Electronic Supplementary Information

Sensitive detection of polynucleotide kinase using rolling circle amplification-induced chemiluminescence

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EXPERIMENTAL SECTION

Materials. All oligonucleotides were synthesized and HPLC purified by Sangon Biotech Co., Ltd (Shanghai, China). The sequences of oligonucleotides are listed in Table S1. T4 polynucleotide kinase (PNK), T4 DNA Ligase, phi29 DNA polymerase, deoxynucleotide (dNTP) solution mixture, and adenosine 5’-Triphosphate (ATP) were purchased from New England Biolabs (Beverly, MA, USA). Hemin, luminol, dithiothreitol, spermine and adenosine 5’-diphosphate sodium salt (ADP) were obtained from Sigma-Aldrich Co (St. Louis, MO, USA). Hemin (1 mM) stock solution was prepared with dimethylsulfoxide (DMSO) and stored at -20°C in the dark, and was further diluted with 10 mM HEPES-NaOH (pH 9.0) in the experiments. Luminol stock solution (20 mM) was prepared with 0.1 M NaOH, and was further diluted with 10 mM Tris-HCl (pH 8.0) in the experiments. All the other chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultrapure water was obtained from a Milli-Q system (Millipore, Billerica, MA) and used throughout the experiments.
**Table S1.** Sequences of the substrates, the padlock probe, and the synthesized DNAzyme

<table>
<thead>
<tr>
<th>oligonucleotide</th>
<th>sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>substrate-1</td>
<td>GAC CGA CGT AAC ACA CAT TTT TCC CAA CCC GCC</td>
</tr>
<tr>
<td></td>
<td><strong>CTA CCC ATT TTT TTA CCC ATC CCG CCC AAC CCT TTT</strong></td>
</tr>
<tr>
<td></td>
<td>TCA CAC ACT CCC GAT CC</td>
</tr>
<tr>
<td>substrate-2</td>
<td>GAC CGA CGT AAC ACA CAT TTT TCC CAA CCC GCC</td>
</tr>
<tr>
<td></td>
<td><strong>CTA CCC TTT TTC ACA CAC TCC CGA TCC</strong></td>
</tr>
<tr>
<td>padlock probe</td>
<td>TAC GTC GGT CGG ATC GGG AGT GTG T</td>
</tr>
<tr>
<td>synthesized DNAzyme</td>
<td>GGG TAG GGC GGG TTG GG</td>
</tr>
</tbody>
</table>

*In the substrate DNA, the underlined and boldfaced regions indicate the binding site of padlock probe and the complementary site of DNAzyme, respectively. The 3’ and 5’ terminal of substrate DNA are hydroxyl.

**Phosphorylation and Ligation Reaction.** The phosphorylation of single stranded DNA substrate was carried out in 20 μL of reaction mixture containing various amounts of PNK, 100 nM DNA substrate, 0.5 mM ATP, 1× PNK buffer (70 mM Tris–HCl (pH 7.6), 10 mM MgCl₂, 1 mM Dithiothreitol). The mixture was incubated at 37 °C for 30 min, followed by inactivation at 75 °C for 10 min. The ligation reaction was carried out in the mixture of phosphorylated substrate and padlock probe (136 nM). The mixture was incubated at 95 °C for 5 min, followed by slowly cooling to the room temperature. With the addition of 2 U ligase, the ligation reaction was carried out at 37 °C for 60 min. The reaction solution was heated to 75 °C for 10 min to deactivate the ligase.
RCA Reaction. The ligation products were directly used for RCA reaction. RCA reaction was performed in 20 μL of reaction solution containing 12 μL of ligation products, 1× phi29 DNA polymerase buffer (50 mM Tris–HCl (pH 7.5), 10 mM MgCl2, 10 mM (NH4)2SO4, 1 mM dNTP and 2 U phi29 DNA polymerase at 37 °C for 2.5 h. The reaction solution was heated to 75 °C for 10 min to deactivate the phi29 DNA polymerase.

Chemiluminescence Assay. The 10 μL of RCA products was added into a mixture containing 20 μL of 5× HEPES-NH4OH buffer (125 mM HEPES, 100 mM KCl, and 1 M NaCl, pH 8.0), 5 μL of hemin (2 μM), 12.5 μL of luminol (2 mM), 10 μL of 1× TE buffer (10 mM Tris–HCl (pH 8.0), 1 mM EDTA), and 42.5 μL of H2O. The mixture was incubated at room temperature for 30 min. After adding 50 μL of H2O2 (5 mM) to the mixture, the chemiluminescence signal was recorded on a GloMax 96 Microplate Luminometer (Promega, Madison, WI, USA) with a time delay of 1.5 s and an integration time of 0.5 s.

Activation and Inhibition Assay. For the activation assay, 0.01 U/mL PNK was mixed with spermine at various concentrations. In the control group, there was neither PNK nor spermine. The activation reaction was performed at 37 °C for 30 min, followed by heating to 75 °C for 10 min to deactivate the PNK. Subsequently, spermine was adjusted with the same concentration to prevent the influence of different-concentration spermine on the chemiluminescence assay. After adding the padlock probes, the mixture was incubated at 95 °C for 5 min, followed by slowly cooling to the room temperature. The ligation reaction was performed in 10 μL of reaction solution containing 1 μL of products, 8 μL of 1× PNK buffer, and 1 μL of ligase (1 U/μL). The ligation
products were directly used for RCA reaction, and the chemiluminescence assay was performed as described before. The inhibition assay was performed with the similar procedures. In inhibition assay, the PNK concentration was 0.02 U/mL, and the ADP concentrations were 0, 1, 2, 4, 6, 8, and 10 mM, respectively. The relative activity in Figures 3A and 3B was obtained by normalization of chemiluminescence signal in the presence of ADP/spermine to that in the absence of ADP/spermine.

Real Sample Analysis. Human embryonic kidney cells (HEK293T cells) were cultured in DMEM with 10% v/v fetal bovine serum. HEK293T cells were pre-incubated in DMEM without fetal bovine serum for 2 h after seeding for 24 h. H$_2$O$_2$ was diluted with DMEM before use. The cells were treated with 0.2 mM H$_2$O$_2$ for 1 h. Then, the nucleoproteins were extracted with a cell nucleoprotein extract kit (Sangon) according to the manufacturer’s protocol. The protein concentrations were measured using the BCA protein assay kit (Novagen) according to the manufacturer’s protocol. The nucleoproteins were directly used for PNK activity assay.

Western Blotting Assay. The 15 μg of nucleoprotein extracts was separated by 12% SDS/PAGE and transferred to nitrocellulose. Subsequently, the membrane was blocked with 5% low fat milk in Tris buffered saline TBS-0.05% Tween-20, and incubated at room temperature for 2 h with either anti-tubulin monoclonal antibody (1:1000) or anti-PNK antibody (sc-271505, Santa, 1:200). After washing, the membrane was incubated with HRP-coupled anti-mouse IgG antibody (1:5000) for 1 h at room temperature, followed by washing the membrane and detecting the immune complexes according to the ECL method on an Image Station 4000MM (Kodak).
OPTIMIZATION OF EXPERIMENTAL CONDITION

Optimization of Hemin, Luminol and H$_2$O$_2$ Concentration. To investigate the influence of hemin, luminol and H$_2$O$_2$ concentrations upon the chemiluminescence signal, a synthesized DNAzyme (Table S1) was used to imitate the products of RCA reaction. Firstly, to optimize the hemin concentration, we examined the variance of $I/I_0$ value with the concentration of hemin, where $I$ and $I_0$ are the chemiluminescence intensity in the presence and in the absence of synthesized DNAzyme, respectively. As shown in Fig. S1A, the value of $I/I_0$ increases with the increase of hemin concentrations from 50 nM to 100 nM, followed by the decrease beyond the hemin concentration of 100 nM. Therefore, 100 nM of hemin was used in the subsequent research.

Secondly, the concentration of luminol was further optimized. As shown in Fig. S1B, the value of $I/I_0$ increases with the increase of luminol concentration from 0.05 mM to 0.25 mM, followed by the decrease beyond the concentration of 0.25 mM. As a result, the $I/I_0$ value reaches the maximum at the luminol concentration of 0.25 mM. Thus, we choose 0.25 mM luminol as the optimal concentration in the following research.

Thirdly, we optimized the H$_2$O$_2$ concentration. As shown in Fig. S1C, the value of $I/I_0$ increases with the increase of H$_2$O$_2$ concentration from 1 mM to 5 mM, but decreases with the H$_2$O$_2$ concentration from 5 mM to 20 mM. Therefore, 5 mM H$_2$O$_2$ was selected in the subsequent experiments.
Fig. S1 Variance of the \( I/I_0 \) value with the concentration of hemin (A), luminal (B), and \( \text{H}_2\text{O}_2 \) (C).

Error bars show the standard deviation of three experiments.

**Optimization of Substrate DNA, Ploymerase and dNTP Concentration.** To ensure the good amplification performance of the proposed method, we studied the influence of substrate DNA, polymerase and dNTP upon the RCA reaction. First, two substrate DNAs of substrate-1 and
substrate-2 with different length were optimized (Table S1). The substrate-1 contains two complete G-quadruplex sequences with seven thymines separating each other, while the substrate-2 includes only one complete G-quadruplex sequence. We investigated the chemiluminescence signal of substrate-1 and substrate-2 in the test group with 1 U/mL PNK ($I$) and in the control group without PNK ($I_0$). As shown in Fig. S2A, the $I/I_0$ value of substrate-1 group is more than twice of substrate-2 group. Therefore, the substrate-1 was chosen in the subsequent research. The amount of phi29 DNA polymerase for the RCA reaction was further optimized. As shown in Fig. S2B, the chemiluminescence intensity increases rapidly with the increase of amount of phi29 DNA polymerase from 0.1 U to 2 U, followed by increasing slowly beyond the amount of 2 U. Therefore, 2 U polymerase was used in the following experiments. Finally, the concentration of dNTP was optimized for the RCA reaction. As shown in Fig. S2C, the chemiluminescence intensity increases with the increase of dNTP concentration from 0.25 mM to 1 mM, followed by leveling off beyond the concentration of 1 mM. Thus, the optimized concentration of dNTP is 1 mM.
Fig. S2  (A) Variance of the $I/I_0$ value with the different substrates. (B) Variance of the chemiluminescence intensity with the amount of phi29 DNA polymerase. (C) Variance of the chemiluminescence intensity with the concentration of dNTP. The concentration of PNK is 1 U/mL. Error bars show the standard deviation of three experiments.

**Detection Selectivity.** To investigate the selectivity of the proposed method for PNK assay, we further measured the chemiluminescence signal in response to the nonspecific adenylate kinase
(AK) and pyruvate kinase (PK). As shown in Fig. S3, a significant chemiluminescence enhancement is observed only in the presence of PNK. In contrast, no distinct chemiluminescence signal is observed in the presence of either AK or PK, suggesting the high selectivity of the proposed method for PNK assay.

**Fig. S3** Selectivity of the proposed method for PNK assay. Each concentration of PNK, AK and PK is 1 U/mL. Error bars show the standard deviation of three experiments.