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Journal:	<i>Genome</i>
Manuscript ID	gen-2014-0185.R4
Manuscript Type:	Techniques
Date Submitted by the Author:	15-Jul-2015
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Keyword:	single-nucleotide extension, BS-OLE, bisulfite conversion, fluorescent dCTP/dUTP, DNA methylation

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Fluorescence polarization-based method with bisulfite conversion-specific one-label extension for quantification of single CpG dinucleotide methylation

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Abstract

To quantify the methylation at individual CpG dinucleotide sites in large biological or clinical samples, we develop a bisulfite conversion-specific one-label extension (BS-OLE) method using visualization by fluorescence polarization (FP) measurement of methylation at single CpG site in small amounts of genomic DNA. Genomic DNA is treated with sodium bisulfite to convert unmethylated cytosine to uracil leaving 5-methylcytosine unaltered and BS-PCR is used to generate DNA template containing target CpG sites. BS-OLE uses a BS-primer hybridized immediately upstream of the target CpG site being examined and then fluorescent dCTP or dUTP is incorporated into the methylated (CpG) or unmethylated (TpG) form of the target site through single-nucleotide chain extension, yielding an FP ratio between the fluorescent dCTP- and dUTP-incorporated products as a measure of methylation. This provides stable estimates of the methylation level of human genomic DNA and of a 250 bp plasmid DNA segment containing a single TCGA *TaqI* cleavage site, in accordance with the results of a combined bisulfite restriction analysis method. We use BS-OLE to measure dose-dependent DNA hypomethylation in human embryonic kidney 293T cells treated with the DNA methyltransferase inhibitor 5-aza-dC. BS-OLE is well suited to high-throughput multi-sample applications in biological and medical studies.

Keywords: single-nucleotide extension, BS-OLE, bisulfite conversion, fluorescent dCTP/dUTP, DNA methylation

Introduction

DNA cytosine methylation is a dynamic epigenetic modification, and is generally associated with transcriptional silencing and plays an important role in diverse cellular processes. These processes include gene regulation, suppression of transposable elements, cellular differentiation, embryonic development, genomic imprinting, X-chromosome inactivation and chromosome stability (Jones and Takai 2001; Schubeler 2015). The conversion of cytosine to 5-methylcytosine is catalyzed by DNA methyltransferases via transferring a methyl group from *S*-adenosylmethionine to a cytosine base. DNA methylation displays differential patterns between plant and mammalian genomes. Plants have extensive methylations of cytosine bases in the symmetric CG, CNG and asymmetric CHH sequence contexts where N denotes any nucleotide and H denotes A, C or T (Henderson and Jacobsen 2007; Law and Jacobsen 2010), which are also observed in embryonic stem cells in mammals (Lister et al. 2009; Lee et al. 2010). In contrast, most of mammals have methylations of cytosine bases in the symmetric CG contexts, which are estimated to occur at ~70-80% of CG dinucleotides throughout the genome (Law and Jacobsen 2010). The CpG dinucleotides in dense clusters known as CpG islands are mostly observed in the promoter regions and transposons such as long interspersed nucleotide elements (LINE elements) and Alu repetitive elements (He et al. 2014). Aberrant DNA methylations are involved in the aging process and diseases, including cancer and psychiatric disorders such as schizophrenia (Feinberg 2007; Pun et al. 2011), and can lead to pleiotropic morphological defects in plants (Law and Jacobsen 2010). A

method for rapid measurement of 5-methylcytosine will aid our understanding of the roles of DNA methylation in biology and medicine.

Several techniques are available for determination of methylation of a single cytosine nucleotide on the basis of bisulfite conversion methods, which convert unmethylated cytosine to uracil leaving 5-methylcytosine unaltered. The sequence differences between methylated and unmethylated cytosine after bisulfite conversion can be determined by microarray- or deep sequencing-based methods for genome-wide analysis, or by pyrosequencing, MALDI/TOF/Sequenom, combined bisulfite restriction analysis (COBRA), methylation-sensitive single-nucleotide primer extension (Ms-SNuPE) and methylation-specific PCR (MSP) for locus-specific analysis (Umer and Herceg 2013). Bisulfite sequencing-based methods give most accurate information about methylation status at individual CpG site in locus-specific or genome-wide regions, however, the requirements for large amounts of DNA, labor-intensive procedures and costly machines limit their use in general laboratories. PCR-based methods such as COBRA, Ms-SNuPE and MSP are considered practical with merits of very low sample consumption, even though these methods may yield false-positive results or sequence-specific bias due to incomplete enzymatic digestion or PCR artifacts due to over-amplification.

We describe a bisulfite conversion-specific one-label extension (BS-OLE) method for quantification of methylation at individual CpG sites. The method uses template-directed primer extension with incorporation of fluorescence-labeled dCTP

or dUTP measured by fluorescence polarization (FP) to quantify the levels of methylated and unmethylated cytosine after bisulfite conversion. The principle of FP is based on the observation that, when a small fluorescent molecule is excited by plane polarized light, the emitted light is more highly polarized if the molecule is stationary throughout the excited state (Sun et al. 2014). In BS-OLE, incorporation of fluorescence-labeled dCTP or dUTP into the extension primer displays relatively little movement during excitation and the emitted light will remain highly polarized. By contrast, the unincorporated fluorescent dCTP or dUTP molecules rotate and tumble rapidly, such that the emitted light is more depolarized relative to the excitation plane. The FP technique has been used in the analysis of molecular interactions of specific DNA sequences (Murakami et al. 1991; Ju et al. 2009) and of methylation status (Wu et al. 2010) based on hybridization with fluorescent probes. FP is used in DNA genotyping (Hsu et al. 2001; Akula et al. 2002; Yu et al. 2006; Sun et al. 2014) and genome-wide DNA methylation (Zhao and Xue 2012) based on single-nucleotide extension with fluorescent nucleotides. To our knowledge, however, no application of FP to the estimation of DNA methylation at the single base level has been reported. The FP measurements can be performed with DNA on 96- or 384-well plates without the need for post-extension purification steps and, therefore, BS-OLE is well suited to high-throughput multi-sample applications in biological and medical studies.

Materials and Methods

Human genomic DNA preparation and bisulfite conversion

Human glioblastoma cell lines of U87, A172, T98G and M059K, as well as human embryonic kidney 293T (HEK293T) cell line, are cultured using standard methods. The HEK293T cells are treated with 5-aza-2'-deoxycytidine (5-aza-dC; Sigma–Aldrich, Sweden) for 3 days prior to being harvested. Genomic DNA from cell lines and human peripheral blood cells is extracted with DNAzol[®] Reagent (Invitrogen, USA) and is employed for methylation analysis after bisulfite conversion.

Bisulfite conversion of genomic DNA is treated by denaturing 1µg genomic DNA with 0.3M NaOH at 42°C for 20 min, followed by 95°C for 3min and 0°C for 1 min, and incubating at pH 5.0 with sodium metabisulfite (2.0 M) and hydroquinone (0.5 mM) at 55°C for 16h in the dark overlaid with mineral oil (Pun et al. 2011). Converted DNA is purified with Promega Wizard DNA Clean Up System (Promega, USA). The eluted DNA is incubated with NaOH (0.3M) at 37°C for 15 min and neutralized by 3M NH₄-acetate to pH 7.0. The neutralized DNA is precipitated by 75% ethanol and recovered in 20 µl of TE buffer. Bisulfite-converted DNA is stored at -20°C until ready for BS-PCR.

BS-PCR amplification

BS-PCR is employed to amplify target DNA segment of bisulfite-converted DNA covering the 250 bp stretches within the promoter of the LINE1 element (NCBI Reference Sequence X58075, **Supplementary Figure 1A**) using BS-PCR primers of

BSF sense primer 5'-TTTTTTGAGTTAGGTGTGGG-3' and BSR antisense primer 5'-CATCTCACTAAAAAATACCAAACAA-3' (Gonzalgo and Liang 2007). Amplification is done in a 20 µl reaction mixture containing 25 ng of bisulfite-converted DNA, 75 nM of each primer, 50 nM each of dNTP, PCR buffer and 1 U *Taq* DNA polymerase (TaKaRa, China). The PCR reaction protocol is: denaturation step for 2 min at 95°C then 35 cycles each of 1 min at 95°C, 1 min at 60°C and 1 min at 72°C, plus a final extension step for 5 min at 72°C. PCR products are resolved by electrophoresis in 1.5% (w/v) agarose gels then stained with ethidium bromide to confirm the presence of products of expected 250-bp sizes and the absence of non-specific products. Meanwhile, non-BS primers are employed to amplify the LINE1 element from the bisulfite-converted DNA for evaluation of BS-conversion efficiency (**Supplementary Figure 2**). To remove unincorporated primers and nucleotides, 15 µl of verified PCR products is treated in a 40 µl of reaction mixture containing 3.6 U Shrimp Alkaline Phosphatase (SAP, GE Healthcare, UK), 2.4 U exonuclease I (TaKaRa) and SAP buffer (20 mM Tris-HCl, pH 8.0 and 10 mM MgCl₂) for 45 min at 37°C followed by 15 min at 85°C.

Methylated or unmethylated control DNA generation

In order to get control DNAs with unmethylation or 100% methylation at the target CpG dinucleotide (S1 site), 250-bp LINE BS-PCR products from bisulfite-converted human genomic DNA are cloned into the pMD18T vector by standard TA cloning according to manufacture protocol (TaKaRa). Because unmethylated C is replaced by T after BS-PCR amplification, any presence of C on the sequence represents a

methyated C allele at CpG dinucleotides in the bisulfite-converted DNA. The methylation status (viz. TpG or CpG) at the target S1 site is identified by sequencing recombinant plasmid DNA from a single clone. The clone with a TpG dinucleotide at the S1 site represents the unmethylated DNA, and the clone with a CpG dinucleotide at the S1 site represents 100% methylated DNA (**Supplementary Figure 1B**). The plasmids are purified using a Wizard DNA Miniprep Kit (Promega), the amount of DNA is quantified by measuring the optical density (O.D.) at 260nm with Thermo Scientific Nanodrop 2000 Spectrophotometer (ThermoFisher Scientific Inc. USA). Mixtures of 100% methylated (CpG at the S1 site) and unmethylated (TpG at the S1 site) plasmid DNA in various proportions are employed for methylation analysis with BS-OLE or COBRA.

BS-OLE assay

The BS-OLE assay is performed in two separate reactions to quantify the level of methylated (viz.C) or unmethylated (viz. T) nucleotide, respectively, at the target S1 site as illustrated by Figure 1. Each reaction is performed in a 20 µl reaction mixture comprising 28-450 ng of PCR products of bisulfite-converted DNA or the recombinant plasmid control DNA, 0.3 U *Taq* DNA polymerase, 250 nM BS-OLE extension primer GGTGGGAGTGATT for the target site, which is complementary to the template DNA immediately 3' of the target site, PCR buffer (10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂ and 50 mM KCl) and 100 nM 5-propargylamino-dCTP-5/6-TAMRA (TAMRA-dCTP; Jena Bioscience, Germany) corresponding to the methylated C allele (Fig. 1, Reaction 1) at the target CpG site on

the DNA template or 50 nM 5-propargylamino-dUTP-5/6-TAMRA (TAMRA-dUTP; Jena Bioscience) corresponding to the unmethylated T allele (Fig. 1, Reaction 2) at the target TpG site. After an initial denaturation step for 3 min at 95°C, primer extension is carried out by 30 thermal cycles consisting of 30 s at 95°C then 30 s at 55°C.

The FP value of each reaction is measured on 96- or 384-well plates using a Multimode Plate Reader Victor™ X5 (PerkinElmer, USA) with excitation wavelength 544 nm (Filter FP544/15) and emission wavelength 595 nm (Filter 595/60) without the need for purification or separation of the reaction products. After correction for the blank FP reading, FP readings of the dCTP- and dUTP-incorporated samples yield the extent of methylation in the sample:

$$\text{Methylation} = 100 \times \text{dCTP}/(\text{dCTP}+\text{dUTP}) \%$$

where dCTP or dUTP represents the blank-corrected FP readings from the TAMRA-dCTP or -dUTP incorporations.

COBRA assay

Because the methylated form of the target S1 site in the 250 bp LINE PCR product of bisulfite-converted DNA is accordant with *Taq* I cleavage TCGA site (the underlined CG for the target S1 site, **Supplementary Figure 1B**) and its unmethylated form (TTGA) cannot be restricted by *Taq* I, the methylation level of the target S1 site examined by BS-OLE can be validated by COBRA assay with *Taq* I restriction enzyme (Xiong and Laird 1997). In COBRA assay, the 250 bp LINE PCR product of

bisulfite-converted DNA is restricted with 2 U *TaqI* (TaKaRa) by incubation for 6 h at 60°C. The PCR product containing a single TCGA *TaqI* cleavage site for the methylated form can be cut into 163 bp and 87 bp fragments by *TaqI*, whereas the product containing a TTGA sequence for the unmethylated form cannot be restricted. *TaqI*-restricted and unrestricted 250 bp PCR products are dissolved in 2% ethidium bromide-agarose gel and electrophoresed for 20 min at 100 V. Bands are quantified using a Gel-image Analyzer (Jiapeng, China) on the basis of the intensities of their UV absorbance.

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Results

The BS-OLE method is based partly on the principle of OLE (Sun et al. 2014), which has been used for the detection of gene mutations. Genomic DNA is treated with sodium bisulfite to convert unmethylated cytosine to uracil leaving 5-methylcytosine unaltered (**Figure 1**), followed by BS-PCR to generate a DNA template containing target CpG sites for methylation analysis. The BS-OLE method initially examines a primer complementary to the template DNA immediately 3' of the target CpG site after specific amplification of bisulfite-converted DNA target sequences. Two separate reactions are used in the BS-OLE method. Each reaction contains a TAMRA-dCTP or -dUTP corresponding to either the methylated form (CpG) or unmethylated form (TpG) of the target site. Once the extension primer have annealed the target sequences, a single-nucleotide chain extension enables incorporation of the fluorescent TAMRA-dCTP into the methylated CpG form of the target site in one reaction and of the fluorescent TAMRA-dUTP into the unmethylated TpG form in another reaction. The reaction products are quantified by measurement of their fluorescent polarization. Thus, $dCTP/(dCTP+dUTP)$ gives the fraction of the methylated CpG site being examined.

Stabilization and accuracy of BS-OLE

The BS-OLE assay is used to determine the methylation level of the target TCGA site (S1 site) in the LINE1 element with various amounts of PCR products from bisulfite-converted human genomic DNA of peripheral blood cells (**Figure 2A**). An estimated $57 \pm 1\%$ methylation for this target site is provided by the regression

analysis (broken line), which has a slight negative slope of $-0.0011\% \text{ ng}^{-1}$ over the concentration range of 28-450 ng DNA/assay. The BS-OLE assay is further used for various amounts of PCR products from plasmid DNA with 25% methylation at the S1 site, which is a mixture of 100% methylated (CpG at the S1 site) and unmethylated (TpG at the S1 site) plasmid DNA as described in Materials and Methods. The percentage methylation of the S1 site is estimated at 12.5-200 ng DNA/assay by the regression analysis (**Figure 2B**, broken line with open circles), which has a slight positive slope of $0.0053\% \text{ ng}^{-1}$, leading to a fairly stable percentage methylation estimate of $33 \pm 1\%$ over this range of DNA concentration, slightly higher than the expected level of 25% methylation. Mixtures (100 ng of methylated and unmethylated plasmid DNA at the S1 site in various proportions) are analyzed by BS-OLE (**Figure 2C**). The estimated level of methylation at the S1 site is $26 \pm 5\%$, $40 \pm 4\%$, $53 \pm 5\%$ and $76 \pm 2\%$ for the 20%, 40%, 60% and 80% methylated preparations, respectively ($R^2 = 0.96$ for the linear regression line), demonstrating the applicability of BS-OLE over this range of DNA methylation levels.

Validation of BS-OLE by the COBRA assay

The validity of BS-OLE assay is assessed against COBRA analysis. The *TaqI*-digested and undigested 250 bp LINE1 PCR products from plasmid DNA preparations with different ratio of TpG (unmethylated form) over CpG (methylated form) at the S1 site are subjected to agarose gel electrophoresis. The amount of undigested 250 bp DNA is obtained by measuring the intensity of the top band (**Figure 2E**), and the amount of *TaqI*-digested methylated 250 bp fragments is obtained as the sum of the intensities of

the second and third bands from the top, corresponding to 163 bp and 87 bp digested fragments, respectively. Measurement of the sum of the 163 bp and 87 bp fragments by Gel-Image software yields the total of methylated 250 bp DNA at the S1 site that is digested by *TaqI*, which corresponds to the percentage methylation in the mixture. The COBRA method gives estimates of $23 \pm 0\%$, $45 \pm 3\%$, $62\% \pm 3\%$ and $78 \pm 6\%$ for the preparations with 20%, 40%, 60% and 80% methylation at the S1 site, respectively (**Figure 2D**). When the BS-OLE estimated percentages are plotted against the COBRA-estimated values, extensive agreement is obtained by linear regression analysis ($R^2 = 0.9581$, $P < 0.0001$), which provides significant cross-validation of the BS-OLE method by the independent COBRA method.

Analysis of DNA methylation from cancer cell lines

The BS-OLE assay is used to analyze DNA hypomethylation of HEK293T cells induced by 5-aza-dC, a known DNA methylation inhibitor (**Figure 3A**). It is shown that the DNA methylation level at the S1 CpG site of the cellular LINE1 DNA is inhibited to 89% of control by 0.25 μM of 5-aza-dC ($P = 0.03$, one-tailed t-test) and to 80% of the control by 0.5 μM of 5-aza-dC ($P = 0.04$, one-tailed t-test). When the BS-OLE assay is applied to human genomic DNA extracted from five human glioblastoma cell lines and a HEK293T cell line (**Figure 3B**), estimated methylation levels ranging from 30% to 50% at the S1 site in LINE1 are $49 \pm 2\%$, $41 \pm 3\%$, $39 \pm 1\%$, $34 \pm 3\%$, $31 \pm 2\%$ and $37 \pm 4\%$ for cell lines U87, M059K, A172, U2OS, T98G and HEK293T, respectively.

Discussion

DNA methylation is a key epigenetic mechanism in eukaryotic gene regulation, development and disease. Understanding the role of DNA methylation in development and disease requires knowledge of the status of these modifications in the genome. DNA methylation usually occurs at the C5 position of cytosine within CG dinucleotides in mammalian genomes. In contrast, in plant genomes, 5-methylcytosine in CNG or CHH triplets is as frequent as in CG dinucleotides, and is recently found in embryonic stem cells in mammals (Lister et al. 2009; Lee et al. 2010). The BS-OLE method developed in the present study can determine the methylated C in the CG, CNG or CHH sites whatever occurs in a symmetrical or asymmetrical context, because this method examines the methylated C at a single strand DNA through a single-nucleotide primer extension. BS-OLE, a robust technique to determine the level of the target cytosine in various contexts, can be widely used to methylation study in plant and mammalian fields when a proper extension primer is employed.

Primer extension-based methods are powerful tools for DNA methylation analysis at the single base level. After PCR amplification of bisulfite converted genomic DNA, the single-nucleotide extension is initiated with a primer through incorporation of radioactively or chemically labeled dCTP or dUTP to determine the methylated or unmethylated cytosine in the target CpG site. In assessing labeled reaction products, the BS-OLE procedure avoids the need for radioactive material or complex protocols

such as separation and washing to remove unincorporated nucleotide by Ms-SNuPE (Gonzalzo and Liang 2007), four-step pyrosequencing (Karimi et al. 2006), or prelabeling of PCR amplicons for FRET measurement (Kaminsky et al. 2005; Feng et al. 2010). Thus, the entire BS-OLE assay can be performed on a 96- or 384-well microtiter plate and is suitable for high-throughput multi-sample analysis.

In the present study, BS-OLE provides stable and accurate estimates of methylation level of plasmid DNA and human genomic DNA over sub-microgram ranges (**Figure 2**). Extensive agreement between these two methods in examining different methylation levels of DNA preparations provides valuable cross-validation of the BS-OLE method by the independent COBRA method (**Figure 2**). Limitations of the BS-OLE method include only a single CpG site that can be measured per reaction and know methylation sites can be detected. Because BS-OLE primer cannot contain any potential methylcytosine in the original sequences (unconverted template DNA), the requirement for BS-OLE primer design is different among plants, embryonic stem cells and other mammals. In this study, we demonstrate that BS-OLE assay will not be applicable for the S1 site if examination would have been employed in plants or embryonic stem cells due to the presence of 5-methylcytosine in the CHG and CHH contexts just before the S1 site (**Figure 1**). Thus, BS-OLE primers involving PCR or extension primers cannot contain any CG, CHG or CHH sequences when employed in mammalian embryonic stem cells or plant genomes. In addition, BS-OLE cannot examine the 5-methylcytosine within the CpG island if there were not enough space before the target CpG site to design a proper extension primer. Furthermore, BS-OLE

primer should be 18-25 bp in length and have annealing temperature of 55-65°C, as a general rule. Thus, some sites may be out of reach for this technique due to primer design limitations. In conclusion, BS-OLE method provides a facile high-throughput procedure for measuring cytosine methylation of single CpG site in DNA preparations as well as in cellular genomes of mammals or plants.

Acknowledgements

We thank the Talents Program in Higher Education of Guangdong (grant No. 2050205), the Guangdong Natural Science Foundation (grant No. S2012010009664) and the National Undergraduate Training Program (grant No.201312121021) of China for financial support.

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Figure legends

Figure 1. Detailed outline of the BS-OLE assay. BS-OLE consists of a standard bisulfite conversion and PCR amplification followed by one-label extension and fluorescence polarization measurement. During the first step, unmethylated C is replaced by T after bisulfite-PCR, whereas the presence of any C remaining in the sequence represents a methylated C at CpG dinucleotides. The methylation level (viz. ratio of C over T) at the target site is examined by incorporation of TAMRA-dCTP (Reaction 1) or TAMRA-dUTP (Reaction 2) into the methylated (CpG) or unmethylated (TpG) form of the target site through single-nucleotide chain extension. The FP ratio between TAMRA-dCTP- and TAMRA-dUTP-incorporated products gives the fraction of the methylated CpG dinucleotides being examined.

Figure 2. Robustness and accuracy of the BS-OLE assay. Application of the BS-OLE assay to the LINE1 PCR product from human genomic DNA (**A**) and plasmid DNA (**B**). The accuracy of the BS-OLE assay is investigated with different methylation of plasmid DNA (**C**) and validated by the COBRA method (**D** and **E**). Unmethylated (T nucleotide at the target TC/TGA S1 site) and 100% methylated (C nucleotide at the target S1 site) plasmid DNAs are mixed to prepare samples with different levels of methylation at the S1 site. Amounts ranging from 12.5 to 200 ng of 25% methylated mixture (**B**) and 100 ng of each preparation with 0%, 20%, 25%, 40%, 60%, 80% or 100% methylation at the S1 site (**C**) are assayed by BS-OLE, and analyzed by gel electrophoresis (**E**). E, Lanes from left to right: *TaqI*-restricted 250 bp DNA with 0%, 20%, 40%, 60%, 80% or 100% methylation at the S1 site. The

positions of three bands are marked with arrows representing from top to bottom: unrestricted 250 bp unmethylated DNA, 163 bp and 87 bp of enzyme-restricted methylated DNA fragments. The percentage methylation of the S1 site (represented by open circles) is obtained from the ratio of FP readings of the TAMRA-dCTP-labeled DNA (filled triangles with standard error indicated by a bar) and TAMRA-dUTP-labeled DNA (open triangles with standard error indicated by a bar) after correction for the blank in the absence of DNA. The broken regression line representing percentage methylation (**A** and **B**) or representing accuracy (**C** and **D**) obtained with different DNA mixtures (open circles) is calculated using GraphPad Prism V5 software. All data points are the average of two or three independent duplicate experiments.

Figure 3. Application of the BS-OLE assay to different cancer cell lines. (**A**) Analysis of 5-aza-dC-induced hypomethylation of the S1 site. DNAs from cultured HEK293T cells after treatment with 0, 0.25 or 0.5 μ M 5-aza-dC for 3d are analyzed by BS-OLE. The percentage methylation of the S1 site values relative to the untreated control are shown in columns with standard error represented by a bar. Significant difference between treated cells and untreated control is indicated as * ($P < 0.05$) based on one-tailed t-test. (**B**) The percentage methylation values of the S1 site values are analyzed by BS-OLE with DNAs from cultured U87, M059K, A172, U2OS, T98G and HEK293T cell lines and are shown in columns with standard error represented by a bar.

A

B