

Rapid quantification of DNA methylation through dNMP analysis following bisulfite-PCR

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ABSTRACT

We report a novel method for rapid quantification of the degree of DNA methylation of a specific gene. Our method combined bisulfite-mediated PCR and quantification of deoxyribonucleoside monophosphate (dNMP) contents in the PCR product through capillary electrophoresis. A specific bisulfite-PCR product was enzymatically hydrolyzed to dNMP monomers which were quantitatively analyzed through subsequent capillary electrophoresis. PCR following bisulfite treatment converts unmethylated cytosines to thymines while leaving methyl-cytosines unchanged. Then the ratio of cytosine to thymine determined by capillary electrophoresis represents the ratio of methyl-cytosine to cytosine in genomic locus of interest. Pure oligonucleotides with known sequences were processed in parallel as standards for normalization of dNMP peaks in capillary electrophoresis. Sources of quantification uncertainty such as carryovers of dNTPs or primers and incomplete hydrolysis were examined and ruled out. When the method was applied to samples with known methylation levels (by bisulfite-mediated sequencing) as a validation, deviations were within $\pm 5\%$. After bisulfite-PCR, the analytical procedure can be completed within 1.5 h.

INTRODUCTION

Cytosine methylation is a well-known epigenetic gene regulation mechanism which plays crucial roles in normal development and differentiation in vertebrates (1–7). Disregulation of DNA methylation could result in abnormalities in a variety of cellular functions including cancers (8). Developments of

many cancers are closely associated with changes in DNA methylation status of specific tumor suppressor genes, repair genes and growth regulatory genes (9–14). In addition to roles in cancer developments, aberrant DNA methylation was also implicated in several imprinting disorders, diseases with tri-nucleotide expansions and in process of aging (8,15).

A variety of different analytical methods for DNA methylation have been developed. Traditionally, methylation-sensitive restriction enzymes (MSRE) were widely used to investigate methylation status of a specific gene. Southern blot analysis following MSRE digestion is accepted as one to yield most authentic methylation information as the method does not deal with the amplified DNA but the genomic DNA itself. However, in spite of directness and quantitiveness of the method, methylation-sensitive Southern blot analysis was hampered by several drawbacks such as low-throughput and limited applicability depending on the existence of MSRE recognition sites. Throughput of MSRE-based methods could be greatly improved by employing PCR amplification following MSRE digestion (16).

Methods employing chromatography such as high performance liquid chromatography (HPLC) and capillary electrophoresis (CE) focus on direct quantification of methyl-cytosine contents in genome (17–19). Those chromatographic methods give quantitative results by measuring overall methyl-cytosine contents from appropriately hydrolyzed DNA samples. Quantitative analyses using chromatography are fast, accurate and readily automatable. However, they require relatively large amounts of genomic DNA, limiting the applicability of the methods in clinical laboratories. Furthermore, they could only provide simple information on net methylation status of genome where gene-specific information would be masked. Recently, a report has introduced a sensitive method adopting laser-induced fluorescence system to lower the sample amount required for analysis (20).

A revolutionary method employing bisulfite treatment of genomic DNA and subsequent PCR has been introduced relatively recently (21,22). Through bisulfite treatment and

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a subsequent PCR, methylation information is transformed to sequence information. Sequence determination following bisulfite-PCR will reveal a precise map of methyl-cytosines in a specific target locus. Quantitative information could also be obtained by sequencing a number of individual clones of bisulfite-PCR product. In spite of richness of information from bisulfite-mediated sequencing, the method is relatively expensive and time-consuming. In that sense, several alternative methods based on bisulfite treatment and subsequent PCR have been developed to overcome the high cost of sequencing. Combined bisulfite restriction analysis [COBRA (23)] and methylation-specific PCR [MS-PCR (24)] methods are fast and cost-effective, which would be suitable for screening or discrimination of a large set of samples. Employment of real-time PCR in MS-PCR could improve quantitiveness of MS-PCR (25,26). Schatz *et al.* have reported a matrix assisted laser desorption ionization-time of flight (MALDI-TOF)-based method for RNA-mediated analysis of methylation status of individual CpGs (27). In addition to methods mentioned above, many other approaches depending on bisulfite treatment were introduced and are being developed for pursuit of specific analytical advantages (9).

We introduce a method for quantification of methylation based on bisulfite-PCR and CE. Our strategy for quantitative analysis of the methylation level of a specific target DNA locus was to measure the relative cytosine contents of a bisulfite-PCR product through CE. Employment of CE system could give further advantages in that CE requires lesser sample amount and shorter analysis time (S.-M. Jang *et al.*, manuscript submitted). In our approach, bisulfite-PCR products were purified, digested and then analyzed by CE to yield quantification errors about $\pm 5\%$ in an additional analysis time of 90 min.

MATERIALS AND METHODS

Bisulfite treatment

pUC19 plasmid DNA (New England Biolabs, Ipswich, MA, USA) was used as a model DNA for establishment of the method. Genomic DNAs from four liver cell lines were used later for validation. EcoR I-digested plasmid DNA samples were methylated *in vitro* using Sss I and Hha I methylases (New England Biolabs). Bisulfite treatment was performed as described by Clark *et al.*, with a few modifications (22). In brief, 100 ng plasmid DNA or 1 μ g genomic DNA from liver cell lines was denatured in 0.3 M NaOH for 15 min at 37°C. After adding freshly prepared 3.5 M sodium bisulfite (Sigma, St Louis, MO, USA) and 1 mM Hydroquinone (Sigma) solution, samples were subjected to 16 h incubation at 55°C under exclusion of light. Then the samples were purified using Microcon YM-30 columns (Millipore, Bedford, MA, USA). Recovered samples were desulfonated in 0.3 M NaOH for 15 min at 37°C, neutralized and purified again using Microcon YM-30 columns.

PCR, cleanup and hydrolysis

PCRs were performed using a commercial pre-mixture kit (PremixTM; Bioneer, Daejeon, Korea). A 350 bp fragment

harboring 25 CpGs was amplified from the bisulfite-treated pUC19 DNA samples (puc19bs-f: AAGTGTAAGTTTGGGGTGTAA and puc19bs-r: AACCTTTACTCACAT-ATTCTTCCTAC) while a nested primer PCR was performed to amplify the 280 bp fragment of CDKN2A (p16) gene harboring 37 CpGs from liver genomic DNA samples (p16bs-f: GATTAAAAGAAGAAGTTATAT, p16bs-r: TTC-AAATCTTCTCAACATTC, p16nest-f: GTTGGTTGGTTA-TTAGAGG and p16nest-r: TCATTCCTCTCCTTAACT). PCR products were cleaned up to remove residual dNTPs and primers using a conventional PCR purification kit (Qiagen, Hilden, Germany). Purified DNA samples (100–500 ng) in 1 \times PCR buffer (Qiagen) were hydrolyzed using 0.005 U of snake venom phosphodiesterase (SVPD) (Amersham, Buckinghamshire, UK) for 1 h at 37°C. Following incubation at 80°C for 20 min for inactivation of the enzyme, samples were directly applied to CE analysis. For MS-PCR of CDKN2A, primers as following were used: p16ms-m-f: TTATTAGAGGGTGGGGCGGA, p16ms-m-r: ACCCCGAACCGCGACCGTAA, p16ms-u-f: TTATTAGAGGGTGGG GTGGA and p16-ms-u-r: CAACCCCAAACC-ACAACCAT.

Cloning and sequencing

Bisulfite-PCR products were sub-cloned into PCRScript II (Stratagene, La Jolla, CA, USA) according to the manufacturer's protocol. Inserted DNA fragments were amplified from 30 independent colonies using M13 forward and reverse sequencing primers and subjected to cycle sequencing reactions (ABI, Foster City, CA, USA). Sequences were determined using an ABI 310 sequence analyzer (ABI).

Capillary electrophoresis

Enzymatically hydrolyzed samples were analyzed by a CE system with a ultraviolet (UV) detector (270 model, ABI). The instrument was operated in a reverse polarity mode with a cationic surfactant, cetyltrimethylammonium bromide (CTAB, Sigma). The fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) with an I.D. of 50 μ m and length to detection window of 60 cm was preconditioned by treatment with 0.1 N NaOH for 1 h. Then the capillary was filled with freshly prepared background electrolyte (8 mM CTAB, 100 mM 2-amino-2-methyl-1-propanol; AMP and 46 mM NaCl with pH of 10.1) using a vacuum pump and equilibrated until the stability of detector was achieved. Samples in 1 \times PCR buffer were electro-kinetically injected for 5–30 s under -5 kV and separated by applying -30 kV using the background buffer mentioned above. Peaks were detected by measuring the absorbance at 265 nm and the data were processed using a homemade software package based on a 16 bit ADC capture board (ComputerBoards, Middleboro, MA, USA).

Calibration

Synthetic oligonucleotides purified through polyacrylamide gel electrophoresis were used as standards for calibration in the radiometric quantification of deoxyribonucleoside monophosphates (dNMPs). Oligonucleotide sequences were as following: T7: TAATACGACTCACTATAGGG and P16r: GTCTGCTGAAACTGCCAACA. The standard oligos

were analyzed by the same procedures as were for PCR products except for the PCR purification step. Theoretical C/T and G/C ratios calculated from sequences of the oligos were divided by C/T and G/C values measured in CE to obtain calibration factors. C/T and G/A values obtained from CE analysis of PCR samples were multiplied by the calibration factors to produce calibrated C/T and G/A ratios which would be the indicatives of methyl-cytosine contents in the target locus.

RESULTS

CE condition

For establishment of the new method, CE condition for dNMP separation was first optimized using commercial 5'-dNMP mixtures (Sigma). dNMPs were separated in a reverse electroosmotic flow (EOF) mode employing CTAB. Analysis of dNMPs through the reverse polarity mode produced sharper peaks with better resolutions in a much shortened separation time compared with those through a normal EOF mode. As dNMPs are negatively charged, the reverse mode where analytes move from cathode to anode is apparently favorable in regard of analysis time. Major parameters for optimization were CTAB concentrations, pH and salt concentrations (S.-M. Jang *et al.*, manuscript submitted). In summary of the optimization experiments, separability of peaks was generally enhanced by elevating CTAB concentration although some peak broadenings with delayed migration times were accompanied. The peak-broadening effect by CTAB was compensated by elevating salt concentration which might have interfered with CTAB-dNMPs interactions. Then, pH of the background buffer was adjusted to be 10.1 because peak separations were facilitated by a pH > 9.7. Secondary deprotonations of dTMP and dGMP at a pH > 9.7 were thought to be the major cause of the enhanced separability. Optimization of CE conditions resulted in a successful separation of the four dNMPs in <6 min of electrophoresis (Figure 1A).

The next step was to investigate the quantification characteristics of CE analysis for dNMPs. Linearity and repeatability are the most important points to be examined in a

quantification experiment. Figure 1B and C show the linearity and the repeatability of ratiometric quantification of dNMPs. For artificially mixed C/T or G/A samples of which molar ratios ranging from 0.3 to 1.0 (total concentration of dNMPs was 32.5 μ M for ratio of 0.3 and 50 μ M for ratio of 1.0), the CE process resulted in a good linearity ($R^2 > 0.999$) and a moderate level of repeatability where maximal coefficients of variation (CV) were <3% for C/T and 5% for G/A. A good linearity of quantification through CE implies that the method would be applicable for samples with a wide range of C/T and G/A ratios. Major cause of variations up to 5% in repetition of CE analysis was thought to be the relatively low signal-to-noise ratio which was inevitable when analyzing a very small amount of sample. If a higher amount of sample is injected or a high sensitivity detector is employed, quantification errors resulting from the low signal-to-noise ratio could be reduced. When the concentrations of dNMP mixtures were increased to 325–500 μ M, CV values <1% were obtained (data not shown). The total dNMP concentration from a single tube PCR would be in the range of 15–75 μ M for 100–500 ng of purified PCR product in 20 μ l. Therefore, in analyses of real PCR products, the quantification errors as represented by CV values are expected to be equivalent to the values obtained from reference samples of 32.5–50 μ M.

Cleanup and hydrolysis

Carryovers of PCR components such as dNTPs and primers are potential sources of dNMPs which might interfere with the quantification of dNMPs from PCR products. Quantities of dNMPs originated from dNTPs were 5–10-times larger than those from a PCR product of interest when quantified through a CE with spiking of deoxyinosine 5'-monophosphate (dIMP) as an internal standard (data not shown). Therefore, it is essential to eliminate a trace of carryover chemicals by an appropriate cleanup process. Cleanup using a conventional PCR purification kit was tested previously and resulted to be satisfactory. The efficiency of the cleanup using conventional PCR purification kit is demonstrated in Figure 2. To examine the removal of dNTPs through cleanup, primers and template were omitted from the PCR mixture so that

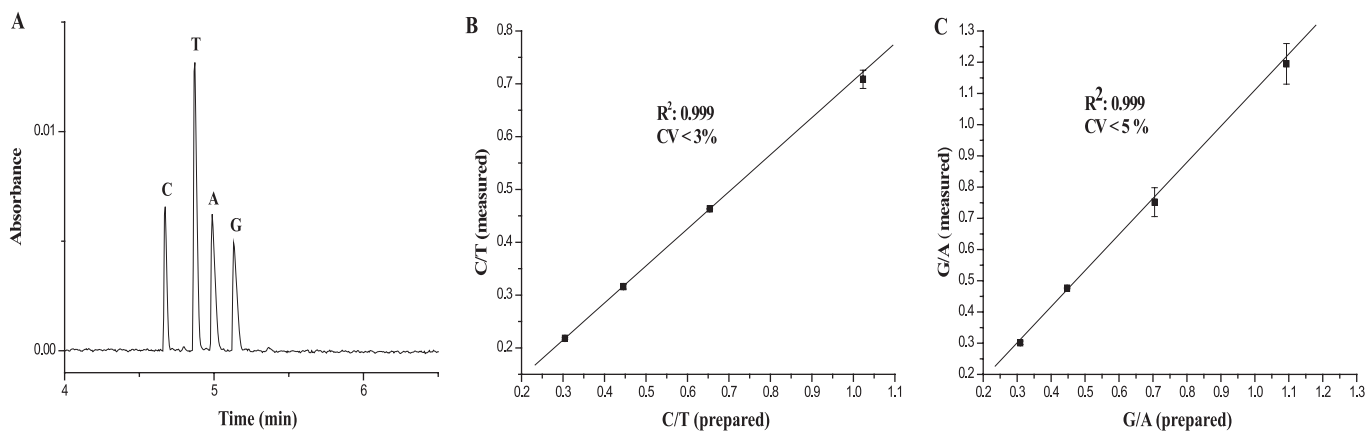


Figure 1. CE condition for quantitative analysis of dNMPs. (A) Separation profile of dNMPs under the optimized CE condition. (B). Linearity and repeatability of quantification of C/T ratio through CE. CVs in triplicate analyses of 32.5–50 μ M dNMP mixtures were <3%. (C) Linearity and repeatability of quantification of G/A ratio through CE. CVs were <5%.

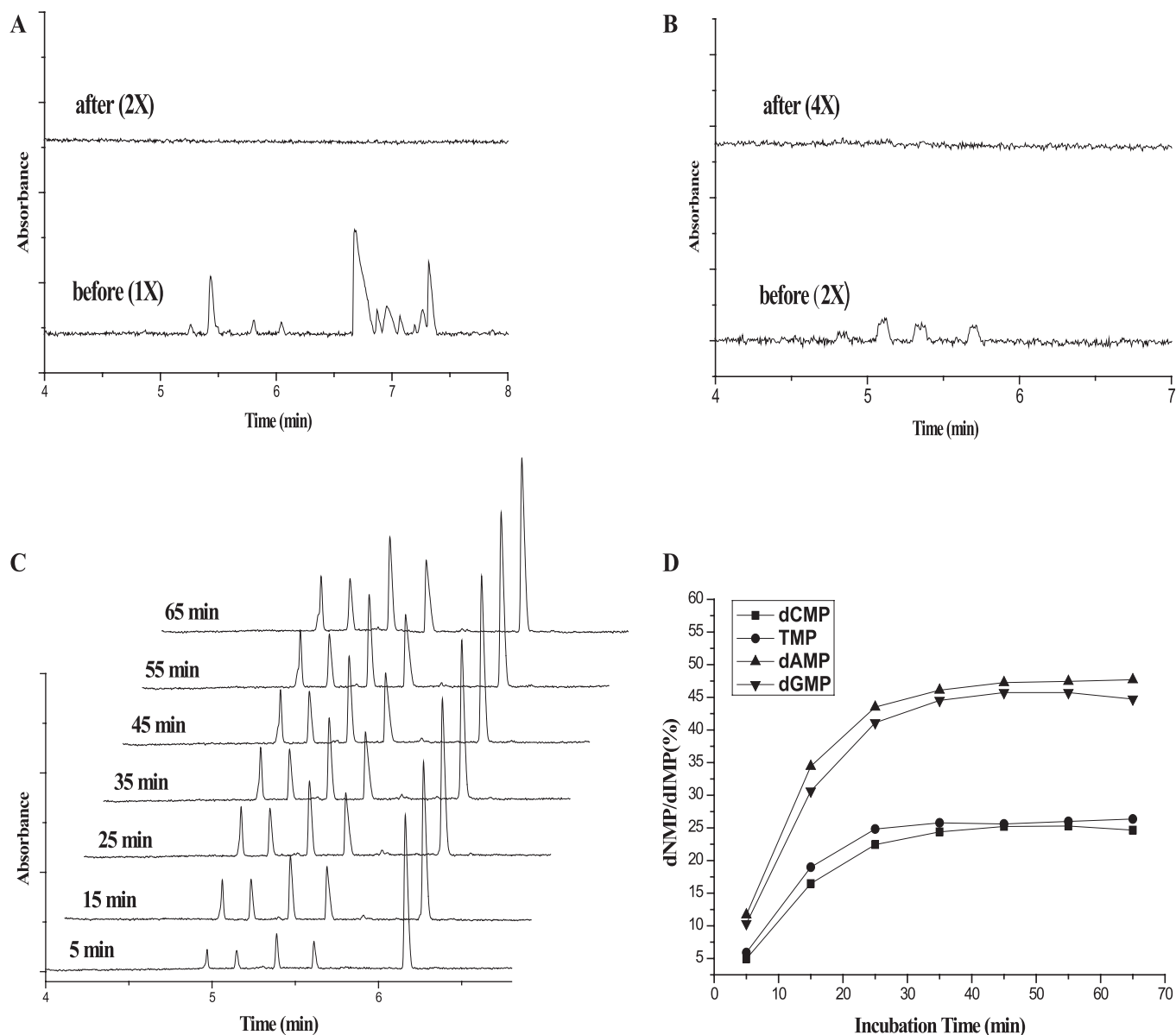


Figure 2. Confirmation of cleanup and enzymatic hydrolysis. (A) Removal of dNTPs. Samples were subjected to CE before and after a conventional PCR purification process. No peak was detected even in the 2-fold concentrated sample after purification. (B) Removal of primers. dNMP peaks resulting from residual primers were not detected in the 4-fold concentrated sample after purification. (C) CE profile of hydrolysis of a PCR product during a time course of phosphodiesterase treatment. dIMP was spiked as an internal standard for comparison. (D) Normalized dNMP peak intensities during a time course of phosphodiesterase treatment.

dNTPs were the only sources of dNMPs in the samples. From duplicated PCR mixtures deprived of primers and templates, one was directly applied to CE without purification and digestion processes while the other was normally purified, digested and applied to CE. It is obvious that dNTPs and dNMPs thereof were successfully eliminated by the cleanup process producing no apparent peak even when a 2-fold concentrated sample was analyzed (Figure 2A). A similar demonstration of the removal of residual primers from the PCR mixture is presented in Figure 2B. PCR mixtures with primers but deprived of dNTPs and templates were processed. In purified samples, no peak was detected even when a 4-fold concentrated sample was analyzed. In the analysis of the 2-fold concentrated sample, which was not purified, minimal peaks were detected

implying quantification errors resulting from unsuccessful removal of primers would be negligible. Regarding the PCR purification, it is important to confirm that the PCR products for analysis are devoid of any non-specific byproducts which would not be easily removed by a conventional purification. In the case with byproducts, the contaminating byproducts must be removed either by excising out the specific PCR band from the gel or by a nested primer PCR.

Complete hydrolysis of a PCR product to dNMP monomers is essential for accurate quantification of methylation in this approach. It was not easy to directly confirm completeness of hydrolysis by SVPD treatment because signals of incompleteness such as short oligomers were not detectable in our CE condition. Instead, we applied an indirect

confirmation approach by monitoring the saturation of peaks in a time course of enzyme treatment. Figure 2C shows a time profile of dNMP peaks during SVPD treatment. The fifth peak at the migration time around 6.3 min represents dIMP which was spiked as an internal standard for normalization of peak intensities. Normalized peak intensities during a time course are represented in Figure 2D. No significant rises of peaks were observed after 45 min of treatment. Three independent experiments led to a same indication that 45 min of enzyme treatment was enough for completion of enzyme digestion. In addition, as will be mentioned later, the fact that C/T ratio was equal to G/A ratio for a double stranded PCR product was another indication of complete digestion to dNMP monomers.

Calibration standards

dNMPs have different molar UV extinction coefficients with each other depending on their structures (28). It means that the ratios of peak intensities as measured through a UV detector do not accurately correlate to the molar ratios of dNMPs. Therefore, a calibration process was needed to correctly assign the molar ratios of dNMPs from the peak ratios. Initially, standard dNMP mixtures of certain molar concentrations were prepared by gravimetry. However, preparation of standard mixtures by weighing resulted in significant variations in molar concentrations of dNMPs in the mixtures owing to hygroscopic natures of the chemicals (data not shown). The alternative choice was to use a sequence-defined pure oligonucleotide as a standard for measurement of dNMP ratios. This strategy was valid only as we were not interested in absolute amount but interested in ratios of dNMPs. Two PAGE-purified synthetic oligonucleotides were employed as standards in this study. Calibration factors calculated from analysis of standard oligonucleotides are presented in Table 1. Based on our CE and UV detector system, C/T ratio was determined to be 1.13 and G/A to be 1.03 in terms of the ratios of peak areas. Small differences between calibration factors derived from different standard oligonucleotides (1.12 versus 1.13 for C/T and 1.02 versus 1.03 for G/A) lied within a range of experimental variation of the CE analysis whose repeatability was measured to be about 3 and 5% of CVs (Figure 1B). It is notable that the calibration factors determined from our analysis were not equal to those calculated from previously known UV extinction coefficients of dNMPs. Calibration factors from recently-revised UV extinction coefficients were 1.21 and 1.24 for C/T and G/A, respectively (28). A major cause of the discrepancy is thought to be the difference of the wavelength of UV used for

measurement. The wavelength in our UV detector was set to be 265 nm while extinction coefficients of dNMPs were determined at under 260 nm. The differences could also have resulted from the differences in measurement environment, such as electrolytes and buffer matrices.

Validation

As a validation of the established method, we compared the methylation levels of *in vitro* methylated DNA fragments measured through our method and through bisulfite sequencing. Bisulfite-PCR products from four differentially methylated plasmid fragments were sub-cloned, and sequences of single representative clones were determined. Methylation states of individual CpG sites in those clones as determined by sequencing are presented in Figure 3A. Then the specific clones were subjected to CE analysis for ratiometric quantification of dNMP components. Electropherograms in Figure 3B show apparent increases of C and G peaks compared with T and A peaks in more heavily methylated clones. Quantification results are summarized in Table 2.

In contrast to the cases of single strand oligonucleotides, C/T ratios measured by CE do not directly indicate actual methyl-cytosine/cytosine ratios in double stranded PCR products. Guanines also contribute to C levels in CE because their counterparts are cytosines in the opposite strand. The C/T ratio in CE represents $(C + G)/(T + A)$ ratio in the original PCR product. Therefore the base composition of the target PCR product should be taken into account for the calculation of actual methyl-cytosine/cytosine ratio. The fact that target DNA is double stranded provides advantageous information in analysis. Owing to the base pairing nature, C/T ratio should always be equal to G/A ratio in the analysis of PCR products. This relationship could be utilized to grossly confirm the validity of the overall processes including completeness of digestion and correctness of calibration factors determined. Any significant level of incomplete digestion, incorrect calibration or insufficient removal of impurities will result in substantial inequality between C/T and G/A values. Our results were equivalent within 2% level, which satisfies an essential requirement for validity of the analytical procedure (Table 2).

The results in Table 2 show <3% differences between methylation levels quantified by sequencing and by our CE methods. The difference <3% correlated well with the degree of variation of CE process itself of which maximal CV was ~3% for C/T. All of the inaccuracy contributions originating from individual processes should be comprehensively reflected in the final accuracy. The fact that overall accuracy levels were equivalent with the variation levels solely from the CE process implies that inaccuracy contributions from other processes such as purification, enzymatic digestion and determination of calibration factors were negligible throughout the analysis.

We also applied our method to quantify the methylation level of P16 promoter regions harboring 37 CpGs from various liver cell lines. Three different analytical approaches, MS-PCR, bisulfite sequencing and quantitative CE were comparatively performed on the same region. MS-PCR results simply indicated hypomethylation of P16 promoter regions in Chang and Hep3B cell lines while hypermethylations in

Table 1. Determination of calibration factors for quantification of dNMPs in CE

| Std. oligo | C/T | | G/A | |
|---------------------|-------------|-------------|-------------|-------------|
| | Oligo 1 | Oligo 2 | Oligo 1 | Oligo 2 |
| Calculated | 1.00 | 1.50 | 0.57 | 0.50 |
| Measured* | 0.89 ± 0.02 | 1.32 ± 0.02 | 0.56 ± 0.02 | 0.48 ± 0.02 |
| Calib. factor | 1.12 | 1.13 | 1.02 | 1.04 |
| Calib. factor (ave) | | 1.13 | | 1.03 |

*Values obtained from three independent analyses with triplicate CE runs (3×3 CE runs). Std. Oligo: standard oligonucleotides of known sequences, calculated: calculated from the sequences, measured: measured through CE.

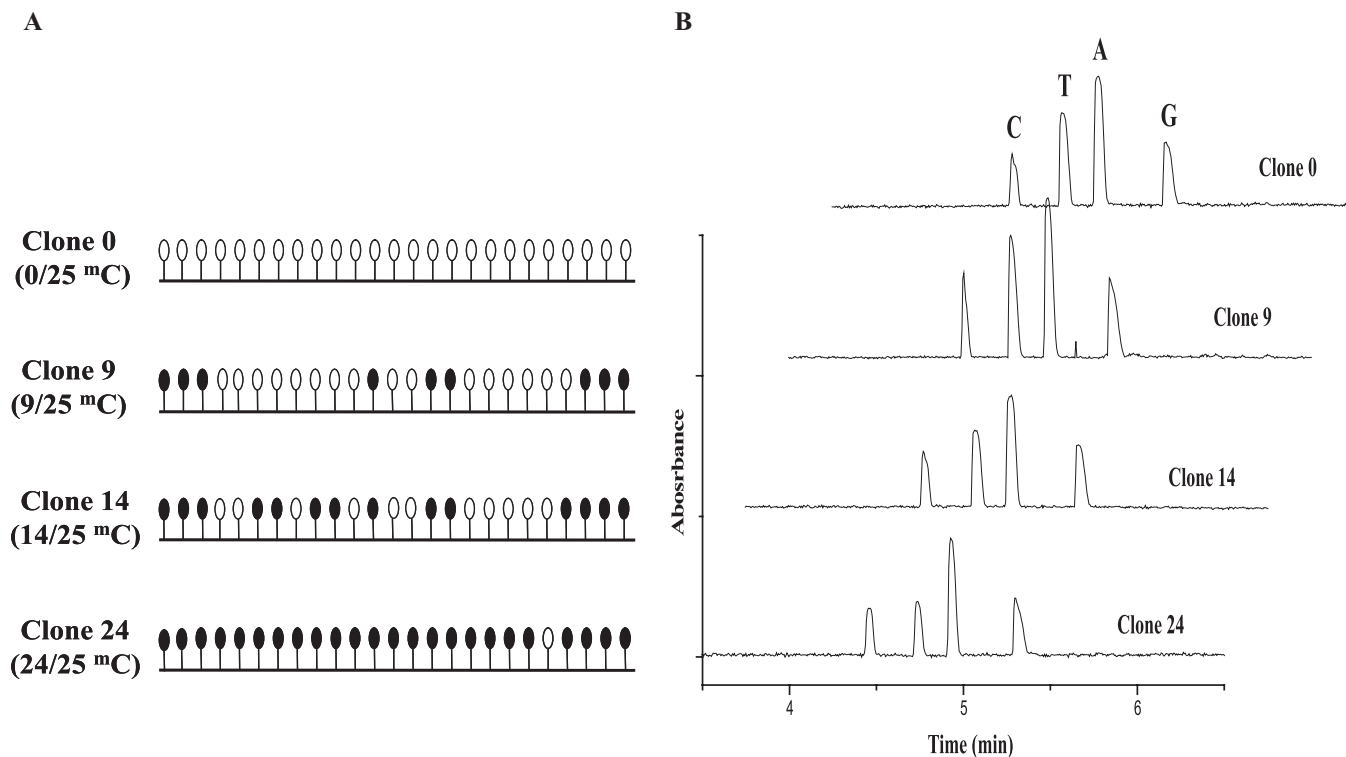


Figure 3. Validation of the method with clones of *in vitro* methylated DNAs. (A) Methyl CpG profiles of tested clones. *In vitro* methylated DNAs were bisulfite-treated, PCR-amplified, sub-cloned and sequenced for determination of methylation states. Number of methyl CpGs are denoted in the name of clones. Closed circles are methylated CpGs. (B) CE profiles of test clones. Higher C/T ratio in the CE profile correlates with heavier methylation of a clone.

Table 2. Quantification of methylation status of bisulfite-PCR clones

| Clone | C0 | | C9 | | C14 | | C24 | |
|----------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| | C/T | G/A | C/T | G/A | C/T | G/A | C/T | G/A |
| Calculated | 0.43 | 0.43 | 0.50 | 0.50 | 0.54 | 0.54 | 0.62 | 0.62 |
| Measured* | 0.37 ± 0.01 | 0.42 ± 0.01 | 0.44 ± 0.02 | 0.48 ± 0.02 | 0.47 ± 0.01 | 0.53 ± 0.02 | 0.55 ± 0.02 | 0.61 ± 0.02 |
| Calibrated | 0.42 | 0.43 | 0.50 | 0.50 | 0.53 | 0.55 | 0.62 | 0.63 |
| Difference (%) | -2.9 | 0.0 | -1.5 | -0.6 | -3.0 | 0.7 | -1.1 | 0.8 |

*2×3 CE runs. Calculated: calculated from bisulfite sequencing; Measured: measured through CE; Calibrated: calibrated using the calibration factors in Table 1; Difference (%): differences of the calibrated values from the calculated

SNU475 and SNU886 cell lines (Figure 4A). The same conclusion on methylation status could be derived from a simple interpretation of the CE profiles (Figure 4B). Peaks for C are significantly higher in profiles of SNU cell lines. Quantitative information from the CE analysis is summarized in Table 3. Bisulfite sequencing results are also supplemented for comparison. According to the results of sequencing, the P16 region analyzed in this study was extremely hypomethylated in Chang and Hep3B while almost fully methylated in SNU475 and SNU886 cells. From analyses of 30 independent clones for each sample, the estimated CpG methylation status were 0, 0.27, 95.6 and 97.5% for Chang, Hep3B, SNU886 and SNU475 cells, respectively (data not shown). Considering the base composition of the P16 PCR product, 0 and 0.3% CpG methylations lead to C/T and G/A ratio of 0.811 while 95.6 and 97.5% CpG methylation lead to ratio of 1.09 and 1.11, respectively. C/T or G/A ratios obtained from our CE results were in the range of 0.77–0.80 for hypomethylated Hep3B and Chang cells while in 1.06–1.10 for hypermethylated SNU cells (Table 3). The differences

of estimated base ratios between bisulfite sequencing and CE analyses were 1.4–4.6%. Although the values estimated from the CE analyses were slightly lower than those from sequencing, the differences lie within or close to the variation range of the CE process. Therefore, it was concluded that quantification results from our CE method and from bisulfite sequencing agreed within ±5%.

DISCUSSION

Accuracy and precision are conventional criteria in evaluating the performance or uncertainty of an analytical method. Accuracy is practically represented by difference of the result from a method and that from a reference one while precision is represented by the degree of variations of the results as expressed in CV. For quantitative analysis of DNA methylation, no reference method with which other methods could be compared is available yet. Bisulfite-mediated sequencing is currently accepted as one to produce most detailed and

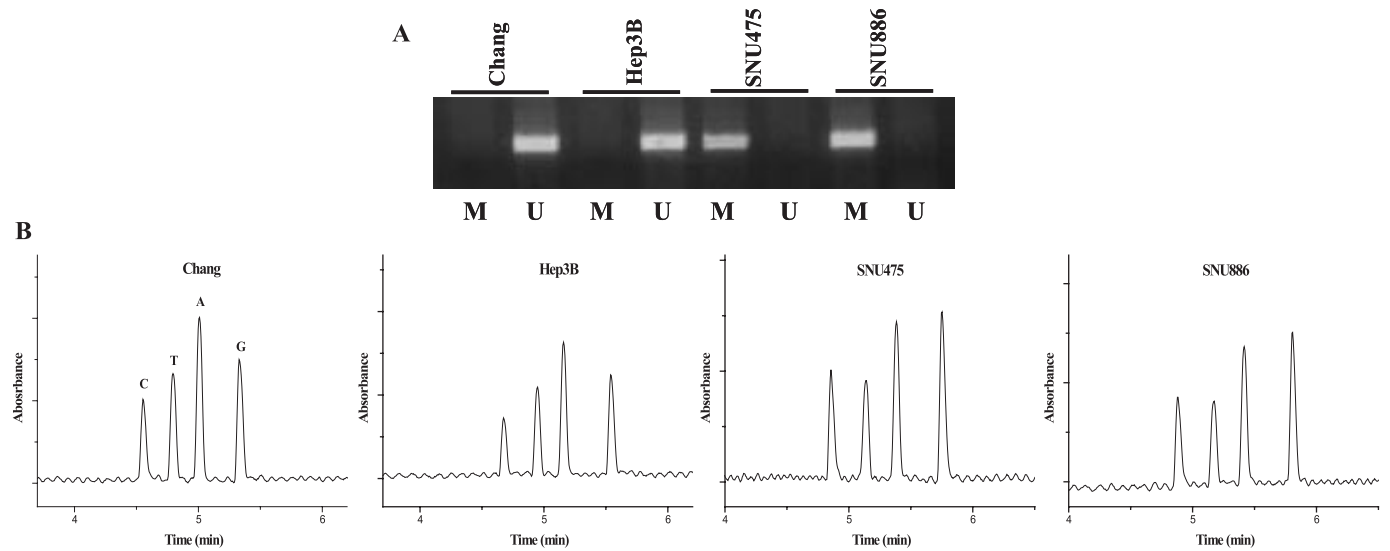


Figure 4. Application of the method to quantify methylation states of P16 promoter regions from liver cell lines. (A) MS-PCR results representing positive or negative type of methylation information. M, methylated; U, unmethylated. (B) CE profiles representing quantitative information on methylation states of the same loci.

Table 3. Summary of DNA methylation analyses of P16 promoter regions in liver cell lines

| Cell line | Chang | | Hep3B | | SNU475 | | SNU886 | |
|-----------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|-------------|
| Base ratio | C/T | G/A | C/T | G/A | C/T | G/A | C/T | G/A |
| MS-PCR | U | U | U | U | M | M | M | M |
| Sequencing* | 0.81 | 0.81 | 0.81 | 0.81 | 1.11 | 1.11 | 1.09 | 1.09 |
| Measured (CE)** | 0.70 ± 0.03 | 0.78 ± 0.01 | 0.70 ± 0.02 | 0.75 ± 0.01 | 0.95 ± 0.03 | 1.02 ± 0.03 | 0.98 ± 0.01 | 1.04 ± 0.01 |
| Calibrated (CE) | 0.79 | 0.80 | 0.79 | 0.77 | 1.07 | 1.06 | 1.10 | 1.08 |
| Difference (%) | -2.6 | -1.4 | -2.3 | -4.6 | -4.0 | -4.4 | 1.0 | -1.0 |

* Values obtained from sequencing of 30 independent clones following bisulfite-PCR and cloning.

** 2x3 CE runs. U: unmethylated, M: methylated.

quantitative information on DNA methylation. Nonetheless, the bisulfite sequencing itself is not an absolute or acceptable reference method because it employs several manipulation processes through which distortion of information could be accompanied. Incompleteness of bisulfite-mediated conversion of bases as well as biases in PCR, sub-cloning and sequencing processes are major potential sources of analytical uncertainties. Owing to unavailability of a reference method, the overall accuracy of our method for quantification of DNA methylation could not be directly assessed. Instead, only comparisons with the result of bisulfite-mediated sequencing are presented as indirect indicators while the shortcoming of the approach is fully acknowledged. Compared with bisulfite sequencing, our method does not include sub-cloning and sequencing procedures albeit the same bisulfite conversion and PCR procedures are included. Therefore, our method is expected to yield a quantification performance with a smaller or similar level of measurement uncertainty compared with that from bisulfite sequencing.

In contrast, however, some later parts of the proposed analytical procedure could be accurately evaluated using an artificial construct with a defined base composition as a reference material. For artificial constructs, the analytical procedure consisted only of hydrolysis, purification and CE analysis.

Deviations from the theoretically calculated values should be highly reliable indicators of the accuracy of the involved procedures.

As shown in Table 2, the differences of the CE results from the calculated values were <3%. It means that provided with a bisulfite-PCR product, our CE method could be applied to quantify the methylation content of the sample with an accuracy level of ±3%.

As for precision, our method currently exhibits 3 and 5% of maximal variations for C/T and G/A, respectively (Figure 1B and C). In real situations such as at clinical settings, however, single measurement is commonly made for speed and convenience. For this reason, the range of the maximum possible scattering of any single measurement is an important consideration. Applying 't-test' for a 95% confidence level (29), the range of the maximum possible scattering of the results of 95% of any single measurements was calculated as ±7.8% of the mean value for the case of the worst data scattering (C/T ratio of cell line Chang in Table 3). In this particular dataset of six data points, the maximum scattering was +5.7%, which is safely within the calculated range of the maximum possible scattering. Therefore, it seems fair to declare that the ranges of the maximum possible scattering of the results of 95% of any single

measurements of the proposed method would be $<\pm 10\%$ of the mean values.

Throughout all analyses in this work, variations of the final results were maintained under these variation limits derived from CE processes. This observation led to a conclusion that CE process itself was the dominant source of analytical uncertainty while contributions from other sources were negligible. Insufficient sensitivity of the detection system for small amount samples seems to be the major cause of the variations. A substantial improvement is expected as the sensitivity of the CE method could be enhanced by adopting an on-column sample concentration technique as well as upgrading the detection system.

Regarding the practicability, our CE-based approach provides an attractive addition to current methods for analysis of DNA methylation. The speed, sensitivity and simplicity of our method are comparable with conventional screening methods such as MS-PCR. Analysis can be performed with sample amounts from single tube PCRs, and batch processing of multiple samples is also possible. Quantitative outputs are provided directly from PCR products within 1.5 h of analysis time. The quantitative information provided by our method would be of great value especially when supplemented onto a simple positive or negative result from MS-PCR. Another advantage of our method is that it provides quantitative information from the full stretch of a PCR product. MS-PCR and real-time PCR-based approaches discriminate the methylation status of a limited number of CpGs located in the regions where primers or probes bind. In those cases, additional processes for verification of methylation status of entire region are inevitable. Our method would be useful in verifying the methylation status in the full stretch of a PCR product following primary screening. As demonstrated through this article, analysis of DNA methylation through CE following bisulfite treatment and PCR provides a fast, accurate, sensitive and flexible choice for applications in clinical and analytical fields. We are currently preparing for a large-scale experimental scheme for confirming the usefulness of the method for versatile applications.

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Conflict of interest statement. None declared.

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