Electronic Supplementary Information

One-step ultrasensitive detection of microRNAs with loop-mediated isothermal amplification (LAMP)

Cuiping Li, [a] Zhengping Li,* [a] Hongxia Jia [a] and Jingli Yan [a]

[a]Key Laboratory of Medical Chemistry and Molecular Diagnosis, Ministry of Education; College of Chemistry and Environmental Science, Hebei University, Baoding 071002, Hebei Province, P. R. China. Email: lzpbd@hbu.edu.cn; Fax: +86 312 5079403

Experimental Section

Materials and Reagents

Bst DNA polymerase large fragment and 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) were purchased from New England Biolabs (USA). Betaine was obtained from Sigma Co. (USA). SYBR Green I (20× stock solution in DMSO, 20 μg/mL) was purchased from Xiamen Bio-Vision Biotechnology (Xiamen, China). The human lung total RNA sample (1 μg/μL) was obtained from Ambion Co. (USA), which was serially diluted to 10 ng/μL with DEPC-treated water immediately prior to use. HPLC-purified RNAs, RNase inhibitor, dNTPs and DEPC-treated water were purchased from TaKaRa Biotechnology Co. Ltd. (Dalian, China). The DNA oligonucleotides including FIP, BIP, B3 primers were synthesized and purified with PAGE by Sangon Biotech Co. Ltd. (Shanghai, China). The template DNA was synthesized by Integrated DNA Technologies, Inc. (IDT, USA). All solutions for miRNAs detection were prepared with DEPC-treated water. The sequences of RNA and DNA oligonucleotides used were listed as follows:

Let-7a miRNA: 5′-ugagguaguagguuguauaguu-3′

Primer FIP: 5′-ACAACGTCGTGACTGGGAAAACCCT-TTTT-GTGCGGGCCTCTTCGCTATTAC-3′

Primer BIP: 5′-CGACTCTAGAGGATCCCCGGGTAC-TTTT-TGTTGTGTGGAATTGTGAGCGGAT-3′

Primer B3: 5′-ACTTTATGCTTCCGGCTCGTA-3′

Template DNA (198 nt): 5′-ACTTTATGCTTCCGGCTCGTA-TGTTGTGTGGAATTGTGAGC
B3 B2
GGATAACAATTTCACACAGGAAACGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGC
B1

Supplementary Material (ESI) for Chemical Communications

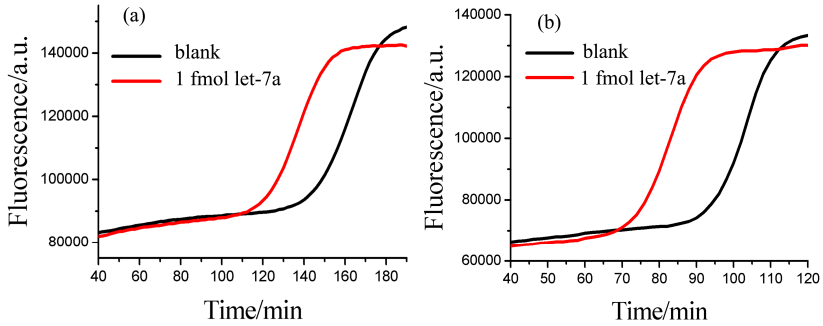
ATGCAAGCTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGGTA
F1c
ATAGCGAAGAGGCCCGCAC-AACTATACAACCTACTACCTCA-3′
F2c M

Experimental procedures for miRNA detection

0.8 μL 10× ThermoPol Reaction Buffer, 0.5 μL 1 nM B3 primer, 0.3 μL 20 μM FIP and BIP primers, 0.5 μL 1 nM DNA template, 0.2 μL 100 mM $MgSO_4$, 2μL 5M betaine, 0.2 μL 40 U/μL RNase inhibitor, 0.8 μL 2.5 mM each of dNTPs and miRNAs with different amounts of synthetic miRNA or 10 ng total RNA sample were mixed with final volume of 8.5 μL. The mixture solution was heated at 95 °C for 5 min and at 50 °C for 10 min. After naturally cooling to room temperature, the mixture solution was set on the ice and mixed with 1.5 μL mixture solution containing 0.2 μL 10× ThermoPol Reaction Buffer, 0.2 μL 20× SYBR Green I, 4 U Bst DNA polymerase large fragment. The final mixture solution was immediately put into the StepOne Real-Time PCR System (Applied Biosystems, USA) to perform the LAMP reaction at 55 °C. The real-time fluorescence intensity was simultaneously monitored at intervals of 2 min.

Optimization of the temperature for LAMP reaction.

To investigate the influence of the temperature for LAMP reaction on miRNA detection, 1.0 fmol let-7a miRNA was detected by the LAMP-based assay at 50 °C, 55 °C and 60 °C, respectively. As shown in Fig. S1, with elevating the temperature of LAMP reaction, the reaction time is shortened and the difference of POI value between 1 fmol let-7a and the blank is decreased. However, when the temperature is elevated from 55 °C to 60 °C, the reaction time is shortened little and the difference of POI value between let-7a and blank is decreased very much. When the temperature is dropped from 55 °C to 50 °C, the reaction time is increased very much and the different of the POI values is increased little. Based on the consideration of both the reaction time required for miRNA detection and the signal produced by target miRNA, 55 °C was selected as the temperature of LAMP reaction for miRNA detection.



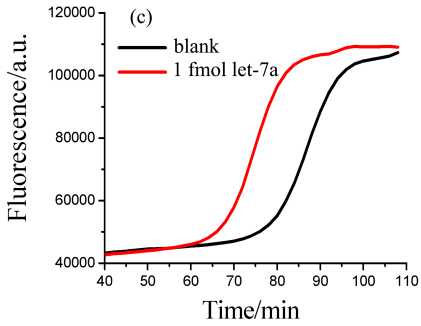
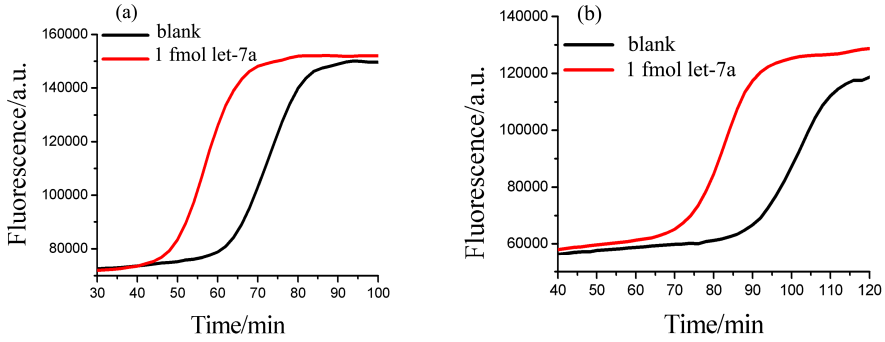


Fig. S1 *The influence of the temperature of LAMP reaction on miRNA detection. 1 fmol let-7a miRNA and the blank without miRNA were detected according to the experimental procedure described above except the temperature of LAMP reaction. The LAMP reactions were performed at (a) 50□, (b) 55□ and (c) 60□, respectively.*

Effect of the concentration of FIP and BIP primers on miRNA detection.

Fig. S2 shows the effect of the concentration of FIP and BIP primers used in LAMP reaction on miRNA detection. One can see from Fig. S2, with increasing the concentration of FIP and BIP primers used in LAMP reaction, the reaction time required for miRNA detection is shortened and the difference of POI value between 1 fmol let-7a miRNA and the blank is decreased. However, the difference of POI value between 1 fmol let-7a miRNA and the blank is shortened very little and the reaction time required for miRNA detection is shortened very much when the concentration of FIP and BIP primers is increased from 0.4 μM to 0.6 μM. When the concentration of FIP and BIP primers is increased from 0.6 μM to 0.8 μM, the reaction time is shortened about 30 min, but the difference of POI value between 1 fmol let-7a miRNA and the blank is decreased very much. Therefore, 0.6 μM each FIP primer and BIP primer was used in the LAMP reaction for miRNA detection.



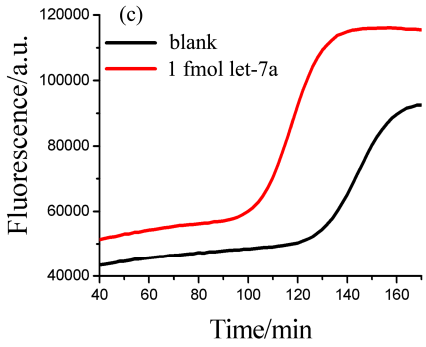
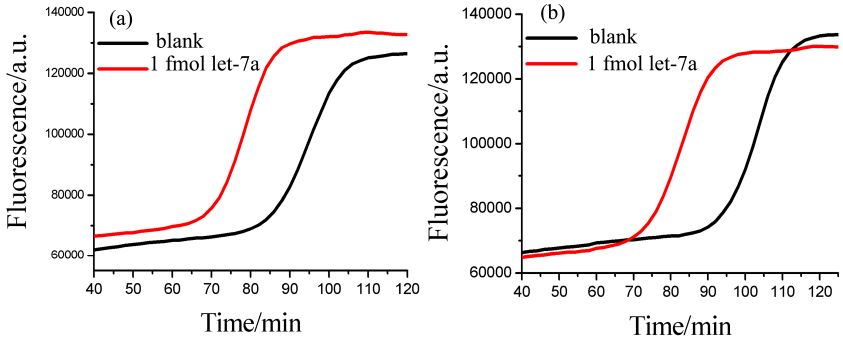


Fig. S2 *The effect of the concentration of FIP and BIP primers on miRNA detection. 1 fmol let-7a miRNA and the blank without miRNA were detected as described in the experimental procedure except the concentration of FIP and BIP primers used in the LAMP reaction, which is used as (a) 0.8 μM, (b) 0.6 μM and (c) 0.4 μM, respectively.*

Effect of B3 primer concentration on miRNA detection.

To investigate the effect of B3 primer concentration on miRNA detection, 1 fmol let-7a was detected by the LAMP-based assay by using different concentration of B3 primer in the LAMP reaction. As shown in Fig. S3, with decreasing the B3 primer concentration, the reaction time required for miRNA detection was increased. However, the increase of the reaction time was very little when the B3 primer concentration was less than 100 pM. The difference of the POI values between 1 fmol let-7a and the blank was increased when the B3 primer concentration was decreased from 1 nM to 100 pM, and almost kept a constant when the B3 primer concentration was in the range from 100 pM to 10 pM. In this paper, 50 pM B3 primer was used in the LAMP reaction for miRNA detection.



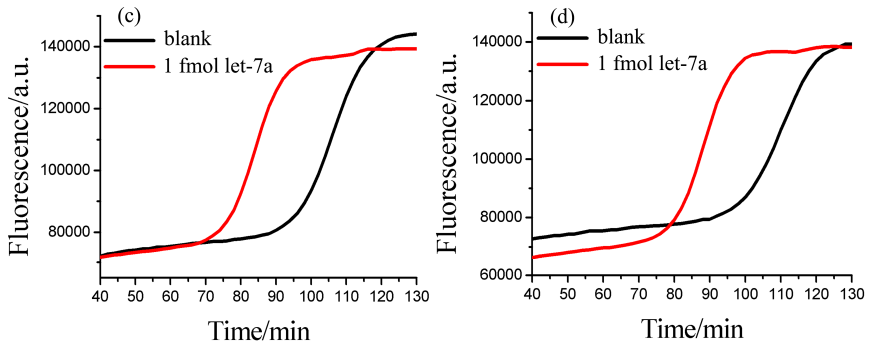
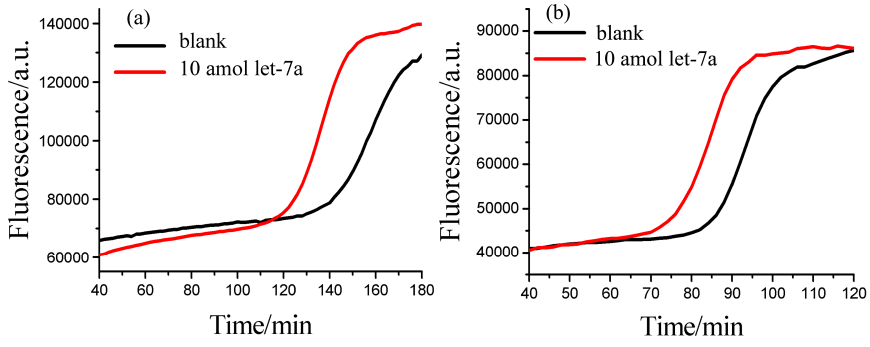


Fig. S3. *The effect of B3 primer concentration used in LAMP reaction on miRNA detection. The real-time fluorescence curves of the LAMP reaction were produced by 1 fmol let-7a miRNA and the blank. The concentration of B3 primer used in the LAMP reaction was (a) 1 nM, (b) 100 pM, (c) 50 pM, and (d) 10 pM, respectively. Other experimental conditions were the same as described in the experimental procedure.*

Influence of the amount of *Bst* DNA polymerase large fragment on miRNA detection.

Fig. S4 showed the influence of the amount of the *Bst* DNA polymerase large fragment used in the LAMP reaction on miRNA detection. With increasing the amount of *Bst* DNA polymerase large fragment, the reaction time required for miRNA detection was decreased and the difference of POI values between 10 amol miRNA and the blank was also decreased. Although the difference of the POI values reached its maximum when 2 U *Bst* DNA polymerase large fragment was used, the reaction time required for miRNA detection was very long. When the amount of *Bst* DNA polymerase large fragment was increased from 4 U to 6 U, the reaction time required for miRNA detection was shortened little. Therefore, 4 U *Bst* DNA polymerase large fragment was used in the LAMP reaction for miRNA detection.



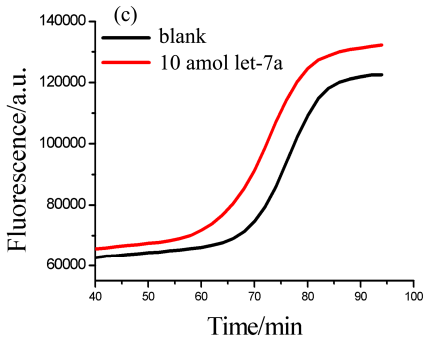
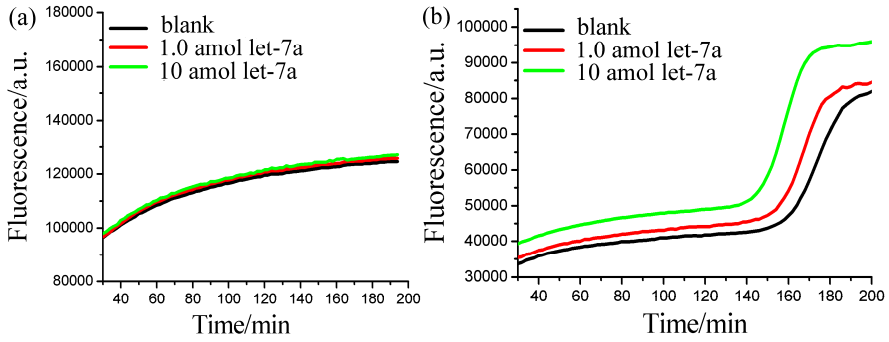


Fig. S4. *The influence of the amount of Bst DNA polymerase large fragment on miRNA detection. The real-time fluorescence curves were produced by 10 amol let-7a miRNA and the blank with LAMP reaction. The amount of Bst DNA polymerase large fragment used in the LAMP reaction was (a) 2U, (b) 4U, and (c) 6U, respectively. Other experimental conditions were the same as described in the experimental procedure.*

Influence of the fluorescence dye on miRNA detection

To investigate the influence of the fluorescence dye used for real-time measurement on miRNA detection, oligreen, picogreen and SYBR Green I (SG) are tested for miRNA detection with real-time measurement of LAMP products. Oligreen is a sensitive fluorescence dye to detect single-stranded (ss) DNA.[1] According to the principle of LAMP, the products of LAMP mainly are double-stranded (ds) DNA except the loop portion. Therefore, as shown in Fig. S5a, oligreen shows very weak signals for the real-time measurements, leading to low sensitivity for miRNA detection. Both picogreen and SG are very sensitive fluorescence dyes for detection of dsDNA with similar sensitivity.[2,3] However, SG shows higher selectivity for staining dsDNA.[4] As shown in Fig. S5b and Fig. S5c, the sensitivity for miRNA detection by using picogreen is similar to that by using SG. However, the reaction time required for miRNA detection is greatly increased by using picogreen. We attribute this time-increase to the very strong interaction between picogreen and dsDNA, which may inhibit the speed of DNA displacement. In addition, picogreen is more expensive than SG. Therefore, SG is adopted as the fluorescence dye for miRNA detection with the LAMP-based assay.



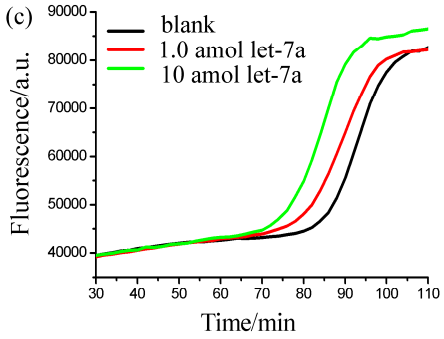


Fig. S5. *The influence of the fluorescence dyes on miRNA detection. The real-time fluorescence curves were produced by 10 amol let-7a miRNA, 1.0 amol let-7a and the blank with LAMP reaction. The fluorescence dye used for the real-time measurements was (a) oligreen, (b) picogreen, and (c) SYBR Green I, respectively. Other experimental conditions were the same as described in the experimental procedure.*

Estimation of interference for let-7a detection arisen from let-7b, c, d, and e.

From Fig. 2b, the correlation equation for let-7a determination in the amount range from 1.0 amol to 1.0 pmol can be obtained as $POI = -33.25 - 6.89\lg A_{miRNA}$. As shown in Fig. 3a, when the M sequence in template DNA is perfectly complementary to let-7a, the same amount of let-7a , b, c, d and e produced different real-time fluorescence signals. We supposed the *POI* corresponding to the signals of let-7a, b, c, d, and e is POI_a, POI_b, POI_c, POI_d and POI_e, respectively. According to the correlation equation, we suppose that POI_a, POI_b, POI_c, POI_d and POI_e are corresponding to let-7a amount as A_a, A_b, A_c, A_d and A_e, respectively. According to the correlation equation mentioned above, following equations can be obtained.

$$POI_b - POI_a = -6.89(\lg A_b - \lg A_a) \qquad (1)$$

$$\lg \frac{A_b}{A_a} = -\frac{POI_b - POI_a}{6.89} \qquad (2)$$

From Fig. 3a, one can see that POI_a, POI_b, POI_c, POI_d and POI_e are 68, 90, 90, 82 and 76, respectively. Therefore, $\frac{A_b}{A_a}$ can be calculated as 0.06%. According the same process, $\frac{A_c}{A_a}$, $\frac{A_d}{A_a}$, and $\frac{A_e}{A_a}$ can be calculated as 0.06%, 0.9% and 6.9%, respectively. Therefore, the interference for detection of let-7a arisen from the signals produced by let-7b, c, d and e was estimated to be 0.06%, 0.06%, 0.9%, 6.9%, respectively.

Determination of let-7a in total RNA sample

Human lung total RNA (1 μg/μL) was diluted to 100 ng/μL with DEPC-treated water. Then the 100 ng/μL total RNA sample was diluted to 10 ng/μL immediately prior to use. 1μL diluted RNA sample

solution containing 10 ng/μL total RNA was added to the mixture solution with the final volume 10 μL according to the experimental procedure described above. Additionally, 1 μL solutions containing 10 ng total RNA sample and 200 amol synthetic let-7a miRNA were used for the LAMP-based assay to further confirm the result. Simultaneously, 1.0, 50, 100, 500 and 1000 amol synthetic let-7a miRNA were used to constructed calibration curve under the same conditions. As demonstrated in Fig. S6a, the well-defined signal of let-7a in 10 ng total RNA samples can be detected. The determination results of let-7a content and the average recovery are demonstrated in the text of this paper.

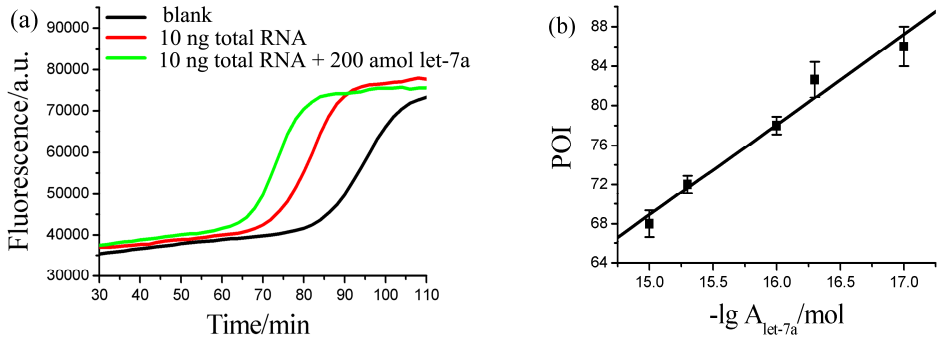


Fig.S6 *Detection of let-7a miRNA in total RNA sample. (a) The real-time fluorescence curves for detection of let-7a miRNA in 10 ng lung total RNA sample and 200 amol synthetic let-7a miRNA + 10 ng lung total RNA sample. (b) The relationship between POI values and –lg amounts of let-7a miRNA (1.0, 50, 100, 500 and 1000 amol). Values were obtained from triplicate determinations and shown with errors bars indicating the standard errors. The experimental conditions were the same as described in the experimental procedure described as above.*

Table S1. The comparison among LAMP, LCR, EXPAR, RCA, PCR, Northern blotting, and Northern blotting-based assays for miRNA detection.

Methods	Detection limit	Total RNA	Analysis time	Cost	Ref*
LAMP-based assay	1.0 amol	10 ng	2h	Lowest[a]	This work
LCR assay	3.5 amol	40 ng	4 h	Lower[b]	15
EXPAR assay	0.1 zmol	10 pg	1.5 h	Lower[c]	14
Branched RCA	8.5 amol	3 ng	8.5 h	Lower	13
Stem-loop RT-PCR	0.01 zmol	25 pg	6.5 h	High[d]	12a
LNA-blotting	1 fmol	2.5 μg	Over 48 h	High	5a
Microarray	0.2 amol	120 ng	Overnight	High	4c

Note: Some contents in the table were adapted from Ref. 15. [a,b,c] The cost per sample was about 0.54 USD for the LAMP-based assay, 2.2 USD for the LCR-based assay, and 1.2 USD for the EXPAR-based assay, respctively, which was calculated based on the selling price of the enzymes

and DNA primers and templates.[d] The cost was calculated as 7.5 USD per sample according to the selling price in China of the Kit for let-7a detection with stem-loop RT-PCR. Other costs were estimated based on the enzymes, DNA probes, and the instrumentation. *Ref represents the cited references in the text of this paper.

Reference

(1) H. Rhinn, , D. Schermana, and V. Escriou, *Anal. Biochem.,* 2008, **372**, 116-118

(2) F. Vitzthum, G. Geiger, H. Bisswanger, H. Brunner, and J. Bernhagen, *Anal. Biochem.*, 1999, **276**, 59-64.

(3) K. Matsui, N. Ishii, M. Honjo, Z. Kawabata, *Aquat. Microb. Ecol.,* 2004, **36**, 99-105.

(4) J. Wang, B. Liu, *Chem. Comm.*, 2008, 4759-4761.