Scientific article

EpiTect Methyl II PCR Array System: A simple tool for screening regional DNA methylation of a large number of genes or samples without bisulfite conversion

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Abstract: The EpiTect® Methyl II PCR Array System is a novel technology enabling simple, fast, and reliable screening of CpG island DNA methylation for a large number of genes or samples simultaneously, using MethylScreen™ technology. The system uses selective digestion of sample DNA with methylation-sensitive and methylation-dependent restriction enzymes, followed by quantification of the remaining DNA by real-time PCR. The relative amounts of differentially methylated DNA species from the targeted regions are determined with a comparative ΔC_T calculation. As little as 2 µg genomic DNA can be used to profile the methylation status of up to 94 gene targets, and methylated DNA can be reliably detected in heterogeneous samples in amounts as low as 6% of the total sample. This technology yields data comparable to bisulfite Sanger sequencing and Illumina® Infinium® BeadChip® assays, without bisulfite conversion. Here we present the basic principles of the EpiTect Methyl II PCR System, along with performance, verification, and application data to demonstrate its robust potential for methylation profiling in various biological systems and for screening of DNA methylation biomarkers.

Introduction

Epigenetic mechanisms, including DNA methylation and histone modifications, can modify gene expression patterns without changing the DNA sequence. Gene silencing via methylation of gene promoters is a well-known occurrence in neoplastic cells and also plays important roles in normal cell differentiation and development (1). This process occurs almost exclusively in the context of CpG dinucleotides and involves the covalent attachment of a methyl group to the cytosine residue. Approximately 60–70% of all human gene promoters overlap with CpG islands, which are regions of DNA with an elevated GC content and a high frequency of CpG sites (2). Most highly expressed genes seem to correlate with unmethylated promoter regions, despite the high percentage of GC content in their promoter regions.

Several technologies have been developed for the quantitative and qualitative analysis of DNA methylation status. The most commonly used methods involve pretreating DNA with sodium bisulfite, a very powerful and versatile technique in which

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unmethylated cytosines are converted to uracils, while methylated cytosines remain unchanged. Subsequent analysis of the bisulfite converted DNA can easily provide extremely high-resolution information about the methylation status of a DNA segment. Such techniques include methylation-specific PCR (3), high resolution melting (HRM) analysis, bisulfite sequencing (4), Pyrosequencing® (5), next-generation sequencing (6), combined bisulfite restriction analysis (COBRA) (7), fluorescence-based real-time MethyLight PCR (8), matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (9), and hybridization-based promoter and CpG island microarrays. Although these technologies can provide very high-resolution methylation data, they are not all well-suited for higher throughput applications.

The EpiTect Methyl II PCR Array System takes methylation analysis to a biology-focused level that is also affordable. This innovative technology enables fast and reliable DNA methylation profiling of target regions from disease- or pathway-focused gene panels, as well as individual genes, without the need for bisulfite modification or specialized equipment. This technology is based on a simple restriction enzyme digest followed by quantitative SYBR® Green real-time PCR (10). As a result, the EpiTect Methyl II PCR system is a highly suitable screening tool for DNA methylation profiling and potential biomarker discovery in a range of scientific fields, including transcription regulation, stem cell research, cell differentiation and development, drug discovery, angiogenesis, cancer, and other human diseases.

The EpiTect Methyl II PCR System allows for simultaneous DNA methylation profiling of multiple genes in convenient 96-well or 384-well formats. Profiling biological DNA samples with these arrays allows the correlation of CpG island methylation levels with biological phenotypes, and the results can provide insight into the molecular mechanisms and biological pathways critical for disease development. Each individual PCR assay on a PCR array is uniquely designed for use in SYBR Green real-time PCR analysis, and its specificity is guaranteed when used with RT^2 SYBR Green qPCR Master Mixes. Furthermore, QIAGEN® offers these predesigned assays for various mammalian genomes, including human, mouse, and rat. The simplicity of the procedure makes it possible to screen the methylation status of many individual genes within a biological pathway in a large number of samples in just a single day.

Principle

The EpiTect Methyl II PCR procedure is based on the quantitative detection of remaining input DNA within a sample population after treatment with a methylation-sensitive (MSRE) and a methylation-dependent (MDRE) restriction enzyme (11, 12). Each DNA sample is divided into four fractions for four different restriction enzyme digestions: no-enzyme mock (Mo), methylation-sensitive (Ms), methylation-dependent (Md), and methylation-sensitive plus methylation-dependent double (Msd). In principle, the MSRE selectively digests unmethylated and partially methylated DNA copies, since cleavage is blocked when the cytosine residue in CpG dinucleotides within its recognition site is methylated. Consequently, the amount of DNA remaining in this reaction represents the fraction of fully methylated DNA within the sample population. In contrast, the fraction of unmethylated DNA is determined by the MDRE digest. This methylation-dependent enzyme requires two methylated half-sites, which are usually separated by 40 to 3000 base pairs, for cleavage. Therefore, the fraction of input DNA remaining after MDRE treatment indicates the amount of unmethylated DNA. The amount of remaining input DNA in each digest is then normalized to the total amount of input DNA, which can be easily assessed by the mock-treated DNA fraction. The relative amounts of methylated and unmethylated DNA within a heterogeneous sample can be determined using a comparative threshold (ΔC_T) method (10). Using the same target region primer set in each of the four enzyme reactions minimizes any calculation bias that could arise from variations in PCR efficiencies. Additionally, the relative amount of partially methylated DNA can be derived from the assumption that all fractions will add up to 100% of the total input DNA. The double digest reaction determines the fraction of input DNA that is not susceptible to digestion by the restriction enzymes. The difference in C_T values between the double digest and the mock reactions represents the analytical window of the assay. On our free, downloadable Microsoft® Excel® based data analysis template, results are simply displayed as percentage of unmethylated fraction (UM) and methylated fraction (M), which includes both the fully methylated and partially methylated fractions.

A schematic view of the EpiTect Methyl II PCR Array procedure is depicted in Figure 1. After isolation, 1 µg of genomic DNA is used to set up the four independent restriction enzyme digests, which are incubated at 37°C for 6–16 hours. Immediately after incubation, the restriction enzymes are heat-inactivated at 65°C for 20 minutes, and the digests are mixed with QIAGEN's ready-

to-use RT² SYBR Green qPCR Master Mix and are dispensed into a PCR array plate containing pre-aliquoted gene-specific primers. After running the real-time PCR, C_T values for each reaction can be calculated. The real-time PCR quantifies the relative amount of DNA remaining after each enzyme digestion, so the methylation status of individual genes and the methylation profile across a gene panel can be reliably and easily calculated using the data analysis template.

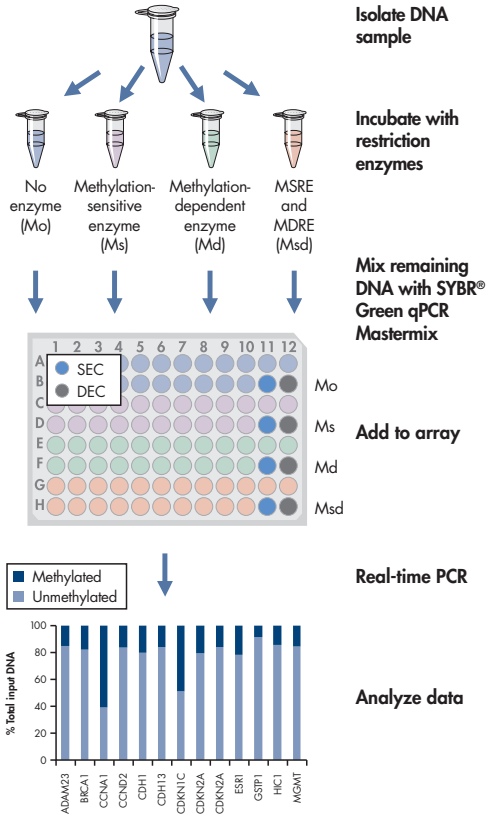


Figure 1. Flowchart of the EpiTect Methyl II PCR System procedure. The system relies on the differential cleavage of target sequences by 2 different restriction endonucleases. After various steps, the enzyme reactions are mixed with a qPCR master mix and are dispensed into a PCR plate containing pre-dispensed primer mixes. Real-time PCR allows the quantification of the relative amounts of remaining input DNA in each reaction.

EpiTect Methyl II PCR Assay and Array results express a target's methylation status as percentage unmethylated (UM) and percentage methylated (M) fraction of input DNA. A pictorial explanation of the results is depicted in Figure 2, in which "UM" represents the percentage of input genomic DNA containing no methylated CpG sites in the targeted region of a gene, and "M" represents the percentage of input genomic DNA containing two or more methylated CpG sites in the targeted region of a gene. The number of CpG sites methylated in a targeted region can vary within the fraction of methylated input DNA.

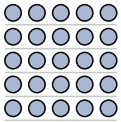
Example #	Input genomic DNA	Final results	
		Unmethylated (UM)	Methylated (M)
1		100%	0%
2		60%	40%
3		0%	100%
4		0%	100% (fully methylated)

Figure 2. Pictorial explanations of results. EpiTect Methyl II PCR Assays and Arrays provide gene methylation status as percentage unmethylated (UM) and percentage methylated (M) fraction of input DNA. Few representative examples are shown. Each horizontal bar represents the targeted region of a gene from one genome. Biological samples usually contain many genomes derived from many cell types. For simplicity, five such genomes are depicted. Light and dark circles represent unmethylated and methylated CpG sites, respectively. In example 2, the targeted region of a gene has two or more methylated CpG sites in two out of five genomes. Thus, the EpiTect Methyl II PCR Assay data reveal that this gene is 60% unmethylated and 40% methylated.

Target site selection and primer design

Most biologically relevant changes in DNA methylation are known to occur at multiple CpG sites simultaneously, rather than at single sites, suggesting that a regional analysis may better represent the methylation status of a CpG island within a region of interest (2, 13, 14) than an analysis of single sites. In addition, the average methylation level has been shown to be bimodally distributed, with an enrichment of highly methylated and unmethylated sequences (2, 13, 14). It has been shown that approximately 60% of the analyzed CpG islands were unmethylated, roughly 20% were fully methylated (2, 13, 14), and the remaining 20% were intermediately methylated. In designing the EpiTect Methyl II PCR Assays, we include CpG islands or CpG-dense areas associated with a gene's transcription start site (TSS), and the region from 5 kb upstream to 3 kb downstream of the TSS. CpG island predictions and TSS definitions are based on data available from the UCSC Genome Bioinformatics Site (15). According to the CpG islands predicted by UCSC's human genome assembly version hg19, which is

comparable to the NCBI 37 genome build, 70% of CpG islands sit on TSSs and can be referred to as promoter CpG islands. Currently QIAGEN offers EpiTect Methyl II PCR Assays for most promoters associated with CpG islands in the human, mouse, and rat genomes. Each target region is selected within one CpG island or CpG-dense area predicted from both the UCSC database and published data with functional annotation. In order to use a universal PCR condition for amplifying up to 94 different targets simultaneously, we developed a unique algorithm for primer design. Our design algorithm also ensures that every amplicon contains sufficient cutting sites for both the methylation-sensitive and methylation-dependent restriction enzymes. As a result, the length of most target amplicons ranges from 150–440 base pairs, with an average amplicon size of 240 base pairs.

Performance data

PCR amplification efficiency and specificity

PCR conditions for EpiTect Methyl II PCR Assays have been extensively optimized in combination with QIAGEN's RT² SYBR Green Real-Time qPCR Master Mixes to guarantee maximum performance. Our unique PCR conditions also ensure proper PCR amplification efficiency in the presence of highly methylated templates.

In Figure 3A, the PCR efficiency was plotted against the amplicon length in base pairs for 380 mouse and 380 human EpiTect Methyl II PCR Assays. This plot demonstrates that under our amplification conditions, PCR efficiency is not dependent on the length of the amplicon. The amplification efficiency was calculated using the amplification plots generated during real-time PCR (Figure 3B) and analyzing the change in fluorescence throughout the linear phase of the amplification according to the DART method (16). In general, only a small fraction of first round primer assays had amplification efficiencies of less than 80%. Since our quality control criteria require that all assays have amplification efficiency greater than 80%, any assay outside this cutoff is redesigned. We also carefully analyzed the dissociation curves of the same amplicons to ensure that only a single peak was produced per amplicon (Figure 3C). These results clearly demonstrate the high efficiency of PCR and the specificity of the primer assays.

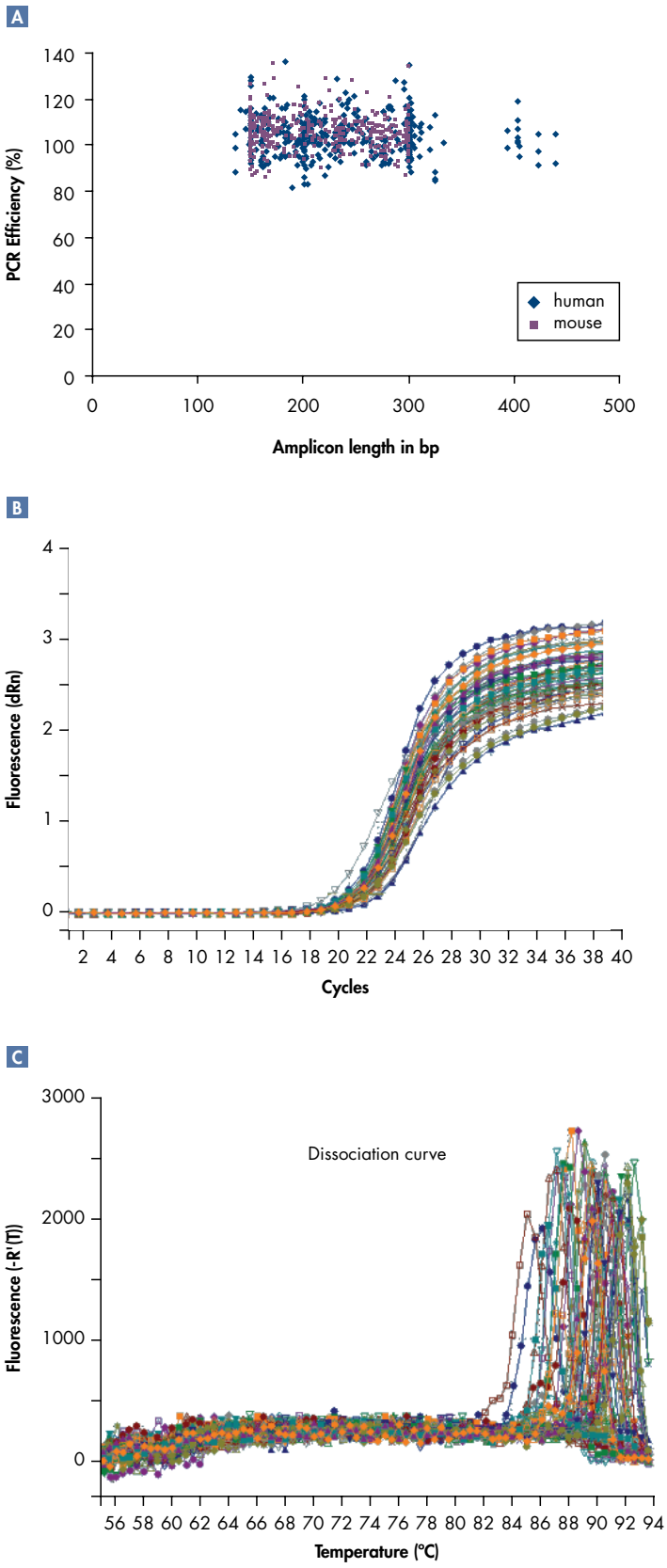


Figure 3. PCR amplification efficiency and specificity of the EpiTect Methyl II PCR System. A The amplification efficiency was plotted against amplicon length in base pairs for 380 mouse and 380 human EpiTect Methyl II PCR Assays. B Examples of amplification curves and C corresponding dissociation curves. The results clearly demonstrate high PCR efficiency and specificity of the primer assays.

Enzyme digestion efficiency

The success of the EpiTect Methyl II PCR System depends upon the cutting efficiencies of the MSRE and the MDRE. Specific controls — methylation-sensitive enzyme control (SEC) and methylation-dependent enzyme control (DEC) — are included in EpiTect Methyl II PCR Arrays to ensure high cutting efficiency of both enzymes. The 5X Restriction Digestion Buffer included in the EpiTect Methyl II DNA Restriction Kit (cat. no. 335452) is spiked with in-house produced and quality-controlled completely methylated plasmid DNA and completely unmethylated plasmid DNA. Each plasmid has specific enzyme-sensitive target regions flanked by unique primer regions. SEC- and DEC-specific primers are included in each PCR array and are also available as individual primer assays. To ensure high DNA digestion efficiency and to pass quality control, the difference in C_T values between the methylation-sensitive and mock digests should be equal to or greater than 4 (ΔC_T (Ms – Mo) ≥ 4) for the SEC. Likewise, the difference in C_T values between the methylation-dependent and mock digests should be equal or greater than 4 (ΔC_T (Md – Mo) ≥ 4) for the DEC. To "pass" quality control means more than 93.6% of control plasmid DNA in the 5X Restriction Digestion Buffer was digested, confirming that the restriction enzyme is active and can digest DNA efficiently. Once the DNA is digested and the PCR is complete, the resulting C_T values are pasted into the data analysis spreadsheet to obtain "pass" or "fail" results for SEC and DEC.

Sensitivity

The analytical sensitivity of the EpiTect Methyl II PCR Assay was empirically determined by spiking genomic DNA from the breast cancer cell line SKBR3 into normal genomic DNA from peripheral blood leukocytes (Figure 4). In this test, the methylation status of HIC-1 was determined using an EpiTect Methyl II PCR Assay, and the experiment was performed in triplicate. The HIC-1 gene has been previously described as a candidate tumor suppressor gene that is silenced by promoter methylation, and it is generally methylated in various human cancers, including breast cancer (17). As shown in Figure 4, the methylated HIC-1 is reliably detected in the DNA mixture, down to as low as 6% of total DNA. Similar results were obtained with 5 other genes. These results demonstrate the high sensitivity and specificity of the EpiTect Methyl II PCR Assay in a heterogeneous DNA population.

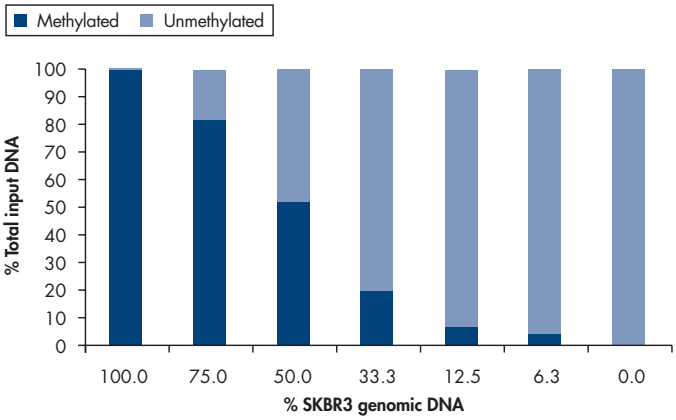
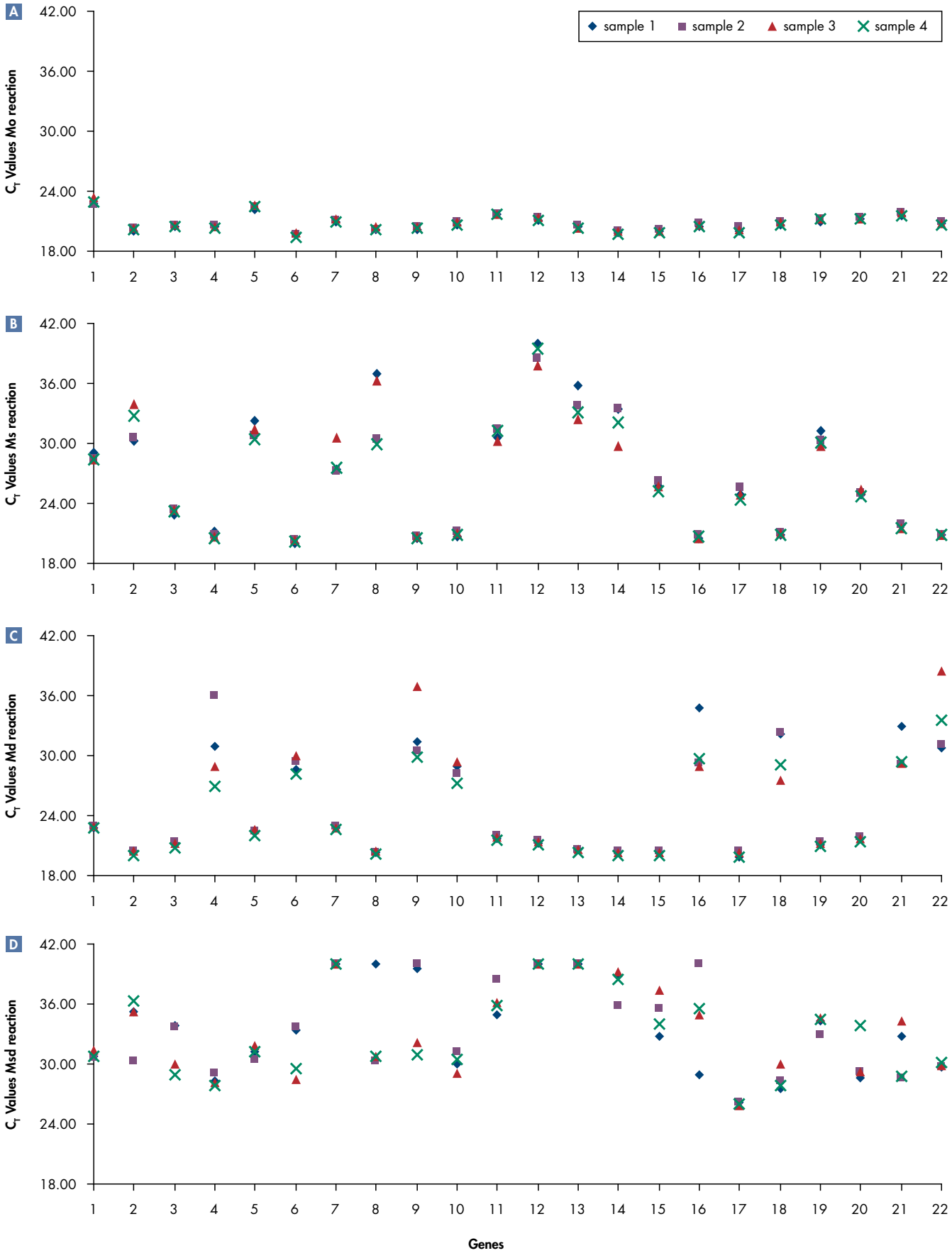


Figure 4. Methylation detection in heterogeneous samples. The analytical sensitivity of the EpiTect Methyl II PCR Assay was tested using a serial dilution of SKBR3 cell line genomic DNA and peripheral blood leukocyte genomic DNA. Using the EpiTect Methyl II PCR Assay for human HIC1, the percentage of methylated HIC1 relative to the total amount of input DNA was detectable in each mixture, even down to only 6% of the total DNA sample. The HIC1 gene promoter is methylated in cancer cells and unmethylated in normal cells.

Reproducibility

The reproducibility of EpiTect Methyl II PCR Assays was demonstrated by simultaneously measuring the methylation status of 22 human gene promoter regions in a custom array. The experiment was performed using the same batch of genomic DNA from breast cancer cell line MCF-7 and was repeated 4 times to generate technical replicates. Figure 5 (panels A through D) depicts the actual C_T values for the four restriction enzyme reactions (Mo, Ms, Md and Msd) and shows the high reproducibility between C_T values for the same gene across four independent experiments. When comparing the C_T values of the double digest, we observe a higher variation between replicates, which is not unexpected, since the remaining amount of template DNA in the double digest reaction is minimal, and results in an increase of the C_T values outside the linear range of a PCR.

In all four experiments, the calculated fraction of methylated and unmethylated DNA per gene is similar, as shown in Figure 5E. A repeated measures one-way ANOVA (analysis of variance) test showed that the means of the groups among 4 independent experiments are not significantly different, with a P-value greater than 0.05. Similar results were obtained for a second human catalog array (data not shown). More importantly, unmethylated and methylated genes are consistently detected by the EpiTect Methyl II PCR Array.


A
B
C
D
sample 1
sample 2
sample 3
sample 4
C_T Values Mo reaction
C_T Values Ms reaction
C_T Values Md reaction
C_T Values Msd reaction
42.00
36.00
30.00
24.00
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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22
Genes

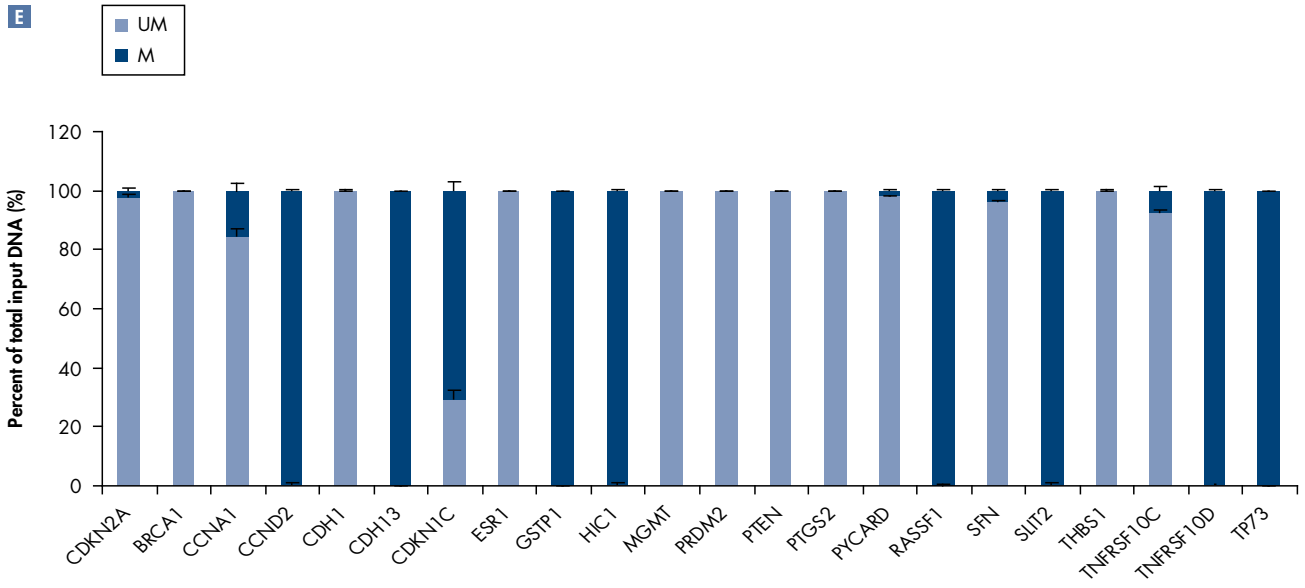
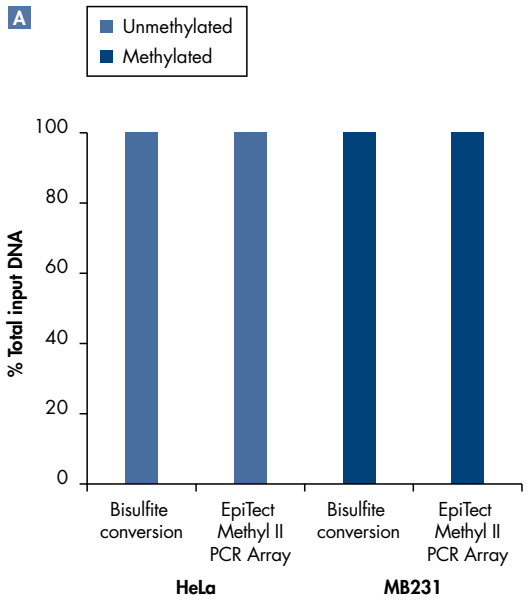


Figure 5. Reproducibility of EpiTect Methyl II PCR Assays. The reproducibility was tested with four technical replicates using a human custom array of 22 promoter region assays. Actual C_T values for the Mo A, Ms B, Md C, and Msd D reactions are represented. The difference in C_T values between the Mo reaction and the Msd reaction represents the analytical window of the assay. The percent methylated and unmethylated target DNA is calculated based on the C_T values as described in the *EpiTect Methyl II PCR Assay Handbook* and is represented as the mean plus or minus the standard error E. M: methylated; UM: unmethylated.

Reliability

To verify the reliability of the EpiTect Methyl II PCR System, we carried out parallel comparisons of this method with bisulfite Sanger sequencing and Infinium BeadChip experiments. It has been previously demonstrated that the promoter regions of the CDH13 gene are aberrantly methylated in breast cancer (18, 19). The methylation status of CDH13 in the breast cancer cell line MB231 and cervical cell line HeLa was measured using EpiTect Methyl II PCR Assays, according to the procedure in the handbook. The calculated fractions of methylated and unmethylated DNA relative to the total amount of input DNA are depicted in Figure 6A. In HeLa cells, our results show that CDH13 is completely unmethylated, whereas in the breast cancer cell line MB231, CDH13 is > 99% methylated.



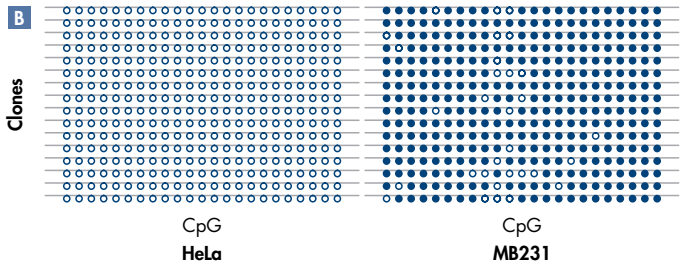


Figure 6. Comparison with bisulfite Sanger sequencing. The methylation levels in the promoter region of the CDH13 gene were analyzed using either EpiTect Methyl II PCR Assays or bisulfite Sanger sequencing. All steps for the EpiTect Methyl II PCR Assays were performed according to the protocol described in the *EpiTect Methyl II PCR Assay Handbook* A. For bisulfite sequencing, genomic DNA was bisulfite converted and amplified with gene-specific methylation-independent primers that included the amplicon region designed for the EpiTect Methyl II PCR Assay. PCR products were purified and sub-cloned, and 16 colonies for each gene in each cell line were sequenced B. M: methylated; UM: unmethylated. Open circles [o] indicate unmethylated CpG sites, while closed circles [●] indicate methylated CpG sites.

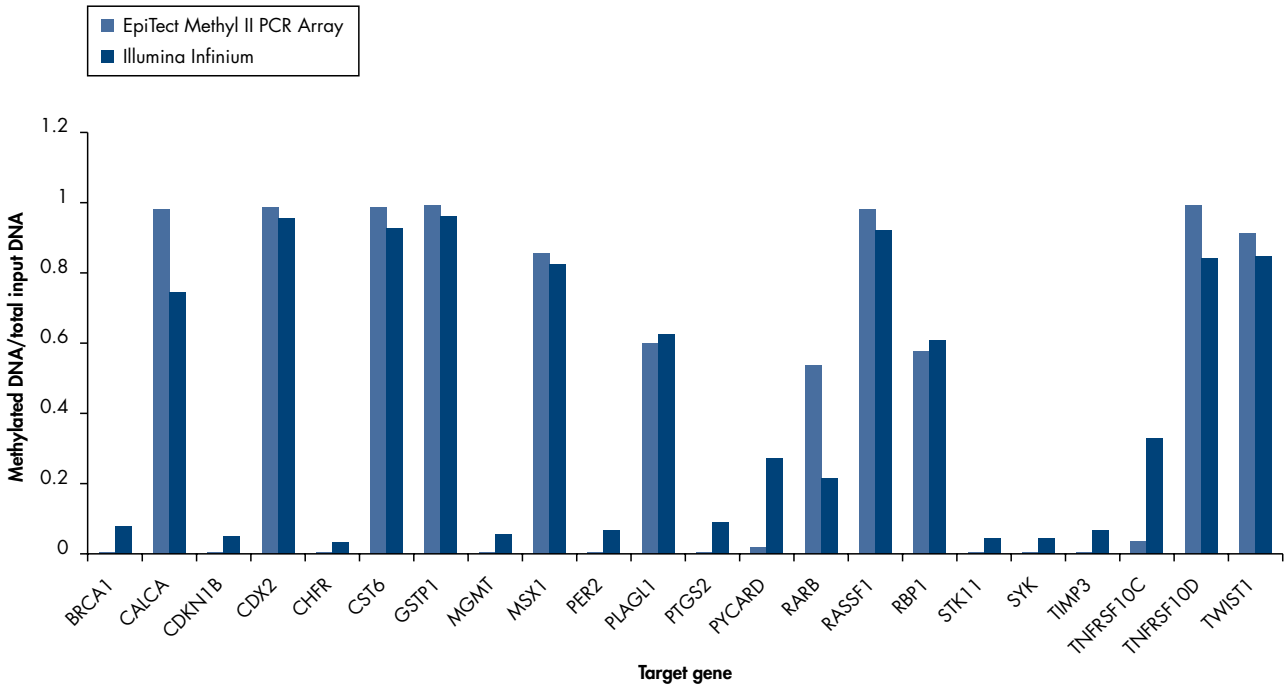


Figure 7. EpiTect Methyl II PCR Arrays generate data comparable to that from BeadChip platforms. EpiTect Methyl II PCR Array and Illumina Infinium Human Methylation 27 BeadChip assays were performed on MCF-7 cells. A representative analysis of 22 genes is shown. For better comparison, results from the EpiTect Methyl PCR Array were converted to averaged beta values, in which 0 means completely unmethylated and 1 means completely methylated.

These results were verified by performing bisulfite Sanger sequencing analysis of a region that includes the complete EpiTect Methyl II PCR target amplicon for CDH13. In this experiment, 1 µg of genomic DNA from each of the three cell lines was bisulfite converted and then amplified with methylation-independent primers specific for the CDH13 target regions. The PCR products were subsequently gel-purified and subcloned, and 16 colonies for each gene in each cell line were sequenced. The actual bisulfite sequencing data is outlined in Figure 6B. The target region analyzed included 23 CDH13-associated CpG sites. The methylation density was calculated by dividing the total amount of methylated CpG sites by the total amount of CpG sites. In order to compare them side by side, we defined the methylation status of any given DNA clone with more than 20% of the CpG dinucleotides modified by a methyl group as "methylated" and others as "unmethylated". As shown in Figure 6B, the unmethylated status of CDH13 in the HeLa cell line was rather clear, since none of the CpG dinucleotides are methylated. However, CDH13 was methylated in the MB231 cell line (Figure 6B). These data clearly indicate that EpiTect Methyl II PCR Assays can yield results highly consistent with those obtained from bisulfite sequencing.

For comparing our method with the Infinium BeadChip, we analyzed a panel of 94 breast cancer targets in MCF-7 cells with an EpiTect Methyl II PCR Array. In parallel, the MCF-7 cells were also analyzed with an Illumina Infinium Human Methylation27 BeadChip, with 27,578 distinct CpG dinucleotides associated with 14,000 genes, according to the manufacturer's protocol. The BeadChip methylation assays provide the methylation status as averaged beta values, so for a better comparison, we converted the data from the EpiTect Methyl II PCR Array to an averaged beta value, where 0 means completely unmethylated and 1 means completely methylated. A representative comparison of 22 genes is shown in Figure 7. The methylation pattern of the panel of genes observed with the EpiTect Methyl II PCR Array was consistent with that observed with the BeadChip assay. Only a small percentage of targets displayed some degree of difference between the two methods, as indicated by TNFRSF10C in Figure 7. This is not unexpected, considering that the principles for these two methods differ significantly. Only a single CpG dinucleotide is chosen to represent each target for most BeadChip assays, while EpiTect Methyl II PCR Arrays screen for multiple CpG sites simultaneously in a regional analysis.

Application data

In order to demonstrate that EpiTect Methyl II PCR Arrays can be used for verification of methylation biomarkers, we first scanned published results to design a custom PCR array representing a panel of the 94 most frequently methylated genes in human breast cancer (www.sabiosciences.com/dna_methylation_array.php). All PCR amplicons were designed based on reported promoter methylation target regions. We then analyzed the methylation profile of this gene panel in 10 different breast cancer cell lines, 1 prostate cancer cell line (VCaP), and normal adult breast tissue. For each sample, 2 µg genomic DNA was used for 4 digestion reactions (Mo, Ms, Md, and Msd). All further steps were performed according to the protocol described in the EpiTect Methyl II PCR Array Handbook.

The results shown in Figure 8 further strengthen the correlation of the selected 94-gene panel with breast cancer. The unsupervised hierarchical clustering clearly demonstrates that the differential methylation patterns of the candidate genes among the various cell lines might be useful for molecular classification or disease stratification for diagnosis and/or prognosis analysis when combined with clinical data. Furthermore the results revealed unmethylated genes like TGFβ1, ESR1, and RB1 across most of the tested cancer cell lines, as well as methylated genes like MUC2 (20), HIC1 (17), and MSX1 (21).

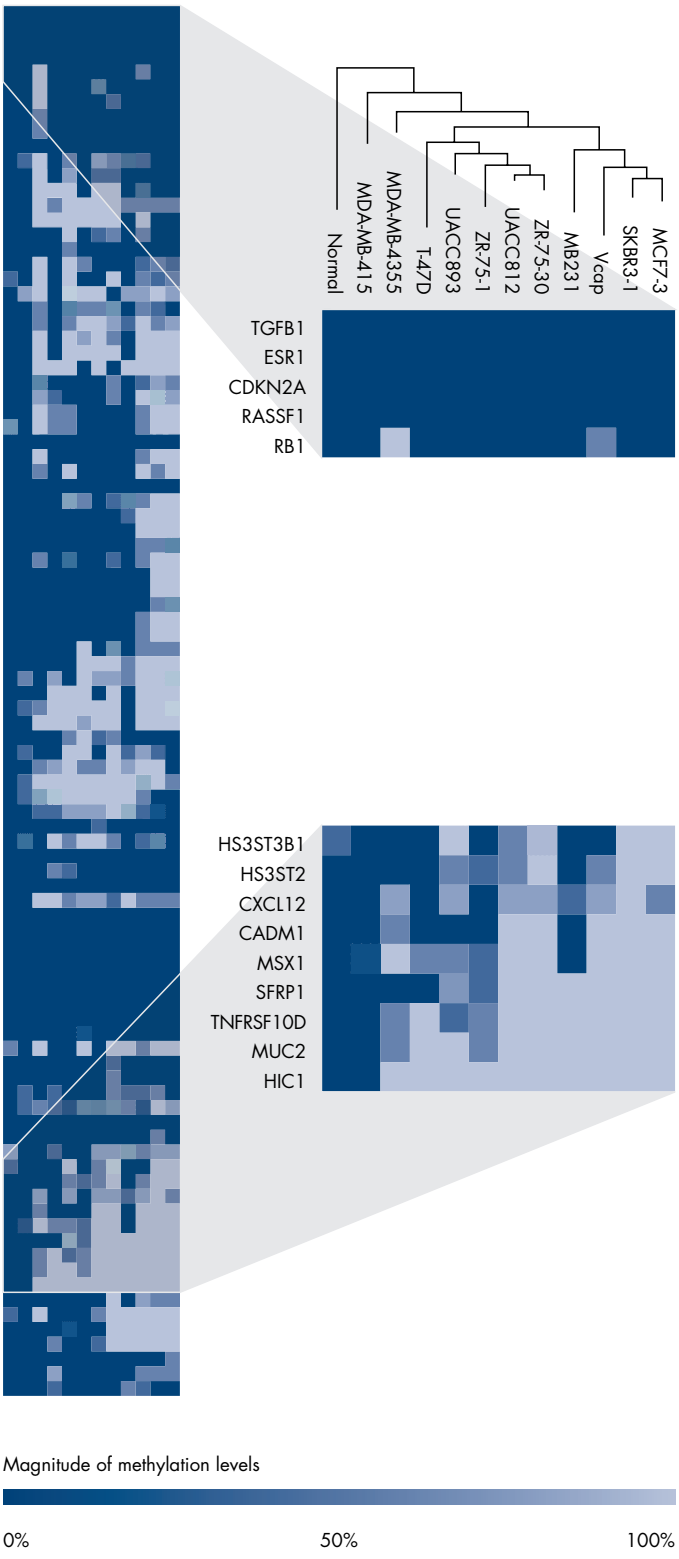


Figure 8. Hierarchical cluster analysis of methylation levels in cancer cell lines. Genomic DNA from 10 breast cancer cell lines and 1 prostate cancer cell line (VCaP) was isolated and analyzed with the EpiTect Methyl II PCR Arrays. This heat map compares the methylation levels of the 96 genes represented in the Human Breast Cancer EpiTect Methyl II Array.

Summary

The EpiTect Methyl II PCR System is highly suited for methylation status screening for large numbers of genes or samples in a pathway-focused manner, for both basic research applications and biomarker screening. While still delivering similar results and sensitivity, the simple procedure is considerably faster and easier, and is more amenable to higher throughput applications than bisulfite Sanger sequencing and other commonly used methods. The DNA methylation status of a target region can be consistently and reliably detected by the EpiTect Methyl II PCR System, even at very low levels within heterogeneous samples. In addition to the significant time savings, since no subcloning and sequencing are required, the EpiTect Methyl II PCR System provides the following benefits:

- Simple, fast, and reliable: results comparable to bisulfite Sanger sequencing in just 3 easy steps, without bisulfite conversion
- Pathway-focused: simultaneous profiling of DNA methylation patterns of gene panels specifically relevant to human diseases and biological pathways
- Fully customizable arrays: genomewide coverage for multiple mammalian genomes for all your candidate genes

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Ordering Information

Product	Contents	Cat. no.
EpiTect Methyl II PCR Arrays	Biological pathway- or disease-focused panels of promoter methylation assays	Varies*
EpiTect Methyl II Custom PCR Arrays	Custom panels of promoter methylation assays	Varies*
EpiTect Methyl II PCR Primer Assays	qPCR primers for individual genes	Varies*
EpiTect Methyl II DNA Restriction Kit	Enzymes and buffer for up to 12 samples	335452
RT^2 SYBR Green qPCR Master Mixes	Instrument-specific qPCR master mixes in several sizes	Varies*

*Visit http://www.sabiosciences.com/dna_methylation_array.php to view individual catalog numbers for these products.

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