Electronic Supplementary Information

Ultrasensitive detection of site-specific DNA methylation with loop-mediated isothermal amplification

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1. Table S1. The sequences of target DNA and primers used in the work

Name	Sequence (5'-3' direction)
Primer FIP	GCGGGCTACTGAGCATCCC-GTGCCACTGCCACCG
Primer BIP	CCGCGATCCTGTGTTCCTCG- GGACTCCCACAGCACCAT
Primer F3	GGTTTGGCTGCTGCTTCC
Primer B3	CCAGCTGCTGCACTGC
Outer sense primer	GTCCCTGGATGTTGCTGAAACTCTC
Outer antisense primer	CTGGATAGAGGTGGGTGGTATTAGG
Inner sense primer	CTCGAGATCATGCGCGGGTT
Inner antisense primer	AAAAGCCCTCGCAAAGTGTCCA
Forward primer	GGGGTTGAGTTTAGTTTTTGGAT
Backward primer	AACTCCCACAACACCATAACTAATT
Target DNA (226 nt)	CTCGAGATCATGCGCGGGTTTGGCTGCTGCTTCCC B3 CGCCGGGTGCCACTGCCACCGCCGCCGCCTCTGCT B2 GCCGCCGTCCGCGGGATGCTCAGTAGCCCGCTGC**C** B1 CGGCCCCCGCGATCCTGTGTTCCTCGGAAGCCGTT F1c TGCTGCTGCAGAGTTGCACGAACTAGTCATGGTGC TGTGGGAGTCCCCGCGGCAGTGCAGCAGCTGGAC F2c F3c ACTTTGCGAGGGCTTTT

Note: Primer FIP, BIP, F3 and B3 were used for LAMP reaction; Outer sense primer and Outer antisense primer, Inner sense primer and Inner antisense primer were respectively used for the first round and second round of the nested-PCR for preparation of the target DNA from genomic DNA.[1] Forward primer and Backward primer were used for bisulfite sequencing. In target DNA, the red color-highlighted CCGG was the recognition sequence of HpaII endonuclease, in which the cytosine in

the CpG dinucleotide was the methylated site.

2. Gel electrophoresis result of the PCR amplification

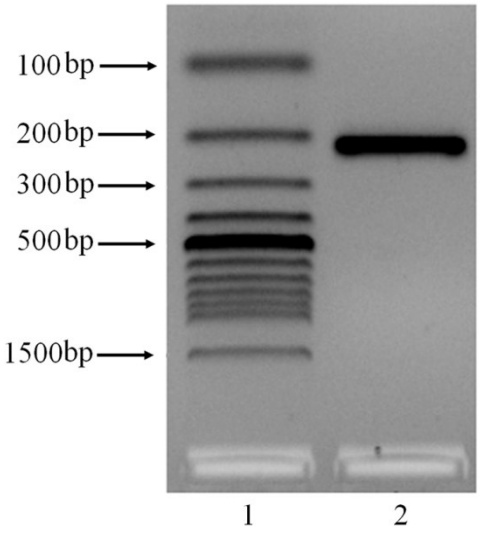

Fig. S1. Agarose electrophoresis analysis of the nested-PCR products. Lane 1, DNA ladder; Lane 2, product of the nested-PCR for preparation of the target DNA from genomic DNA.

After PCR, agarose electrophoresis analysis was performed to check the nested-PCR products, which was carried out in 2% (w/v) agarose gels with TAE buffer (pH 8.5). The mixtures containing 1 μL of 6× loading buffer and 5 μL of PCR products were prepared, then 5 μL of mixtures were loaded on the gel. Electrophoresis was run at a constant voltage 100 V for 40 min, the gel was stained by 4S Red Plus Nucleic Acid Stain and visualized on a Gel Doc EZ Imager.

The result shown in Fig. S1 has obviously demonstrated that the target DNA carried 226-bp in the promoter of *HPP1* gene was amplified and prepared successfully.

3. Fig. S2 Real-time fluorescence curves of five replicative measurements of blank control in the LAMP reaction

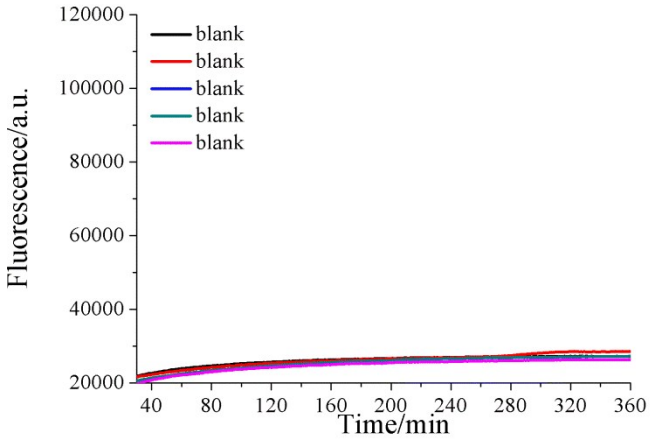

Fig. S2. Real-time fluorescence curves of five replicative measurements of blank control in the LAMP reaction.

4. Effect of the amount of HpaII endonuclease on detection of DNA methylation

The amount of HpaII endonuclease used to cleave unmethylated DNA is a crucial factor for achieving high selectivity to discriminate between methylated and unmethylated DNA. To test the effect of the amount of HpaII endonuclease on detection of DNA methylation, the blank, 100 aM, 10 fM target M and 10 fM target N were simultaneously detected with LAMP-based methylation assay by using different amounts of HpaII. As depicted in Fig. S3 (a~c), there was almost no changes of POI values in the real-time fluorescence curves produced by target M when the HpaII was increased from 10 U to 30 U, indicating that the real-time fluorescence signals produced by methylated DNA were not affected by the HpaII treatment. However, when HpaII was increased from 10 U to 20 U, the POI value of target N were obviously increased, resulting in increase of the POI difference between target M and target N. One can see from Fig. S3 that the real-time fluorescence curves of blank control were straight lines. So the real-time fluorescence signal of target N could be produced by the small amount of uncleaved target N. Compared with use of 10 U HpaII, it is obvious that the unmethylated DNA could be cleaved more thoroughly by using 20 U HpaII. Nevertheless, there was almost no increase of the POI value difference between target M and target N when HpaII was increased from 20 U to 30

U, indicating that the target N could be cleaved to its minimum by using 20 U HpaII. Therefore, 20 U was used for the LAMP-based methylation assay.

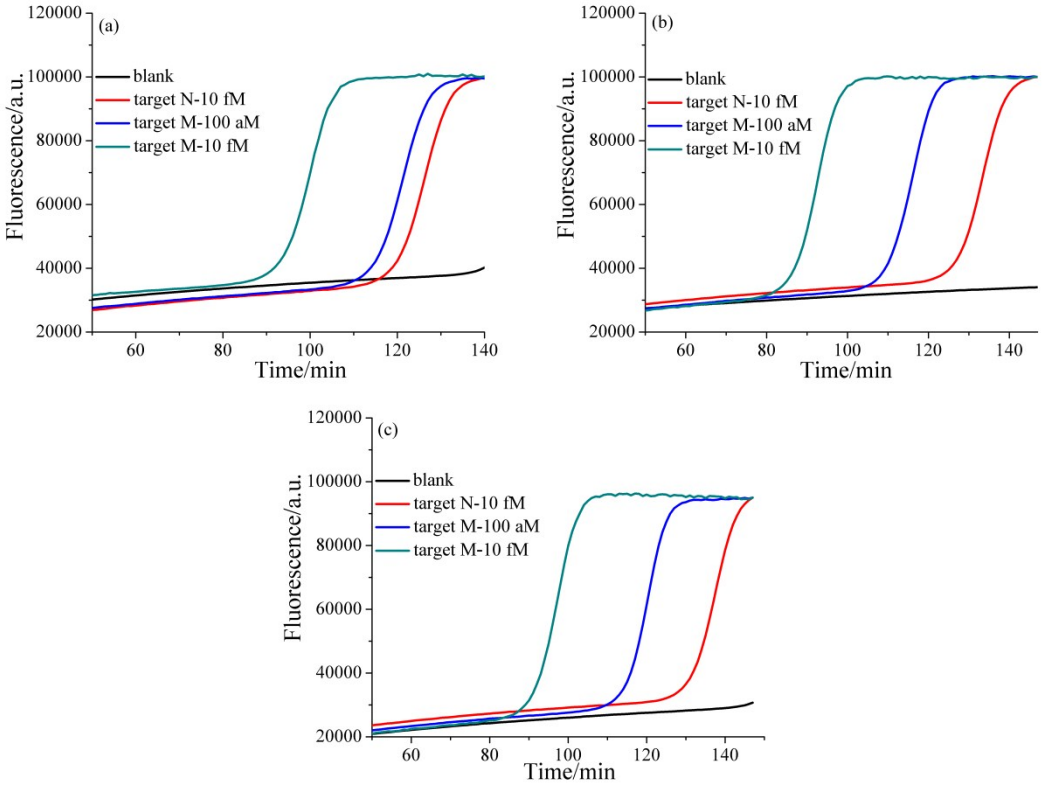

Fig. S3. The effect of the amount of HpaII endonuclease on detection of DNA methylation. The real-time fluorescence signals were produced by blank, 100 aM, 10 fM target M and 10 fM target N with the LAMP-based methylation assay according to procedures as described in the experimental section except the amount of HpaII endonuclease, which was (a) 10 U, (b) 20 U, (c) and 30 U, respectively.

5. Optimization of the temperature of LAMP reaction

The temperature of LAMP reaction was closely related to the sensitivity and selectivity for detection of DNA methylation. The influence of the temperature of LAMP reaction was investigated by simultaneously detecting the blank, 100 aM, 10 fM target M and 10 fM target N with the LAMP-based methylation assay at different reaction temperatures. As shown in Fig. S4 (a~c), when the LAMP reaction was performed at 55 °C, 60 °C and 65 °C, both 100 aM target M and 10 fM target M could produce well-defined real-time fluorescence signals. However, the POI values

of the real-time fluorescence curves reduced with raising the temperature of LAMP reaction, indicating that the LAMP reaction speed could be increased at higher temperature. Meanwhile, the real-time fluorescence signals of blank control at different temperature were all near to zero. On the other hand, the difference of POI values between target M and target N reached its maximum when the LAMP reaction was performed at 65 °C. When the temperature of LAMP reaction was increased to 70 °C, as shown in Fig. S4d, the target M could not produce defined real-time fluorescence signals, indicating that no LAMP reaction occured at 70 °C because the temperature was higher than the melting temperatures of FIP, BIP primers and target DNAs. Based on the consideration of both reaction time and discrimination between methylated and unmethylated DNA, 65 °C was selected as the optimized temperature of LAMP reaction in this work.

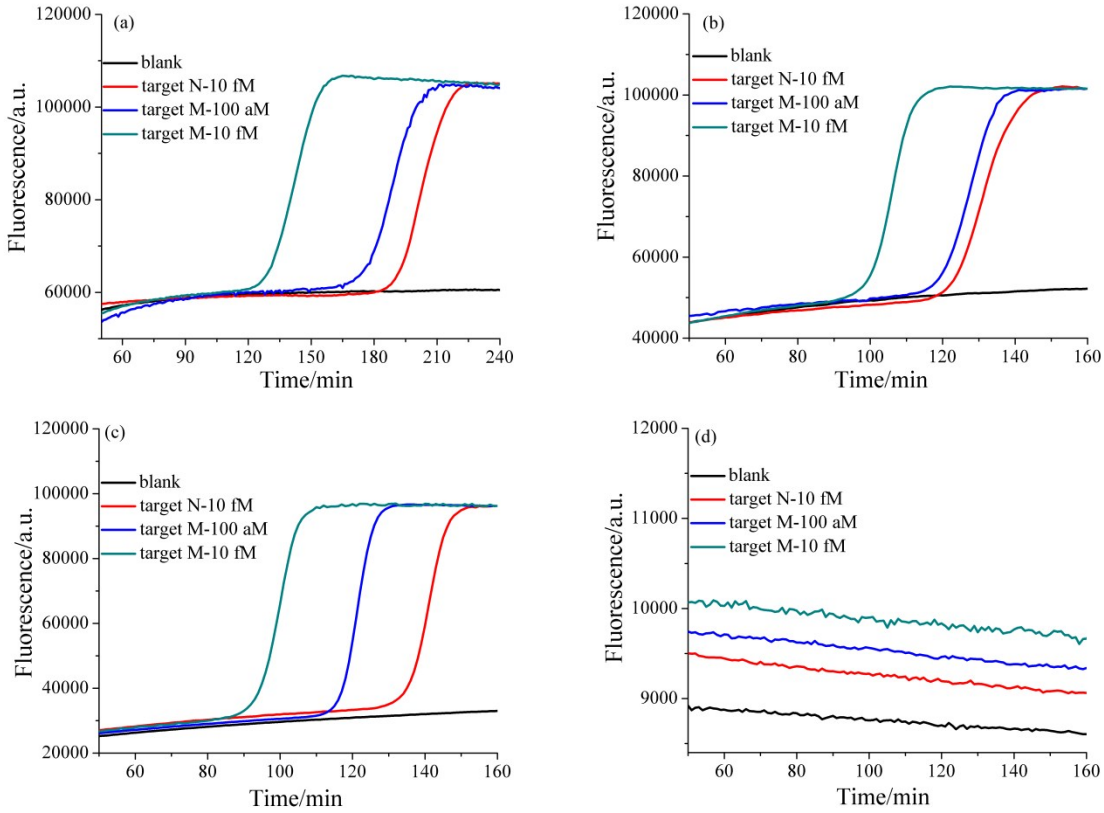

Fig. S4. The influence of the temperature of LAMP reaction on methylated DNA detection. The blank, 100 aM, 10 fM target M and 10 fM target N were simultaneously detected with the LAMP-based methylation assay. The temperature in LAMP reaction was (a) 55 ºC, (b) 60 ºC, (c) 65 ºC and (d) 70 ºC, respectively. The

assay was performed according to the procedures as described in experimental section except the temperature.

6. Optimization of the amount of *Bst* DNA polymerase in LAMP reaction

The LAMP-based DNA methylation assay relied on auto-cycling strand displacement DNA synthesis that was catalyzed by *Bst* DNA polymerase with high strand displacement activity. Therefore, the amount of *Bst* DNA polymerase was another critical parameter for the LAMP-based methylation assay. The effect of the amount of *Bst* DNA polymerase was investigated in the range of 2.4~7.2 U by detection of blank, target M, and target N with the LAMP-based methylation assay and the experimental results were presented in Fig. S5 (a~c). Firstly, there was almost no change of the blank signals as increasing the amount of *Bst* DNA polymerase and all blank signals were near to zero, indicating no non-specific LAMP reaction in the blank control. Secondly, the POI values of the real-time fluorescence curves produced by target M were gradually shortened with increasing the amount of *Bst* DNA polymerase in LAMP reaction. It is reasonable that increase of *Bst* DNA polymerase could accelerate the LAMP reaction. Finally, the difference of POI values produced by target M and target N reached its maximum when *Bst* DNA polymerase used in LAMP reaction was 4.8 U. In order to achieve both high sensitivity and specificity for detection of methylated DNA, 4.8 U was employed as the optimum amount of *Bst* DNA polymerase in LAMP reaction.

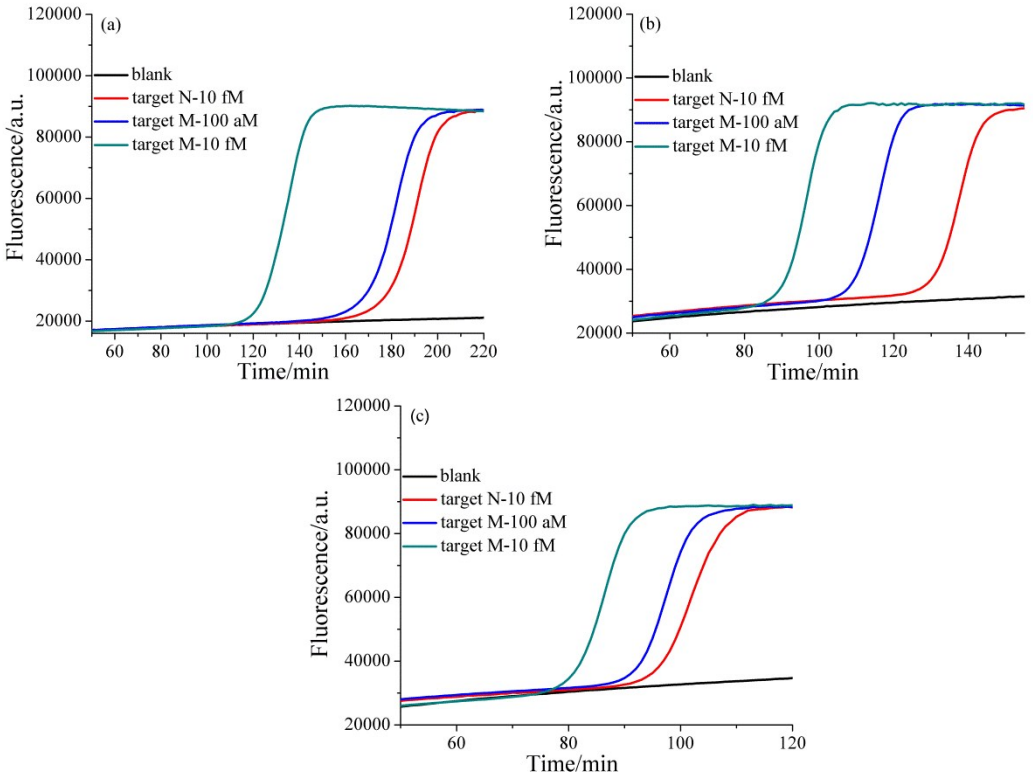

Fig. S5. The influence of the amount of *Bst* DNA polymerase on detection of methylated DNA. The real-time fluorescence curves were respectively produced by the blank, 100 aM, 10 fM target M and 10 fM target N with LAMP-based methylation assay. The amount of *Bst* DNA polymerase used in the LAMP reaction was (a) 2.4 U, (b) 4.8 U, and (c) 7.2 U. Other experimental conditions were the same as described in the experimental section.

7. The real-time fluorescence curves for the detection of genomic DNA

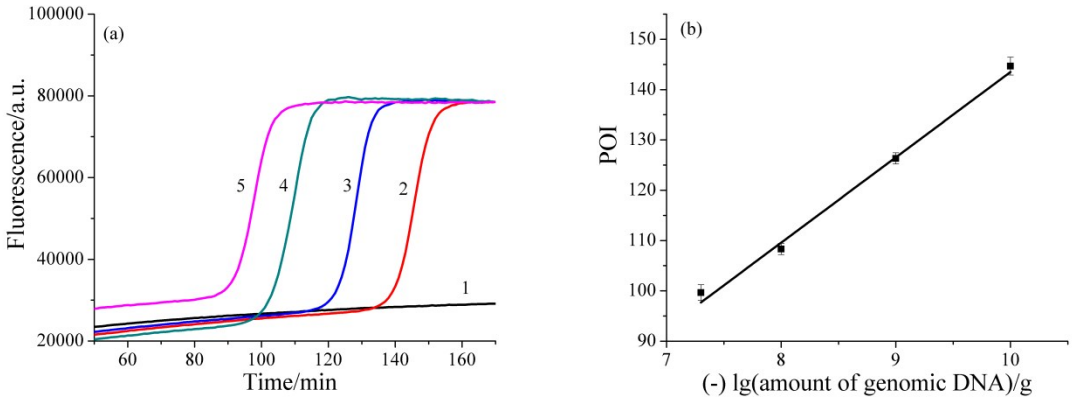

Fig. S6. (a) The real-time fluorescence curves for detection of (1) blank, (2) 100 pg, (3) 1 ng, (4) 10 ng, (5) 50 ng genomic DNA extracted from human colon cancer cells

HCT116. (b) The relationship between the POI values and lg of the amount of genomic DNA. Error bars were estimated from the standard deviation of three replicative measurements. The experiment conditions were the same as described in the experimental section.

8. Bisulfite-sequencing result

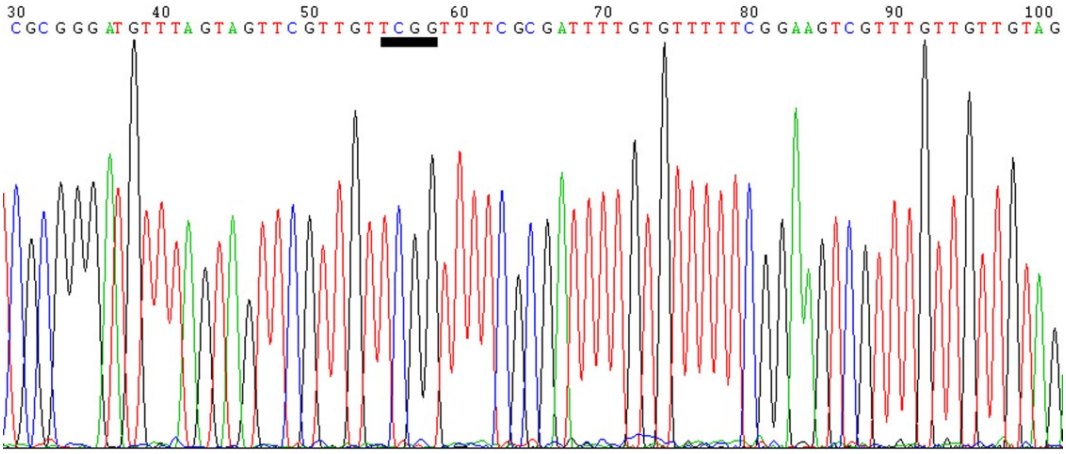

Fig. S7. The sequencing result of bisulfite-treated genomic DNA in the promoter region of *HPP1* gene in which the sequence of the detected methylation site was underlined.

Genomic DNA was extracted from human colon cancer cell lines HCT116 using TIANamp Genomic DNA Kit according to the manufacture's protocol and then was treated with sodium bisulfite and purified according to the protocols of EpiTect® Bisulfite kit. PCR amplification of the bisulfite treated genomic DNA was performed in 50 μL aqueous solution containing 0.2 mM dNTPs, 0.4 μM each of forward primer and reverse primer (see Table S1), PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), and 2.5 U of JumpStart Taq DNA polymerase in a thermocycler with the following procedures: 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 45 s, and 72 °C for 45s , and finally incubated at 72 °C for 5 min. The PCR product was sequenced with an ABI 3730xl DNA analyser (Applied Biosystems) by Life Technologies (Beijing, China).

The sequencing result shown in Fig. S7 indicated that the sequence of CCGG in genomic DNA at the detected methylation site was TCGG, demonstrating that the

cytosine in the CpG dinucleotide was methylated and the first cytosine base in the CCGG was not methylated.

References

1 F. D. Feng, L. B. Liu and S. Wang, *Nat. protoc.*, 2010, **5**, 1255-1388.