# Electronic Supplementary Information

## Ultrasensitive multiplexed microRNA detection with multiplex ligation-dependent probe amplification (MLPA) using ribonucleotide-modified DNA probes

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# Supporting Information

## 1. Materials and reagents.

Taq Platinum DNA Polymerase was purchased from Tiangen Biotech Co., Ltd. (Beijing, China). T4 RNA ligase 2 and T4 RNA ligase reaction buffer were obtained from New England Biolabs. HPLC-purified miRNAs, ribonucleotide-modified DNA probe, dNTPs, Ribonuclease inhibitor and DEPC-treated water were purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). PAGE-purified DNA oligonucleotides were obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The sequences of all RNA and DNA used were listed in Table S1 and S2. SYBR Green I (20 × stock solution in dimethyl sulfoxide, 20 μg/mL) was purchased from Xiamen Bio-Vision Biotechnology (Xiamen, China). The Hi-Di formamide and the GeneScan LIZ 120 Size Standard were purchased from Applied Biosystems, USA. All the solutions for ligation reactions were prepared in DEPC-treated deionized water. All other reagents were of analytical reagent grade and used as purchased without further purification.

**Table S1. The sequences of let-7 miRNA family members and miRNAs used for multiplex ligation reactions.**

| miRNA | sequence |
|---|---|
| let-7a | 5′-UGAGGUAGUAGGUUGUAUAGUU-3′ |
| let-7b | 5′-UGAGGUAGUAGGUUGUGUGGUU-3′ |
| let-7c | 5′-UGAGGUAGUAGGUUGUAUGGUU-3′ |
| let-7d | 5′-AGAGGUAGUAGGUUGCAUAGU-3′ |
| let-7e | 5′-UGAGGUAGGAGGUUGUAUAGU-3′ |
| let-7f | 5′-UGAGGUAGUAGAUUGUAUAGUU-3′ |
| let-7g | 5′-UGAGGUAGUAGUUUGUACAGU-3′ |
| let-7i | 5′-UGAGGUAGUAGUUUGUGCUGU-3′ |
| mir-92a | 5′-UAUUGCACUUGUCCCGGCCUGU-3′ |
| mir-31 | 5′-AGGCAAGAUGCUGGCAUAGCU-3′ |
| mir-143 | 5′-UGAGAUGAAGCACUGUAGCUC-3′ |
| mir-145 | 5′-GUCCAGUUUUCCCAGGAAUCCCU-3′ |

**Table S2. The sequences of DNA probes used for multiplex ligation reactions, length of ligation products, and the primers for multiplex PCR amplification.**

| Target | Probe A | Probe B | Length of ligation product |
|---|---|---|---|
| mir-92a | 5′-GGGATACTGGAACCTGATGATGACATCTAACAGGCCGGrGrA-3′[α] | 5′-phosphate-CAAGTGCAATAATTCTCACACTCTATGCTTGCTACCGTCG-3′ | 80nt |
| mir-31 | 5′-GGGATACTGGAACCTGATGATGACATTGACAGCTATGCrCrA-3′[α] | 5′-phosphate-GCATCTTGCCTCACGACACTATCACACTCTATGCTTGCTACCGTCG-3′ | 86nt |
| mir-143 | 5′-GGGATACTGGAACCTGATGATGACAATAACGAGCTACArGrT-3′[α] | 5′-phosphate-GCTTCATCTCACTACACATTACCTCATTCACACTCTATGCTTGCTACCGTCG-3′ | 92nt |
| mir-145 | 5′-GGGATACTGGAACCTGATGATGACACGTAAGGGATTCCrTrG-3′[α] | 5′-phosphate-GGAAAACTGGACATACAACCACTATACACAACCTCACACTCTATGCTTGCTACCGTCG-3′ | 98nt |
| let-7a | 5′-GGGATACTGGAACCTGATGATGACTAACTAACTACCTGGCTGATAACTATACArArC-3′[α] | 5′-phosphate-CTACTACCTCACCTTTGCTTTACTACTCTCACACACTCTATGCTTGCTACCGTCG-3′ | 110nt |
| forward primer | 5′-CGACGGTAGCAAGCATAGAGTGTG-3′ | | |
| reverse primer | 5′-FITC-GGGATACTGGAACCTGATGATGAC-3′ | | |
| Internal standard | 5′-FITC-AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA-3′ | | |

Note. The underlined bases are primer specific sequences, the dotted bases are target specific sequences and the double underlined bases are stuffer sequences. The length of ligation products is equal to the length of PCR products.

[α] The letter "r" indicates "ribonucleotide".

## 2. Total RNA extraction

Human lung adenocarcinoma epithelial cell line A549 was obtained from Hebei Medical University and cultured in RPMI 1640 (GIBCO, Cat. 31800-022), which contains 10% (v/v) fetal bovine serum, 0.2% $NaHCO_3$, 100 U/mL penicillin, 100 μg/mL streptomycin and 3 mmol/L L-glutamine. Total RNA was isolated from cells using Trizol® Reagent (Invitrogen, Beijing, China) following the manufacturer's protocol. The concentration was determined by the absorption at 260 nm with TU-1901 UV-VIS spectrophotometer (PGeneral, Beijing, China).

## 3. Standard experimental procedures for MLPA-based multiplexed miRNA assay.

**Ligation of DNA probes templated by miRNA**.

The ligation reaction mixture contains 1 ~ 4 nM probe A and probe B which are corresponding to the five target miRNAs: mir-92a, mir-31, mir-143, mir-145 and let-7a, respectively, 20 U Ribonuclease inhibitor and 0.5 μL ligation buffer (50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, 400 μM ATP). After addition of five target miRNAs or total RNA samples, the reaction mixture was heated for 2 min at 65°C and then incubated for 30 min at 39°C for hybridization. After hybridization, 1 U T4 RNA ligase 2 and 0.5 μL ligation buffer was added into the mixture to a final volume of 10 μL. Then the reaction was performed by incubation for 35 min at 39°C, followed by heat inactivation of the ligase at 90°C for 4 min. The ligation products were put on ice immediately, ready to be amplified by PCR.

**Multiplex PCR**.

Multiplex PCR was performed to amplify the ligation product using universal primers (see the sequences in Table S2) in a single tube. 2 μL of the ligation product was added to PCR reaction mixture with a final volume of 20 μL. The PCR reaction mixture consisted of 1 U Taq Platinum DNA Polymerase, 0.2 μM forward and reverse primer, 50 μM dNTPs, 50 nM Internal Standard in a reaction buffer (200 mM Tris-HCl, pH 8.4, 200 mM KCl ,100 mM $(NH_4)_2SO_4$, 15 mM $MgCl_2$ ). The PCR reaction was performed with a 2720 thermocycler (Applied Biosystems, USA) at following thermal cycles: denaturation at 94°C for 5 min, followed by 32 cycles of 94°C for 30 s and 60°C for 30 s.

**Analysis of PCR products.**

The PCR products were diluted 100 times using sterilized water, and a volume of 1 μL was taken out and then mixed with 19 μL Hi-Di formamide and 0.1 μL of GeneScan 120 LIZ Size Standard (Applied Biosystems). The sample was first incubated at 95°C for 5 min for denaturation and then allowed to stand on ice for 10 minutes. Afterward, capillary electrophoresis was performed on ABI 310 Genetic Analyzer (Applied Biosystems, USA), and the parameters for each run are: injection time 10 s, injection voltage 7 kV , run voltage 10 kV, temperature 60°C, and run time 26 min.

## 4. The electropherograms of individual miRNA detection by using the MLPA-based assay

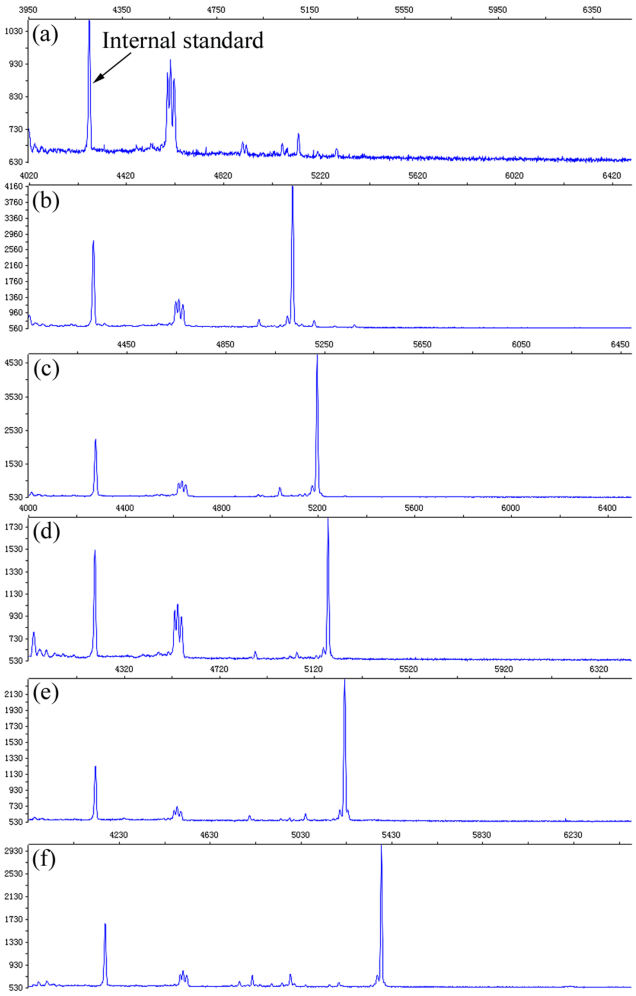


**Fig. S1.** The electropherograms of individual detection of mir-92a, mir-31, mir-143, mir-145 and let-7a, respectively. The concentration of each miRNA is 20 fM. The procedures of the individual detection are the same as described in the experiment section mentioned above, expect that one reaction contains only one miRNA target.

## 5. Optimization of temperature for ligation reaction.

Due to the high similarity among miRNA sequences, the high specificity of miRNA assay for discriminating one-base differences between miRNAs is very important. The specificity of our MLPA-based miRNA assay largely depends on the selectivity of ligation reaction. The temperature plays an important role for the selectivity of ligation reaction. Therefore, the optimization of the temperature for the ligation reaction was investigated. Let-7a and let-7c are chosen as the model targets for the optimization because they are different only by one-base (see the sequences in Table S1) and they are most difficult to be discriminated among the let-7 miRNA

family members. The ligation reaction of probe A and probe B, in which the target-specific sequences are complementary to let-7a, is performed at 37°C, 39°C, 40°C and 41°C, respectively, templated by let-7a and let-7c. After ligation and PCR amplification, the PCR products are separated and detected by capillary electrophoresis. As shown in Fig. S2, when the ligation temperature is increased from 37°C to 39°C, the signal of let-7a decreases to 77.47%. However, the signal ratio of let-7a to let-7c is greatly improved from 44.25% to 25.53%. When the ligation temperature is continuously increased from 39°C to 41°C, the signal of let-7a gradually decreases, which can result in decreased sensitivity. However, no obvious improvement was observed for the signal ratio of let-7a to let-7c. Taking into account of both specificity and sensitivity for miRNA detection, 39°C is selected as the ligation temperature in this study.

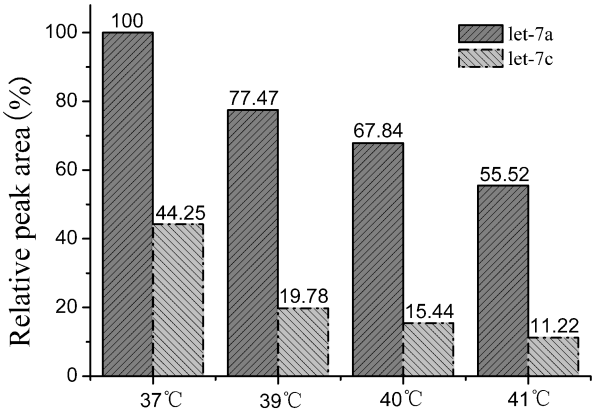


**Fig. S2.** Effect of the temperature of ligation reaction on the specificity of miRNA detection. The relative peak area is calculated from the electropherograms of each PCR product. The relative peak area produced by let-7a at the ligation temperature of 37°C is defined as 100%.

**6. The electropherograms of a series dilution of let-7a miRNA analyzed with the proposed assay.**

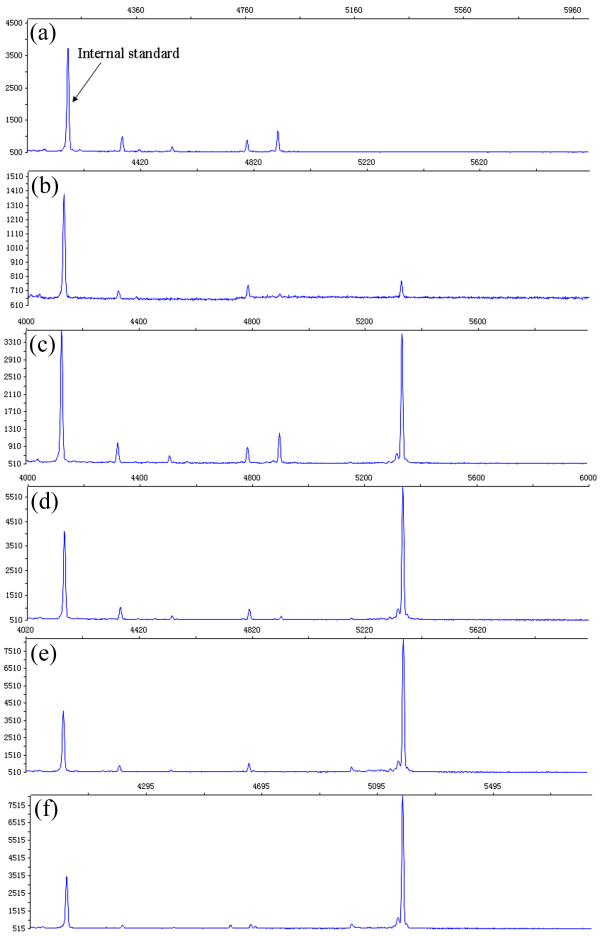


**Fig. S3.** The electropherograms for detection of let-7a at different concentrations. (a) blank, (b) 0.2 fM, (c) 1.0 fM, (d) 2.0 fM, (e) 10 fM, (f) 20 fM. The blank is detected in the same procedure but without addition of miRNA target. The experimental procedure is the same as described in Fig. S1 for let-7a detection.

**7. The linear relationship between relative peak area and log of let-7a concentration.**

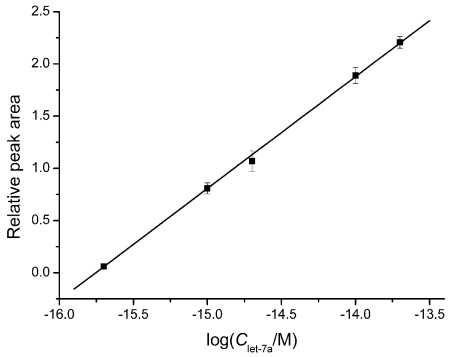

**Fig. S4.** The relationship between relative peak area and log of let-7a concentration (M). The concentration of let-7a is 0.2, 1.0, 2.0, 10, 20 fM, respectively. Error bars are estimated from four replicate measurements.