Supporting information

Molecular beacon-peptide probe based double recycling amplification for multiplexed detection of serum exosomal microRNAs

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Table 1S. The nucleotide sequence used in this study.

Name	Sequence
miR-21	UAGCUUAUCAGACUGAUGUUGA
SM miR-21	UAGCUUAUCAAACUGAUGUUGA
DM miR-21	UAGCUUAUCATCCUGAUGUUGA
miR-191	CAACGGAAUCCCAAAAGCAGCUG
SM miR-191	CAACGGAAUCCCGAAAGCAGCUG
DM miR-191	CAACGGAAUCCAGAAAGCAGCUG
miR-451a	AAACCGUUACCAUUUACUGAGUU
SM miR-451a	AAACCGUUACCAGUUACUGAGUU
DM miR-451a	AAACCGUUACCCGGUUACUGAGUU
Random RNA	CGAUUAGCGGUAUCGGUACUAA
Primer	*T*CTTGGAC
Probe 21	*A*AGCTGAGG/iDBCOdT/CTTGGACA TCAACATCAGTCTGATAAGCTATGTCCAAGA-Biotin
FAM-probe-21	*A*AGCTGAGG/FAMdT/CTTGGACA TCAACATCAGTCTGATAAGCTATGTCCAAGA-Dabcyl
Probe-191	*A*AGCTGAGG/iDBCOdT/CTTGGACA CAGCTGCTTTTGGGATTCCGTTGTGTCCAAGA-Biotin
Probe-451a	*A*AGCTGAGG/iDBCOdT/CTTGGACA AACTCAGTAATGGTAACGGTTT-TGTCCAAGA-Biotin

SM: single-base mismatched RNA, DM: double-base mismatched RNA, the mismatched positions are underlined, * Bases are Phosphorothioates.

Table S2. Accuracy and precision for QC samples.

Target	Nominal concentration	0.100 fM	0.300 fM	50.0 fM	8.00 pM
miR-21	Mean	0.0921	0.312	56.4	8.14
	%Bias	-7.86	4.34	12.8	1.76
	Intra-day Precision (%CV)	13.2	9.28	7.73	8.16
	Inter-day Precision (%CV)	14.3	10.7	6.35	9.68
miR-191	Mean	0.112	0.321	54.21	8.27
	%Bias	12.3	7.36	8.42	3.38
	Intra-day Precision (%CV)	14.3	8.21	7.56	9.14
	Inter-day Precision (%CV)	12.9	12.5	6.32	7.28
miR-451a	Mean	0.109	0.335	48.87	7.68
	%Bias	9.28	11.7	-2.26	-4.29
	Intra-day Precision (%CV)	16.9	12.94	7.34	8.44
	Inter-day Precision (%CV)	15.7	10.58	9.25	9.28
	n	18	18	18	18
	Number of Runs	3	3	3	3

Figure S1

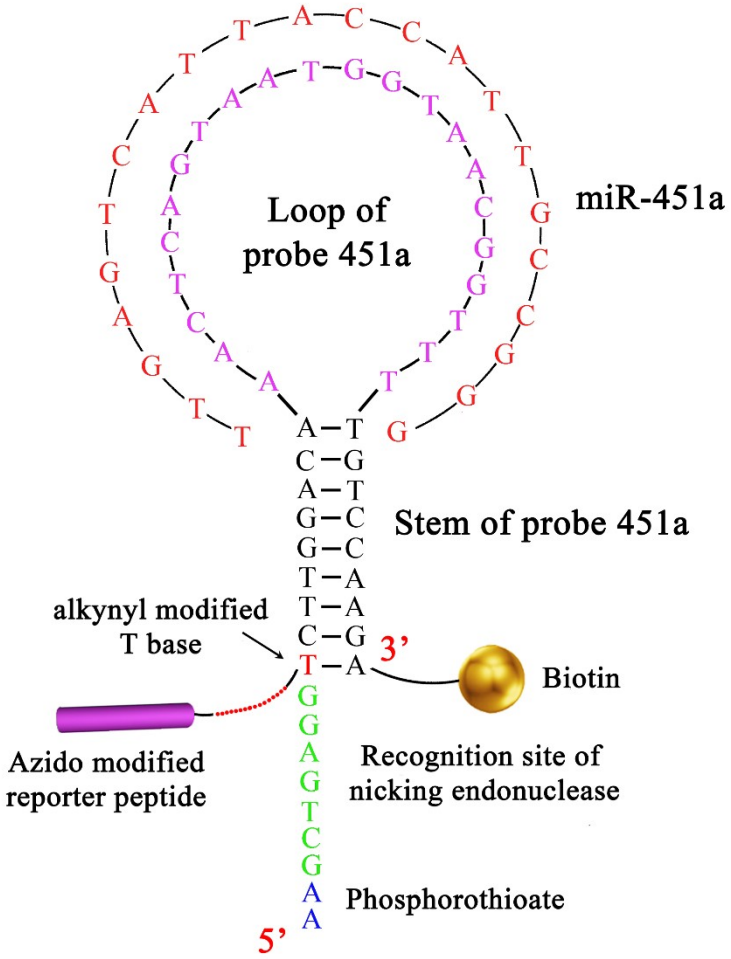

Figure S1. Structure of MBP probe. The probe contains three essential functional domains, including a loop region that allows for complementary binding of the target miRNA, a substrate peptide that serves as the mass reporter signal, a specific sequence for the recognition site of Nt.BbvCI nicking endonuclease. The DNA sequence connects with the azido modified substrate peptide via alkynyl-azido click chemistry reaction.

Figure S2

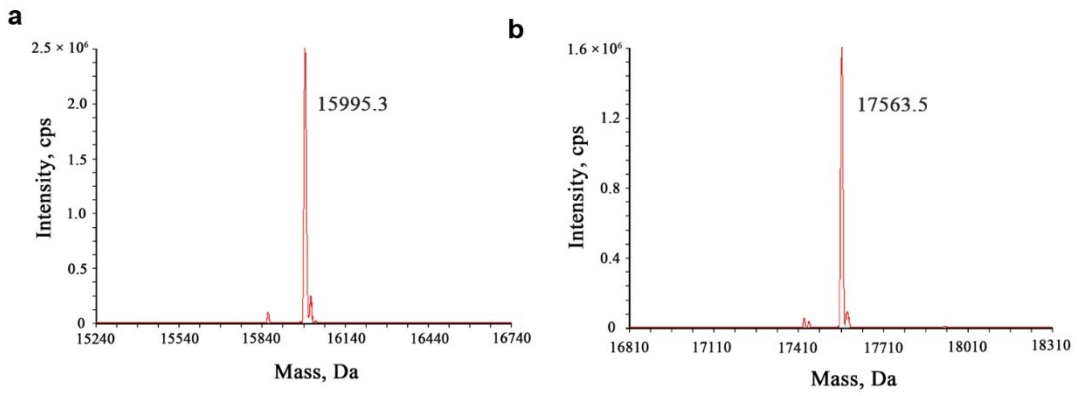

Figure S2. Mass spectra of (A) the molecular beacon and (B) molecular beacon-peptide probe. MS detection was performed using a Thermo LCQ Deca XP Plus ion trap MS, which was operated with electrospray ionization in the positive mode, Probe-21 was used as an example.

Figure S3

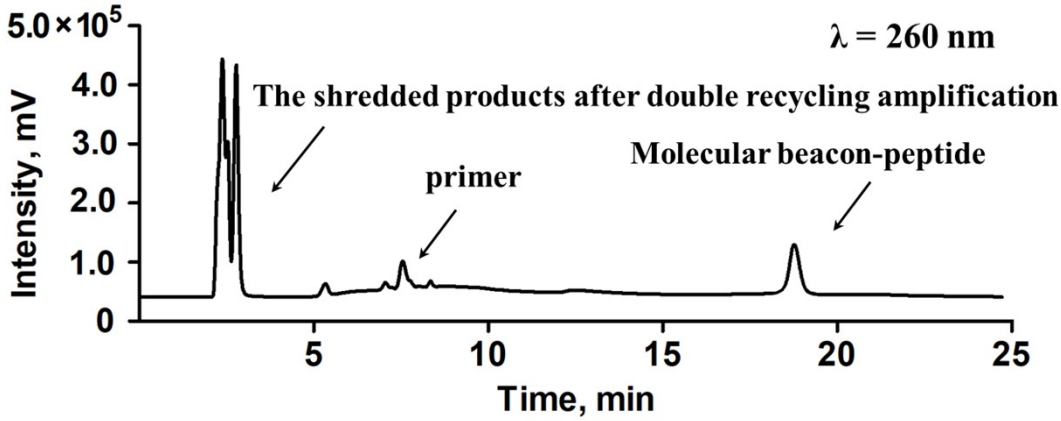

Figure S3. HPLC chromatograms of the shredded products after double recycling amplification, using probe-21 as an example. The amplification reaction was performed in 200 μL 50 mM Tris–HCl (pH 8.0) buffer, consisting of 500 nM probe, 2 μM primer, 64 U Bst DNA polymerase, 60 U nicking endonuclease, 50 U lambda exonuclease, 400 μM dNTPs, 5 mM $MgCl_2$ and 1 mM DL-Dithiothreitol (DTT). The target miR-21 was added to this reaction system with a final concentration of 10 pM, the reaction was stopped at 30 min. Under this concentration the target miR-21, it was undetectable in the chromatogram, so we could not see the miR-21 target peak.

Figure S4

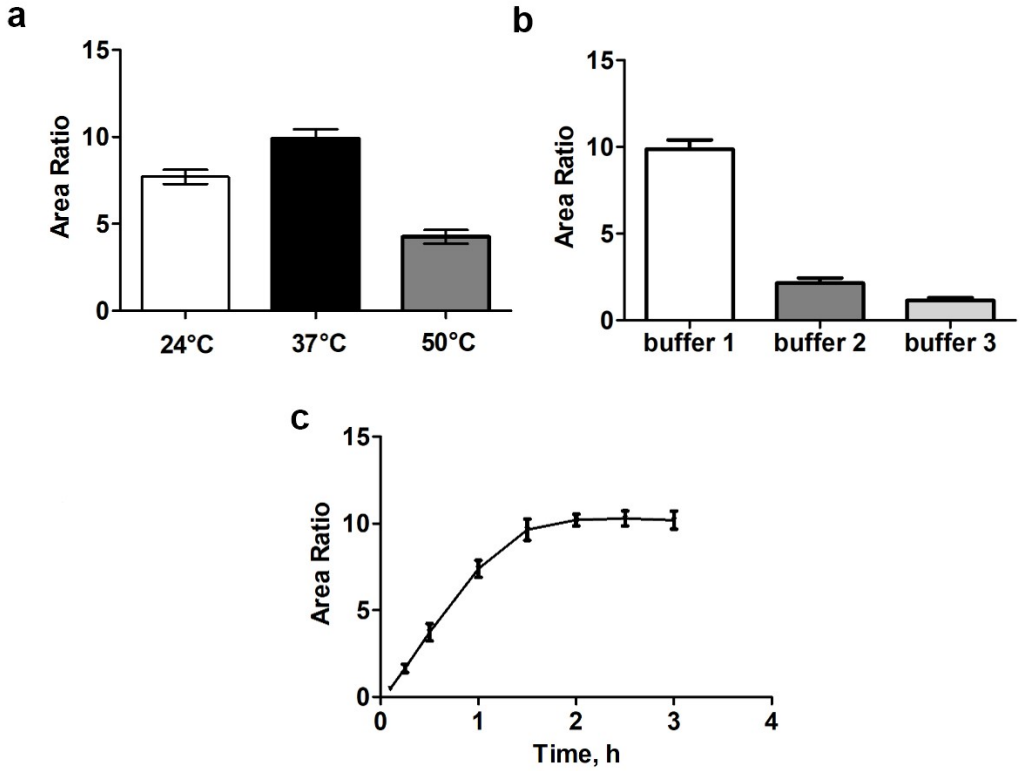

Figure S4. Optimal conditions for the molecular beacon-peptide based double recycling amplification (MBPDRA). (a) the effect of temperature; (b) the effect of buffer; (c) the reaction time. In these reactions, the test concentration of target miR-21 was 1 pM.

Figure S5

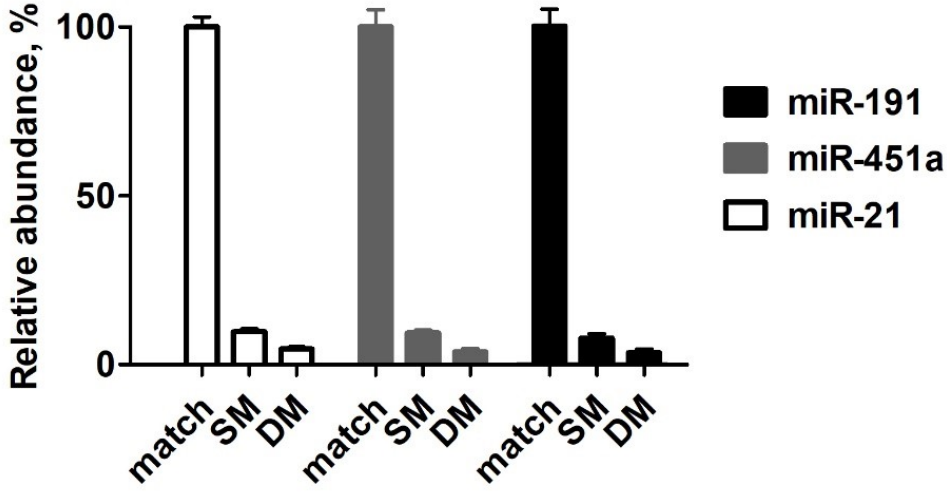

Figure S5. The specificity investigations of the molecule beacon-peptide probe-based double recycling amplification (MBPDRA) assay.

Figure S6

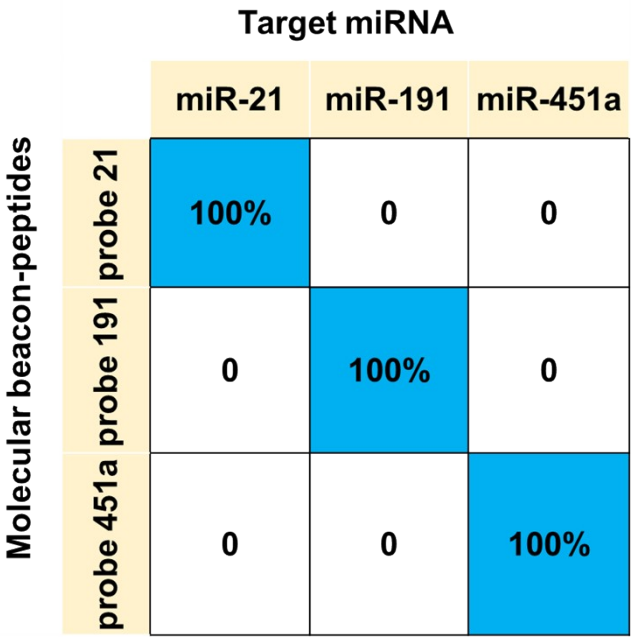

Figure S6. Cross-hybridization of the multiplex MBPDCA assay. Signals were reported as the relative percentages of the intended miRNAs of each molecular beacon-peptide probe (taken as 100%).

Figure S7

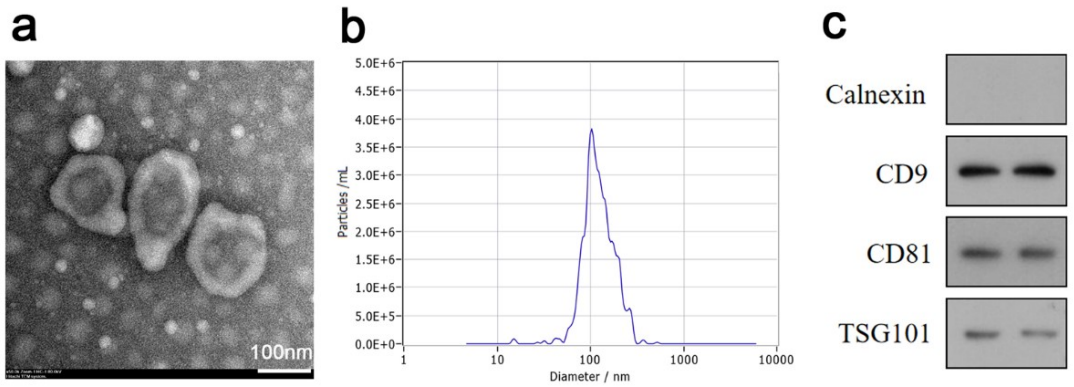

Figure S7. (a) TEM and (b) NTA measurement of the size distribution of exosomes (c) western blot of the specific protein of exosome, two sample was tested. CD9, CD81, and TSG101 are specific protein markers for exosomes. Calnexin is a negative marker for exosomes.

Figure S8

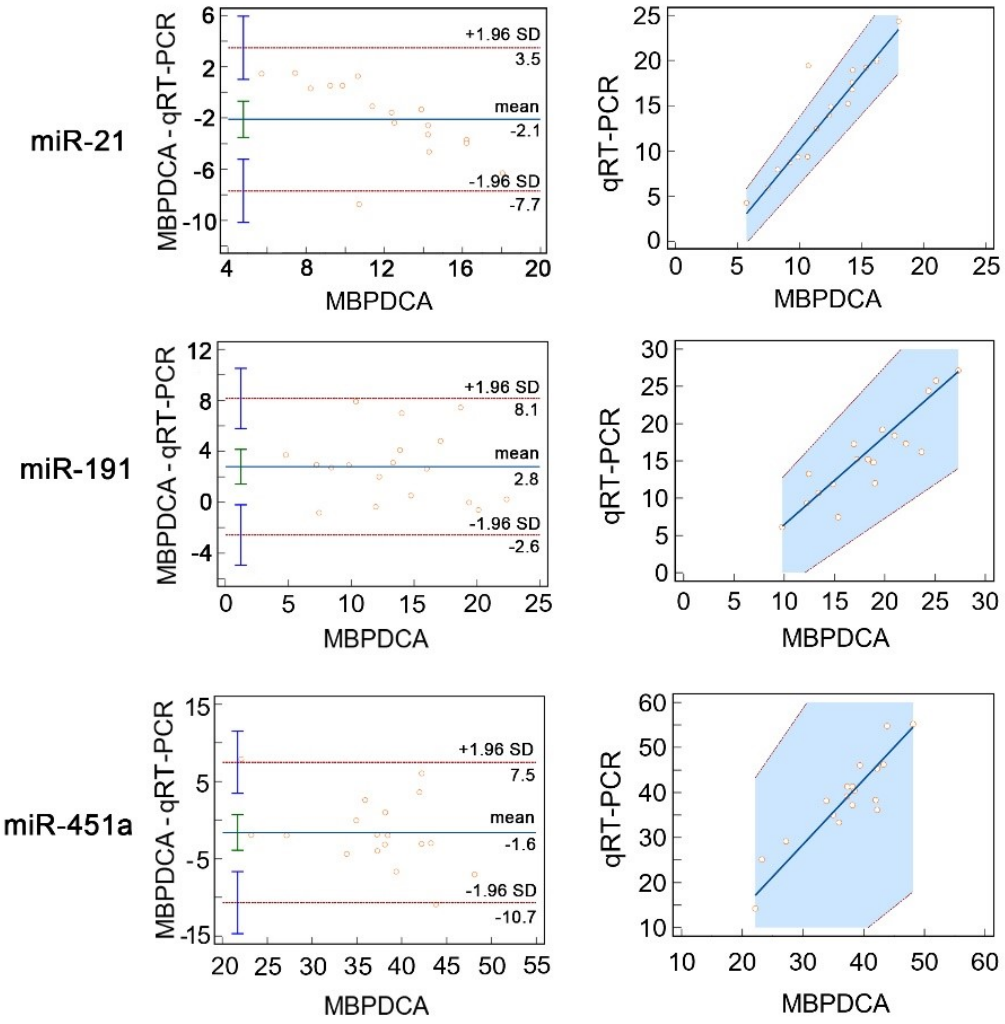

Figure S8. Bland-Altman plot analysis and the corresponding Passing-Bablok regression analysis of three target exmiRNAs of pancreatic cancer serum samples for MBPDRA *vs.* qRT-PCR. Dashed lines represent the limits of agreement (± 1.96 × standard deviation (SD)) in the Bland-Altman plot and the 95% confidence interval for the regression line in the Passing-Bablok regression plot. The solid line corresponds to the regression line.

S1. Chemicals and reagents

Peptides including azido-modified substrate peptide ((6-Azido)-GDRAVQLGVDPFR, (6-Azido)-GDRAVDLGVDPFR, (6-Azido)-GDRAVLGVDPFR), reporter peptide (AVQLGVDPFR, AVDLGVDPFR, AVLGVDPFR) and stable isotope-labeled internal standard (A*VQLGVDPFR) were synthesized by ChinaPeptides Co., Ltd. (Suzhou, China). DNA sequences were custom synthesized by sangon biotech (Shanghai, China), and miRNAs were purchased from Ribobio (Guangzhou, China). Sequencing-grade trypsin was purchased from Promega (Madison, WI, USA). Streptavidin agarose were supplied by Thermo Fisher Scientific (Waltham, MA, USA). Bst DNA polymerase (large fragment), Nb.BbvCI nicking endonuclease, Lambda exonuclease, NEB buffer 2 were purchased from New England BioLabs (Ipswich, MA, USA). Bovine serum albumin (BSA), dNTPs, DNA loading buffer, diethylpyrocarbonate (DEPC)-treated water, RNase inhibitor were supplied by TaKaRa Bio. ExoQuick Exosome extraction Solution was System Biosciences, Mountain View, CA, USA). HPLC-grade acetonitrile, methanol (MeOH), formic acid (FA), were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Deionized water was supplied by a Clever-Q system obtained from Zhiang (Shanghai, China).

S2. Blood sample collection

Blood samples were obtained from both healthy donors (as control) and patients with pancreatic cancer at Zhongnan Hospital of Wuhan University, following approval from the ethical review committee (Approval Number: 2021033). All participants gained written and voluntary informed consent prior to their inclusion in this study. The peripheral blood was collected into serum-separator tubes and centrifuged at 3,000× *g* for 10 minutes. The upper serum was transferred into new tubes and subsequently stored in a refrigerator at -80 °C.

S3. Preparation of Stock Solutions, Calibration Standards and Quality Controls

(QCs)

Stock solutions (1 mM) of each target miRNA (miR-21, miR-191, miR-451a) were first dissolved in deionized water and stored at -80 °C. The mixed miRNA stock solution (100 μM) was prepared by diluting each stock solutions using water. The calibration standard of miRNA was prepared by serially diluting the mixed stock solutions using wate. The concentrations of the calibration standard were 0.1 fM, 1 fM, 10 fM, 100 fM, 1 pM, and 10 pM. Correspondingly, the QC standards (i.e., lower limit of quantification (LLOQ), low QC, mid QC and high QC) were set at 0.1 fM, 0.3 fM, 500 fM and 8 pM and frozen prior to use.

S4. HPLC conditions

Chromatographic evaluation of the synthesized probe was performed using a Shimadzu HPLC system equipped with an LC-20AT solvent delivery pump and an SPD-20A UV detector. Separation was achieved on an Elite C8 column (4.6 mm × 150 mm, 5 μm; Dalian, China) maintained at a temperature of 40 °C. The mobile phase A was 50 mM triethylamine in water, pH = 7.6 and mobile phase B was acetonitrile (ACN). A linear gradient flow rate of 1 mL/min was applied as follows: B 5% (0 min) → 16% (5 min) → 20% (20 min) → 5% (25 min) → 20% (25 min). The injection volume was set as 20 μL. Detection wavelength for analysis was set at 260 nm.

Peptide chromatographic separation was carried out using an Elite C18 column (4.6 mm × 150 mm, 5 μm; sourced from Dalian, China). The mobile phase for analysis consisted of solvent A (0.1% TFA in water) and solvent B (0.1% TFA in ACN). A gradient program was implemented as follows: B 10% (0 min) → 10% (1 min) → 50% (16 min) → 10% (20 min) → 10% (21 min). The detection wavelength was set at 220 nm.

S5. LC-MS/MS conditions

Samples were analyzed using an LC-30A UPLC system coupled with an LCMS-8050 triple-quadrupole mass spectrometer from Shimadzu (Japan). The mass

spectrometer was equipped with an electrospray ion source operating in positive mode. UPLC separation was conducted on an Agilent SB C18 column (2.7 mm, 30 mm × 2.1 mm) maintained at a temperature of 40 °C. The mobile phase consisted of water (0.1% FA) as mobile phase A and ACN (0.1% FA) as mobile phase B. The flow rate was set at 0.3 mL/min, and the injection volume was 5 μL. The gradient elution started with 10% B, held for 1 min, then increased to 90% B over 4 min, maintained for 4 min, and finally returned to 10% B after 1 min (all concentrations refer to mobile phase B). For sample analysis, the following mass parameters were employed: ESI desolvation temperature was set at 350 °C, and the gas flow rate was 10 L/min. Q1 and Q3 were set at unit resolution. The capillary voltage was set at 4 kV, and the nebulizer pressure was set to 35 psi. Data collection and analysis were performed using LabSolution software.

S6. qRT-PCR

The reverse transcription reaction was conducted using the following components: 5 nM of the stem-loop reverse transcription primer (Bulge-LoopTM miRNA primer, sourced from Ribobio in Guangzhou, China), in a solution containing 0.8 U/μL of reverse transcriptase, 4 U/μL of RNase inhibitor, and 0.2 mM of dNTPs in a 25 μL volume. This solution was prepared using the RevertAid^{TM} First-strand cDNA Synthesis Kit from Thermo Scientific in Massachusetts, USA, following the manufacturer's instructions. To create a calibration curve ranging from 10 fM to 1nM of synthetic target miRNA, it was generated in parallel with the biological samples. This curve allowed for the estimation of the absolute amount of the target miRNAs (specifically, miR-21, miR-191, and miR-451a) in the samples. For quantitative real-time PCR (qRT-PCR), the assays were performed on a 7500 real-time PCR system from Applied Biosystems in California, USA. Each 20 μL PCR reaction mixture consisted of 2 μL of the reverse transcription product, 10 μL of SYBR Green Mix, 0.2 μM of forward primers, and 0.2 μM of reverse primers. The cycling parameters involved an initial step at 95 °C for 10 minutes, followed by 40 cycles at 95 °C for 15

seconds and 60 °C for 30 seconds. All PCR reactions were conducted in triplicate, which included no-template controls.

S7. Transmission electron microscopy

The exosomal pellets were resuspended in 50 μL of phosphate-buffered saline, and a small aliquot of this suspension was carefully placed on a piece of parafilm. Subsequently, a copper grid was gently positioned on top of the droplet and allowed to incubate at room temperature for 5-10 minutes. Any excess liquid was carefully removed using filter paper. Following the blotting step and air-drying, the samples were stained with 10 μL of a 2% uranyl acetate solution. Finally, high-resolution images were acquired using a HT7700 transmission electron microscope (HITACHI, Japan).

S8. Western blot

The protein concentrations of the exosomes were determined using a BCA assay kit (Beyotime, China), following the guidelines provided by the manufacturer. The exosomes were lysed using ice-cold RIPA lysis buffer (Beyotime, China), which was supplemented with a protease inhibitor cocktail from Sigma (USA). Equal amounts of protein lysates were subsequently separated through SDS-PAGE (Biorad, USA) and then transferred onto PVDF membranes supplied by Pall Corporation (USA). After several rinses with TBS (Signalway Antibody, USA) and blocking with 5% non-fat milk (BBI, China), the membranes were incubated overnight with primary antibodies, including anti-CD9 (Duolaimi biotechnology, DL210002, China), anti-CD81 (Proteintech, 66866-1-Ig, USA), and anti-TSG101 (Duolaimi biotechnology, DL210005, China). Following thorough washing, the membranes were exposed to HRP-conjugated secondary antibodies (Signalway Antibody, L3012, USA) in darkness for 1 hour. Immunoreactive protein bands were visualized using an ECL reagent (Tanon, China), and the ChemiDoc MP imaging system (BioRad, USA) was employed for the analysis.