

Electronic Supplementary Information (ESI)

**Multiplex miRNA Assay Using Lanthanide-tagged Probes and Duplex-Specific
Nuclease Amplification Strategy**

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Experimental Section

Chemicals and Materials. Magnetic microparticles (Dynabeads M-280 Streptavidin, Dynabeads Myone Streptavidin C1) and separation magnets (DynaMag-2) were commercially available from Invitrogen Co. (Shanghai, China), as shown in Fig. S1. 1,4,7,10-tetraazacyclododecane-1,4,7-tris-aceticacid-10-maleimidoethylacetamide (MMA-DOTA) was purchased from Macrocyclics (Dallas, TX). DSN was obtained from Evrogen Joint Stock Company (Russia). RNase inhibitor was purchased from Thermo Scientific Inc. (MA, USA). PrCl_3 , $\text{TbCl}_3 \cdot 6\text{H}_2\text{O}$, and $\text{TmCl}_3 \cdot 6\text{H}_2\text{O}$ were purchased from Aladdin Inc. (Shanghai, CHINA). An RNase-free environment was created throughout the experiments by using DEPC-treated water and RNase-free tips and tubes.

The sequence information of all the oligonucleotides was listed in Tab. S1. miRNAs were purchased from Sangon Inc. (Shanghai, CHINA). The sequence information of miRNA was listed in Tab. S2.

Table S1. Sequence information of oligonucleotide probes,

| Name | Sequence (5' - 3') |
|----------|-------------------------------------------------------------|
| P141-rep | AGA CAG TGT TA -SH -141Pr |
| P141-cap | biotin- AAAAAAAAAA CCA TCT TTA CC |
| P141 | 141Pr- SH- CCA TCT TTA CCA GAC AGT GTT A TTTTTTTTTT -biotin |
| P7d | 159Tb- SH- AAC TAT GCA ACC TAC TAC CTC T TTTTTTTTTT -biotin |
| P21 | 169Tm- SH- TCA ACA TCA GTC TGA TAA GCT A TTTTTTTTTT -biotin |

Table S2. Sequence information of miRNA

| Name | Sequence (5' - 3') |
|---------|-------------------------------|
| miR-141 | UAA CAC UGU CUG GUA AAG AUG G |
| let-7d | AGA GGU AGU AGG UUG CAU AGU U |
| miR-21 | UAG CUU AUC AGA CUG AUG UUG A |

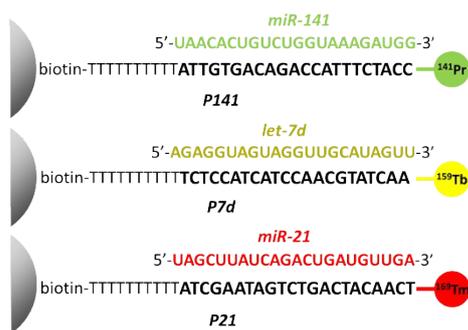
| | |
|--------|-------------------------------|
| let-7a | UGA GGU AGU AGG UUG UAU AGU U |
| let-7c | UGA GGU AGU AGG UUG UAU GGU U |
| let-7b | UGA GGU AGU AGG UUG UGU GGU U |

For miRNA sandwich hybridization assay, 5'-end biotinylated capture probes (P141-cap) and 3'-end ¹⁴¹Pr tagged report probes (P141-rep) were designed to hybridize with miR-141. P141-cap was the capture probe for miRNA sandwich hybridization assay. P141-rep was the reporter probe for miRNA sandwich hybridization assay and further labeled with ¹⁴¹Pr at 3'-end. For multiplex miRNA assay based on DSN amplification strategy, P141, P7d, and P21 were oligonucleotide probe complimentary with miR-141, let-7d, and miR-21, respectively. P141, P7d, and P21 were functionalized with thiol group (-SH) at 5'-end as the reporter part and biotin at 3'-end as the capture part to bind streptavidin-coated MMPs. The reporter part with lanthanide at 5'-end can be detected by ICP-MS and further labeled with ¹⁴¹Pr, ¹⁵⁹Tb, and ¹⁶⁹Tm, respectively. All sequence information can be seen in Scheme S1.

miRNA Sandwich Hybridization Assay



Multiplex miRNA Assay with DSN Amplification



Scheme S1. Sequence information of ICP-MS based miRNA sandwich hybridization assay and multiplex miRNA assay based on DSN amplification strategy.

Labeling oligonucleotide probes with lanthanide tags. First, oligonucleotide-sulfydryl-3' or 5'-sulfydryl- oligonucleotide-biotin-3' probe was dissolved with NH₄Ac buffer (0.5 M, pH 5.8) to 100 μM, and 25-fold excess MMA-DOTA was added to the solution. The -SH group of oligonucleotide probe and maleimide group of MMA-DOTA were allowed to react for 2 h with gentle shaking at 37 °C. Then, the product was purified by HPLC and tested by MALDI-TOF-MS to verify the reaction. Lanthanides were dissolved with NH₄Ac buffer (0.5 M, pH 5.8), and diluted to 10 mM by NH₄Ac buffer (0.5 M, pH 5.8) as elemental stock solutions. Then, each of MMA-DOTA-activated sequence-specific oligonucleotide probes was mixed with 5-fold excess elemental solution required, and allowed to react for 1 h with gentle shaking at 37 °C. After that, stoichiometric amount (4 times the amount of probes) of EDTA solution (5 mM) was added to integrate the excess unchelated metal ions for 15 min. The product of lanthanide-tagged oligonucleotide probe was desalted and purified by HPLC¹.

MMPs functionalization with oligonucleotides. Dynabeads Myone Streptavidin C1 (7–10×10⁹ beads/mL, 0.2 mL) was used for basic sandwich miRNA assay. Dynabeads M-280 Streptavidin (6–7×10⁸ beads/mL, 0.2 mL) was used for DSN amplification miRNA assay. Dynabeads were washed twice with Solution A (0.1 M NaOH, 0.05 M NaCl) and once with Solution B (0.1 M NaCl), and further magnetically separated and washed once with 'Binding and Washing' (B&W) buffer (10 mM Tris-HCl, 1 mM EDTA, 2 M NaCl, pH 7.5). Successively, these beads were resuspended in 400 μL RNase free 2× B&W buffer. For immobilization, 40 μL 100 μM oligonucleotide probes and 360 μL DEPC-treated water were added and incubated for 1 h at room temperature under gentle rotation. The biotinylated oligonucleotide coated beads was separated with a magnet for 2-3 min and washed twice with RNase free 1× B&W buffer and once with hybridization buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8.5). Finally, dynabeads were suspended to 100 μL RNase free hybridization buffer for miRNA sandwich assay and stored at 4 °C.

miRNA Sandwich Hybridization Assay Procedures. P141 cap-functionalized Dynabeads (5 μL), P141-rep (20 μL), and hybridization buffer (45 μL) were mixed in a tube. Then, miR-141 (10 μL) of different concentration (50 nM, 100 nM, 200 nM, 500 nM, 1 μM, 2 μM) was added. The mixture was gently shaken at 20 °C, which is below the T_m for miR-141 (44.1 °C), for 2 h².

Subsequently, the sandwich complexes were isolated by the magnetic field and washed three times with hybridization buffer (0.5 mL) to remove excess lanthanide-tagged probes. Then, the buffer-washed sandwich complexes were diluted with deionized water (0.2 mL), gently shaken, and heated to 75 °C for 15 min to allow lanthanide-tagged probes to release from the sandwich complexes. Finally, the supernatant was diluted to 1.5 mL and measured by ICP-MS.

miRNA Assay Procedures based on DSN amplification strategy. For miR-141 assay based on DSN amplification strategy, A volume of 40 μ L reaction mixture containing 4 μ L P141 functionalized Dynabeads, 4 μ L 10 \times DSN buffer (50 mM Tris-HCl, 5 mM MgCl₂, pH 8.0), 0.5 μ L RNase inhibitor (40 U/ μ L), 10 μ L miR-141 of different concentration (1 nM, 5 nM, 10 nM, 20 nM, 50 nM, 100 nM), 1 μ L DSN (0.1 U/ μ L, in 50% 25 mM Tris-HCl, pH 8.0; 50% glycerol), and 20 μ L DEPC-treated water was incubated at 60 °C for 1 h. Subsequently, 40 μ L of 10 mM EDTA was added into the reaction mixture and incubated at 65 °C for 5 min to inactivate DSN enzyme. Finally, the supernatant was diluted to 1.5 mL and measured by ICP-MS.

For multiplex miRNA assay based on DSN amplification strategy, the procedure was the same as single miRNA detection method described above, except for adding lanthanide probe functionalized Dynabeads (3 μ L each, 9 μ L in total), and less DEPC-treated water (16 μ L). For HeLa cell lysate experiment, 2.5 μ L lysate was added.

ICP-MS Detection. An X series ICP-MS (Thermo Electron Corp., Winsford, UK) equipped with a glass concentric nebulizer and an impact bead spray chamber for aerosol generation and filtration was used for the experiments. Before each measurement, the operating parameters of instruments were optimized by the standard solution of 10 μ g/L Rh, as shown in Tab. S3.

Table S3. The operating parameters of ICP-MS instrument.

| Parameter | Value |
|----------------------------|-------|
| RF power (W) | 1200 |
| Cool gas flow (L/min) | 13 |
| Auxiliary gas flow (L/min) | 0.8 |
| Nebulizer gas flow (L/min) | 0.82 |

| | |
|-------------------------------|--------------------|
| Sample uptake rate (mL/min) | 0.5 |
| Torch | Shield torch |
| Cones | Nickel, HPI design |
| Dwell time (ms) | 10 |
| Resolution | Standard |
| Analogue detector voltage (V) | 3750 |
| PC detector voltage (V) | 1950 |

Cell Culture. Human cervical cancer cell lines (HeLa) were obtained from the Cell Resource Center, IBMS, CAMS/PUMC. HeLa cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and 100 IU/mL penicillin-streptomycin. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO₂. Cells were passaged by trypsinization with 0.25% trypsin in phosphate-buffered saline (PBS). The cell density was determined using a hemocytometer.

Preparation of Cellular Lysate. 2.5×10^6 cells were washed with PBS buffer and harvest by 0.25% trypsin (2.2 mM EDTA, 1 x sodium bicarbonate) and washed with DMEM medium. The cell pellet was suspended in 350 μ L Lysis Buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 0.2 mM EDTA, 1% Triton x-100). After incubating for 15 min on ice, the lysed cellular suspension was briefly mixed on a vortex and centrifuged for 15 min, 12,000 rpm at 4 °C. Then the supernatant was collected.

All the buffers used were prepared as follows.

For coupling of nucleic acids:

Solution A: DEPC-treated 0.1 M NaOH, DEPC-treated 0.05 M NaCl.

Solution B: DEPC-treated 0.1 M NaCl.

Binding and Washing (B&W) Buffer (2 \times): 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2 M NaCl, DEPC-treated water.

1× DSN Buffer: 50 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, DEPC-treated water.

Hybridization Buffer: 10 mM Tris-HCl (pH 8.5), 1 mM EDTA, 100 mM NaCl, DEPC-treated water.

For miRNA sandwich assay:

Hybridization Buffer: 10 mM Tris-HCl, pH 8.0; 1 mM EDTA, 100 mM NaCl, DEPC-treated water.

For miRNA assay using DSN amplification strategy

10× DSN Buffer: 500 mM Tris-HCl, pH 8.0; 50 mM MgCl₂, 1 mM DTT, DEPC-treated water.

Supporting Figures and Discussions

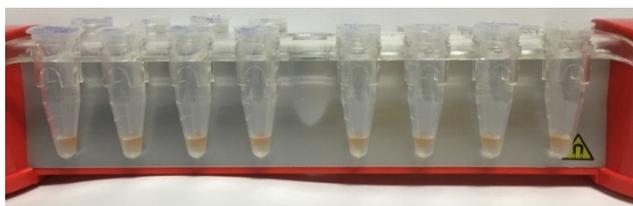


Figure S1. Magnetic microparticles were separated with separation magnets after DSN amplification for miRNA assay

Selectivity of Lanthanide-labeled Oligonucleotide Probes

To prove the lanthanide-labeled oligonucleotide probes are available for multiplex assay, we also hybridized the designed lanthanide-tagged oligonucleotide probe with other miRNAs to investigate the selectivity of the probes. The specificity of lanthanide-tagged oligonucleotide probes was validated by ICP-MS. Blank solution, or 0.5 pmol of different miRNA targets (miR-141, let-7d, miR-21) were added into suspensions containing P141-¹⁴¹Pr (Fig. S2a), P7d-¹⁵⁹Tb (Fig. S2b), and P21-¹⁶⁹Tm (Fig. S2c) functionalized MMPs. Strong signal could be clearly observed when the miRNA matches the probe-functionalized MMPs, for example miR-141 and P141-141Pr functionalized MMPs (Fig. S2a). Negligible crosslink signal was found between the miRNA and probes designed for other miRNAs (Fig. S2), which shows the designed probes were viable for multiplex detection. Negligible crosslink signal was found between the miRNA and

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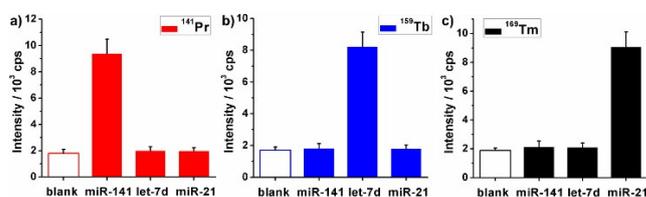


Figure S2. Selectivity of multiplex miRNA assay. Blank solution, or 0.5 pmol of different miRNA targets (miR-141, let-7d, miR-21) were added into suspensions containing a) P141-¹⁴¹Pr, b) P7d-¹⁵⁹Tb, and c) P21-¹⁶⁹Tm functionalized MMPs.

The ability to distinguish miRNA family members was also tested. let-7d and three other miRNAs from let-7 miRNA family (let-7a, let-7c, and let-7b) were chosen to evaluate the sequence-specificity of the proposed assay. The sequence of let-7a, let-7c, and let-7b differs from let-7d by 2 to 4 nucleotides. Different concentrations of let-7d, let-7a, let-7c, and let-7b were hybridized with P7d-¹⁵⁹Tb functionalized MMPs respectively, and ¹⁵⁹Tb signal was collected by ICP-MS. As can be seen in Fig. S3, the proposed method can distinguish let-7d from other let-7 family members and discriminate between perfect-matched miRNA and two base-mismatched miRNA.

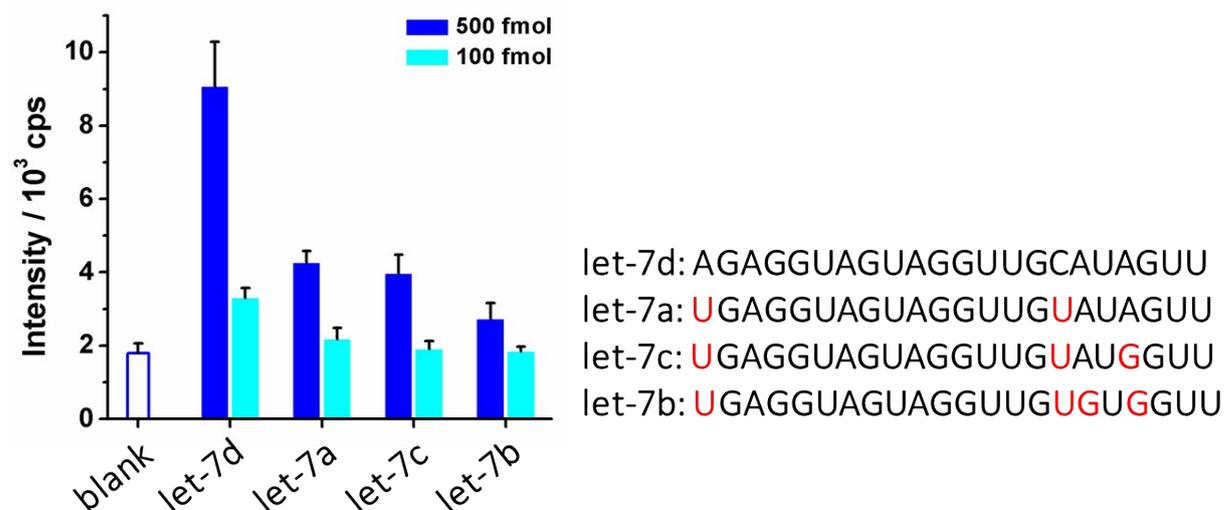


Figure S3. Specificity of the method. let-7d, let-7a, let-7c, and let-7b from let-7 family at two concentrations of 500 fmol and 100 fmol were added into suspensions of P7d-¹⁵⁹Tb functionalized

MMPs to perform DSN amplification assay.

Optimization of the Reaction Time.

The reaction time was optimized to be 1 h to achieve the highest signal/noise ratio (Fig. S4).

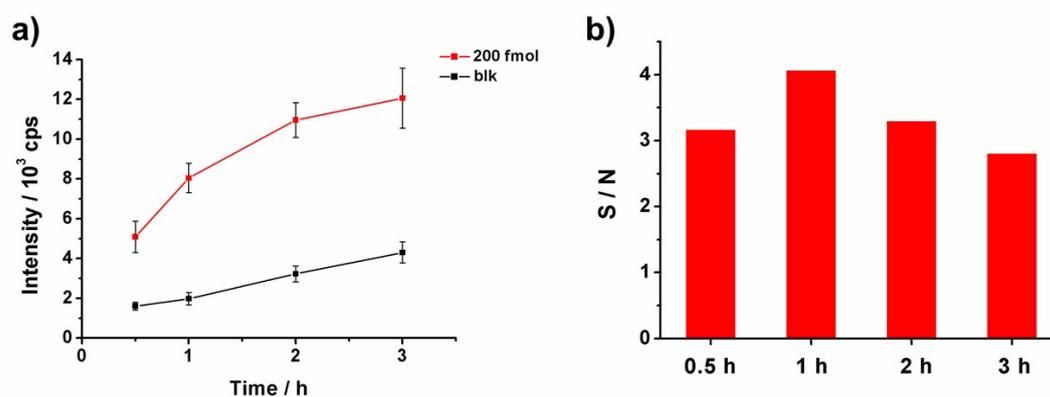


Figure S4. a) Comparison of the intensity of 200 fmol miR-141 and blank sample. b) The relationship between S/N ratio and reaction time.

Optimization of the Amount of DSN.

The amount of DSN was also optimized to be 0.1 U to achieve the highest signal/noise ratio (Fig. S5).

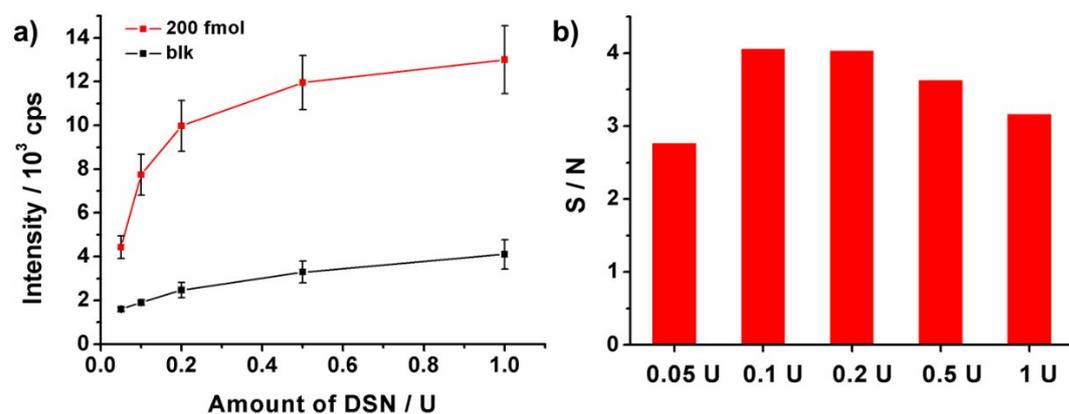
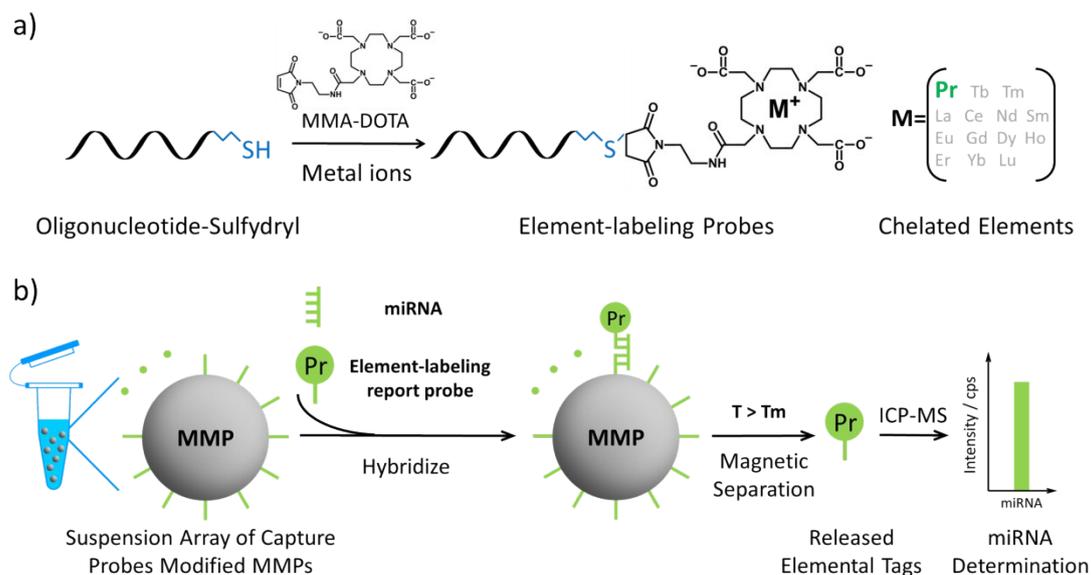


Figure S5. a) Comparison of the intensity of 200 fmol miR-141 and blank sample. b) The relationship between S/N ratio and the amount of DSN.

miRNA Sandwich Hybridization Assay



Scheme S2. a) Labeling oligonucleotide with lanthanide tag. The oligonucleotide with a $-\text{SH}$ group on 3'-end was conjugated with MMA-DOTA, and then rare earth elements were chelated in the macrocyclic DOTA. b) Schematic of sandwich hybridization assay. miRNA and lanthanide-tagged report probe were added to the capture-probe functionalized MMPs to form sandwich structure. After the hybridization procedure, by magnetic separation and raising temperature above the melting temperature (T_m), lanthanide-tagged report probe could be released to the supernatant and then quantified by ICP-MS.

For sandwich assay, a lanthanide-tagged report probe named P141-rep was designed for miR-141 detection. As can be seen in Scheme S2a, thiol-modified oligonucleotide was firstly conjugate with MMA-DOTA by thiol-maleimide chemistry. DOTA is a macrocyclic chelator which is thermodynamically stable and kinetically inert with lanthanide ions. Thus, DOTA-lanthanide is commonly used as tag for bioanalytical purposes³. Then, the MMA-DOTA tagged oligonucleotide was labeled with lanthanide and purified by HPLC. The identity of P141-rep was verified by MALDI-TOF-MS (Fig. S6).

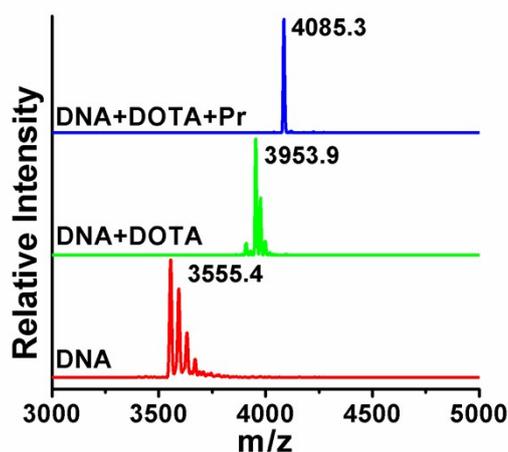


Figure S6. MALDI-TOF-MS results of synthesizing P141-rep probe.

The miRNA hybridization assay workflow is demonstrated in Scheme S2b. A biotinylated capture probe named P141-cap was used to immobilize on streptavidin-coated MMPs, while the lanthanide tagged report probe (P141-rep) labeled with ^{141}Pr was used for ICP-MS quantification. First, P141-cap was stabilized on streptavidin-coated MMPs. Then, ^{141}Pr tagged report probe (P141-rep) and target miR-141 were mixed with capture probe-modified MMPs. After the hybridization procedure, by raising the temperature over the melting temperature (T_m), P141-rep was released in the supernatant and ^{141}Pr was measured by ICP-MS.

Experimentally, 0, 0.1, 1, 2, 5, 10, and 20 pmol miR-141 were detected through sandwich hybridization assay. The intensities of ^{141}Pr signal and concentrations of miR-141 in sandwich hybridization assay showed good linear relationship, as shown in Fig. S7. The linear range was about 1–20 pmol of miR-141, while the limit of detection (LOD) is calculated to be 0.84 pmol by $3 \times \text{SD of blank/slope of calibration curve}$.

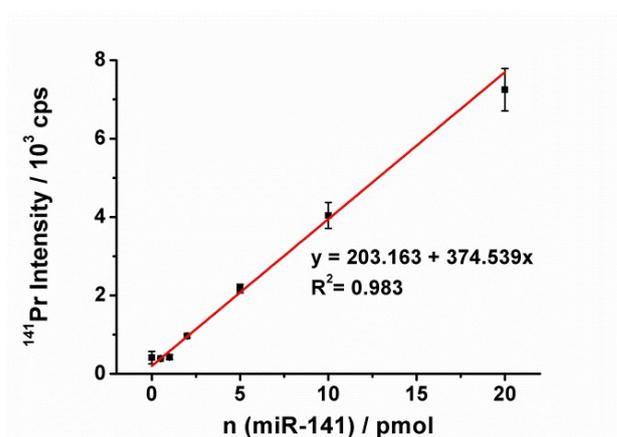


Figure S7. Relationship between the intensities of ¹⁴¹Pr and the concentrations of miR-141 in sandwich hybridization assay. Error bars represent the standard deviation of triplicates.

Since ICP-MS based miRNA assay using lanthanide tags was unaffected by sample matrix, we applied the proposed method to multiplex detection of miRNAs in complex biological matrix (cell lysate sample from cervical cancer cell line of HeLa). In HeLa cell lysates, no signal of the three miRNAs was observed because of the low expression levels of the three miRNA in HeLa cell⁴. Experimentally, we added 0.5 pmol of miR-141, let-7d, and miR-21 to HeLa cell lysates. ICP-MS results indicate that the detection of miR-141, let-7d, and miR-21 were unaffected by components in HeLa cell lysate (Fig. S8), thus the proposed method has the potential for application in real biological samples.

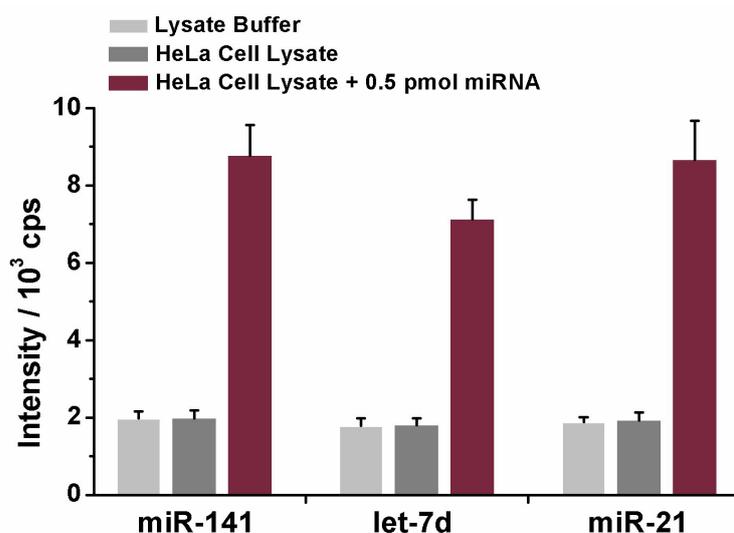


Figure S8. Biological feasibility of the method. miR-141, let-7d, and miR-21 of 0.5 pmol were added into HeLa cell lysates and detected by ICP-MS.

Discussions

The reaction steps of this method is simpler than multiplex miRNAs assay based on ligase chain reaction⁵, hybridization chain reaction⁶, and rolling circle amplification⁷. Since DSN amplification is a one-step method while the other three methods need an extra hybridization step.

The sensitivity of the method is comparable to multiplex miRNA detection using lanthanide-labeled DNA probes and laser ablation ICP-MS (femtomol range)⁸, but is higher than other fluorescence-based method⁴ and electrochemistry-based method⁹. The main limit for sensitivity of our method is the background signals which were considered to be originated from the non-specific interaction between MMPs and lanthanide tagged probes or the surface memory of the vessels and tubing.

The selectivity is comparable to the methods mentioned above. Although we didn't test the selectivity of single-base mismatch target, this method is capable of distinguishing miRNA from let-7 family.

The main advantage of this method is the potential high-level multiplex ability. Fluorescence-based methods were mostly applied for multiplex miRNA assay. But the spectral overlap restricts their application in high-level multiplex quantification¹⁰.

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