Electronic Supplementary Information

Sensitive detection of alkaline phosphatase by dephosphorylation-initiated transcription reaction-mediated dual signal amplification

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EXPERIMENTAL SECTION

Chemicals and Materials. All oligonucleotides (Table S1) are synthesized by Sangong Biotechnology (Shanghai, China). Alkaline phosphatase (ALP), 10× CutSmart buffer (500 mM KAc, 200 mM Tris-Ac, 100 mM $Mg(Ac)_2$, 1000 μg/ml BSA, pH 7.9), protein phosphatase (PP), uracil DNA glycosylase (UDG), CpG methyltransfease (M. SssI), lambda exonuclease (λ exo), 10× λ exo buffer (670 mM Glycine-KOH, 25 mM $MgCl_2$, 50 μg/mL BSA, pH 9.4), RNase inhibitor and streptavidin (SA) are obtained from New England BioLabs (Beverly, MA, USA).

The T7 RiboMAX express large scale RNA production system was obtained from Promega (Madison, WI, USA). Duplex-specific nuclease (DSN) and 10× DSN master buffer (500 mM Tris-HCl, 50 mM $MgCl_2$ and 10 mM DTT, pH 8.0) are obtained from Evrogen Joint Stock Company (Moscow, Russia). Diethylpyrocarbonate (DEPC) treated water (RNase free) are obtained from TaKaRa Bio. Inc. (Dalian, China). Sodium orthovanadate (Na_3VO_4), bovine serum albumin (BSA), catalase, and glucose oxidase (GO) are purchased from Sigma-Aldrich (St. Louis, Missouri, USA). SYBR Gold is purchased from Invitrogen Corporation (California, CA, USA).

Table S1. Sequence of synthesized oligonucleotides [a]

note	sequence (5'-3')
T7 promoter	P-TAA TAC GAC TCA CTA TAG GG
template	TAA CAC TGT CTG GTA AAG ATG GCC CTA TAG TGA GTC GTA TTA
signal probe	FAM-TAA CAC TGT CTG GTA AAG ATG G-Eclipse

[a] The letter P in T7 promoter indicates the PO_4 modification at the 5' end.

Preparation of Stock Solutions. All oligonucleotides were diluted with 1× Tris-EDTA buffer to prepare the stock solutions. The T7 promoter/template duplex were prepared by incubating 1 μM T7 promoter strand with 1 μM template strand in annealing buffer (5 mM Tris−HCl, 50 mM NaCl , pH 8.0) at 95 °C for 5 min, followed by slowly cooling to room temperature. The obtained dsDNA substrates were stored at -20 °C for further use.

Fluorescent Detection of ALP. Dephosphorylation was performed in 20 μL of reaction mixture

containing different-concentration ALP, 100 nM dsDNA substrates (T7 promoter/template duplex), 1× CutSmart buffer (50 mM KAc, 20 mM Tris-Ac, 10 mM $Mg(Ac)_2$, 100 μg/mL BSA, pH 7.9), and incubated at 37 °C for 30 min, followed by inactivation at 65 ℃ for 5 min. Then, 1 U of λ exo was added to the mixture and incubated for another 30 min at 37 °C, followed by heating at 90 °C for 5 min to inactive λ exo. The transcription reaction was carried out in 30 μL of solution containing 6 μL of λ exo digestion products at 37 °C for 60 min in T7 RiboMAX express large scale RNA production system. Then 5 μL of DNA transcription products were incubated with a solution containing 1× DSN master buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, and 1 mM DTT, pH 8.0), 0.1 U of DSN, 20 U of RNase inhibitor, and 600 nM Taqman probe in a final volume of 30 μL at 55℃ for 40 min, and then the sample was subjected to fluorescence measurement. The fluorescence spectra were measured at room temperature using an F-7000 fluorescence spectrophotometer (Hitachi, Japan) equipped with a xenon lamp as the excitation source. The spectra were recorded in the range from 500 to 650 nm at an excitation wavelength of 490 nm. The excitation and emission slits were set for 5.0 and 5.0 nm, respectively. The fluorescence intensity at 520 nm was used for data analysis.

Gel Electrophoresis. The reaction products were analyzed by a 12% nondenaturing polyacrylamide gel (PAGE) in 1× TBE buffer (9 mM Tris-HCl, 9 mM boric acid, 0.2 mM EDTA, pH 7.9) at a 110 V constant voltage for 50 min at room temperature. After being stained by SYBR Gold, the gel was imaged by a Bio-Rad ChemiDoc MP Imaging System (Hercules, CA).

Inhibition Assay. To evaluate the effect of ALP inhibitor, various-concentration Na_3VO_4 was incubated with the mixture of 5 U/L ALP, 100 nM dsDNA substrates, 1× CutSmart buffer (50 mM KAc, 20 mM Tris-Ac, 10 mM $Mg(Ac)_2$, 100 μg/mL BSA, pH 7.9) at 37 °C for 30 min, followed

by inactivation at 65 ℃ for 5 min, the ALP activity was measured by the proposed method. The relative activity (RA) of ALP was measured according to:

$$\text{RA}\ (\%) = \frac{F_\text{i} - F_0}{F_\text{t} - F_0} \times 100\%$$

where F_0 is the fluorescence intensity in the absence of ALP, F_t is the fluorescence intensity in the presence of 5 U/L ALP, and F_i is the fluorescence intensity in the presence of 5 U/L ALP and Na_3VO_4. The IC_{50} value was calculated from the curve of RA versus the Na_3VO_4 concentration.

Kinetic Analysis. To evaluate the enzyme kinetic parameters of ALP, we measured the initial velocity in the presence of 5 U/L ALP and different-concentration DNA substrate in 5 min reaction at 37 °C. The kinetic parameter is fitted to the Michaelis−Menten equation:

$$V = \frac{V_\text{max}[S]}{K_\text{m} + [S]}$$

where V_max is the maximum initial velocity, and $[S]$ is the concentration of T7 promoter substrate, and K_m is the Michaelis−Menten constant.

Recovery Assay. A total volume of 20 μL of reaction mixture containing 1% fetal bovine serum (FBS) spiked with varying-concentration ALP, 100 nM dsDNA substrates, 1× CutSmart buffer (50 mM KAc, 20 mM Tris-Ac, 10 mM $Mg(Ac)_2$, 100 μg/mL BSA, pH 7.9), and incubated at 37 °C for 30 min, followed by inactivation at 65℃ for 5 min. The subsequent reactions and measurement followed the above procedures.

Cell Culture and Preparation of Cell Extracts. Human cervical carcinoma cell line (HeLa cells) and human embryonic kidney 293 cell line (HEK cells) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, USA) and 1% penicillin-streptomycin in 5% CO_2 incubator at 37°C. The number of cells was measured by Countstar cell counter. The cells were collected with trypsinization, washed

twice with ice-cold PBS (Gibco, USA, pH 7.4), and centrifuged at 1000 rpm for 5 min. Then the cells were suspended in 100 μL of lysis buffer (10 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.25 mM sodium deoxycholate, 1.0% glycerol, and 0.1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, pH 8.0), and incubated on ice for 30 min, followed by centrifugation at 12000 rpm for 20 min at 4°C. The supernatant was transferred into a fresh tube and stored at −80°C.

Optimization of Experimental Conditions. To achieve the best assay performance, various experimental conditions including ALP incubation time, the amount of λ exo, the amount of DSN, DSN reaction temperature and DSN incubation time are optimized. The ratio of F/F_0 was used for assessing the assay performance, where F is the fluorescence intensity in the presence of 0.5 U/L ALP, and F_0 is the fluorescence intensity in the absence of ALP.

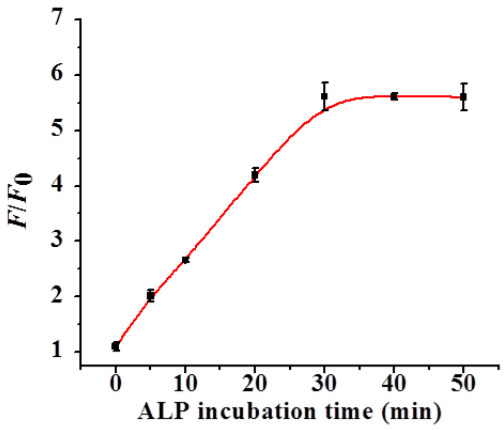

Fig. S1 Variance of the F/F_0 value with the ALP incubation time. Error bars show the standard deviation of three experiments.

We investigated the effect of ALP incubation time upon the assay performance. After incubation with 0.5 U/L ALP from 0 to 50 min, the samples were measured by the proposed method. As shown in Fig. S1, the F/F_0 value enhances with the incubation time from 0 to 30 min,

and levels off beyond 30 min. This can be explained by either the complete loss of ALP activity after 30 min incubation or the consumption of all available phosphorylated T7 promoter substrates. Therefore, the 30 min is used as the optimized ALP incubation time in the following experiments.

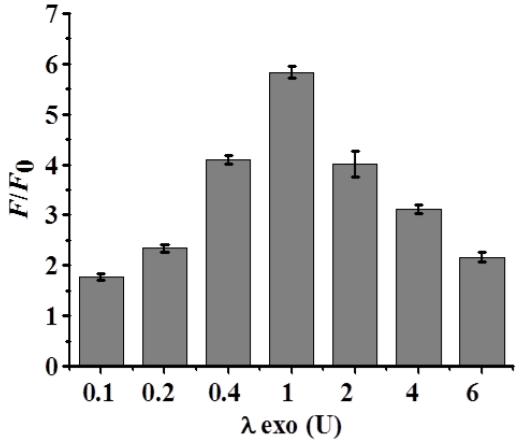

Fig. S2 Variance of the F/F_0 value with the amount of λ exo. Error bars show the standard deviation of three experiments.

The protection of dephosphorylated T7 promoter substrates from λ exo digestion is a key step in the proposed assay. Therefore, the amount of λ exo should be optimized. As shown in Fig. S2, the F/F_0 value enhances with the increasing concentration of λ exo from 0.1 to 1 U, followed by the decrease beyond the concentration of 1 U. This may be explained by that although 5'-phosphorylated dsDNA is the preferred substrate of λ exo, it can also degrade the non-phosphorylated substrates at a greatly reduced rate.[1] Therefore, 1 U of λ exo is used in the subsequent researches.

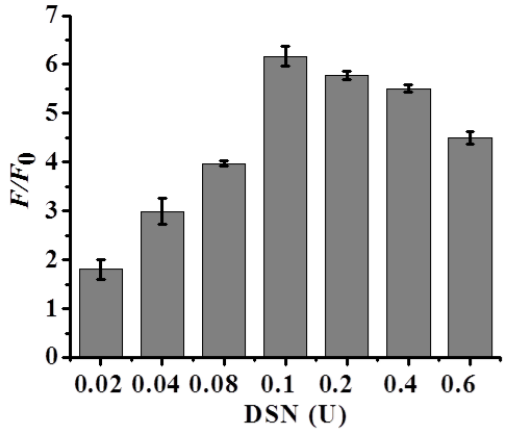

Fig. S3 Variance of the F/F_0 value with the the amount of DSN. Error bars show the standard deviation of three experiments.

In the proposed assay, DSN-assisted signal amplification is employed for quantitative detection of ALP-induced RNA transcription products. Larger amount of DSN can cleave more signal probes to generate high fluorescence signal, but excess DSN may induce the increase of background signal due to non-specific cleavage of single-stranded Taqman probe.[2] As shown in Fig. S3, the F/F_0 value enhances with the increase of DSN amount from 0.02 to 0.1 U, and slightly decreases when DNS amount is further increased. Therefore, 0.1 U of DSN is used in the subsequent researches.

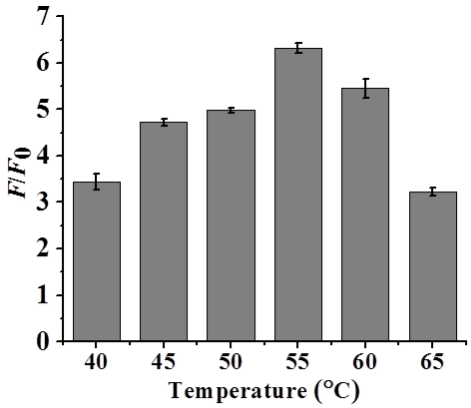

Fig. S4 Variance of the F/F_0 value with DSN reaction temperature. Error bars show the standard deviation of three experiments.

It is reported that higher temperature can facilitate faster hybridization and subsequent dissociation of RNA in the DNS-mediated cleavage reaction, but it may also weaken the enzyme activity of DSN. We further optimize the temperature of DSN reaction. As shown in Fig. S4, the F/F_0 value enhances with the increase of reaction temperature from 40 to 55 ℃, followed by the decrease beyond 55 ℃. This sharp decrease may be explained by that the double-strand RNA/DNA substrate required for DSN reaction is denaturated when the reaction temperature is higher than 55 ℃.[3] Therefore, the optimal temperature of DSN reaction is set as 55 °C in the following experiments.

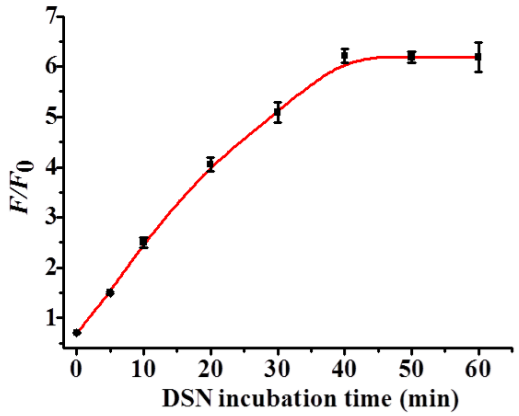

Fig. S5 Variance of the F/F_0 value with DSN incubation time. Error bars show the standard deviation of three experiments.

Finally, the effect of DSN incubation time upon assay performance is investigated. As shown in Fig. S5, the F/F_0 value enhances with the increase of reaction temperature from 0 to 40 min, and reaches a plateau beyond 40 min. This may be ascribed to either the complete loss of DSN activity after 40 min incubation or the consumption of all available RNA transcripts. Therefore, 40 min is used as the optimal DSN incubation time in the subsequent researches.

Table S2. Comparison of the proposed method with the reported ALP assays

strategy	signal model	assay time*	live cell assay	linear range (U/L)	LOD (U/L)	ref.
target-induced dual signal amplification	fluorescence	~3 h	yes	0.05 – 1	0.02	this work
excimer/monomer conversion	fluorescence	over 4 days	no	0 –100	0.1	4
DNA-scaffolded silver nanocluster-based assay	fluorescence	~ 3 h	no	30 – 240	5	5
graphene quantum dots-based assay	fluorescence	~ 1 h	no	1 – 90	0.45	6
target-responsive NIR probe	fluorescence	over 1 day	yes	10 – 2000	3	7
carbon quantum dot-based assay	fluorescence	over 3 days	no	4.6 – 383.3	1.4	8
excited-SIPT and aggregation-induced emission	fluorescence	~ 1 h	yes	0 – 150	0.15	9
nucleic acid-controlled quantum dots aggregation	fluorescence	~5 h	no	0 – 3	0.1	10
gold nanocluster-based assay	fluorescence	~ 12 h	no	1 – 200	0.05	11

copper nanoparticle-based assay	fluorescence	~ 2 h	no	0.3 – 7.5	0.035	12
exonuclease-mediated signal amplification	electrochemistry	~ 20 h	no	1,000 – 20,000	100	13
hairpin probe-based assay	electrochemistry	~ 9 h	no	0.1 – 10	0.1	14
target-induced gold nanoparticle aggregation	electrochemistry	~ 1 h	no	32 – 100	32	15
gold/silver core/shell nanorod-based assay	electrochemistry	~ 4 h	no	5 – 100	3.3	16
target-induced aggregation of Ag coated AuNPs	SERS	~2 h	no	0.5 – 10	0.1	17

[a]LOD: limit of detection (U/L). Ref: references, ICP: infinite coordination polymer, SIPT: state intramolecular proton transfer, NIR: near-infrared, AuNP: gold nanoparticles, SERS: surface-enhanced Raman scattering.

*Assay time includes the preparation time.

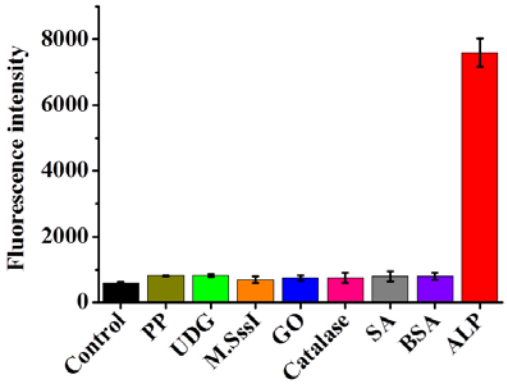

Fig. S6 Measurement of fluorescence intensity in responses to 5 U/L PP, 5 U/L UDG, 5 U/L M.SssI, 10 mg/L GO, 10 mg/L catalase, 10 mg/L SA, 10 mg/L BSA, and 5 U/L ALP, respectively. Sample without any treatment was used as the control. Error bars show the standard deviation of three experiments.

Table S3. Recovery of ALP spiked in fetal bovine serum (1%) samples

added (U/L)	measured (U/L)	recovery (%)	RSD (%)
1	1.01	101	2.75
2	1.98	99	1.54
5	5.02	100.4	1.33

Assay Specificity. To investigate the specifictity of the proposed method, protein phosphatase (PP), uracil DNA glycosylase (UDG, 5 U/L), CpG methyltransfease (M. SssI, 5 U/L), glucose oxidase (GO, 10 mg/L), catalase (10 mg/L), streptavidin (SA, 10 mg/L), and bovine serum albumin (BSA, 10 mg/L) were used as the interferences. PP catalyzes the release of phosphate groups from phosphorylated serine, threonine and tyrosine residues in proteins.[18] UDG is a DNA

glycosylase responsible for excising uracil base form the damaged DNA.[19] M. SssI is a DNA methyltransfease from *Spiropiasma sp. strain MQ1* that specifically methylates all cytosine residues (C^5) within CpG sequences.[20] Catalas catalyzes the decomposition of hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2).[21] SA and BSA are two commly used irrelevant proteins. Theoretically, none of these samples can remove 5' phosphate groups of the phosphated T7 promoter strand to protect T7 promoter from digestion by λ exo, thus no transcription reaction occurs and no fluoresce signal can be detected. As shown in Fig. S6, a high fluorescence signal can only be obeserved in the presence of target ALP (Fig. S6, red column). In contrast, extremly low flurescence signals are detected in response to PP (Fig. S6, brown column), UDG (Fig. S6, green column), M. SssI (Fig. S6, orange column), GO (Fig. S6, blue column), catalase (Fig. S6, pink column), SA (Fig. S6, gray column), and BSA (Fig. S6, purple column), with no obvious changes compared to that obtained from the control group without any treatment (Fig. S6, black column), indicating these samples cannot interfere the detection of ALP. To further investigate the assay performance in real sample analysis, we performed recovery studies by adding varying-concentration ALP (1, 2, and 5 U/L) to the ALP-free fetal bovine serum samples.[9] As shown in Table S3, a quantitative recovery rate ranging from 99% to 101% is obtained, demonstrating that this assay is not affected by the complex biological matrix. Our assay is suprior to the state intramolecular proton transfer (ESIPT)-based assay,[9] in which the fluorescence signal produced by detection probe is strongly interfered by FBS. These results demonstrate the high accuracy and good selectivity of the proposed method.

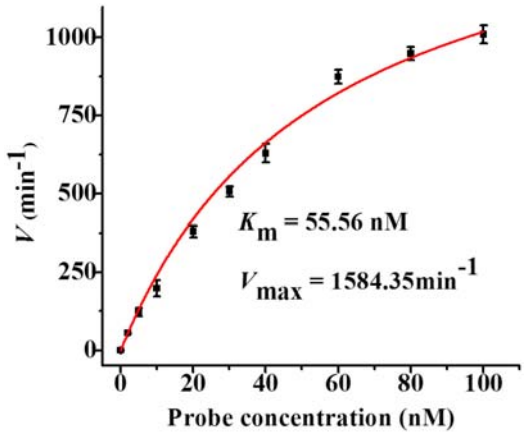

Fig. S7 Analysis of Michaelis−Menten kinetic parameters by the initial-rate method. The ALP concentration is 5 U/L. The time for ALP incubation is 5 min. Error bars show the standard deviation of three experiments.

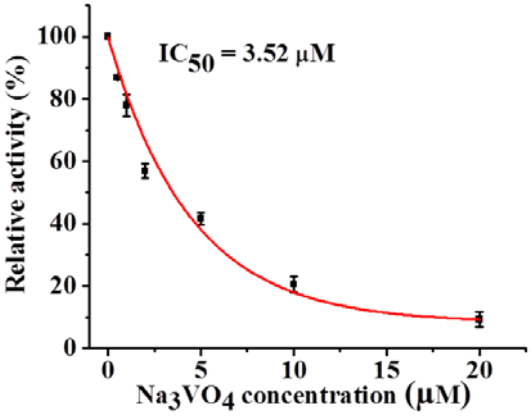

Fig. S8 Variance of the reactive activity in response to the different-concentration NA_3VO_4. The ALP concentration is 5 U/L. Error bars show the standard deviation of three experiments.

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