Electronic Supplementary Information (ESI)

A Ligation-Based Loop-Mediated Isothermal Amplification (Ligation-LAMP) Strategy for Highly Selective MicroRNA Detection

Wenfang Du, Mengmei Lv, Junjie Li, Ruqin Yu, and Jianhui Jiang **

State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, P. R. China.

* Corresponding authors. E-mail: jianhuijiang@hnu.edu.cn; rqyu@hnu.edu.cn. Tel.: 86-731-88822577; Fax: 86-731-88822872.

Experimental Section

Materials and Reagents: *E. coli* poly(A) polymerase, M-MuLV reverse transcriptase, *Taq* DNA ligase, T4 DNA ligase, *Bst* DNA polymerase (large fragment), and ATP were obtained from New England Biolabs (Ipswich, MA, USA). HPLC-purified miRNAs, RNase inhibitor and RNase-free water were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The DNA probes, 5× TBE buffer (225 mM Tris-Boric Acid, 50 mM EDTA, pH 8.0) and dNTPs were obtained from Sangon Biotech Co., Ltd. (Shanghai, China). SYBR Green I (10,000× stock solution in dimethyl sulfoxide) was purchased from Life Technologies Corporation (USA). GeneRuler Ultra Low Range DNA Ladder was obtained from Thermo Fisher Scientific Inc. (Waltham, MA, USA). RPMI 1640 medium, penicillin, 15% heat-inactivated fetal bovine serum, streptomycin and DMEM high glucose medium were obtained from Thermo Scientific HyClone (MA, USA). Sequences of DNA probes and miRNAs are given in Table S1. All other reagents were of analytical grade and used without additional purification. RNase-free water was used in all experiments.

Detection of miRNA using Ligation-LAMP assay: The reverse transcription reaction was carried out in a volume of 10 μL containing 1 μL 10× M-MuLV Reverse Transcriptase Reaction Buffer (500 mM Tris-HCl, 750 mM KCl, 30 mM $MgCl_2$, 100 mM DTT, pH 8.3), 0.1 μL RNase inhibitor, 1 μL primer (100 nM), 1 μL ATP (10 mM), 1 μL dNTPs (10 mM), 1 μL *E. coli* poly(A) polymerase, 1 μL M-MuLV reverse transcriptase and different concentrations of target miRNA or extracted total RNA. The reaction was incubated at 37 °C for 1 h, 65 °C for 20 min. The ligation procedure was performed at 37 °C for 30 min, 95 °C for 5 min and 4 °C for 15 min in a 20 μL reaction mixture containing 1× *Taq* DNA ligase Reaction Buffer (20 mM Tris-HCl，25 mM KAc，10 mM $Mg(Ac)_2$, 10 mM DTT, 1 mM NAD, 0.1% Triton X-100, pH 7.6), 1 nM H1, 1 nM H2, 2 U/μL *Taq* DNA ligase and 10 μL reverse transcription products. After that, 12.5 μL of RNase-free water, 5 μL 10× themoPol® Reaction Buffer (200 mM Tris-HCl, 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM $MgSO_4$, 1% Triton® X-100, pH 8.8), 3 μL FP (5 μM), 3 μL BP (5 μM), 2.5 μL 10×

SG I, 3 μL dNTPs (10 mM), and 1 μL *Bst* DNA polymerase were added. The real-time fluorescence intensity was monitored at 63 °C in a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA) with a CFX96 in situ detection system at intervals of 30 s for 120 cycles employing the FAM/SYBR Green channel.

The 50 μL product obtained after 30 min was diluted to 100 μL using RNase-free water for typical fluorescence spectral measurements, which were recorded using a quartz cuvette on an F-7000 fluorescence spectrophotometer (Hitachi, Japan) at room temperature. The excitation wavelength was set at 495 nm, and the spectra were recorded from 505 nm to 600 nm with a slit of 5 nm for both excitation and emission.

Gel electrophoresis analysis: The Ligation-LAMP reaction products obtained after 30 min were analyzed using 3% agarose gel electrophoresis which was stained by 0.5 μg/mL GoldView and 0.5 μg/mL ethidium bromide. The gel Electrophoresis was carried out at a constant voltage of 101 V for 90 min in 0.5× TBE buffer at room temperature with a load of 10 μL of each sample into the lanes. The image of the gel was visualized using a Tanon 4200SF gel imaging system (Tanon Science & Technology Co., Ltd., China).

Cell culture and total RNA extraction: Human mammary epithelial cell line (MCF-10A), human fibrosarcoma cell line (HT 1080), human cervical carcinoma cell line (HeLa), and human breast cancer cell line (MCF-7) were cultured. MCF-7, MCF-10A, and HeLa cells were cultured in RPMI 1640 medium supplemented with 15% heat-inactivated fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. HT 1080 cells were cultured in DMEM high glucose medium. All of the cells were cultured at 37 °C in a humidified incubator containing 5% CO_2. Total RNA were extracted from each cell line by employing the UNlQ-10 column Trizol total RNA purification kit (Sangon Biotech Co., Ltd, Shanghai, China). The extracts were stored at -80 °C for analysis by utilizing our proposed method.

Detection of miRNA using qRT-PCR assay: The cDNA synthesis reaction was carried out using AMV First Strand cDNA Synthesis Kit (BBI, Toronto, Canada) in a volume of 20 μL containing 4 μL 5× Reaction Buffer, 2 μL total RNA, 1 μL reverse transcription primer (5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGA

GTCAACATCA-3′), 2 μL dNTPs, 1 μL RNase inhibitor, and 2 μL AMV reverse transcriptase. The reaction was incubated at 37 °C for 5 min, 42 °C for 60 min, 70 °C for 10 min. The qRT-PCR was performed in a volume of 20 μL containing 10 μL SybrGreen qPCR Master Mix，1 μL 10 μM primer F (5′-ACACTCCAGCTGGGTAGCTTATCAGACTG-3′), 1 μL 10 μM primer R (TGGTGTCGTGGAGTCG), 1 μL reverse transcription products, and 7 μL RNase-free water. The reaction was incubated at 95 °C for 2 min followed by 40 cycles of 95 °C for 10 s, 60 °C for 40 s.

Table S1. Sequences of miRNAs and DNA probes

Name	Sequence (5'-3')
miR-21	UAGCUUAUCAGACUGAUGUUGA
cmiR-21	UCAACAUCAGUCUGAUAAGCUA
SM miR-21	UAGCUUAUCACACUGAUGUUGA
2M miR-21	UAGCUUAUCACACUGAUUUUGA
let-7a	UGAGGUAGUAGGUUGUAUAGUU
miR-141	UAACACUGUCUGGUAAAGAUGG
primer	TTTTTTTTTTTCAACA
H1	AAGCCCCATTGGAGGCATATCCTTGGATCTTGCGGATGTCA CCTGTCTTGTCTATATGCCTCCAATGGGGCTTTTTTAGCTTA TCAG
H2	phosphate-ACTGATGTTGATTTCTGGGCATATTCGGGAGCAATTTTGAG GTTCTTGCCGAGGTGTATACATTGACTGCTTGCTCCCGAATA TGCCCAG
FP	CTGGGCATATTCGGGAGCAAGCAGTCAATGTATACACCTCG
BP	AAGCCCCATTGGAGGCATATCCTTGGATCTTGCGGATG

The underlined bases in SM miR-21 and 2M miR-21 are the mismatched bases. The sequences of H1 and H2 marked in purple are complementary to the cDNA of the target miRNA. The green region of H1 is the same with BP. The red bases of H2 are complementary to FP. The stem regions of H1 and H2 are underlined and double underlined, respectively.

Table S2. Comparison of the proposed method with other assays

Methods	Sensitivity (limit of detection)	Specificity	Complexity	Experimental cost	Ref.
Hairpin Probe-Based Circular Exponential Amplification Assay	0.38 pM	High specificity	Complex design of multiple probes	High cost: dual labeling probe	1
Silver Nanocluster DNA Probe	20 nM	High specificity toward detecting specific miRNA sequences	Complex synthesis of silver nanocluster	Not defined	2
Electrochemical Biosensing Strategy Based on Hybridization Chain Reaction	1 pM	High specificity	Complex electrode pretreatment	Low cost	3
LAMP	1 amol	High selectivity with single mismatch discrimination	Low complexity	Low cost	4
Duplex-Specific Nuclease Signal Amplification Using Taqman probe	100 fM	High specificity with discrimination between similar sequences	Low complexity	High cost: dual labeling probe	5
DNA−Gold Nanoparticle Probes	5 - 8 pM	Not defined	Complex preparation of oligonucleotide-functionalized AuNPs	High cost: fluorescently labeled DNA probe	6
Cyclic Enzymatic Amplification Method	9 pM	High specificity with single base selectivity	Low complexity	High cost: probe labeling	7
Duplex Specific Nuclease Signal Amplification Using Molecular Beacons	0.4 pM	High specificity with discrimination between similar sequences	Complex labeling of DNA probe	High cost: probe labeling	8
SERS Based on Silver Nanorod Array	28 nM	Not defined	Complex synthesis and treatment of silver nanorod	High cost	9
Ligation-LAMP	0.2 fM (2 zmol)	Very high specificity with a discrimination ratio over 300 for single-base mismatch	Low complexity with simple probe design but three incubation steps	Not high cost, three enzymes but label-free probes	This method

References

1. G.-l. Wang and C.-y. Zhang, *Anal. Chem.*, 2012, **84**, 7037-7042.
2. S. W. Yang and T. Vosch, *Anal. Chem.*, 2011, **83**, 6935-6939.
3. T. Hou, W. Li, X. Liu and F. Li, *Anal. Chem.*, 2015, **87**, 11368-11374.

4. C. Li, Z. Li, H. Jia and J. Yan, *Chem. Commun.* 2011, **47**, 2595-2597.
5. B.-C. Yin, Y.-Q. Liu and B.-C. Ye, *J. Am. Chem. Soc.*, 2012, **134**, 5064-5067.
6. F. Degliangeli, P. Kshirsagar, V. Brunetti, P. P. Pompa and R. Fiammengo, *J. Am. Chem. Soc.*, 2014, **136**, 2264-2267.
7. L. Cui, X. Lin, N. Lin, Y. Song, Z. Zhu, X. Chen and C. J. Yang, *Chem. Commun.*, 2012, **48**, 194-196.
8. X. Lin, C. Zhang, Y. Huang, Z. Zhu, X. Chen and C. J. Yang, *Chem. Commun.*, 2013, **49**, 7243-7245.
9. J. D. Driskell and R. A. Tripp, *Chem. Commun.*, 2010, **46**, 3298-3300.

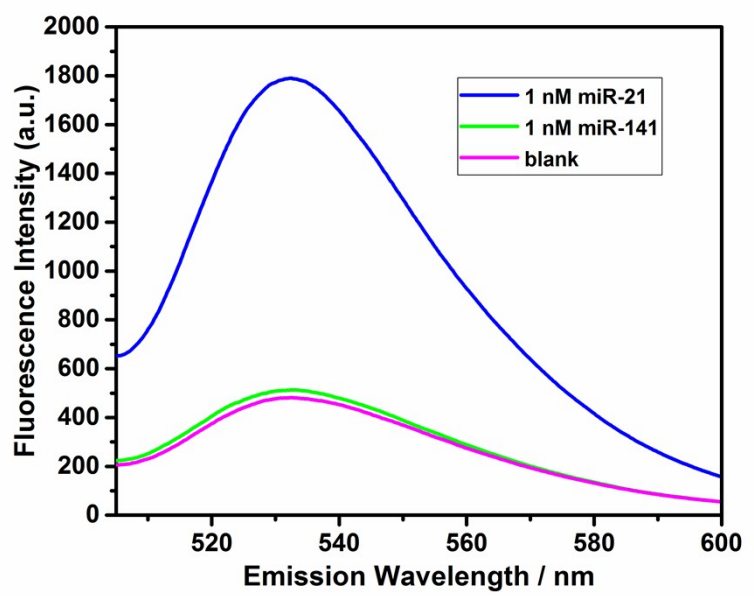

Fig. S1 Typical fluorescence spectra upon 1 nM miR-21, 1 nM miR-141 and no miRNA target, respectively. (Reaction time was 30 min).

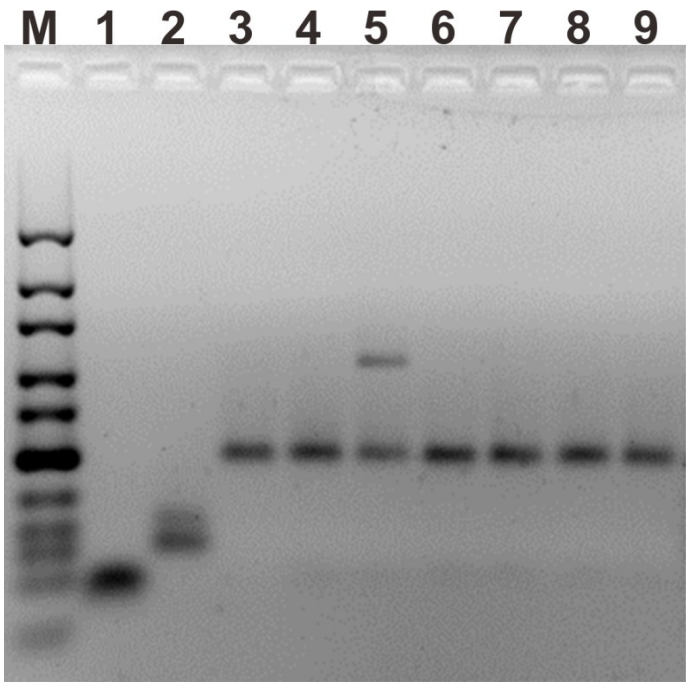

Fig. S2 Gel electrophoresis image for the ligation products of H1 and H2. Lane 1: 5 μM cDNA of miR-21; Lane 2: 1 μM FP + 1 μM BP; Lane 3: 0.2 μM H1 + 0.2 μM H2; Lane 4: 0.2 μM H1 + 0.2 μM H2 + 1 μM cDNA of miR-21; Lane 5: 0.2 μM H1 + 0.2 μM H2 + 1 μM cDNA of miR-21 + 2 U/μL *Taq* DNA ligase; Lane 6: 0.2 μM H1 + 0.2 μM H2 + 1 μM cDNA of SM-21; Lane 7: 0.2 μM H1 + 0.2 μM H2 + 1 μM cDNA of SM-21 + 2 U/μL *Taq* DNA ligase; Lane 8: 0.2 μM H1 + 0.2 μM H2 + 1 μM cDNA of 2M-21; Lane 9: 0.2 μM H1 + 0.2 μM H2 + 1 μM cDNA of 2M-21 + 2 U/μL *Taq* DNA ligase; Lane M: DNA marker (10-300 bp).

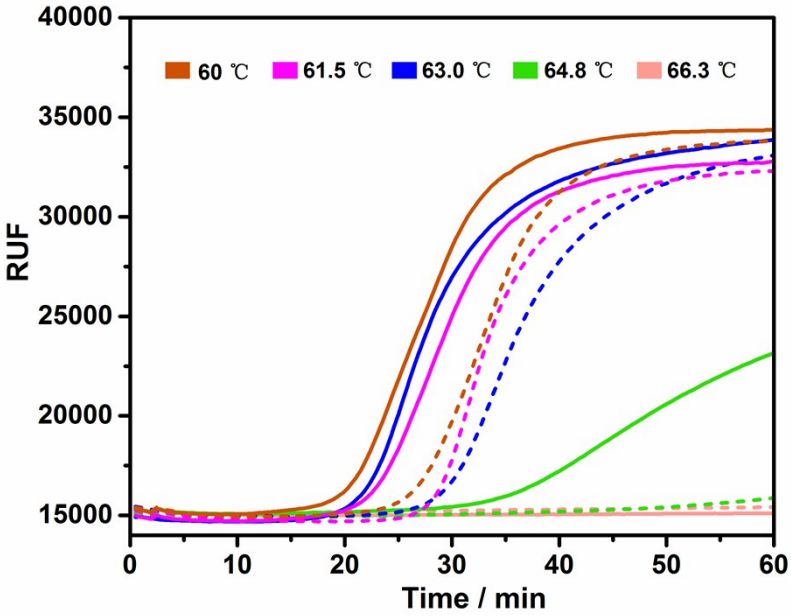

Fig. S3 The representation of the Ligation-LAMP reaction upon different temperatures in the presence (solid line) or absence (dash line) of miR-21 (1 pM).

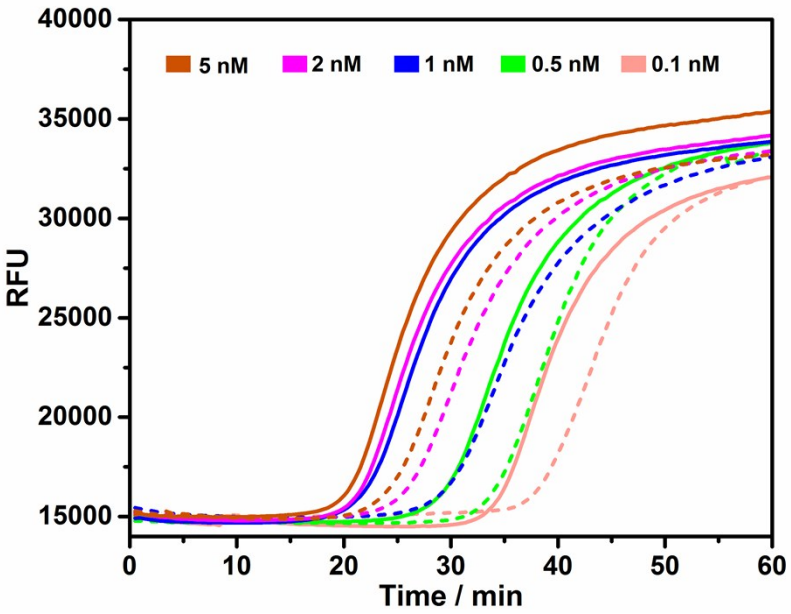

Fig. S4 The representation of the Ligation-LAMP reaction upon various concentrations of probes H1 and H2 in the presence (solid line) or absence (dash line) of miR-21 (1 pM).

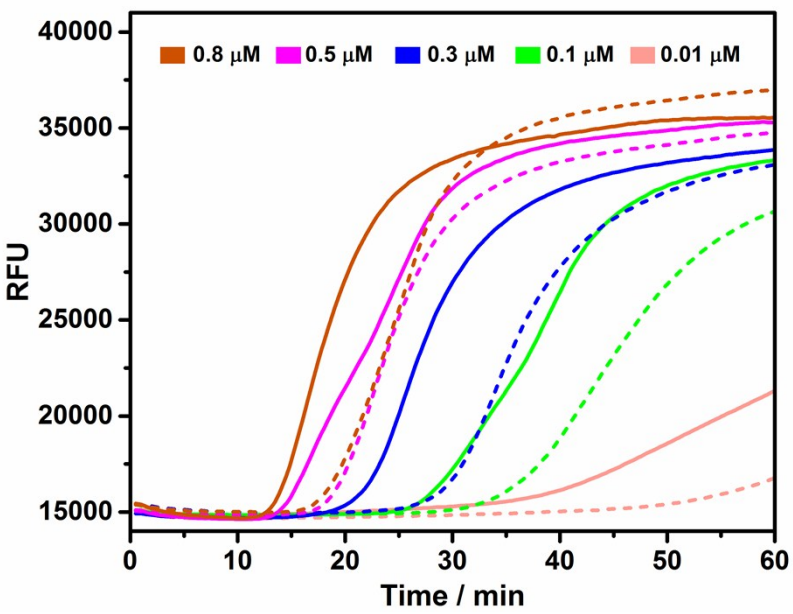

Fig. S5 The representation of the Ligation-LAMP reaction upon different concentrations of FP and BP in the presence (solid line) or absence (dash line) of miR-21 (1 pM).

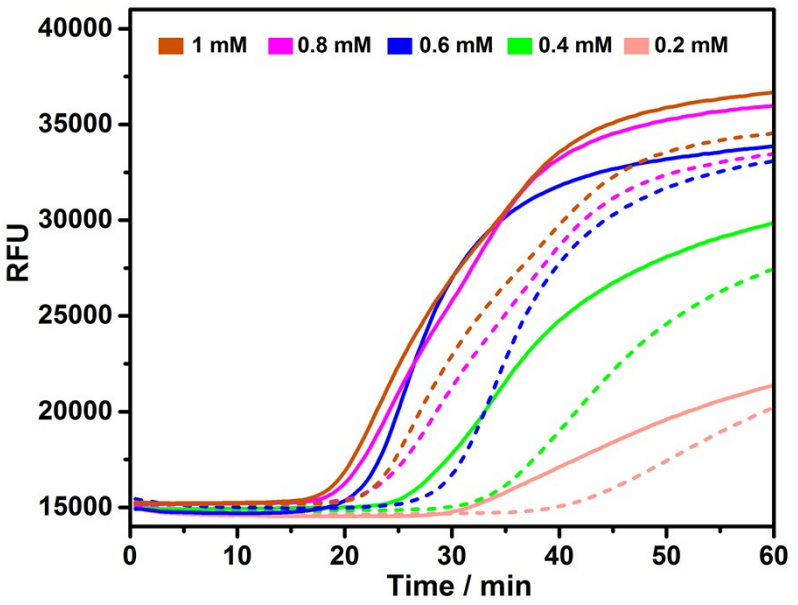

Fig. S6 The representation of the Ligation-LAMP reaction upon different concentrations of dNTPs in the presence (solid line) or absence (dash line) of miR-21 (1 pM).

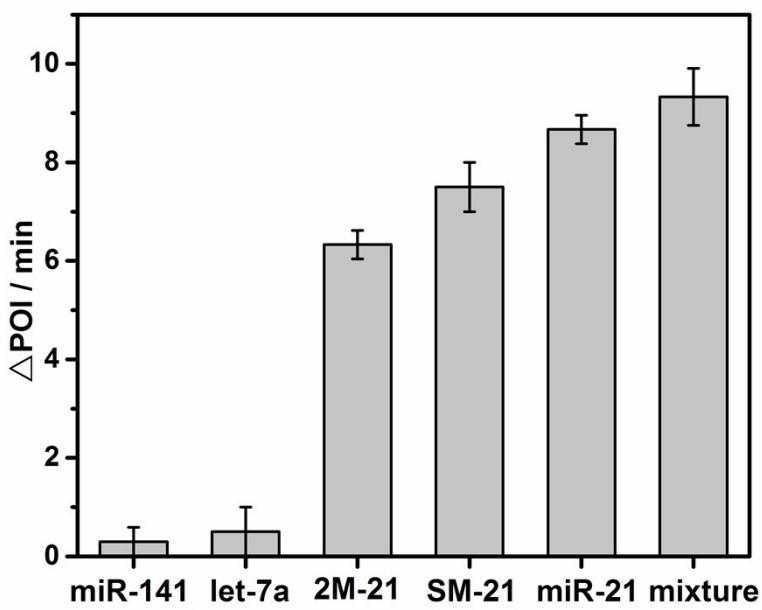

Fig. S7 Selectivity of the Ligation-LAMP assay using T4 DNA ligase for miRNA detection. MiRNA concentration is 1 pM. Error bars are standard deviation of three repetitive experiments.

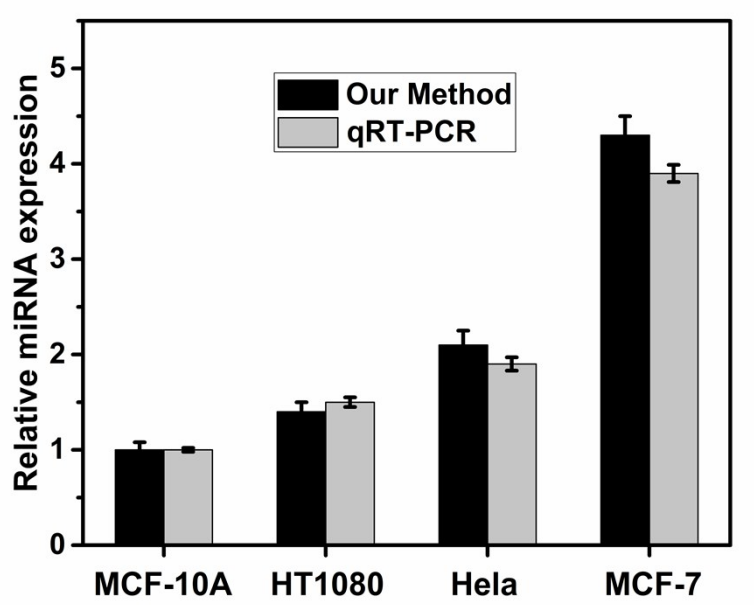

Fig. S8 Detection of the relative expression levels of miR-21 in total RNA samples from four human cancer cell lines. Bars represent the relative expression levels of different cell lines versus MCF-10A cell line determined using our method and qRT-PCR method. Error bars are the standard deviation of three repetitive experiments.