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Homogeneous Multiplexed Digital Detection of MicroRNA with Ligation-Rolling Circle Amplification

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Experimental

Materials and Instruments

Padlock DNAs, fluorescent oligo probes and microRNAs were purchased from GenScript (Nanjing, China) and Rui Biotech (Beijing, China). The sequences of those nucleic acid chains were listed in Table S1-S4. The microRNA and DNA were dissolved by diethyprocarbonated (DEPC)-treated deionized and purified by HPLC. And the DEPC-treated deionized water was bought from Invitrogen Co and used in all experiments. T4 RNA ligase 2, phi29 DNA polymerase and dNTPs mixtures were obtained from New England Biolabs (Ipswich, MA). As for gel electrophoresis, the agarose and 40% polyacrylamide gel electrophoresis (PAGE (37.5:1 Acrylamide-bisacrylamide)) were bought from Baygene Biotechnology Company (Shanghai, China). TAE (50X), TBE (10X), loading buffer, DNA ladders and the nucleic acid dye Goldview were obtained from BioDee Biotech.Co.Ltd (Beijing, China). The human embryonic liver normal cells (CCC-HEL-1), breast cancer cell lines (MCF-7), cervical cancer cell lines (Hela) and hepatocellular carcinoma cell lines (HepG-2) were purchased from National Infrastructure of Cell Line Resource (Beijing, China). And we got the serums of people from Chinese People's Liberation Army General Hospital.

The morphologies and sizes of RCA nanoflower ball were characterized by transmission electron microscope (TEM, Hitachi-7700, 100 kV), scanning electron microscope (SEM, Hitachi S-4800) and atomic force microscope (AFM, Bruker Multimode8). The real-time burst traces are monitored by nano flow cytometry (NanoFCM, Xiamen). The Nanoflower balls of targets were counted and quantified by flow cytometry (LSRFortessa, BD) (APC and FITC fluorescent channel). The picture of DNA agarose gel (3.0%) and Page gel (40%) was recorded by Ultra-Violet Products CCD camera (Bio-RAD ChemiDOCTM XRS+ imaging system). The excitation and emission spectra of FAM and Cy5 were detected by a Hitachi F-7000 fluorescence spectrometer.

L-RCA reaction

Preparation of DNA circle-templates. The ligation has been carried out by T4 RNA Ligase 2, which was better than traditional DNA ligase for microRNA detection. And the procedure was referred to some literatures[1-3]. We firstly prepared a solution containing 1× T4 RNA Ligase 2 buffer (50 mM Tris-HCl, 2 mM $MgCl_2$, 1 mM DTT, 400 μM ATP, pH 7.5), 10 U T4 RNA Ligase 2, 100 nM Padlock DNA and different amount of target miRNA in volume of 20 μL. For the ligation reaction with high efficiency, it was necessary that the target microRNA and padlock has been denatured in 5 minutes at 75 ^{o}C. After cooling down to the room temperature, the T4 RNA Ligase 2 and buffer were added into the tube and incubated for optimized time at 37 ^{o}C. The mixture was heated in 5 minutes at 75 ^{o}C for ending the reaction.

The procedure of RCA has followed to reported protocols[4-5]. The ligation products were quickly mixed with optimized phi29 DNA polymerase (10 U) and dNTPs (400 μM). And 5 μL 10× polymerase buffer (330 mM Tris-acetate (pH 7.9 at 37 °C), 100 mM Mg-acetate, 660 mM K-acetate, 1% (v/v) Tween 20, 10 mM DTT) has been added. The fluorescent DNA probes (200 nM) was injected into the mixture. After that, the DEPC-water was added until to 50 μL. Unless otherwise specified, the polymerization has been operated at 37 ^{o}C. The reaction was determined at 65 ^{o}C. As for the multiplexed detection, the fluorescent probes (200 nM P1 and P2, Table S3) were added into the solution after RCA reaction. Before the hybridization, heat step (65 °C, 10 min) needed to be applied. And the labeling procedure was handled in hybridization buffer (1× SSC, 0.1% Tween-20). In addition, a spiking test was carried out with serum samples and the procedure was be coincident with above.

Measurement of Fluorescent Cytometry

After the RCA reaction, a series of samples were gently shacked and resuspended by 1 mL DEPC-water respectively. The solution was loaded into cytometry tube and analyzed directly by flow cytometry (LSRFortessa, BD). The Alexa Fluor 488 and FAM dye were monitored by FITC channel (λ_{ex}: 488 nm, λ_{em}: 525 nm). And the Cy5 dye were monitored by APC channel (λ_{ex}:635 nm, λ_{em}: 675nm). Each sample was collected in same flow rate (180 μL/min) and the date was recorded for 2 minutes. We directly analyzed those date by flow cytometry software Flow Jo.

Agarose and PAGE gel electrophoresis

In this work, the products of rolling circle amplification were analyzed by 3.0% agarose gel electrophoresis. And the ligation products have been characterized by PAGE gel electrophoresis. Agarose gel electrophoresis was prepared in 1× TAE buffer at 120 V for 1 h and PAGE gel electrophoresis was prepared in 1× TBE buffer at 100 V for 2 h. As for synthesis of PAGE gel electrophoresis, 7.5 mL 40% (37.5:1 Acrylamide-bisacrylamide), 1.5 mL 10× TBE, 10 μL TEMED, 100 μL (100 mg/mL) $(NH_4)_2S_2O_8$ and 6 mL water were directly mixed together. After reacting 40 min, the target samples were loaded on gel. Finally, the results of gel electrophoresis were pictured through the Ultra-Violet Products CCD camera.

Characterization of nanoflower balls (NFBs)

To character the NFBs using SEM, TEM and AFM, the RCA products were deposited on silicone matrices. And then, the silicone matrices were dried in oven at 75 ºC for 1 h. The dried samples needed to be coated with Au in prior to SEM. In addition, the 10 μL RCA products of 4 h-8 h has been diluted into 1 mL DEPC-Water. The real-time burst traces are obtained by nano flow cytometry. And the time and SSC intensity has been recorded in 500 milliseconds.

Cell and RNA preparation

Four human normal and cancer cell lines including human embryonic liver normal cells (CCC-HEL-1), breast cancer cell lines (MCF-7), cervical cancer cell lines (Hela) and hepatocellular carcinoma cell lines (HepG-2) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C, 5% CO_2 inside an incubator (Thermo Scientific). Total RNA was extracted from each cell line using microRNA extraction kit (Takara Biotechnology Co., Ltd) according to the kit protocols. The total RNA was quantified by Nanodrop 2000c (Thermo Scientific). The RNA was reversed transcribed into cDNA by Revert Aid Reverse Transcriptase (Thermo Scientific). Afterwards, the Real-time PCR reaction was performed in QPCR instrument (ABI 7500). (The sequence of RT-primer: GCGCGTGAGCAGGCTGGAGAAATTAACCACGCGCCTACCT; The sequence of Forward primer: GGCAAAGTGCTTAC; The sequence of Reverse primer: GAGCAGGCTGGAGAA)

DNA sequences and modification

Table S1. The sequences of micro RNA

Name	Sequences	5'-Modified
Micro 141	UAACACUGUCUGGUAAAGAUGG	None
Micro 21	UAGCUUAUCAGACUGAUGUUGA	None
Let-7a	UGAGGUAGUAGGUUGUAUAGUU	None

Table S2. The sequences of Micro 21 Padlock DNA and fluorescent probe

Name	Sequences	5'-Modified
Model-Padlock	CTGATAAGCTATTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGAGTCTGCCTTTGCAT CCAGGTCTTTTTTTCAACATCAGT	PO_4^{3-}
Signal-AF488	TGAGTCTGCCTTTGCATCCAGGTC	Alexa Fluor 488

Table S3. The sequences of fluorescent DNA probes for multiplexed detection

Name	Sequences	5'-Modified	3'-Modified
P1	AAACCTTCCCACCTCCGTCCTTG	FAM	PO_4^{3-}
P2	AAATGCCGTCTCCCAATCTTCGA	Cy5	PO_4^{3-}

Table S4. The sequences of multiplexed Padlock DNAs

Name	Sequences	5'-Modified	GC
Micro 141-Padlock	AGACAGTGTTAtttCCTTCCCACCTCCGTCCTTGtttTGCC GTCTCCCAATCTTCGAtttCCATCTTTACC	PO_4^{3-}	46.5 %
Let-7a-Padlock	CTACTACCTCAtttCTCAATTCTGCTACTGTACTtttCCTTC CCACCTCCGTCCTTGtttAACTATACAAC	PO_4^{3-}	40.8 %
Micro 21-Padlock	CTGATAAGCTCtCTCAATTCTTTTACTGTACTtttttTGCC GTCTCCCAATCTTCGAtttTCAACATCAGT	PO_4^{3-}	39.4 %

Blue: sequence of fluorescent probe recognition

Red: sequence of microRNA recognition

Characterization of RCA nanoflower ball

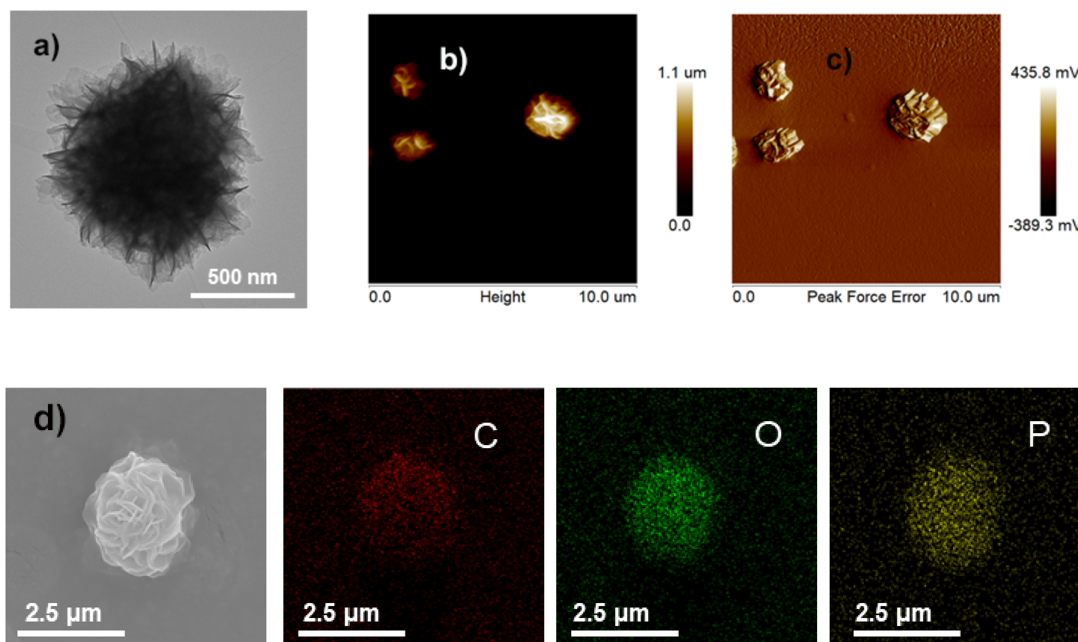

Figure S1 a) The TEM image of RCA nanoflower balls (NFBs) (scale bar 500 nm); b) and c) Characterization of RCA NFBs using AFM. And the AFM images display that monodisperse and micrometer NFBs. The height of NFBs is about 1.1 μm. d) is the SEM image of single RCA nanoflower ball along with the corresponding EDS element maps for C element, O element and element (scale bar 2.5 μm).

Autofluorescence and background fluorescence of RCA nanoflower ball

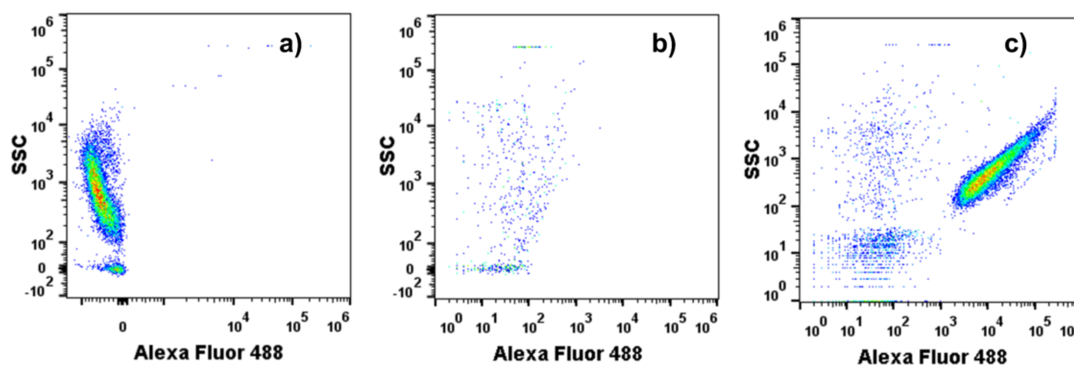

Figure S2 a) Flow cytometric profiles of the RCA products without signal-AF488 DNA probe. b) The ligation of padlock DNA is lack of the target micro RNA-21 and there have rare particles in flow cytometry diagram after rolling circle amplification. c) Flow cytometric profiles of the RCA products with both Signal-AF488 DNA probe and microRNA-21.

Agarose gel electrophoresis (3.0%) for time optimization

Figure S3 Agarose gel electrophoresis (3.0%) of RCA products synthesized by different RCA reacting times using linear padlock probe (Model-padlock, table S2). Ladder：100 bp – 10 kbp Lane 1~8: 1h, 2h, 4h, 6h, 8h, 12h, 16h, 24h. RCA amplicons are already reaching very large size within 1h (the RCA products are with a length of over 10 kb).

TEM characterization for time optimization

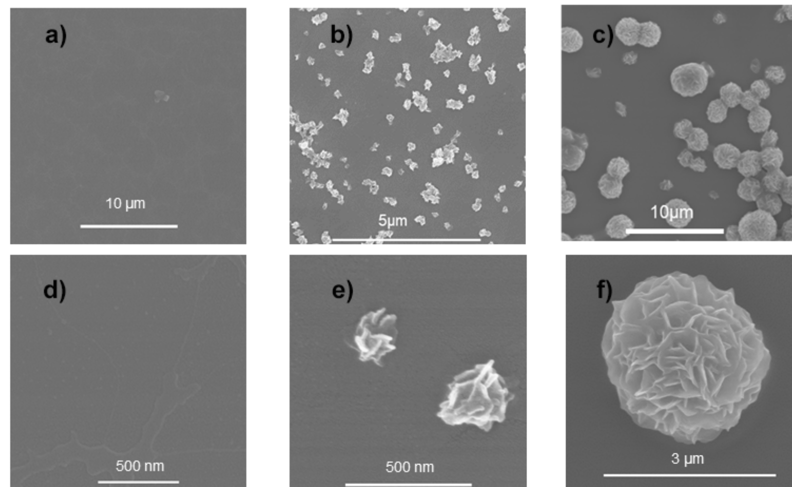

Figure S4 SEM imaging of the morphology and size of NFBs during the growth process with reaction time 0.5 h, 4 h and 12 h. a) RCA products couldn't be observed at 30 minutes. b) Nano level small particles emerge from silicone matrices at 4 hours and the size of those nano particle is about 200 nm. It is obviously that some rumple occurs on the RCA products, like flower balls. b) the particle grows great after RCA reaction 12 hours. And the size of nanoflowers is about 3 µm which is like bacteria and suitable for flow cytometry detection.

dNTPs concentration optimization

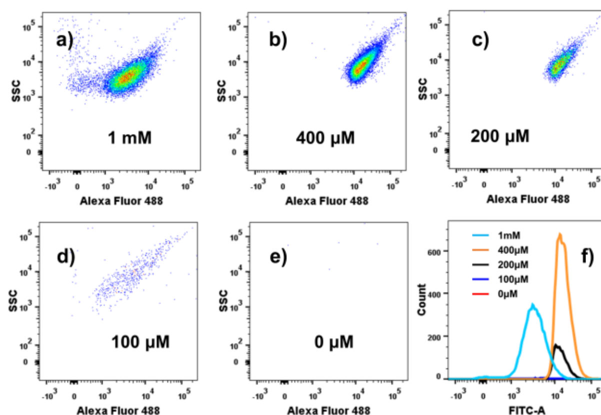

Figure S5 The flow cytometric profiles of dNTPs concentration optimization. The concentration of dNTPs in a) ~ e) was 1 mM, 400 μM, 200 μM, 100 μM and 0 μM. f) Compiled fluorescent intensity distribution histograms for individual samples of five concentrations of dNTPs measured separately by flow cytometry.

As the concentration of dNTPs increased, the number, size and fluorescent intensity of NFBs increased. Because the number of fluorescent probes in the solution was determined. As shown in Figure S5f, when the concentration of dNTPs was higher than 0.4 mM, excessive RCA products would introduce a decrease in the number of fluorescent probes on each NFBs. So that the average fluorescence intensity of Alexa Fluor 488 on NFBs reduced. The 0.4 mM of dNTPs was optimal.

phi 29 DNA polymerase optimization

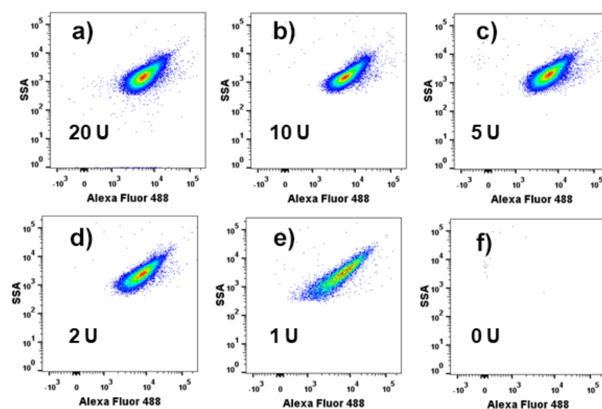

Figure S6 The flow cytometric profiles of amount of phi29 DNA polymerase optimization. The number of dNTPs in a) ~ e) is 20 U, 10 U, 5 U, 2 U, 1 U and 0 U in 50 μL.

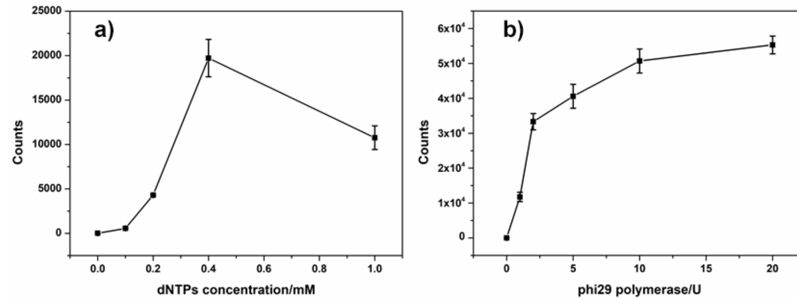

Figure S7 The relationship between the counts of RCPs and the concentration of dNTPs a) and phi29 DNA polymerase b). The experimental concentration of micro 21 RNA was 4 nM. The RCA reactions were performed with 100 nM padlock probe in a reaction volume of 50 μL at 37 ^{o}C. Error bars represent the SD (standard deviation) of three replicates experimental.

In Figure S5-S7, the influence of dNTPs concentration was complicated. When the dNTPs concentration was higher than 0.4 mM, the RCA reaction products were difficult to be detected in FCM. This was perhaps excessive products would motivate the aggregation of monodisperse particles forming DNA gels in the solution [6]. The number of NFBs decreased, and the flow cytometry instrument was easily blocked. Therefore, 0.4 mM was recognized as the optimal concentration of dNTPs for following detection. Furthermore, the Phi29 DNA polymerase was another important role which affected the amplification reaction. It was clear that when the polymerase increased until 10 U in the mixture, the number of particles reached the platform in flow cytometric profile. In order to optimal detection sensitivity, the 10 U of DNA polymerase was selected.

SNP (single nucleotide polymorphisms) detection and Recovery

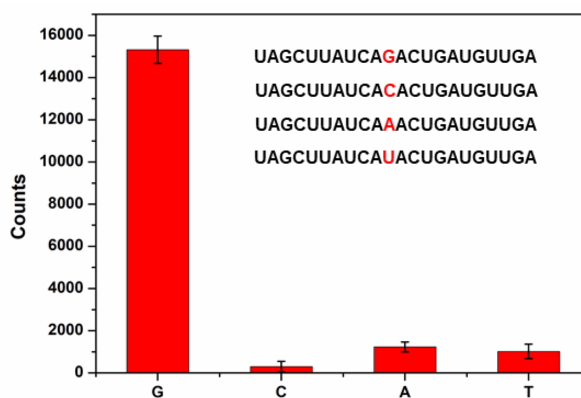

Figure S8 Single nucleotide polymorphism detection of the proposed method for micro RNA 21.

Table S5. Recoveries of microRNA-21 in human serums (concentration mean ± S.D., n=3)

Added (pM)	Found (pM)	Recovery (%)
50	51.2±2.0	102.2
100	93.1±5.3	93.2
200	185.3±6.9	92.7
400	415.3±6.5	104.3

Emission spectrometry of Cy5 probe and FAM probe

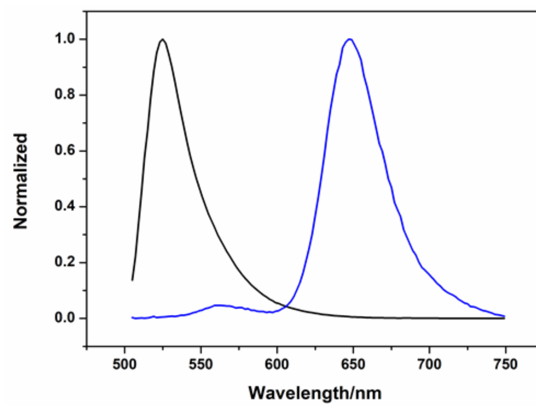

Figure S9 The emission spectra of Cy5 (P2 probe, Table S3) and FAM (P1 probe, Table S3). The mix emission wavelength of FAM is 525 nm and the mix emission wavelength of Cy5 is 660 nm. There are almost no spectra overlapping of two fluorescent DNA probe.

Padlock DNA probe encoding strategy

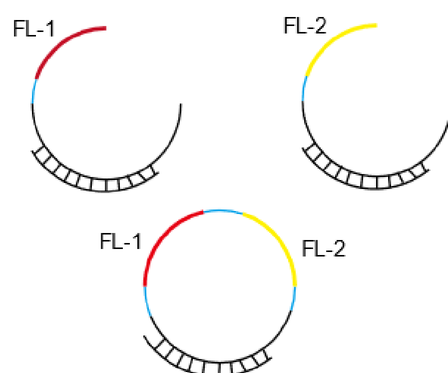

Figure S10 Multiplexed microRNAs detection based on the dFC-LRCA strategy. The scheme displays the multiplexed padlock DNA probes. One-color encoding padlock DNA (FAM or Cy5) and two-color encoding padlock DNA (FAM and Cy5).

DNA secondary structure of multiplexed padlock DNA

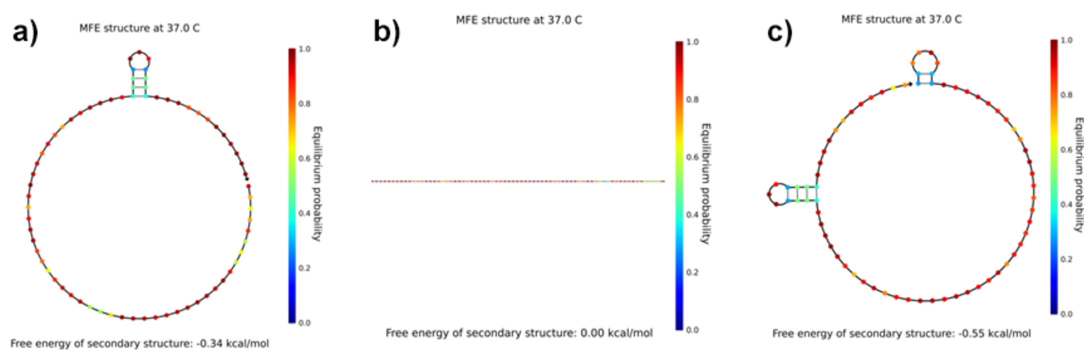

Figure S11 The secondary structure of multiplex padlock probes for one color encoding (a) and b)) and two color encoding (c)). The picture a), b) and c) is Micro 141-Padlock, Let-7a-Padlock and Micro 21-Padlock, respectively. The structure has been predicted by NUPACK [4]. The free energy of three padlock DNAs is recorded on bottom of the picture. These images indicate the padlock DNAs for multiplex detection have no or minor secondary structure, which is benefit for rolling circle amplification.

Micro 141, Let-7a RCA products Characterization

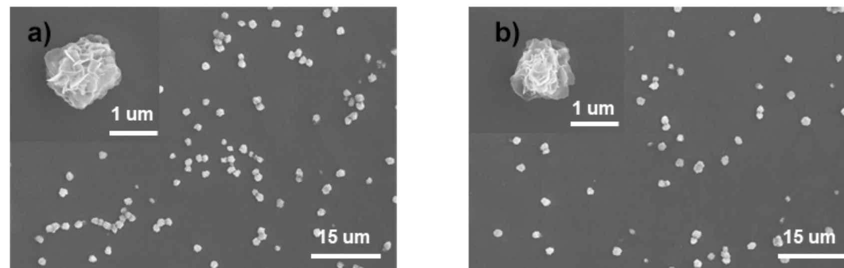

Figure S12 SEM image of RCA products introduced by microRNA 141 (a)) and Let-7a (b)).

Quantitative of Multiplex microRNA

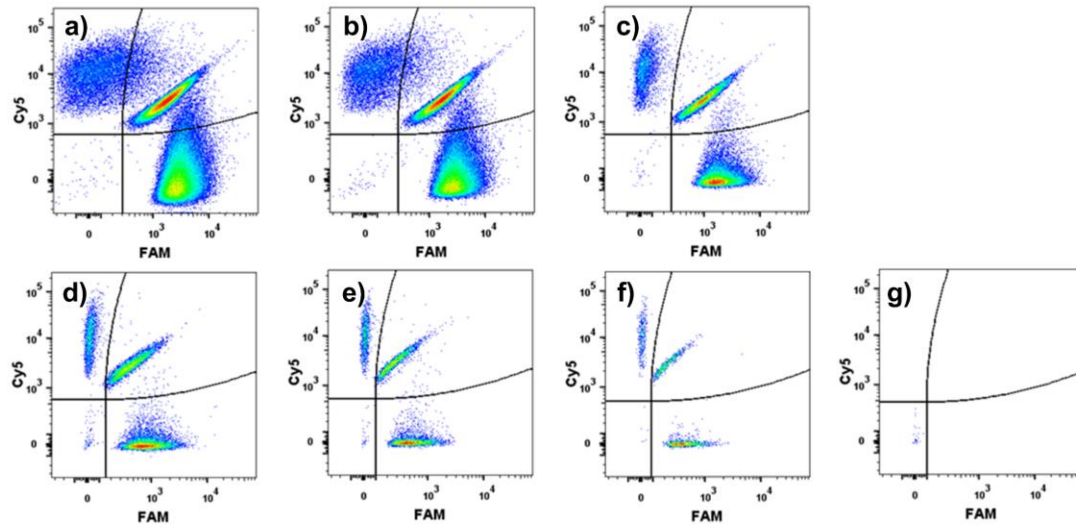

Figure S13 The flow cytometric profiles of multiplex microRNA detection. The concentration of microRNAs is 1400 pM, 1000 pM, 400 pM, 200 pM, 100 pM, 40 pM and 0 pM (a) ~ g)).

Real sample detection

Table S6. RT-PCR reagents

Reagents name	Reagents dosage
SYBR Green I mix (2X) (TransGen Biotech, AQ601-24)	5ul
Primer-F(10uM)	0.3ul
Primer-R(10uM)	0.3ul
cDNA	0.2ul
ddH_2O	Up to 10ul

Table S7. RT-PCR reaction program designing

Temperature	Time	procedure
95℃	10 min	
95℃	5 s	Cycle 40 times
60℃	15s	
72℃	10s	
4℃	storage	

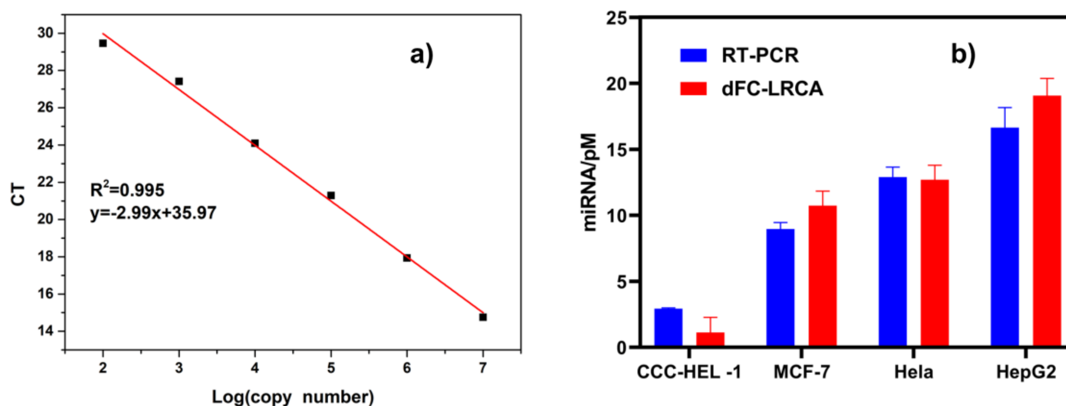

Figure S14 a) Standard curve of quantitative reverse transcription PCR (qRT-PCR); b) Detection of microRNA-21 levels in each cell (CCC-HEL-1, MCF-7, Hela, HepG2) by the proposed RCA method and RT-PCR.

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