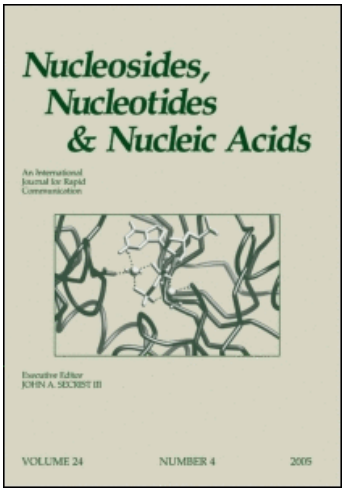

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Methylation-Dependent Fragment Separation: Novel Analysis Of 5-Methyl Cytosine By Capillary Electrophoresis Of Amplified Dna Using Pcr Incorporation Of Chemically Modified Dctp

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METHYLATION-DEPENDENT FRAGMENT SEPARATION: NOVEL ANALYSIS OF 5-METHYL CYTOSINE BY CAPILLARY ELECTROPHORESIS OF AMPLIFIED DNA USING PCR INCORPORATION OF CHEMICALLY MODIFIED dCTP

Victoria L. Boyd, Kristina I. Moody, Achim E. Karger, Kenneth J. Livak, Gerald Zon, and John W. Burns □ *Genetic Analysis R&D, Applied Biosystems, Foster City, California, USA*

□ *Methylation of the cytosine (C) ring to form 5-methyl cytosine (MeC) in normally unmethylated CpG-rich regions of promoters in genes is associated with transcriptional silencing. Quantification of MeC is of current interest in findining new biomarkers for cancer. To this end, and for basic research in epigenomics, we have investigated a new method for relatively simple measurement of MeC content by capillary electrophoresis (CE). PCR amplicons for CE analysis are generated from bisulfite-converted DNA [C → uracil (U)] using fluorescently labeled primers that anneal independent of methylation status. Resultant incorporation of C vs. T at original MeC vs. C positions can lead to separate CE peaks for signal integration that is proportional to MeC content. Furthermore, these PCR products are suitable for additional methylation analyses by sequencing, single-base extension, or TaqMan®. Interestingly, PCR using α-thio-dCTP led to greater CE separations.*

Keywords 5-Methyl cytosine; bisulfite conversion; methylated DNA analysis; capillary electrophoresis

INTRODUCTION

Bisulfite deamination of C to give U has been extensively studied,[1] and under proper conditions can exhibit remarkably high chemoselectivity. Thus, denatured DNA at pH 5, ~50°C for ~15 hours undergoes conversion of C to U without significant deamination of adenosine (A) or guanosine (G). Moreover, MeC is not converted to thymidine (T). Bisulfite adduct formation is generally assumed to be operative for this deamination process,[1] and discrimination against MeC can be rationalized by steric interference between Me and bisulfite during *cis*-addition. Regardless of the origin of this chemoselectivity, its utilization for analysis of MeC in DNA by sequencing[2] has become the "gold standard" in epigenomics. Methylation of DNA in

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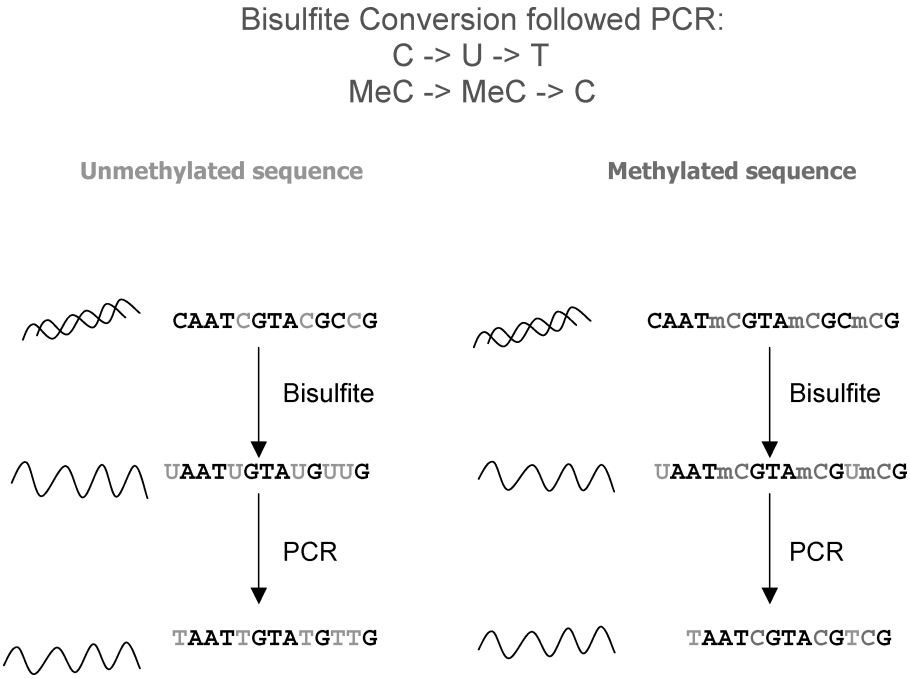

FIGURE 1 CpG (and other C) sites in unmethylated DNA strand are converted to U by bisulfite conversion and, following PCR, give rise to a T-rich strand. MeCpG (mCpG) sites in methylated DNA strand remain as mCpG upon treatment with bisulfite and, following PCR, give rise to a C-rich strand, while other C sites give rise to T.

CpG-rich sequences in promoter regions of genes function as structural signals to block expression. This "silencing" of tumor suppressor genes has been associated with various cancers. Consequently, quantitative analysis of methylated DNA levels for particular genes is of current interest as clinically useful biomarkers.[3] For adequate signal intensity, DNA methylation analyses employ PCR amplification, following bisulfite conversion of the original DNA sample. As shown in Figure 1, resultant amplicons contain TpG at original CpG sites, but have CpG at original MeCpG sites. This C versus T change following PCR can be detected by sequencing or various hybridization-based methods.[4] However, we hypothesized that aggregate C versus T content might be easily measured directly by CE peak area intensities, as depicted in Figure 2. Initial findings have been reported recently.[4]

MATERIALS AND METHODS

Bisulfite Conversion

Human genomic DNA (gDNA; 300 ng) in 45 μL of water was mixed with 5 μL of "M-dilution buffer" (Zymo Research, Orange, CA, USA) and

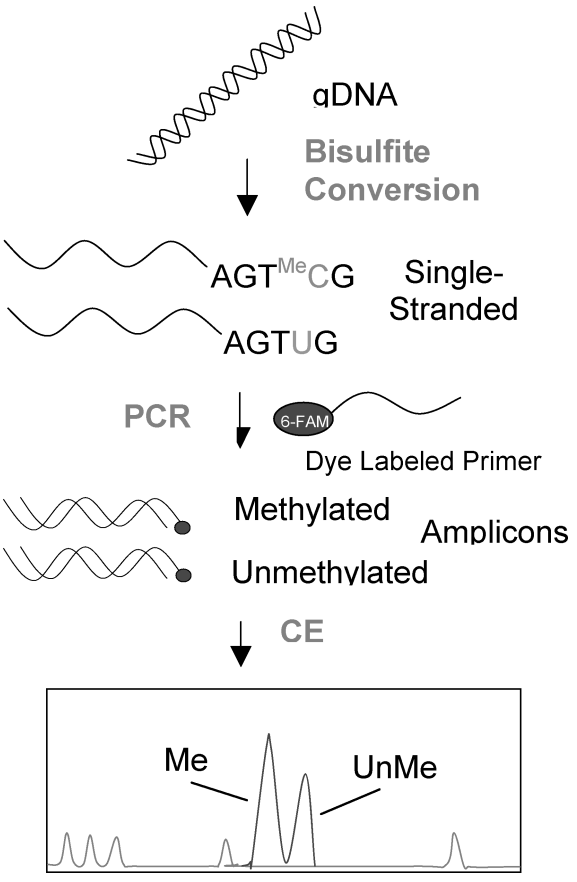

FIGURE 2 Workflow for methylation-dependent fragment separation. Step 1, bisulfite conversion of CpG to UpG (and other Cs to Us). Step 2, PCR amplification of region of interest using fluorescent dye-labeled (6-FAM) primers leads to differential T versus C content, as shown in Figure 1. Step 3, CE separation of faster migrating C-rich strand from slower migrating T-rich strand derived from methylated (Me) and unmethylated (UnMe) DNA, respectively.

the solution was heated for 15 minutes at 37°C and then kept at 37°C until ready for use. To the resultant denatured gDNA, 150 μL of bisulfite "CT conversion reagent" (Zymo) was added to give a final volume of 200 μL, and the reaction was incubated at 50°C for 15 hours. The solution was transferred to a Microcon 100 device (Millipore, Bellerica, MA, USA), mixed with 150 μL of water and then centrifuged 15–20 minutes at 500 × g (2800 rpm in an Eppendorf 5415) until just or nearly dry. Water (350 μL) was added to the upper chamber and centrifugation was resumed until nearly dry. This step was repeated. For desulfonation (*in situ*), 350 μL of 0.1 M NaOH was added to the upper chamber, and after 5 minutes at room temperature, the solution was centrifuged until nearly dry. Water (350 μL) was added and centrifugation was continued until near dryness. TE buffer (50 μL

of 10 mM Tris-0.1 mM EDTA, pH 8, Teknova) was added to the upper chamber, and the liquid mixed by pipeting. The resultant TE solution of bisulfite-converted gDNA was removed and stored at 4°C. Quantitation of the bisulfite-converted gDNA was carried out using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

PCR

Primer sequences were tailed with the –21 M13 forward and reverse sequences, and were selected with the aid of MethPrimer, freely accessible at http://www.urogene.org/methprimer/. Chosen primer pairs were based on the criteria that they contain no CpG in the primers, and no regions with poly T $\geq$ 9 in the amplicon. The gene specific portion of the primer typically had a Tm of 55 $\pm$ 5°C based on theoretical calculations at http://www.basic.northwestern.edu/biotools/oligocalc.html. The forward primer was dye-labeled (6-FAM) for detection during CE. The primer pair (0.25 μL forward primer, 0.25 μL reverse primer, 5 μM each) was combined with 0.5 μL bisulfite-treated gDNA (3 ng/μL, assuming 100% recovery of unfragmented gDNA after the bisulfite conversion), 1 μL AmpliTaq Gold 10X buffer (Applied Biosystems, Foster City, CA, USA), 0.8 μL dNTPs (2.5 mM each), 0.8 μL $MgCl_2$ (25 mM), 0.2 μL AmpliTaq Gold polymerase (5 U/μL, Applied Biosystems), and 6.2 μL water. The thermal cycling conditions were 5 minutes at 95°C, 5 cycles of 95°C/30 seconds, 60°C/2 minutes, 72°C/3 minutes; 30 cycles of 95°C/30 seconds, 65°C/1 minutes, 72°C/3 minutes, hold at 60°C/85 minutes, and stored at 4°C. The optimum annealing temperature for the initial 5 cycles was chosen to be ~5°C above the calculated Tm.

RESULTS

Primers for bisulfite-converted gDNA were designed to amplify a region regardless of methylation state by annealing to non-CpG sequences flanking regions of high CpG-content. An amplicon from fully unmethylated (UnMe) gDNA will contain all TpG motifs (in the forward strand) while an amplicon from a fully methylated (Me) gDNA will contain all CpG motifs; all other Cs in UnMe or Me gDNA give rise to Ts in Me and UnMe amplicons. (Note: "Me and UnMe amplicons" refer to amplicons from the bisulfite-converted gDNA that originally had MeCpG or CpG sites, respectively.) CE results (see below) demonstrated that fully Me and fully UnMe amplicons are resolvable, with the C-rich amplicon (from fully Me gDNA) migrating faster than the T-rich amplicon (from fully UnMe gDNA). Migration times during CE in a denaturing, sieving media are related to the number of nucleotides N as expressed by $t_{mig} = k + n\ N$, wherein k is a constant offset and n is a coefficient relating t_{mig} and size. While ODN length

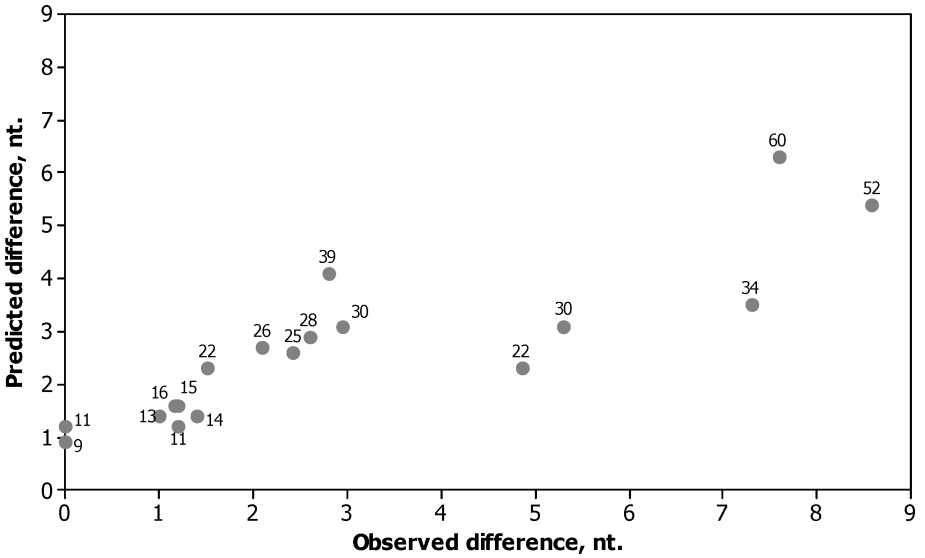

FIGURE 3 Observed (by CE) versus predicted (calculated) size-difference for Me and UnMe amplicons using unmodified dNTPs. The number of CpG moieties (in gDNA before bisulfite conversion) per amplicon is given next to each data point. CE migration-times for pairs of Me and UnMe amplicons are shown in Figure 4 for four representative cases; co-injection of size standards (not shown in Figure 4) was used to convert CE migration times into observed difference in nt-equivalents. Predicted difference in nt-equivalents was obtained for each pair of amplicons using the equation given by size = 4.010 + 0.819 A + 1.180 G + 0.916 T + 0.812 C (see text for details).

is linearly related to t_{mig} as expressed, it is well known[5] that A-, G-, T-, and C-content affects migration time, in which case a variable is assigned to each nucleotide, that is, $t_{mig} = k + a\,A + g\,G + t\,T + c\,C$, wherein A, G, T, and C are the numbers of nucleotides and a, g, t, and c are the base-specific coefficients. Measurement of t_{mig} for a set of $\geq$5 ODNs of known base composition allows calculation of coefficients k, a, g, t, and c, which can then be used to calculate (predict) t_{mig} of any ODN. For better precision of the regression analysis, we replaced the independent variable t_mig with fragment size, as determined using co-injected size standards and analysis by GeneScan software. Coefficients a, g, t, and c, thus, were determined for a set of synthetic ODNs with sizes from 19 to 61 nt; size = 4.010 + 0.819 A + 1.180 G + 0.916 T + 0.812 C. Predicted sizes for Me and UnMe amplicons were then calculated and compared to measured sizes, as shown in Figure 3. For a larger data set, more accurate sizing and size difference predictions should be possible by adjusting the algorithm.

Livak and coworkers[6] previously used a biotinylated dCTP derivative having a 36-carbon linker to induce a measurable shift in electrophoretic mobility of a PCR amplicon to detect single nucleotide polymorphisms in DNA. We found that this biotin derivative had an analogous effect on our CE measurements (data not shown). Interestingly, use of a minimally

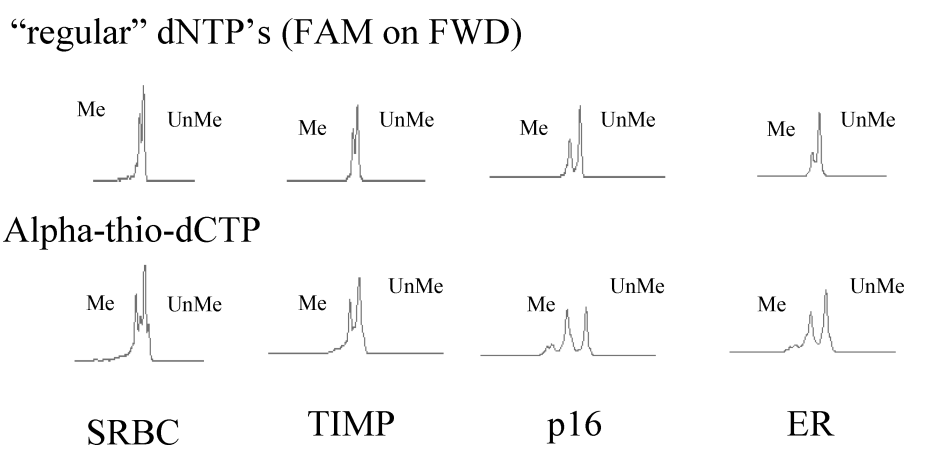

FIGURE 4 CE traces for labeled (6-FAM) forward-primer amplicons derived from Me and UnMe DNA, following bisulfite conversion and use of either dCTP ("regular") or α-thio-dCTP during PCR. Gene names are indicated by SRBC, TIMP, p16, and ER.

substituted dCTP derivative, namely α-thio-dCTP, in PCR following bisulfite conversion also led to CE-mobility shifts. This is evident from results given in Figure 4 for amplicons derived from representative CpG-rich loci in four different genes. Moreover, in each of these examples, the CE-mobility difference between pairs of Me and UnMe amplicons was greater with α-thio-dCTP. Other PCR-compatible modifications, such as α-borano-dCTP, will be investigated in the future.

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