Three Optimized Workflows for CpG Island Methylation Profiling

DNA methylation is involved in the regulation of many cellular processes including X chromosome inactivation, chromosome stability, chromatin structure, embryonic development, and transcription. In mammals only the 5' carbons of cytosines adjacent to guanines (CpG dinucleotides: cytosine-phospho-guanine) are substrates for DNA methylation.

Importance of CpG Islands

CpG islands are 300–3000 base pair stretches of DNA that are CG rich, and are often found within unmethylated regions of promoters. Profiling of CpG island methylation indicates that some genes are more frequently methylated than others [1]. Hence, CpG islands play a critical role in regulating gene expression. CpG islands found outside promoter regions are commonly methylated, and are believed to be responsible for silencing transcription of repetitive sequences and parasitic sequence elements, such as viral DNA. Aberrant methylation can lead to either silencing of critical genes or increased expression of detrimental factors, which has been associated with many human diseases, including cancer [2]. This phenomenon makes the discovery of CpG islands and the determination of their methylation status the subject of intense medical research [3].

Three Workflows for the Discovery of CpG Islands

Bisulfite DNA conversion is one of the most frequently used techniques for methylation studies because of its relative simplicity, whereas other methods are cumbersome and require significant optimization. The bisulfite conversion method allows precise analysis of methylation in a target region by converting all non-methylated cytosines into uracils, while methylated cytosines remain unchanged. The three workflows described here provide an effective solution for methylation analysis with straightforward protocols.

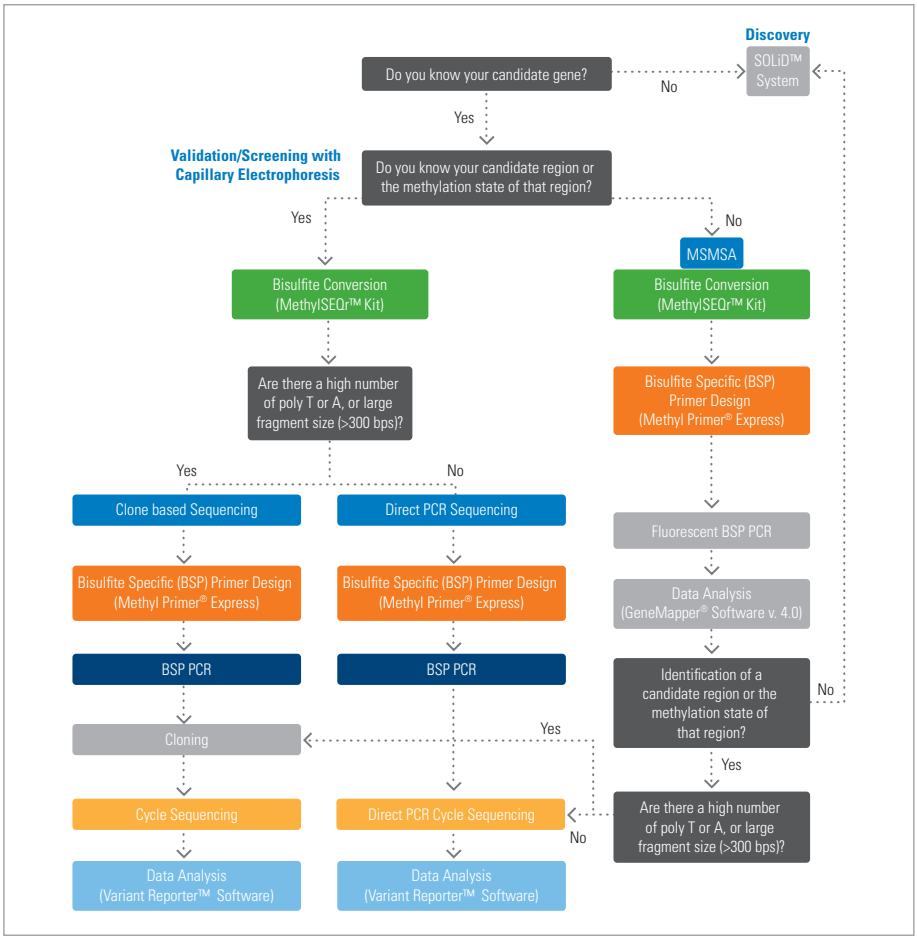

Figure 1. Decision Tree Outlining the Process for Choosing an Application. When choosing a methylation workflow, it is necessary to make a clear decision about the research goals first, and then decide what technical strategy will fit best.

1. Bisulfite Specific Methylation Sensitive Mobility Shift Assay (Figure 2)
2. Bisulfite Specific PCR-based Sequencing (Figure 4)
3. Bisulfite Specific Cloning-based Sequencing (Figure 6)

Figure 1 outlines the process to assist in choosing a workflow that is well-suited for a specific application.

The general steps for all three workflows are as follows:

Step 1: DNA Extraction

Prepare high quality genomic template using RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE Tissues, MELT ™ Total Nucleic Acid Isolation System, NucPrep® Chemistry for Tissues, or BloodPrep® DNA Chemistry for Cultured Cells and Blood.

Tip: Since purity of gDNA is critical to the success of complete bisulfite conversion, a proteinase K digestion step is recommended to remove all proteins prior to bisulfite conversion.

Step 2: Bisulfite Treatment

Use MethylSEQr™ Bisulfite Conversion Kit to convert non-methylated cytosines to discriminate from methylated cytosines.

Step 3: Primer Design and Amplification

Design primers using Methyl Primer Express® Software. For a free download of this software, visit **www.appliedbiosystems.com/methylprimerexpress**. Amplify targets using AmpliTaq Gold® DNA Polymerase and Applied Biosystems Thermal Cyclers.

Tips: Design short amplicons for better results (150–250 bp for sequencing, 450 bp for cloning). Avoid more than 8 to 9 Ts/As in a stretch to reduce slippage. Also, use M13-tailed primers for PCR, so that the same primers can be used for sequencing.

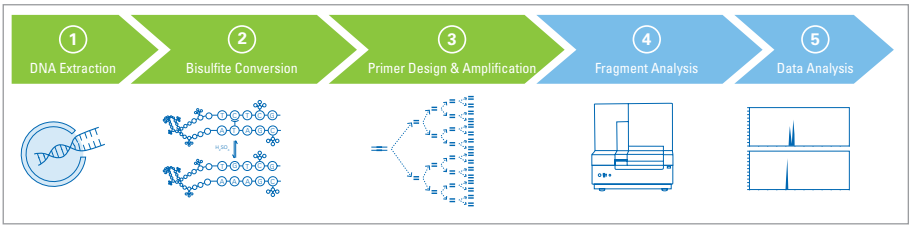

Figure 2. Bisulfite Specific Methylation Sensitive Mobility Shift Assay Workflow.

Step 4: Cycle Sequencing and Cleanup

For sequencing specific workflows, perform cycle sequencing of the specific target using BigDye Terminator Sequencing Kits. The reactions will then require removal of unincorporated dye terminators and unused primers using a reliable clean up kit.

Tips: For fast and reliable sequencing clean-up, use Big Dye® XTerminator™ Kits for cleanup of sequencing reactions.

Step 5: Data Collection and Analysis

Use Applied Biosystems capillary electrophoresis instruments for data collection. Perform application specific analysis of data and interpretation of results to evaluate or confirm the changes in the methylation status. For secondary analysis of fragment analysis-based workflows, use GeneMapper® Software v4.0 or Peak Scanner™ Software (Figure 2). For secondary analysis of sequencing-based workflows, use Variant Reporter™ Software v1.0 or SeqScape® Software v2.6 (Figure 4, 6).

Workflow 1: Bisulfite Specific Methylation Sensitive Mobility Shift Assay (MSMSA)

MSMSA is a DNA fragment analysis application used to assess the degree of methylation present in a given amplicon. The first 3 steps in Figure 2 are similar to the general workflow described above. Samples from a fragment analysis assay can also be used in a downstream sequencing step, making this application ideal for screening. The assay primers are designed like bisulfite sequencing primers (ie: designed to regions without CpGs) using Methyl Primer Express Software, so that PCR amplification is not dependent on methylation status. The forward primer has a fluorescent dye label (FAM™). The capillary electrophoresis step takes advantage of the mobility differences between C/T. Hence, methylated C-rich fragments migrate faster than unmethylated T-rich fragments (Figure 3). GeneScan™ 600 LIZ® Size Standard is used as a sizing marker. For data analysis, GeneMapper Software v4.0 or Peak Scanner Software v1.0 is used.

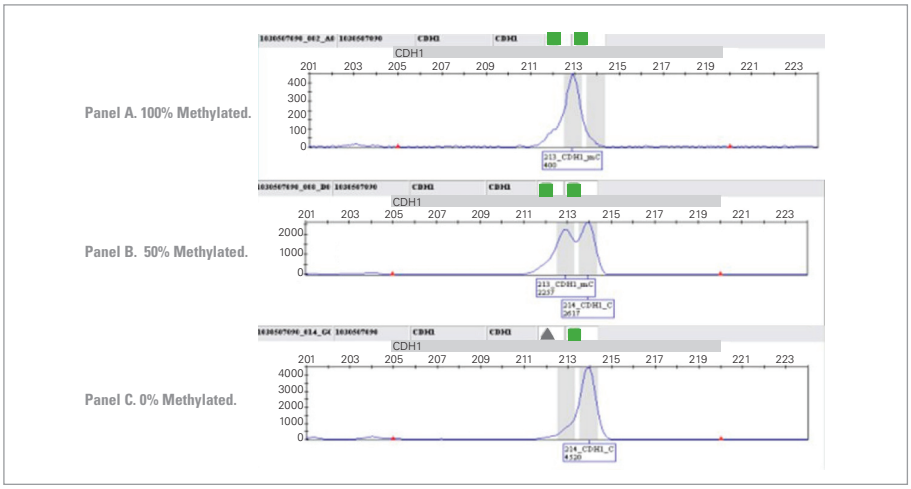

Figure 3. Cumulative Estimation for Methylation State of CpG Islands within an Amplicon. Methylated fragments migrate faster (Panel A, B) than those that are unmethylated (Panel C).

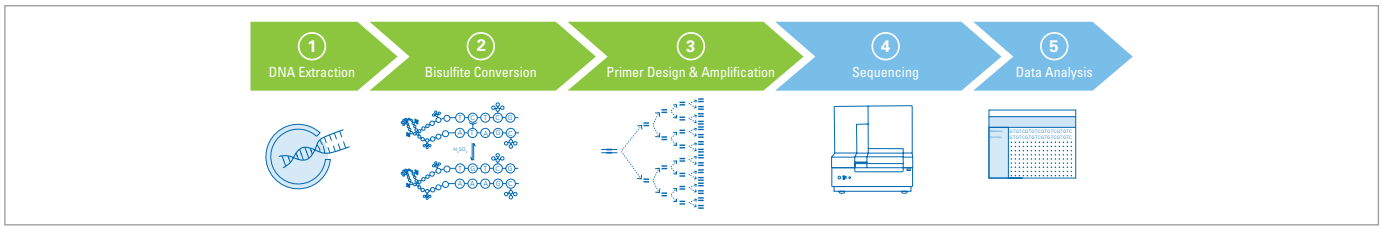

Figure 4. Bisulfite Specific PCR-based Sequencing.

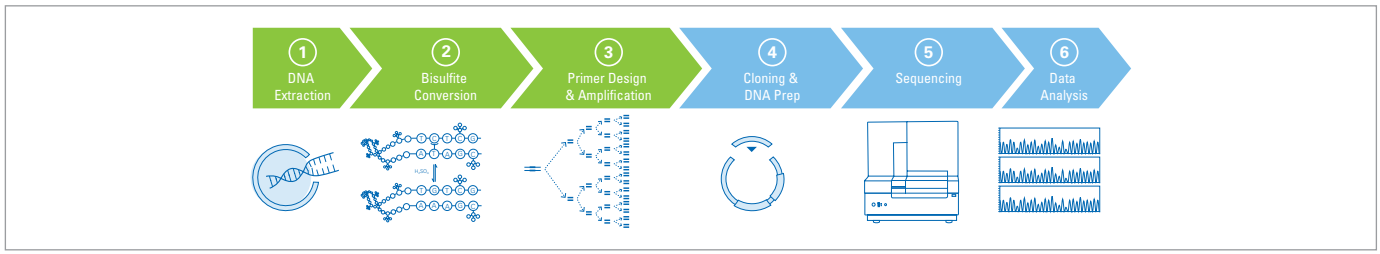

Figure 6. Bisulfite Specific Cloning-based Sequencing.

Workflow 2: Bisulfite Specific PCR-based Sequencing

This application is used to identify which CpG dinucleotides are important for the biological process under investigation. The first 3 steps of the workflow in Figure 4 are described in the general workflow above. Direct PCR sequencing of a pool of fragments takes place providing qualitative results and information on which CpG dinucleotide has changed. Figure 5 provides qualitative information for each CpG loci across the entire amplicon. For data analysis, Variant Reporter Software v1.0 or SeqScape Software v2.6 is used.

Workflow 3: Bisulfite Specific Cloning-based Sequencing

In this application, a population of fragments is segregated by cloning, providing quantitative results. The first 3 steps of the workflow in Figure 6 are described in the general workflow above. This is followed by sequencing and separation of single samples/clones. Analysis can determine the percentage of methylation observed for specific CpG dinucleotides in a candidate region. Figure 7 provides quantitative results to understand the percentage of methylation per CpG dinucleotide in mixed samples.

For data analysis, Variant Reporter Software v1.0 or SeqScape Software v2.6 is used.

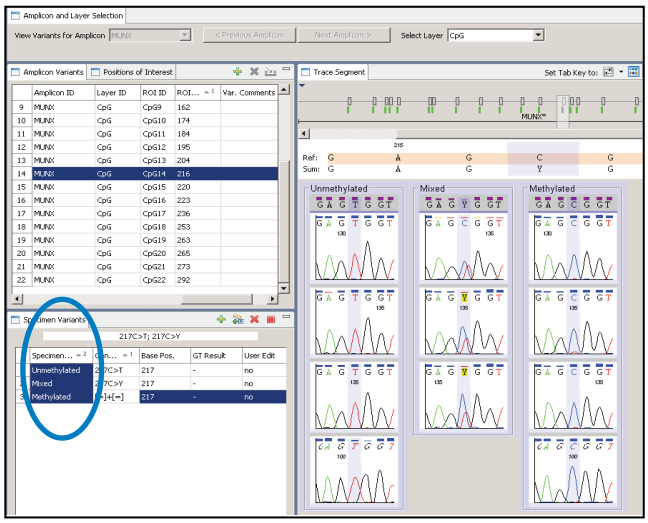

Figure 5. Qualitative Information for each CpG Loci across the Entire Amplicon. For a given CpG dinucleotide the result of the sample will be mixed, non-methylated, or methylated.

Complete Solution

These three methylation profiling workflows provide a complete solution from DNA extraction to data analysis provided by Applied Biosystems kits, reagents, instruments, and software. The user can expect cumulative, qualitative, and quantitative analysis using these workflows.

References

1. Feltus FA, Lee EK, Costello JF, Plass C, Vertino PM (2003) Predicting aberrant CpG island methylation, *Proc Natl Acad Sci* **100(21):**12253–12258.
2. Jones PA, Baylin SB (2002) The fundamental role of epigenetic events in cancer, *Nat Rev Genet* **3(6):**4z15–428.
3. Shi H, Wang MX, Caldwell CW (2007) CpG islands: their potential as biomarkers for cancer, *Expert Rev Mol Diagn* **7(5):**519–531.

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