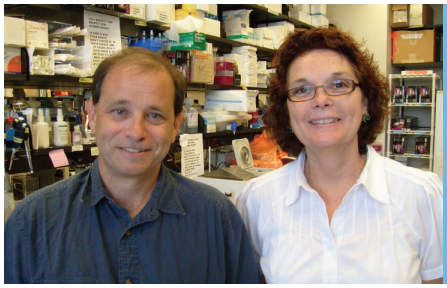
Amplification of Long GC-Rich Sequences

Applied Biosystems AmpliTaq Gold® 360 Master Mix



National Institutes of Health (NIH)
Lawrence Brody, Ph.D.
Patricia Porter-Gill

Scientific Focus
Effects of folic acid on methylation status

Applications
*CpG-island amplification
*Methylation analysis

Technologies
AmpliTaq Gold® 360 Master Mix

One way scientists study epigenetic variations in the human genome is by focusing on changes in DNA methylation. Modification of DNA by methylation is believed to affect gene expression levels (see the sidebar, *Importance of Correlating DNA Methylation and Gene Expression*). One of the goals of this work is to discover the connection between DNA methylation and disease states or phenotypes, since changes in methylation status could promote or influence tumor formation or growth.

Dr Lawrence Brody and Ms Patricia Porter-Gill, at the National Institutes of Health (NIH) in Bethesda, Maryland, are currently studying the effects of folic acid (folate) and vitamin B12 pathways on specific disease processes. These pathways convert folate compounds found in food to methyl groups, which are used by the body in hundreds of biochemical reactions. Since DNA methylation status can change with age and diet, Brody and Porter-Gill are specifically looking at how dietary and age-related changes effect methylation of DNA and where in the genome this methylation is taking place [1].

Amplification of Long CpG Islands

Many areas of disease associated methylation are found to be GC-rich, with some spanning 1000 bp or longer. Studying these areas involves amplifying long GC-rich sequences. However, the polymerases currently used for DNA amplification often have difficulty getting through these sequence segments. Thus,

IMPORTANCE OF CORRELATING DNA METHYLATION AND GENE EXPRESSION

A portion of the folic acid found in food is used as a substrate for DNA methyltransferases. These enzymes add extra methyl groups to specific cytosine (C) residues, most often in the context of CpG dinucleotides. In the genome, there are many CpG-islands [2] characterized by long GC-rich sequences. These clusters of CpG dinucleotides are mostly ~1000 bp long, but can be as long as 3000 bases and may contain as much as 85% GC content. A large number of CpG-islands are found in the 5' promoter regions and the first intron of genes, where methylation influences transcription levels. Research supports the fact that methylation within the promoter region can inactivate gene expression [3].

Methylation also serves as a marker of gene expression and a window into what is happening at the chromatin level. As histones [4] are modified and genomic regions are condensed, the DNA is often methylated. These methylation marks are carried throughout DNA replication and transferred to daughter cells. By looking at the methylation status, we can tell whether specific genes have been turned on or off.

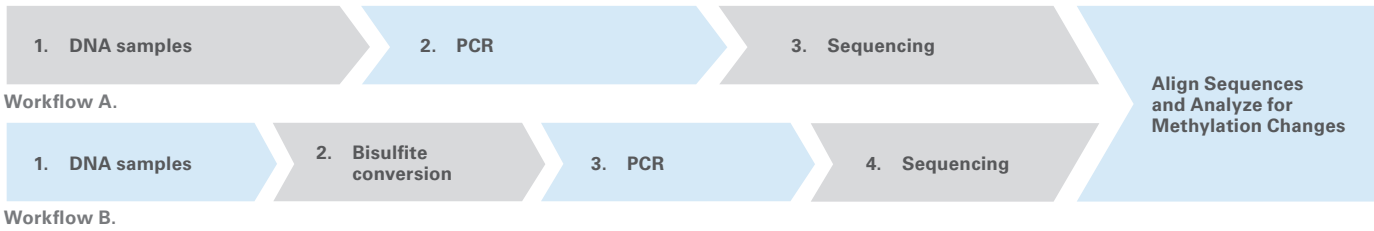


Figure 1. Workflow for non-bisulfite (A) and bisulfite-converted (B) DNA samples.

very few researchers have carried out detailed analyses of large GC-rich regions. Brody and Porter-Gill have selected the Applied Biosystems AmpliTaq Gold® 360 Master Mix to drive amplification through these large stretches of the genome in order to better understand the relationship between methylation and diet.

The PCR amplification in the NIH study was two-fold:

1. Amplification of the selected GC-rich regions of DNA before-bisulfite treatment of wild-type, methylated, or unmethylated DNA.

2. Amplification of the exact same amplicon region after-bisulfite treatment using the appropriate bisulfite-converted primers specific for the converted sequence.

Bisulfite Conversion of DNA and Subsequent Amplification of Long CpG Islands

Many methylation assays are based on bisulfite conversion of DNA [5]. Treatment of DNA with bisulfite (Figure 1) converts unmethylated cytosine (C) residues ultimately to thymidine (T) residues. By converting all the unmethylated Cs to T residues, one can infer that any remaining Cs were methylated. Bisulfite-treated DNA is usually not as stable as native DNA, since bisulfite treatment tends to render the DNA single-stranded. Dr Brody reports that, "Because bisulfite-treated DNA is relatively unstable and most polymerases have difficulty moving through these long CpG islands, it makes amplification of such large stretches of the genome difficult to achieve." The AmpliTaq Gold 360 Master Mix successfully amplifies these long GC-rich targets. When the amplicons were sequenced, Brody and Porter-Gill observed C bases in the sequence at the CpG locations where methylation is occurring, and were able to quantitate the degree of methylation.

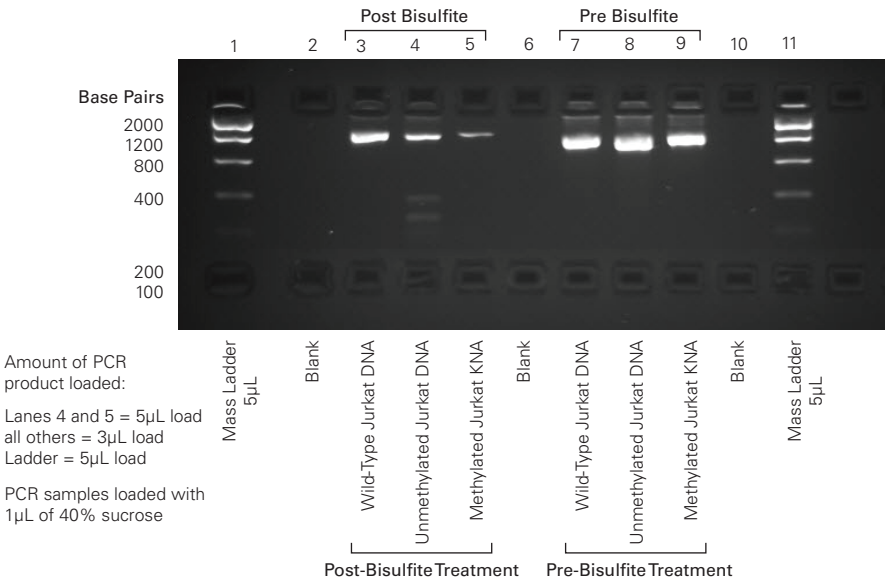
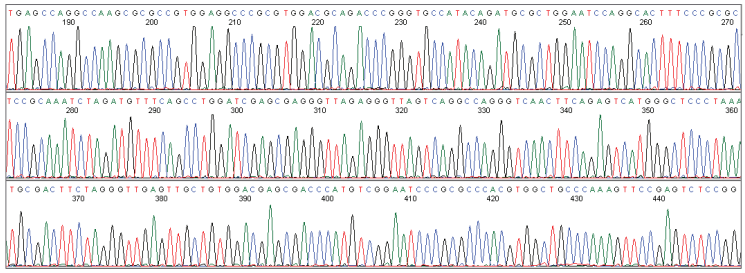


Figure 2. Amplification of Long CpG Islands Before and After Bisulfite Treatment. AmpliTaq Gold® 360 Master Mix was used to successfully amplify a set of DNA samples with various levels of methylation. Jurkat DNA with 3 levels of methylation (unmethylated, wild-type, and methylated) were amplified before and after bisulfite treatment. After the bisulfite treatment, the primers were modified to amplify the same region resulting in the same amplicon size.

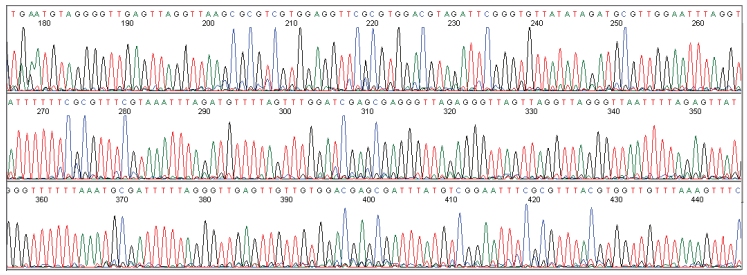
Today, 800–900 bp CpG islands are easily amplified using AmpliTaq Gold 360 Master Mix, both before and after the bisulfite treatment of DNA; and as shown in Figure 2, an 1100 bp target was also successfully amplified. Methylation analysis by bisulfite treatment and subsequent sequencing proves to be an interesting test of PCR chemistry. This is because the template has a high GC content, then becomes more AT-rich after the bisulfite treatment, and, as Dr Brody mentions above, the bisulfite-treated DNA is less stable. The PCR product, however, has to be very specific because methylation analysis requires a high-quality DNA sequence read. This transition from high-GC to high-AT content is illustrated by the following example: A coding strand has 425 C residues. With the CpG sites fully methylated, a total of 274 C residues can be converted to T residues. Thus, the GC content is reduced to approximately 50%. If the DNA is completely unmethylated, the GC content of the fragment is further reduced to 37%. Figure 2 shows examples of the same amplicon region before and after bisulfite treatment. The AmpliTaq Gold 360 Master Mix successfully amplifies each of the DNA samples independent of GC content due to methylation status.

NIH Investigates Promoter Methylation

Dr Brody's lab performs an additional analysis to assess the methylation status of the CpG islands that involves MALDI TOF mass spectrometry. The PCR products are transcribed and cleaved at thymine or cytosine residues and subsequently analyzed on the mass



Panel A. Before Bisulfite Conversion.



Panel B. After Bisulfite Conversion.

Figure 3. Trace File Results Before (Panel A) and After (Panel B) Bisulfite Conversion. All non-methylated cytosines appear as thymines in Panel B. The samples used were completely methylated DNA. To determine the methylation patterns, the sequences in Panel A and B need to be aligned. Base Position 190 in Panel A can be aligned to Base Position 195 in Panel B. For example, starting at Base Position 190 in Panel A, the sequence changes from 'GGCCAA' to 'GGTTAA', due to the bisulfite conversion of Cs that are not methylated.

spectrometer. Using both PCR combined with DNA sequencing and mass spectrometry, Brody and Porter-Gill are investigating the methylation profiles of promoter regions by analyzing large target amplicons of 1000 bp or more. In the study of these large amplicons, Brody and Porter-Gill have been using the Applied Biosystems AmpliTaq Gold 360 Master Mix to test small sample sets (20 samples at a time) and different primer sets for genes of specific interest. Sample sizes range anywhere from 900–2000 bp.

Replicates of DNA samples, before and after bisulfite treatment, were amplified using the Applied Biosystems AmpliTaq Gold 360 Master Mix and purified to remove dNTPs and primers. The resulting amplicons were sequenced using the Applied Biosystems BigDye® Terminator Kit v3.1 and cleaned up with the Applied Biosystems BigDye® XTerminator™ Purification Kit. The purified sequencing reactions were sequenced on an Applied Biosystems 3730*xl* DNA Analyzer. (For PCR and sequencing protocols, see sidebar, *Protocols for PCR Amplification and Sequencing*.)

Figure 3 shows an example of a GC-rich 905 bp amplicon that was successfully PCR amplified and sequenced both before (Panel A) and after (Panel B) bisulfite treatment of the DNA. Note that the Cs are seen as Ts in the amplicon formed after bisulfite treatment, except for Cs adjacent to Gs.

Large Amplicon Analysis Paves the Way for Whole Genome Studies

Concurrently, NIH associates of Brody and Porter-Gill are performing bioinformatics studies. When supported with the data generated from these 1000+ bp, CpG-island amplicons, it will allow the researchers to demonstrate the methylation status of specific sections of the genome. To date, the results suggest

CYCLE SEQUENCING

The cycle sequencing reactions were purified with the Applied Biosystems BigDye® XTerminator™ Purification Kit, per kit instructions. The amplicons were sequenced on an Applied Biosystems 3730*xl* DNA Analyzer. Unmethylated and methylated DNA controls were sequenced successfully along with the sample DNA.

PROTOCOLS FOR PCR AMPLIFICATION AND SEQUENCING

PCR PARAMETERS

PCR was performed at the NIH by Patricia Porter-Gill using the Applied Biosystems AmpliTaq Gold® 360 Master Mix, a new product designed to amplify a larger range of targets. Primers were tailed with M13 tags for subsequent sequence analysis.

Amplification Conditions:

Reagent	Volume for 50µL Reaction
2X AmpliTaq Gold® 360 Master Mix	25
Forward primer 10 µM	1
Reverse primer 10 µM	1
Nuclease-free H_2O	18
Modified DNA template	5
Or WT DNA (50 ng/µL)	

PCR - Thermal Cycling Conditions:

Step	Temp/Time
Initial Denaturation	95°C for 10 minutes
# of Cycles	40 cycles
Denaturation	95°C for 30 sec
Annealing	60°C for 2:00 min
Extension	72°C for 1:00 min
Post-Extension Hold	72°C for 7:00 min
Storage	4°C

SEQUENCING PARAMETERS

Each of the PCR amplicons shown in Figure 2 were purified after amplification with the Agencourt® AMPure® Kit to remove the unincorporated dNTPs and primers. The PCR was diluted 1:5, and 0.5 µL was used in the cycle sequencing reaction:

Amplification Conditions:

Reagent	Amount
BigDye® Terminator v3.1	4 µL
M13 forward or reverse primer 3.2 µM	0.5 µL
Diluted amplicon	0.5 µL
H_2O	5 µL

Thermal Cycling Conditions:

Initial Denaturation	96°C/1 min
# of Cycles	25 cycles
Denaturation	96°C/10 sec
Annealing	50°C/4 min
Storage	4°C

that methylation can occur at sites other than CpG dinucleotides and that clusters of CpG dinucleotides have varied methylation patterns. Brody and Porter-Gill plan to create a methylation profile for genes of interest for each sample and look at how it differs from person to person, diet to diet, and across the ages of the sample donors.

In the final analysis, Dr Brody and Patricia Porter-Gill point out that "routine amplification of a 1000 bp CpG-island target is considered an amazing feat. This newly acquired capability allows us to start asking bigger questions for future studies. Ultimately, we would like to have a methylation map for each gene of interest, showing exactly where in the promoter region methylation occurs."

The views expressed in this article do not represent those of NIH. The authors declined the $500 Share Your Data credit offered by Applied Biosystems.

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ORDERING INFORMATION

Description	Quantity	Reactions	P/N
Applied Biosystems AmpliTaq Gold® 360 Master Mix (1 mL)	1 Kit	40 reactions	4398876
Applied Biosystems AmpliTaq Gold® 360 Master Mix (5 mL)	1 Kit	200 reactions	4398881
Applied Biosystems AmpliTaq Gold® 360 Master Mix (10 x 5 mL)	1 Kit	2000 reactions	4398901
Applied Biosystems AmpliTaq Gold® 360 Master Mix (50 mL)	1 Kit	2000 reactions	4398886
Applied Biosystems BigDye® XTerminator™ Purification Kit	1 Kit	1000	4376487
Applied Biosystems BigDye® Terminator Cycle Sequencing Kit	10 x 800 µL	1000	4337456
Applied Biosystems Sequencing Analysis Software V.5.3.1	1 Package	—	*
Applied Biosystems 3730*xl* DNA Analyzer	1 Instrument	—	*

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