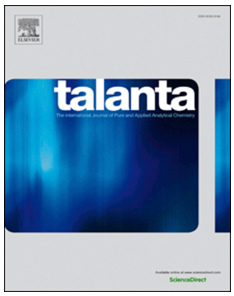
Accepted Manuscript



Detection of DNA 3'-phosphatase activity based on exonuclease III-assisted cascade recycling amplification reaction

Yuan Zhang, Yue Wang, Syed Faheem Askari Rizvi, Yida Zhang, Yintang Zhang, Xiaoyan Liu, Haixia Zhang

PII: S0039-9140(19)30652-6

DOI: https://doi.org/10.1016/j.talanta.2019.06.027

Reference: TAL 20027

To appear in: *Talanta*

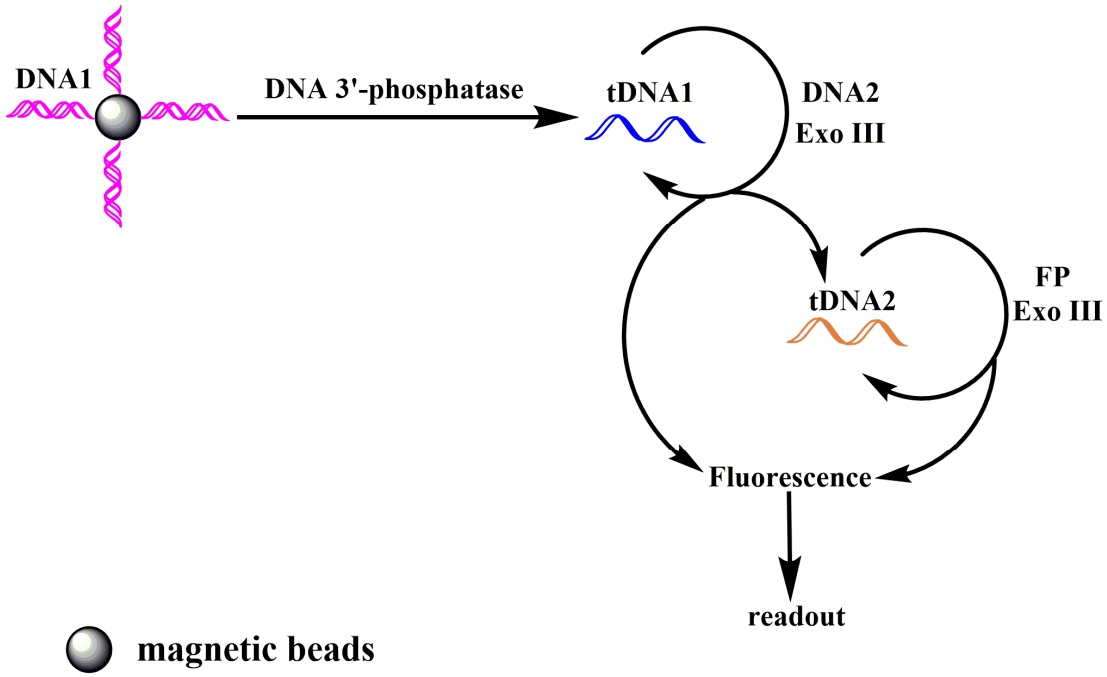
Received Date: 12 March 2019

Revised Date: 4 June 2019

Accepted Date: 8 June 2019

Please cite this article as: Y. Zhang, Y. Wang, S.F. Askari Rizvi, Y. Zhang, Y. Zhang, X. Liu, H. Zhang, Detection of DNA 3'-phosphatase activity based on exonuclease III-assisted cascade recycling amplification reaction, *Talanta* (2019), doi: https://doi.org/10.1016/j.talanta.2019.06.027.

Graphical Abstract



Detection of DNA 3'-phosphatase activity based on exonuclease III-assisted cascade recycling amplification reaction

Yuan Zhang[a], Yue Wang[a], Syed Faheem Askari Rizvi[a], Yida Zhang[a], Yintang Zhang[b], Xiaoyan Liu[a], Haixia Zhang[a,*]

[a] Key Laboratory of Nonferrous Metals Chemistry and Resources Utilization of Gansu Province, State Key Laboratory of Applied Organic Chemistry, College of Chemistry and Chemical Engineering, Lanzhou University, Lanzhou 730000, PR China

[b] Henan Key Laboratory of Biomolecular Recognition and Sensing, College of Chemistry and Chemical Engineering, Shangqiu Normal University, Shangqiu 476000, China

*Corresponding author: zhanghx@lzu.edu.cn

Abstract

DNA 3'-phosphatase is an essential enzyme, which plays a pivotal role in repairing DNA damage. The peculiar activity of DNA 3'-phosphatase has been proved to associate with a variety of human pathologies. Therefore, sensitive determination of DNA 3'-phosphatase is necessary for clinical diagnosis and therapy. Here, we develop a simple, sensitive, and specific fluorescent biosensor including three DNA chains of hairpin DNA1, hairpin DNA2 and fluorescence probe DNA (FP) for detecting the activity of DNA 3'-phosphatase. First, biotin-modified hairpin DNA1 binds with streptavidin-modified magnetic beads (MB) to get MB-DNA1. DNA 3'-phosphatase can hydrolyze phosphate groups on MB-DNA1 to form hydroxyl groups, which leads to the polymerization extension and nicking endonuclease cleavage reaction to obtain the trigger DNA1 fragment (tDNA1). Next, two cyclic amplification reactions are designed. In cycle I, the tDNA1 hybridizes with the hairpin DNA2, which leads the hairpin structure of DNA2 opened and the fluorescence signal of 6-carboxy-fluorescein (FAM) labeled on hairpin DNA2 turned on. This cyclic reaction is amplified by exonuclease III (Exo III). At the same time,

the trigger DNA2 fragment (tDNA2) is obtained. In cycle II, similarly, the tDNA2 hybridizes with FP. Thus, the fluorescence signal of FAM labeled on FP released, which multiplies with the fluorescence signal from cycle I. Finally, this strategy is applied to determine two typical DNA 3'-phosphatases including T4 polynucleotide kinase (T4 PNK) and alkaline phosphatase (ALP) with the detection limit (LOD) of 0.0033 and 0.00037 U/mL, respectively. The method provides a promising platform to evaluate the DNA 3'-phosphatase activity in the complicated biological samples and can be potentially applied in the relevant fields such as biomedical research, drug discovery and clinical diagnosis.

Keywords: T4 polynucleotide kinase; alkaline phosphatase; fluorescence; exonuclease III; cascade recycling amplification reaction.

1. Introduction

The breakdown of DNA strands caused by many endogenous or exogenous factors, such as exogenous genotoxic agents, ionizing radiation, endogenous stress induction, is considered as an important damage type [1]. The phosphate group at the 3' end of DNA is a ubiquitous product of DNA damage, which induces mutations in the gene to affect the organism adversely. Therefore, before repairing DNA strands, the 3' phosphate ends must be converted to hydroxyl groups [2-3]. In general, this task can be accomplished by the DNA 3'-phosphatase due to its phosphodiesterase activity. Thus DNA 3'-phosphatase plays a very important role in many biological processes, such as gene transcription, cell metabolism and neurological function. On account of its biological significance, sensitive detection of DNA 3'-phosphatase is of great value for diagnosis and treatment of disease.

T4 polynucleotide kinase (T4 PNK) and alkaline phosphatase (ALP) are two typical DNA 3'-phosphatases. T4 PNK is a bifunctional enzyme with both kinase and phosphatase activities [4], which not only transfers the γ-position phosphate group on adenosine triphosphate or other nucleoside triphosphates to the 5' end of DNA or RNA, but also removes the phosphate group at the 3' end of DNA or RNA. ALP widely distributes on various cell membranes of the human body and can hydrolyze various phosphate esters under alkaline conditions. It shows relatively lower activity and lower specificity in comparison with T4 PNK.

Traditional methods to detect DNA 3'-phosphatase include absorption spectrophotometry [5], immunoassay [6], ground state isotope labeling, polyacrylamide gel electrophoresis and ray self-tracking [7-9], which are partly limited in practical application due to the complex operation or radioactive hazards. Therefore, some new determination methods for DNA 3'-phosphatase have been established, including electrochemistry, chemiluminescence, fluorescence and colorimetry [10-14]. In response to the high throughput and high sensitivity [15-18], fluorescence analysis approaches are developed well. For example, Zhu [19] used graphene oxide (GO) as platform and a hairpin primer and polymerase as extended

reaction system for the detection of DNA 3'-phosphatase and its inhibitors. Song [20] developed a high-throughput detection platform for real-time quantification of DNA 3'-phosphatase based on universal molecular beacons. Although these methods have made great progress, it still needs to further improve the analytical performance and detection sensitivity for DNA 3'-phosphatase.

DNA cyclic amplification is a cost-effective method to improve the detection sensitivity with easy coding operational rule. However, non-specific amplification often occurs during the process, resulting in high background signal and low signal-to-noise ratio. Therefore, it is important to design a DNA cyclic amplification method to avoid non-specific hybridization.

Under guidance of this requirements and principles, we propose a new method for the detection of DNA 3'-phosphatase based on exonuclease III (Exo III)-assisted cascade recycling amplification reaction. Two kinds of DNA sequences (hairpin DNA2 and FP) are designed with the modification of the fluorophore 6-carboxy-fluorescein (FAM) and the quenching group Black Hole Quencher 1 (BHQ1), respectively. Since FAM is close to BHQ1, fluorescence resonance energy transfer (FRET) occurs and it causes the fluorescence of FAM quenched. However, when DNA 3'-phosphatase is existed, a series of Exo III-assisted cleavage reactions make the hairpin DNA2 and FP fragmented and the fluorescence of FAM turned on. In this way, the fluorescence signals of the system are superimposed and the detection limit for DNA 3'-phosphatase can be improved. Finally, this strategy is applied to perform T4 PNK and ALP spiked experiments in HeLa cell extract and diluted human serum, respectively.

2. Experimental

2.1 Chemicals and materials

Streptavidin-modified magnetic beads (MB, 5 mg/mL, 1.0 μm) were purchased from Tianjin Baseline ChromTech Research Centre (Tianjin, China). T4 polynucleotide kinase (T4 PNK, 10000 U/mL) and its diluting solvent (70 mM Tris-HCl, 10 mM $MgCl_2$, 5 mM DTT), alkaline phosphatase (ALP, 1000 U/mL) and

its diluting solvent (50 mM KAc, 20 mM Tris-Ac, 10 mM $Mg(Ac)_2$, 100 μg/mL BSA), the nicking endonuclease (Nb.BbvCl, 10000 U/mL), Klenow fragment polymerase (KF polymerase, without 3' to 5' exonuclease activity, 5000 U/mL), and exonuclease III (Exo III, 10000 U/mL) were bought from New England Biolabs Inc. (Beijing, China). N,N,N',N'-tetramethyldiethylamine (TEMED), Na_3VO_4 were obtained from Energy Chemical (Shanghai, China). 6 × DNA loading buffer, 20 bp DNA Ladder, and SYBR Gold were purchased from Takara Bio Inc. (Dalian, China). Deoxynucleotide solution mixture (dNTPs), 30% acrylamide/methylene bisacrylamide solution and DNA sequences were ordered from Sangon Biotech Co., Ltd. (Shanghai, China). The reagents used in the experiments were of analytical grade and used without further purification. The water was provided by Milli-Q Ultrapure Water System (Millipore). The sequences of oligonucleotide probes used in this work are listed in Table 1.

Table 1 DNA sequences used in the experiments

Oligonucleotides name	Sequence (5'-3')	Description
hairpin DNA1 (5.0×10^{-6} M)	Biotin-GAC GTT CTT TCA CTC ACT CAT CAT CTA CCT CAG CGC TTA TCG AAA AAA AAA ACG ATA AGC-P	The underlined letters indicate a sequence that can complement each other, the same below
hairpin DNA2 (3.0×10^{-5} M)	BHQ1-**ATG ATG AGT GAG TGA AAG CTC AAA AAA AAA A***CT TTC ACT CAC TCA TCA T*-*FAM*-*CTA CCT CA*	The italicized letters represent the complementary sequence of tDNA1; the **bold** letters represent the tDNA2 sequence; the **blue** letters represent the sequence complementary to FP
FP (3.0×10^{-5} M)	FAM-TTG AGC TTT C-BHQ1	

2.2 Apparatus

All fluorescence measurement experiments were performed on a RF5301PC fluorescence spectrometer. The excitation wavelength adjusted at 490 nm and the emission wavelength at 520 nm. The excitation slit and emission slit were 5 nm and 3 nm.

2.3 Coupling hairpin DNA1 with MB

First, the magnetic beads (MB) modified with streptavidin were dispersed using an ultrasonic cleaner for 10 min. Then, 10 μL of MB dispersion was taken out and washed for three times with each 100 μL Tris-HCl buffer (TH buffer, 70 mM, pH 7.9). The MB was dispersed again in 10 μL of the TH buffer, then 5 μL of 5.0×10^{-6} M biotin-modified hairpin DNA1 was added. The mixture was incubated at 37 °C for 2 h. After magnetic separation and washing three times with the TH buffer, the MB-DNA1 were re-suspended in 10 μL of the TH buffer and stored at 4°C before further use.

2.4 DNA 3'-phosphatase catalyzed polymerization nicking reaction

Ten μL of MB-DNA1, 20 μL of 1 × T4 PNK buffer with different concentrations of T4 PNK were incubated at 37°C for 40 min and further at 65°C for 20 min. Then, 3.0 U Klenow fragment polymerase, 300 μM dNTPs, and 6.0 U Nb.BbvCl were added. The mixture was fixed to 100 μL and incubated at 37°C for 90 min and further at 80°C for 20 min. Finally, the magnetic separation was used to collect the supernatant.

2.5 Exo III-assisted reaction (cycle I)

The supernatant (from 2.4 part) was mixed with 5 μL of 3.0×10^{-5} M hairpin DNA2 and 20.0 U Exo III. The mixture was fixed to 200 μL and incubated at 37°C for 60 min. The relationship between T4 PNK concentration and the fluorescence signal from FAM on hairpin DNA2 was studied.

2.6 Exo III-assisted cascade recycling amplification reaction (cycle I and cycle II)

The supernatant (from 2.4 part) was added to the solution containing 5 μL of 3.0×10^{-5} M hairpin DNA2, 5 μL of 3.0×10^{-5} M FP and 35.0 U Exo III. The mixture was fixed to 200 μL and incubated at 37°C for 100 min. The relationship between T4 PNK concentration and the fluorescence signal from FAM on both hairpin DNA2 and FP was studied.

2.7 Inhibition of the dephosphorylation reaction

Na_2HPO_4 was selected as an inhibitor to study the inhibitory effect of T4 PNK catalytic dephosphorylation. Ten μL of MB-DNA1, 1.0 U/mL T4 PNK, 20 μL of 1 × T4 PNK buffer, and different concentrations of Na_2HPO_4 were incubated at 37°C for

40 min and further at 65°C for 20 min. The residual steps were the same as for detecting the concentration of T4 PNK.

2.8 Real sample testing

T4 PNK activity was detected in diluted cell extracts. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere with 5% CO_2 incubator. HeLa cells (1×10^6) were collected during the exponential phase of growth and washed twice with ice-cold phosphate buffered solution (PBS, 0.01 M, pH 7.4). Then the cells were suspended in 1.0 mL of cell lysis buffer and incubated for 30 min on ice. The mixture was centrifuged at 12000 rpm for 30 min at 4ºC and the supernatant was collected and 100 times - diluted with the TH buffer. The dilution was analyzed with the same procedure as the detection of T4 PNK in buffer.

3. Results and discussion

3.1 Detection principle

The principle for detecting DNA 3'-phosphatase activity based on Exo III-assisted cycling reaction is shown in Fig. 1. First, the hairpin DNA1 modified with biotin at the 5' end is linked to streptavidin-modified magnetic beads (MB) to form multifunctional magnetic probes (MB-DNA1). In the presence of DNA 3'-phosphatase, the 3'-phosphate group of the hairpin DNA1 is hydrolyzed to a 3'-hydroxyl group. In the presence of Klenow fragment polymerase and dNTPs, the 3' end of the hairpin DNA1 can undergo growth extension to produce a complete duplex [21]. The nicking endonuclease (Nb.BbvCl) can recognize and cleave the complementary sequence of 5'-CCTCAGC-3' of complete duplex [22-24]. Furthermore, the complete duplex is formed again in the presence of Klenow fragment polymerase and dNTPs. Simultaneously, tDNA1 is released and accumulated continuously.

After magnetic separation, tDNA1 can open hairpin DNA2 via a front-end mediated strand displacement reaction (SDR) to form a new double-stranded DNA. Before the hairpin DNA2 is opened, the fluorescent group FAM labeled on the hairpin

DNA2 is close to the quenching group BHQ1, fluorescence resonance energy transfer (FRET) occurs between the FAM and BHQ1, and the fluorescence signal of the FAM is quenched [25] (the structures of FAM and BHQ1 are shown in Fig. S1). After the hairpin of DNA2 is opened by tDNA1, FAM is separated from BHQ1 and its fluorescence signal is turned on.

Exonuclease III (Exo III) as a nuclease, can selectively catalyze the hydrolyzation of mononucleotides from the blunt or the recessed 3'-hydroxyl termini of duplex DNA [26]. When Exo III is added, it can remove the mononucleotides from the 3' terminus of the hairpin DNA2 and release more tDNA1 and tDNA2. The tDNA1 will continue to open the hairpin of DNA2 and initiate the cycling reaction (cycle I). Meanwhile, tDNA2 is released continuously along with the cycle I.

Herein, tDNA2 can hybridize with FP to form double-stranded DNA. With the existence of Exo III, FP is cleaved and more tDNA2 are obtained to continue the new cycling reaction (cycle II). As the results, the FAM modified FP is separated from BHQ1 and the fluorescence signal of the FAM is restored [27-28]. The fluorescence signal from FAM is accumulated on account of the two cycling reactions.

If DNA 3'-phosphatase does not exist in the reaction system, the phosphate group at the 3' end of the hairpin DNA1 cannot hydrolyzed to the hydroxyl group and all the above reaction cannot be happened.

Here, the hairpin DNA2 and FP are designed based on FRET mechanism, which must meet two conditions simultaneously. First, the emission spectrum of one fluorophore (donor, FAM) overlaps with the absorption spectrum of another group (acceptor, BHQ1). Second, the distance between both fluorescent groups is close enough (generally less than 10 nm). The maximum emission wavelength of FAM is 520 nm (see Fig. S2), and the absorption wavelength range of BHQ1 is 480-580 nm. Moreover, the distance between BHQ1 and FAM is short due to the hairpin structure of DNA2 and the short length of FP (3.4 nm [29]). Therefore, the FRET can be happened easily.

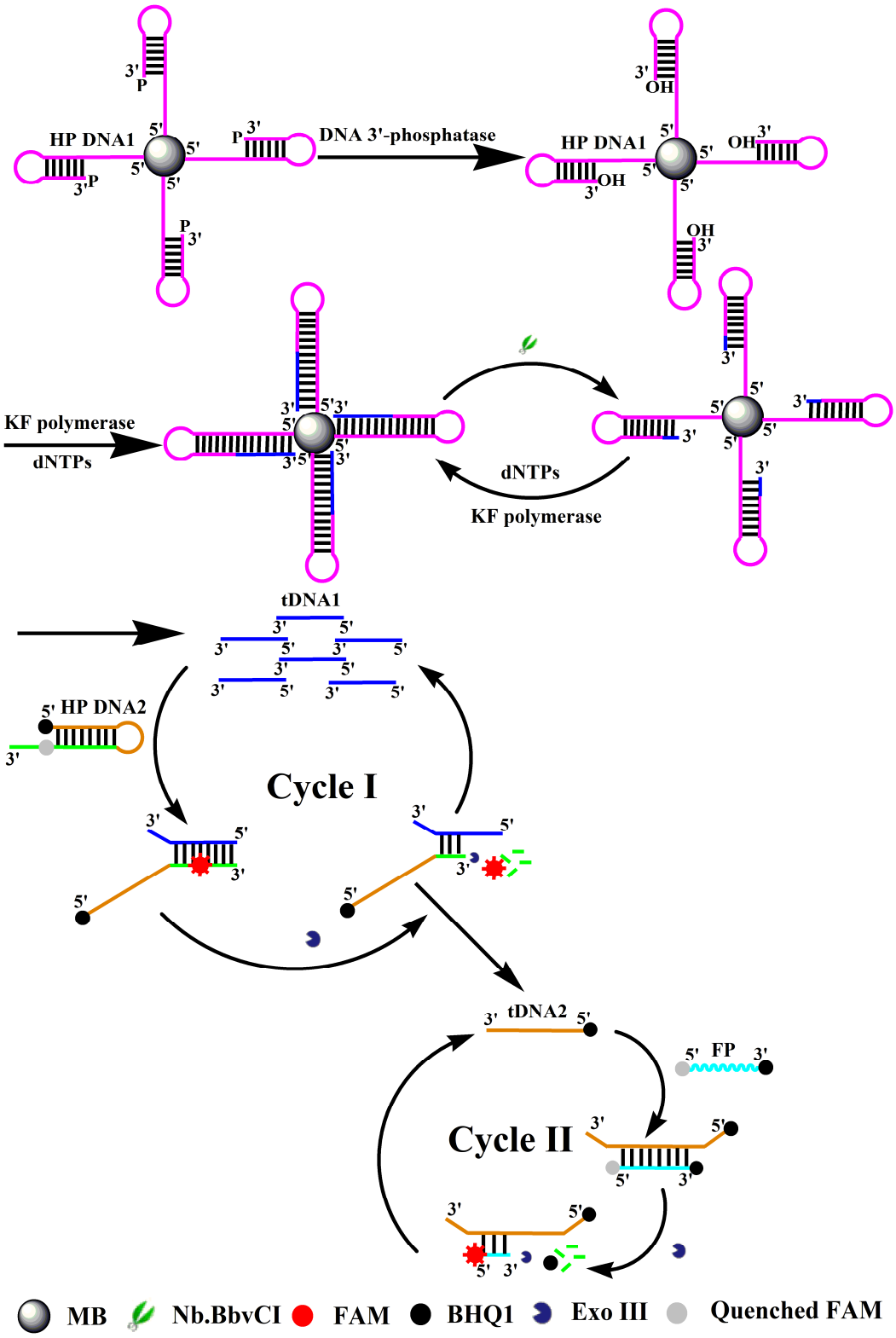


Fig. 1 Schematic detection of DNA 3'-phosphatase activity.

3.2 Experimental feasibility

To verify the detection feasibility of T4 PNK activity, the fluorescence experiments were performed to assess the necessity of each component in the reaction

system. In Fig. S2, the fluorescence spectra of FAM shows that the emission wavelength is around 520 nm under the excitation wavelength at 490 nm. In Fig. 2, curve 'a' exhibits the result of the reaction system (part 2.4 and 2.5), showing the largest fluorescence signal; curve 'b' represents the reaction system without adding Exo III, and the less fluorescence signal indicates the necessity of Exo III for the sensitive measurement; curves 'c' and 'd' depict the result of the reaction system in the absence of Klenow fragment polymerase and Nb.BbvCl, respectively, meaning their importance for the reaction system. Curve 'e' represents the reaction system in the absence of T4 PNK and only weak signal can be observed. It may be attributed to the non-special cleaving of the Exo III product. Curve 'f' represents the pure hairpin DNA2. Curve 'g' represents the reaction system without adding hairpin DNA2. Both systems show no fluorescence signals. The above results prove that the T4 PNK is the only factor to trigger fluorescence changes of cycle I.

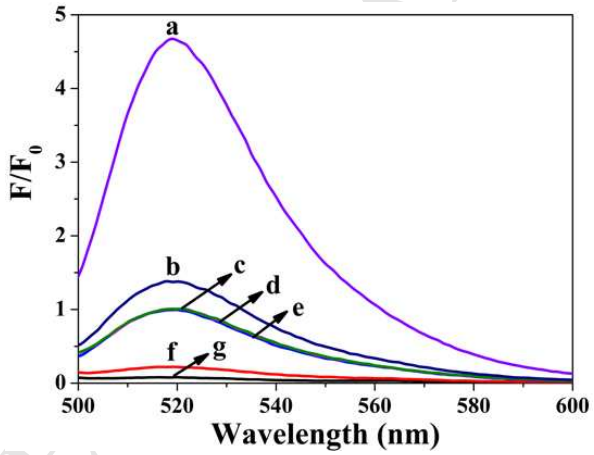


Fig. 2 Fluorescence signals of the reaction system with changed components.
(a) The reaction system (with 10.0 U/mL T4 PNK); (b) to (e) the reaction system without Exo III, Klenow fragment polymerase, Nb.BbvCl and T4 PNK, respectively; (f) only hairpin DNA2; (g) the reaction system without hairpin DNA2.

The experimental feasibility was further confirmed by polyacrylamide gel electrophoresis (PAGE). As indicated in Fig. S3, a new strip is generated in lane 4, which means the 3' end of hairpin DNA1 is extended and forms a complete duplex under the existence of Klenow fragment polymerase. While in the presence of all components (lane 5), the strips of the hairpin DNA1 and hairpin DNA2 become

narrower and darker (compared with lane 6, absence of T4 PNK), indicating that they are consumed and the reactions are happened.

All these lines of evidence prove that the experimental design is feasible.

3.3 Optimization of the reaction conditions

The experimental conditions were optimized with Simple Variable Method including the critical concentrations and reaction times. The reaction system was kept a total of 200 μL with 8 components. As shown in Fig. 3, the variables include the concentrations of the dNTPs (curve 'a') and hairpin DNA1 (curve 'b'); the dosage of Klenow fragment polymerase (curve 'c'), nicking endonuclease Nb.BbvCl (curve 'd'), and Exo III (curve 'e'); the reaction times of dephosphorylation (curve 'f'), polymerization nicking (curve 'g') and Exo III (curve 'h'). The results show that the optimal experimental system includes 300 μM dNTPs, 5 μM hairpin DNA1, 3.0 U Klenow fragment polymerase, 6.0 U Nb.BbvCl, 20.0 U Exo III, and the reaction times of dephosphorylation, polymerization nicking, and the Exo III are 40, 90 and 60 min, respectively.

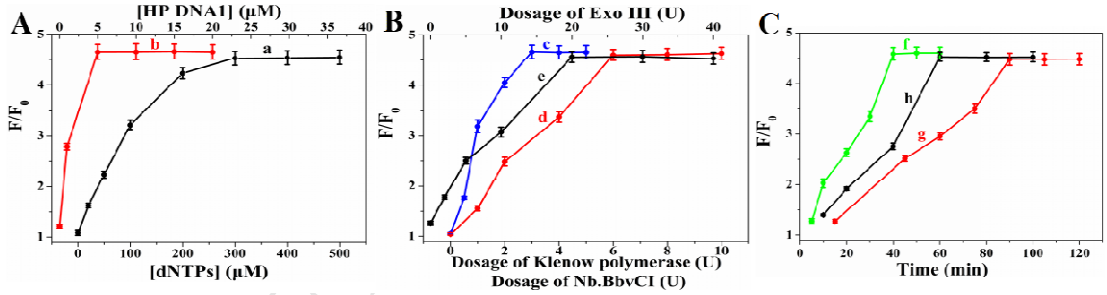


Fig. 3 Effect of the various factors on the efficiency of the first cycling reaction.

(A) The concentration of dNTPs (a) and hairpin DNA1 (b); (B) the dosage of Klenow fragment polymerase (c), Nb.BbvCl (d) and Exo III (e); (C) the reaction times of dephosphorylation (f), polymerization nicking (g) and Exo III (h). The concentration of T4 PNK is 10.0 U/mL. Error bars are standard deviation of three repetitive measurements, the following are same as n=3.

3.4 Fluorescence measurement of T4 PNK activity via the first cycling reaction (cycle I)

Under the optimal experimental conditions, a series of different concentrations of T4 PNK were added. The results show a positive correlation between the ratio of

fluorescence intensity and the T4 PNK at the concentrations ranging from 0 to 10.0 U/mL (Fig. 4A). As shown in Fig. 4B, when the concentration of T4 PNK is increased from 0.02 to 1.0 U/mL, the ratio of fluorescence intensity (F/F_0) of the reaction system have a good linear relationship with the concentration of T4 PNK, where F and F_0 indicate the fluorescence intensity in the presence and absence of T4 PNK in the reaction system, respectively. The linear regression equation is $F/F_0 = 1.5247\ C + 1.2829$ with a correlation coefficient of $R^2 = 0.9930$, where C is the concentration of T4 PNK. The LOD for T4 PNK is estimated to be 0.018 U/mL.

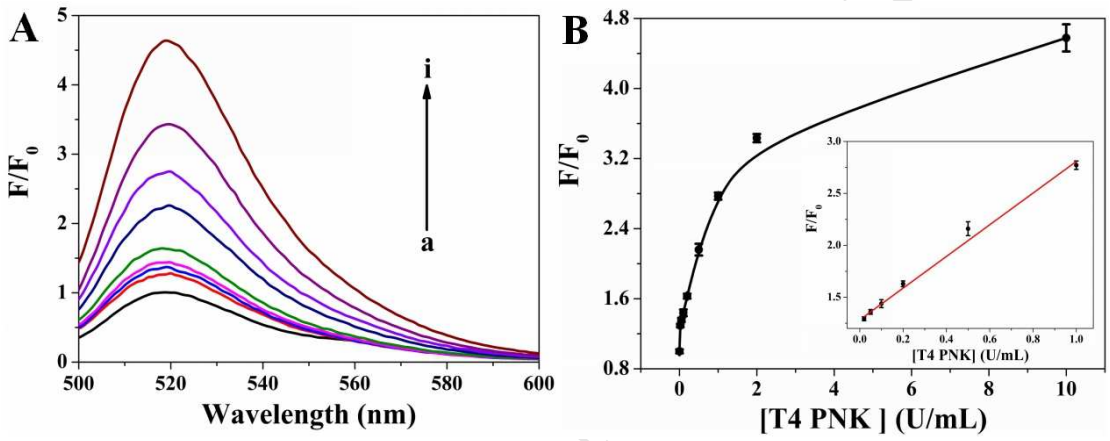


Fig. 4 Fluorescence signals of the reaction system with different concentrations of T4 PNK. (A) The fluorescence spectra (a) 0; (b) 0.02; (c) 0.05; (d) 0.1; (e) 0.2; (f) 0.5; (g) 1.0; (h) 2.0; (i) 10.0 U/mL. (B) The relationship between T4 PNK concentration and the ratio of fluorescence intensity. (n=3)

3.5 Principle of two cycling reactions

In the light of above investigation, we demonstrated that the activity of T4 PNK can be detected on the basis of Exo III-assisted cycling reaction. The tDNA2 released from the first cycling reaction (cycle I) can participate in the second cycling reaction (cycle II) and the fluorescence signal is further enlarged (Fig. 1), which will make a positive contribution to the analysis sensitivity of T4 PNK. The experimental feasibility and gel electrophoresis results are shown in the Fig. S4 and Fig. S5.

3.6 Fluorescence measurement of T4 PNK activity via two cycling reactions

Certainly, Exo III should be consumed more in two cycling reactions than in the first cycling reaction. Therefore, the dosage of Exo III and the reaction time of Exo III

are optimized. The optimal conditions are 35.0 U and 100 min, respectively. (Fig. S6).

Under these optimal experimental conditions, the fluorescence signals were measured at the different concentrations of T4 PNK. The results are shown in Fig. 5A. The fluorescence intensity increases with the increased concentration of T4 PNK from 0 to 1.0 U/mL. As shown in Fig. 5B, a good linear relationship is obtained between the ratio of fluorescence intensity and the T4 PNK concentration (C) ranging from 0.005 to 0.2 U/mL, with a regression equation of $F/F_0 = 3.7450\ C + 1.1239$ ($R^2 = 0.9958$). The LOD for T4 PNK is as low as 0.0033 U/mL, lower than the most reported (Table 2). This is due to two main reasons. First, the magnetic separation can effectively avoid the non-specific hybridization between DNA1 and DNA2 or FP. Second, the two cycling reactions triggered by Exo III benefit to enhance the analysis sensitivity of T4 PNK.

As shown in Table 2, the LOD reported in reference [33] is lowest and the linear range is wide, which is mainly attributed to the hyperbranched rolling circle amplification reaction, which is highly efficient and lead to the high sensitivity. In our work the cyclic amplification reaction initiated by Exo III is much more economical because the raw materials can be recycled repeatedly. In addition, the method is simple and has advantage on a shorter total operation time.

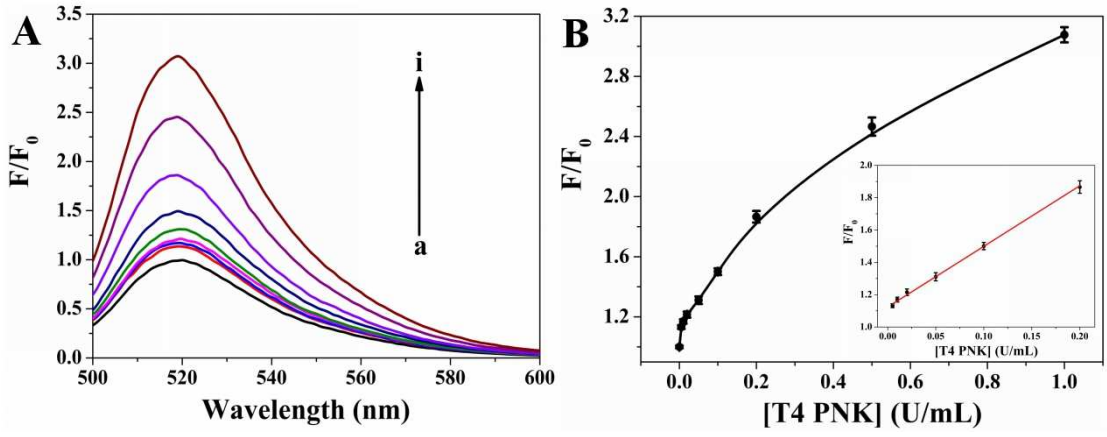


Fig. 5 Fluorescence signals of the reaction system with different concentrations of T4 PNK. (A) The fluorescence spectra (a) 0; (b) 0.005; (c) 0.01; (d) 0.02; (e) 0.05; (f) 0.1; (g) 0.2; (h) 0.5; (i) 1.0 U/mL. (B) The relationship between T4 PNK concentration and the ratio of fluorescence intensity. (n=3)

Table 2 Comparison of the previous detection methods for T4 PNK activity

Signal readout	LOD (U/mL)	Linear range (U/mL)	Ref.
electrochemistry	0.01	0.01-0.1	[30]
electrochemistry	0.02	0.05-10.0	[31]
colorimetry	0.06	0.1-100.0	[32]
fluorescence	0.0000436	0.0001-1.0	[33]
fluorescence	0.001	0.001-0.1	[34]
fluorescence	0.005	0.005-0.2	[35]
fluorescence	0.01	0.01-10.0	[36]
fluorescence	0.02	0-0.25	[37]
fluorescence	0.05	0.05-10.0	[38]
fluorescence	0.0033	0.005-0.2	This work

3.7 Selectivity of T4 PNK activity assay

To verify the specificity of the method for T4 PNK, the influence of other proteins on the assay has been investigated. The same concentration (1.0 U/mL) of DNA adenine methylation methyltransferase (Dam), methylation-sensitive restriction endonuclease (DpnI), uracil-DNA glycosylase (UDG), T4 polynucleotide kinase (T4 PNK) and 100 mg/mL of bovine serum albumin (BSA) and chymotrypsin (CHT), as well as the blank experiment (not added T4 PNK) were tested, respectively. The results are shown in Fig. 6. The ratio of the fluorescence signal (F/F_0) upon addition of T4 PNK is far higher than that of other substances. This proves that the experimental method has a good selectivity.

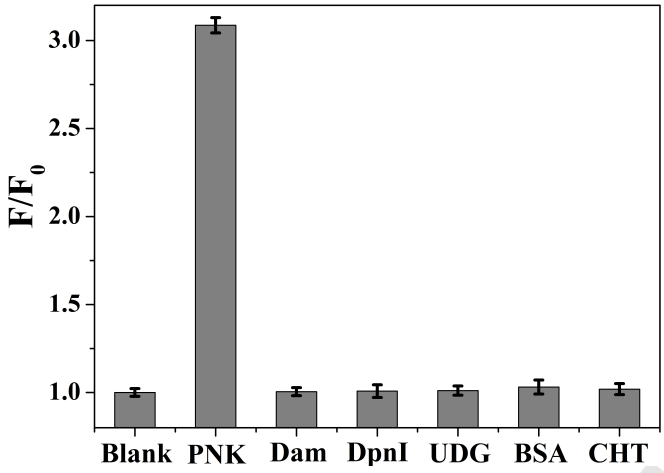


Fig. 6 Fluorescence signals of the different reaction systems (n=3).

3.8 Inhibition evaluation

Disodium hydrogen phosphate (Na_2HPO_4) is selected as a representative inhibitor to study the inhibitory effect on T4 PNK [39]. Fig. S7 shows the effect of Na_2HPO_4 on the activity of T4 PNK. IC_{50} values (which represents the concentration of the inhibitor required, when half of the enzyme activity is inhibited) of Na_2HPO_4 is found to be 19.0 mM. The results indicate that the proposed method is feasible for evaluating the action of the DNA 3'-phosphatase inhibitor.

3.9 Assay of phosphatase in biological sample

In order to verify the practicability of the experimental method in real sample, T4 PNK in diluted cell extracts was detected by standard addition method. As shown in Fig. 7, the results in the buffer and in diluted cell extracts are similar, which indicates the method has a good practicability. The spiked recovery of T4 PNK in diluted cell extracts are tested as between 94.0% and 110.0% with the RSD lower than 5.3% (Table 3).

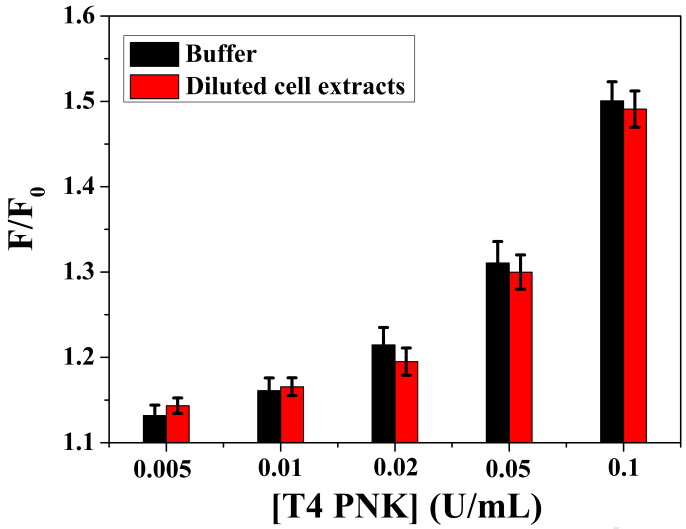


Fig. 7 Detection of T4 PNK in buffer and 1% HeLa cell extract (n=3).

Table 3 Recovery of T4 PNK in spiked cell dilution samples (n=3)

Number	Added (U/mL)	Found (U/mL)	Recovery (%)	RSD (%)
1	0.005	0.0052	104	3.3
2	0.010	0.011	110	3.5
3	0.020	0.019	95	5.3
4	0.050	0.047	94	4.3
5	0.10	0.098	98	3.1

At last, the similar experiments on detection of ALP were performed and their results were shown in supporting information.

4. Conclusion

In this work, a method with good sensitivity and selectivity was designed to detect the activity of DNA 3'-phosphatasey using the characteristics of polymerization-nicking reaction and Exo III-assisted cascade recycling amplification reaction. The lower LOD for the enzymes originates from magnetic beads separation and the tandem cyclic amplification reaction. The DNA 3'-phosphatase activity can be detected in buffer as well as in biological samples. However, the sensitivity is not enough yet for determination of DNA 3'-phosphatase in the dilution of biological samples and the method must be improved further for practical application.

Acknowledgment

This research was financially supported by the National Natural Science Foundation of China (No. 21575055) and Henan Key Laboratory of Biomolecular Recognition and Sensing (HKLBRSK1802).

Supporting Information

Other supplementary materials related to this article can be found in the supporting information.

References

[1] R. A. Deshpande, T. E. Wilson, Identification of DNA 3'-phosphatase active site residues and their differential role in DNA binding, Mg^{2+} coordination, and catalysis, Biochemistry. 43 (2004) 8579-8589.

[2] W. D. Henner, L. O. Rodriguez, S. M. Hecht, W. A. Haseltine, Gamma ray induced deoxyribonucleic acid strand breaks 3' glycolate termini, Journal of Biological Chemistry. 258 (1983) 711-713.

[3] B. N. Ames, L. S. Gold, W. C. Willett, The causes and prevention of cancer, Proceedings of the National Academy of Sciences of the United States of America. 92 (1995) 5258-5265.

[4] F. Karimi-Busheri, G. Daly, P. Robins, B. Canas, D. J. C. Pappin, J. Sgourous, G. G. Miller, H. Fakhrai, E. M. Davis, M. M. L. Beau, M. Weinfeld, Molecular characterization of a human DNA kinase, Journal of Biological Chemistry. 274 (1999) 24187-24194.

[5] V. Cameron, O. C. Uhlenbeck, 3'-Phosphatase activity in T4 polynucleotide kinase, Biochemistry. 16 (1977) 5120-5126.

[6] F. Karimi-Busheri, A. Rasouli-Nia, J. Allalunis-Turner, M. Weinfeld, Human polynucleotide kinase participates in repair of DNA double-strand breaks by nonhomologous end joining but not homologous recombination, Cancer Research. 67 (2007) 6619-6625.

[7] A. Rasouli-Nia, F. Karimi-Busheri, M. Weinfeld, Stable down-regulation of human polynucleotide kinase enhances spontaneous mutation frequency and sensitizes cells to genotoxic agents, Proceedings of the National Academy of Sciences of the United States of America. 101 (2004) 6905-6910.

[8] M. Meijer, F. Karimi-Busheri, T. Y. Huang, M. Weinfeld, D. Young, Pnk1, a DNA kinase/phosphatase required for normal response to DNA damage by γ-radiation or camptothecin in schizosaccharomyces pombe, Journal of Biological Chemistry. 277 (2002) 4050-4055.

[9] L. K. Wang, S. Shuman, Domain structure and mutational analysis of T4 polynucleotide kinase, Journal of Biological Chemistry. 276 (2001) 26868-26874.

[10] J. Y. Zhuang, W. Q. Lai, M. D. Xu, Q. Zhou, D. P. Tang, Plasmonic AuNP/g-C_3N_4 nanohybrid-based photoelectrochemical sensing platform for ultrasensitive monitoring of polynucleotide kinase activity accompanying DNAzyme-catalyzed precipitation amplification, Acs Applied Materials and Interfaces. 7 (2015) 8330-8338.

[11] Q. M. Zhang, Z. Li, Y. L. Zhou, X. Li, B. C. Li, H. S. Yin, S. Y. Ai, Electrochemical biosensors for polynucleotide kinase activity assay and inhibition screening based on phosphorylation reaction triggered λ exonuclease and exonuclease I cleavage, Sensors and Actuators B: Chemical. 225 (2016) 151-157.

[12] W. Tang, G. C. Zhu, C. Y. Zhang, Sensitive detection of polynucleotide kinase using rolling circle amplification-induced chemiluminescence, Chemical Communications. 50 (2014) 4733-4735.

[13] Z. L. Shi , X. F. Zhang, R. Cheng, B. X. Li, Y. Jin, A label-free cyclic assembly of G-quadruplex nanowires for cascade amplification detection of T4 polynucleotide kinase activity and inhibition, Analyst. 140 (2015) 6124-6130.

[14] J. J. Cheng, Y. Sun, L. Zhou, K. C. Zhang, J. Wang, Z. Y. Wu, R. J. Pei, Phosphorylation triggered poly-nanoparticle assembly for naked-eye distinguishable T4 polynucleotide kinase detection, RSC Advances. 4 (2014) 56731-56735.

[15] Y. Cen, W. J. Deng, R. Q. Yu, X. Chu, Sensitive fluorescence sensing of T4 polynucleotide kinase activity and inhibition based on DNA/polydopamine nanospheres platform, Talanta. 180 (2018) 271-276.

[16] H. Z. Zhao, Q. Liu, M. Liu, Y. Jin, B. X. Li, Label-free fluorescent assay of T4 polynucleotide kinase phosphatase activity based on G-quadruplexe-thioflavin T complex, Talanta. 165 (2017) 653-658.

[17] Z. Z. Dong, L. Zhang, M. Qiao, J. Ge, A. L. Liu, Z. H. Li, A label-free assay for T4 polynucleotide kinase/phosphatase activity and its inhibitors based on poly(thymine)-templated copper nanoparticles, Talanta. 146 (2016) 253-258.

[18] C. Y. Lee, K. S. Park, H. G. Park, A simple, sensitive, and label-free assay for alkaline phosphatase activity based on target-promoted exponential strand displacement amplification, Sensors and Actuators B: Chemical. 262 (2018) 1001-1005.

[19] W. P. Zhu, Z. W. Zhao, Z. Li, J. H. Jiang, G. L. Shen, R. Q. Yu, A graphene oxide platform for the assay of DNA 3′-phosphatases and their inhibitors based on hairpin primer and polymerase elongation, Journal of Materials Chemistry B. 1 (2013) 361-367.

[20] C. Song, C. Zhang, M. P. Zhao, Development of a high□throughput screening platform for DNA 3′□phosphatases and their inhibitors based on a universal molecular beacon and quantitative real□time PCR, Chemistry - An Asian Journal. 5 (2010) 1146-1151.

[21] Z. F. Shen, F. Li, Y. F. Jiang, C. Chen, H. Xu, C. C. Li, Z. Yang, and Z. S. Wu, Palindromic molecule beacon-based cascade amplification for colorimetric detection of cancer genes, Analytical Chemistry. 90 (2018) 3335-3340.

[22] W. L. Song, Q. Zhang, X. X. Xie, S. S. Zhang, Fluorescence aptameric sensor for isothermal circular strand-displacement polymerization amplification detection of adenosine triphosphate, Biosensors and Bioelectronics. 61 (2014) 51-56.

[23] W. L. Song, Z. Zhu, Y. N. Mao, S. S. Zhang, A sensitive quartz crystal microbalance assay of adenosine triphosphate via DNAzyme-activated and aptamer-based target-triggering circular

amplification, Biosensors and Bioelectronics. 53 (2014) 288-294.

[24] C. X. Li, J. Lin, Y. S. Guo, S. S. Zhang, A novel electrochemiluminescent reagent of cyclometalated iridium complex-based DNA biosensor and its application in cancer cell detection, Chemical Communications. 47 (2011) 4442-4444.

[25] X. W. Xing, F. Tang, J. Wu, J. M. Chu, Y. Q. Feng, X. Zhou, B. F. Yuan, Sensitive detection of DNA methyltransferase activity based on exonuclease-mediated target recycling, Analytical Chemistry. 86 (2014) 11269-11274.

[26] I. V. Shevelev, U. Hübscher, The 3′–5′ exonucleases, Nature Reviews Molecular Cell Biology, 3 (2002) 364-367.

[27] L. Li, J. Feng, Y. Y. Fan, B. Tang, Simultaneous imaging of Zn^{2+} and Cu^{2+} in living cells based on DNAzyme modified gold nanoparticle, Analytical Chemistry. 87 (2015) 4829-4835.

[28] X. Z. Wang, T. Hou, T. T. Lu, F. Li, Autonomous exonuclease III-assisted isothermal cycling signal amplification: a facile and highly sensitive fluorescence DNA glycosylase activity assay, Analytical Chemistry. 86 (2014) 9626-9631.

[29] M. Singh-Zocchi, S. Dixit, V. Ivanov, G. Zocchi, Single-molecule detection of DNA hybridization, Proceedings of the National Academy of Sciences. 100 (2003) 7605-7610.

[30] Y. H. Wang, X. X. He, K. M. Wang, X. Q. Ni, J. Su, Z. F. Chen, Ferrocene-functionalized SWCNT for electrochemical detection of T4 polynucleotide kinase activity, Biosensors and Bioelectronics. 32 (2012) 213-218.

[31] T. Hou, X. Z. Wang, X. L. Liu, C. Pan, F. Li, Sensitive electrochemical assay for T4 polynucleotide kinase activity based on dual-signaling amplification coupled with exonuclease reaction, Sensors and Actuators B: Chemical. 202 (2014) 588-593.

[32] C. Jiang, C. Y. Yan, J. H. Jiang, R. Q. Yu, Colorimetric assay for T4 polynucleotide kinase activity based on the horseradish peroxidase-mimicking DNAzyme combined with λ exonuclease cleavage, Analytica Chimica Acta. 766 (2013) 88-93.

[33] X. Li, X. W. Xu, J. Song, Q. W. Xue, C. Z. Li, W. Jiang, Sensitive detection of T4 polynucleotide kinase activity based on multifunctional magnetic probes and polymerization nicking reactions mediated hyperbranched rolling circle amplification, Biosensors and Bioelectronics. 91 (2017) 631-636.

[34] T. Hou, X. Z. Wang, X. J. Liu, T. T. Lu, S. F. Liu, F. Li, Amplified detection of T4 polynucleotide kinase activity by the coupled λ exonuclease cleavage reaction and catalytic assembly of bimolecular beacons, Analytical Chemistry. 86 (2014) 884-890.

[35] S. F. Liu, J. J. Ming, Y. Lin, C. F. Wang, T. Liu, C. B. Cheng, F. Li, Amplified detection of T4 polynucleotide kinase activity based on a λ-exonuclease cleavage-induced DNAzyme releasing strategy, Sensors and Actuators B: Chemical. 192 (2014) 157-163.

[36] C. B Ma, H. S. Liu, J. Y. Du, H. C. Chen, H. L. He, S. X. Jin, K. M. Wang, J. Wang, Quencher-free hairpin probes for real-time detection of T4 polynucleotide kinase activity, Analytical Biochemistry. 494 (2016) 1-3.

[37] C. X. Song, X. H. Yang, K. M. Wang, Q. Wang, J. B. Liu, J. Huang, L. L. He, P. Liu, Z. H. Qing, W. Liu, A sensitive detection of T4 polynucleotide kinase activity based on β-cyclodextrin polymer enhanced fluorescence combined with an exonuclease reaction, Chemical. Communications. 51 (2015) 1815-1818.

[38] L. Lin, Y. Liu, X. Zhao, J. H. Li, Sensitive and rapid screening of T4 polynucleotide kinase activity and inhibition based on coupled exonuclease reaction and graphene oxide platform, Analytical Chemistry. 83 (2011) 8396-8402.

[39] J. Xu, Y. F. Gao, B. X. Li, Y. Jin, Cyclic up-regulation fluorescence of pyrene excimer for studying polynucleotide kinase activity based on dual amplification, Biosensors and Bioelectronics. 80 (2016) 91-97.

Highlights

1. Magnetic beads are introduced to avoid non-specific hybridization.

2. The tandem cyclic amplification reaction increases the sensitivity.

3. Magnetic separation achieves the separation quickly and effectively.

4. This method has a good selectivity for analyte.