

MethylPath™ MeDIP-on-chip

Comprehensive discovery of methylated DNA regions across whole genomes

Active Motif has developed a comprehensive, genome-wide methylated DNA assay using a combination of methylated DNA immunoprecipitation (MeDIP) and Affymetrix whole-genome tiling arrays. In this assay, DNA from cells or tissue samples is IP'd with an antibody against 5-methylcytosine using IP protocols optimized to give maximum sensitivity and minimal background. The resulting DNA is analyzed on a whole-genome basis using Active Motif's MethylPath™ MeDIP-on-chip analysis, or on a gene-by-gene basis using Active Motif's MethylPath™ MeDIP qPCR analysis. In this whole-genome assay, the IP'd DNA is amplified, labeled, and hybridized to one or more Affymetrix GeneChip® Tiling Arrays. These arrays are typically Human or Mouse Tiling 2.0R Arrays, which contain non-repetitive tiling probes of 25 nucleotides separated by 10-base pair gaps across sets of 7 arrays/genome. Tiling arrays for other species or other types of arrays may also be used, including the Affymetrix Human or Mouse Promoter 1.0R Arrays.

MeDIP-on-chip Identifies Methylated DNA Regions Across the Genome

Methylation of eukaryotic DNA is a major mechanism by which epigenetic changes regulate gene expression. DNA methylation occurs exclusively on cytosines that are followed immediately by a guanine. This configuration, referred to as a CpG, occurs in the genome less frequently than expected due to the propensity of methylated cytosines to mutate to thymidines. However, there are regions of DNA called CpG islands in which CpGs are overrepresented. CpG islands are typically 200-2000 bp in length, have a G+C content of > 50%, and have an observed-to-expected CpG dinucleotide ratio of > 0.6. CpG islands are found in the promoters of approximately 40% of human genes but also often appear within repetitive DNA and exonic sequences. In general, methylation of promoter CpG islands is correlated with inactivation of gene transcription. To advance the understanding of gene regulation involving epigenetic changes, assays to discover and track cytosine methylation with good sensitivity, accuracy, and reproducibility are needed for a wide variety of applications.

Methods already in use to determine methylation status of individual cytosines or broader genomic regions include bisulfite conversion of cytosine to uracil followed by sequencing, methylation-specific PCR (MSP, which also requires bisulfite conversion), and enrichment or depletion of methylated sequences using methylation-specific restriction enzymes followed by analysis on microarrays. These methods have significant limitations, e.g., bisulfite sequencing and MSP are not well suited for high-throughput analysis, while restriction enzyme-mediated enrichment or depletion is not comprehensive due to the need for restriction sites at the thousands of regions to be analyzed.

The ability of Active Motif's Methylated DNA Assays to specifically detect and enrich for methylated DNA was initially shown using DNA from MDA-MB-231 cells, an estrogen recep-

tor-negative breast cancer cell line. Following the IP, qPCR was carried out using primer pairs that amplify DNA regions previously reported to be methylated in this cell line. These regions included the CpG island at the 5' end of the CDH1 (E-cadherin) gene and the extensive CpG island covering the entire HIC1 gene (Hypermethylated In Cancer). As illustrated in Figure 1, signals for these two regions were significantly elevated compared to unmethylated Untr12 and Untr20 negative control regions. Following amplification of the IP'd DNA prep to generate adequate DNA for array hybridization, qPCR again showed enrichment of the CDH1 and HIC1 regions compared to Untr12 and Untr20.

Amplified DNA was labeled using Affymetrix's dsDNA terminal labeling kit and hybridized to the GeneChip® Human Tiling 2.0R Arrays. Arrays were washed and scanned according to Affymetrix standard procedures. Raw data from the scans were analyzed using Affymetrix's Tiling Analysis Software (TAS) and the results were both viewed in Affymetrix's Integrated Genome Browser (IGB) and compiled further using Active Motif's proprietary analysis software.

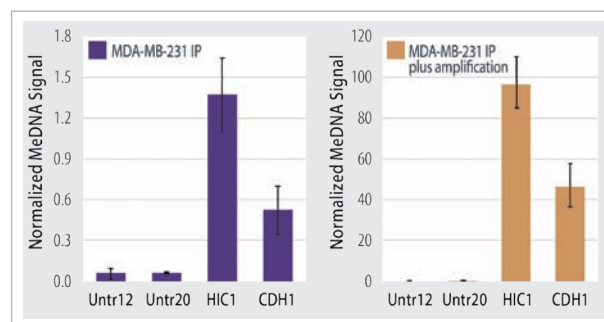


Figure 1. MeDIP qPCR of IP'd DNA showing enrichment of regions known to be methylated compared to negative control regions (Untr12 and Untr20) in MDA-MB-231 cells. qPCR was carried out after the IP (left) and after the IP and amplification (right).

Identification of Methylated Regions Throughout the Genome

Analysis of the MeDIP-on-CHIP results obtained using Array F (human chromosomes 8, 11, and 12) showed methylated DNA regions at numerous locations. Figure 2 illustrates peaks corresponding to methylated DNA regions surrounding the ST14 gene. Overall, 997 methylated DNA regions were identified on chromosomes 8, 11, and 12 (Table 1). Extrapolation across the entire genome suggests an estimated 6,979 methylated regions in MDA-MB-231 cells. Further analysis on the locations of these regions relative to Build 34 genomic annotation showed that many of the methylated DNA regions identified correspond to CpG islands (Table 1). Of the 997 methylated regions, 233 were in or close to a CpG island (+/- 200 bp). In addition, 123 were in promoters, 584 were inside genes, 137 were in or close to exons (+/- 100 bp), and 216 were in unannotated regions. To further demonstrate how the MeDIP-on-chip assay identifies valid regions, Figure 3 shows the genomic region surrounding the TBX3 gene. Six methylated DNA regions were identified by peaks, and all six correlate well with CpG islands as shown by uploading the CpG locations from the UCSC browser into IGB.

Peaks on the array were next verified as methylated DNA regions using Active Motif's MethylPath MeDIP qPCR assay. Twenty peaks representing sites of DNA methylation, of which 10 exhibited large peaks and 10 exhibited moderate peaks, were examined. As illustrated in Figure 4, all 20 sites gave qPCR signals above the negative control regions (Untr12, Untr20, and ACTB). In general, regions with large peaks gave higher signals by qPCR than regions with moderate peaks (it should be noted that the regions tested by qPCR for FZD7, MGC45477, and LRSAM1 were represented in more than one copy per haploid genome, which may contribute to the higher signals for those regions). A more detailed analysis of the NMNAT2 region is shown in Figure 5, in which the qPCR results for six subregions overlapping and surrounding the NMNAT2 methylated DNA array peak are overlaid onto the array probe signals. A strong correlation can be seen between array hybridization signal and qPCR signal.

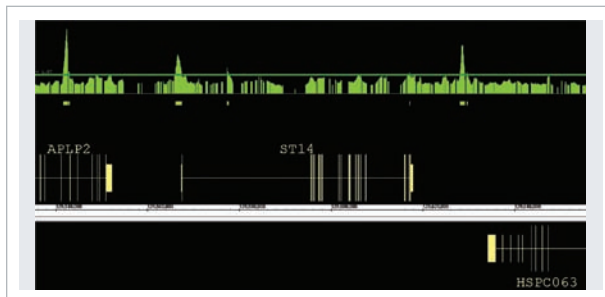


Figure 2. Array peaks (green) on IGB indicating methylated DNA regions near the transcription start site (TSS) of the ST14 gene, in the middle of the APLP2 gene (both genes transcribed left to right), and between the 3' ends of the ST14 and HSPC063 genes (latter transcribed right to left).

Table 1. Locations of peaks representing methylated DNA

Peaks on Array F (chromosomes 8, 11, 12)	Number	Percent
Total number on Array F	997	
Extrapolated to entire genome	6979	
In CpG Islands +/- 200 bp	233	23.4%
In promoters (-1 to -10,000 bp from TSS*)	123	12.3%
In genes	584	58.6%
In exons +/- 100 bp	137	13.7%
Unannotated (no gene within 10 kb)	216	22.0%

*TSS = Transcription Start Site



Figure 3. Array peaks (green) on IGB indicating methylated DNA regions in and around the TBX3 gene (transcribed right to left). Green bars below the peaks denote areas of signal above the threshold (green horizontal line). Blue blocks at the top represent CpG islands from the UCSC browser.

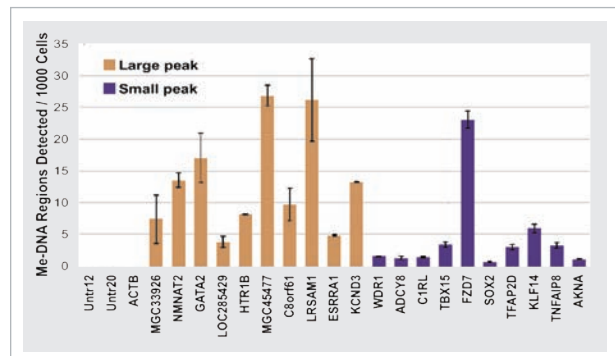


Figure 4. Verification by MeDIP qPCR that peaks observed on the arrays represent methylated DNA regions. Untr12, Untr20, and ACTB represent negative control regions. Regions for FZD7, MGC45477, and LRSAM1 were found to be present in > 1 copy/haploid genome.

Validation that MeDIP Detects Methylated DNA

The methylation status of individual CpG dinucleotides was further ascertained using bisulfite treatment, which converts unmethylated cytosines to uracils, followed by DNA sequencing. Bisulfite sequencing was performed on 7-10 clones each from nine different genomic regions, seven of which were positive on the tiling arrays and two of which were negative. Of all possible CpGs in the seven methylated genomic regions, 93% were found to be methylated (examples in Figure 6). In contrast, the two genomic regions chosen as negative controls (GAPDH and ACTB) showed methylation on only 0.3% of CpGs (Figure 6). These data thus show an absolute correlation between methylated DNA regions detected by MeDIP-on-ChIP and methylation status as determined by bisulfite sequencing.

Further validation of methylated DNA regions detected on arrays was undertaken using 5-azacytidine treatment of the MDA-MB-231 cells. 5-Azacytidine (AZA) is a methyltransferase inhibitor that causes decreased methylation in treated cells. As shown in Figure 7 (upper), AZA-treated MDA-MB-231 cells showed a 52%-67% reduction in methylated DNA levels at four genomic regions relative to untreated cells. Because a decrease in methylation would be expected to result in an increase in gene expression, the same genes were tested for RNA levels by RT-PCR. As expected, all four genes with decreased methylated DNA levels after AZA treatment also showed increased RNA (Figure 7, lower). In contrast, the KLF14 gene showed no change in methylation level after AZA treatment and no change in RNA levels. Controls included ACTB and GAPDH, which have always been found to be non-methylated (see ACTB in Figure 4). Upon testing by RT-PCR for RNA expression levels, these two genes showed high expression levels that were unaffected by AZA treatment (data not shown).

The global effect of AZA was further demonstrated by performing MeDIP-on-chip using AZA-treated versus untreated MDA-MB-231 cells. Results using Array F showed a general decrease in methylated DNA signals in the AZA-treated cells. Examples of affected methylated DNA regions over a region of approximately 300 kb that includes the RAB35, PXN, and MSII genes are shown in Fig. 8. Of the four major peaks with high signal in the untreated cells (upper green), three were significantly reduced after AZA treatment, while the fourth peak showed only a minor reduction.

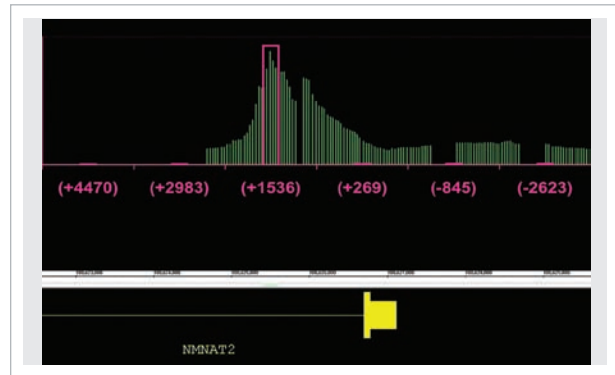


Figure 5. Individual probe signals (vertical green lines) at the 5' end of the NMNAT2 gene showing a peak that represents a methylated DNA region. qPCR results for six subregions, along with their locations relative to the gene, are overlaid in pink, showing correlation with the array results.

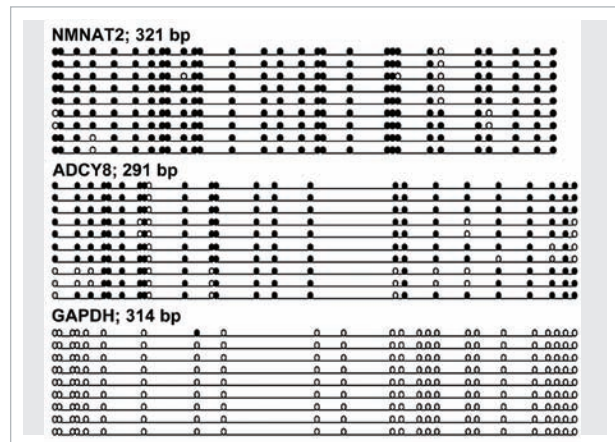


Figure 6. Genomic regions subjected to bisulfite sequencing showing that the DNA regions detected by MeDIP-on-chip (NMNAT2 and ADCYB are representative examples) are methylated on their CpGs, whereas the negative control CpG island upstream of the GAPDH gene is unmethylated. Each line represents an independently sequenced clone. Closed and open circles represent methylated and unmethylated CpGs, respectively.

Genome-Wide Discovery of Methylated DNA Regions With High Sensitivity and Specificity

Active Motif's MethylPath MeDIP-on-chip Assays provide an accurate and reproducible means for examining global DNA methylation patterns without limitations. No other DNA methylation technique detects methylated regions on a whole-genome scale without discrimination against certain types of sequences. As shown in the above data, DNA methylation occurs not only in promoters, CpG islands, and other expected regions, but also in genomic regions that do not have defined sequence or annotation features. Active Motif's assays can also discern different methylation levels when comparing multiple samples. Active Motif's MethylPath Assays will provide valuable information to scientists who are working to decipher how methylated DNA patterns are involved in gene transcription and regulation.

Overview of Active Motif Services

Active Motif services have wide utility in the discovery and development of targeted drugs, novel diagnostic biomarkers, and the identification of causal disease and pharmacological pathways.

We offer a complete suite of epigenetic services for understanding gene regulation and genetic pathways:

- **FactorPath™** for discovery of transcription factor binding sites
- **TranscriptionPath™** for discovery of genomic DNA sequences undergoing active transcription in cells
- **MethylPath™** and **HistonePath™** for discovery and analysis of epigenetic events such as DNA methylation and histone modification
- **Bisulfite Sequencing** for determining the methylation status of cytosines at specific CpGs
- **Antibody Qualification** to validate those antibodies that are suitable for use in ChIP, ChIP-Seq and ChIP-on-chip

All of our services include expert consultation on experimental design, quality controls at multiple steps and comprehensive data analysis. Please contact us with any questions you might have about initiating a project in your cell system.

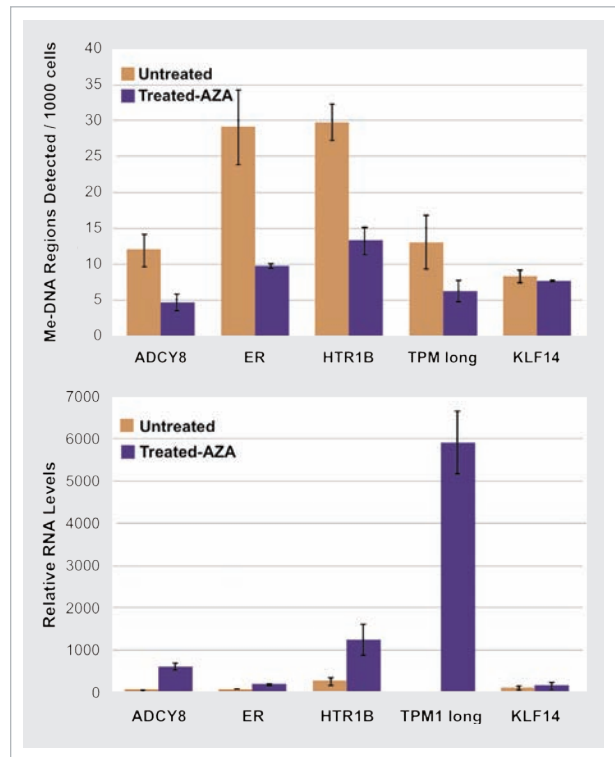


Figure 7. (Upper) MeDIP qPCR results demonstrating that MDA-MB-231 cells treated with 5-azacytidine exhibit decreased methylation levels at many but not all regions. (Lower) RT-PCR in the same cells showing increased RNA levels for the AZA-affected genes.

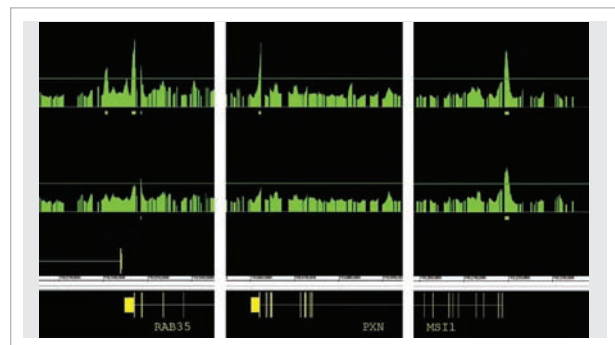


Figure 8. Array peaks on IGB demonstrating decreases in methylated DNA levels as a result of 5-azacytidine treatment (lower) compared to untreated cells (upper). Subregions shown span a genomic region of approximately 300 kb.