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Supplementary Information

A hybridization-based dual-colorimetric kit for circulating cancer miRNA detection

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1. Materials and Instruments

The miRNA-200b and miRNA-223 were purchased from Sangon Biotech Co., Ltd. The DNA1, DNA2, DNA3 and DNA4 were purchased from were purchased from Sangon Biotech Co., Ltd. The streptavidin and horseradish peroxidase (HRP) were purchased from Beijing Solarbio Science ＆ Technology Co., Ltd. The GelRed was purchased from biosharp life sciences Co., Ltd. The experimental water used was Mill-Q secondary ultrapure water (18.2 MΩ•cm). The chemical reagents used in the experiment were analytical grade and without purification. DNA Mixer was purchased from Ningbo Scientz Biotechnology Co., Ltd. Peiqing JS-680D Automatic digital gel imaging analysis system was purchased from Peiqing Science ＆ Technology Co., Ltd.

2. Methods

2.1 The Synthesis of Fe_3O_4-COOH Nanoparticles (NPs)

Ferric chloride (0.65 g, 4 mmol) and trisodium citrate (0.2 g, 0.68 mmol) were dissolved in 20 ml of ethylene glycol, stirring at room temperature to dissolve the ferric chloride. Then, anhydrous sodium acetate (1.2 g) was added to the reaction system, and continued stirring for 30 min. Subsequently, the solution was transferred into a Teflon-lined stainless steel autoclave, and heated to 200 °C for 10 h.[1] Fe_3O_4-COOH was obtained by magnetic separation from the reaction system, and was washed three times with absolute ethanol and water.

2.2 The Synthesis of Fe_3O_4-Streptavidin Nanoparticles

At first, Fe_3O_4-COOH NPs (1 mg) were washed two times with 200 μL MEST solution (100 mM MES, pH 5.0, 0.05% Tween 20). Next, Fe_3O_4-COOH was obtained by magnetic separation and dissolved in a fresh buffer containing 100 μL EDC (10 mg/mL, the solvent is MEST buffer) and 100 μL NHS (10 mg/mL, the solvent is MEST buffer). The mixed solution was stirring at room temperature and reacting for 30 min. After that, the carboxyl group is activated. Then the activated Fe_3O_4-COOH NPs and streptavidin (30-50 μg/mg) were mixed in a microcentrifuge tube with mixing slowly (Solvent is PBS buffer, pH≈8.0, 0.05% tween 20 can be added to improve the dispersion of Fe_3O_4-COOH NPs). After cross-linking for 2 h at 25 ℃, the mixture was kept in 4 ℃ overnight. Then the Fe_3O_4-streptavidin NPs were obtained by magnetic separation, and the supernatant was discarded. Next, the Fe_3O_4-streptavidin NPs were treated with PBST solution containing 1% BSA (blocking buffer) for blocking. Pour off the blocking buffer and rinse briefly with PBS buffer three times, the final Fe_3O_4-streptavidin NPs were obtained after that.

2.3 The Synthesis of Au NPs.

This work uses the classical sodium citrate reduction method to synthesize the 20 nm Au NPs. All glassware used was pretreated with aqua regia (HNO_3 / HCl=3:1, v / v). The solution of $HAuCl_4$ (0.01%, 100 mL) was heated to boiling and refluxed 10 min. Then added trisodium citrate (1%, 3.5 mL) quickly under stirring. After a few minutes, the colors of solution turns from colorless to blue-violet and finally to burgundy. And the reaction was maintained 15 min after the color changed into burgundy. Subsequently, this reaction was stopped heating and continued stirring until it cools to room temperature. The colloidal gold solution was filtered with 0.45 μm Millipore membrane filter and stored in 4 ℃ for using after then. The size of Au NPs was measured by Transmission electron microscopy (TEM) and Dynamic light scattering (DLS). The concentration of Au NPs was calculated based on their extinction coefficient ($\varepsilon = 2.7\times10^8$ L mol^{-1} cm^{-1}) at 524 nm.

2.4 DNA Functionalized Au NPs.

DNA3 was purchased by sangon biotech Ltd. At first, DNA3 (50 nM of final concentration)

was mixed with Gold nanoparticle (1 nM) solution, and the mixture was slowly shaken at 180 rpm, 25 °C. After 4 hours, 0.1% SDS solution was added to the reaction system (final concentration of SDS is 0.01%) and shaken at 25 °C overnight. Then the prepared NaCl solution (1 M) that was dissolved in PBS buffer (pH = 7.4) was added to the reaction over 8 h period and the final concentration of NaCl was 100 nM. The mixture reacted at 25 °C with shaking 180 rpm for 12 hours, and then remove it from the shaker. After aging treatment for 48 hours, the Au-DNA3 NPs were achieved by centrifugation (30 min, 4 °C, 13, 000 rpm). The NPs were stored at 4 ° C in the dark for next using.

2.5 Horseradish Peroxidase (HRP) Conjugated-DNA4[2].

Buffers used in this work: Buffer A: 0.1 M NaCl, 0.1 M sodium phosphate buffer, pH 7.3, 0.05% Tween-20.

Buffer B: 0.1 M sodium borate buffer, pH 9.2.

To 30 μL of 1 OD amine-DNA in Millipore water, 30 μL of Buffer B were added and mixed. This solution was further mixed with 20 mg of p-phenylene diisothiocyanate (PDITC) dissolved in 1 mL DMF. The resulting solution was placed on a shaker and react in the dark for 2 hours at room temperature. After that, the solution was mixed with 3 mL of Millipore water and 3 mL 1-butanol. The upper organic phase was discarded after centrifuging for 10000 rpm, 10 min. The aqueous phase was then extracted with 2 mL 1-butanol three times, and purified by Amicon-10K using Buffer A without Tween-20 for 8 times to produce a PDITC-activated amine-DNA solution. Then, 1 mg of HRP were added to the activated DNA solution in Buffer A without Tween-20 to reach a final concentration about 5 mg / mL. The resulting solution was reacted at room temperature for 48 hours. To remove unreacted PDITC-activated amine-DNA, the solution was purified by Amicon-100K 3-5 times using Buffer A without Tween-20.

2.6 The Detection of miRNA-223.

Fe_3O_4-streptavidin NPs (1 mg) and biotin-labeled DNA1 (the final concentration is 1 μM) were mixed in a 1.5 mL centrifuge tube and mixing in DNA Mixer at room temperature for 20 min. Then the Fe_3O_4-streptavidin-biotin DNA1 NPs (FSBD1 NPs) were washed with PBS buffer for three times and obtained by magnetic separation. Briefly, the FSBD NPs (0.1 mg) and Au-DNA3 (3 nM) were mixed with samples with or without the target miRNA-223 in PBS buffer. After an incubation time of 15 min at room temperature, magnetic nanoparticles were removed by magnetic separation, and then the color change of the supernatant was observed.

2.7 The Detection of miRNA-200b.

Fe_3O_4-streptavidin NPs (1 mg) and biotin-labeled DNA2 (the final concentration is 1 μM) were mixed in a 1.5 mL microcentrifuge tube and stirred in DNA Mixer at room temperature for 20 min. Then the Fe_3O_4-streptavidin-biotin DNA2 NPs (FSBD2 NPs) were obtained by magnetic separation after washing with PBS buffer for three times. Originally, the FSBD2 NPs (0.1 mg) and HRP-DNA4 (the final concentration is 0.5 μM) were mixed with samples with or without the target miRNA-200b in PBS buffer. After an incubation time of 15 min at room temperature, the supernatant was removed under magnetic field. And the sediment was washed with PBST buffer 2 times, and suspended in the solution containing TMB and H_2O_2. After 2 minutes, the colorimetric changes were observed by naked-eye and recorded by taking pictures.

2.8 The Simultaneous Detection of miRNA-223 and miRNA-200b.

FSBD1 NPs, FSBD2 NPs, Au-DNA3 NPs and HRP-DNA4 were mixed with simples with or without the two target miRNAs in a 1.5 mL microcentrifuge tube and stirred in DNA Mixer at

room temperature for 15 min. Under the action of magnetic field, the color changes of supernatant were observed as the detection results of miRNA-223. Then the supernatant was removed, and the sediment was washed with PBST buffer for 2 times, and then suspended in the solution containing TMB and H_2O_2. After 2 minutes, the colorimetric changes were observed by naked-eye and recorded by taking pictures. The above procedures were repeated for three times.

2.9 Polyacrylamide Gel Electrophoresis

To verify the miRNAs-initiated hybridization reaction, 15% Polyacrylamide gel and 1 X TBE buffer were prepared, firstly. Next, the DNA1, DNA2, DNA3, and DNA4 were dissolved in PBS (final concentration is 10 μM) containing 50 mmol NaCl in 1.5 mL tubes, respectively. After heating this DNA tubes into 95 ℃ for maintaining 5 min, these DNAs were annealing by cooling to room temperature. Then DNA1, DNA3 were mixed with miRNA-223 and DNA2, DNA4 were mixed with miRNA-200b in PBS buffer, and the mixture were reacted at room temperature for 15 min. Afterwards, the samples were prepared via mixing 9 μL of reaction solution with 1 μL 10 X loading buffer. Next, the gel was running under constant pressure120 mV for 90 min. After that, the gel was dyeing by GelRed for 30 min. The gel images were scanned on peiqing JS-680D automatic digital gel imaging analysis system.

2.10 The detection of human serum samples

FSBD1 NPs, FSBD2 NPs, Au-DNA3 NPs and HRP-DNA4 were mixed with 50 μL serum samples and stirred in DNA Mixer at room temperature for 15 min. After that, the color change of supernatant after magnetic separation was obtained as the detection results of miRNA-223. Then the supernatant was removed, and the sediment was washed with PBST buffer for 2 times, and then suspended in the solution containing TMB and H_2O_2. After 2 minutes, the colorimetric changes were observed by naked-eye and recorded by taking pictures. The above procedures were repeated for three times.

2.11 Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of Shandong Normal University and written informed consents were signed by all patients. Experiments of human serum samples were carried out in accordance with the approved guidelines of China.

3. Supporting Figures and Tables

Table S1. The comparison of the method in this work and traditional methods

	Time	Steps	Temperature	Apparatus
Northern blotting	More than 2 h	More than 10 steps	90 ℃, 42 ℃	Developing device
Microarray hybridization	More than 2 h	More than 10 steps	70 ℃, 42 ℃, 65 ℃	CCD chip scanner
qRT-PCR	More than 2 h	More than 10 steps	95 ℃, 68 ℃, 37 ℃	Fluorescence quantitative PCR instrument
Dual-colorimetric kit (This work)	Within 20 min	Within 5 steps	Room temperature	No

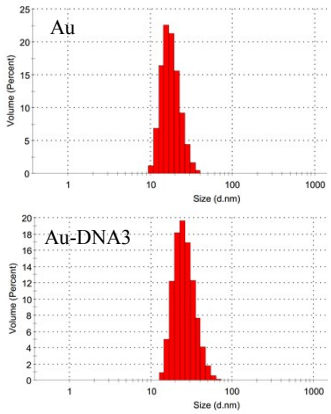


Figure S1. The DLS measurement of hydrodynamic sizes of Au and Au-DNA3 NPs.

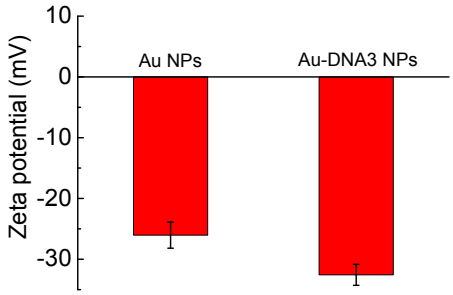


Figure S2. Zeta potentials of the Au and Au-DNA3 NPs.

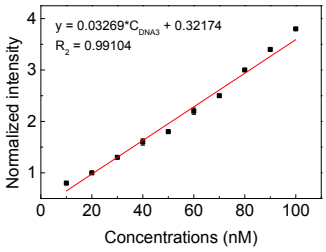


Figure S3. Standard linear calibration curve of DNA3.

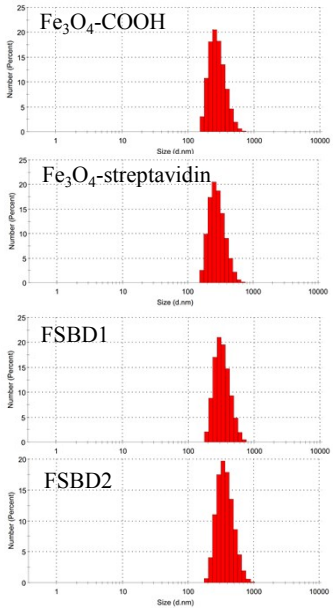


Figure S4. The DLS measurements of Fe_3O_4-COOH, Fe_3O_4-streptavidin, FSBD1, and FSBD2 NPs.

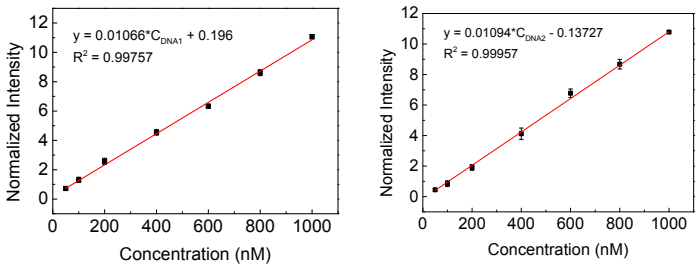


Figure S5. The standard curves of DAN1 (left) and DNA2 (right).

Table S2. The sequences used in this work.

Sequence (5'-3')
miRNA-200b: 5'-UCUUACUGGGCAGCAUUGGA-3'
miRNA-223: 5'-UGUAU UUGAC AAGCU GAGUU-3'
miRNA-205: 5'-UCCUUCAUUCCACCGGAGUCU-3'
miRNA-21: 5'-GCUUAUCAGACUGAUGUUGA-3'
DNA1: 5'-Biotin-AAAAAAAACTCAGCTTGTCAAATATGGTGTGAGTTATTTGACAAG-3'
DNA2: 5'-Biotin-AAAAAATCCAATGCTGCCCAGTAATGGTGTGAGTTTACTGGGCAG-3'
DNA3: 5'-SH-AAAAATGTCAAATAACTCACACCATATTTGACAAGTGGTGTGAGTT-3
New DNA3: 5'-TAMRA-AAAAATGTCAAATAACTCACACCATATTTGACAAGTGGTGTGAGTT-3'
DNA4: 5'-NH2-5'-AAAAAAGGGGAGTA AACTCACACCATTACTGGGCAGTGGTGTGAGTT-3'

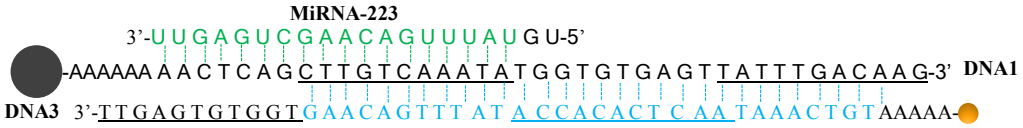
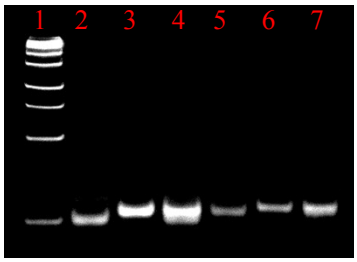


Figure S6. The hybridization scheme of miRNA-223 induced chain polymerization reaction.



1: Marker, 2: DNA1, 3: new DNA3,
4: DNA1+new DNA3, 5: DNA2,
6: DNA4, 7: DNA2+DNA4

Figure S7. Native-PAGE image of DNAs.

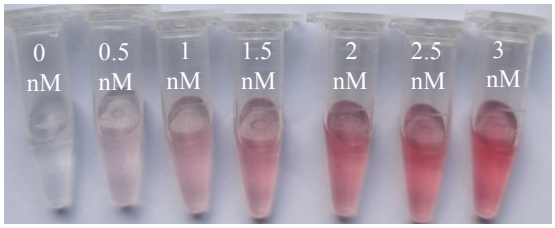


Figure S8. The picture of Au NPs solutions with different concentration.

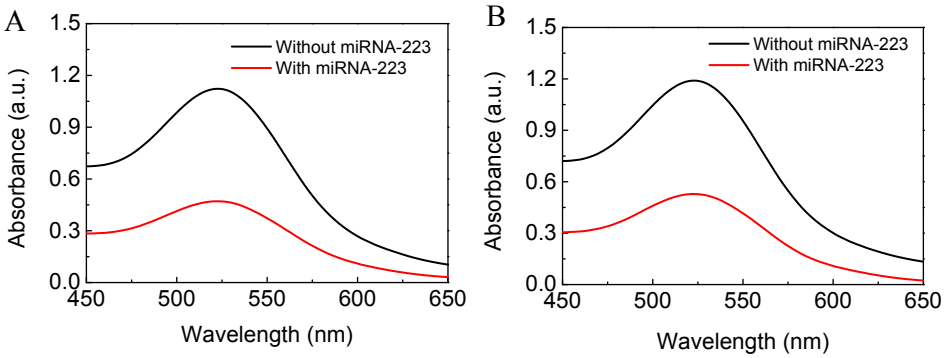


Figure S9. The UV-absorption charts. (A) The miRNA-223 in PBS. (B) The miRNA-223 in serum.

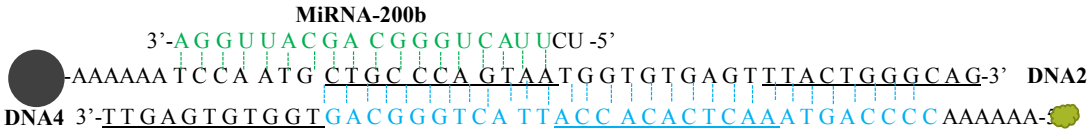


Figure S10. The hybridization scheme of miRNA-200b induced chain displacement reaction.

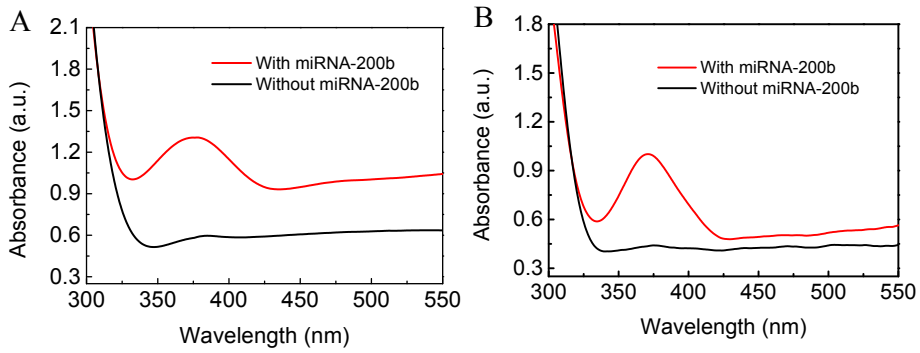
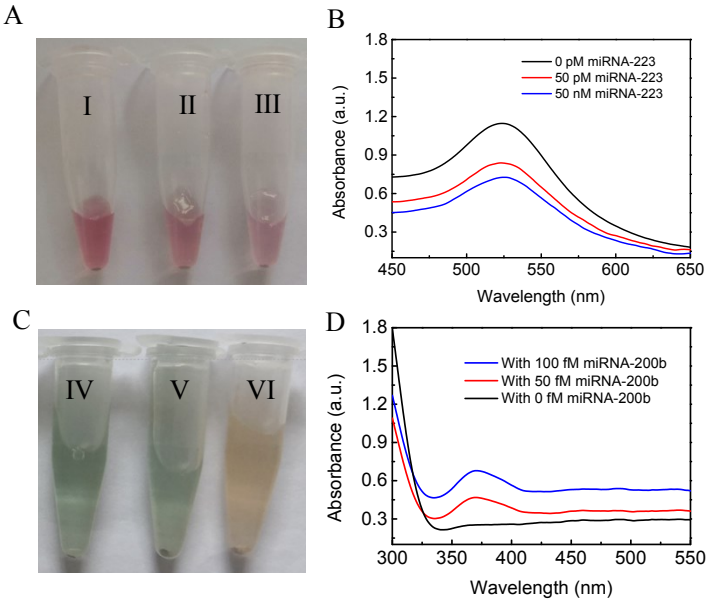


Figure S11. The UV-absorption charts. (A) The miRNA-200b in PBS. (B) The miRNA-200b in serum.



I: 100 nM miR-21; 100 nM miR-205; 100nM miR-200b
II: 50 pM miR-223; 100 nM miR-21; 100 nM miR-205; 100nM miR-200b
III: 50 nM miR-223; 100 nM miR-21; 100 nM miR-205; 100nM miR-200b
IV: 100 fM miR-200b; 100 nM miR-223; 100 nM miR-21; 100 nM miR-205
V: 50 fM miR-200b; 100 nM miR-223; 100 nM miR-21; 100 nM miR-205
VI: 100 nM miR-223; 100 nM miR-21; 100 nM miR-205

Figure S12. Specificity experiment based on the detection route (1) (A, B) and detection route (2) (C, D).

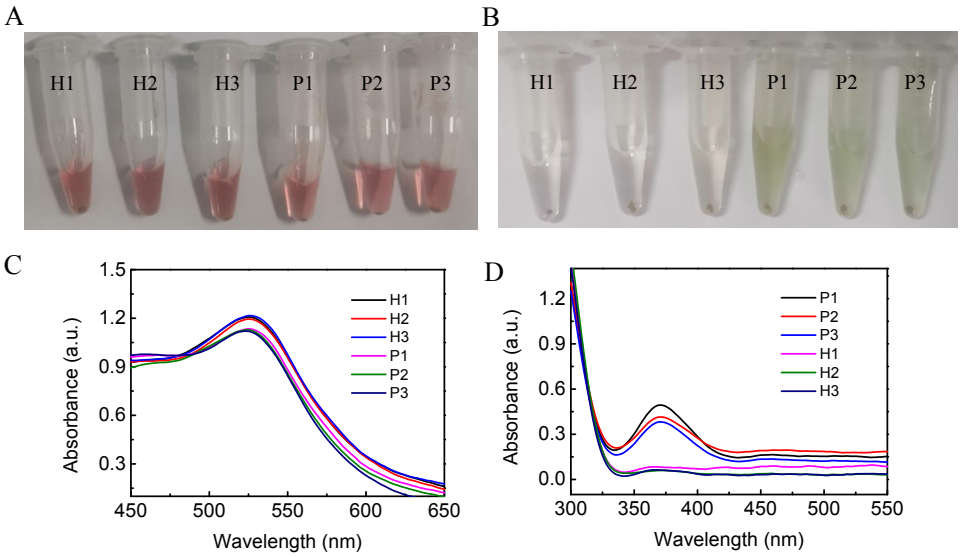


Figure S13. The detection of serum samples. The colorimetric detection of miRNA-223 (A) and miRNA-200b (B), using 6 serum samples suffering from 3 healthy individuals (H1-H3) and 3 lung cancer patients (P1-P3). (C)The UV-absorption charts of the supernatant corresponding to A. (D) The UV-absorption charts of the supernatant corresponding to B.

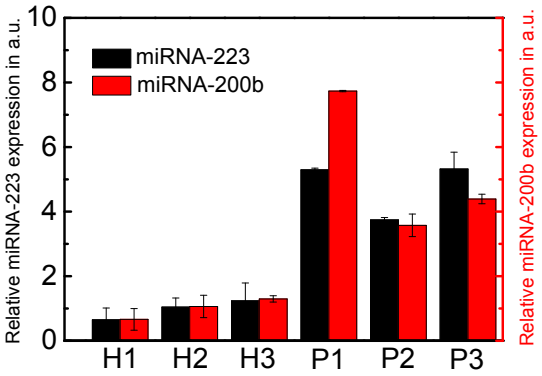


Figure S14. The qRT-PCR analysis of clinical serum samples. H1-H3 as the control groups, the error bars of measurements represent ± SEM of n=3 measurements.

Table S3. Detection performance comparison of miRNA colorimetric detection methods

Detection performance	This work	Other colorimetric methods
MiRNA target of simultaneous detection	Two	One[3-12]
Limit of detection (LOD)	10 pM and 10 fM	50 pM[3], 36.2 fM[4], 3.7 fM[7], 2.60 nM[8],1.06 nM[9], 260 fM[10]
Type of Colorimetric	Two	One[3-11], Two[12]
Detection time	17 min	2 h[3,7], 0.5 h[4], 3 h[5], 30 min[8], 35 min[9], 45 min[10], 40 min[11]
Reaction temperature	Room temperature	37 °C[3-4, 8], Room temperature[5, 9], 30 °C[6], 55 °C[10],
Application	Human serum sample	Cell lysates[3, 5-6, 11-12], Synthesized miRNA[4-5, 7-8], Human serum sample[9], Tissue sample[10], Mouse serum sample[11].

4. Reference

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