SUPPORTING INFORMATION

Digital LAMP-based absolute methylation quantification revealed hypermethylated DAPK1 in cervical cancer patients

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Table of Contents

ADDITIONAL EXPERIMENTAL SECTION

Gene information. Homo sapiens death associated protein kinase 1 (DAPK1): RefSeqGene on chromosome 9, NCBI Reference Sequence: NG_029883.1, Source: Homo sapiens (human)； Homo sapiens actin beta (ACTB): RefSeqGene on chromosome 7, NCBI Reference Sequence: NG_007992.1, Source: Homo sapiens (human).

Preparation of methylated and unmethylated DNA targets. The prepared target DAPK1 was divided into two equal parts. One part of them was not treated and used as the unmethylated DAPK1 target (target U). At the same time, the other part was used them as the methylated DAPK1 target (target M) with the M.SssI treatment according to the following steps: 12.8 nmol of SAM, 10 μL of 10× NEB buffer 2 (500 mM NaCl, 100 mM Tris-HCl, 100 mM $MgCl_2$, 10 mM DTT, pH 7.9@25 °C) and 3 U of M.SssI were added to the target DNA at a final volume of 100 μL, and were incubated at 37 °C. After 5 h of incubation, the reaction mixture was supplemented with 3 U of M.SssI of SAM and 12.8 nmol and incubated at 37 °C for another 12 h to ensure complete methylation. Then the M.SssI was heated at 65 °C for 20 min to inactivate it.

Cleavage of the DNA with HpaII endonuclease. Digestion of unmethylated and methylated DNA targets or DNA samples by HpaII endonuclease with a final volume of 50 μL. And the digestion process was performed in CutSmart buffer (10 mM Bis Tris propane-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.0@25 °C) at 37 °C with 20 U of HpaII endonuclease, after 12 h of the reaction and heat-inactivated at 85 °C for 15 min.

Dilution of methylated DNA at different ratios. In order to investigate the specific detection ability of this method for the level of methylated DNA in the mixture, we prepared a series of artificial mixtures of unmethylated and methylated DNA with different proportions. The total concentration of unmethylated DAPK1 and methylated DAPK1 is 2.5 pM, and the mixture contains 0%, 0.01%, 0.1%, 1%, 5%, 10%, 50% and 100% methylated DAPK1. And a series of samples (600 copies/μL) with known methylation levels (1%, 5%, 10%, 25%, 50%, 75%, and 100%) was prepared by mixing fully methylated and unmethylated DAPK1.

Preparation of cell genomic DNA. HeLa and L-02 cells were purchased from the cell bank of Central Laboratory of Xiangya Hospital (Changsha, China) and cultured in the DMEM medium supplemented with 10% fetal calf serum, 100 mg/mL streptomycin and 100 U/mL penicillin. The cells were maintained at 37 °C in a humidified atmosphere (5% CO_2 and 95% air). After trypsinization, 10^6 adherent HeLa and L-02 cells were collected in a 1.5 mL microfuge tube. At room temperature, the HeLa and L-02 cells were pelleted by centrifugation at 11200g for 1 min and the supernatant were carefully discarded. According to the manufacturer's instruction, the genomic

DNA was extracted by using All-In-One DNA/RNA Mini-Preps Kit. Then, the genomic DNA was stored at -80 °C for further analysis.

Paraffin Genomic DNA extraction and bisulfite conversion. DNA was extracted with Ezup Column FFPE DNA Purification Kit following the manufacturer's protocol. Then, the extracts were modified using EpiTect® Bisulfite kit following the protocol provided by the manufacturer. The concentration of purified DNA samples was measured with an ultraviolet-visible spectrophotometer. These bisulfite-treated DNA was eluted in 20 μL TE buffer and stored at -20 °C for further test.

Quantitative methylation-specific PCR (qMSP). Quantitative MSP was performed with a ABI StepOnePlus qPCR instrument. The forward and reverse primers for qMSP assay are 5'-ATTCGTAGCGGTAGGGTTTG -3' and 5'- GACCATAAACGCCAACGC -3', respectively. The qMSP experiment was performed in 10 μL reaction mixture containing 5 μL 2× SybrGreen qPCR Master Mix, 1 μL synthetic template or HCV cDNA and 0.2 μL of 10 μM forward and reverse primers on an ABI StepOnePlus qPCR instrument. Thermal cycling procedures were as follows: 3 min at 95 °C followed by 45 cycles of 5 s at 95 °C and 30 s at 60 °C. Fluorescence data were collected during the annealing/extension step for the determination of the cycle threshold (Ct).

Agarose gel electrophoresis analysis. The products of LAMP reaction were used for 3% agarose gel electrophoresis by adding 10 μL of each sample into the lanes. The agarose gel was stained with 0.5 μg/mL ethidium bromide and 0.5 μg/mL Goldview. The electrophoresis was carried out in 0.5× TBE buffer (90 mM Tris-HCl, 90 mM boric acid, and 2 mM EDTA, pH 8.0) at a constant voltage of 90 V for 1.5 h at room temperature. The images of the gel were visualized with Tanon 4200SF gel imaging system.

Microfluidic chip fabrication. Y-shape droplet microfluidic chip patterns were drawn using AutoCAD software and printed on the dark field mask film. The microchannels on masks transfer to glass surfaces by photolithography and etched to produce a channel cross-section at junction 55 μm (w) × 50 μm (d) and wide channel cross-section 250 μm (w) × 50 μm (d), in the stirred tank filled with diluted $HF/NH_4F/HNO_3$ solution. Five holes with a diameter of 1.8 mm were etched at the end of the channel by mechanical drill through etching glass. The etched glass was held in a muffle furnace at 580 °C for 2 hours to maintain the same thickness as the uncharacteristic glass substrate. After bonding, the microchannels were rinsed with isopropyl alcohol, acetone, piranha solution (H_2SO_4/H_2O_2, 3:1) and deionized water in sequence, and then dried with nitrogen. Finally, we add a cover, which was bonded to the glass plate by normal temperature bonding process. Then the glass channel was treated with dry toluene solution containing 0.1 % octadecyltrichlorosilane for 5 min.

The treated channels were successively washed with dry toluene, isopropanol, and deionized water, and dried at 150 °C for 2 h. The chip was then used for experiments.

The droplet counting microwell chip was designed by AutoCAD software to collect droplets. The droplet counting microwell chip with hydrophilic channels was prepared by using standard multi-step photolithography and wet chemical etching technology, and chromium film was deposited on the 1.6 mm thick 20 × 60 mm borosilicate glass substrate. The chip was composed of the main channel (54.7 mm length, 6.4 mm wide, and 50 μm deep), an inlet, and an outlet. After UV exposure, the photomask pattern was transferred to the glass plate. In a fully stirred bath containing diluted $HF/NH_4F/HNO_3$, the microchannels were etched on the glass substrate with an etching depth of 50 μm. Then, we completely removed the remaining chromium layer with the previous chromium etching solution. The glass plate was ultrasonically cleaned with acetone, household washing powder, ultrapure water and ethanol, and dried in an oven at 150 °C for 2 h. Finally, we bonded the cover plate to the glass plate by room temperature bonding process. Finally, the whole surface of the droplet counting microwell chip was subjected to salinization with 1% octadecyltrichlorosilane in isooctane (v/v), and the hydrophobic surface was obtained. After cleaning and drying, we fabricated a hydrophobic coating around the droplet counting microwell chip. These microchips can be used repeatedly.

Ethical approval. All the experiments with clinical samples were performed in accordance with The Third Xiangya Hospital of Central South University. The relevant ethical approvals were obtained from the ethical committee of The Third Xiangya Hospital of Central South University.

Confocal fluorescence imaging. We incubated droplet counting microwell chip at 63 °C for 60 min and acquired fluorescence images by using an inverted confocal laser scanning microscope under a 10× objective. We used 488 nm, 560 nm, and 640 nm laser as the excitation source simultaneously. Then, we analyzed the pictures and calculated different positive droplets with NIS-Elements software and then counted the template copies according to the Poisson distribution.

Droplet counting with NIS-Element software:

1. Open the captured image in NIS-Element software.

2. Click tool bar, choose View → Analysis Controls → Automated Measurement → Thresholding → Per channel, we choose the suitable threshold and size, then enter and analyze it.

3. Click tool bar, choose View → Analysis Controls → Automated Measurement Results, then we can get the number of droplets in each fluorescence channel of the pictures.

Table S1. DNA primers and templates for HEADLAMP analysis.

Name	Sequence (5'-3')
DAPK1*-F3	AAAAGGCGGCAAGGAGC
DAPK1-B3	TCGGAGGCCGACCATAGG
DAPK1-FIP	GAGTTGCCGAGTCCCCTCCGCTTCGGAGTGTGAGGAGGAC
DAPK1-BIP	GATCTGCGCCCCCCACTCAAGCGCCGGAGACTAGC
DAPK1-LP	AAAGTCCCCGGCGTTGG
DAPK1-LMB	Cy5-AGCGCATATCTCAGCGCT(BHQ2)CTCCCTAGCTGTGTTCCCGC
β-actin#-F3	TGGCCTACTGGCAGAGAG
β-actin-B3	AGGTCATCTCAGTAGCCTCC
β-actin-FIP	TAGAACCACCCCAGAGAGCCCGTGGTACCCTTCCTCCTGT
β-actin-BIP	TGATTTCTGCAGTGGTGAGCCCACCGTAGAGTGGTCACTCAA
β-actin-LP	TCAGGGGACCTCCAAGATAAC
β-actin-LMB	TAMRA-AGCGCATATCTCAGCGCT(BHQ2)GCCACTTAATCATTCAACCCCCATT
B-DAPK1-F3	GGCGGTAAGGAGTCGAGAG
B-DAPK1-B3	ACCGACCATAAACGCCAAC
B-DAPK1-FIP	CCGAATCCCCTCCGCGAAAAAAGTTGTTTCGGAGTGTGAGGA
B-DAPK1-BIP	GTCGGCGTTTGGGAGGGATT-CTAACCGAAACGACGACGAA
B-DAPK1-LP	ACGTTAACTCGATCCGACTATCC
B-DAPK1-LMB	Cy5-AGCGCATATCTCAGCGCT(BHQ2)ATTTATTTTTTAGTTGTGTT

B represent the bisulfite treatment of methylated DAPK1.

***** Beginning position: 4983, ending position: 5234.

Beginning position: 9604, ending position: 9891.

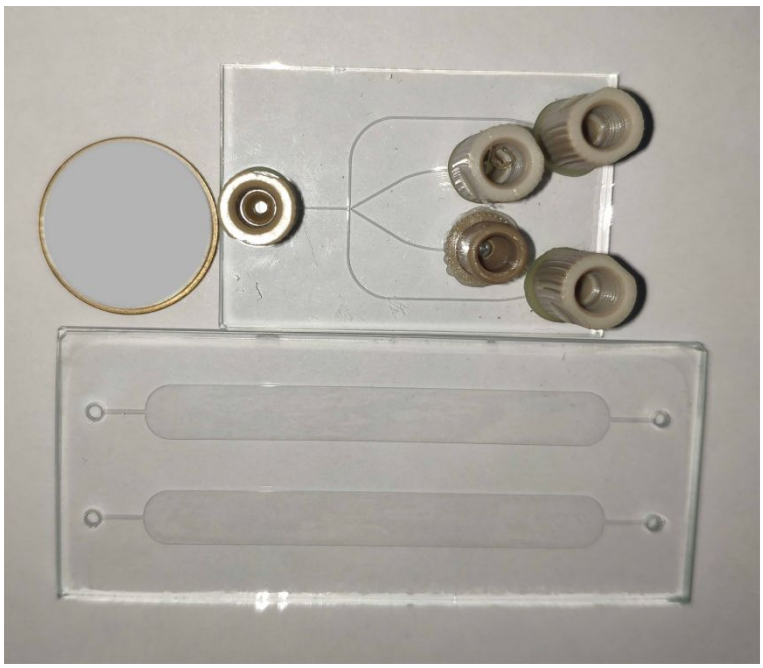

Figure S1. Image of Y-shape droplet-generating microfluidic chip and droplet counting microwell chip. The diameter of the coin is 20.5 mm.

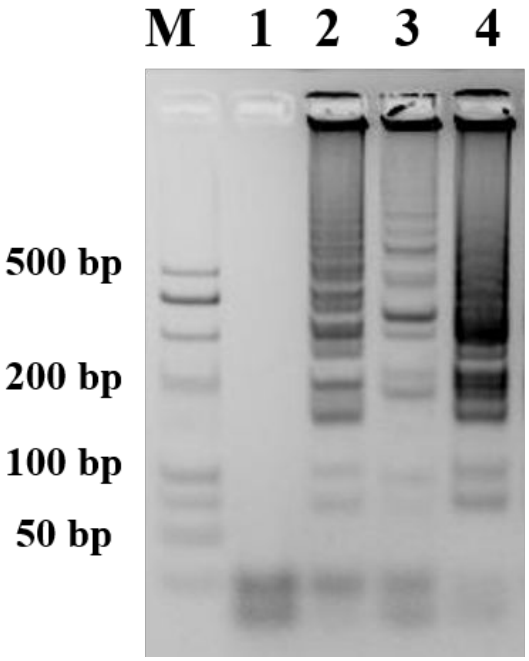

Figure S2. Gel electrophoresis of the HpaII-edited LAMP amplified products. M: DNA marker (25-500 bp). Lane 1: blank; Lane 2: 10 fM β-actin; Lane 3: 10 fM methylated DAPK1; Lane 4: 10 fM β-actin and 10 fM methylated DAPK1.

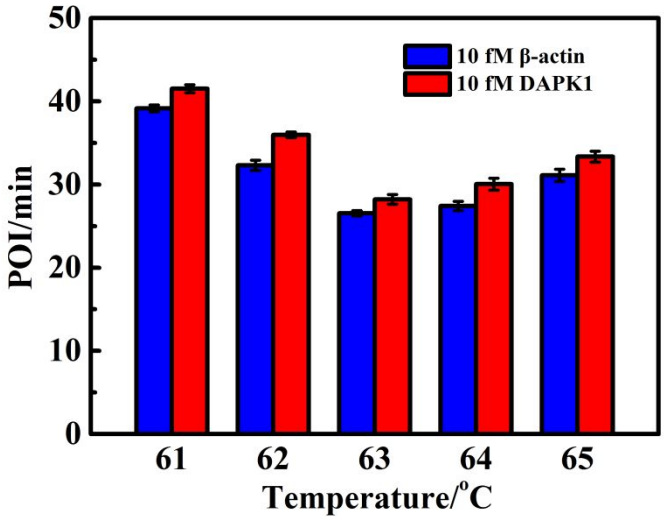

Figure S3. Optimization of reaction temperature for the HpaII-edited LAMP assay. Error bars represent mean ± SD.

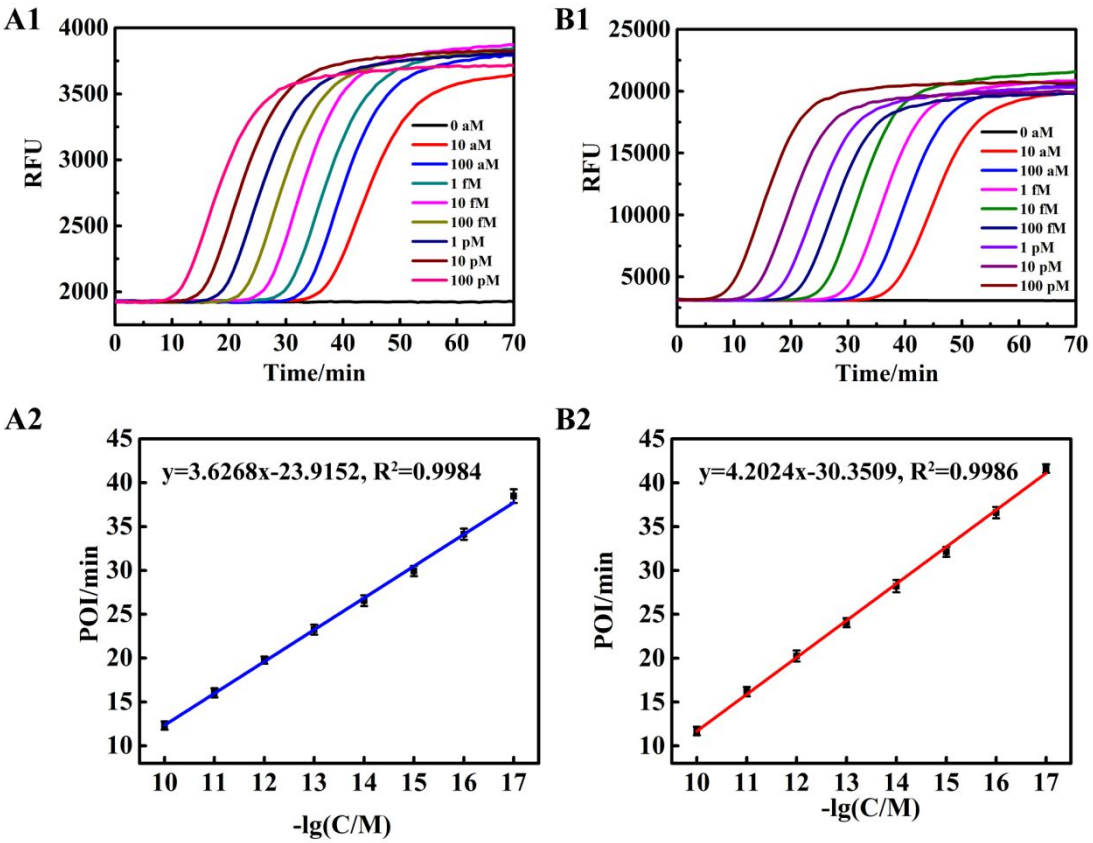

Figure S4. Sensitivity of the HpaII-edited LAMP assay. (A1, B1) Real-time fluorescence curves of HpaII-edited LAMP reactions at various concentrations for β-actin and methylated DAPK1. (A2, B2) Linear relationship between the POI value and the logarithmic concentration of β-actin and methylated DAPK1. Error bars represent mean ± SD.

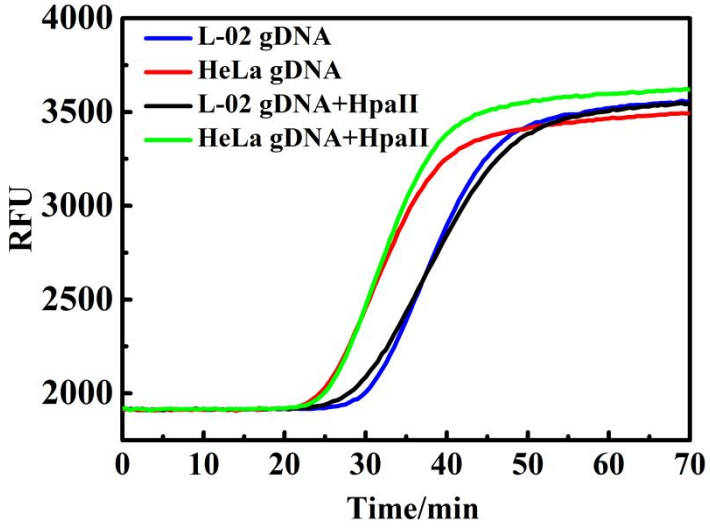

Figure S5. HpaII-edited LAMP assay for the detection of β-actin in L-02 and HeLa cells.

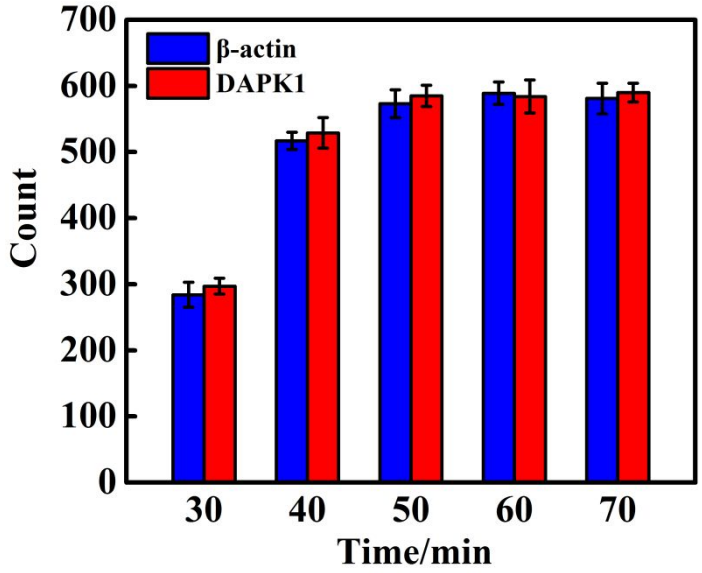

Figure S6. Optimization of the reaction time for HEADLAMP assay. Error bars represent mean ± SD.

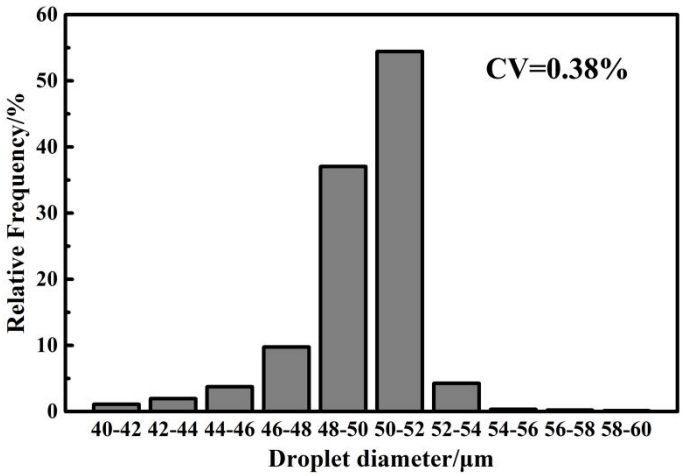

Figure S7. The diameter distribution of the generated droplets.

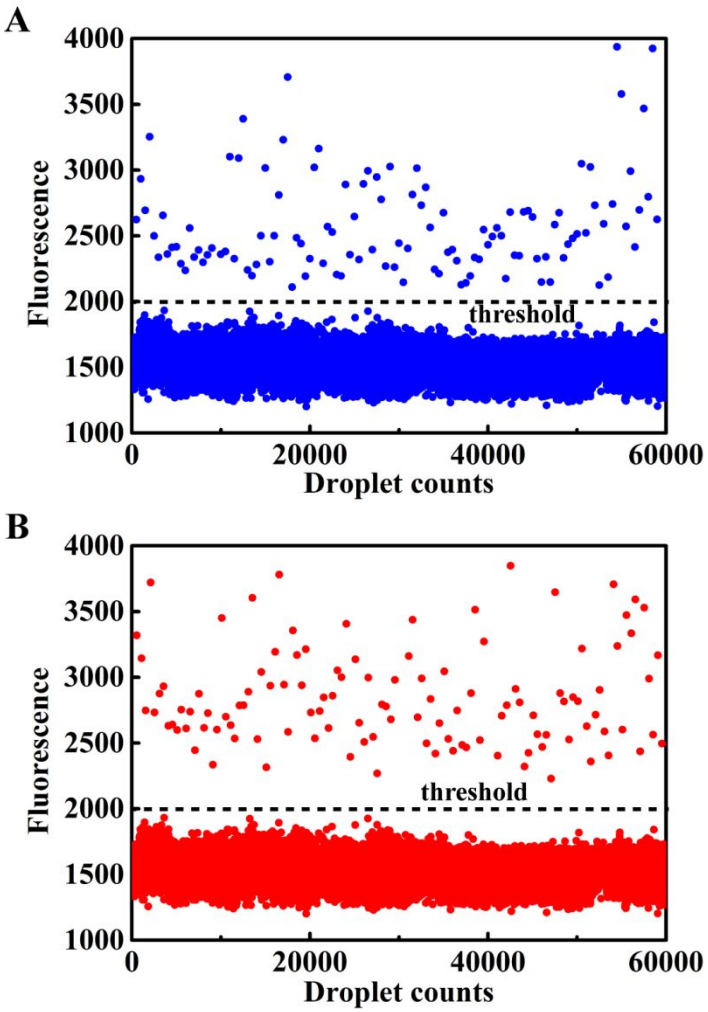

Figure S8. Scatter plot of fluorescence intensities of droplets. Distribution of fluorescence intensities of droplets in (A) TAMRA channel (β-actin) and (B) Cy5 channel (DAPK1). The threshold was set at 2000 in both channel.

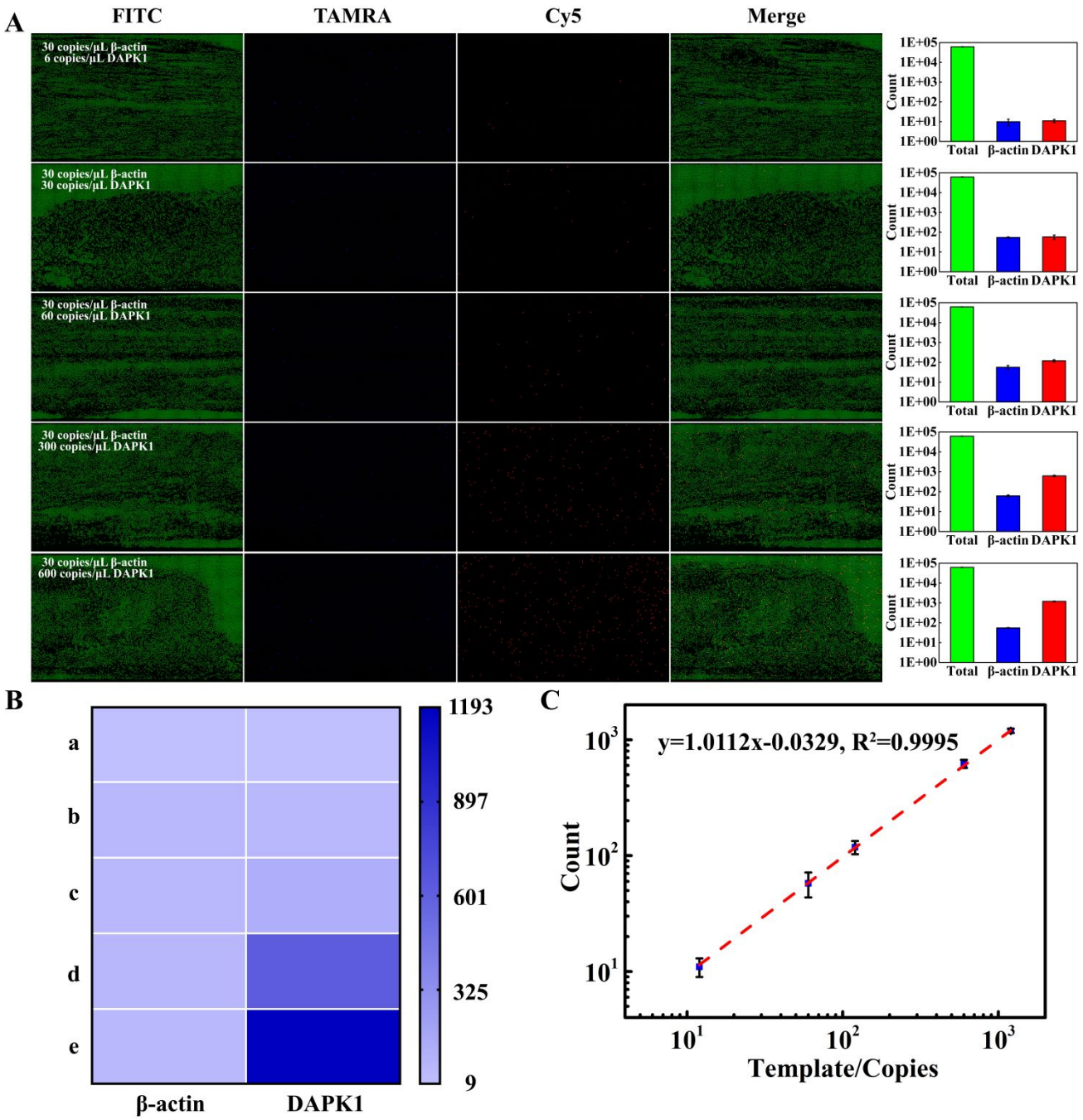

Figure S9. Quantitative detection of β-actin and methylated DAPK1 using HEADLAMP. (A) Counting of fluorescent droplets. (B) Heat map of the concentration of β-actin and methylated DAPK1. (a) 30 copies/μL β-actin and 6 copies/μL DAPK1; (b) 30 copies/μL β-actin and 30 copies/μL DAPK1; (c) 30 copies/μL β-actin and 60 copies/μL DAPK1; (d) 30 copies/μL β-actin and 300 copies/μL DAPK1; (e) 30 copies/μL β-actin and 600 copies/μL DAPK1. (C) Relationship between the estimated copy numbers for methylated DAPK1 with the actual copy numbers of methylated DAPK1 targets. Error bars represent mean ± SD.

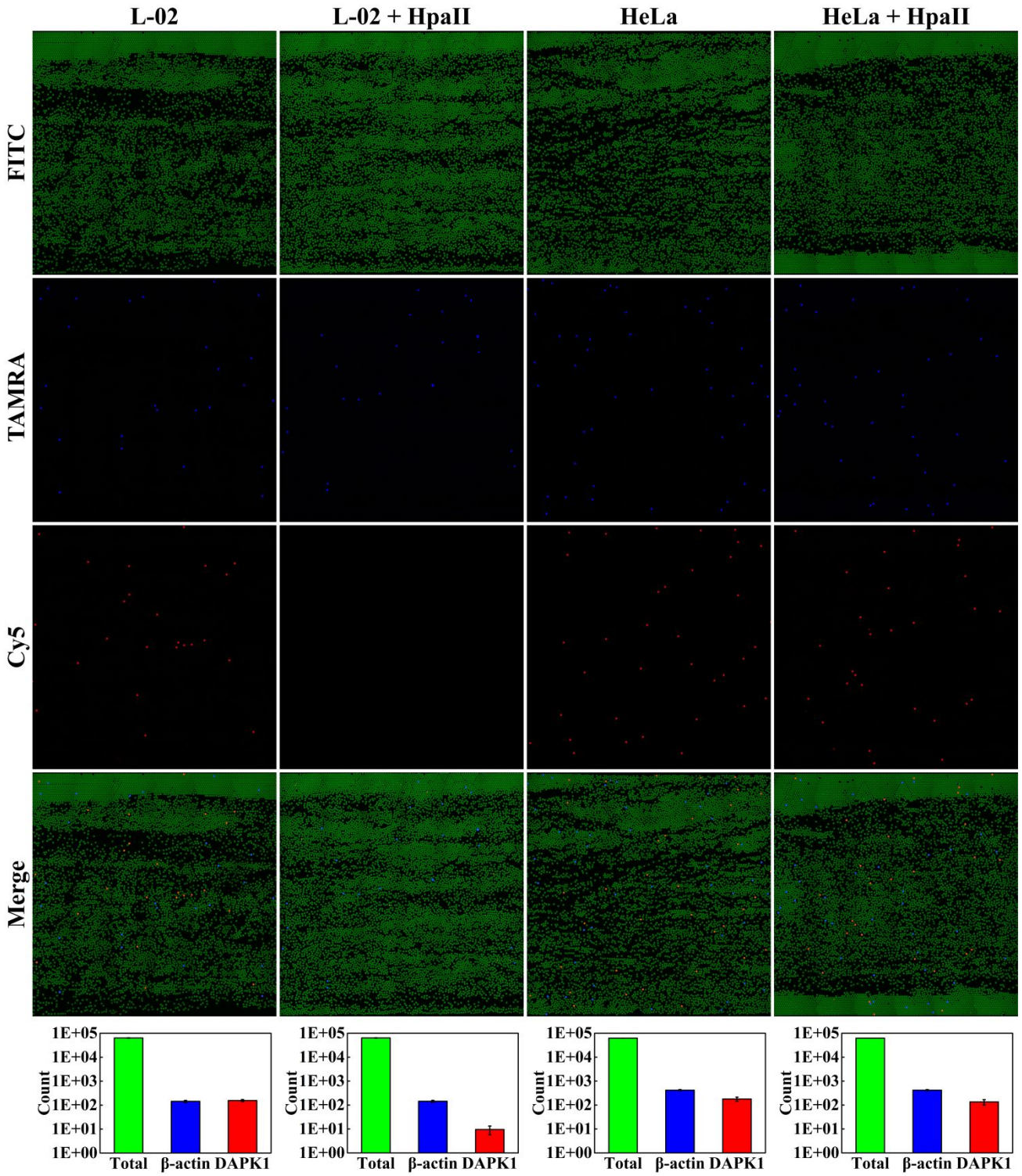

Figure S10. Detection of DAPK1 methylation levels in L-02 and HeLa cells with HEADLAMP. Error bars represent mean ± SD.

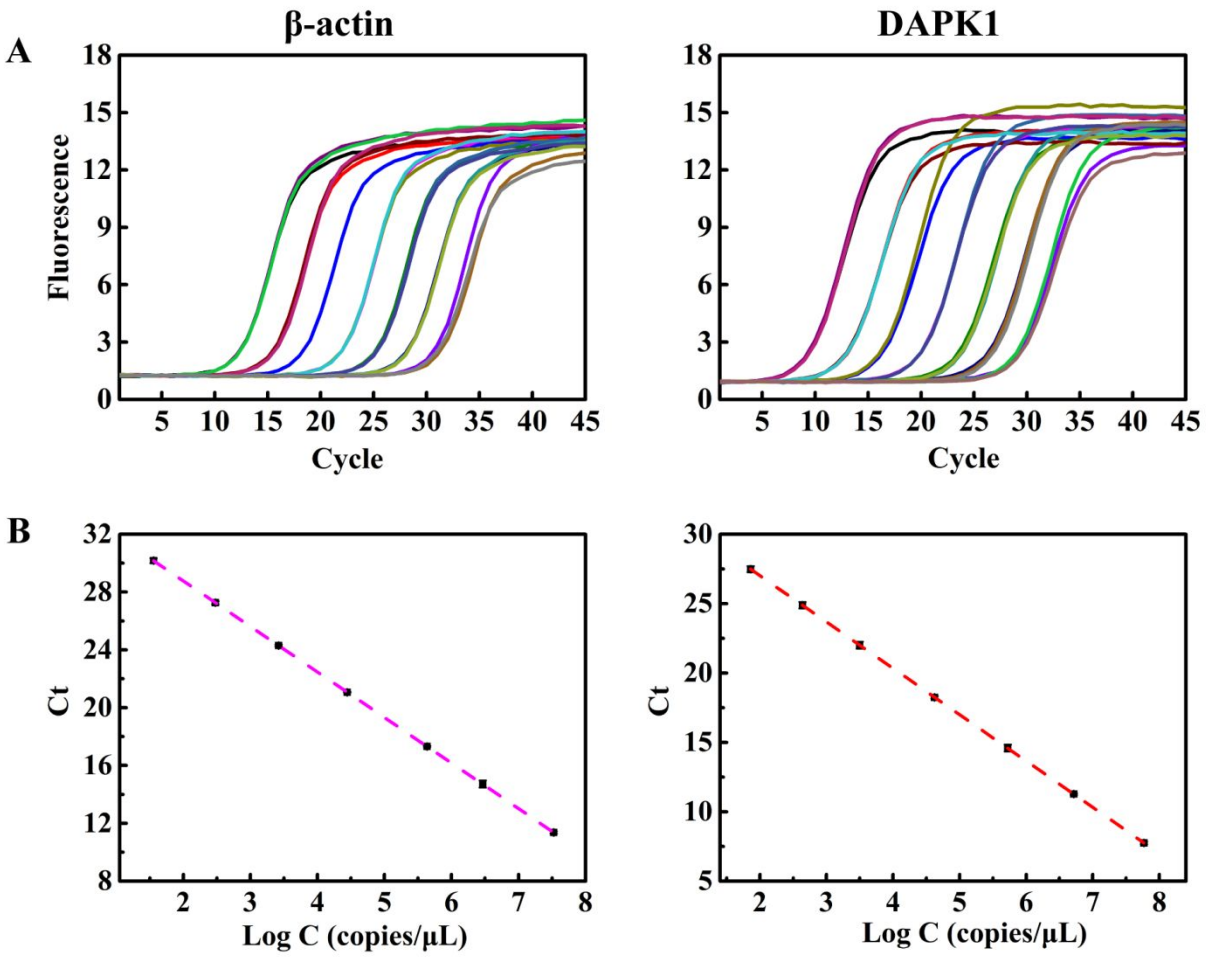

Figure S11. qMSP analysis of DAPK1 methylation for genomic DNA extracted from tissue samples. (A) Real-time fluorescence PCR curves for β-actin of different concentrations (36 ~ 3.4 × 10^7 copies/μL) and DAPK1 of different concentrations (72 ~ 5.8 × 10^7 copies/μL). (B) Ct values versus logarithmic cDNA concentrations. Error bars represent mean ± SD.

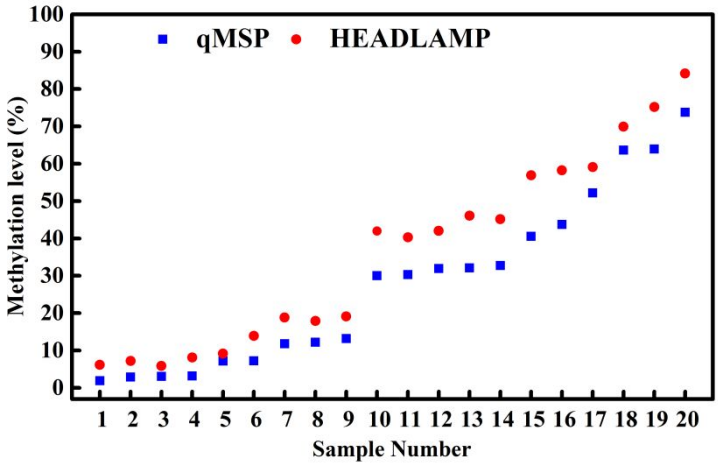

Figure S12. Comparison between bisulfite conversion-based qMSP and HEADLAMP in detecting DNA methylation in clinical tissue samples.