

Electronic Supporting Information

Cyclic Enzymatic Repairing-Mediated Dual-Signal Amplification for Real-Time Monitoring Thymine DNA Glycosylase

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MATERIALS AND METHODS

Materials. All oligonucleotides (Table 1) were synthesized and purified by HPLC Takara Biotechnology Co. Ltd. (Dalian, China). Thymine DNA glycosylase (TDG) was bought from R&D System (Minneapolis, Minnesota, U.S.A.). Uracil-DNA glycosylase (UDG), 10× UDG reaction buffer (200 mM trizma hydrochloride (Tris-HCl), 10 mM DL-Dithiothreitol (DTT), 10 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0), endonuclease IV (Endo IV), 10× NEBuffer 3 (1000 mM sodium chloride (NaCl), 500 mM Tris-HCl, 100 mM magnesium chloride (MgCl₂), 10 mM DTT, pH 7.9), and 10× NEBuffer 4 (500 mM potassium acetate, 200 mM tris-acetate, 100 mM magnesium acetate, 10 mM DTT, pH 7.9) were bought from New England Biolabs (Beijing, China). Bst. DNA polymerase (large fragment), 10× ThermoPol reaction buffer (200 mM Tris-HCl,

100 mM ammonium sulfate (NH₄)₂SO₄), 100 mM potassium chloride (KCl), 20 mM magnesium sulfate (MgSO₄), 1% Triton X-100, pH 8.8), T4 DNA ligase, 10× T4 DNA ligase reaction buffer (500 mM Tris-HCl, 100 mM MgCl₂, 10 mM adenosine 5'-triphosphate (ATP), 100 mM DTT, pH 7.5), human 8-oxoguanine-DNA glycosylase 1 (hOGG1) and deoxynucleotide (dNTP) solution set were purchased from New England Biolabs (Beijing, China). 2'-deoxyuridine-5'-triphosphate (dUTP) was from Takara Biotechnology Co., Ltd. (Dalian, China). SYBR Gold was obtained from Life Technologies (Carlsbad, CA, U.S.A.). Bovine serum albumin (BSA) was from Sigma Aldrich Company (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade and used without further purification. Ultrapure water was prepared by a Millipore filtration system (Millipore, Milford, MA, U.S.A.).

Table 1. Sequences of the Oligonucleotides^a

Note	Sequence (5'-3')
linear probe	GAA GGC GGG CGA CAG <i>TGC AAC GAA GAA AAA AC</i>
linear padlock probe	P- <u>CTG TCG CGT</u> TGC TTC GAT TGC TGC GTC CCT TCC CGC CCT GCC CTT <u>CTC TTC CGC CCG</u>
signal probe	TGC TGC GTC CCT <u>T</u> (FAM)CC CXC <u>CCT</u> (BHQ1) GCC CT
ligation probe	GAA GGC GGG CGA CAG CG

^aIn linear probe, the italicized “T” denotes the strategically designed deoxythymidine mismatched with guanine. The underlined “C” symbolizes 2',3'-dideoxycytidine. In linear padlock probe, the “P” denotes a 5'-phosphate modification, and the underlined regions signify the binding region of ligation probe. In signal probe, the underlined bases “T” are modified with fluorescein (FAM) and

black hole quencher 1 (BHQ1), respectively. The “X” denotes the tetrahydrofuran abasic site mimic.

Preparation of Circular Templates. Ligation probes and linear padlock probes were diluted with 1× Tris-EDTA buffer to 10 μM, and denatured at 95 °C for 5 min. Then 2 μL of each ligation probes and linear padlock probes were added into 20 μL of ligation buffer containing 1× T4 ligation buffer (6.6 mM MgCl₂, 10 mM DTT, 0.1 mM ATP, 66 mM Tris-HCl (pH 7.6)), 50 U T4 DNA ligase, incubated at 16 °C overnight. After ligation reaction, 10 μL of ligation products was transferred into 10 μL of digestion buffer containing 1 mM DTT, 6.7 mM MgCl₂, 67 mM glycine-KOH (pH 9.5), 10 U Exonuclease I, and 20 U Exonuclease III, incubated at 37 °C for 2 h and then inactivated at 95 °C for 10 min. The resultant digestions were further purified by Dr. GentLE precipitation carrier from Takara Biotechnology Co., Ltd. (Dalian, China).

Cyclic Enzymatic Repairing-Mediated Dual-Signal Amplification. The TDG assay involves two consecutive steps. Firstly, the linear probe was diluted with 1× Tris-EDTA buffer to 100 nM. Then 2 μL of ligation probes was added into 20 μL of excision buffer containing 1× NEBuffer 4 (50 mM potassium acetate, 20 mM tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH 7.9) and 2 U TDG, then incubated at 65 °C for 50 min. Secondly, 2 μL of excision products was added into 50 μL of amplification reaction mixture containing 500 μM of dNTPs (dATP, dUTP, dGTP, dCTP each), 5 U Bst. DNA polymerase, 10 U UDG, 20 U Endo IV, 5 μL of 10× ThermoPol reaction buffer, 5 μL of 10× UDG reaction buffer, 5 μL of 10× NEBuffer 3 and 900 nM signal probes, and incubated at 37°C for 50 min. For the kinetic analysis, the first-step excision time is

determined to be 10 min for the measurement of initial rates.

Gel Electrophoresis Analysis. After reaction, 10 μL of the products was analyzed using 2% agarose gel and electrophoresis in 1 \times TAE buffer (40 mM tris-acetate, 1 mM EDTA, pH 8.0) at 110 V constant voltages for 40 min at room temperature. After electrophoresis, the gel was stained with 1 \times SYBR gold and visualized by a ChemiDoc MP Imaging system (Hercules, California, U.S.A.).

Measurement of Fluorescence Intensity. The 25 μL of amplification products were diluted to a final volume of 60 μL with ultrapure water. All fluorescence spectra were measured using a quartz cuvette on a Hitachi F-7000 fluorescence spectrophotometer (Tokyo, Japan). The excitation wavelength was 492 nm, and the emission spectra were recorded over the wavelength range of 492 to 600 nm with a slit width of 5 nm for both excitation and emission. In real-time fluorescence measurement, the emission wavelength is fixed at 520 nm, and the fluorescence intensity was monitored at a time interval of 10 s.

Cell Culture and Preparation of Cell Extracts. Human cervical carcinoma cell line (HeLa) and human breast cancer cell line (MCF-7) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were cultured at 37 $^{\circ}\text{C}$ in a humidified atmosphere containing 5% CO_2 . In the exponential phase of growth, cells were collected with trypsinization, washed twice with ice-cold phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4) and then

centrifuged at 800 rpm at 4 °C. The obtained cells were suspended in 100 µL of lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 1% (w/v) NP-40, 0.25 mM sodium deoxycholate, 1% (w/v) glycerol and 0.1 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride, pH 8.0) and incubated on ice for 30 min with simultaneously vortexing for 30 s every 5 min. After lysis, the cell debris was centrifuged at 12,000 rpm for 20 min at 4 °C, and the resultant supernatant was transferred into a fresh tube and used immediately for TDG assay.

SUPPLEMENTARY RESULTS

Optimization of TDG-induced excision reaction time. TDG-induced thymine excision repair reaction plays a critical role in this assay, and it may directly affect the final result of overall reaction. Therefore, the reaction time should be optimized. As shown in Fig. S1, the fluorescence intensity increases with the reaction time, and reaches a plateau within 50 min. Thus, 50 min was selected as the appropriate reaction time in subsequent experiments.

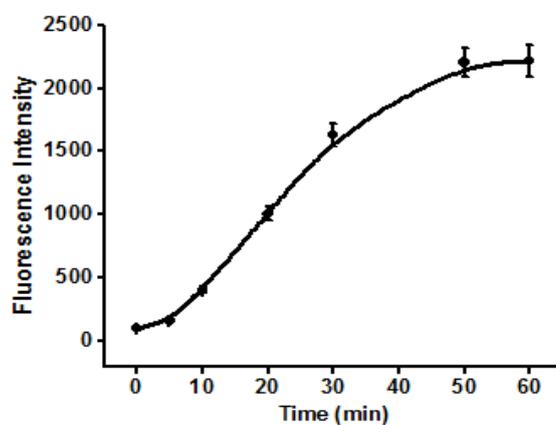


Fig. S1 Variance of fluorescence intensity with the reaction time. Error bars show the standard deviation of three independent experiments.

Optimization of UDG concentration. In this assay, UDG is responsible for the performance of uracil excision-assisted cyclic rolling circle amplification (RCA) reaction. The amount of UDG may affect the detection sensitivity, and thus we optimized the concentration of UDG. As shown in Fig. S2, the fluorescence intensity enhances with the increasing concentration of UDG, and reaches a plateau beyond the concentration of 10 U. Therefore, 10 U UDG was used in the subsequent experiments.

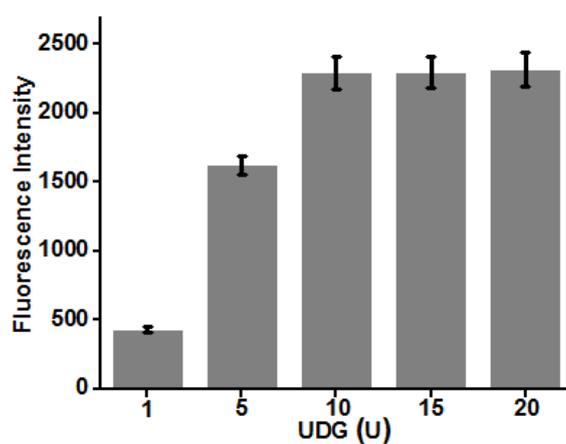


Fig. S2 Variance of fluorescence intensity with the concentration of UDG. Error bars show the standard deviation of three experiments.

Optimization of Endo IV concentration. In this assay, repairing enzyme Endo IV plays a critical role in the dual-signal amplification reaction, and the amount of Endo IV may directly affect the amplification efficiency. As shown in Fig. S3, the fluorescence intensity enhances with the increasing concentration of Endo IV, and reaches a plateau beyond the concentration of 20 U. Thus, 20 U Endo IV was used in the subsequent experiments.

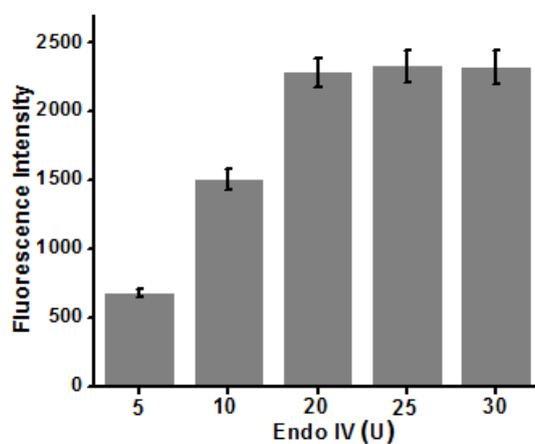


Fig. S3 Variance of fluorescence intensity with the concentration of Endo IV. Error bars represent the standard deviations of three experiments.

Optimization of the concentration of signal probe. In this assay, the amount of signal probe has a close relationship with the detection sensitivity, and the concentration of signal probe should be optimized. As shown in Fig. S4, the fluorescence intensity increases gradually in the range from 100 nM to 900 nM, and reaches the highest value at 900 nM. Thus, 900 nM signal probe was used in the subsequent experiments.

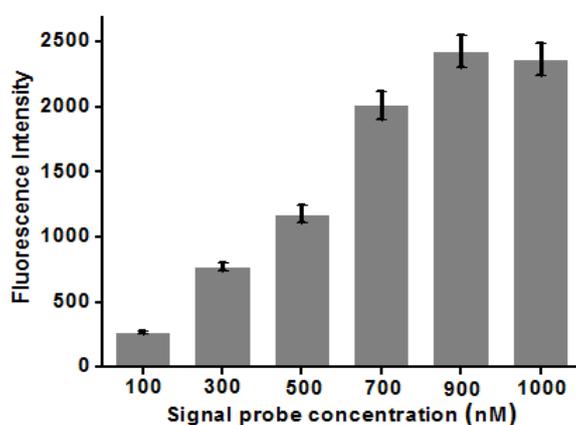


Fig. S4 Variance of fluorescence intensity with the concentration of signal probe. Error bars show the standard deviations of three independent replicates.

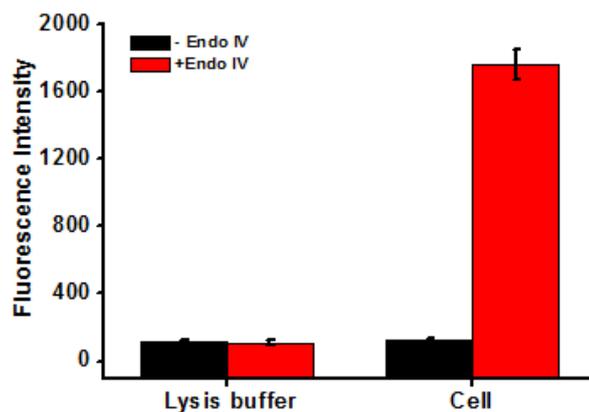


Fig. S5 Variance of fluorescence intensity in response to lysis buffer and Hela cell sample. The black and red columns indicate fluorescence intensity in the absence and in the presence of Endo IV, respectively. This result indicates that the AP site-excised repair proteins such as APE1 which exist in cancer cells do not interfere the detection of TDG in real sample analysis. Error bars show the standard deviation of three independent experiments.