Supporting information for

Amplified detection of genome-containing biological targets using terminal deoxynucleotidyl transferase-assisted rolling circle amplification

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1 Experimenal section

1.1 Materials and Reagents

Table S1. The oligonucleotides used in this work

Oligonucleotide	Sequence (5′→3′)
HP	P-ATTCGTAGA*CCCTAACCCTAACCCTAACCC*TCAGCTTTT TTTTTTTTTTTTTTTTCCTCAGCTACGAAT
N_{11}	AAC TAT ACA CT
N_{11}-A_{10}	AAC TAT ACA CTA AAA AAA AAA
N_{11}-A_{15}	AAC TAT ACA CTA AAA AAA AAA AAA AA
N_{11}-A_{21}	AAC TAT ACA CTA AAA AAA AAA AAA AAA AAA AA
N_{11}-A_{21}-N_4	AAC TAT ACA CTA AAA AAA AAA AAA AAA AAA AA TCGC
N_{11}-A_{25}	AAC TAT ACA CTA AAA AAA AAA AAA AAA AAA AAA AAA
N_{11}-A_{30}	AAC TAT ACA CTA AAA AAA AAA AAA AAA AAA AAA AAA AAA AA
Padlock	P-ATCTCGACTAA*CCCTAACCCTAACCCTAACCC*TCAGCTT TTTTTTTTTTTTTTTTTTTGTCTCGGAT
Ligation template	TAGTCGAGATATCCGAGACA
HP-right	P-GAATGCATGTGAGAGAAACTATACAACCTACTACCTC A*CCCAATCCCAATCCCAATCCC*AATCTCTCAC
HP-left	P-ATGCATTCGTGAGAGAAACTATACAACCTACTACCTC A*CCCAATCCCAATCCCAATCCC*AATCTCTCAC
MUC-1 aptamer	Biotin-GCA GTT GAT CCT TTG GAT ACC CTG G

The red and underlined sequences in **HP** are complementary sequences that can fold into double-stranded stem of hairpin-like structure. The blue and underlined sequences in **Padlock** are the sequences that can hybridize with **Ligation template**. The red and underlined sequences in **HP-right** and **HP-left** are complementary sequences that can fold into double-stranded stem of hairpin-like structure, and the blue and underlined sequences in them are complementary sequences that can hybridize to each other. P = phosphorylation. Letters with italics are C-rich sequences whose complementary sequences can fold into G-quadruplex.

The oligonucleotides used in this work (Table S1) were synthesized and purified by Sangon Biotech. Co. Ltd. (Shanghai, China). Terminal deoxynucleotidyl transferase (TdT), T4 DNA ligase, phi29 DNA polymerase, exonuclease I (Exo I), exonuclease III (Exo III), deoxyribonuclease I (DNase I), deoxyadenosine triphosphate (dATP) and deoxyribonucleoside 5′-triphosphate mixture (dNTPs) were obtained from New England Biolabs (Beijing, China). Plasmid pBR322 DNA was purchased from TaKaRa Biotechnology (Dalian). Tissue/Cell genomic DNA extract kit was bought from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Gel Red was bought from KeyGEN BioTECH. Ethidium bromide (EB) was purchased from Tiangen Biotech. Co. Ltd. (Beijing, China). Thioflavin (ThT, 3,6-dimethyl-2-(4-dimethylaminophenyl) benzo-thiazolium cation) was obtained from Sigma. Streptavidin-coupled Dynabeads were purchased from Invitrogen (Dynabeads MyOne Streptavidin T1, Catalog number 65601). All chemical reagents were of analytical grade and used without further purification. *Cryptococcus neoformans* (*C. neoformans*) var. neoformans strain JEC21 (serotype D) was the generous gift of Prof. Xudong Zhu (Nankai University, Tianjin, China).

1.2. Cell culture and sample preparation

HeLa cells, MCF-7 cells and 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL) and 10% fetal bovine serum. The cells were maintained at 37 ^{o}C in a humidified atmosphere of 5% CO_2 and 95% air. Then, approximately 8×10^5 cells were dispensed in a 1.5 mL centrifuge tube, washed twice with PBS buffer and centrifuged at 2000 rpm for 3 min to discard the buffer.

1.3. Specific capture of cancer cells by apatmer-functionalized magnetic beads

Streptavidin-coupled magnetic beads were first washed with Washing Buffer for 3 times, then incubated with the biotinylated DNA MUC-1 aptamer (Table S1) for 1h, and washed three times with the help of a magnet. Different numbers of cells were incubated with 10 μL aptamer-functionalized magnetic beads in PBS buffer at 37 °C for 30 min in the presence of 5% CO_2. The magnetic beads were then separated via an external magnetic field. After being washed with PBS buffer, and the captured cells were used for genomic DNA extraction and TdT-assisted RCA assay.

1.4. DNase I-assisted fragmentation of genomic or plasmid DNA

Genomic DNA extraction from HeLa cells or pathogen *C. neoformans* was conducted according to the protocol of Tissue/Cell genomic DNA extract kit. The extracted genomic DNA or purchased pBR322 DNA was fragmented by 10 U DNase I in the presence of 1 × DNase buffer (10 mM Tris-HCl, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, pH 7.6). The DNA fragmentation reaction was conducted at 37 °C for 10 min, then terminated by heating the mixture at 80 °C for 10 min.

1.5. DNA fragment extension by TdT

Above-prepared DNA fragments were mixed with 1 × TdT buffer (50 mM KAc, 20 mM Tris-Ac, 10 mM $Mg(Ac)_2$, 0.25 mM $CoCl_2$, pH 7.9), 2 mM dATP and 10 U TdT enzyme. The total volume of the mixture was 25 μL. The extension reaction was performed at 37 °C for 3 h, and then terminated by heating the solution at 75 °C for 10 min.

1.6. Preparation of RCA templates

Preparation of dumbbell-like template: Reaction mixture containing 5 μL of **HP** (Table S1) solution (10 μM), 5 μL 10 × T4 DNA ligase reaction buffer (500 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM dithiothreitol, 10 mM ATP, pH 7.5) and 38 μL H_2O

was incubated at 95 °C for 5 min, and then cooled to 25 °C to ensure the oligonucleotide **HP** can fold into hairpin-like structure. Then, 2 μL of T4 DNA ligase (10 U/μL) was added and the ligation reacton was conducted at 16 °C for 2 h.

Preparation of traditional circular template: Reaction mixture containing 5 μL **Padlock** (Table S1) solution (10 μM), 5 μL **Ligation template** (Table S1) solution (10 μM), 5 μL 10 × T4 DNA ligase reaction buffer and 38 μL H_2O was incubated at 95 °C for 5 min, and then cooled to 25 °C to ensure the full hybridization between **Padlock** and **Ligation template**. Then, 2 μL of T4 DNA ligase (10 U/μL) was added and the ligation reacton was conducted at 16 °C for 2 h. After that, 1 μL of Exo I (20 U/μL) and 1 μL of Exo III (100 U/μL) were added, and the mixture was incubated at 37 °C for 1 h to digest **Ligation template**. The enzymes were then inactivated by heating the mixture at 80 °C for 20 min.

1.7. RCA amplification and detection

End-point RCA: 100 μL reaction mixture containing 25 μL of prepared dumbbell template, 10 μL of TdT extension product, 7.5 μL of 10 × phi29 DNA polymerase reaction buffer (500 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, 40 mM dithiothreitol), 1 μL BSA (10 mg/mL), 4 μL dNTPs (10 mM), 5 μL ThT (100 μM) and 0.5 μL phi29 DNA polymerase (10 U/μL) was prepared. After incubation at 30 °C for 5 h, the fluorescence spectrum of the mixture was recorded on a Shimadzu RF-5301 fluorescence spectrometer (λ_{ex} = 425 nm). Excitation and emission slit widths were both set to 5 nm.

Real-time RCA: 30 μL reaction mixture containing 15 μL of prepared dumbbell template, 5 μL of TdT polymerization buffer, 2.5 μL of 10 × phi29 DNA polymerase reaction buffer, 0.3 μL BSA (10 mg/mL), 2 μL dNTPs (10 mM), 2.5 μL ThT (100 μM) and 0.3 μL phi29 DNA polymerase (10 U/μL) was perpared. The reaction mixture was incubated at 30 °C on the ABI StepOne Plus real-time PCR system (Applied Biosystems), and time-dependent fluorescence change was monitored at an interval of 1 min.

1.8 Polyacrylamide gel electrophoresis (PAGE) analysis of RCA templates

RCA template was prepared as described in **1.5** section but the oligonucleotide concentration was changed from 1 μM to 10 μM (final concentration), and 200 U T4 ligase was used. The ligation product was analyzed by using 15% polyacrylamide gel electrophoresis (PAGE) in 1 × TBE buffer (89 mM Tris-boric acid, 2.0 mM EDTA, pH 8.3) at a 110 V constant voltage for 50 min. The gel was stained with 2 × Gel Red and photographed using a Gel Documentation system (Huifuxingye, Beijing, China).

1.9 Agarose electrophoresis analysis of RCA products

15 μL RCA reaction solution was mixed with 5 μL loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol, 10 mM EDTA). The mixture was loaded onto a 1% agarose gel containing 1 × EB dye, and electrophoresis analysis was carried out in TBE buffer (89 mM Tris-boric acid, 2.0 mM EDTA, pH 8.3) at room temperature. The electrophoresis was run at a constant potential of 70 V for 1 h, and the gel was photographed by a Gel Documentation system (Huifuxingye, Beijing, China).

2. Preparation of dumbbell-like RCA template

Polyacrylamide gel electrophoresis (PAGE) analysis was carried out to verify the formation of dumbbell-like RCA template. Ligation was carried out in the presence of T4 DNA ligase that can ligate both sticky ends of DNA and blunt-ended DNA. Therefore, we assumed that two molecules of hairpin **HP** (Table S1) could be connected via blunt-end ligation reaction, forming the dumbbell-like circular template **HPHP** (Figure S1, Lane 2). To test and verify the rationality of this assumption, we designed two another two hairpins, **HP-left** and **HP-right** (Table S1), each with 8-nucleotide overhang extended from double-stranded stem. **HP-left** and **HP-right** can be ligated through sticky-end ligation, forming dumbbell-like circular DNA (Figure S1, Lane 7). After addition of Exo I and Exo III to the ligation reaction mixture, the band corresponding to closed dumbbell DNA remained intact (Figure S1, Lane 3 and Lane 8). These experimental results demonstrated that dumbbell template **HPHP** could be successfully produced with high efficiency.

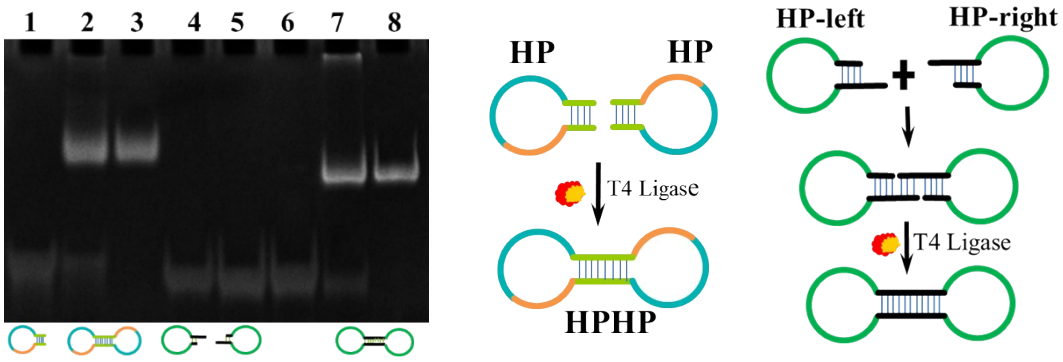


Figure S1. Gel electrophoresis analysis of dumbbell-like RCA template. (lane 1) **HP**; (lane 2) **HP** + T4 DNA ligase; (lane 3) **HP** + T4 DNA ligase + Exo I + Exo III; (lane 4) **HP-left**; (lane 5) **HP-right**; (lane 6) **HP-left** + **HP-right**; (lane 7) **HP-left** + **HP-right** + T4 DNA ligase; (lane 8) **HP-left** + **HP-right** + T4 DNA ligase + Exo I + ExoIII.

The performances of dumbbell template and traditional circular template in RCA-based sensing systems were compared. A synthetic oligonucleotide $\mathbf{N_{11}}$-$\mathbf{A_{21}}$ was used to simulate the extension product of $\mathbf{N_{11}}$ by TdT. As shown in Figure S2, both two templates can bind with $\mathbf{N_{11}}$-$\mathbf{A_{21}}$ to trigger subsequent RCA reaction, resulting in the fluorescence increase of the sensing systems. However, under the same conditions, the sensing system using the dumbbell template gave about 75 times fluorescence increase compared to the blank control, the one using traditional circular template could only give 10-fold signal increase due to the higher background fluorescence. The relatively higher background fluorescence given by traditional circular template can be ascribed to the incomplete degradation of **Ligation template**, which can in turn act as primer to trigger RCA reaction, resulting in the increase of background. Compared to traditional RCA system, our proposed dumbbell template preparation strategy gave a much lower background signal, thus showing more excellent sensing performance.

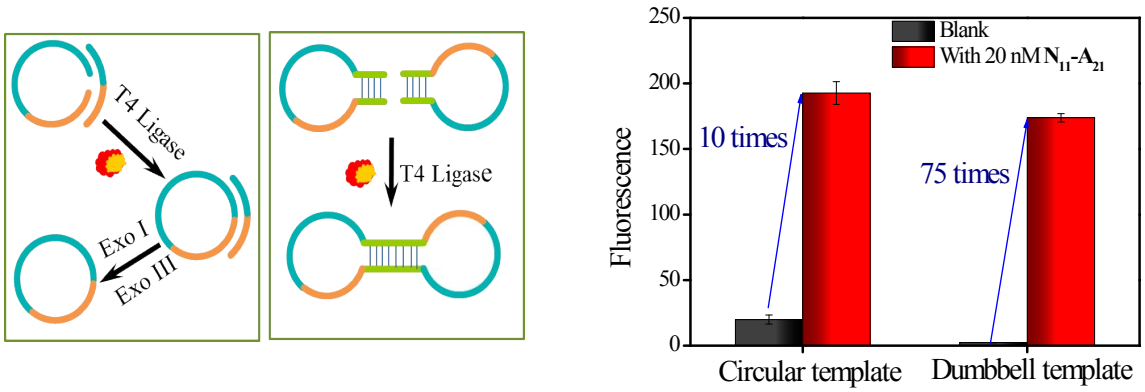
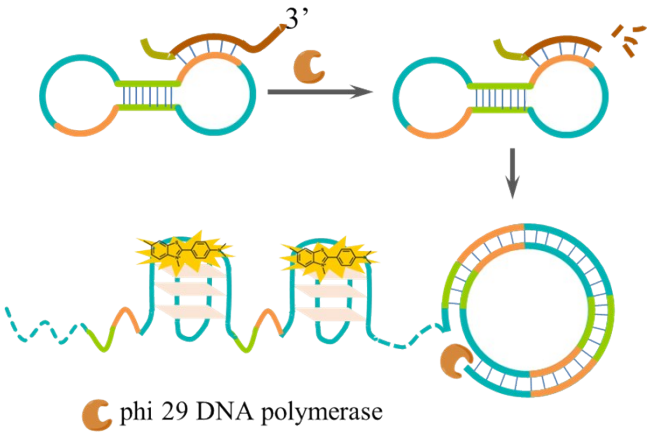


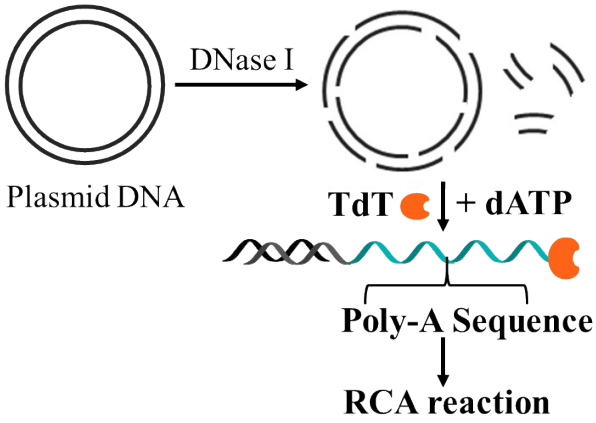
Figure S2. Preparation of traditional circular template and dumbbell-like template (Left) and the sensing performance comparison of the two templates (Right). Traditional circular template is prepared by **Padlock** and **Ligation template** (Table S1), and dumbbell-like template is prepared by **HP** (Table S1). 20 nM $\mathbf{N_{11}}$-$\mathbf{A_{21}}$ is used as the primer.

3. 3′-overhang can be degraded by phi29 DNA polymerase



Scheme S1. Schematic representation of how the 3′-overhang is degraded by phi29 DNA polymerase.

4. Schematic presentation of pBR322 DNA quantitation by TdT-RCA.



Scheme S2. Schematic representation of pBR322 DNA quantitation by TdT-RCA.

5. Methods for long-stranded DNA fragmentation

The results show that both DNase I digestion and ultrasound treatment could break circular pBR322 DNA into short fragments, which was reflected by the disappearance of DNA band in agarose gel electrophoresis analysis. However, only significant fluorescence enhancement was observed for the sensing system with DNase I digestion treatment (Figure S3).

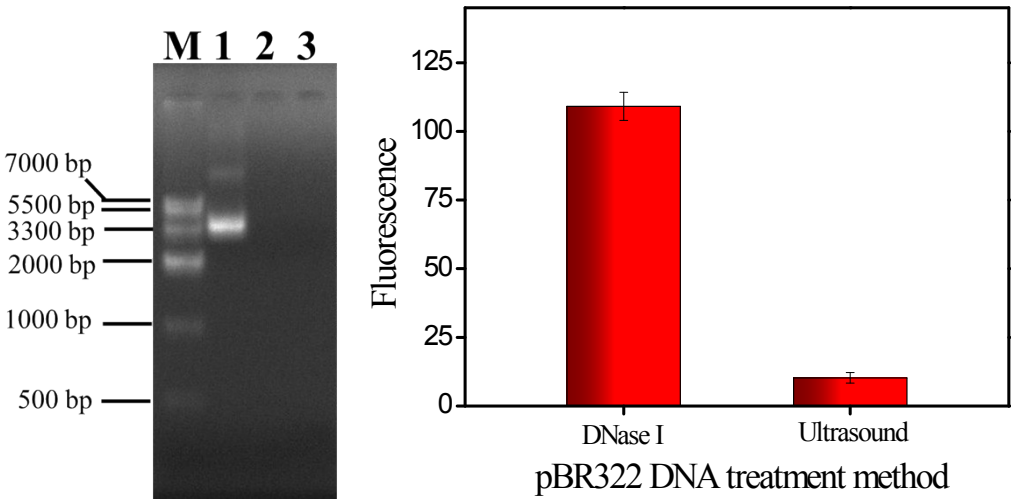


Figure S3. pBR322 DNA treatment by DNase I or ultrasound. (Left) Electrophoresis analysis. (M) DNA marker; (Lane 1) pBR322 DNA; (Lane 2) pBR322 DNA treated with 10 U DNase I for 10 min; (Lane 3) pBR322 DNA treated with ultrasound for 2 h (40 KHz). (Right) TdT-RCA analysis of the obtained DNA fragments.

6. Amplfication efficiency estimination of DNase I-triggered long-stranded DNA breakage

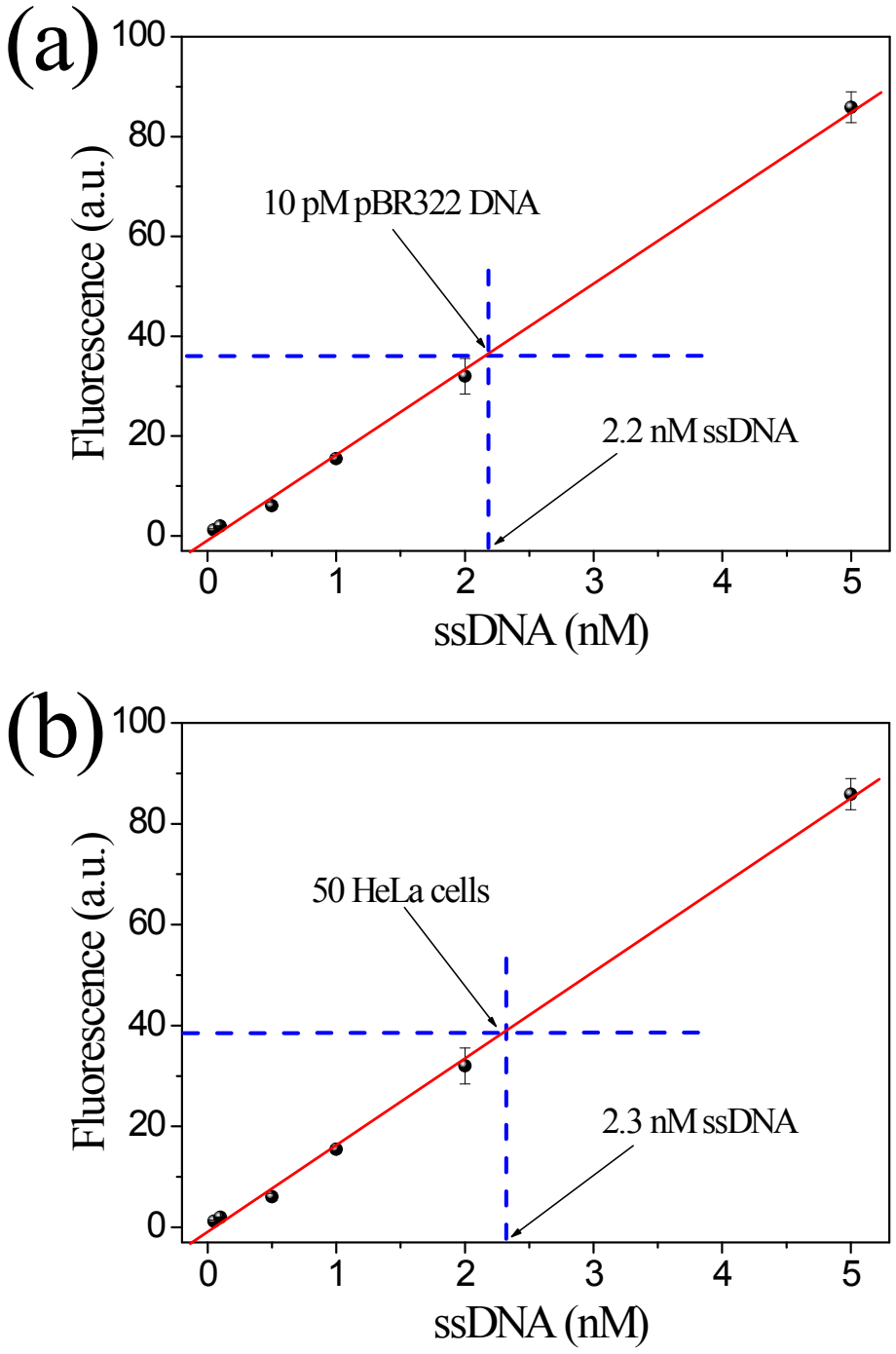


Figure S4. Fitting the fluorescence signals given by (a) 10 pM pBR322 DNA or (b) 50 HeLa cells into the standard curves constructed by ssDNA. The sequence of ssDNA is: 5′-TAGTCGAGATATCCGAG ACA.

7. *C. neoformans* quantitation by TdT-RCA

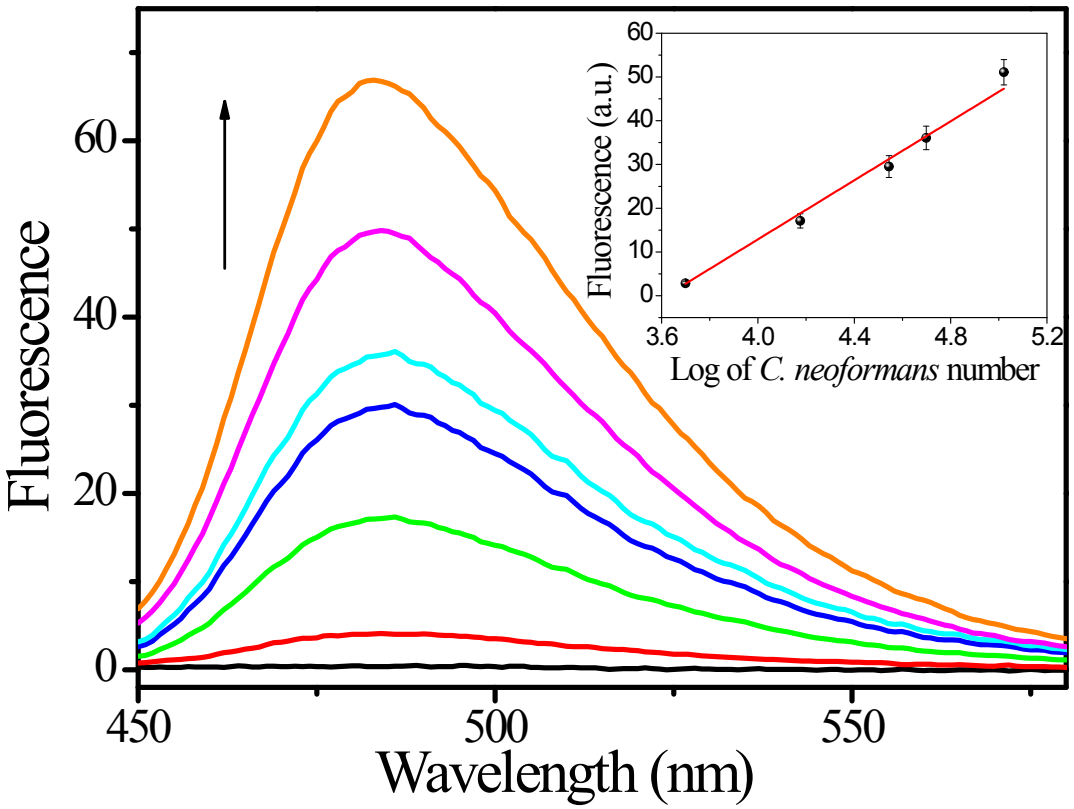


Figure S5. *C. neoformans* number-dependent fluorescence spectral change of TdT-RCA sensing system. The *C. neoformans* numbers (arrow direction) are 0, 5000, 15000, 35000, 50000, 75000 and 105000. The insert shows the linear relationship between the fluorescence intensity at 487 nm and the *C. Neoformans* number.

8. Optimization of TdT-RCA-based cancer cell-sensing platform

According to the results shown in Figure S6, the optimal DNase I amount and digestion time were selected as 10 units and 10 min, respectively.

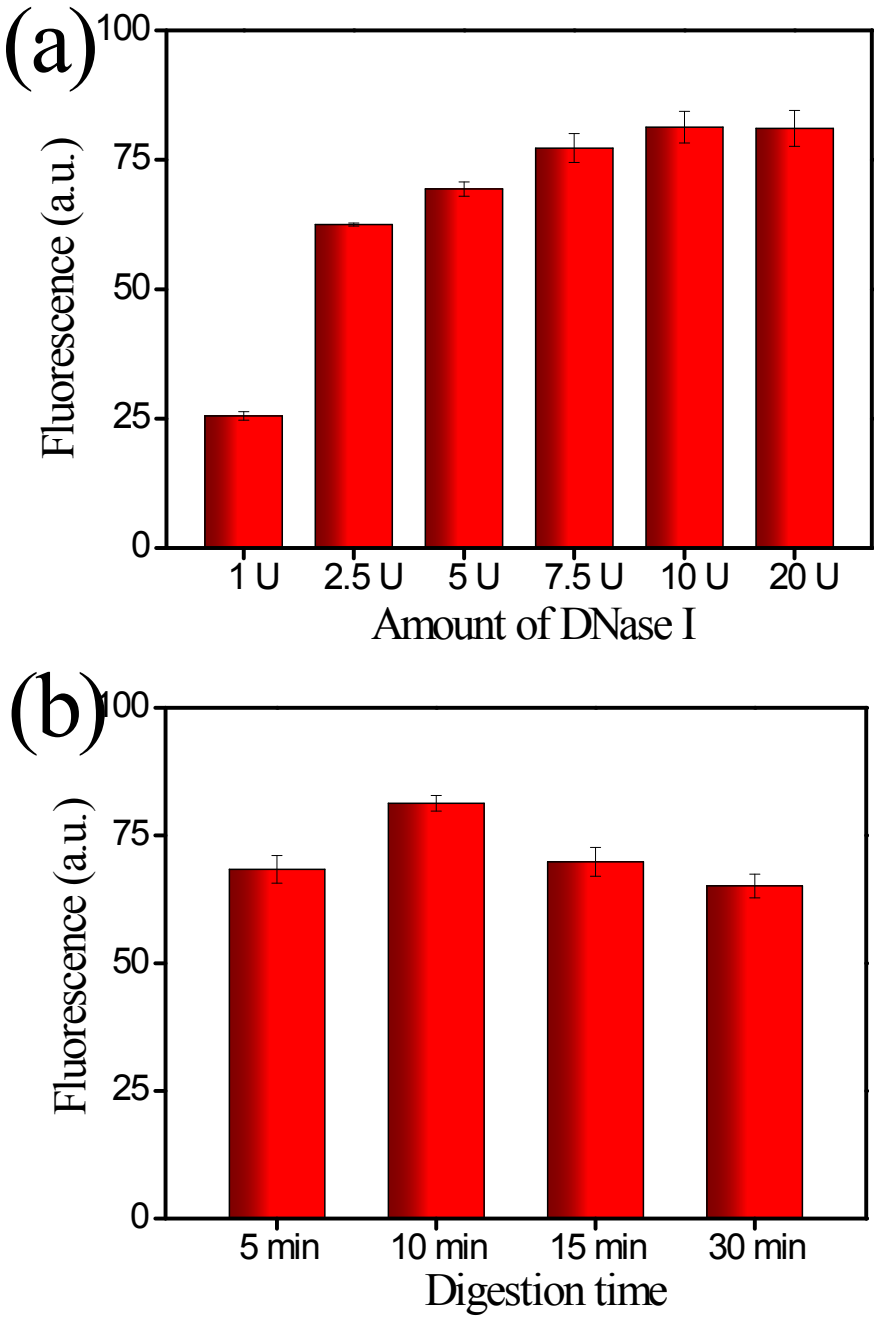


Figure S6. Effects of (a) DNase I amount and (b) digestion time on cancer cell detection. Herein, 2000 HeLa cells were used.

9.Detection of captured cells with TdT-RCA sensing platform

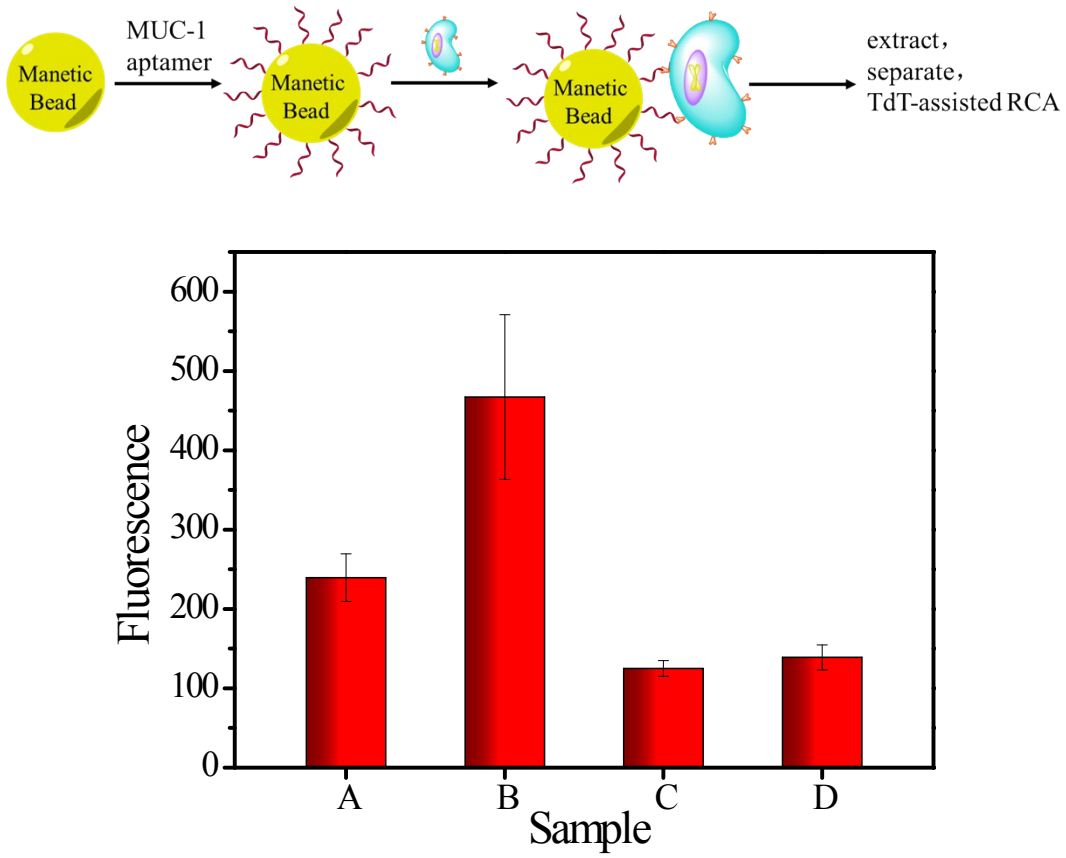


Figure S7. Cancer cells-specific detection by TdT-RCA assay. Cancer cells are specifically captured and seperated by aptamer-functionalized magnetic beads, and then captured cancer cells are quantified by TdT-RCA. A: 1000 MCF-7 cells, B: 10000 MCF-7 cells, C: No cell. D: 10000 3T3 cells