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Supporting information

A DNA-linker-DNA bifunctional probe for simultaneous SERS detection of miRNAs via symmetric signal amplification

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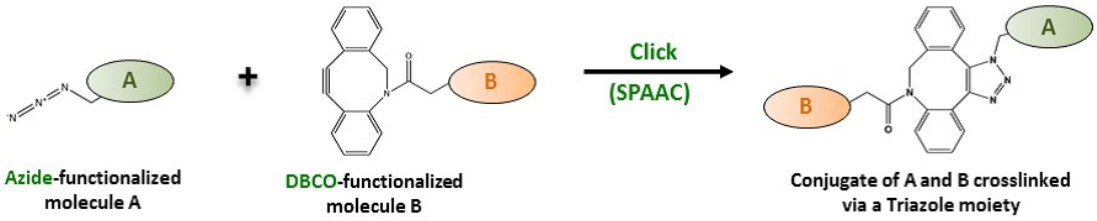
Experimental section

Materials and reagents: All oligonucleotides designed in this study were synthesized by Sangon Biotech Co., Ltd. (Shanghai China). Their sequences are listed in Table S-1. Magnetic microbeads (MBs) modified with carboxyl group (1.0-2.0 μm, 10 mg/mL) were commercially available from Baseline Chrom Tech Research Center (Tianjin, China). 1-Ethyl-3- (3-dimethylaminopropyl) carbodiimide (EDC) and Chloroauric acid ($HAuCl_4.3H_2O$) were obtained from Sigma-Aldrich (St. Louis, MO). The washing buffer was phosphate-buffered saline (PBS) solution (10 mM phosphate buffer, 0.14 M NaCl, 2.7 mM KCl, pH 7.4). All chemical reagents employed were of analytical grade and used without further purification. Doubly distilled deionized water was used throughout the experiments.

The structure of linker:



Apparatus: Transmission electron microscopy (TEM) images were recorded on a JEM-2100 transmission electron microscope (JEOL, Japan). UV/Vis absorption spectra were obtained with a Cary 50 Series spectrophotometer (Varian, USA). SERS was performed on an inVia Raman microscope (Renishaw, England).

Preparation of gold nanoparticles (AuNPs): AuNPs were synthesized by reduction of tetrachloroauric acid ($HAuCl_4$) with trisodium citrate. Briefly, 100 mL of 0.01% (w/w) $HAuCl_4$ solution was boiled with vigorous stirring, and then 1.5 mL of 1%

(w/w) trisodium citrate solution was rapidly added to the boiling solution. The resulting reaction solution was maintained at its boiling point for 30 min. The color of the solution changed from faint-yellow to purple before a wine-red color was reached, indicating the formation of AuNPs. The resulting colloidal suspension was allowed to naturally cool to room temperature with continuous stirring. The synthesis of AuNPs was characterized by TEM (Figure. S1). The prepared gold colloidal solutions were stored in brown glass at 4 °C untill use.

Preparation of AuNP-Functionalized Raman probes (Rox bio-barcode and Cy3 bio-barcode): The Rox bio-barcodes were prepared as follows, 10 μL of 1 × 10^{-7} M 5′-thiol modified capture DNA (B3) and 50 μL of 1 × 10^{-6} M Rox-DNA (5′-thiol and 3′-Rox, C1) was added to freshly prepared AuNPs (1 mL) and shaken gently overnight (approximately 20 h) at 37 °C. And the Cy3 bio-barcodes were prepared as follows, 10 μL of 1 × 10^{-7} M 5′-thiol modified capture DNA (B4) and 50 μL of 1 × 10^{-6} M Cy3-DNA (5′-thiol and 3′-Cy3, C2) were added to freshly prepared AuNPs (1 mL) and shaken gently overnight (approximately 20 h) at 37 °C. Subsequently, the DNA-AuNPs conjugates were aged in 0.05 M salts solution (NaCl, 200 μL) for 6 h and in 0.1 M salts solution (NaCl, 200 μL) for 6 h, respectively. Excess reagents were removed by centrifugation at 10,000 rpm for 30 min. After the supernatant was removed, the red precipitate was washed and centrifuged three times. The resulting bio-barcode probe was finally dispersed into 100 μL of 0.01 M pH 7.4 phosphate buffer (PBS, 0.01 M, pH 7.4) and stored at 4 °C.

Immobilization of two kinds of hairpin DNAs onto MBs: Hairpin DNAs（B1

and B2） were heated to 90 °C for 5 min and then allowed to cool to room temperature for 1 h before use. First, a 10 μL suspension of carboxylated MBs were placed in a 0.5 mL Eppendorf tube (EP tube) and separated from the solution on a magnetic rack. After the MBs were washed three times with 100 μL of 0.1 M imidazol-HCl buffer (pH 6.8), they were activated in 100 μL of 0.1 M imidazol-HCl buffer (pH 6.8) containing 0.1 M EDC at 37 °C for 30 min. After been washed three times with 100 μL of 0.01 M PBS (pH 7.4), amino-group modified B1 DNA (0.1 μM, 50 μL) and B2 DNA (0.1 μM, 50 μL) were added to the freshly activated MBs and incubated at 37 °C overnight. Finally, the excess DNAs were removed by magnetic separation. The resulting DNA-conjugated MBs were rinsed three times with 200 μL of 0.01 M PBS (pH 7.4), resuspended in 50 μL of 0.01 M PBS and stored at 4 °C for use.

Assay procedure of miR-141 and miR-21: The microRNA analysis was carried out as follows. Firstly, the DNA-linker-DNA (S1) was heated at the water bath 90 °C for 5 min and allowed to cool to room temperature for 1 h. Then, miR-141 and miR-21 samples at specific concentration (10 μL), Klenow polymerase (1 μL), dNTPs (5 μL), Nb.BbvCI (1 μL) and buffer were added into the heated aptamer solution(10 μL). Finally, the whole system was incubated for 2 h at 37 °C. After that, the reacted solution and bio-barcode (10 μL) were added into DNA-conjugated MBs for 2 h at 37 °C. Then the MBs incorporated Raman probes were performed through magnetically controlled separation to remove the excess bio-barcodes, washed with PBS for three times and redispersed in 20 μL of 0.01 M PBS (pH 7.4, 0.3 M NaCl) .

Cell culture: The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with heat-inactivated bovine serum (10%), 100 U/mL penicillin, and 100 U/mL streptomycin at 37 °C in a humidified atmosphere (5% CO_2 and 95% air). The cells were grown in 25-cm^2 cell culture flasks at a density of 6 × 10^5 cells/dish and were allowed to adhere for 12 h.

Preparation of cell lysates: The cells were collected in culture medium and centrifuged at 3,000 rpm for 5 min, washed once with PBS buffer and twice with buffer solution, and then centrifuged at 3,000 rpm for 5 min. The cell pellets were suspended in 600 μL of PBS buffer. Then, the cells were centrifuged at 12,000 rpm for 5 min at 4 °C. After the supernatant was removed, the cell precipitate was washed and centrifuged three times. The cell precipitate was finally dispersed into 30 μL of 0.01 M PBS buffer (PBS, pH 7.4) and stored at 4 °C (the concentration of cell is 100 cells/μL).

SERS measurements: Two microliters of the MB-incorporated Raman molecular probe solution was pipetted onto the surface of gold film (glass slide with gold plating) and air-dried at room temperature. Raman spectra were measured using a Raman spectrometer at an excitation laser of 633 nm. The laser power was 5 mW. The acquisition time for each spectrum was 10 s. Eleven spectra were obtained from different cites of each sample and averaged to represent the SERS results, and the experiments were carried out in triplicate. Error bars show the standard deviation of the eleven experiments.

Results and discussions

Characterization of AuNPs: The AuNPs synthesized were charactered by TEM (Figure S-1).

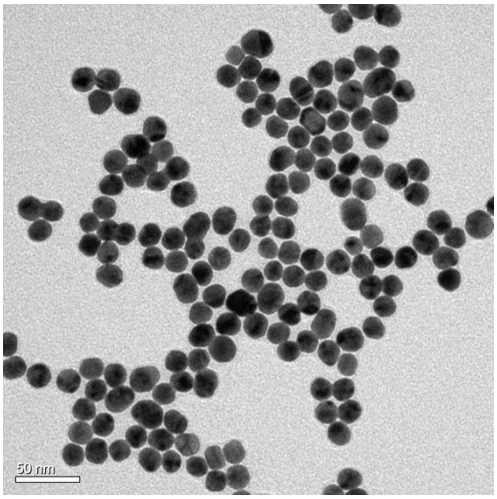


Figure S-1. TEM image of AuNPs synthesized (approximately 16 nm)

UV-visible spectra of the Rox-DNA and Cy3-DNA conjugates: The UV-visible spectra of the signal probe (Rox-DNA, Cy3-DNA), capture probe, AuNPs and bio-bar codes were carried using a Cary 50 UV/Vis-NIR spectrophotometer. As shown in Figure S-2 (A), curve a, b, c exhibited the characteristic absorbance of capture DNA (~260 nm), Rox-DNA (~260 nm and two characteristic absorbance at 500~600 nm) and AuNPs (~520 nm), respectively. Curve d, which exhibited the characteristic absorbance of both Rox-DNA and AuNPs, indicated that Rox-DNA had successfully conjugated with AuNPs. The results in Figure S-2 (B) indicated that Cy3-DNA was also conjugated with AuNPs.

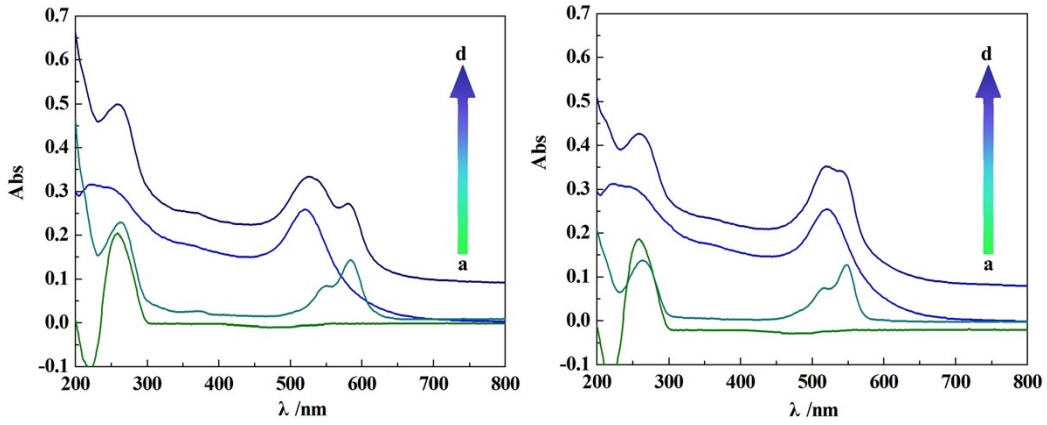
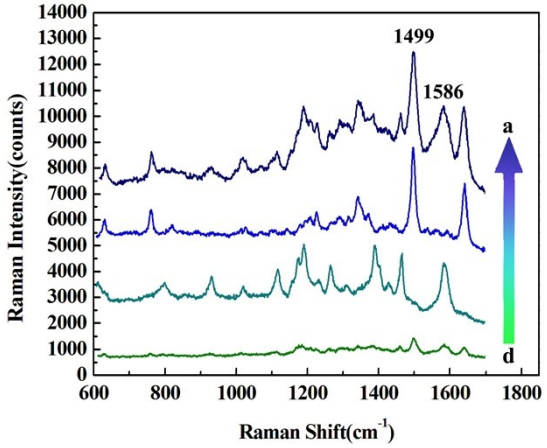


Figure S-2. (A) UV spectra of capture DNA1 (a), Rox-DNA (b), Au colloid (c) and Rox bio–barcode (d); (B) UV spectra of capture DNA2 (a), Cy3-DNA (b), Au colloid (c) and Cy3 bio–barcode.

The feasibility of this strategy:



- **Figure S-3.** Raman intensity of different target. (a) both miR-21 and miR-141; (b) only miR-141; (c) only miR-21; (d) no target. (miR, 10^{-13} M).

Optimization of the reaction temperature and pH: The temperature and pH of reaction solutions strongly influence the efficiency of DNA hybridization and the activity of enzyme. So they are the two most important parameters for optimizing the analysis system. We therefore investigated the intensity of Raman signal under different temperature and pH conditions. The influence of pH values ranging from 5.5 to 8.5 for the Raman-signal intensity produced by 1.0×10^{-13} M miR-141 in Figure S-

4A, the Raman intensity (ΔI) reached a maximum at pH 7.4. Thus, we selected pH 7.4 as the optimum condition. As shown in Figure S-4B, the Raman intensity (ΔI) increased with the temperature from 20 °C to 50 °C, which reached a maximum at 37 °C. After that, the intensity decreased gradually. Thus, 37 °C was chosen as the optimal temperature.

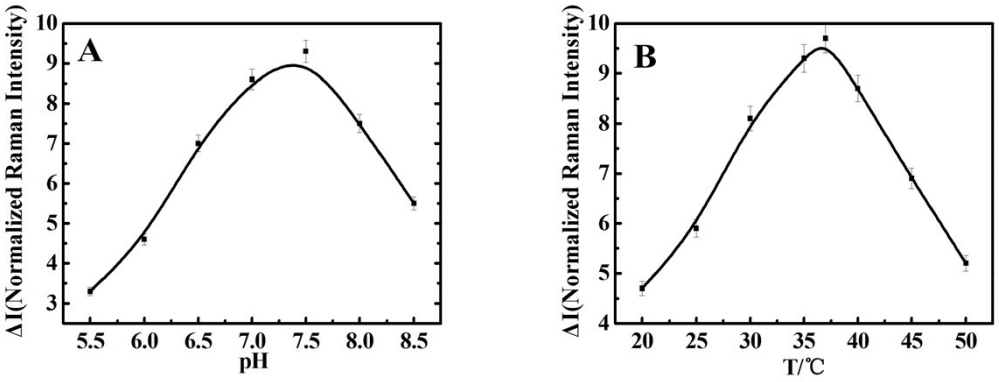


Figure S-4. Influence of pH (A) and temperature (B) of the reaction on the ΔI signal (miR-141, 10^{-13} M).

Optimization of the incubation time: The incubation time was investigated. Figure S-5 shows the changes of Raman signals generated by performing the experiments at different time intervals. The results revealed that the Raman intensity increased rapidly as the incubation time prolonged and reached a plateau after 120 min. We therefore deduced that 120 min was the best incubation time for the assay.

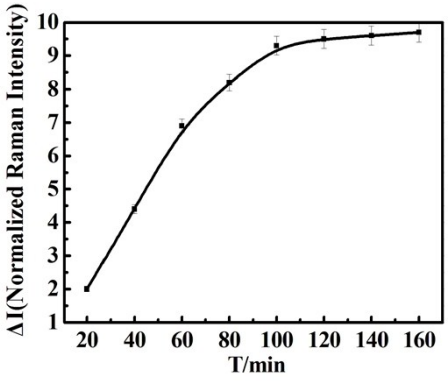


Figure S-5. Effect of the time of the reaction on the ΔI signal (miR-141, 10^{-13} M)

Optimization of the amount of nicking endonuclease and Klenow polymerase: The amount of nicking endonuclease and Klenow polymerase has effected on the Raman intensity. To increase the sensitivity of Raman detection, we designed a series of control experiments to optimize the amount of Nb.BbvCI and Klenow polymerase. The results demonstrated that the Raman intensities enhanced rapidly with the increase of the amount of Nb.BbvCI up to 0.50 $U\mu L^{-1}$ (Figure S-6A), followed by a plateau. Therefore 0.50 $U\mu L^{-1}$ of Nb.BbvCI was adopted to be the optimum amount for the system polymerization. Similarly, as it could be seen in Figure S-6B, 0.45 $U\mu L^{-1}$ of the polymerase was the optimum.

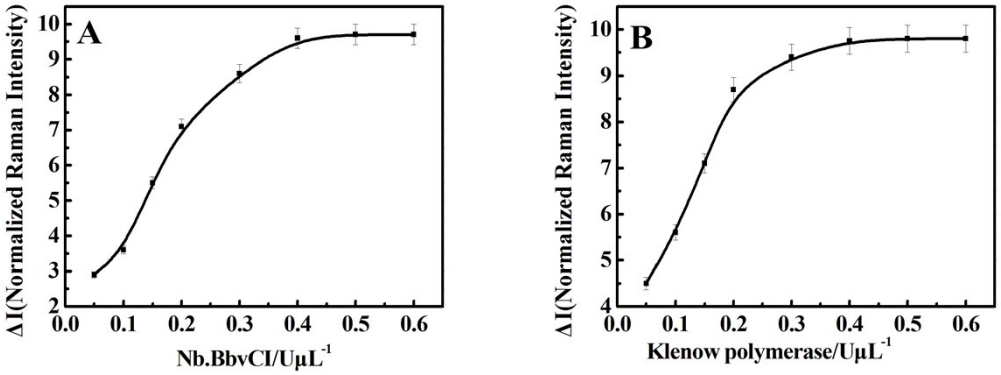


Figure S-6. Effect of the amount of nicking endonuclease Nb.BbvCI (A) and the Klenow polymerase (B) on the ΔI signal (miR-141, 10^{-13} M).

Optimization of the ratio and concentrations of two kinds of probes: For the preparation of bio-barcode probe, the Raman intensity is also influenced by the concentrations and proportion of the signal DNA probes (B3, B4) and the capture probe (C1,C2) immobilized on the Au-NPs. To improve the sensitivity of SERS quantification of DNA, the concentrations and proportion of the capture probe and the signal DNA probes were optimized. Figure S-7 shows the variance of SERS intensity

with the concentration of capture probe (B4) from 1.0×10^{-8} M to 1.0×10^{-6} M. It can be seen that the SERS intensity increases with the increase of B4 concentration from 1.0×10^{-8} M to 1.0×10^{-7} M, and reaches a maximum at 1.0×10^{-7} M, upon analyzing target miR-21 at a concentration corresponding to 1.0×10^{-10} M, and then it starts to level off, attributed to the steric and electrostatic hindrance arising from the more tightly packed probe, which were necessary for highly hybridization efficiency. Therefore, the concentration of hairpin probes 1.0×10^{-7} M was employed in the following work.

In addition, Figure S-7 shows the variance of Raman intensity with the proportion of the signal DNA probes and the capture probe, at the series of the concentrations of B4, 10^{-8} M, 10^{-7} M and 10^{-6} M. It was obviously that Raman intensity increased upon raising the proportion from 1:30 to 1:50 (B4 : C2), and then it started to level off. Thus the ratio of 1:50 was selected for the subsequent assays.

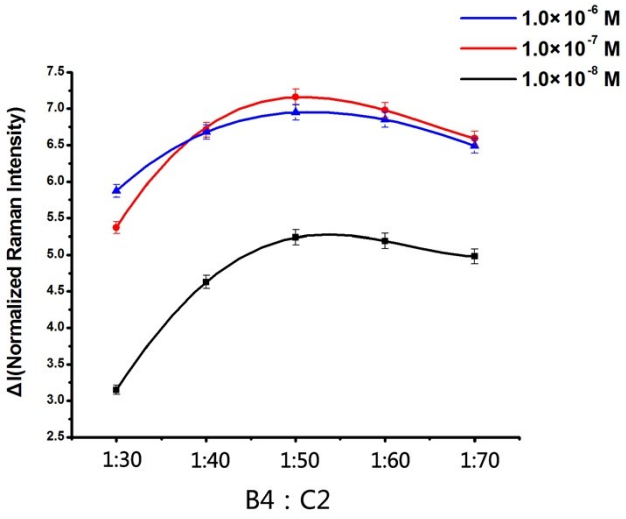


Figure S-7. The variance of Raman intensity with the concentrations and proportions of the signal DNA probes and the capture probes (B4, 1.0×10^{-8} M, 1.0×10^{-7} M and 1.0×10^{-6} M).

The practicability of the method in the presence of different concentration ratios of two kind of miRNA mixture: A series of controlled experiment was executed. A mixtures of miR-21 and miR-141 with different concentration ratio of 1:1, 1:5, 1:10 and 1:100 were detected using this method. The SERS intensity were presented in the concentration of miR-21, respectively for 10^{-14} M, 10^{-13} M, 10^{-12} M. From Figure S-8A-C, it can be seen that in the presence of the mixture with different concentration ratios, no obvious difference in SERS signals of column from 1:1 to 1:100 was observed, suggesting the relative high concentration miR-141 had no significant effect on the detection of miR-21, in the linear range of the method. The analysis shows the present method has a good stability and practicability for multiple miRNAs detection with the mixed analytes of different concentration ratios .

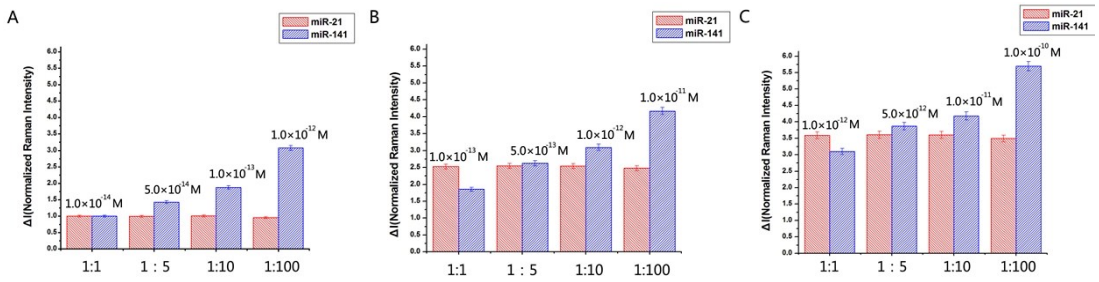


Figure S-8. The SERS intensity of a mixture of miR-21 and miR-141 with different concentration ratio of 1:1, 1:5, 1:10 and 1:100.

The comparison of SERS intensity of the method with DLD probe versus the intensity from the method with unlinker probes： In this work, the DLD probe is designed to minimize the uneven signal amplification through two separate probes, with easy operation, high sensitivity and specificity. To prove the advantage of the DLD probe, two experiments of 10 replicate measurements of 1.0×10^{-13} M miR-141 and miR-21 were performed with DLD probe and two same sequence single DNA

probe, respectively.

As shown in Figure S-9A, B, compared with the method using the single two DNA probes, the intensity distributions with the method using DLD probe are relatively homogeneous. Relative standard deviations (RSD) of 1.93% and 1.71% for miR-141 and miR-21, respectively, were obtained from the 10 replicate measurements using the DLD probe under the RSDs of 3.89% and 3.16% for miR-141 and miR-21, from the measurements using the two single probe. So the SSA method with DLD probe has very good stability and accuracy for simultaneous detection.

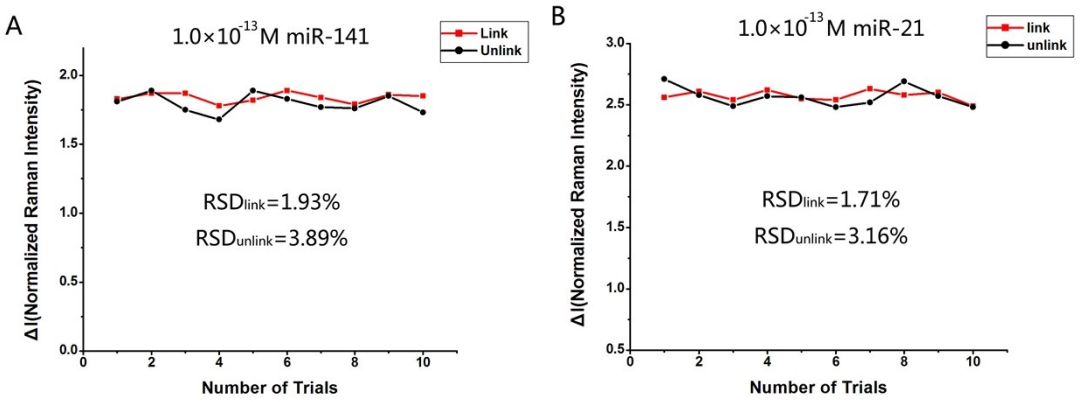


Figure S-9. Multiple test results based on DLD probes and unlinker probes (two same sequence single DNA probes).

Table S-1. Oligonucleotide sequences used in our experiments.

Oligonucleotide name	Sequences (5' to 3')
S1	5'-CCATCTTTACCAGACAGTGTTAGTCGACTCCGCTCCTGGGTAACACTGTCTGGGCGGAGT-3'-**linker**-5'-TCAACATCAGTCTGATAAGCTAGTCGACTCCGCTCCTGGGTAGCTTATCAGACGCGGAGT-3'
B1	NH_2-TTTTTTCCGGAGAACAACCATCTTTACCAGACAGTGTTAGTCGAATTGTTCTAATC
B2	NH_2-TTTTTTAGGGCATTAAATCAACATCAGTCTGATAAGCTAGTCGAATTTAATGATGG
B3	SH-GATTAGAACAA
B4	SH-CCATCATTAAA
C1	Rox-TTTTTTCCTAGCGAC-SH
C2	Cy3-TTTTTTCCTAGCGAC-SH
miR-21	UAGCUUAUCAGACUAAUGUUGA
miR-141	UAACACUGUCUGGUA AAGAUGG
miR-200b	UAAUACUGCCUGGUAAUGAUGA
miR-203	GUGAAAUGUUUAGGACCACUAG
miR-21-1bm	UAGCUCAUCAGACUAAUGUUGA
miR-141-1bm	UAACACCGUCUGGUA AAGAUGG

Table S-2. The comparison of different methods for miRNA detection

Method	Transducer	Detection limit
Two distinguishable CdSe@ZnS and CdTe quantum dot (QD) probes by target recycling amplification strategy	Fluorscence	miR-21, 1 pM [1] miR-141, 1 pM [1]
Using target catalyzed hairpin assembly (CHA) signal amplification	Fluorscence	miR-21, 3.5 pM [2]
Chronocoulometric detection based on electrocatalytic nanoporous superparamagnetic nanocubes.	CV	miR-21, 100 fM[3]
Netlike rolling circle amplification (NRCA)	paper-based point-of-care testing (POCT)	miR-21, 9.3 fM[4]
Duplex specific nuclease (DSN) signal amplification	Fluorscence	miR-141, 1.03 pM [5]
Duplex specific nuclease (DSN) signal amplification	ICP-MS	miR-141, 0.84 pM [6]
Using CdSe/ZnS quantum dots (QDs) modified by FRET Quencher-functionalized nucleic acids and DSN-mediated cleavage	Fluorscence	MiR-141, 0.28 pM [7]
This work	SERS	miR-21, 5.6 fM miR-141, 7.5 fM

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