Electronic Supporting Information (ESI†) for

Sensitive and Homogeneous microRNA Detection Using Branched Cascade Enzymatic Amplification

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EXPERIMENTAL SECTION

Reagents and Materials

Polymerase klenow fragment exo$^-$ (KFexo$^-$) and terminal deoxynucleotidyl transferase (TdTase) were purchased from New England Biolabs (NEB). SYBR Green I (SG, 20×stock solution in dimethyl sulfoxide, 20 mg/mL) was purchased from Xiamen Bio-vision Biotechnology Co. Ltd. (Xiamen, China). All the synthetic DNA, RNA, deoxyadenosine triphosphates (dATPs), doxythymidine triphosphates (dTTPs) and RNase inhibitor were obtained from TaKaRa Biotechnology Co. Ltd. (Dalian, China). All the oligonucleotides were purified by HPLC. MCF-7 breast cancer cells were obtained from the second affiliated hospital of Nanchang University. DMEM high glucose medium, penicillin, streptomycin and fetal bovine serum were purchased from Thermo Scientific HyClone (MA, USA). DEPC-treated deionized water was used in all experiments. The sequences of the oligonucleotiedes and miRNAs are listed in Table S1.

Table S1. Sequence Information for the Oligonucleotides and miRNAs Used in This Study.

Note	Sequence (5'to 3')
pDNA	TTTTTTTTTTTTTTTTCAACATCAGTCTGA-C6-NH_2
miR-21	UAGCUUAUCAGACUGAUGUUGA
second primer	UUUUUUUUUUUUUUU
SM miR-21	UAGCUUAUAAGACUGAUGUUGA
miR-210	CUGUGCGUGUGACAGCGGCUGA

Apparatus

The fluorescence measurements were performed with a Hitachi F-7000 spectrofluorimeter (Tokyo, Japan) equipped with a xenon lamp. Gel electrophoresis images were acquired with a VersaDoc 4000 imaging system (Bio-Rad, USA). Atomic force microscopy (AFM) image was recorded under the mode of ScanAsyst using Bruker MultiMode-8 atomic force microscopy (Bruker, USA).

BCEA Reaction

The reaction mixtures for amplification reaction were prepared separately on ice as part A and part B. Part A consisted of buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$), 5 U of polymerase KFexo$^-$, 1 mM dATPs, 50 nM pDNA and an appropriate amount of the target miRNA in a reaction volume of 20 μL. Part B consisted of 20 mM Tris-Ac (pH 7.9), 50 mM KAc, 10 mM $Mg(Ac)_2$, 0.25 mM $CoCl_2$, 100 μM dTTPs, 500 nM second primers and 9 U of TdTase in a reaction volume of 10 μL. Before KFexo$^-$ and dATPs were added, Part A was first denatured at 80 °C for 5 min and cooled slowly to room temperature. Then, followed by addition of the polymerase KFexo$^-$ and dATPs, Part A was incubated at 37 °C for 30 min. After the reaction finished, part B was added immediately, and the amplification reaction of part A with part B was performed at 37 °C for 1.5 h.

Measurement of Fluorescent Spectra

Aliquots of 25 μL products and 5 μL SG (20×concentrate) were combined in a 0.5 mL centrifuge tube and diluted to 200 μL with 10 mM Tris-HCl buffer (pH 7.5). After incubation for 10 min at room temperature, the fluorescence spectra were measured in a 1×1 cm quartz cuvette. The excitation wavelength was 490 nm, and the spectra were recorded between 510 and 600 nm. The fluorescence emission intensity was measured at the peak wavelength of 525 nm.

Gel Electrophoresis Analysis

The BCEA products used was the same as the reaction solution was analyzed with 1.8% (w/w) agarose gel electrophoresis and the effect of polymerase on the progress of the BCEA reaction was investigated by 15% urea denaturing gel electrophoresis, running in

0.5×TBE (22.5 mM Tris-Boric acid, 5 mM EDTA, pH 8.0) at room temperature. Electrophoresis was performed at a constant potential of 110 V.

AFM Analysis

Two microliters of freshly prepared BCEA products were dripped on mica substrates in a confined environment to dry. Then, the AFM analysis was performed.

Cell Cultures

MCF-7 breast cancer cell line was selected to check the BCEA performance. It was grown at 37 °C in a humidified atmosphere containing 5% CO_2. MCF-7 cells were maintained in high-glucose DMEM, supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin.

miRNAs Extraction

Total RNA samples were extracted by using the UNlQ-10 column Trizol total RNA purification kit (Sangon Biotech Co., Ltd, Shanghai, China). The total RNA quality and concentration were evaluated by measuring the absorbance at 260, 230 and 280 nm with spectrophotometer. In all cases, the expected 260/280 (～2.0) and 260/230 (2.0-2.2) ratio values accepted for pure RNA were obtained. [1,2]

Optimization of BCEA Reaction Conditions

The polymerization reaction usually promises ultrahigh sensitivity, but the sensitivity and detection limit of polymerization reaction is mainly hindered by nonspecific background amplification, which especially appears to be affected by the concentration of the probe. Thus, it is important to optimize the probe concentration to improve the signal/background ratio of BCEA. To this end, the fluorescence ratio (F/F_0) was applied to evaluate the performance of the test factors, where F and F_0 are the fluorescence intensities at 525 nm under excitation at 490 nm obtained from the presence and the absence of miR-21, respectively, in this study. It should be noted that a higher fluorescence ratio (F/F_0) value is more favorable to obtain higher sensitivity of the detection method. The probe concentrations were investigated for four concentrations of 20, 50, 100, and 200 nM. As shown in Fig. S1A, the fluorescence emission spectra with different probe concentrations in the absence and the presence of miR-21 were

recorded. It can be found that signal increased along with the concentration increment of probe. In addition, excessive probe significantly increased the background signal as well, which decreased the signal/background ratio, and more importantly, made it difficult to quantify miRNA concentrations. This significant increase in background signal was attributable to the common phenomenon of nonspecific background amplification. The reasons may be primer template-independent polymerization, false priming at sites where primer dimers are formed, or inherent technical difficulty.[3] To discriminate the signal from noise to achieve good sensitivity, 50 nM probe concentration was chose using the highest fluorescence ratio (F/F_0) value (Fig. S1B) for the subsequent experiments.

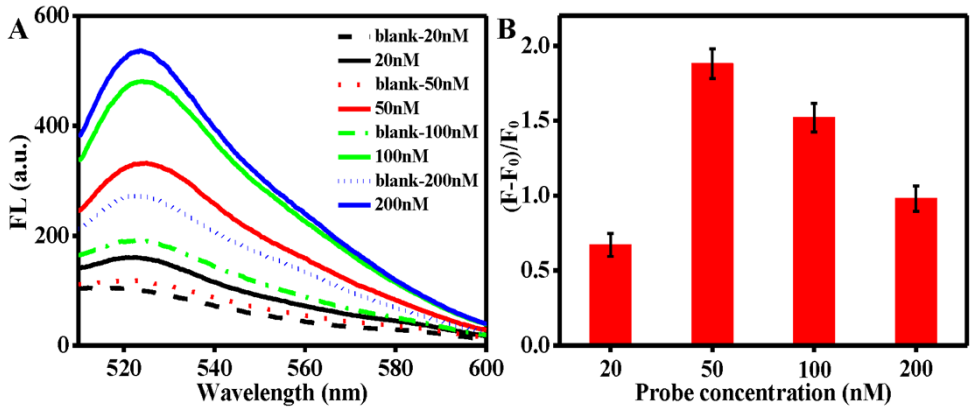

Fig. S1 Effect of probe concentration on the performance of the proposed method. (A) Fluorescence emission spectra responses probe with different concentrations in the absence and the presence of miR-21. (B) Bar representing fluorescence ratio (F/F_0) responses in the presence of the probe with different concentrations, where F and F_0 are fluorescence intensities of amplification products by BCEA in the presence and absence of miR-21, respectively. The concentration of miR-21 was 5 fM.

To obtain high amplification efficiency of BCEA reactions, several detection conditions such as the concentrations of dNTPs, the second primers, and polymerases were experimentally optimized. In this reaction, two dNTPs (dATPs and dTTPs) were used. To investigate the influence of concentration of dNTPs, firstly, the concentration of dATPs from 200 μM to 2000 μM was investigated, as shown in Fig. S2. As the concentration of dATPs increased, the fluorescence ratio (F/F_0) value increased gradually until 1000 μM. Then, the concentration of dTTPs was further investigated (Fig. S3). The fluorescence ratio (F/F_0) value increased gradually until 100 μM dTTPs.

Thus, 1000 μM dATPs and 100 μM dTTPs were selected as the optimum concentrations of dNTPs in the following research. With the decrease of the second primer concentrations from 2000 nM to 200 nM, the fluorescence ratio (F/F_0) values raised until 500 nM (Fig. S4), suggesting that low second primer concentration leads to improved discrimination against the miR-21. This can be explained by the fact that high-concentration exogenous primers might adversely cause primer dimerization and nonspecific amplification.[4] Therefore, 500 nM was selected as the optimum concentration of second primers in the subsequent research. The concentration of polymerase is another factor which affects the amplification reaction. When different concentrations of KFexo$^-$ polymerase were employed, the fluorescence signals were analyzed, and the data are shown in Fig. S5. There was little change in fluorescence ratio (F/F_0) value with increasing concentration of KFexo$^-$ polymerase. The effect of TdTase amount on the assay was also assessed. The data are shown in Fig. S6. The produced fluorescence ratio (F/F_0) value increased with increasing the concentration of TdTase polymerase until the concentration reached 9 U. In order to obtain optimal detection sensitivity, 5 U KFexo$^-$ and 9 U TdTase were selected, respectively.

In addition, the temperature has a crucial effect on the reactivity of enzyme and the hybridization efficiency of nucleic acids. The effect of the temperature was also investigated by comparing fluorescence ratio (F/F_0) value (Fig. S7). Several temperatures (25, 30, 37 and 39 °C) were compared to obtain higher detection sensitivity. Finally, 37 °C was considered to be the optimal temperature.

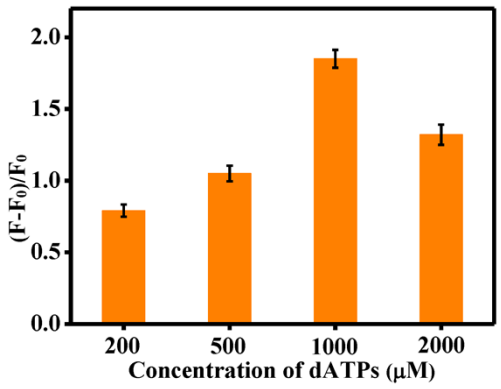

Fig. S2 The relationship between the fluorescence intensity and the concentration of dATPs.

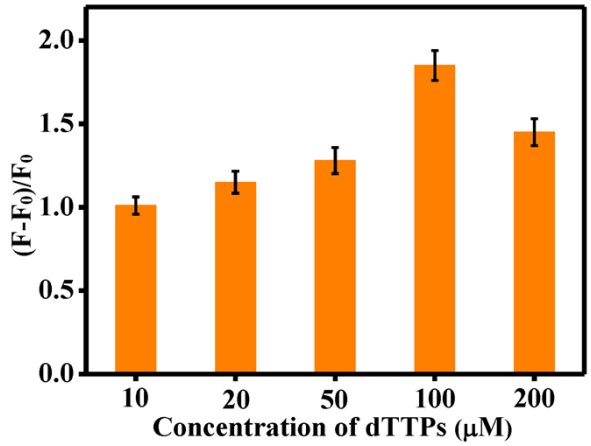

Fig. S3 The relationship between the fluorescence intensity and the concentration of dTTPs.

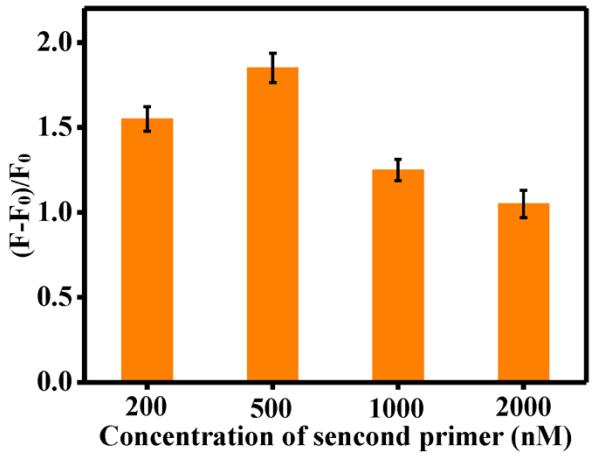

Fig. S4 The relationship between the fluorescence intensity and the concentration of second primer.

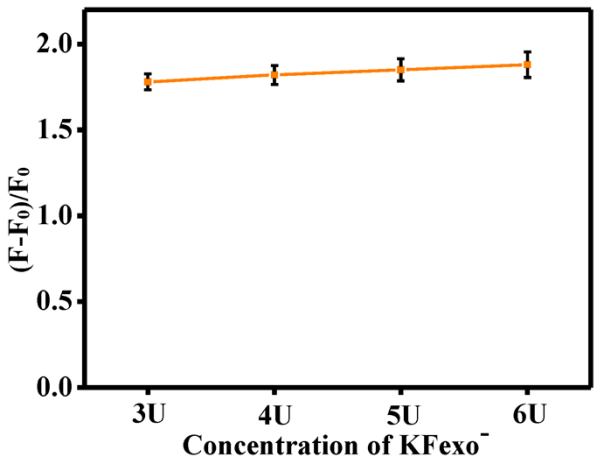

Fig. S5 The relationship between the fluorescence intensity and the concentration of KFexo$^-$.

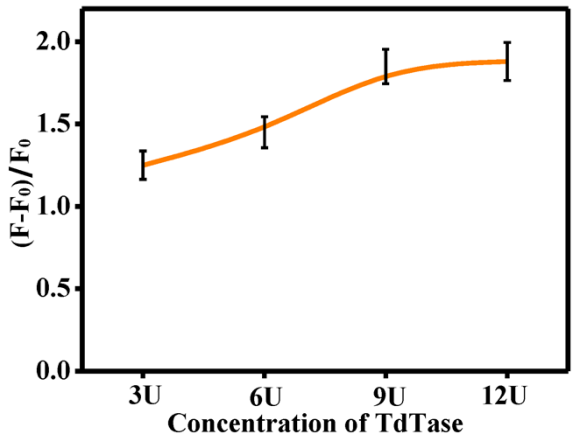

Fig. S6 The relationship between the fluorescence intensity and the concentration of TdTase.

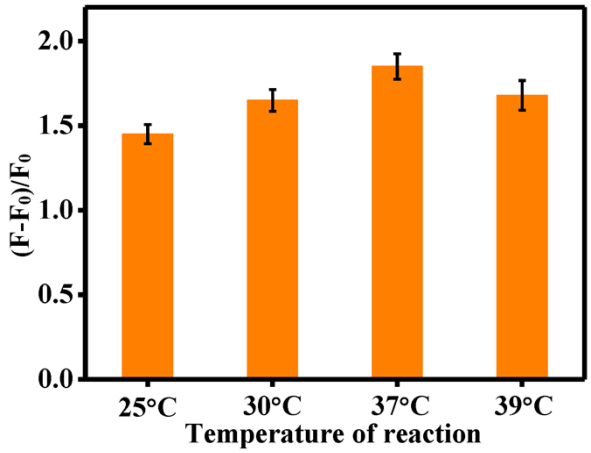

Fig. S7 The influence of reaction temperature.

Sensitivity of the Assay

The correlation equation was Y = 813.09 + 204.84 $\log_{10}$ X, where Y was the fluorescence intensity at 525 nm and X was the concentration of miR-21, with a correlation coefficient r = 0.9994.

Specificity of the Assay

Distinguishing among members from the miRNA family is of great importance for better understanding the biological functions of individual miRNA. Since miRNA families often possess closely related sequences with high homology,[5] it is a great challenge to discriminate the differences between miRNA family members that differ by only a few nucleotides. To evaluate the specificity of the proposed assay, we performed a series of contrast experiments using miR-210 and single-base mismatched miR-21 (SM) as negative controls and a sample without target as a blank control

(fluorescence spectra were shown in Fig. S8). The results (Fig. S9, red histogram) showed that the fluorescence signals from miR-210 scarcely changed compared with the blank control, while the signals of SM miR-21 showed slight increases compared with the blank control. Nevertheless, perfectly matched miR-21 (PM) exhibited a much stronger response than the blank control, which could be easily discriminated from the control signals. Moreover, we applied this sensor to detect the target in human serum, the most challenging mediums containing many complex components. The measurements were conducted in 1:100 dilutions of serum samples. Compared with fluorescence signals observed in the buffer under the same experimental condition, signals were increased a little when the sensor was introduced to human serum samples (Fig. S9, green histogram), which may be related to the interference of the sample matrix in the reaction environment affecting the enzymatic reaction and fluorescence signals.

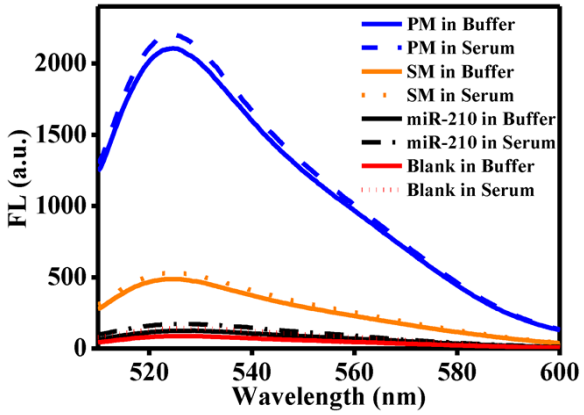

Fig. S8 Specificity investigation of BCEA assay in buffer and serum, respectively. Fluorescence emission spectra response to a series of contrast experiments in buffer and diluted serum (1:100) using 100 pM single-base mismatched miR-21 (SM) and miR-210 as negative controls, and a sample without target as blank control experiment (blank) and 100 pM perfectly matched miR-21 (PM).

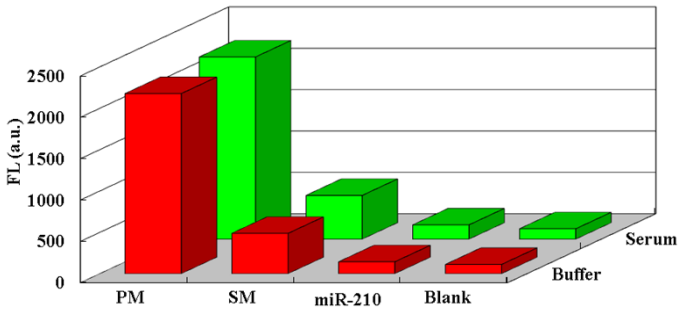

Fig. S9 Specificity investigation of BCEA assay in buffer and serum, respectively. A series of contrast experiments in buffer and diluted serum (1:100) using 100 pM single-base mismatched miR-21 (SM) and miR-210 as negative controls, and a sample without target as blank control experiment (blank) and 100 pM perfectly matched miR-21 (PM).

Table S2. Comparison of Analytical Properties of BCEA Technique with Other miRNA Detection Methods.

Method description	miRNA	Biological sample	LOD	Ref.
Pb^{2+}-induced DNAzyme-assisted target recycling and RCA based ECL biosensor	miR-155	cell lysates	0.3 fM	6
cyclic enzyme amplification and resonance energy transfer based ratiometric ECL biosensor	miR-21	cell lysates	0.24 fM	7
solid-phase RCA and chronocoulometry for electrochemical detection	miR-143	blood samples	10 fM	8
in situ DNA-templated synthesis of silver nanoclusters and HCR for electrochemical detection	miR-199a	serum	0.64 fM	9
RCA-based DNA machine coupling catalytic hairpin assembly with DNAzyme formation for colorimetric analysis	let-7	complex sample matrix (salmon sperm DNA)	0.68 fM	10
quantum dot-based point mutation assays using primer generation-mediated RCA for fluorescence assay	miR-196a2	total RNA	50.9 aM	11
RCA-based visualization of miRNA expression patterns at the single-cell level for fluorescence assay	miR-222	single cell	-	12
isothermal nucleic acid amplification strategy by cyclic enzymatic repairing for fluorescence assay	miR-21	cell lysates	0.1 fM	13
DNA−gold nanoparticle probes and endonuclease DSN-based target recycling for fluorescence assay	miR-203	cell lysates	0.2 fM	14
branched cascade enzymatic amplification (BCEA) for fluorescence assay	miR-21	cell lysates	0.1 fM	this work

Notes and references

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