Electronic Supplementary Information

Label-Free and Ultrasensitive Detection of Polynucleotide Kinase Activity at the Single-Cell Level

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MATERIALS AND METHODS

Materials. All oligonucleotides (Table S1) were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). The T4 polynucleotide kinase (PNK), lambda exonuclease (λ exo), the Klenow fragment polymerase (3'→5' exo-, KF polymerase), the nicking enzyme Nt.BbvCI, adenosine 5'-triphosphate (ATP), deoxynucleotide (dNTP) solution mix, BSA and NEBuffer 2 were obtained from New England Biolabs (Ipswich, MA, USA). SYBR Green I was purchased from Life Technologies (Carlsbad, CA, USA). All other reagents were of analytical grade and used as received without further purification. Ultrapure water obtained from a Millipore filtration system was used throughout all experiments.

Table S1. Sequence of the Oligonucleotides [a]

note	sequence (5'-3')
probe-S-1	GTT GAG CAT CTG GCT TGT TTC ATA GTT TTT T
probe-S-2	GTT GAG CAT CTG GCT TGT TTC ATA GCA GTT TTT T
probe-S-3	GTT GAG CAT CTG GCT TGT TTC ATA GCA GAT GTT TTT T
probe-S-4	GTT GAG CAT CTG GCT TGT TTC ATA GCA GAT GCG CTT TTT T
probe-A	P-G*C*A* CAA AGG ACT GAG GCT GAG GGT TGC GCA TCT GCT ATG AAA CAA GCC AGA TGC TCA AC
template	GCA CAA AGG ACT GAG GCT GAG GGC ACA AAG GAC TGA G-P

[a] The asterisks indicate the phosphorothioate modification. The letter P represents the phosphate modification. The letter S refers to the sense strand of the probe. The letter A refers to the antisense strand of the probe.

Preparation of the Probes. To obtain the double-strand probes, 10 μM probe-S and 10 μM probe-A were mixed in the hybridization buffer containing 100 mM NaCl, 1 mM EDTA and 50 mM Tris−HCl (pH 8.0). After heating at 95 °C for 5 min, the mixture was slowly cooled to room temperature within 30 min to form the duplex structure. The obtained stock solutions were stored at −20 °C prior to the use.

PNK Activity Assay and Inhibition Assay. The assay was carried out in 20 μL of solution containing 0.2 μL of the probes (10 μM), 0.2 μL of ATP (10 mM), 1 μL of λ exo (5000 U/mL), indicated-concentration PNK, and 2 μL of 10 × reaction buffer (700 mM Tris-HCl, 100 mM $MgCl_2$, pH 8.0). The samples were incubated at 37 °C for 30 min to perform the phosphorylation

and digestion reaction. The reaction was terminated by heating at 75 °C for 10 min, followed by slowly cooling to the room temperature to form the probe-A with hairpin structure for next isothermal amplification step. For PNK inhibition assay, we used ADP, $(NH_4)_2SO_4$ and Na_2HPO_4 as the model inhibitors. After the annealing of probe-A and probe-S, various-concentration inhibitors were mixed with the probe duplexes and incubated for 10 min prior to the phosphorylation and digestion reaction.

Isothermal Amplification Reaction. The reaction mixtures were prepared separately on ice as part A and part B. Part A consisted of 2 μL of the post-digestion mixture, 50 nM template, 2 μL of 10 × NEBuffer 2 (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, pH 7.9). Part B consisted of 0.1 mg/mL BSA, 0.2 U/μL Nt.BbvCI nicking endonuclease, 0.1 U/μL KF polymerase, 250 μM deoxynucleotide triphosphates (dNTPs) and 2 μL of 10× SYBR green I. The reaction was initiated by mixing part A and part B, and then immediately processed at 37 °C in a Bio-Rad CFX connect Real-Time System. The fluorescence intensity was monitored at intervals of 30 s.

Preparation of Cell Extracts. The human embryonic kidney cells (HEK293T cells) were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, USA) with 10% fetal bovine serum (FBS, Life Technologies, USA) and 50 U/mL of penicillin plus 50 μg/mL streptomycin at 37 °C with 5% CO_2. For real sample analysis, the cell extracts were prepared using a nucleoprotein extraction kit (Shanghai Sangon, China) according to the manufacturer's protocol, and the protein concentration was measured using a NanoDrop 2000c Spectrophotometer (Thermo Scientific, Wilmington, Delaware, USA).

Gel Electrophoresis. The reaction products were analyzed by 12% nondenaturing polyacrylamide

gel electrophoresis (PAGE) in 1× TBE (9 mM Tris−HCl (pH 7.9), 9 mM boric acid, 0.2 mM ethylenediaminetetraacetic acid, EDTA) at a 100 V constant voltage at the room temperature for 40 min. The gels were stained by SYBR gold and analyzed by a Bio-Rad ChemiDoc MP Imaging System (USA).

SUPPLEMENTARY RESULTS

Table S2. Comparison of current method with the reported methods for PNK assay.

Strategy	Assay time*	Requirement of labels	Detection limit (U/mL)	Cell analysis	Reference
EXPAR and λ exonuclease cleavage	~ 2 h	No	0.0002	Yes	this work
Bimolecular beacons and λ exonuclease cleavage	~ 20 min	Fluorescent label (FAM)	0.04	No	1
Catalytic assembly of bimolecular beacons and λ exonuclease cleavage	~ 4 h	Fluorescent label (FAM)	0.001	No	2
Graphene Oxide and λ exonuclease cleavage	~ 6 h	Fluorescent label (FAM)	0.05	No	3
Paper-based fluorescence assay and λ exonuclease assistance	~ 5.5 h	Fluorescent label (Cy5)	0.0001 U	Yes	4
DNAzyme and λ exonuclease cleavage	~ 1.5 h	No	0.06	No	5
Ferrocene-functionalized SWCNT	over 24 h	No	0.01	No	6
Nanochannel and λ exonuclease cleavage	~ 3.5 h	Biotin label	0.01	No	7

Perylene probe and λ exonuclease cleavage	~ 1.5 h	No	0.003	No	8
Copper nanoparticle and λ exonuclease cleavage	~ 1.5 h	No	0.49 U/mL	No	9
Nicking reactions-mediated hyperbranched rolling circle amplification	~ 9 h	No	0.0000436	No	10

*Assay time includes the preparation time.

Optimization of Experimental Conditions. To achieve the best performance, we optimized the experimental conditions including the length of probe-S (Fig. S1A), the concentration of λ exo (Fig. S1B), the reaction time of PNK-catalyzed phosphorylation and λ exo-meditated digestion (Fig. S1C), and the concentration of ATP (Fig. S1D). We used the point of inflection (POI) and the ΔPOI for quantitative analysis. The POI is defined as the time corresponding to the maximum slope in the sigmoidal curve,[11] and the ΔPOI is defined as the difference in the POI value between the sample and the control without PNK. We firstly investigated the effect of the length of probe-S upon the assay performance. Long length of probe-S may benefit the maintenance of the unfold structure of probe-A and eventually the reduction of background signal. However, too long length of probe-S might prevent the phosphorylated probe-S from being digested by λ exo and the subsequent release of free probe-AS. We designed four probe-S with different lengths (Table S1) to optimize the length of probe-S. As shown in Fig. S1A, the ΔPOI value enhances with the

increasing length of probe-S from 31 to 40 nt, and levels off beyond the length of 37 nt. Therefore, the probe-S-3 with a length of 37 nt is used in the subsequent researches.

The λ exo is key for the digestion of phosphorylated probe. Although 5'-phosphorylated dsDNA is the preferred substrate of λ exo, it can degrade the non-phosphorylated substrates at a reduced rate as well.[7] Moreover, despite the high-concentration λ exo can lead to the digestion of more phosphorylated probe-S for the generation of high signal, it may cause the digestion of more non-phosphorylated probe-S in the control group, leading to high background signal. Thus, the concentration of λ exo should be optimized. As shown in Fig. S1B, the ΔPOI value enhances with the increasing concentration of λ exo from 0.05 to 0.25 U/μL, and levels off beyond the concentration of 0.25 U/μL. Therefore, 0.25 U/μL λ exo is used in the subsequent researches.

We further investigated the influence of reaction time of PNK-catalyzed phosphorylation and λ exo-meditated digestion upon the assay performance. As shown in Fig. S1C, the ΔPOI value increases rapidly with the reaction time, and reaches a plateau within 30 min, indicating the complete phosphorylation and the digestion of all available probes. Therefore, the reaction time of 30 min is used in the subsequent researches.

The ATP is the substrate of PNK and it provides the phosphate group during the PNK-catalyzed phosphorylation. We further investigated the effect of ATP concentration upon the assay performance. As shown in Fig. S1D, the ΔPOI value improves with the increasing concentration of ATP from 0.1 to 1 mM, followed by the decrease beyond the concentration of 2 mM. This can be explained by that excess ATP can reduce the activity of T4 PNK by partially blocking its binding sites.[2] Therefore, 1 mM ATP is used in the subsequent researches.

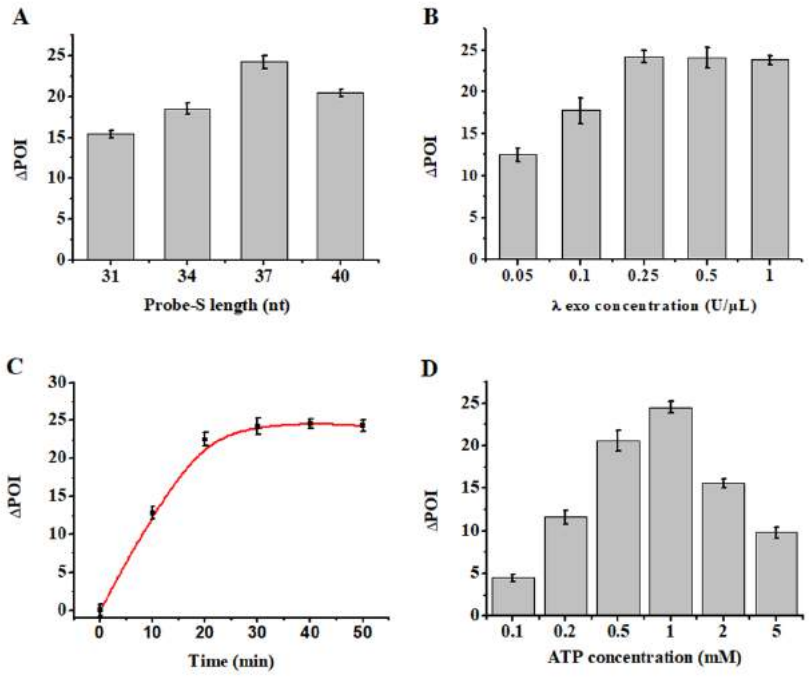

Fig. S1 (A) Variance of the ΔPOI value with the length of probe-S. (B) Variance of the ΔPOI value with concentration of λ exo. (C) Variance of the ΔPOI value with the reaction time of PNK-catalyzed phosphorylation and λ exo-meditated digestion. (D) Variance of the ΔPOI value with the concentration of ATP. The 1 U/mL PNK, 100 nM probe and 50 nM template were used in this research. The error bars show the standard deviations of three experiments.

Assay Specificity. To investigate the assay specificity, we used six enzymes as the interference enzymes, including HhaI, Dam, uracil-DNA glycosylase (UDG), thymine-DNA glycosylase (TDG), and human8-oxoguanine DNA glycosylase (hOGG1). HhaI is a DNA restriction endonuclease isolated from *Haemophilus haemolyticus*.[12] Dam is a DNA methyltransferase that catalyzes methylation on the N^6 position of the adenine residue.[13] UDG, TDG and hOGG1 are three kinds of DNA glycosylases. UDG catalyzes the release of free uracil from the uracil-containing DNA;[14] TDG removes thymine or uracil from the guanine/thymine and guanine/uracil mismatches in the damaged DNA;[15]

hOGG1 is responsible for the excision of 8-oxoguanine (8-oxoG).[16] In theory, none of these enzymes can initiate the phosphorylation-digestion reaction, and thus no isothermal amplification reaction can occur. We used the ΔPOI to investigate the detection specificity. The ΔPOI is defined as the difference in the POI values between a sample and the negative control without PNK. As shown in Fig. S2, the ΔPOI of PNK (Fig. S2, red column) is 33.2-fold higher than that of HhaI (Fig. S2, green column), 67.8-fold higher than that of Dam (Fig. S2, purple column), 20.6-fold higher than that of UDG (Fig. S2, brown column), 10.2-fold higher than that of TDG (Fig. S2, blue column), and 62.4-fold higher than that of hOGG1 (Fig. S2, cyan column). These results demonstrate the high specificity of the proposed method toward PNK.

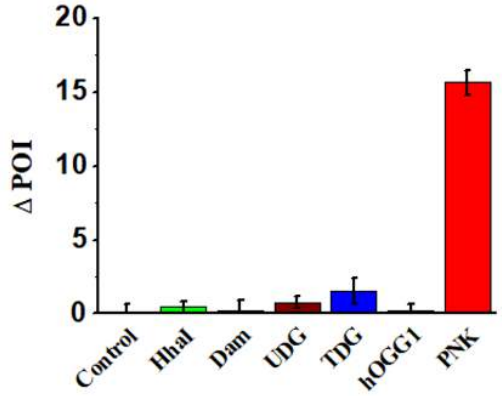

Fig. S2 The comparison of ΔPOI values in response to 0.5 U/mL HhaI (green column), 0.5 U/mL Dam (purple column), 0.5 U/mL UDG (brown column), 0.5 U/mL TDG (blue column), 0.5 U/mL hOGG1 (cyan column), 0.5 U/mL PNK (red column), and the control group without PNK (black column), respectively. The probe concentration is 100 nM, and the template concentration is 50 nM. Error bars show the standard deviation of three experiments.

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