Electronic Supplementary Information

A Versatile Size-Coded Flow Cytometric Bead Assay for Simultaneous Detection of Multiple MicroRNAs Coupled with a Two-Step Cascading Signal Amplification

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1. Materials and Reagents

Dynabeads® M-270 Streptavidin (diameter of 2.8 μm) and Dynabeads® Myone Streptavidin C1 (diameter of 1.0 μm) were purchased from Thermo Fisher Scientific (Invitrogen, Oslo, Norway). All of the DNA and miRNA sequences were custom synthesized and purified by Takara Biotechnology (Dalian, China). The detailed sequences of the DNA or RNA oligonucleotides used in this study are listed below. Duplex-specific nuclease (DSN) and 10× DSN reaction buffer (500 mM Tris-HCl, 50 mM $MgCl_2$,10 mM DTT, pH 8.0) were obtained from Evrogen (Moscow, Russia). Terminal deoxynucleotidyl transferase (TdT) and 5× TdT buffer (125 mM Tris-HCl, 1 M potassium cacodylate, 0.05% (v/v) Triton X-100, 5 mM $CoCl_2$, pH 7.2) were supplied by beyotime Biotechnology (Shanghai, China). All of other reagents used in this work were of analytical grade and were used as received without further purification.

The DNA and miRNA sequences used in this study are listed as follows (5′→3′ direction):

Let-7a: UGAGGUAGUAGGUUGUAUAGUU

mir143: UGAGAUGAAGCACUGUAGCUC

mir155: UUAAUGCUAAUCGUGAUAGGGGU

mir21: UAGCUUAUCAGACUGAUGUUGA

mir122: UGGAGUGUGACAAUGGUGUUUG

mir92a: UAUUGCACUUGUCCCGGCCUGU

anti-let-7a-P: biotin-*CCCCCCCCCC*(spacer)-AACTATACAACCTACTACCTCA-PO_4

anti-mir21-P: biotin-*CCCCCCCCCC*(spacer)-TCAACATCAGTCTGATAAGCTA-PO_4

FAM-poly(A)$_{25}$: AAAAAAAAAAAAAAAAAAAAAAAAA-FAM

2. Standard procedures of the proposed flow cytometric bead assay (FCBA) for miRNA analysis

Firstly, we will take the detection of let-7a miRNA as a proof-of-concept target to describe the standard assay procedures of the proposed FCBA strategy by using Dynabeads® M-270 Streptavidin (abbreviated as M-270 MBs) as the reaction carrier. The stocking vial of M-270 MBs was firstly vortexed to make sure that the MBs were homogeneously dispersed, and then 2 μL slurry of the MBs was pipetted out. After thoroughly washing of the MBs to remove the preservative, 10 μL of Tris-HCl buffer (20 mM Tris-HCl containing 300 mM NaCl, pH 7.4) containing 20 nM anti-let-7a-P was mixed with the M-270 MBs and incubated at room temperature for 0.5 h under vigorous shaking.

After magnetic isolation and washing, the anti-let-7a-P-conjugated M-270 MBs were dispersed in 10 μL of 1× DSN buffer as the stock solution for subsequent use.

For a typical DSN-based target recycling reaction, 1 μL of the as-prepared anti-let-7a-P-conjugated M-270 MBs (corresponding to 0.2 μL of the original MB slurry, ~1.2×10^5 beads) was incubated with 0.5 U DSN and serious dilutions of let-7a target in a total 10 μL of 1× DSN buffer at 52°C for 2 h in a home-made shaking incubator to perform the DSN cleavage reaction (the concentrations of miRNAs are all calculated in the 10 μL DSN reaction system throughout this work). Afterward, the MBs were magnetically isolated and washed three times with Tris-HCl buffer. Such MBs were further suspended in 10 μL of 1× TdT buffer containing 1 mM dTTP and 10 U TdT, and the mixture was further incubated at 37°C for 1 h under shaking to conduct the TdT-catalyzed extension reaction. After magnetic purification, the MBs were further incubated with excess FAM-labeled poly$(A)_{25}$ ssDNA (500 nM) in 10 μL hybridization buffer (50 mM phosphate buffer containing 750 mM NaCl) at room temperature for 1 h. Finally, each sample was directly diluted to 500 μL and immediately subject to flow cytometry (FCM) analysis on a FACSCalibur Flow Cytometer (BD Biosciences). During FCM analysis, 10000 MBs were collected for each sample and the fluorescence signals of the MBs were detected by the FL1 (FAM/FITC) channel under the 488 nm laser excitation. For each sample, the mean fluorescence intensity (MFI) of all the detected MBs was statistically analyzed for the quantitative analysis of let-7a. Furthermore, after being separated from the matrix with the help of a magnet, fluorescence imaging test of the FAM-loaded MBs was also performed on a FV-1200 laser-scanning fluorescence confocal microscope (Olympus) by dropping the MBs onto a 0.17 mm glass slide. Fluorescence images of such MBs (Fig. 3 in the main text) were acquired by collecting the fluorescence emissions in the range of 500~600 nm under the excitation of a 488 nm laser (other acquisition parameters of the fluorescence microscope: HV, 350 V; gain, 1; offset, 20%).

The anti-mir21-P-functionalized Myone MBs (Dynabeads® Myone Streptavidin, 1.0 μm) was prepared according to the same protocols as the preparation of anti-let-7a-P-functionalized M-270 MBs. As for the simultaneous detection of let-7a and mir21 in this study, the anti-let-7a-P-functionalized M-270 MBs (~3×10^5 beads, 2.8 μm) and the anti-mir21-P-functionalized Myone MBs (~3×10^5 beads, 1.0 μm) were firstly mixed together, then the subsequent DSN-catalyzed target recycling reaction, TdT-mediated signal amplification reaction, FAM-poly$(A)_{25}$ hybridization and the flow cytometry analysis were conducted following essentially the same procedures stated above. The signals of FSC, SSC and FL1 channels were recorded simultaneously by the flow cytometer where the FSC/SSC signals were used for

bead size-decoding and the FL1 signals were used for the quantification of let-7a and mir21.

3. Optimization of anti-let-7a-P density immobilized on the surface of M-270 MBs for let-7a analysis

Several experimental parameters, such as the anti-miRNA-P probe density on the MBs, the reaction temperature as well as the dosage of DSN, and the concentration of TdT, can all influence the analytical performance of the proposed strategy. Thus we have systematically optimized the experimental conditions by using let-7a as a model target.

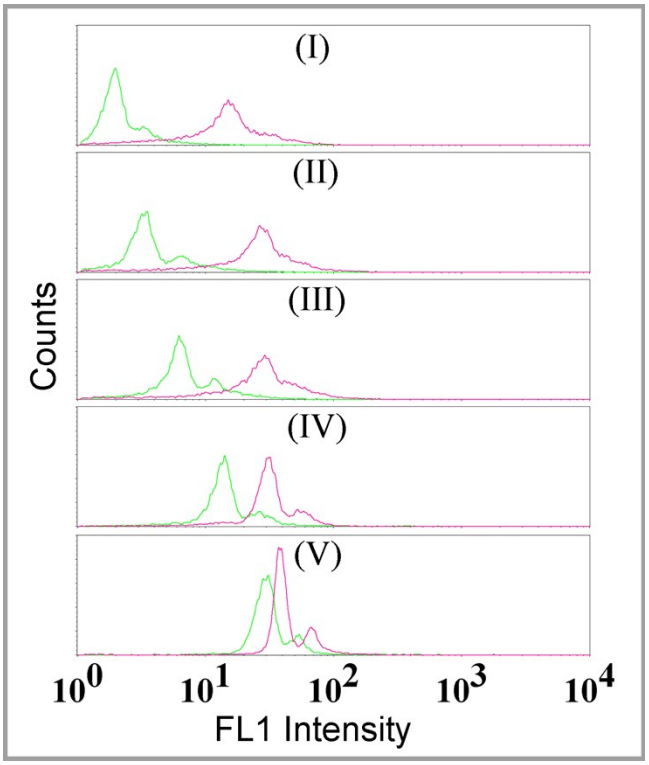

Fig. S1. Effect of the anti-let-7a-P density of the M-270 MBs on the FCBA method for the detection of let-7a. 100 pM of let-7a was used for this optimization (pink lines) in comparison with blank control (green lines). Anti-let-7a-P density (calculated for the functionalization of 0.2 μL M-270 MBs in a 10 μL volume): (I) 1 nM; (II) 2 nM; (III) 5 nM; (IV) 10 nM; (V) 20 nM. Other conditions: DSN dosage, 0.2 U; DSN reaction temperature, 52 °C; TdT, 2 U; dTTP, 1 mM, FL1 Voltage for the FCM measurement, 315 V.

In this study, 0.2 μL of the original M-270 MBs were used for each reaction, which can theoretically capture ~0.4 pmols (~40 nM calculated in a 10 μL solution) biotinylated ssDNA according to the product instruction of M-270 MBs. We firstly investigated the effect of the density of anti-let-7a-P probes immobilized on M-270 MBs on the

proposed FCBA strategy by varying the amount of anti-let-7a-P from 1 nM to 20 nM (calculated in a 10 μL binding solution, 20 nM is corresponding to 50% of the theoretical coverage rate of the used MBs). As can be seen from the FCM results displayed in Fig. S1, with the increase of the anti-let-7a-P density on M-270 MBs, the fluorescence signals produced by 100 pM of let-7a display a slow increase tendency while the signals of the blank (without let-7a target) increase more sharply. The reason may be that the higher density of anti-let-7a-P on the M-270 MBs may lead to higher risk of nonspecific enzymatic cleavage by DSN. Therefore, to ensure the best discrimination between the target-produced signal with the blank background and to achieve lower detection limit, 2 nM of anti-let-7a-P (calculated in a 10 μL volume) is considered to be the optimum amount to be immobilized on the M-270 MBs in this study.

4. Optimization of the experimental conditions for the DSN-catalyzed target recycling reaction

The reaction temperature of the DSN-based cleavage step is crucial for the proposed miRNA assay because the temperature would affect both the activity of DSN and DNA/miRNA hybridization. So we have optimized the reaction temperature of DSN in detail for let-7a analysis. As can be seen from Fig. S2a, 100 pM let-7a-produced fluorescence signals of the MBs increase gradually when the reaction temperature rises from 48 °C to 52 °C. This is because the optimal reaction temperature of DSN is about 60 °C and the DSN activity rise with the increasing temperature in this temperature range. Then the fluorescence intensity shows a gradually decreasing tendency when the temperature is further elevated from 52 °C to 61 °C. This is probably because the DNA/miRNA hybrid duplexes are not stable at high temperature close to its melting temperature (Tm ~56.8 °C, estimated from the website: http://sg.idtdna.com/calc/analyzer), and this can lead to low efficiency of the DSN reaction. Meanwhile, the DSN reaction temperature exhibits little effect on the fluorescence signal of the blank control without let-7a. As shown in Fig. S2b, the highest signal-to-blank ratios of the MFI (mean fluorescence intensity of the MBs) is obtained at 52 °C, so 52 °C is chosen to be the optimum temperature of DSN reaction in this study.

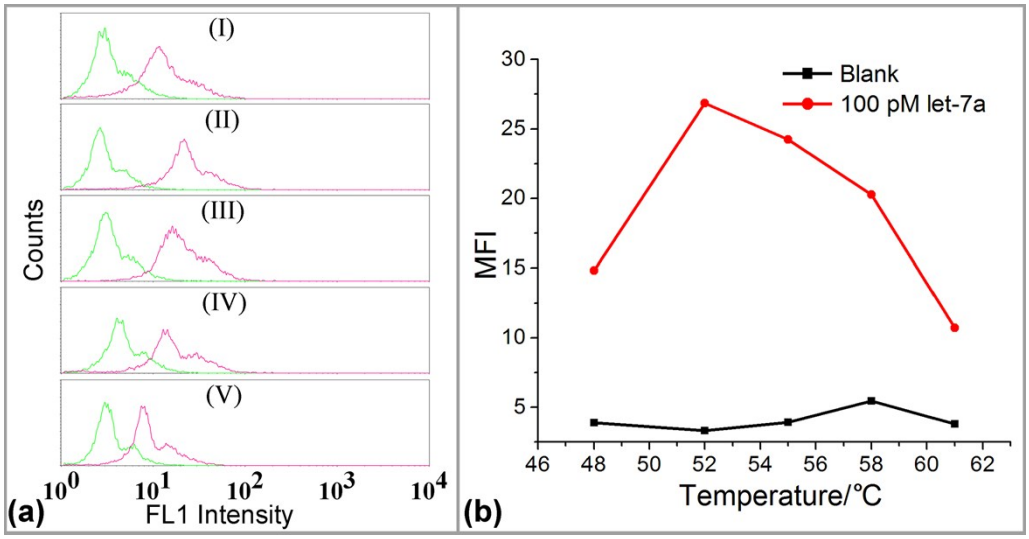

Fig. S2. Optimization of DSN reaction temperature for the detection of let-7a. **(a)** Histograms of fluorescence responses of the MBs treated with 100 pM let-7a (pink lines) in comparison with those of blank control (green lines) under different temperature of DSN-catalyzed reaction. (I) 48 °C; (II) 52 °C; (III) 55 °C; (IV) 58 °C; (V) 61 °C. **(b)** The corresponding MFI values of the histograms shown in image (a). Other conditions: DSN dosage, 0.2 U; TdT, 2 U; dTTP, 1 mM, FL1 Voltage, 315 V.

The concentration of DSN is another crucial factor for this proposed miRNA assay, which was further optimized. It can be seen from Fig. S3 that the fluorescence signals of the MBs produced by let-7a increase sharply as the DSN concentration increases from 0.05 U to 0.5 U, and then will keep almost stable from 0.5 U to 1.0 U. Meanwhile, the fluorescence signals of the blank control without let-7a almost keep constant irrespective of the variation of DSN concentrations. Thus, 0.5 U DSN is selected as the optimum for the DSN-based cleavage reaction in this work.

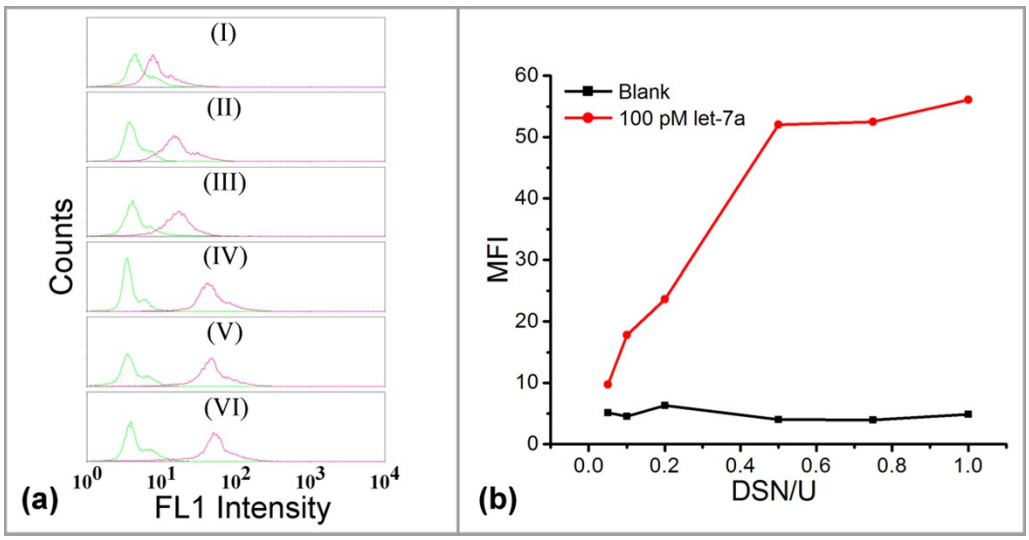

Fig. S3. Optimization of DSN dosage for the detection of let-7a. **(a)** Histograms of fluorescence responses of the MBs

treated with 100 pM let-7a (pink lines) in comparison with those of blank control (green lines) in the presence of different DSN dosages. (I) 0.05 U; (II) 0.1 U; (III) 0.2 U; (IV) 0.5 U; (V) 0.75 U; (VI) 1.0 U. **(b)** the corresponding MFI values of the histograms shown in image (a). Other conditions: DSN reaction temperature, 52 °C; TdT, 2 U; dTTP, 1 mM, FL1 Voltage, 315 V.

5. Optimization of the amount of TdT enzyme

To achieve the best analytical performance of the proposed method, the influence of the amount of TdT enzyme was further investigated by varying the TdT dosage from 1.0 U to 30 U after the DSN-catalyzed target-recycling reaction. As show in Fig. S4, the let-7a-produced fluorescence intensities of the MBs increase sharply with the increase of TdT dosage from 1.0 U to 10 U and then the increasing trend will become leveled off in the TdT dosage range of 10 U to 30 U. Meanwhile, the fluorescence signals of the blank (without let-7a) also display an increasing tendency with the increase of TdT dosage. Taking into consideration of both high target-induced signal and relative low background to achieve better discrimination between the target and the blank, 10 U of TdT is selected for miRNA analysis in this study.

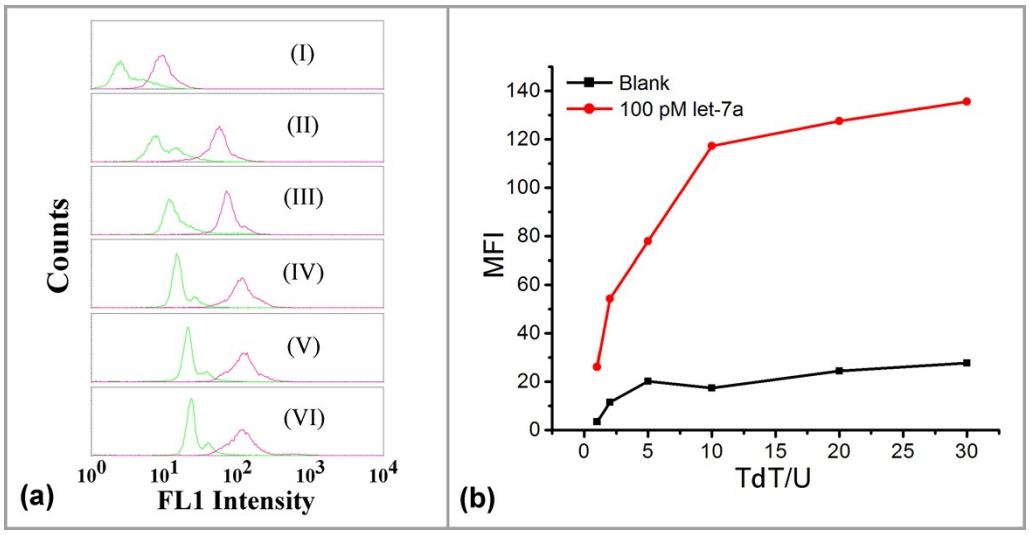

Fig. S4. Optimization of TdT dosage for the detection of let-7a. **(a)** Histograms of fluorescence responses of the MBs treated with 100 pM let-7a (pink lines) in comparison with those of blank control (green lines) in the presence of different amount of TdT. (I) 1.0 U; (II) 2.0 U; (III) 5.0 U; (IV) 10 U; (V) 20 U; (VI) 30 U. **(b)** the corresponding MFI values of the histograms shown in Fig. S4a. Other conditions: DSN dosage, 0.5 U; DSN reaction temperature, 52°C; dTTP, 1 mM; FL1 Voltage, 325 V.

It should be noted that if 10 U TdT is used for miRNA analysis, the blank control shows a relatively high signal

under the FL1 voltage of 325 V for FCM measurements (as shown in image IV of Fig. S4a). So when evaluating the analytical performance of the proposed FCBA under the optimized experimental conditions (Fig. 2 in the main text), the FL1 Voltage for the FCM analysis was rationally lowered down to 280 V to ensure a low blank signal and a wider dynamic range for miRNA analysis.

6. Specificity evaluation of the proposed miRNA assay

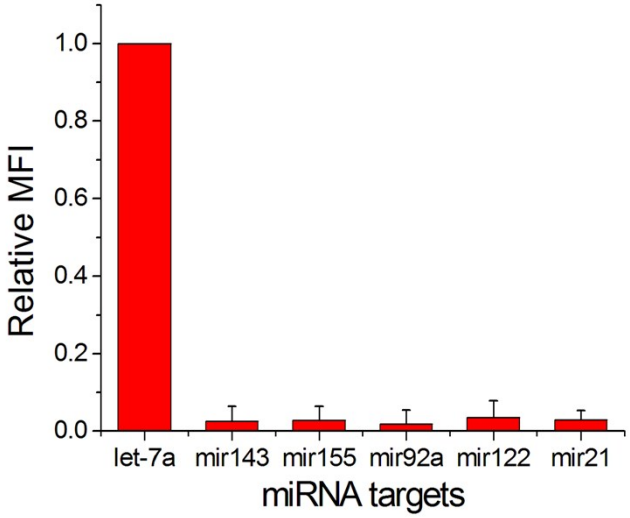

Fig. S5. Specificity evaluation of the proposed method by using let-7a-specific probe. All of the miRNAs used in this study are 100 pM. The fluorescence response of the let-7a is normalized to be 1.

7. Comparison of the analytical performance of different DSN-based miRNA assays.

Table S1. Comparison of the analytical performance of the proposed method for miRNA analysis with other DSN-based strategy.

Method	Signal Readout	Detection Limit	Absolute Amount	Ref.
DSN/catalytic hairpin assembly	Electrochemilumine-scence (ECL)	5.4 fM	1.1 amol	[S1]
DSN/backbone-modified MB	Fluorescence	0.4 pM	80 amol	[S2]
DSN/Taqman probe	Fluorescence	100 fM	3 amol	[S3]
DSN/WS_2 nanosheet as Fluorescence Quencher	Fluorescence	300 fM	30 amol	[S4]

DSN/AuNPs aggregation	Colorimetric	16 pM	1.6 fmol	[S5]
DSN/molecular beacon	Fluorescence	3.8 pM	760 amol	[S6]
DSN/DNA-gold nanoparticle probes	Fluorescence	5 pM	200 amol	[S7]
DSN/terbium as the fluorescence probe	Fluorescence	8 fM	2 amol	[S8]
DSN/GO as the Fluorescence Quencher	Fluorescence	60 pM	12 fmol	[S9]
DSN/lanthanide tags	ICP-MS	~5 nM	~ 50 fmol	[S10]
DSN/TdT-based FCBA	Fluorescence/FCM	100 fM	1 amol	This work

Note: Since different methods use different final reaction volume, the absolute miRNA amount is also calculated to evaluate the detection limits of each method.

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