

Tagging the Rolling Circle Products with Nanocrystal Clusters for Cascade Signal Increase in Detection of miRNA

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Supporting Information

Experimental

Reagents and materials

All oligonucleotides and miRNAs were purchased from Integrated DNA Technologies (Coralville, IA) and were used as received. The 5' end of the padlock probes were phosphorylated for the ligation and the primer were labeled with amine groups for microsphere conjugation. The detection probe (DP) was tagged with FAM. 100bp DNA ladder was from Promega Corp. (Madison, WI). The RepliPHI™ phi29 DNA polymerase (100 U/μL) and the mixture of deoxyribonucleotides (dNTPs) were purchased from Epicentre Biotechnologies (Madison, WI). T4 DNA ligase was from New England Biolabs Inc. (Ipswich, MA). Carboxyl magnetic particles (2.5% w/v) with a nominal diameter of 4.35 μm were acquired from Spherotech Inc. (Lake Forest, IL). 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), and silver nitrate were purchased from Acros Organics (Pittsburgh, PA). Streptavidin, sulfo-N-hydroxysuccinimide (sulfo-NHS), dithiothreitol (DTT), NHS-SS-diazirine (SDAD), and the salts used to prepare buffer solutions were supplied by Fisher Scientific (Pittsburg, PA). Bovine serum albumin (BSA), adenosine 5'-triphosphate disodium (ATP) and Tween-20 were obtained from Sigma Aldrich (St. Louis, MO). Horseradish peroxidase (HRP)-streptavidin conjugate, biotin-14-dATP, Human Brain Total RNA and FluoZin-3 were from Invitrogen (Carlsbad, CA). Super AquaBlue ELISA substrate for HRP color development was ordered from eBioscience Inc. (San Diego, CA). The water used for preparation of all solutions was produced by the Milli-Q water purification system from Millipore (Billerica, MA). The amine-coated ZnS nanocrystal clusters were produced through hydrothermal approach³⁶, using thiourea to control cluster growth.

Conjugation of Microspheres and Nanoclusters with biomolecules

Coupling the primers to the surface of microspheres was performed as follows. First, 2 nmol of the primer was added to 1 mL of the linking buffer (0.02 M phosphate buffer (pH 7.2)) containing around 2.5×10^7 magnetic particles. The carboxyl groups on magnetic particles (MNPs) were activated with the presence of 0.1 mg EDC and 0.1 mg sulfo-NHS. The reaction was incubated at RT for 3 hr. A MACSmix tube rotator (MiltenyiBioten Inc., Auburn, CA) was used to agitate the reaction tubes to prevent bead aggregation. Then the MNPs were pelleted with a permanent magnet. After removal of the supernatant, the microspheres were dispersed in 100 μL of the linking buffer and stored at 4 °C.

Streptavidin was coupled to the amine-coated ZnS NCCs also through the amide bond with the presence of EDC and sulfo-NHS. Forty microliters of the stock ZnS NCCs ($\sim 4 \times 10^{11}$ particles) were washed with 0.01 % Tween-20 solution by centrifugation at 16.1k X g for 15 min before conjugation. The carboxyl group on streptavidin was activated by mixing 40 μL of 1 mg/mL streptavidin with 6 mg EDC and 5 mg sulfo-NHS dissolved in 100 μL of linking buffer. After 15 min activation, the residual EDC was quenched by 1.2 μL of 2-mercaptoethanol to prevent protein cross-linking. Then, ZnS NCCs were added to the activated streptavidin, and the mixture was gently vortexed for 3 hr at RT. Finally, the ZnS-streptavidin conjugate was precipitated with centrifugation at 16.1k X g for 12 min and dispersed in 100 μL of linking buffer. ZnS-streptavidin products were blocked by 10 mL of 5% BSA/PBS solution before being used in the assay to reduce the nonspecific adsorption.

RCA detection

The padlock probe and the primer were carefully designed to ensure that there is no secondary structure at RT so that all the hybridization can be conveniently performed at RT. In a typical experiment, hsa-let-7a solutions of different concentrations were hybridized to the 5' and 3' termini of 100 nM linear padlock probe in 20 μL of ligation buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 10 mM DTT, and 1 mM ATP). The reaction mixture was denatured at 85 °C for 3 min and then incubated at RT for 1 hr. Then, 2 mM ATP

and 0.1 U T4 DNA ligase were introduced to trigger the ligation reaction. After 1 hr of ligation at RT, 1 μ L of MNP-primer was added to the reaction mixture to capture the circular probe. Again, the reaction mixture was denatured at 85 $^{\circ}$ C for 3 min and incubated at RT for 1 hr. Then, the beads were separated with a magnet and re-dispersed in 40 μ L of phi29 reaction buffer (40 mM Tris-HCl, pH 7.5, 50 mM KCl, 10 mM MgCl₂, 5 mM (NH₄)₂SO₄, 4 mM DTT) containing 5% BSA. Beads were then blocked with BSA for 30 min at RT to minimize the nonspecific adsorption. Polymerization was carried out at 37 $^{\circ}$ C for 50 min with the addition of 50 U/ μ L phi29 DNA polymerase, 1 μ M dNTP mixture and 200 nM Biotin-14-dATP. Afterwards, the on-beads biotinylated RCA products were incubated with ZnS-streptavidin particles in 100 μ L of 5% BSA/PBS buffer for 1 hr at RT on the tube rotator. Finally, the beads were washed twice with 200 μ L 0.01% Tween-20/PBS buffer to remove the unbound ZnS-streptavidin particles.

CXamp and Fluorescence Measurements

It has been demonstrated that microwave-assisted cation exchange (MWCX) reaction can greatly improve the Zn release efficiency in ZnS NCCs³⁶. In our test, CX was performed in a 100 μ L of 500 μ M AgNO₃ and 3 μ M FluoZin-3 solution, with 2 min microwave irradiation in a household microwave oven (Hairer, Model: MWM0701TB, New York, NY). Next, microspheres were removed by the magnet and the supernatant was transferred to a 96-well plate. Fluorescence signals were measured in the Victor II Microplate reader (Perkin-Elmer, Waltham, MA) using an excitation wavelength filter of 485 nm and an emission filter of 530/30 nm.

For miRNA detection with DP-FAM probe, 2 mM dNTP was used during polymerization and other RCA reaction conditions were kept same. After that, 5 μ M DP-FAM was hybridized with MNPs-RCA product for 1 hr at RT in 100 μ L of hybridization buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, and 0.01% Tween-20). The FAM fluorescence was recorded under the same measurement wavelength as for CXamp. For HRP-streptavidin detection system, MNPs-biotin-RCA products were incubated with 100 μ L of 0.42 μ g/mL streptavidin-HRP 5% BSA/PBS solution at RT for 1 hr. Color development was carried out in 100 μ L of Super AquaBlue ELISA substrate with 20 min incubation. The green product was measured by the absorbance at 405 nm using the same Victor II Microplate reader.

Polyacrylamide gel electrophoresis

To verify the biotin-RCA reaction occurring on the surface of MNPs, gel electrophoresis was conducted to analyze the RCA reaction product. Large MNPs of 4.35 μ m cannot go through the gel preventing the entry of the attached ssDNA into the gel. It is necessary to cleave the RCA product from the beads before the gel electrophoresis. Accordingly, SDAD was chosen as the crosslinker because it contains cleavable S-S bond. Typically, 25 μ M of the primer was incubated with 700 μ M of SDAD in 100 μ L of 0.1 M phosphate buffer (pH 7.2) at RT for 30 min. The N-hydroxysuccinimide (NHS) esters on SDAD react with amine groups on the primer to form stable amide bonds upon release of NHS. Then, the excess SDAD was quenched with 7.5 μ L of 1M Tris-HCl (pH 8.0) for 5 min. About magnetic particles ($\sim 2.5 \times 10^7$) was dispersed in the SDAD-primer mixture and illuminated with UV light (365 nm, Spectronics Corp. Westbury, NY) for 15 min. Photoactivation of diazirine creates reactive carbene intermediates, which can form covalent bonds with the carboxyl carbons on the MNPs. The beads are then washed three times with 0.1 M phosphate buffer (pH 7.2) and disperse in 100 μ L of the phosphate buffer. After the biotin-RCA reaction on MNPs, the elongated ssDNA was cleaved through S-S bond by 10 mM DTT at 37 $^{\circ}$ C for 30 min and then analyzed with 8% denaturing polyacrylamide gel. The gel image was scanned with an Amersham Typhoon 9410 Imager (GE Healthcare, Madison, WI). (Note: With SDAD serving as the crosslinker, DTT was not included in the polymerase buffer in order to protect the stability of MNP-primer conjugate. However, as an active enzyme preservative, 4 mM DTT is recommended for RCA reaction. Therefore, to pursue the maximal Phi29 enzyme activity, EDC/Sulfo-NHS was selected as the cross-linking reagent in the assay test as it is compatible to DTT.)

Characterization of ZnS NCCs

In the test, ZnS NCCs of semi-hollow core-shell structures were synthesized through a simple hydrothermal reaction by using zinc acetate dihydrate and thiourea. ZnS NCCs dispersed in ethanol were deposited onto a carbon-coated copper grid, followed by evaporation under vacuum at RT. High-resolution TEM (HRTEM) images were obtained using a Tecnai G2 S-Twin electron microscope operated at 200 kV. The histogram of ZnS NCCs sizes was obtained by measuring over 160 units of NCCs. TEM image and size distribution show that the synthesized Zn NCCs have a nominal diameter of 40 nm. The cluster structure was measured on a Bruker D8 Advance X-ray diffractometer (XRD) with a Cu_{K α} radiation ($\lambda = 1.5415 \text{ \AA}$). The data was collected at a scan rate of 0.03 $2\theta \text{ s}^{-1}$ per point. ZnS NCCs exhibited the same XRD patterns as ZnS (JCPDS 36-1450), with the main diffraction peaks were found to be (002), (110), and (112).

Table S1. Sequences of RNA and DNA Probes Used in Our Assay. Mutated Nucleotide in miRNA Were Presented in Bold

Strand name	sequence
hsa-let-7a	5'-rUrGrArGrGrUrArGrUrArGrGrUrUrGrUrArUrArGrUrU-3'
hsa-let-7e	5'-rUrGrArGrGrUrArGr GrGr ArGrGrUrUrGrUrArUrArGrUrU-3'
hsa-let-7i	5'-rUrGrArGrGrUrArGrUrArGr UrUrUrGrUrGrCrUr GrUrU-3'
padlock probe (56 nt)	5'-phosphate-ACTACCTCATAACCACATAAGATACCGCATAAAG TTCTCACCTATTCTATACAACCT-3'
Primer (30 nt)	5'-NH ₂ -C ₁₂ -A ₁₅ -AT GCG GTA TCT TATG-3'

The letter "r" indicates "ribose nucleotide".

Table S2. RCA primers (CP) or RCP was cleaved off MPs at the disulfide bond of SDAD and determined by SYBR Green II in 110 μ l 10 mM DTT solution.

Sample	SYBR Green II fluorescence intensity	Relative Concentration of ssDNA equivalents(nM)	Number of MPs	RCA amplification fold
Blank	854	0	0	---
RCA primers cleaved from MPs	1221	6.55	2.5×10^6	---
Amplified RCP cleaved from MPs	4833	71.05	6.2×10^4	440.1

The number of CP per MP was calculated as follows:

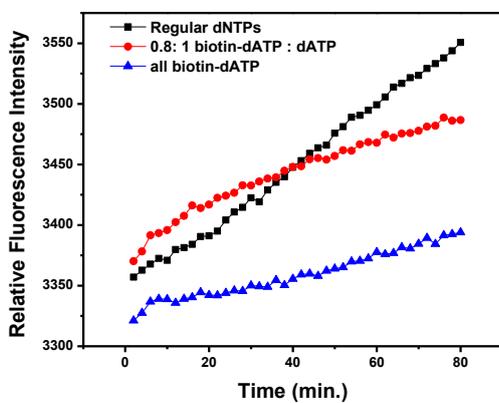
$$\text{Total number of CP} = 6.55 \times 10^{-9} \times 110 \times 10^{-6} \times 6.02 \times 10^{23} = 4.33 \times 10^{11}.$$

$$\text{The number of CP per MP} = 4.33 \times 10^{11} / (2.5 \times 10^6) = 1.7 \times 10^5$$

$$\text{RCA amplification fold considering the length difference between the primer and the padlock probe} = 71.05 / 6.55 \times [(2.5 \times 10^6) / (6.2 \times 10^4)] \times (30 \text{ nt} / 56 \text{ nt}) = 236$$

Figure S1. A) Decrease of enzyme activity detected by a real-time PCR machine. The RCA reaction was carried out in the same way as in the detection assay, but the signal was read out by the SYBR-gold dye. Continuous production of the RCP increased the fluorescence of SYBR-gold linearly when only the regular dATP was used in the reaction. However, with more and more substitution of the biotin-dATP with the regular dATP, the slope of the fluorescence increase vs. time became smaller and smaller, indicating the drop of enzyme activity. B) The detection performance in the presence of 1 fmol of *let-7a* was examined with different ratio of Biotin-14-dATP/dATP.

A)



B)

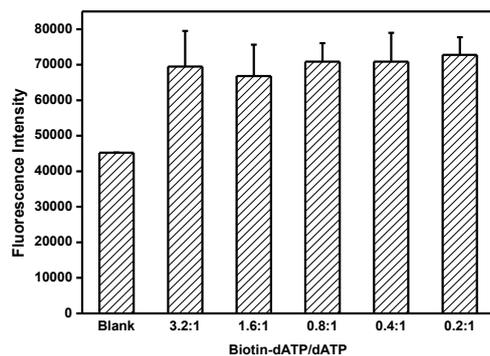


Figure S2. Calibration curve of ssDNA primer detected with intercalated SYBR Green II fluorescent dye.

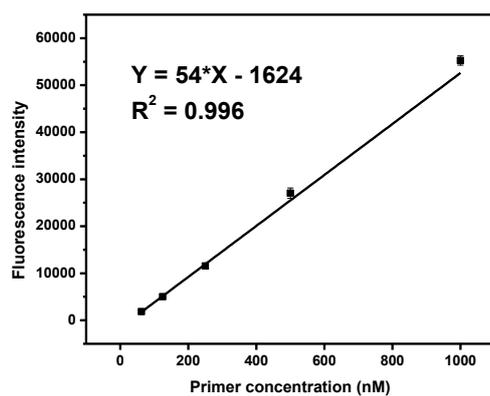


Figure S3. Transmission electron microscope (TEM) image (a), size distribution (b) and X-ray diffraction pattern (XRD) (c) of the ZnS NCCs.

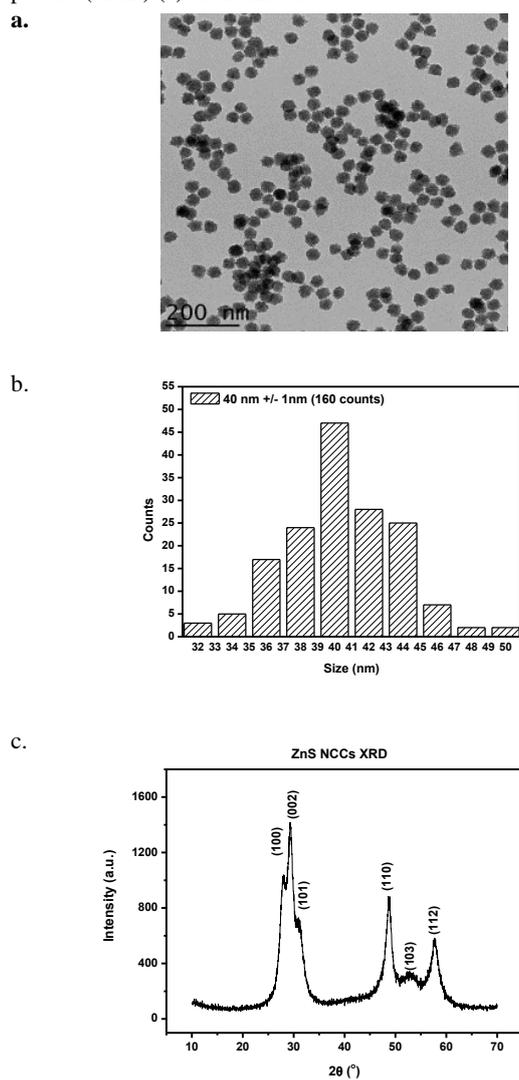


Figure S4. The Zn^{2+} release performance was compared with two methods, i.e., CX and microwave assisted CX (MWCX). Firstly, total $[Zn^{2+}]$ was determined by the nitric acid digestion, which can completely dissolve the entire ZnS NCCs but involves tedious pH neutralization steps for Zn^{2+} titration. During the measurement, ZnS NCCs of different concentrations were dissolved in 100 μ L of 46 mM nitric acid and the solution was neutralized by 10 μ L of 0.5 N NaOH, and the solution pH was maintained at 7.2 by adding 10 μ L of 1.0 M phosphate buffer; The released Zn^{2+} concentration was obtained from fluorescent measurement and plotted against the total $[Zn^{2+}]$. Finally, 10 μ L of 100 mM HEPES buffer containing 30 μ M of FluoZin-3 was introduced into the solution for fluorescence measurements. In order to determine the final Zn concentration in the ZnS NCCs solution after the acid dissolution, the standard calibration curve (a) was obtained with solutions prepared by the exact same procedure but replacing the ZnS NCCs with $Zn(Ac)_2$. Meanwhile, after the CX reaction with the addition of Ag^+ , the released Zn^{2+} was determined with FluoZin-3. Then, we plot these CX-released Zn^{2+} against the total Zn^{2+} and calculated the Zn release efficiency, as shown in (b). 28.9% of Zn can be freed from ZnS NCCs and the release efficiency was increased to 66.1% when CX was assisted with only 2 min of microwave irradiation. Therefore, microwave heating could help to increase the release efficiency by 2.3 fold.

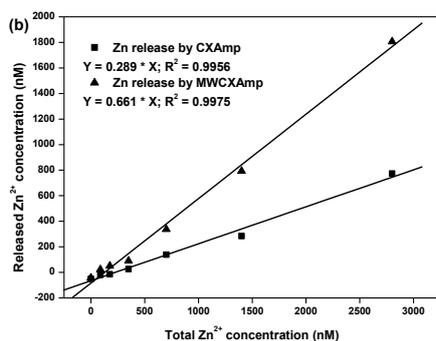
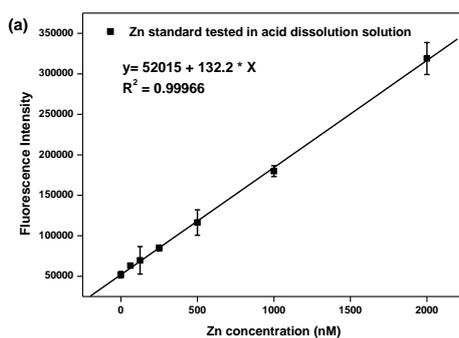


Figure S5. The T4 DNA ligation was performed with the presence of 100 pmol *let-7a* and the ligated product was analyzed by 20% denaturing polyacrylamide gel. The gel was stained with ethidium bromide followed by being scanned with the Typhoon 9410 Imager. The DNA band intensity and area were analyzed by ImageJ software, showing 80% of the ligation efficiency was achieved by T4 DNA ligase.

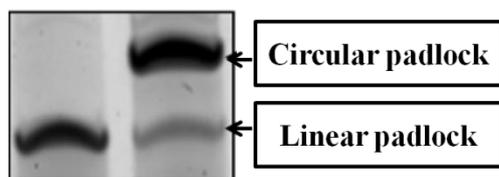


Figure S6. Rolling circle reaction duration was optimized with the presence of 1 fmol *let-7a*.

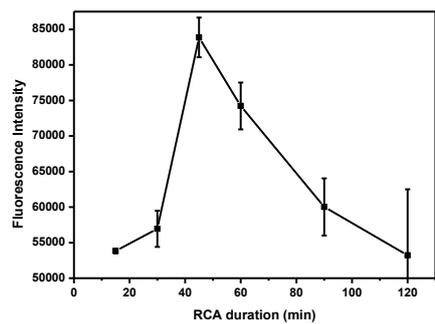


Figure S7. The detection performance in the presence of 1 fmol of *let-7a* was compared with different amount of BSA, i.e. 2% and 5%. Decreased background signal and improved reproducibility was obtained with increased amount of BSA due to the reduced nonspecific adsorption.

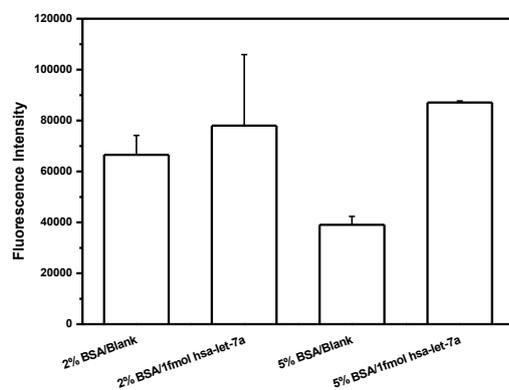


Figure S8. Specificity was demonstrated with *let-7a*, *let-7e* and *let-7i*. The sequence of *let-7e* and *let-7i* differ from that of the *-7a* by one and four bases, respectively.

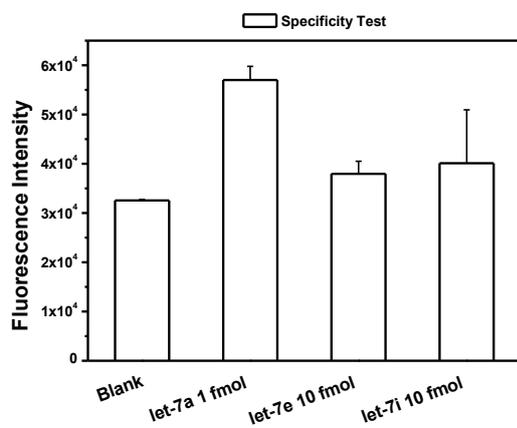


Figure S9. Quantification of *hsa-mir-Let-7a* in Human Breast Total RNA extract. The solid line represented the calibration curve measured at the same time. The red lines indicated the fluorescence intensities measured with 50 ng total RNA sample itself and spiked with 500 amol *let-7a*, and their corresponding RNA amounts.

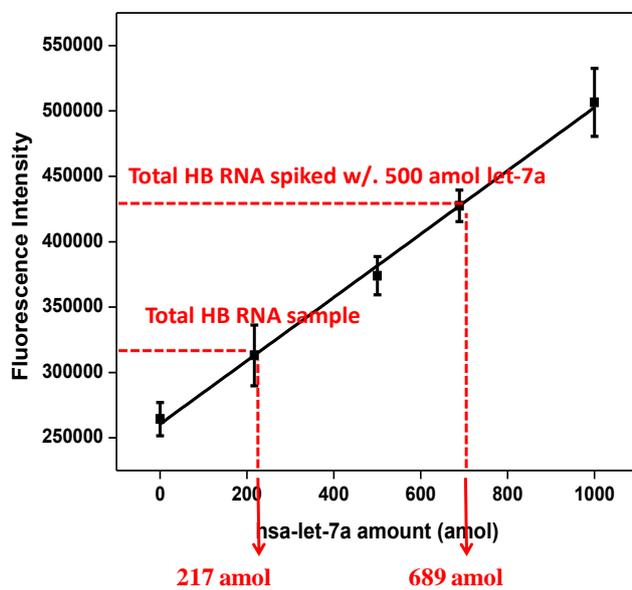


Figure S10. Detection performance at various amount of let-7a with (a) HRP-Streptavidin; or (b) Qdots as the RCP labels. RCA conditions were the same as in detection with ZnS NCCs, except for switching the NCCs solution with the HRP or Qdots solutions in the last step of the assay.

