Supporting Information

Template-free multiple signal amplification for highly sensitive detection of cancer cell-derived exosomes

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1. Experiment section

1.1. Reagents and materials

DNase I and TdT enzymes are purchased from Beyotime Biotechnology. All customized DNA strands used in this study are synthesized and purified from Sangon Biotech (Shanghai) Co., Ltd. These DNA sequences are displayed in Table S1. dATP is purchase from Takara. Magnetic beads modified with streptavidin (SA-MBs) are purchased from Beaver Biosciences Inc. Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) are obtained from Thermo Fisher Scientific Inc. SYBR Green I (SGI) dye is purchased from Jiangsu KeyGEN BioTECH Co., Ltd.

1.2. Cell culture

HeLa and HT29 cells purchased from ATCC are cultured in high-glucose DMEM medium with 1% (v/v) penicillin−streptomycin and 10% (v/v) FBS. These cells are maintained at the temperature of 37 °C with 5% CO_2 in a humidified atmosphere. When these cells grow at 70 confluency, the cell culture medium is removed and the cells are gently rinsed twice with phosphate-buffered saline (PBS), followed by incubation for another 48 h in exosomes-free DMEM medium with 1% (v/v) penicillin−streptomycin. Finally, the medium of these cells is collected for the purification of exosomes.

1.3. Exosomes extraction from cell medium

Firstly, the collected supernatant is centrifuged at the speed of 3000 g for 20 min to remove large particles and cellular debris. Then, the microvesicles of the suspension medium are discarded by centrifuged at 10000 g for 30 min. To get rid of other large cell-secreted vesicles, the cell supernatant is filtered via a 0.22 μm syringe filter. Finally, exosomes can be received in the bottom of centrifuge tube by centrifugation at 120000 for 2 h and the sediment is resuspended with 200 μL of PBS. The purified exosomes are stored at -80 °C for further use.

1.4. The characterization of exosomes

The transmission electron microscopy (TEM) image of the isolated exosomes is performed via negative staining using 1% sodium phosphotungstate (dissolved in PBS buffer) with modification. Briefly, 5 μL of the purified exosomes are gently dropped

onto carbon-coated copper grids for 5 min at room temperature. The residual buffer is discarded and the exosomes can be absorbed on the grids. Then, 5 μL of sodium phosphotungstate (dissolved in PBS buffer) is dropped onto the grids and sustained for 2 min. After removal of the staining solution, the grids are quickly rinsed with 10 μL of H_2O to get rid of redundant saline substance. Finally, the prepared samples are dried at room temperature and then imaged by Hitachi H-7650 TEM. The concentration and size distribution of exosomes is measured via nanoparticle tracking analysis.

1.5 Exosomes detection

50 μL of SA-MBs (10 mg/mL) is incubated with 5 μL of biotin-modified CD63 antibody (0.5 mg/mL) at the temperature for 1 h. Then the antibody-modified MBs are washed three times with PBS buffer, following by incubation with 100 μL of 10% BSA to block MBs. The prepared MBs is diluted with PBS buffer and stored in 4 °C for further use.

For the exosomes detection, 2.5 μL of Apt_{MUC1} (1 μM), 1 μL of $MgCl_2$ (200 mM) and 5 μL of exosomes are incubated with 2.5 μL antibody-modified MBs in 40 μL of 0.01% PBST at the temperature of 37 °C for 1 h. After being washed three times with 0.05% PBST buffer, exosomes-captured MBs can be performed with HCR for 2 h at 37 °C by adding PBS buffer containing H1 and H2 strands. The unbound DNA strands of MBs are removed via washing three times with 0.05% PBST buffer. Next, 1 μL of DNase I (1 U/μL) is incubated with MBs in 20 μL of reaction buffer for 1 h at 37 °C. After magnetic separation, the MBs are discarded and DNase I in the supernatant are inactivated via treated with 85 °C for 20 min. Afterwards, 2 μL of KAc (1 M), 2 μL of $CoCl_2$ (10 mM), 2 μL of of dATP (10 mM), and 1 μL of TdT (5 U/μL) are incubated with short ssDNA in the supernatant for 1.5 h at 37 °C. Finally, the TdT-mediated reaction is terminated by heat-inactivation with 75 °C for 10 min. The fluorescence signal of the reaction solution is measured via F-7000 spectrometer or microplate reader after the addition of T_{25}, SGI dye and PBS buffer to meet a volume of 100 μL.

1.6. Clinical sample analysis

Clinical plasma samples of patients and healthy individuals are firstly centrifuged at the speed of 3500 g for 20 min to remove cellular debris and large particles. Then, after

being filtered via a 0.22 μm syringe filter to get rid of other large cell-derived vesicles and diluted by five times using PBS buffer, the supernatant is analyzed by this proposed method. The inclusion criteria of patients: colorectal cancer is confirmed by colonoscopy and pathology with no previous history of other malignancies. Meanwhile, patients diagnosed with systemic infectious diseases, mental diseases, and other serious organic diseases are excluded. All experiments are performed in compliance with the scientific ethical committee of Nanjing University.

1.7. Electrophoresis experiments

For the analysis of the HCR, 100 nM Apt_{MUC1} is incubated with 500 nM H1 and H2 for 1 h at 37 °C. After that, 1 μL of DNase I (1 U/μL) is added into the product of HCR and incubated at 37 °C for 0.5 h. Subsequently, the above samples stained with GelRed dye are loaded into 10% PAGE and the electrophoresis is performed in TBE buffer (90 mM Tris, 90 mM Boric acid, and 2 mM EDTA; pH 8.2) for 1 h at 90 V. Finally, the gel image is obtained via Gel Imaging System.

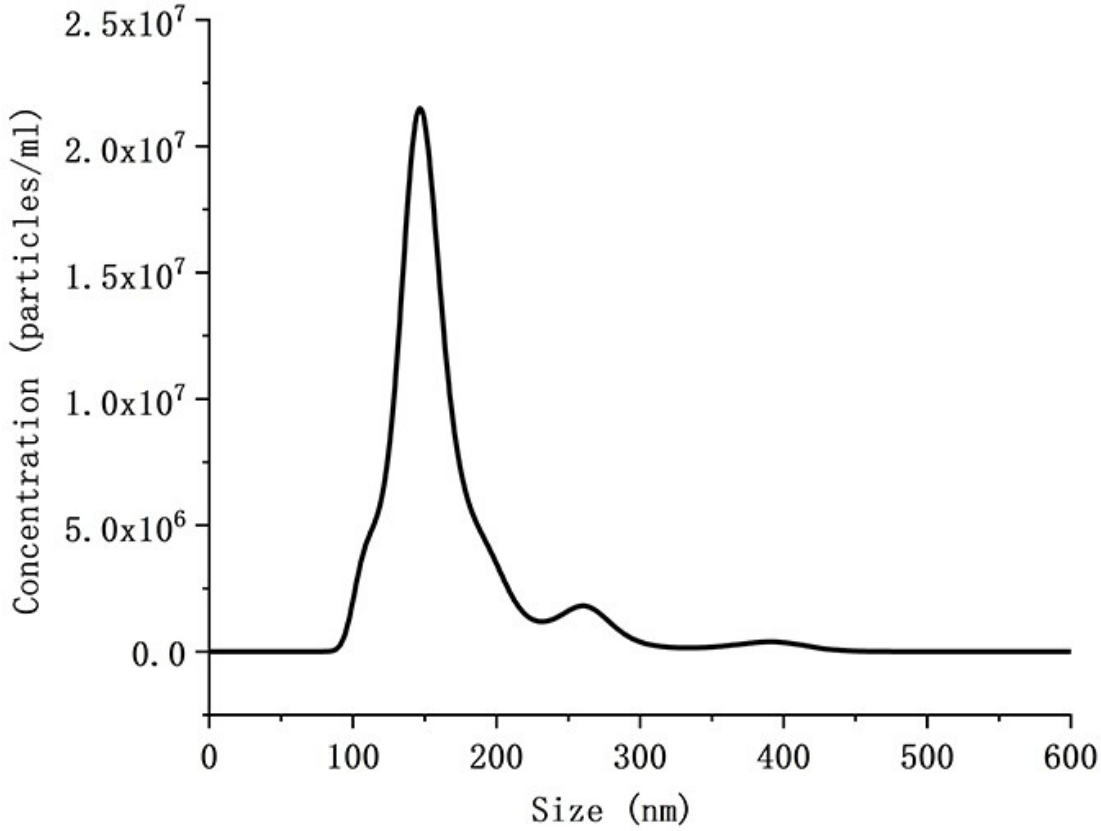

Figure S1. The size distribution characterization of exosomes using NanoSight. The purified exosomes are diluted 10 times with PBS buffer before measured.

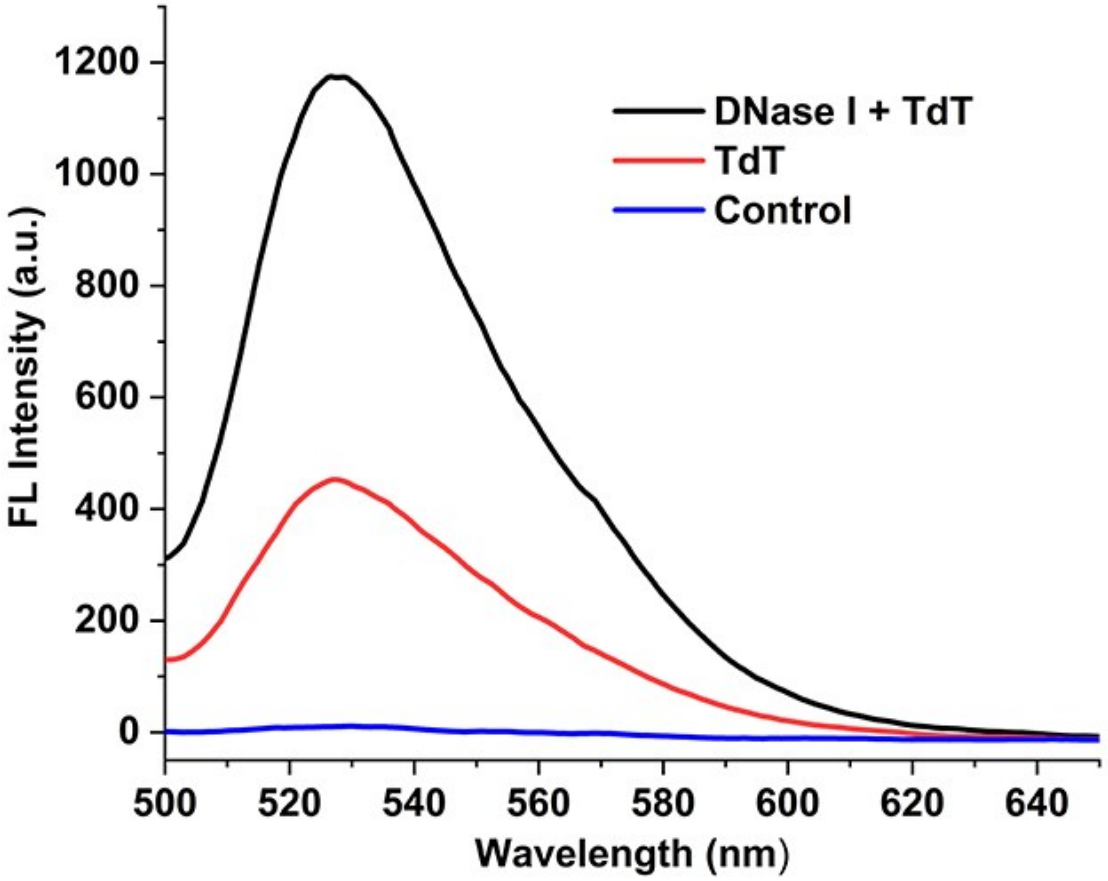

Figure S2. Verification of signal amplification by TdT and DNase I. Blue, red, and black curves denote generated fluorescence intensity corresponding to without enzyme, with TdT enzyme, and with TdT + DNase I enzymes, respectively. The substrates of enzymes are random ssDNA and dATP. The fluorescence is generated by incubation with T_{25} and SGI. The content of TdT and DNase I enzyme is 5 U and 0.1 U, respectively. The experiment is conducted at 37 °C for 80 min.

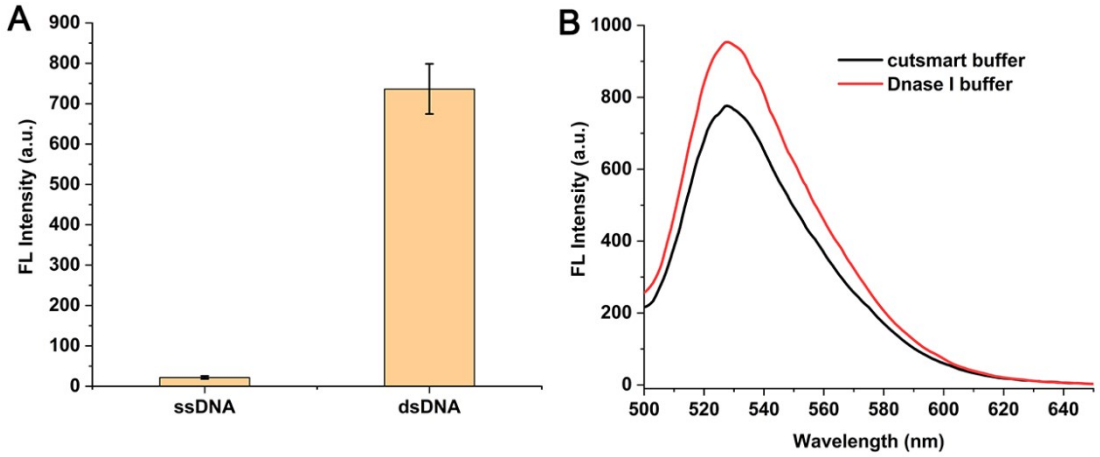

Figure S3. (A) Comparison of fluorescence intensity of SYBR Green I dye combined with ssDNA and dsDNA. (B) Comparison of TdT activity in cutsamart and Dnase I buffer. The content of TdT enzyme is 5 U, and the experiment is conducted at 37 ℃ for 60 min.

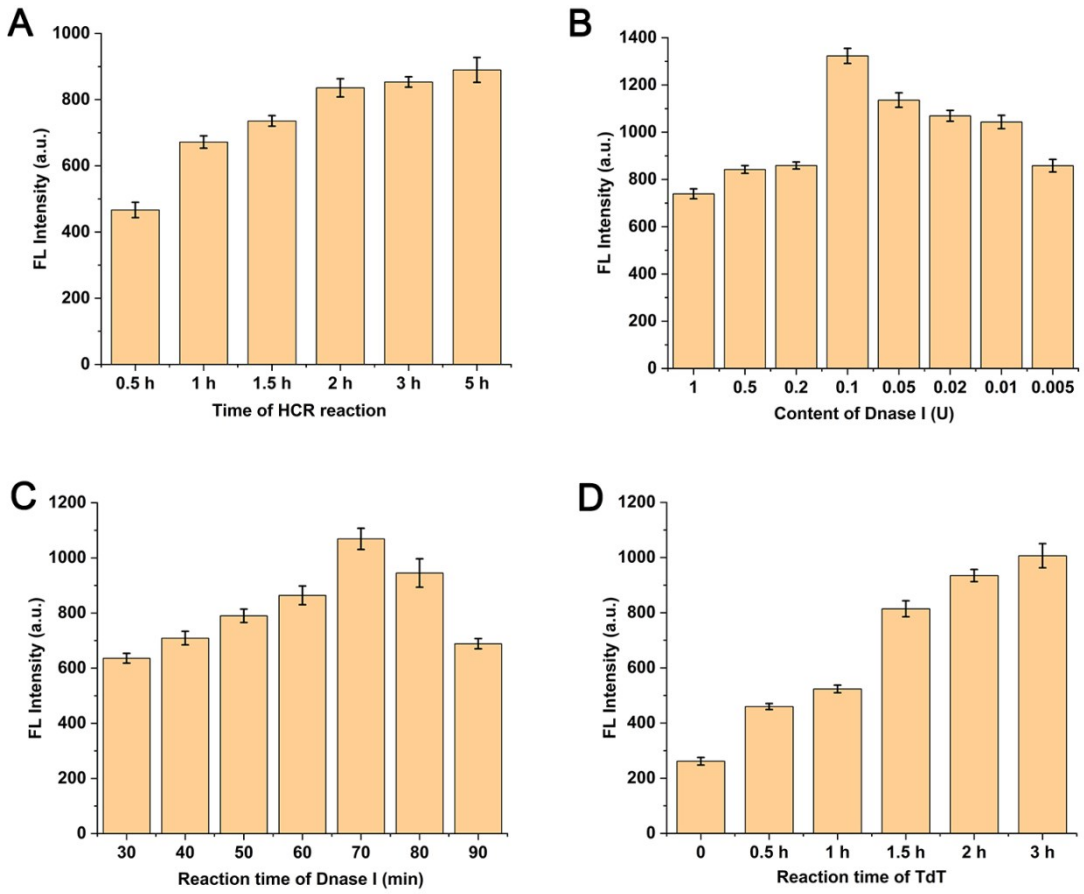

Figure S4. The optimization of experimental conditions. (A) The relation between the fluorescence signal and the incubation time of HCR. (B) The optimization of the content of Dnase I where the reaction time of Dnase I is 1 h. (C) The relation between the fluorescence signal and the incubation time of Dnase I where the content of Dnase I is 0.1 U. (D) The optimization of reaction time of TdT where the content of TdT is 5 U. Error bars represent the standard deviation of three repeat experiments.

Table S1. The nucleic acids sequence used in this assay.

Name	Sequence（5’ - 3’）
Apt_{MUC1}	GCAGTTGATCCTTTGGATACCCTGGTTTTTTTGAGGTAGTAGGTTGTATAGTT
H1	AGTAGGTTGTATAGTTCAAAGTAACTATACAACCTACTACCTCA
H2	ACTTTGAACTATACAACCTACTTGAGGTAGTAGGTTGTATAGTT
T_{25}	TTTTTTTTTTTTTTTTTTTTTTTTT
random ssDNA	CATCTGAGTACTGA

Table S2. Comparison of different assays for the detection of exosomes.

Method	**Signal label**	**Signal output**	**Detection limit (particles/μL)**	**Reference**
dual-signal amplification	fluorescent dye	fluorescence	1×10^2	[1]
paper-supported aptasensor	gold nanorods	fluorescence	1.1×10^3	[2]
multipedal DNA walker	methylene blue	electrochemistry	6	[3]
magneto−electrochemical detection	HRP	electrochemistry	3×10^3	[4]
stimuli-responsive DNA microcapsules	label-free	electrochemiluminescence	21	[5]
electrochemical ratiometric	ferrocene	electrochemistry	13	[6]
template-free multiple signal amplification	label-free	fluorescence	10	this work

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