# Supplementary Information

# A photo-elutable 8-17 DNAzyme labeling and PCR-free colorimetric quantification strategy for 5-hydroxymethylcytosine in mammalian genomic DNA

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## EXPERIMENTAL SECTION

**Reagents and Materials.** The model 5hmC-ssDNA was synthesized and HPLC purified by Takara Biomedical Technology Co., Ltd. (Beijing, China), which were derived from the segment of the T53 MPRM10823 promoter with 50-nt. The other primers including PC-linker-containing 5'-FAM-ssDNA with or without a 3'-Biotin end, rA-linker, DBCO-DNAzyme with PC-linker, and model ssDNA without 5hmC were HPLC purified and synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). All the sequences were listed below. Genomic DNA samples were extracted from the designated tissues of SPF-grade ICR mice utilizing the TIANamp Genomic DNA Kit (Catalog No. DP304) provided by TIANGEN Biotech (Beijing) Co., Ltd. UDP-6-azido-6-deoxy-D-glucose (UDP-$N_3$-Glu) was supplied by Biosynth Carbosynth Corporation (UK). T4 Phage $\beta$-glucosyltransferase (T4 $\beta$GT) and its NEBuffer 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate and 1 mM dithiothreitol, pH 7.9) were purchased from New England Biolabs (USA). The biotin-11-dUTP, terminal deoxynucleotidyl transferase (TdT) with its buffer (5×), BeyoMag™ Streptavidin Magnetic Beads (SA-MB, 10 mg/mL) were provided by Beyotime Biotechnology (Shanghai, China). The 1×phosphate buffer saline (PBS, pH 7.4) and Oligo DNA Purification Kits (UNIQ 10) were ordered from Sangon Biotech Co., Ltd. (Shanghai, China). Other regents of analytical grade were obtained from local suppliers and used without further purification. All aqueous solutions were prepared by Millipore Milli-Q water purification system (USA) with an electrical resistance > 18.2 MΩ.

**Apparatus.** Polyacrylamide gel electrophoreses (PAGE) were operated by Mini-PROTEAN Tetra electrophoresis instrument (Bio-Rad, USA) and imaged by DigiGenius Gel Imaging System (Syngene, UK). The UV-vis spectra were tested on UV-2600 spectrophotometer (Shimadzu, Japan). All concentrations of DNA samples were determined by NanoPhotometer™ P-Class (IMPLEN, Germany). The ELISA measurements were conducted using a Spectrophotometer Model 1510 (Thermo Fisher, USA). The cleavage of the PC-Linker was conducted using 365 nm UV light

from a 24W UV lamp.

**Functionalization of AuNPs.** AuNPs were prepared by the classical method of reducing $HAuCl_4$ with trisodium citrate according previous reports.[1, 2] and the prepared AuNPs were functionalization by thiol modified oligonucleotides (SH-Arm1 and SH-Arm2) respectively. The specific procedure was as follows: The concentration of AuNPs was obtained by absorbance at 450 nm,[3] and mixed with 200 times the amount of SH-Arm1 or SH-Arm2, and placed at 4 °C. After 24 h, an appropriate amount of PB buffer (0.2 M, pH 7.4) was added to bring the final phosphate concentration to 0.01 M, and the NaCl (4 M) was added several times for 48 h to bring the final concentration to 0.1 M. Finally, the dark red oil was obtained at the bottom of tube after centrifugation (18000 rpm or 21000 g), and the upper clear and colorless supernatant was discarded. The AuNPs-Arm1 or AuNPs-Arm2 was washed with 0.01 M PB buffer (containing 0.1 M NaCl) through centrifugation for 3 times, finally dispersed in wash solution and stored at 4 °C before use.

**Biotinylation at 3'-OH Termini of DNA Samples.** As an essential pretreatment, the 3'-OH termini of all DNA samples were biotinylated using biotin-11-dUTP and TdT. This process facilitates the subsequent removal of excess reactive materials during modifications to 5hmC. In detail, the reaction system consisted of biotin-11-dUTP and 3'-OH terminated DNA at a molar ratio of 5:1, with 10 U of TdT enzyme added. The DNA sample amount was controlled to not exceed 100 pmol or 2 μg. DEPC-treated water was added to adjust the final volume of the reaction mixture to 100 μL, while ensuring the TdT buffer concentration was 1×. After incubation at 37 °C for 4 hours, the reaction mixture was heated to 70 °C and maintained for 20 minutes to inactivate TdT. The resulting product was precipitated with ethanol to remove excess biotin-11-dUTP.[4] For shorter DNA samples and models, purification could be performed using a spin column (for instance the UNIQ-10 Kit) according to standard protocols.

**Labeling and purification of 5hmC using 8-17 DNAzyme with PC-Linker and DBCO-end (DBCO-DNAzyme), and release of the 5hmC-specific DNAzyme.** According to our previous work,[5] for the 2 $\mu$M of biotinylated model ssDNA with certain molecular weight, the 5hmC modification was performed in a 30 $\mu$L solution containing 20 U T4 $\beta$GT with its 1×NEBuffer 4, and 200 nM UDP-$N_3$-Glu. The mixture was subsequently incubated at 37 °C for a period of 24 hours. Following the completion of the reaction, the strands were purified using the UNIQ-10 Kit to eliminate residual reactants. For genomic samples, given the low levels of 5hmC and the stringent requirements of ethanol precipitation, the total sample amount for a single treatment should not be less than 1 $\mu$g. All steps and reagent amounts are consistent with the model chains, except for the purification step to remove the UDP-$N_3$-Glu, which employs the ethanol precipitation method. For the subsequent "Click" reaction, 10×PBS buffer and a 50-fold molar excess of DBCO-DNAzyme was added to the purified solution. The final solution was then diluted to 1×PBS and incubated at 37 °C for an additional 24 h.

Since all samples were pre-biotinylated, SA-MB was added at a ratio of 375 μg per 100 pmol of biotinylated model ssDNA to remove the excess unreacted DBCO-DNAzyme. For genomic DNA samples, the amount was adjusted to 10 $\mu$g DNA per 375 $\mu$g SA-MB. Following this, it should be incubated at room temperature (25°C) for 1 hour, during which manual inversion and mixing should be performed every 10 minutes. It should be noted that SA-MB must be washed three times with 1×TE buffer prior to its addition to the reaction system. Following the binding process, all DNA samples, including those modified with DNAzyme-targeted 5hmC, were immobilized on the surface of the magnetic beads. Excess reactants especially DBCO-DNAzyme were subsequently removed via magnetic separation, and the beads were washed at least three times with TE buffer. Finally, the SA-MB was dispersed in an appropriate volume of TE buffer to achieve a final DNA sample concentration of 30 ng/μL. Since the DNAzyme at the 5hmC site was modified with a PC-Linker, this tube with the lid off was then irradiated with a 365 nm UV lamp for 15 min. During this period, the

tubes were manually mixed at 2 min intervals to ensure uniform irradiation. Subsequently, magnetic separation was performed to isolate the supernatant containing quantifiable 5hmC-DNAzyme, which was designated as solution H.

**Measurement of 5hmC level by this colorimetric strategy.** AuNPs-Arm1 and AuNPs-Arm2 were washed and dispersed with Tris buffer (0.02 M, pH 7.4, contain 0.1 M NaCl).[6] Then, 10 $\mu$L of each AuNPs solution, 1.2 $\mu$L of the rA-linker (1 $\mu$M), 0.5 $\mu$L of $Pb(OAc)_2$ (2 mM), and 10 $\mu$L of 5×Tris buffer (0.1 M, pH 7.4, contain 0.5 M NaCl) were added to the 10 μL of 5hmC-specific DNAzyme solution (to ensure the testing sample input was 300 ng) released from the magnetic beads.[7] Following adjustment of the mixture volume to 50 μL with deionized water, the solution was subsequently heated to 95 °C for 3 minutes to denature the secondary structures of all nucleotide chains. Finally, after standing at room temperature for 2 hours, the absorption spectrum and absorbance of the obtained AuNPs solution were measured. The maximum absorbance was found to be proportional to the concentration of DNAzyme, thereby providing a quantitative reflection of the 5hmC content.

**THE SEQUENCES OF ALL NUCLEOTIDES**

**Table 1.** The sequences of all used nucleotides

| Name of mentioned nucleotides | Sequences (5'-3') |
|---|---|
| model 5hmC-ssDNA | CCGGAGTCCGCTTTCCTCTTC$^{5hm}$CGGAAAATG TAAGCCGAACCTAAAGCAATCACC |
| model C-ssDNA | CCGGAGTCCGCTTTCCTCTTCCGGAAAATGTA AGCCGAACCTAAAGCAATCACC |
| PC-Linker-containing 5'-FAM-ssDNA | FAM-CCGGAGTCCGCTTTCCTCTTCCGGAAAATGTA AGCCGAACCTAAAGCAATCA-(PC-Linker)-CCTTTTTT-Biotin |
| rA-linker | GAGAAGGAGGAGTGCACTCACTAT-(rA)-GGAAGAGATGGAGAGAGAGTGAGAC |
| SH-Arm1 | SH-TTTTTTTGTCTCACTCTCTCTC |
| SH-Arm2 | GCACTCCTCCTTCTCTTTTTTT-SH |
| DBCO-DNAzyme | DBCO-TT-(PC-Linker)-TTCATCTCTTCTCCGAGCCGGTCGAAATAGTG AGT |

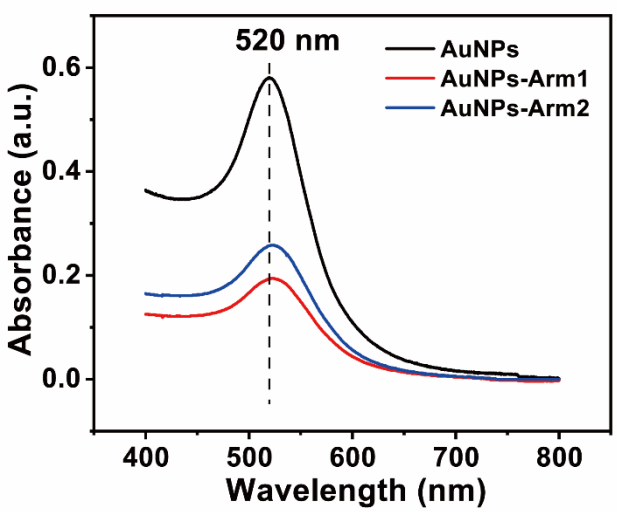


**Figure S1.** The absorption spectra of AuNPs, AuNPs-Arm1, and AuNPs-Arm2

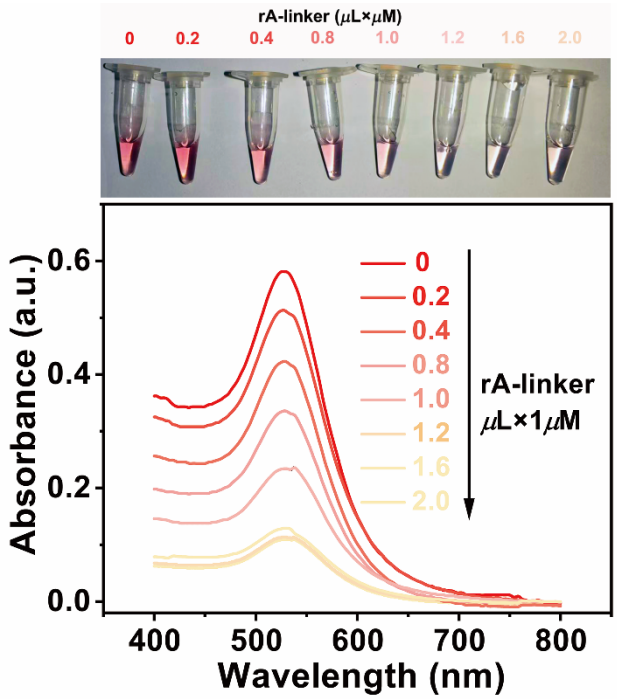


**Figure S2.** Optimization of rA-linker. (UV-visible absorption spectra, numbers are the volume of 1 μM rA-linker solution added to the mixture containing $Pb^{2+}$, AuNPs-Arm1, and AuNPs-Arm2. The above photograph displays the color changes in the system at the corresponding adding volumes.)

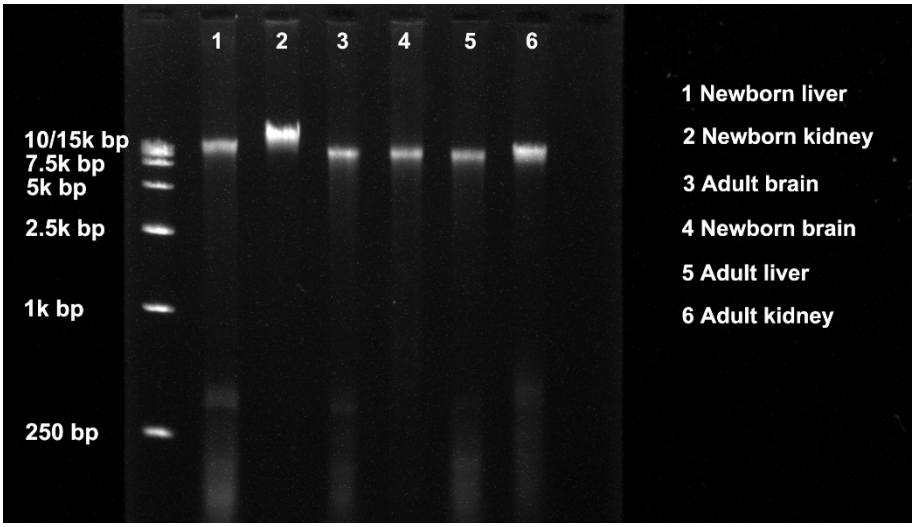


**Figure S3.** The agarose gel electrophoresis (0.8%) analysis of the several ICR mouse genomic DNA.

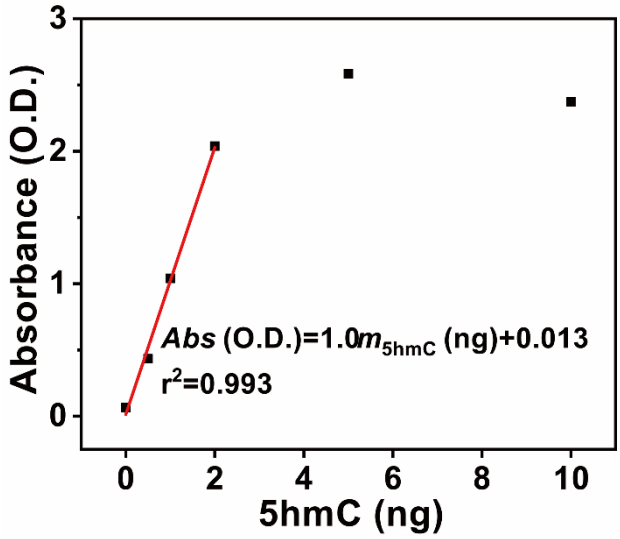


**Figure S4.** The standard curve of ELISA to quantify 5hmC.

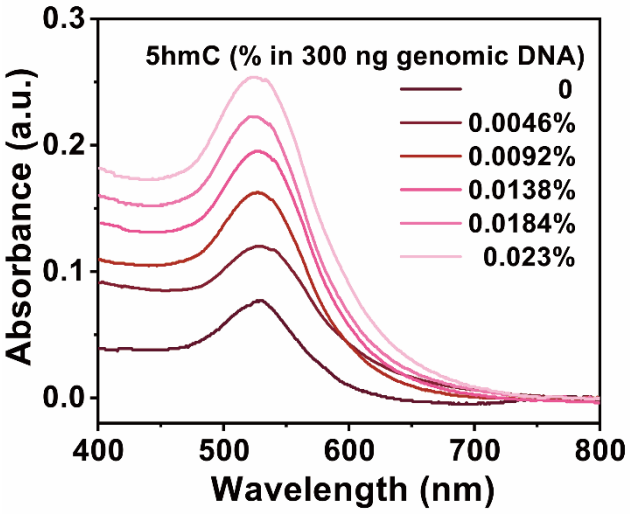


**Figure S5.** Absorption spectra of genomic DNA with varying levels of 5hmC from the AB strain obtained by using this method.

## References


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