

Electronic Supplementary Information

Direct Detection of Circulating MicroRNA in Cancer Patient Serum by using Protein-Facilitated Specific Enrichment and Rolling Circle Amplification

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EXPERIMENTAL SECTION

Materials

MiRNA-21, miRNA-16, miRNA-155 and Janus probe were synthesized and purified by TaKaRa Biotechnology Co. (Dalian, China). Circular template, detection probe, diethylprocarbonated (DEPC)-treated deionized water, dNTPs and SYBR Green I were purchased from Shanghai Sangon Biotechnology Co. (Shanghai, China). The miRNA and DNA sequences are given in Table S1. Phi29 DNA polymerase, p19 protein, chitin magnetic beads and magnetic separation rack were purchased from New England BioLabs. All solutions were prepared with diethylprocarbonated (DEPC)-treated deionized water. The buffers used in this work were as follows: binding buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1 mM TCEP, 0.02% Tween-20), washing buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 100 μ g/ml BSA). The fluorescence measurements were performed with a Hitachi F-4600 fluorimeter (Hitachi Co. Ltd., Japan) equipped with a xenon lamp under room temperature.

Preparation of P19 Protein functionalized Chitin Magnetic Beads

Before use, the chitin magnetic beads were suspended in binding buffer with a brief vortex. Then 3 mL of p19 protein was added to 10 mL of magnetic beads suspension. The mixtures were incubated on a bench top shaker for 30 minutes at room temperature. The p19 protein has the C-terminal fusion of the chitin binding domain, which allows p19 protein to bind tightly to chitin magnetic beads. After incubation, the p19 proteins were immobilized on magnetic beads. Then the p19 protein-functionalized magnetic beads were drawn to the side of the microfuge tube using the magnetic separation rack and the supernatant was carefully removed with a micropipetor. Excessive p19 proteins were removed by washing two times in 1.5 mL binding buffer one time in 1.5 mL DEPC-treated deionized water. Finally, the p19 protein-functionalized magnetic beads were resuspended in 10 mL binding buffer and stored at 4 °C.

Binding of the Janus Probe-MiRNA Complex to P19 Protein-functionalized Magnetic Beads

10 μL of Janus probe (20 nM) and 500 μL of different concentration of target miRNA-21 were added into 490 μL of binding buffer and incubated at 30 °C for 2 h. Then 15 μL of p19 protein-functionalized magnetic beads suspension was added into the above 1 mL of Janus probe-miRNA complex. The binding reaction was performed by shaking for 2 h at room temperature. Unbound Janus probes were removed by washing five times in 500 μL washing buffer and one time in 500 μL DEPC-treated deionized water. Finally, the magnetic beads were drawn to the side of the microfuge tube using the magnetic separation rack and the supernatant was carefully removed with a micropipetor.

Collection of Janus Probe-MiRNA Complex

After the washing step, magnetic beads were resuspended in 20 μL DEPC-treated deionized water. The suspension was then heated at 90 °C for 15 min to release the Janus probe-miRNA complex. Subsequently, Janus probe-miRNA complex solution was collected quickly by a magnetic separation and transferred into a microcentrifuge tube.

RCA Reaction

For RCA, the 10 μL of collected Janus probe-miRNA complex was added to the 8 μL of 10 mM Tris-HCl buffer (pH 7.5) containing 2 mM MgCl_2 , 2 mM $(\text{NH}_4)_2\text{SO}_4$, 0.4 mM DTT, 1 mM of each dNTP and circle template. The RCA reaction was initiated by addition of 2 μL of DNA polymerase and performed at 30 °C for 3 h. Then the resulting mixture was heated to 65 °C for another 15 min to inactivate the RCA process. After the resulting mixture cooled to room temperature, 3 μL of 1 μM detection probe was added and hybridized to the RCA product. The reaction mixture was incubated at 30 °C for 1 h.

Measurement of Fluorescent Spectra

The RCA products and 3 μ L SG (20 \times concentrate) were combined in a 1.5 mL centrifuge tube and diluted to 600 μ L with 10 mM phosphate buffer (pH 7.5). After incubation for 15 min at room temperature, the fluorescent spectra were measured. The excitation wavelength was 480 nm, and the spectra are recorded between 500 and 650 nm. The fluorescence emission intensity was measured at 530 nm.

Detection of Target MiRNA in Human Serum Samples

Human serum samples of breast cancer patients and healthy donors (confirmed by pathological examinations) were obtained from Fujian Medical University (Fujian, China). Signed informed consent was obtained from each patient participating in the study before surgery. Serum samples were heated at 95 $^{\circ}$ C for 10 min and then used for analysis according to the above procedure.

Table S1. Sequence of oligonucleotides probes used in this work

oligonucleotide	sequence (from 5' to 3')
Janus probe	UCAACAUCAGUCUGAU <i>AAGCUAAAATTGCGAAATGCTAAACC</i>
miRNA-21	UAGCUUAUCAGACUGAUGUUGA
circle template	GTCTCACAGTGTGTCCTCATTTCAGTT <u>TACGGTTTAGCATTTCGCAATTTT</u>
detection probe	TCTCACAGTGTGTCCTCA
miRNA-155	UUA AUGCUAAUCGUGAUAGGGGU
miRNA-16	UAGCAGCACGUAAAUAUUGGCG

The bold fragments of Janus probe are complementary sequences to target miRNA-21. The italic fragments of Janus probe are complementary sequences to the underlined fragments of circle template.

Evaluate the enrichment ability of PFMBs

Magnetic beads, as a special biomolecular immobilizing carrier, were useful to purify the analysts and enhance the sensitivity in biosensing.^{1,2} As shown in Figure S1, the fluorescence signal produced by 100 fM target miRNA after p19 protein-facilitated magnetic beads specific enrichment and rolling circle amplification was about 94.8% of that produced by 10 pM Janus Probe with rolling circle amplification. The results indicated that the PFMBs-based method can achieve at least 100-fold in vitro target enrichment and has potential for detecting rare circulating miRNA from a large amount of blood serum samples.

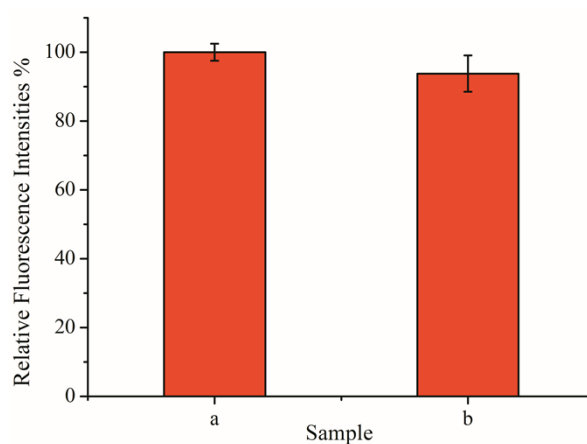


Figure S1. The relative fluorescence intensities of amplification products by RCA of 10 pM Janus Probe (a), and 100 fM target miRNA-21 with magnetic beads enrichment (b). The illustrated error bars represent the standard deviation of five repetitive measurements.

Optimization of Janus Probe Concentration

The Janus probe can hybrid to target miRNA-21 and form Janus probe-miRNA complex, which can selectively bind to PFMBs. Therefore, we first evaluated the optimization of Janus probe concentration. The effect of concentrations of Janus probe (from 0 to 200 nM) on the fluorescent signal for 10 pM target miRNA after RCA reaction was shown in Figure S2. The fluorescence intensity increased significantly as the concentrations increased up to 20 nM, suggesting that the target miRNA-21 would effectively hybrid to Janus probe and form Janus probe-miRNA complex. However, the signal did not increase obviously when the concentrations of Janus probe were up to 50 nM. Therefore, 20 nM was chosen as the optimum Janus probe concentration in this study.

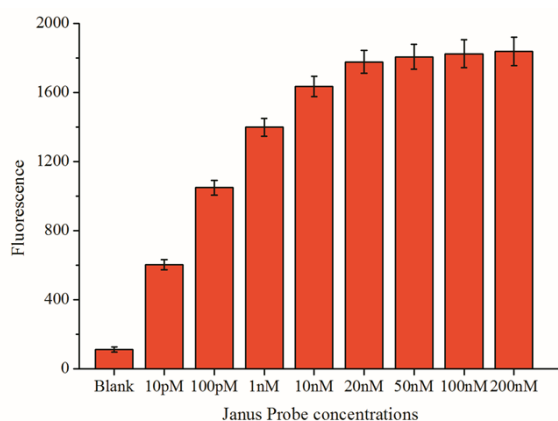


Figure S2. Effects of the concentrations of Janus probe for detection of 10 pM target miRNA-21. Different concentrations of Janus probe were used (0, 10 pM, 100 pM, 1 nM, 10 nM, 20 nM, 50 nM, 100 nM and 200 nM). The illustrated error bars represent the standard deviation of five repetitive measurements.

Optimization of Circular Template Concentration

As demonstrated in Figure S3, the RCA signals increased along with the concentration increment of circular templates. When the concentration of the circular template was 10 nM, fluorescence intensity for low-concentration targets (1 fM) were almost indistinguishable from the background, which led to relatively poor sensitivity as compared to the use of other concentrations of circular template. However, excessive circular template (100 nM) significantly increased the background, which made it difficult to quantify miRNA concentrations. We found that 25 nM of circular template possessed the highest S/N ratio. Therefore, 25 nM was chosen as the optimum circular template concentration in this study.

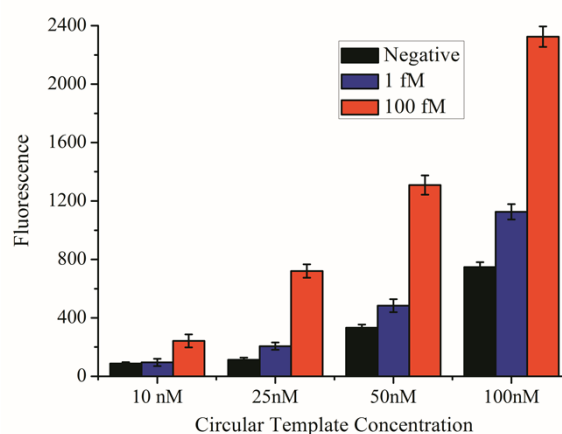


Figure S3. The concentration effects of the circular template on fluorescence intensities. The concentrations of circular template were varied from 10 nM to 100 nM. The illustrated error bars represent the standard deviation of five repetitive measurements.

Viability of the Method for Different miRNA Detection

To explore the generalizability of our design, we applied this method to detect other miRNA. We employed miRNA-16 as the model target analyte. The bifunctional Janus probe contain two regions. One region of the Janus probe is the complementary RNA sequence of target miRNA. The other region of the Janus probe is DNA sequence, which can be used as primer for RCA. When it comes to miRNA-16 detection, we just replace the RNA sequence ‘UCAACAUCAGUCUGAUAAGCUA’ to ‘CGCCAAUAUUUACGUGCUGCUA’. While the DNA sequence in the Janus probe doesn’t need to be changed. The result shown in Figure S4 demonstrated that proposed method is general for different target miRNA detection.

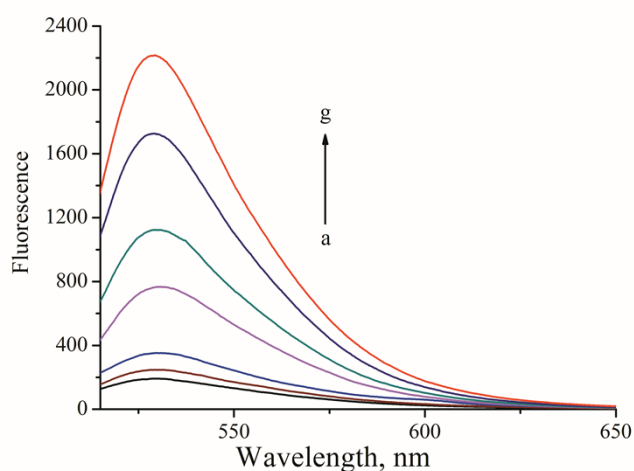


Figure S4. Fluorescence emission spectra upon addition of different target miRNA-16 concentrations: (a) 0 M, (b) 1 fM, (c) 10 fM, (d) 100 fM, (e) 1 pM, (f) 10 pM, and (g) 100 pM. The Janus probe is: 5’-CGCCAAUAUUUACGUGCUGCUAAAATTGCGAAATGCTAAACC-3’. The illustrated error bars represent the standard deviation of five repetitive measurements.

Determination of miRNA-21 in Human Serum Samples

The standard addition method was used to determination of miRNA-21 in the breast cancer patient serums. We choose cancer patient serum sample 1' as an example. A series of synthetic miRNA-21 at concentrations of 0, 10, 20, 40, 60, 80, and 100 fM were spiked into serum sample 1', respectively, with equal volume to establish a calibration curve (in this case, the concentration of serum sample 1' and synthetic miRNA-21 were both equivalent to be diluted 2-fold). The other steps were followed by the same experimental procedures described in experimental section. From the Figure S5, the fluorescent intensities of the RCA products increased with increasing the spiked concentration of the synthetic miRNA-21 and exhibited a fairly good linear relationship in the range from 5 to 50 fM. According to the standard addition method, the concentration of miRNA-21 in the diluted serum sample 1' was estimated to be 30.01 fM. Thus, the content of miRNA-21 in the original serum sample 1' was calculated to be 60.02 fM.

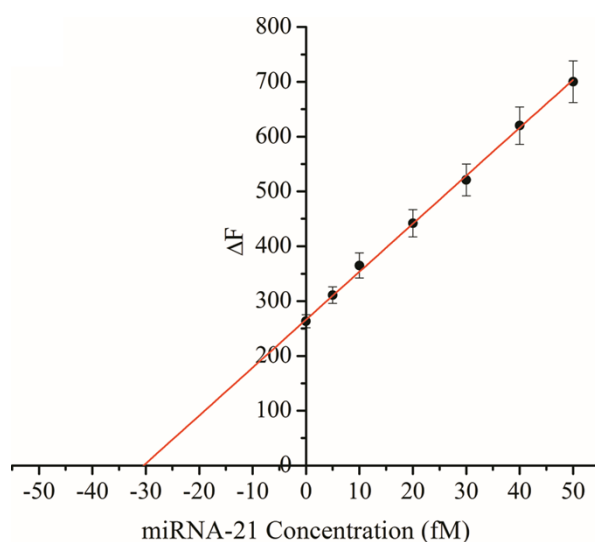


Figure S5. Relationship between the fluorescence intensity and the standard addition of synthetic miRNA-21 into diluted serum sample 1'. The data shown here represent the average of three independent experiments.

Comparison of proposed method and qPCR detection methods

As references, expression levels of the breast cancer patient serum miRNA-21 were simultaneously quantified by a commercial qRT-PCR kit. The results obtained with the proposed are in good agreement with those obtained by qRT-PCR on the same samples.

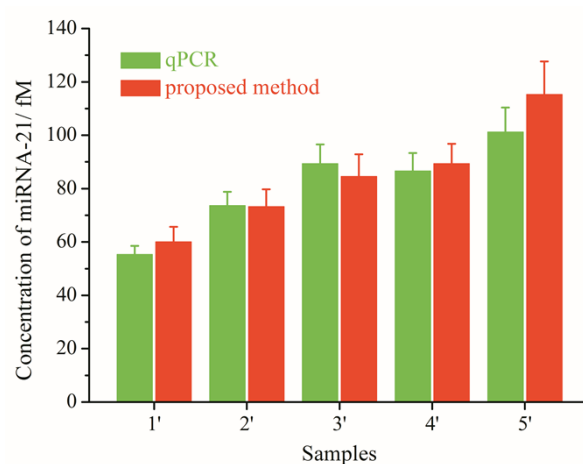


Figure S6. Bars represent the expressions of miRNA-21 in serum samples detected by the proposed method (red bars) and qRT-PCR (green bars), respectively. Error bars represent standard deviations for measurements taken from at least five independent experiments.

References

- (1) Chen, A. Q.; Bao, Y. W.; Ge, X. X.; Shin, Y. S.; Du, D.; Lin, Y. H. *RSC Adv.*, **2012**, *2*, 11029-11034.
- (2) Munge, B. S.; Coffey, A. L.; Doucette, J. M.; Somba, B. K.; Malhotra, R.; Patel, V.; Gutkind, J. S.; Rusling, J. F. *Angew. Chem., Int. Ed.* **2011**, *123*, 8061-8064