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Supporting Information

One-Step Detection of MicroRNA with High Sensitivity and Specificity via Target-triggered Loop-Mediated Isothermal Amplification (TT-LAMP)

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1. Experimental Section

Materials and Apparatus. Bst 2.0 WarmStart DNA polymerase was purchased from New England BioLabs (U.S.A.). HPLC-purified oligonucleotides including stem-loop template DNA (SLTD), stem-loop primer (SLP), forward primer (FP), backward primer (BP), target miRNAs, the dNTPs, Ribonuclease Inhibitor, 6× nucleic acid sample loading buffer and RNase-free water used in this study were all obtained from Takara Biotechnology Co., Ltd. (Dalian, China). The sequences of all RNA and DNA are listed in Table S1. The betaine was obtained from Sigma (U.S.A.). SYBR Green I (20 ng/μL stock solution in DMSO) was purchased from Xiamen Bio-Vision Biotechnology Co. Ltd. (Xiamen, China). Acrylamide, bisacrylamide, N, N, N′, N′-tetramethyl ethylenediamine (TEMED), ammonium persulfate (APS), trihydroxymethyl aminomethane (Tris), ethylene diamine tetraacetic acid (EDTA), boric acid and 4S Red Plus nucleic acid stain for the PAGE analysis were all obtained from Sangon Biotech Co. Ltd. (Shanghai, China). The StepOne Real-Time PCR system (Applied Biosystems, U.S.A.) was employed for real-time fluorescence measurement. All the solutions for reactions were prepared in RNase-free water.

Table S1. The sequences of the nucleic acids used in this work.

name	sequence
let-7a	5′-UGAGGUAGUAGGUUGUAUAGUU-3′
let-7b	5′-UGAGGUAGUAGGUUGUGUGGUU-3′
let-7c	5′-UGAGGUAGUAGGUUGUAUGGUU-3′
let-7d	5′-AGAGGUAGUAGGUUGCAUAGUU-3′
let-7e	5′-UGAGGUAGGAGGUUGUAUAGUU-3′
let-7f	5′-UGAGGUAGUAGAUUGUAUAGUU-3′
let-7g	5′-UGAGGUAGUAGUUUGUACAGUU-3′
let-7i	5′-UGAGGUAGUAGUUUGUGCUGUU-3′
mir-122	5′- UGGAGUGUGACAAUGGUGUUUG-3′
SLTD	5′-CGACAGCAGAGGATTTGTTGTGTGGAAGTGTGAGCGGATTTTCCTCTG F1c F2 F1 CTGTCGTTTTCATAATCCCTAGACCAGACATCACACTTAACTATACAACC SHS TACTACCTCA-phosphate-3′

SLTD-122	5′-CGACAGCAGAGGATTTGTTGTGTGGAAGTGTGAGCGGATTTTCCTCTG F1c F2 F1 CTGTCGTTTTCATAATCCCTAGACCAGACATCACACTTCAAACACCATTG SHS TCACACTCCA-phosphate-3′
SLP	5′-ATCGTCGTGACTGTTTGTAATAGGACAGAGCCCCGCACTTTCAGTCAC B1c B2 B1 GACGATTTTATGTCTGGTCTAGGGATTATG-3′
FP	5′-CGACAGCAGAGGATTTGTTGTGTGGAAGTGTGAGCGGA-3′ F1c F2
BP	5′-ATCGTCGTGACTGTTTGTAATAGGACAGAGCCCCGCAC-3′ B1c B2

MiRNA target-triggered LAMP reaction and real-time fluorescence measurement. The TT-LAMP reaction was performed in a 10 μL mixture which was prepared separately as the part A and part B solutions. The part A solution consisted of 500 pM SLTD, 500 pM SLP, reaction buffer (20 mM Tris-HCl, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 4 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8 @25°C), 4 U Ribonuclease Inhibitor, 0.2 mM dNTPs and different amounts of target miRNA or total RNA sample in a volume of 6 μL. The part B solution consisted of 0.4 μM FP and 0.4 μM BP, 1 mM betaine, SYBR Green I, 1.6 U Bst 2.0 WarmStart DNA polymerase and reaction buffer in a volume of 4 μL. The part A was firstly heated at 50 °C for 10 min. After naturally cooling to room temperature, the part A was then put on the ice and mixed with the part B solution. The final mixture solution was put into the Step-One Real-Time PCR system to perform the TT-LAMP at 55 °C and the real-time fluorescence intensity was monitored at intervals of 1 min.

Cell culture the total RNA extraction from the cells. The human colon cancer cell lines (HCT-116) was purchased from the cell bank of Chinese Academy of Sciences (Shanghai, China) which was maintained in DMEM Medium (GBICO, Cat. 12100-046) supplemented with 10% (v/v) fetal calf serum (GBICO, Cat. 1600036), 1% $NaHCO_3$, 100 U/mL penicillin and 100 μg/mL streptomycin. The cell was cultured at 37 °C under a humid atmosphere containing 5% CO_2. The total RNA sample was extracted from the cell by using of Trizol Reagent (Invitrogen, Beijing, China). The amount of extracted total RNA was quantified with a Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, WA).

2. Influence of the amount of Bst 2.0 WarmStart DNA polymerase on miRNA detection

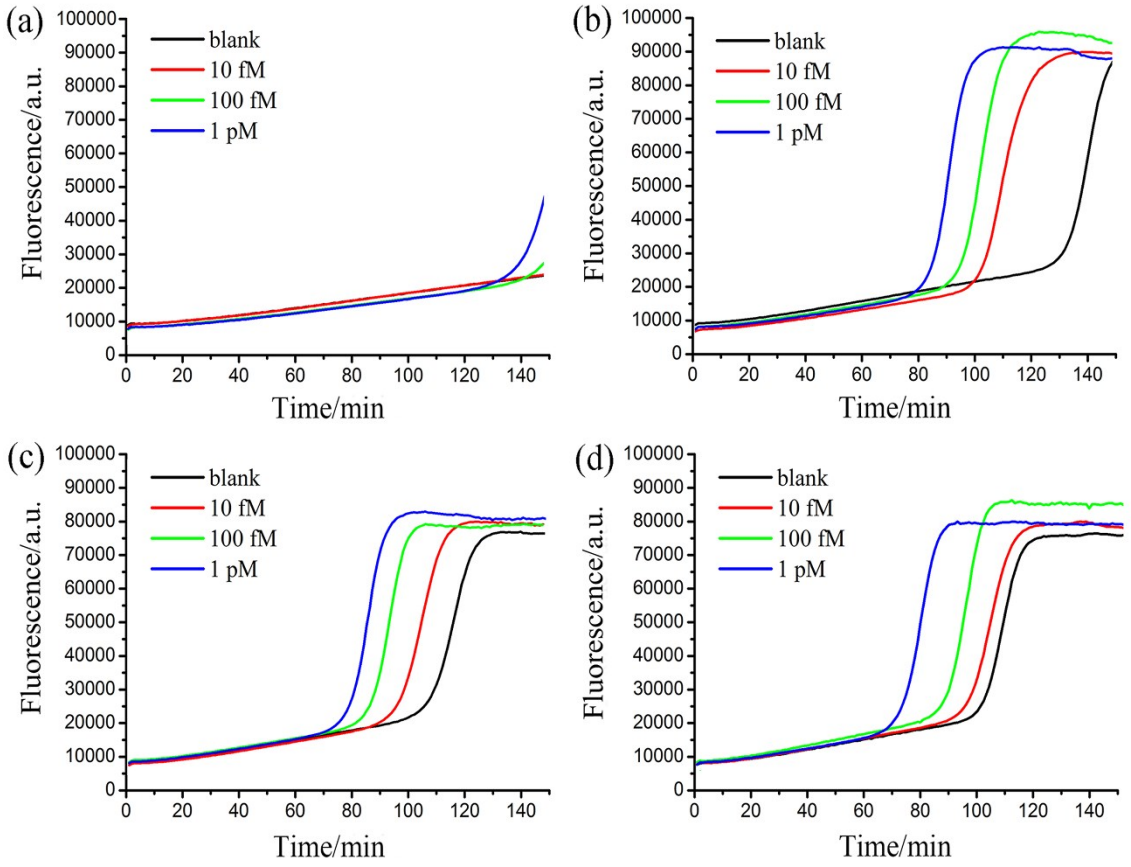

Fig. S1 The influence of the amount of Bst 2.0 WarmStart DNA polymerase on the TT-LAMP-based miRNA assay. The real-time fluorescence curves produced by let-7a miRNA of 0, 10 fM, 100 fM and 1 pM were recorded respectively under the catalysis of (a) 0.8 U, (b) 1.6 U, (c) 2.4 U and (d) 3.2 U Bst 2.0 WarmStart DNA polymerase. Other experimental conditions were the same as described in the experimental procedure.

The TT-LAMP reaction relies on the catalysis of the DNA polymerase with high displacement activity. In the one-step miRNA assay, the amplification efficiency will be primarily affected by the amount of DNA polymerase. The effect of different amounts of Bst 2.0 WarmStart DNA polymerase ranging from 0.8 U to 3.2 U on the proposed assay were investigated. As shown in Fig. S1, when less than 0.8 U of Bst 2.0 WarmStart DNA polymerase is employed, almost no fluorescence for exponential amplification is detected within the reaction time of 150 min, indicating that the amount of Bst 2.0 WarmStart DNA polymerase is insufficient to produce efficient DNA extension. When the Bst 2.0 WarmStart DNA polymerase is increased to 1.6 U, 2.4 U and 3.6 U, the TT-LAMP reaction is gradually accelerated with

the increase of polymerase dosage. Nonetheless, the difference of POI (point of inflection) values, which is defined as the time corresponding to the maximum slope in the fluorescence curve, between 10 fM let-7a miRNA and the blank are significantly decreased. One can see from Fig. S1 that the difference of POI values produced by the target miRNA and blank reaches the maximum with 1.6 U Bst 2.0 WarmStart DNA polymerase. In this regard, the amount of 1.6 U Bst 2.0 WarmStart DNA polymerase is chosen as the optimal amount for the TT-LAMP-based miRNA assay.

3. Effect of the concentration of the SLTD and SLP on the proposed miRNA assay

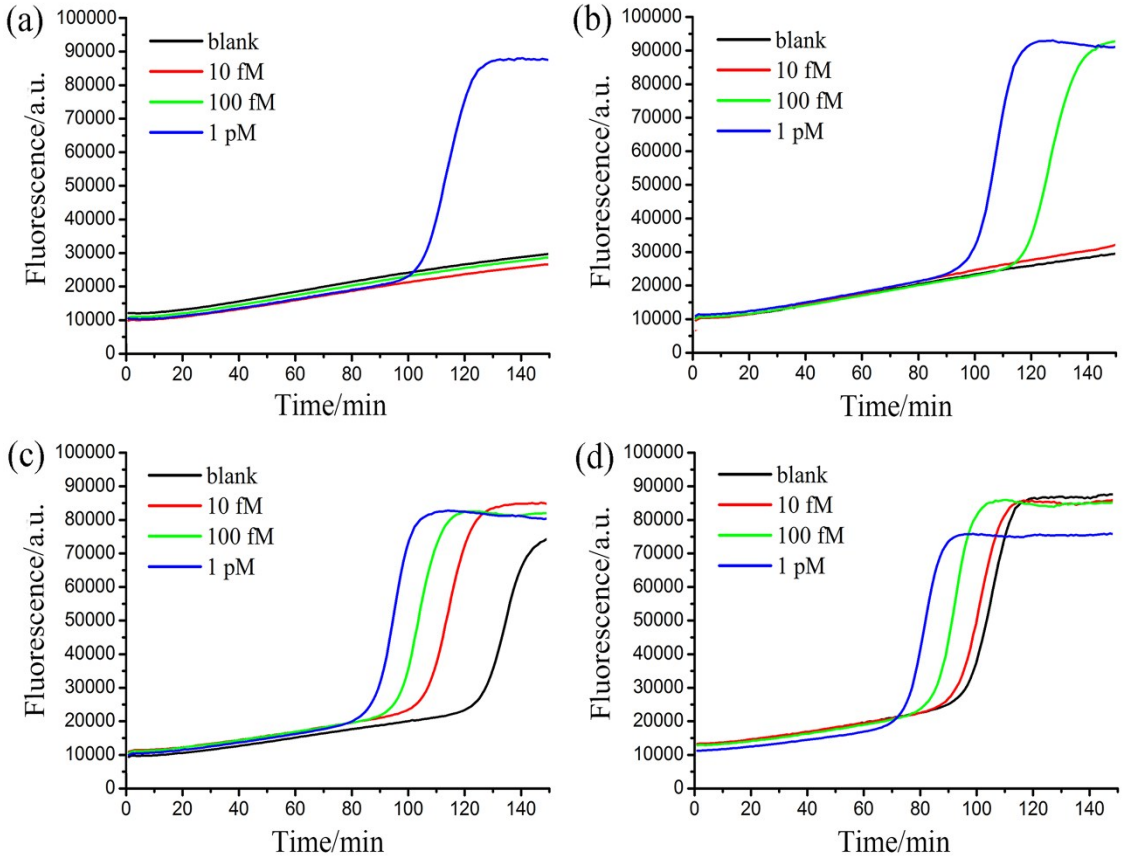

Fig. S2 The effect of the concentrations of the SLTD and SLP on the detection of miRNA. The TT-LAMP reaction and real-time fluorescence measurement were carried out with different SLTD/SLP concentrations of (a) 50 pM, (b) 100 pM, (c) 500 pM and (d) 2 nM, respectively. Other experimental conditions were the same as described in the experimental procedure.

The concentration of the SLTD and SLP is a critical factor to affect the formation of the double stem-loop structure, the essential element for the subsequent LAMP amplification. So the amount of SLTD

and SLP (keep the same concentration in this study) was optimized with the different concentrations in the range of 50 pM to 2 nM. As depicted in Fig. S2a~2b, when the concentration of SLTD/SLP is lower than 100 pM, the fluorescence signals produced by 10 fM (Fig. S2a, b) or even 100 fM (Fig. S2a) let-7a cannot be detectable. The reason may be that low concentrations of SLTD/SLP will lead to low efficiency of TT-LAMP, particularly at the low levels of the target miRNA. With increasing the concentration of SLTD/SLP to 500 pM (Fig. S2c), 10 fM let-7a target can be clearly detected. However, when the SLTD/SLP dosage is further increased from 500 pM to 2 nM, one can see from Fig. S2d that the difference of POI value between 10 fM let-7a and the blank is significantly reduced. This indicates that too excessive SLTD and SLP may cause much target-independent nonspecific extension and amplification. Therefore, 500 pM of SLTD and SLP is employed for the TT-LAMP miRNA assay in this work.

4. Optimization of the reaction temperature for TT-LAMP

To investigate the influence of the reaction temperature on the TT-LAMP miRNA assay, the reaction was performed at the temperature of 50 °C, 55 °C and 60 °C, respectively. As shown in Fig. S3a, when the TT-LAMP is performed at 50 °C, no detectable exponential amplification signals are observed at any concentration of let-7a miRNA, which may be attributed to the low activity of enzyme at the lower temperature. With increasing temperature from 50 °C to 60 °C (Fig. S3a~3c), the TT-LAMP reaction is correspondingly sped up probably due to the gradually enhanced activity of the polymerase (with a recommended temperature of 65 °C). At the reaction temperature of 60 °C, though the TT-LAMP reaction is obviously accelerated, the non-specific amplification significantly increases so that the difference of POI value between 10 fM let-7a and the blank is decreased (Fig. S3c). When the TT-LAMP is conducted at 55 °C (Fig. S3b), the difference of POI value between 10 fM let-7a and the blank reaches the maximum value, which should be beneficial to enhance the detection sensitivity. Based on the consideration of both the reaction time and the sensitivity, 55 °C is selected as the optimal temperature of TT-LAMP in this study.

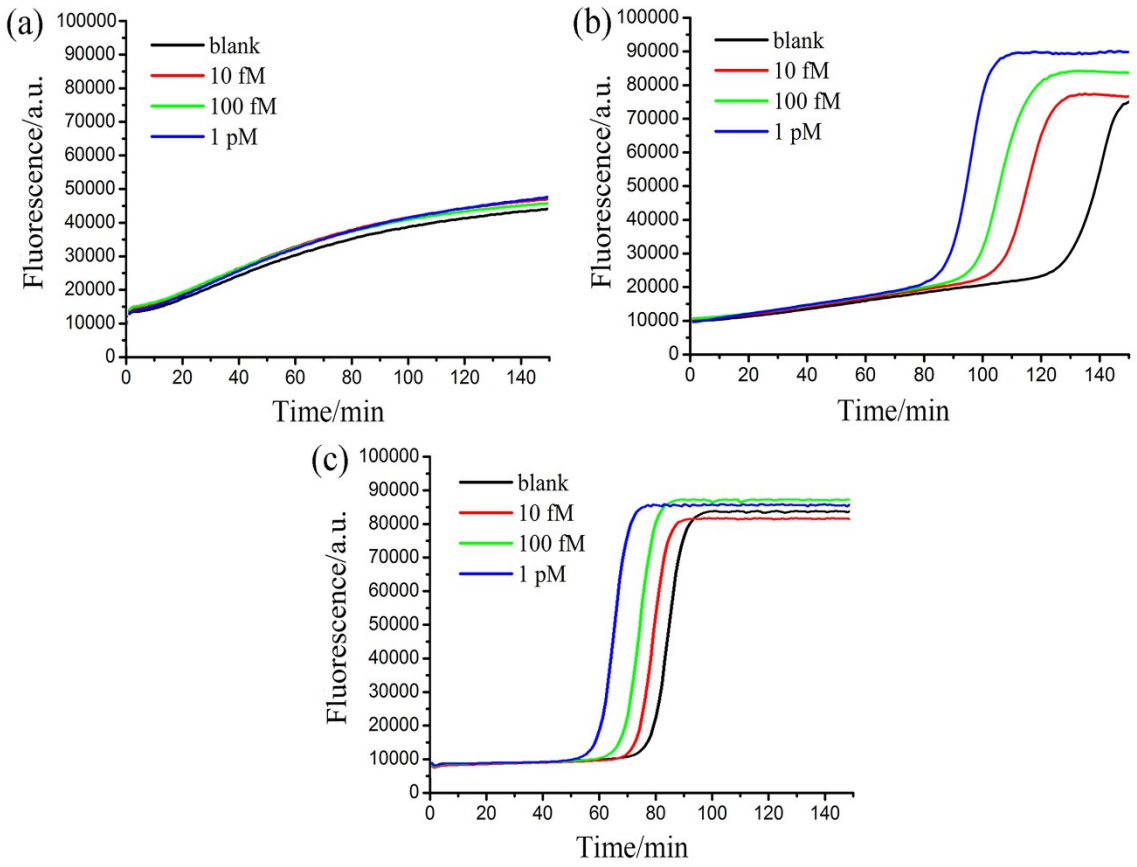

Fig. S3 The influence of the reaction temperature on the TT-LAMP miRNA assay. Real-time fluorescence signals produced by let-7a miRNA were recorded with different TT-LAMP temperatures of (a) 50 °C, (b) 55 °C, (c) 60 °C. From right to left, the let-7a concentration successively is 0, 10 fM, 100 fM and 1 pM in each image.

5. Polyacrylamide gel electrophoresis result of the TT-LAMP assay

In order to further verify the feasibility of the proposed TT-LAMP assay, the non-denaturing polyacrylamide gel electrophoresis (PAGE) has been conducted to characterize the TT-LAMP products. After the TT-LAMP reaction was conducted for 112 min in the presence of different concentrations of let-7a miRNA, the final products were respectively subject to PAGE analysis, and the results are presented in Fig. S4. It can be seen from Fig. S4 that with the reaction time of 112 min, the blank control without let-7a did not produce any observable bands of amplification products (lane 4). By contrast, in the presence of let-7a target, ladder-like multiple bands can be clearly observed (lane 5~7), and the target dose-responsive pixel intensity increase of the ladder bands can be also identified. According to the

principle of TT-LAMP, the lengths of the target-generated products are not identical because they consist of a mixture of stem-loop DNAs with varying stem lengths. So the ladder-like patterns of the PAGE results were consistent with the prediction of TT-LAMP products. The acquired PAGE results agreed well with the real-time fluorescence detection results (Fig. 2a) of the TT-LAMP miRNA assay, suggesting the proposed TT-LAMP is feasible and reliable for miRNA analysis.

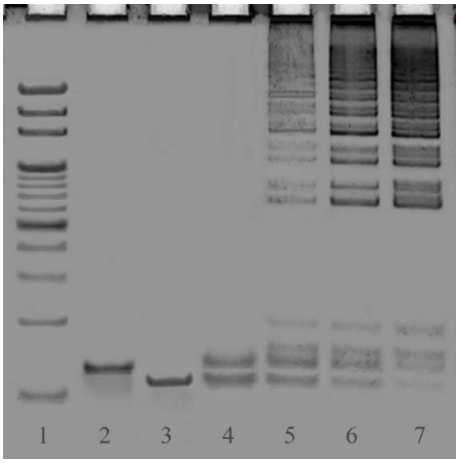

Fig. S4 The non-denaturing polyacrylamide gel electrophoresis analysis of the TT-LAMP miRNA assay. Lane 1: DNA markers (20 bp DNA ladder); lane 2: FP; lane 3: BP; lane 4 to 7: TT-LAMP reaction where the concentration of let-7a successively is 0 (blank), 10 fM, 1 pM, 100 pM. The blank is characterized with the same experimental procedure but without miRNA. The experimental condition: SLTD and SLP, 500 pM; FP and BP, 0.4 μM. Electrophoresis apparatus (Beijing Liuyi Biotechnology, China) and Gel DoxTM EZ Gel imaging system (BIO-RAD, USA) were used to perform electrophoresis separation and imaging analysis of TT-LAMP products.

6. Estimation of the interference of other let-7 miRNA members for let-7a detection

From the results of Fig. 2, we can obtain the correlation equation for the determination of let-7a that is $POI = -23.42 - 10.08 \lg C_{let\text{-}7a}/M$. As displayed in Fig. S5, the real-time fluorescence signal produced by let-7a could be obviously separated from those produced by any other let-7 family miRNAs with the let-7a-specific SLTD in the TT-LAMP reaction. The POI values of let-7 family members could be obtained from the corresponding real-time fluorescence curves, which are supposed as POI_a, POI_b, POI_c, POI_d, POI_e, POI_f, POI_g and POI_i, respectively. On the basis of the correlation equation in Fig. 2, we

presume that the POI_a, POI_b, POI_c, POI_d, POI_e, POI_f, POI_g and POI_i corresponding to the amount of let-7a are recorded as A_a, A_b, A_c, A_d, A_e, A_f, A_g and A_i, respectively. If the detection value of the let-7a (A_a) is normalized to be 100%, the relative detection of other miRNAs ($A_{other\ miRNA}/A_a$) can be calculated respectively.

According to the above-mentioned correlation equation, the following equation could be gained.

$$POI_b - POI_a = -10.08\ (\lg A_b - \lg A_a) \quad (1)$$

$$\lg \frac{A_b}{A_a} = -\frac{POI_b - POI_a}{10.08} \quad (2)$$

For example, we can see that the POI_a, POI_b are 90 and 128 respectively in Fig. S5. As a consequence, $\frac{A_b}{A_a}$ could be calculated as 0.02% through the above constructed equations. In order to further improve the accuracy of the detection, the final results could be obtained by using the average values with three repetitive experiments. In the same way, $\frac{A_c}{A_a}$, $\frac{A_d}{A_a}$, $\frac{A_e}{A_a}$, $\frac{A_f}{A_a}$, $\frac{A_g}{A_a}$ and $\frac{A_i}{A_a}$ could be calculated as 1.58%, 6.56%, 23.67%, 2.49%, 0.02% and 0.02%, respectively. With the proposed method, except let-7e, the interference produced by others let-7 family members are all less than 7%. Therefore, the TT-LAMP reaction exhibits high specificity.

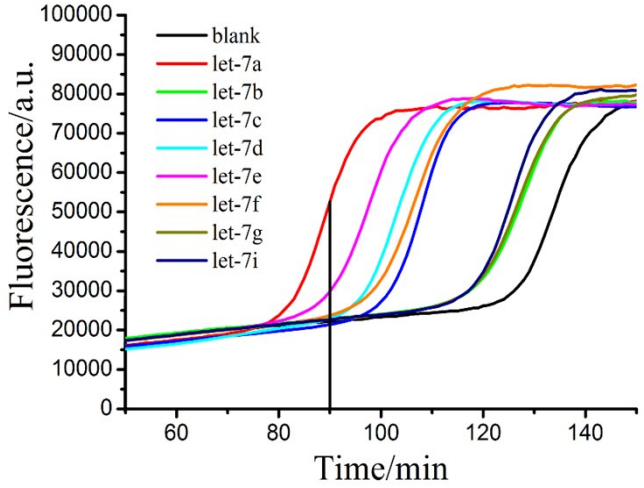

Fig. S5 Real-time fluorescence curves with the TT-LAMP amplification method produced by let-7a to 7g, and let-7i (The concentration of each miRNA is 1 pM).

7. Determination of let-7a in real cell samples with TT-LAMP method

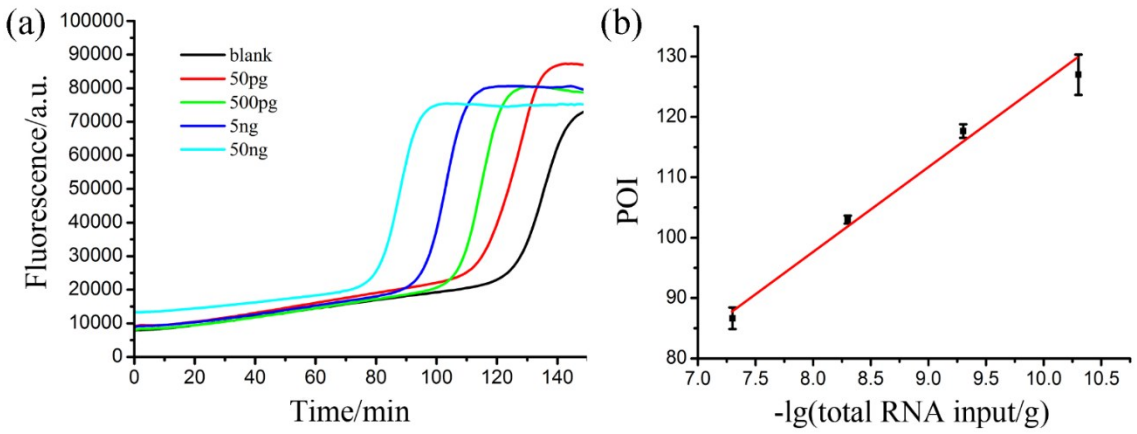

Fig. S6 Let-7a analysis in total RNA samples. (a) The real-time fluorescence curves produced by different amounts of total RNA input (50 pg ~50 ng). (b) The relationship between the POI values and the logarithm (lg) of the different amounts of total RNA input. Error bars were calculated from three replicate measurements.

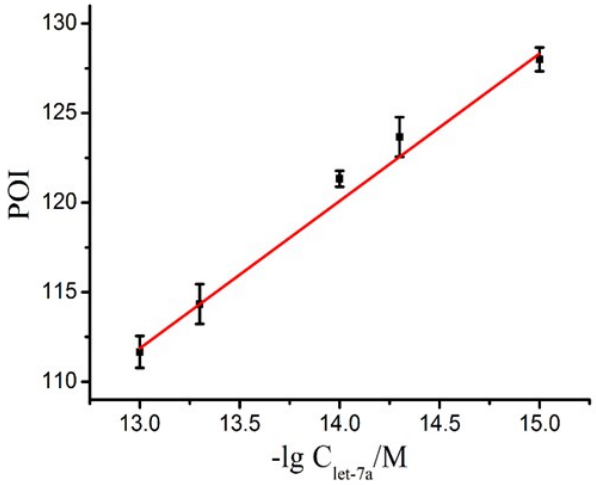

Fig. S7 The relationship between the POI values and the logarithm (lg) of the concentrations of standard let-7a miRNA. The concentrations of let-7a are 1, 5, 10, 50 and 100 fM, respectively. Error bars were estimated from three replicate measurements.

The total RNA sample was extracted from the human colorectal cancer cells (HCT-116). With the TT-LAMP assay, let-7a level in the total RNA sample was detected according to the standard experiment

procedure described in the experimental section except that the let-7a miRNA was replaced by the total RNA sample. As shown in Fig. S6a, the amount of HCT-116 total RNA samples ranging from 50 pg to 50 ng can be accurately detected by the TT-LAMP-based assay. There is a good linear relationship between the POI values and the logarithm (lg) of the total RNA input ranging from 50 pg to 50 ng. The correlation equation is *POI = –17.55 – 14.49 lg (total RNA input/g)* with the corresponding correlation coefficient R^2 of 0.9946. Therefore, as small as 50 pg total RNA can be clearly determined with our proposed TT-LAMP assay, suggesting a high sensitivity for the detection of miRNAs in real biological samples. Meanwhile, the concentrations of 1, 5, 10, 50 and 100 fM synthetic let-7a standard were used to construct the calibration curve under the same conditions (Fig. S7). Then according to the simultaneously constructed calibration curve, the amount of let-7a in the total RNA samples can be exactly determined.

8. Quantification of let-7a in real cell samples by stem-loop RT-PCR method

The stem-loop RT-PCR protocol is adopted from the literature method,[S1] which contains two steps including reverse transcription (RT) and real-time PCR. First, the stem-loop RT primer hybridized with the target miRNA and inversely transcribed with the template of miRNA under the catalysis of reverse transcriptase. Afterward, the reverse transcription products were detected with real-time quantitative PCR by using SYBR Green I as the fluorescent dye. It is worth noting that such RT-PCR-based method for miRNA analysis needs two-step reactions and two types of enzymes, meanwhile the precise thermal cycling process is also inevitable. The detailed experimental procedures are described as follows:

Reverse transcription reaction. The reverse transcription reaction was carried out in the 5 μL mixture including 50 nM stem–loop RT-primer (5′-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACT GGATACGACAACTA-3′), RT buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$, pH 8.3 @25°C), 0.25 mM dNTPs, 40 U ProtoScrip Ⅱ reverse transcriptase, 4 U RNase inhibitor and series dilutions of synthetic let-7a standard or total RNA samples. The mixture was incubated following the procedure: 30 min at 16°C, 30 min at 42°C, 5 min at 85°C and then held at 4°C.

Real-time quantitative PCR. A volume of 1 μL of the reverse transcription products was transferred to the PCR reaction mixture with a final volume of 10 μL. The PCR reaction mixture contained 200 nM

forward primer (5′-GCCGCTGAGGTAGTAGGTTGTA-3′) and 200 nM reverse primer (5′-GTGCAGGGTCCGAGGT-3′), 0.2 mM dNTPs, SYBR Green I, 0.5 U JumpStart^{TM} Taq DNA Polymerase and PCR buffer (10 mM Tris-HCl pH 8.3@25°C, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin). The PCR reaction was carried out on a StepOne Real-Time PCR System (Applied Biosystems, USA) by firstly hot start at 94°C for 2 min, followed by 50 cycles of 94°C for 20 s, 60°C for 30 s and 72°C for 20 s. As can be seen from Fig. S8, the calibration curve between Ct values and the logarithm (lg) of let-7a concentrations was obtained by using synthetic standard let-7a. Then accordingly, the let-7a content in 50 pg of the same batch of total RNA samples is calculated to be 1.90 fM, which agrees well with the value determined by the proposed TT-LAMP assay.

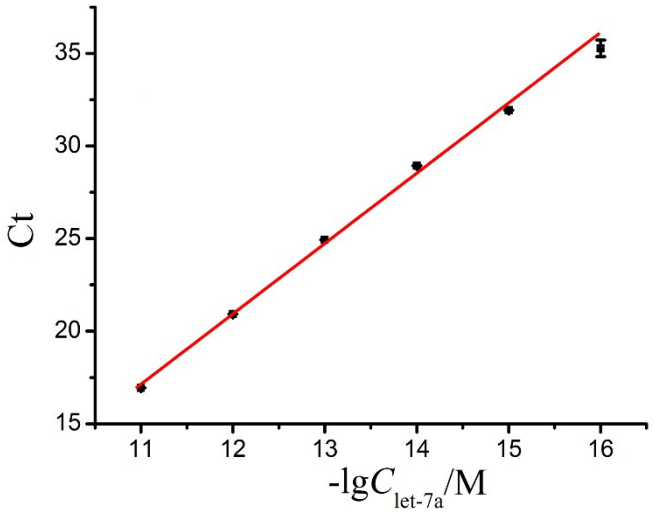

Fig. S8 The relationship between Ct values and the logarithm (lg) of the concentrations of standard let-7a by stem-loop RT-PCR.

9. The comparison of the proposed TT-LAMP approach with other widely used miRNA assays

Table S2. The comparison of the proposed TT-LAMP approach with other widely used miRNA assays.

Detection strategy	Temperature control	Operation and procedures	Enzymes involved	Detection limit	Reference
RCA with fluorescence detection	isothermal	Pre-ligation reaction step is essential before the RCA; signal amplification and fluorescence detection are conducted separately	Both ligase and polymerase are required	10 fM (6 amol in 600 μL)	S2

EXPAR with real-time fluorescence detection	isothermal	Signal amplification and detection are accomplished in one step	Both polymerase and nicking enzyme are required	10 aM (0.1 zmol in 10 μL)	S3
3 WJ structure and quadratic isothermal DNA machine with fluorescence detection	isothermal	Signal amplification and endpoint fluorescence detection are conducted separately	Both polymerase and nicking enzyme are required	100 fM (2 amol in 20 μL)	S4
SDA with fluorescence detection	isothermal	Signal amplification and signal detection are performed separately	Both polymerase and nicking enzyme are required	1.6 fM (16 zmol in 10 μL)	S5
DSN-assisted recycling with fluorescence detection	isothermal	Fluorescently-labeled DNA probes should be conjugated with AuNPs	Only DSN is needed	5 pM (0.2 fmol in 40 μL)	S6
Graphene fluorescence switch-based cooperative amplification with fluorescence detection	isothermal	Signal amplification and detection must be performed separately	Both polymerase and nicking enzyme are required	10.8 fM	S7
LAMP with real-time fluorescence detection	isothermal	One-step assay with sophisticated multiple linear primers	Only polymerase is needed	100 fM (1 amol in 10 μL)	S8
PCR by base-stacking with real-time fluorescence detection	thermal cycle	The signal amplification and detection are accomplished in one step	Only polymerase is needed	500 fM (5 amol in 10 μL)	S9
RT-PCR with real-time fluorescence detection	thermal cycle	Stem-loop primer design and reverse transcription reaction are essential before PCR	Both reverse transcriptase and polymerase are required	1.3 aM (0.013 zmol in 10 μL)	S1
Ligation-based-PCR with real-time fluorescence detection	thermal cycle	Pre-ligation reaction step is essential before PCR	Both ligase and polymerase are required	0.2 fM (4 zmol in 20 μL)	S10
Ligation-based LCR with gel electrophoresis analysis	thermal cycle	Pre-ligation reaction step is essential before LCR; the LCR and signal readout by gel electrophoresis are	Two-types of ligase are needed	0.7 pM (14 amol in 20 μL)	S11

		conducted separately			
TT-LAMP with real-time fluorescence detection	isothermal	The signal amplification and detection are all accomplished in one step	Only polymerase is needed	100 aM (1 zmol in 10 μL)	This work

Note: It is worth noting that in different literatures, the detection limits of miRNA target are presented either as concentrations or absolute quantities, which are both provided in this table if the corresponding reaction volume is available from the original literature.

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