Electronic Supplementary Information

A DNA logic gate with dual-anchored proximity aptamers for accurate identification of circulating tumor cells

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Materials and Methods

Chemicals and Materials. All DNA oligonucleotides were synthesized by Sangon Biotechnology Inc. (Shanghai, China) and purified by high-performance liquid chromatography (HPLC). The sequences are shown in Table S1. The primary antibodies of MUC1, EpCAM and GAPDH, and the second antibodies of mouse IgG (HRP-labeled and Alexa-488 conjugated) and rabbit IgG (HRP-labeled and Alexa-647 conjugated) were obtained from Abcam (Cambridge, MA, USA). T4 DNA Ligase, phi29 DNA polymerase, Exonuclease Ⅰ and Exonuclease Ⅲ were purchased from New England Biolabs Inc. Deoxynucleotide solution mixture (dNTPs) and 6 × loading buffer were obtained from Takara Biotechnology Co., Ltd. (Dalian, China). All other chemicals were of analytical grade and were purchased from Sigma Aldrich (St. Louis, MO, USA). All solutions were prepared with Milli-Q water (18.2 MΩ cm^{-1}) from a Milli-Q purification system (Millipore, Milford, MA, USA).

Gel Electrophoretic Analysis. Agarose gel electrophoresis was performed for the characterization of oligonucleotides samples. A volume of 5 μL of the samples with 1 μL of 6 × loading buffer was loaded onto a 3% nondenaturing agarose gel. The electrophoretic experiments were carried out in 1 × Tris-acetate-EDTA (TAE) at 80 V for 30 min. Subsequently, the gel was stained with SYBR Green I for 30 min. And the RCA products were stained with SYBR Green II for 30 min. The imaging of the gel and Fluorescence imaging wer performed using a Gel Doc XR Imaging System.

Preparation of PBA modified aptamers. Firstly, 20 μM 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and 20 μM N-hydroxy succinimidepurum (NHS) were added into 10 μM carboxyl-modified "A1" or "A2" DNA probe and incubated together at 37 ℃ for 1 h. Then, the mixture was further added with 100 μM 3-aminophenylboronic acid (AAPBA) and incubated at 37 ℃ for 3 h. The products were purified using an amicon filtration device 3,000 cut-off (Millipore, USA) by centrifuge ultrafiltration, and characterized using VERTEX 70 Fourier transform infrared spectrometry (FTIR, Bruker Co. Ltd., Bergisch Gladbach, Germany) (Figure S5).

Cell Culture. All cells were obtained from the Institute of Biochemistry and Cell Biology (Chinese Academy of Science). MCF-7 (human breast cancer cell, MUC1-positive, EpCAM-positive), HepG2 (human hepatocellular carcinoma cell, MUC1-negative, EpCAM-positive) and HeLa (human cervical cancer cell, MUC1-positive, EpCAM-negative) were cultured in DMEM medium (KeyGEN, Nanjing, China) supplemented with 10% FBS (Gibco, Invitrogen), 100 U/mL penicillin, and 100 g/mL streptomycin. All cells were maintained in a humidified incubator at 37 ℃ in a humidified atmosphere of 5% CO_2 and 95% air. Cells were collected at the end of the log phase and then counted with an automated cell counter (Bio-Rad, US) for the following experiments.

DNA logical operation. Cells were seeded in confocal dishes and incubated at 37 ℃ for 24 h. After removing the medium by washing with phosphate-buffered saline (PBS) briefly, the cells were fixed with 4% paraformaldehyde (PFA), and blocked with 2% bovine serum albumin (BSA). Then cells were incubated with "A1" and "A2" probes in a binding buffer (PBS containing 0.1 mg/mL yeast tRNA and 1 mg/mL BSA) at 37 ℃ for 1 h. The length of "A1" and "A2" was adopted after experimental optimization (Figure S6 & S7). Then, the cells were incubated with 100 nM double-nicking padlock probes "C" and "L" in PBS (pH 7.4, containing 1 mM ATP and 100 U of T4 DNA ligase) at 37 ℃ for 1 h to ligate the padlock probes. Subsequently, cells were incubated in a solution containing 8 U phi29 DNA polymerase, 400 μM dNTPs and 400 μg/mL BSA at 37 ℃ for 0 ~ 120 min to perform RCA. Then the cells were incubated with 100 nM FAM-labeled DNA probe at 37 ℃ for 30 min. Between each reactions, the cells were washed 3 times with PBS buffer containing 0.05% Tween-20 (PBST). Finally, the cells were ready for imaging using a green filter. The cell nucleuses were stained with 4', 6-diamidino-2-phenylindole (DAPI) for 10 min and imaged using a blue filter. Fluorescent observations were performed under a LSM 710 confocal laser scanning microscope (Zeiss, Germany).

For flow cytometry, the cells were trypsinized into a single cell suspension, and all the steps shown above were performed in tubes. Finally the cells were analyzed using CytoFLEX Flow Cytometer (Beckman Coulter, USA).

Immunofluorescence assay of MUC1 and EpCAM. Cells were seeded in confocal dishes and incubated at 37 ℃ for 24 h. After washing with PBS, cells were fixed with 4% PFA and blocked with 2% BSA. Then cells were incubated with the primary antibodies of MUC1 and EpCAM at room temperature for 2 h. The Alexa-488 conjugated second antibody and Alexa-647 conjugated second antibody are added into dishes together at room temperature for 1 h. The cell nucleuses were stained with DAPI for 10 min. Finally the cells were analyzed using a confocal laser scanning microscope and a Flow Cytometer.

Western blotting analysis. Cells were lysed with RIPA Lysis Buffer (KeyGEN, Nanjing, China) at 4 ℃ for 30 min and centrifuged at 15000 rpm for 20 min to get supernatant solution. Then the lysates (3 mg/mL) were resolved by 12% SDS-PAGE gel electrophoresis and transferred to pure nitrocellulose blotting membranes (Millipore, Milford, MA, USA). The membranes were blocked with 5% BSA at room temperature for 2 h. Then, the membranes were incubated with the primary antibodies of MUC1, EpCAM and GAPDH at at 4 ℃ overnight. Afterward, the membranes were incubated with secondary antibody at room temperature for 2 h. Finally the membranes were observed using a ChemiDocTM XRS Plus luminescent image analyzer (Bio-Rad, United States).

Analysis of artificial CTCs in whole blood samples. The whole blood samples were collected in EDTA tubes from healthy donors in Shanghai Ninth People's Hospital

under approved protocol and were processed within 24 h. All experiments were performed in compliance with the relevant laws and guidelines, and were approved by Ethics Committee of School of Life Sciences of Shanghai University. For experiment, the whole blood was pretreated by lysing the red blood cells with 6 × the volume of ACK Lysis Buffer (KeyGEN, Nanjing, China) at room temperature for 10 min. After centrifugation at 15000 rpm for 10 minutes, the supernatant was discarded and the cell pellet was re-suspended in an equivalent volume of PBS to obtain a solution of WBC, whose numbers were counted with the automated cell counter. As the artificial CTCs, 1000 MCF-7 cells were mixed with 500,000 WBC for immunofluorescence assay or DNA logical operation. Finally the artificial CTCs were analyzed by fluorescent observations and flow cytometry.

Table S1. Sequences of oligonucleotides.

Oligonucleotides	Sequence (5'-3')
Padlock probe 1 (C)	P- TCCCCACCCTCCAACCACCAAGGCAATGTACACGAATTCGCCGA ACGCACGCGATCCGCAT
Padlock probe 2 (L)	P-CTGTCAATGTGGAAAATCAATCTAGCAGT
Aptamer 1 (A1)	COOH- GCAGTTGATCCTTTGGATACCCTGGTTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTGAGGGTGGGGAACTGCTAGATT
Aptamer 2 (A2)	COOH- AGCGTCGAATACCACTACAGAGGTTGCGTCTGTCCCACGTTGTC ATGGGGGGTTGGCCTGCTAATGGAGCTCGTGGTCAGTTTTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTTTTTTCCACATTGACAGATGC GGATCGC
Aptamer 1s (A1s)	COOH- GCAGTTGATCCTTTGGATACCCTGGTTTTTTTTTTTTTTTTTTG AGGGTGGGGAACTGCTAGATT
Aptamer 2s (A2s)	COOH- AGCGTCGAATACCACTACAGAGGTTGCGTCTGTCCCACGTTGTC ATGGGGGGTTGGCCTGCTAATGGAGCTCGTGGTCAGTTTTTTTTT TTTTTTTTTTCCACATTGACAGATGCGGATCGC
Aptamer 1l (A1l)	COOH- GCAGTTGATCCTTTGGATACCCTGGTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGAGGGTGGG GAACTGCTAGATT
Aptamer 2l (A2l)	COOH- AGCGTCGAATACCACTACAGAGGTTGCGTCTGTCCCACGTTGTC ATGGGGGGTTGGCCTGCTAATGGAGCTCGTGGTCAGTTTTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT

	TTCCACATTGACAGATGCGGATCGC
FAM-labeled probe	FAM-ACCAAGGCAATGTACACGAATTC
FAM-labeled C	FAM-TCCCCACCCTCCAACCACCAAGGCAATGTACACGAATTCGCCGAACGCACGCGATCCGCAT

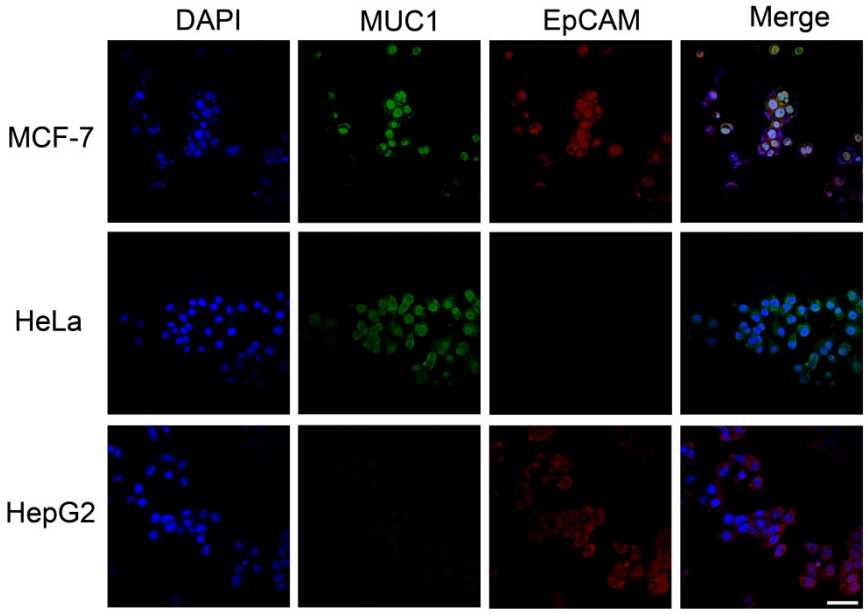

Fig. S1. Confocal microscopy images of immunofluorescence assay of MUC1 and EpCAM in MCF-7, HeLa, and HepG2 cells. Scale bar = 50 μm.

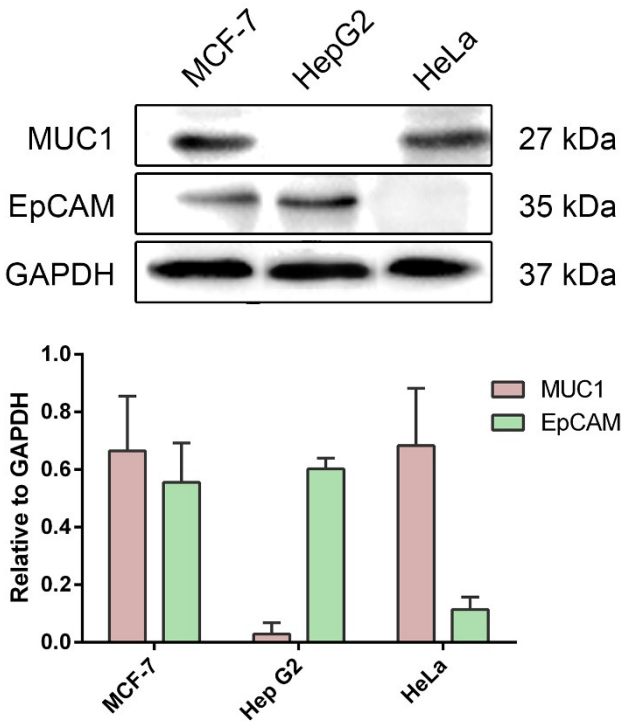

Fig. S2. Western blotting results of MUC1 and EpCAM expression in MCF-7, HeLa, and HepG2 cells.

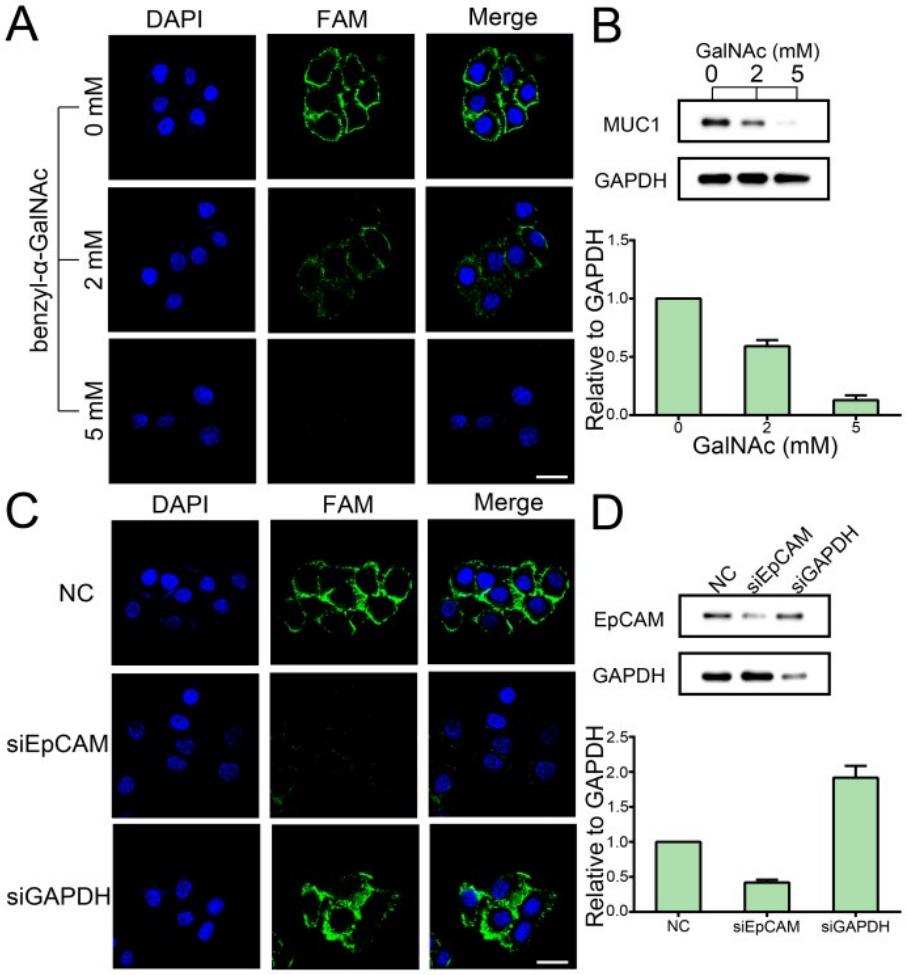

Fig. S3 (A) Confocal microscopy images of the operation of the DNA logic gate on MCF-7 cells which had been pretreated with different concentrations of benzyl-α-GalNAc. Scale bar = 20 μm. (B) Western blotting patterns and relative quantity of MUC1 in MCF-7 cells which had been pretreated with different concentrations of benzyl-α-GalNAc. (C) Confocal microscopy images of the operation of the DNA logic gate on MCF-7 which had been pretreated with different siRNA transfection. Scale bar = 20 μm. (D) Western blotting patterns and relative quantity of EpCAM in MCF-7 cells which had been pretreated with different siRNA transfection.

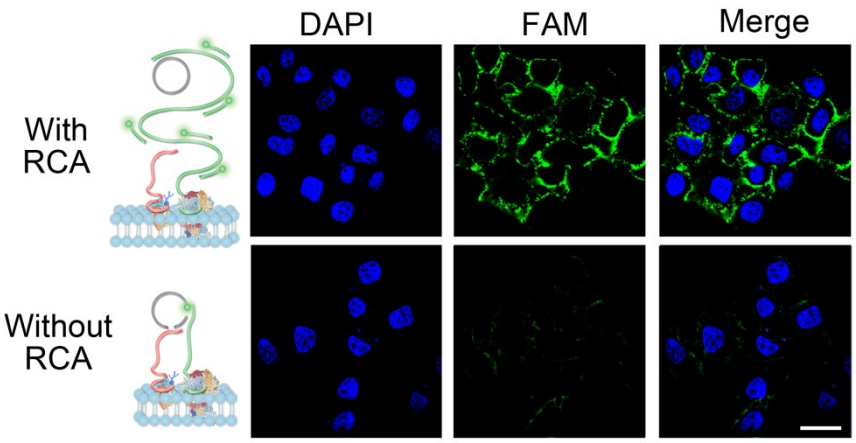

Fig. S4. Confocal microscopy images of MCF-7 cells on which the DNA logic gate was performed with or without RCA reaction .Scale bar = 20 μm.

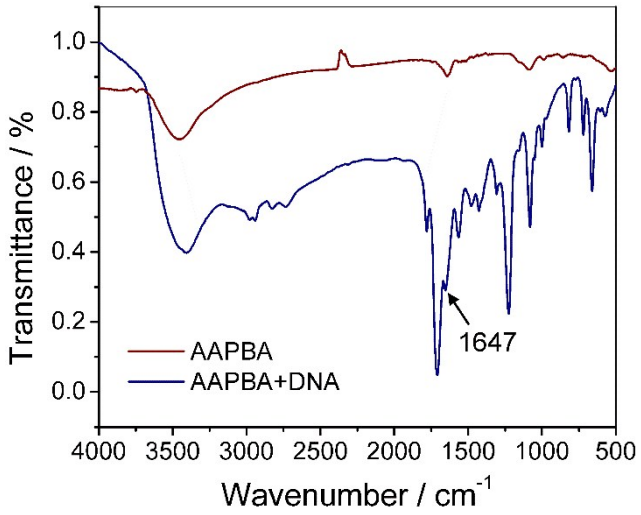

Fig. S5. Fourier transform infrared spectrometry of DNA modified with 3-aminophenylboronic acid (AAPBA).

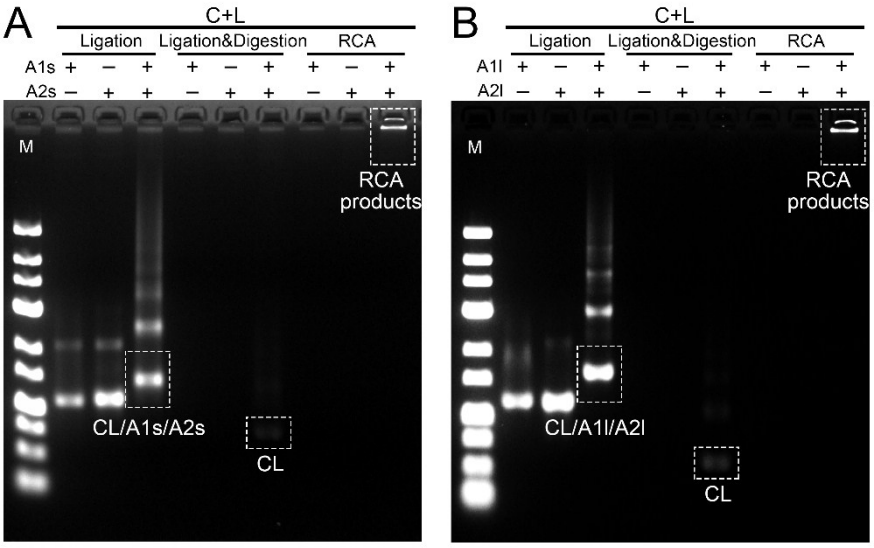

Fig. S6. (A) Agarose gel electrophoretic pattens of ligation, exonucleolytic digestion and RCA products in the presence of "A1s" or "A2s". (B) Agarose gel electrophoretic pattens of ligation, exonucleolytic digestion and RCA products in the presence of "A1l" or "A2l". These results show that all of the "A1" and "A2" probes with different lengths of the linker region can be adopted for ligation and RCA.

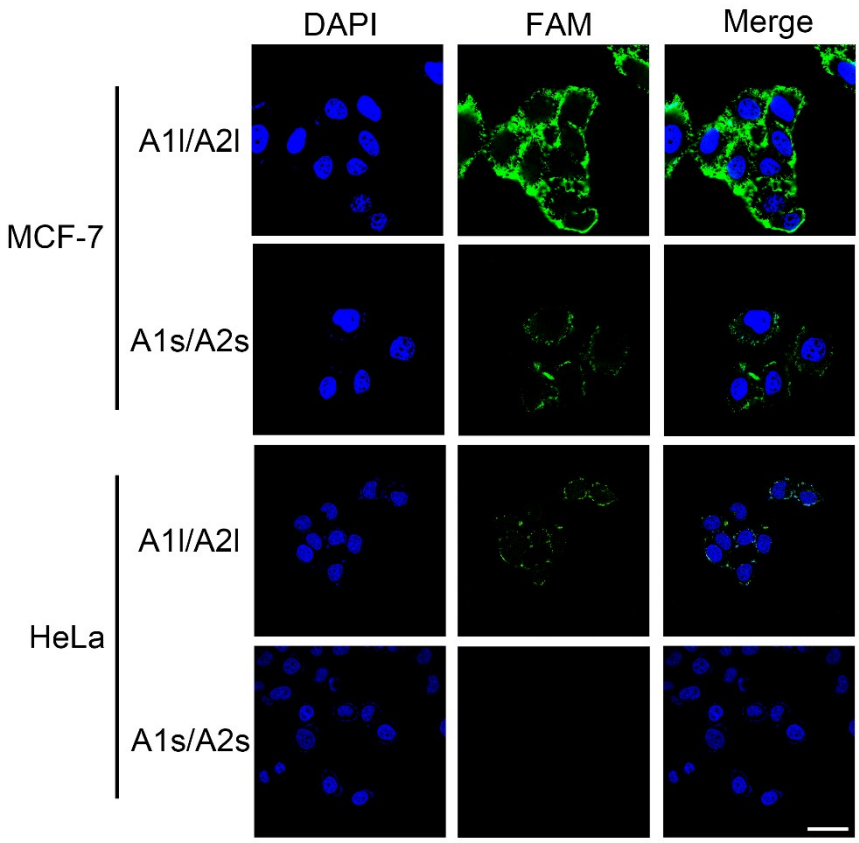

Fig. S7. Confocal microscopy images of MCF-7 and HeLa after logical operation using different length of "A1" and "A2" probes. This result shows that shorter probes ("A1s" and "A2s") will result in the weak fluorescence singals, while longer probes ("A1l" and "A2l") will result in the unexpected background signals in single positive Hela cells. So, "A1" and "A2" with medium-length linker was adopted, whose results were shown in the main text.