Supplementary Material

Microchip electrophoretic sensing of multiplex microRNAs based on a dual nucleic acid amplification

Qihui Xie, Shuang Tang, Jianan Lv, Xing Geng, Fan Zhang*, Qingjiang Wang*

School of Chemistry and Molecular Engineering, East China Normal University, 500 Dongchuan Road, Shanghai, 200241, PR China

*Corresponding author.

Tel.: +86 21 54340100. E-mail address: qjwang@chem.ecnu.edu.cn (Q. Wang)

Tel.: +86 21 54340049. E-mail address: fzhang@chem.ecnu.edu.cn (F. Zhang)

1. Experimental section

1.1. Material and apparatus

F-12K medium, fetal bovine serum (FBS), trypsin/EDTA solution, penicillin/streptomycin (P/S) and Dulbecco's phosphate buffered saline (PBS) were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). A549 were obtained from Chinese Academy of Sciences (Shanghai, China). SanPrep Column microRNA Extraction Kit, RNase Inhibitor, DEPC was provided by Shanghai Sangon Biotechnology Co. Ltd. Apurinic/apyrimidinic Endonuclease 1(APE 1) (10000 U/mL) with 10×NEBuffer 4(500 mM Potassium Acetate, 200 mM Tris-acetate, 100 mM Magnesium Acetate, 10 mM DTT, pH 7.9 at 25°C) were acquired from New England BioLabs (Beijing, China). Duplex-specific nuclease (DSN) (1000 U/mL) with 10×DSN master buffer (500 mM Tris-HCl, 50 mM $MgCl_2$, 10mM DTT, pH 8.0 at 25°C) was purchased from Evrogen (Moscow, Russia). TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH = 8.0), Water-DEPC Treated Water, NTP mixture (25 mM) was provided by Shanghai Sangon Biotechnology Co. Ltd. The DNA-500kit was purchased from Genesci Medical technology Co., Ltd. (Shanghai, China). SYBR Gold solution was purchased from Invitrogen Corporation (Karlsruhe, Germany). Ultrapure water (DI, ≥18.2 MΩ·cm) was obtained from Milli-Q water purification system (Millipore, Bedford, MA, USA). Avidin-modified magnetic beads (500 nm) were purchased from Bio Basic Inc. (BBI, Shanghai, China).

All DNA and miRNA oligonucleotides used in this study were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China) and purified by HPLC. DNA oligo was dissolved in TE buffer and miRNA oligo was dissolved in DEPC treated water. Their respective sequences are listed in **Table 1**.

1.2. Instruments

A549 cells were cultivated in a humidified incubator (NU-5500E, NuAire, America). The high speed refrigerated centrifuge was provided by Shanghai Anke Scientific Instrument Co., LTD (Shanghai, China). The metal bath and constant temperature incubator shaker were obtained from Shanghai Yiheng Scientific Instrument Co., LTD (Shanghai, China). The portable rapid fluorescent PCR instrument was purchased by Tianlong Technology Co., LTD (Xian, China). Mastersizer 3000+ laser particle size analyzer was obtained from Malvern Panalytical (Shanghai, China). B-600 ultramicrospectrophotometer was obtained from

Shanghai Metash Instruments Co., LTD (Shanghai, China).

The MultiNA MCE-202 (Shimadzu, Kyoto, Japan) was used to separate and detect all the DNA and RNA. Quartz microchips with cross-shaped microchannels were also purchased from Shimadzu. A blue LED (470 nm; 20 mA) apparatus was installed in the MCE instrument along with four microchips that were used for the simultaneous detection of samples. The microchannels were 23 mm in length for separation, 104 μm in width and 48 μm in depth. The MCE chip platform consists of a separation channel and four buffer reservoirs: the sample reservoir (S), sample waste reservoir (SW), separation buffer reservoir (B), and separation buffer waste reservoir (BW), which correspond to voltages V1, V2, V3, and V4, respectively. The instrument analyses the sample as follows: injection voltage V1 = 280 V, V2 = 510 V, V3 = 320 V, V4 =0 V, injection time is 50 s; separation detection voltage is V1 =250 V, V2 =250 V, V3 =0 V, V4 =1000 V, separation detection time is 135 s.

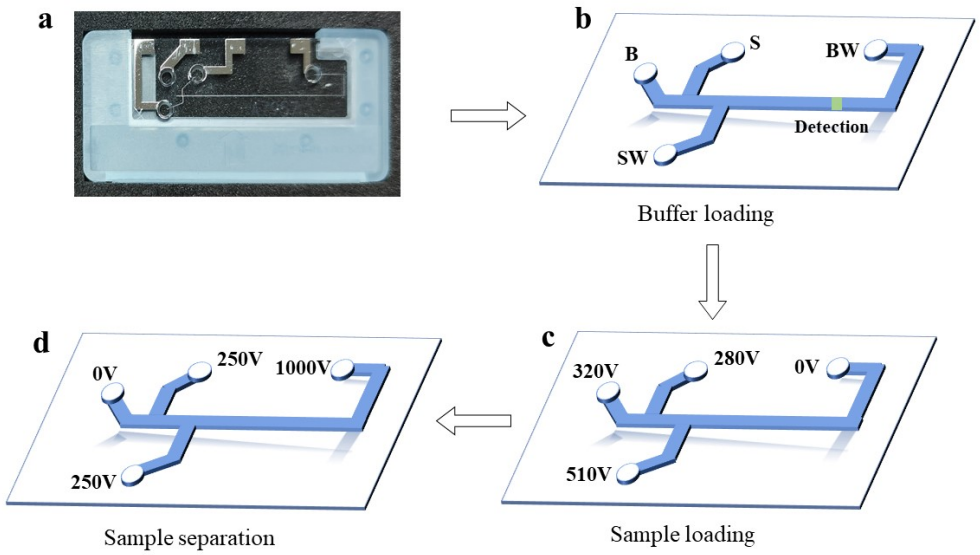

Fig. S1. Procedure for the MCE platform to detect DNA. (a) Appearance of the microchip. (b) Buffer loading. (c) Sample loading. (d) Sample separation and detection. S: sample reservoir; SW: sample waste reservoir; B: buffer reservoir; BW: buffer waste reservoir.

1.3. Cell culture

A549 cells were cultured at 37°C, 5% CO_2 in a carbon dioxide incubator for more than three generations, and then used for extraction experiments after all the cell components became stable. Afterward, the cells in monolayer cultures were collected.

1.4. MiRNAs extraction

The cells were lysed directly in the culture vessel (the culture area was 10 cm2, the number of cells was 10^7 approximately). SanPrep Column microRNA Extraction Kit was used to extract total miRNAs (the instruction of this Kit was listed in the Supplementary Material). 1 mL of miRNAExtractor was added per 10 cm2 in the culture vessel and blown up with a pipette. Cells were not cleaned before miRNAExtractor was added to avoid miRNA degradation. Then the lysed homogenate was placed at room temperature for 5–10 min to completely separate nuclear protein and nucleic acid. Next, 0.2 mL of chloroform was added, shaken vigorously for 30 s, placed at room temperature for 3 min, and centrifuged at 12,000 rpm for 10 min at 4 °C. Under experimental conditions, the relative centrifugal force is about 9117 g. After that, the sample will be divided into three layers: an upper aqueous phase, an intermediate and a lower organic phase, with the miRNA in the upper aqueous phase. After continuous elution with different proportions of ethanol, the miRNA was finally enriched in the center of the adsorption membrane in adsorption column and dissolved in 30 μL RNase-free water. The resulting miRNAs solution was stored at −80 °C or used for subsequent experiments.

2. Results and discussion

2.1. Analysis of DSN mediated cycle

As shown in **Fig. S2A**, the capture probe (pro-21) demonstrated excellent target-binding specificity through Watson-Crick base pairing. In a 1:1 probe-miRNA molar ratio system (lane b), 0.5 μM pro-21 completely hybridized with equimolar of miR-21, with no residual probe signal detected. Under conditions of miRNA excess (2:1 molar ratio, lane c), unbound probe residues were observed. Negative controls (lane d) confirmed no nonspecific cleavage of free probes by DSN enzyme (comparison of lanes a and d), ensuring a low false-positive signal. In the final reaction system (lane e), DSN-mediated specific cleavage generated a distinct product band.

Fig. S2B evaluated the target recycling capacity of DSN. In an equimolar reaction system (0.5 μM, lane b), DSN cleavage produced detectable products, and intact miR-21 was efficiently recycled (compared to the standard miRNA in lane d, 0.5 μM), with most of the target re-entering the reaction cycle. Increasing the concentration to 1 μM (lane c) enhanced

both product yield and miRNA recovery efficiency.

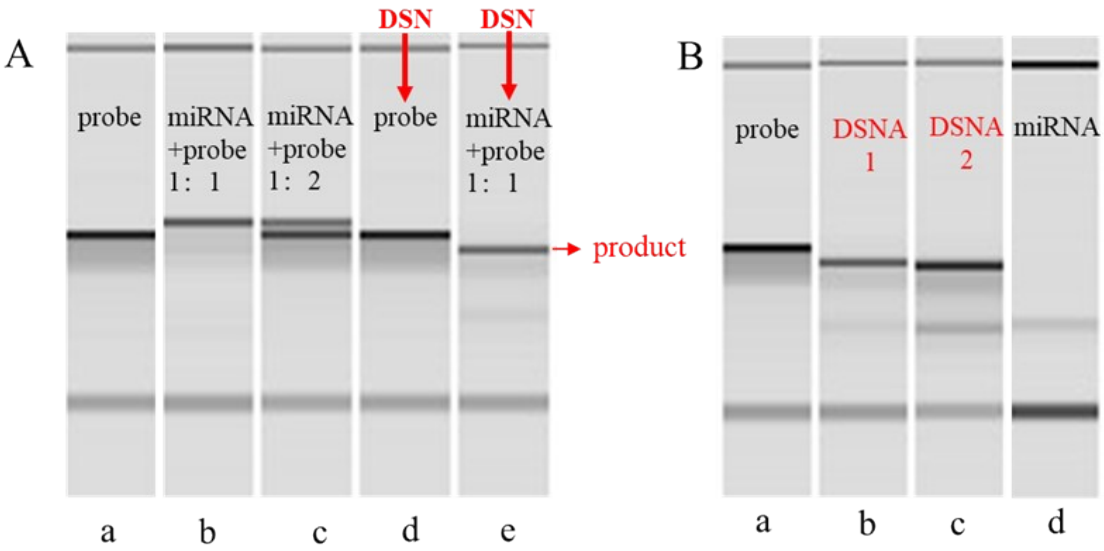

Fig. S2. Analysis of DSN-mediated recycling. (A) Gel images of the products at each step: (a) 1μM probe; (b) 0.5μM probe + 0.5μM miRNA; (c) 1μM probe + 0.5μM miRNA; (d) 1μM probe + DSN; (e) 0.5μM probe + 0.5μM miRNA+ DSN; (B) Gel images at each step: (a) 1μM probe; (b) 0.5μM probe + 0.5μM miRNA+ DSN +buffer; (c) 1μM probe + 1μM miRNA+ DSN +buffer; (d) 0.5μM miRNA.

2.2. Simulation concentration of the complex

It was difficult to achieve 100% hybridization, even with fully complementary sequences. NUPACK can provide this data of the preferential binding between nucleotide strands that may occur in practice. The data was about the binding situations, respectively, when the seven bipedal walking legs (DLS1) and S1 were in a 1:1 ratio of 1μM, with products including individual probes, individual walkers, double-strand complexes, triple-strand complexes, and other secondary structures. At a fixed total concentration, if there are more double-strands, there are fewer triple-strands, which does not allow for the maximization of walker efficiency. It demonstrated the ability of DLS1-4, DLS1, and DLS1-5 to form ternary complexes with S1, with the highest concentration of DLS1.

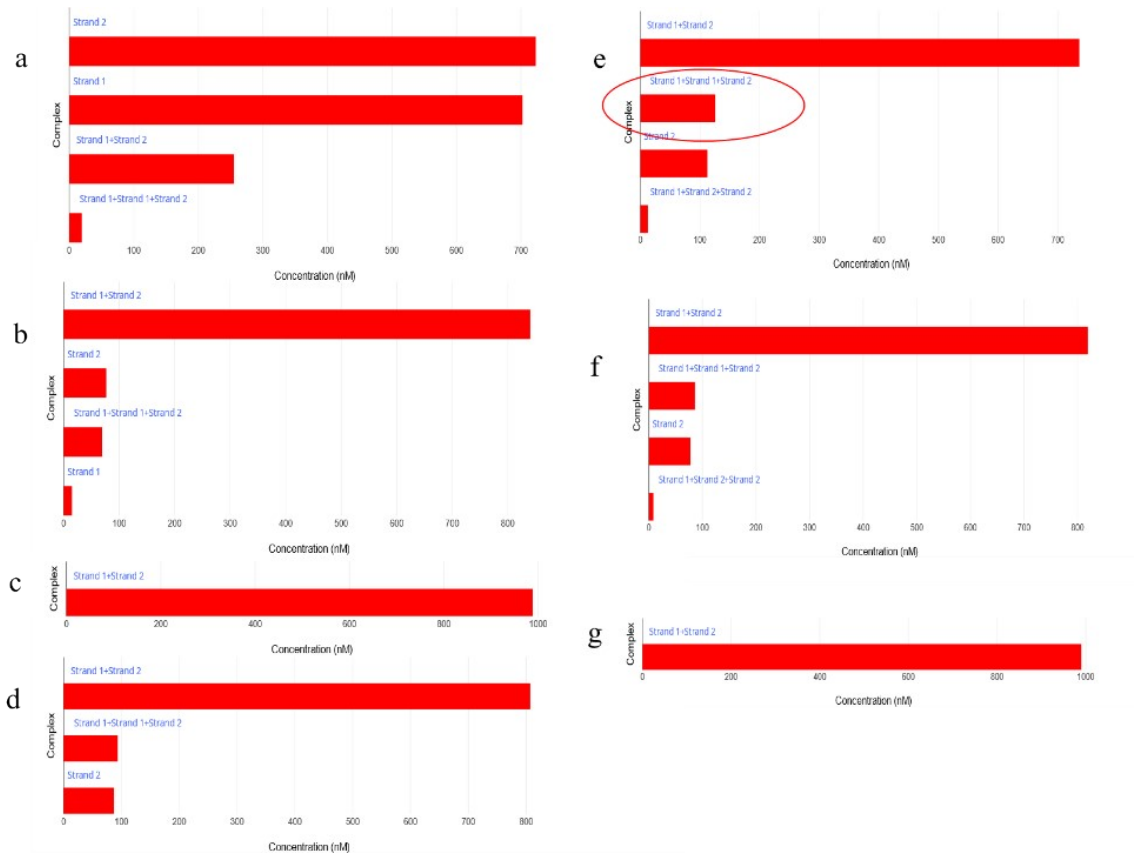

Fig. S3. The theoretical concentration of the secondary structures proved by NUPACK:(a) DLS1-1; (b) DLS1-2; (c) DLS1-3; (d) DLS1-4; (e) DLS1; (f) DLS1-5; (g) DLS1-6. Strand 1 represents S1and Strand 2 represents DLS1.

2.3. Simulation analysis of secondary structures

To ensure the affinity of the bipedal DNA walker, the binding structures of the substrate probe (S1and S2) and the walking legs (DLS1and DLS2) were simulated. The bipedal walking legs can form binary complexes as well as ternary complexes with the substrate probe. The simulated data of NUPACK are shown in **Fig. S4**.

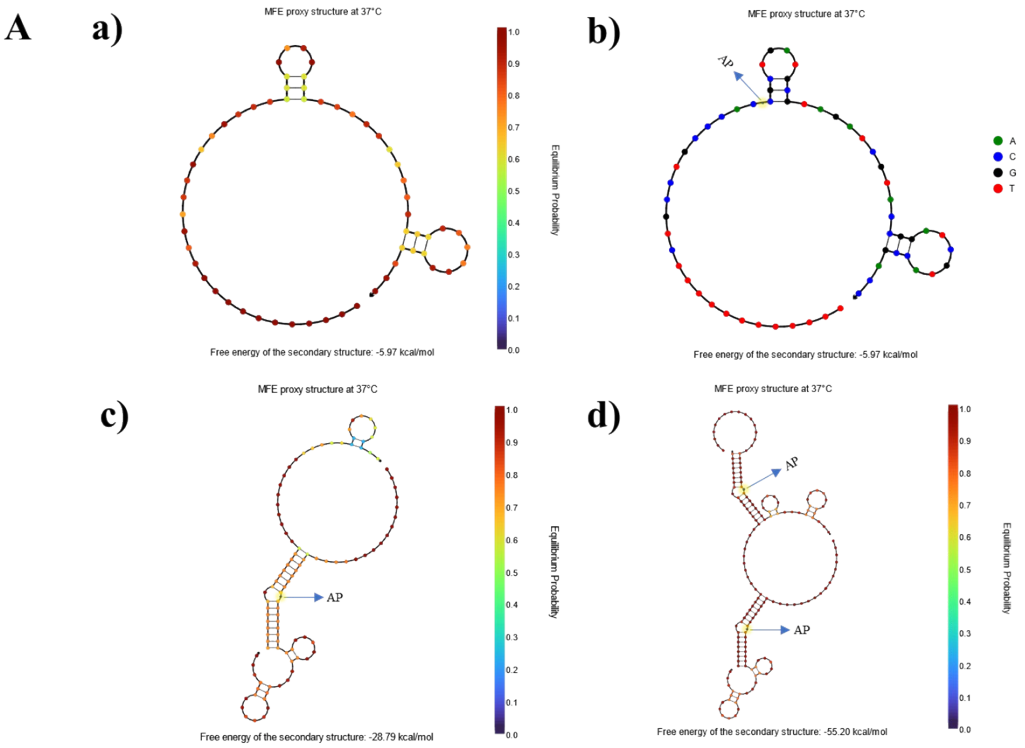

Fig. S4. Simulation analysis of secondary structures at 37℃. (A):(a) the structure of S1; (b) the nucleotide sequence of S1; (c) the structure of DLS1-S1; (d) the structure of S1-DLS1-S1.