Electronic Supplementary Information

Sensitive Detection of MicroRNAs by Duplex Specific Nuclease-Assisted Target Recycling and Pyrene Excimer Switching

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MATERIALS AND METHODS

Materials. All oligonucleotides (Table S1) were purchased from TaKaRa Bio. Inc. (Dalian, China). Duplex-specific nuclease (DSN) and 10× DSN master buffer (500 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$ and 10 mM DTT) were obtained from Evrogen Joint Stock Company (Moscow, Russia). The RNase inhibitor was obtained from New England Biolabs, Inc. (Ipswich, MA, USA). Diethylpyrocarbonate (DEPC) treated water (RNase free) was obtained from TaKaRa Bio. Inc. (Dalian, China). SYBR Gold was purchased from Life Technologies (Carlsbad, CA, USA). The silver staining kit was obtained from

Tiandz, Inc. (Beijing, China). All other chemicals were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO, USA).

Table S1. Sequence of the Oligonucleotides

note	sequence (5′−3′)
let-7a	UGA GGU AGU AGG UUG UAU AGU U
let-7b	UGA GGU AGU AGG UUG UGU GGU U
let-7c	UGA GGU AGU AGG UUG UAU AGU U
probe	AAC TATA CAA CCT ACT ACC TCA
reporter	Pyrene-CCT AGC TGA GGT AGT AGG TTG TAT AGT TGC TAG G-Pyrene

MicroRNA Assay. The experiments were performed in 20 μL of reaction solution containing DEPC treated water, 1× DSN master buffer (50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$ and 1 mM DTT), 50 mM NaCl, 0.1 U DSN, 20 U RNase inhibitor, 2 μM probe and different-concentration target microRNA, and incubated at 55 °C for 30 min. The reaction was terminated by 5 mM EDTA at 55 °C for 5 min to inactivate DSN enzyme activity. Then 20 μL of reaction products was incubated with 800 nM pyrene excimer-reporter reaction buffer containing 2.5 M NaCl at 95 °C for 4 min, followed by slowly cooling to the room temperature. The fluorescence spectra were measured by an F-7000 fluorescence spectrophotometer (Hitachi, Japan) equipped with a xenon lamp as the excitation source. The spectra were recorded in the range from 350 to 600 nm at an excitation wavelength of

340 nm. The excitation and emission slits were set for 10.0 and 10.0 nm, respectively. The fluorescence intensity at 398 nm and 485 nm was used for data analysis.

Gel Electrophoresis Analysis. The 12% nondenaturating polyacrylamide gel electrophoresis (PAGE) analysis was carried out in 1× TBE buffer (9 mM Tris-HCl, pH 8.0, 9 mM boric acid, 0.2 mM EDTA) at a 110 V constant voltage for 50 min at room temperature. The gel was stained with a silver staining kit (Tiandz Inc., Beijing, China) and visualized by a Kodak Image Station 4000 MM (Rochester, NY, U.S.A.).

Cell Culture and Preparation of Total RNA Samples. Human cervical cancer cell lines (Hela cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were incubated at 37 °C in a humidified chamber with 5% CO_2. The total RNA was extracted from Hela cells using the miRNeasy Mini Kit (Qiagen, German) according to the manufacturer's procedures, and the concentration of total RNA was determined by the NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE).

Recovery Assay. A total volume of 20 μL of sample mixture containing 1% serum spiked with various concentration target microRNA, 1× DSN master buffer (50 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$ and 1mM DTT), 50 mM NaCl, 0.1 U DSN, 20 U RNase inhibitor, 2 μM probe were incubated at 55°C for 30 min. The reaction was terminated by 5 mM EDTA at 55 °C for 5 min to inactivate DSN enzyme activity. Then 20 μL of reaction products was

incubated with 800 nM pyrene excimer-reporter and 26 μL of reaction buffer (22.4 μL of H_2O, 3.6 μL of 2.5 M NaCl) at 95 °C for 4 min, followed by slowly cooling to room temperature. The measurement was performed with same protocols described above.

SUPPLEMENTARY RESULTS

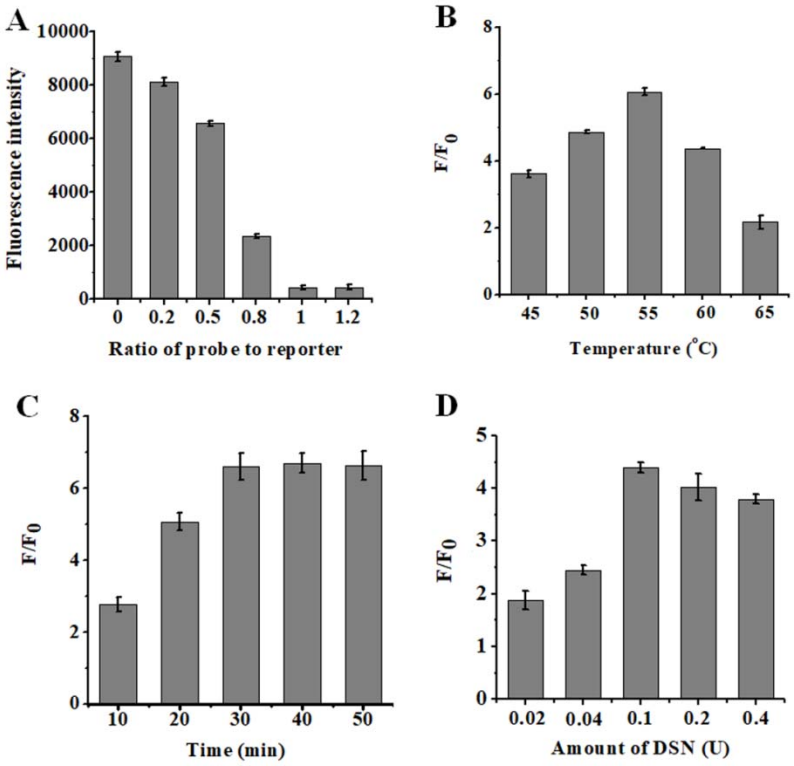

Fig. S1 (A) Variance of fluoresce intensity with the ratio of the probe to the reporter. (B) Variance of the F/F_0 ratio value with the reaction temperature. (C) Variance of the F/F_0 ratio value with reaction time. (D) Variance of the F/F_0 ratio value with the amount of DSN. Error bars show standard deviation of three independent experiments.

Under the optimally experimental conditions, the hybridization of the reporter with the probe may prevent the formation of pyrene excimer (Scheme 1). Consequently, the ratio of

the probe to the reporter may significantly influence the background signal and should be optimized. As shown in Fig. S1A, the fluorescence intensity decreases with the increasing probe-to-reporter ratio from 0 to 1:1 at a fixed reporter concentration of 800 nM, and levels off at the ratio of 1:1, suggesting the transformation of all on-state reporters to off-state ones at the ratio of 1:1. Therefore, the probe-to-reporter ratio of 1:1 is used in the subsequent experiments.

We further optimized the reaction temperature. As shown in Fig. S1B, the F/F_0 ratio value (where F and F_0 are the fluorescence intensity in the presence and in the absence of target let-7a, respectively) enhances with the increasing reaction temperature from 40 to 55 °C, followed by the decrease beyond 60 °C. This may be explained by the loss of DSN activity at high temperature beyond 60 °C. Therefore, the reaction temperature of 55 °C is used in the subsequent experiments.

We investigated the effects of DSN amount on the assay performance as well. As shown in Fig. S1C, the F/F_0 ratio value improves with the increasing DSN amount from 0.02 to 0.1 U, followed by the decrease beyond the amount of 0.1 U. Therefore, 0.1 U DSN is used in the subsequent experiments.

We further investigated the influence of reaction time on the assay performance. As shown in Fig. S1D, F/F_0 ratio value increases with reaction time, and reaches a plateau within 30 min. Therefore, the reaction time of 30 min is used in the subsequent experiment. Notably, the reaction time involved in this research is much shorter than those of the reported isothermal nucleic acid amplification-based approaches, such as branched

RCA-based assay (8 h),[1] LAMP-based assay (100 min),[2] and hairpin probe-based RCA assay (60 min).[3]

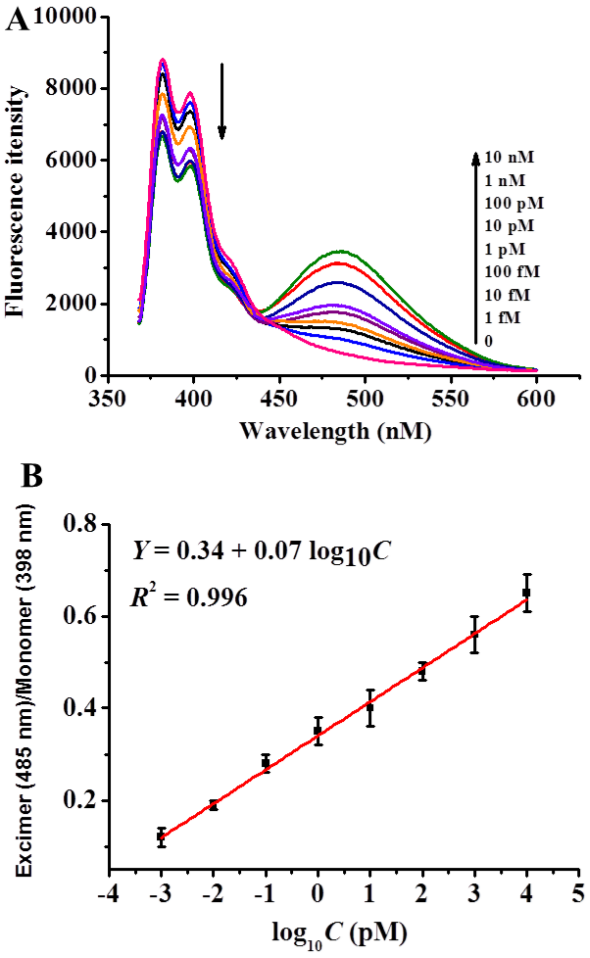

Fig. S2 (A) Fluorescence emission spectra of pyrene excimer and monomer in response to different-concentration let-7a. (B) Linear relationship between the pyrene excimer-to-monomer emission ratio and the logarithm of let-7a concentration. Error bars show the standard deviation of three independent experiments.

We further investigated the whole spectra including both pyrene excimer and monomer emission. As shown in Fig. S2, the fluorescence emission of pyrene excimer at 485 nm improves with the increasing concentration of let-7a. Meanwhile, the fluorescence emissions of pyrene

monomer at 375 and 398 nm decrease with the increasing concentration of let-7a. Moreover, the excimer (485 nm)-to-monomer (398 nm) ratio has a linear correlation with the logarithm of let-7a concentration in the range from 1 fM to 10 nM. The correlation equation is $Y = 0.34 + 0.07 \log_{10} C$ with a correlation coefficient of 0.996, where Y is the excimer-to-monomer ratio and C is the let-7a concentration (pM). The detection limit is calculated to be 0.22 fM based on 3σ/K, where σ is the standard deviation of control group and K is the slope of linear regression curve. Notably, the detection limit derived from this ratiometric analysis is comparable to that from excimer emission analysis (Fig. 2B). These results clearly demonstrated that the proposed method can be used for ratiometric analysis as well. Notably, to simplify the data analysis, pyrene excimer emission analysis is frequently used in the pyrene probe-based assays for the detection of DNA,[4] Zirconium,[5] thrombin,[6] and cocaine.[7]

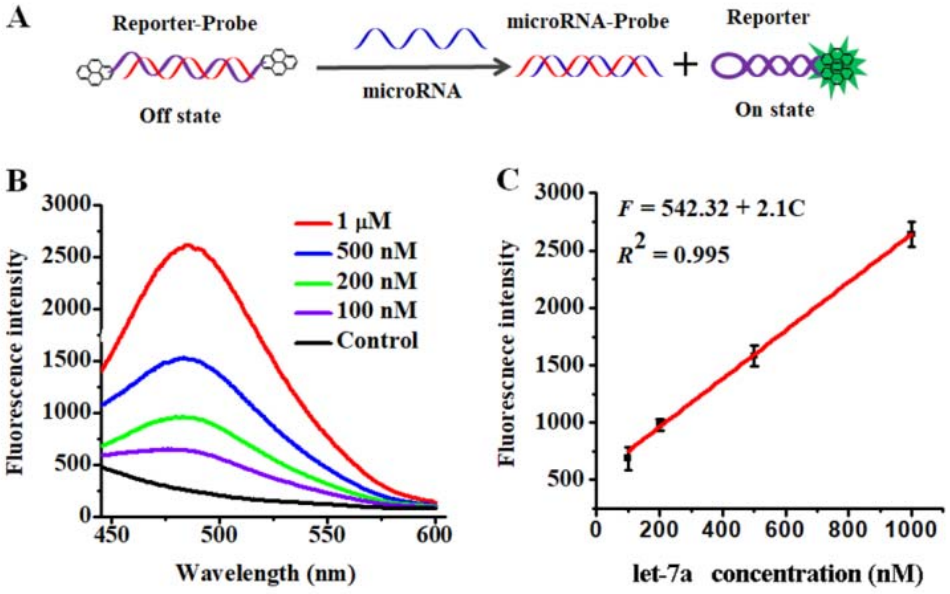

Fig. S3 Detection sensitivity without DSN. (A) Schematic illustration of microRNA assay without DSN. (B) The fluorescence emission spectra of pyrene excimer in response to different-concentration let-7a. (C) Linear relationship between the fluorescence intensity of

of pyrene excimer and the let-7a concentration ranging from 100 nM to 1 μM. Error bars show the standard deviation of three independent experiments.

To verify the improvement of detection sensitivity induced by DNS, we performed the control experiments without the involvement of DSN (Fig. S3A). In the absence of target microRNA, no pyrene excimer is formed, and the reporter remains off-state due to the hybridization of the probe with the reporter. In the presence of target microRNA, it can competitively bind to the probe, enabling the release of the reporter. The released reporter may fold into a stem-loop structure with the formation of pyrene excimer, switching to the on-state (Fig. S3A). As shown in Fig. S2B, the fluorescence emission of pyrene excimer at 485 nm improves with the increasing let-7a concentration. Moreover, the fluorescence intensity exhibits a linear correlation with the let-7a concentration in the ranges from 100 nM to 1 μM (Fig. S3C). The correlation equation is $F = 542.32 + 2.1\ C$, with a correlation coefficient of 0.995, where F is the fluorescence intensity of pyrene excimer at 485 nm and C is the let-7a concentration (nM). The detection limit is calculated to be 15.2 nM based on 3σ/K. Notably, the sensitivity of DSN-assisted approach (Fig. 2B) has improved by 7 orders of magnitude compared with that without the involvement of DSN (Fig. S3C), suggesting that the introduction of DSN may greatly amplify the signal and improves the detection sensitivity. In addition, the DSN-assisted signal amplification strategy has a much simpler reaction scheme, and it can eliminate the risk of nonspecific amplification in the conventional nucleic acid amplification-based approaches.

Table S2 Recovery Studies in Serum Samples

added (nM)	measured (nM)	recovery (%)	RSD (%)
50	49.88	98.76	2.27
100	100.35	102.35	2.07
200	199.04	99.52	3.34

To further investigate the performance of the proposed method in complex biological fluids, we added different-amount let-7a into 100-fold diluted serum sample, in which no detectable endogenous let-7a existed,[8] and measured the corresponding recovery rates. As shown in Table S2, a quantitative recovery ranging from 98.76 % to 102.35 % is obtained, with comparable recovery obtained by the double-strand displacement-based assay,[9] suggesting the feasibility of the proposed method for accurate microRNA assay in complex biological samples and its potential applications in clinical diagnosis and prognosis.

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