Supporting information for:

An enzyme-powered microRNA discriminator for subtype-specific diagnosis of breast cancer

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Experimental Section

Chemicals and Materials

T7 Exo, NEBuffer, and streptavidin-functionalized magnetic beads were purchased from New England Biolabs (Beijing, China). Fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM), and RPMI 1640 were purchased from Gibco Co., Ltd. (Beijing, China). Mammary epithelial cell medium (MEpiCM) was obtained from ScienCell (Shanghai, China). MiR-21 mimic (micrONhsa-miR-21-5p mimic) and mir-21 inhibitor (micrOFF hsa-miR-21-5p inhibitor) were purchased from Guangzhou RiboBio Co., Ltd. (Guangzhou, China). Mir-210 inhibitor (targapremir-210) was purchased MedChemExpress LLC (Shanghai, China). CB [7] was purchased from Sigma (Shanghai, China). The oligonucleotides were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China) and their sequences were listed in Table S4.

Fluorescence analysis of toehold-mediated strand displacement reactions

For fluorescence analysis, 100 μL of reaction samples with a final concentration of 1 μM were prepared, including A/FAM-B/BHQ1-C, JOE-A/B/BHQ1-C, and the mixture of A/FAM-B/BHQ1-C and miR-21, JOE-A/B/BHQ1-C and miR-21, A/FAM-B/BHQ1-C and miR-210, JOE-A/B/BHQ1-C and miR-210, A/FAM-B/BHQ1-C and both miRNAs, and JOE-A/B/BHQ1-C and both miRNAs. The FAM and JOE fluorescence was determined by fluorescence spectrometer at excitation wavelength of 498 nm and 520 nm, respectively. To prepare fluorescent magnetic beads loading with A/FAM-B/CY5-C, streptavidin-functionalized magnetic beads were incubated with biotin-A at 25 ^{o}C for 30 min, and hybridized with CY5-C and FAM-B at 37°C for 2 h. The labeled magnetic beads then reacted with miR-21, miR-210, and both miRNAs at 37 ^{o}C for 1 h, and washed with PBS for three times before use.

Electrophoresis analysis of T7 Exo-powered strand displacement reactions

50 μL of different DNA samples were prepared for electrophoresis analysis. In detail, strands A, B and C were first heated at 95^{o}C for 5 min and naturally cooled to room temperature to prepare three-stranded signal probe A/B/C. After that, 5 μL of the A/B/C (10 μM) was mixed with 5 μL of different miRNA inputs (10 μM) and 1 μL of T7

Exo (10 units) in NEBuffer, and incubated at the room temperature for 40 min to sustain T7 Exo-powered strand displacement reactions. For gel electrophoresis, 8 μL of the prepared DNA samples were mixed with 2 μL of 5 × loading buffer, and the mixture was loaded onto 15% polyacrylamide gel. Electrophoresis separation was carried out in 1 × Tris-borate-EDTA buffer at 120 V for 90 min, and the gel was finally photographed using a GelDoc XR$^+$ system (Bio-Rad, USA).

Electrochemical determination of miRNA based on T7 Exo-powered miRNA discriminator

For electrochemical determination of miRNA, electroactive signal probe A/B/C was prepared by mixing 3 μL of strand A (100 μM) that was labelled by MB and Fc molecule at each end, 3 μL of strand B (100 μM), 3 μL of strand C (100 μM) and 21 μL of PBS in a centrifuge tube, followed by being annealed at 95^{o}C for 5 min and naturally cooled to room temperate. Then, 30 μL of the prepared electroactive A/B/C was reacted with 15 μL of sample solution containing different miRNAs with a desired concentration and 1 μL of T7 Exo (10 units) in NEBuffer. The reaction was performed at 25^{o}C for 40 min, and EDTA (20 mM) was added to terminate the digestion for 15 min. Afterward, the reaction mixture was incubated with CB[7]-functionalized graphite electrode, which was prepared according to our previous work.[S1] After being kept for 1 h, the electrode that enriched MB and Fc molecules was used together with a platinum wire and a calomel electrode for electrochemical measurements on a CHI-660C workstation. The electrochemical responses of MB and Fc molecules were recorded by square wave voltammetry (SWV) in the potential range of -0.6 V to +0.7 V.

Cell culture and miRNA determination

MCF-7, BT-474, MCF-10A, and MDA-MB-231 cells were purchased from the Institute of Biochemistry and Cell Biology of Chinese Academy of Science (Shanghai, China). All cells were cultured in a humidified environment containing 5% CO_2 at 37°C. MCF-7 and MDA-MB-231 cells were grown in DMEM containing 10% FBS, MCF-10A cells were grown in MEpiCM containing 10% FBS, and BT-474 cells were grown in RPMI 1640 containing 10% FBS. All the cells were collected at the end of the logarithmic growth phase. The RNA extract was obtained from the cell lysis by using an RNA

Extraction Kit according to the manufacturer's instruction (TransGen Biotech, Beijing, China). For the electrochemical measurements, the RNA extracts (15 μL, 200 ng/μL) from normal breast cells and breast cancer cells with different subtypes were incubated with the prepared electroactive A/B/C and T7 Exo in NEBuffer and reacted as that described for the electrochemical determination of miRNA. qRT-PCR measurements were performed to quantify the expression of miR-21 and miR-210 according to previous work.[S2] For the upregulation of miR-21 expression, 10 μL of miR-21 mimic (20 μM), 120 μL of 1× buffer, and 12 μL of transfection reagent were added into the cell culture medium, and incubated with the MCF-10A cells for 24 h at 37°C. For the inhibition of miR-21 expression, 15 μL of miR-21 inhibitor (20 μM), 120 μL of 1× buffer, and 12 μL of transfection reagent were added into the cell culture medium, and incubated with the desired cells for 24 h at 37°C. For the inhibition of miR-210 expression in MDA-MB-231 cells, 6 μL of miR-210 inhibitor (100 μM) was added to DMEM, and incubated with the MDA-MB-231 cells at 37°C for 24 h.

Electrochemical analysis of real tissue samples from mice model

Mice experiments were approved by the Ethical Committee of the Shanghai University and performed in compliance with the policy of the Ethical Committee on animal use. Specifically, tumour and normal breast tissues were obtained from the tumor-bearing mice with the subcutaneous injection of MDA-MB-231 cells, which were established according to previous work.[S3] Afterward, the tissues were digested with trypsin (0.25%) and Dnase I (1 mg/mL), and the digested cells at the number of 10^5 were collected by centrifuging at 500 rpm for 5 min and washed with PBS for three times. Finally, RNA extracts were obtained from the digested cells by using the RNA extraction kits and incubated with the electroactive A/B/C and T7 Exo in NEBuffer for electrochemical determination.

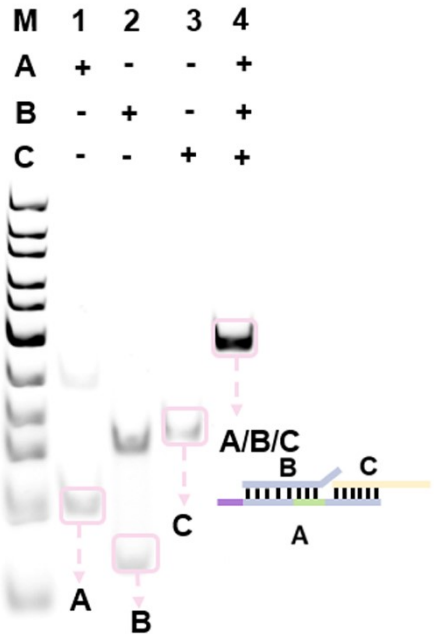

Fig. S1 Gel electrophoresis analysis result of signal probe A/B/C.

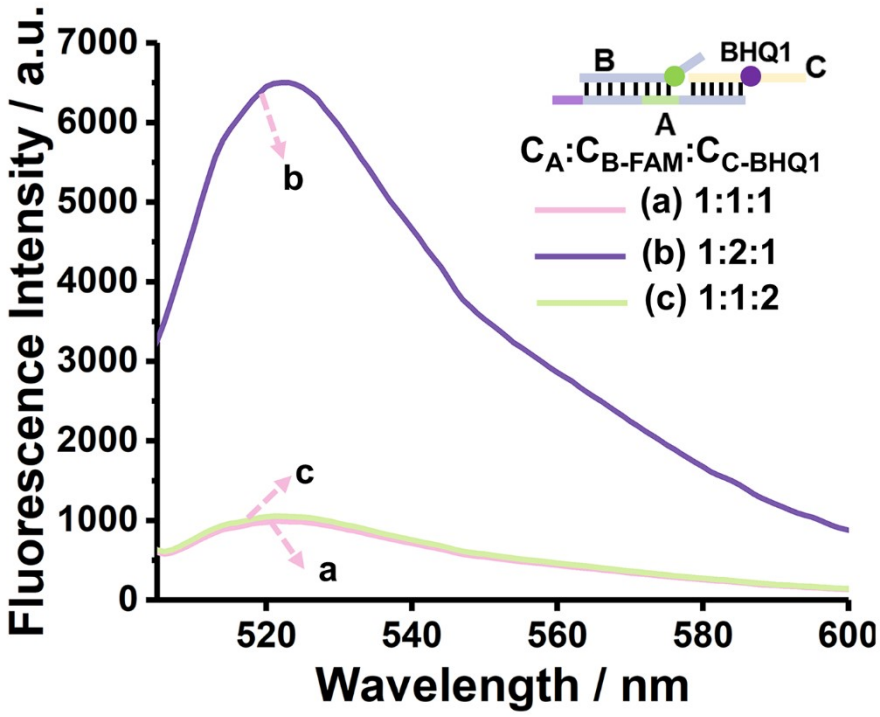

Fig. S2 Fluorescence spectrum obtained after the assembly of A, FAM-B, BHQ1-C in different hybridization ratios.

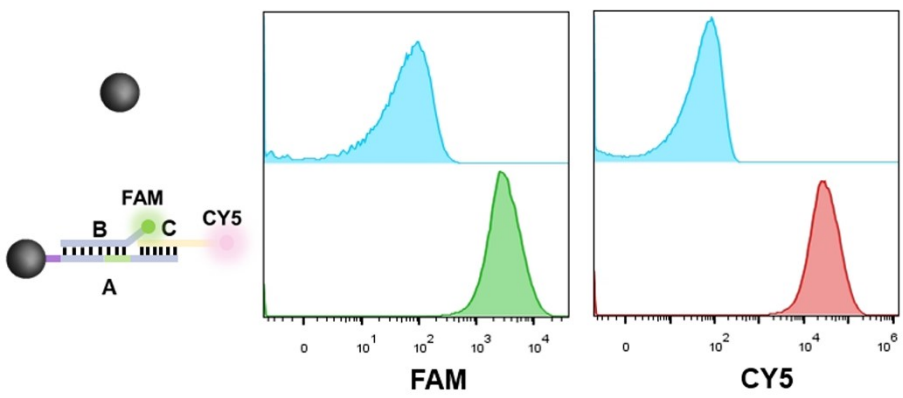

Fig. S3 Flow cytometry analysis of the magnetic beads functionalized by A/FAM-B/CY5-C.

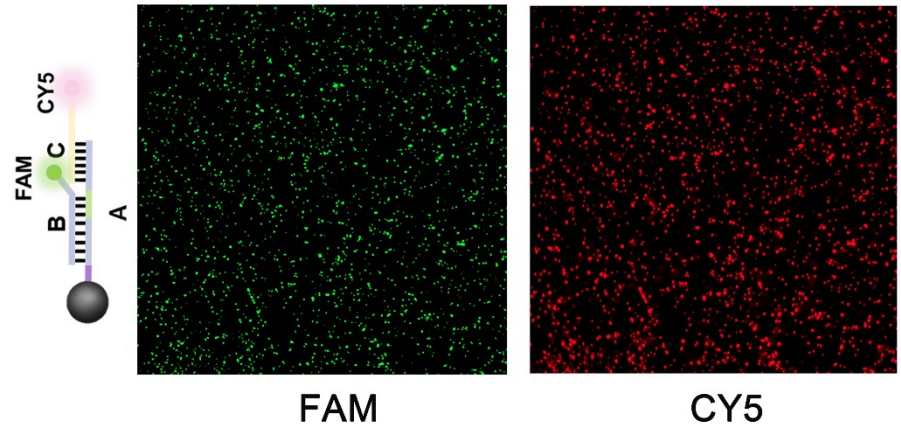

Fig. S4 Fluorescence microscope images of the magnetic beads functionalized by A/FAM-B/CY5-C.

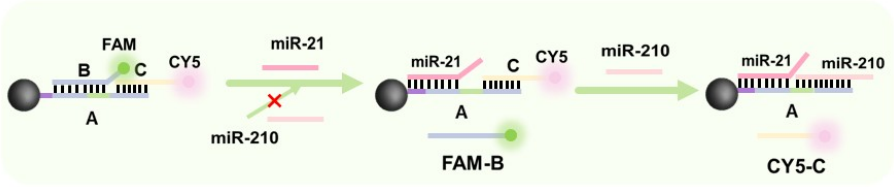

Fig. S5 Schematic diagram of strand displacement reactions at the magnetic beads that are labeled by signal probe A/FAM-B/CY5-C.

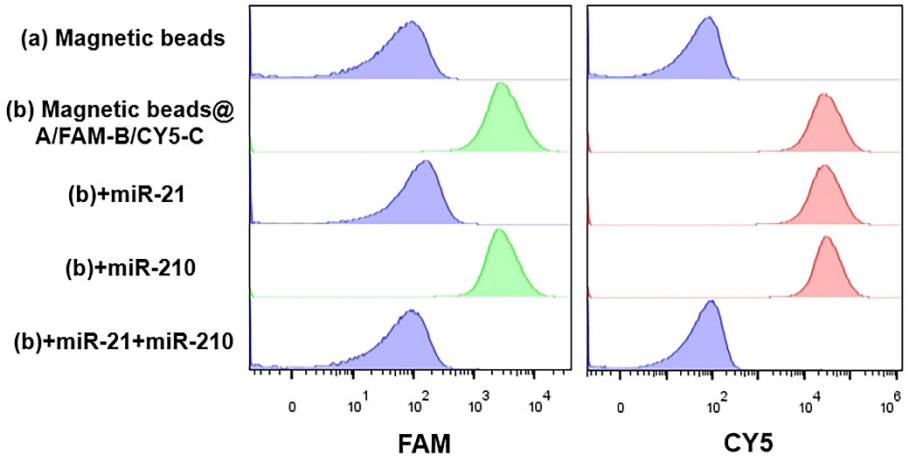

Fig. S6 Flow cytometry analysis of FAM and CY5 fluorescence at the magnetic beads with different miRNA inputs.

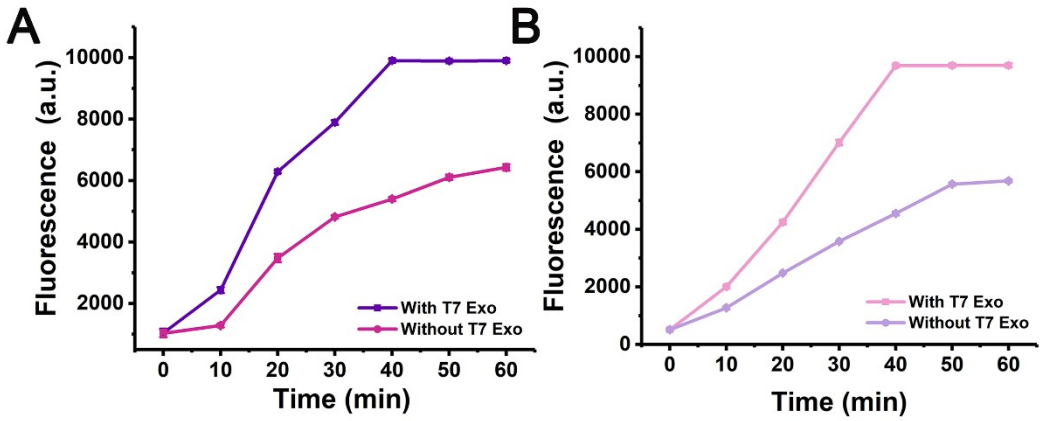

Fig. S7 Fluorescence intensities of (A) FAM and (B) JOE obtained with or without T7 Exo.

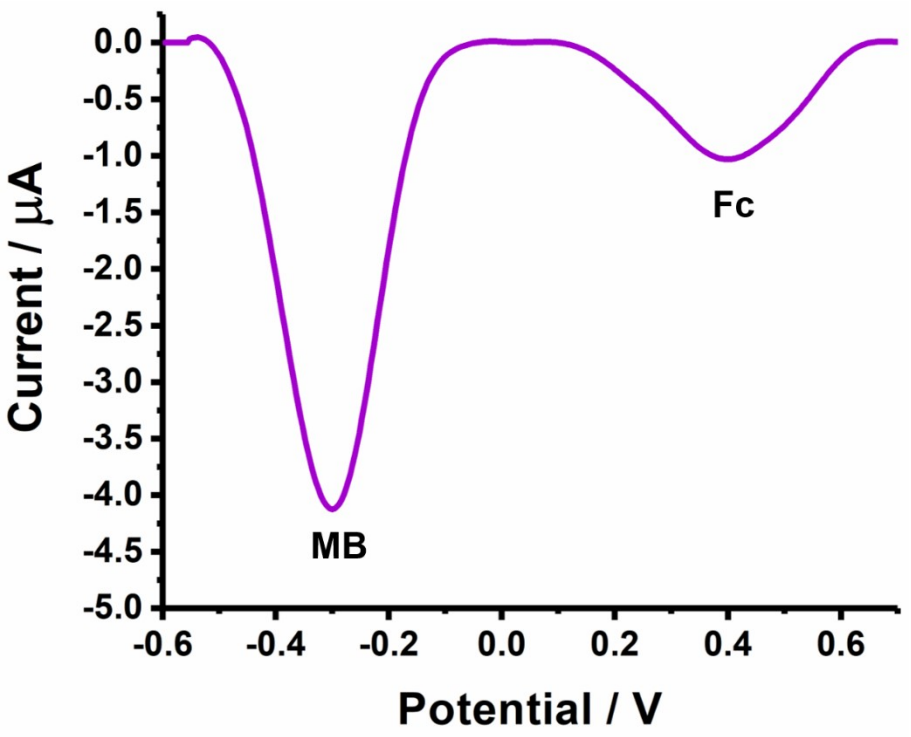

Fig. S8 Electrochemical response of free MB and Fc molecules at CB[7]-functionalized electrode.

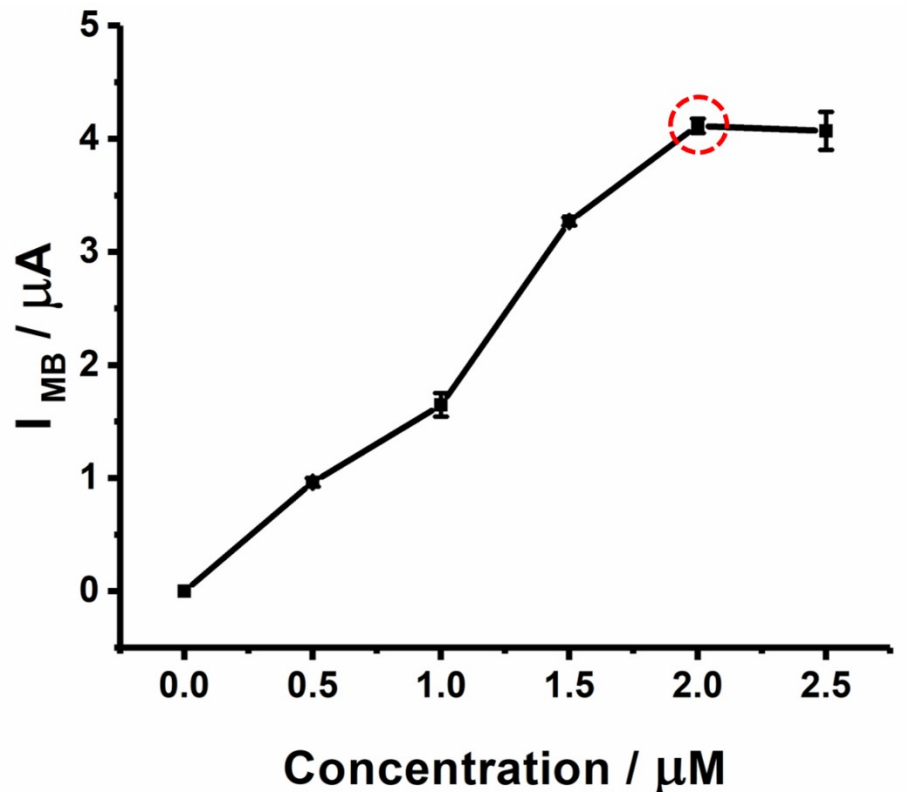

Fig. S9 I_{MB} obtained with different concentrations of the signal probe.

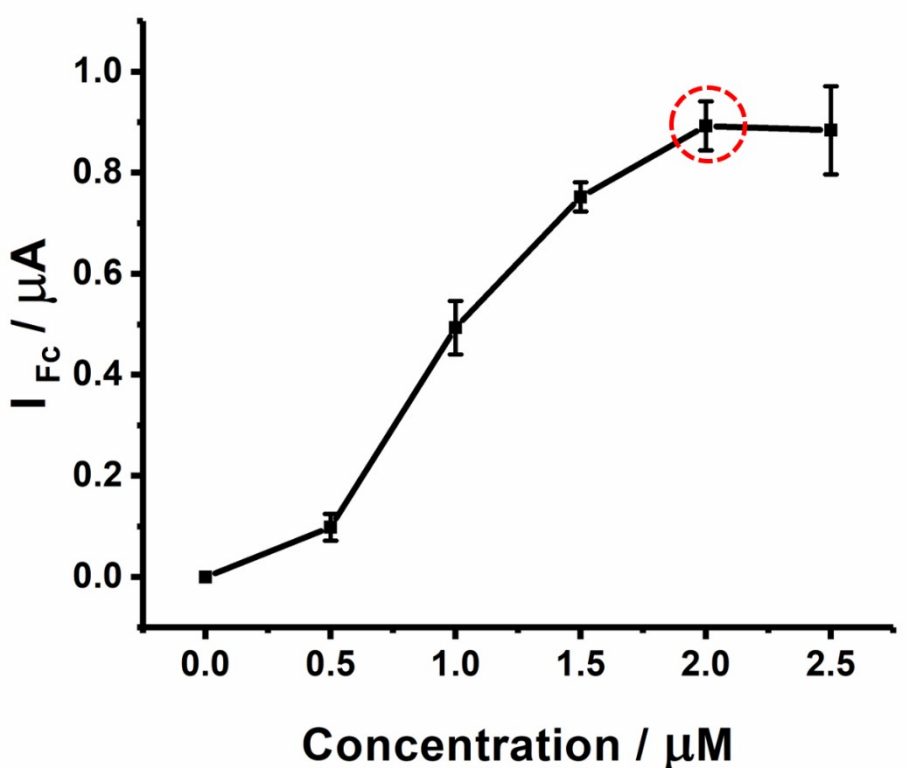

Fig. S10 I_{Fc} obtained with different concentrations of the signal probe.

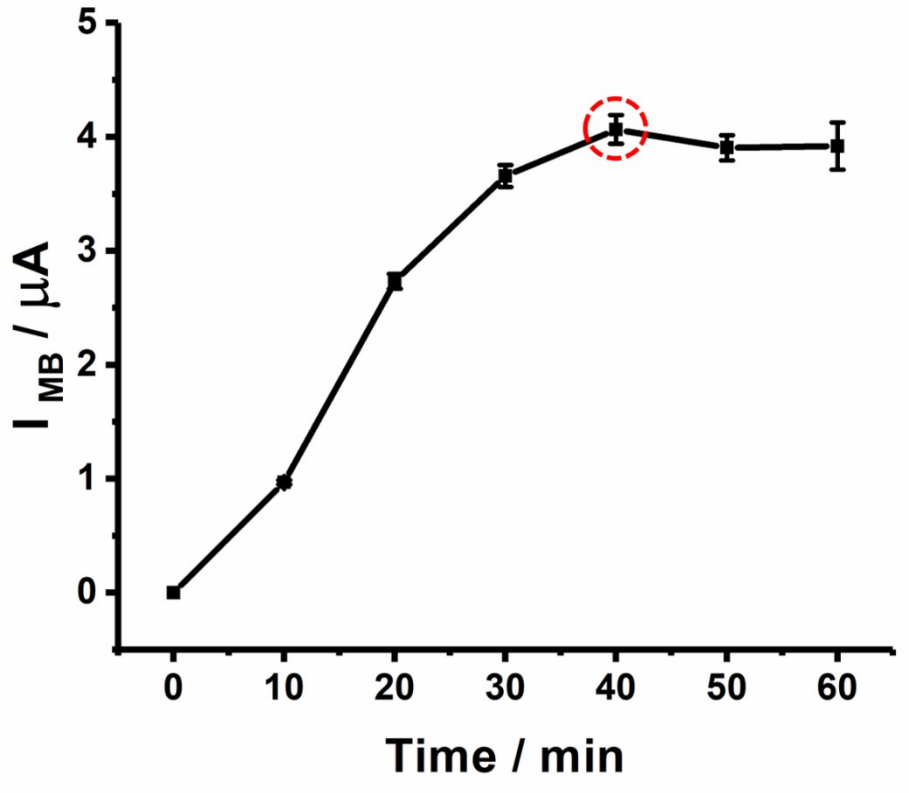

Fig. S11 I_{MB} obtained after different reaction time for T7 Exo-powered strand displacement reactions.

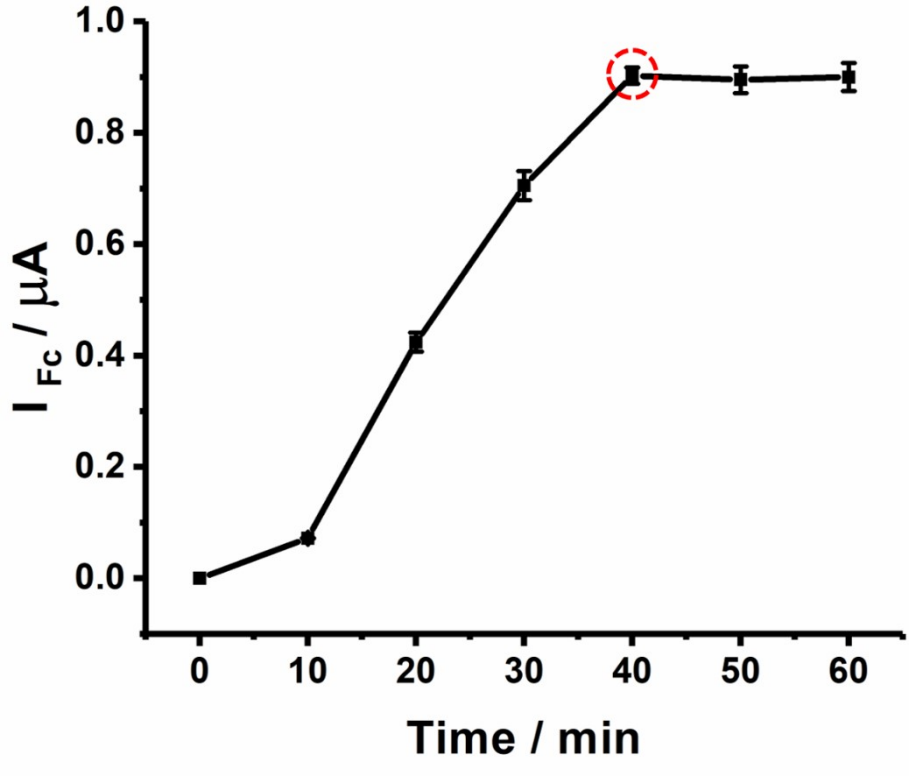

Fig. S12 I_{Fc} obtained after different reaction time for T7 Exo-powered strand displacement reactions.

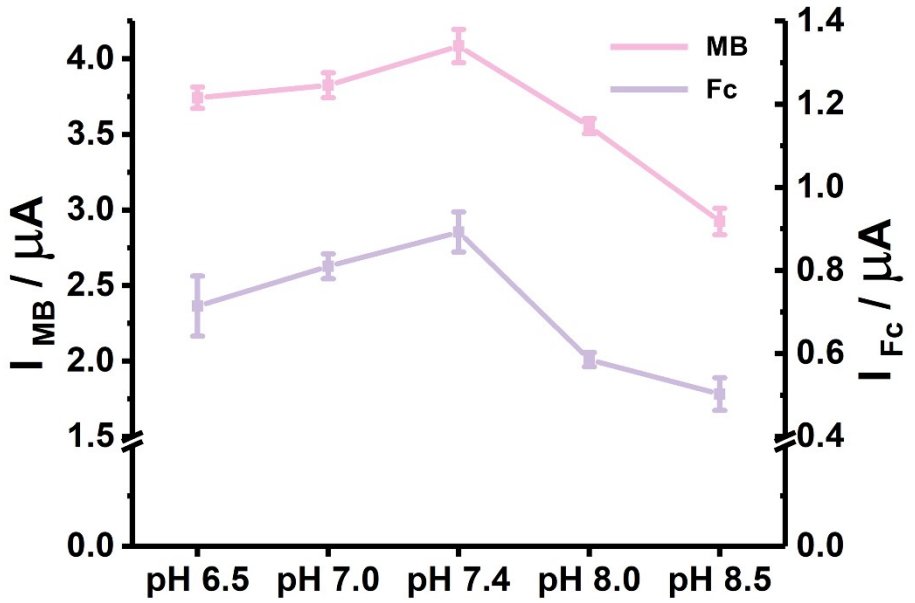

Fig. S13 I_{MB} and I_{Fc} obtained at different pH for T7 Exo-powered strand displacement reactions.

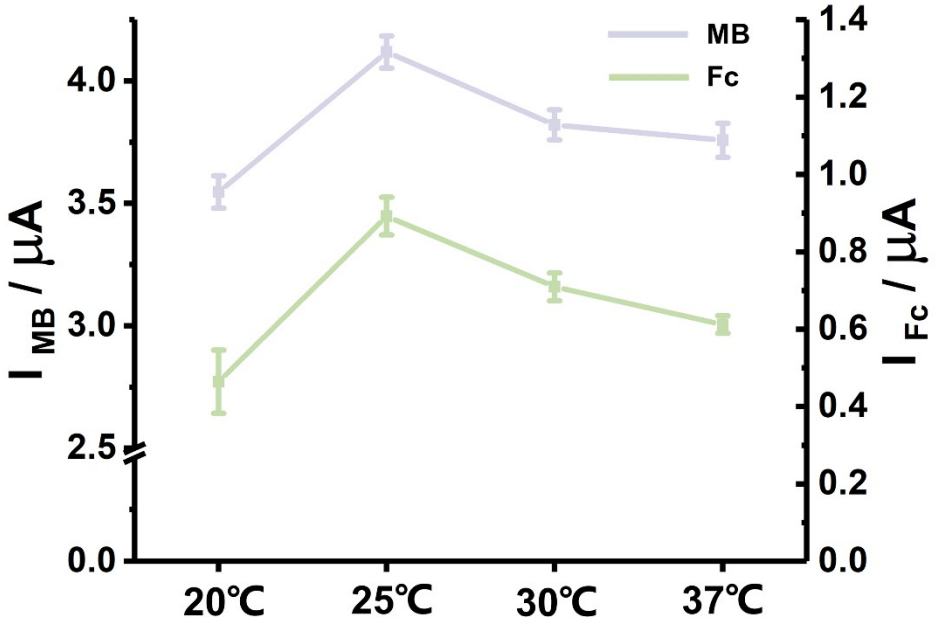

Fig. S14 I_{MB} and I_{Fc} obtained at different reaction temperature for T7 Exo-powered strand displacement reactions.

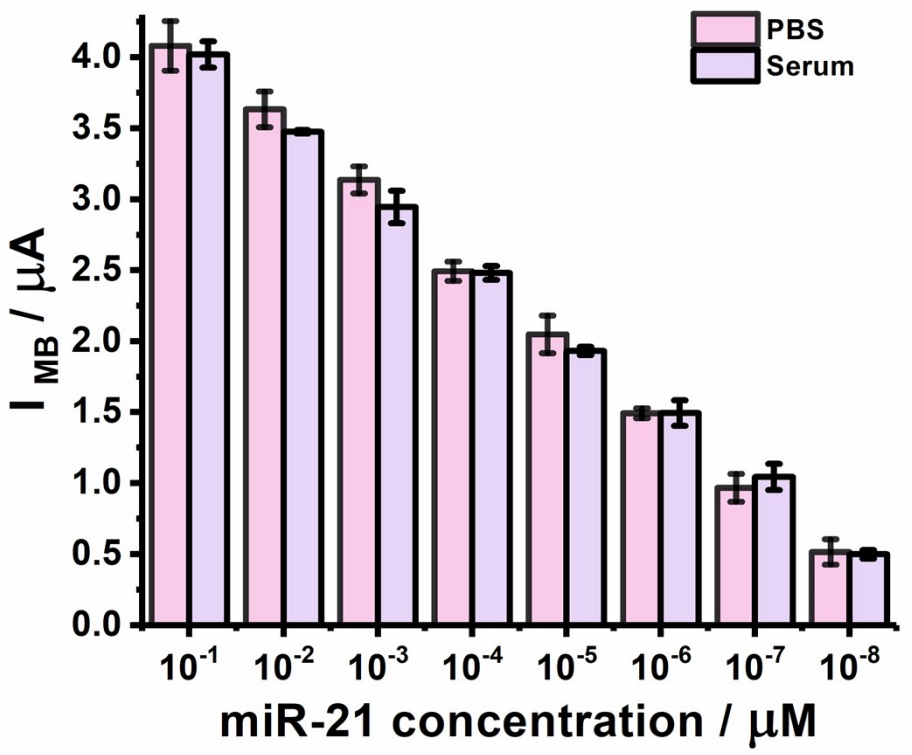

Fig. S15 I_{MB} obtained in the buffer and serum samples with different miR-21 concentrations.

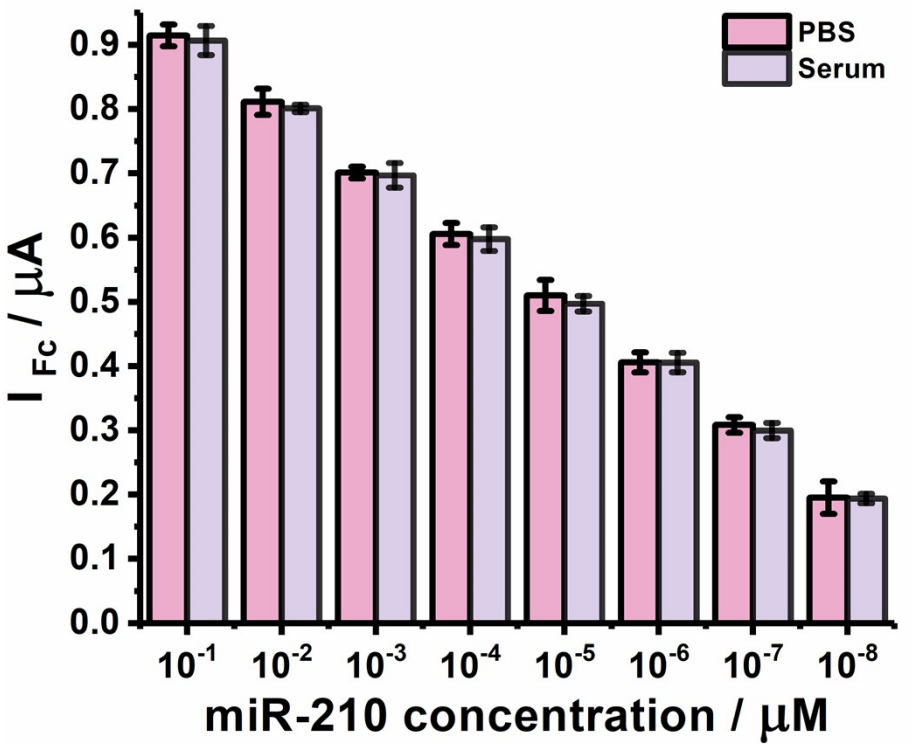

Fig. S16 I_{Fc} obtained in the buffer and serum samples with different miR-210 concentrations.

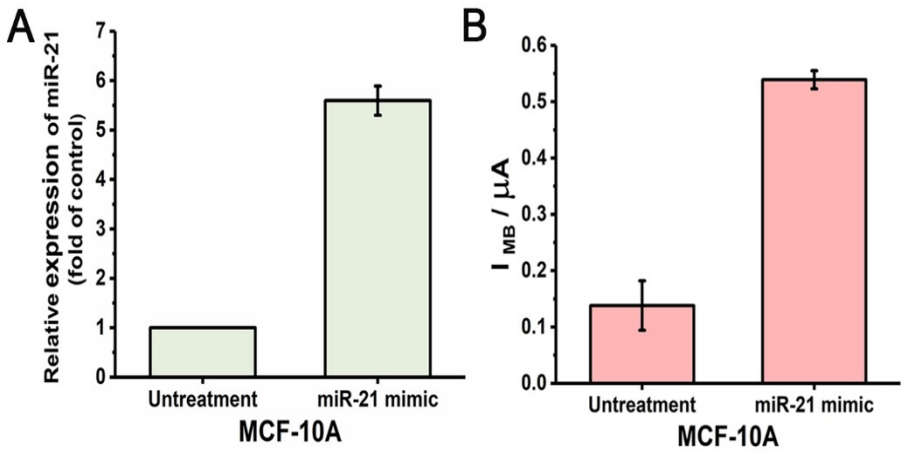

Fig. S17 qRT-PCR results and I_{MB} obtained for MCF-10A cells without or with pre-treatment with miR-21 mimic.

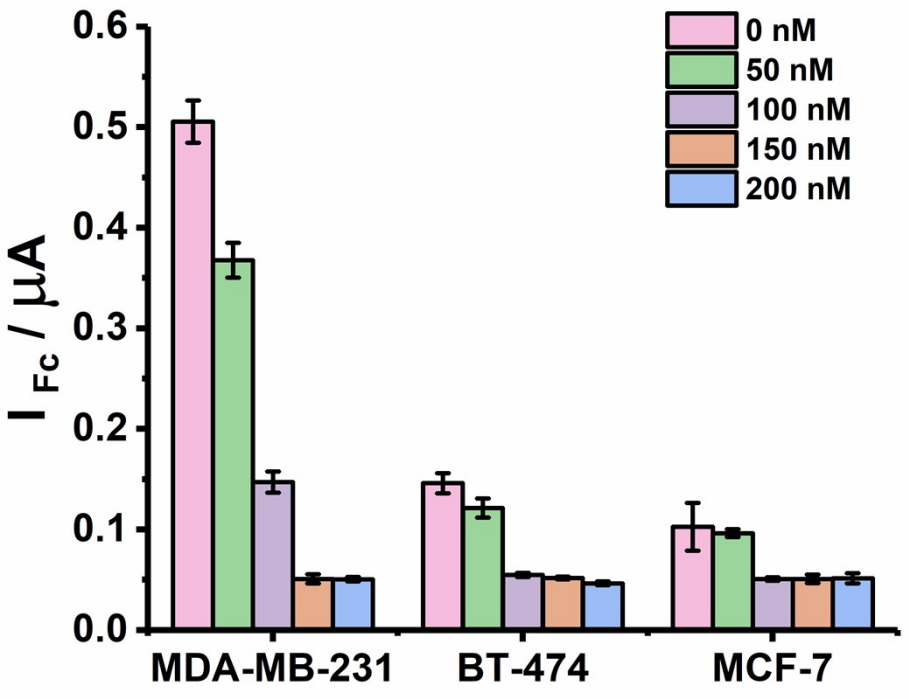

Fig. S18 I_{Fc} obtained for MCF-7, BT-474, and MDA-MB-231 cells that were pre-treated with different concentrations of miR-21 inhibitor.

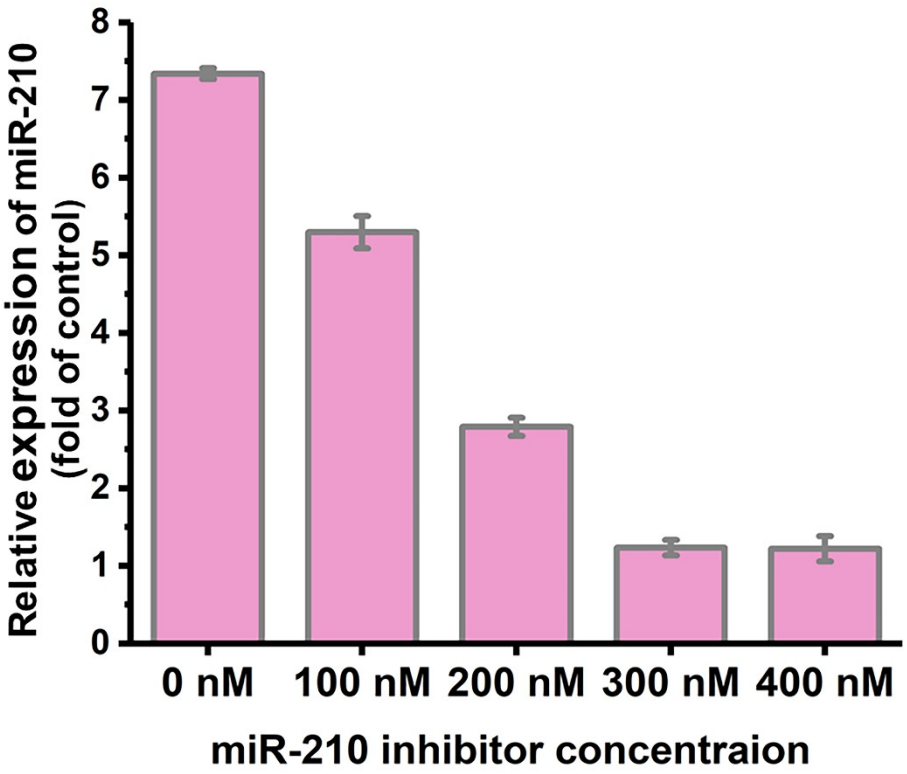

Fig. S19 qRT-PCR quantification of miR-210 expression in MDA-MB-231 cells treated with different concentrations of miR-210 inhibitor.

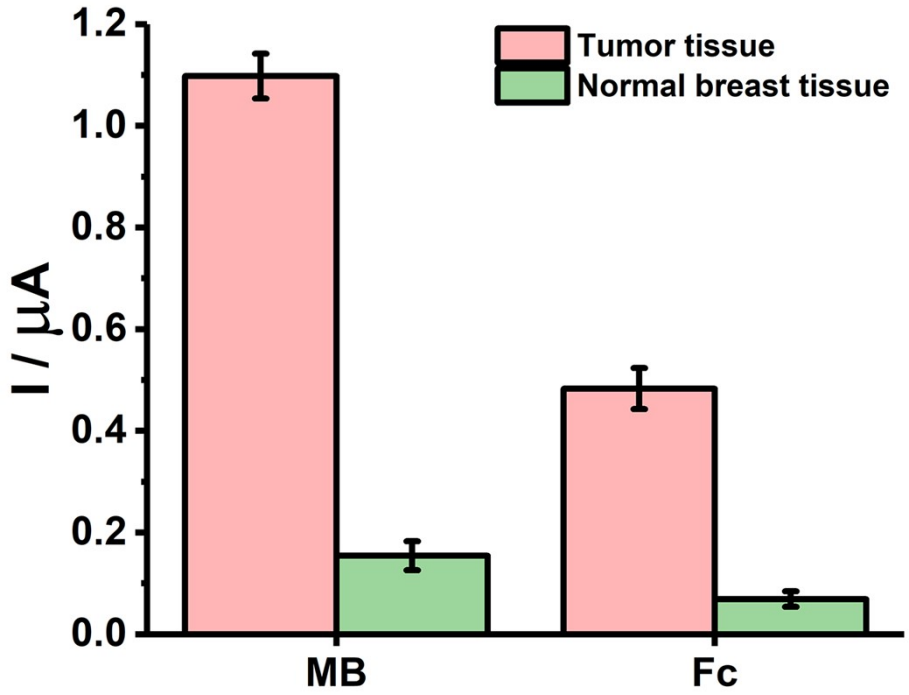

Fig. S20 I_{MB} and I_{Fc} obtained in the tumor tissue and normal breast tissue that were obtained from tumor-bearing mice with the subcutaneous injection of MDA-MB-231 cells.

Table S1 Comparison of different methods for the detection of miRNA

Method	Liner range	Detection limit	References
Electrochemical method using tetrahedral DNA framework based CRISPR	10 pM – 10 nM	10 pM	S4
Electrochemical method based on 2D metal-organic framework nanozyme	1 pM - 1 μM	120 fM	S5
Electrochemical method based on a host–guest assembly strategy	1 pM - 1 nM	365 fM	S6
Photoelectrochemical method based on a small reaction chamber and target-triggered hybridization chain reaction	100 fM - 1 nM	45 fM	S7
Simultaneous electro-optical nanopore sensing based on size-encoded molecular probes	0.1 pM - 100 pM	130 fM for miR-375-3p 100 fM for miR-141-3p	S8
Electrochemical method based on DNAzyme-cleavage cycling amplification and hybridization chain reaction amplification	20 fM - 5 nM	5.68 fM	S9
Electrochemical method using T7 Exo-powered miRNA discriminator	10 fM - 100 nM	3.0 fM for miR-21 1.81 fM for miR-210	This work

Table S2 MiR-21 concentration determined by our method in the serum samples.

Samples	Added concentration	Detected concentration	Recovery (%)
1	10.0 fM	10.82 fM	108.2
2	10.0 pM	10.22 pM	102.2
3	10.0 nM	10.10 nM	101.0

Table S3 MiR-210 concentration determined by our method in the serum samples.

Samples	Added concentration	Detected concentration	Recovery (%)
1	10.0 fM	9.607 fM	96.07
2	10.0 pM	9.667 pM	96.67
3	10.0 nM	9.933 nM	99.33

Table S4 Sequences of oligonucleotides used in this work

Oligonucleotides	Sequence (from 5' to 3')
A	TCAACATCAGTCTGATTCAGCCGCTGTCACACG
B	TTTTTTTCTGAATCAGACTG
C	TTTTTTTCGTGTGACAGCG
A1	TCAGCCGCTGTCACACG
JOE-A	TCAACATCAGTCTGATTCAGCCGCTGTCACACG-JOE
FAM-B	TTTTTTT-FAM-CTGAATCAGACTG
BHQ1-C	TTTTTTT-BHQ1-CGTGTGACAGCG
Biotin-A	Biotin-TCAACATCAGTCTGATTCAGCCGCTGTCACACG
CY5-C	CY5-TTTTTTTCGTGTGACAGCG
FAM-A-CY5	FAM-TCAACATCAGTCTGATTCAGCCGCTGTCACACG-CY5
Biotin-C	Biotin-TTTTTTTCGTGTGACAGCG
MB-A-Fc	MB-TCAACATCAGTCTGATTCAGCCGCTGTCACACG-Fc
miR-21	UAGCUUAUCAGACUGAUGUUGA
miR-210	CUGUGCGUGUGACAGCGGCUGA
miR-21 (T) for gel analysis	TAGCTTATCAGACTGATGTTGA
miR-210 (T) for gel analysis	CTGTGCGTGTGACAGCGGCTGA
miR-375	UUUGUUCGUUCGGCUCGCGUGA
miR-188	CAUCCCUUGCAUGGUGGAGGG
miR-21-SM1	UAGCUUAUCAGACUGAUGUUCA
miR-21-SM2	UAGCUUAUCAGACUGAUCUUGA
miR-21-DM	UAGCUUAUCAGACUGAUCUUCA
miR-21-TM	UAGCUUAUCAGACUGUUCUUCA
miR-210-SM1	CUGUGCGUGUGACAGCGGCAGA
miR-210-SM2	CUGUGCGUGUGACAGCGCCUGA
miR-210-DM	CUGUGCGUGUGACAGCGGGAGA
miR-210-TM	CUGUGCGUGUGACAGCGCGAGA

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