Supplementary Information

Split G-quadruplex programmed label-free CRISPR-Cas12a sensing system

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Experimental details

1.1 Chemicals and reagents

Streptavidin beads were purchased from Abmart. Cas12a and 10× NEBuffer 2.1 were purchased from New England Biolabs (Beijing). All reagents were used as received without further purification.Diethyl pyrocarbonate (DEPC) treated water and high fat transfection reagent were purchased from Sangon biotechnology company. Anti-CD63 mouse monoclonal antibody was purchase from Santa Cruz Biotechnology. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), 0.25% trypsin-EDTA, and antibiotics (penicillin-streptomycin) were purchased from Gibco (Grand Island, NY). ThT was purchased from Aladdin Reagent Corporation (Shanghai, China). Cell lines, including liver cancer HepG2, breast cancer MCF-7, cervical cancer HeLa were obtained from American Type Culture Collection.

Apparatus

UV1800PC spectrophotometer (Shanghai, China) was used to quantify Oligonucleotide. Fluorescence signals were recorded on a RF-5301PC (Shimadzu) fluorospectro photometer. All DNA samples were annealed on an Applied Biosystems 96-well thermocycler. Native polyacrylamide gel electrophoresis (PAGE) was utilized to characterize the nucleic acids.

1.2 Nucleic acids

All DNA oligonucleotides including 5-biotin-labeled aptamers CD63 (CD63-Apt-bio), epithelial cell adhesion molecule (EpCAM-Apt), prostate-specific membrane antigen (PSMA-Apt), and carcinoembryonic antigen (CEA-Apt), 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 Fig. S1.

1.3 Exosomes isolation and characterization

Exosomes secreted by MCF-7, HeLa, and HepG2 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), 1000 *g* (4 ℃, 10 min), and 10000 *g* (4 ℃, 30 min). Then the supernatant was subjected to ultracentrifugation at 100000 *g* (4 ℃, 70 min). The resulting supernatant was removed. The precipitate was dispersed in sterile PBS (pH 7.4) to perform the ultracentrifugation 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).

1.4 Native polyacrylamide gel electrophoresis (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 split G4 assembly process characterization: 6a, 6b, L4 were utilized here as the G4-a, G4-b, and Link, respectively. Equivalent of component strands (1 μM each) were annealed in 1× TAMg buffer from 95 ℃ to 4 ℃. The resulting samples were then analyzed with 8 % native (PAGE).

For the Cas12a *trans*-cleavage activity characterization: Cas12a (200 nM)/EpCAM-sgRNA (1 μM) complex was pre-assembled in 1× NEBuffer 2.1. After adding EpCAM apt (1 μM) and substrate chain (Link strand, 1 μM), the obtained solution was incubated at 37 °C for 40 min before subjected for native PAGE.

1.5 The investigation of spacer length

The Link strand (1 μM) with different spacer lengths (L0, L4, L8, L12, L16, L20, L24), 6a (1 μM) and 6b (1 μM) were added into the 1× NEBuffer 2.1 buffer containing 300 mM KCl with 5 μM ThT. The resulting solution was incubated for 20 min before the fluorescence signals were recorded (λ_{ex}: 405 nm, λ_{em}: 485 nm).

1.6 Optimization of the ThT/G4 ratio

6a, 6b, L4 were utilized as the G4-a, G4-b, and Link, respectively. 6a, 6b, and L4 (1 μM each) were added into the 1× NEBuffer 2.1 buffer containing 300 mM KCl. Different amounts of ThT (0.2 μM, 0.4 μM, 0.6 μM, 0.8 μM, 1 μM, 1.5 μM, 2 μM, 3 μM, 5 μM, 10 μM, 20 μM, 30 μM) were added into the resulting split G4 complex. The fluorescence signals were recorded after 20 min (λ_{ex}: 405 nm, λ_{em}: 485 nm).

1.7 Exosomes detection

The CD63 Apt-bio strand (4 μM) was incubated with exosomes at room temperature for 30 min. Then streptavidin magnetic beads were added for incubation (1 h) before magnetic separation. The beads were then dispersed in 1× NEBuffer 2.1 buffer. Atp 2 (EpCAM Apt, PSMA Apt or CEA Apt) was added to the above solution to reach a final concentration of 0.1 μM. After 20 min reaction at 37℃, the magnetic beads were discarded. The residual supernatant was collected for Cas12a reaction. Taking EpCAM detection as an example, Cas12a (200 nM)/EpCAM-sgRNA (1 μM) complex was pre-assembled in 1× NEBuffer 2.1 before adding to the supernatant with L4 strand, which served as the substrate of Cas12a. After incubation at 37℃ for 40 min, 6a (1 μM), 6b (1 μM) and ThT (5 μM) were added into the solution along with 300 mM KCl. Six minutes later, the fluorescence was measured.

1.8 Clinical serum samples detection

Serum samples, from healthy volunteers and breast cancer patients,

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 from volunteers 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.

1.9 Cell culture

MCF-7 and HeLa cells were cultured with DMEM complete medium, and HepG2 cells were cultured with 1640 complete medium (DMEM basic medium or 1640 basic medium with 10% fetal bovine serum and 1% penicillin and 100 μg/mL streptomycin mixed evenly). After dispersing the cells evenly in DMEM or 1640 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 or 1640 complete medium. The dish was washed twice with sterile PBS, and then a certain amount of DMEM basic medium as 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.

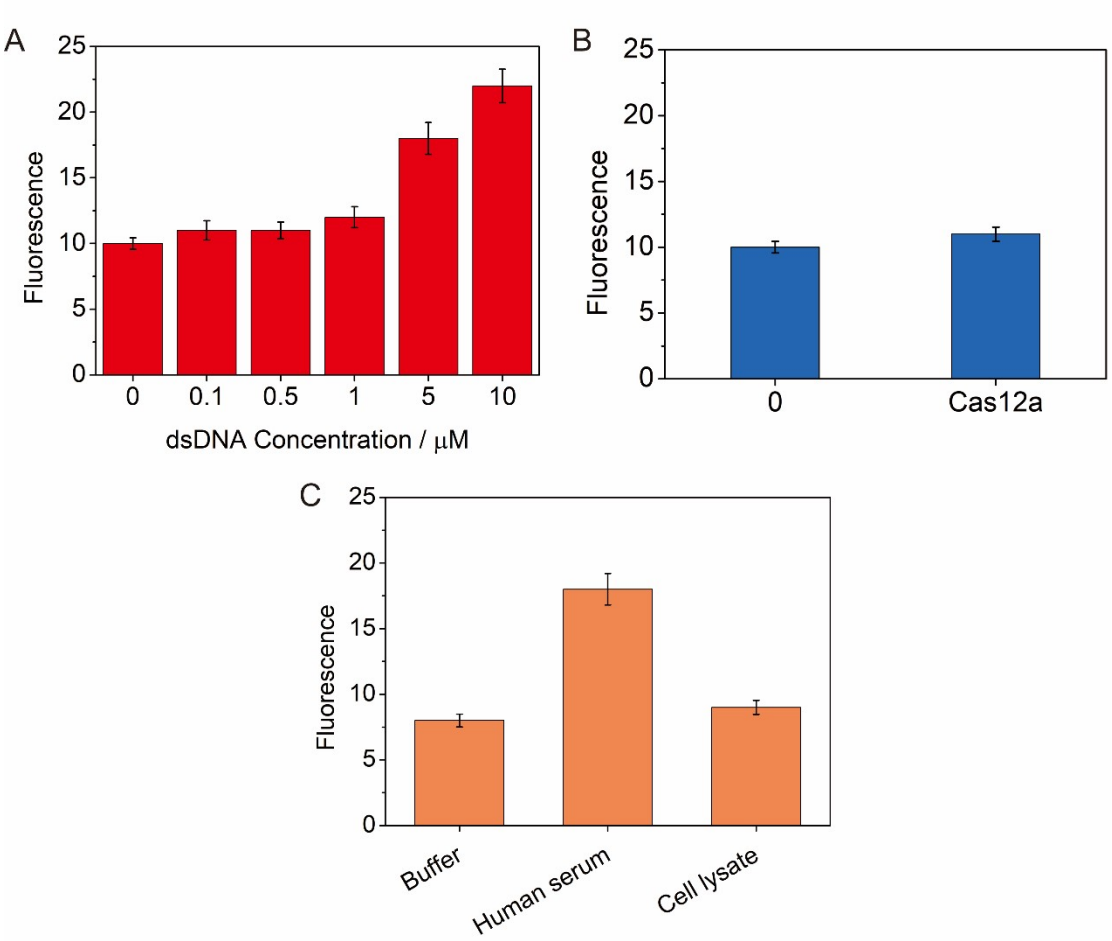

Fig. S1 Nonspecific binding of ThT with dsDNA (A), Cas12a (B) and biological matrix (C). The dsDNA was obtained by annealing L4 and L4-C from 95 ℃ to 4 ℃. 5 μM ThT was utilized throughout the above binding experiments. 200 nM Cas12a was utilized in Fig. S1B. Human serum sample (from healthy volunteer) was obtained from local hospital and stored at -20 ℃ for usage. HeLa cells (1×10^6 mL^{-1}) were lysed via low-frequency ultrasonic wave. For ThT binding assay in Fig. S1C, certain amount of cell lysate or human serum were spiked into the reaction buffer to reach a final volume ratio of 1% (v/v).

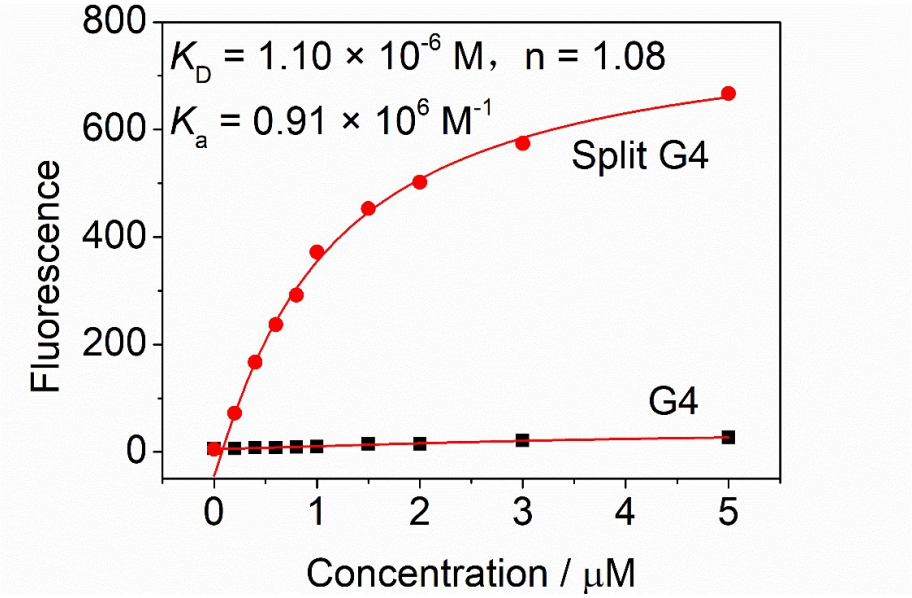

Fig. S2 The ThT affinity with the intact T30695 and the split G4. Different concentrations of intact T30695 or the split G4 (0 μM, 0.2 μM, 0.4 μM, 0.6 μM, 0.8 μM, 1 μM, 1.5 μM, 2 μM, 3 μM and 5 μM) were added into the ThT solution (5 μM) containing 300 mM K^+, respectively. After incubation at 37 ℃ for 6 min, the fluorescence signals were measured (λ_{ex}: 405 nm, λ_{em}: 485 nm). The ThT/split G4 binding curve was fitted with the equation: $y = START + (END - START)\frac{x^n}{k^n + x^n}$.

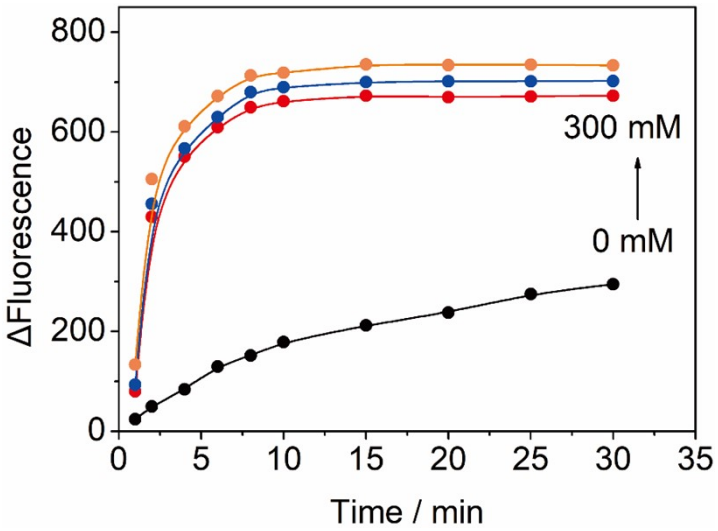

Fig. S3 The influence of K^+ on the fluorescence signal of split G4/ThT. Five-fold ThT was added into the pre-annealed split G4 solutions (G4-a, G4-b and L4, 1 μM each) containing 0 mM, 100 mM, 200 mM, and 300 mM K^+, respectively. The fluorescence was recorded at an interval of 2 min (0-10 min) or 5 min (10-30 min).

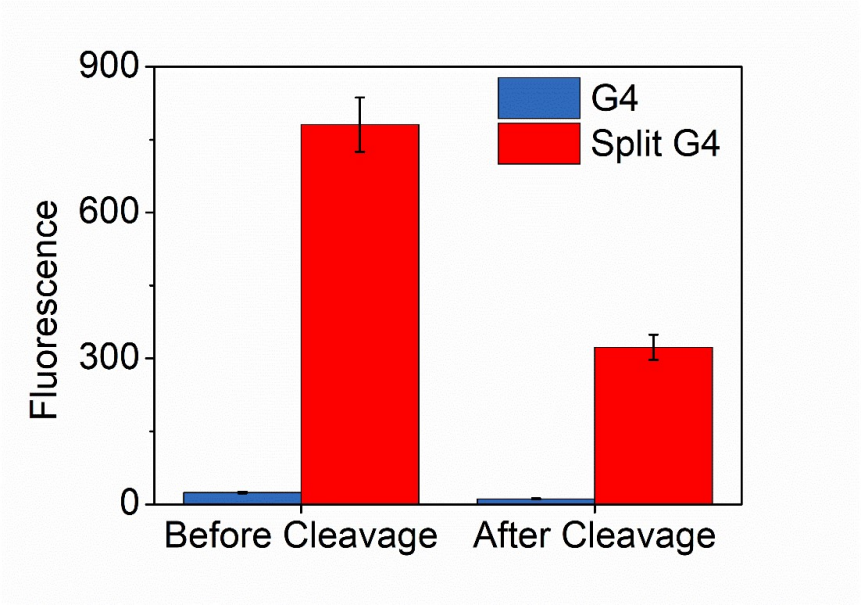

Fig. S4 The cleavage of G4 and split G4 with Cas12a. Cas12a (200 nM)/EpCAM-sgRNA (1 μM) complex was pre-assembled in 1× NEBuffer 2.1 before adding G4 or link (without K^+). After incubation at 37℃ for 40 min, 6a (1 μM), 6b (1 μM) and ThT (5 μM) were added into the solution along with 300 mM KCl. Six minutes later, the fluorescence was measured.

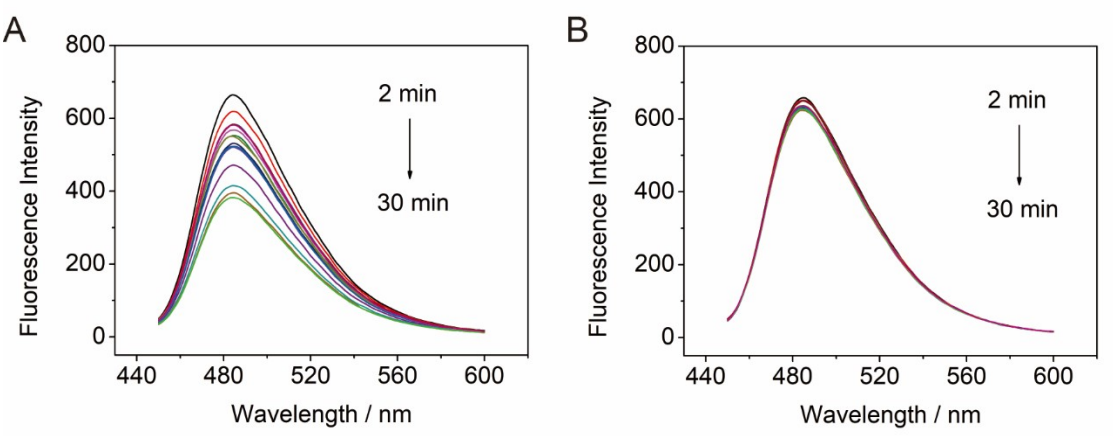

Fig. S5 The stability of split G4/ThT under the cleavage of activated Cas12a in the presence of 100 mM (A) and 300 mM K^+ (B). The fluorescence spectra were recorded at an interval of 2 min after the addition of Cas12a into the solution of split G4/ThT.

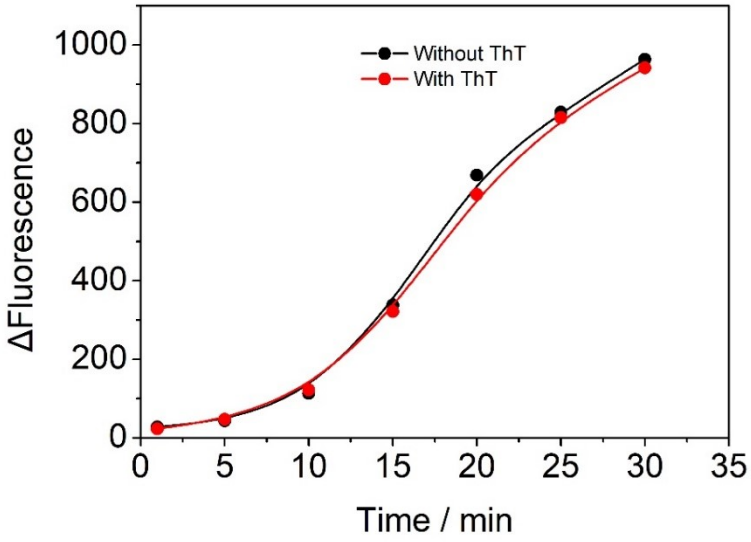

Fig. S6 The influence of ThT on the trans-cleavage activity of Cas12a. FQ-labelled ssDNA (5'-FAM-TTATT-BHQ1-3') was utilized as the reporter substrate.

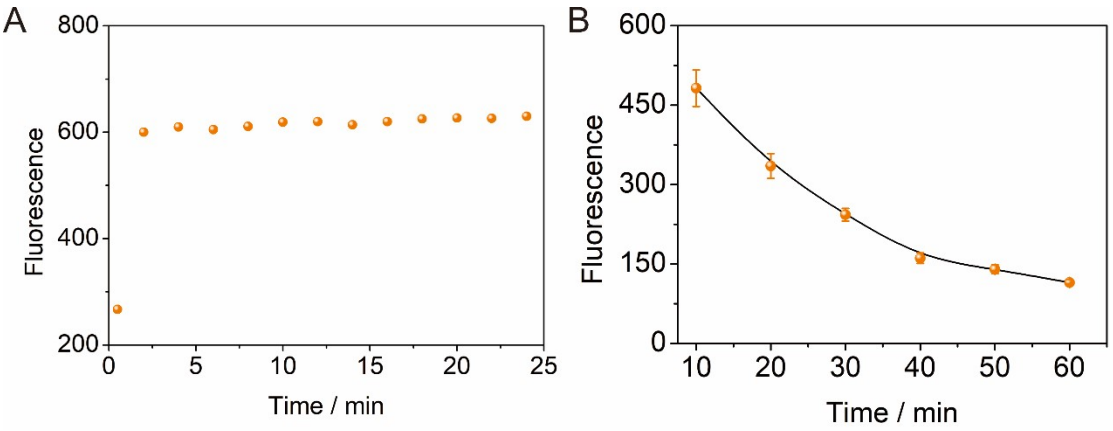

Fig. S7 Time optimization of the reaction system. (A) Time optimization for split G4/ThT complex formation. Plot of the fluorescence intensity against the time after addition of 6a, 6b, L4 and ThT to the solution of activated Cas12a. (B) The *trans*-cleavage kinetic plot of Cas12a. Cas12a was pre-assembled with EpCAM-sgRNA. Then EpCAM-Apt and the L4 strand were added to the solution simultaneously to trigger the cleavage process. After cleavage for 10, 20, 30, 40, 50, and 60 min, respectively, 6a (1 μM), 6b (1 μM) and ThT (5μM) were added into solution along with 300 mM K^+. After incubation for 6 min, the fluorescence intensities were measured. The error bars represented the standard deviations of three repetitive experiments.

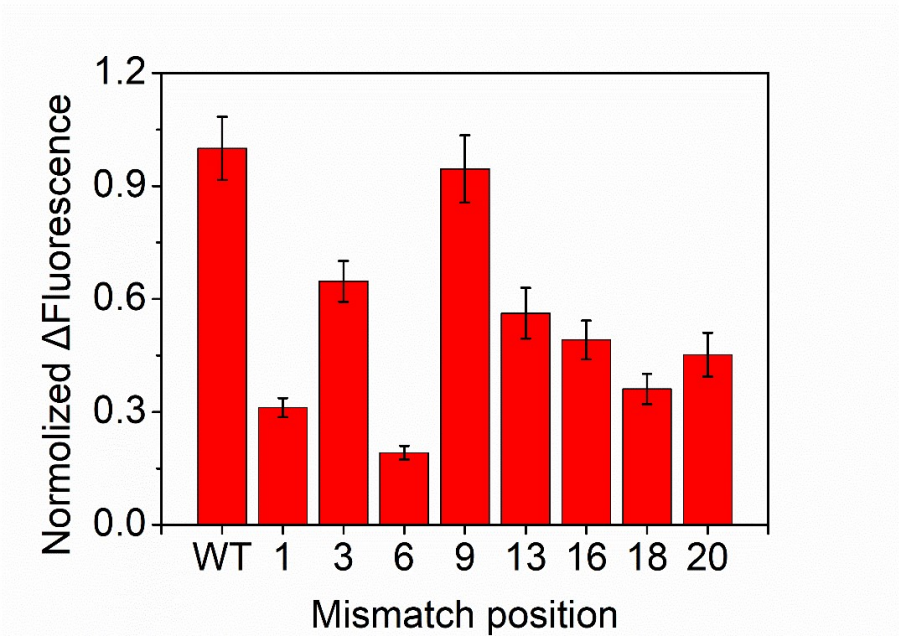

Fig. S8 Specificity of the split G4 programmed Cas12a sensing system. Error bars represent mean ± SD, where n = 3 replicates. WT: wild type sequence without mismatch. The mismatch position was numbered from 5’ to 3’.

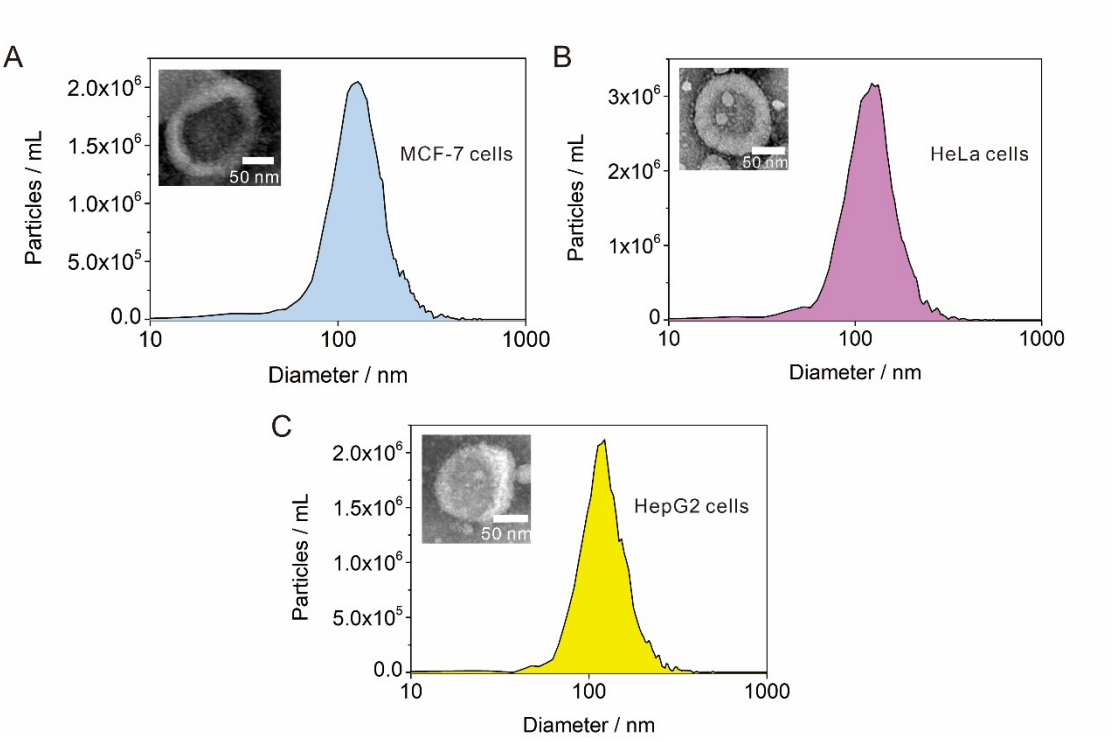

Fig. S9 TEM and NTA characterization of exosomes derived from MCF-7 cells (A), HeLa cells (B) and HepG2 cells (C).

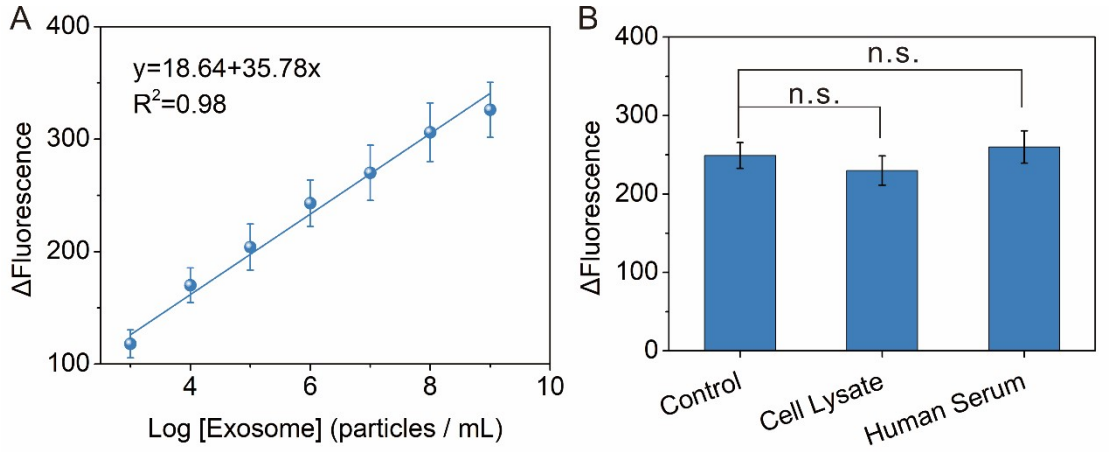

Fig. S10 Exosomal protein detection with the split G4-programmed Cas12a strategy. (A) Calculation curve between the increased fluorescence intensity (ΔF) and the logarithm of the number of exosomes. (B) Detection of the tumor-derived exosomes in biological matrix spiked with 1% cell lysate or ultracentrifuged human serum. All measurements were repeated three times, and the data arewere shown as the mean ± standard deviation.

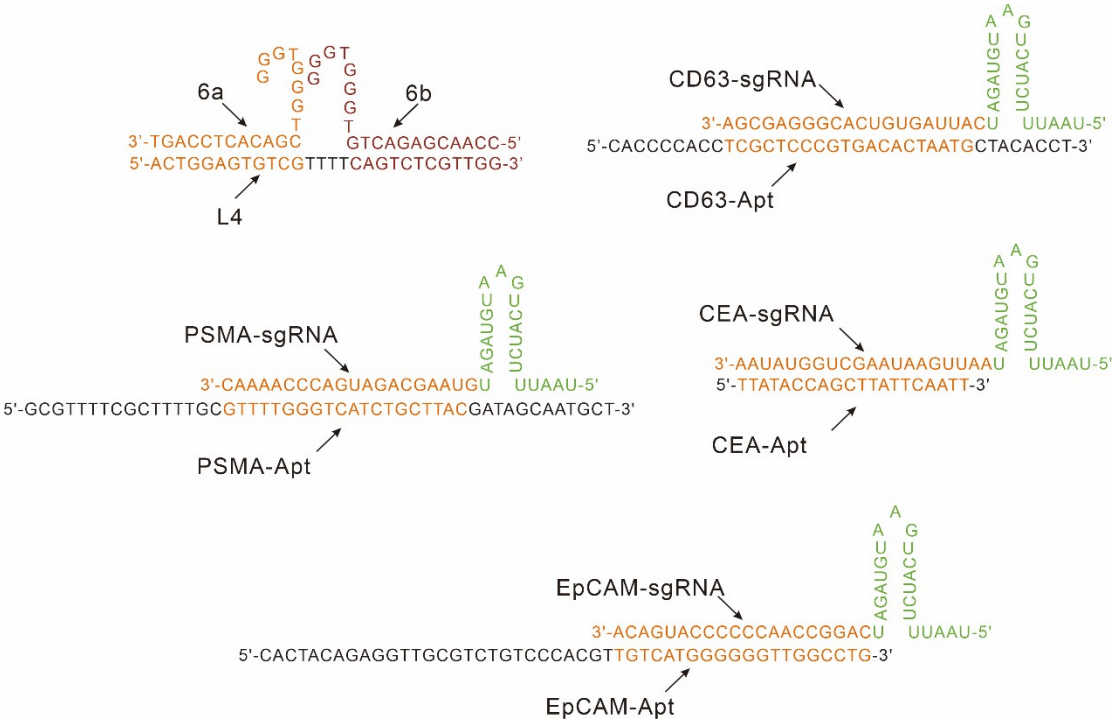

Fig. S11 The illustration of the nucleic acids used in this work.

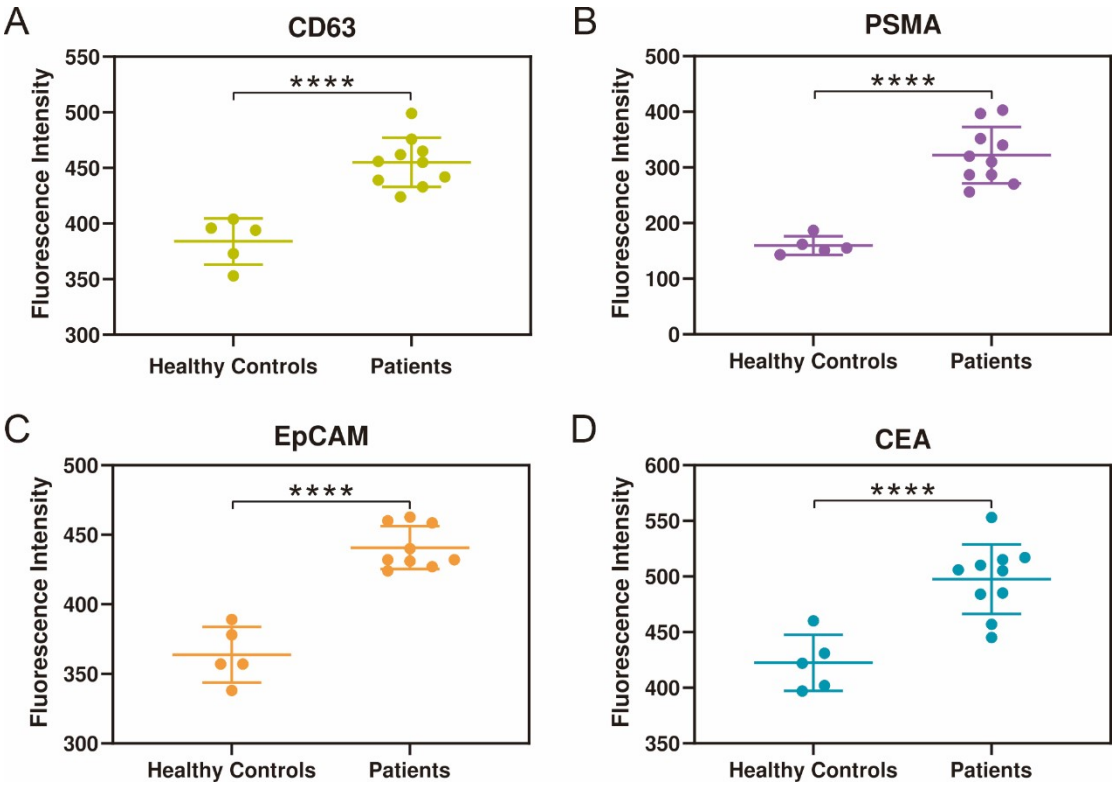

Fig. S12 Exosomal protein profiling of clinical serum samples. The expression-level of CD63 (A), PSMA (B), EpCAM (C), and CEA (D) in the serum samples from healthy controls and breast cancer patients. (****, $P < 0.0001$).

Table S1. Sequences and oligonucleotides used in this work.

Name	Sequences (5' to 3')
G4	GGGTGGGTGGGTGGGT
6a	CCAACGAGACTGTGGGTGGG
6b	GGGTGGGTCGA CAC TCC AGT
8a	CCAACGAGACTGTGGGTGGGTGG
4b	GTGGGTCGA CAC TCC AGT
1a	CCAACGAGACTGTG
11b	GGTGGGTGGGTGGGTCGA CAC TCC AGT
2a	CCAACGAGACTGTGG
10b	GTGGGTGGGTGGGTCGA CAC TCC AGT
3a	CCAACGAGACTGTGGG
9b	GGGTGGGTGGGTCGA CAC TCC AGT
5a	CCAACGAGACTGTGGGTGG
7b	GTGGGTGGGTCGA CAC TCC AGT
L0	ACTGGAGTGTCGCAGTCTCGTTGG
L4	ACTGGAGTGTCGTTTTCAGTCTCGTTGG
L4-C	CCAACGAGACTGAAAACGACACTCCAGT
L8	ACTGGAGTGTCGTTTTTTTTCAGTCTCGTTGG
L12	ACTGGAGTGTCGTTTTTTTTTTTTCAGTCTCGTTGG
L16	ACTGGAGTGTCGTTTTTTTTTTTTTTTTCAGTCTCGTTGG
L20	ACTGGAGTGTCGTTTTTTTTTTTTTTTTTTTCAGTCTCGTTGG
L24	ACTGGAGTGTCGTTTTTTTTTTTTTTTTTTTT

	TTTTCAGTCTCGTTGG
EpCAM-Apt	CAC TAC AGA GGT TGC GTC TGT CCC ACG TTG TCA TGG GGG GTT GGC CTG
EpCAM-Apt-M1	CACTACAGAGGTTGCGTCTGTCCCACGTAG TCATGGGGGGTTGGCCTG
EpCAM-Apt-M3	CACTACAGAGGTTGCGTCTGTCCCACGTTG ACATGGGGGGTTGGCCTG
EpCAM-Apt-M6	CACTACAGAGGTTGCGTCTGTCCCACGTTG TCAAGGGGGGTTGGCCTG
EpCAM-Apt-M9	CACTACAGAGGTTGCGTCTGTCCCACGTTG TCATGGCGGGTTGGCCTG
EpCAM-Apt-M13	CACTACAGAGGTTGCGTCTGTCCCACGTTG TCATGGGGGGATGGCCTG
EpCAM-Apt-M16	CACTACAGAGGTTGCGTCTGTCCCACGTTG TCATGGGGGGTTGCCCTG
EpCAM-Apt-M18	CACTACAGAGGTTGCGTCTGTCCCACGTTG TCATGGGGGGTTGGCGTG
EpCAM-Apt-M20	CACTACAGAGGTTGCGTCTGTCCCACGTTG TCATGGGGGGTTGGCCTC
EpCAM-Apt-bio	Bio-TTTTTTTTTTTTTTCACTACAGAGGTTGCG TCTGTCCCACGTTGTCATGGGGGGTTGGCC TG
EpCAM-sgRNA	UAA UUU CUA CUA AGU GUA GAU CAG GCC AAC CCC CCA UGA CA
CD63-Atp	CACCCCACCTCGCTCCCGT GAC ACT AAT GCT ACA CCT

CD63-Atp-bio	Bio- TTTTTTTTTTTTTTCACCCCACCTCGCTCCC GT GAC ACT AAT GCT ACA CCT
CD63-sgRNA	UAAUUUCUACUAAGUGUAGAU C AUU AGU GUC ACG GGA GCG A
CEA-Apt	TTATA CCA GCT TAT TCA ATT
CEA-sgRNA	UAA UUU CUA CUA AGU GUA GAU AAU UGA AUA AGC UGG UAU AA
PSMA-Apt	GCG TTT TCG CTT TTG CGT TTT GGG TCA TCT GCT TAC GAT AGC AAT GCT
PSMA-sgRNA	UAA UUU CUA CUA AGU GUA GAU GUA AGC AGA UGA CCC AAA AC
FQ-labelled ssDNA	FAM-TTATT-BHQ1

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

No.	Method	Sample type	LOD (particles/mL)	Dynamic range (particles/mL)	Reference
1	DNA motor	MCF-7 cell-derived exosomes in PBS	8.2×10^{3}	$20\sim2\times10^{6}$	[1]
2	ExoAPP[a]	HepG2 cell-derived exosomes in PBS	1.6×10^{5}	$1.6\times10^{5}\sim1.6\times10^{8}$	[2]
3	CRISPR/Cas12a	A549 cell-derived exosomes in PBS	Not Provided	$3\times10^{3}\sim6\times10^{7}$	[3]
4	Covalent organic frameworks based nanoprobe	Colo 205 cell-Derived Exosome in PBS	1.60×10^{5}	$5\times10^{5}\sim10^{10}$	[4]
5	zirconium-mediated fluorescence method	HeLa cell-derived exosomes in PBS	7.6×10^{6}	$1.68\times10^{7}\sim4.2\times10^{10}$	[5]
6	DNAzyme walker-amplified electrochemical method	MCF-7 cell-derived exosomes in PBS	1.3×10^{4}	$5\times10^{4}\sim1.0\times10^{10}$	[6]
7	Split G4 programmed Cas12a	MCF-7 cell-derived exosomes in PBS	258.8	$10^{3}\sim10^{11}$	Our work

[a] exosome-oriented, aptamer nanoprobe-based profiling assay.

Table S3. Standard addition analysis for tumor-derived exosomes in 1% ultracentrifuged human serum.

Samples	Added (Particles / mL)	Recovered (Particles / mL)	Rate of recovery (%)	RSD (%)
1	1.0×10^{8}	0.946×10^{8}	94.6	8.4
2	1.0×10^{6}	1.052×10^{6}	105.2	9.7
3	1.0×10^{4}	0.954×10^{4}	95.4	10.5

[a] Values represented mean of three measurements.

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