Electronic Supplementary Information (ESI)

Hybridization chain reaction on silica coated Qbeads for colorimetric detection of multiplex microRNAs†

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1. Experimental procedures

1.1 Design of Probes and Hairpins. The targets, the capture probes, and hairpin species were synthesized by Invitrogen (Shanghai, China), and their sequences are listed in Table S1. The stem-loop structured capture probes are modified with amino group at the 5' end. C12 and 12(dT) are added as a spacer between the amino group and the probe sequence. Specifically, the region underlined for each probe form the 12-bp stem. The capture probes have the common initiator sequence at the 3' end, which is complementary to part of H1. Both of the two hairpin species (H1 and H2) have 6-base loops and 13-bp stems.

1.2 Preparation and Characterization of Qbead@SiO_2. Blue-emitting ZnCdS@ZnS QDs (EM: 440 nm) and green-emitting CdSe@ZnS QDs (EM: 516 nm) were synthesized and purified following the methods in literatures.[1] PSDM microbeads were prepared *via* seeded copolymerization using styrene, divinylbenzene and methylacrylic acid as monomers.[2] The polymeric microbeads suffered from an extraction with methylene chloride for 48 h to obtain mesoporous structures.[3] The Qbeads were produced by doping the porous microbeads with dual-colored QDs following a swelling-evaporation approach.[4] By the use of three intensities of blue-emitting QDs and two intensities of green-emitting QDs, Qbeads with blue-to-green intensity ratios of 2:1, 1:2, 1:1, and 0:1, respectively, were prepared.

Encapsulation of Qbeads with silica shell was accomplished by a modified

Stöber method.[5] Typically, 4 mg of Qbeads were dispersed in 20 mL of ethanol solution of poly(vinylpyrrolidone) (PVP, 30 mg/mL), and the solution was stirred overnight. The resulting Qbead@PVP precipitates were redispersed in 20 mL of 99.5% ethanol, followed by addition of 400 μL of 33% NH_4OH. 1 mL of 99.99% TEOS was slowly added to initiate silica condensation, and the mixture was stirred for 12 h. The resulting Qbead@SiO_2-OH were rinsed repeatedly with ethanol and isolated by centrifugation. Thereafter, they were redispersed in 10 mL of ethanol followed by the addition of 2% (w/v) APTMS, and the mixture was stirred for 12 h. Subsequently, the resulting Qbead@SiO_2-NH_2 precipitates were dispersed in 10 mL of anhydrous DMSO followed by the addition of 100 mg of succinic anhydride (99%), and the mixture was stirred overnight. After repeated washing with DMSO and isolation by centrifugation, the Qbead@SiO_2-COOH precipitates were obtained. Qbead@SiO_2 with blue-to-green intensity ratios of 2:1, 1:2, 1:1, and 0:1, respectively, were used as the four barcodes.

The photostability of microbeads was characterized by challenging Qbeads and Qbead@SiO_2-COOH with different experimental conditions and recording the fluorescence intensities at 444 nm and 522 nm on a fluorescence spectrometer (Hitachi F-4600, Japan). For the pH stability study, the two sets of Qbeads were incubated in solutions with pH ranging from 4 to 11 for 12 h, respectively. For the stability against crosslinking reagents, the two sets of Qbeads were incubated with five chemicals often used in bioconjugation for 12 h at room temperature, respectively. The concentrations of the chemicals used were as follow, 5 mM of EDC or NHS, 0.05 mg/mL of biotin, avidin or streptavidin. For studying the stability against GelRed, the two sets of Qbeads were incubated in the hybridization buffer containing different concentrations (dilution coefficients) of GelRed for 2 h, respectively. The dilution coefficients used were 1×10^{-5}, 1×10^{-5}, 1×10^{-4}, and 5×10^{-4}. The time stability was investigated by incubating the two sets of Qbeads in hybridization buffer (10 mM Tris-HCl, 500 mM NaCl, 1% fetal bovin serum, pH 7.4) at 37 °C with shaking for different time (up to 10 h), respectively, and recording the fluorescence intensities at 444 nm and 522 nm.

1.3 Bioconjugation. The bioconjugation of Qbead@SiO_2-COOH with capture probes was *via* the carbodiimide reaction.[6] Typically, 4 mg of Qbead@SiO_2-COOH were suspended in 1 mL 2-morpholinoethanesulfonic acid buffer (pH 6.0), followed by addition of 100 μL buffer solutions of 10 μM capture probes and 50 mg/mL EDC, respectively, and the mixture was stirred for 2 h. An additional 100 μL buffer solution of 50 mg/mL EDC was added, and the mixture was allowed to be stirred for another 2 h. Subsequently, 500 μL of Tris-HCl buffer (pH 7.4) containing 0.1% Tween-20 was added, and the mixture was stirred for 30 min. The barcodes were collected by centrifugation, and re-suspended in hybridization buffer. To estimate the amount of capture probes per Qbead@SiO_2, 6-carboxyfluorescein (FAM)-labeled probes were used to conjugate with blue-emitting QDs encoded Qbead@SiO_2 at identical condition. The fluorescence signal of FAM in the supernatant before/after the carbodiimide reaction was recorded and compared with the standard curve produced by fluorescence signals of eight known probe concentrations.

1.4 Target binding, HCR and Staining on Qbead@SiO_2. Target binding and HCR were performed in a volume of 10 μL of hybridization buffer (containing approximately 1000 number of a barcode) at 37 °C. For target binding, certain abundance of target miRNA was added into the hybridization buffer of a barcode with shaking for 30 min. To estimate the binding efficiency, the absorbance of target in the supernatant before/after binding was recorded and compared with the standard curve produced by absorbance of eight known target concentrations.

Thereafter, 7 μM H1 and 7 μM H2 were added simultaneously, with continuous shaking for 2 h. The resulting Qbead@SiO_2—HCR products were then stained by the addition of GelRed (with the dilution coefficient of 5×10^{-4} in hybridization buffer) at room temperature for 30 min. Finally, the stained Qbead@SiO_2—HCR products were collected by centrifugation, and purified with washing buffer (10 mM Tris-HCl, 0.01% Tween, 200 mM NaCl, pH 7.4).

1.5 Fluorescence Microscopy Measurements. Fluorescent imaging tests of the

stained Qbead@SiO_2—HCR products were performed on an Olympus IX73 inverted fluorescence microscope. The excitation wavelength for QDs is 405 nm and that for GelRed is 530 nm. For quantification experiments, the Qbead@SiO_2—HCR products were prepared by incubating each barcode with varying abundance of the corresponding target from 0 to 15 nM, followed by the presence of 7 μM of H1 + H2. For the specificity experiments, the Qbead@SiO_2—HCR products were prepared by incubating barcode 1:1 with 3 nM of miRNA-10b, miRNA-10a, miRNA-21, miRNA-155, and miRNA-373, respectively, followed by the presence of 7 μM of H1 + H2. For the accuracy experiments, the Qbead@SiO_2—HCR products were prepared by incubating barcode 1:1 with varying abundance of miRNA-10b spiked in 100 ng of total RNA, followed by the presence of 7 μM of H1 + H2.

1.6 Calculation of Chromaticity Coordinates. The *XYZ* tristimulus values and the (*x*,*y*) chromaticity coordinates were calculated with the following equations: [7]

$$\begin{bmatrix} X \\ Y \\ Z \end{bmatrix} = \frac{1}{0.17697} \begin{bmatrix} 0.49 & 0.31 & 0.20 \\ 0.17697 & 0.81240 & 0.01063 \\ 0.00 & 0.01 & 0.99 \end{bmatrix} \begin{bmatrix} R \\ G \\ B \end{bmatrix} \quad (1)$$

$$x = \frac{X}{X + Y + Z} \quad (2)$$

$$y = \frac{Y}{X + Y + Z} \quad (3)$$

where R, G and B values were directly obtained from the fluorescent images by ImageJ software.

1.7 Flow Cytometric Detection. The stained Qbead@SiO_2—HCR products for each barcode were subjected to flow cytometric detection. Fluorescence signals were detected in PE-Texas Red channel at 615/30 nm using an ACEA NovoCyte D2070R flow-cytometer with 488 nm laser excitation, and the flow cytometry data were acquired using NovoExpress software.

1.8 Cell lysis and Colorimetric Detection. MCF-7 cells were cultured in a Dulbecco's modified Eagle's medium supplemented with 10% fetal bovin serum, 80 U/mL

penicillin, and 80 μg/ mL streptomycin at 37 °C in 5% CO_2 atmosphere. Cell number was counted by using a hemocytometer. After counting, cells were washed by phosphate-buffered saline, detached by trypsin, and collected by centrifugation. Then the cell pellet was suspended in SingleShot™ Cell Lysis Buffer containing proteinase K. After incubating for 10 min, the lyzed cellular suspension was mixed on a vortex and centrifuged. Then the supernatant was collected.

Cell lysates from varying number of MCF-7 cells (1-10^5 cells) were incubated with the array of four barcodes (the 1.0×10^3 number for each barcode), respectively, for 30 min at 37 °C. After HCR and staining, the stained Qbead@SiO_2—HCR products were imaged. Afterwards, (*x*,*y*) chromaticity coordinates of each barcode were calculated and compared with the constructed calibration curves. Abundances of the four miRNAs were calculated, and their relationships with cell number in the lysates were plotted.

1.9 qRT-PCR. Calibration curves of the four target miRNAs were generated by the use of varying abundance of synthetic miRNAs (10^4-10^9 copies). The synthetic miRNAs and cell lysates (from 10^3 cells) were reverse transcribed with RevertAid™ First-strand cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's protocol. The PCR amplification of the cDNAs was then performed in 96-well plates with SYBR Green Master (Applied Biosystems), forward primers, reverse primers and cDNAs in a 20 μL reaction volume on a StepOnePlus™ Real time-PCR System (Applied Biosystems).

1.10 Spike/recovery Evaluation. Spike/recovery was evaluated by incubating the array of four barcodes (the 1.0×10^3 number for each barcode) with cell lysates (from 10^4 cells) spiked with 10, 100 and 500 pM of the four synthetic miRNAs, respectively. After HCR and staining, the stained Qbead@SiO_2—HCR products were imaged. The abundances of the four miRNAs were calculated using the procedure described in Section 1.8.

2. Supporting Tables and Figures

Table S1. Sequences for the targets, probes and DNA hairpins (H).

Name	Sequences
miRNA-21 probe	NH_2-C12-TTTTTTTTTTTTGACCCTTTTAGTTCAACATCAGTCTGATAAGCTAACTAAAAGGGTCTGAGGGT
miRNA-21	UAGCUUAUCAGACUGAUGUUGA
miRNA-155 probe	NH_2-C12-TTTTTTTTTTTTGACCCTTTTAGTACCCCTATCACGATTAGCATTAACTAAAAGGGTCTGAGGGT
miRNA-155	UUAAUGCUAAUCGUGAUAGGGGU
miRNA-10b probe	NH_2-C12-TTTTTTTTTTTTGACCCTTTTAGTCACAAATTCGGTTCTACAGGGTAACTAAAAGGGTCTGAGGGT
miRNA-10b	UACCCUGUAGAACCGAAUUUGUG
miRNA-10a	UACCCUGUAGA**U**CCGAAUUUGUG
miRNA-373 probe	NH_2-C12-TTTTTTTTTTTTGACCCTTTTAGTGGAAAGCGCCCCCATTTTGAGTACTAAAAGGGTCTGAGGGT
miRNA-373	ACUCAAAAUGGGGGCGCUUUCC
H1	GCGTAGACTAAAAGGGTCT*ACCCTC*AGACCCTTTTAGT
H2	AGACCCTTTTAGT*CTACGC*ACTAAAAGGGTCTGAGGGT

Table S2. LODs and dynamic ranges of the four target miRNAs by the Qbead@SiO_2 assays with/without HCR.

target	detection limit		dynamic range	
	with HCR	without HCR	with HCR	without HCR
miRNA-21	65 fM	90 pM	0.15 pM-3 nM	150 pM-3 nM
miRNA-155	70 fM	100 pM	0.15 pM-3 nM	150 pM-3 nM
miRNA-10b	60 fM	80 pM	0.15 pM-3 nM	150 pM-3 nM

miRNA-373	80 fM	130 pM	0.15 pM-3 nM	150 pM-3 nM

Table S3. Comparison of LOD and dynamic range for beads and nanoparticles-based HCR assays.

platform	LOD	dynamic range	reference
Qbead	70 fM	0.15 pM-3 nM	this work
magnetic bead	0.5 pM	1 pM-3 nM	14a
magnetic bead	68 fM	0.1 pM-1 nM	14b
Au nanoparticle	0.6 pM	1 pM-100 nM	14c
Ag nanocluster	0.78 nM	1.56-400 nM	14d
graphene oxide	0.18 pM	0-200 pM	14e
graphene oxide	1 pM	1 pM-5 nM	14f

Table S4. Spike/recovery in MCF-7 cell lysates.

platform	endogenous (pM)	spiked (pM)	measured (pM)	recovery (%)
miRNA-21	260.5	10.0	264.0	97.6
		100.0	375.5	104.0
		500.0	730.6	96.1
miRNA-155	1.3	10.0	10.8	95.6
		100.0	103.0	101.7
		500.0	515.7	102.7
miRNA-10b	101.8	10.0	106.5	94.8

		100.0	196.1	97.1
		500.0	594.6	98.7
miRNA-373	6.5	10.0	15.8	95.8
		100.0	112.4	105.1
		500.0	502.0	99.1

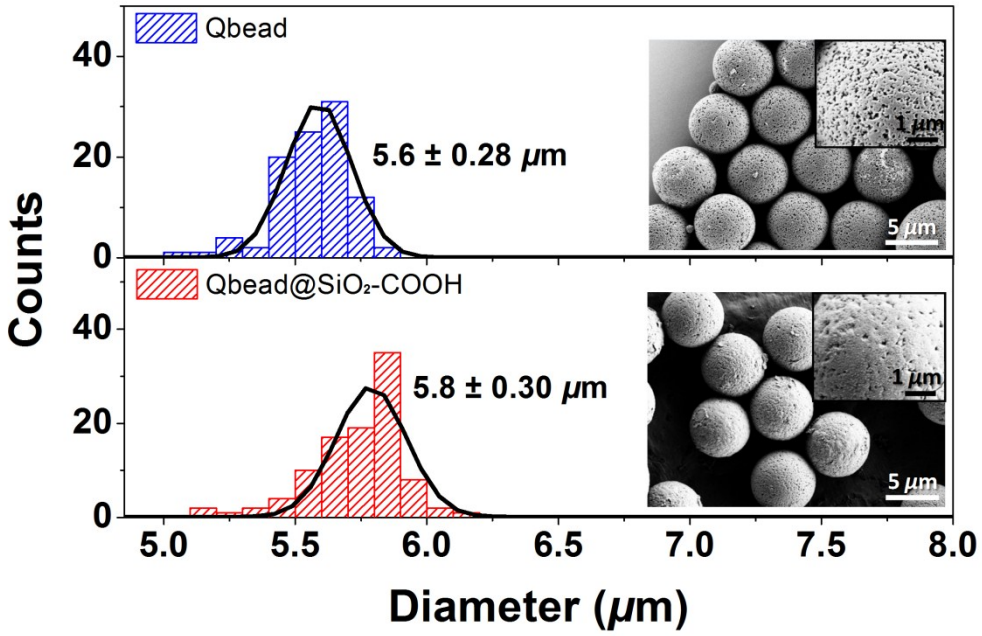

Fig. S1 Diameter measurements of Qbead and Qbead@SiO_2-COOH represented in histograms with Gaussion curves fitted on the distributions. The insets are corresponding SEM images.

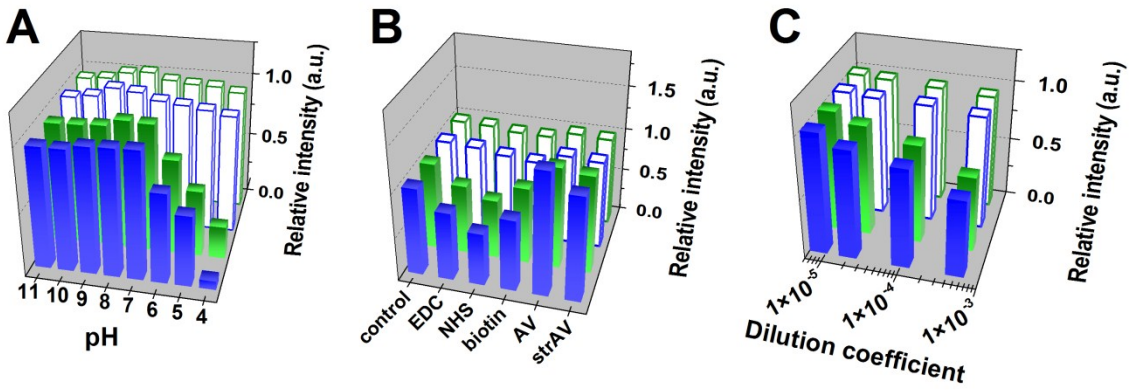

Fig. S2 Relative intensities of bare Qbeads (solid) and Qbead@SiO_2 (open) upon (A) suspension in aqueous buffer solutions with pH ranging from 4 to 11, (B) treating with various crosslinking reagents, (C) addition of GelRed with varying dilution coefficients. Blue and green bars represent fluorescence intensities at 444 nm and 522 nm, respectively.

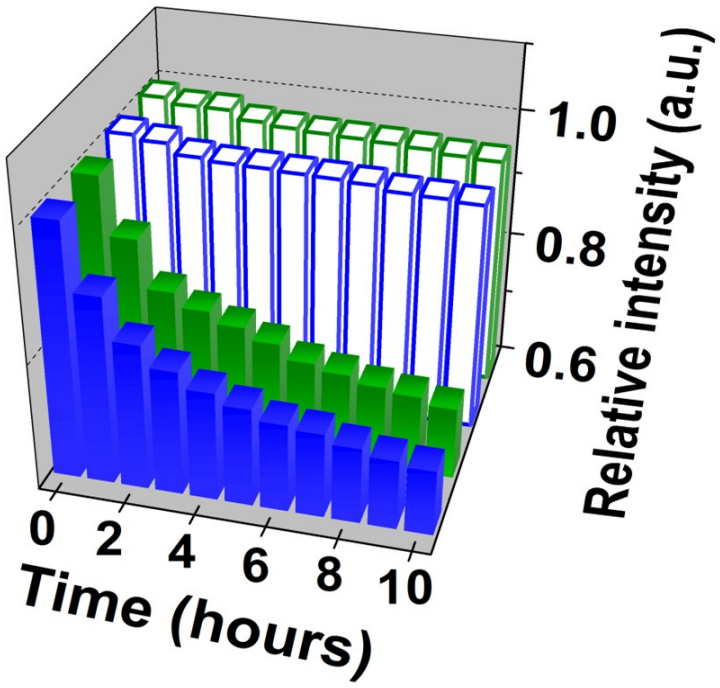

Fig. S3 The photostability of bare Qbeads (solid) and Qbead@SiO_2 (open) against time in hybridization buffer at 37 °C with shaking. Blue and green bars represent fluorescence intensities at 444 nm and 522 nm, respectively.

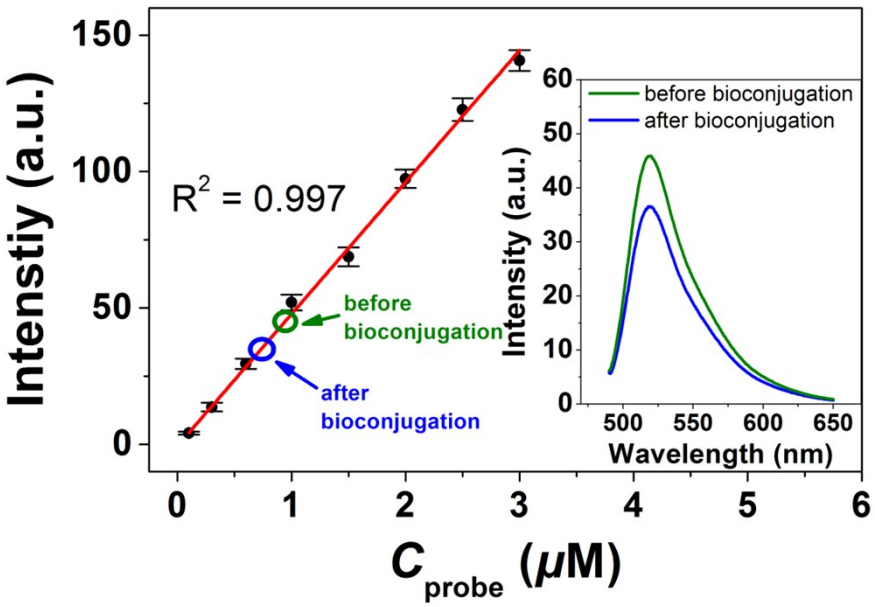

Fig. S4 Standard curve produced from eight known concentrations of FAM-labeled miRNA-155 probe. The intensities of the probe in the supernatant before/after bioconjugation to Qbead@SiO_2 are plotted on the curve. The corresponding fluorescence spectra are shown in the inset.

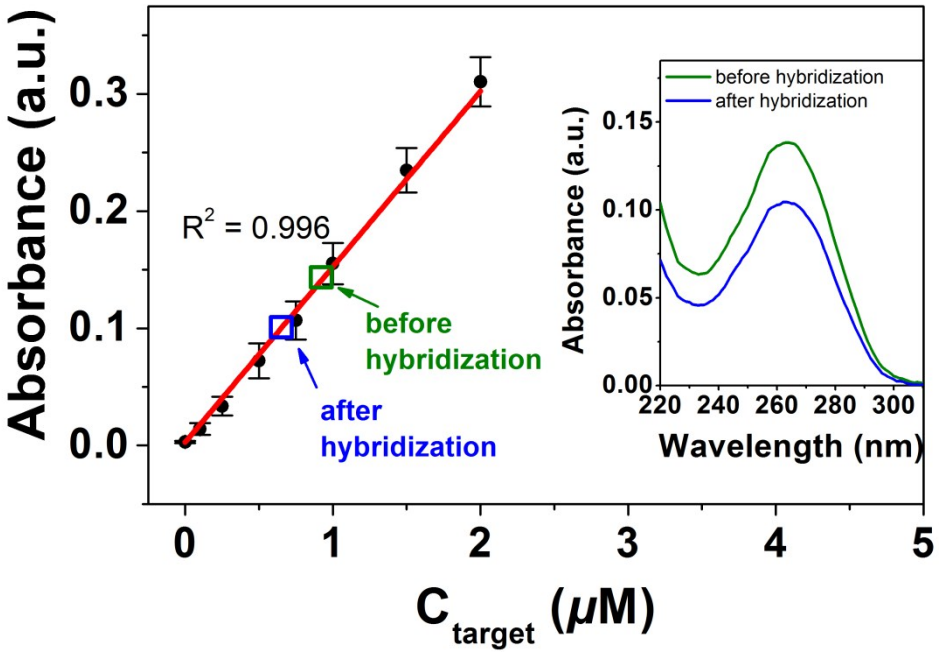

Fig. S5 Standard curve produced from eight known concentrations of miRNA-155. The absorbance of miRNA-155 in the supernatant before/after binding are plotted on the curve. The corresponding absorption spectra are shown in the inset.

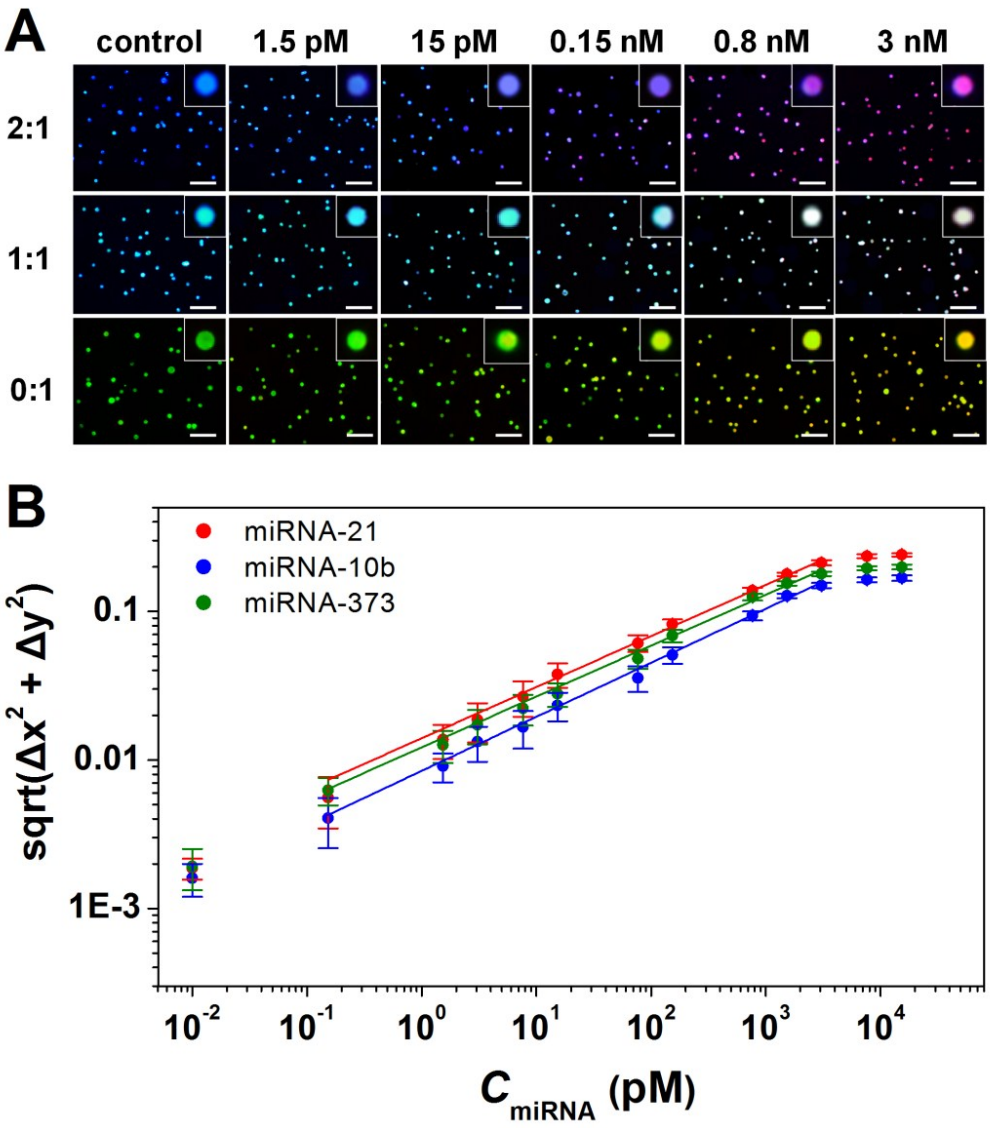

Fig. S6 (A) Fluorescent images of barcodes 2:1, 1:1 and 0:1 as a function of the abundance of miRNA-21, miRNA-10b and miRNA-373, respectively. Scale bar: 50 μm. The inset shows 10-fold amplified image of single Qbead@SiO_2—HCR product. (B) Relationship between the color-shift distance and the miRNA abundance.

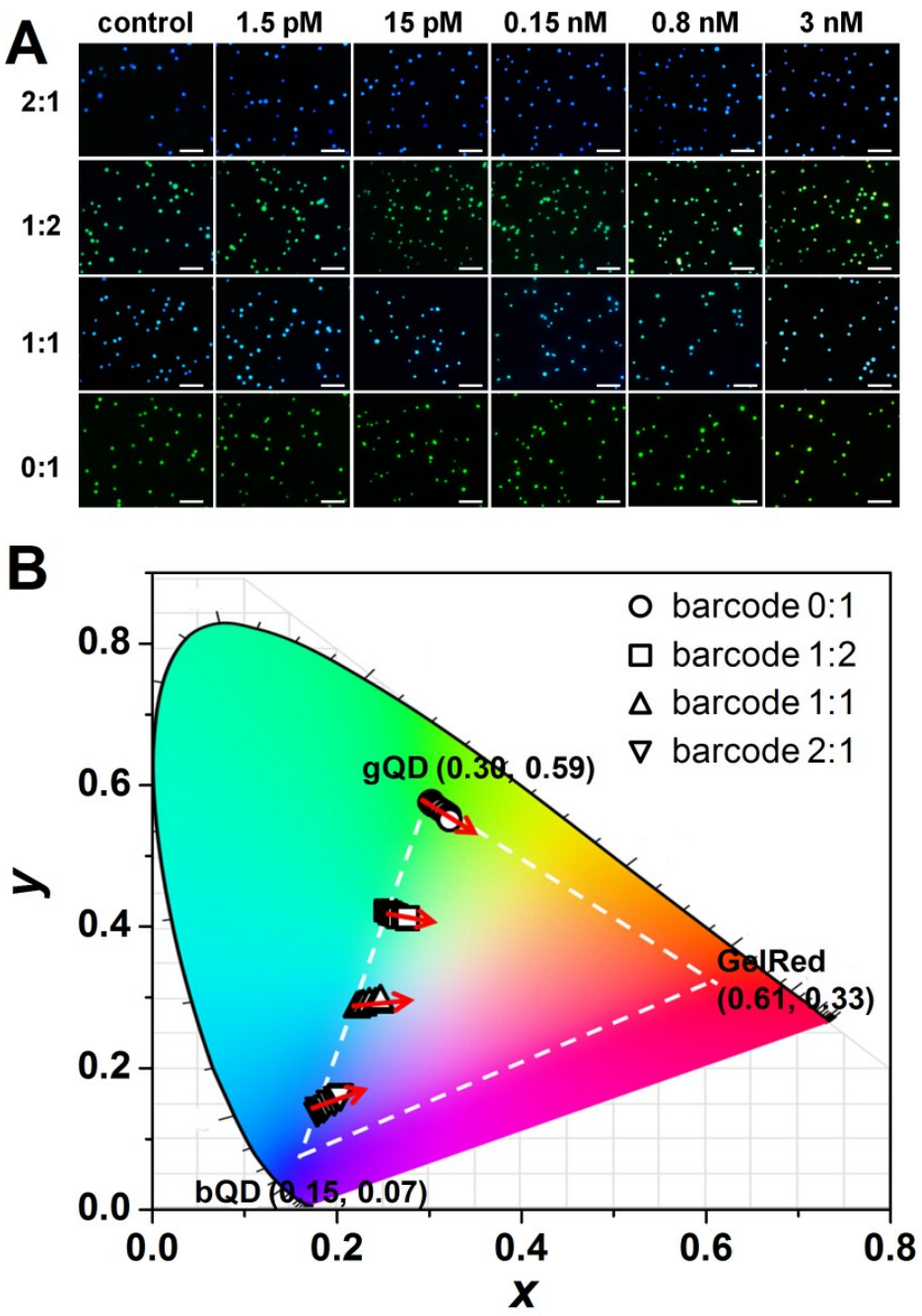

Fig. S7 (A) Fluorescent images of the four barcodes upon binding with varying abundance of 4 target miRNAs, respectively, without HCR and with staining. Scale bar: 50 μm. (B) CIE chromaticity diagram showing the color shifts of four barcodes with increasing abundance of four miRNAs.

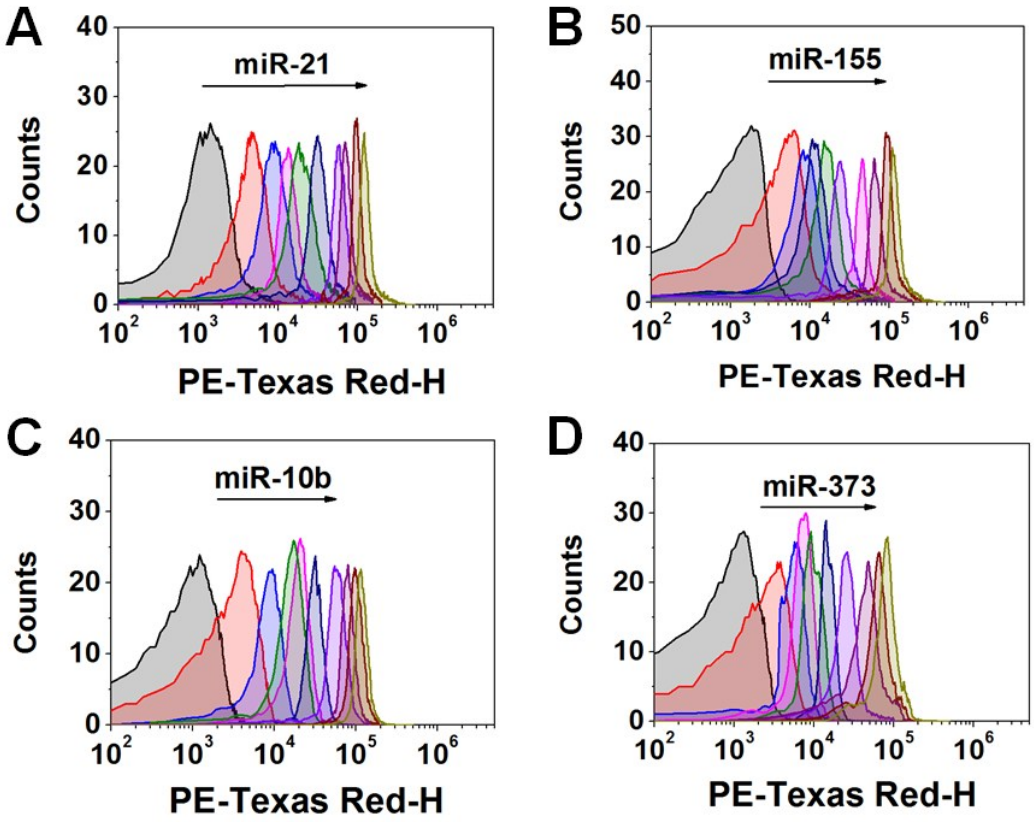

Fig. S8 Fluorescence histograms of stained Qbead@SiO_2—HCR products for quantification of the four target miRNAs by using barcodes 2:1, 1:2, 1:1 and 0:1, respectively. The target concentration from left to right: 0 (control), 0.15 pM, 1.5 pM, 8 pM, 15 pM, 80 pM, 0.15 nM, 0.8 nM, 3 nM and 15 nM, respectively.

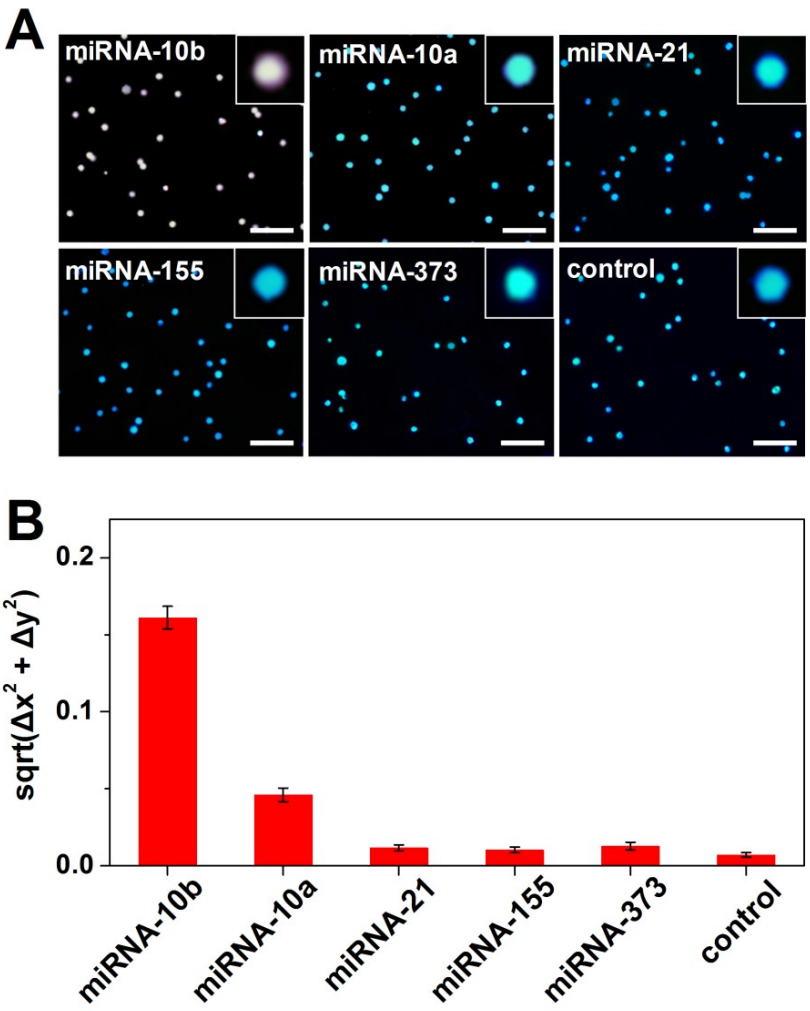

Fig. S9 (A) Fluorescent images of barcode 1:1, by the presence of 3 nM of miRNA-10b, miRNA-10a, miRNA-21, miRNA-155 and miRNA-373, respectively. Scale bar: 50 μm. The inset shows 10-fold amplified image of single Qbead@SiO_2—HCR product. (B) Histogram on the color-shift distance in (A).

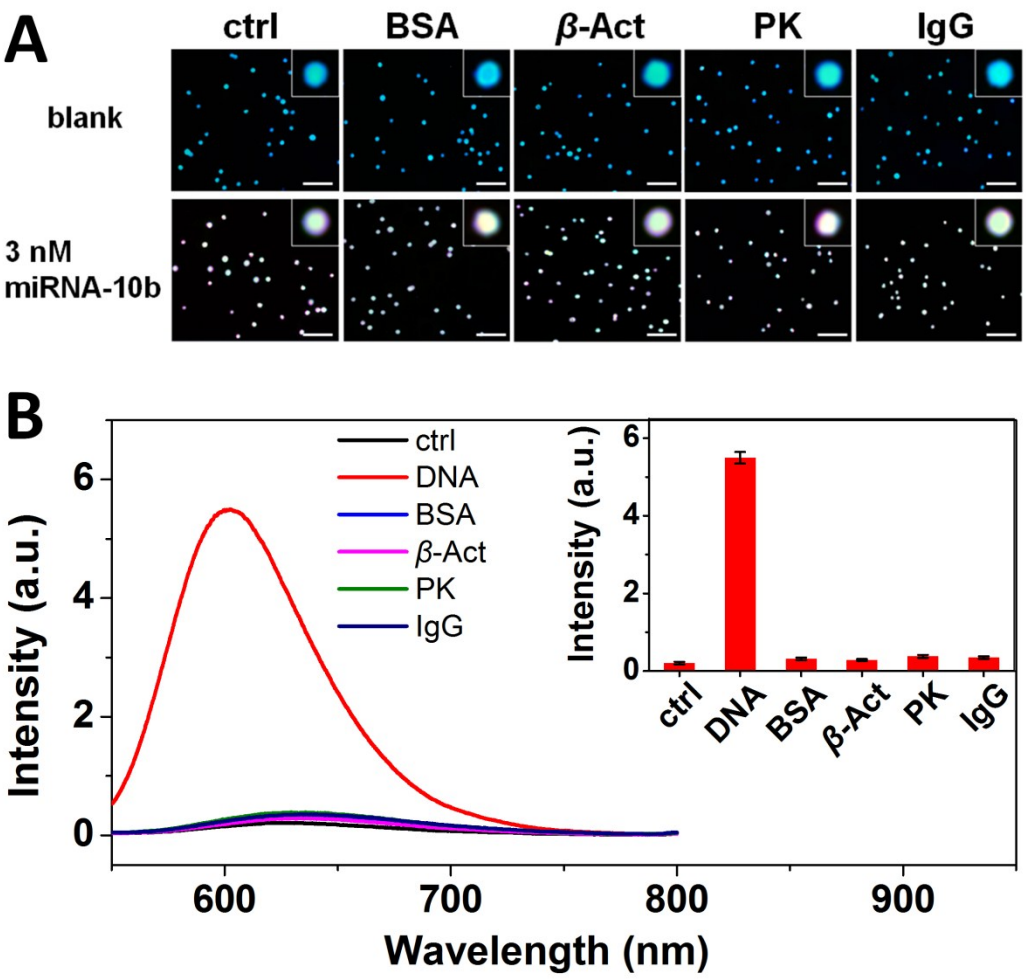

Fig. S10 (A) Fluorescent images of barcode 1:1 in hybridization buffer spiked with 7 μM of bovine serum albumin (BSA), *β*-actin (*β*-Act), proteinase K (PK) and immunoglobulin G (IgG), respectively. Scale bar: 50 μm. The inset shows 10-fold amplified image of single barcode. (B) Fluorescence spectra of GelRed in the presence of DNA and protein/ proteinase. The inset shows the intensities at 605 nm.

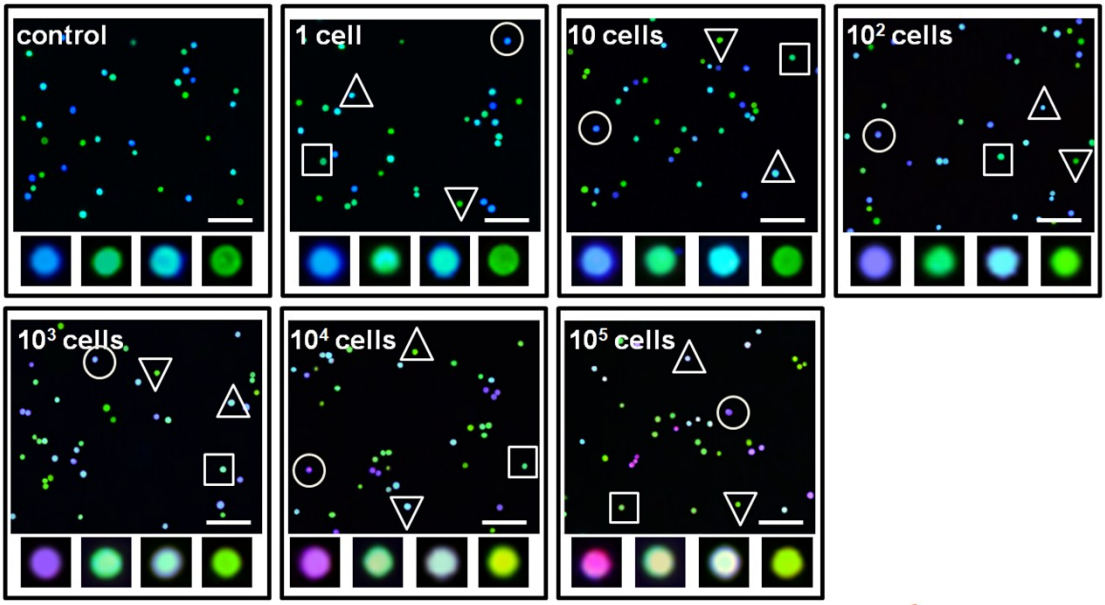

Fig. S11 Fluorescent images for quantification of target miRNAs in MCF-7 cell lysates. Symbols of "○", "□", "△" and "▽" highlight colour changes of barcodes 2:1, 1:2, 1:1 and 0:1, respectively. For clarity, 10-fold amplified images of single Qbead@SiO_2—HCR product for barcodes 2:1, 1:2, 1:1 and 0:1 (from left to right), are displayed at the bottom.

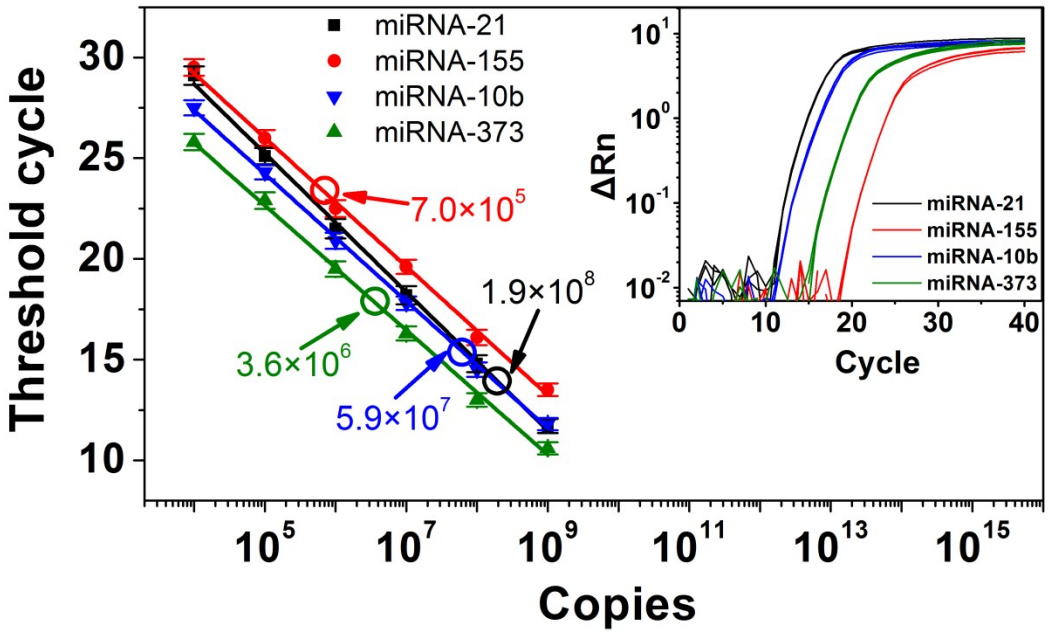

Fig. S12 Calibration curves for quantification of miRNAs by qRT-PCR. The threshold cycle values of the four miRNAs are plotted on the curves. The inset shows the corresponding amplification curves of the four target miRNAs in cell lysate (10^3 cells).

3. Supporting references

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