR and not restriction (data not shown). We have repeatedly observed using this *GSTP1* assay that as little as 8 ng genomic DNA (e.g., 2 ng per restriction and all into PCR) can yield a result indistinguishable from that obtained with 1 µg restriction reactions and 20 ng PCRs (H.H., M.S., and J.A.J., unpublished observation). Assays employing <1 ng genomic DNA as template in the PCR did not have this capability (data not shown). Because the assay effectiveness dropped in dilution along with the efficiency of the quantitative PCR primers, its likely assay performance is impacted by primer efficiency most at low PCR input rather than restriction enzyme limitation.

The MethylScreen approach offers distinct advantages over existing methodologies in that the assay requires only a small amount of input DNA and the sample DNA does not have to go through bisulfite mutagenesis. The assay is adaptable to very simple automation. Finally, the inclusion of the MDRE alone or in combination with a double-digest offers additional valuable information on intramolecular heterogeneity of the cytosine methylation present within the population of molecules studied (i.e., methylation density). It should be noted, however, that because this detection capacity relies upon a change in C_t between the double-digest and either the MSRE or MDRE for detection, the ability to resolve intermediately methylated molecular populations will be sensitive to the amount of unmethylated DNA present. That is, the density monitoring capacity should decrease in dilution. We have confirmed this hypothesis using dilutions of DU145 into placental DNA (data not shown).

Cytosine Methylation Density Monitoring Using Quantitative PCR

Unlike MSP, MethylScreen allows for the detection of nonhomogenously methylated as well as partially methylated states (i.e., intermediate methylation states) within complex populations of molecules. An adaptation of qMSP (called QAMA), using probes specific to different states, has been developed and successfully employed

(29). However, even that assay can survey only a few different states. A locus consisting of 24 CpG sites contains 16,777,216 (2^{24}) possible different methylation configurations. Making primer and probe sets capable of monitoring all of them would seem to be impossible due to interference between the probes and the constraints governing the ability to perform mismatch detection with highly similar probes. Having the capability to monitor subtle differences in methylation might be very relevant. Subtle differences between methylation states are perhaps indicative of disease progression, as DU145 seemed to possess a methylation state closer to that obtained from the tumor samples than that obtained from LNCaP (Figure 3B, Table 1B, and Figure 5A). Because recent investigations have demonstrated that adjacent nonmalignant clinical tissues exhibit cytosine methylation abnormalities at *GSTP1* (30), it remains tantalizing to speculate that the ability of MethylScreen to monitor heterogeneously methylated populations may offer a detection advantage. However, only further study and analysis will be able to formally establish such a relationship.

MethylScreen as a Clinical DNA Methylation Monitoring Platform

Gene-silencing occurs through the construction of localized heterochromatin (see Reference 31 for a review). ^{m}CG sites are maintained as methylated at a frequency lower than the fidelity of DNA replication (32,33). A single histone-octomer containing the modified H3 connoting the silent state (e.g., mK9) placed anywhere in the genome encompasses more than 150 bp DNA. Therefore, the biological unit of CpG methylation is rarely a single CpG, rather the density of the methylation per region is what has been most often associated with gene silencing (24). Resolving the molecular differences within complex populations should allow binary data like methylation presence or absence per site to be translated into continuous data for a region.

In conclusion, we have developed a highly sensitive, quantitative methylation detection assay. By combining treatments of both methylation-sensitive and methylation-dependent enzymes,

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this assay not only has built-in controls for enzyme performance but it also yields additional important information about methylation density. This test can readily be incorporated into a clinical laboratory setting as it relies on reagents and instrumentation already in use and can be adapted to a single pipeting event format. Such studies are currently under way.

COMPETING INTERESTS STATEMENT

Orion Genomics, LLC markets MethylScreen technology. All authors of this work recognize a competing interest in the work as employees and shareholders of Orion Genomics, LLC.

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