Supporting Information

Amplification-Free and Mix-and-Read Analysis of Multiplexed MicroRNAs on a Single Plasmonic Microbead

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1. Experimental section

Materials and reagents S9.6 antibody (mouse anti-DNA/RNA hybrid antibody), $HAuCl_4 \cdot 3H_2O$ (≥99.9 %), bovine serum albumin (BSA), 4-mercaptobenzonitrile (4-MBN), 2-naphthalenethiol (2-NT), 4-nitrothiophenol (4-NTP) were purchased from Sigma-Aldrich. Trisodium citrate, Tween-20, sodium borate decahydrate, 20× PBS solution (pH=7.4), NaBr and 20×PB solution (200 mM, pH=7.0) were obtained from Sangon Biotech (Shanghai, China). The Streptavidin-functionalized magnetic microbeads (SA-microbeads, 90 ± 2 μm in diameter) were purchased from GE Healthcare (Uppsala, Sweden). Dynabeads® M-270 (carboxyl microbeads, 2.8 μm in diameter) were purchased from Thermo Fisher Scientific (Invitrogen, Oslo, Norway). The DNA oligonucleotides used in this study were synthesized and purified by Sangon Biotech (Shanghai, China). The RNA sequences were obtained from Takara Biotechnology (Dalian, China). The HepG2 cell lines were purchased from the cell bank of Chinese Academy of Sciences (Shanghai, China). All of the other reagents used in this work were of analytical grade and used as received without further purification. The solutions used in this study were prepared by RNase free water.

The detailed components of the used buffers in this study are listed below:

1× PBS (10 mM, pH 7.4, containing 137 mM NaCl and 2.7 mM KCl)

1× PBST (1× PBS with 0.1% Tween-20)

1× borate buffer solution (BBS, 2 mM, pH 9.2)

Table S1 The sequences used in the work

Name of the nucleic acid	sequence (from 5' to 3')
miRNA-21	UAGCUUAUCAGACUGAUGUUGA
miRNA-24	UGGCUCAGUUCAGCAGGAACAG
miRNA-122	UGGAGUGUGACAAUGGUGUUUG
$P_{miRNA-21}$	SH-TTTTTTTTTTTCAACATCAGTCTGATAAGCTA
$P_{miRNA-24}$	SH-TTTTTTTTTTCTGTTCCTGCTGAACTGAGCCA
$P_{miRNA-122}$	SH-TTTTTTTTTTCAAACACCATTGTCACACTCCA
dilution DNA	SH-TTTTTTTTTTTTTTTTTTT

biotin-DNA linker	Biotin-TTTTTTTTTTTTTTTTTTTTTTTTT-SH

The synthesis of GNPs GNPs were prepared according to the previous literatures of Frens et al.[S1] Generally, all of the glassware were soak in chromic acid lotion for at least 24 hours, then rinsed with abundant Millipore-filtered water, and oven-dried prior to use. Afterwards, 100 mL 0.01% $HAuCl_4$ solution was injected into the three-necked round-bottomed flask with a condenser. Then it was heated with magnetic stirring. Once the aqueous solution began to reflux, 1 mL of 1% sodium citrate was quickly added. The color should change from pale yellow to red in 1 min. After keep heating for another 15 min, the solution will cool down to room temperature slowly. The solution was filtered by a 0.22 μm nitrocellulose membrane and then kept at 4 °C for long-term storage, and the particle molar concentration of the as-prepared colloidal GNPs was about 78 pM indicated by NTA (ZetaView Nanoparticle tracking analyzer, Particle Matrix). The as-prepared GNPs were also characterized by transmittance electron microscopy (TEM, Tecnai G2 F20, FEI), which shows that the GNPs are uniform with an average size of ~40 nm (Figure S1).

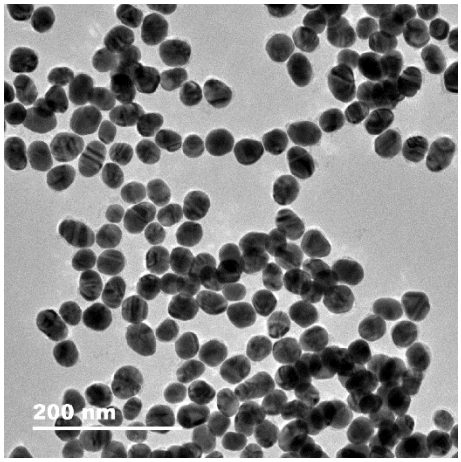

Figure S1. The TEM image of the as-synthesized GNPs.

Preparation of the plasmonic layer GNPs and SERS reporter GNPs 4 μg S9.6 antibody was incubated with 1 mL GNPs at room temperature for 12 hours in a total 1200 μL borate buffer solution (BBS, 2 mM, pH 9.2). Then 20 μL of 50 μM biotin-DNA linker was mixed with the S9.6 antibody modified GNPs for another 12 hours at ambient temperature. After that, 15 μL of 5% Tween-20, 75 μL of 200 mM

PB buffer (pH 7.0) and 190 μL water were added into the above aqueous system and kept at room temperature for 12 hours. Sequentially, 200 μL of 3 M NaBr solution and 300 μL water were slowly added. 12 hours later, 40 μL 10% BSA was added to the system to block the empty sites of GNPs. After another 12 hours, the aqueous solution was centrifuged at 6000 rpm for 15 min and the precipitate was washed with 1× PBST buffer twice. Finally, the prepared plasmonic layer GNPs were dispersed in 100 μL 1× PBST buffer and kept at 4 °C for the future use.

With regard to the SERS reporter GNPs for miRNA-21 analysis, 1 mL GNPs was incubated with 10 μL 10 μM $P_{miRNA-21}$ and 20 μL 50 μM dilution DNA at ambient temperature for 12 hours. After that, 15 μL of 5% Tween-20, 75 μL of 200 mM PB buffer (pH 7.0) and 380 μL water were added into the above aqueous system and kept at room temperature for 12 hours. Sequentially, 200 μL of 3 M NaBr solution and 300 μL water were slowly added. 12 hours later, 50 μL of 3 mM 4-MBN ethanol solution (20%) was gradually added into the above aqueous GNPs. After 12 hours, 40 μL of 10% BSA aqueous solution was used to seal the vacancy on the GNPs. After standing for another 12 hours the reaction solution was centrifuged at 6000 rpm for 15 min and the precipitate was washed with 1× PBST buffer for two times. Finally, the SERS reporter GNPs were dispersed in 100 μL 1× PBST buffer and kept at 4 °C for further use.

The SERS reporter GNPs for miRNA-24 and miRNA-122 analysis were prepared in the same protocol except that the DNA probes and Raman reporter molecules are replaced accordingly. Specifically, $P_{miRNA-24}$ and 4-NTP were used to functionalize the SERS tag GNPs for miRNA-24, and $P_{miRNA-122}$ and 2-NT were used to functionalize the SERS tags for miRNA-122.

Standard protocol for miRNA analysis Firstly, target miRNAs hybridized with the P_{miRNA} on the SERS reporter GNPs. In detail, 1 μL SERS reporter GNPs and target miRNA with different concentrations were thoroughly mixed in a total 10 μL 1 × PBST solution and reacted for an hour at room temperature. Then, a plasmonic microbead was manipulated as illustrated in our previous studies.[S2–S5] Briefly, after

thoroughly blending, 1 μL slurry of the microbeads were pipetted into a 200 μL centrifuge tube, then washed twice with 1× PBST buffer via magnetic isolation and resuspended in 200 μL 1× PBST buffer for the subsequent using. Then, a Narishige micromanipulator system equipped on an Olympus IX53 inverted microscope with a monitor was applied to capture and manipulate the single microbead. For each sample, in a total 10 μL reaction system, a single microbead was reacted with 1 μL of the as-prepared plasmonic layer GNPs in 1× PBST buffer at room temperature for 60 min under mild shaking. After that, these single microbeads were carefully washed twice with 1× PBST buffer. Subsequently, the reaction solution of SERS reporter GNPs and target miRNA were added into the tube with the obtained single plasmonic microbead for another two hours at ambient temperature under shaking. Finally, the single microbeads of each reaction were washed twice with 1× PBST buffer and taken for SERS imaging.

Multiplexed miRNA analysis For the multiplexed assay of various kinds of miRNA targets, a SERS reporter GNP pool was prepared. Specifically, the 4-MBN functionalized GNPs are designated as miRNA-21-specific SERS reporters, the 2-NT ones are specific for miRNA-122, and the 4-NTP ones are assigned to miRNA-24. Firstly, 1 μL of each SERS reporter GNPs and target miRNAs with different concentrations were thoroughly mixed in a total 10 μL 1 × PBST solution and reacted for an hour at room temperature. Then, a single plasmonic microbead was further introduced and incubated for another two hours at ambient temperature under shaking. Finally, these single microbeads were washed twice with 1× PBST buffer.

SERS signal mapping An XploRA PLUS (Horiba) confocal Raman microscope with the LabSpec6 software was employed to obtain the SERS mapping images of the individual microbead dropped on a piece of glass slide and analyze the data. For the measurements, 638 nm/2.43 mW excitation laser, 1200 gr/mm optical grating, and 50× long-working-distance lens (NA = 0.5, WD = 10.6 mm) were selected. Besides, the hole and slit were both set as 100 μm. SWIFT mapping technology with EMCCD were used in the process of SERS mapping. Particularly, the single spot acquisition

time was 0.05 s, the degree of EM gain was 800 and the step was 2 μm at both X and Y directions. With the help of SWIFT mapping technology and EMCCD, it takes about 3 min for mapping a single microbead and the SERS mapping image can be obtained simultaneously. All the SERS mapping images and the averaged Raman spectra were obtained after baseline correction of the original spectra by using the Labspec6 software. In detail, the SERS images in this work were acquired by confocal laser scanning of the whole equatorial plane of each microbead, and the SERS spectra that reflect the spatially averaged SERS signal (per pixel) across the scanned region were processed by the Labspec6 software.

Single-point based SERS measurements on ensemble microbeads Firstly, target miRNA-21 hybridized with the $P_{miRNA\text{-}21}$ on the SERS reporter GNPs. In detail, 1 μL SERS reporter GNPs and 1 μL target miRNA-21 were thoroughly mixed in a total 10 μL 1 × PBST solution and reacted for an hour at room temperature.

Then, in a total 10 μL reaction system, 6×10^4 of 2.8 μm microbeads was reacted with 1 μL of the as-prepared plasmonic layer GNPs in 1× PBST buffer at room temperature for 60 min under mild shaking. After that, these 2.8 μm microbeads were carefully washed twice with 1× PBST buffer. Subsequently, the solution of miRNA-21-loaded SERS reporter GNPs were mixed with the microbeads for another two hours at ambient temperature under shaking. Finally, these 2.8 μm microbeads were washed twice with 1× PBST buffer, magnetically isolated and then measured by a confocal Raman microscope.

Cell culture and exosome isolation The HepG2 cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM, Life). All the media were supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C under a humidified atmosphere containing 5% CO_2 for 4 days. Then, the cells were washed twice with 1 × PBS buffer and cultured in the vesicle-depleted medium which is 3.5 mL DMEM medium containing 1% penicillin-streptomycin at 37 °C under a humidified atmosphere containing 5% CO_2 for 48 hours. To isolate exosomes derived from cell lines, the cell culture media were

collected.

The collected media was processed by membrane filtration (0.22 μm, Millipore, USA). Then the exosome isolation was conducted according to the procedures of exoEasy Maxi Kit (QIAGEN, Germany) and the concentration of the obtained exosomes was quantified by NTA.

Total small RNA extraction Total small RNA samples were extracted from the obtained exosomes by using RNAiso for Small RNA Kit (Takara, China) according to the manufacturer's instructions.

Reverse transcription reaction The reverse transcription reaction was carried out in the mixture with 1 μL target miRNA (or total small RNA sample), 1.2 μL of RNase-free water, 1 μL of 5 × RT buffer (50 mM Tris-HCl, 75 mM KCl, 3 mM $MgCl_2$), 1 μL of 2.5 mM dNTPs, 0.2 μL of 200 U/μL ProtoScrip II reverse transcriptase, 0.5 μL of 1 μM stem-loop RT-Probe (see detailed sequence in Table S2), and 0.1 μL of 40 U/μL RNase inhibitor. The 5 μL mixture was treated with following conditions: 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C and then held at 4 °C.

Table S2 Nucleic acid sequences used in stem-loop RT-PCR method

name of the nucleic acid	sequence（from 5' to 3'）
stem-loop RT-Probe	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGT TGAGTCAACATC
forward primer	ACACTCCAGCTGGGTAGCTTATCAGACT
reverse primer	CTCAACTGGTGTCGTGGAGT

Quantitative real-time PCR analysis 5 μL transcription product was added into the PCR reaction mixture with a final volume of 10 μL. The PCR reaction mixture consists of 200 nM forward primer and 200 nM reverse primer, 250 μM dNTPs, 0.4 × SYBR Green I, 0.5 U JumpStartTM Taq DNA Polymerase, and 1 × PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, and 0.001(w/v) gelatin, pH 8.3). The 10 μL PCR reaction mixture was incubated in a StepOne Real-Time PCR System (Applied Biosystems, USA) according to the following thermal cycling conditions: hot start at 94 °C for 2 min, followed by 50 cycles of 94 °C for 15 s, 60 °C for 1 min,

and 72 °C for 20 s.

2. Verification of the specific binding between DNA/miRNA hybrid and S9.6 antibody

As a supplement of the results in Figure 2, to more clearly prove the specificity of S9.6 antibody, they were directly modified on the surface of carboxyl-microbeads (DynaBeads) without the coating of plasmonic GNP layer. As shown in Figure S2a, under SEM the microbead has a smooth surface after S9.6 antibody functionalization. After adding miRNA-21, the SERS reporter GNPs are captured on the microbead's surface (Figure S2b) in a high density. Nevertheless, negligible SERS reporter GNPs can be captured on the microbead's surface if non-target miRNA molecules (let-7a) are added (Figure S2c).

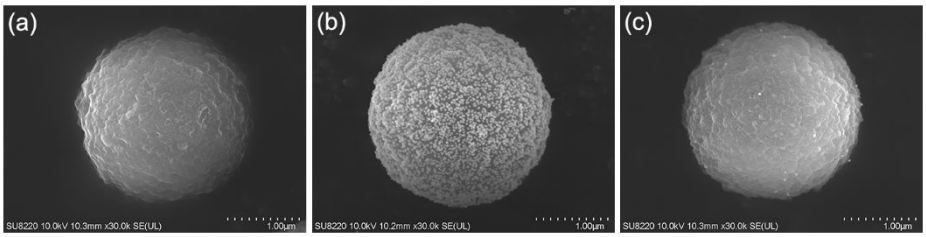

Figure S2. SEM images of the microbeads for the verification of the specific conjugation of DNA/miRNA duplexes and S9.6 antibody. The S9.6 antibody molecules were directly modified on the microbeads (a). After adding target miRNA (miRNA-21), the corresponding SERS reporter GNPs can be captured onto the microbead (b). If the non-target miRNA let-7a is added, no SERS reporter GNPs can be captured (c).

3. Optimization of the experimental conditions

As the amount of S9.6 antibodies and $P_{miRNA\text{-}21}$ on the GNPs as well as the temperature for binding will impose an obvious influence on the performance of the proposed strategy for miRNA-21 analysis. Therefore, we have optimized the dosage of S9.6 antibodies functionalized on the plasmonic layer GNPs, the ratio of $P_{miRNA\text{-}21}$ and dilution DNA on the SERS reporter GNPs, as well as the temperature for the binding of S9.6 antibody and DNA/miRNA hybrid. In the first place, the amount of S9.6 antibody was optimized by conducting the standard assay protocol for detecting miRNA-21 (fixed at 50 pM) and tracking the SERS signal at 2225 cm^{-1}. In this

experiment, the ratio of $P_{miRNA-21}$ and dilution DNA was 1:10 while the binding temperature is 25 °C. As can be seen from Figure S3a, without adding miRNA-21 in the reaction system, the blank control signal (gray bars) kept stable when tuning the amount of S9.6 antibody. While when 4 μg of S9.6 antibody was used, the SERS signal induced by the 50 pM miRNA-21 reached the maximum value.

Afterward, the ratio of $P_{miRNA-21}$ and dilution DNA was optimized by fixing the amount of S9.6 antibody at 4 μg and the binding temperature at 25°C. As Figure S3b shows, the ratio of $P_{miRNA-21}$ and dilution DNA did not show obvious influence on the blank control groups (gray bars). The strongest SERS intensity aroused by 50 pM miRNA-21 was obtained when the ratio of $P_{miRNA-21}$ and dilution DNA was around 1:10.

Then, the temperature for binding was optimized by fixing the amount of S9.6 antibody at 4 μg, and the ratio of $P_{miRNA-21}$ and dilution DNA at 1:10. As Figure S3c shows, the temperature for binding did not show any evident influence on the blank control groups (gray bars). The strongest SERS intensity aroused by 50 pM miRNA-21 was obtained when the binding temperature is 25°C. Therefore, for the best assay performance, the S9.6 antibody dosage, and the ratio of $P_{miRNA-21}$/dilution DNA were selected as 4 μg and 1:10 respectively, and the temperature for binding was selected as 25°C.

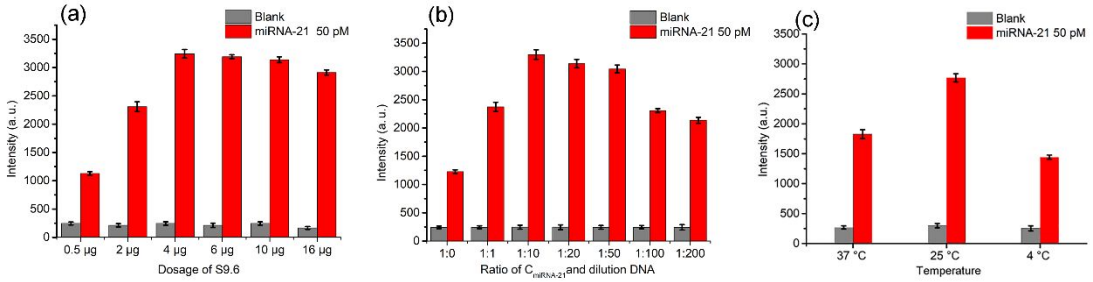

Figure S3. Optimization of the experimental conditions. (a) Effect of the dosage of S9.6 antibody for the proposed miRNA assay. Other conditions: the ratio of $P_{miRNA-21}$ and dilution DNA is 1:10, the binding temperature is 25 °C. (b) Optimization of the ratio of $P_{miRNA-21}$ and dilution DNA. Other conditions: the dosage of S9.6 antibody is 4 μg and the binding temperature is 25 °C. (c) Optimization of the temperature for DNA/miRNA duplex-S9.6 antibody binding. Other conditions: the dosage of S9.6 antibody is 4 μg and the ratio of $P_{miRNA-21}$ and dilution DNA is 1:10. The intensity refers to the Raman peak at 2225 cm^{-1}. The error bars represent the standard deviation

of three measurements.

4. The generality of the single microbead-based SERS mapping strategy

By simply altering the combination of the ssDNA probes and the SERS reporter molecules on the SERS reporter GNPs, the proposed method can be generally employed to the quantitative analysis of a library of miRNAs. For example, the SERS reporter GNPs for miRNA-24 were co-functionalized with 4-NTP and $P_{miRNA\text{-}24}$ while the plasmonic layer GNPs remain unchanged. The detailed assay processes were the same as those for miRNA-21 analysis without any adjustment. As shown in Figure S4a, the per-pixel mean SERS intensity exhibits an increasing trend with the concentration of miRNA-24 altering from 0.1 pM to 100 pM. The corresponding relationship between the miRNA-24 concentration and the mean SERS intensity at 1334 cm^{-1} (the characteristic peak of 4-NTP) is lg (ΔI) = 0.39 lg $C_{miRNA\text{-}24}$ (pM) + 2.78 with a correlation coefficient of R^2 = 0.9960 (Figure S4b). The SERS mapping images of the single microbead with each concentration gradient are inserted in Figure S4b.

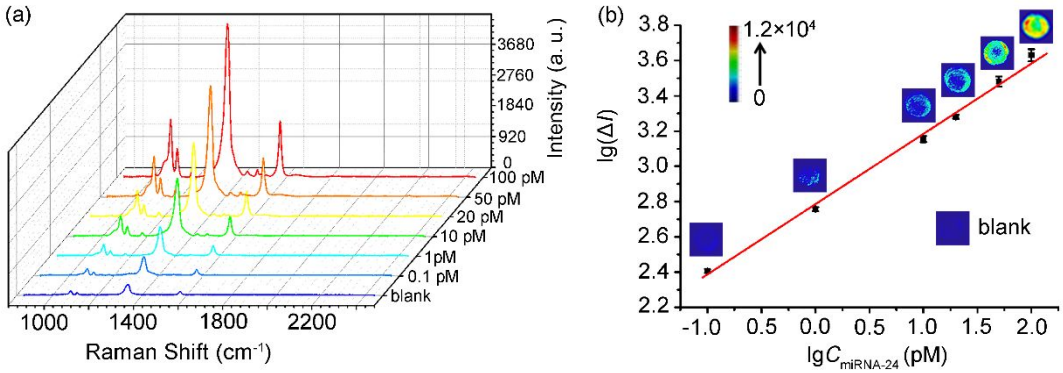

Figure S4. (a) The averaged spectra of the SERS signals on the individual single plasmonic microbead treated with different concentrations of miRNA-24. From near to far: 0 (blank control), 0.1 pM, 1 pM, 10 pM, 20 pM, 50 pM, and 100 pM. (b) The corresponding relationship between the miRNA-24 dosage and the per-pixel mean SERS intensity at 1334 cm^{-1}. The SERS mapping images with each miRNA-24 concentration gradient are displayed.

5. Comparison of stem-loop RT-PCR and the proposed single microbead-based SERS mapping strategy

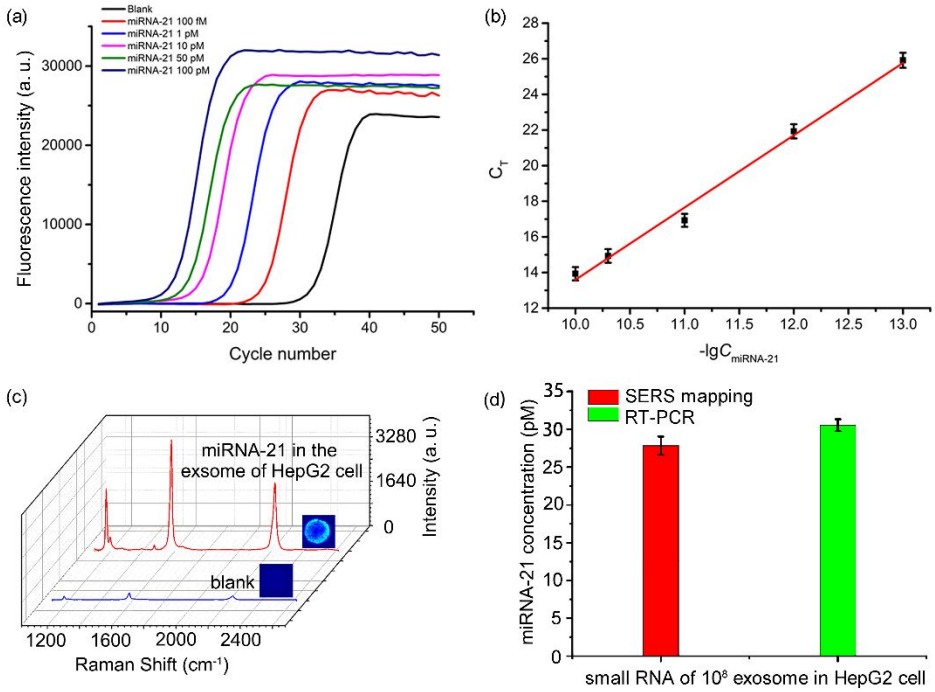

Figure S5. (a) Real-time fluorescence curves for the detection of miRNA-21 by the stem-loop RT-PCR. From right to left, the concentration of the miRNA-21 (synthetic standard) is 0, 0.1 pM, 1 pM, 10 pM, 50 pM, and 100 pM, respectively. (b) Standard calibration curve of the stem-loop RT-PCR protocol for the detection of miRNA-21. The error bar was obtained from triplicate measurements. (c) The assay result of miRNA-21 in 10^8 exosome of liver cancer cell HepG2 by using the single microbead-based SERS mapping strategy. (d) The comparison of the determined amount of miRNA-21 in the same batch of real sample by using the stem-loop RT-PCR protocol and the proposed single microbead-based SERS mapping strategy.

The stem-loop reverse-transcription PCR (RT-PCR) protocol is inherited from literatures with some modifications,[S6, S7] which consists of two processes, namely, reverse transcription (RT) and real-time PCR. For the first process, the stem-loop RT-probe is hybridized to the target miRNA, and then reversely transcribed. Then the reverse transcription products are amplified and detected by fluorescence quantitative real-time fluorescence PCR using SYBR Green I as the signal reporter.

The real-time fluorescence spectra and the standard calibration curve established by the RT-PCR method (using synthetic miRNA as the standard) are shown in Figure S5a and b, respectively. The miRNA-21 level in small RNA sample in 10^8 HepG2 exosome was evaluated by the proposed single microbead-based SERS mapping strategy, and the results are shown in Figure S5c. The comparison of the exosomal

miRNA-21 detection results by using the two methods is shown in Figure S5d.

Reference:

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