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Supporting Information

Modified exponential amplification reaction (EXPAR) with improved signal-to-noise ratio for ultrasensitive detection of polynucleotide kinase

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1. Experimenal section

1.1. Materials and reagents

All HPLC-purified DNA oligonucleotides (Table S1) were obtained from Sangon Biotech. Co. Ltd. (Shanghai, China). T4 polynucleotide kinase (T4 PNK), nicking endonuclease Nb.BbvCI, Klenow fragment polymerase (KFP), and deoxyribonucleoside 5'-triphosphate mixture (dNTPs) were obtained from New England Biolabs (Beijing, China). Thioflavin T (ThT) was obtained from Sigma. All chemical reagents were of analytical grade and used without further purification. SYBR Green I (20 × in DMSO) was obtained from keyGEN BioTECH (Jiangsu, China). Ultrapure water obtained from a Millipore filtration system was used in all experiments.

Table S1. The oligonucleotides used in this work.

Oligonucleotides	Sequence (5′-3′)
T&P	CAACATTACTCCTCAGCAACATTACTCCTCATT CACAACATTTTTTTTTTAATGTTGTG-*p*
rC&T	ATCCCTATCCCTATCCCTATCCCTACCTCAGCA ACATTACTCCTCA
P	TTCTTCTTCTTCTTCTTC-*p*
rC&cP	CCCTATCCCTATCCCTATCCCTATCCTCAGCGA AGAAGAAGAAGAAGAA
T&P'	GCCCCTATCCCTATCCCTATCCCTCAGCCCCTA TCCCTATCCCTATCCCTCATTCACAACATTTTTT TTTTAATGTTGTG-*p*
Synthetic G-rich product	TGAGGTAGGGATAGGGATAGGGATAGGGAT
Synthetic EXPAR product	TGAGGAGTAATGTTG

The letter *p* represents the phosphate modification. The recognition sequences for Nb.BbvCI in **T&P**, **rC&T, rC&cP** and **T&P'** are underlined.

1.2. T4 PNK activity detection

Modified EXPAR-based sensing system. 6 nM **T&P** and 160 nM **rC&T** were mixed with different concentrations of T4 PNK, 0.4 mM dNTPs, 0.5 mM KCl, 5 μM ThT, 1.5 U KFP and 4.5 U Nb.BbvCI in 1 × T4 PNK reaction buffer (70 mM Tris-HCl (pH=7.6), 5 mM DL-Dithiothreitol, 10 mM $MgCl_2$). The mixture was incubated at 37 °C for 40 min, and then its fluorescence spectrum was recorded in the range of 430 ~ 600 nm under the excitation at 425 nm. The fluorescence intensity at 485 nm was used for T4 PNK activity detection.

Traditional EXPAR-based sensing system. 50 nM **T&P** was mixed with different concentrations of T4 PNK, 0.4 mM dNTPs, 5 μL SYBR Green I, 0.075 U KFP and 1 U Nb.BbvCI in 1 × T4 PNK reaction buffer. The mixture was incubated at 37 °C for 15 min, and then its fluorescence spectrum was recorded in the range of 460 ~ 620 nm under the excitation at 497 nm. The fluorescence intensity at 520 nm was used for T4 PNK activity detection.

SDA-based sensing system. 50 nM **P** and 300 nM **rC&cP** were mixed with different

concentrations of T4 PNK, 0.4 mM dNTPs, 0.5 mM KCl, 5 μM ThT, 1 U KFP and 3.5 U Nb.BbvCI in 1 × T4 PNK reaction buffer. The mixture was incubated at 37 °C for 100 min, and then its fluorescence spectrum was recorded in the range of 460 ~ 550 nm under the excitation at 425 nm. The fluorescence intensity at 485 nm was used for T4 PNK activity detection.

1.3. Denatured polyacrylamide gel electrophoresis (PAGE) analysis

25 μL of reaction mixture containing 1 × reaction buffer, 1.6 μM of **T&P**, 3.2 μM of **rC&T**, 0.4 U of T4 PNK, 10 mM of dNTPs, 1 U of KFP and 3 U of Nb.BbvCI was prepared and incubated at 37 °C for 3 h. Then, 15 μL of reaction solution was sufficiently mixed with 14 μL formamide and 1 μL 6 × loading buffer. The mixture was analyzed by 15% denaturing PAGE in 1 × TBE buffer (89 mM Tris-boric acid, 2.0 mM EDTA, pH 8.3) at a 120 V constant voltage for 110 min. The gel was photographed by a gel documentation system (Huifuxingye, Beijing, China) after staining by ethidium bromide (EB) solution for 20 min.

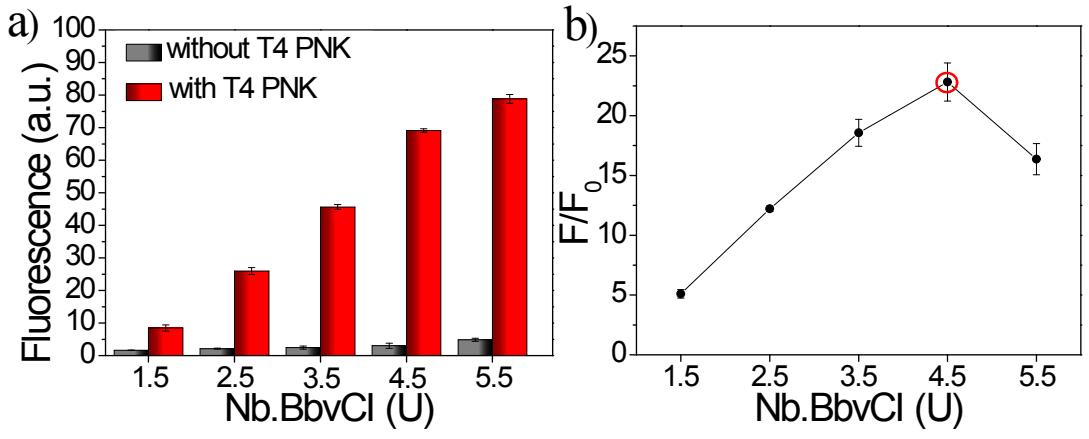
1.4. Preparation of cell lysates

About 1×10^7 HeLa or MDCK cells were dispensed in a 1.5 mL centrifuge tube. The cells were then washed with PBS buffer twice and centrifuged at 2000 rpm for 3 min. 500 μL lysis buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, sodium pyrophosphate, β-glycerophosphate, EDTA, Na_3VO_4 and leupeptin) was added into the cell residues. The mixture was incubated on ice for 30 min with vortexing for 15 s every 5 min. The resulting mixture was centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was used immediately for PNK activity assay.

1.5. T4 PNK inhibition study

$(NH_4)_2SO_4$ and Na_2HPO_4 were used as the model inhibitors of T4 PNK to perform the inhibition study. The experimental steps were the same as those of modified EXPAR-based sensing operation except that different concentrations of $(NH_4)_2SO_4$ (or Na_2HPO_4) were added in the sensing system together with 0.1 U mL^{-1} T4 PNK.

2. Optimization of modified EXPAR-based sensing system



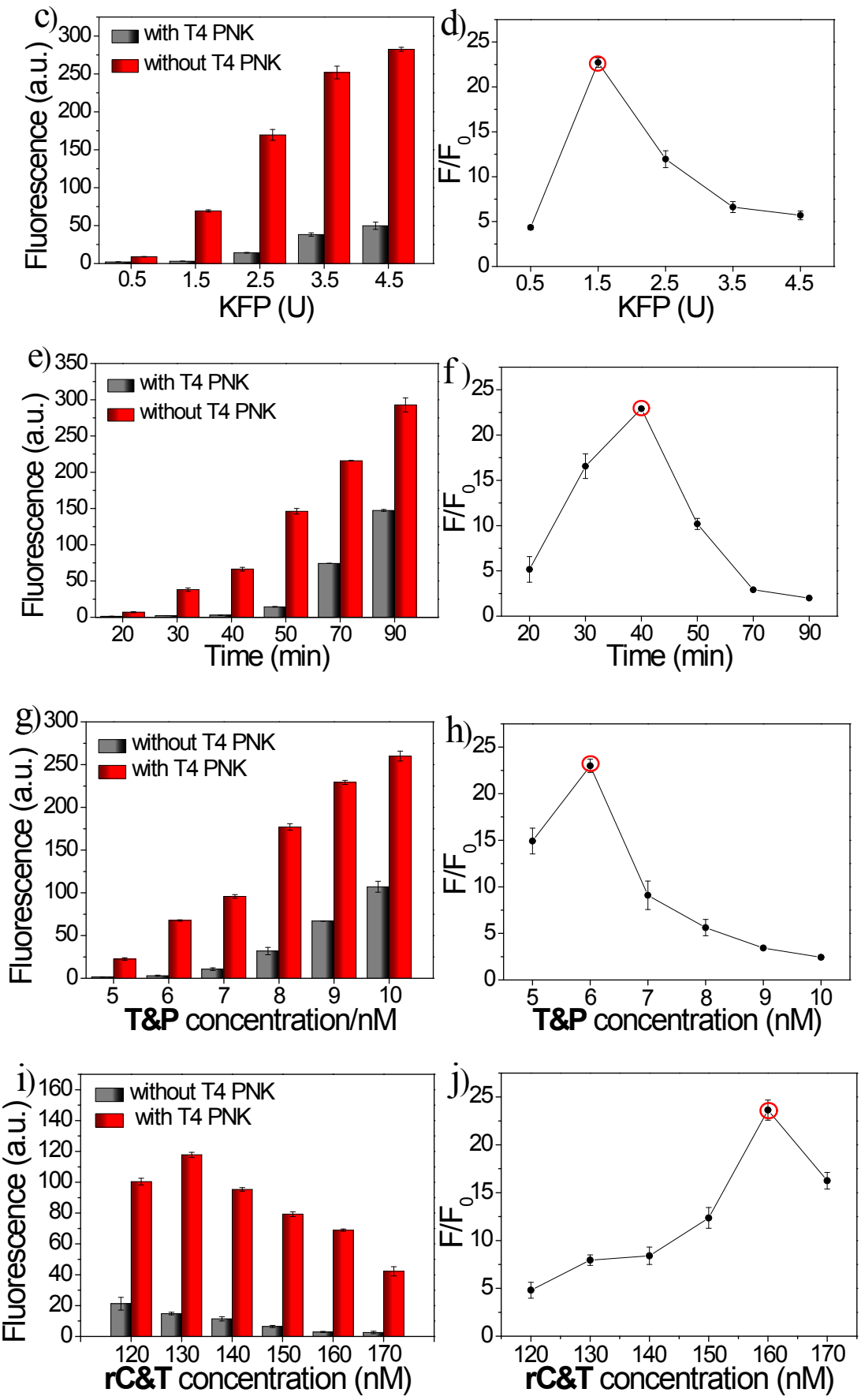


Figure S1. Fluorescence intensity change of the sensing system with (a) Nb.BbvCI concentration,

(c) KFP concentration, (e) reaction time, (g) **T&P** concentration and (i) **rC&T** concentration in the absence or presence of T4 PNK. Signal-to-noise ratio (F/F_0) change of the sensing system with (b) Nb.BbvCI concentration, (d) KFP concentration, (f) reaction time, (h) **T&P** concentration and (j) **rC&T** concentration. The concentration of T4 PNK is 0.01 U mL^{-1}.

3. T&P'-based T4 PNK-sensing system

We also tried to integrate the functions of **T&P** and **rC&T** into one oligonucleotide **T&P'** (Table S1). The template sequence in **T&P'** was designed as C-rich sequence. Thus, the T4 PNK-triggered SDA reaction could give G-rich product. We hoped that the product could play two roles: primer of EXPAR to initiate exponential amplification and substrate of ThT to give fluorescence signal output. However, almost no ThT fluorescence increase was given by corresponding sensing system even when the reaction time was extended to 3h (Fig. S2). The reason might be that the G-rich amplification product might fold into stable G-quadruplex, thus cannot efficiently trigger exponential EXPAR.

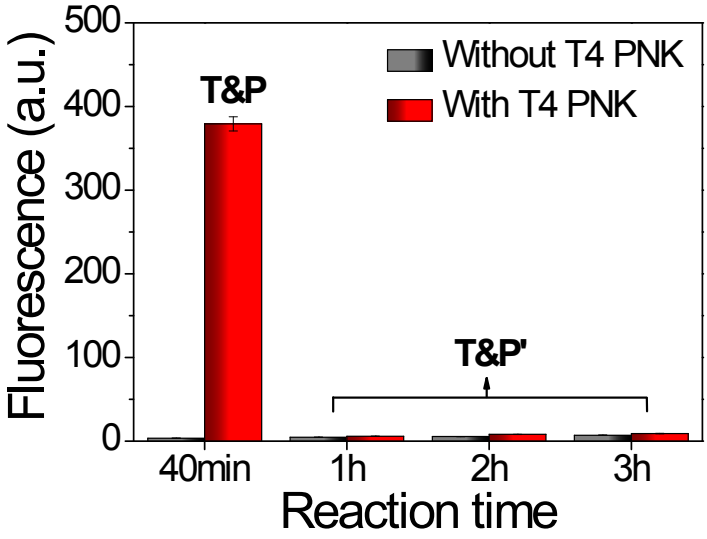


Figure S2. Comparison of T&P or T&P'-based T4 PNK-sensing systems. The concentration of T4 PNK is 1.0 U mL^{-1}.

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

Table S2. Comparison of several PNK detection methods

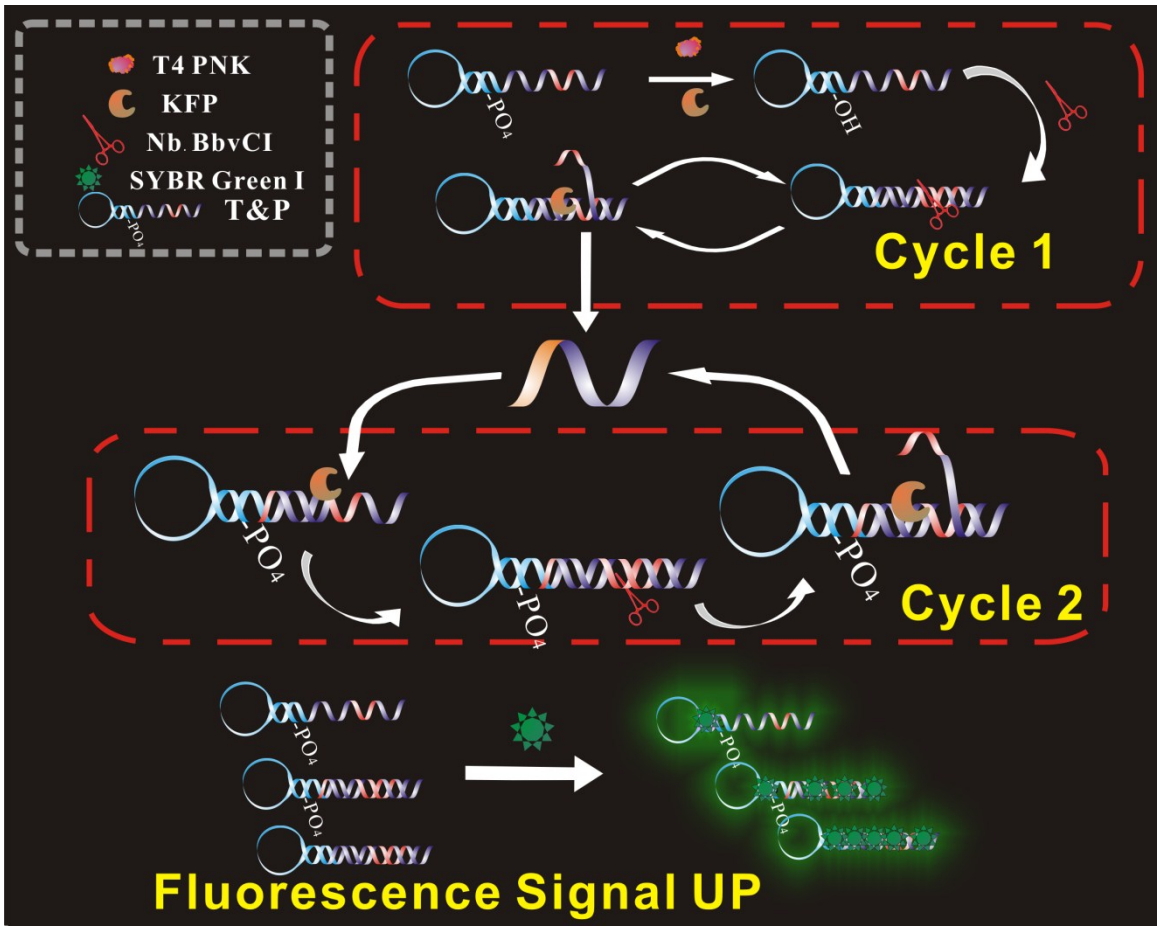
Method	Operation and procedures	Detection limit (U mL^{-1})	Cell analysis	Reference[a]
Fluorescence	Flow cytometric bead assay	4×10^{-6}	No	24
Fluorescence	Phosphorylation triggered isothermal exponential amplification	2.00×10^{-4}	Yes	33
Fluorescence	Single-molecule detection of PNK based on phosphorylation recovery of fluorescence quenched by Au nanoparticle	9.77×10^{-5}	Yes	25
Fluorescence	Multifunctional magnetic probes and polymerization nicking reactions mediated hyperbranched rolling circle amplification	4.36×10^{-5}	No	27
Fluorescence	Paper-based fluorescence assay with λ exonuclease assistance	1×10^{-4}	Yes	17
Electrochemistry	Signal amplification and electrochemistry detection are conducted separately	7.76×10^{-4}	No	22
Fluorescence	Allosteric aptamer probe consisting of streptavidin aptamer and the complementary DNA	0.01	Yes	26
Fluorescence	exponential amplification reaction	7.9×10^{-4}	no	34
Fluorescence	SDA-mediated exponential amplification reaction	1.5×10^{-6}	Yes	This work

[a]The references are listed in the main text.

5. Traditional EXPAR-based T4 PNK-sensing strategy

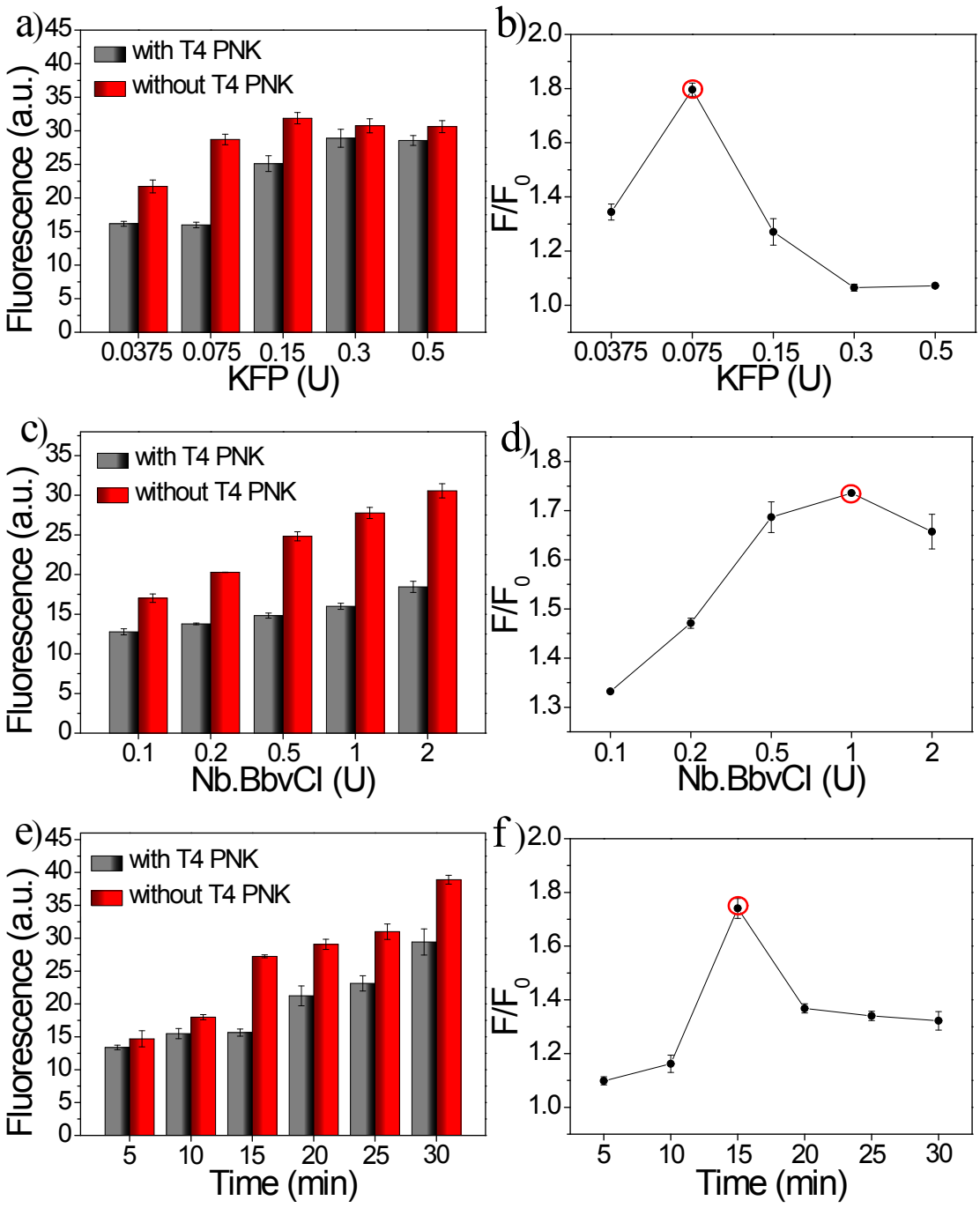
5.1 Working mechanism of the traditional EXPAR-based T4 PNK-sensing strategy

As we all know, the SYBR Green I dye will generate a fluorescent signal after inserting into the double-stranded DNAs. As a result, the traditional EXPAR reaction commonly uses SYBR Green I to monitor the progress of the reaction.[S1-S4] In order to highlight the advantages of our proposed method, we also designed a T4 PNK sensor utilizing traditional EXPAR strategy, whose working principle is illustrated in Scheme S1. In this sensing system, only **T&P** was used. T4 PNK can convert the 3′-phosphate group of **T&P** to 3′-OH, thus initiate EXPAR reaction, producing exponentially amplified EXPAR products. By utilizing the fluorescence response of SYBR Green I to DNA strands (including double-stranded and single-stranded ones), T4 PNK activity can be detected.



Scheme S1. Working mechanism of traditional EXPAR-based T4 PNK-sensing strategy.

5.2 Optimization of traditional EXPAR-based sensing system



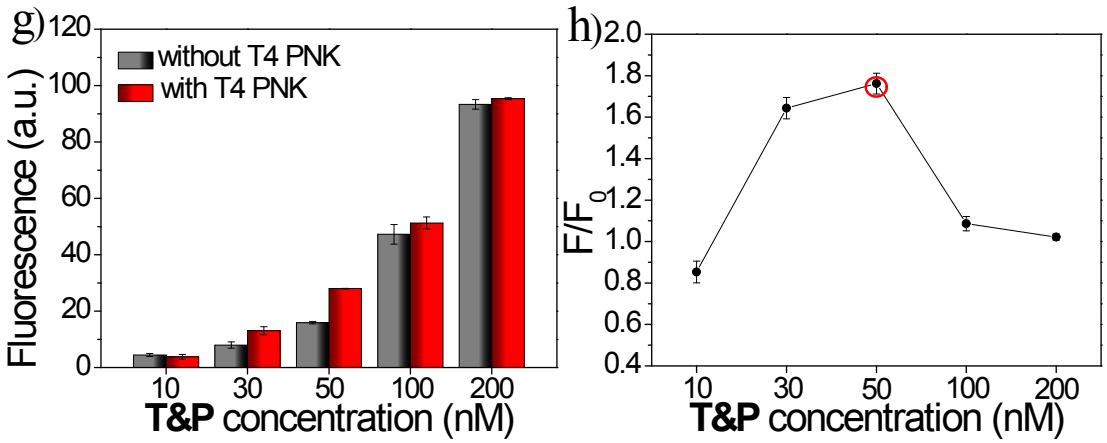


Figure S3. Fluorescence intensity change of the sensing system with (a) KFP concentration, (c) Nb.BbvCI concentration, (e) reaction time and (g) **T&P** concentration in the absence or presence of T4 PNK. Signal-to-noise ratio (F/F_0) change of the sensing system with (b) KFP concentration, (d) Nb.BbvCI concentration, (f) reaction time and (h) **T&P** concentration. The concentration of T4 PNK is 0.5 U mL^{-1}.

5.3. Sensitivity of the traditional EXPAR-based sensing system

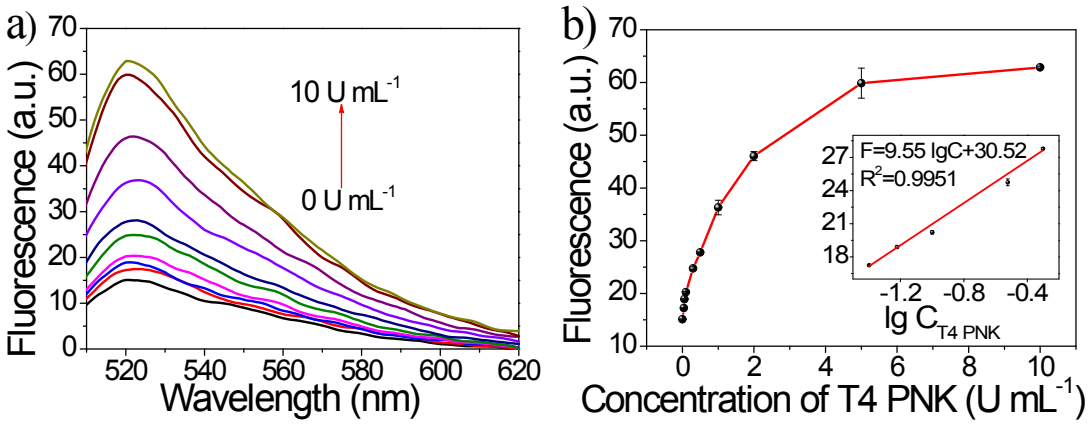
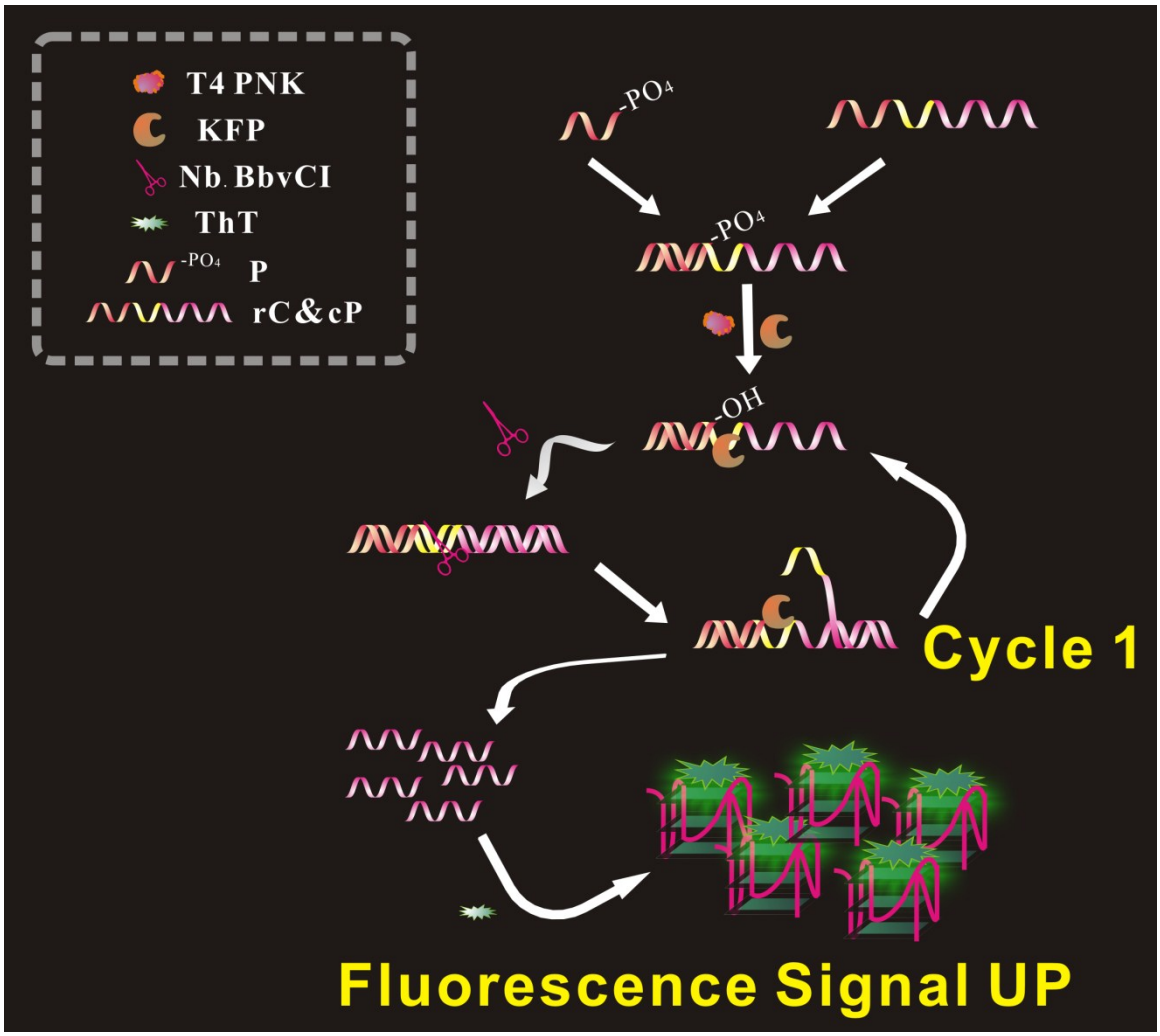


Figure S4. (a) Fluorescence spectra of the sensing systems containing different amounts of T4 PNK; (b) T4 PNK activity-dependent fluorescence change at 520 nm. The insert shows the linear relationship between the fluorescence intensity and the logarithm of T4 PNK amount in the range of 0.04 ~ 0.5 U mL^{-1}.

6. SDA-based T4 PNK-sensing strategy

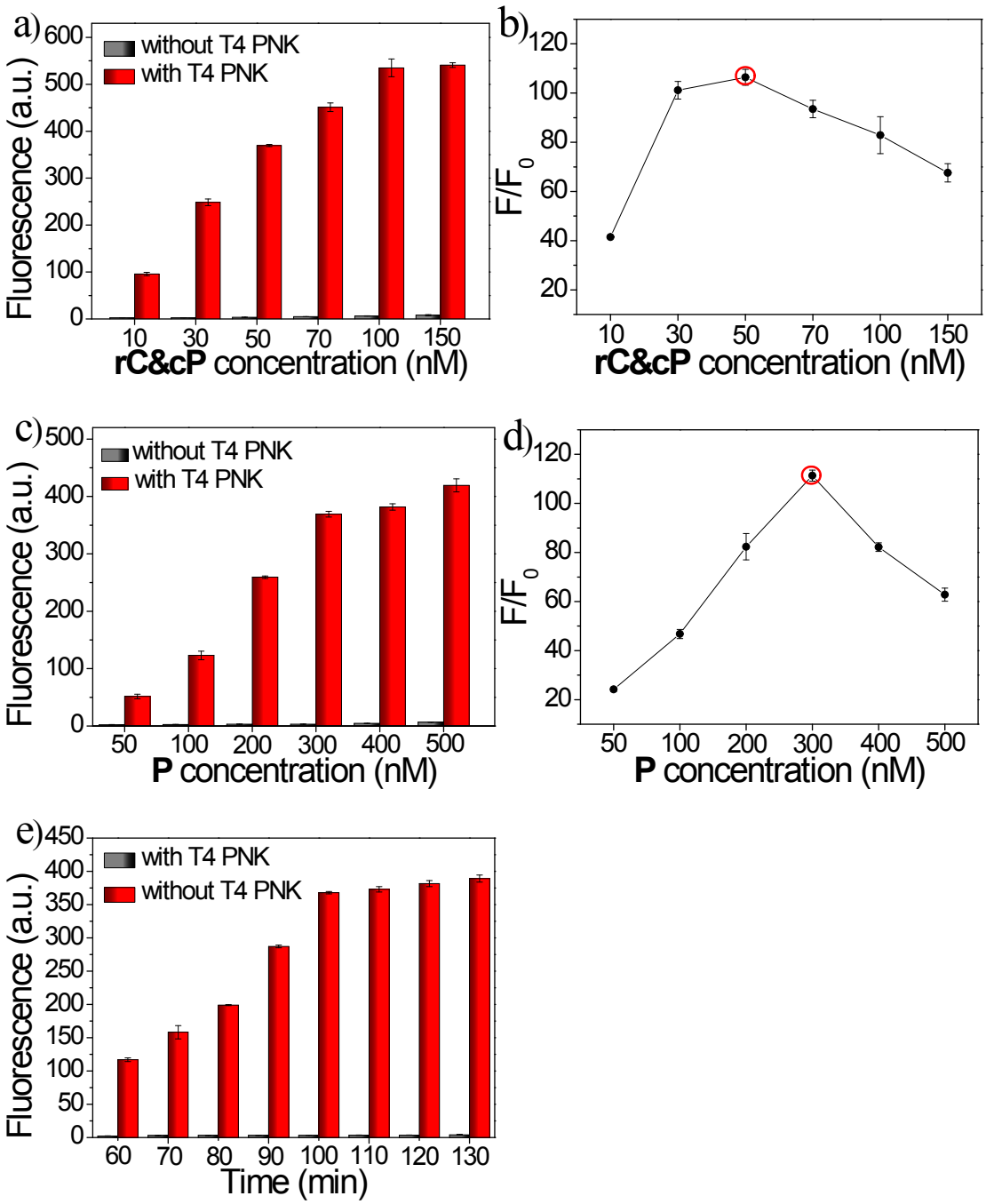
6.1 Working mechanism of SDA-based T4 PNK-sensing strategy

In order to highlight the powerful signal amplification capability of EXPAR, we established a T4 PNK sensor utilizing SDA strategy. Scheme S2 illustrates the working principle of the SDA-based T4 PNK-sensing method. The oligonucleotides of **P** and **rC&cP** were used as the primer and template, respectively. **P** can hybridize with **rC&cP** but cannot be extended along **rC&cP** due to the presence of 3′-phosphate group. In the presence of T4 PNK, the 3′-phosphate group of **P** is converted to 3′-OH. As result, **P** is extended along **rC&cP** by KFP and triggers the nicking-polymerization-displacement cycle of the SDA reaction in the presence of Nb.BbvCI, producing G-rich amplification products that can be specifically probed by ThT.



Scheme S2. Working mechanism of SDA-based T4 PNK-sensing strategy.

6.2 Optimization of SDA-based sensing system



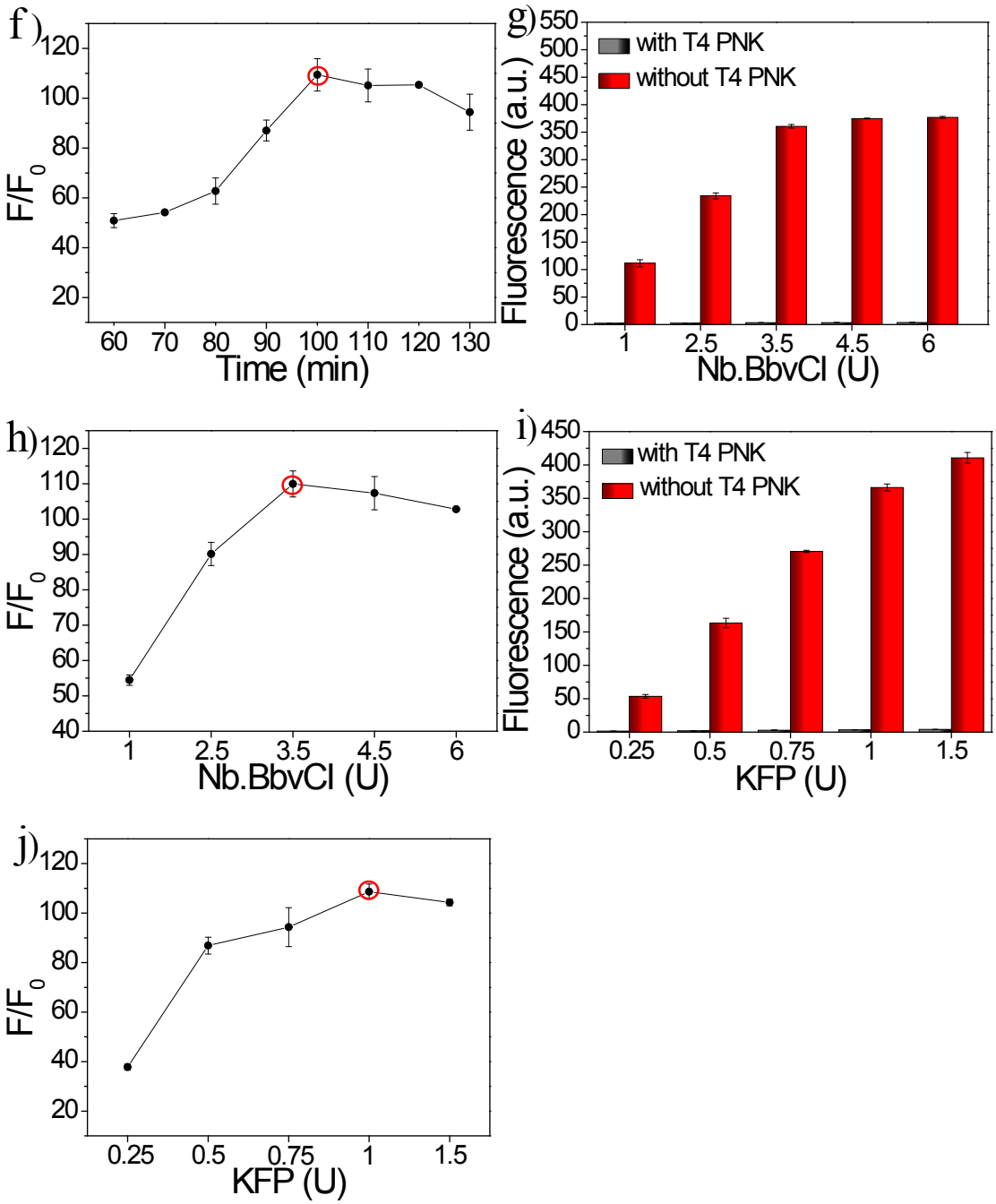


Figure S5. Fluorescence intensity change of the sensing system with (a) **rC&cP** concentration, (c) **P** concentration, (e) reaction time, (g) Nb.BbvCI concentration and (i) KFP concentration in the absence or presence of T4 PNK. Signal-to-noise ratio (F/F_0) change of the sensing system with (b) **rC&cP** concentration, (d) **P** concentration, (f) reaction time, (h) Nb.BbvCI concentration and (j) KFP concentration. The concentration of T4 PNK is 0.06 U mL^{-1}.

6.3 Sensitivity of SDA-based sensing system

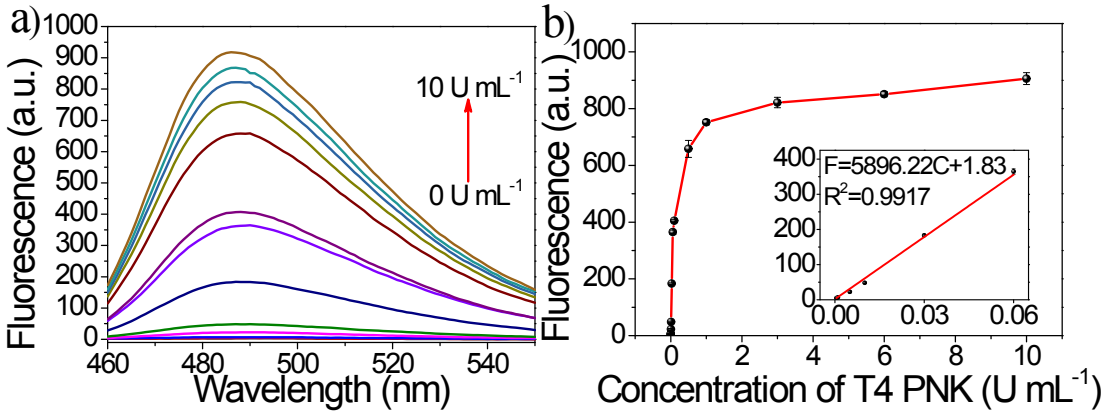


Figure S6. T4 TNK activity detection utilizing SDA strategy. (a) Fluorescence spectra of the sensing systems containing different amounts of T4 PNK. (b) T4 PNK activity-dependent fluorescence change at 485 nm. The insert shows the linear relationship between the fluorescence intensity and T4 PNK amounts in the range of $5 \times 10^{-4} \sim 0.06$ U mL^{-1}.

7. Selectivity of our modified EXPAR-based T4 PNK-sensing system

As shown in Fig. S7, our modified EXPAR-based T4 PNK-sensing system could discriminate PNK from other enzymes clearly. Different from T4 PNK that gave greatly increased fluorescence output, none of terminal deoxynucleotidyl transferase (TdT), uracil-DNA glycosylase (UDG), T4 DNA ligase and heat-inactived T4 PNK could give detectable fluorescence increase compared to the blank control.

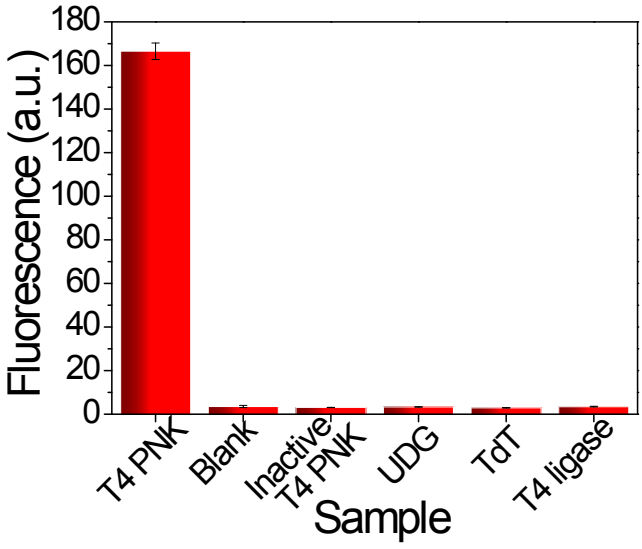


Figure S7. Selectivity of the modified EXPAR-based T4 PNK-sensing assay. The concentration of T4 PNK is 0.1 U mL^{-1}, The concentrations of TdT, UDG and T4 ligase are all 10 U mL^{-1}. Inactive T4 PNK is prepared by heating this enzyme at 95 ^{o}C for 20 min.

References

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S3. X. Wang, H. Wang, C. Liu, H. Wang and Z. Li, *Chem. Commun.,* 2017, ***53*****,** 1124-1127.

S4. M. Huang, X. Zhou, H. Wang and D. Xing, *Anal. Chem.,* 2018, ***90*****,** 2193-2200.