Supporting Information

Bisulfite-free and quantitative detection of 5-formylcytosine in DNA through qPCR

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1. Materials and methods

Materials and chemicals. All chemicals were purchased from Adamasbeta® (Shanghai, China) or Beijing Innochem Sci. & Tech. Co. Ltd. (Beijing, China) or unless mentioned otherwise. The shorter oligonucleotides 15-DNA-5fC, 15-DNA-5mC, 15-DNA-5hmC, 15-DNA-fU, 15-DNA-AP and dNTPs mix (N = A, T, C, G) were bought from Takara Biotechnology Co., Ltd. (Dalian, China). The nucleic acid stains (Super GelRed, NO.: S-2001) were ordered from US Everbright Inc. (Suzhou, China). 2× Hieff® qPCR SYBR® Green Master Mix were ordered from YEASEN (Shanghai, China). SplintR® ligase, phi 29 polymerase, Bst DNA polymerase and T4 DNA ligase were purchased from New England BioLabs (USA). DNA Degradase Plus™ was purchase from Zymo. All of the unmodified oligonucleotides were synthesized and purified by GeneCreate Co., Ltd. (Wuhan, China).

Chemical labelling reaction. For the reaction between 15-DNA-X (X =C, 5mC, 5hmC, 5fC, 5fU and AP) and malononitrile (MA), 10 μL of 15-DNA-X (100 μM) was incubated with 100 mM of MA (dissolved in H_2O) in 100 mL at 37 °C for 20 hours in an Eppendorf tube in a thermos-shaker (Ningbo Biocotek Scientific Instrument Co., Ltd., China, 200 r.p.m.). After reaction the product was purified by diethyl ether extraction and mini quick spin oligo columns (Roche).

Ligation reaction of 15-DNA-L and 15-DNA-R. 1 μL of 15-DNA-X (X = T, C, 5mC, 5hmC, 5fC, 5fU and AP each 0.1 μM) with or without MA labelling were added to the ligation reaction system containing 1.5 μL of 15-DNA-L (0.1 μM), 1.5 μL of 15-DNA-R (0.1 μM), 1 μL of SplintR ligase (1 U/μL), 1 μL of SplintR reaction buffer (10x) and 4 μL of H_2O in a PCR tube. Then the PCR tube was heated at 37 °C for 10 min and quenched at 80 °C for 10 min.

The protocol of qPCR analysis of 5fC. qPCR was performed using a CFX-96 Real-Time System (Bio-Rad, USA). The ligation products were directly diluted 100-fold. The mixture contained 1 μL of template diluted ligation products, 1 μL of qPCR forward primer (10 μM), 1 μL of qPCR reverse primer (10 μM), 10 μL of 2× Hieff™ PCR SYBR® Green Master Mix and 7 μL of ddH_2O to give a final volume of 20 μL. The mixture was subject to qPCR according to the following thermal cycle: 95°C for 5 min, 40 cycles of (95°C for 10 s, 60°C for 30 s, 72°C for 20 s).

Ligation reaction of 15-DNA-RCA. 1 μL of 15-DNA-X (X =C, 5mC, 5hmC, 5fC, 5fU and AP each 0.3 μM) with or without MA labelling were added to the ligation reaction system containing 1 μL of 15-DNA-RCA (1 μM), 1 μL of SplintR ligase (5 U/μL), 1 μL of SplintR reaction buffer (10x) and 6 μL of H_2O in a PCR tube. Then the PCR tube was heated at 37 °C for 30 min and quenched at 80 °C for 10 min.

The protocol of rolling circle amplification (RCA). 5 μL of ligation products of cycle DNA was mixed with 1 μL of dNTPs (10 mM), 1 μL of phi 29 polymerase (1 U/μL), 1 μL of phi 29 reaction buffer (10x) and 2 μL of H_2O in a PCR tube. Then the PCR tube was heated at 30 °C for 12 hours and quenched at 80 °C for 10 min.

Fluorescence measurement of RCA products. 5 μL of RCA products were incubated with 5 μL

of SYBR green I (10x) and 40 μL of H_2O at room temperature for 5 min. Fluorescence signals were measured on PerkinElmer LS 55 (PerkinElmer, USA) with the excitation of 454 nm wavelength light.

Quantification of chemical labelling efficiency and recovery of DNAs. Purified MA reaction products DNAs in the presence of 10 x Degradase Plus reaction buffer (2.5 μL) and Degradase Plus (1 μL) in a final volume of 25 μL were digested to its corresponding nucleosides by incubation at 37 °C for 2 hours. The mixture was high speed centrifugation then the supernatant was applied for LC-MS analysis.

2. The sequences used in this study

Table S1. The sequences of the oligo DNA used in this study.

ODNs	Sequence
15-DNA-C	GACTCAACAGCCGTA
15-DNA-T	GACTCAATAGCCGTA
15-DNA-fC	GACTCAA5fCAGCCGTA
15-DNA-5mC	GACTCAA5mC AGCCGTA
15-DNA-5hmC	GACTCAA5hmC AGCCGTA
15-DNA-fU	GACTCAA5fUAGCCGTA
15-DNA-AP	GACTCAAAPAGCCGTA
15-DNA-L	GCTCGCTTTGTTGGTTTCCTTGTTCTCTGTCCGAGTACGGCTA
15-DNA-L-G	GCTCGCTTTGTTGGTTTCCTTGTTCTCTGTCCGAGTACGGCTG
15-DNA-L-noA	GCTCGCTTTGTTGGTTTCCTTGTTCTCTGTCCGAGTACGGCT
15-DNA-R	5'Phosphorylation-TTGAGTCGGGTGAAAGCAGCGCGAGCAAGCGAGACAGGACAC
15-DNA-RCA	5'Phosphorylation-TTGAGTCTCGTATTAACGTACCAACAAGAGAGTTCAAGTCCATCTACAATCTAAAAGTGGTGGGTGTGACCCTAAAATGTAGATGGACTTGAACTCTTTAGAGGCTACGGCTA-3'
15-DNA-RCA-G	5'Phosphorylation-TTGAGTCTCGTATTAACGTACCAACAAGAGAGTTCAAGTCCATCTACAATCTAAAAGTGGTGGGTGTGACCCTAAAATGTAGATGGACTTGAACTCTTTAGAGGCTACGGCTG-3'
qPCR forward primer	GCTCGCTTTGTTGGTTTCCTTGTTCTCTGT
qPCR reverse primer	GTGTCCTGTCTCGCTTGCTCGCGCTGCTTT
15-DNA-fC-2	ATCGTCT5fCTGTCAGC
15-DNA-C-2	ATCGTCTCTGTCAGC
15-DNA-L-2	GCTCGCTTTGTTGGTTTCCTTGTTCTCTGTGCTGACAA
15-DNA-R-2	5'Phosphorylation-AGACGATAAAGCAGCGCGAGCAAGCGAGACAGGACAC
40-DNA-C	CGACGTAGTCGATCGTTACCGGTCTAGTCAGTTAGCTGCC
40-DNA-fC	CGACGTAGTCGATCGTTAC5fCGGTCTAGTCAGTTAGCTGCC

40-DNA-L	GCTCGCTTTGTTGGTTTCCTTGTTCTCTGTCTAGACCA
40-DNA-R	5'Phosphorylation-GTAACGAAAAGCAGCGCGAGCAAGCGAGACAGGACAC

3. Evaluating the feasibility of qPCR-based and RCA-based 5fC detection method

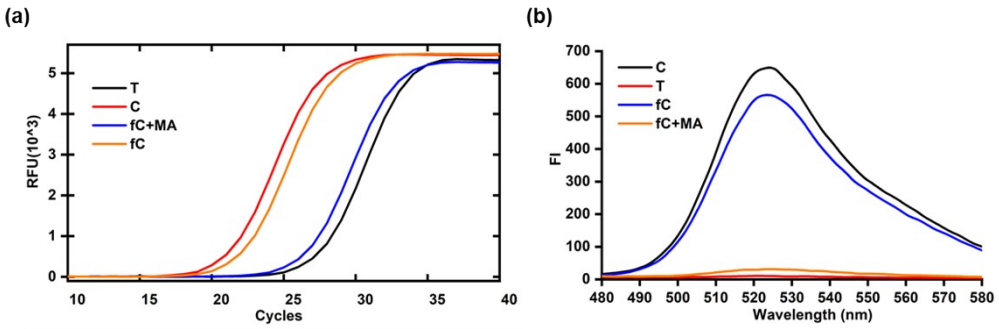

Figure S1. 15-DNA-L and 15-DNA-RCA were changed into 15-DNA-L-G and 15-DNA-RCA-G (both with a guanine base at the 3' end) in 5fC detection method. (a) The qPCR curves of 15-DNA-T, 15-DNA-C and 15-DNA-fC with (blue line) or without (yellow line) MA labelling. (b) The RCA fluorescence curves of 15-DNA-T, 15-DNA-C and 15-DNA-fC with (yellow line) or without (blue line) MA labelling.

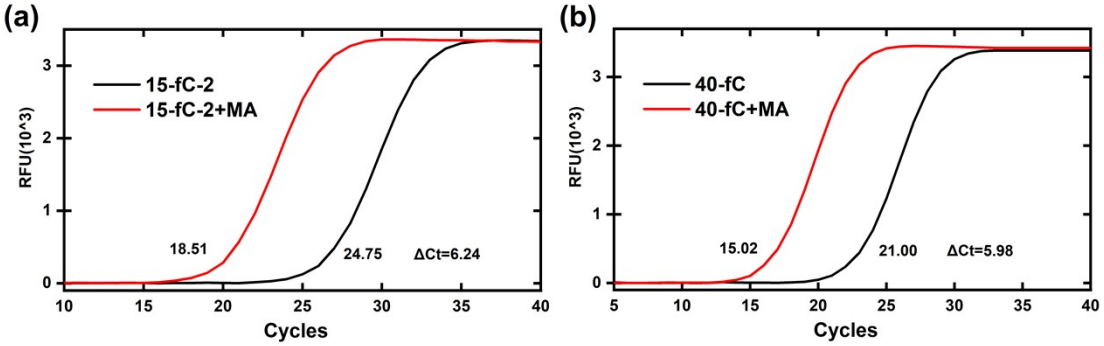

Figure S2. To evaluate whether the qPCR method has any bias in sequence or length of DNA, 15-DNA-fC was changed into 15-DNA-fC-2 (different sequence) and 40-DNA-fC (different sequence and length). (a) qPCR curves of 15-DNA-fC-2 and 15-DNA-fC-2+MA reflected the ligation condition of 15-DNA-L-2 and 15-DNA-R-2. (b) qPCR curves of 40-DNA-fC and 40-DNA-fC+MA reflected the ligation condition of 40-DNA-L and 40-DNA-R.

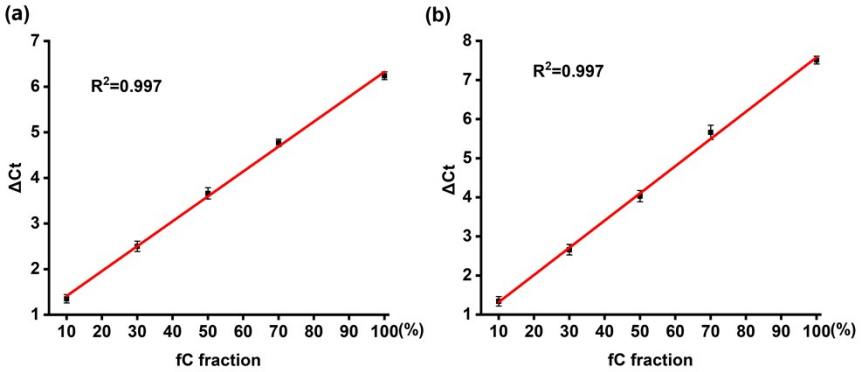

Figure S3. We tested the qPCR-based quantitative capability of 5fC with 15-DNA-fC-2 and 40-DNA-fC. DNAs with different fractions of 5fC were used at the same concentration (0.1 μM) to

quantify 5fC with the LOD of 0.01 pmol. (a) Linear relationship between the 5fC fraction in 15-DNA-fC-2 and the ΔC_t value. (b) Linear relationship between the 5fC fraction in 40-DNA-fC and the ΔC_t value.

4. Elongation-ligation 5fC detection method

15-DNA-L was changed into 15-DNA-L-noA (without the adenosine base at the 3' end) and the reaction contains two step. In the step 1, the Bst DNA polymerase elongated 15-DNA-L-noA with the addition of dATP. Then in the step 2, the nick ligation between the elongated 15-DNA-L-noA and 15-DNA-R were mediated by T4 DNA ligase. The final ligation products were carried into qPCR reaction to quantitation. We explored the different reaction condition by changing the reaction buffer, ligation reaction time and concentration of enzyme. What's more, the specificity of this method for 5fC and the linear relationship between 5fC fraction and ΔC_t value were also tested.

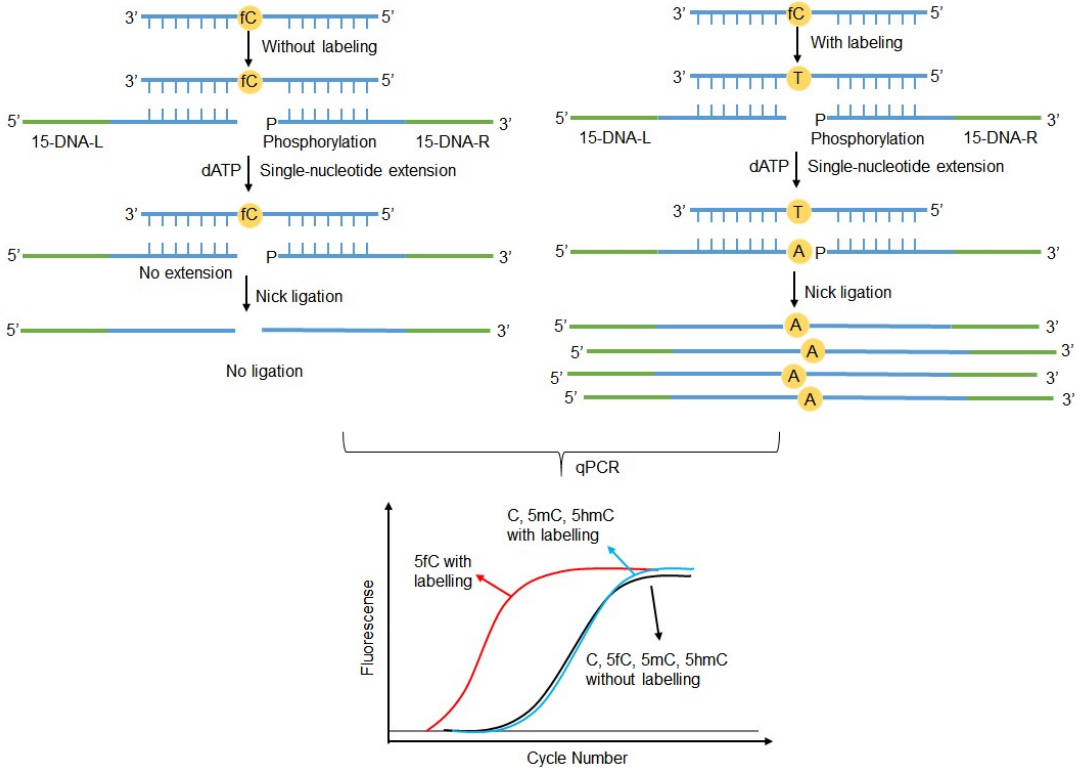

Figure S4. Scheme for qPCR-based detection of 5fC by elongation-ligation strategy.

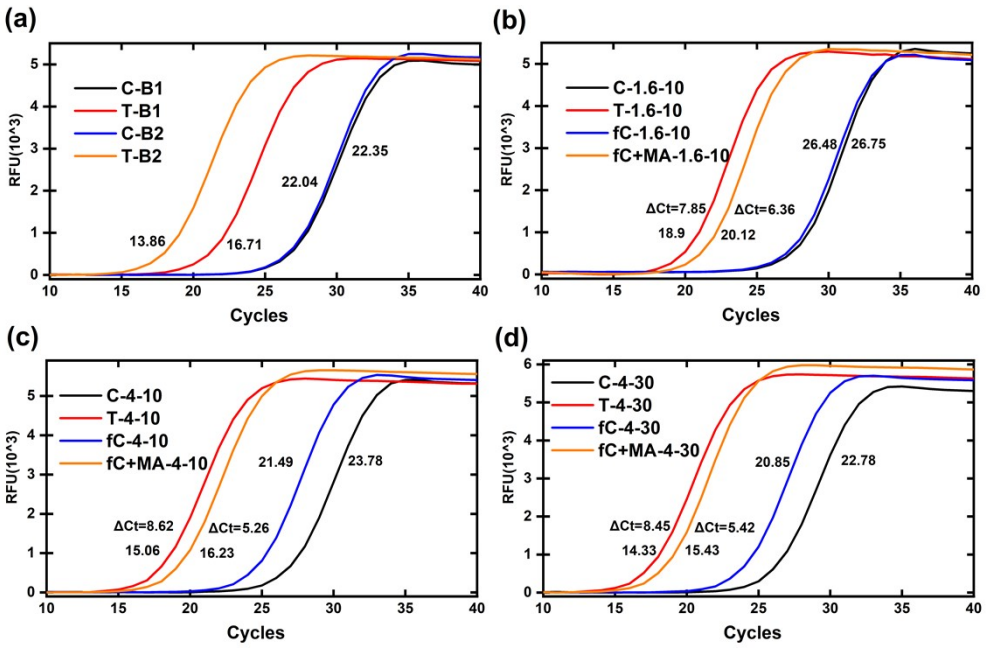

Figure S5. (a) Different reaction buffer. B1 represents the mixture of Bst and T4 buffer. B2 represents the T4 buffer. (b) Different reaction time and concentration of Bst DNA polymerase. The reaction was incubated at 37 °C in 10 min with 1.6 U Bst DNA polymerase and 1 U T4 DNA ligase. (c) The reaction was incubated at 37 °C in 10 min with 4 U Bst DNA polymerase and 1 U T4 DNA ligase. (d) The reaction was incubated at 37 °C in 30 min with 4 U Bst DNA polymerase and 1 U T4 DNA ligase.

Finally, we choose this reaction condition. 1 μL of 15-DNA-X (X = T, C, 5mC, 5hmC and 5fC each 0.1 μM) with or without MA labelling were added into the ligation reaction system containing 1.5 μL of 15- DNA-L-noA (0.1 μM), 1.5 μL of 15- DNA-R (0.1 μM), 1.5 μL of dATP (100 μM), 1 μL of T4 DNA ligase (1 U/μL), 1 μL of Bst ligase (1.6 U/μL), 1 μL of T4 DNA reaction buffer (10x) and 1.5 μL of H_2O in a PCR tube. Then the PCR tube was heated at 37 °C for 10 min and quenched at 80 °C for 10 min.

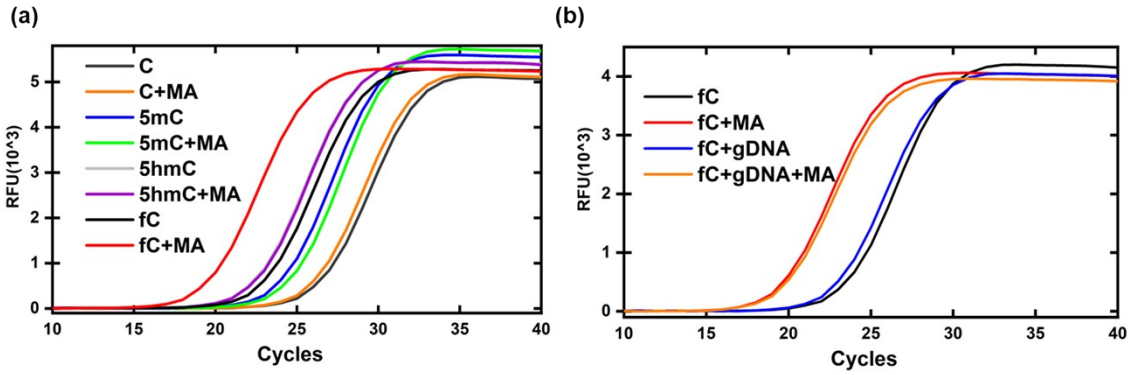

Figure S6. Specificity of elongation-ligation method for 5fC. (a) The qPCR curves of 15-DNA containing a C, 5mC, 5hmC or 5fC with or without MA labelling. (b) The qPCR curves of 15-DNA-fC mixed with genomic DNA of 293T cells with or without MA labelling.

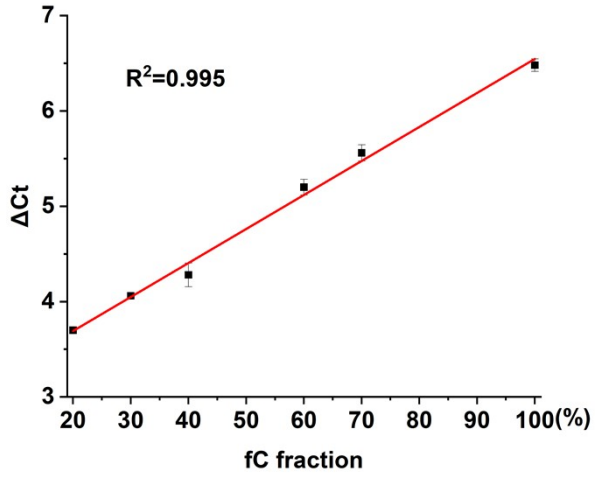

Figure S7. The linear relationship between 5fC fraction and ΔC_t value in elongation-ligation method. The equal concentration (0.1 uM) 15-DNA-C and 15-DNA-fC were mixed with varying molar ratios (10:0, 8:2, 7:3, 6:4, 4:6, 3:7, 0:10) as the fC fractions varied from 0 to 100% with the same final concentration (0.1 uM).

5. Detection and quantification of 5fC in RCA method

A long DNA oligo (named 15-RCA) with an A base at the 3' end and phosphorylation modification at the 5' end complementarily pairs to 15-DNA-fC at the head and tail. The nick of 15-RCA was closed by SplintR ligase after the C-to-T conversion in 15-DNA-fC with MA treatment, forming circular DNA as the template for rolling circle amplification (RCA). Under the catalysis of phi 29 polymerase, 15-DNA-fC as the primer yields abundant DNA products that emit fluorescence after incubation with SYBR GREEN I. Thus, simple RCA-based operations can be performed to detect and quantify 5fC-modified target template DNA. RCA-based method also performed well in terms of specificity towards 5fC and ability to quantify 5fC

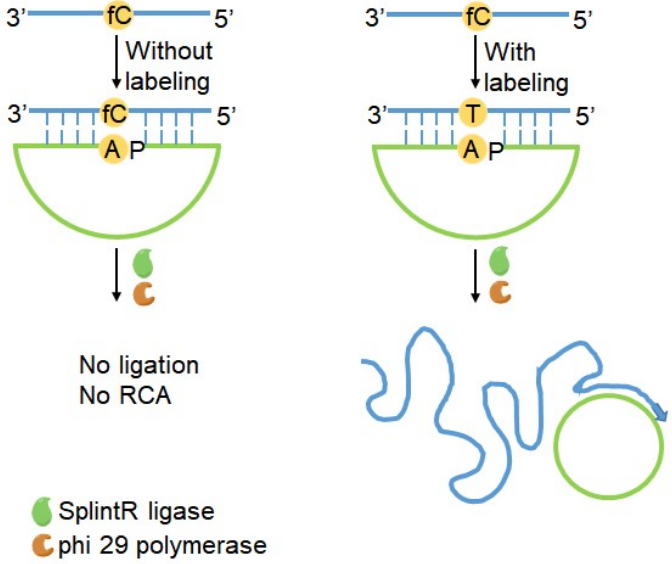

Figure S8. Illustration of 5fC detection based on malononitrile-mediated RCA.

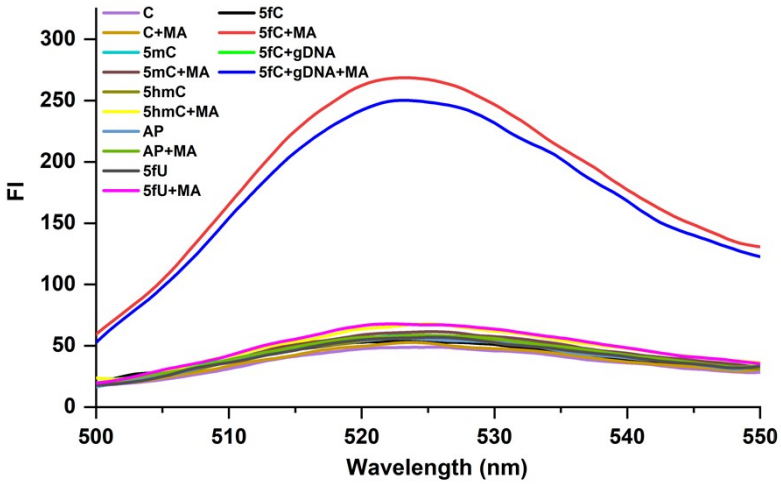

Figure S9. Fluorescence curves of 15-DNA-C, 15-DNA-fC, 15-DNA-5mC, 15-DNA-5hmC，15-DNA-fU, 15-DNA-AP and 15-DNA-fC mixed with 293T cell genomic DNA with or without MA labelling.

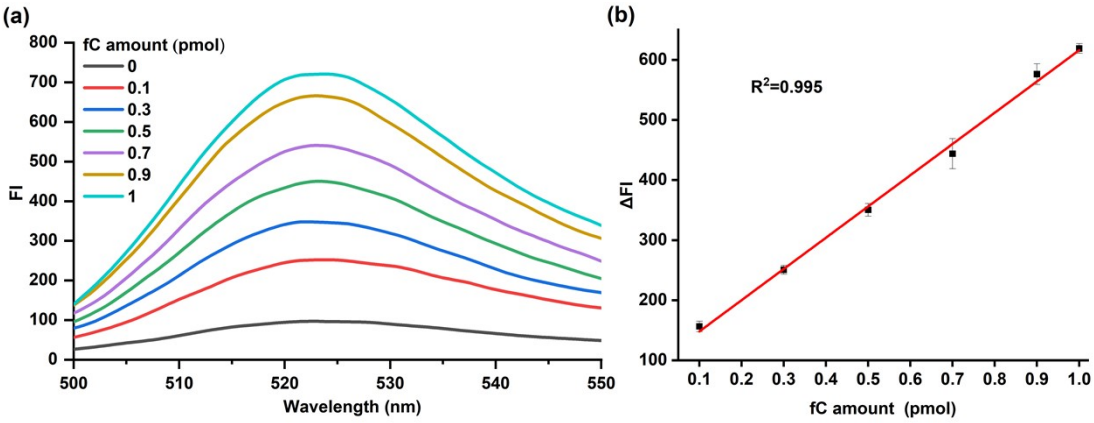

Figure S10. (a) Fluorescence curves of varying amounts of MA-labelled 15-DNA-fC. (b) Linear relationship between the amount of 5fC in the template and the ΔFI value.

6. Evaluating the malononitrile (MA) treatment

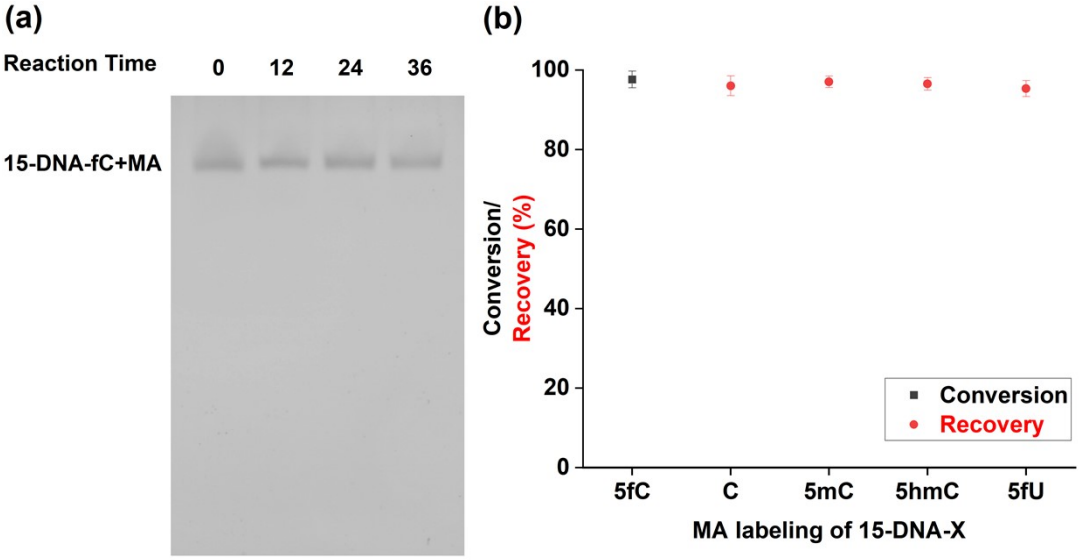

Figure S11. (a)15-DNA-fC were incubated with MA at 37 °C for 12, 24, 36 hours. Polyacrylamide gel electrophoresis analysis showed no noticeable degradation after reaction at different time point. (b)Conversion (for 5fC) and recovery (for C, 5mC, 5hmC and 5fU) of 15-DNA-X after MA treatment