

## Supporting Information

### **Ultrasensitive determination of 5-methylcytosine (5'- mdC) and 5-hydroxymethylcytosine (5'- hmdC) in genomic DNA by sheathless interfaced capillary electrophoresis-mass spectrometry**

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## Experimental Section

### Reagents

5'-dC, 5'-dG, 5'-mdC and 5'-hmdC standards were purchased from Berry & Associates (Dexter, MI). Acetic acid (CH<sub>3</sub>COOH, 99.9985 %, metal basis) was purchased from Alfa Aesar (Ward Hill, MA, USA), and ammonium acetate (CH<sub>3</sub>COONH<sub>4</sub>, LC-MS grade) were purchased from sigma-Aldrich-Fluka (St Louis, MO). Methanol was LC-MS Grade, purchase from J.T.Baker. The water used throughout this work was ultrapure water purified using 0.45 µm MF-membrane filter (Merck Millipore, Germany).

### CE-ESI-MS

The CE-ESI-MS experiments were carried out with CESI-8000 capillary electrophoresis (CE) system from Beckman Coulter (Brea, California, USA) coupled with a Thermo Q-Exactive Mass Spectrometer (Thermo, USA) through an modified Nanosprayed II interface. Bare fused-silica capillaries etched with a porous tip were made available by Beckman Coulter (Brea, California, USA), which could be inserted into the sheathless nanospray interface. The separation capillary was 100 cm long with an internal diameter of 30 µm and an outside diameter of 150 µm.

The capillary was flushed with methanol for 10 min at 100 psi, followed by water, 0.1 M sodium hydroxide, 0.1 M hydrochloric acid and water for 10 min each at 100 psi, and finally by the background electrolyte (BGE) of 10 % acetic acid (pH 2.2) for 10 min at 100 psi before first used. The BGE was also used as conductive liquid in the conductive liquid capillary. Before each run, the conductive liquid capillary was rinsed with BGE for 5 min at 100 psi. Samples for detection were stored at 5°C in the CE system. Hydrodynamic injections were used in this study, and about 100 nL sample was injected into the separation system for each analysis. A voltage of +25 kV was applied during the separation and the current was between 3.0 to 3.2 µA. The electrospray voltage was optimized to get the best nanospray stability and efficiency and +1.2 kV was good enough for this study.

Data were acquired over the MS scan range of 150 m/z to 400 m/z. The temperature of the ion transportation capillary was set at 200 °C in order to protect the porous tip. The mass

resolution was 70000. Maximum inject time: 100 ms; Automatic gain control (AGC) target was  $3 \times 10^6$ .

## **DNA Extraction and Enzymatic Digestion**

Two cancer tissue samples were analyzed to evaluate the feasibility of this CE-ESI-MS system dealing with practical sample. The sample was 400  $\mu\text{L}$  tissue homogenate which contained 100 mg tissue. The cancer tissue DNA was extracted using the Takara MiniBEST universal Genomic DNA Extraction Kit Ver 5.0 (Takara, Biotechnology Co., Ltd. Dalian, China). The concentration of the purified genomic DNA which was extracted from one tissue sample was determined by Nanodrop 2000 spectrophotometer from Thermo (Thermo, USA). Typically, 30 ng DNA was obtained from 200  $\mu\text{L}$  sample solution.

Genomic DNA from mouse embryonic stem cells (ESC) was diluted to be 125 pg in 16  $\mu\text{L}$   $\text{H}_2\text{O}$  using ultrapure water. The enzymatic digestion protocol for each genomic DNA sample was the same.

Genomic DNA (all in 16  $\mu\text{L}$   $\text{H}_2\text{O}$ ) was first denatured by heating at 95  $^\circ\text{C}$  for 5 min and then transferred into ice water, quick-cooling for 2 min. After that, 2  $\mu\text{L}$  of  $10 \times \text{S1}$  nuclease buffer (30 nM  $\text{CH}_3\text{COONa}$ , pH 4.6, 260 mM NaCl, 1 mM  $\text{ZnSO}_4$ ) and 360 units (2  $\mu\text{L}$ ) of S1 nuclease were added into the DNA solution. The mixture (20  $\mu\text{L}$ ) was then incubated at 37  $^\circ\text{C}$  for 4 hours. Then 69  $\mu\text{L}$  of  $\text{H}_2\text{O}$ , 10  $\mu\text{L}$  of  $10 \times$  alkaline phosphatase buffer (50 mM Tris-HCl, 10 mM  $\text{MgCl}_2$ , pH 9.0), 1  $\mu\text{L}$  of alkaline phosphatase were added into the DNA digestion solution. And the incubation was continued at 37  $^\circ\text{C}$  for an additional 4 hours. The digested solution was filtered using ultrafiltration tube (10 kDa, cutoff, Amicon, Millipore) to remove the proteins and the resulting solution was stored at 4  $^\circ\text{C}$  before analysis.

## **Method Validation and Quality Control.**

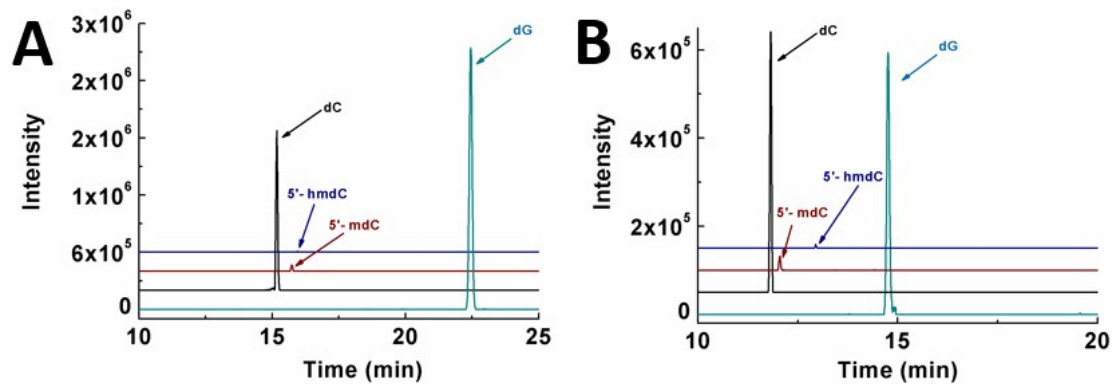
To validate the sheathless CE-ESI-MS method, the standard solutions of 5'-mdC and 5'-hmdC standards of varying concentration (2.0, 4.0, 8.0, 16.0, 32.0, 64.0 nM) mixed with 50 nM dC standard (as internal standard) were prepared and analyzed using CE-ESI-MS method described above. Each sample was analyzed at least three times. The intra-day and inter-day

precision of the CE-ESI-MS method was investigated by quantification of 50 nM dC standard per day for three consecutive days.

The quantification calibration curves of 5'-mdC and 5'-hmdC were constructed by plotting the peak area ratios of 5'-mdC / dG and 5'-hmdC / dG. A 16-mer oligodeoxynucleotide (5'-GTAGGTCGTCATGAGG – 3') was mixed with a 16-mer oligodeoxynucleotide (5' – GTAGGT (5'-mdC) GTGATGAGG – 3') at different amount to make the molar ratios of 5'-mdC / dG range from  $5.0 \times 10^{-3}$  – 0.1. And a 39-mer oligodeoxynucleotide (5'-GAGTGGTATGGGATGGGCAGG GAAAGGTGAGTCGAGTGC – 3') was mixed with a 13-mer oligodeoxynucleotide (5'-CGTCCA (5'-hmdC) GTCTAC) at different amount to make the molar ratios of 5'-hmdC / dG range from  $1 \times 10^{-4}$  –  $5 \times 10^{-3}$ . All these quantification samples were digested using enzymatic method mentioned above and analyzed by CE-ESI-MS method at least three times.

### **Determination of ESC genomic DNA sample and cancer tissue sample**

The resulting solutions of ESC genomic DNA sample and cancer tissue sample were directly measured by CE-ESI-MS. Each sample was analyzed for three times. The 5'-mdC / dG and 5'-hmdC / dG results were compared with data reported by others to validate the precision of our method.



**Figure S1** CE-ESI-MS chromatograms for genomic DNA sample of mouse ESC (A) and genomic DNA sample of one of the human liver cancer tissue (B).

Table S1 Comparison of the LODs of 5'- mdC and 5'- hmdC obtained in different analytical methods

Analytical method	LOD	
	5'- mdC	5'- hmdC
HILIC-ESI-MS/MS <sup>1</sup>	220 amol	220 amol
Online trapping – capillary HILIC-ESI-Q-TOF-MS <sup>2</sup>	60 amol	190 amol
HPLC-ESI-MS/MS with ammonium bicarbonate enhancement <sup>3</sup>	25 amol	250 amol
HPLC-ESI-MS/MS with derivatization <sup>4</sup>	100 amol	60 amol
CE-ESI-MS (this work)	5 amol	10 amol

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Table S2 The intra-day and inter-day precisions for the determination of 5'- mdC and 5'- hmdC in

450 pg mESC gDNA sample.

	Intra-day RSD (n=3)	Inter-day RSD (n=3)
5'- mdC / dG	4.3%	7.1%
5'- hmdC / dG	6.2%	7.9%