

Electronic Supporting Information

Phosphorylation-**Induced** Hybridization Chain Reaction on Beads: An Ultrasensitive Flow Cytometric Assay for the Detection of T4 Polynucleotide Kinase Activity

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1. Experimental Section

Materials and Reagents. T4 PNK (10000 U/mL) and λ exonuclease (λ exo, 5 U/ μ L) were obtained from New England Biolabs (UK). Adenosine triphosphate (ATP) was purchased from Takara. Dynabeads M-270 Streptavidin (STV-MBs) were supplied by Life Technologies. All other reagents used in this study were of analytical grade and used directly without further purification. All of the DNA sequences were adapted from the literature^[1] and custom synthesized by Sangon Biotech (Shanghai, China) and their sequences are listed as follows (5'→3', the sticky ends of H1 and H2 hairpins are underlined while the loops are italicized):

H1: 5'-AGTCTAGGATTCGGCGTGGGTTAACACGCCGAATCCTAGACTACTTTG- FAM-3'

H2: 5'-FAM-TTAACCCACGCCGAATCCTAGACTCAAAGTAGTCTAGGATTCGGCGTG- 3'

HCR Initiator: 5'-biotin-TTTTTTTTTTCAAAGTAGTCTAGGATTCGGCGTG-3'

Blocking DNA: 5'-CACGCCGAATCCTAGACTACTTTG-3'

Preparation of HCR Initiator-Conjugated Microbeads (DNA-MBs). Dynabeads M-270 Streptavidin (STV-MBs, 2.8 μ m) were used throughout this work. The stocking vial of STV-MBs was firstly vortexed to make sure that the MBs were homogeneously dispersed, and then 5 μ L slurry of the MBs was pipetted out. After thoroughly washing of the MBs to remove the preservative, 10 μ L of Tris-HCl buffer (70 mM Tris-HCl, 10 mM MgCl₂, pH 7.6) containing excess biotinylated HCR Initiator (1 μ M) was mixed with the STV-MBs and incubated at 37°C for 0.5 h under shaking. Subsequently, 20 μ L of 1 μ M Blocking DNA was added into the above solution and the mixture was further incubated at 37°C for 1 h under vigorous shaking. Therefore the HCR Initiator sequences immobilized on the MBs were all hybridized with the Blocking DNA. After that, the MBs were magnetically purified to remove the unbound HCR Initiator and the excess Blocking DNA. Finally, the HCR Initiator-conjugated MBs (DNA-MBs) were dispersed in 50 μ L of Tris-HCl buffer as the stock solution for subsequent use.

Standard Procedures of the Proposed FCBA for Assaying T4 PNK Activity. Typically, in a total 20 μ L reaction buffer (70 mM Tris-HCl, 10 mM MgCl₂, pH 7.6), 1 μ L of the as prepared DNA-MBs ($\sim 7 \times 10^4$ beads)

was incubated with 1 mM ATP, 0.1 U of λ exo, and certain concentration of T4 PNK at 37°C for 1 h under vigorous shaking to simultaneously perform the phosphorylation and λ exo-based DNA digestion. Afterward, the MBs were magnetically isolated and washed three times with Tris-HCl buffer. Subsequently, such MBs were further incubated with 250 nM H1 and 250 nM H2 respectively in 20 μ L of saline sodium phosphate buffer (SSPB, 50 mM phosphate buffer containing 750 mM NaCl, pH 7.4) at 37°C for 3 h to conduct the HCR reaction. Finally, each sample was directly diluted to 500 μ L and immediately analyzed by an Accuri C6 flow cytometer (BD Biosciences). During flow cytometry analysis, 10000 MBs were collected for each sample under the standard high-speed running conditions, and the fluorescence signals of the HCR products-accumulated MBs were detected by the FL1 (FAM/FITC) channel under the 488 nm laser excitation. For each sample, the mean fluorescence intensity (MFI) of all the detected MBs was used for the quantitative analysis of T4 PNK activity.

T4 PNK Inhibition Study. Several model compounds, including adenosine diphosphate (ADP), $(\text{NH}_4)_2\text{SO}_4$ and Na_2HPO_4 , were used in this work to investigate the effects of inhibitors on the T4 PNK-catalyzed DNA phosphorylation. The inhibition studies were carried out with the similar procedures as those for T4 PNK assay stated above except that the T4 PNK (fixed at 0.01 U/mL) was pre-mixed with varied concentrations of inhibitor.

2. Optimization of the Experimental Conditions

During the T4 PNK-catalyzed phosphorylation process, ATP is indispensable because it acts as the donor of the phosphoryl group. So the effect of ATP concentration is investigated in this study. As shown in Fig. S1a, the MFI value produced by 0.001 U/mL T4 PNK increases gradually as the ATP concentration increases from 0 to 1.0 mM. However, the MFI values would be obviously decreased when ATP concentration is higher than 1 mM, suggesting that at high ATP concentrations, the phosphorylation might be inhibited due to the competitive binding to T4 PNK between DNA and ATP, which has been reported previously.^[2] Meanwhile, the MFI values

of the blank control without T4 PNK almost keep stable irrespective of the variation of ATP concentrations. Thus, 1 mM ATP is selected as the optimum for the phosphorylation process in this work.

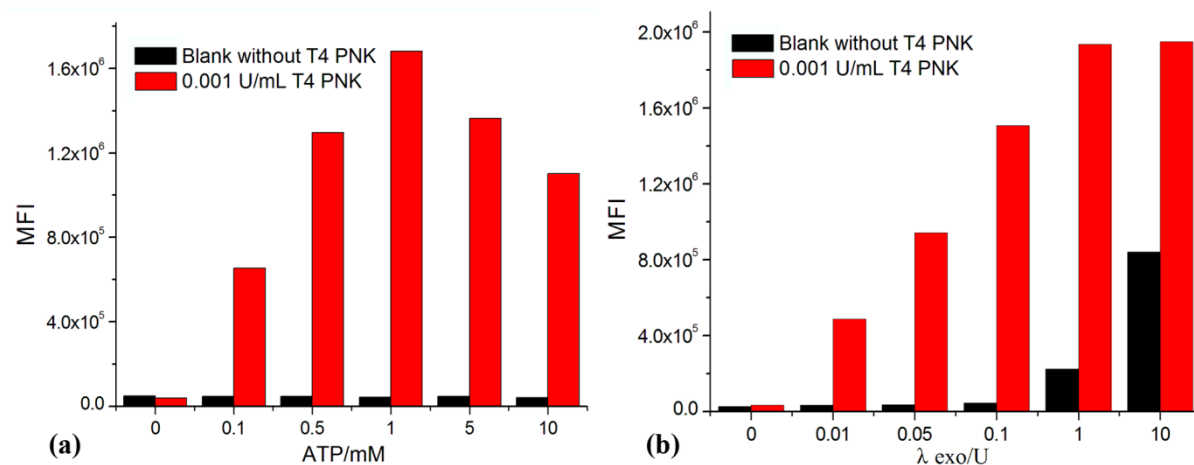


Fig. S1. (a) The effect of ATP concentration on the proposed FCBA system. 0.1 U of λ exo was used for this study; (b) optimization of λ exo concentration for the FCBA system by fixing ATP at 1 mM.

Furthermore, according to the design mechanism, the concentration of λ exo is crucial for this proposed T4 PNK assay. To find the optimal amount of λ exo, a series of DNA-MBs are treated with a fixed concentration of T4 PNK (0.001 U/mL) and different doses of λ exo. Meanwhile, the DNA-MBs reacted with only such varying amount of λ exo but without T4 PNK are used as the blank control. As can be seen from Fig. S1b that in the presence of T4 PNK, the MFI values increase sharply with the increase of λ exo from 0 to 1 U. However, no obvious MFI increase is observed when further elevating λ exo concentration. In contrast, for the control samples treated with only λ exo but without T4 PNK, only slight MFI value changes are observed with the increase of λ exo from 0 to 0.1 U. But the MFI of the blank control will be remarkably enhanced when the λ exo further increases to 1 U or 10 U, indicating that excess λ exo will accelerate nonspecific DNA digestion towards 5'-OH termini of the Blocking DNA. Therefore, 0.1 U is selected as the optimal amount of λ exo for further analytical applications in this study.

3. Verification of the Feasibility of the FCBA system for the Detection of T4 PNK Activity

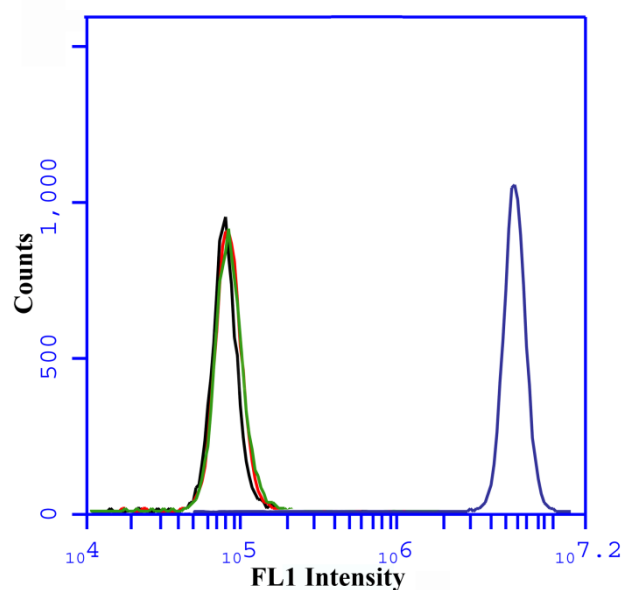


Fig. S2. Histograms of fluorescence signals (FL1 channel) of the FCBA system treated with different reaction components. Black line: ATP + λ exo; Blue line: ATP + T4 PNK + λ exo; Green line: T4 PNK + ATP; Red line: T4 PNK + λ exo without ATP. Other experimental conditions: T4 PNK, 1 U/ml; λ exo, 0.1 U; ATP, 1 mM.

The results shown in Fig. S2 clearly verify the feasibility of this proposed FCBA for assaying T4 PNK activity. One can see that when the DNA-MBs are incubated with only λ exo (black line) or only T4 PNK (green line), the MFI values of the MBs population are both around 8.0×10^4 , which is almost the same as that of pure DNA-MBs (data not shown). In contrast, when the DNA-MBs are treated with both T4 PNK and λ exo, the MFI value increases sharply to 5.9×10^6 with a significant 75-fold enhancement (blue line). In a further control experiment, when the DNA-MBs are reacted with the combination of T4 PNK/ λ exo but without ATP (in such case T4 PNK cannot phosphorylate the DNA), the change of MFI is also negligible (red line). These results indicate that the MFI increase can be only aroused by first T4 PNK-catalyzed phosphorylation and then λ exo-based DNA cleavage. It is clear that T4 PNK-induced phosphorylation of the Blocking DNA is the prerequisite and rate determining step for the λ exo digestion and subsequent HCR amplification. Therefore, by measuring the changes of MFI values, quantitative determination of T4 PNK activity can be achieved.

4. Detection of T4 PNK Activity by the 1:1 Binding Mode Without HCR

The efficient fluorescence accumulation on the MBs *via* HCR coupled with the powerful flow cytometry detection may be the main driving force to achieve the ultrahigh sensitivity for the proposed FCBA strategy. As depicted in Fig. 1, after the phosphorylation and λ exo digestion reaction, if only H1 instead of H1/H2 is added as the fuel, each liberated HCR Initiator on the MBs will only capture one H1 hairpin and the hybridization reaction will be stopped (1:1 binding). In such cases, as shown in Fig. S3, the detection limit of T4 PNK without HCR is only around 0.001 U/mL. These results clearly suggest that the efficient HCR amplification makes the detection sensitivity two orders of magnitude higher.

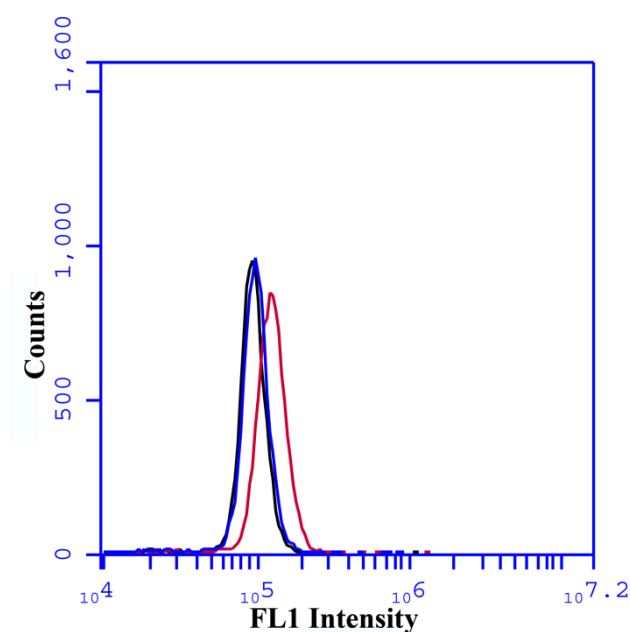


Fig. S3. Fluorescence histograms of the MBs-based 1:1 binding assay for T4 PNK by using only H1 as the fuel. T4 PNK activity: black line, 0 U/mL (blank control); blue line, 0.0005 U/ml; red line, 0.001 U/ml.

5. Specificity Evaluation of the FCBA for the Detection of T4 PNK Activity.

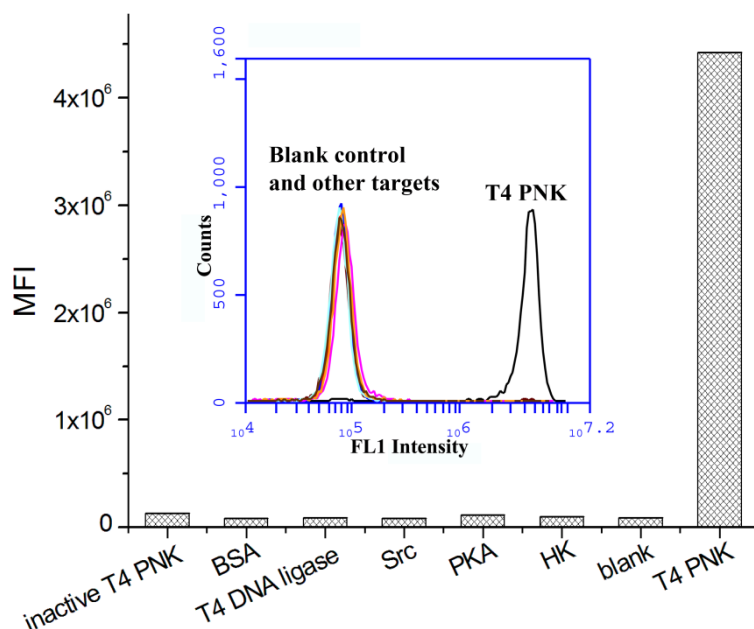


Fig. S4. Specificity evaluation of the proposed FCBA strategy for T4 PNK analysis. (inset) histograms of the FCBA system challenged by different targets. The histograms of other targets except T4 PNK are almost completely overlapped with that of blank control. Other experimental conditions: BSA, 0.5 μ M; Src, 1 μ g/mL; The concentration of T4 DNA ligase, PKA, HK, T4 PNK, and heat-inactivated T4 PNK were all 0.01 U/mL; Heat-inactivated T4 PNK was prepared by heating the sample at 85 $^{\circ}$ C for 10 min.

6. Detection of T4 PNK Activity in Complex Samples.

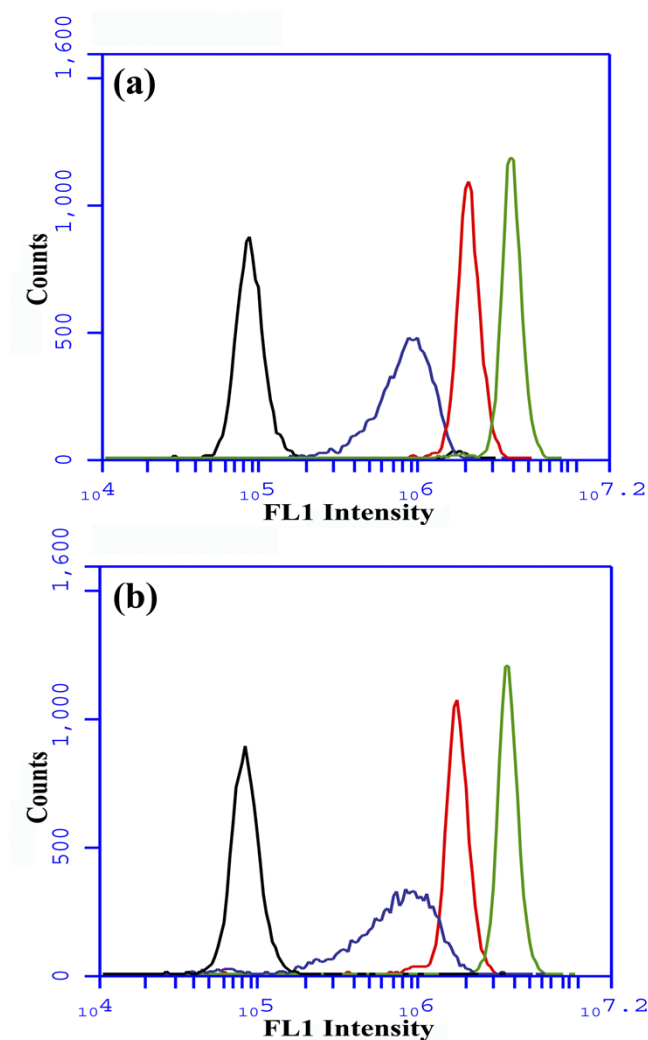


Fig. S5. (a) Responses of the FCBA system to different concentrations of T4 PNK in clean reaction buffer; (b) responses of the FCBA to different concentrations of T4 PNK in complex DMEM matrix. Black line, blank control; blue line, 0.0005 U/ml; red line, 0.002 U/ml; green line, 0.01 U/ml.

7. Inhibition Effects of Na_2HPO_4 and ADP on T4 PNK

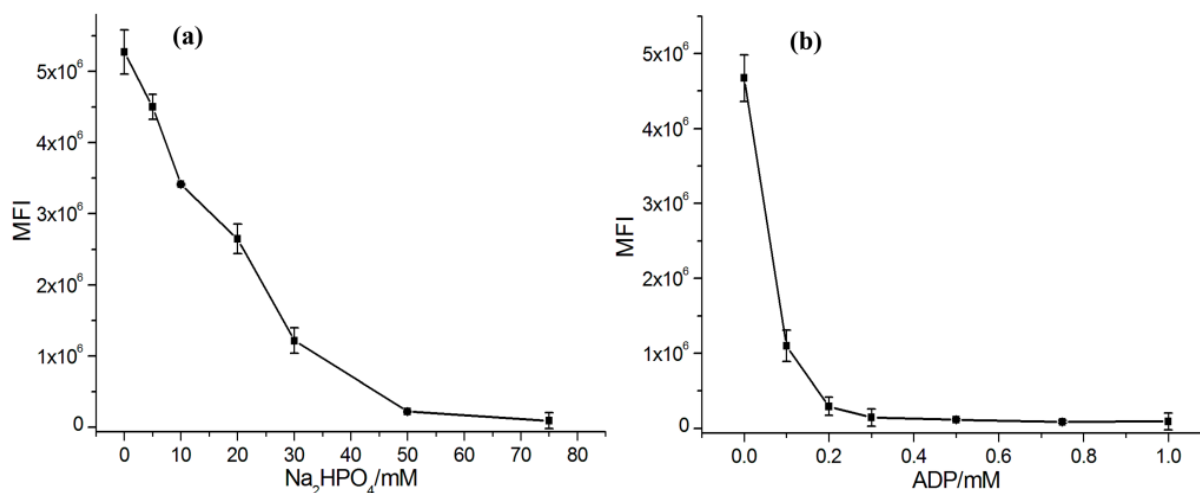


Fig. S6. Inhibition effects of Na_2HPO_4 (a) and ADP (b) on T4 PNK activity. T4 PNK, 0.01 U/mL.

References

- [1] Dirks, R. M.; Pierce, N. A. *Proc. Natl. Acad. Sci. U.S.A.* **2004**, *101*, 15275–15278.
- [2] Song, C.; Zhao, M. *Anal. Chem.*, **2009**, *81*, 1383–1388.