Electronic Supporting Information

Single Quantum Dot-Based Nanosensor for Rapid and Sensitive Detection of Terminal Deoxynucleotidyl Transferase

Li-Juan Wang,[a, †] Ming-Li Luo,[a, †] Qianyi Zhang, [b, †] Bo Tang,[a, *] and Chun-Yang Zhang [a, *]

[a] College of Chemistry, Chemical Engineering and Materials Science, Collaborative Innovation Center of Functionalized Probes for Chemical Imaging in Universities of Shandong, Key Laboratory of Molecular and Nano Probes, Ministry of Education, Shandong Provincial Key Laboratory of Clean Production of Fine Chemicals, Shandong Normal University, Jinan 250014, P. R. China

[b] Nantou High School Shenzhen, Shenzhen, 518052, China

† These authors contributed equally

* To whom correspondence should be addressed. E-mail: cyzhang@sdnu.edu.cn; tangbo@sdnu.edu.cn

MATERIALS AND METHODS

Chemicals and Materials. All oligonucleotides were obtained from Takara Biotechnology Co. Ltd. (Dalian, China). Terminal deoxynucleotidyl transferase (TdT), 10× terminal deoxynucleotidyl transferase reaction buffer (500 mM potassium acetate (KAc), 200 mM tris-acetate (Tris-Ac), 100 mM magnesium acetate (Mg(Ac)$_2$), pH 7.9), cobalt(ll) chloride (CoCl$_2$), exonuclease III (Exo III), 10× NEBuffer 1 (100 mM bis-tris-propane-hydrochloride，100 mM magnesium chloride (MgCl$_2$), 10 mM DL-dithiothreitol (DTT), pH 7.0), deoxynucleotide (dNTP) solution set (dATP, dTTP, dGTP and dCTP), adenosine 5'-triphosphate (ATP), guanosine 5'-triphosphate (GTP), and

cytidine 5'-triphosphate (CTP) were purchased from New England Biolabs (Ipswich, MA, USA).

Bst. DNA polymerase (large fragment), uracil-DNA glycosylase (UDG), endonuclease IV (Endo

IV) and T4 DNA ligase were obtained from New England Biolabs (Ipswich, MA, USA). $MgCl_2$,

trizma hydrochloride (Tris-HCl), ethylenediaminetetraacetic acid (EDTA), sodium chloride (NaCl),

ammonium sulfate $((NH_4)_2SO_4)$, glycerol, glycine, potassium hydroxide (KOH), bovine serum

albumin (BSA), trolox, glucose oxidase, D-glucose and catalase were obtained from

Sigma-Aldrich Company (St. Louis, MO, USA). The streptavidin-conjugated CdSe/ZnS QDs with

a maximum emission at 605 nm (605QDs) were obtained from Invitrogen Corporation (California,

USA). SYBR Gold was bought from Life Technologies (Carlsbad, CA, USA). The human serum

was purchased from Dingguo Biotech. Co. (Beijing, China). Other chemicals were of analytical

grade and used without further purification. The ultrapure water used in all experiments was

obtained from a Millipore filtration system (Millipore, Milford, MA, USA).

Table 1. Sequences of the Oligonucleotides[α]

note	sequence (5'-3')
DNA primer	ATG GGC GGC A
capture probe	<u>A</u>AA AAA AAA AA<u>A</u>
synthesized product	ATG GGC GGC ATT TTT TTT TTT TTT TTT T

[α]In capture probe, the underlined "A" bases at the 5′ and 3′ ends are modified with biotin and Cy5,

respectively.

Polymerization-Induced Exo III-Mediated Cyclic Release of Cy5 from the Cy5-dsDNA-QD

Nanostructure. All oligonucleotides were diluted with 10× Tris-EDTA buffer to prepare the stock

solutions (1 μM). The detection of TdT activity involves three consecutive steps. First, 100 nM

DNA primer was added into 20 μL of polymerization reaction solution containing

various-concentration TdT, 0.25 mM $CoCl_2$, 5 mM dTTP and 1× terminal deoxynucleotidyl

transferase reaction buffer (50 mM KAc, 20 mM Tris-Ac,10 mM $Mg(Ac)_2$, pH 7.9). After

incubation at 37 °C for 30 min, the reaction was terminated by heating at 75 °C for 10 min.

Second, 10 μL of TdT-catalyzed polymerization products was added into the incubation solution

(0.1 μM capture probes, 2.78 nM 605QDs, 3 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, and 100 mM

Tris-HCl, pH 8.0) and incubated at room temperature for 10 min to form the Cy5-dsDNA-605QD

nanostructures. Third, 20 U of Exo III and 6 μL of 10× NEB buffer 1 (100 mM

bis-tris-propane-HCl, 100 mM $MgCl_2$, 10 mM DTT, pH 7.0) were added to the incubation mixture

and incubated at 37 °C for 20 min, enabling the release of Cy5 molecules from the

Cy5-dsDNA-605QD nanostructures.

Gel Electrophoresis and Fluorescence Measurement. The TdT-catalyzed polymerization

reaction and the subsequent hybridization reaction were monitored by 2% agarose gels at 110 V in

1× TAE buffer (40 mM tris-acetate, 1 mM EDTA, pH 8.0) at 110 V constant voltages for 50 min at

room temperature. After electrophoresis, the gels were stained with 1× SYBR Gold and imaged by

the ChemiDoc MP Imaging system (Hercules, California, USA). The fluorescence spectra of the

605QDs and Cy5 were measured with a HitachiF-7000 fluorescence spectrophotometer (Tokyo,

Japan) at the excitation wavelength of 488 nm.

Single-Molecule Detection and Data Analysis. The reaction products were diluted with the

imaging buffer (67 mM glycine-KOH (pH 9.4), 2.5 mM MgCl$_2$, 50 µg/mL BSA, 1 mg/mL trolox)

with the addition of oxygen-scavenging buffer (1 mg/mL glucose oxidase, 0.04% mg/mL catalase,

and 0.4% (w/v) D-glucose) to prevent the photobleaching. The samples were excited by a 488-nm

argon laser (Coherent, USA). The photons from the 605QDs and Cy5 were collected by an Andor

Ixon DU897 EMCCD with an exposure time of 100 ms, and subsequently analyzed by the image J

software (version 1.41). A region of interest (600 × 600 pixels) was selected for Cy5 molecule

counting. The reduction of Cy5 counts were calculated according to equation 1:

$$N_{reduction} = N_{control} - N_{TdT} \qquad (1)$$

where $N_{reduction}$ represents the reduction of Cy5 counts, $N_{control}$ represents the Cy5 counts in the

absence of TdT, and N_{TdT} represents the Cy5 counts in the presence of TdT.

Inhibition Assay. Different-concentration ATP, GTP and CTP were added into the reaction system,

followed by same measurement procedure described above. The relative activity (*RA*) of TdT was

calculated according to equation 2:

$$RA = \frac{N_i - N_o}{N_t - N_o} \qquad (2)$$

where N_o, N_t and N_i represent the Cy5 counts in the absence of TdT, in the presence of TdT (0.01

U/µL), and in the presence of TdT (0.01 U/µL) and various-concentration ATP, GTP and CTP,

respectively. The relative activity was plotted against the concentration of ATP, GTP and CTP,

respectively, and the half-inhibition concentration (IC$_{50}$) values were calculated from the obtained

fitting curves.

Cell Culture and Preparation of Cell Extracts. Human cervical cancer cell line (HeLa cells) and

human leukemic T-cell line (Molt-4 cells) were cultured with 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin−streptomycin (PS, Gibco, USA) in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) and RPMI medium 1640 (Gibco, USA), respectively, in a humidified chamber containing 5% CO_2 at 37 °C. At the exponential phase of growth, cells were collected by washing 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 1000 rpm for 5 min. The collected cells were suspended in 500 μL of lysis buffer (150 mM NaCl, 100 mM EDTA, 50 mM Tris, 1% Triton), incubated on ice for 30 min and vortexed for 30 s every 5 min. Then the lysed products were centrifuged at 12000 rpm for 20 min at 4 °C, with the supernatant being transferred into a fresh tube for TdT assay. Moreover, to detect the variance of Cy5 counts with different amounts of TdT enzymes from the cancer cells, the proteins were extracted with a commercial protein extraction kit (BSP022, Sangon Biotech, Shanghai, China), and the protein concentration was quantified by a modified Lowry protein assay kit (SK4041, Sangon Biotech, Shanghai, China).

Cellular TdT Detection with Enzyme-Linked Immunosorbent Assay (ELISA). We compared side-by-side our method with a conventional ELISA assay for the measurement of TdT activity in cancer cells. Molt-4 cells were collected at the exponential phase of growth, and the proteins were obtained according to the procedures of human TdT ELISA Kit (SBJ-H1214, SenBeiJia Biotech, Nanjing, China). The TdT activity was quantified by using ELISA Kit and the color change was measured on a SpectraMax i3x multi-mode detection platform (Molecular Devices, Sunnyvale, CA, USA) at a wavelength of 450 nm. As shown in Fig. 5B, the absorbance value increases with the increasing number of Molt-4 cells, accompanied by the obvious color changes. A linear

relationship is obtained between the absorbance value and the logarithm of the number of Molt-4 cells. The correlation equation is $A = 0.2 \log_{10} C - 0.2$ ($R^2 = 0.9545$), where A represents the absorbance value and C represents the number of Molt-4 cells, respectively. The detection limit is calculated to be 10 cells. In comparison with the conventional ELISA method, single QD-based FRET nanosensor has significant advantages of good correlation ($R^2 = 0.9985$) and high sensitivity with a detection limit of 5 cells (Fig. 5A)

SUPPLEMENTARY RESULTS

Formation of Cy5-dsDNA-605QD Nanostructure. The capture probe (black color) is a 15-adenine (A) base sequence modified with Cy5 and biotin at the 3′ and 5′ ends, respectively. The capture probes may assemble on the surface of the 605QD through specific biotin-streptavidin interaction to form a Cy5-ssDNA-605QD nanostructure. In the presence of TdT, 2′-deoxythymidine 5′-triphosphates (dTTPs) will be constantly polymerized into the 3′-OH terminus of 10-base DNA primers (Fig. S1, green color), producing a long chain of poly-thymidine (T) sequence (Fig. S1, blue color). With the addition of capture probe-modified 605QDs, the T-rich sequences may specifically hybridize with the A-rich capture probes to form stable dsDNA duplexes with 3′ recessed ends, consequently producing the Cy5-dsDNA-605QD nanostructures (Fig. S1).
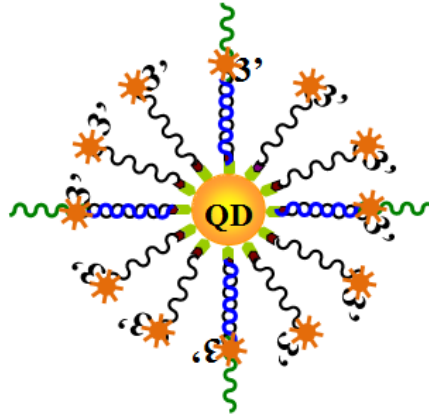
Fig. S1 Structure of the Cy5-dsDNA-605QD nanostructure.

Variance of Fluorescence Intensity with Different-Concentration TdT. To further demonstrate the feasibility of the proposed method, the variance of fluorescence intensity in response to different-concentration TdT is monitored. As shown in Fig. S2A, the 605QD fluorescence intensity increases with the increasing TdT concentration, accompanied by the corresponding decrease of Cy5 fluorescence intensity. On the basis of Scheme 1, the more the TdT concentration, the more the Cy5-labeled capture probes digested by Exo III, and consequently the more Cy5 molecules being released from the Cy5-dsDNA-605QD nanostructures. Consequently, less FRET between the 605QD donor and Cy5 acceptor occurs. Moreover, the variance of Cy5 fluorescence intensity in response to different-concentration TdT is also monitored (Fig. S2B). The reduction of Cy5 fluorescence intensity is defined according to equation 3:

$$F_{\text{reduction}} = F_{\text{control}} - F_{\text{TdT}} \qquad (3)$$

where $F_{\text{reduction}}$ represents the reduction of Cy5 fluorescence intensity, F_{control} represents the Cy5 fluorescence intensity in the absence of TdT, and F_{TdT} represents the Cy5 fluorescence intensity in the presence of TdT. As shown in Fig. S2B, the more the TdT concentration, the greater the reduction of Cy5 fluorescence intensity. Thus, the reduction of Cy5 signals can be used to
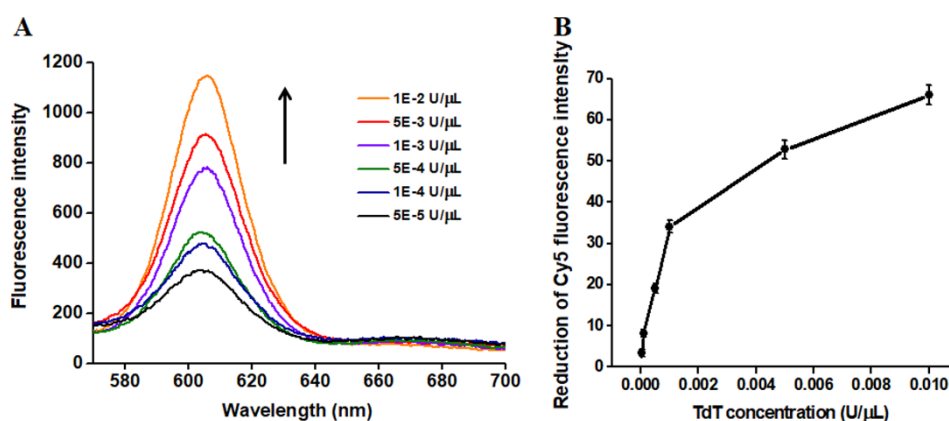
quantify the TdT activity.

Fig. S2 Variances of fluorescence intensity with different-concentration TdT. Error bars show the standard deviations of three independent experiments.

Optimization of the Ratio of Cy5-labeled Capture Probe to the 605QD. Since a single 605QD can accommodate multiple Cy5 receptors,[1] the molar ratio of Cy5-labeled capture probe to the 605QD should be optimized to achieve the highest FRET efficiency. We measured the Cy5 fluorescence intensity at the excitation wavelength of 488 nm. The obtained Cy5 fluorescence intensities are plotted against the molar ratio of Cy5-labeled capture probe to the 605QD. As shown in Fig. S3, the maximum Cy5 fluorescence intensity is obtained at the Cy5-labeled capture probe-to-605QD ratio of 36:1, and the Cy5 fluorescence intensity decreases beyond the molar ratio of 36:1. This may be ascribed to the inner-filter effect that may induce the decrease of fluorescence emission and the distortion of band shape.[2] In contrast, the FRET efficiency enhances with the increasing ratio of capture probe to the 605QD (above the ratio of 36:1), implying that excess Cy5 (above the ratio of 36:1) may induce the quenching of the 605QDs no matter whether they are assembled on the surface of the 605QDs or not.[3] To avoid the false positivity, the optimal Cy5-labeled capture probe-to-605QD ratio is 36:1. The FRET efficiency (E)

is calculated based on equation 4:

$$E\,(\%) = (1 - F_{DA}/F_D) \times 100 \qquad (4)$$

where F_{DA} is the 605QD fluorescence intensity in the presence of Cy5, and F_D is the 605QD

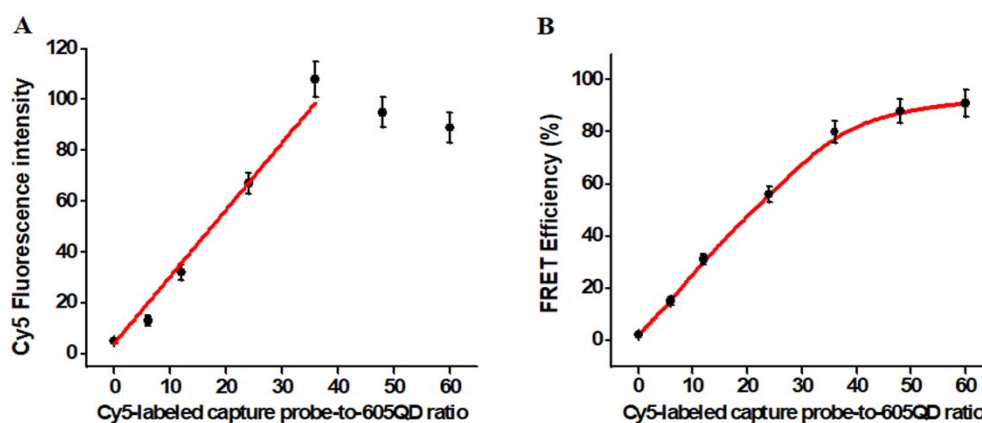fluorescence intensity in the absence of Cy5.

Fig. S3 Variance of Cy5 fluorescence intensity (A) and FRET efficiency (B) with different

Cy5-labeled capture probe-to-QD ratio in the ensemble measurement. Error bars show the

standard deviation of three experiments.

Optimization of the Digestion Time of Exo III. The digestion time of Exo III has a direct effect

on the detection sensitivity in this assay. Exo III is a multifunctional enzyme, and it exhibits not

only the 3′→5′ exonuclease but also Ribonuclease H, 3′-phosphatase and apurinic/apyrimidinic

endonuclease activities.[4] To avoid unfavorable cleavage resulting from excessive digestion, the

digestion time of Exo III should be optimized. As shown in Fig. S4, the reduction of Cy5

fluorescence intensity increases with the digestion time, and reaches a plateau at 20 min,

indicating that the digestion process has been completely accomplished. Therefore, the optimal
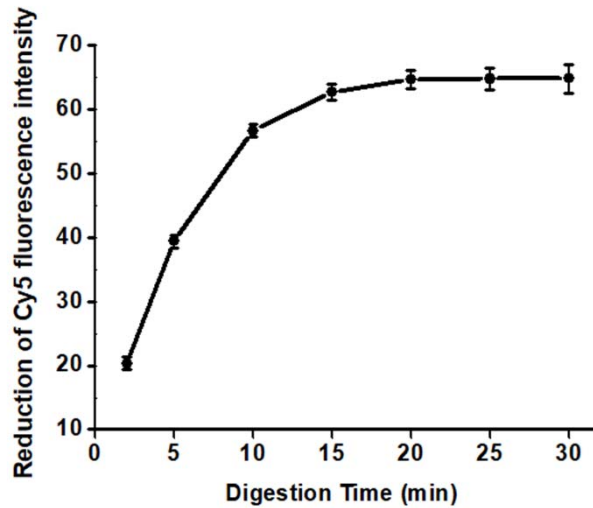
digestion time is selected to be 20 min.

Fig. S4 Variance of Cy5 fluorescence intensity with the digestion time. Error bars show the standard deviation of three independent experiments.

Optimization of the Amount of Exo III. The amount of Exo III is an important parameter in this assay, and it may influence the amount of Cy5 molecules cleaved from the Cy5-dsDNA-605QD nanostructures. As shown in Fig. S5, the reduction of Cy5 fluorescence intensity increases with the increasing concentration of Exo III, and reaches the highest value at the concentration of 20 U. Thus, 20 U is selected as the optimal concentration of Exo III.
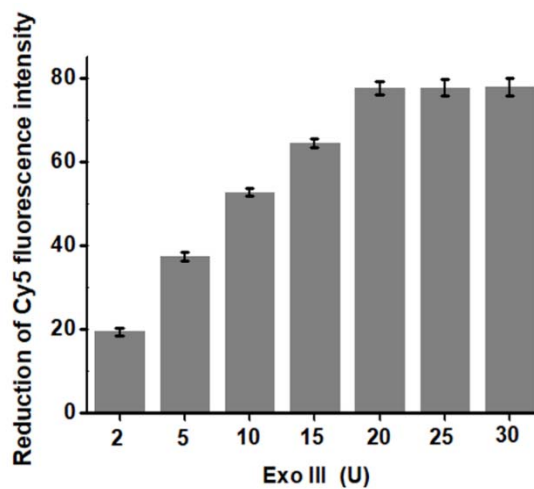
Fig. S5 Variances of Cy5 fluorescence intensity with the amount of Exo III. Error bars show the standard deviations of three independent experiments.

Selectivity of TdT Assay. We used Bst. DNA polymerase and four nonspecific DNA-modifying enzymes (i.e., Endo IV, Exo III, T4 DNA ligase and UDG) as the negative controls to investigate the selectivity of this assay. The Bst. DNA polymerase and four nonspecific DNA-modifying enzymes cannot initiate the polymerization reaction without a DNA template, while TdT can catalyze the continuous addition of dTTP to the ssDNA primer and generates a poly-T DNA product, inducing Exo III-mediated cyclic release of Cy5 molecules from the Cy5-dsDNA-605QD nanostructures. Consequently, TdT may induce great reduction of Cy5 counts (Fig. S6, red column). In contrast, no significant reduction of Cy5 counts is observed in the control groups with reaction buffer, Bst. DNA polymerase, Endo IV, Exo III, T4 DNA ligase and UDG (Fig. S6). These results demonstrate the good selectivity of the proposed method towards TdT.
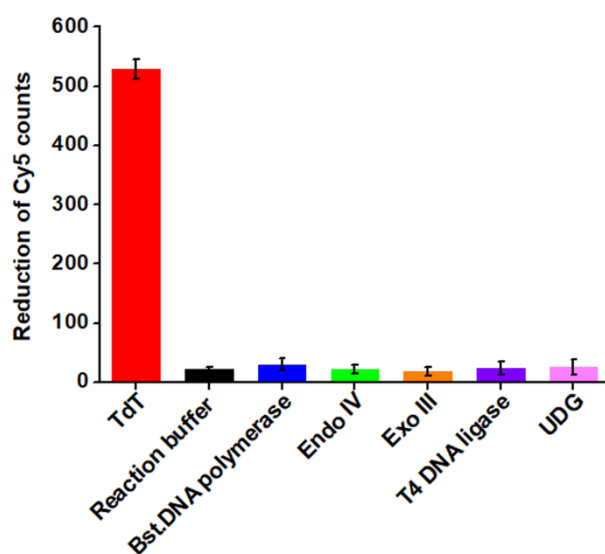

Fig. S6 Measurement of the reduction of Cy5 counts in response to 0.01 U/μL TdT, reaction buffer, 0.01 U/μL Bst. DNA polymerase, 0.01 U/μL Endo IV, 0.01 U/μL Exo III, 0.01 U/μL T4 DNA ligase, and 0.01 U/μL UDG, respectively. Error bars show the standard deviation of three independent experiments.

Inhibition Effect of ATP, GTP and CTP upon TdT Activity.

To demonstrate the feasibility of this method for the screening of TdT inhibitors, we used ATP as a model inhibitor. ATP is a competitive inhibitor that can strongly prevent the polymerization of dNTP through blocking the deoxyribotriphosphate binding site of the enzyme.[5] We monitored the variance of the relative activity of TdT with different concentrations of ATP, and found that the relative activity of TdT decreases with increasing concentration of ATP (Fig. S7). The IC_{50} value is calculated to be 29.96 mM, consistent with that obtained by the silver cluster-based fluorescence assay (IC_{50} = 35.55 mM),[5] suggesting the feasibility of this method for TdT inhibitor screening. In addition, we investigated the inhibition effects of GTP and CTP on TdT activity, respectively. As shown in Fig. S8, with the increasing concentration of GTP (Fig. S8A) and CTP (Fig. S8B), the relative activities of TdT decrease correspondingly. The IC_{50} values of GTP and CTP are determined to be 81.53 μM and 52.53 μM, respectively.
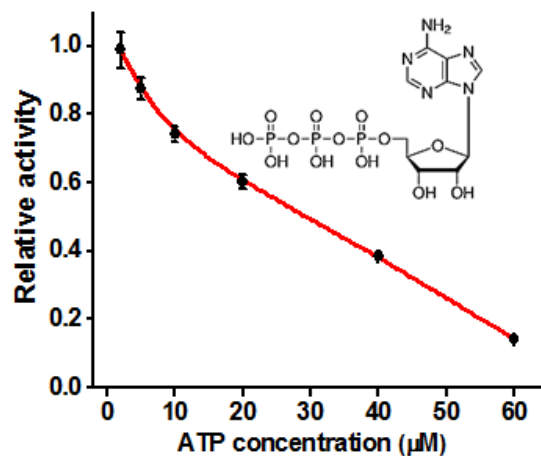

Fig. S7 Variance of the relative activity of TdT with the ATP concentration. The inset shows the chemical structure of ATP. The TdT concentration is 0.01 U/μL. Error bars show the standard deviation of three experiments.
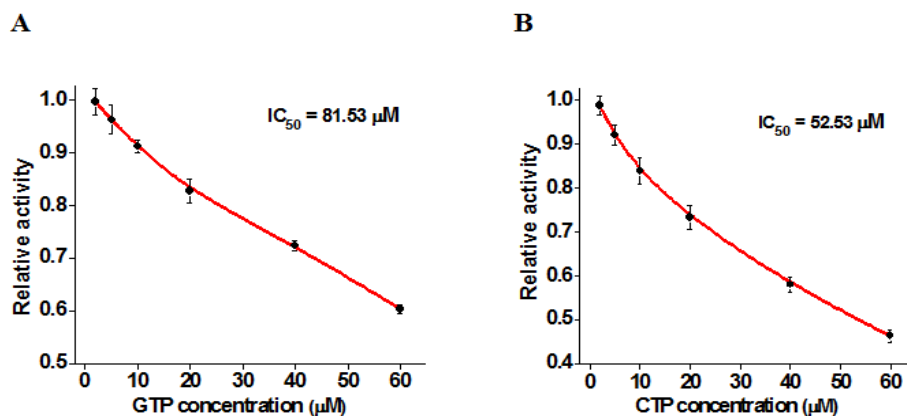
A

B

Fig. S8 Variance of relative activity of TdT with different-concentration GTP (A) and CTP (B).

The TdT concentration is 0.01 U/μL. Error bars show the standard deviation of three experiments.

Measurement of TdT Activity in Cell Lysates. To investigate the feasibility of the proposed

method for the detection of TdT activity in different cancer cells, we measured TdT activity in

HeLa and Molt-4 cells, respectively. As shown in Fig. S9, great reduction of Cy5 counts is

observed in Molt-4 cells (Fig. S9, red column), while no obvious reduction of Cy5 counts is

detected in both control group (Fig. S9, black column) and HeLa cells (Fig. S9, blue column),

suggesting that the capability of this method to discriminate leukemic cells from other cancer cells

in clinical diagnosis.. Furthermore, to investigate the variances of Cy5 counts in response to

different amounts of TdT enzymes, we extracted the proteins from 5, 10, 100, 1000, and 10000

Molt-4 cancer cells, respectively. The protein concentration is quantified with a modified Lowry

protein assay kit. As shown in Fig. S10, the reduction of Cy5 counts increases with the increasing

concentration of proteins extracted from 5, 10, 100, 1000, and 10000 Molt-4 cancer cells. In the

logarithm scale, the reduction of Cy5 counts shows a linear correlation with the protein

concentration. The correlation equation is $N = 242.6 \log_{10} C + 73.9$ with a correlation coefficient

of 0.9991, where C represents the concentration of proteins extracted from Molt-4 cancer cells and

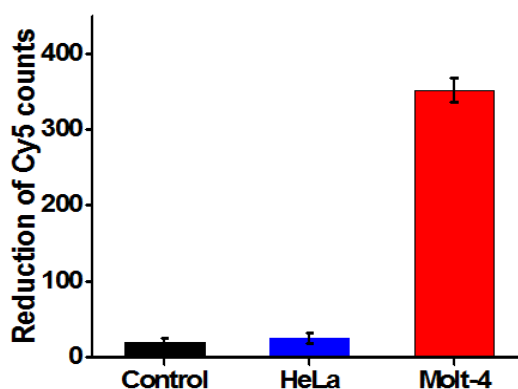*N* represents the reduction of Cy5 counts, respectively.

Fig. S9 Measurement of the reduction of Cy5 counts in response to the control group with only lysis buffer (black column), 1000 HeLa cells (blue column), and 1000 Molt-4 cells (red column), respectively. Error bars represent the standard deviations of three experiments.
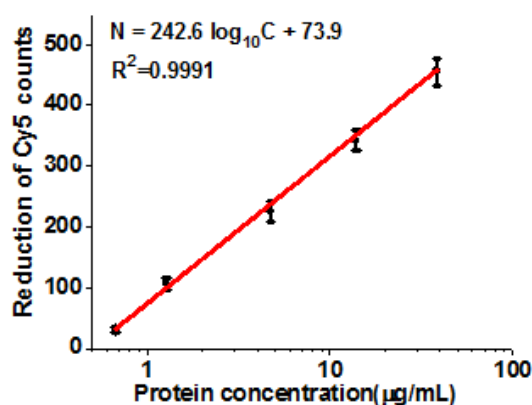

Fig. S10 Variance of Cy5 counts with the concentration of proteins extracted from Molt-4 cells. Error bars represent the standard deviations of three experiments.

Sensitivity and Specificity of TdT Assay in Human Blood Serum. We investigated the sensitivity of the proposed method in 10% human blood serum samples. As shown in Fig. S11, the reduction of Cy5 counts increases gradually with the increasing concentration of TdT in human blood serum samples, and shows a linear correlation with the logarithm of TdT concentration in the range from 1×10^{-6} to 1×10^{-3} U/µL. The correlation equation is $N = 103.8 \log_{10} C + 643.7$

(R^2 = 0.9948), where N is the reduction of Cy5 counts and C is the TdT concentration, respectively. The detection limit is determined to be 1×10^{-6} U/μL, consistent with the value measured with pure TdT enzymes (Fig. 3). We further investigated the specificity of the proposed method in 10% human blood serum samples. As shown in Fig. S12, only TdT can induce great reduction of Cy5 counts, while no reduction of Cy5 counts is observed in reaction buffer and other interferences (i.e., Bst. DNA polymerase, Endo IV, Exo III, T4 DNA ligase and UDG), consistent with the result measured with pure TdT enzymes (Fig. S6). These results clearly demonstrate that the proposed method exhibit high sensitivity and good specificity in complex samples.
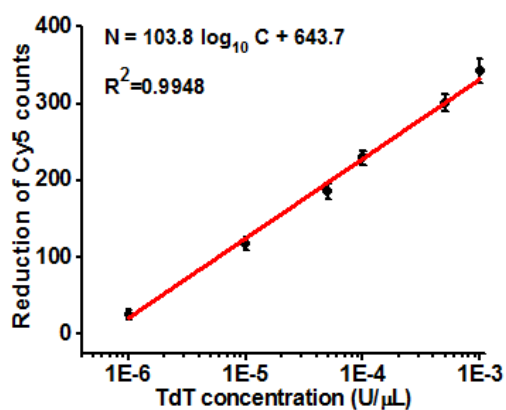

Fig. S11 Variance of the reduction of Cy5 counts with increasing concentration of TdT. Error bars show the standard deviation of three experiments.
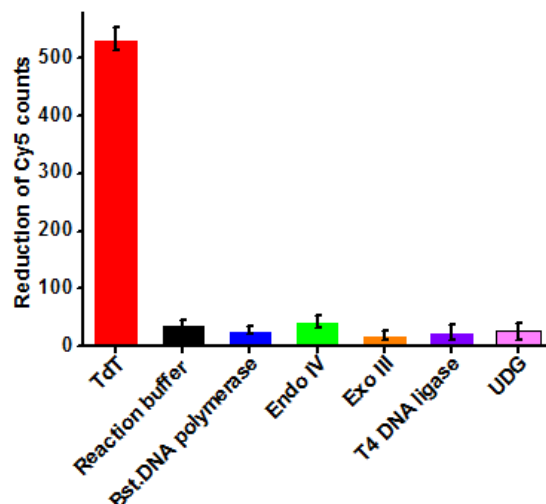
Fig. S12 Variance of the reduction of Cy5 counts in response to 0.01 U/μL TdT, reaction buffer, 0.01 U/μL Bst. DNA polymerase, 0.01 U/μL Endo IV, 0.01 U/μL Exo III, 0.01 U/μL T4 DNA ligase, and 0.01 U/μL UDG, respectively. Error bars show the standard deviation of three independent experiments.

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