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Supplementary Information

Simultaneous and multiplexed phenotyping of circulating exosomes with orthogonal CRISPR-Cas platform

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Experimental details

1. Chemicals and reagents

Streptavidin beads were purchased from Sangon Biotech (Shanghai, China). Cas12a and 10× NEBuffer 2.1 were purchased from New England Biolabs (Beijing). Cas13a and Buffer were purchased from Kemike Biotech (Wuhan). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and PBS solution (pH 7.4) were purchased from Thermo Fisher Scientific (China). 0.25% trypsin-EDTA and antibiotics (penicillin-streptomycin) were purchased from Gibco (Grand Island, NY). The breast cancer MCF-7 cell lines were obtained from the American Type Culture Collection. All reagents were used as received without further purification. Diethyl pyrocarbonate (DEPC) treated water were purchased from Sangon biotechnology company. The oligonucleotides were designed with the help of Integrated DNA Technology and synthesized and purified by Sangon biotechnology company. The ELISA kits for CD63 and EpCAM detection were purchased from Jiangsu Meimian Industrial Co., Ltd.

2. Apparatus

UV1800PC spectrophotometer (Shanghai, China) was used to quantify the nucleic acids. Fluorescence signals were recorded on a RF-5301PC (Shimadzu) fluorospectrophotometer. All DNA samples were annealed on an Applied Biosystems 96-well thermocycler. Tanon EPS300

electrophoresis apparatus was used for native polyacrylamide gel electrophoresis (PAGE). The PAGE results were imaged with a Tanon 4600SF gel imaging system under UV light.

3. Nucleic acids

All DNA oligonucleotides, including 5-biotin-labeled CD63 aptamers (CD63-apt), epithelial cell adhesion molecule (EpCAM-apt), Cas12a-T, Cas13a-T, Cas13a-sgRNA, Cas12a-sgRNA, were synthesized and purified by Sangon Biotech (Shanghai, China). Tris-acetic acid-magnesium (1×TAMg) buffer (45 mM Tris-acetic acid and 7.6 mM magnesium acetate, pH 8.0) was used for all nucleic acids self-assemblies. All the nucleic acids used in this work were listed and illustrated in Table S1 and Figure S1.

4. Cell culture

MCF-7 cells were cultured with DMEM complete medium. After dispersing the cells evenly in DMEM complete medium, the cell culture dish was placed in the cell culture box (37 ℃ humid environment, 5% CO_2) for proliferation. When the cells covered 80% ~ 90% of the cell culture dish, remove the DMEM complete medium. The dish was washed twice with sterile PBS, and then a certain amount of DMEM basic medium was added before placing in the cell culture box. After 48 hours of culture, all the liquid in the cell culture dish were taken out for exosomes isolation.

5. Exosomes isolation and characterization

Exosomes secreted by MCF-7 cells were isolated from the supernatant of cell culture dish by differential centrifugation. Impurities such as cells, dead cells and cell fragments were removed through 300 *g* centrifugation (4 ℃, 10 min). Then the supernatant was subjected to centrifugation at 1000 *g* (4 ℃, 10 min), and 10000 *g* (4 ℃, 30 min) before purified by ultracentrifugation at 100000 *g* (4 ℃, 70 min). The resulting supernatant was removed. The precipitate was dispersed in sterile PBS (pH 7.4) to centrifugate at 100000 *g* (4 ℃, 70 min) again. Finally, the supernatant was removed, and the bottom precipitate was dispersed in 200 μL sterile PBS (pH 7.4) and stored at -80 ℃ until use. The concentration of exosomes was determined by nanoparticle tracking analysis (NTA) and their morphology was characterized with transmission electron microscopy (TEM).

6. Native PAGE characterization

8% native PAGE was utilized in this work. The electrophoresis was conducted in 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). A constant voltage of 90 V was used for 80 min of electrophoresis before the gels were stained with 4S Red for imaging.

For the Cas13a *trans*-cleavage activity characterization: Cas13a (200 nM)/Cas13a-sgRNA (1 μM) complex was pre-assembled in 1× NEBuffer 2.1. After adding Cas13a-T (1 μM) and substrate chain (ssRNA,1 μM), the

obtained solution was incubated at 39 °C for 40 min before subjected for native PAGE.

For the Cas12a *trans*-cleavage activity characterization: Cas12a (200 nM)/Cas12a-sgRNA (1 μM) complex was pre-assembled in 1× NEBuffer 2.1. After adding Cas12a-T (1 μM) and substrate chain (ssDNA,1 μM), the obtained solution was incubated at 39 °C for 40 min before subjected for native PAGE characterization.

7. Fluorescence characterization

Validation of the *trans*-cleavage preferences of Cas13a: The Cas13a (200 nM)/Cas13a-sgRNA (1 μM) complex was pre-assembled in 1× NEBuffer 2.1. Cas12a (200 nM)/ Cas12a-sgRNA (1 μM) complex was pre-assembled in 1× NEBuffer 2.1. Subsequently, different concentrations of Cas13a-T (0 nM, 10 nM), and substrate chain (ssRNA and ssDNA, 1 μM each) were added to the Cas13a/Cas13a-sgRNA and Cas12a/Cas12a-sgRNA mixture. After 40 min incubation at 37 °C, fluorescence signal was recorded with a fluorescence spectrophotometer (λex(Cy5): 643 nm，λem(Cy5): 660 nm，λex(FAM): 485 nm，λem(FAM): 520 nm).

Validation of the *trans*-cleavage preferences of Cas12a: The Cas13a (200 nM)/Cas13a-sgRNA (1 μM) complex was pre-assembled in 1× NEBuffer 2.1. Cas12a (200 nM)/ Cas12a-sgRNA (1 μM) complex was pre-assembled in 1× NEBuffer 2.1. Subsequently, different concentrations of Cas12a-T (0 nM, 10 nM), and substrate chain (ssRNA and ssDNA, 1 μM

each) were added to the Cas13a/Cas13a-sgRNA and Cas12a/Cas12a-sgRNA mixture. After 40 min incubation at 37 °C, fluorescence signal was recorded with a fluorescence spectrophotometer (λex(Cy5): 643 nm，λem(Cy5): 660 nm，λex(FAM): 485 nm，λem(FAM): 520 nm).

8. Optimization of experimental conditions

Optimization of reaction temperature: the Cas13a (200 nM)/Cas13a-sgRNA (1 μM) complex was pre-assembled in 1× NEBuffer 2.1. The Cas12a (200 nM)/ Cas12a-sgRNA (1 μM) complex was pre-assembled in 1× NEBuffer 2.1. Subsequently, Cas13a-T, Cas12a-T, and substrate chain (ssRNA and ssDNA, 1 μM each) were added to the Cas13a/Cas13a-sgRNA and Cas12a/Cas12a-sgRNA mixture. After 40 min incubation at different temperatures (34°C, 37°C, 40°C, 43°C), fluorescence signals were recorded with a fluorescence spectrophotometer (λex(Cy5): 643 nm，λem(Cy5): 660 nm，λex(FAM): 485 nm，λem(FAM): 520 nm).

Optimization of reaction time: Cas13a (200 nM)/Cas13a-sgRNA (1 μM) complex was pre-assembled in 1× NEBuffer 2.1, the Cas12a (200 nM)/ Cas12a-sgRNA (1 μM) complex was pre-assembled in 1× NEBuffer 2.1. Subsequently, Cas13a-T, Cas12a-T, and substrate chain (ssRNA and ssDNA, 1 μM each) were added. After incubation at 40°C for different time (10 min, 20 min, 30 min, 40 min), fluorescence signals was recorded with a fluorescence spectrophotometer (λex(Cy5): 643 nm，λem(Cy5): 660 nm，λex(FAM): 485 nm，λem(FAM): 520 nm).

9. Exosomes detection

CD63apt (2 μM) /Cas12a-T (1 μM) or EpCAM-apt (2 μM)/Cas13a-T (1 μM) were annealed from 95°C to 4°C in 1× TAMg buffer. Then streptavidin magnetic beads were added. After 1 h incubation (1 h), the magnetic beads were magnetically separated. Exosomes were then incubated with the modified magnetic beads at room temperature for 30 min. After magnetic separation, the supernatant was collected for Cas12a/Cas13a system reaction. Pre-assembled Cas13a (200 nM)/Cas13a-sgRNA (1 μM) complex and pre-assembled Cas12a (200 nM)/Cas12a-sgRNA (1 μM) complex was mixed before supernatant and substrate chain (ssDNA and ssRNA, 1 μM each) addition. After incubation at 40℃ for 40 min, fluorescence signal was recorded with a fluorescence spectrophotometer (λex(Cy5): 643 nm，λem(Cy5): 660 nm，λex(FAM): 485 nm，λem(FAM): 520 nm).

10. Clinical serum samples detection

Serum samples were provided by the Affiliated Hospital of Nantong University (Nantong, China). All experiments using human serum samples were performed in compliance with Nantong University's policy and ethics about human subjects. Human serum samples were collected under the consent of the donors. These samples cannot be traced back to a specific person and the authors do not know the identity of the person(s) providing

the sample. The samples were diluted with PBS before detected with the same procedure for exosome detections.

11. ELISA analysis

Initially, standard samples or exosome samples, and HRP-conjugated detection antibodies were added to pre-coated wells with CD63 antibody and EpCAM, followed by an incubation at 37℃ and thorough washing. Subsequently, TMB substrate was added for colour development; TMB turns blue catalyzed by horseradish peroxidase and transitions to yellow upon acidification. Lastly, the absorbance (OD value) was measured at 450nm wavelength using a spectrophotometer to calculate sample concentrations. Exosome samples were incubated with the modified magnetic beads at room temperature for 30 min, before magnetic separation and subsequent ELISA procedures.

12. Characterization of the detection specificity with antibody blocking methods

To block the proteins CD63 and EpCAM on exosomes, we incubated exosomes separately with different concentrations of CD63 antibody and EpCAM antibody at 37°C overnight. Subsequently, they were incubated with the modified magnetic beads at room temperature for 30 min. Magnetic separation was then performed, and the resulting supernatant was analyzed with the Cas12a/Cas13a system.

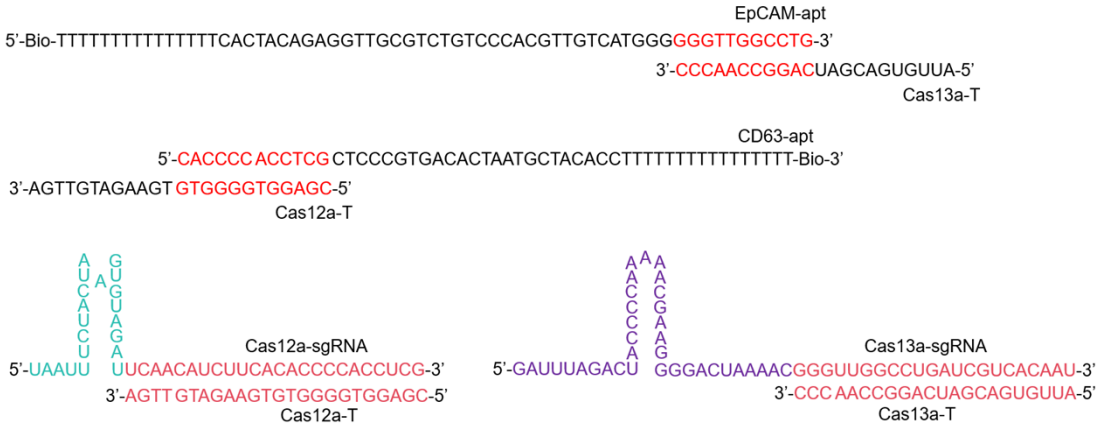

Figure S1 Schematic illustration of the nucleic acids used in this work.

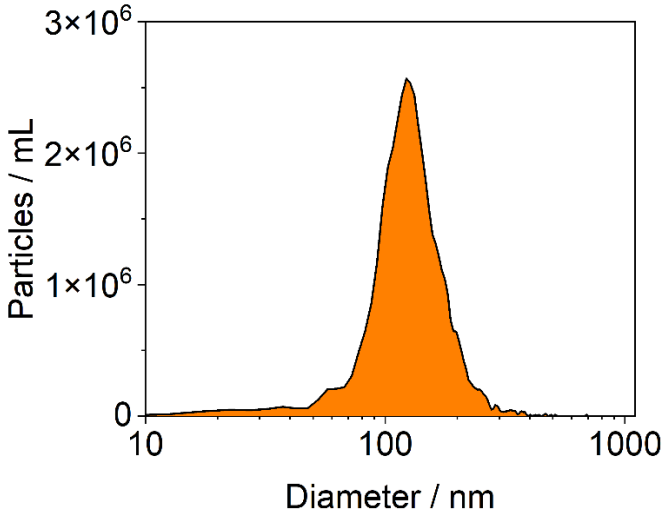

Figure S2 NTA characterization of exosomes derived from MCF-7 cells.

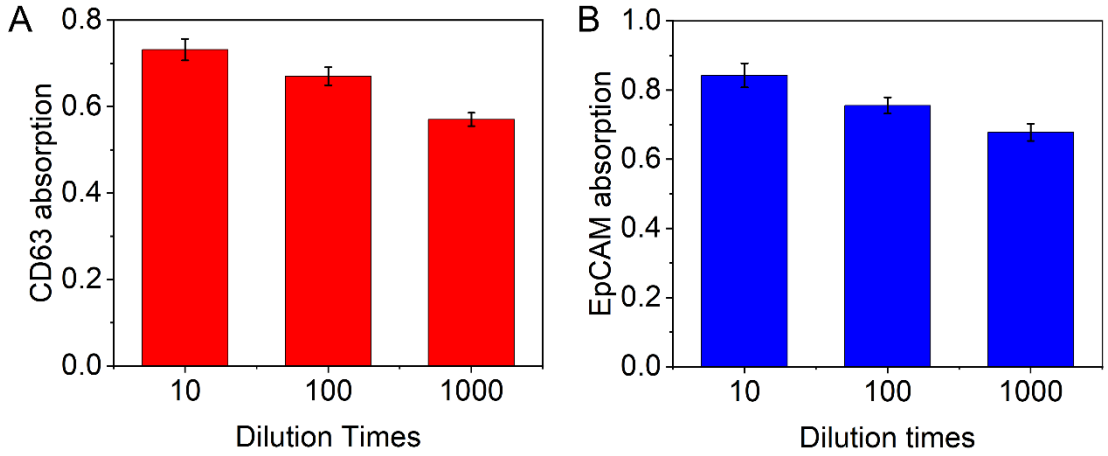

Figure S3 Characterization of the expression of CD63 (A) and EpCAM (B) protein on the exosome surface with ELISA. The exosome stock solution was diluted different times before analyzed with CD63 and EpCAM ELISA kits, respectively. We could find the signals decreased along with the increase of exosome dilution times for both CD63 and EpCAM, demonstrating the expression of CD63 and EpCAM protein on the exosome surface.

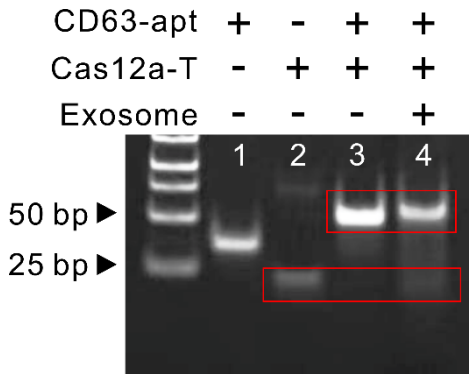

Figure S4 PAGE characterization of the recognition capability of exosomes with CD63-apt.

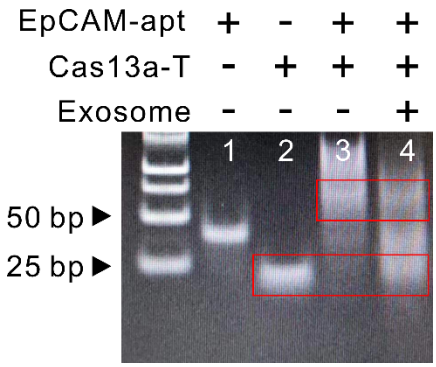

Figure S5 PAGE characterization of the recognition capability of exosomes with EpCAM-apt.

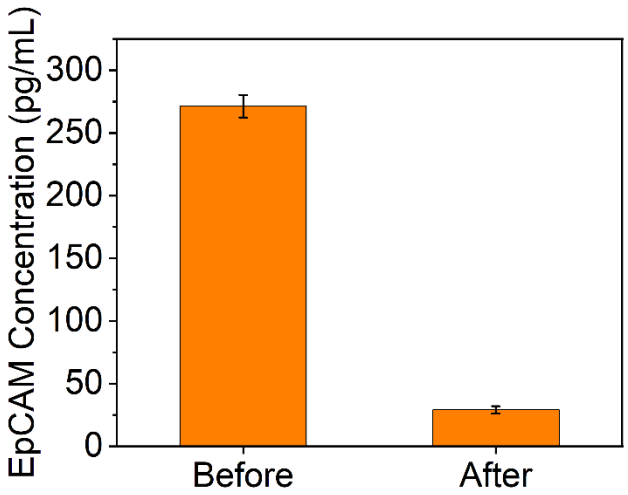

Figure S6. The calculated concentrations of the EpCAM protein detected through ELISA before or after incubation with the magnetic beads. Before: ccertain concentration of EVs (10^5 particles/mL) was analyzed with EpCAM ELISA kit. After: the same concentration of EVs were incubated with aptamer/target functionalized magnetic beads. After magnetic separation, the EVs in supernatant were then analyzed with EpCAM ELISA kit. The EpCAM concentrations were calculated through the established linear regression curve.

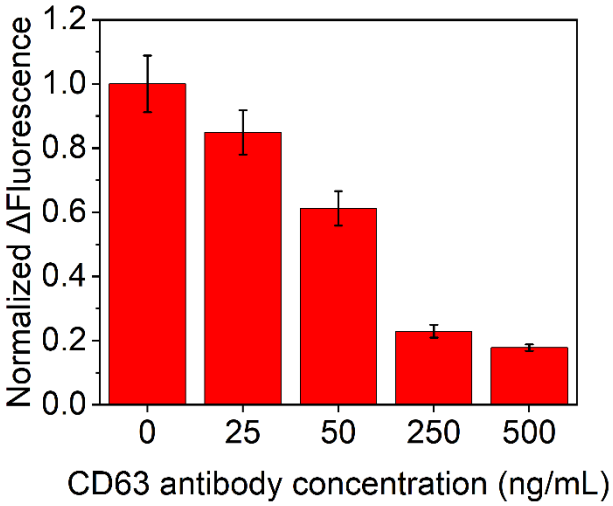

Figure S7. Specificity analysis using CD63 antibody blocking. We investigated whether other proteins on the exosome surface than CD63 could trigger the Cas12a cleavage. Through blocking exosome with CD63 antibody, the Cas12a fluorescence signals showed a concentration-dependent decrease, demonstrating less CD63 was available for aptamer binding. And other proteins on the exosome surface cannot be recognized by the CD63-apt. The fluorescence signals were normalized so that the highest signal was 1.

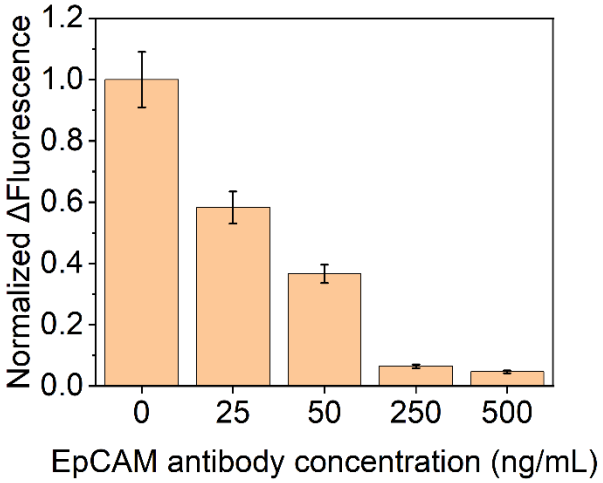

Figure S8. Specificity analysis using EpCAM antibody blocking. We investigated whether other proteins on the exosome surface than EpCAM could trigger the Cas13a cleavage. Through blocking exosome with EpCAM antibody, the Cas13a fluorescence signals showed a concentration-dependent decrease, demonstrating less EpCAM was available for aptamer binding. And other proteins on the exosome surface cannot be recognized by the EpCAM-apt. The fluorescence signals were normalized so that the highest signal was 1.

Table S1 Nucleic acids used in this work.

Oligonucleotides	Sequences （5'→3'）
EpCAM-apt	Bio-TTTTTTTTTTTTTTTCACTACAGAGGTTGCGTCTGTCCCACGTTGTCATGGGGGGTTGGCCTG
Cas13a-T	AUUGUGACGAUCAGGCCAACCC
Cas13a-sgRNA	GAUUUAGACUACCCCAAAAACGAAGGGGACUAAAAC GG GUU GGC CUG AUCGUCACAAU
CD63-atp	CACCCCACCTCGCTCCCGTGACACTAATGCTACA CCTTTTTTTTTTTTTTT-Bio
Cas12a-T	CG AGG TGG GGT GTGAAGATGTTGA
Cas12a-sgRNA	UAAUUUCUACUAAGUGUAGAUUCAACAUCUUCACACCCCACCUCG
ssDNA	6-FAM-AATAA-HBQ1
ssRNA	Cy5- UUUUU-BHQ-2

Table S2. Comparison with reported methods for the exosome detection.

No.	Method	Recognition type	Capability of multiplex detection	Dynamic range (particles/mL)	Reference
1	Simultaneous multiplexed in situ detection of exosomal	Antibody and Nucleic acids	Yes	10^5~10^9	[1]
2	Printed Divisional Optical Biochip	Antibody	Yes	6×10^7~6×10^9	[2]
3	Nanoplasmonic Sandwich Immunoassay	Antibody	No	1×10^3~750×10^3	[3]
4	DNA motor	Aptamer	No	20~2×10^6	[4]
5	DNAzyme walker-amplified electrochemical method	Aptamer	No	5×10^4~1.0×10^{10}	[5]
6	Orthogonal CRISPR-Cas	Aptamer	Yes	10^3 ~10^9	Our work

Table S3. Data for Figure 4D.

	Cas12a			Cas13a		
Control	124	115	100	155	181	163
Serum	122	138	114	130	143	165
Lysate	129	100	110	208	160	181

References

[1] S. Cho, H.C. Yang, W.J. Rhee, Simultaneous multiplexed detection of exosomal microRNAs and surface proteins for prostate cancer diagnosis, Biosensors and Bioelectronics 146 (2019).

[2] X. Yang, Z. Zhang, Y. Wu, H. Wang, Y. Yun, Y. Sun, H. Xie, B. Bogdanov, P. Senyushkin, J. Chi, Z. Lian, D. Wu, M. Su, Y. Song, Printed Divisional Optical Biochip for Multiplex Visualizable Exosome Analysis at Point-of-Care, Advanced Materials (2023).

[3] C. Wang, C.-H. Huang, Z. Gao, J. Shen, J. He, A. MacLachlan, C. Ma, Y. Chang, W. Yang, Y. Cai, Y. Lou, S. Dai, W. Chen, F. Li, P. Chen, Nanoplasmonic Sandwich Immunoassay for Tumor-Derived Exosome Detection and Exosomal PD-L1 Profiling, Acs Sensors 6(9) (2021) 3308-3319.

[4] Y. Yu, W.S. Zhang, Y. Guo, H. Peng, M. Zhu, D. Miao, G. Su, Engineering of exosome-triggered enzyme-powered DNA motors for highly sensitive fluorescence detection of tumor-derived exosomes, Biosensors and Bioelectronics 167 (2020) 112482.

[5] L. Zhao, R.J. Sun, P. He, X.R. Zhang, Ultrasensitive Detection of Exosomes by Target-Triggered Three-Dimensional DNA Walking Machine and Exonuclease III-Assisted Electrochemical Ratiometric Biosensing, Analytical Chemistry 91(22) (2019) 14773-14779.