Supporting Information

Targeted Detection Method for Locus-specific m⁶A Modifications in Low-Abundance Transcripts Based on Chemical Conversion

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Materials

RNA oligonucleotides used in this work were synthesized from Accurate Biology and DNA primers used in this work were purchased from Genecreate, the sequences information were listed in Table.

Experimental Procedures

RNA Demethylation

The 100 ng RNA were mixed with 5X FTO reaction buffer(250 mM Tris-HCl, pH 7.5, 5 mM αKG, 5 mM L-Asc), 0.5 μL FTO RNA Demethylase(NEB,M0616S), 2.5 μL 750 μM Fe(Ⅱ) solution and nuclease-free water to 25μL. The reaction is incubated for 2 hour at 37°C. It is imperative that the RNA substrate be EDTA-free. Presence of EDTA will reduce FTO activity.The product RNA is purified using RNA Clean and Concentrator-5 Kit (Zymo Research, R1016).

Sodium nitrite and glyoxal treatment

200 ng RNA with m^6A modification and 200 ng RNA without m^6A modification were added into 50 μL of reaction buffer containing 13 μL glyoxal solution, 10 μL DMSO, 10 μL 5 M $NaNO_2$, 5 μL saturated H_3BO_3 solution, 4 μL 500 mM MES (pH 6.0) and RNase-free water. The mixture was incubated for 10 min at 50°C. RNA was purified by ethanol precipitation. The pellets were dissolved by 50 μL deprotection buffer with 2 mM EDTA. The mixture was incubated at 95°C for 10 min. (20 mL deprotection buffer combined 10 mL 1 M triethylammonium acetate solution (pH 8.6) with 9.5 mL deionized formamide, then add RNase-free water to 20 mL. This solution is dispensed and stored at −20°C, protected from light. EDTA may be added before use at a final concentration of 2 mM).RNA was purified by ethanol precipitation.

Reverse transcription

500 ng RNA sample were mixed with 1.0 μL 10 μM Random primer (Takara, 3801). 1 μL 10 mM dNTP solution mix (Takara, 4019), RNase-free water to 14.5 μL. The reaction was mixed well and incubated at 70°C for 2 min. To this mixture, 4 μL 5X RT Buffer (250 mM Tris-HCl (pH 8.3 at 25 °C), 375 mM KCl, 15 mM $MgCl_2$, 50 mM DTT.), 0.5 μL Ribolock RNase Inhibitor (Vazyme, R301-01) and 1 μL Maxima H Minus Reverse Transcriptase (Thermo, EP0753) were added. The reaction was mixed well and incubated at 50 °C for 1 h, 70°C for 15 min, followed by adding 1 μL RNase H (5 U/μL) (Thermo, EN0202) and incubating at 37°C for 20 min. The reaction mixture was heated at 70°C for 5 min and then the cDNA was purified using oligo Clean and Concentrator (Zymo Research, D4061). The eluted cDNAs (25 μL) were stored at −20°C.

Ligation-based qPCR method

The 1 μL cDNA were mixed with 1.5 μL 0.1 μM Up probe, 1.5 μL 0.1 μM Down probe, 1 μL 10X HiFi Taq DNA ligase reaction buffer and 4 μL RNase-free water. The cDNA and primers were annealed by incubating the mixture at a temperature gradient: 90°C for 1 min, 80°C for 1 min, 70°C for 1 min, 60°C for 1 min, 50°C for 1 min, and then 40°C for 6 min. Subsequently, 1 μL 2 U HiFi Taq DNA ligase (NEB, M0647S) was added to the former mixture. The final reaction mixture was incubated at 70°C for 30 min, denatured at 95°C for 5 min and kept at 12°C.

Afterwards, quantitative realtime PCR (qPCR) reaction was performed in a CFX-96 RealTime

System (Bio-Rad). All qPCR reactions were performed as 20 μL reactions using 2 × SupRealQ Purple Universal SYBR qPCR Master Mix (Vazyme, Q412-02), 1 μL 10 μM qPCR-FP, 1 μL 10 μM qPCR-RP and 1 μL reaction mixture and ddH_2O. qPCR was run under the following condition: 95℃ for 5 min; (95℃ for 10 s; 60℃ for 35 s) × 40 cycles; 95℃ for 15 s; 60℃ for 1 min; 95℃ for 15 s (collect fluorescence at a ramping rate of 0.05℃/s); 4℃ hold.

Cell culture and RNA extraction

HEK293T and HeLa cells were maintained in Dulbecco's modified eagle's medium (DMEM) (High Glucose) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (Beijing Ding-guo changsheng Biotechnology Co., Ltd, GA3502) at 37℃ with 5% CO_2. Total RNA was extracted using VeZol reagent (Vazyme, R411-02) according to the manufacturer's instructions. PolyA-RNA was isolated from total RNA through two rounds of poly(A)+ selection with Oligo(dT)25 magnetic beads (NEB, S1419S).

PCR procedure

The cDNA were mixed with 1 μL 10 μM PCR FP, 1 μL 10 μM PCR RP, 25 μL 2× *TransStart FastPfu* Fly PCR SuperMix(-dye)(Transgene biotech, AS231-01) and 22 μL RNase-free water. The reaction was mixed well and incubated at 98℃ 1 min, (98℃ 10 s, 60℃ 5 s, 72℃ 30 s)× 35 cycles; 72℃ 1 min, 12℃ hold.

Western blot

The protein level of *METTL3* in control and *METTL3*$^{+/-}$ heterozygous HeLa cells were measured by western blotting. In brief, ctrl and *METTL3*$^{+/-}$ cell were collected, mixed with 1×SDS loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.1% bromophonol blue) and then incubated at 95°C for 10 min. After centrifugation, samples were separated by SDS PAGE and transferred from gel to PVDF membrane. Antibody staining was carried out with *METTL3* antibody(Abclonal, A8370) and β-Actin antibody(Proteintech, 20536-1-AP). Finally, membrane was imaged on Molecular Imager ChemiDocTM XRS+ Imaging System (Bio-Rad) after incubation with Rhea ECL (US Everbright, Inc.).

SELECT method

RNA were mixed with 40 nM Up Primer, 40 nM Down Primer and 5 μM dNTP in 17μL 1×CutSmart buffer (50 mM KAc, 20 mM Tris-HAc, 10 mM $MgAc_2$, 100 μg/mL BSA, pH 7.9). The RNA and primers were annealed by incubating mixture at a temperature gradient: 90°C for 1min, 80°C for 1min, 70°C for 1min, 60°C for 1min, 50°C for 1min, and then 40°C for 6min. Subsequently, a 3 μL of mixture containing 0.01 U Bst 2.0 DNA polymerase, 0.5 U SplintR ligase and 10 nmol ATP was added in the former mixture to the final volume 20 μL. The final reaction mixture was incubated at 40°C for 20 min, denatured at 80°C for 20 min and kept at 4°C. Afterwards, quantitative realtime PCR (qPCR) reaction was performed in a CFX-96 RealTime System (Bio-Rad). All qPCR reactions were performed as 20 μL reactions using 2 × SupRealQ Purple Universal SYBR qPCR Master Mix (Vazyme, Q412-02), 1 μL 10 μM qPCR-FP, 1 μL 10 μM qPCR-RP and 2 μL of the final reaction mixture and ddH_2O. qPCR was run under the following condition: 95°C, 5min; (95°C, 10s; 60°C, 35s)×40 cycles; 95°C, 15s; 60°C, 1min; 95°C, 15s (collect fluorescence at a ramping rate of 0.05°C/s); 4°C, hold.

m^6A Dot blot

RNA was isolated from HEK293T cells. After treatment with FTO enzyme， equal amounts of RNA were dropped on a nylon membrane followed by cross-linking under conditions of ultraviolet light at 254 nm, 0.12 J cm^{-2}. The membrane was blocked in 5% BSA (5% BSA in TBST) for 1 h and subsequently incubated with N6-methyladenosine / m^6A Rabbit pAb (Abclonal, A17924) overnight at 4℃. After washing three times in TBST buffer, the membrane was blotted with secondary antibody HRP-conjugated Goat anti-Rabbit IgG (H+L) (Abclonal, AS014) at 37℃ 70rpm for 1 h. The dot blotting signal was visualized after reaction with enhanced chemiluminescence.

HPLC-MS/MS

After being treated by the FTO enzyme, 15 RNA m^6A was purified by ice ethanol precipitation. 50 ng RNA is dissolved in 17 μL RNase-free water, 2 μL Nuclease P1 Buffer and 1 μL Nuclease P1(NEB, M0660S). The mix was incubated at 37℃ 12 h. To this mixture, 2.5 μL 1 M NH_4HCO_3 and 1 μL CIAP(Takara, 2250A) were added. The reaction was mixed well and incubated at 37℃ 2 h. Then the sample were centrifugated at 12000 g for 5 min and diluted supernatant to 50 μL. The HPLC-MS/MS analysis of the digested RNA was carried out on a triple quadrupole mass spectrometer(QTRAP 6500+). Five nucleosides were quantified based on the nucleoside to base transitions: 282.0 to 150.2 (m^6A), 268.1 to 136.0 (rA), 245.0 to 112.9 (rU), 284.2 to 152.1 (rG), 244.1 to 112.2 (rC).

Results and Discussion

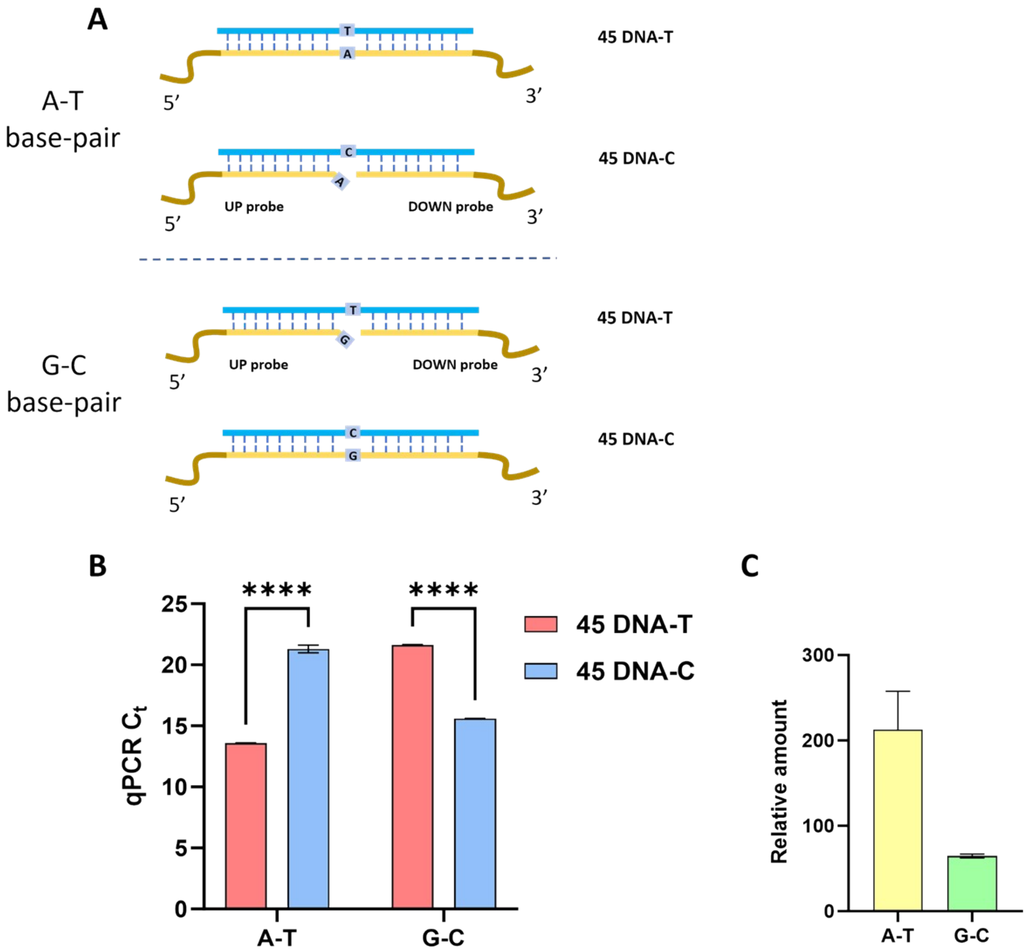

Figure. S1 The detection efficacy differences between the two base-pairing modes (A–T and G–C). **(A)** Schematic diagram of base-pairing. The DNA simulated the cDNA sequence derived from reverse transcription products of sodium nitrite and glyoxal-treated RNA containing either m^6A (represented by 45 DNA-T) and A (represented by 45 DNA-C). **(B-C)** The corresponding cycle threshold differences (left) and calculated relative amount (right). 10 ng DNA was carried out for analysis. Error bars indicate mean ± s.d. for three technical replicates. *P < 0.05; **P < 0.01; ***P < 0.001; ns, non-significant by t-test (two-tailed).

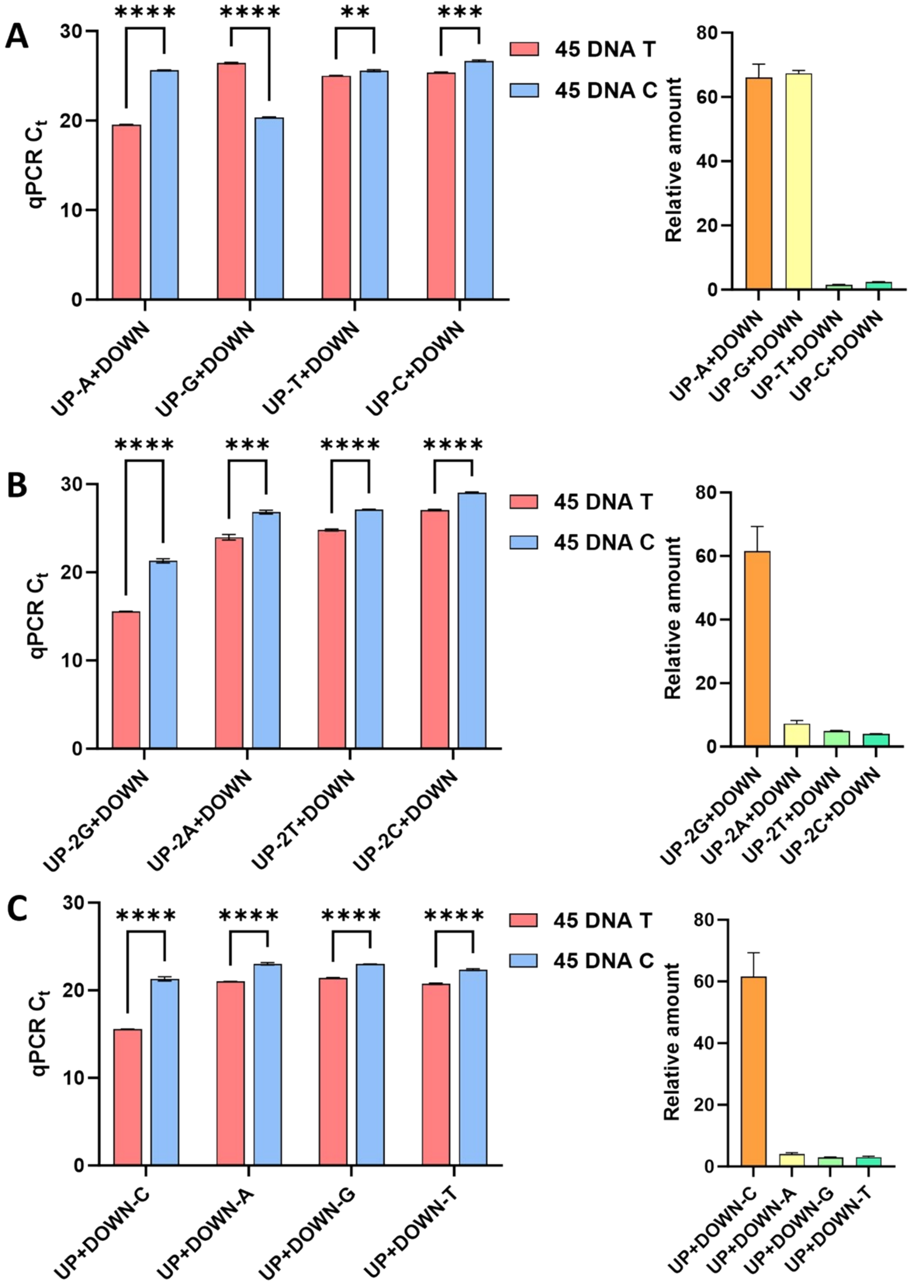

Figure. S2 Impact of terminal base mismatches in UP and DOWN probes on the generation of ligation products. **(A)** Altering the terminal base of the UP probe. **(B)** Altering of the penultimate base in the UP probe. **(C)** Altering the terminal base of the DOWN probe. 10 ng DNA was carried out for analysis. Error bars indicate mean ± s.d. for three technical replicates. *P < 0.05; **P < 0.01; ***P < 0.001; ns, non-significant by t-test (two-tailed).

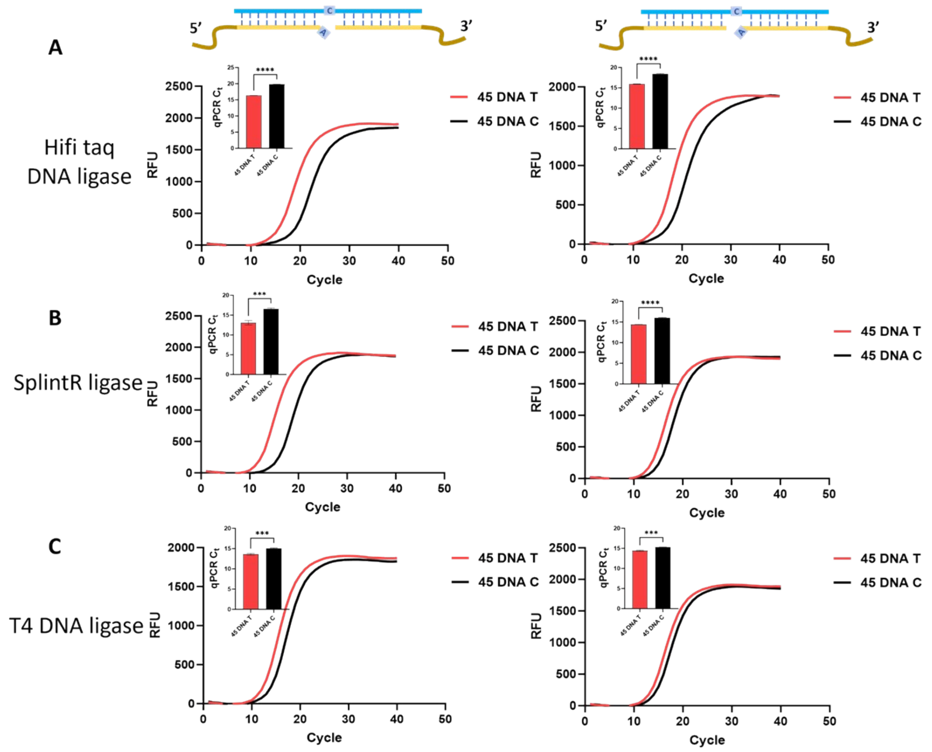

Figure. S3 Comparison of ligation efficiency placing the cognate A at either 3' end of the UP probe or the 5' end of the DOWN probe. Ligation efficiency at the 3' terminus (left) or the 5' terminus (right) of the paired base. **(A)** HiFi Taq DNA Ligase. **(B)** SplintR Ligase. **(C)** T4 DNA ligase. 10 ng DNA was carried out for analysis. Error bars indicate mean ± s.d. for three technical replicates. *P < 0.05; **P < 0.01; ***P < 0.001; ns, non-significant by t-test (two-tailed).

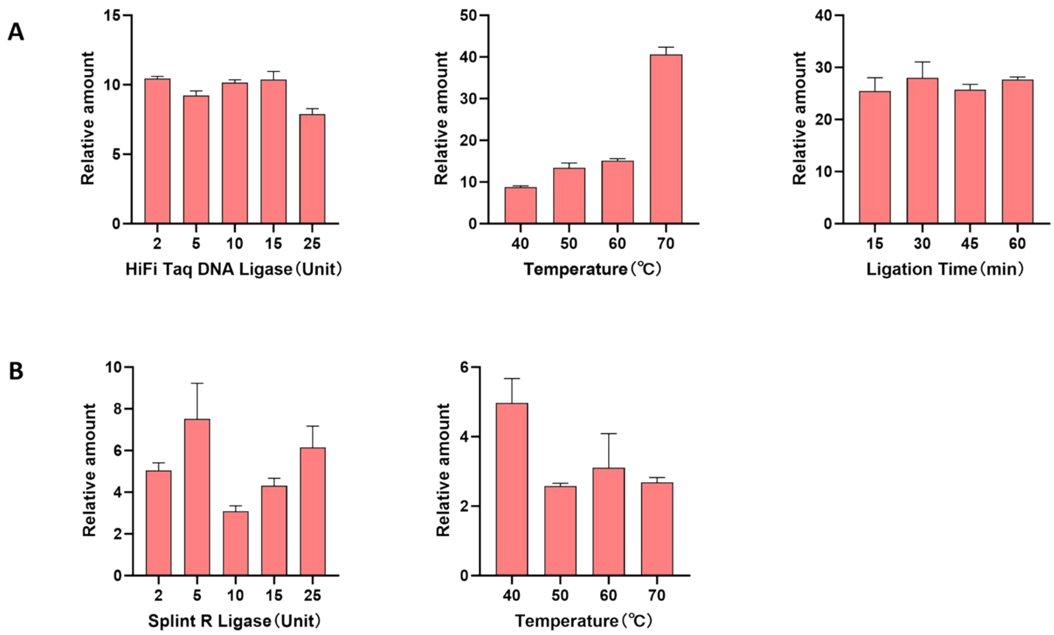

Figure. S4 Preliminary experimental optimization of our method. **(A)** Optimization of the enzyme concentration, ligation temperature, and ligation time for HiFi Taq DNA Ligase. **(B)** Optimization of the enzyme concentration, ligation temperature for SplintR Ligase. 10 ng DNA was carried out for analysis. Error bars indicate mean ± s.d. for three technical replicates.

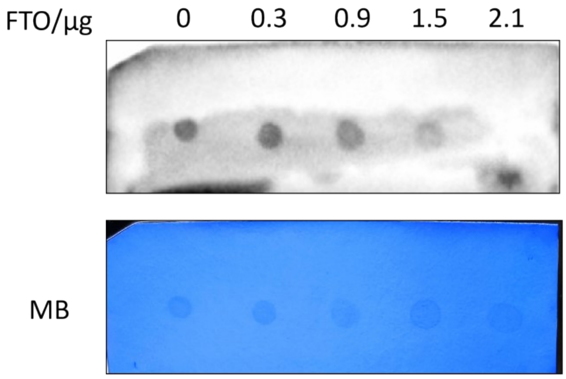

Figure. S5 Investigation of FTO enzyme dosage. Top: Chemiluminescence imaging; Bottom: Methylene blue staining.

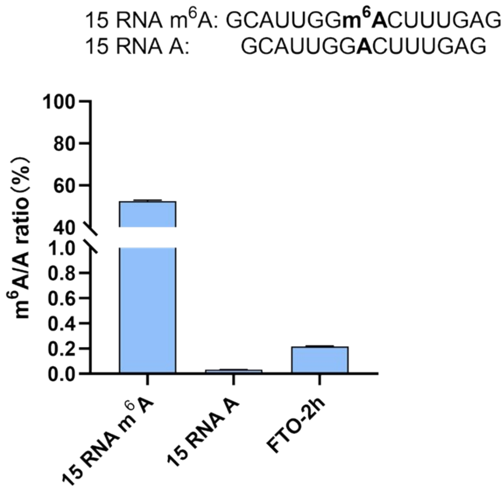

Figure. S6 The level of m^{6}A/A in RNA after FTO treatment. Error bars indicate mean $\pm$ s.d. for three technical replicates.

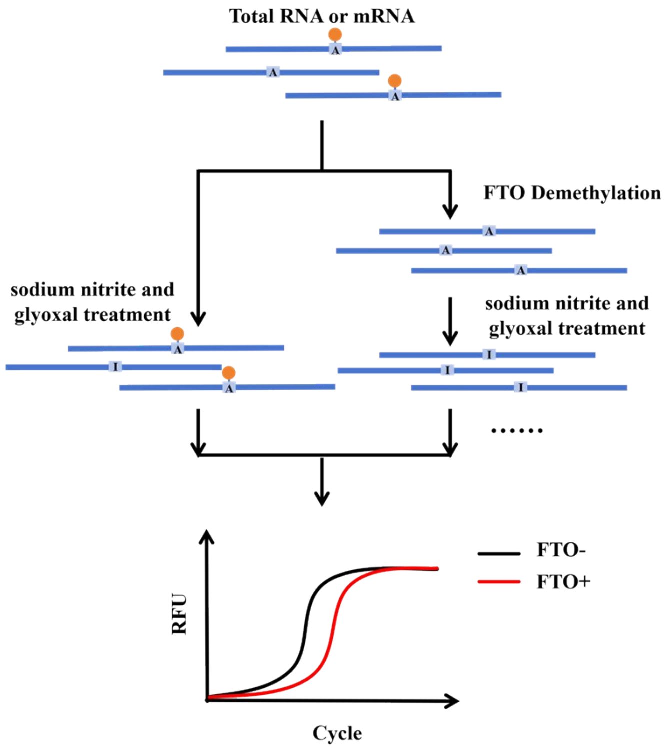

Figure. S7 Schematic diagram of the method for processing RNA samples with FTO enzyme.

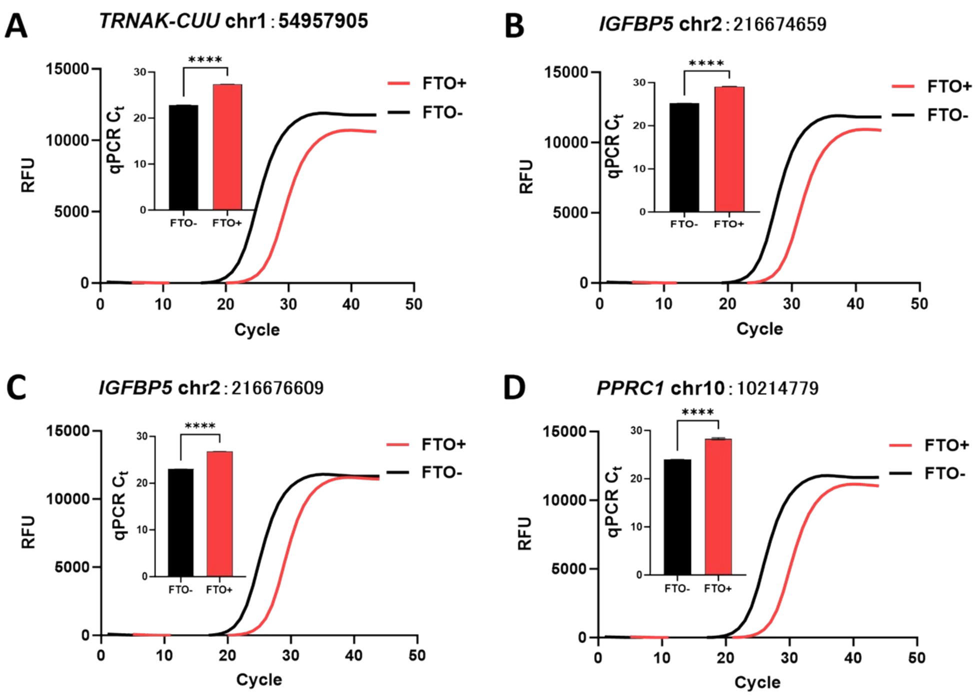

Figure. S8 Validation of reported m⁶A sites on tRNA and mRNA through FTO-mediated demethylation. **(A-D)** Detection results of m⁶A sites presented by real-time amplification curves. Error bars indicate mean ± s.d. for three technical replicates. *P < 0.05; **P < 0.01; ***P < 0.001; ns, non-significant by t-test (two-tailed).

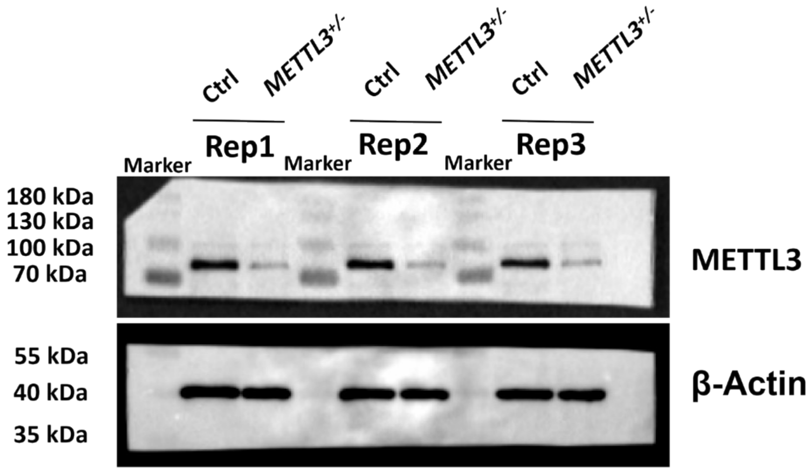

Figure. S9 Full uncropped images of Western blot for Fig. 5A.

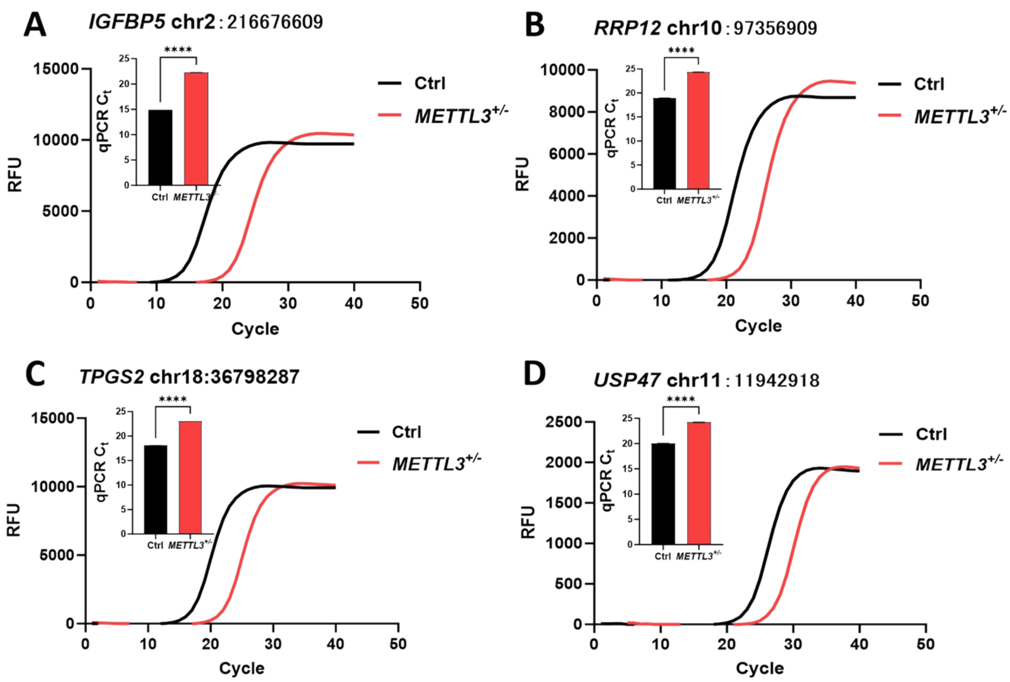

Figure. S10 Detection of m⁶A sites in RNA from *METTL3*$^{+/-}$ HeLa cells and wild-type controls. Error bars indicate mean ± s.d. for three technical replicates. *P < 0.05; **P < 0.01; ***P < 0.001; ns, non-significant by t-test (two-tailed).

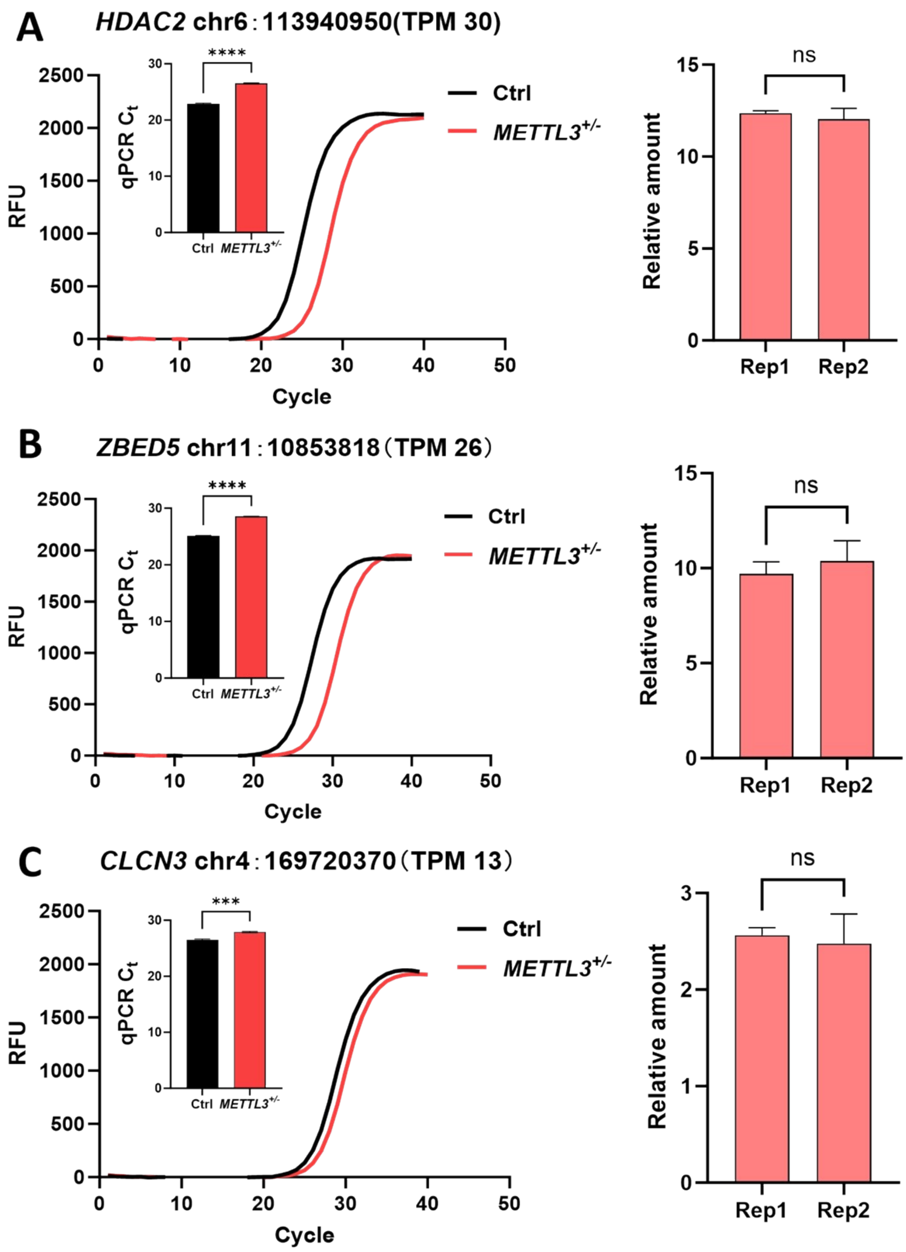

Figure. S11 Detection of m⁶A sites in low-abundance transcripts from RNA of *METTL3*$^{+/-}$ HeLa cells. **(A-C)** Real-time fluorescence amplification curves and calculated relative amount showed the detection results of mRNA. Error bars indicate mean ± s.d. for three technical replicates. Each experiment was replicated twice (Rep1 and Rep2). *P < 0.05; **P < 0.01; ***P < 0.001; ns, non-significant by t-test (two-tailed).

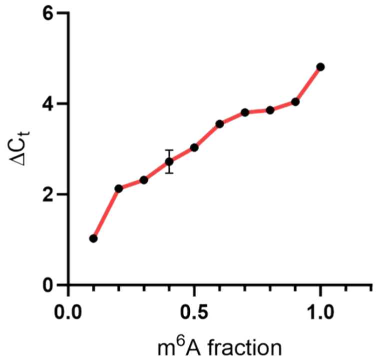

Figure. S12 A series with 0.31 fmol of the standard RNA mixture with a known m^6A fraction obtained by mixing 45 RNA m^6A with 45 RNA A were subjected for our method. Error bars indicate mean ± s.d. for three technical replicates.

The sequences used for this study

Table S1. Oligo templates and qPCR primers

Name	sequence(5'-3')
45 DNA T	GAACCACCCCCGCCACGAAAGTCCCCCACACCCCCCAAACCAAGC
45 DNA C	GAACCACCCCCGCCACGAAAGCCCCCCACACCCCCCAAACCAAGC
45 RNA A	GCUUAGUUUGAAAAAUGUGAAGGACUUUCGUAACGGAAGUAAUUC
45 RNA m^6A	GCUUAGUUUGAAAAAUGUGAAGGm^6**A**CUUUCGUAACGGAAGUAAUUC
15 RNA m^6A	GCAUUGGm^6**A**CUUUGAG
15 RNA A	GCAUUGGACUUUGAG
UP A(UP-2G)	GTTCAGAGTTCTACAGTCCGACGATCGCTTGGTTTGGGGGGTGTGGGGGA
DOWN C	CTTTCGTGGCGGGGGTGGTTCACACGACGCTCTTCCGATCT
5 UP	GTTCAGAGTTCTACAGTCCGACGATCGCTTGGTTTGGGGGGTGTGGGGG
5 DOWN	ACTTTCGTGGCGGGGGTGGTTCACACGACGCTCTTCCGATCT
GLORI 2515 PCR FP	GCTTGGTTTGGGGGGTGT
GLORI 2515 PCR RP	GAACCACCCCCGCCAC
qPCR FP	AGATCGGAAGAGCGTCGTGT
qPCR RP	GTTCAGAGTTCTACAGTCCGACGATC
GAPDH-qPCR-FP	CGCACCACCAACCGCCCAGC
GAPDH-qPCR-RP	GGCGTGGGCTGTGGTCGTGGG
UP-T	GTTCAGAGTTCTACAGTCCGACGATCGCTTGGTTTGGGGGGTGTGGGGGT
UP-G	GTTCAGAGTTCTACAGTCCGACGATCGCTTGGTTTGGGGGGTGTGGGGGG
UP-C	GTTCAGAGTTCTACAGTCCGACGATCGCTTGGTTTGGGGGGTGTGGGGGC
UP-2A	GTTCAGAGTTCTACAGTCCGACGATCGCTTGGTTTGGGGGGTGTGGGGAA
UP-2T	GTTCAGAGTTCTACAGTCCGACGATCGCTTGGTTTGGGGGGTGTGGGGTA
UP-2C	GTTCAGAGTTCTACAGTCCGACGATCGCTTGGTTTGGGGGGTGTGGGGCA
DOWN-A	ATTTCGTGGCGGGGGTGGTTCACACGACGCTCTTCCGATCT
DOWN-T	TTTTCGTGGCGGGGGTGGTTCACACGACGCTCTTCCGATCT
DOWN-G	GTTTCGTGGCGGGGGTGGTTCACACGACGCTCTTCCGATCT

Table S2. UP and DOWN probes at each site after chemical treatment

Name	sequence(5'-3')
GLORI 4190 UP	GTTCAGAGTTCTACAGTCCGACGATCGGCGGTGCGCCTGTCGGGCGGTGA
GLORI 4190 DOWN	CGCGGGTGTCCTGGGGCGGGCTACACGACGCTCTTCCGATCT
GLORI 2577 UP	GTTCAGAGTTCTACAGTCCGACGATCCCGGCTTGGTGTTTTTGCGTTGGA
GLORI 2577 DOWN	CTTTGGGTTGGGGTTGTTTTTACACGACGCTCTTCCGATCT
GLORI A2511 UP	GTTCAGAGTTCTACAGTCCGACGATCGGTGGCTTGGTTTGGGGGGTGTGA
GLORI A2511 DOWN	GGGGCTTTCGTGGCGGGGGTGGTTCACACGACGCTCTTCCGATCT
GLORI 18S A1835 UP	GTTCAGAGTTCTACAGTCCGACGATCGGGGTGGGGGTCGTGACGG
GLORI 18S A1835 DOWN	GGTTTCCGTGGGTGGGCCTACACGACGCTCTTCCGATCT
GLORI A4194 UP	GTTCAGAGTTCTACAGTCCGACGATCCGGTGCGCCTGTCGGGCGGTGGCGCA
GLORI A4194 DOWN	GGTGTCCTGGGGCGGGCTACACGACGCTCTTCCGATCT
GLORI *IGFBP5*-2 UP	GTTCAGAGTTCTACAGTCCGACGATCCTGTCTGTTTGGGGGGGCTGGGGA
GLORI *IGFBP5*-2 DOWN	CCTCGGGGTCTCTGGCGGGACACGACGCTCTTCCGATCT

GLORI *IGFBP5*-1 UP	GTTCAGAGTTCTACAGTCCGACGATCTCGGCCGGTGGGTGGCCTTGTGA
GLORI *IGFBP5*-1 DOWN	CTGTTCTTTGGGGCTGGGGGTGTCCACACGACGCTCTTCCGATCT
GLORI *PPRC1* UP	GTTCAGAGTTCTACAGTCCGACGATCGCGTGTGTGTCTGCTGGGGGTGTGA
GLORI *PPRC1* DOWN	CTGTTGGGGGGCCTGCGTCACACGACGCTCTTCCGATCT
GLORI *TRNAK-CUU* UP	GTTCAGAGTTCTACAGTCCGACGATCGTCGGTGGGGCGTGGGGCTCTTA
GLORI *TRNAK-CUU* DOWN	GTCTCGGGGTCGTGGGTTTGGGCACACGACGCTCTTCCGATCT
GLORI *xist* UP	GTTCAGAGTTCTACAGTCCGACGATCGTGGGGGGCCCGGGGTGTGGGA
GLORI *xist* DOWN	CGGGTGGTTTGGGGCTTGGGGCACACGACGCTCTTCCGATCT
GLORI *RRP12* UP	GTTCAGAGTTCTACAGTCCGACGATCCCGGGTGGGGTCGCCCTTCGGGGA
GLORI *RRP12* DOWN	CGTCCCTGGTGCCTGGGGGTGGGACACGACGCTCTTCCGATCT
GLORI *TPGS2* UP	GTTCAGAGTTCTACAGTCCGACGATCCGGGGCGGGTGGTTCCGGTGGA
GLORI *TPGS2* DOWN	CGGCCTCGCTTTGCTCGTCTGGTTGACACGACGCTCTTCCGATCT
GLORI *USP47* UP	GTTCAGAGTTCTACAGTCCGACGATCCTCGGGTCCCTTTGGCTGGTGGGCTTGA
GLORI *USP47* DOWN	CTCTCGCGGTGTCGCGGGTGGACACGACGCTCTTCCGATCT
GLORI *HDAC2* UP	GTTCAGAGTTCTACAGTCCGACGATCTTCGTTTTGTGCTGCTTTGGCGTGGA
GLORI *HDAC2* DOWN	CTGTGTTTGTTTTCGGGTGGCTTTTTCGACACGACGCTCTTCCGATCT
GLORI *CLCN3* UP	GTTCAGAGTTCTACAGTCCGACGATCTGCTCCGGCGTTGCGGGGA
GLORI *CLCN3* DOWN	CGCGTTGTCGGTCCCTGTTTCTGGACACGACGCTCTTCCGATCT
GLORI *ZBED5* UP	GTTCAGAGTTCTACAGTCCGACGATCGGTGGTCGCTGCCTGTTGTGCGGA
GLORI *ZBED5* DOWN	CGTGCGCTGGCGGTTGGGGACACGACGCTCTTCCGATCT
SELECT 2515 UP	GTTCAGAGTTCTACAGTCCGACGATCGAATTACTTCCGTTACGAAAG
SELECT 2515 DOWN	CCTTCACATTTTTCAAACTAAGCACACGACGCTCTTCCGATCT
SELECT *CLCN3* UP	GTTCAGAGTTCTACAGTCCGACGATCCCCTCTAGAAATAGGGACTGATAATGTG
SELECT *CLCN3* DOWN	CTTTGCAATGTTGGAGCAACAGACACGACGCTCTTCCGATCT
SELECT *CLCN3*+ UP	GTTCAGAGTTCTACAGTCCGACGATCAGTAATCCCTCTAGAAATAGGGACTGATAA
SELECT *CLCN3*+ DOWN	GTGTCTTTGCAATGTTGGAGCAAACACGACGCTCTTCCGATCT
2515 Pair UP	ACTCTTGATCTTGAATTACTTCCGTTACGAAA
2515 Pair DOWN	CTTCACATTTTTCAAACTAAGCTACTATATTTAAGGCCTT

Table S3. Internal reference primers at each site

Name	sequence(5'-3')
4194 FP	GGCGGTACACCTGTCAA
4194 RP	AGCTCGCCTTAGGACACC
2511 FP	AGTAGCTTAGTTTGAAAAATGTGAAG
2511 RP	TCTTGAATTACTTCCGTTACGA
PPRC1 FP	GTGTGTCTGCTGAAGGTGTG
PPRC1 RP	CTCTGATGCAGGCTCCTCA
USP47 FP	CAGATCCCTTTGGCTAATGGAC
USP47 RP	CGTTCTTCTACTACTTGTGATACTGTG
TRNAK-CUU FP	CGGTAGAGCATGAGACTCTTA
TRNAK-CUU RP	CTCAAACCCATGACCCTGA
IGFBP5-1 FP	AACCAATGGGTGGCCTT
IGFBP5-1 RP	CCTGGATATCTTCAGCCTTAAAGAA

IGFBP5-2 FP	CTATCTATTTGAGGAAACTGAGGACC
IGFBP5-2 RP	GCCCTTGCTAGAGATTCCG
xist FP	GGATAGAAGGCCCAAAGTATAAG
xist RP	GGTCTCAAGTCTCAAACCATCT
RRP12 FP	CCAAATGGAATCACCCTTCAGA
RRP12 RP	TCTCCAGGCACCAGGG
TPGS2 FP	AGCAAGACAGATGATTCCAATGG
TPGS2 RP	CCACTTAACTAGATGAGTAAAGTGAGG
HDAC2 FP	AAGACTTCTGGCTTCATTTTATACTACT
HDAC2 RP	CGAAAAAGCCATTTGAAAATAAATACAG
CLCN3 FP	CTGTTGCTCCAACATTGCA
CLCN3 RP	GTAATCCCTCTAGAAATAGGGACTG
ZBED5 FP	AGCACCAGTAGTCACTGC
ZBED5 RP	TAGGCATTATTTTAACTGCCAGTG
CLCN3 PCR FP	GTTGCTCCGGCGTTG
CLCN3 PCR RP	CCCCAGAAACAGGGACC