Electronic Supplementary Information (ESI)

Xeno nucleic acid probes mediated methylation-specific PCR for single-base resolution analysis of N^6-methyladenosine in RNAs

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Experimental section

Isolation of total RNA from cells

Human cervical cancer cell line Hela (CCL-2), embryonic kidney cell line HEK293T (CRL-11268) were cultured in DMEM medium (GBICO, Cat.12100-046) supplemented with 10% (v/v) fetal bovine serum (HyClone), 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were maintained at 37 °C under a humidified atmosphere containing 5% CO_2. The DMEM medium was removed from the petri dish while the bottom of the petri dish was filled of cells, after that the cells were washed three times with cold PBS buffer (10 mM sodium phosphate buffer, 0.1 M NaCl, pH 7.4). Take about 5×10^6 cells, centrifuged at 3000 rpm for 5 min, remove the supernatant completely, then 350 μL RLT solution was added in and mixed well, homogenized for 30 sec by homogenizer. 1/2 volume of anhydrous ethanol was added to the cracked sample and mixed well. Put the adsorption column into the collection pipe, all the solution was added into the adsorption column and stilled for 2 minutes, then centrifuged at 8000 rpm for 1 min. The adsorption column was put into the

recovery header, then 500 μL RW solution was added in and stilled for 1 min, centrifuged at 10000 rpm for 1 min. The adsorption column was put into the recovery header, 500 μL RPE solution was added in, allow it to stand for 2 min, and centrifuged it at 10000 rpm for 1 min, put the adsorption column into the recovery header and centrifuged at 10000 rpm for 2 min. The precipitate was washed once with 75% ethanol and dissolved with DEPC-treated water. Finally, the concentration of total RNA was determined with the Nano Drop 1000 (Thermo Scientific), and stored at -80 °C for subsequent tests.

Extraction of polyA-RNA from total RNA

The structure of most lncRNA is similar to that of mRNA containing a polyA tail. The m6A to be detected is located in MALAT1 lncRNA, ACTB and TPT1 mRNA can be obtained by the extraction of polyA-RNAs. 5' ends phosphate modified oligos binding on Fe_3O_4@UiO-66-NH_4. Firstly, 500 μL Fe_3O_4@UiO-66-NH_4 and 5 μL phosphate modified oligos (capture probe 10 nM, Close probe 50 nM) were added into the tube. Then the tube was placed at 4 °C for 4 h with slight shaking, and thus the phosphate modified oligos bound on Fe_3O_4@UiO-66-NH_4. The free phosphate modified oligos was washed out by washing buffer (10 mM Tris-HCl, 150 mM NaCl). Finally, 500 μL 2 × binding buffer (300 mM KCl, 3 mM $MgCl_2$, 20 mM Tris-HCl) was added into the tube.

Hybridization with polyA-RNA: The magnetic beads and total RNA (the volume was 500 μL) were heated at 85 °C for 3 min respectively, after that the magnetic beads were transferred into the total RNA and the mixture was heated at 85 °C for another 3 min. Next the mixture was shaken for 30 min at room temperature, during that time the polyA region present in polyA-RNA was hybridized with capture probe. Elution of polyA-RNA: The magnetic beads were washed three times with washing buffer. Then the polyA-RNAs attached to the magnetic beads were eluted by DEPC-treated water. The concentration of polyA-RNAs were determined with the Nano Drop 1000 (Thermo Scientific) and then stored at -80 °C.

FTO-mediated m6A demethylation reaction

Total RNA or polyA-RNA was treated with FTO protein following previously reported method[1]. In brief, 50 μg total RNA or 2 μg polyA-RNA were mixed with FTO, 50 mM HEPES (pH 7.0), 2 mM L-ascorbic acid, 300 μM α-KG, 280 μM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ and 0.2 U/μL RiboLock RNase inhibitor (ThermoFisher Scientific) to react at 37 °C for 1 h. Reaction quenched by adding 20 mM EDTA. Note that for control (FTO-EDTA), 20 mM EDTA was added prior to demethylation reaction. After 95 °C inactivation for 5 min, followed by extraction with phenol/chloroform and ethanol precipitation. Subsequently RNA was quantified by Qubit (Thermo Fisher Scientific) with Qubit™ RNA HS Assay Kit (Thermo Fisher Scientific) and subject to the next reaction.

RT-qPCR amplification reaction and real-time fluorescence measurement

The RT-qPCR reaction was carried out with a One Step Real-Time PCR System (Applied Biosystems, USA), 20 μL reaction mixture contained reverse transcription primer, PCR forward primer, reverse primer, (each concentration was 200 nM), 50 fM XNA probes, and PCR buffer (100 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.001% (w/v) gelatin, 250 μM dNTPs, 0.4 × Super Green I, 0.5 U JumpStart™ Taq DNA Polymerase). The thermal cycling conditions were as follows: 55 °C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s and 4 °C-∞. The real-time fluorescence intensity was monitored at 60 °C.

Synthesis of Fe_3O_4 nanoparticles.

Fe_3O_4 nanoparticles were synthesized according to a literature method with slight modifications[2]. 2 g of $FeCl_3 \cdot 6H_2O$ was completely dissolved in 20 mL of ethylene glycol to form a uniform solution under ultrasonic and vigorous stirring. Then, 20 mL diethylene glycol was added into the mixture and stirred until well mixed. 3 g sodium acetate trihydrate and 2 g Poly (ethylene glycol) were added in and ultrasonicated for half an hour to fully mix and then sealed in a teflon-lined stainless-steel autoclave (30 mL capacity). The autoclave was heated at 200 °C for 6 h and then allowed to cool to room temperature. The black products were separated magnetically and washed five times with ethanol and de-ionized water to eliminate organic and inorganic impurities

before dispersing in 50 mL DMF for next experimentation.

Synthesis of Fe_3O_4@UIO-66-NH_2 core-shell composites.

5 mL of as prepared Fe_3O_4 was exchanged into 20 mL N, N-dimethyllformamide (DMF) through three repeated magnetic separation and re-dispersion in a round-bottom flask. After that, $ZrOCl_2 \cdot 8H_2O$ (80 mg), 2-aminoterephthalic acid (NH_2-BDC, 50 mg) and 2.5 mL glacial acetic acid were added successively. The mixture was mechanically stirred at 120 °C for 12 h. The brown products (Fe_3O_4@ UIO-66-NH_2) were magnetic separation and washed several times with DMF and ethanol successively. The purified Fe_3O_4@UIO-66-NH_2 core-shell composites were dispersed in phosphate buffer solution (PBS) and stored at 4 °C for next tests.

Table S1. List of the oligonucleotides used in this study.

oligonucleotides	**Sequences (from 5' to 3')**
Oligo1	UAAUUCAAGAUCAAGAGUAAUUACCAACUUAAUG UUUUUGCAUUGGX(X=m6A or A)CUUUGAGUUAAGA
XNA-probe1	TCTTAACTCAAAGT+C/NH_2
XNA-probe2	TCTTAACTCAAA+GT+C/NH_2
XNA-probe3	TCTTAACTCA+AA+GT+C/NH_2
XNA-probe4	TCTTAACT+CA+AA+GT+C/NH_2
XNA-probe5	TCTTAA+CT+CA+AA+GT+C/NH_2
RT-primer1	TCTTAACTCAAAGTCCAATG
RT-primer2	TCTTAACTCAAAGTCCAATGC
RT-primer3	TCTTAACTCAAAGTCCAATGCA
RT-primer4	TCTTAACTCAAAGTCCAATGCAA
RT-primer5	TCTTAACTCAAAGTCCAATGCAAA
qPCR-R1	TCTTAACTCAAAGTCCAATGCA
qPCR-F1	TAATTCAAGATCAAGAGTAATTA
Oligo2	UAAUUCAAGAUCAAGAGUAAUUACCAACUUAAUG UUUUUGCAUUAA X(X=m6A or A)CUUUGAGUUAAGA
Oligo3	UAAUUCAAGAUCAAGAGUAAUUACCAACUUAAUG UUUUUGCAUUGG X(X=m6A or A)CCUUGAGUUAAGA
Oligo4	TAGTTTGAAAAATGTGAAGGACTTTCGTAACGGAA GTAATTCAAGATCAAGAGTAATTACCAACTTAATGT TTTTGCATTGG X(X=m6A or A)CTTTGAGTTAAGA
qPCR-F2	TAGTTTGAAAAATGTGAAGGAC

MALAT1-m6A-2515:	XNA : CCGTTAC+GA+AA+GT+C/NH_2
	qPCR-R: CCGTTACGAAAGTCCTTCACA
	qPCR-F: GGCTTTTGGAAGAGTTAGAAGAA
	T7-2515-m6A-R(T7-2511-m6A-R):TAAGCTACTATATTTAAGGCCTTCATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC
MALAT1-A-2511:	XNA: TTACGAA+AG+TC+CT+T/NH_2
	qPCR-R : TTACGAAAGTCCTTCACATTTTTC
	qPCR-F: GGCTTTTGGAAGAGTTAGAAGAA
ACTB-m6A-1216:	XNA: ACTAAGT+CA+TA+GT+C/NH_2
	qPCR-R: ACTAAGTCATAGTCCGCCTAGA
	qPCR-F: CAGCAAGCAGGAGTATGACGA
	T7-1216-m6A-R:GTCCGGCCCCTCCATCGTCCACCGATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC
TPT1-m6A-687:	XNA: ATTTGTC+TT+AA+GT+C/NH_2
	qPCR-R: ATTTGTCTTAAGTCCTGGTGT
	qPCR-F: ATGTTAACAAATGTGGCAATTAT
	T7-687-m6A-R(T7-703-m6A-R):AGTTATGATGACAGGTGATAGATCATCTACACTTAGTAGAAATTACCCTATAGTGAGTCGTATTAATTTC
TPT1-A-703:	XNA: AGATGAC+AT+CA+GT+C/NH_2
	qPCR-R: AGATGACATCAGTCCCATTTGT
	qPCR-F: ATGTTAACAAATGTGGCAATTAT
MALAT1-Capture probe	Phos/TTTTTTTTTTTTTTTTTTTTTCCCCAATCAAGATTTTTTT
ACTB- Capture probe	Phos/TTTTTTTTTTTTTTTTCATTTTTAAGGTGTGCACTTTTAT
TPT1- Capture probe	Phos/TTTTTTTTTTTTTTTTTATCAGTGTTCTCTCAAGT

	TGGTT
Close probe	Phos/TTTTTTTTTTTTTTT

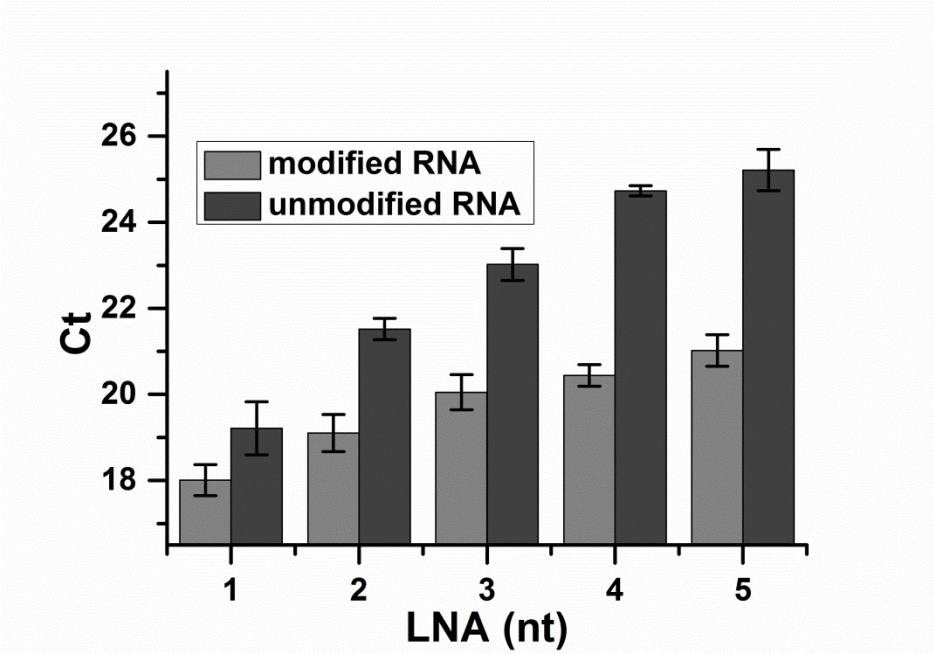

Fig S1. Optimization of the number of LNA bases. Error bars, mean ± s.d. for 3 biological replicates × 2 technical replicates.

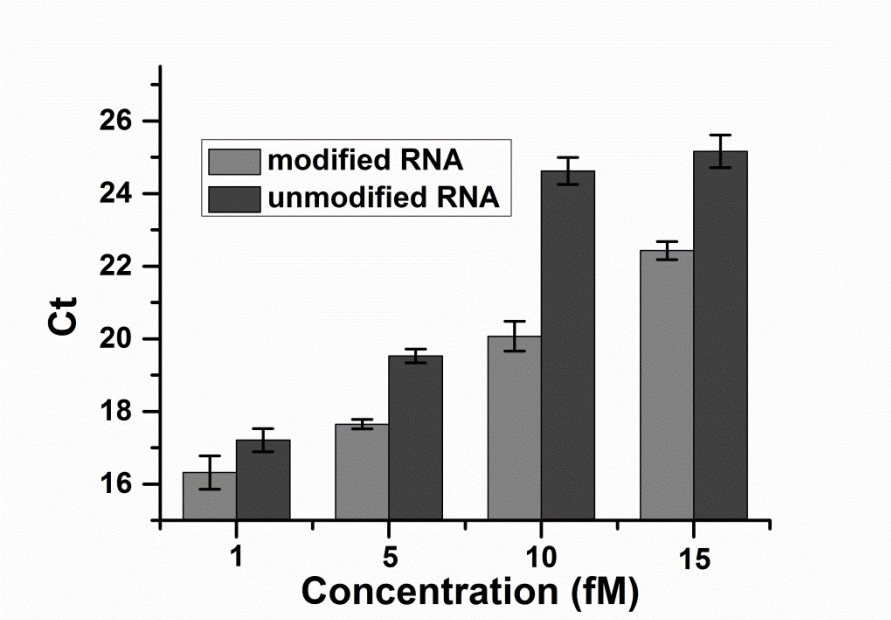

Fig S2. Optimization of the concentration of XNA probe. Error bars, mean ± s.d. for 3 biological replicates × 2 technical replicates.

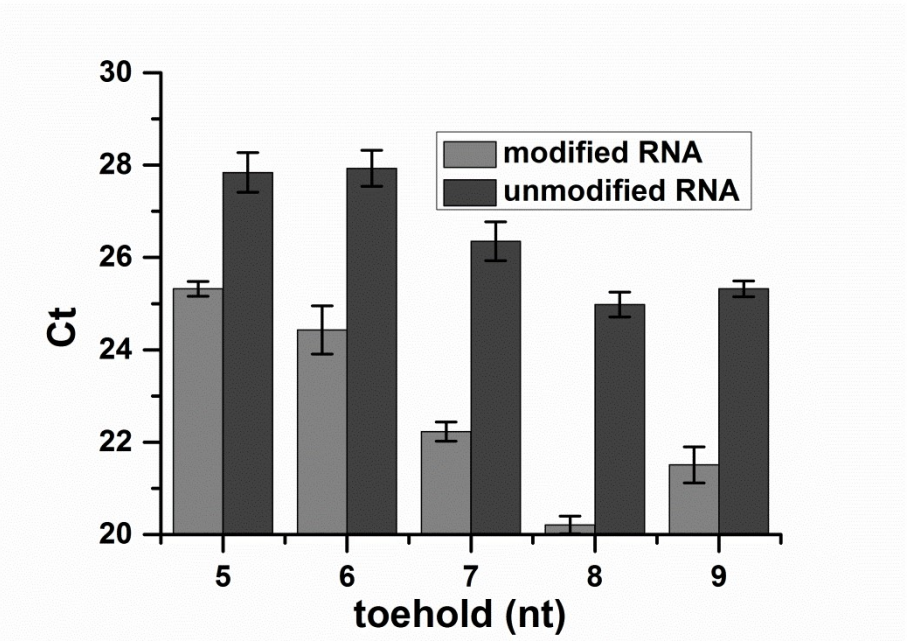

Fig S3. Optimization of the toehold length of RT primers. Error bars, mean ± s.d. for 3 biological replicates × 2 technical replicates.

Table S2. Reproducibility results of three different m6A fraction samples.

Samples	m6A-RNA/A-RNA	measured	RSD (%)
1	0.8	0.81	3.25
2	0.6	0.58	2.71
3	0.2	0.23	4.69

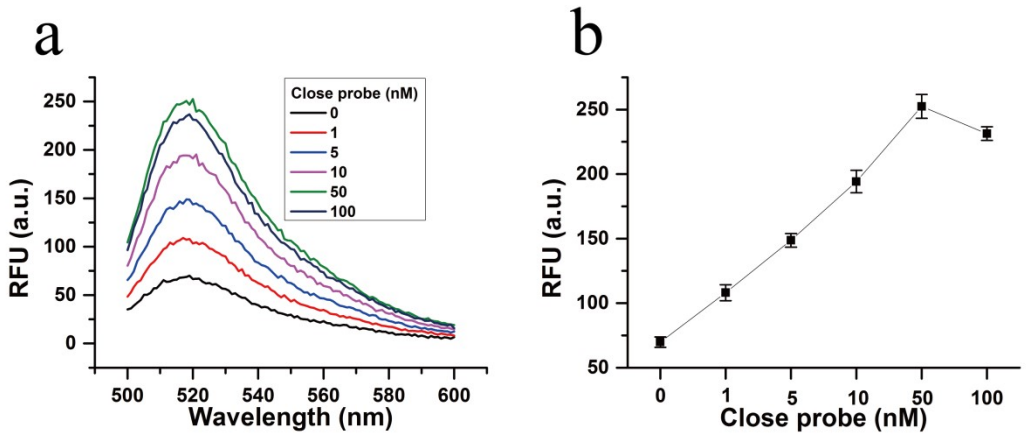

Fig S4. Optimization of the proportion of the capture probe and close probe. (a) Fluorescence emission spectra in response to two different concentrations of probe, the 3' end of the capture probe was labeled FAM fluorescence, and the concentration

of capture probe is 10 nM, and the concentration of close probe is 0~100 nM. (b) The effect of the concentration of close probe on the value of spectra.

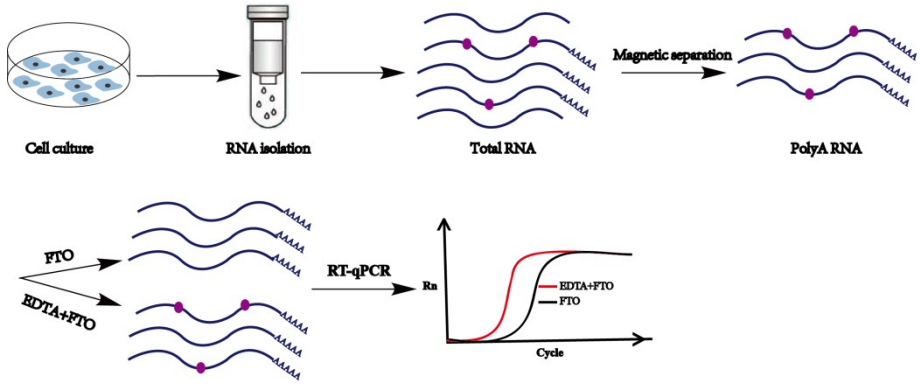

Fig S5. Schematic of the combination of MsRT-qPCR assay with an FTO-assisted demethylation step to identify the m6A sites in cell RNA samples.

References

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2 S. Yang, Y. Guo, J. Fan, Y. Yang, C. Zuo, S. Bai, S. Sheng, J. Li and G. Xie, *Mikrochim Acta*, 2020, **187**, 188-197.