

DNA Methylation Analysis

Illumina provides a broad portfolio of DNA Methylation Analysis solutions. Each product supports different ranges of multiplex and sample throughput levels for use in any experimental design.

Introduction

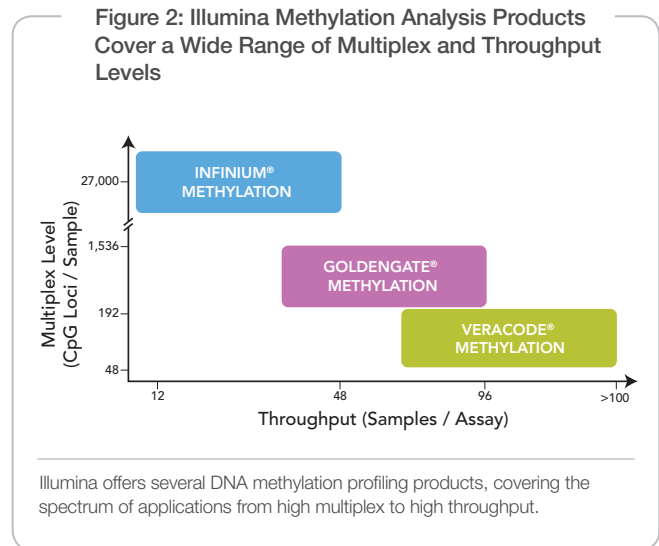
Epigenetic mechanisms play important roles during normal development¹, aging², and a variety of disease conditions. Numerous studies have implicated aberrant methylation in the etiology of common human disease, including cancer, MS, diabetes, and schizophrenia^{3,4}. Hypermethylation of CpG islands located in the promoter regions of tumor suppressor genes is firmly established as the most frequent mechanism for gene activation in cancers^{5,6}.

Methylation of the 5' carbon of cytosine is a form of epigenetic modification that does not affect the primary DNA sequence, but affects secondary interactions that play a critical role in the regulation of gene expression. Aberrant DNA methylation may suppress transcription and subsequently gene expression. DNA methylation has typically been studied in a locus-targeted manner because effective tools for performing global analyses were unavailable.

Now with Illumina high-throughput profiling assays, true genome-wide analysis of DNA methylation and its widespread impacts on gene regulation can be easily studied. Illumina has developed a portfolio of products with unprecedented capabilities for studying DNA methylation—covering the spectrum from genome-wide scans to focused studies and biomarker validation—to provide unique insights into the role of epigenetics in gene regulation (Figure 2).

Illumina DNA Methylation Analysis Portfolio

Illumina offers a broad portfolio of methylation analysis products to satisfy the needs of different research goals and experimental designs. Sensitive and reproducible genome-wide screening of DNA methylation patterns is enabled with the trusted Infinium® Assay. The GoldenGate® Assay for Methylation, deployed on BeadArray™ or VeraCode® platforms, interrogates methylation patterns with equally high sensitiv-



ity and accuracy, but with higher sample throughput (Figure 2 and Table 1). The GoldenGate Assay also provides researchers additional flexibility to create custom content panels for targeted interrogation of DNA methylation.

Both GoldenGate and Infinium Assay genotyping technologies have been proven in labs worldwide. They are now the foundation for the most comprehensive methylation analysis tools available. Users of other Illumina products will find that the high similarity between assay workflows enable fast generation of DNA methylation data.

Single-Site Resolution

All three Illumina Methylation Profiling technologies are based on genotyping bisulfite-converted DNA. Treatment of DNA samples with a convenient methylation kit from Zyro Research converts unmethylated cytosines to uracils, whereas methylated cytosines are protected and remain cytosine (Figure 1). After this conversion step, determining whether the base at a given locus was converted or not converted provides information on its original methylation status. Methylation status of the interrogated CpG site is then calculated as the ratio of signal from a methylated probe relative to the sum of both methylated and unmethylated probes. This value, known as β , ranges continuously from 0 (unmethylated) to 1 (fully methylated).

Comprehensive Genome-wide DNA Methylation Profiling

The HumanMethylation27 BeadChip provides an efficient solution for surveying genome-wide DNA methylation profiles across many samples. Powered by Illumina's revolutionary Infinium Assay, the HumanMethylation27 BeadChip allows researchers to interrogate

Figure 1: DNA Methylation

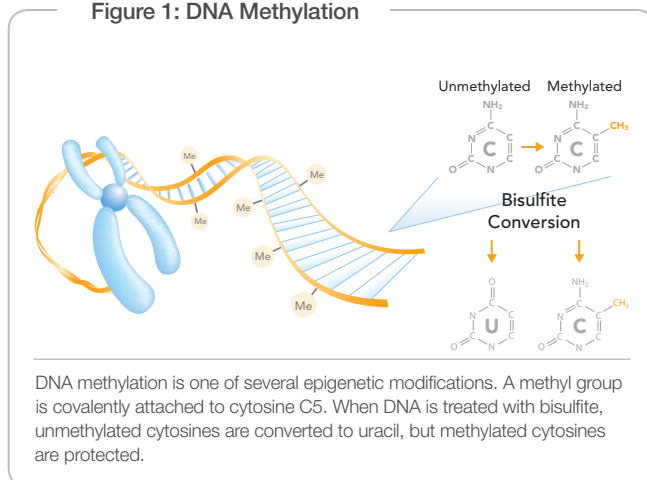


Table 1: Illumina Methylation Analysis Options

	Infinium Methylation	Goldengate Methylation	Veracode Methylation
Products	HumanMethylation27	Cancer Panel I Custom Design	Custom Design
Assay	Infinium	GoldenGate	GoldenGate
Platform	BeadArray	BeadArray	BeadXpress
Content	Genome-wide	Custom or Cancer	Custom
CpG Sites	27,578	up to 1,536	96–384
Sample Throughput	12 / BeadChip	96 / SAM	80 / hr (@ 96-plex)
Reproducibility	$r^2 > 0.98$	$r^2 > 0.98$	$r^2 > 0.99$
Inter-Product Concordance	$r^2 > 0.8$		$r^2 = 0.97$
Internal Controls	✓	✓	✓
FFPE Compatible		✓	✓
Analysis Software	BeadStudio	BeadStudio	BeadStudio
Integrate with Expression	✓	✓	✓

more than 27,000 highly informative CpG sites per sample at single-site resolution. Methylation profiles can be compared between different samples and results can easily be integrated with RNA expression profiling experiments using BeadStudio software.

The robust Infinium Assay workflow is highly streamlined, does not require PCR, and contains all assay reagents are supplied in one convenient kit (except the Zymo bisulfite conversion kit). Plus, each BeadChip can assay 12 independent samples simultaneously for fast project completion. Low sample input requirements (as low as 500 ng for two assays, pre-bisulfite conversion) for the Infinium Methylation Assay enables analysis of valuable samples derived from limited DNA sources.

HumanMethylation27 BeadChip Content

The Infinium Assay allows great design flexibility for rationally selecting loci and designing probes. With it, Illumina scientists were able to create a carefully chosen panel of high-value methylation sites, including promoters and methylation hotspots. Probe design flexibility is particularly important for methylation applications, since bisulfite treatment reduces the complexity of the genome, making unique sites even more valuable.

Within each CpG island targeted, optimal CpG sites were chosen for markers on the HumanMethylation27 BeadChip. These sites were determined by holistically analyzing the anticipated assay performance, their location in an island, and their proximity to the transcription start site (TSS). To maximize genome-wide coverage, probe design leverages the finding that adjacent CpG sites have similar methylation status⁸, a well-supported hypothesis assumed by many other methylation technologies. Thus, most CpG islands can be interrogated sufficiently

Figure 3: Infinium Humanmethylation27 Beadchip

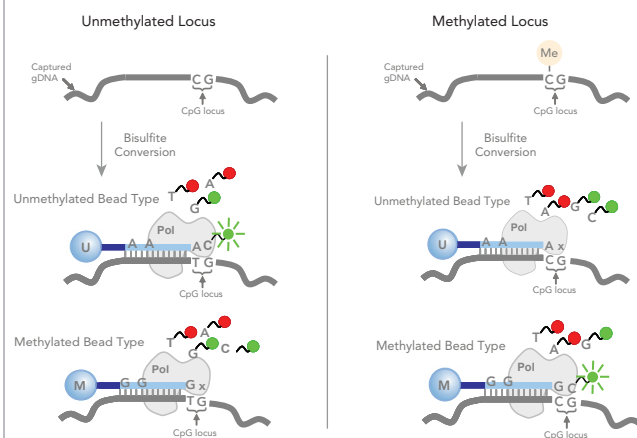


Researchers can assay > 27,000 CpG sites per sample and interrogate up to 12 samples on a single BeadChip.

Humanmethylation27 Beadchip Highlights

- **High Throughput:**
> 27,000 CpG assays per sample, 12 samples per BeadChip
- **Specific:**
Single CpG resolution
- **Streamlined Workflow:**
PCR-free protocol
- **Low Sample Input:**
500 ng manual or 1 µg automated processing
- **Product Integration:**
Content overlap with Illumina gene expression BeadChips, integrated analysis supported by BeadStudio Software

Figure 4: Infinium Assay for Methylation



The Infinium Assay for Methylation detects methylation status at individual CpG loci by typing bisulfite-converted DNA. Unmethylated C is converted to T (left), whereas methylation protects C from conversion (right). A pair of bead-bound probes are used to detect the presence of T or C by hybridization followed by single-base extension with a labeled nucleotide.

using an average of two markers each. Other select high-value areas have been targeted on the HumanMethylation27 BeadChip with additional markers for higher resolution analysis.

The HumanMethylation27 panel targets CpG sites covering over 14,000 genes, including almost 13,000 genes in the NCBI CCDS Database⁹ (Genome Build 36). Additional markers provide increased resolution focused on 144 established cancer genes that have shown differential methylation patterns. To ensure the most important loci were represented, Illumina designed markers to cover nearly 1,000 cancer-related genes described in published literature.

Of all genes represented on the HumanMethylation27 BeadChip, more than 12,700 are also present on the HumanRef-6 Gene Expression BeadChip, permitting the integrated analysis of global methylation status and gene expression levels.

Not only are coding gene promoters targeted, but CpG sites located within 110 miRNA promoter regions complete the broad content. Expression profiling information for all of these miRNA can be integrated with methylation status by using Illumina's microRNA Profiling Panel.

Table 2: Infinium Methylation Content

Markers	Description	Avg Coverage
14,475	Total RefSeq Genes	1.9 sites
12,833	Well-annotated genes described in the NCBI CCDS database ⁹ (Genome Build 36)	1.9 sites
144	Methylation hotspots in cancer genes	7.6 sites
982	Cancer-related targets	1.9 sites
110	miRNA promoters	2.3 sites

Goldengate Methylation on Beadarray Highlights

- **High Throughput:**
Up to 1,536 CpG sites assayed simultaneously
- **Flexible Content:**
Standard and custom panels
- **Streamlined Workflow:**
96 samples per array, with a convenient 3-day workflow
- **Robust Performance:**
High sensitivity and reproducibility

Infinium Assay for Methylation

The Infinium Assay for Methylation (Figure 4) is based on highly multiplexed genotyping of bisulfite-converted genomic DNA. First, genomic DNA is treated with a bisulfite conversion kit. The result is DNA that contains unconverted cytosines (if they were methylated) or uracils (derived from unmethylated cytosine). This differentially converted DNA is interrogated simultaneously at each locus by two probes, one designed against the unmethylated site and one against the methylated site. After hybridization, allele-specific single-base extension provides another level of specificity and incorporates a fluorescent label for detection.

The level of methylation is determined at each locus by the intensity of the two possible fluorescent signals, from the C (methylated) and T (unmethylated) alleles. The relative level of methylation (β), is calculated as the ratio of methylated-probe signal to total locus signal intensity. This assay enables sensitive and reproducible methylation profiling, and provides quantitative measurements of the degree of methylation, which is often dynamic during disease progression¹⁰.

GoldenGate Methylation Profiling on the BeadArray Platform

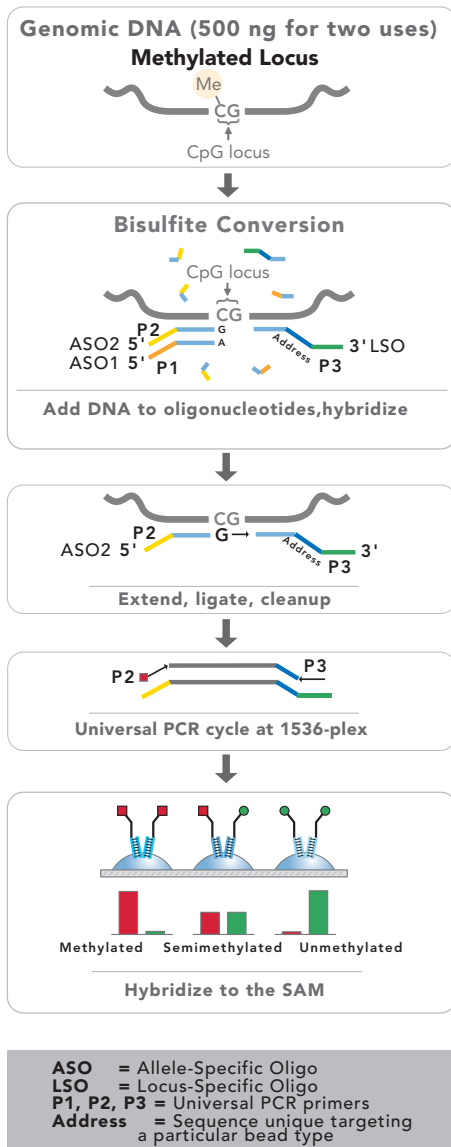
For more focused DNA Methylation profiling than the broad whole-genome Infinium HumanMethylation27 BeadChip, Illumina offers methylation profiling based on the GoldenGate Assay. This profiling technology advances methylation research and biomarker discovery with high reproducibility (average $r^2 > 0.98$), flexible content design, strong correlation to methylation-specific PCR, and the sensitivity to detect small changes in methylation status between biological samples.

Figure 5: 96-Sample GoldenGate Assay for Methylation



The GoldenGate Assay allows processing 96 samples on a single SAM.

Figure 6: GoldenGate for Methylation Assay



Bisulfite-treated genomic DNA is mixed with assay oligos. Each ASO is complementary to either the converted U (unmethylated site) or the protected C (methylated site). Following hybridization, primers are extended and ligated to the LSO creating a template for universal PCR. Labeled PCR primers are used to create a detectable product. Individual assays localize to specific bead types on the SAM by hybridization of address sequences.

The Illumina GoldenGate Assay for Methylation on the BeadArray platform provides a powerful means of assessing the methylation status of up to 1,536 independent CpG sites simultaneously across each of 96 samples on either standard or custom content oligonucleotide sets. Differential methylation analysis can be accomplished with confidence on hundreds of samples in less than a week from bisulfite conversion to data analysis completion. Even higher throughput is achievable using the VeraCode Platform (see below).

Figure 7: BeadXpress Reader



The BeadXpress Reader enables the high-throughput assay readout of the GoldenGate Methylation Assay on VeraCode Technology.

Cancer-Focused Methylation Analysis

GoldenGate Methylation Cancer Panel I covers 1,505 CpG loci selected from 807 genes where 71.4% contain two or more CpG assays per gene. Targeted genes were selected from several high-value classes (Table 3).

All genes targeted on the GoldenGate Methylation Cancer Panel I are present on the Illumina Human-6 v2 Expression BeadChip. BeadStudio analysis software lets researchers combine methylation and gene expression data sets for integrated studies.

Custom Methylation Profiling

Customers can design custom GoldenGate Methylation Assays to target specific CpG loci near genes or in regions of interest. Illumina scientists and a proprietary Assay Design Tool help customers create successful content panels. Custom content designs can be submitted using any of the following formats: sequence, accession number, gene symbol, gene ID, GI number, or chromosomal region. For more information about custom content submission, please contact Illumina technical support or read the technical note, Designing Custom GoldenGate Methylation Profiling Panels⁹.

GoldenGate Assay for Methylation

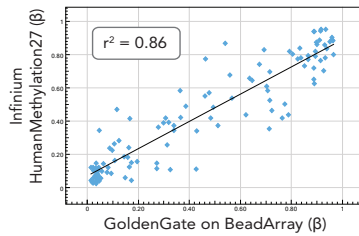
Illumina's GoldenGate Assay is also based on genotyping bisulfite-converted genomic DNA (Figure 6). Ideal for focused studies or whole-genome data validation, this assay procedure is similar to that described previously for standard SNP genotyping¹¹, with a few modifications⁷.

Table 3: Cancer Panel I Target Groups

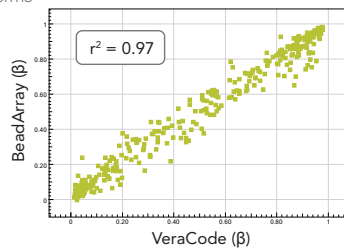
- Tumor suppressor genes
- Oncogenes
- DNA repair
- Cell cycle control
- Differentiation
- Apoptosis
- X-linked
- Imprinted genes

Figure 8: Assay Consistency Across Illumina Products

A: Concordance between Infinium and GoldenGate Methylation



B: Concordance between GoldenGate Methylation on BeadArray and VeraCode Platforms



Bisulfite-treated genomic DNA is mixed with assay oligos. Each ASO is complimentary to either the converted U (unmethylated site) or the protected C (methylated site). Following hybridization, primers are extended and ligated to the LSO creating a template for universal PCR. Labeled PCR primers are used to create a detectable product. Individual assays localize to specific bead types on the SAM by hybridization of address sequences.

GoldenGate Assays are deployed on SAMs that utilize BeadArray technology, which combines a bead-based array platform with a high level of assay multiplexing. Using a four-probe design, the assay is able to differentiate between methylated and unmethylated sequences. High specificity is achieved by hybridization followed by two enzymatic discrimination steps: allele-specific extension and ligation. DNA targets are generated using a pair of allele-specific (ASO) and locus-specific (LSO) oligonucleotides (Figure 6c-e). Amplification using universal primers creates products that are hybridized to a SAM at sites bearing complementary address sequences (Figure 6f). Amplification also incorporates a fluorescent label that denotes a methylated or unmethylated state for a given assay.

High-Throughput Methylation Profiling on VeraCode Platform

For methylation profiling applications that demand even higher throughput, such as biomarker validation and screening or diagnostic development, the GoldenGate Assay for Methylation can be performed using the VeraCode Technology. Researchers with a refined panel of 96–384 highly informative DNA methylation sites have the option to use the VeraCode Technology on the BeadXpress™ Reader.

VeraCode Technology

VeraCode microbeads are glass cylinders where each bead type is uniquely identified by an inscribed holographic code. VeraCode beads with immobilized universal capture oligonucleotides are an

ideal high-throughput readout platform for the GoldenGate Assay for Methylation.

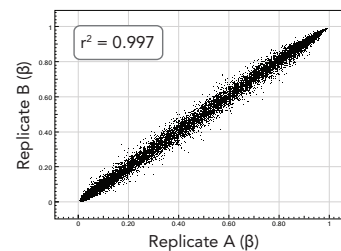
The BeadXpress Reader is an automated fluidics and multi-laser imaging device that rapidly scans the bioassays deployed on VeraCode beads. Individual methylation assay signals are quantified by the standard fluorescent readout of the GoldenGate Assay while the identity of the assayed locus is detected by the code image within the bead.

Bisulfite conversion and GoldenGate chemistry proceed just as on the BeadArray platform, but VeraCode solution-based kinetics support a faster assay protocol, reducing total time to two days.

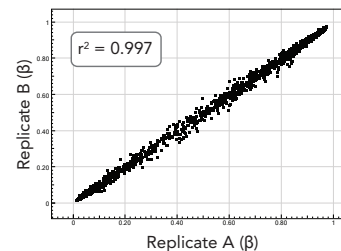
Since the assay is identical, researchers can be confident they will not sacrifice accuracy for higher throughput (Figure 8C). Internal testing showed that assays on VeraCode and BeadArray platforms have highly concordant results (avg $r^2 > 0.97$) using a panel of 384 markers with replicate samples from several different cell types (Figure 8B).

Figure 9: Assay Reproducibility

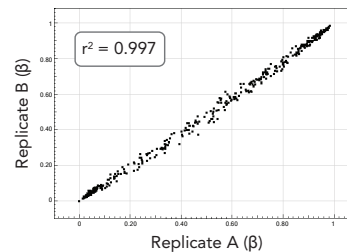
A: Infinium Methylation



B: GoldenGate Methylation

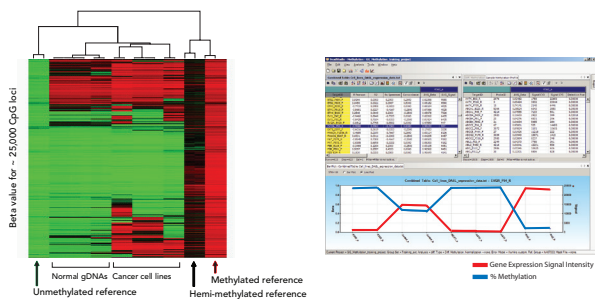


C: GoldenGate Methylation on VeraCode



Using any Illumina DNA Methylation product, users can be confident of robust data. Representative plots from internal testing show r^2 correlations greater than 0.99.

Figure 10: Data Analysis with Illumina Beadstudio Software



BeadStudio supports DNA methylation analysis on any platform. Data are displayed in intuitive graphics. Gene expression data can be integrated easily with methylation projects (plotted on right).

High Quality Data

Regardless of assay technology or deployment platform chosen, researchers achieve industry-leading quality data using Illumina methylation profiling assays. Illumina scientists rigorously test every product to ensure strong and reproducible performance.

Precision and Accuracy

Reproducibility has been determined with replication experiments (Figure 9). All products show correlation (r^2) greater than 0.98. Such high reproducibility is driven in part by the inherent 15- to 18-fold average feature redundancy of Illumina Infinium HumanMethylation27 BeadChip and average 30-fold redundancy of GoldenGate assays. Since each probe is represented with high redundancy, all products yield highly accurate and quantitative methylation measurements.

Further independent testing was conducted by comparing results that were derived independently between Infinium and GoldenGate products. Since these products are based on different chemistries and are

deployed on different substrates, their comparison provides an ideal orthogonal quality test. Very high correlations ($r^2 > 0.8$) were found between Infinium and GoldenGate Assays (Figure 8A).

Sensitivity

By comparing the results of replicate experiments (10 replicates each of 8 biological samples), Illumina scientists have shown that the HumanMethylation27 BeadChip reliably detects a difference in beta of less than 0.2 with a lower than 1% false positive rate.

Internal Quality Controls

Products based on both the Infinium and GoldenGate Assays for Methylation have several sample-dependent and sample-independent controls so researchers have confidence in producing the highest quality data. Negative controls are particularly important in methylation analysis assays since sequence complexity is decreased after bisulfite conversion. The BeadStudio Methylation Module Software has an integrated Controls Dashboard where the performance of all controls can be monitored easily.

Integrated Analysis Software

Infinium and GoldenGate Methylation data analysis is supported by the powerful and intuitive BeadStudio Methylation Module. The Methylation Module enables researchers to effortlessly perform methylation or differential methylation analysis across samples (Figure 10). The BeadStudio software features advanced visualization tools that enable researchers to view vast amounts of data in a single graph, such as heat maps, scatter plots, and line plots. These tools and the BeadStudio Genome Browser (Figure 11) display valuable information such as chromosomal coordinates, percent GC, location in a CpG Island, and methylation β values.

Illumina's methylation assay technology is consistent with other Illumina applications, including gene expression profiling. This enables researchers to perform cross-application analysis such as integrating gene expression data with DNA methylation data (Figure 10b).

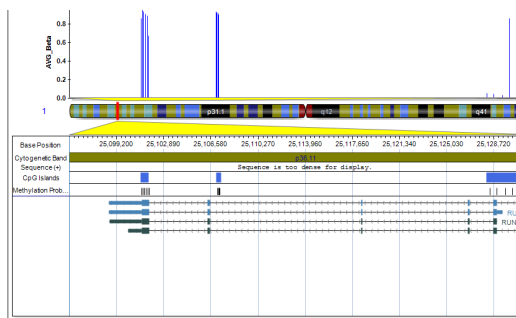
Summary

Understanding the impact of DNA methylation on gene regulation requires robust and flexible technologies. The Infinium HumanMethylation27 BeadChip, together with the GoldenGate Methylation Cancer Panel and Custom options on BeadArray or VeraCode platforms, facilitate robust methylation profiling. By creating the most comprehensive set of offerings to the epigenetics field, Illumina has empowered researchers to perform a wide range of experiments without trade-offs. At any multiplex level or sample throughput desired, Illumina assays provide high accuracy, easy workflows, and low input DNA requirements.

Additional Information

For more information about the whole-genome Infinium HumanMethylation27 BeadChip or the flexible GoldenGate Assay for focused methylation detection on BeadArray or VeraCode platforms visit www.illumina.com/methylation or contact us at the address on the last page.

Figure 11: Methylation Hotspots Visualization on the Beadstudio Chromosome Browser



The chromosome browser in BeadStudio provides easy visualization of methylation hotspots. Nearby genomic elements are also displayed to facilitate interpretation.

Ordering Information

Catalog No.	Product	Samples	Description
Infinium Methylation BeadChip			
WG-311-2201	HumanMethylation27, Rev B (2 BeadChips)	24 samples	The Infinium HumanMethylation27 BeadChip targets over 27,000 CpG loci and each kit contains 2 BeadChips and reagents for processing 24 samples.
WG-311-2202	HumanMethylation27, Rev B (4 BeadChips)	48 samples	Each kit contains 4 BeadChips and reagents for processing 48 samples.
GoldenGate Methylation on BeadArray Platform (product was discontinued as of March 1, 2010)			
GM-17-211	Methylation Cancer Panel I	96 samples	Experimentally validated Oligo pool for Methylation Assay (OMA) for 1,505 CpG sites selected in promoter regions and/or first exon of over 800 genes. Sufficient for the analysis of 96 samples.
GM-95-201	Single-Use Activation Kit*	576 samples	Used in combination with the GoldenGate Assay Kit. Contains reagents for six 96-well plates of samples.
GM-95-205	GoldenGate Assay Kit with UDG*	96 samples	Prepares genotyping reactions for 96 DNA samples. Contains UDG enzyme for contamination control.
GM-12-109	Sentrix® Universal-96 Array Matrix	96 samples	Processes 96 samples with up to 1,536 assays per sample.
GoldenGate Methylation on VeraCode Technology			
VC-201-3096	VeraCode GoldenGate Methylation Analysis, 96-plex Kit	480 samples	Kit includes reagents, a custom 96-plex oligo pool (OMA), and 96-plex VeraCode bead plates.
VC-201-3384	VeraCode GoldenGate Methylation Analysis, 384-plex Kit	480 samples	Kit includes reagents, a custom 384-plex oligo pool (OMA), and 384-plex VeraCode bead plates.
Related Products			
D5001†	EZ DNA Methylation Kit		50 reactions (single column format)
D5002†	EZ DNA Methylation Kit		200 reactions (single column format)
D5004†	EZ-96 DNA Methylation Kit		2x96 reactions (deep-well format)

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