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A novel combined bisulfite UDG assay for selective 5-methylcytosine detection

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ABSTRACT

DNA modification, a significant epigenetic event, largely affects genes' binding with the transcription factors and some other DNA binding proteins. Among DNA modifications, methylation, especially cytosine methylation is of great importance and attracts extensive studies as it leads to the silence of tumor-suppressor gene expression. In this work, a novel combined bisulfite Uracil-DNA glycosylase (UDG) assay has been developed on the basis of bisulfite modification to generate uracil from cytosine, subsequent UDG-mediated uracil elimination and ultimate DNA cleavage in alkaline condition. This strategy can be used to selectively detect exact number and loci of 5-methylcytosine residues regardless of sequence context. Moreover, it provides linear quantitative results of DNA methylation level across a wide range.

1. Introduction

We are accustomed to the idea that the coding potential of the genome lies within the arrangement of the four bases adenine, cytosine, guanine and thymine; however, additional information affecting phenotypes is stored in the distribution of the modified base 5-methylcytosine [1–3]. Special methyltransferases replace the H atom at the 5 position by a methyl group to form methylcytosine (^{5Me}dC), which frequently appears in CpG islands. During early mammalian development, cytosine methylation exhibits dramatic global changes [4]. It has become apparent in recent years that the transcriptional silencing associated with 5-methylcytosine is important in mammalian development, protection against intragenomic parasites, genomic imprinting, X chromosome inactivation, mental health and cancer [5–9]. It has also been shown that global hypomethylation of genomic DNA can increase the incidence of various diseases, especially cancers. Identification of the DNA methylation level and status of various genes is consequently the focus of intensive research efforts [10,11].

Many molecular biological techniques have been developed to quantify the cytosine methylation of genomic DNA. Methods that have been used to date include bisulfite sequencing [12,13], combined bisulfite restriction analysis (COBRA) [14], methylation-specific PCR amplification [15], single molecule real time sequencing (SMRT) [16,17] and several others. At the same time, the differential reactivity of some chemical compounds towards dC and ^{5Me}dC has been utilized to selectively distinguish ^{5Me}dC from dC. Reagents useful in such assays include hydrazine [18], OsO_4 [19], the V_2O_5/LiBr pair [20] and N-halo-N-sodiobenzenesulfonamide [21]. Bisulfite conversion is the most classic of these methods. It is used in combination with amplification and sequencing to profile the methylation status of the genome. The broad utility of this method is a result of its easy accessibility, high selectivity and yield, and quantitative accuracy [22].

Uracil-DNA glycosylase (UDG) is an enzyme ubiquitous among prokaryotes and eukaryotes, and it is present even in some DNA viruses. One important function of UDG is the prevention of mutagenesis through elimination of uracil from DNA molecules. This achieved through cleavage of N-glycosidic bond and initiation of base-excision repair (BER) [23]. It was reported recently that deamination-coupled glycosylase-related BER has emerged as an alternative demethylation pathway [24,25].

We have developed a novel combined bisulfite UDG assay to detect 5-methylcytosine selectively in DNA sequences (Scheme 1). This assay entails initial modification of DNA by sodium bisulfite, converting all unmethylated (but not methylated) cytosine to uracil through a deamination reaction. The assay takes advantage of such sequence differences, as treatment with UDG removes uracil to generate an apyrimidinic site and leaves unreacted substrates intact. Cleavage of the abasic sites under mild alkaline conditions allows for specific detection of the products after denaturing

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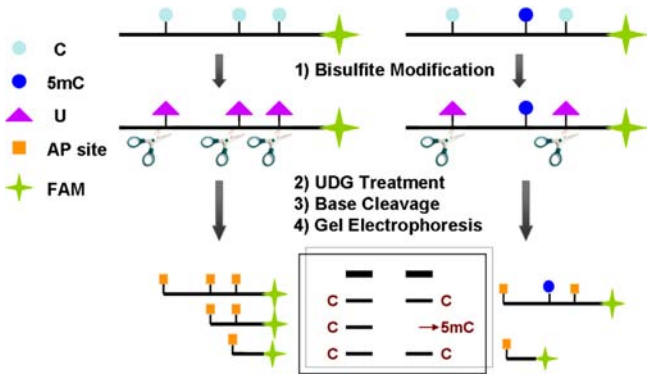

Scheme 1. Combined bisulfite UDG assay for 5-methyl-cytosine detection.

gel electrophoresis. Finally, by comparing the corresponding gel band deletions, the number and respective loci of methylated cytosine residues can be obtained easily in a precise and intuitive way.

2. Materials and methods

2.1. Materials and equipments

All solvents and reagents were commercially available and used without further purification unless for special needs. Sodium bisulfite, sodium hydroxide, hydroquinone, mineral oil, absolute ethyl alcohol, piperidine, dimethylsulfate and ethanethiol were bought from Sangon Biotechnology Co., Ltd. (Shanghai, China). TE buffers, sodium acetate buffer were prepared and confirmed by pH meter. All the oligomers labeled with 6-Carboxyfluorescein (FAM) were purchased from TaKaRa Biotech (China). We scanned the denaturing polyacrylamide gel electrophoresis products with Pharos FX Molecular imager (Bio-Rad, USA) operated in the fluorescence mode. Uracil DNA glycosylase was purchased from BioLabs (NEW ENGLAND).

2.2. Bisulfite modification

Approximately 2 μg of DNA was incubated with 0.3 M of NaOH for 30 min at 42 °C. Sodium bisulfite was then added to achieve a final concentration of 2.5 μM. Hydroquinone was added to achieve a final concentration of 0.5 mM. The reaction was heated in a mineral oil bath at 50 °C for 16 h. Samples were desalted three times each with Millipore membranes, subjected to ethanol precipitation and resuspended in TE buffer, pH 7.5.

2.3. Uracil DNA glycosylase treatment

Unless otherwise noted, 100 ng of bisulfite-modified DNA was treated with 1 unit of UDG (New England Biolabs, Beverly, MA) in 1 × UDG buffer for 1 h at 37 °C. The mixture was then treated with hot 10% piperidine for 30 min at 90 °C. The reaction was quenched and subjected to ethanol precipitation with 10 mM of sodium acetate (pH 5.0) and 2 μl of glycogen at −80 °C for 2 h. The precipitated DNA was dried in vacuo and dissolved in 80% deionised formamide (10 μl) for subsequent dPAGE analysis.

2.4. Denaturing polyacrylamide gel electrophoresis

The DNA samples were loaded on a 24 cm × 24 cm 20% polyacrylamide gel for electrophoresis at 20 V/cm for 90 min and analyzed by Pharos FX Molecular Imager (Bio-Rad, USA).

2.5. Preparation of G-ladder

1 μl of DNA solution (100 μM), 10 μl of TE buffer (100 mM, pH 7.5) and 89 μl of distilled water were added to the G reaction tube. While 40 μl of distilled water, 20 μl of sodium acetate buffer (1 M, pH 5.0), and 40 μl of ethanethiol were added into the other tube to prepare the DMS stop buffer. Then, 2 μl of DMS was added to the G reaction tube. The solution was mixed and incubated at room temperature for 8 min, followed by addition of the DMS stop buffer. The mixture was subjected to ethanol precipitation for 2 h and then treated with 10% piperidine for 30 min at 90 °C. DNA was subjected to a second ethanol precipitation, dried in vacuo and dissolved in 80% deionised formamide (100 μl) for dPAGE analysis.

3. Results and discussion

3.1. PAGE analysis of DNA with a single cytosine in the middle of the sequence

As a proof of concept, two 30-mer single-strand oligodeoxynucleotides labeled with a fluorophore (FAM) were synthesized with a single internal modified cytosine residue embedded in a CpG motif (ODN S30-C is unmethylated, while ODN S30-mC is methylated). These two ODNs were subjected to traditional bisulfite treatment according to the general procedure [15]. Hence, ODN S30-C generated dU, while the ^{5Me}dC residue in ODN S30-mC was retained. The ODNs were treated with the uracil excision protein UDG, followed by base-induced cleavage, and the products were analyzed by denaturing polyacrylamide gel electrophoresis (dPAGE) as shown in Fig. 1. The enzyme substrate ODN S30-C was completely converted into a 15-mer fragment product that appears in the lower position on the gel (Lane 2), showing both highly efficient uracil formation and thorough UDG-mediated base excision. On the other hand, no cleavage was observed for ODN S30-mC in Lane 4, as ^{5Me}dC remained intact throughout the experiment. Therefore, the lack of a lower band in Lane 4 reveals the location of the methylated cytosine.

3.2. PAGE analysis of single- and double-stranded DNA containing multiple dC and ^{5Me}dC residues

To ensure that the discrimination against ^{5Me}dC was not limited to the context of a single sequence, we further applied this method to single- and double-stranded DNA containing multiple dC and

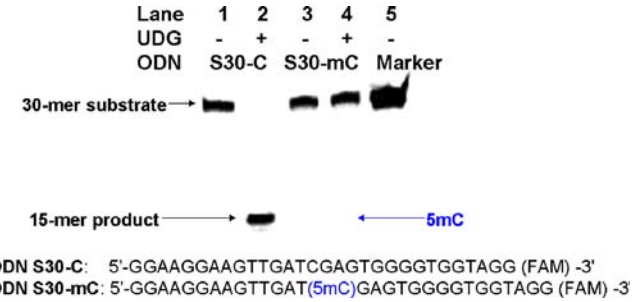

Fig. 1. PAGE analysis of FAM-labeled 30-mer ODN S30-C and ODN S30-mC with a single cytosine in the middle of the sequence. All the samples in Lane 1–4 were subjected to the same bisulfite modification and hot piperidine treatment as described in experimental section. Lane 1 and 2 refer to ODN S30-C; Lane 3 and 4 refer to ODN S30-mC; Lane 5 is a 30-mer DNA marker. Lane 1 and 3 are samples without UDG treatment; Lane 2 and 4 are samples with 1 unit UDG treatment for 1 h at 37 °C. Lane 2: a complete cleavage product at the dC site of ODN S30-C was observed; Lane 4: no cleavage product at the ^{5Me}dC site of ODN S30-C was observed.

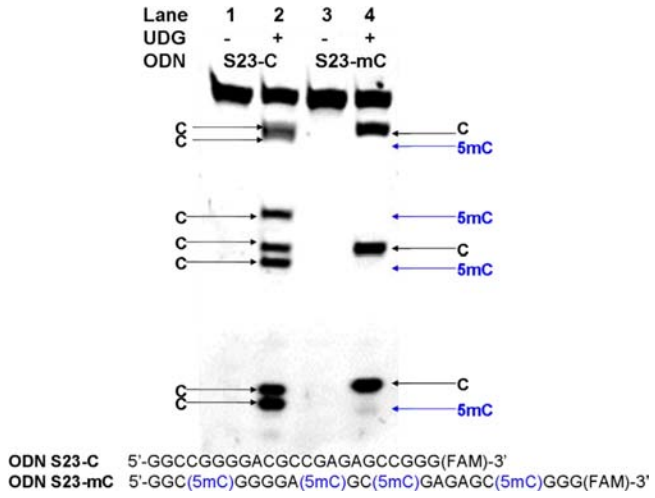

Fig. 2. PAGE analysis of FAM-labeled single-stranded 23-mer ODN S23-C and ODN S23-mC. ODN S23-C has seven dC residues; ODN S23-mC has three dC residues and four ^{5Me}dC residues. All the samples in Lane 1–4 were subjected to the same bisulfite modification and hot piperidine treatment as described in experimental section. Lane 1 and 2 refer to ODN S23-C; Lane 3 and 4 refer to ODN S23-mC. Lane 1 and 3 are samples without UDG treatment; Lane 2 and 4 are samples with 1 unit UDG treatment for 1 h at 37 °C. Lane 2: seven cleavage fragments at the dC sites of ODN S23-C were observed; Lane 4: only three cleavage fragments were observed, referring to dC sites of ODN S30-mC. The locations of the four missing bands refer to ^{5Me}dC sites of ODN S30-mC.

^{5Me}dC residues (Fig. 2 and Fig. S1). We chose two sets of FAM-labeled oligodeoxynucleotides, ODN S23-C/ ODN S23-mC and ODN DS23/ODN DS23-mC as our model sequences (Supplementary Table 1). ODN S23 is a short sequence from the CpG island of *RASSF1A* (Ras association domain family 1 isoform A) gene, a tumor suppressor for which inactivation is implicated in the development of many human cancers. [26] The variant ODN S23-C has seven dC residues, while the variant ODN S23-mC has three dC residues and four ^{5Me}dC residues in the CpG context. Fig. 2 shows that the product fragments were effectively separated by dPAGE after the combined bisulfite UDG assay. As expected, seven cleavage bands appeared for ODN S23-C (Lane 2) upon UDG treatment; however, only three new bands were observed for ODN S23-mC (Lane 4) under the same conditions. This result clearly indicates that the locations of the four missing bands represent four ^{5Me}dC sites in ODN S23-mC. When this assay was performed directly on the double-stranded DNA sequences (ODN DS23/ ODN DS23-mC), we observed the cleavage patterns shown in Fig. S1; these are consistent with those found for the single-stranded DNA constructs (ODN S23-C/ ODN S23-mC). The presence of a guanine base paired to cytosine in the double-stranded DNA did not affect bisulfite conversion or UDG excision. Taken together, these results illustrate the utility of our combined bisulfite UDG assay in identifying the exact number and loci of multiple methylated cytosine residues in both single- and double-strand DNA.

3.3. Determination of the quantitative accuracy of combined bisulfite UDG assay

We next examined whether our method would provide quantitative results across a wide range of DNA methylation levels. Mixtures of unmethylated ODN S23-C and methylated ODN S23-mC in various molar ratios were prepared prior to bisulfite treatment and processed as independent samples throughout the entire procedure. The results are shown in Fig. 3. The integrated density volume of the 14 bp band was plotted as a function of DNA methylation percentage. Linear regression analysis, with a correlation coefficient of 0.976, confirms that the combined bisulfite UDG assay yields reliable information on DNA methylation levels in a linearly quantitative fashion.

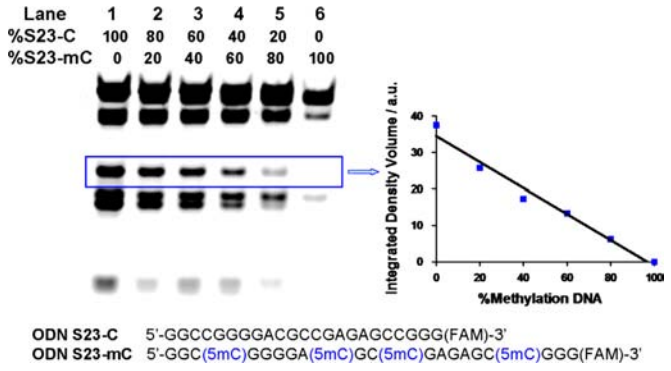

Fig. 3. Determination of the quantitative accuracy of combined bisulfite UDG assay. ODN S23-C and ODN S23-mC were pre-mixed at different molar fraction (0%, 20%, 40%, 60%, 80% and 100%) before bisulfite modification, and then treated under the same reaction conditions described in Fig. 2. The integrated density volume of the 14 bp band shows a linear relationship with ODN S23-mC percentage.

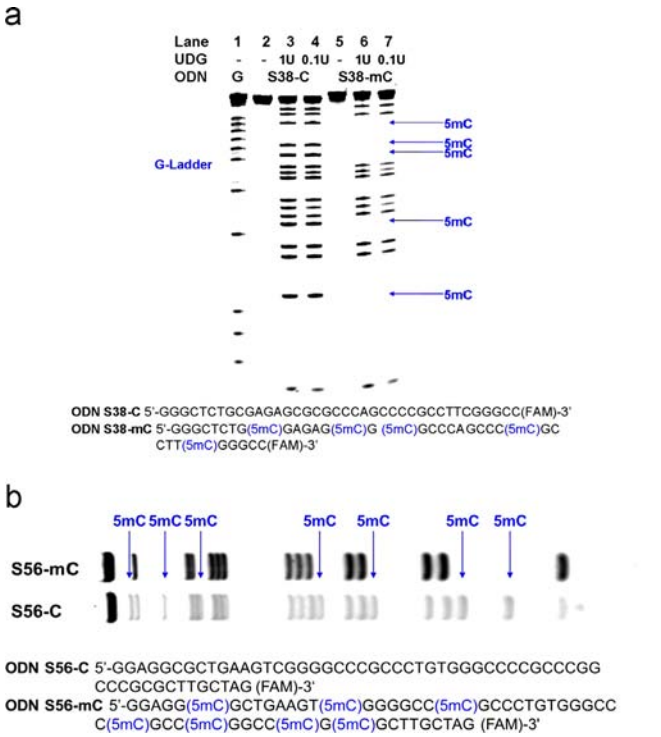

Fig. 4. (a) PAGE analysis of FAM-labeled single-stranded 38-mer ODN S38-C and ODN S38-mC. ODN S38-C has 17 dC residues; ODN S38-mC has 12 dC residues and five ^{5Me}dC residues. All the samples in Lane 1–7 were subjected to the same bisulfite modification and hot piperidine treatment as described in experimental section. Lane 1 is Maxam–Gilbert G sequencing lane; Lane 2–4 refer to ODN S38-C; Lane 5–7 refer to ODN S38-mC. Lane 2 and 5 are samples without UDG treatment; Lane 3 and 6 are samples with 1 unit UDG treatment for 1 h at 37 °C. Lane 4 and 7 are samples with 0.1 unit UDG treatment for 1 h at 37 °C. Lane 3 and 4: 17 cleavage fragments at the dC sites of ODN S38-C were observed; Lane 6 and 7: only twelve cleavage fragments were observed, referring to dC sites of ODN S38-mC. The locations of the five missing bands refer to ^{5Me}dC sites of ODN S38-mC. (b) PAGE analysis of FAM-labeled single-stranded 56-mer ODN S56-C and ODN S56-mC. ODN S56-C has 22 dC residues; ODN S56-mC has 15 dC residues and seven ^{5Me}dC residues. Two samples were treated with 1 unit UDG. The locations of the seven missing bands refer to ^{5Me}dC sites of ODN S56-mC.

3.4. PAGE analysis of the location of ^{5Me}dC in longer sequences

To verify the feasibility of our approach in longer sequence context, two random regions on the CpG island of the *RASSF1A*

gene were then excerpted as target sequences: a 38-mer ODN with five ^{5Me}dC residues and a 56-mer ODN with seven ^{5Me}dC residues (Sequences of oligomers are listed in Table S1). After bisulfite treatment under the same conditions described above, the UDG cleavage behavior was analyzed. The result in Fig. 4(a) and (b) confirm that all dC residues can be cleaved efficiently with the retention of ^{5Me}dC residues irrespective of the DNA environment. As shown in Fig. 4(a), the deletion of fragments in Lane 7 when compared with the corresponding products from unmethylated DNA in Lane 4 accurately reveals the exact positions of the five ^{5Me}dC residues in ODN S38-mC. It is worth mentioning that the G ladder in Lane 1 facilitates the determination of the relative position of methylated cytosine in the CpG context; this ladder was prepared according to the reported Maxam–Gilbert method [18]. More importantly, the amount of UDG enzyme had an effect on this assay, which can be observed in Lane 6 (1 unit UDG) and Lane 7 (0.1 unit UDG). Higher concentrations of UDG increased the rate of uracil removal, which led to higher yields of cleavage fragments.

4. Conclusions

The combined bisulfite UDG assay described here can be used to detect effectively and specifically the amount and location of 5-methylcytosine residues regardless of sequence context. This approach provides linearly quantitative assessments of DNA methylation across a wide range of methylation levels. This assay has the potential to enable profiling of methylation levels in genomic DNA, which will be pursued in future work.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.09.026.

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