Electronic Supplementary Information

Real-Time Monitoring of Small Biological Molecules by Ligation-Mediated Polymerase Chain Reaction

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EXPERIMENTAL SECTION

Materials. All oligonucleotides (Table S1), T4 DNA ligase, and *E. coli* DNA ligase were obtained from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Exo I and Exo III were purchased from New England BioLabs (Beverly, MA, USA). TransStart Top Green qPCR SuperMix was obtained from TransGen Biotech Co., Ltd. (Beijing, China). Adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine 5′-monophosphate (AMP), uridine triphosphate (UTP), cytidine triphosphate (CTP), and guanosine triphosphate (GTP) were purchased from the Sangon Biotechnology Co., Ltd. (Shanghai, China). Nicotinamide adenine dinucleotide (NAD^+), the reduced form of nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADP) and nicotinamide adenine dinucleotide phosphate hydride (NADPH) were

obtained from Sigma-Aldrich (St. Louis, MO, USA). SYBR gold was obtained from Invitrogen (Carlsbad, CA, USA). All other reagents were of analytical grade and used without additional purification. Ultrapure water obtained from a Millipore filtration system was used throughout all experiments.

Table S1. Sequences of the Oligonucleotides[α]

note	sequence (5′-3′)
probe 1 (P1)	TTA TCA CTA ATA CAT ATC ACG GTA TAG TCA CCA AC
probe 2 (P2)	AGC GAA GTG GAT ACG AAG CGT TTC AGG CAC ATC AAT GCA CCT CAA GTT GGT GAC TAT ACC
probe 3 (P3)	PO_4-GTG ATA TGT ATT AGT GA**T AA**
reverse primer	AGC GAA GTG GAT ACG AAG CGT TTC

[α]The bold region of P3 indicates the phosphorothioate modification.

Ligation Reaction and Digestion Reaction. The 66 mM Tris-HCl (pH 7.6) buffer solution, 6.6 mM $MgCl_2$, and 10 mM dithiothreitol (DTT) were used for ligation reaction. Firstly, 10 μL of DNA probes solution (final concentration of 1 μM) was prepared by mixing equal volumes of P1, P2 and P3 solutions. Then the solution was denatured at 95°C for 5 min and cooled slowly to room temperature. For ATP detection, the ATP at various concentrations was mixed with 1 μL of 1 μM DNA probes and T4 DNA ligase (final concentration of 0.1 U/μL) in 10 μL of ligation buffer, and incubated at 37°C for 60 min. After the ligation, 10 μL of products was added to 10 μL of exonuclease mixture containing Exo I and Exo III (final concentration of 1 U/μL) and 2 μL of 10

× NEB buffer 1, and the mixture was incubated at 37°C for 30 min to digest the unligated DNA probes, followed by inactivation at 80°C for 20 min. For NAD^+ detection, a procedure similar to that used for ATP detection was performed except for the use of *E. coli* DNA ligase (final concentration of 0.2 U/μL) instead of T4 DNA ligase.

Real-Time PCR. Real-time PCR was performed on Bio-Rad CFX 96 Real-Time PCR instrument (BIO-RAD, USA). For ATP detection, 1 μL of digestion products containing DNA probes (final concentration of 2.5 nM) as the PCR templates, 1× qPCR supermix, 100 nM forward primer, 100 nM reverse primer were mixed and fixed to a final volume of 20 μL. The reactions were incubated in a 96-well plate at 94 °C for 30s, followed by 35 cycles of 94 °C for 5 s, 60 °C for 30 s. All reactions were performed in triplicate. For NAD^+ detection, a procedure similar to that used for ATP detection was performed except for using 1.5 μL of digestion products with DNA probes (final concentration of 3.75 nM) as the PCR templates The threshold cycle (C_t) was defined as the fractional cycle number at which the fluorescence passed a fixed threshold.

Gel Electrophoresis. The digestion products were analyzed by 12% nondenaturating polyacrylamide gel electrophoresis (PAGE) in 1× TBE (9 mM Tris−HCl, pH 7.9, 9 mM boric acid, 0.2 mM EDTA) at a 120 V constant voltage for 40 min at room temperature. The gel was stained by SYBR gold and analyzed by a Kodak Image station 4000MM (Carestream Health, Rochester, NY, USA).

Analysis of Complex Biological Samples. The RPMI 1640 medium (GIBCO) supplemented with 10% fetal bovine serum (FBS) (heat inactivated, GIBCO) was used as the cell media. Human serum samples were obtained from Sigma-Aldrich (St. Louis, MO, USA). These samples (1:10 dilution ratio with ligase buffer) were spiked with different-concentration ATP and analyzed by the

method described above.

Supplementary Results

Optimization of experimental conditions for ATP assay. The ATP-triggered ligation reaction may significantly influence the subsequent real-time PCR. To achieve the best performance, we optimized the incubation time of Exo I and Exo III, the concentrations of T4 DNA ligase, Exo I and Exo III, and DNA probes. In this study, we used the value change of ΔC_t ($\Delta C_t = C_{t,\ blank} - C_{t,\ ATP}$) between the experimental sample and the blank to quantify the signals. The concentration of T4 DNA ligase was evaluated using a fixed concentration of ATP (1×10^{-8} M). As shown in Fig. S1A, the value of ΔC_t increases with the increasing concentration of T4 DNA ligase, and levels off at 0.1 U/μL. Thus, 0.1 U/μL is selected as the optimal concentration of T4 DNA ligase. Exo I and Exo III can digest the unsealed DNA probes to reduce the background signal. The concentration and incubation time of Exo I and Exo III were also investigated. As shown in Fig. S1B and C, 1 U/μL Exo I and Exo III and incubation time of 30 min are the optimal conditions. The DNA template is a key factor for efficient PCR reaction. The concentration of DNA probes was evaluated as well. As shown in Fig. S1D, the optimal concentration of DNA probes is 2.5 nM.

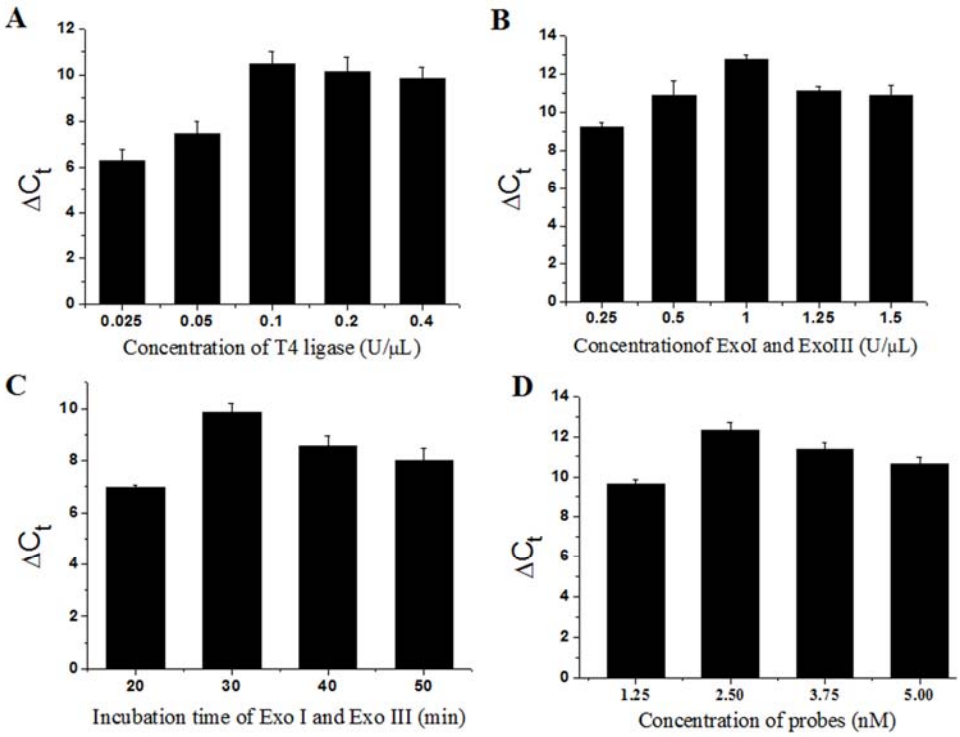

Fig. S1 Effect of the concentrations of T4 DNA ligase (A), Exo I and Exo III (B), the incubation time of Exo I and Exo III (C), the concentration of DNA probes (D) upon the ΔC_t value. The concentration of ATP is 1×10^{-8} M. Error bars show the standard deviation of three experiments.

Detection of ATP in Complex Samples. To evaluate the robustness of the proposed method, we measured ATP in cell media and human serum. We spiked the test samples (1:10 dilution ratio with ligase buffer) with different-concentration ATP and performed the measurement in one 96-well PCR plate. In the logarithmic scales, the ΔC_t value exhibits a linear correlation with the concentration of ATP over a large dynamic range from 1×10^{-13} M to 1×10^{-8} M.. The regression equation is $\Delta C_t = 1.9051 \log_{10} C + 26.7526$ with a correlation coefficient of 0.9900 in cell media (Fig. S2A), and $\Delta C_t = 1.9629 \log_{10} C + 27.6372$ with a correlation coefficient of 0.9987 in human serum (Fig. S2B), where ΔC_t is the threshold cycle change value ($\Delta C_t = C_{t,\ blank} - C_{t,\ ATP}$) and C is the concentration of ATP (molar), respectively. The detection limit is calculated to be 2.99×10^{-14} M

in cell media and 2.14×10^{-14} M in human serum (3σ/slope), with no much difference from that in pure buffer.

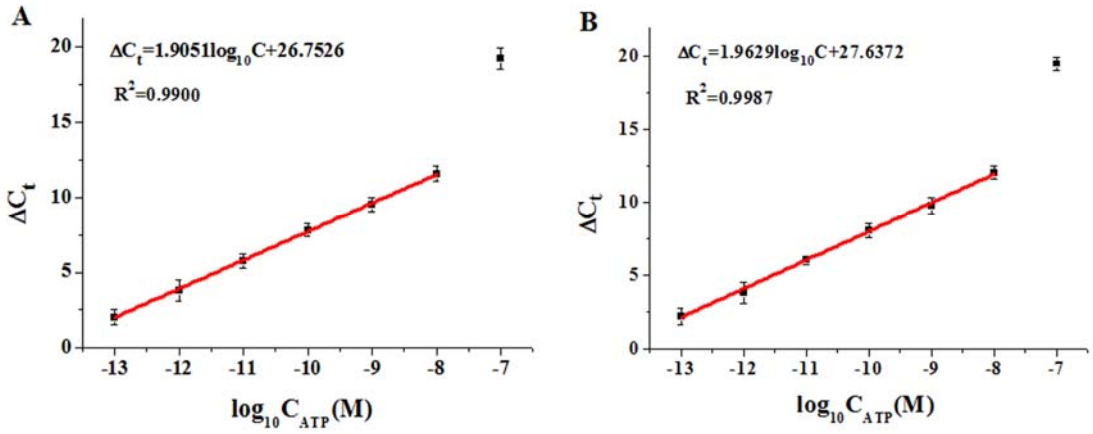

Fig. S2 Detection of ATP in complex biological samples including (A) cell media and (B) human serum. Error bars show the standard deviation of three experiments.

Detection of NAD$^+$. To demonstrate the generality of the proposed method, we further measured NAD^+ using *E. coli* DNA ligase instead of T4 DNA ligase. To achieve the best performance, we optimized the experimental conditions as follows: 0.2 U/μL *E. coli* DNA ligase, 1 U/μL Exo I and Exo III, 30 min digestion time, 3.75 nM DNA probes (Fig. S3). *E. coli* DNA ligase together with the cofactor NAD^+ can catalyze the ligation of P2 and P3 to form a long DNA strand which is resistant to Exo III digestion due to the protection of phosphorothioate bases near the 3′-end of P3. The sealed DNA strands may be subsequently amplified by real-time PCR and generate an enhanced fluorescence signal. As shown in Fig. S4, the C_t value gradually increases with the decrease of NAD^+ concentration from 1×10^{-7} M to 1×10^{-13} M.

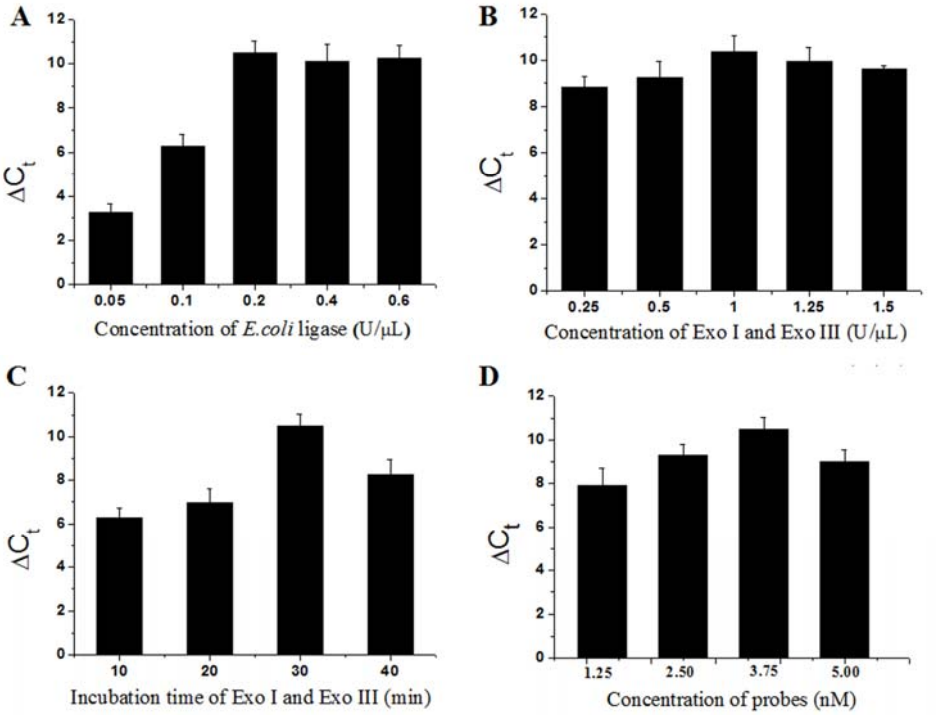

Fig. S3 Effect of the concentrations of *E. coli* DNA ligase (A), Exo I and Exo III (B), the incubation time of Exo I and Exo III (C), and the concentration of DNA probes (D) upon the ΔC_t value. The concentration of NAD^+ is 1×10^{-7} M. Error bars show the standard deviation of three experiments.

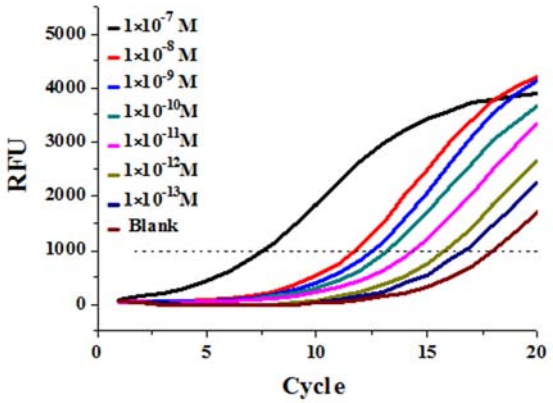

Fig. S4 Real-time fluorescence curves in response to different NAD^+ concentration.