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Supporting Information

DNAzyme-RCA based colorimetric and lateral flow dipsticks assays for the point-of-care testing of exosomal m5C-miRNA-21

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1. Experimental section

1.1 Instruments and materials

All DNA oligonucleotides (Table S1) were obtained from Bioengineering Co., Ltd. (Shanghai, China) and purified using standard 10% polyacrylamide gel electrophoresis (PAGE). The DNA sequences were designed using the computer program NUPACK. The nicking endonuclease *Nb.BbvCI,* T4 polynucleotide kinase (PNK), T4 DNA ligase, and phi29 DNA polymerase were purchased from Bioengineering Co., Ltd. (Shanghai, China). γ-[32P] ATP was purchased from PerkinElmer. Sodium chloride (NaCl) was obtained from Shanghai McLean. BCA protein concentration assay kit was purchased from Beyotime Co., Ltd. (Shanghai, China). MethylFlash™ m5C RNA Methylation ELISA Easy Kit (Fluorometric) was purchased from Epigentek Group Inc. (New York, USA). RPMI-1640 liquid medium, 1×PBS buffer (sterile), 0.25% trypsin, and penicillin-streptomycin solution were purchased from HyClone. Fetal bovine serum (FBS) was purchased from Sijiqing. All other chemicals were purchased from Sigma-Aldrich (Oakville, Canada) and used without further purification. The gel images were obtained using a Typhoon 9200 variable mode imager (GE Healthcare) and analyzed using Image Quant software (Molecular Dynamics). Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, USA) and Edinburgh fluorescence spectrophotometer (FLS 900, UK) were used for analysis. Transmission electron microscopy (TEM) analysis was conducted on the Hitachi HT7700 electron microscope. Ultraviolet-visible (UV-Vis) spectra were performed on a Hitachi U-4100 spectrophotometer.

Table S1 Oligonucleotides Sequence (5’-3’).

Oligonucleotides	Sequence (5’-3’)
miRNA-21	UAGCUU AUCA GACU GAUG UUGA
Methylated miRNA-21	UAGCUUAU-methyC-AGACUGAUGUUGA
miRNA-181	AACAUUCAACGCUGUCGGUGAGU
Methylated miRNA-181	AACAUUCAACG-methyC-UGUCGGUGAGU
miRNA-203	AGUGGUUCUUAACAGUUCAACAGUU
Methylated miRNA-203	AGUGGUUCUUAA-methyC-AGUUCAACAGUU
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT
mmu-miRNA-21-RT	CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCAACATC
mmu-miRNA-21-S	ACACTCCAGCTGGGTAGCTTATCAGACTGA
primer-A	TGGTGTCGTGGAGTCG
DNAzymes	TACCATCAACATCAGTCGGTAGTAGGATGGCGATCCGATAAGCTATGGTAT
RCA fragments	AGCAATCCAATCCTCAATTCAACATCACTCTGACCCAACCAATCCAATCTAAGCTACCTC
bio-ssDNA 1	GAGTGATGTTGAATTGAGGATTGGATTGCT-biotin
bio-ssDNA 2	GAGGTAGCTTAGATTGGATTGGTTGGGTCA-biotin

AuNPs-DNA	TGACCCAACCAATCCAATCTAAGCTACCTC-HS
Padlock	ACTCTGACCCAACCAATCCAATCTAAGCTACCAATCCAATCCTCAATTCAACATC
AuNPs hairpin probes	SH-ATCGATGGATTGGTAGCTTAGATTCCCATCGAT
Primer-1	TCAGACTGATGTTGA
Primer-2	TAGCTTA
Anti-miRNA-21	TCAACATCAGTCTGATAAGCTA

1.2 The preparation of gold nanoparticles

A solution of 13 nm diameter AuNPs was prepared following the previously described literature. Briefly, all glassware was cleaned with aqua regia (3 parts HCl, 1 part HNO_3), thoroughly rinsed with distilled water, and air-dried before use. Aqueous solutions of 0.01% (wt/vol) chloroauric acid and 1% (wt/vol) sodium citrate were prepared. The 0.01% chloroauric acid solution (100 mL) was placed in a 2-neck round-bottomed flask equipped with a magnetic stirrer and heated to reflux with an oil-bath thermostat. A 1% sodium citrate solution (3 mL) was added to the flask with constant stirring at the maximum speed to obtain a 13 nm AuNPs solution. The reaction was carried out for 10 minutes, during which the solution turned red. The resulting AuNPs solution was cooled to room temperature and further cooled to 4°C for storage before use.

1.3 The preparation of AuNPs-DNA and magnetic beads

AuNPs prepared were functionalized with thiolated short nucleic acid strands which modified with phosphorothioate bonds. The AuNPs and magnetic beads were mixed with aminated single-stranded DNA in a ratio of 1:3.5 and incubated for 12 h in 0.5 TBE (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) and 50 mM NaCl. The samples were then centrifuged at 8000 g for 10 min to remove any uncoupled oligonucleotide from the solution. The supernatant was removed, and the pellet was resuspended in water.

1.4 Cell culture

The MCF-10A, MCF-7 and HCT 116 cells were cultured in DMEM, RPMI-1640 and McCoy's medium with 10% FBS and penicillin–streptomycin, respectively. All cells were incubated at 37 °C in an atmosphere supplemented with 5% CO_2.

1.5 The isolation of exosomes

Cell culture medium: Exosomes-depleted FBS was prepared by centrifugation at 100,000 g for 20 h, then filtered through a 0.2 μm filter. Cells were first seeded in regular medium and then switched to exosomes-depleted medium (contain 10% exosomes-depleted FBS) for 48 h before harvesting. Briefly, medium or serum was first cleared by differential centrifuged at 1000 g for 10 minutes, 3000 g for 15 minutes and 10,000 g for 30 minutes to remove cellular debris, apoptotic bodies and large vesicles. Then, cleared medium was passed through 0.2 μm filter and ultracentrifuged at 100,000 g for 90 minutes to wash contaminating protein. Purified exosomes were resuspended in particle-free PBS and store in − 80 °C until use.

Blood: The blood was first cleared by differential centrifuged at 1000 g for 10 minutes, 3000 g for 15 minutes and 10,000 g for 30 minutes to remove plasma, blood cells and large vesicles, yielding the cleared serum. Then, the cleared serum was passed through 0.2 μm filter and ultracentrifuged at 100,000 g for 90 minutes to wash contaminating protein. Purified exosomes were resuspended in particle-free PBS and store in − 80 °C until use.

1.6 The BCA protein concentration assay

According to the manufacturer's instructions, the concentration and extraction efficiency of exosomes can be

determined using a BCA protein concentration assay kit. The procedure involves mixing solution A and solution B in a 50:1 ratio. Subsequently, 200 µL of the mixed solution should be transferred into the wells of a 96-well plate, followed by the addition of 20 µL of the exosome solution. The mixture should then be incubated at 37 °C for 20-30 minutes. Finally, the absorbance at a wavelength of 562 nm should be measured using an enzyme-linked immunosorbent assay (ELISA) reader.

1.7 The capture of miRNA-21

Total RNA was extracted from cells and exosomes using TRIzol reagent according to the manufacturer's instructions. Small RNAs (< 100 nt) including miRNA were isolated from total RNA using the High Pure miRNA Isolation Kit, and annealed to single-strand DNA oligonucleotides complementary to target miRNAs. The mixture was heated to 95 °C, then gradually cooled to 30 °C. The miRNA-DNA complex was incubated with Dynabeads M-270 Amine at 4 °C for 1 h, followed by magnetic separation. After heat-eluting the mixture (miRNA-DNA complex/Dynabeads) and conducting magnetic separation again, miRNA21 could be obtained in the supernatant for subsequent experiments.

1.8 The ELISA assay for m5C-miRNA-21

The ELISA assay for m5C-miRNA-21 was performed using an MethylFlash™ m5C RNA Methylation ELISA Easy Kit (Fluorometric) (EpigenTek, P-9009) according to the manufacturer's instructions. The extracted miRNA-21 or the m5C-miRNA-21 standard was added to the wells of the 96-well plate and incubated at 37 ℃ for 90 minutes for immunoprecipitation. During the last 10 minutes of sample incubation, the m5C detection complex solution was prepared as follows: In each 1 mL of diluted wash buffer, 1 µL of anti-m5C-antibody was added and mixed, followed by the addition of 1 µL of signal indicator and 1 µL of enhancer solution. After the 90-minute incubation, the binding solution was removed from each well, and each well was washed three times with 150 µL of diluted wash buffer each time. Washing was done by pipetting diluted wash buffer in and out of the wells. Subsequently, 50 µL of the m5C detection complex solution was added to each well, covered, and incubated at room temperature for 50 minutes. The m5C detection complex solution was then removed, and each well was washed five times with 150 µL of diluted wash buffer each time. Finally, 50 µL of fluorescence development solution was added to each well and incubated at room temperature for 2 to 4 minutes away from direct light. The fluorescence was then read on an ELISA reader within 2 to 10 minutes at $530_{ex}/590_{em}$ nm.

1.9 The cleavage reaction of DNAzyme

All reactions were performed in a reaction buffer consisting of 5 mM Tris-HCl, 0.5 mM EDTA, 100 mM NaCl, 20 mM KCl, and 50 mM $MgCl_2$ at pH 8.0. DNAzyme-mediated RNA cleavage reactions were carried out at 37 ℃ using 1 µM DNAzyme and 40 nM m5C-miRNA-21 with reaction times of 0 h, 3 h, and 6 h.

1.10 The cyclization of padlock DNA

Phosphorylated padlock DNA (100 pmol) was mixed with DNA primer1 (200 pmol) in 20 µL of PBS. The mixture was heated to 90 °C for 5 minutes and then cooled to room temperature for 15 minutes. To this mixture, 10 µL of 10X T4 DNA ligase buffer (50 mM Tris−HCl, 400 mM ATP, 2 mM $MgCl_2$, 10 mM DTT, pH 7.5) and 10 U of T4 DNA ligase were added, along with water to a total volume of 50 µL. The resulting mixture was incubated at 4 °C overnight. The ligated padlock DNA were concentrated by standard ethanol precipitation and purified by 10% denaturing PAGE.

1.11 The reaction of RCA

The RCA reaction of the linear padlock DNA was performed in 20 µL of reaction mixture containing 1× buffer, 0.8 U of RNase inhibitor, 100 pM ligated padlock DNA, and different concentrations of miRNA. The ligated padlock DNA and miRNA mixtures were first denatured at 65 °C for 5 min and then cooled to room temperature slowly. Next, 0.4 U of Phi*29* DNA polymerase, 400 µM dNTP, and 20 mM Tris−HCl buffer (containing 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 6 mM $MgSO_4$, 0.1% Triton X-100) were added into 10 µL of ligation reaction products to form a 20 µL mixture. After the mixture was incubated at 37 °C for 3 h, the RCA products can be obtained.

1.12 The native polyacrylamide gel electrophoresis

For each individual reaction, the DNA solutions were mixed with 6X loading buffer (TEK buffer, pH 8.0, 50% glycerol, 0.25% bromophenol blue) and analyzed by 10% native polyacrylamide gel electrophoresis. The electrophoresis was carried out in 1X TBE (pH 7.4) at a constant voltage of 120 V for 1 hour. The gels were scanned using a Typhoon 9200 variable mode imager (GE Healthcare) to visualize DNA species.

1.13 Patient Sample Collection.

Blood samples were received from Shandong Provincial Qianfoshan Hospital. The experiments using blood were agreed by the Shandong Provincial Qianfoshan Hospital Institutional Review Board (YXLL-KY2022(039)).

1.14 The real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

qRT-PCR was typically performed in a volume of 20 µL. It included 10 µL of the isolated miRNA molecules, 0.5 µM each of the forward primer (FP) and reverse primer 1 (RP1), 200 µM dNTPs, 1x qRT-PCR buffer (75 mM Tris-HCl, pH 9.0, 2 mM $MgCl_2$, 50 mM KCl, 20 mM $(NH_4)_2SO_4$), and 2.5 units of Thermus thermophilus DNA polymerase (Biotools, through Mandel Scientific; Guelph, ON, Canada). The amplification was carried out using the following thermocycling program: one cycle of 95 °C for 1 minute; 13 cycles of 95 °C for 30 seconds, 50 °C for 45 seconds, and 72 °C for 45 seconds (the number of amplification cycles varied between different selection rounds, typically between 13 and 15 cycles, to achieve full amplification as assessed by 2% agarose gel electrophoresis); followed by one cycle of 72 °C for 1 minute.

Subsequently, we substituted the obtained Ct data into the standard curve (Ct = -3.074X + 34.64) to calculate the copy number of the measured samples. Then, we determined the sample concentration using the formula C = (Ct - 34.64)/(-3.074*20*10*500/NA*1000).

1.15 Fluorescence analysis

All fluorescence measurements were conducted in low volume cuvettes with a sample volume of 100 µL using an Edinburgh fluorescence spectrophotometer (FLS 900, UK). The slit widths for excitation and emission were both set at 5.0 nm. The excitation wavelength was set at 475 nm, and emission was scanned from 500 nm to 580 nm in 1 nm increments. For fluorescence detection of RCA amplification products, FAM-labeled hairpin probes with different concentrations were added, and the reactions proceeded for 1 hour before fluorescence was monitored. Spectra were not corrected for instrument artifacts. Concentration-response plots were generated using the integrated fluorescence intensity across the emission range, with all samples measured in triplicate. Data were fitted to a log-linear regression model, and detection limits were determined based on the minimum concentration required to produce a signal 3σ (standard deviation) above the baseline error.

1.16 The colorimetric assay

In the colorimetric assay, 96-well plates containing the RCA products were mixed with 200 µL of an AuNPs solution. The solutions were thoroughly mixed and visually observed over time. Additionally, the UV-vis absorption spectra of the mixtures were measured. A concentration series was performed for the target m5C-miRNA-21, including concentration points of 100 nmol/L, 10 nmol/L, 1 nmol/L, 100 pmol/L, 10 pmol/L, and 1 pmol/L. Blank negative controls were also included for the RCA reaction. Colorimetric assays and UV-vis analyses were performed to study the interaction and effects of the RCA products on the AuNPs, which could be observed visually and quantitatively through the UV-vis absorption spectra.

1. 17 The asembly of lateral flow dipsticks (LFDs)

The LFDs were assembled using five components: a backing card, printed nitrocellulose membrane (NCM), a sample pad, a conjugate pad, and an absorbent pad. The TL-DNA and CL-DNA sequences were printed onto the NCM as follows:

1. A mixture of 5 µM streptavidin and 25 µM ssDNA 1 or ssDNA 2 in 200 µL of PBS (pH 7.4) was incubated at room

temperature for 30 minutes.

2. The streptavidin and bio-ssDNA conjugate was passed through a centrifugal column with a 50K molecular cutoff size for 10 minutes at 14,000 rpm.

3. The conjugate was washed twice with 200 µL of PBS.

4. The concentrated streptavidin and bio-ssDNA were recovered by inverting the filter device into a fresh microcentrifuge tube and centrifuging at 1,000 rpm for 2 minutes.

5. The recovered streptavidin and bio-ssDNA were diluted to a final volume of 100 µL in PBS.

6. The streptavidin-DNA conjugates were printed onto the nitrocellulose membrane using a non-contact microarray printer.

The NCM was pre-cut into pieces measuring 25X 300 mm and preconditioned at 60% humidity in the printer chamber. The control and test lines were printed on the NCM, approximately 22 mm (CL) and 17 mm (TL) below the top edge, respectively, with an interline distance of 5 mm. Each line had a diameter of 0.5 mm. The NCM was air-dried for 1 hour after printing. The printed NCM was then attached to the middle of a backing card and cut into 4 mm wide strips using the Biodot Guillotine cutter. The absorbent pad was cut into sizes of 4X 20 mm and attached to the backing card just above the control line of the strips, with a 0.5 mm overlap with the NCM. Glass fiber was cut into sizes of 4X 10 mm, with one piece used as a sample pad and the other as a conjugate pad. The conjugate pad was immersed in a gold nanoparticle suspension, while the sample pad glass fiber was soaked in a resuspension solution containing 0.1% SDS. Both pads were dried for 2 hours at room temperature. The conjugate pad was attached just below the test line of the strip, with a 0.5 mm overlap with the NCM. The sample pad was attached just below the conjugate pad, with a 0.5 mm overlap with the conjugate pad. The final assembled LFDs consisted of the following layers, from bottom to top: sample pad, conjugate pad, printed NCM, and absorbent pad. The assembled strips were stored in a desiccator at room temperature until use.

2. Supplemental figures

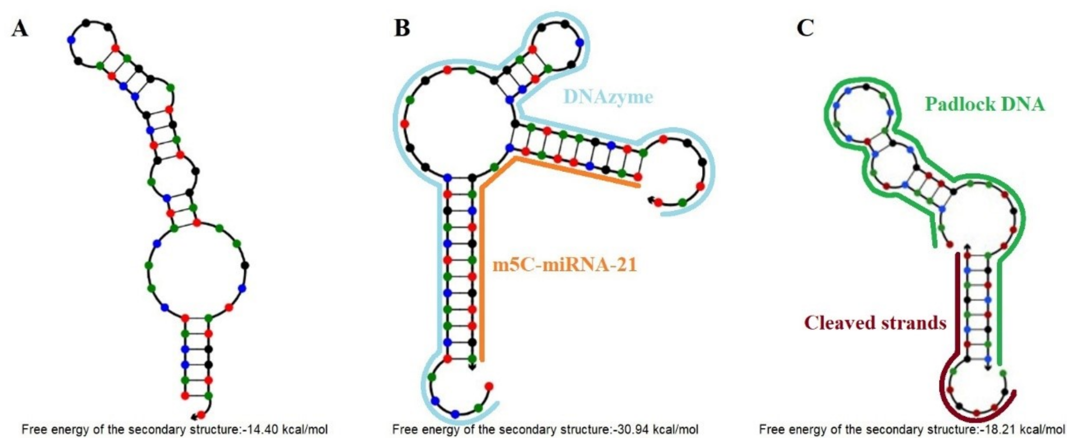

Figure S1. The simulation of DNA structure and binding by NUPACK. (A) The structural stability of DNAzyme (conformation 1) and (B) the DNAzyme & m5C-miRNA-21 (conformation 2) was calculated using NUPACK at 37 °C. (C) The structural stability of padlock DNA combined with the cleaved strands of m5C-miRNA-21 at 37 °C.

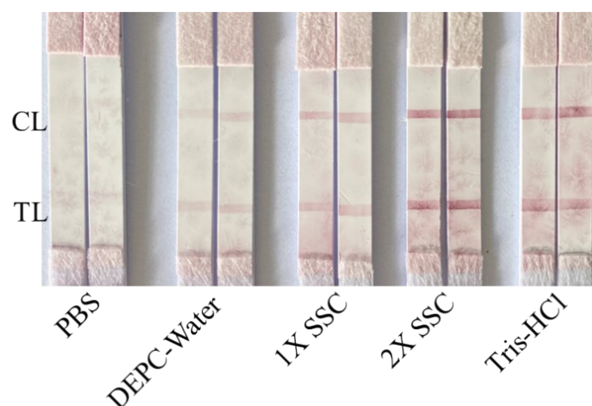

Figure S2. The optimization of running buffer. Four common types of running buffers (PBS, SSC, DEPC-Water and tris-HCl) were used to evaluate the performance of the LFDs. PBS: Phosphate Buffer Saline; DEPC-Water: Diethyl pyrocarbonate water; SSC: Sodium Chloride-Sodium Citrate Buffer; Tris-HCl: Tris (Hydroxymethyl) Aminomethane Hydrochloride. pH of running buffer: 7.4; Concentration of m5C-miRNA-21: 10 pM.

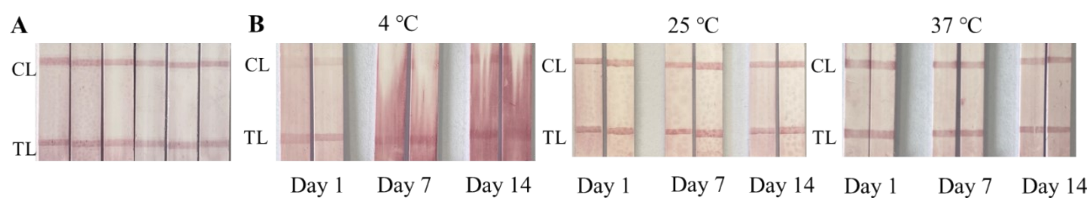

Figure S3. The repeatability and stability of the LFDs. A) Six repeated measurements of m5C-miRNA-21 were performed using LFDs at a concentration of 10 pM. B) LFDs were incubated under different temperatures (4°C, 25°C, 37°C) for 14 days with a concentration of 10 pM.

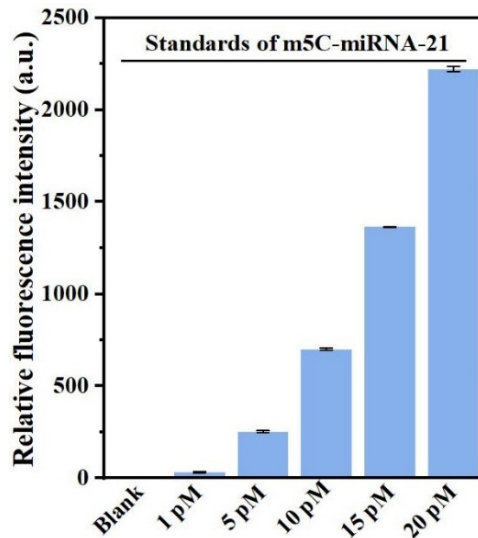

Figure S4. The ELISA assay for m5C-miRNA-21. We used ELISA assay to measure the standards of m5C-miRNA-21 at concentrations of 1 pM, 5pM,10 pM, 15 pM and 20 pM.

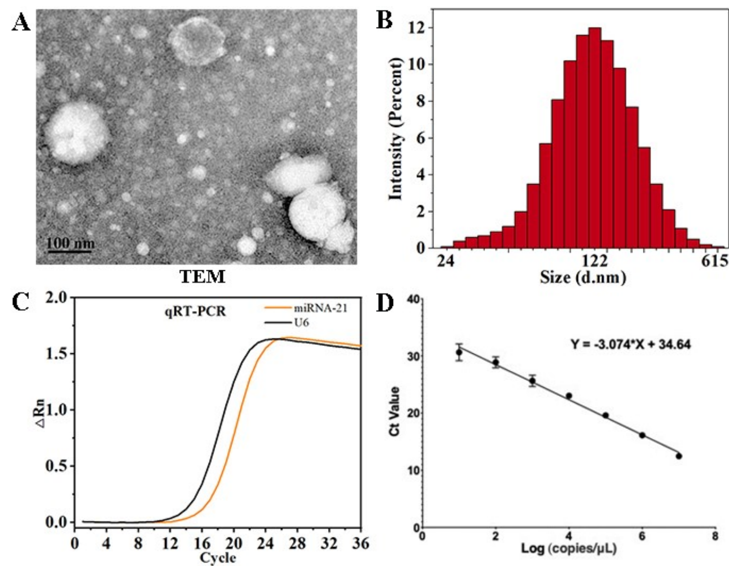

Figure S5. The detection of exosomes in the blood of patients with colorectal cancer. (A) TEM image and (B) size distribution measured by DLS of exosomes from clinical blood samples. (C) The qRT-PCR detection of exosomal miRNA-21 from clinical blood samples, and (D) the corresponding standard curve.

Table S2. Comparison the methods for detection of exosomal miRNAs.

Methods	Limit of detection	References
Fluorescence spectrometry	160 fM	2
	0.36 fM	3
Electrochemical sensing	500 aM	4
	33.4 aM	5
Surface-enhanced Raman scattering	1 aM	6
	0.24 aM	7
LFDs	58.90 fM	8
Colorimetric	44.6 fM	9
Colorimetric	1.00 pM (the lowest concentration can be detected)	This
LFDs	0.10 pM (the lowest concentration can be detected)	work

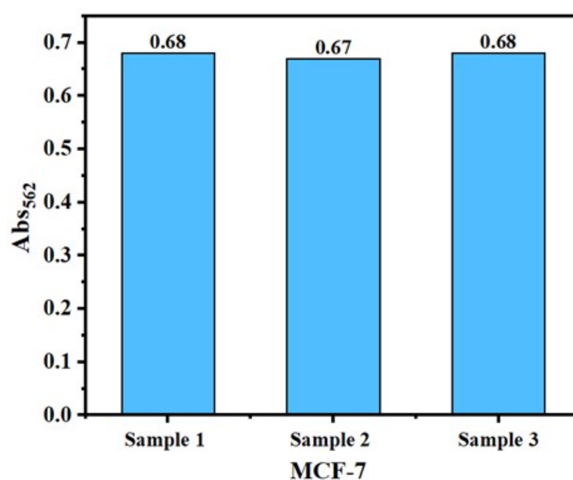

Figure S6. The stability of exosome extraction. We used BCA protein concentration assay kit to detect exosomes extracted from the identical cell culture medium.

Table S3 Clinicopathological characteristics of patients and healthy individuals.

Sample (No.)	Age	Sex	Disease stage
Healthy individual 1	62	F	Healthy
Healthy individual 2	72	F	Healthy
Healthy individual 3	35	M	Healthy
Healthy individual 4	44	F	Healthy
Colon polyp patient 1	53	M	Adenomatous polyp
Colon polyp patient 2	57	M	Focal adenocarcinoma transformation
Colon polyp patient 3	44	F	Focal adenocarcinoma transformation
Colon polyp patient 4	65	M	Focal adenocarcinoma transformation
Colon cancer patient 1	50	M	Middle
Colon cancer patient 2	51	F	Terminal
Colon cancer patient 3	63	M	Early
Colon cancer patient 4	62	M	Early
Colon cancer patient 5	64	F	Terminal
Colon cancer patient 6	45	M	Early

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