Supporting Information

Dual enzyme-assisted one-step isothermal real-time amplification assay for ultrasensitive detection of polynucleotide kinase activity

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1. Experimenal section

1.1. Materials and Reagents

Both Recognition probe (TTC TTC TTC TTC TTC TTC-P, P = phosphorylation) and Signal probe (FAM-TTT TTT TTT TTT-BHQ1) were purchased from Sangon Biotech. Co. Ltd. (Shanghai, China). Their concentrations were represented as single-stranded concentrations, which were calculated by the UV absorbance at 260 nm. Molar extinction coefficient was determined by OligoAnalyzer 3.1 software provided in the following website (http://sg.idtdna.com/calc/analyzer). Ammonium sulfate ($(NH_4)_2SO_4$) and sodium hydrogen phosphate (Na_2HPO_4) were obtained from MACKLIN (Shanghai, China) and HEOWNS (Tianjin, China), respectively. T4 polynucleotide kinase (T4 PNK), 10 × reaction buffer (200 mM Tris-HAc, pH 7.9, 500 mM KAc, 100 mM $Mg(Ac)_2$, 50 mM DTT), T7 Exonuclease (T7 Exo) and terminal deoxynucleotidyl transferase (TdT) were obtained from New England Biolabs (Beijing, China). Deoxyadenosine triphosphate (dATP), ethidium bromide (EB) and DNA ladder marker were obtained from Tiangen Biotech. Co. Ltd. (Beijing, China). N,N,N′,N′-tetramethylethylenediamine (TEMED) was obtained from Beyotime (Shanghai, China). 30% acrylamide-N,N′-methylenebisacrylamide (Acr-Bis, 29:1) was obtained CWBIO (Beijing, China). 6 × loading buffer (0.25% of bromophenol blue, 0.25% of xylene cyanol, 30% of glycerol and 10 mM of EDTA) was obtained from Takara (Dalian, China). All chemical reagents were of analytical grade and used without further purification. Ultrapure water (resistance > 18 $\Omega M \cdot cm^{-1}$) was obtained through a Millipore filtration system.

1.2. T4 PNK activity assay

1 μL of 10 μM Recognition probe and 1 μL of 10 μM Signal probe were diluted in 1 × reaction buffer. Then, 1 μL of 100 mM dATP, 10 U of TdT, 5 U of T7 Exo and different amounts of T4 PNK were added. In end-point detection mode, the obtained 100 μL reaction mixture was incubated at 37 °C for 3 h, and the fluorescence signal was recorded on a Shimadzu RF-5301pc fluorescence spectrometer (Shimadzu Ltd., Japan) in the range of 500 ~ 600 nm by setting the excitation wavelength at 490 nm. The fluorescence signal intensity at 518 nm was used for quantitative analysis of T4 PNK activity. In real-time detection mode, 30 μL reaction mixture was prepared and incubated at 37 °C for 3 h in a commercial StepOnePlus™ Real-Time PCR instrument. The fluorescence detection channel set for FAM was used to collect the fluorescence signal, and the fluorescence intensity was monitored at intervals of 1 min.

1.3. Non-denaturing polyacrylamide gel electrophoresis (PAGE) analysis

50 μL of reaction mixture containing 1 × reaction buffer, 1 μM of FAM-labelled Recognition probe (FAM-TTC TTC TTC TTC TTC TTC-P), 10 U of T4 PNK, 10 mM of dATP, 25 U of TdT was prepared and incubated at 37 °C for 3 h. The mixture was heated at 95 °C for 5 min to inactivate TdT and then cooled to 37 °C. After addition of 5 μL of 100 μM FAM-labelled Signal probe (FAM-TTT TTT TTT TTT) and 25 U of T7 Exo, the mixture was incubated at incubated at 37 °C for another 3 h. 10 μL of reaction solution was sufficiently mixed with 2 μL 6 × loading buffer. The mixture was analyzed by 10% PAGE in 1 × TBE buffer (89 mM Tris-boric acid, 2.0 mM EDTA, pH 8.3) at a 120 V constant voltage for 45 min. The gel was

photographed by a gel documentation system (Huifuxingye, Beijing, China) after staining by EB solution for 20 min.

1.4. PNK inhibition study

The T4 PNK activity inhibition study was performed by using $(NH_4)_2SO_4$ and Na_2HPO_4 as the model inhibitors. The experimental procedures were similar to those mentioned above except that T4 PNK was pre-incubated with different concentrations of each inhibitor for 10 min before addition in sensing systems.

1.5. PNK activity analysis in cell extracts

In a humidified atmosphere of 5% CO_2 and 95% air, HeLa cells (human cervical cancer cell line) were cultured at 37 °C in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal calf serum (Sijiqing) and 1% penicillin-streptomycin (Gibco). The cell extracts were prepared using a nucleoprotein extraction kit (Sangon Biotech, Shanghai, China) according to the manufacturer's protocol. The collected cell extracts were used for PNK activity assay immediately or stored at -80 °C until use. The detection procedures were same as those described above but cell extracts were added in the sensing systems instead of T4 PNK.

2. Optimization of experimental conditions

2.1. Reaction temperature

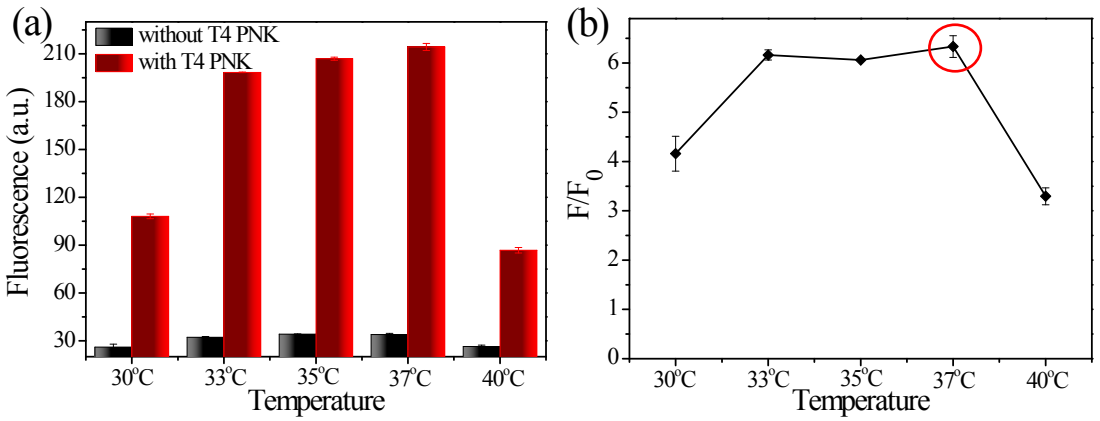

Figure S1. Variance of (a) fluorescence intensity and (b) signal-to-noise ratio (F/F_0) with reaction temperature. F and F_0 are the fluorescence intensities at 518 nm in the presence and absence of T4 PNK, respectively. [Recognition probe] = [Signal probe] =100 nM. The amouts of T7 Exo and TdT are 5 U and 10 U, respectively. No $CoCl_2$ is added.

2.2. Recognition probe concentration

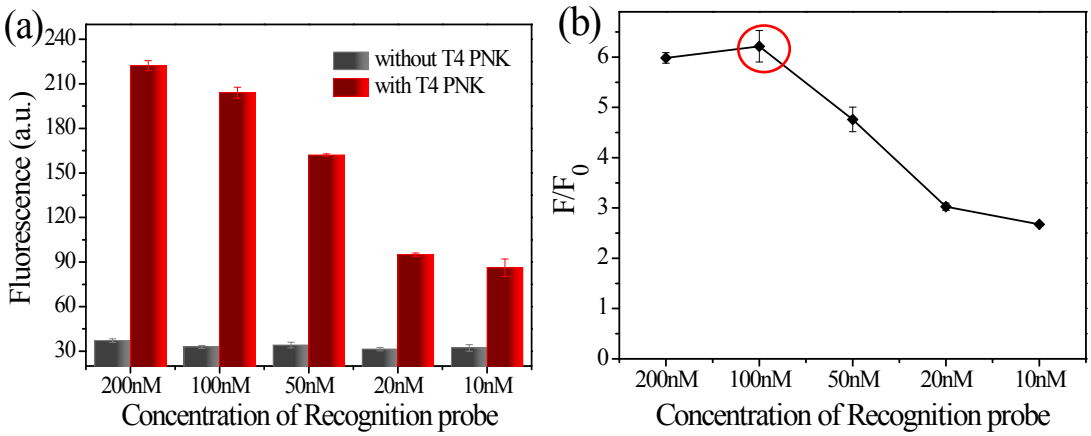

Figure S2. Variance of (a) fluorescence intensity and (b) signal-to-noise ratio (F/F_0) with the concentration of Recognition probe. F and F_0 are the fluorescence intensities at 518 nm in the presence and absence of T4 PNK, respectively. [Signal probe] =100 nM. The amouts of T7 Exo and TdT are 5 U and 10 U, respectively. No $CoCl_2$ is added. Reaction temperature is 37 °C.

2.3. Signal probe concentration

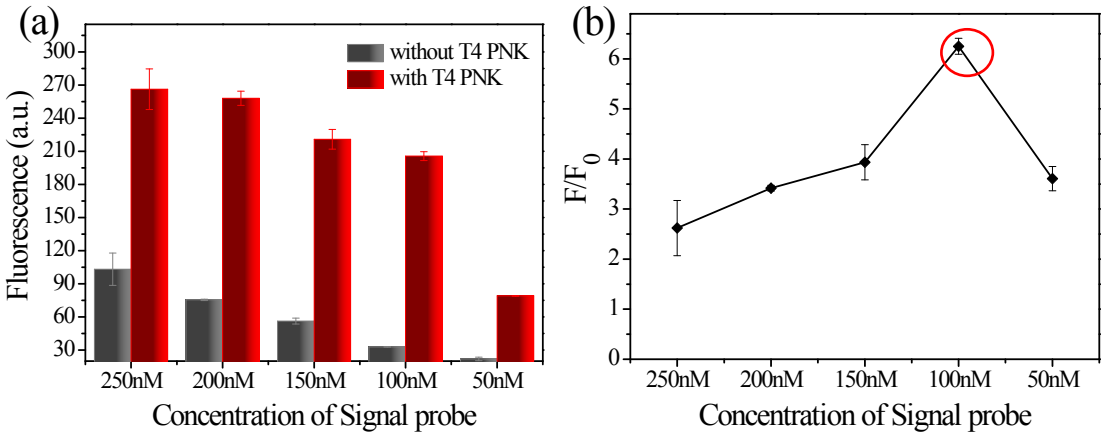

Figure S3. Variance of (a) fluorescence intensity and (b) signal-to-noise ratio (F/F_0) with the concentration of Signal probe. F and F_0 are the fluorescence intensities at 518 nm in the presence and absence of T4 PNK, respectively. [Recognition probe] = 100 nM. The amouts of T7 Exo and TdT are 5 U and 10 U, respectively. No $CoCl_2$ is added. Reaction temperature is 37 °C.

2.4. TdT amount

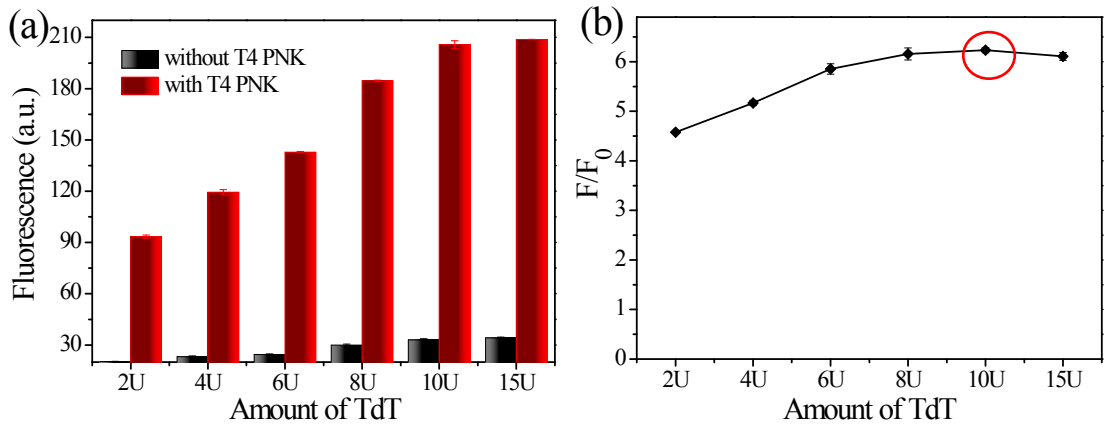

Figure S4 Variance of (a) fluorescence intensity and (b) signal-to-noise ratio (F/F_0) with TdT amount. F and F_0 are the fluorescence intensities at 518 nm in the presence and absence of T4 PNK, respectively. [Recognition probe] = [Signal probe] =100 nM. The amout of T7 Exo is 5 U. No $CoCl_2$ is added. Reaction temperature is 37 °C.

2.5. T7 Exo amount

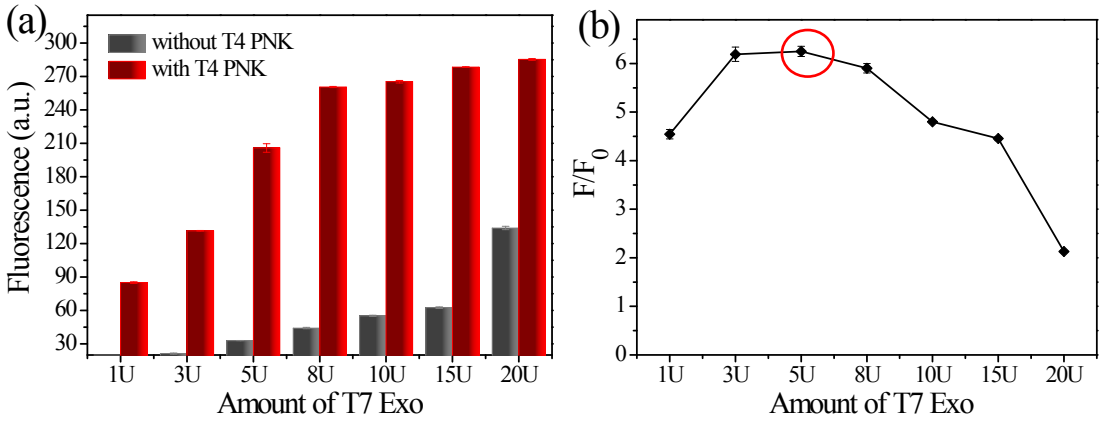

Figure S5. Variance of (a) fluorescence intensity and (b) signal-to-noise ratio (F/F_0) with T7 Exo amount. F and F_0 are the fluorescence intensities at 518 nm in the presence and absence of T4 PNK, respectively. [Recognition probe] = [Signal probe] =100 nM. The amout of TdT is 10 U. No $CoCl_2$ is added. Reaction temperature is 37 ^oC.

2.6. $CoCl_2$ concentration

It is reported that $CoCl_2$ can make TdT more efficient, especially when double-stranded DNA is used as the substrate of TdT.[S1] However, because the addition of $CoCl_2$ can quench the fluorescence of FAM in some degree, 0 mM of $CoCl_2$ was selected according to the criterion of F/F_0.

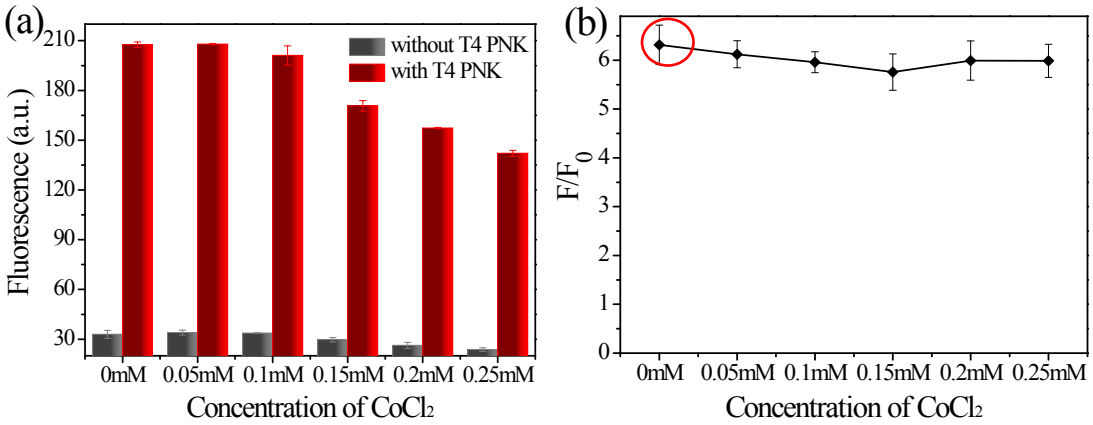

Figure S6 Variance of (a) fluorescence intensity and (b) signal-to-noise ratio (F/F_0) with the concentration of $CoCl_2$. F and F_0 are the fluorescence intensities at 518 nm in the presence and absence of T4 PNK, respectively. [Recognition probe] = [Signal probe] =100 nM. The amouts of T7 Exo and TdT are 5 U and 10 U, respectively. Reaction temperature is 37 ^oC.

3. PNK activity detection in real-time mode

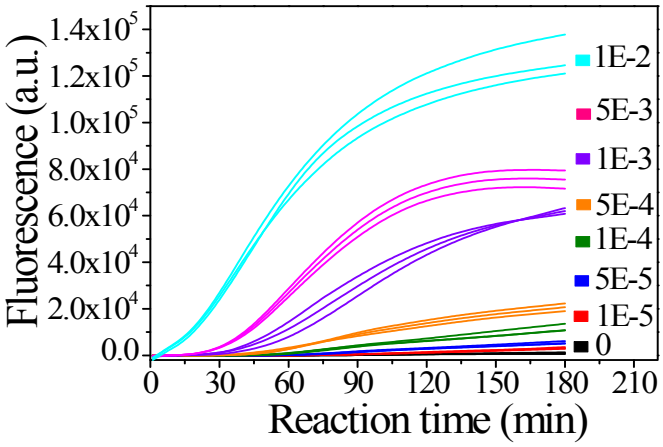

Figure S7 Fluorescence ~ time plot given by the sensing systems containing different amounts of T4 PNK. The T4 PNK concentrations are labelled in the figure.

4. Comparison of our PNK detection method with other reported ones

Table S1. Comparison of several PNK detection methods

Method	Operation and procedures	Detection limit (U/mL)	Cell analysis	Reference
Fluorescence	Signal amplification and endpoint fluorescence detection are conducted separately	1.00×10^{-3}	No	[16]
Fluorescence	Pre-preparation of the Probe; the signal amplification and real-time detection are executed	2.00×10^{-4}	Yes	[11a]
Fluorescence	Pre-preparation of the AuNPs-DNA probe; signal amplification and fluorescence detection are conducted separately	6.70×10^{-3}	No	[11c]
Fluorescence	Magnetic separation step is essential before the RCA; amplification and fluorescence detection are conducted separately	4.36×10^{-5}	No	[15]
Fluorescence	Pre-preparation of the AuNPs-DNA probe; signal amplification and single-molecule imaging are conducted	9.77×10^{-5}	Yes	[21]
Electrochemistry	Signal amplification and electrochemistry detection are conducted separately	7.76×10^{-4}	No	[8a]
Chemiluminesce-nce	Multi-step operations with different reaction temperature	2.20×10^{-4}	Yes	[9]
Colorimetry	One-step assay without amplification	0.06	No	[10]
Fluorescence	The signal amplification and real-time detection are all accomplished in one step	1.00×10^{-5}	Yes	This work

5. Effects of T4 PNK inhibitors on different enzymes

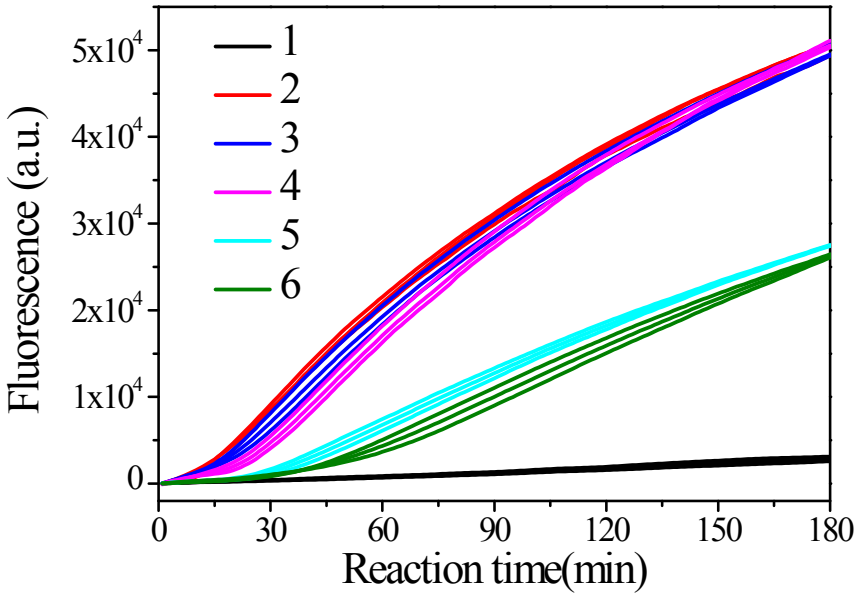

Figure S8. Fluorescence ~ time curves given by the sensing systems with or without inhibitors. (1): without T4 PNK and inhibitors; (2): only T4 PNK; (3): $(NH_4)_2SO_4$ was added after T4 PNK-catalyzed dephosphorylation of Recognition probe; (4): Na_2HPO_4 was added after T4 PNK-catalyzed dephosphorylation of Recognition probe; (5): treatment of T4 PNK with $(NH_4)_2SO_4$ before adding into the system; (6): treatment of T4 PNK with Na_2HPO_4 before adding into the system. [T4 PNK] = 0.001 U/mL; $[(NH_4)_2SO_4] = [Na_2HPO_4]$ = 12 mM.

6. PNK inhibition study

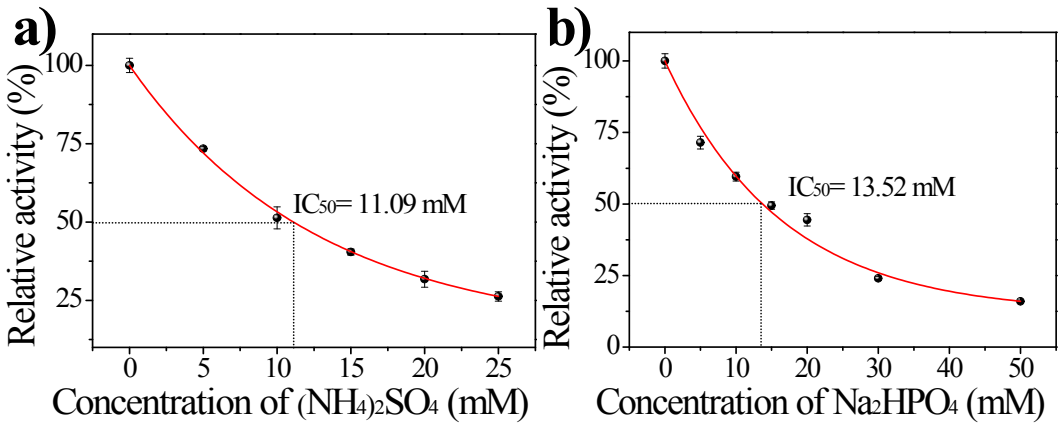

Figure S9 Relative activity of T4 PNK in the presence of increasing concentrations of (a) $(NH_4)_2SO_4$ and (b) Na_2HPO_4, respectively.

7. Reference

S1. Roychoudhury, R.; Jay, E.; Wu, R. Terminal labeling and addition of homopolymer tracts to duplex DNA fragments by terminal deoxynucleotidyl transferase. *Nucleic Acids Res.* **1976**, 3, 101-116.