



## A sensitive mass spectrometry method for simultaneous quantification of DNA methylation and hydroxymethylation levels in biological samples

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### ABSTRACT

The recent discovery of 5-hydroxymethyl-cytosine (5hmC) in embryonic stem cells and postmitotic neurons has triggered the need for quantitative measurements of both 5-methyl-cytosine (5mC) and 5hmC in the same sample. We have developed a method using liquid chromatography electrospray ionization tandem mass spectrometry with multiple reaction monitoring (LC–ESI–MS/MS–MRM) to simultaneously measure levels of 5mC and 5hmC in digested genomic DNA. This method is fast, robust, and accurate, and it is more sensitive than the current 5hmC quantitation methods such as end labeling with thin layer chromatography and radiolabeling by glycosylation. Only 50 ng of digested genomic DNA is required to measure the presence of 0.1% 5hmC in DNA from mouse embryonic stem cells. Using this procedure, we show that human induced pluripotent stem cells exhibit a dramatic increase in 5mC and 5hmC levels compared with parental fibroblast cells, suggesting a dynamic regulation of DNA methylation and hydroxymethylation during cellular reprogramming.

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The pattern of methylated cytosine residues in DNA provides an inheritable epigenetic code that regulates gene expression during development. The covalent addition of a methyl group at the 5-position of cytosine primarily occurs in the CpG dinucleotide and is catalyzed by a family of DNA methyltransferases (Dnmts),<sup>3</sup> including maintenance Dnmt1 and *de novo* Dnmt3a and Dnmt3b. DNA methylation is involved in various biological processes such as genomic imprinting, silencing of retroviral transposons, X chromosome inactivation, and cellular differentiation. Mechanistically, promoter

methylation can lead to transcriptional repression directly by inhibiting transcriptional binding or indirectly by recruiting various proteins, including methyl CpG binding domain proteins (MBDs), corepressors, and histone modification enzymes involved in chromatin remodeling [1–6]. Importantly, many studies have shown that DNA methylation is a dynamic process in cellular proliferation and differentiation and is tightly regulated in normal development. Aberrant DNA methylation patterns and mechanisms are deleterious to the developing central nervous system (CNS) [6–9].

Recently, there has been renewed interest in another related mammalian DNA modification, 5-hydroxymethyl-cytosine (5hmC). Significant levels of 5hmC are found in the developed murine CNS and in embryonic stem cells [10–13]. The *in vivo* addition of a hydroxyl group onto 5-methyl-cytosine (5mC) is catalyzed by 2-oxoglutarate oxygenase Tet1, Tet2, and Tet3 [10,13]. There are also reports that 5hmC can be formed by other mechanisms besides the Tet pathway, including ultraviolet (UV) irradiation of 5mC in aerated aqueous solution [14] and DNA methyltransferase reaction of cytosine with formaldehyde [15]. To date, only the Tet pathway has been demonstrated to produce 5hmC in mammalian genomic DNA.

Speculation that 5hmC is involved in the DNA demethylation pathway comes from the two reported mechanisms of converting 5hmC into cytosine (C). Bacterial DNA methyltransferases catalyze the removal of formaldehyde from 5hmC, thereby converting

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<sup>3</sup> Abbreviations used: Dnmt, DNA methyltransferase; CNS, central nervous system; 5hmC, 5-hydroxymethyl-cytosine; 5mC, 5-methyl-cytosine; UV, ultraviolet; C, cytosine; LC–ESI–MS/MS–MRM, liquid chromatography electrospray ionization tandem mass spectrometry with multiple reaction monitoring; mESC, mouse embryonic stem cell; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LIF, leukemia inhibitory factor; hESC, human embryonic stem cell; MEF, mouse embryonic fibroblast; iPSC, induced pluripotent stem cell; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PCR, polymerase chain reaction; UPLC, ultra-performance liquid chromatography; dC, deoxycytidine; cDNA, complementary DNA; BSA, bovine serum albumin; DAPI, 4',6-diamidino-2-phenylindole; SSC, salt sodium citrate; RT, reverse transcription; 5mC, 5-methyl-2'-deoxycytidine; 5hmC, 5-hydroxymethyl-2'-deoxycytidine.

5hmC to C [15]. Another deformylation mechanism involves the photochemical hydration of 5hmC in basic solution [14]. However, these two possible DNA demethylation mechanisms have yet to be confirmed in mammalian models.

Some of the most commonly used methods for profiling and quantification of DNA methylation, such as bisulfite sequencing and methylation-sensitive enzyme-based assays, are unable to distinguish between 5hmC and 5mC [10,16]. Several methods have been used to measure the 5hmC levels in the genome; these include end labeling followed by thin layer chromatography [10], high-performance liquid chromatography (HPLC) with UV detection [15], enzymatic radioactive glycosylation labeling [17], and single-molecule real-time sequencing [18]. The thin layer chromatography method has the advantage of being low cost and simple, but it requires the availability of radioactive substrates and the accuracy is not comparable to that of other available methods. The specificity of UV detection relies heavily on the chromatographic separation to avoid coelution of other components, including other DNA and RNA nucleotides that may be present in biological samples. The glycosylation method is based on enzymatic incorporation of radiolabeled glucose into genomic 5hmC, with quantification by radioactive counting. However, a complete enzymatic reaction cannot be readily ensured, and 5mC levels cannot be measured simultaneously. Independent measurement of 5hmC is possible with next generation sequencing, but the technology has yet to be perfected for accurate quantitation of many low-abundant nucleotides, including 5hmC.

Previous work has demonstrated the precision, selectivity, and sensitivity of liquid chromatography tandem mass spectrometry for measuring 5mC in biological samples and as a diagnostic tool for cancer [19–25]. Using this technique, all known DNA (excluding 5hmC) and RNA components have been separated, distinguished, and independently quantitated [24]. This approach allows DNA methylation to be measured at both the global [20,21,23,24] and gene promoter [22] regions. However, none of the previous reports included 5hmC. We were prompted to develop a fast, sensitive, and accurate method to measure both 5mC and 5hmC levels to support ongoing work on epigenetic control of stem cells and neural development. Here we report the use of liquid chromatography electrospray ionization tandem mass spectrometry with multiple reaction monitoring (LC–ESI–MS/MS–MRM) for the determination of genomic DNA methylation and hydroxymethylation. Separation of the deoxyribonucleosides is achieved within 6 min using sub-2- $\mu$ m particle size reverse phase chromatography columns. In addition, mass-based detection discriminates among the three nucleoside bases of interest: 5hmC, 5mC, and C. The combination of LC and MS minimizes any possible cross-talk between the measurements of low-abundant molecules (5hmC and 5mC) in the face of a chemically similar abundant species (C). Together, our data indicate that the MRM method provides unambiguous and independent quantification of 5hmC, 5mC, and C with high reproducibility and low limits of detection of approximately 0.5 fmol per sample. This limit of detection can be equated to 50 ng of digested genomic DNA to measure 5hmC levels at the 0.1% level. Furthermore, the method is relatively fast, requiring less than 48 h from extracting genomic DNA (few hours to a day), to digesting genomic DNA into nucleoside components (1–2 h), and measuring the 5hmC and 5mC levels using the MRM method (6 min per sample).

## Materials and methods

### Cell culture

Mouse embryonic stem cells (mESCs) of wild-type J1, *Dnmt1*<sup>−/−</sup> (cc), double knockout *Dnmt3a*<sup>−/−</sup>;*Dnmt3b*<sup>−/−</sup> (DKO), and triple knockout *Dnmt1*<sup>−/−</sup>;*Dnmt3a*<sup>−/−</sup>;*Dnmt3b*<sup>−/−</sup> (TKO) were

maintained on gelatin-coated plates in mESC medium containing Dulbecco's modified Eagle's medium (DMEM, Invitrogen), 10% fetal bovine serum (FBS, Gibco), 100  $\mu$ M 2-mercaptoethanol (Sigma), 0.1 mM nonessential amino acids (Gibco), 1 mM sodium pyruvate, 1  $\times$  L-glutamine (Gibco), 1  $\times$  penicillin/streptomycin (MP Biomedicals), and 100 U/ml leukemia inhibitory factor (LIF).

Human BJ fibroblasts from neonatal foreskin were purchased from American Type Culture Collection (ATCC). These were maintained in medium containing DMEM, 1  $\times$  penicillin/streptomycin, 1  $\times$  glutamine, and 10% FBS. Fibroblasts were passaged every 3–5 days using 0.05% trypsin (Invitrogen).

Undifferentiated human embryonic stem cells (hESCs, HSF1) were maintained on a feeder layer of mitomycin C (Sigma)-treated mouse embryonic fibroblasts (MEFs) in hESC medium containing DMEM/F12 (Invitrogen) supplemented with 20% Knockout Serum Replacement (KSR, Gibco), 1  $\times$  glutamax, 1  $\times$  nonessential amino acids, 0.11 mM  $\beta$ -mercaptoethanol (Sigma), 1  $\times$  penicillin/streptomycin, and 10 ng/ml basic fibroblast growth factor (bFGF, Pepro-Tech). hESCs were passaged every 5–7 days using collagenase IV (Gibco) and dispase (Gibco) at a final concentration of 1 mg/ml in hESC medium. All cells were cultured under a protocol approved by the Chancellor's Animal Research Committee and Embryonic Stem Cell Research Oversight Committee at the University of California, Los Angeles.

### Derivation of human iPSCs

The production of human induced pluripotent stem cells (iPSCs) followed a published protocol [26] with slight modifications. Specifically, retroviruses containing OCT4, SOX2, KLF4, and c-MYC were produced in the Platinum-E (PLAT-E) retrovirus packaging cell line (Cell Biolabs). Viral supernatants were collected 48 and 72 h after transfection and were filtered through a 0.45- $\mu$ m polyvinylidene difluoride (PVDF) filter (Millipore). The Slc7a1-expressing human BJ fibroblasts were plated at  $1.5 \times 10^5$  cells per well on a 6-well plate on day 1. On day 2, each retroviral supernatant was added into the fibroblasts in the presence of 4  $\mu$ g/ml polybrene (Sigma). A second round of transduction was performed on day 3. Infection efficiency was monitored by fluorescence microscopy of cells transduced by retrovirus-carrying green fluorescent protein (GFP). On day 5, cells were trypsinized and replated in a density of  $1 \times 10^5$  per 10-cm plate on mitomycin (Sigma)-treated MEFs. On day 6, the medium was changed to hESC medium. iPSC colonies were picked 3 weeks after infection. The picked colonies were cultured and passaged according to standard culturing protocols. All plasmids used for the derivation of iPSCs, including pMXs–OCT4, pMXs–SOX2, pMXs–KLF4, pMXs–c-MYC, and pLenti6/Ubc/mSlc7a1, were purchased from Addgene.

### DNA extraction and hydrolysis

hESC and iPSC colonies were harvested and passed through a cell strainer (BD Falcon) to remove the feeder cells. Colonies were washed with phosphate-buffered saline (PBS), treated with 500  $\mu$ l of DNA lysis buffer (100 mM Tris–HCl [pH 8.0], 5 mM ethylenediaminetetraacetic acid [EDTA], 200 mM NaCl, and 0.2% sodium dodecyl sulfate [SDS]), 5  $\mu$ l of proteinase K (100 mg/ml, Roche), and 5  $\mu$ l of RNase A (10 mg/ml, Roche), and incubated overnight at 37 °C in a shaking incubator. Genomic DNA was purified by a standard phenol/chloroform extraction followed by precipitation with 2 volumes of cold 100% ethanol. Subsequently, the extracted genomic DNA was redissolved in TE buffer (10 mM Tris–HCl and 1 mM EDTA, pH 8.0). Genomic DNA was quantified spectrophotometrically at 260 nm (Thermo Scientific NanoDrop).

DNA hydrolysis was performed by using DNA Degradase Plus (Zymo Research). Briefly, 1  $\mu$ g of genomic DNA was mixed with

2.5  $\mu\text{l}$  of 10 $\times$  DNA Degradase Reaction Buffer, 1  $\mu\text{l}$  of DNA Degradase Plus, and water to make a total reaction volume of 25  $\mu\text{l}$ . The reaction mixture was incubated in 37 °C for more than 1 h. Finally, the reaction was inactivated by adding 175  $\mu\text{l}$  of 0.1% formic acid to yield a final concentration of 5 ng digested DNA/ $\mu\text{l}$ .

#### DNA standards

Three 897-bp DNA standards, each homogeneous for either unmodified C, 5mC, or 5hmC, were purchased from Zymo Research and used to generate a calibration curve. The standards were prepared by polymerase chain reaction (PCR) using the appropriate nucleotides and were spin column purified by the manufacturer to obtain 50-ng/ $\mu\text{l}$  solutions. By MRM criteria, all of these standards were more than 99.6% pure (see [Supplementary Fig. 1](#) in supplementary material).

#### MRM quantitation

DNA hydrolysis samples (10  $\mu\text{l}$  typically containing 50 ng of digested DNA) were injected onto a reverse phase ultra-performance liquid chromatography (UPLC) column (Eclipse C18, 2.1  $\times$  50 mm, 1.8  $\mu\text{m}$  particle size, Agilent) equilibrated and eluted (100  $\mu\text{l}/\text{min}$ ) with water/methanol/formic acid (95:5:0.1, v/v/v). The effluent from the column was directed to an electrospray ion source (Agilent Jet Stream) connected to a triple quadrupole mass spectrometer (Agilent 6460 QQQ) operating in the positive ion MRM mode using previously optimized conditions, and the intensities of specific  $\text{MH}^+ \rightarrow$  fragment ion transitions were recorded (5mC  $m/z$  242.1  $\rightarrow$  126.1, 5hmC  $m/z$  258.1  $\rightarrow$  142.1, and deoxycytidine (dC)  $m/z$  228.1  $\rightarrow$  112.1). The measured percentage of 5mC and 5hmC in each experimental sample was calculated from the MRM peak area divided by the combined peak areas for 5mC plus 5hmC plus C (total cytosine pool).

With each batch of experimental samples, a series of standard samples was simultaneously prepared using the 897-bp DNA standards and run. The standard samples contained increasing amounts of 5mC and 5hmC in the presence of the same amount of C (0–10% for 5mC and 0–2% for 5hmC). Calibration curves were constructed for 5mC and 5hmC from the data obtained from the standard samples (measured 5mC or 5hmC peak area/total cytosine pool plotted against actual percentage of either 5mC or 5hmC in the samples). The measured percentage of 5mC and 5hmC in each experimental sample was then converted to actual percentage 5mC and 5hmC by interpolation from the calibration curves. This provided a correction for any differences that might exist in the molar MRM responses of the various nucleosides.

#### TET1 cloning, virus production and infection

The Flag-tagged TET1 catalytic domain (NM\_030625) was amplified from hESC complementary DNA (cDNA) using PCR with HotStar Taq polymerase (Qiagen). Primers used for the amplification of TET1 are described in [Supplementary Table 1](#) (see Supplementary material). PCR products were purified and cloned into a pCR4-TOPO plasmid using a TOPO TA Cloning Kit (Invitrogen) following the manufacturer's protocol. TET1 sequences were verified by DNA sequencing. The TET1 catalytic domain was digested and ligated into *Bam*HI and *Eco*RI sites of lentiviral plasmid, FUIGW (Addgene).

For lentivirus production, 293T cells were plated at  $8 \times 10^6$  cells per 10-cm plate and incubated at 37 °C overnight. Cells were transfected with 9  $\mu\text{g}$  of FUIGW-Flag-TET1-GFP or FUIGW along with 4.5  $\mu\text{g}$  of pMLDg/pRRE (Addgene), 1.8  $\mu\text{g}$  of pRSV-Rev (Addgene), and 2.7  $\mu\text{g}$  of pCMV-VSVG (Addgene) by Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Next, 48

and 72 h after transfection, viral supernatants were collected and filtered through a 0.45- $\mu\text{m}$  PVDF filter (Millipore). Fresh viral supernatants were infected into 293T cells in the presence of 4  $\mu\text{g}/\text{ml}$  polybrene (Sigma) overnight. Infected cells were analyzed by immunostaining and extracted for LC-ESI-MS/MS-MRM.

#### Immunocytochemistry

Antibodies used for immunostaining were OCT4 (1:20, Santa Cruz), NANOG (1:100, Abcam), TRA1-60 (1:200, Chemicon), SSEA4 (1:200, Chemicon), SOX2 (1:200, Cell Signaling), and DNMT3B (1:500, a gift from En Li). hESCs and iPSCs were plated on sterile coverglasses in 6-well plates and cultured for 24–48 h. The medium was aspirated, and cells were washed once with PBS and fixed with 4% paraformaldehyde/PBS for 30 min at room temperature. Cells were washed three times with 0.2% Tween 20/PBS and then permeabilized with 0.2% Triton X-100/PBS for 30 min at room temperature and washed once with 0.2% Tween 20/PBS. Blocking was performed for 1 h at room temperature with 2% bovine serum albumin (BSA)/0.1% Tween 20/PBS. Primary antibodies diluted in blocking solution were incubated for 1 h at room temperature. Cells were washed three times with 0.2% Tween 20/PBS. Cy2- and Cy3-conjugated secondary antibodies diluted in blocking solution were incubated at room temperature for 30 min. Cells were washed three times with 0.2% Tween 20/PBS, stained with 4',6'-diamidino-2-phenylindole (DAPI), and mounted on glass slides (Fisher Scientific). Images were analyzed on a Nikon Eclipse 80i inverted microscope equipped with a charge-coupled device (CCD) camera by using Spot Advance imaging software (Diagnostic Instruments).

#### Southern blot analysis

Genomic DNA (5  $\mu\text{g}$ ) was digested with *Bst*BI (NEB) overnight at 37 °C, separated on a 1% agarose gel, and transferred to a Hybond-N<sup>+</sup> membrane (Amersham) in 10 $\times$  salt sodium citrate (SSC). The membrane was hybridized with P<sup>32</sup>-end-labeled oligo probes for *Sat 2* and *Sat 3* in QuikHyb solution (Stratagene) at 42 °C for 2 h. The hybridized membrane was washed twice in 2 $\times$  SSC/0.1% SDS at room temperature and washed once in 0.1 $\times$  SSC/0.1% SDS at 60 °C. The membrane was exposed to a Kodak BioMax MS film. Oligo probes are described in [Supplementary Table 1](#).

#### Bisulfite sequencing

Genomic DNA (2  $\mu\text{g}$ ) was subjected to bisulfite conversion using an EZ DNA Methylation Kit (Zymo Research) following the manufacturer's protocol. Subsequently, PCR was carried out with HotStar Taq polymerase (Qiagen). Primers (OCT4 and NANOG) and PCR conditions are described in [Supplementary Table 1](#). PCR products were purified by a Wizard SV Gel and PCR Clean-Up Kit (Promega) and cloned into pCR4-TOPO plasmid using a TOPO TA Cloning Kit (Invitrogen) following the manufacturer's protocol. Following transformation, 10–12 colonies were subjected to direct sequencing with the M13 reverse primer, followed by inoculations and minipreps.

## Results

#### MS characterization of 5hmC, 5mC, and C

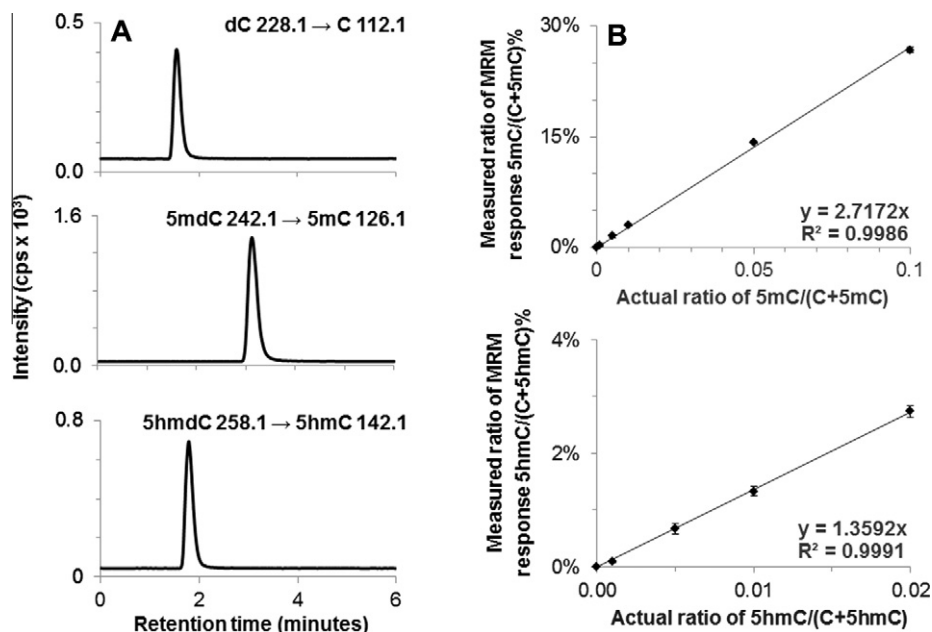
An equal molar mixture of three commercial 897-bp standard DNA fragments (Zymo Research), each homogeneous for either unmodified C, 5mC, or 5hmC, was prepared and digested into the nucleoside components. This mixture produced ions during ESI

corresponding to the protonated nucleosides dC, 5-methyl-2'-deoxycytidine (5mdC), and 5-hydroxymethyl-2'-deoxycytidine (5hmdC) at  $m/z$  228.1, 242.1, and 258.1, respectively. Collisionally induced dissociation (CID) of these protonated nucleosides produced a number of fragments, the most abundant of which correspond to the protonated bases liberated by cleavage of the glycosidic bond at  $m/z$  112.1 (C), 126.1 (5mC), and 142.1 (5hmC). Therefore, mass-based distinction between these nucleosides is possible because the parent masses are unique, as are the corresponding bases that result from glycosidic cleavage. The gas phase glycosidic cleavage of nucleosides is efficient, and the intensity of transitions of the protonated nucleosides to their corresponding bases can be used in the MRM mode for independent quantification:  $m/z$  228.1  $\rightarrow$  112.1, 242.1  $\rightarrow$  126.1, and 258.1  $\rightarrow$  142.1 for dC to C, 5mdC to 5mC, and 5hmdC to 5hmC, respectively.

When the equivalent of 50 ng DNA was analyzed by LC-ESI-MS/MS-MRM, the sequentially eluting symmetrical peaks corresponding to dC to C, 5mdC to 5mC, and 5hmdC to 5hmC transitions revealed no detectable cross-talk (Fig. 1A). Using the same commercial DNA fragments, the linearity of the response was tested by preparing and analyzing samples with varying amounts of 5mC and 5hmC in the presence of a constant amount of C containing DNA. Calibration curves constructed from this data set for both 5mC and 5hmC were linear (Fig. 1B), and were used to calculate the percentage DNA methylation and hydroxymethylation in experimental samples.

#### Validation of the MRM method

The method was then used to measure the percentage of 5mC and 5hmC in some mESC lines. The 5mC level of *Dnmt1*<sup>-/-</sup> mESC is approximately 25% of the wild-type 5mC level. In addition, the double knockout *Dnmt3a*<sup>-/-</sup>;*Dnmt3b*<sup>-/-</sup> mESC at passage 35 (P35) has a 5mC level of approximately 16% of the wild-type (Fig. 2). These results are consistent with previous studies that used nearest neighbor analysis and bisulfite next generation sequencing [27,28].



**Fig. 1.** (A) LC-MS/MS-MRM chromatograms of nucleosides derived from an equal molar mixture of three commercial 948-bp standard DNA fragments showing peaks corresponding to the response obtained from gas phase transitions of dC to C, 5hmdC to 5hmC, and 5mdC to 5mC. (B) Standard curves for 5mC and 5hmC. Percentages DNA methylation and hydroxymethylation are plotted against the known ratios of methylated and hydroxymethylated DNA, respectively, to the total pool of cytosine in the standard sample. cps, counts per second.

A comparison of the 5mC and 5hmC levels in various mESC lines shows a strong correlation (Fig. 2). This correlation is consistent with the biological conversion of 5mC to 5hmC by oxygenase TET enzyme [10,13]. A higher 5mC level would favor more 5hmC conversion and, thus, raise the global level of 5hmC.

To confirm another previous study, the FLAG-tagged TET1 catalytic domain was overexpressed in 293T cells (Fig. 3A). Using the MRM method, a drastic increase in 5hmC level was recorded accompanied by an approximately 50% loss of 5mC level compared with control cells (Fig. 3B). This observation was consistent with the previous study using 5mC antibody fluorescence immunocytochemistry showing that transfected 293T cells have 55% of the DNA methylation level found in control cells [10].

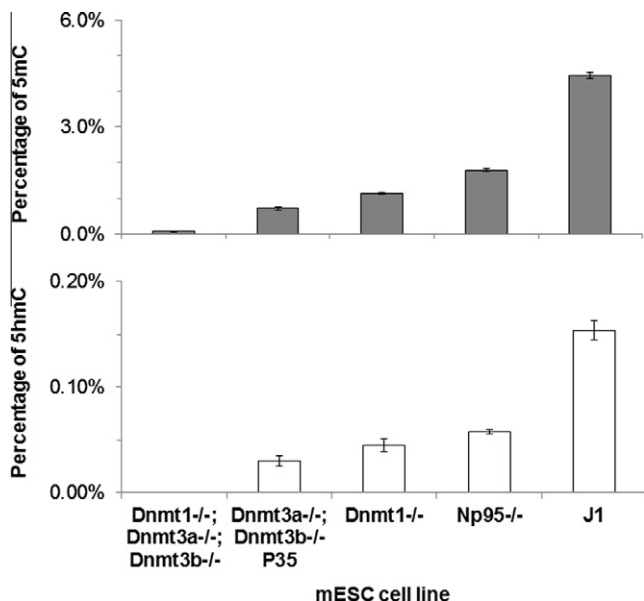
#### Measuring 5hmC and 5mC in somatic and iPSCs

BJ fibroblasts were used to generate iPSCs by retrovirally introducing *Oct4*, *Sox2*, *Klf4*, and *c-Myc* (Supplementary Fig. 2). Two iPSC colonies (BJ iPS 7 and BJ iPS 8) were picked and expanded for further analysis. Both iPSC lines showed a significant increase in 5mC after reprogramming from BJ fibroblasts (Fig. 4A). This 5mC increase was accompanied by a significant increase in the 5hmC level.

Southern blot was performed on *Sat 2* and *Sat 3* repetitive sequences of BJ fibroblasts, BJ iPS 7 and BJ iPS 8, and showed an increase in DNA methylation at *Bst*BI sites (TTCGAA) in the repetitive regions (Fig. 4B). These results are consistent with the MRM result. However, the promoter regions of both *Nanog* and *Oct4* underwent DNA demethylation (Fig. 4C), suggesting that the 5mC level increase occurs on selective gene regions.

#### Discussion

We have reported the details of a fast and reliable method for measuring the relative levels of 5mC and 5hmC in small samples of digested DNA. Through the use of UPLC with sub-micron particle size packing, the analysis time is reduced to 6 min per sample.



**Fig.2.** Percentages of 5hmC and 5mC in mESC DNA. 5mC and 5hmC contents are expressed as the percentages of 5mC and 5hmC, respectively, in the total pool of cytosine. Data are the means ± standard deviations from triplicate analyses.

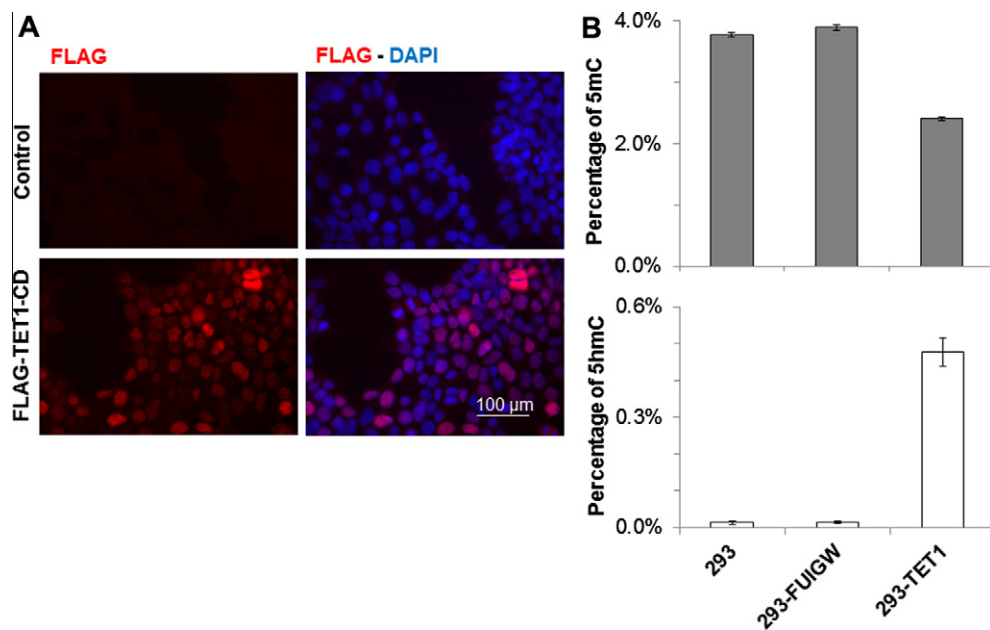
Using this method, the limit of detection for these two nucleosides is approximately 0.5 fmol injected on-column. The linearity of the response is demonstrated across one order of magnitude, which is more than sufficient for biological samples and is probably much greater, and the levels of 5mC and 5hmC have been measured in 10 different cell lines. Experience has shown that batches exceeding 100 samples can be analyzed without any noticeable change or deterioration in chromatographic performance and MRM response. The durability of the UPLC columns used in this work is such that hundreds of samples have been analyzed on the same column,

although as a precaution high organic washes every 20–30 samples are used to avoid any complications that could arise from the accumulation of materials not eluted during the isocratic analyses.

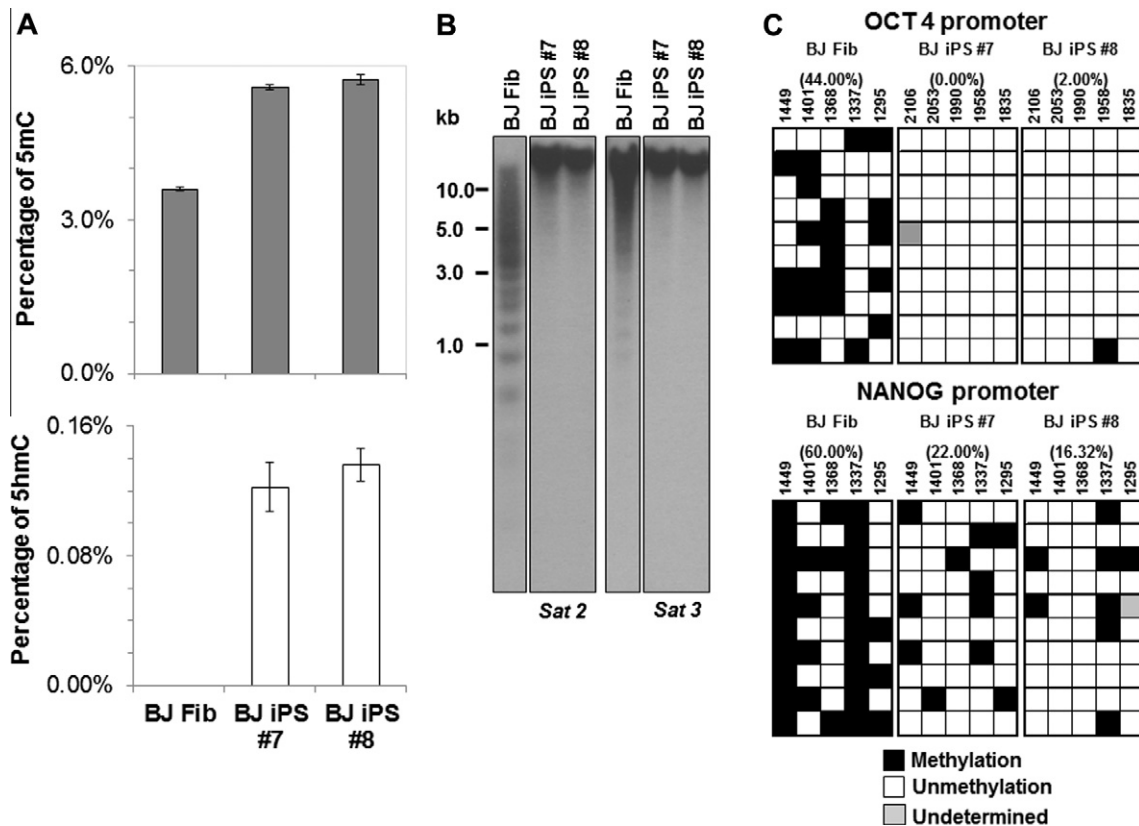
Both internal [23] and external standards [20–22,24] have been used for quantitative measurements of DNA methylation. External standards that mimic the processing of biological samples were used here. This was done by preparing premixed standard DNA samples and then processing them through the entire workup and digestion. The resulting standard curves reflect the unavoidable errors that arise during sample workup such as ion suppression that might arise from components used in the reaction solutions. Consistent with the report of Song and coworkers [24], our results reveal no evidence that small variations in the completeness of DNA hydrolysis adversely affect the linearity of the observed responses.

In this article, the DNA methylation levels in iPSCs are not similar to those in ESCs and fibroblasts. Various findings have already indicated that there are epigenetic differences between normal ESCs and iPSCs, particularly in DNA methylation patterns [29–33]. Our preliminary results on the comparison between iPSCs and parental somatic cells show that a significant number of genes undergo increased DNA methylation during reprogramming (Shen et al., unpublished data). It has been reported by others that the epigenetic mechanism of DNA methylation is a limiting factor in the reprogramming process and that the DNA methylation pattern might not truly emulate the pattern found in ESCs [31,34]. For example, treatment of DNA methyltransferase inhibitor, 5-azacytidine, facilitated the transition of partially reprogrammed cells to iPSCs [34]. Interestingly, the level of 5hmC in iPSCs from reprogrammed fibroblasts reported here appears to be restored to the levels found in ESCs.

In conclusion, we have established an accurate and robust assay for the simultaneous quantification of 5hmC and 5mC levels in biological samples. LC-ESI-MS/MS-MRM is acknowledged as a “gold standard” in quantitation methodology, and the method described here will have widespread applicability and is sufficiently flexible for expansion to include other rare nucleosides.



**Fig.3.** Overexpression of human TET1 catalytic domain in 293T cells. (A) 293T cells overexpressing FLAG-catalytic domain of TET1 were costained for FLAG antibodies and DAPI. Scale bar = 100 μm. (B) Percentages of 5hmC and 5mC in DNA from TET1-transfected cells, mock-transfected cells, and untransfected cells. Data are the means ± standard deviations from triplicate analyses.



**Fig. 4.** Reprogramming BJ fibroblasts into iPSCs. (A) Percentages of 5mC and 5hmC in DNA from BJ fibroblasts and two BJ iPSC lines, namely 7 and 8. (B) Southern blot analysis of DNA methylation in BJ and two BJ iPSC lines. DNA was digested with methyl-sensitive *Bst*BI, separated on agarose gel, transferred to the membrane, and hybridized to probes of the repetitive regions of *Sat 2* and *Sat 3*. Small DNA fragments of BJ fibroblasts are indicative of DNA hypomethylation in *Sat 2* and *Sat 3* repetitive region. (C) Bisulfite sequencing of *Oct4* and *Nanog* promoter of BJ fibroblasts and two BJ iPSC lines. Each row represents one clonal analysis, and each box represents a CpG site where the site number is indicated above. The methylation analysis is displayed according to the key. The overall percentage methylation of the gene promoter is indicated for each sample.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2011.01.026.

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