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Electronic Supplementary Information

Double blocking gap-filling-ligation coupled with cascade isothermal amplification for ultrasensitive quantifying N^6-methyladenosine

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1. Materials and reagents

RNA-A and RNA-m⁶A segments were synthesized by Integrated DNA Technologies (America). DEPC-treated water, RNAiso (lysis buffer), dTTP, UTP, dNTPs, padlock probe, SLP, FIP, and BIP were obtained from TaKaRa Biotechnology Co. Ltd. (Dalian, China). T3 DNA Ligase and Bst DNA Polymerase (Large Fragment) were purchased from New England Biolabs (Beijing, China). PolyATract® mRNA Isolation System III was purchased from Promega (Beijing) Biotech Co., Ltd. SYBR Green I was purchased from Xiamen Zeesan Biotech Co., Ltd. (Xiamen, China). Trichloromethane, isopropyl alcohol, and ethanol were purchased from Sinopharm Chemical Reagent Co. Ltd. (Beijing, China). All the solutions for reactions were prepared with DEPC-treated water and the sequences of synthetic RNA and DNA used in this study are listed in Table S1.

Table S1 The sequences of the synthetic RNA and DNA used in this study.

Name	Sequence (5'-3')
RNA-A	CUUAAUGUUUUUGCAUUGGACUUUGAGUUAAGAUUAUUUUUUAAAU 2577th site 2583rd site CCUGAGGACUAGCAUUAAUUGACAGCUG
RNA-m^6A	CUUAAUGUUUUUGCAUUGGm^6ACUUUGAGUUAAGAUUAUUUUUUAA 2577th site 2583rd site AUCCUGAGGACUAGCAUUAAUUGACAGCUG
padlock probe for 2577th site	po_4CCAATGCAAAAACATTAATTTTCGACACGACACGAAAAAGGAACTC TGCTCGACGGATTAAAATAATACAGTCTGCCCACAACCTTTTAATAAT CTTAACTCAAAG
padlock probe for 2583rd site	po_4CAAAGTCCAATGCAAAAATTTTCGACACGACACGAAAAAGGAACTC TGCTCGACGGATTAAAATAATACAGTCTGCCCACAACCTTTTAAAAA ATAATCTTAAC
padlock probe-U	po_4CCAATGCAAAAACATTAATTTTCGACACGACACGAAAAAGGAACTC TGCTCGACGGATTAAAATAATACAGTCTGCCCACAACCTTTTAATAAT CTTAACTCAAAGU
SLP	ATCGTCGTGACTGTTTTCCCTAACCCTAACCCTAACCCTTTTCAGTCACG ACGATTTTTTAATACAGTCTGCCCACAACC
FIP	CGACACGACACGATTTTAATCCGTCGAGCAGAGTTCC
BIP	ATCGTCGTGACTGTTTTCCCTAACCCTAACCCTAACCC

Note: The 'po_4' indicates as phosphate group.

2. Extraction of poly $^{A+}$ RNA from HeLa (HEK293T) cell

HeLa or HEK293T cells 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℃ under a humidified atmosphere containing 5% CO_2.

When the bottom of the petri dish was filled of cells, remove the DMEM medium from the petri dish. Then the cells were washed three times with cold PBS buffer (10 mM sodium phosphate buffer, 0.1 M NaCl, pH 7.4 @25°C). Next, 6 mL RNAiso solution was added into the petri dish and incubated at room temperature for 2 min to lyse the cells. The cell lysates were collected into a centrifuge tube which contained 1.2 mL trichloromethane, and shaken slightly at room temperature for 2 min. After that, the cell lysates were centrifuged for 15 min at 12000 rpm at 4℃ and the aqueous phase was collected in a new tube. A volume of 3 mL isopropyl alcohol was added into the aqueous phase and the mixture was incubated at room temperature for 10 min and centrifuged at 12000 rpm at 4℃ for 20 min. Next, the precipitate (total RNA) was washed once with 75% ethanol and dissolved with DEPC-treated water. Finally, the $poly^{A+}$ RNA was extracted from the total RNA by using PolyATract® mRNA Isolation System III according to the instruction manual. The concentration of $poly^{A+}$ RNA was determined with the Nano Drop 2000 (Thermo Scientific).

3. Standard procedures of the proposed assay

In the proposed assay, the gap-filling and ligation of the padlock probe were first conducted. Typically, 10 μL reaction system contained 1× buffer (80 mM Tris–HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 10 mM $MgSO_4$, 2 mM ATP, 1 mM DTT, 3.75% PEG 6000 and 0.1% Triton X-100, pH 8.8), 2 nM padlock probe, 250 μM UTP, 0.8 U Bst DNA polymerase, 2 U T3 DNA ligase and appropriate amount of target RNA. The reaction mixture was incubated at 40°C for 20 min to complete the gap-filling-ligation reaction. Then the mixture were incubated at 85°C for 10 min to inactive the Bst DNA polymerase and T3 DNA ligase.

Finally, a volume of 2 μL of the reaction products were pipetted into the

amplification reaction mixture containing 100 nM SLP, 0.8 μM FIP/BIP, 1 M betaine, 2 mM dNTPs, 4 U Bst DNA polymerase and 0.4 ng/μL SYBR Green I and 1× ThermoPol reaction buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$ and 0.1% Triton X-100, pH 8.8) with a final 10 μL volume. The final mixture solution was immediately put into the StepOne Real-Time PCR System (Applied Biosystems, USA) to perform the RCA and LAMP amplification reaction at 65℃. The fluorescence intensity was simultaneously monitored at intervals of 30 s.

4. Principle of the LAMP amplification

The mechanism of LAMP is illustrated in Fig. S1. This amplification reaction relies on auto-cycling strand displacement DNA synthesis performed by the Bst DNA polymerase and two inner primers. The inner primers are called the forward inner primer (FIP) and the backward inner primer (BIP), respectively. The FIP is made up of F1c and a complement sequence (F2) to the F2c. The BIP is made up of B1c and a complement sequence (B2) to the B2c.

During the RCA reaction many double stem-loop DNAs with varying stem lengths are generated, which will serve as the starting material for the amplification of LAMP. Here we take one stem-loop DNA as an example to illustrate the process of LAMP reaction. At first, the double stem-loop DNA auto-starts the self-primed DNA synthesis from its 3' end to form the stem-loop DNA (structure II). Then FIP hybridizes to the loop (F2c) in the stem-loop DNA (structure II) and primes strand displacement DNA synthesis, generating an intermediate stem-loop DNA (structure III) with an additional inverted copy of the structure I sequence in the stem and a ssDNA loop (B2c) formed at the opposite end. Next, the structure III auto-starts the self-primed DNA synthesis from its 3' end and yields a complementary structure of the original dumb-bell form DNA (structure V) and structure IV. Similarly, the structure V can also yield the structure I and structure VIII by displacement DNA synthesis in the presence of BIP, which means that the cycle reaction between the structure I and the structure V is established. Besides the structure IV, structure VIII can also serve as the template for BIP, FIP in the subsequent displacement DNA synthesis respectively, and produce

longer stem-loop DNAs. Therefore, by the elongation and recycling DNA synthesis reaction, a mixture of stem-loop DNAs with different stem lengths are produced.

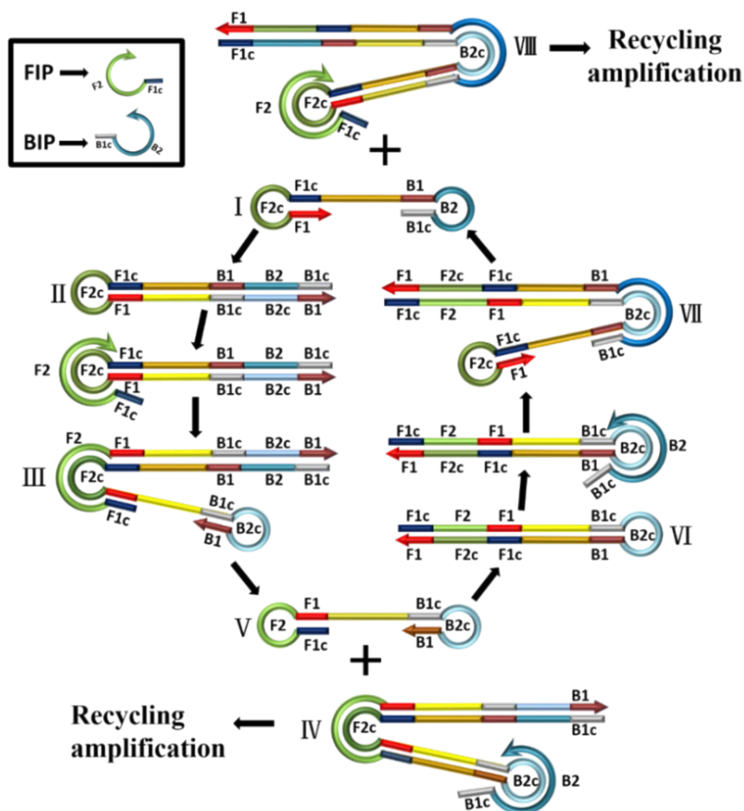

Fig. S1 Principle of the LAMP amplification.

5. The POI values of the fluorescence curves from Fig. 2

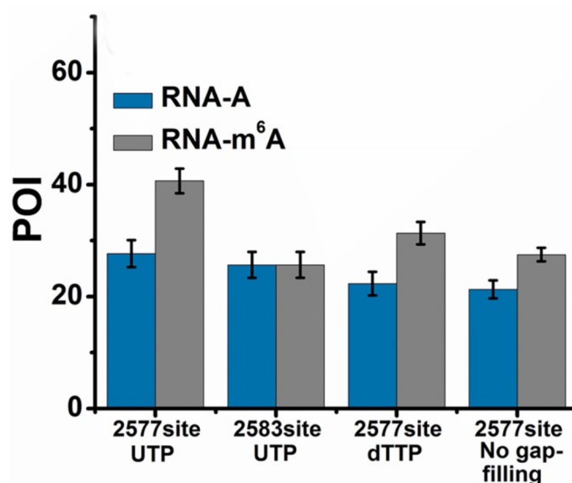

Fig. S2 The POI values of the fluorescence curves from Fig. 2b to 2e. The error bars were calculated from three independent sample measurements.

6. Characterization of circular DNA templates, RCA and LAMP products

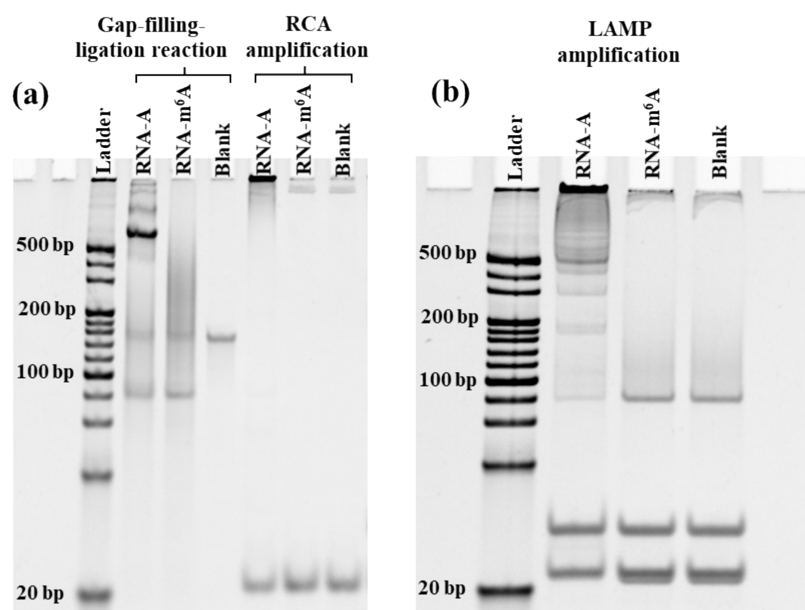

Fig. S3 Characterization of circular DNA templates, RCA and LAMP products by using polyacrylamide gel electrophoresis. (a) The polyacrylamide gel (16%) electrophoretogram of the circular DNA templates produced in the gap-filling-ligation and the RCA products produced during the RCA amplification. The target RNA (RNA-A and RNA-m^{6}A) used in the gap-filling-ligation was 1 μM and in the RCA amplification was 10 nM. (b) The polyacrylamide gel (16%) electrophoretogram of the LAMP amplification products by using 10 nM RNA-A or RNA-m^{6}A serves as the RNA target.

7. Optimization of the experimental conditions

In this work, the formation of circular ssDNA is the basis of the proposed method. Therefore, the gap-filling-ligation reaction is the key step for the differentiation of A and m^{6}A. The experimental conditions such as the dosage of T3 DNA ligase and Bst DNA polymerase, the concentration of the padlock probe, are crucial factors in the gap-filling-ligation reaction. In order to achieve the best assay performance, these parameters were studied and optimized systematically

(1) Optimization of the concentration of the padlock probe in the gap-filling-ligation reaction.

The effect of the amount of padlock probe was investigated with the padlock probe varying from 100 pM to 5 nM, respectively. As shown in Fig. S4, when the concentration of the padlock probe is 100 pM, the fluorescence curves produced by 10 pM target RNA-A and 10 pM target RNA-m^{6}A cannot be clearly discriminated from

each other and close to the curve produced by the Blank, indicating that the number of cyclized padlock probe was very low. By gradually increasing the padlock probe from 100 pM to 5 nM, the fluorescence curves aroused by RNA-A, RNA-m^{6}A and blank can be discriminated obviously, especially when 2 nM padlock probe was employed. When 5 nM padlock probe was used, the fluorescent signal of blank enhanced earlier than that used 100 pM to 2 nM padlock probe, indicating 5 nM was excessive for the ligation reaction to cause a nonspecific ligation. The △POI values between the target RNA-A and target RNA-m^{6}A reaches the maximum when 2 nM padlock probe was used in the assay. Therefore, 2 nM padlock probe is selected as the optimum for the proposed assay.

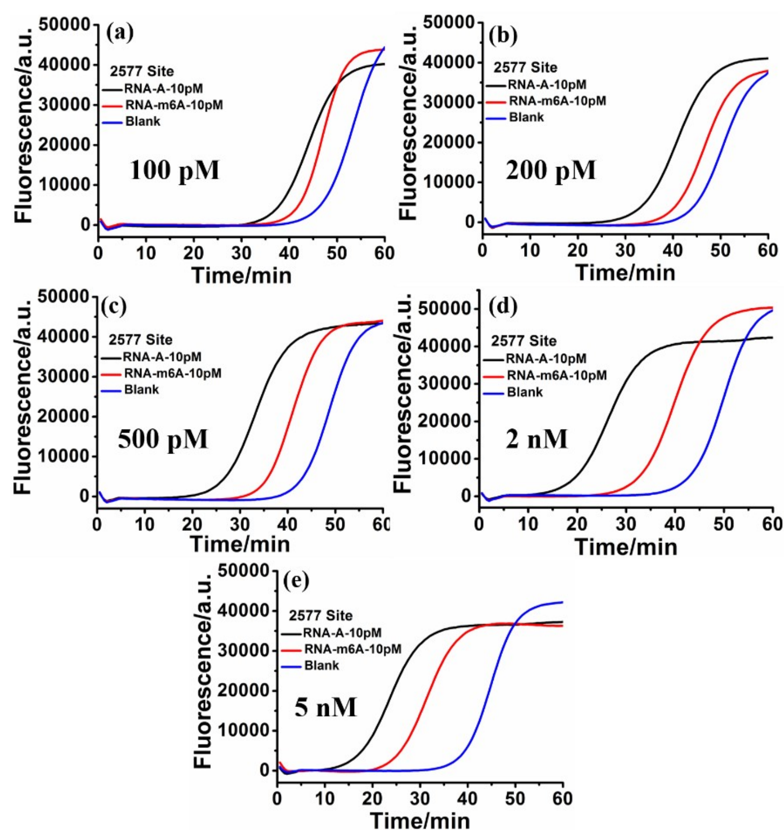

Fig. S4 Optimization of the concentration of the padlock probe in the gap-filling-ligation reaction. The real-time fluorescence curves were respectively produced by 10 pM RNA-A, 10 pM RNA-m^{6}A and blank (no target) with varying padlock probes of (a) 100 pM, (b) 200 pM, (c) 500 pM, (d) 2 nM and (e) 5 nM.

(2) Optimization of the dosage of Bst DNA polymerase in the gap-filling-ligation reaction

To optimize the amount of Bst DNA polymerase in the gap-filling reaction, we conducted the reaction by using 0.4 U, 0.8 U and 1.6 U Bst DNA polymerase,

respectively. As shown in Fig. S5, when the amount of Bst DNA polymerase was increased from 0.4 U to 0.8 U, the △POI values between the fluorescence curves produced by 10 pM RNA-A, 10 pM RNA-m^{6}A increased, which means the number of cyclized ssDNA increased. However, when the amount of Bst DNA polymerase was 1.6 U, the △POI values dropped sharply, because the polymerase amount was excessive so the hindrance effect of m^{6}A for Bst DNA polymerase was reduced. Therefore, 0.8 U Bst DNA polymerase was chosen in the following assay.

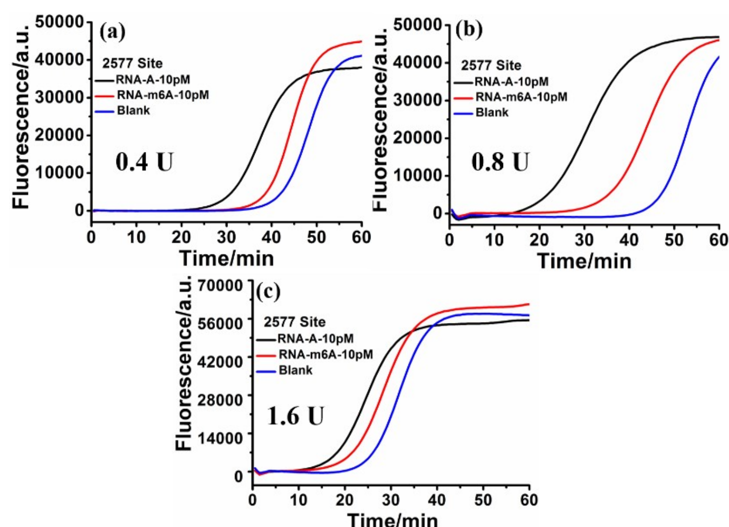

Fig. S5 Optimization of the dosage of Bst DNA polymerase in the gap-filling-ligation reaction. The real-time fluorescence curves were respectively produced by 10 pM RNA-A, RNA-m^{6}A and blank with Bst DNA polymerase dosages of (a) 0.4 U, (b) 0.8 U, and (c) 1.6 U.

(3) Optimization of the dosage of T3 DNA ligase in the gap-filling-ligation reaction

The influence of the amount of T3 DNA ligase was also examined by using 1 U, 2 U and 4 U T3 DNA ligase in the ligation reaction. As can be seen from Fig. S6, the more T3 DNA ligase was added in the ligation reaction, the faster the fluorescence curves appear both in the presence or absence (blank) of the target. From Fig. S6 (c)，when T3 DNA ligase was 4 U, the fluorescence curve of the blank was obviously moved forward, because of the nonspecific ligation in the presence of the excessive T3 DNA ligase. Also because the T3 DNA ligase was excessive, the differentiation between A and m^{6}A decreased. When 2 U T3 DNA ligase was employed, the △POI values between the fluorescence curves produced by 10 pM RNA-A, RNA-m^{6}A reaches the maximum. Thus, 2 U T3 DNA ligase was selected as the optimum amount for the assay.

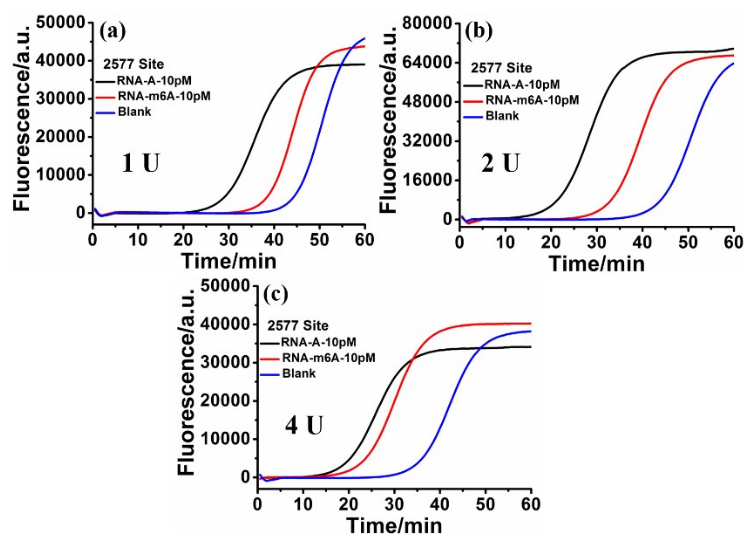

Fig. S6 Optimization of the dosage of T3 DNA ligase in the gap-filling-ligation reaction. The real-time fluorescence curves were respectively produced by 10 pM -RNA-A, RNA-m^{6}A and blank with T3 DNA ligase dosages of (a) 1 U, (b) 2 U, and (c) 4 U.

8. Table S2 Quantitative detection of RNA-m^{6}A in the mixed RNA sample

Sample	1	2	3	4	5	6
Added concentration of RNA-A (fM)	0	5	10	20	50	100
Added concentration of RNA-m^{6}A (fM)	100	95	90	80	50	0
Calculated concentration of RNA-m^{6}A (fM)	96.6±11.5	92.2±9.4	88.4±8.1	78.6±7.5	46.3±4.5	0±0.5

The results are obtained from measurements of three independent samples.

9. Detection of m^{6}A in HeLa and HEK293T cells

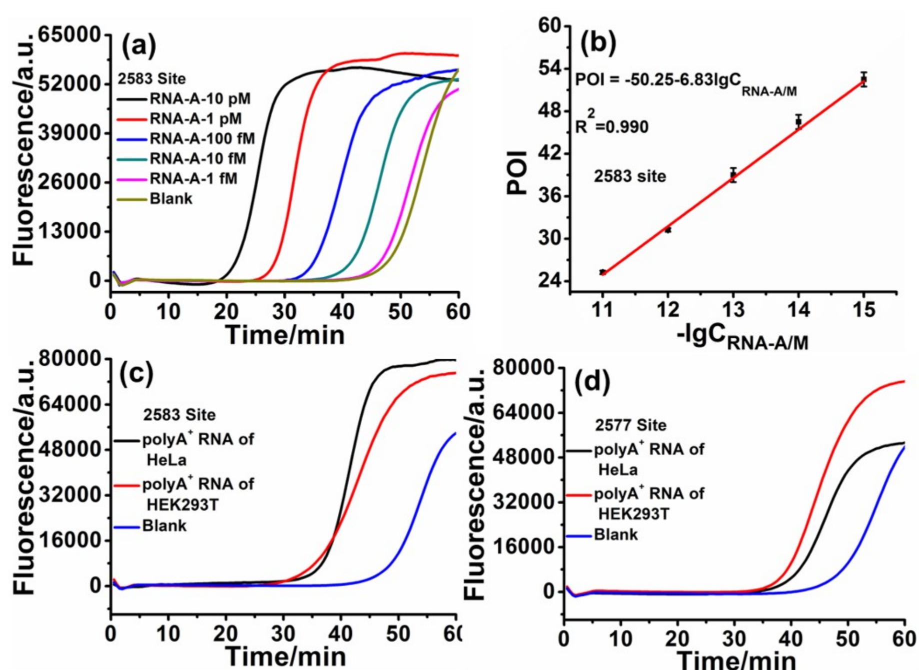

Fig. S7 Determination of m^{6}A fractions in poly^{A+} RNA of HeLa and HEK293T cells. (a) Real-time fluorescence curves produced by different concentrations of RNA-A at 2583rd site. (b) The linear relationship between POI values and logarithm (lg) of RNA-A concentrations calculated from (a). The error bars were calculated from three independent sample measurements. (c) Real-time fluorescence curves produced by MALAT1 lncRNA in the poly^{A+} RNA of HeLa and HEK293T cells at 2583rd site. (d) Real-time fluorescence curves produced by MALAT1 lncRNA in the poly^{A+} RNA of HeLa and HEK293T cells at 2577th site.