Electronic Supplementary Information

Dephosphorylation-directed tricyclic DNA amplification cascades for sensitive detection of protein tyrosine phosphatase

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MATERIALS AND METHODS

Materials. All the oligonucleotides (Table S1) were synthesized by Sangon Biotech Co. Ltd. (Shanghai, China). DNA-peptide conjugate was synthesized by Takara Biotechnology Co. Ltd. (Dalian, China). Peptide substrate (biotin-KGDGVpYAAC) was synthesized by Chinese Peptide Company (Hangzhou, China). Recombinant human PTP1B was purchased from SinoBiological (Beijing, China). Vent (exo-) DNA polymerase, Nb.BbvCI, 10× Cutsmart buffer (200 mM tris-acetate, 500 mM potassium acetate, 100 mM magnesium acetate, 1 mg/mL BSA, pH 7.9), deoxynucleotide solution mixture (dNTPs), protein phosphatase 2A (PP2A) and polynucleotide kinase (PNK) were bought from New England Biolabs (Beverly, MA, USA). Streptavidin-coated

magnetic beads (Dynabeads® M-280 Streptavidin, Dynal) were obtained from Invitrogen Corporation (California, CA, USA). Sodium pyruvate ($CH_3COCOONa$), sodium orthovanadate (Na_3VO_4), (R)-3-hexadecanoyl-5-hydroxymethyltetronic acid (RK-682), 1.4-dithiothreitol (DTT), sodium bicarbonate ($NaHCO_3$), bovine serum albumin (BSA), α-chymotrypsin (CMT) and immunoglobulin G (IgG) were obtained from Sigma-Aldrich Company (St. Louis, MO, USA). Ethylenediaminetetraacetic acid (EDTA), 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid sodium salt (HEPES, 1 M) and 1× PBS (pH 7.4) were purchased from Solarbio Bioscience & Technology Co. Ltd. (Beijing, China). Human embryonic kidney cell line (HEK-293 cells), cervical carcinoma cell line (HeLa cells), breast cancer cell line (MCF-7 cells) and human hepatoma cancer cell line (HepG2 cells) were obtained from Cell Bank, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China).

Table S1. Sequences of oligonucleotides [a]

note	sequences (5'-3')
peptide-DNA substrate	biotin-(COOH)-Lys-Gly-Asp-Gly-Val-pTyr-Ala-Ala-Cys-(NH_2)-GGA
	TCG TCA TGT ACG GCA GCT
DNA template	*CAT GAG ATT CGT TGT AGC TAG CCT CAA C*CC TCA GC*C ATG*
	AGA TTC GTT GTA GCT AGC CTC AAC CCT CAG CTG CCG TAC
	ATG ACG ATC C
signal probe	CAT GAG A(Cy3)TrA rUTC AAC CC(BHQ2)T C
68-nt polymerization product	TGA GGG TTG AGG CTA GCT ACA ACG AAT CTC ATG GCT GAG
	GGT TGA GGC TAG CTA CAA CGA ATC TCA TG
33-nt amplification product	TGA GGG TTG AGG CTA GCT ACA ACG AAT CTC ATG
9-nt cleavage product	CAT GAG ATrA

[a] In the DNA template, the underlined regions indicate the recognition sequences of Nb.BbvCI, and the italic regions indicate the complementary sequences of DNAzyme. In the signal probe, the underlined bases "T" indicate the modification of a fluorescein (Cy3) and a black hole quencher 2 (BHQ2), respectively, and the bases rA and rU indicate adenosine and uracil ribonucleotides, respectively. In the 9-nt cleavage product, the base rA indicates adenosine ribonucleotide.

Conjugation of peptide-DNA substrates with the streptavidin-coated magnetic beads. The coupling of peptide-DNA substrates to the magnetic beads (MBs) was carried out according to the following protocols of Invitrogen Corporation. First, 200 μL of streptavidin-coated MBs solution (10 mg/mL) was added to the centrifuge tube and washed twice with 1× PBS buffer (pH 7.4).

After magnetic separation, the supernatants were removed, and then the MBs were resuspended to 200 μL. Second, 128 μL of resultant MBs solution was mixed with 2 μL of peptide-DNA substrates (100 μM) and 20 μL of 1× PBS buffer (pH 7.4), followed by incubation for 30 min at room temperature. To remove the unconjugated substrates, the mixture was further magnetically separated, followed by washing five times with 1× PBS buffer (pH 7.4). Third, the mixture was resuspended to the total volume of 80 μL with 1× PBS buffer (pH 7.4), and the final concentration of MB-conjugated substrates was determined to be 2.5 μM.

PTP1B-directed tyrosine dephosphorylation and successive cleavage of peptide-DNA substrates. The 12 μL of MB-conjugated substrates was added into the dephosphorylation reaction system (50 μL) containing various-concentration PTP1B and 1× PTP1B reaction buffer (10 mM HEPES, 1 mM DTT, 2 mM EDTA), incubated at 37 °C for 2 h. After dephosphorylation, the reaction was terminated at 80 °C for 20 min. Then 0.6 μL of chymotrypsin (1 mg/mL) was added into the cleavage reaction system (60 μL), followed by incubation at 37 °C for 2 h. After the cleavage reaction, the magnetic separation was performed for 3 min, and then the supernatant solution was collected.

Dephosphorylation-directed DNAzyme-based tricyclic DNA amplification. After dephosphorylation and cleavage reactions, 30 μL of supernatant solution was added into the hybridization reaction system containing 0.7 μL of 5 μM templates, 3.5 μL of 10× annealing buffer (50 mM $MgCl_2$, 100 mM Tris-HCl) and 0.8 μL of H_2O, heated at 95 °C for 5 min, followed by slowly cooling to room temperature to form the double-stranded DNA (dsDNA). After

annealing reaction, 35 μL of annealing products were added into the exponential amplification system (50 μL) containing 400 μM dNTPs, 8 U/mL Vent (exo-) DNA polymerase, 0.3 U/μL Nb. BbvCI, 5 μL of 10× Cutsmart buffer, followed by incubation at 55 °C for 1 h. After exponential amplification reaction, 50 μL of reaction products was mixed with 2.4 μL of 10 μM signal probe, 1 μL of 10× Cutsmart buffer and 6.6 μL of H_2O, and incubated at 37 °C for 1 h to carry out the recycling cleavage of signal probes.

Gel electrophoresis and fluorescence measurement. The products of chymotrypsin-catalyzed cleavage reaction were analyzed by 14% nondenaturating polyacrylamide gel electrophoresis (PAGE) in 1× TBE buffer (9 mM Tris-HCl, 9 mM boric acid, 0.2 mM EDTA, pH 8.3) at a 100 V constant voltage for 90 min. The products of tricyclic DNA amplification reaction were analyzed by 12% nondenaturating PAGE in 1× TBE buffer at a 110 V constant voltage for 45 min. The gels were stained by SYBR gold and imaged by ChemiDoc MP imaging system (Hercules, CA, USA). For fluorescence measurement, 60 μL of amplification products were diluted to a final volume of 80 μL with ultrapure water, and then subjected to the fluorescence measurement. The fluorescence intensity at the emission wavelength of 569 nm was detected with a Hitachi F-7000 spectrometer (Tokyo, Japan) at an excitation wavelength of 512 nm, and the emission spectra were recorded over a wavelength range of 550 – 750 nm with a slit width of 5 nm for both excitation and emission. The value $F_t - F_o$ is used to calculate the concentration of PTP1B based on the obtained regression equation, where F_o and F_t are the fluorescence intensity at 569 nm in the absence and presence of PTP1B, respectively.

Inhibition assay. After the preincubation of PTP1B (4 nM) with varying concentration of RK-682 or Na_3VO_4 at 37 °C for 10 min, the follow experiment was carried out according to the procedures of PTP1B assay described above. The relative activity (*RA*) of PTP1B was calculated according to the equation 1.

$$RA\ (\%) = (F_i - F_o) / (F_t - F_o) \times 100\% \qquad (1)$$

where F_o, F_t and F_i are the fluorescence intensity in the absence of PTP1B, in the presence of PTP1B, and in the presence of both PTP1B and the inhibitor, respectively. The IC_{50} value of inhibitor was obtained from the curve-fitting equation.

Cell culture and preparation of cellular extracts. Human embryonic kidney cell line (HEK-293 cells), cervical carcinoma cell line (HeLa cells) and breast cancer cell line (MCF-7 cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. Human hepatoma cancer cell line (HepG2 cells) was cultured in Minimum Eagle's Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, 0.11 g/L sodium pyruvate ($CH_3COCOONa$) and 1.5 g/L $NaHCO_3$. All cells were cultured at 37 ºC in a humidified atmosphere containing 5% CO_2. For preparing cellular extracts, the cells were collected with trypsinization, washed twice with 1× PBS (pH 7.4), and then centrifuged at 800 rpm for 5 min. The number of cells was measured by Countstar automated cell counter (IC1000, Wilmington, DE, USA). About 1×10^6 cells were suspended in 100 μL of RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS, pH 7.4), and incubated on ice for 10 min, followed by sonication. The resultant lysates were centrifuged at 14000 g for 5 min at 4 ºC, and the obtained

supernatants were analyzed by measuring the absorbance at 280 nm with a Nanodrop 2000C spectrophotometer (Thermo Scientific, Wilmington, DE, USA) to quantify the total protein concentration.

Optimization of reaction conditions of PTP1B-catalyzed dephosphorylation and cleavage of DNA-peptide substrates. The ratio of F_t/F_o is used to assess the assay performance, where F_t is the fluorescence intensity in the presence of 4 nM PTP1B and F_o is the fluorescence intensity in the absence of PTP1B. As shown in Fig. S1A, we optimized the PTP1B reaction time. The F_t/F_o value enhances with the incubation time from 0.5 to 2.5 h, and levels off beyond 2 h. This can be explained by either the complete loss of PTP1B activity after 2 h incubation or the consumption of all available substrates. Therefore, 2 h is used as the optimal PTP1B reaction time in the following experiments.

To ensure the high sensitivity of the proposed method, large amounts of chymotrypsin are added into the reaction system to completely cleave the dephosphorylated peptide-DNA substrates. As shown in the Fig. S1B, the F_t/F_o value enhances with the increasing concentration of chymotrypsin from 2 μg/mL to 10 μg/mL, followed by decreasing beyond 10 μg/mL. Thus, 10 μg/mL is selected as the optimal chymotrypsin concentration. In addition, we optimized the reaction time of chymotrypsin cleavage. As shown in the Fig. S1C, the F_t/F_o value increases with the chymotrypsin cleavage time from 0.5 to 2.5 h, and reaches a plateau beyond 2 h. Therefore, 2 h is used as the optimal reaction time of chymotrypsin in the following research.

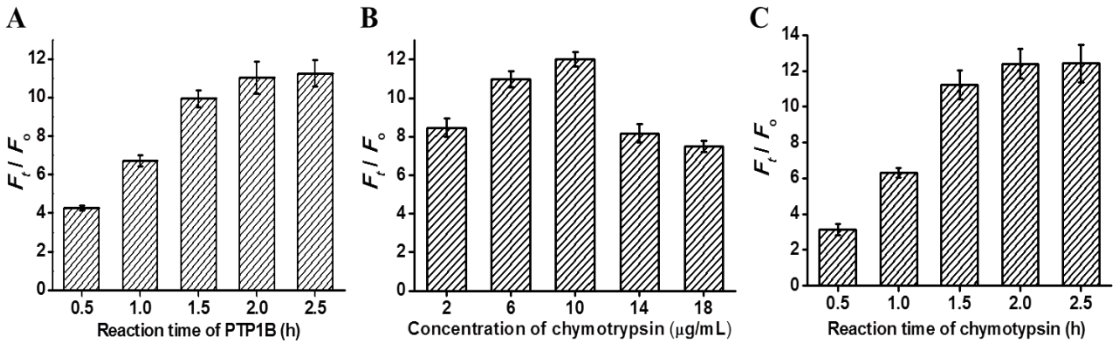

Fig. S1 (A) Variance of the F_t/F_o value with the PTP1B reaction time. (B) Variance of the F_t/F_o value with different concentrations of chymotrypsin. (C) Variance of the F_t/F_o value with the chymotrypsin reaction time. The concentration of PTP1B is 4 nM. Error bars show the standard deviation of three independent experiments.

Optimization of reaction conditions of the exponential strand displacement amplification. To obtain the high amplification efficiency, the experimental conditions including the concentration of DNA template, the amount of Vent (exo-) DNA polymerase, the amount of Nb.BbvCI, the concentration of dNTPs, and the reaction temperature of SDA reaction were experimentally optimized. We investigated the effect of DNA template concentration upon the amplification efficiency of SDA. As shown in Fig. S2A, the F_t/F_o value increases with the increasing concentration of DNA template from 80 to 100 nM, followed by decreasing beyond the concentration of 100 nM. These results can be explained by the dependence of SDA amplification efficiency upon the hybridization of DNA template with the DNA primer. On one hand, high-concentration templates may result in the high hybridization efficiency for the generation of more DNAzymes, but the released DNAzymes can further hybridize with the free DNA templates, leading to the decrease of available templates bound to DNA primers. On the other hand, low-concentration DNA templates may cause the incomplete hybridization of templates with DNA

primers, adversely affecting the amplification efficiency and resulting in the decrease of DNAzymes. Therefore, 100 nM DNA template is used in the subsequent research. The close cooperation of DNA polymerase and nicking enzyme is crucial for efficient nucleic acid amplification.[1] The concentration of Vent (exo-) DNA polymerase influences the efficiency of SDA. As shown in Fig. S2B, the F_t/F_o value enhances with the polymerase amount from 2 to 8 U/mL, and slightly decreases above 8 U/mL. Therefore, 8 U/mL of Vent (exo-) DNA polymerase is used in the subsequent researches. We further investigated the influence of Nb.BbvCI amount upon the F_t/F_o value at a fixed amount of Vent (exo-) DNA polymerase (8 U/mL) (Fig. S2C). The F_t/F_o value improves with the increasing amount of Nb.BbvCI from 0.12 to 0.3 U/μL, followed by level-off beyond 0.3 U/μL. Therefore, 0.3 U/μL Nb.BbvCI is used in the subsequent experiments. We investigated the influence of dNTPs concentration upon the F_t/F_o value as well (Fig. S2D). The F_t/F_o value improves with the increasing concentration of dNTPs from 100 to 400 μM, followed by level-off beyond 400 μM. Therefore, 400 μM dNTPs is used in the subsequent experiments. Moreover, taking into account the reaction temperature requirements of both enzymes and hybridization,[2] we investigated the effect of reaction temperature upon the F_t/F_o value. The F_t/F_o value enhances significantly when the reaction temperature increases from 37 °C to 55 °C, and reduces markedly beyond 55 °C (Fig. S2E). The decrease of F_t/F_o value may be attributed to the denaturation of duplex helixes at high temperature. Therefore, 55 °C is used as the appropriate reaction temperature in the following experiments.

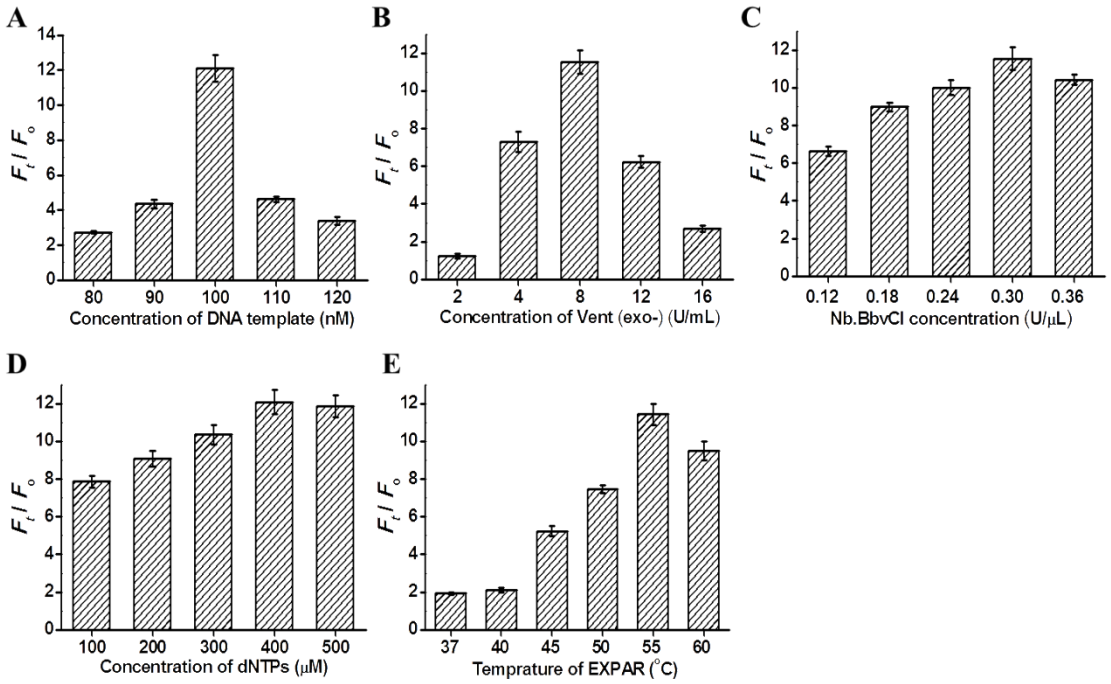

Fig. S2 (A) Variance of the F_t/F_o value with different concentrations of DNA template. (B) Variance of the F_t/F_o value with different amounts of Vent (exo-) DNA polymerase. (C) Variance of the F_t/F_o value with different amounts of Nb.BbvCI. (D) Variance of the F_t/F_o value with different concentrations of dNTPs. (E) Variance of the F_t/F_o value with different temperature. The concentration of PTP1B is 4 nM. Error bars show the standard deviation of three independent experiments.

Optimization of reaction conditions of the recycling DNAzyme cleavage. We investigated the effect of signal probe concentration upon the assay performance. As shown in Fig. S3A, the F_t/F_o value enhances gradually when the signal probe concentration increases from 100 to 500 nM, and reaches the highest value at 400 nM. Thus, 400 nM signal probe is used in the subsequent experiments. Mg^{2+} is a cofactor of 10-23 DNAzyme, and has significant effect upon the 10-23 DNAzyme-mediated recycling cleavage of signal probes.[3] As shown in Fig. S3B, the maximum F_t/F_o value is obtained when 13 mM Mg^{2+} is added to the reaction system. Therefore, 13 mM

Mg^{2+} is used in the subsequent researches.

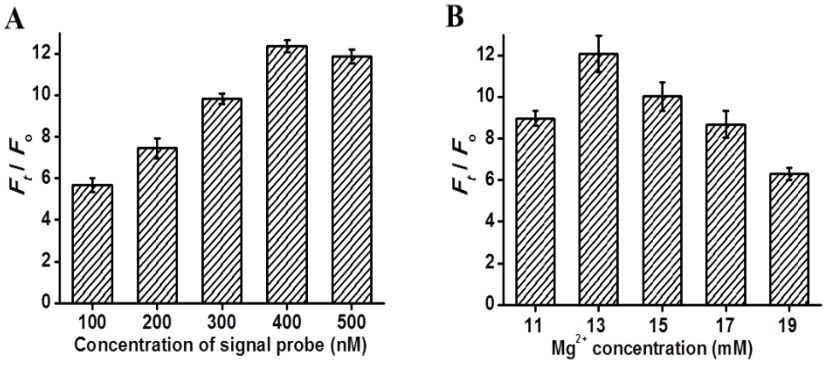

Fig. S3 (A) Variance of the F_t/F_o value with different concentrations of signal probe. (B) Variance of the F_t/F_o value with different concentrations of Mg^{2+}. The concentration of PTP1B is 4 nM. Error bars show the standard deviation of three independent experiments.

Selectivity of PTP assay. To investigate the selectivity of the proposed method, protein phosphatase 2A (PP2A), polynucleotide kinase (PNK), immunoglobulin G (IgG) and bovine serum albumin (BSA) are used as the negative controls. PP2A and PNK involve in the regulation of intracellular phosphorylation pathways. PP2A can catalyze the dephosphorylation of threonine, but has no activity toward tyrosine.[4] PNK can catalyze the transfer of one terminal phosphate group from adenosine triphosphate (ATP) to the 5′-end hydroxyl group of oligonucleotides.[5] IgG and BSA are two nonspecific proteins without enzymatic activity of dephosphorylation. As shown in Fig. S4, no distinct fluorescence signal is detected in response to PP2A, PNK, IgG and BSA, respectively, whereas a high fluorescence signal is detected in response to PTP1B, indicating that only PTP1B can catalyze the tyrosine dephosphorylation and the successive chymotrypsin-mediated cleavage of peptide-DNA substrate can induce tricyclic DNA amplification cascades for the generation of an enhanced fluorescence signal. These results demonstrate that the proposed method has good selectivity toward PTP1B.

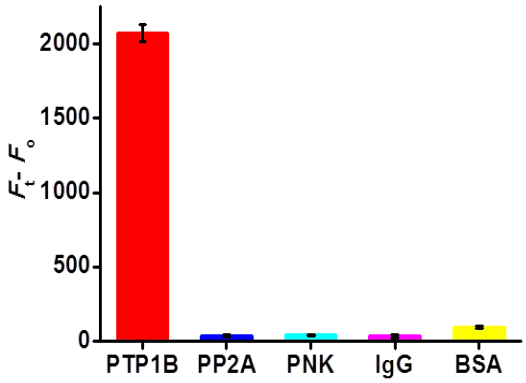

Fig. S4. Variance of fluorescence intensity in response to 4 nM PTP1B, 4 nM PP2A, 40 U/mL PNK, 0.005 g/L IgG, and 0.005 g/L BSA, respectively. Error bars show the standard deviations of three independent experiments.

PTP kinetic analysis. To evaluate the enzyme kinetic parameters of PTP1B, the reaction time for measuring the initial velocity was initially determined to be 10 min to make sure ~80% of the substrates unconsumed (i.e., in the initial-rate regime) (see Fig. S1A). In the presence of 4 nM PTP1B and different concentrations of DNA-peptide substrates, we measured the initial velocity after 10-min PTP1B-catalyzed tyrosine dephosphorylation reaction at 37 °C. As shown in Fig. S5, the enzyme kinetic parameters of PTP1B are obtained by fitting the data to the Michaelis-Menten equation $V = V_{max}[S]/(K_m + [S])$, where V_{max} is the maximum initial velocity, $[S]$ is the concentration of DNA-peptide substrate, and K_m is the Michaelis-Menten constant. The V_{max} value is calculated to be 44.30 min^{-1}, and the K_m value is calculated to be 0.54 μM. The obtained K_m value is consistent with that obtained by the UV-vis spectrometric method (0.73 μM).[6] These results demonstrate that the proposed method can be used to evaluate the kinetic parameters of PTP1B with high accuracy.

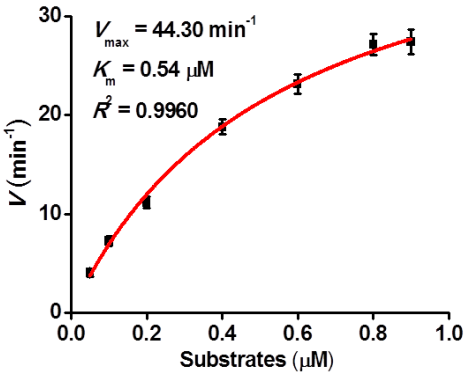

Fig. S5. Variance of initial velocity (*V*) with different concentrations of substrates. The concentration of PTP1B is 4 nM. Error bars show the standard deviations of three independent experiments.

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