Methyl-Seq Direct workflow: a fast method for DNA methylation analysis

Here we show:

- How to perform methylation analysis of DNA using a complete workflow based on Sanger sequencing
- Example sequencing data demonstrating the utility of the workflow for basic, translational, and clinical research
- How the Applied Biosystems™ SeqStudio™ Genetic Analyzer can facilitate DNA methylation analysis

Introduction

The presence or absence of methylation at carbon position 5 in the cytosine base (5-meC) of a CpG dinucleotide motif is a key epigenetic regulator of cellular and developmental processes in higher eukaryotes. For example, aberrant hypomethylation of certain oncogenes or hypermethylation of tumor suppressor genes can lead to cancer due to transcriptional activation or repression, respectively [1]. As a result, there is great interest in analyzing changes in DNA methylation patterns associated with cancer and other pathologies. This has led to the discovery of many genomic regions and specific loci where the presence or absence of CpG methylation influences cell and tissue physiology [2]. These discoveries can be extended by analyzing methylation state differences in the large number of archived clinically relevant samples. However, retrospective studies using these formalin-fixed, paraffin-embedded (FFPE) samples are hampered by the degraded state of the nucleic acids extracted from these samples. A DNA methylation analysis method that is compatible with FFPE tissues could open up avenues of research using this rich resource of retrospective samples.

SeqStudio Genetic Analyzer

The SeqStudio Genetic Analyzer offers a low-cost alternative to high-throughput instruments. This flexible capillary electrophoresis (CE) system can perform both automated Sanger DNA sequencing and high-precision sizing and analysis of multicolor fluorescent DNA fragments. The instrument can accommodate a 96-well plate loaded with sequencing reactions that are sequentially processed in batches of four samples per run. The sequencing samples can be processed in a 30 min “short” run cycle, resulting in DNA sequences of about 450 bases each.

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Next-generation sequencing (NGS) technology has opened the possibility to explore the biological significance of genome methylation on a massive scale. While NGS is useful for whole-genome, discovery-based methylome analysis, researchers utilize faster, easier, and more affordable technology to focus on loci that arise from the discovery-based approaches. To this end, Sanger sequencing is the ideal companion analysis method for follow-up studies. Sanger sequencing has been the gold standard for 5-meC detection for over 25 years [3]. This technique allows the detection of 5-meC at multiple sites along a short genomic DNA (gDNA) region of up to 400 nucleotides.

The most widely used method to detect 5-meC in DNA is bisulfite treatment of gDNA (Figure 1). In the bisulfite reaction, all unmethylated cytosines are deaminated and converted to uracils, while methylated cytosines remain unchanged. The method relies on the different reactivity of methylated vs. unmethylated cytosine to deamination by bisulfite at low pH. PCR is then used to amplify the region of interest, and the amplicons are subsequently sequenced to identify the bisulfite-induced changes. The changes can be read as a C>T change for unmethylated cytosines, and no sequence change for 5-meC, relative to the original sequence [3].

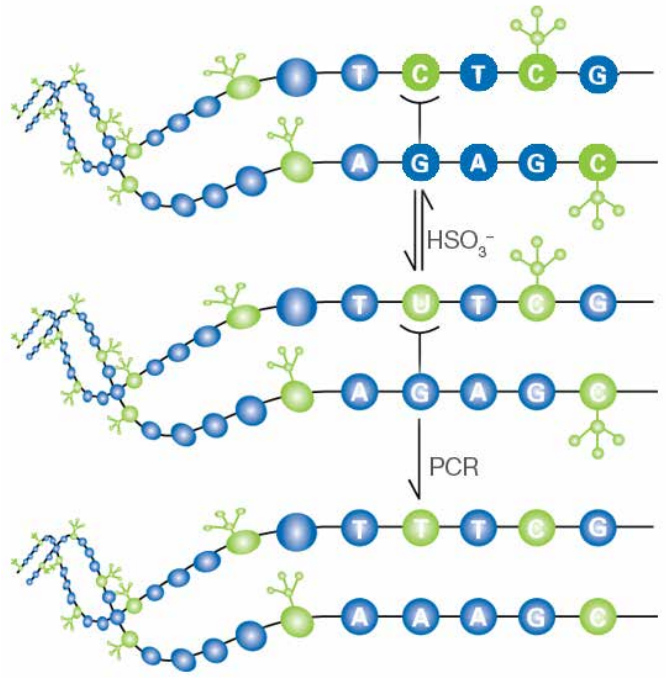


Figure 1. Overview of the bisulfite conversion process for detecting 5-meC. Bisulfite (HSO_3^-) treatment changes unmethylated cytosines to uracil but leaves methylated cytosines unchanged. Through PCR, U is replaced by T and 5-meC is replaced by unmethylated C. These changes can be detected by DNA sequencing of experimental and reference samples.

Here we demonstrate a novel method for bisulfite sequencing, termed the methyl-Seq Direct workflow, using the PCR and sequencing reagents of the Applied Biosystems™ BigDye™ Direct Cycle Sequencing Kit. For this workflow, extracted DNA is bisulfite modified using the Thermo Scientific™ EpiJET™ Bisulfite Conversion Kit. PCR primers specific for bisulfite-converted sequences are designed using Applied Biosystems™ Methyl Primer Express™ Software. PCR using these M13-tagged primers is then used to amplify the region of interest (ROI). The amplicon is sequenced using BigDye Direct Cycle Sequencing Master Mix and purified using the Applied Biosystems™ BigDye XTerminator™ Purification Kit. Sequences can be analyzed on Applied Biosystems™ CE instruments, such as the Applied Biosystems™ SeqStudio™ Genetic Analyzer and 3500 Series Genetic Analyzers. The entire workflow, including bisulfite conversion, PCR amplification, and DNA sequencing, takes about 7 hours, including about an hour of hands-on time. The workflow is compatible with FFPE samples, enabling the use of archived samples for retrospective studies.

Planning and preparing your experiment

Controls are essential for monitoring progress in this multistep procedure. We recommend 3 reference samples that will be valuable controls for the bisulfite conversion process and later data analysis:

- **A reference gDNA sample that is nearly completely methylated at all CpG sites:** This control is available as Thermo Scientific™ CpG Methylated Human Genomic DNA (Cat. No. SD1131). It is useful as a reference sample to identify the location of cytosines in the CpG context and facilitates the alignment of bisulfite-converted DNA sequence to the original, native DNA sequence. It also serves as a reference for determining whether a potential demethylation event has occurred in the sample of interest. If an individual-specific sample is required, the gDNA can be readily methylated nearly completely using Thermo Scientific™ CpG Methyltransferase (M.SssI) (Cat. No. EM0821).

- **A reference gDNA sample that is unmethylated at all CpG sites in the genome:** This control DNA can be generated *in vitro* by whole-genome amplification (WGA) of the gDNA using Thermo Scientific™ EquiPhi29™ DNA Polymerase (Cat. No. A39390). This control sample is useful to determine complete bisulfite conversion from C to T and for alignment with an experimental sample when methylation is expected or possible. Mixing fully methylated and unmethylated reference samples at defined ratios may also provide additional information on the quantitative level of methylation in a test sample.
- **A native control gDNA with "as is" methylation status:** In this control, some CpGs are methylated and others are not when the DNA is prepared from the cell or tissue sample. The Applied Biosystems™ Control DNA (from CEPH Individual 1347-02, Cat. No. 403062) is a reference gDNA prepared from a transformed cell line. This gDNA is derived from a female human, so one X chromosome is inactivated by methylation and the resulting compact chromatin structure. This feature can be a useful control for obtaining mixed sequencing traces for CpG sites at a theoretical 50:50 level when an X-linked region is sequenced after complete bisulfite conversion. YpG (CpG and TpG) base calls should result at former CpG sites with no other cytosines in the sequence trace. It also allows for assessment of any potential PCR or sequencing bias. However, a caveat to the use of gDNA from a transformed cell line is that abnormal methylation changes or patterns are common.

Defining the region of interest (ROI)

Obtain the original (i.e., natural) DNA sequence for your ROI from a genome browser (e.g., NCBI Genome Data Viewer or UCSC Genome Browser). For PCR primer design, we suggest adding about 300 bases up- and downstream of the immediate target area to provide sufficient sequence space for successful primer placement. The DNA sequence of the ROI plus the flanking regions (the PCR primer zones) can then be converted *in silico* to reflect the two most extreme bisulfite-converted DNA states:

- Fully unmethylated state: all C nucleotides are converted to T
- Fully methylated state: all C nucleotides in a CpG context remain C, all other C nucleotides are converted to T

This *in silico* conversion can be done by pasting the original DNA sequence into the methylation primer design program described in the next section. The two bisulfite-converted reference sequences are used later for data analysis (i.e., alignment of the sequencing trace file to each of these reference sequences). To map the location of methylation sites to the original DNA, it is important that the bisulfite-modified reference sequences are exactly derived from the original (i.e., no additions or deletions).

PCR primer design

PCR is the most critical step in the sequencing process for bisulfite-treated DNA. Bisulfite conversion drastically changes the template genome from a 4-base genome to a predominantly 3-base (G, A, T) genome with C (representing the former 5-meC) as a relatively minor component. Hence, PCR primer binding specificity is impaired and must be accounted for by increasing primer length to achieve a desired annealing temperature of 60°C. Detailed tips on optimizing PCR conditions have been described [4,5].

Primer design for bisulfite-converted DNA has many confounding factors to consider. The sequences obtained from methylated gDNA and unmethylated gDNA are fundamentally different after bisulfite conversion. The sequence from methylated gDNA will still have C at CpG sites, while the sequence obtained from unmethylated gDNA will not have C nucleotides. Primers for bisulfite-converted gDNA can be designed to anneal to a sequence that discriminates methylated gDNA from unmethylated gDNA (methylation-specific primers, MSPs). They can also be designed to a region without CpG sites (bisulfite-specific primers, BSPs) so that PCR amplification is not dependent on methylation status, which is the preferred design for direct sequencing. However, primer annealing sites are relatively restricted due to limited availability of regions that span a CpG island yet lack CpG sites in the primer annealing site. Selection of these primers can be further constrained by the relatively large number of closely spaced CpG motifs, which lead to primer sites too short to achieve a desirable T_m.

Another constraint is often the presence of relatively long (>9 bases) poly(T) sequences in bisulfite-converted templates that result in nonspecific primer annealing or poor amplification due to polymerase slippage. These sequences need to be avoided when designing primers. BSPs directed at CpG-flanking sequences in bisulfite-converted gDNA consist of only 3 bases and are T-rich. This often leads to primers >30 bases in length designed to obtain typical T_m values for PCR of ~60°C. These relatively long 3-base, T-rich primers are apparently more prone to mismatch hybridization—multiple amplification products are frequently obtained. The reverse primer is A-rich and thus provides favorable conditions for primer-dimer formation with the T-rich forward primer. Once the first cycle of amplification amplifies a mismatched sequence, subsequent PCR cycles are matched to that sequence, so PCR proceeds as efficiently as it would for the correctly matched sequence. Smaller amplicons from either primer-dimers or mismatched secondary amplicons have greater amplification efficiency and can outcompete the amplicon of interest. The presence of undesired amplicons usually diminishes the PCR efficiency of the intended amplicon. When selecting primers, a SNP database (e.g., dbSNP, SNPCheck) should be consulted to avoid designing primers over a SNP. To this end, the "parental" primer equivalents (i.e., the original primer DNA sequence before bisulfite conversion) must be used.

Methyl Primer Express Software is a free online primer design tool specifically for methylation studies that assists in designing primers for both methylated and unmethylated bisulfite-modified DNA. Users simply cut and paste the selected genomic sequence, then the software performs an *in silico* bisulfite conversion (Cs are converted to Ts) and aids in the selection of primers. Methyl Primer Express Software is available as a free download from Thermo Fisher Scientific (see Table 5).

For the methyl-Seq Direct workflow, the BSP primer design mode should be selected. The target-specific sequences of the PCR primers are then further expanded to include M13 tags at the 5′ ends that serve as binding sites for the sequencing primers. An example is shown in Figure 2. Sequencing primers can be ordered as Invitrogen™ Custom DNA Oligos at **thermofisher.com/oligos**.

Preparing genomic DNA from cells or FFPE slides for bisulfite conversion

DNA purity is a very important factor for successful bisulfite conversion. We recommend using a commercially available kit such as the Thermo Scientific™ GeneJET™ Genomic DNA Purification Kit for general-purpose gDNA purification.

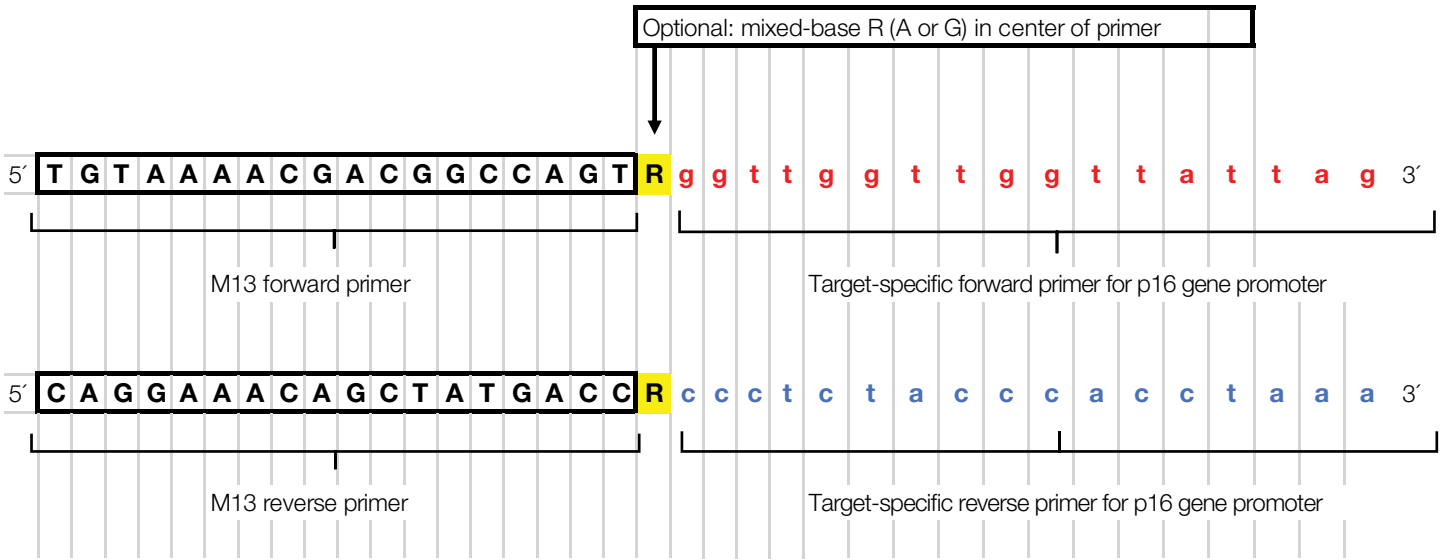


Figure 2. Example of a bisulfite PCR primer pair design for the methyl-Seq Direct workflow. The forward and reverse PCR primers are composed of the M13 sequence tags followed by the target-specific primer sequences. Optional is the inclusion of a mixed-base R (A or G) nucleotide between the M13 and target sequences. The R nucleotide will result in a mixed-base Y (C or T) readout on the strand complementary to the primer. This Y readout is a useful indicator of the incorporation efficiency of a C or T base by the sequencing polymerase into a mixed base such as a heterozygous CpG/5-meCpG allele. Note that the M13 primers contain all 4 bases whereas the target-specific primers only contain 3 bases (g, a, t for the forward primer and c, t, a for the reverse primer). The 4-base composition in the M13 primers is beneficial for base-calling accuracy. The target-specific primer sequences shown are for PCR of the bisulfite-converted p16 gene promoter.

FFPE DNA poses an additional challenge, as the DNA is often covalently crosslinked to proteins or other entities. The Invitrogen™ RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE efficiently purifies gDNA that is suitable for bisulfite conversion and subsequent PCR sequencing. The gDNA of higher organisms is densely and tightly bound by histones and other proteins. To access all cytosine residues in the genome with the bisulfite reagent, it is crucial to remove DNA-binding proteins as much as possible by Proteinase K incubation at 50°C for an extended time (>12 hr for fresh cells and over 40 hr for FFPE samples). Further details are provided in a dedicated product bulletin [6].

Bisulfite conversion

The EpiJET Bisulfite Conversion Kit is designed for easy and reliable bisulfite conversion of DNA for methylation analysis. The thermal DNA denaturation and the bisulfite conversion reaction are integrated into a 2.5 hour run on a PCR thermal cycler. The converted DNA is then bound to the membrane of a micro-column for on-column desulfonation and subsequent DNA purification (washing) steps. The converted DNA is eluted in a low volume of elution buffer (typically ~20 µL) and is suitable for several techniques used for the analysis of methylation status, including PCR and sequencing. For optimal results, we suggest using 200–500 ng of input DNA. Using higher amounts of DNA may result in incomplete bisulfite conversion for some GC-rich regions. Note that since the bisulfite-converted DNA strands are no longer complementary, this DNA stays mainly in single-stranded form and is therefore unsuitable for analysis on agarose gels. To calculate the concentration of converted DNA using a spectrophotometer (1 cm path length), multiply the measured absorbance at 260 nm by 40 µg/mL and the dilution factor.

PCR optimization

For economical PCR and cycle sequencing of a small number of samples (e.g., 1–16 samples), we recommend the use of PCR strip tubes with attached caps and the Applied Biosystems™ ProFlex™ 3 x 32-Well PCR System. This versatile thermal cycler is ideal for optimizing PCR conditions, as it supports flexible temperature zones and allows 3 independent PCR or sequencing experiments in parallel. The PCR products should be checked by agarose electrophoresis prior to sequencing. To this end, we recommend the use of Invitrogen™ E-Gel™ EX Agarose Gels, 2%, and an appropriate size marker such as the Invitrogen™ E-Gel™ Ultra Low Range DNA Ladder. A successful PCR reaction presents as a clearly visible, single band migrating at the predicted size. We recommend loading a 20 µL sample consisting of 1.5–2 µL of the PCR reaction diluted to 20 µL with water. The intensity of the PCR amplicon is expected to be similar to a marker band (~5–10 ng) of similar size. The presence of one or more additional bands on the gel indicates nonspecific amplification or formation of primer-dimers, which will interfere with sequencing. A primer-dimer band typically migrates at 80–100 bp. Primer-dimer formation is often difficult to suppress by varying the PCR conditions and may require redesign of the PCR primer pair or removal of the primer-dimer by preparative gel electrophoresis or ultrafiltration.

The methyl-Seq Direct workflow

The methyl-Seq Direct workflow uses the PCR and sequencing reagents of the BigDye Direct Cycle Sequencing Kit for bisulfite sequencing. Sanger sequencing with the BigDye Direct Cycle Sequencing Kit offers advantages over standard BigDye Terminator kits, including no PCR purification required prior to cycle sequencing and superior resolution at the 5´ end of the sequence for accurate base calling.

The methyl-Seq Direct workflow deviates from the original BigDye Direct protocol by using only a fraction of the PCR material for DNA sequencing. This modification makes the protocol more economical and allows for PCR quality control by agarose gel electrophoresis. In addition to the BigDye Direct kit, a small volume (1 mL) of Applied Biosystems™ BigDye™ Terminator v1.1 & v3.1 5X Sequencing Buffer is needed. This buffer is available as an individual product but also is included with the regular BigDye Terminator v.1.1 or v.3.1 Cycle Sequencing Kit.

PCR of the ROI

The PCR reaction for bisulfite-converted DNA is set up as shown in Table 1. The PCR cycling conditions may have to be optimized for each specific ROI and primer pair. A typical profile used for amplification is shown in Table 2.

Table 1. Reagent setup for a typical PCR reaction using BigDye Direct PCR Master Mix from the BigDye Direct Cycle Sequencing Kit.

Reagent	Volume for 1 PCR reaction (1 specimen)
BigDye Direct PCR Master Mix	5.0 µL
PCR primer pair for ROI with M13 tags (10 µM each)	0.5 µL
Bisulfite-converted DNA (1–10 ng)	1.0 µL (adjustable with water)
Molecular biology–grade water (not included in BigDye Direct kit)	3.5 µL (adjustable with DNA input)
Total volume	10.0 µL

Table 2. General thermal cycling profile for PCR with bisulfite-converted DNA.

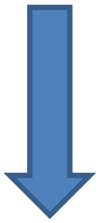
Stage	Temperature	Time
Hold	95°C	5 min
Cycle (5x)	95°C	10 sec
	56–60°C (variable)*	2 min
	72°C	3 min
Cycle (35x)	95°C	10 sec
	65°C	30 sec
	72°C	1 min
Hold	4°C	Indefinitely

* The optimal temperature needs to be determined experimentally [7]. An initial round of 5 cycles is run where the target-specific primers are given time to generate amplicons from the gDNA. This is followed by 35 cycles for more rapid amplification of the primary amplicon material at a higher temperature that exploits the higher melting temperature of the extended M13-tagged primers.

Cycle sequencing

After verification by agarose gel electrophoresis that the expected PCR amplicon is formed in sufficient quality and quantity, the sequencing reaction is set up as shown in Table 3. The thermal cycling conditions for the sequencing reaction are shown in Table 4.

Table 3. Setup of the forward and reverse sequencing reactions using BigDye reagents and sequencing primers.

Reagent	Volume for 1 forward sequencing reaction	Volume for 1 reverse sequencing reaction
Molecular biology grade water (not included in BigDye Direct kit)	5 µL	5 µL
BigDye Terminator v1.1 & v3.1 5X Sequencing Buffer (not included in BigDye Direct kit)	1.5 µL	1.5 µL
BigDye Direct M13 forward primer (included in BigDye Direct kit)	0.5 µL	–
BigDye Direct M13 reverse primer (included in BigDye Direct kit)	–	0.5 µL
BigDye Direct Sequencing Master Mix (included in BigDye Direct kit)	1 µL	1 µL
Total volume	9 µL	9 µL
		
Well or tube 1	9 µL of forward sequencing reaction + 1 µL* of PCR	
Well or tube 2		9 µL of reverse sequencing reaction + 1 µL* of PCR

* The volume of the input PCR product can be increased by 0.5 or 1 µL in case the yield of PCR amplicon was low.

Table 4. Thermal profile for the cycle sequencing reaction on a ProFlex PCR System or other Applied Biosystems™ thermal cycler.

Stage	Temperature	Time
Hold	37°C	15 min
Hold	80°C	2 min
Cycle (25x)	95°C	10 sec
	50°C	5 min
	60°C	1 min
Hold	4°C	Indefinitely

Sequencing reaction cleanup

After cycle sequencing, 50 µL of BigDye XTerminator suspension mix is added to each sequencing reaction to remove unincorporated dye terminators. The suspension mix consists of 1 part of BigDye XTerminator beads (10 µL/sample) and 4 parts of SAM™ Solution (40 µL/sample) and is prepared in bulk for all samples shortly before use. The sequencing plate with the treated samples is sealed and vigorously vortexed for 30 min and then centrifuged for 1 min to pellet the beads; 20 µL of the supernatant is then transferred to a fresh PCR plate, centrifuged for 1 min to remove any residual air bubbles, and then placed on the SeqStudio Genetic Analyzer for CE.

Sequencing by CE on the SeqStudio Genetic Analyzer

The samples can be run using the short (30 min) or medium (45 min) sequencing run module. Note: Do not use a BigDye XTerminator run module, since the supernatant CE sample does not contain a bead bed.

Data analysis

Due to the complexity of mapping sequences in unmethylated CpG regions, the analysis of bisulfite-converted DNA sequencing data can be complicated if no alignment tool is used. Several third-party bioinformatics software providers offer useful gene alignment programs to aid in data analysis. Thermo Fisher Scientific offers several tools for alignment and detection of sequence variants, including those produced by bisulfite conversion. Technically, the absence of a "C" and presence of a "T" in a (formerly) unmethylated CpG locus can be treated just like a common SNP variant. Applied Biosystems™ SeqScape™ Software is the preferred software for high-throughput CpG genotyping of Sanger sequencing trace files in table format. Applied Biosystems™ Variant Reporter™ Software is a user-friendly local application (Microsoft™ Windows™ 7 or 10 Software) for variant detection by alignment of sequence trace files to a reference sequence in text format. Both applications are included with the purchase of a SeqStudio Genetic Analyzer. Applied Biosystems™ Variant Analysis (VA) module is freely available cloud-based software that offers numerous benefits, including the capability to export Sanger sequencing trace files (.ab1) into numerical peak files (.csv) for quantitative studies of mixed-peak heights. Several publications have demonstrated the capability to use the peak height data from direct sequencing of a PCR amplicon derived from a nonclonal gDNA pool for semiquantitative assessment of methylation status [8,9]. Depending on the initial question of whether methylation or demethylation is to be detected, the previously mentioned *in silico* bisulfite-converted (text) sequences can be used as references for variant detection.

Figure 3. Sequencing of the p16 gene promoter region using CEPH individual 1347-02 gDNA as template. Primer sequences for this ROI are shown in Figure 2. **(A)** gDNA template was amplified by WGA prior to bisulfite conversion leading to complete removal of 5-meC (unmethylated control). Note the absence of any (blue) "C" peaks. **(B)** gDNA template was treated prior to bisulfite conversion *in vitro* with M.SssI CpG methyltransferase leading to complete methylation of all CpG dinucleotides (full methylation control). Note the presence of blue "C" peaks upstream of "G" peaks. **(C)** gDNA template "as is" (i.e., the native state of the genome when it was extracted from the pool of cells). Note the differential and partial methylation pattern. The CEPH DNA is derived from a transformed cell line and may therefore display an aberrant methylation pattern reflecting this physiological state.

Example data from the methyl-Seq Direct workflow

Unmethylated CpG is the default state

The cyclin-dependent kinase inhibitor 2A gene *CDKN2A*, also known as the p16 gene, encodes a 16 kDa protein that slows cell division by slowing the progression of the cell cycle from the G1 phase to the S phase, thereby acting as a tumor suppressor. Mutations resulting in malfunction or reduction of function of the *CDKN2A* gene by hypermethylation are associated with increased risk of a wide range of cancers, and alterations of the gene are frequently seen in cancer cell lines.

A 248 bp segment of the p16 gene promoter was amplified from bisulfite-converted DNA and sequenced as previously outlined. Figure 3 shows the results of CEPH control DNAs that were either completely unmethylated by WGA, fully methylated *in vitro*, or processed as is. The control DNAs show the expected presence or absence of methylated cytosines, and the as-is sample reveals some discrete methylation at certain CpG sites, consistent with the transformed nature of the CEPH cell line used as a source for the gDNA.

To demonstrate the feasibility of the protocol with DNA extracted from FFPE tissue using the RecoverAll Total Nucleic Acid Isolation Kit, two pairs of matched normal and tumor tissue samples were processed. Figure 4 shows that discrete changes are apparent in the methylation pattern between normal and tumor tissue.

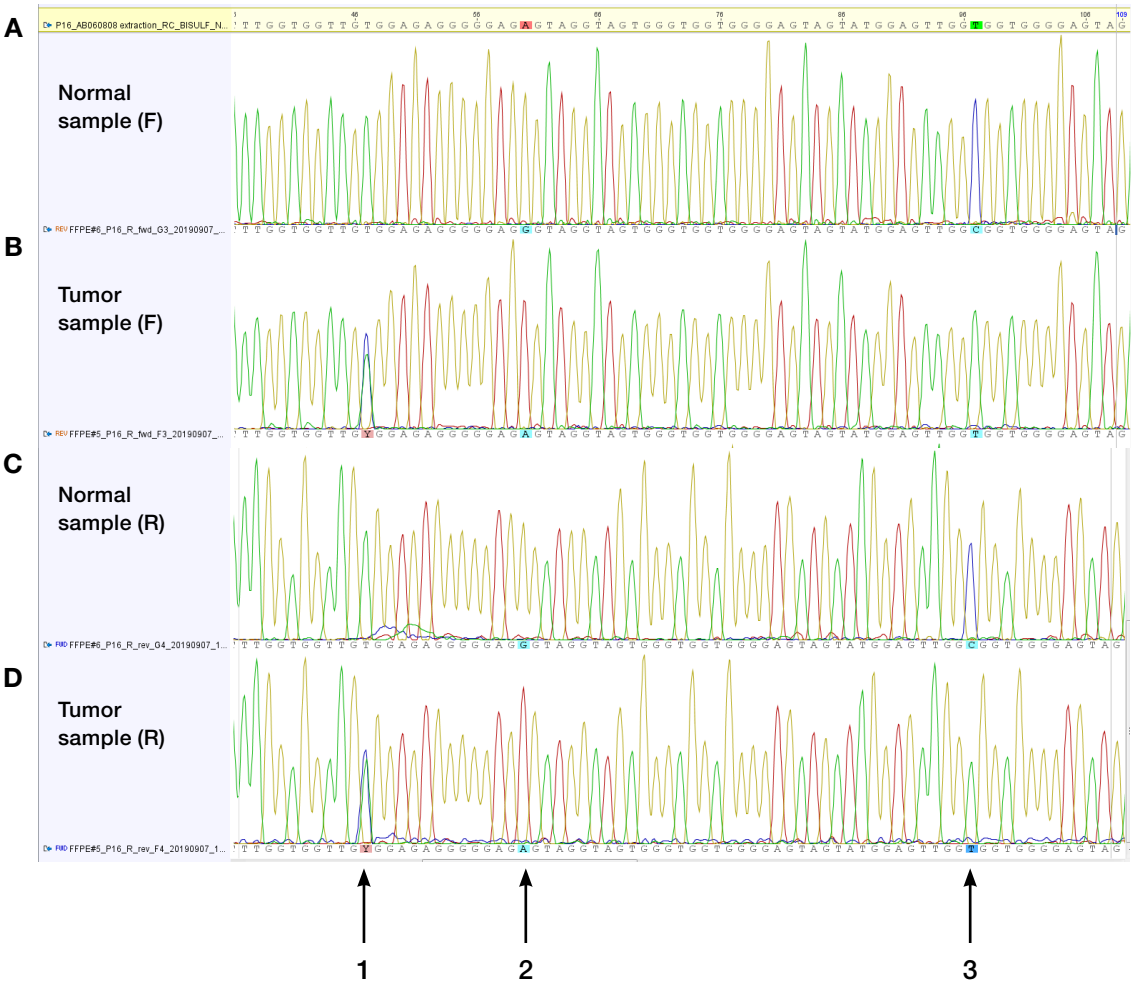


Figure 4. Bisulfite sequencing of the p16 gene promoter using FFPE DNA from a colon normal–tumor pair. (A) Normal sample, forward sequence. **(B)** Tumor sample, forward sequence. **(C)** Normal sample, reverse sequence. **(D)** Tumor sample, reverse sequence. Numbers indicate (1) balanced 50:50 methylation in the tumor sample, (2) a G>A variant not related to methylation, and (3) a demethylation event in the normal sample.

Methylated CpG is the default state

The product of the *MGMT* gene (O^6-methylguanine DNA methyltransferase) repairs mutations of guanine and prevents mismatch and errors during DNA replication and transcription, thus playing an important role in genomic stability. In experimental models of exposure to alkylating agents, lack of MGMT increases carcinogenic risk. Deficiencies of *MGMT* gene function are frequently found in various solid cancer types, including colorectal and brain cancer. Gene expression is epigenetically regulated on multiple levels, including CpG methylation. Loss of CpG methylation correlates with poor prognosis in glioblastoma patients. Figures 5 and 6 are examples of bisulfite sequencing data from the *MGMT* promoter [4]. Note the emerging demethylation of CpG in cells of tumor origin, including the transformed CEPH cell line.

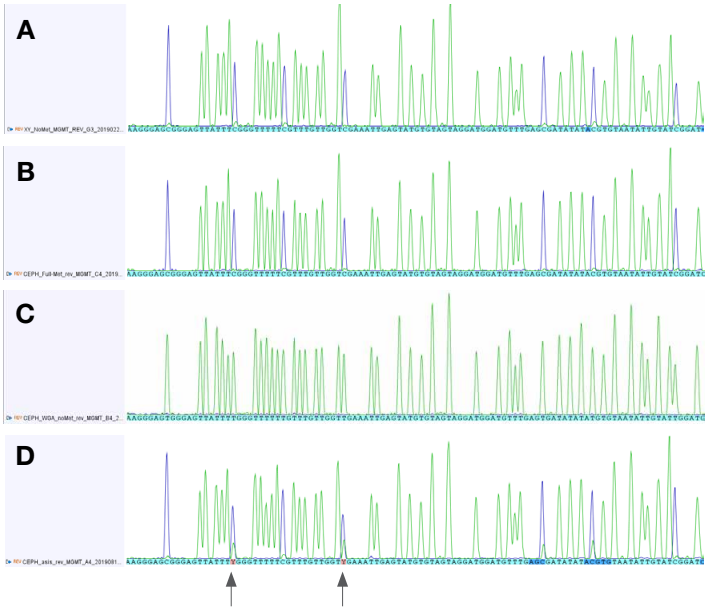


Figure 5. Bisulfite sequencing of the *MGMT* gene promoter. For clarity, only C (blue) and T (green) sequence traces are displayed. **(A)** gDNA isolated from white blood cells, normal male. Note the default state of CpG methylation. **(B)** CEPH DNA fully methylated *in vitro*. **(C)** CEPH DNA amplified by WGA to remove methyl groups from cytosine. **(D)** CEPH DNA "as is" (i.e., as isolated from transformed cell line). Note the emerging demethylation at some CpG sites (arrows).

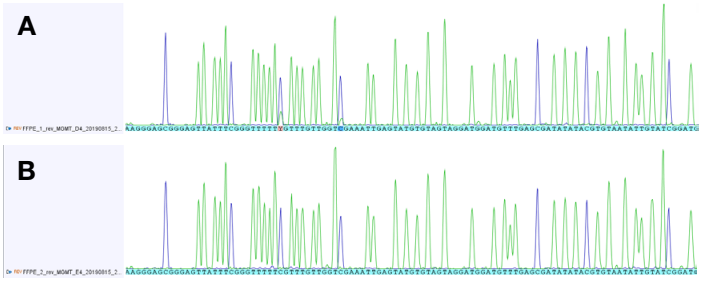


Figure 6. *MGMT* methylation in FFPE samples. (A) FFPE DNA from tissue of a colorectal tumor preparation. **(B)** Matching FFPE DNA from normal adjacent tissue. Note the emerging demethylation in CpG 3 and 4 (from left to right) of the tumor sample **A**, visible as a minor peak.

Conclusions

We have shown the feasibility of a direct PCR sequencing workflow for methylation analysis of bisulfite-converted DNA from cell lines and FFPE samples using reagents, instrumentation, and software available from Thermo Fisher Scientific (summarized in Table 5). The SeqStudio Genetic Analyzer, an affordable instrument capable of both Sanger sequencing and fragment analysis, enables rapid results for follow-up methylation research studies. This application note provides a novel, streamlined Sanger sequencing workflow for DNA methylation analysis that is compatible with archived FFPE samples, opening up the possibility of using such samples for retrospective studies.

Table 5. Recommended products and additional information for the methyl-Seq Direct workflow.

Product	Cat. No.
Preparation of control DNAs	
CpG Methylated Human Genomic DNA	SD1131
CpG Methyltransferase (M.SssI)	EM0821
Exo-Resistant Random Primer	SO181
dNTP Set	R0181
Pyrophosphatase, inorganic	EF0221
Control DNA (from CEPH Individual 1347-02)	403062
Primer design and synthesis	
Methyl Primer Express Software v1.0	4376041
Invitrogen custom oligos	**thermofisher.com/us/en/home/life-science/oligonucleotides-primers-probes-genes.html**
Sample preparation	
GeneJet Genomic DNA Purification Kit	K0721
RecoverAll Total Nucleic Acid Isolation Kit for FFPE	AM1975
Bisulfite conversion	
EpiJET Bisulfite Conversion Kit	K1461
PCR	
Proflex 3 x 32-Well PCR System	4484073
EasyStrip Plus Tube Strip with Attached Flat Caps	AB2000
E-Gel EX Agarose Gels, 2%	G402002
E-Gel Ultra Low Range DNA Ladder	10488096
Cycle sequencing	
BigDye Direct Cycle Sequencing Kit (includes PCR reagent)	4458689
BigDye Terminator v1.1 & v3.1 5X Sequencing Buffer	4336697
BigDye XTerminator Purification Kit	4376486
SeqStudio Genetic Analyzer System with SmartStart Orientation (includes secondary analysis software)	A35645
Data analysis	
SeqScape Software (included with SeqStudio SmartStart Orientation)	4474978
Variant Reporter Software v3.0 (included with SeqStudio SmartStart Orientation)	A38884
Analysis Module Variant Analysis (VA) Software (available for free on cloud-based Connect platform)	A28220

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