Supplementary Information

T7 exo–mediated FRET-breaking combined with DSN–RNAse–TdT for the detection of microRNA with ultrahigh signal-amplification

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Procedure for miRNA Detection

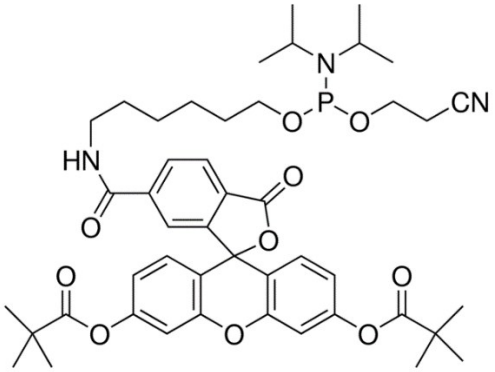
miRNA21:C-miRNA was annealed by heating at 95 °C and then cooling quickly to room temperature. Duplex specific nuclease (0.5 units) in master buffer (50 mM Tris-HCl, pH 8.0; 5 mM $MgCl_2$; 1 mM DTT) was added and then the mixture was incubated at 54 °C for 30 mins. RNAse (1 μL) was added into the sample and then the system was incubated at 37 °C for 30 min. Thereafter, 2 μL of dATP (10 mM), 1.5 μL of 10× TdT buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate), 1.5 μL of $CoCl_2$ (2.5 mM), and 20 units of TdT were added into the mixture (with a total volume of 15 μL) and then the mixture was incubated at 37 °C for 5 h to initiate dATP incorporation. The reaction was stopped by heating at 70 °C for 10 min. Next, 10× NEB Buffer 4 (50 mM Potassium Acetate, 20 mM Tris-acetate,10 mM, Magnesium Acetate, 1 mM DTT) (3 μL), **Probe-P1** (1 μM, 10 μL), and T7 Exo (5 units) were added. The mixture (with a total volume of 30 μL) was incubated for 2 h at room temperature. All fluorescence spectra were recorded using a JASCO FP-6500 spectrofluorometer with excitation at 485 nm; emission spectra were recorded between 500 and 550 nm. The fluorescence intensity at 520 nm was measured for quantitative assaying of miRNA.

Polyacrylamide Gel Electrophoresis (PAGE) Analysis

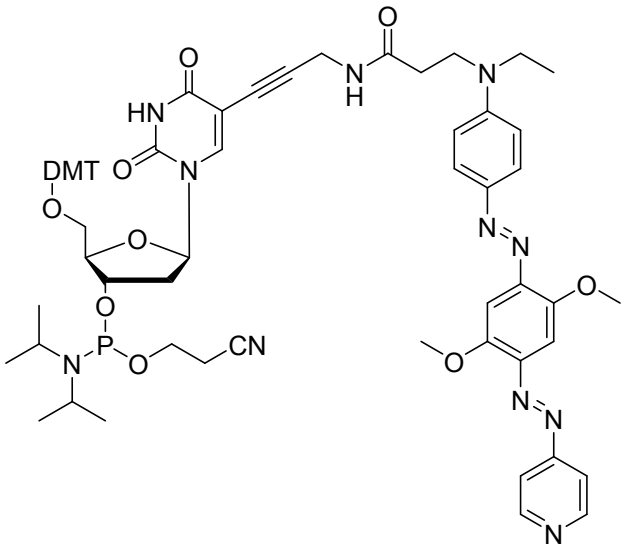
The reaction products were analyzed through 15% polyacrylamide gel electrophoresis (PAGE) in 1× TBE buffer (89 mM Tris-boric acid, 2.0 mM EDTA, pH 8.3) at a constant voltage of 80 V for 3 h. The gel was stained with EtBr solution (0.5 μg/mL) or SYBR™ Green I Nucleic Acid Gel Stain solution and photographed using a UV transilluminator.

Solid-Phase Oligonucleotide Synthesis

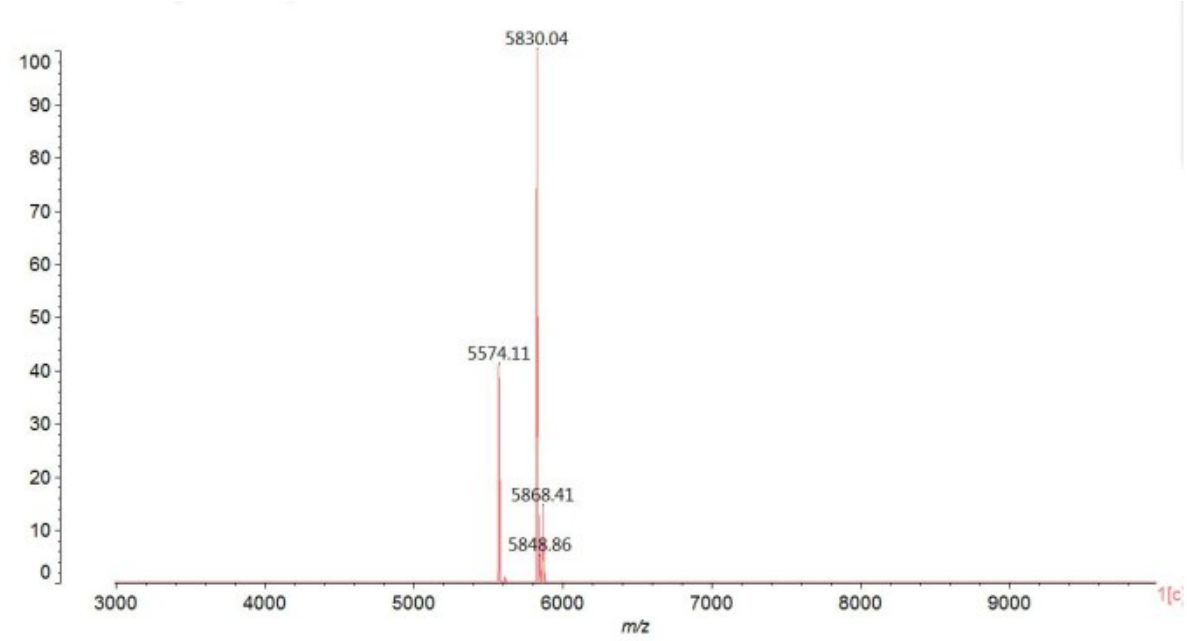
The phosphoramidites were introduced to FAM at the 5′-end and to dT-EBQ at the 3′-end to produce **Probe-P1** on a controlled-pore glass (CPG) solid support (1-*O*-dimethoxytritylpropyldisulfide, 1′-succinyl-lcaa-CPG), using a standard phosphoramidite approach and an automated DNA synthesizer (MERMADE DNA-Synthesizer). The synthesized oligonucleotide was cleaved from the solid support under the influence of 30% aqueous NH_4OH (1.0 mL) for 10 h at 60 °C. The crude product obtained from the automated ODN synthesis was lyophilized and diluted with distilled water (1 mL). The ODN was purified through high-performance liquid chromatography (HPLC; Merck LichoCART C18 column; 10 × 250 mm; 10 μm; pore size: 100 Å). The HPLC mobile phase was held isocratic for 10 min [5% MeCN/0.1 M triethylammonium acetate (TEAA); pH 7.0] at 2.5 mL/min. The gradient was increased linearly over 10 min from 5 to 50% MeCN/0.1 M TEAA at the same flow rate. The fractions containing the purified ODN were cooled and lyophilized. Subsequently, 80% aqueous AcOH was added to the ODN. After 1 h at ambient temperature, the AcOH was lyophilized under reduced pressure. The residue was diluted with water (1 mL); this solution was purified through HPLC using the conditions described above.



Fluorescein Phosphoramidite



dT-EBQ phosphoramidite



MALDI-TOF mass spectrum of the oligonucleotide **Probe-P1**.
Calculated MW of **Probe-P1**: *m*/*z* 5824.0388

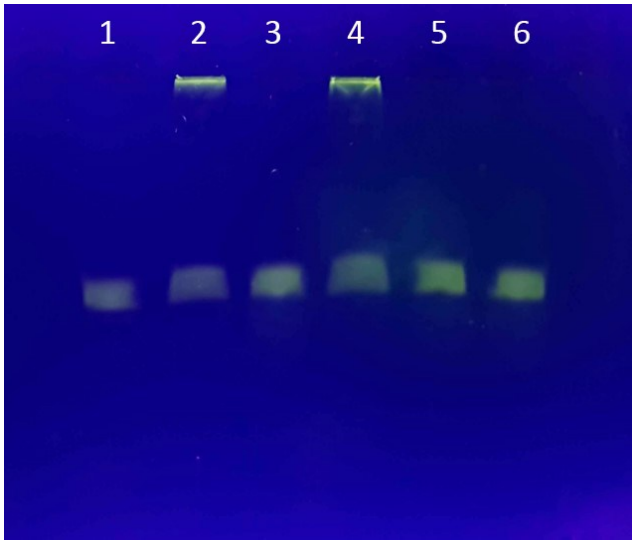


Figure S1: Gel electrophoresis analysis of the tailing reaction with different types of 3'-modified **C-miRNA**. **Lane 1**: C-miRNA-thiol; **lane 2**: tailing reaction with C-miRNA-thiol; **lane 3**: C-miRNA-amine; **lane 4**: tailing reaction with C-miRNA-amine; **lane 5**: C-miRNA-phosphate; **lane 6**: tailing reaction with C-miRNA-phosphate. The gel was stained with SYBR solution for 15 mins.

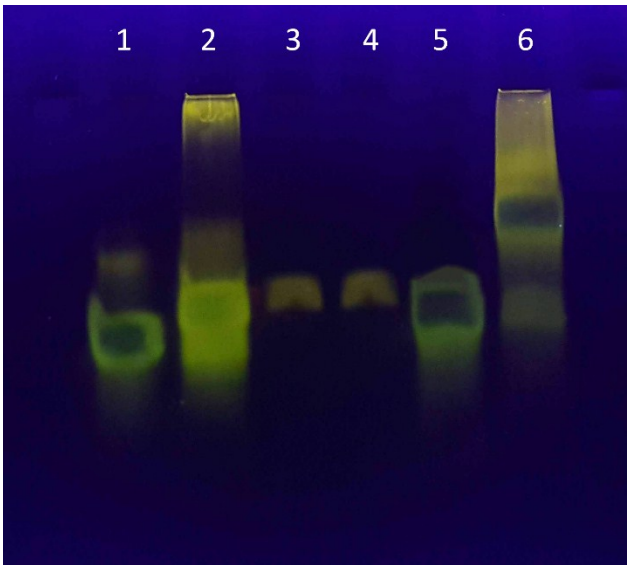


Figure S2 Gel electrophoresis analysis of the tailing reactions with miRNA 21, C-miRNA, and a random oligonucleotide. **Lane 1**: miRNA 21; **lane 2**: miRNA 21with TdT polymerase; **lane 3**: C-miRNA; **lane 4**: C-miRNA with TdT polymerase; **lane 5**: random oligonucleotide; **lane 6**: random oligonucleotide wth TdT polymerase. The gel was stained with SYBR solution for 15 mins.

Figure S3 Electrophoresis gel analysis of the tailing reactions before and after treatment with RNAse. **Lane 1**: miRNA 21; **lane 2**: miRNA 21 + TdT polymerase ; **lane 3**: miRNA 21 + RNAse; **lane 4**: miRNA 21 + RNAse + TdT polymerase. The gel was stained with SYBR solution for 15 mins.

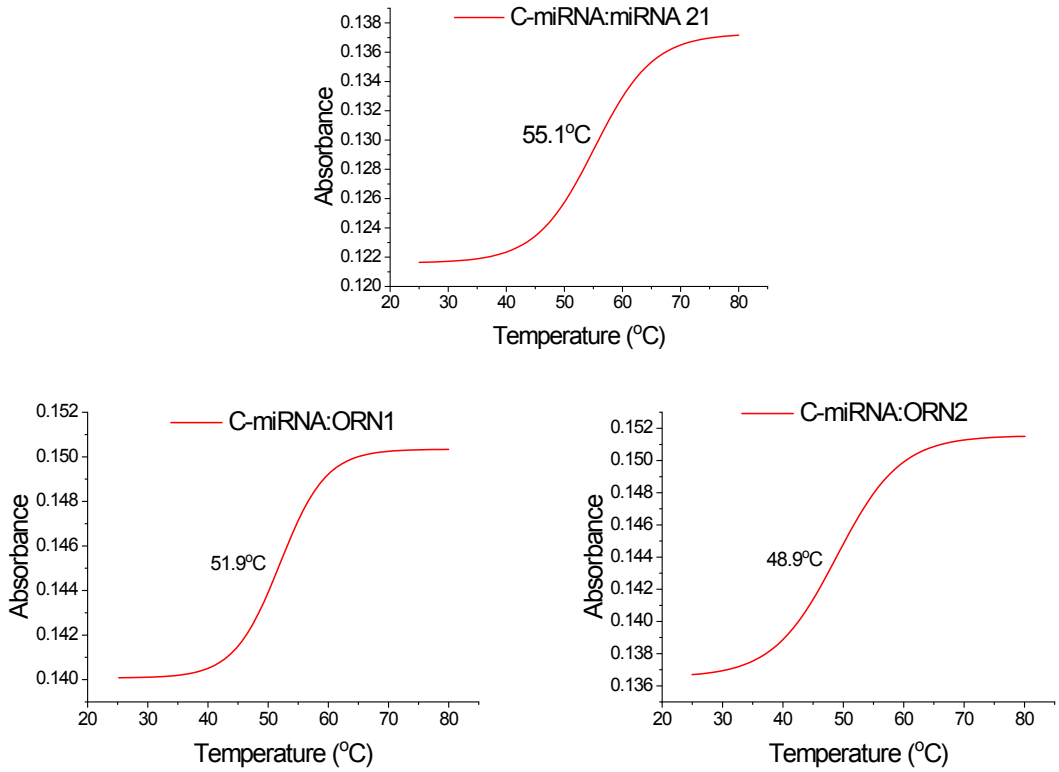


Figure S4 Melting curve analysis of double-strand formation between **C-miRNA** and various miRNAs (miRNA 21 and one-base-mismatched miRNAs 21). **C-miRNA** (0.1 mM) and miRNA (0.1 mM) were annealed to form a **C-miRNA : miRNA** duplex by heating to 95 °C and then cooling to room temperature. All samples were diluted to 1 mL for measurement.

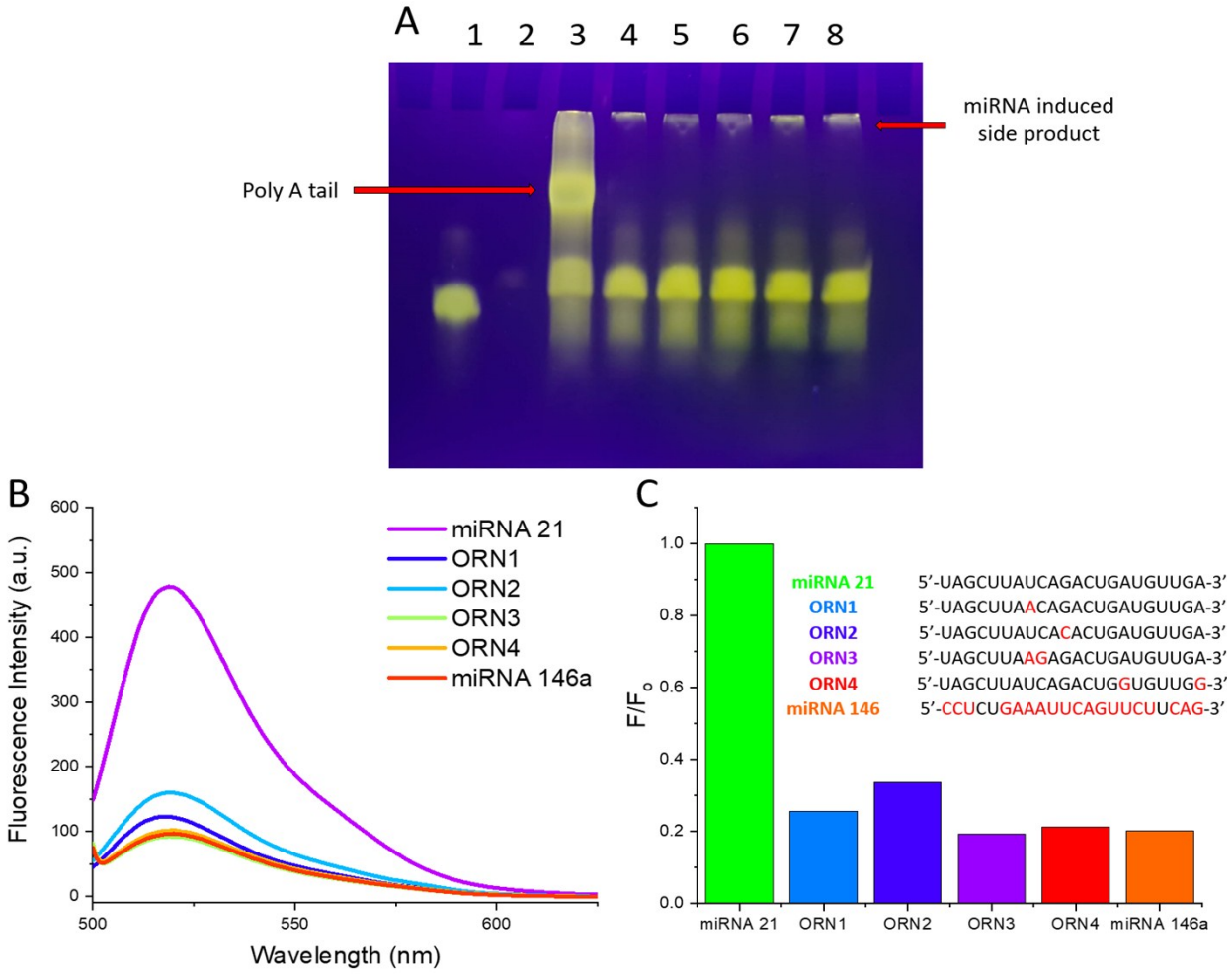


Figure S5 Selectivity of the DSN–TdT–T7 exo strategy for miRNA 21 detection in the absence of RNAse treatment. (A) Denaturing gel electrophoresis analysis of the tailing reactions with miRNA 21 and other mismatched miRNAs. **Lane 1**: miRNA 21; **lane 2**: **C-miRNA**; **lanes 3–8**: tailing reactions of **C-miRNA** with different miRNAs using TdT polymerase: **lane 3**: miRNA 21 (perfect target); **lane 4**: ORN1; **lane 5**: ORN2; **lane 6**: ORN3; **lane 7**: ORN4; **lane 8**: miRNA 146a. (B) Fluorescence spectra recorded in the presence of miRNA 21 and with various mismatched miRNAs. (C) Specificity of the sensing system toward the target miRNA.

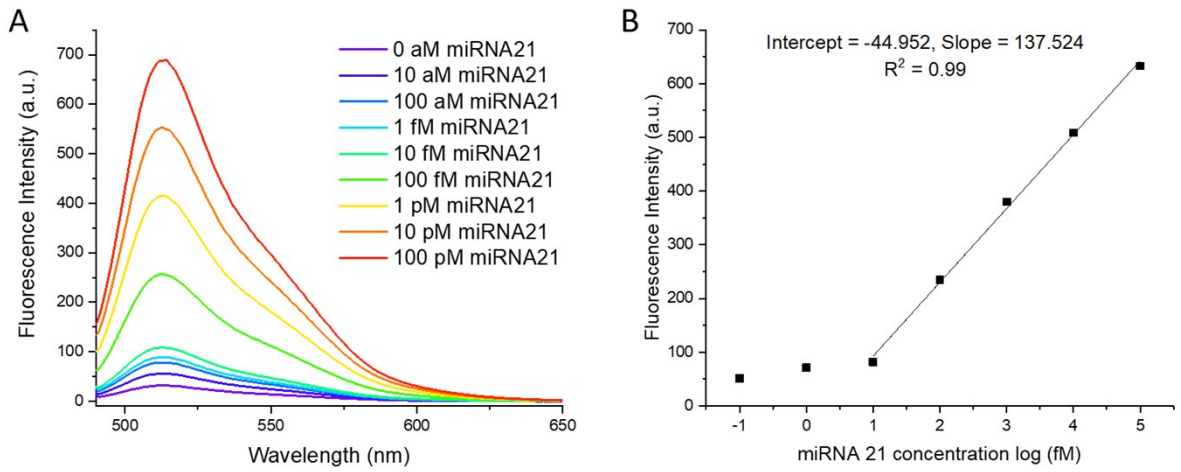


Figure S6 Sensitivity of the DSN–RNAse–TdT–T7 exo system for the detection of miRNA 21 in human serum. (A) Fluorescence spectra recorded with miRNA 21 at various concentrations (0–100 pM) in human serum. (B) Linear relationship between the fluorescence intensity (λ_{em} = 520 nm) and the logarithm of the target miRNA concentration in the range from 10 fM to 100 pM; Limit of detection (LOD): 9.56 fM. LOD was calculated using the 3σ method [LOD = 3(SD/S), where SD is the standard deviation of the response and S is the slope of the standard curve in the linear area].

All samples were prepared in serum solution with different concentration of **miRNA 21.** 2 µL sample was mixed with 2 µL C-miRNA21 and then duplex formation miRNA21:C-miRNA was formed heating at 95 °C and then cooling quickly to room temperature. Duplex specific nuclease (0.5 units) in master buffer (50 mM Tris-HCl, pH 8.0; 5 mM $MgCl_2$; 1 mM DTT) was added and then the mixture was incubated at 54 °C for 30 mins. RNAse (1 µL) was added into the sample and then the system was incubated at 37 °C for 30 min. Thereafter, 2 µL of dATP (10 mM), 1.5 µL of 10× TdT buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate), 1.5 µL of $CoCl_2$ (2.5 mM), and 20 units of TdT were added into the mixture (with a total volume of 15 µL) and then the mixture was incubated at 37 °C for 5 h to initiate dATP incorporation. The reaction was stopped by heating at 70 °C for 10 min. Next, 10× NEB Buffer 4 (50 mM Potassium Acetate, 20 mM Tris-acetate,10 mM, Magnesium Acetate, 1 mM DTT) (3 µL), **Probe-P1** (1 µM, 10 µL), and T7 Exo (5 units) were added. The mixture (with a total volume of 30 µL) was incubated for 2 h at room temperature. All fluorescence spectra were recorded using a JASCO FP-6500 spectrofluorometer with excitation at 485 nm; emission spectra were recorded between 500 and 550 nm. The fluorescence intensity at 520 nm was measured for quantitative assaying of miRNA.

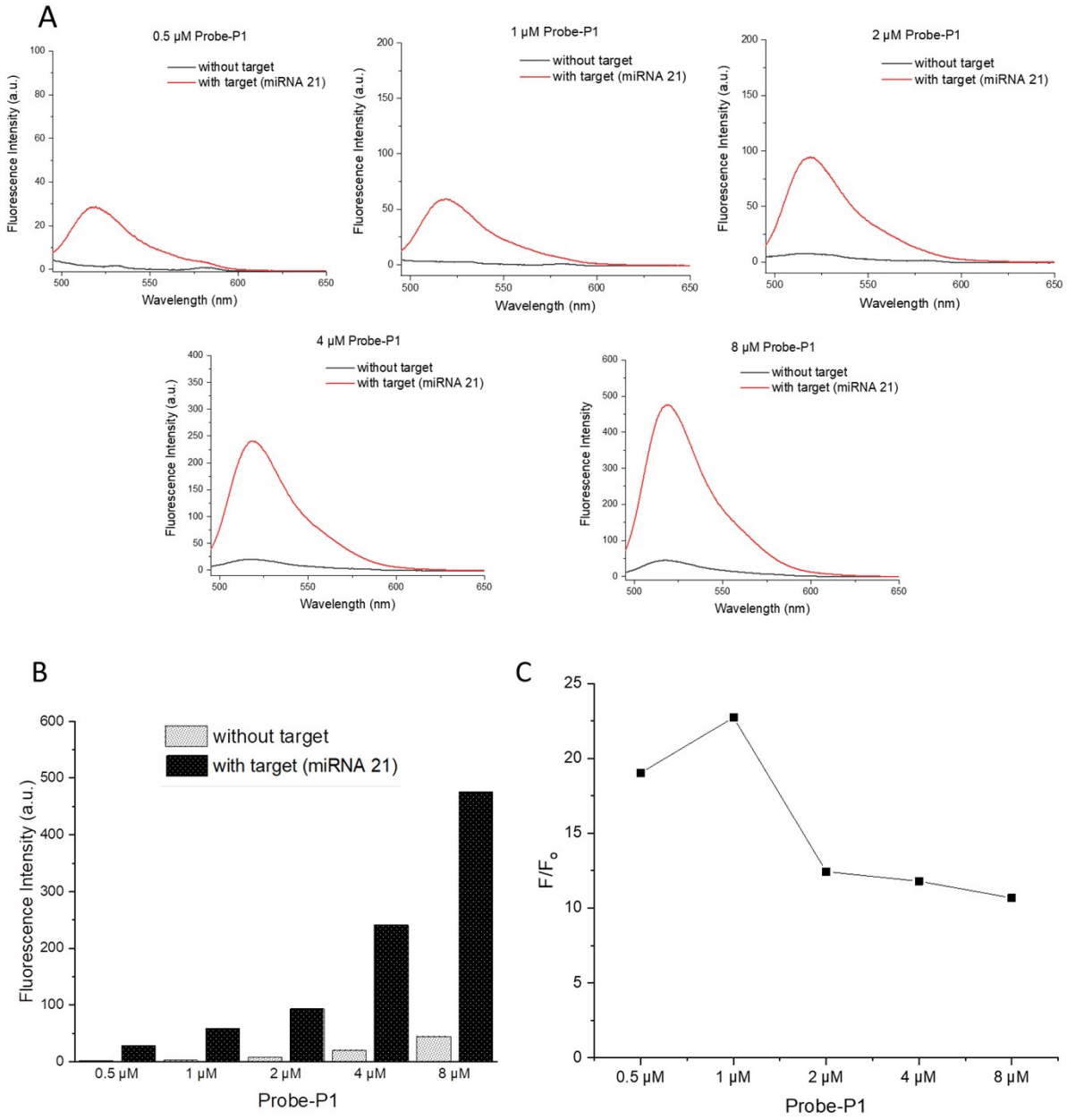


Figure S7 Optimization of concentration of Probe-P1 (A) Fluorescence spectral characterization of miRNA analysis by using various concentrations of Probe-P1. (B) Fluorescence intensity at 520 nm characterization of miRNA analysis by using various concentrations of Probe-P1. (C) Comparison chart of enhancement level of fluorescence intensities at 520 nm with and without target miRNA21 at different concentrations of Probe-P1. Excitation wavelength 485 nm. miRNA21:C-miRNA was annealed by heating at 95 °C and then cooling quickly to room temperature. Duplex specific nuclease (0.5 units) in master buffer (50 mM Tris-HCl, pH 8.0; 5 mM $MgCl_2$; 1 mM DTT) was added and then the mixture was incubated at 54 °C for 30 mins. RNAse (1 μL) was added into the sample and then the system was incubated at 37 °C for 30 min. Thereafter, 2 μL of dATP (10 mM), 1.5 μL of 10× TdT buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate), 1.5 μL of $CoCl_2$ (2.5 mM), and 20 units of TdT were added into the mixture (with a total volume of 15 μL) and then the mixture was incubated at 37 °C for 5 h to initiate dATP incorporation. The reaction was stopped by heating at 70 °C for 10 min. Next, 10× NEB Buffer 4 (50 mM Potassium Acetate, 20 mM Tris-acetate,10 mM, Magnesium Acetate, 1 mM DTT) (3 μL), **Probe-P1** (various concentrations, 10 μL), and T7 Exo (5 units) were added. The mixture (with a total volume of 30 μL) was incubated for 2 h at room temperature.

Table S1. Comparison of the Sensitivity of Different Fluorescence-Based Detection Methods for the Assay of miRNA

Detection strategy	Detection limit	Ref.
DSN signal amplification	100 fM	1
Target-triggered catalytic hairpin assembly and fluorescence enhancement of DNA-silver nanoclusters	0.29 nM	2
DNA-gold nanoparticles	0.01 pM	3
Target-primed and branched rolling-circle amplification	10 fM	4
DNAzyme based amplification	0.68 nM	5
Graphene Quantum Dots and Pyrene-Functionalized Molecular Beacons	100 pM	6
AgNCs Hairpin Probe	1.7 nM	7
Transcription amplification based on Spinach	3 pM	8
Label-free fluorescent DNA dendrimers based on nonlinear hybridization chain reaction-mediated multiple G-Quadruplex	0.7 fM	9
Multiplexed MicroRNA Detection Using a Single Excitation Wavelength	18 pM	10
Dumbbell Probe-Based Hybridization Chain Reaction	3.2 pM	11
Exonuclease III-assisted cycle amplification	6 pM	12
Visible-to-Near-Infrared Fluorescent Graphene Quantum Dots	10 fM	13
Target-fueled DNA walker	58 fM	14
Quantum dot-based FRET	0.4 fM	15
enzymatically engineered primer extension poly-thymine (EPEPT) reaction using copper nanoparticles as nano-dye	100 fM	16
hairpin DNA-templated copper nanoclusters	2.2 pM	17
Nicking-enhanced rolling circle amplification	10 pM	18
Enzyme-free hybridization chain reaction (HCR) with graphene oxide (GO)	0.18 pM	19
Target-initiated labeling for the dual-amplified detection	1 pM to 2.5 nM	20
T7 exo-mediated FRET-breaking combined with DSN-RNAse-TdT	2.5 fM	This work

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