Applied Biosystems

Methylation Analysis Using FFPE Samples—Reliable and Reproducible

DNA methylation is involved in the regulation of many cellular processes, including X chromosome inactivation, chromosome stability, chromatin structure, embryonic development, and transcription. As a key player in regulatory processes, it is not surprising that aberrant DNA methylation has been implicated in many developmental and disease systems, including cancer [1]. DNA methylation is the addition of a single methyl group to the cytosine (C) residue in cytosine-guanine (CG) pairs. This methyl group is not preserved during amplification processes such as PCR or whole genome amplification (WGA). To overcome this limitation, it is possible to pre-treat the DNA with bisulfite, which converts all of the cytosines to uracils (U), but leaves the methylated cytosines unchanged. Bisulfite sequencing is the gold standard in methylation analysis, because only sequencing provides direct detection of methylation events, as well as information across the entire amplified region.

When working with archival material, researchers often need to use DNA from samples that have been stored as formaldehyde- or paraformaldehyde-fixed paraffin embedded (FFPE) tissue blocks. These FFPE samples are a valuable source of information for retrospective research, often providing complete historical and clinical information on treatments, procedures, or outcomes. Maintenance of the tissue structure of FFPE samples is optimized for histological analysis, but collateral damage to the nucleic acid incurred through the fixation, embedding, and storage processes can impede sequencing and methylation analysis.

Applied Biosystems provides a streamlined workflow for bisulfite sequencing that takes researchers from DNA isolation to final results. In this article, we describe step-by-step how to generate suitable high quality DNA and perform bisulfite sequencing on FFPE samples.

Figure 1. Formaldehyde-Fixed, Paraffin-Embedded (FFPE) Methylation Sequencing Workflow.

Ambion®

Step 1. DNA Isolation From FFPE Tissues

Isolating DNA from FFPE tissues provides several challenges for downstream analysis of methylation patterns. Fragmentation, protein contamination and chemical modifications all contribute to poor DNA quality and quantity following extraction from FFPE blocks. DNA isolation methods must be optimized to efficiently remove protein contaminants and extract a wide range of DNA fragments.

Determine Percent Functionality (Optional):

The extent of DNA degradation and protein contamination dramatically impact the efficiency of bisulfite conversion, PCR amplification and sequencing reactions. Unlike fragmentation, DNA modification and protein contamination cannot be assessed by looking at size alone. The amount of functional DNA may be determined by using an assay targeting the single copy gene RNase P. See calculation percentage of Functional Template, page 3.

Case Study: Isolating Superior Quality DNA from Human FFPE Samples

Human FFPE samples comprising a wide range of tissue types (kidney, placenta, pancreas, normal breast, tumor, breast, skin) and block ages (2–18 years) were used for this study (Figure 2).

Genomic DNA (gDNA) was extracted from 20 µM sections of the FFPE samples using the Ambion® RecoverAll™ Total Nucleic Acid Isolation Kit. This kit enables the release and recovery of trapped nucleic acid from the crosslinked matrix, and provides better protein removal when compared to FFPE gDNA isolated using other kits.

The FFPE tissue sections were first deparaffinized using a series of xylene and ethanol washes (Figure 3). Then sections were subjected to a rigorous protease digestion using 400 µL Digestion Buffer and 4 µL Protease and an incubation time tailored for recovery of DNA. The nucleic acid was purified using a rapid glass filter methodology that included an on-filter nuclease treatment: 60 µL RNase Mix (10 µL RNase A + 50 µL nuclease-free water) was added to each Filter Cartridge and incubated for 30 minutes at 37°C. Finally, recovered DNA was eluted into either water or the low salt buffer provided.

Tissue	Block Age (Years)	DNA Percent Functionality
Testis	14	3%
Squamous mucosa	14	13%
Placenta	8	25%
Kidney	2	16%
Pancreas	3	27%
Normal breast	18	7%
Skin	8	5%
Tumor breast	9	4%

Figure 2. FFPE Specimens from Various Tissue Sources.

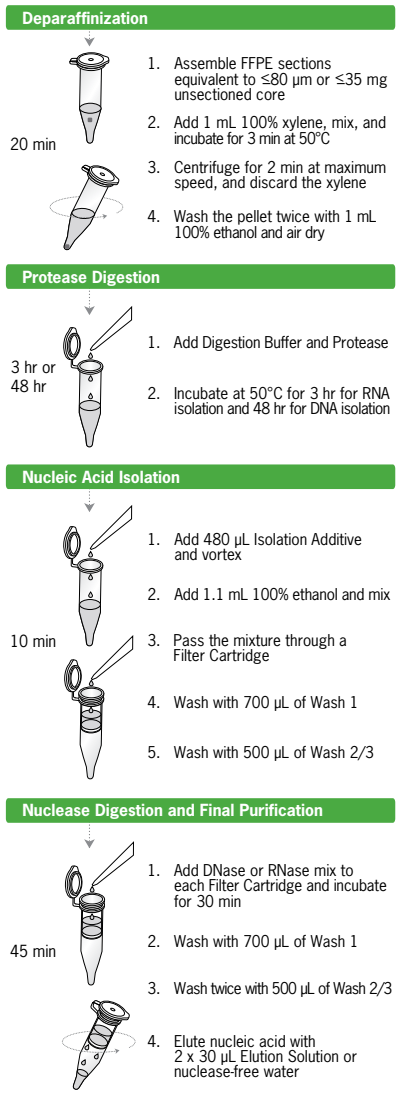


Figure 3. Overview of the Ambion® RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE

TIP: Achieve Higher DNA Quality by Increasing Protease Digestion Incubation Time

Extending the 50°C protease digestion from 3 hours to 16 hours or longer has shown to release substantial amounts of DNA from FFPE samples. For most samples the yield of DNA per mass of tissue will be about the same when comparing overnight and 2-day incubations as was done here. The benefit of the longer incubations is that they provide slightly better quality DNA as determined by TaqMan® RNase P Detection Reagents (data not shown).

Calculating Percentage of Functional Template

A "percent functionality" of the gDNA was calculated to determine which blocks should be included in this experiment. Applied Biosystems TaqMan® RNase P Detection Reagents Kit, which provides the components needed to detect and quantitate genomic copies of the human RNase P gene using the 5' nuclease assay was used for this purpose.

The amount of functional DNA template in each sample was measured by comparison to a standard curve generated with the DNA template standards, RNase P gene primers, and probe provided in the Applied Biosystems TaqMan® DNA Template Reagents Kit and the TaqMan RNase P Detection Reagents Kit. The average percentage of functional template in the recovered FFPE DNA samples was found to be 10.07 ± 7.08%, relative to the total DNA content indicated by A_{260} measurements. This shows that A_{260} values are an unreliable gauge of functional DNA template for FFPE samples.

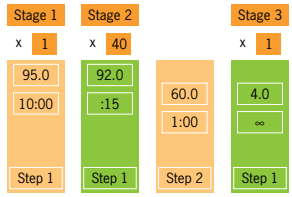
For each DNA sample, 10 ng (as calculated by A_{260} measurement) in a 10 µL volume were amplified in triplicate using the TaqMan RNase P Detection Reagents Kit and TaqMan® Universal PCR Master Mix, No AmpErase® UNG. Quantitative PCR (qPCR) signal intensities (data not shown) were compared to a standard curve to quantify the amount of functional template and to calculate the percentage of functional template in each FFPE DNA sample (Figure 2).

1. **Prepare FFPE DNA samples and control**
   Prepare 10 ng (A_{260}) of each DNA sample in 4.5 µL nuclease-free water. For a positive control, use 10 ng of the Human Genomic Control DNA included in the TaqMan® RNase P Detection Reagents Kit, which provides a calibrated human genomic DNA sample.
2. **Prepare DNA standard curve**
   Using the DNA standards provided in the TaqMan® DNA Template Reagents Kit, prepare a curve with each template in 4.5 µL nuclease-free water (see "Standard Curve for FFPE Samples", below, for more details).
3. **Prepare the PCR plate using the following components:**

Components	Volume/Well (µL)
DNA sample [10 ng (A_{260})] and standards	4.5
2X TaqMan® Universal PCR Master Mix	5
20X RNase P Primer-Probe (FAM™ dye) Mix	0.5
Total	10

   Mix by pipetting up and down 2–3 times. Apply optical adhesive cover and briefly centrifuge reaction plate.

4. **Run the plate on a Real-Time PCR System using the thermal cycler protocol:**



5. **Create a standard curve to quantify the amount of functional template in each FFPE DNA sample, and use this to determine the percentage of functional template.** Quantity (ng)/10 ng*100% = Percent Functional DNA

Figure 4. Overview of the Protocol for Calculating the Percentage of Functional Template

Standard Curve for FFPE Samples

The range of standard curve copy number should bracket anticipated copy numbers of the unknown samples on the same plate. The TaqMan® DNA Template Reagents Kit includes a standard dilution series of human gDNA for easy preparation of a standard curve (Step 2 in figure 4). A range of 6061 copies to 24 copies (20 ng to 24 pg, respectively, of DNA template standard) bracketed the majority of unknown FFPE DNA samples at 10 ng (as calculated by A_{260}) input.

Step 2. Bisulfite Conversion

Treatment of gDNA with bisulfite allows distinction between methylated and unmethylated cytosines (C). It is challenging to achieve complete and highly selective bisulfite conversion of non-methylated Cs without significant side-reactions or extensive cleavage of gDNA. An incomplete conversion will lead to an artificial increase of different methylation patterns, and false positive results.

Case Study: Effective Bisulfite Conversion

In this study, the Applied Biosystems methylSEQr™ Bisulfite Conversion Kit, was used to convert non-methylated cytosines (C) in the DNA sample to uracils. A key feature of this kit is the use of centrifugal filtration for the isolation and purification of gDNA after treatment with bisulfite. This unique method reliably provides a high recovery of shelf-stable, bisulfite-converted gDNA.

The FFPE samples listed in Figure 2 were treated with bisulfite using the methylSEQr Kit. In all cases, 300 ng of input gDNA was used for the reaction, purified using methylSEQr columns, and recovered in a final volume of 50 µL. It is important not to exceed 300 ng of gDNA per conversion reaction, as excess gDNA may result in incomplete bisulfite conversion.

After PCR amplification, the sequence of the bisulfite converted DNA will have cytosine (C) residues only if the C was methylated and occurred adjacent to guanine (G), forming a CpG motif (Figure 5). All other nonmethylated Cs will be detected as thymine (T). An unmethylated gDNA sample will have no Cs in the sequencing data. Comparison of sodium bisulfite-treated DNA sequences with sequences obtained from untreated gDNA allows the precise identification of all methylated cytosines within a stretch of DNA (Figure 9).

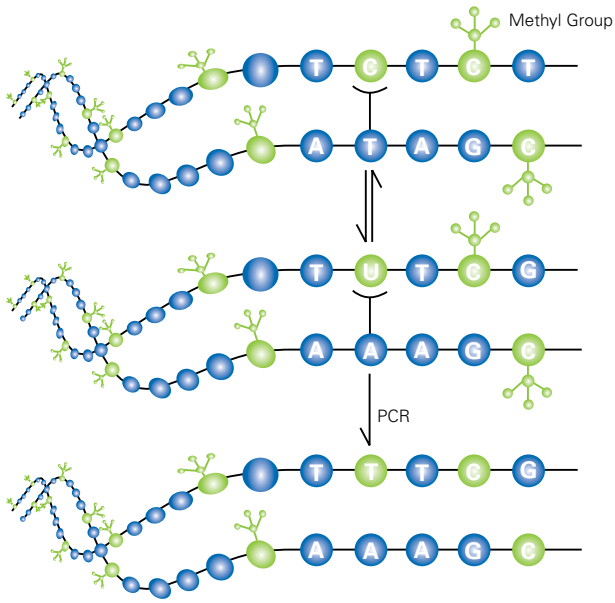


Figure 5. CpG Motif. The methylation-susceptible cytosines occur next to Guanine residues as a CpG dinucleotide. CpG islands (that is, clusters of CpGs with a GC content of >55% in ~500 bp regions) are often found in the regulatory regions of genes.

Veriti™ 96-Well Thermal Cycler

The Veriti 96-Well Thermal Cycler delivers the proven reliability of the Applied Biosystems GeneAmp® PCR System 9700 and more. With the flexibility to run fast or standard PCR, and the capability to perform PCR optimization with VeriFlex™ Blocks, the Veriti Thermal Cycler can handle all your PCR applications. A screen provides a powerful, yet simple user interface geared to simplify both instrument setup and use.

methylSEQr™ Bisulfite Conversion Kit

The methylSEQr Bisulfite Conversion Kit is a robust method for generating high quality bisulfite-converted DNA for optimized methylation analysis. The kit uses size exclusion column purification, increasing your sample yields and reducing the amount of bias seen with resin purification methods. Unbiased DNA recovery of methylated and unmethylated fragments is critical, as it enables more accurate downstream quantitative analyses. The kit's gentler denaturation conditions reduce DNA fragmentation and increase the amount of high quality DNA available for amplification. With low DNA input requirements and increased sample stability, the methylSEQr Kit produces converted DNA uniquely suited to clinical research applications.

Step 3. Assay Selection and Amplification

Assay Design

Several recent studies have indicated that methylation is initiated near the transcription start site, and spreads out over the entire region as loss of gene expression becomes more pronounced. CpG motifs (Figure 5) are underrepresented in the human genome; however, a significant percentage of genes have regions of relatively high CpG-density in the promoter known as CpG islands. The correlation between loss of expression and methylation is most frequently observed the closer the methylation occurs to the transcription start site. To understand methylation status of a particular region of DNA, primers should be designed near the promoter sequence and transcription start site of the target gene of interest.

Case Study: Using Bisulfite-Converted Template in PCR

Four gene targets, often reported to be methylated regions in cancer, were assessed for methylation status (Figure 6). Relatively small amplicon sizes were selected due to the fragmented nature and low percentage of functional template in gDNA from FFPE preserved tissue. Methyl Primer Express® Software v1.0 was used to design primers for the same region before and after bisulfite conversion, which converts Cs to Ts unless methylated.

Based on a 10 µL PCR volume, 4.5 µL bisulfite converted solution (6 ng/µL) provided 27 ng of DNA per well. The thermal cycler program used is listed in Figure 7, step 3.

The gene targets were also amplified from gDNA prior to bisulfite conversion, using the same volume of master mix, gDNA solution (6 ng/µL or 33 ng), and primers. In all cases, the appropriate volume of RecoverAll™ gDNA was diluted into water to give 300 ng in 45 µL, the same as when performing a bisulfite conversion. However, these dilution were used, as is, in PCR. This allowed for direct comparison of the sequence whether before or after bisulfite treatment (Figure 9).

NOTE: PCR master mixes containing uracil DNA glycosylase (UNG) should not be used in bisulfite PCR. UNG cleaves uracil-containing DNA and will degrade uracil-containing template produced by bisulfite conversion of gDNA.

Gene Target	Size of Amplicon	Size with M13 tails	# CpG in Amplicon
RARβ2	111	147	10
GSTP	100	136	7
PTEN	184	220	10
CDH1	195	231	14

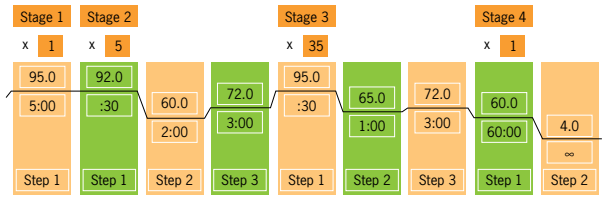
Figure 6. Gene Targets From Methylated Regions.

1. Prepare a solution containing 5 mg/mL BSA and 5% glycerol:
250 µL 20 mg/mL BSA solution
700 µL molecular biology grade water
50 µL molecular biology-certified glycerol

2. Prepare PCR reactions using the following components:

Components	Volume (µL)
AmpliTaq Gold® 10X buffer	1
dNTP 2.5 mM each	0.8
$MgCl_2$ 25 mM	0.8
AmpliTaq Gold® polymerase (5 U/µL)	0.2
Forward Primer 5 µM	0.25
Reverse Primer 5 µM	0.25
Bisulfite-gDNA template (6 ng/µL)	4.5
BSA-glycerol solution	1
Water	1.2

3. Run the plate using the thermal cycler protocol:



4. (Optional Fragment Analysis Step)
Mix 0.5—1 µL PCR Product and 9 µL formamide containing 10 % ROX 500 size standard. Run this on an ABI PRISM® 3100 Genetic Analyzer with POP-4 (See page 9, Figure 11).

5. PCR product Cleanup
If not performing fragment analysis, the PCR product should be cleaned up prior to sequencing.

PCR cleanup: Add 2 µL of ExoSAP-IT® Reagent (USB Corporation), incubate at 37°C for 30 min, followed by heat inactivation at 80°C for 15 min.

Figure 7. Bisulfite PCR Protocol Overview.

Methyl Primer Express® Software v1.0

Applied Biosystems Methyl Primer Express Software v1.0 is a free online primer design tool specifically for methylation studies which assists in designing primers in both methylated and unmethylated bisulfite-modified DNA. The essential steps in designing sequence-specific DNA methylation experiments are substantially automated with Methyl Primer Express. Users simply cut and paste in the selected genomic sequence. The software then performs an in-silico bisulfite conversion (Cs are converted to Ts) and aids in the selection of primers.

Step 4. Detection and Analysis

Bisulfite sequencing is the "gold standard" for the detection and analysis of methylation events and is used for validation of methylation status. Sequencing primers with or without universal tails are generally designed to anneal to non-CpG regions flanking each region of interest, and thus amplify bisulfite-converted gDNA regardless of methylation status. Consequently, if one of the primers is fluorescently labeled at the 5′ end, the resultant amplicon can be analyzed by the Capillary Electrophoresis (CE)-based fragment analysis method on page 7 and also used for sequencing.

An optional fragment analysis experiment will demonstrate the formation of a correct-sized amplicon and indicate the presence of the intended target sequence.

Case Study: Sequencing

All fragments were cycle sequenced using Applied Biosystems BigDye® Terminator v1.1 Cycle Sequencing Kit and M13 tailed primers (Figure 8).

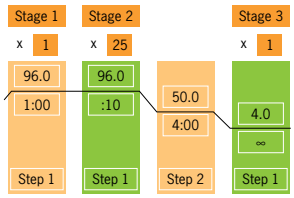
Complete removal of fluorescently labeled dideoxy terminators, dNTPs and salts is needed prior to CE analysis of the sequencing extension products. Bisulfite sequencing reactions can be purified using BigDye® XTerminator™ Kit, Centri-Sep™ columns (96-well plate) or ethanol precipitation. The BigDye XTerminator Purification Kit, which offers a simpler workflow alternative and reduces sample loss was used here. After the sequencing reaction is carried out, a slurry of absorptive, biphasic material is added directly to each sequencing well, which is then sealed and vortexed for 30 min. After centrifugation to settle particulates to the bottom of the wells, the plate is directly used with a a 3730 / 3730*x*l DNA Analyzer or 3130 / 3130*x*l Genetic Analyzer for sequencing. The resulting sequence is free of so-called "dye-blobs" and provides much stronger signal than alternative purification protocols, due to significant desalting of the sample.The cleaned-up cycle sequenced reactions were run on the 3730*x*l DNA Analyzer with POP-7. Sequencing Analysis Software, SeqScape® Software v2.5, or Variant Reporter™ Software v1.0 are recommended for data analysis using the KB™ basecaller software.

Figure 9 is a segment of the RARβ2 gene shown before and after bisulfite conversion. In this example, all Cs were converted to Ts by the bisulfite treatment, so that no Cs are seen in the lower trace. If this sample had methylated cytosines, the Cs, which are adjacent to Gs, would still be detected as Cs.

1. **Add the following reagents to set up the sequencing reaction:**

Components	Volume (in µL)
PCR amplicon (bisulfite treated, 1-5 ng/µL)*	1
BigDye® Terminator v1.1 Ready Reaction Mix	8
Primer (M13 Forward or Reverse, 3.2 µM))	1
DH_2O	10
Total volume	20

2. **Seal the plate and quickly spin down the contents in each well in a centrifuge.**
   Run the following thermal cycler protocol:



3. **Remove unincorporated dye terminators and unused primers with Applied Biosystems BigDye® XTerminator™ Purification Kit.**
   The reactions are now ready for sequencing.

Figure 8. Overview of the Cycle Sequencing Protocol

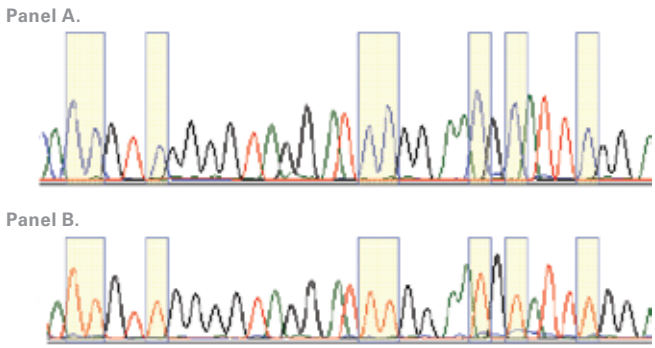


Figure 9. Electropherogram Trace of RARβ2 Before and After Bisulfite Conversion. (A) RARβ2 sequence from an unmethylated sample prior to bisulfite conversion (B) RARβ2 sequence after bisulfite conversion. Highlighted panels show unmethylated C's completely converted to T.

Fragment Analysis (Optional Step)

The forward primer in each of the 4 gene targets included a FAM™ dye. The amplicons from the Bisulfite PCR step were analyzed by Capillary Electrophoresis (CE) based fragment analysis to compare relative yields [2]. The thermal cycling conditions in Figure 10 were used with M13 tailed primers.

The additional 60 minutes at 60°C at the end of the thermal cycling allowed a non-template A to be added at the 3' end of each amplicon-product. When analyzing by fragment analysis, it is important that the A nucleotide is consistently added to all strands to ensure that peaks are sharp and clearly resolved. This step is not necessary if the amplicon is for sequencing only, and thus may be omitted. Figure 11 is an example of PCR amplification of the HDAC region on the X Chromosome for a female gDNA sample and a fully methylated control sample analyzed on an ABI PRISM® 3100 Genetic Analyzer with POP-4 polymer.

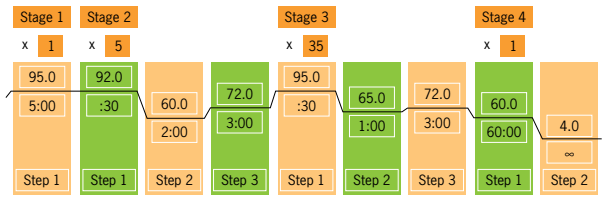
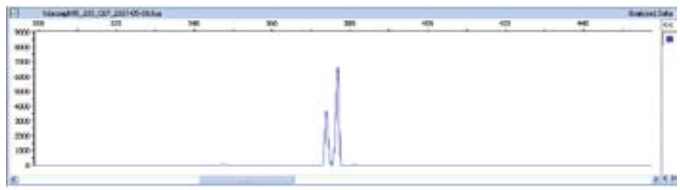
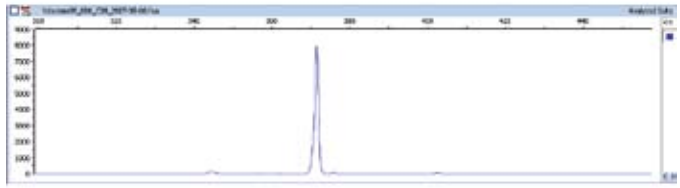


Figure 10. Fragment Analysis Thermal Cycler Protocol



Panel A.



Panel B.

Figure 11. Capillary Electrophoresis (CE) Based Fragment Analysis Results from the Histone Deacetylase (HDAC) Region on the X Chromosome. (A) A sample from a female showing one methylated allele (left) and one unmethylated allele (right) as expected due to normal methylation mediated inactivation of one copy of the X chromosome. (B) A universally methylated sample showing only a methylated peak.

A Complete Solution

Applied Biosystems provides multiple kits for DNA isolation to meet your sample type and throughput needs. The RecoverAll kit provides high quality DNA isolation from FFPE samples. The methylSEQr Bisulfite Conversion Kit uses size exclusion column purification, increasing your sample yields and reducing the amount of bias seen with resin purification methods. The reduced complexity and low melting temperature (Tm) of bisulfite treated DNA makes it difficult to amplify and sequence. Assay and primer design is therefore especially critical to the success of any methylation experiment. Applied Biosystems offers a free tool, Methyl Primer Express® Software v1.0 for the design of PCR Primers specifically tailored for bisulfite sequencing or Methylation Specific PCR (MSP). The Methyl Primer Express algorithm is optimized for methylation and provides a rapid tool for primer design with less PCR failure. Furthermore, with the added control of Veriti™ Thermal Cycler you have six independent temperature blocks for precise control in optimizing annealing conditions. As the leader in sequencing technology, Applied Biosystems provides the highest quality sequencing instruments and reagents for improved performance on difficult bisulfite converted sequences.

For more information about methylation, go to **info.appliedbiosystems.com/methylation**. For more details on bisulfite sequencing, please refer to the following user manual **http://docs.appliedbiosystems.com/pebiodocs/00116560.pdf**

FFPE Methylation Sequencing Workflow

1 DNA Isolation	2 Bisulfite Conversion	3 Assay Selection and Amplification	4 Sequencing
PRODUCTS	PRODUCTS	PRODUCTS	PRODUCTS
• RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE	• methylSEQr™ Bisulfite Conversion Kit	• Veriti™ 96-Well Fast Thermal Cycler	• BigDye® Terminator v1.1 Cycle Sequencing Kit
• TaqMan® DNA Template Reagents Kit		• GeneAmp® PCR System 9700 Thermal Cycler	• BigDye® XTerminator™ Purification Kit
• TaqMan® RNase P Detection Reagents Kit		• 9800 Fast Thermal Cycler	• 3730/3730xl DNA Analyzer
• TaqMan® Universal PCR Master Mix, No AmpErase® UNG		• 2720 Thermal Cycler	• 3130/3130xl Genetic Analyzer

Green: Ambion reagents and kits. Blue: Applied Biosystems reagents and instruments.

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ORDERING INFORMATION

Description	Quantity	Part Number
DNA Isolation		
RecoverAll™ Total Nucleic Acid Isolation Kit for FFPE	40 purifications	AM1975
TaqMan® DNA Template Reagents Kit	1 kit	401970
TaqMan® RNase P Detection Reagents Kit	100 rxns	4316831
TaqMan® Universal PCR Master Mix, No AmpErase® UNG	200 rxns	4324018
Bisulfite Conversion		
methylSEQr™ Bisulfite Conversion Kit	1 kit	4379580
Amplification		
Veriti™ 96-Well Fast Thermal Cycler	1 instrument	4375305
Aluminum 96-Well GeneAmp® PCR System 9700	2 pieces	4314879
9800 Fast Thermal Cycler with 96-well Aluminum Sample Block Module	2 pieces	4352604
2720 Thermal Cycler	1 piece	4359659
Sequencing		
BigDye® Terminator v1.1 Cycle Sequencing Kit	100 rxns	4337450
BigDye® XTerminator™ Purification Kit-2 mL	1 kit	4376486
3730*x*l DNA Analyzer	1 instrument	3730xl
3130 Genetic Analyzer	1 instrument	3130-01

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