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Label-free fluorescence assay coupled exonuclease reaction and SYBR Green I for the detection of T4 polynucleotide kinase activity

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Abstract

A sensitive label-free fluorescence assay for monitoring T4 polynucleotide kinase (T4 PNK) activity and inhibition was developed based on a coupled λ exonuclease cleavage reaction and SYBR Green I. In this assay, a double-stranded DNA (dsDNA) was stained with SYBR Green I and used as a substrate for T4 PNK. After the 5′-hydroxyl termini of the dsDNA was phosphorylated by the T4 PNK, the coupled λ exonuclease began to digest the dsDNA to form mononucletides and single-stranded DNA (ssDNA). At this moment, the fluorescence intensity of the SYBR Green I decreased because of less affinity with ssDNA than dsDNA. The decreasing extent was proportional to the concentration of the T4 PNK. After optimization of the detection conditions, including the concentration of ATP, amount of λ exonuclease and reaction time, the activity of T4 PNK was monitored by the fluorescence measurement, with the limit of detection of 0.11 U/mL and good linear correlation between 0.25-1.00 U/mL (R^2=0.9896). In this assay, no label was needed for fluorescence detection. Moreover, the inhibition behaviors of the T4 PNK's inhibitors were evaluated by this assay. The result indicated the potential of using this assay for monitoring of the phosphorylation-related process.

Keywords

Label-free; phosphorylation; T4 PNK; λ exonuclease; SYBR Green I.

Introduction

Phosphorylation, a transferring of γ -phosphate residues of adenosine triphosphate (ATP) to 5′ -hydroxyl terminus of polynucleotides, plays an important role in DNA replication, DNA repair and recombination.[1, 2] In these normal cellular processes, several factors, such as chemical substances, ionizing radiation[3] and nucleases, [4] would cause nucleic acid lesions that contain 5'-hydroxyl termini. These 5'-hydroxyl termini may lead to failure of 5'-phosphate terminal dependent repair processes for nucleic acids, resulting in severe gene damage and diseases.[5, 6] Therefore, it is highly desirable to monitor the polynucleotide kinase activity and inhibition for further understanding phosphorylation and related biological processes.

Normally, methods used to study the phosphorylation of nucleic acids included radical isotope ^{32}P-labeling, polyacrylamide gel electrophoresis (PAGE) and autoradiography.[6-8] However, most of the methods required radio labelling, bringing a safety concern, time-consuming and complicity for the assays. Recently, several electrochemical and fluorescence sensors for monitoring kinase activities were developed.[9-21] The replacement of radio labelling using fluorescence labelling in these assays eliminated safety concerns; however, labelling fluorophores to nucleic acids increased the cost of the assay. For example, Tang *et al.* reported a fluorescence assay for monitoring phosphorylation process using a dual-label molecular beacon,[12] and Song *et al.* developed a real-time method for the kinase activity detection using a singly labelled molecular beacon.[13] By using graphene oxide as a quencher platform, Lin *et al.* developed a novel method for the kinase activity and inhibition monitoring with a FAM modified dsDNA.[14] These methods provided high sensitivity for detections in spite of labelling requirements. The development of labelling-free methods for the detection of phosphorylation kinase activities and inhibition is still a challenge.

The integrate DNA dyes, such as SYBR Green I (SG), are very powerful tools for fabricating DNA sensors based on their different affinities to the ssDNA and dsDNA.[16, 22, 23] They have been widely used for the detection of DNAs,[24] metal ions,[25-27] proteins and small molecules[28] with high sensitivity and low cost. Therefore, SG could provide a low-cost alternative for label-free detection of phosphorylation kinase activities and inhibition. λ exonuclease (λ exo) is an enzyme that catalyses efficiently cleavage of dsDNA from 5'-phosphate termini to generate ssDNA and mononucleotides. However, the cleavage efficiency of the λ exo is greatly reduced if the 5'-termini of dsDNA is hydroxyl. In this work, we developed a simple, label-free sensitive method for kinase activity and inhibition analysis based on the coupled λ exo cleavage reaction and the SYBR Green I. The label-free method developed in this work can detect phosphorylation kinase activities in the linear range of 0.25 to 1.00 U/mL with limit of detection of 0.11 U/mL.

Experimental

Materials and Apparatus

DNA sequences were purchased from Eurofins MWG Operon (Alabama, USA). The sequences for hair-pin probe were: 5'-TGC CTA CGA CCA ATT CCA CCT CAG CTA CCA GCA ACA GT-3' and 5'- ACT GTT GCT GGT AGC T-3'. The T4 polynucleotide kinase and exonuclease enzyme (λ exo) and 10X NEBuffer 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate and 1 mM DTT, pH 7.9.) were purchased from New England Biolabs, Inc (NEB, U.K.). SYBR Green I was purchased from Fisher Scientific (Pennsylvania, USA). The deionized water (18.3 MΩ•cm) was produced from the Millipore water purification system. Fluorescence experiments were performed using a Fluorolog-3 Spectrofluorometer with a 450 W xenon lamp (Jonin Yvon-Spex, Instruments S.A., Edison, NJ).

T4 PNK Detection and Optimization

In a typical phosphorylation and cleavage assay, 0.1 μL 10x SG, 20 nM dsDNA probe, 0.1 mM ATP, 0.25 units of λ exo, and a series of different concentrations of T4 PNK were added into 100 μL of 1x NEBuffer 4. After 30 min incubation at 37°C, the fluorescence spectra with the excitation wavelength of 490 nm and emission from 505 nm to 650 nm were recorded. The fluorescence intensity at 518 nm was used as the detection signal. The optimal values of phosphorylation time, concentrations of λ exo and ATP were investigated.

T4 PNK Inhibitor Evaluation

In order to investigate effects of the T4 PNK inhibitors on the phosphorylation process, the reactions were performed at various concentrations of inhibitors, including $(NH4)_2SO_4$ (0-120 mM), and Na_2HPO_4 (0-90 mM). All mixtures contained 0.1 μL 10x SG, 20 nM dsDNA probe, 0.1 mM ATP, 0.25 units of λ exo, and 10 U/mL T4 PNK.

Results and Discussion

Scheme of the T4 PNK activity detection

Based on the high affinity of SG to dsDNA and the unique property of λ exo, a label-free method was designed for the detection of T4 PNK activity. The proposed scheme for the PNK activity detection was illustrated in Fig. 1. A double-stranded DNA with one duplex 5'-hydroxyl termini was used as the label-free DNA probe. An intercalating dye molecule, SG, was selected as the fluorescence signal source. The dsDNA coupled with SG would generate strong fluorescence intensity. With the addition of the T4 PNK, the 5'-hydroxyl termini of the dsDNA would be transferred to 5'-phosphate termini in the presence of ATP (Fig. 1, step A). In the following step, the dsDNA probe was digested by the λ exo from the duplex 5'-phosphate termini to generate

mononucletides and ssDNA (Fig.1, step B). The binding force between the ssDNA and SG decreased dramatically, which led to a reduction of the fluorescence intensity. The extent of the fluorescence decrease was proportional to the concentration of T4 PNK. As a control, in the absence of T4 PNK, the λ exo digestion of the 5'-hydroxyl termini dsDNA was limited due to the lack of preferred substrate. As a result, the fluorescence intensity of the SG would have little change. With this strategy, no fluorescence label was needed to perform the detection of T4 PNK activity.

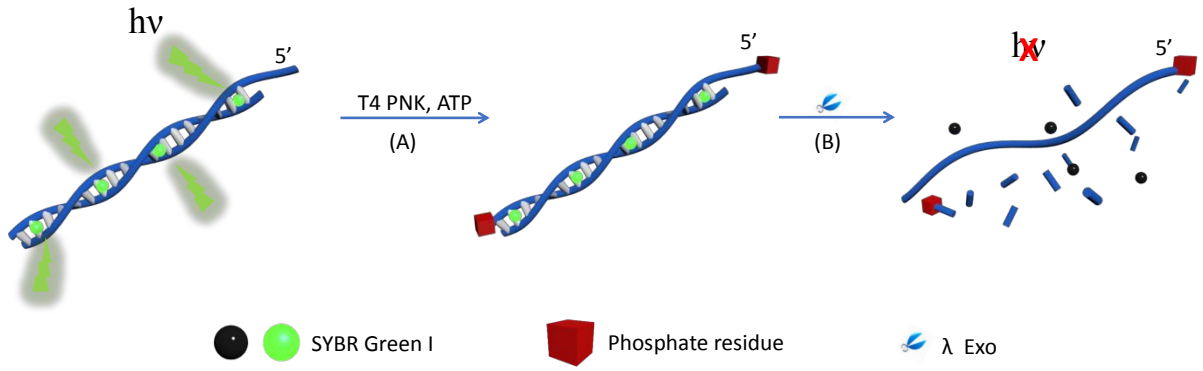
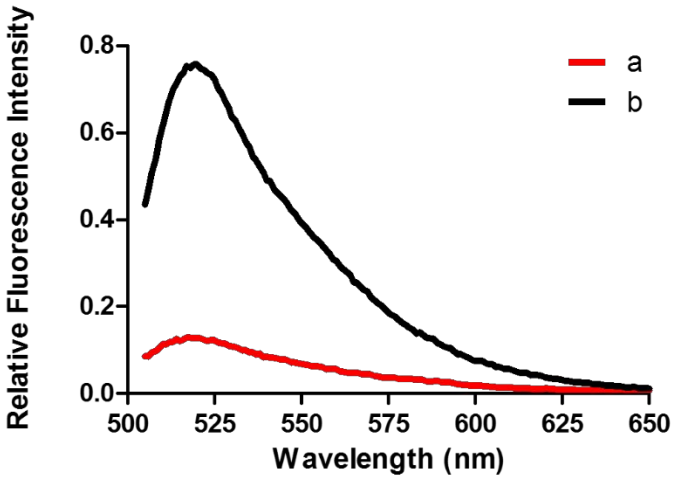


Fig.1. Schematic diagram of monitoring the T4 PNK activity by a label-free DNA probe coupled with SG and λ exo.

The phosphorylation process of T4 PNK could be monitored by measuring fluorescence changes of the system using SG as the intercalating dye. As shown in Fig. 2, when the T4 PNK was added to phosphorylate the 5'-termini of the dsDNA probe followed by the digestion of the λ exo, much lower fluorescence intensity was observed (Fig. 2, curve a). However, when the dsDNA probe was treated with λ exo only without the addition of T4 PNK, it exhibited high fluorescence intensity in the presence of SG (Fig. 2 curve b). Up to 90% fluorescence emission disappeared upon the addition of the 20 U/mL T4 PNK. The main reason was that the phosphorylated 5'-termini dsDNA was the preferred substrate of λ exo. It was reported that the reaction rate of the λ exo for dsDNA with 5'-phosphoral was over 300 times faster than that with 5'-hydroxyl termini.[29]

As a result, the dsDNA probe phosphorylated by the T4 PNK was digested much more rapidly than that without T4 PNK treatment by λ exo. The generated mononucleotides and ssDNA had low affinity to the SG, resulting in low fluorescence emission signal. The dsDNA probe without



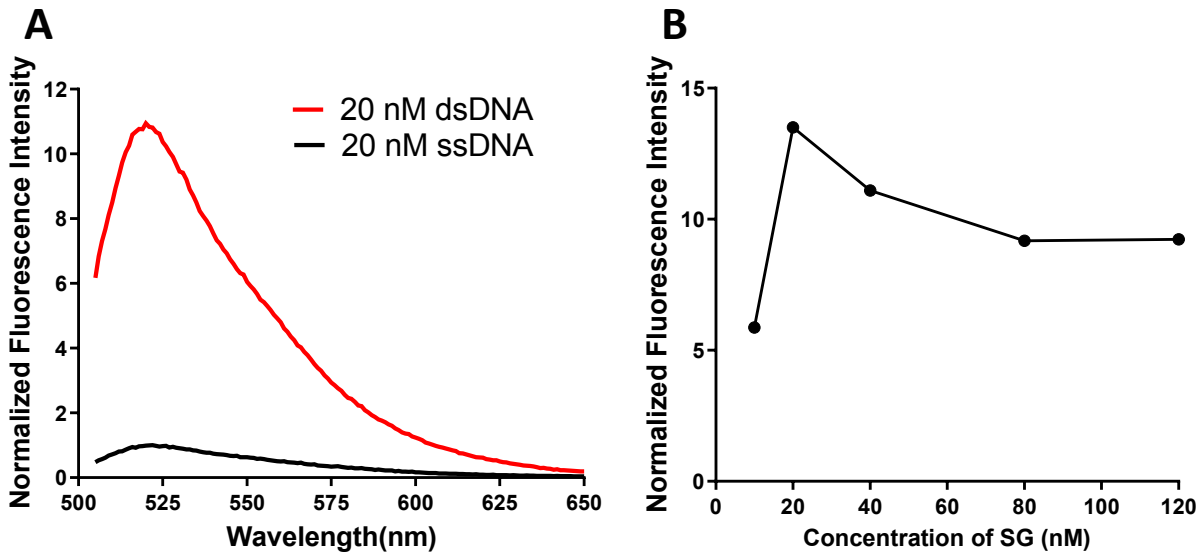
T4 PNK treatment remained the double-stranded structure which emitted high fluorescence signal with the interaction of the SG. The following experimental results showed that the fluorescence decrease was proportional to the activity and concentration of the T4 PNK, which demonstrated that this label-free system could be used for the detection of the T4 PNK activity.

Fig.2. Fluorescence spectra of the dsDNA system with 20 U/mL T4 PNK (a) and without T4 PNK (b). Reactions were performed in 100 μL of 1x NEBuffer 4 (pH = 7.9) with 20 nM dsDNA at 37°C for 30 min. The concentrations of ATP and λ exo were 0.1 mM and 0.25 units, respectively. λ_{ex} = 480 nm.

Optimization of SG concentration

SG played a crucial role in this designed assay. Therefore, the optimal concentration to distinguish ssDNA and dsDNA should be investigated in order to obtain the highest sensitivity of the assay. In the presence of ssDNA, a very limited fluorescence signal was appeared (Fig. 3A, black curve). However, the fluorescence intensity of SG enhanced several times when a dsDNA

was added to the solution (Fig. 3 A, red curve). The dramatic difference was the base for distinguishing an ssDNA and a dsDNA. To obtain the optimal detection ability, the concentration of SG should be optimized since it would affect the signal-to-background ratio of the detection. As shown in Fig. 3B, with the increase of the concentration of SG, both the fluorescence intensities of dsDNA and ssDNA solutions increased. In order to obtain the highest difference between



dsDNA and ssDNA, the fluorescence intensity of dsDNA-to-ssDNA ratio was plotted against the SG concentration as shown in Fig. 3B. The highest fluorescence intensity ratio of dsDNA-to-ssDNA was reached at the concentration of 20 nM. Therefore, 20 nM SG was chosen as the optimal concentration for the following experiments.

Fig.3. (A) Fluorescence spectra of 40 nM SG incubated with 20 nM ssDNA (Curve a) and dsDNA (Curve b). (B) The fluorescence intensity ratio of dsDNA to ssDNA with the change of the concentration of SG. Fluorescence intensity was measured in 100 μL of 1x NEBuffer 4 (pH = 7.9) with 20 nM ssDNA or dsDNA at 37°C. λ_{ex} = 480 nm.

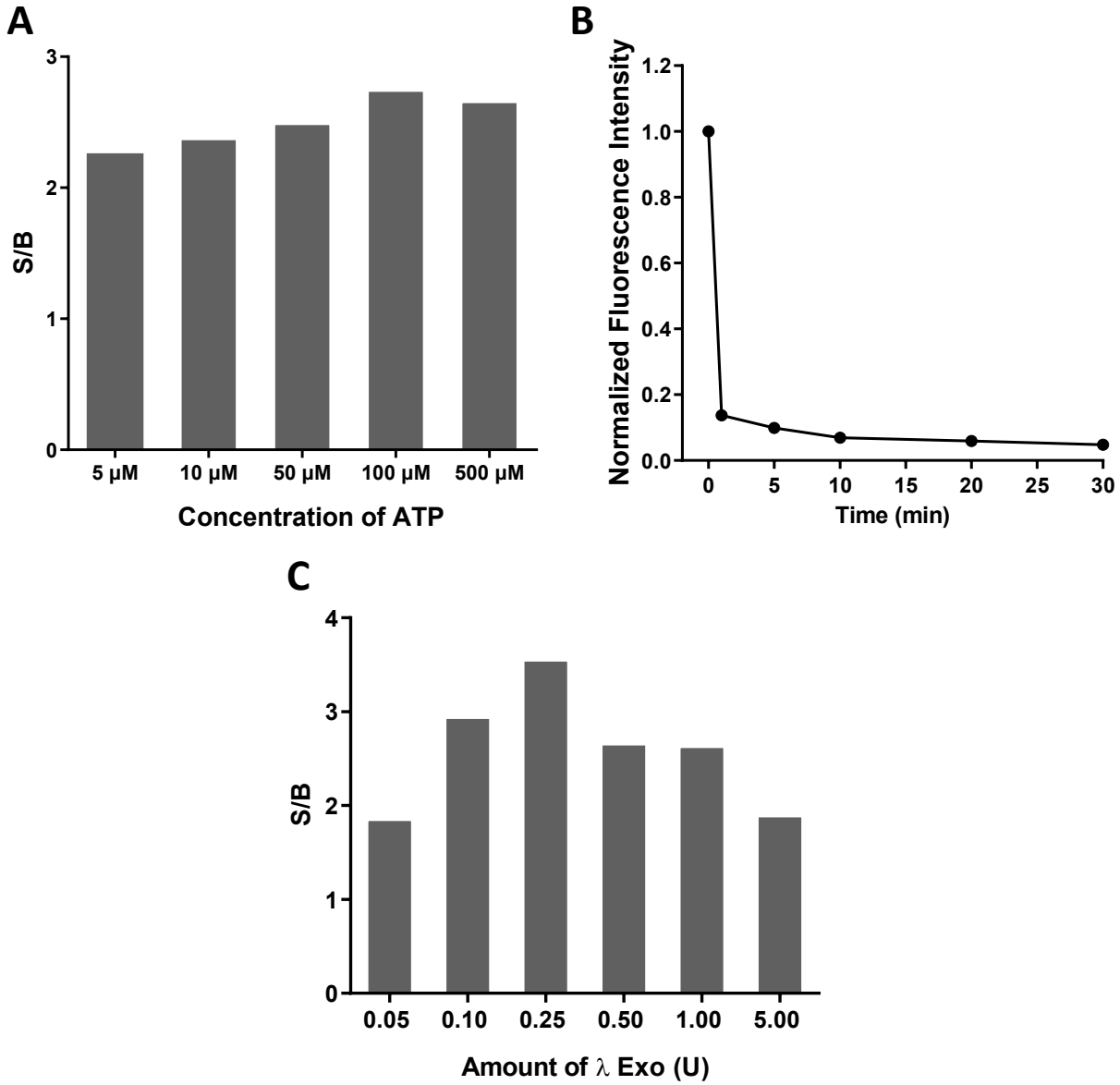
Optimization of the detection parameters

In the phosphorylation process of DNA, there are several factors that will affect the catalysis efficiency. ATP is a co-factor for assisting in T4 PNK phosphorylation because it provides the phosphate group to be modified on the DNA strands. [12] The concentration of ATP may affect the activity of the T4 PNK in the system. In order to gain better analytical performance, the effect of ATP concentration on the fluorescence intensity was studied as shown in Fig. 4A. Because the fluorescence intensity of SG will decrease when the T4 PNK was present, we used the fluorescence intensity change as a measurement to evaluate the effect of ATP on the T4 PNK activity. With the increase of the ATP concentration, the signal-to-background (S/B) ratio reached the highest point when 100 μM ATP was introduced. As a result, 100 μM was chosen as the optimum concentration of ATP in the phosphorylation and cleavage processes.

The reaction time of the T4 PNK catalysed phosphorylation and the coupled λ exo digestion was the other crucial factor. As shown in Fig. 4B, the fluorescence intensity of SG decreased dramatically through the incubation time. The results showed that the fluorescence intensity decreased with the increase of the incubation time to 30 min. In the first 2 min, 87.5 % fluorescence was decreased, followed by the gradual decrease until 30 min. This is due to the rapid catalytic activity of λ exo when T4 PNK was added to phosphorylate the dsDNA. The stable fluorescence intensity after 30 min suggested the complete phosphorylation and digestion. As a result, 30 min was chosen for the incubation time in the following experiments.

In this assay, λ exo was used as an enzyme to digest dsDNA after the phosphorylation by T4 PNK. The concentration of the λ exo should be a primary factor for affecting fluorescence intensity of the system. A higher concentration of λ exo would increase fluorescence background signal by digesting the dsDNA that without the phosphorylation by T4 PNK, which led to the lower signal-to-background ratio (S/B) ratio. In contrast, the lower amount of λ exo was not

enough to digest the phospholated-dsDNA. Therefore, it is necessary to investigate the optimal concentration of λ exo. To get the optimum (S/B), the relationship of the S/B ratio with the amount



of the λ exo was studied. It was shown that the highest S/B value was obtained in the presence of 0.25 units of λ exo (Fig. 4C). Therefore, 0.25 units of λ exo were used in the following experiments.

Fig.4. (A) Optimization of ATP concentration. The concentrations of λ exo, dsDNA, and T4 PNK were 1 unit, 20 nM, and 20 U/mL, respectively. (B) Optimization of the reaction time. The

concentrations of λ exo, dsDNA, ATP and T4 PNK were 0.25 units, 20 nM, 0.1 mM and 20 U/mL, respectively. λ_{ex} = 480 nm. (C) The relationship of signal-to-background ratio (S/B) with the amount of λ exo. The concentrations of dsDNA, ATP and T4 PNK were 20 nM, 0.1 mM and 20 U/mL, respectively.

Monitoring of the phosphorylation process of T4 PNK

Under the optimized conditions, a series of concentrations of T4 PNK from 0 to 20 U/mL was applied to quantify the kinase activity of the T4 PNK. As shown in Fig. 5A, it is demonstrated that the fluorescence signals gradually decreased through varying the concentration of T4 PNK from 0 to 20 U/mL. The relative fluorescence intensity reached the saturation when the T4 PNK concentration reached 10 U/mL. The calibration curve between the fluorescence intensity change and T4 PNK concentration was shown in Fig. 5B. There was a good linear range between 0 to 1.00 U/mL (Figure 5 B inset). The linear relationship can be demonstrated as Y = -0.4384X + 0.7364 with a correlation coefficient of 0.9887. Y and X represented the relative fluorescence intensity and concentration of T4 PNK in units of U/mL, respectively. The limit of detection (LOD) was 0.11 U/mL according to the definition of 3σ. This result demonstrated is a potential simple, quantitative and label-free strategy for polynucleotide kinase activity assay.

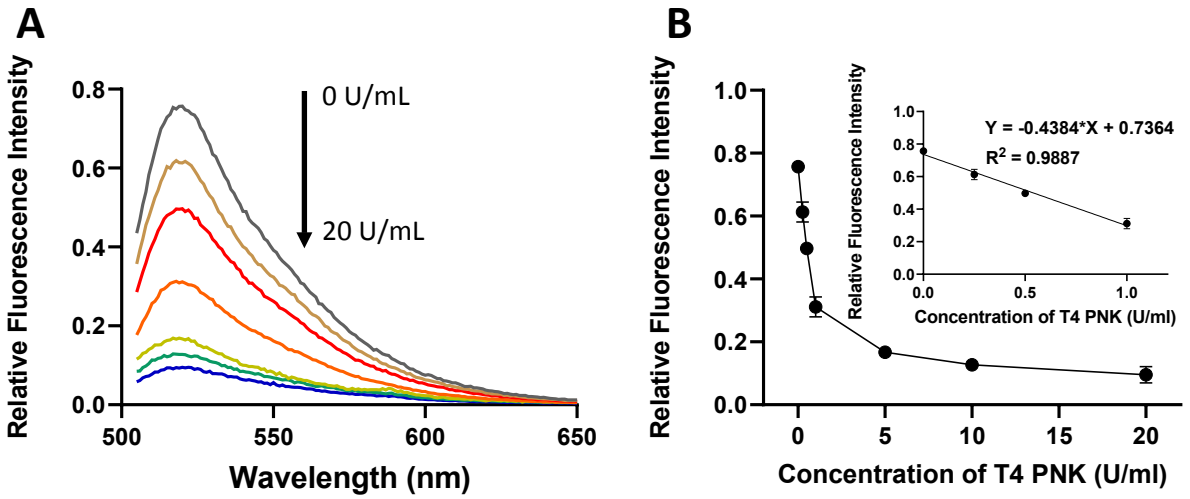


Fig.5. Monitoring the T4 PNK activity. (A) Relative fluorescence intensity with different concentrations of T4 PNK (top to bottom, 0, 0.25, 0.50, 1.00, 5.00, 10.00, 20.00 U/mL). (B) Concentration curve for T4 PNK. Inset indicated the linear relationship between fluorescence intensity change and the concentration of T4 PNK from 0 to 1.00 U/mL. Fluorescence intensity was measured in 100 μL of 1x NEBuffer 4 (pH = 7.9) at 37°C after 30 min incubation. The concentrations of λ exo, dsDNA, and ATP were 0.25 units, 20 nM, and 0.1 mM, respectively. λ_{ex} = 480 nm.

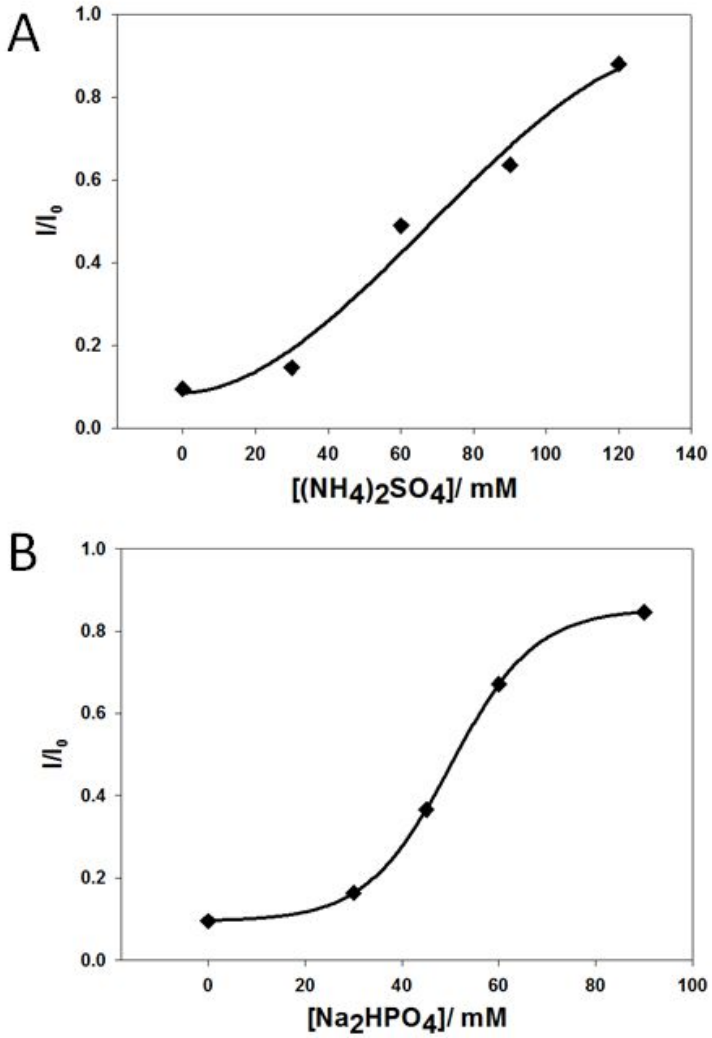


Fig.6. The effect of different concentrations of T4 PNK inhibitor. (A) Effect of $(NH_4)_2SO_4$ (0-120 mM); (B) Effect of Na_2HPO_4 (0-90 mM). Fluorescence intensity was measured in 100 µL of 1x NEBuffer 4 (pH = 7.9) at 37°C after 30 min incubation. The concentrations of λ exo, dsDNA, ATP and T4 PNK were 0.25 units, 20 nM, 0.1 mM, and 10 U/mL respectively. λ_{ex} = 480 nm.

Inhibition investigation

Since the phosphorylation plays important roles on several bioprocesses, the inhibition to the T4 PNK activity was also important to evaluate. Therefore, the inhibition effects of the ammonium sulphate, and sodium hydrogen phosphate on phosphorylation were also investigated

through this label-free fluorescence assay. As shown in Fig. 6, the phosphorylation activity of T4 PNK was inhibited by both ammonium sulphate and sodium hydrogen phosphate depending on the concentration of the inhibitors. With the increase of the concentration of the inhibitors, the relative fluorescence intensity of the system increased which indicated that the phosphorylation process was inhibited by the high concentration inhibitors. The results showed that this assay could investigate the various effects on phosphorylation quantitatively.

Conclusions

In conclusion, we have developed a facile, quantitative, cost-effective and label-free fluorescence assay for the detection of T4 PNK activity and inhibition coupled with λ exo and SG. In this sensing platform, the 5'-hydroxyl group of the dsDNA was phosphorylated by T4 PNK in the presence of ATP, the resulting 5'-phosphoryl termini dsDNA was digested by the λ exo in the following step. As a result, the SG integrated in the dsDNA was released from the dsDNA which lead to the fluorescence intensity decrease of the system. This assay offered a new opportunity in the development of phosphorylation monitoring and related drug screening and clinical diagnostics.

Acknowledgements

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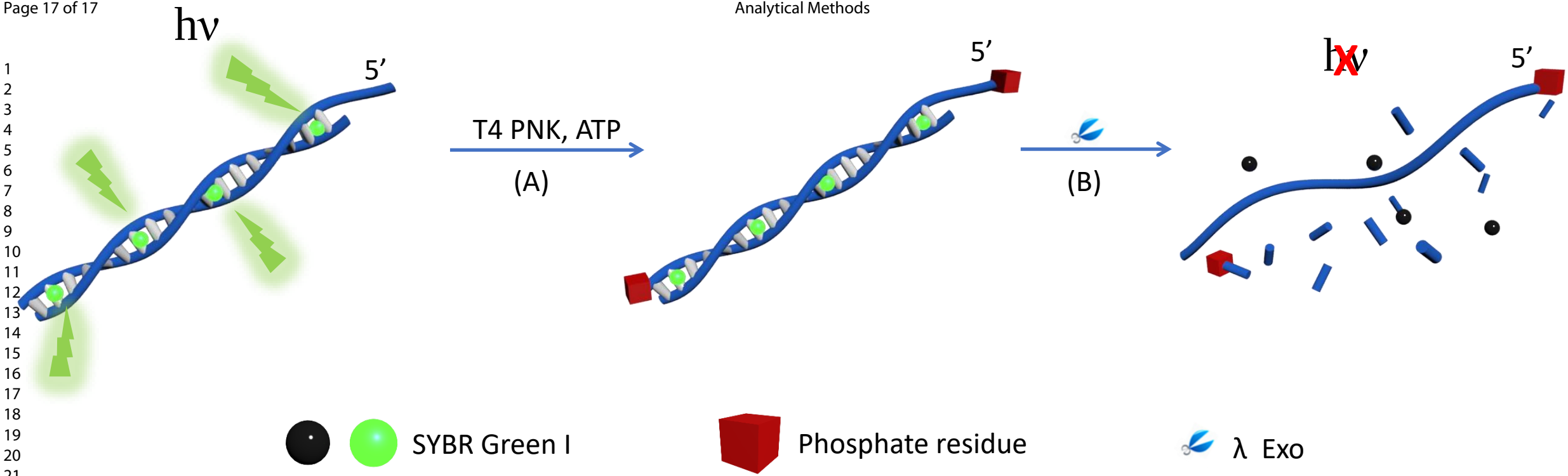
References

1. A. Rasouli-Nia, F. Karimi-Busheri and M. Weinfeld, *Proc. Natl. Acad. Sci.,* 2004, **101**, 6905-6910.
2. F. Karimi-Busheri, A. Rasouli-Nia, J. Allalunis-Turner and M. Weinfeld, *Cancer. Res.*, 2007, **67**, 6619-6625.
3. W. D. Henner, L. O. Rodriguez, S. M. Hecht and W. A. Haseltine, *J. Biol. Chem.*, 1983, **258**, 711-713.
4. A. Torriglia, P. Perani, J. Y. Brossas, E. Chaudun, J. Treton, Y. Courtois and M. F. Counis, *Mol. Cell. Biol.*, 1998, **18**, 3612-3619.
5. L. K. Wang, C. D. Lima and S. Shuman, *EMBO. J.*, 2002, **21**, 3873-3880.
6. C. J. Whitehouse, R. M. Taylor, A. Thistlethwaite, H. Zhang, F. Karimi-Busheri, D. D. Lasko, M. Weinfeld and K. W. Caldecott, *Cell* 2001, **104**, 107-117.
7. L. K. Wang and S. Shuman, *J. Biol. Chem.*, 2001, **276**, 26868-26874.
8. F. Karimi-Busheri, G. Daly, P. Robins, B. Canas, D. J. Pappin, J. Sgouros, G. G. Miller, H. Fakhrai, E. M. Davis, M. M. Le Beau and M. Weinfeld, *J. Biol. Chem.*, 1999, **274**, 24187-24194.
9. T. Wu, S. Liu, H. Li, L. Wang and X. Sun, *J. Nanosci. Nanotechnol.*, 2011, **11**, 10078-10081.
10. C. Ma and E. S. Yeung, *Anal. Bioanal. Chem.*, 2010, **397**, 2279-2284.
11. Y. Wang, X. He, K. Wang, X. Ni, J. Su and Z. Chen, *Biosens. Bioelectron.*, 2012, **32**, 213-218.
12. Z. Tang, K. Wang, W. Tan, C. Ma, J. Li, L. Liu, Q. Guo and X. Meng, *Nucleic. Acids. Res.,* 2005, **33**, e97.
13. C. Song and M. Zhao, *Anal. Chem.*, 2009, **81**, 1383-1388.
14. L. Lin, Y. Liu, X. Zhao and J. Li, *Anal. Chem.*, 2011, **83**, 8396-8402.
15. C. Ma, H. Liu, J. Du, H. Chen, H. He, S. Jin, K. Wang and J. Wang, *Anal. Biochem.*, 2016, **494**, 1-3.
16. S. Lian, C. Liu, X. Zhang, H. Wang and Z. Li, *Biosens. Bioelectron.*, 2015, **66**, 316-320.
17. Y. Cen, W. J. Deng, R. Q. Yu and X. Chu, *Talanta*, 2018, **180**, 271-276.
18. X. Li, X. Xu, J. Song, Q. Xue, C. Li and W. Jiang, *Biosens. Bioelectron.*, 2017, **91**, 631-636.
19. L. J. Wang, Q. Zhang, B. Tang and C. Y. Zhang, *Anal. Chem.*, 2017, **89**, 7255-7261.

20. M. Liu, F. Ma, Q. Zhang and C. Y. Zhang, *Chem. Commun.*, 2018, **54**, 1583-1586.
21. F. Ma, H. Liu, C. C. Li and C. Y. Zhang, *Chem. Commun.*, 2018, **54**, 12638-12641.
22. J. R. Webster, M. A. Burns, D. T. Burke and C. H. Mastrangelo, *Anal. Chem.*, 2001, **73**, 1622-1626.
23. C. P. Diggle, J. Bentley and A. E. Kiltie, *Nucleic. Acids. Res.,* 2003, **31**, e83.
24. H. Zipper, H. Brunner, J. Bernhagen and F. Vitzthum, *Nucleic. Acids. Res.,* 2004, **32**, e103.
25. H. Xu, S. Gao, Q. Liu, D. Pan, L. Wang, S. Ren, M. Ding, J. Chen and G. Liu, *Sensors*, 2011, **11**, 10187-10196.
26. X. Zhu, X. Zhou and D. Xing, *Biosens. Bioelectron.*, 2011, **26**, 2666-2669.
27. N. Dave, M. Y. Chan, P. J. Huang, B. D. Smith and J. Liu, *J. Am. Chem. Soc.*, 2010, **132**, 12668-12673.
28. W. Pu, H. Zhao, C. Huang, L. Wu and D. J. M. A. Xua, *Microchimica. Acta.*, 2012, **177**, 137-144.
29. J. W. Little, *J Biol Chem*, 1967, **242**, 679-686.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T4 PNK, ATP
(A)
5’
(B)
hν
5’
SYBR Green I
Phosphate residue
λ Exo