Electronic Supplementary Information

Sensitive and label-free discrimination of 5-hydroxymethylcytosine and 5-methylcytosine in DNA by ligation-mediated rolling circle amplification

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EXPERIMENTAL SECTION

Materials. All oligonucleotides (Table S1) were synthesized by Sangon Biotechnology Co. Ltd. (Shanghai, China). The Vent® DNA polymerase, 10× ThermoPol reaction buffer (200 mM Tris-HCl, 100 mM ammonium sulfate ($(NH_4)_2SO_4$), 100 mM potassium chloride (KCl), 20 mM magnesium sulfate ($MgSO_4$), 1% Triton X-100, pH 8.8), T4 DNA ligase, 10× T4 DNA ligase reaction buffer (500 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM adenosine 5'-triphosphate (ATP), 100 mM DTT, pH 7.5), and deoxynucleotide solution mixture (dNTPs) solution set were purchased from New England Biolabs (Ipswich, MA, USA). The SYBR Green II was purchased from Solarbio Life Sciences (Beijing, China). The fetal bovine serum was purchased from Thermo

Fisher Scientific (Waltham, MA, USA). All other chemicals were of analytical grade and obtained from Sigma-Aldrich Company (St. Louis, MO, USA). The ultrapure water was prepared by a Millipore filtration system (Millipore, Milford, MA, USA).

Table S1. Sequences of the Oligonucleotides[α]

note	sequences (5'-3')
5hmC-DNA-1 with one 5hmC site	CAT ACC ATT TAA ^{hm}CGA TAA ATT ACA A
5mC-DNA-1 with one 5mC site	CAT ACC ATT TAA ^{m}CGA TAA ATT ACA A
padlock probe-1	P-**TTA AAT GGT ATG** GAA TGC TAA TTC GAG TCT GTT GAT AAG TAG AAT GCT A**TT GTA ATT TAT CA**
5hmC-DNA-2 with two 5hmC sites	CAT ACC ATT TAA ^{hm}CGA TAA ATT ACA ATA A^{hm}CG TA
5mC-DNA-2 with two 5mC sites	CAT ACC ATT TAA ^{m}CGA TAA ATT ACA ATA A^{m}CG TA
padlock probe-2	**P-TTA AAT GGT ATG** GAA TGC TAA TTC GAG TCT GTT GAT AAG TAG AAT GC**T ACA TTA TTG TAA TTT ATC A**

[α]In padlock probe, the "P" indicates the phosphate group (PO_4) modification at the 5′ end. The binding region for the target 5hmC-DNA / 5mC-DNA is shown in boldface.

DNA Oxidation. The 1 μg of DNA was dissolved in 0.05 M NaOH with a total volume of 24 μL on ice, and then 1 μL of $KRuO_4$ solution with final concentration of 15 mM was added. The reaction was performed for 1 h with occasional vortexing on ice, followed by purification with a mini quick spin oligo column (Roche, Basel, Switzerland) and washing four times with 600 μL of

H_2O.

Bisulfite Treatment of DNA. Bisulfite treatment of DNA was performed according to the reported procedure.[1] First, 1 μg of DNA was denatured in 0.35M NaOH for 20 min at 37℃. Then the reaction was carried out in 3.2 M $NaHSO_3$ (freshly prepared) and 0.5 mM hydroquinone (freshly prepared) at 50 ℃ for 16 ~ 18 h. Then the DNA was recovered by a desalting column (Promega Inc., Madison, USA), and the modification was completed in 0.3 M NaOH for 15 min at 37 ℃, followed by neutralization with ammonium acetate, precipitation with ethanol and drying. Finally, the DNA was resuspended in water and used immediately or stored at −20 ℃.

Ligation and RCA Reactions. After oxidation and bisulfite treatment, the ligation reaction of padlock probe with target 5hmC-DNA or 5mC-DNA was performed in 10 μL of reaction solution containing 1× ligation buffer (50 mM Tris-HCl, 1 mM ATP, 10 mM $MgCl_2$, 10 mM DTT, pH 7.5), 4U/μL T4 DNA ligase, 300 nM padlock probe, and different-concentration target 5hmC-DNA or 5mC-DNA DNA. The mixtures were incubated at 16 ℃ overnight. The RCA reaction was performed at 55 ℃ for 25 min in 10 μL of reaction solution containing 1× ThermoPol buffer (20 mM Tris-HCl, 10 mM KCl, 2 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 2 mM $MgSO_4$, pH 8.8), 400 μM dNTPs, 0.4 U of Vent® DNA polymerase, and 1 μL of ligation products.

Gel Electrophoresis Analysis. The reaction product of RCA reaction was analyzed on 2% agarose gel with 1× SYBR Gold as the fluorescent indicator and performed in 1× Tris-acetate-EDTA (TAE) buffer (40 mM Tris−ethylic acid, 2 mM EDTA) at 110 V for 50 min. The gel was visualized by a ChemiDoc MP Imaging system (Hercules, California, USA).

Measurement of Fluorescence Spectra. The 10 μL of amplification products and 1 μL of 10× SYBR Green II were added into 40 μL of deionized water. Fluorescence spectra were measured using Hitachi F-7000 fluorometer (Tokyo, Japan). The excitation wavelength was 485 nm and the spectra were recorded from 500 nm to 700 nm. The maximum fluorescence emission at 525 nm was used for data analysis.

Detection of 5hmC DNA in Serum Samples. The 5hmC-DNA-spiked serum samples were prepared by adding different-concentration 5hmC-DNA in 100-fold diluted serum samples. The RCA reaction and fluorescence measurement followed the procedures described above.

SUPPLEMENTARY RESULTS

Optimization of polymerization time.

As shown in Figure S1, the $F - F_0$ value increases with the polymerization time (F and F_0 are the fluorescence intensity in the presence and absence of 5hmC-DNA, respectively) and reaches the highest value at 25 min, followed by the decrease beyond 25 min. Thus, 25 min is selected as the optimal polymerization time in subsequent experiments.

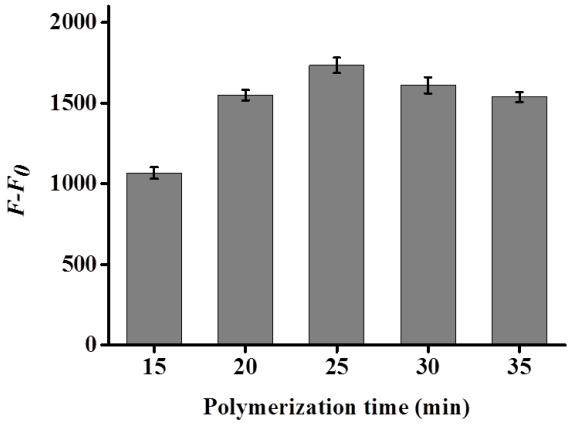

Figure S1. Variance of the F - F_0 value with the polymerization time. The 5hmC concentration is 1 nM, and the 5mC concentration is 1 nM. Error bars show the standard deviation of three independent experiments.

Optimization of the amount of DNA polymerase.

As shown in Figure S2, the F - F_0 value enhances with the increasing amount of DNA polymerase from 0.2 U to 0.3 U (F and F_0 are the fluorescence intensity in the presence and absence of 5hmC-DNA, respectively), followed by the decrease beyond the amount of 0.3 U. Thus, 0.3 U is chosen as the optimal amount of DNA polymerase in subsequent experiments.

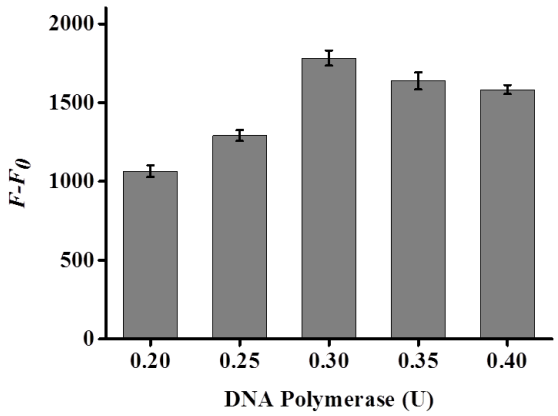

Figure S2. Variance of the F - F_0 value with the amount of DNA polymerase. The 5hmC

concentration is 1 nM, and the 5mC concentration is 1 nM. Error bars show the standard deviation of three independent experiments.

Detection of 5hmC-DNA-2 with two 5hmC sites.

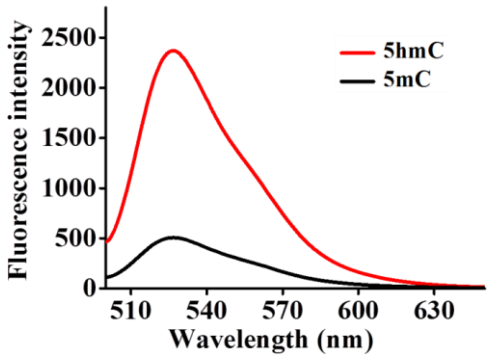

Figure S3. Fluorescence emission spectra of reaction products in response to 10 nM 5hmC-DNA-2 (red line) and 10 nM 5mC-DNA-2 (black line).

Reference

1. M. J. Booth, M. R. Branco, G. Ficz, D. Oxley, F. Krueger, W. Reik and S. Balasubramanian, Science, 2012, 336, 934.