

Supplementary Information

In vitro selection of DNA-cleaving deoxyribozyme with site-specific thymidine excision activity

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1. Method

1.1 General information

T4 polynucleotide kinase(PNK), T4 ligase, and FastAP™ were purchased from MBI Fermentas. 9 N DNA ligase was purchased from NEB (New England Biolabs). Klentaq DNA polymerase was purchased from Huagene Biosciences, Co., Ltd. (Fujian, China). [γ -³²P]ATP, were purchased from Furui Biological Engineering(Beijing, China). ATP, dCTP and all of the DNA oligonucleotides were PAGE-purified by Sangon BiotechCo., Ltd. (Shanghai, China). All reagents and chemicals such as H₂O¹⁸, NaN₃, DABCO, DMSO, H₂O₂, DTT and *t*-BuOH were of analytical grade and used without further purifications.

1.2 Labeling reactions

Oligonucleotides were incubated with 10 units of Polynucleotide kinase (PNK) at 37 °C for 0.5 h for DNA phosphorylation in a reaction mixture containing 50 mM Tris-HCl, pH 7.8 at 23 °C, 40 mM NaCl, 10 mM MgCl₂, 1 mg/mL BSA, and 10 μ Ci [γ -³²P]ATP. The labeled product was purified by 10% denaturing PAGE.

1.3 Standard conditions of deoxyribozyme cleavage assays

Trans-cleaving reaction were carried out with 4.5 μ M deoxyribozyme was combined with 15 nM substrate in reaction buffer (50 mM HEPES pH 7.4, 400 mM NaCl, 100 mM KCl, 10mM MgCl₂, 7.5 mM MnCl₂, 50 μ M CuCl₂) at 37 °C. Deoxyribozyme-substrate was first heated in H₂O at 90 °C for 30 s, and it was then cooled at room temperature for 5 min. Reaction was initiated by adding the same volume of 2 \times Reaction Buffer, and it was stopped after designated period of time by adding EDTA (pH 8.0) to 30 mM. Cleavage products form *trans* reaction were separated by 10 % denaturing PAGE and quantitated by phosphorimager.

1.4 Kinetic analyses

Time courses for each deoxyribozyme were conducted at least twice using over 8 time points for each. The experimental data was fit to $Y = Y_{\max}(1 - e^{-(k_{\text{obs}}t)})$ exponential equation using non-linear regression analysis in GraphPad Prism 4, from which the observed rate constant (k_{obs}) and maximum cleavage yield (Y_{\max}) were determined.

2. In Vitro Selection Procedure

Each round of in vitro selection consists of steps I-VI illustrated in *Figure S2.1*.

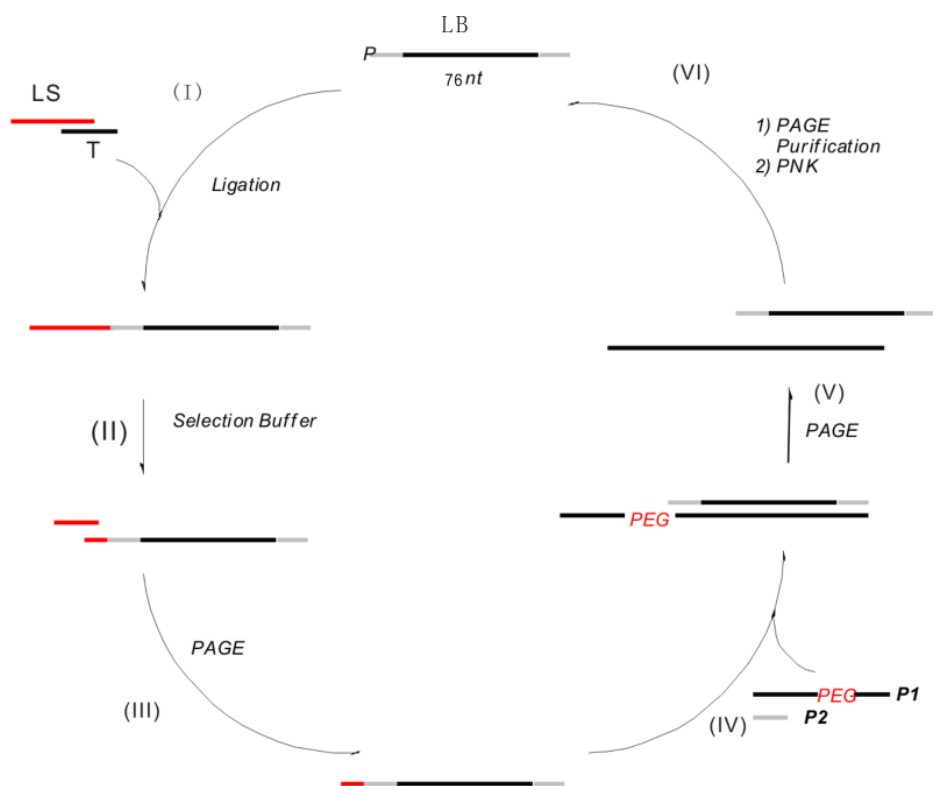


Figure S2.1: In vitro selection scheme. Each round of in vitro selection consists of steps I-VI: (I) The LB-LS c are constructed by ligation reaction and purified with PAGE. (II) The full library are incubated in the reaction buffer for a designed period of time, followed by quenching with EDTA.(III) The catalytic oligonucleotides are isolated by Gel based separation. (IV) The catalytic DNA oligonucleotides are amplified by PCR with primers P1 and P2.(V) The catalytic single-stranded DNA is isolated by 10% denaturing PAGE. (VI) recycled DNA from step V is phosphorylated by T4 polynucleotide kinase and feed into the next round of selection.

Step I: The 5'-phosphorylated 76 nt library LB (5'-A GCT TGT ACT AGT GTT CC N40 TGG ATC GGC ATG ACT AAC) was ligated to 55 nt LS substrate (5'- AAA AAA AAA AAA AAA AA CCT CTG CCG ATC CAT ACT GCG GAA CAC TTC ATG CG) to yield the LB-LS construct. The reaction mixture containing 1nM of LB, 1nM of LS, and 1 nM of template T (5'-GGA ACA CTA GTA CAA GCT CGC ATG AAG TGT TCC GCA GTA TGG ATC GGC AGA GG) was heated to 94 °C for 1 min and then cooled to room temperature. The 10× ligase buffer 5 ul (Fermentas) and 2 ul of T4 DNA ligase (10 μ/μL) were added to the mixture (50 μM final volume). The reaction mixture was incubated for 12 h at 37 °C. After ethanol precipitation, full library LB-LS was purified by 10% denaturing PAGE.

Step II: The purified LB-LS dissolved into 100 μL H₂O, and a equal volume of 2× Reaction Buffer (composed of 50 mM HEPES, 400 mM NaCl, 100 mM KCl, 10 mM MgCl₂, 7.5 mM MnCl₂, 50 μM CuCl₂) was added. The resultant reaction mixture (with DNA concentration at 7 μM) was allowed to at 37 °C for 12 h. A 2 × volume of stop buffer (45 mM EDTA, pH 8.5) was added to the reaction mixture to quench the reaction.

Step III: The cleavage products were separated by 10% denaturing PAGE. A 5'-P³² labeled synthetic oligonucleotide (95nt) with similar length with possible cleaved product (92-98nt) was applied as marker on the gel to help recycle the cleavage product through elution and ethanol precipitation.

Step IV: PCR was used to amplify the recovered cleavage fragments. The PCR in a volume of 100 μ L containing 75 mM Tris-HCl (pH 9.0), 2 mM MgCl₂, 50 mM KCl, 20 mM (NH₄)₂SO₄, 0.5 mM each of four dNTPs, 5 U Tag DNA polymerase, 1 μ M primer P1 (5'- AAA AAA AAA AAA AAA AAA AA-PEG- GTT AGT CAT GCC GAT CCA) and 1 μ M primer P2 (5'- AGC TTG TAC TAG TGT TCC). The PCR (Figure S2.1) was carried out for 28 thermo cycles during which the temperature was altered in the following order: 30 s at 94 $^{\circ}$ C (1 min for the first cycle), 45 s at 59 $^{\circ}$ C, and 45 s at 72 $^{\circ}$ C.

Step V: The amplified DNA from PCR reaction above was recovered by ethanol precipitation. The targeted single-stranded DNA was purified by 10% denaturing PAGE.

Step VI: The recovered DNA molecules were incubated with 10 units of PNK in a 10 μ L reaction mixture containing 50 mM Tris-HCl, pH 7.6 at 25 $^{\circ}$ C, 10 mM MgCl₂, 5 mM DTT, 1 mM spermidine, and 5 μ Ci [γ -³²P]ATP for 30 min, and then added 1 μ l 100mM ATP for 10 min .The 5'-phosphorylated DNA was used for the next round of selection.

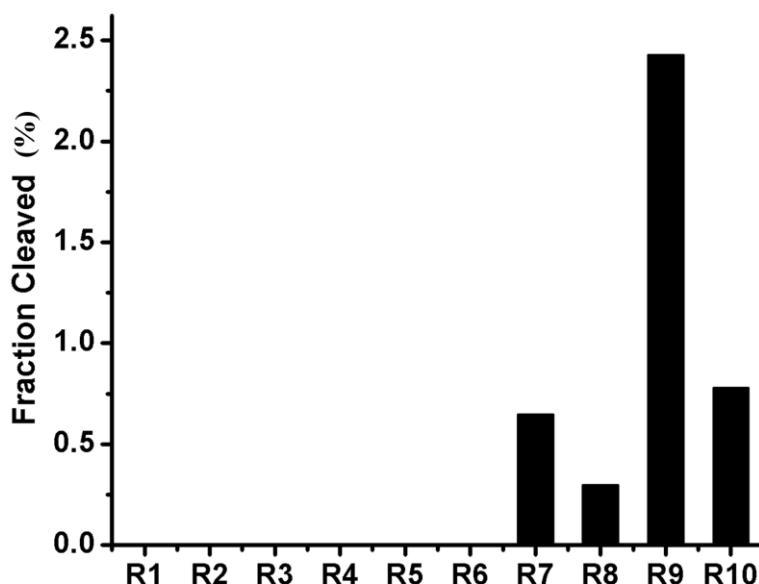


Figure S2.2 In vitro selection of self-cleaving deoxyribozymes. Plot of the fraction of DNA cleaved versus the population (R1 through R10), Clear cleaving band appeared from R7. The reaction time of cleaving reaction from R1 to R7 is 12h. The reaction was decreased gradually in following selections: 6h, 2h and 1h for the 8, 9 and 10 round respectively.

3. Sequences of the new deoxyribozyme

deoxyribozyme	enzyme region sequence
F-8	5'-AGTGTTCGGTGGATGGAGCAATAGTCTCCCGGGTCCGTATGGATCGGC-3'
substrate	sequence
LS-1	5'-CCTCTGCCGATCCATACTGCGGAACACT-3'

Figure S3 Sequence alignments of the deoxyribozyme **F-8** and substrate **LS-1**.

4. Denaturing PAGE images of F-8-catalyzed DNA cleavage.

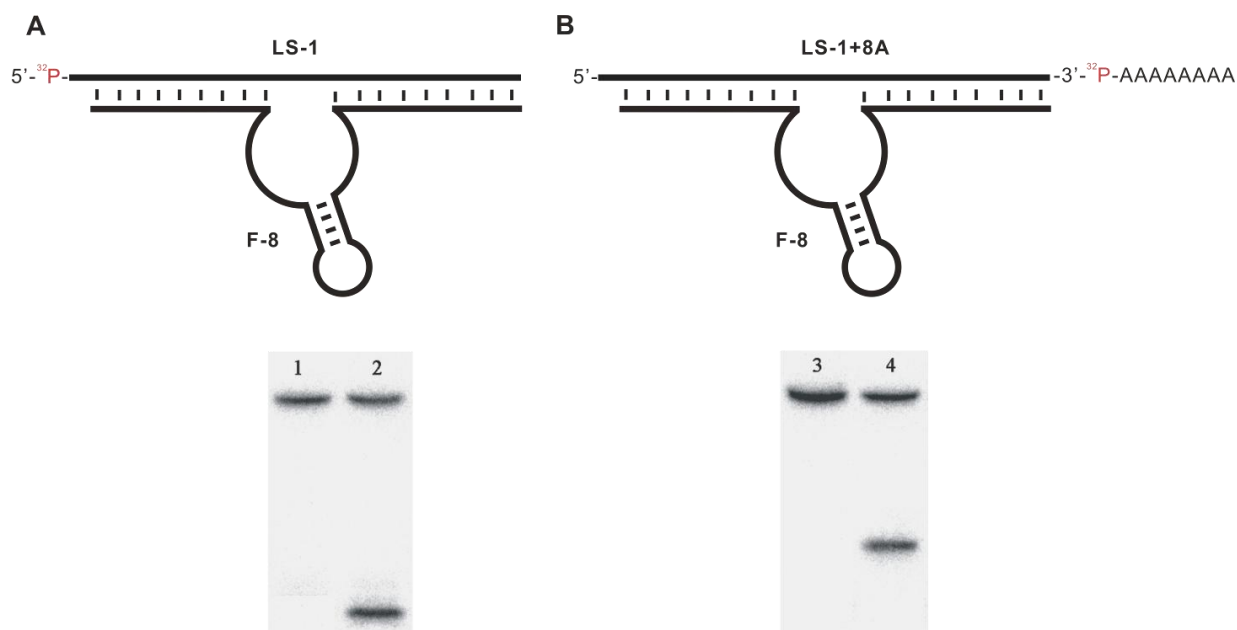


Figure S4. Cleavage of 5'- (A) and 3'-³²P-radiolabeled (B) **LS-1** substrates by **F-8**. 5'-³²P-radiolabeled substrate **LS-1** was prepared using T4 PNK and 3'-³²P substrate **LS-1+8A** was prepared by ligating a labeled oligonucleotide (5'-³²P-AAAAAAAA-3') using a DNA splint and T4 DNA ligase. All the substrates were purified by 10% denaturing PAGE. Lane 1, DNA substrate (**LS-1**) control; Lane 2, cleavage by **F-8**; Lane 3, DNA substrate (**LS-1+8A**) control; Lane 4, cleavage by **F-8**. Cleavage assays were under standard single-turnover conditions.

5. Analysis of DNA Cleavage site of F-8.

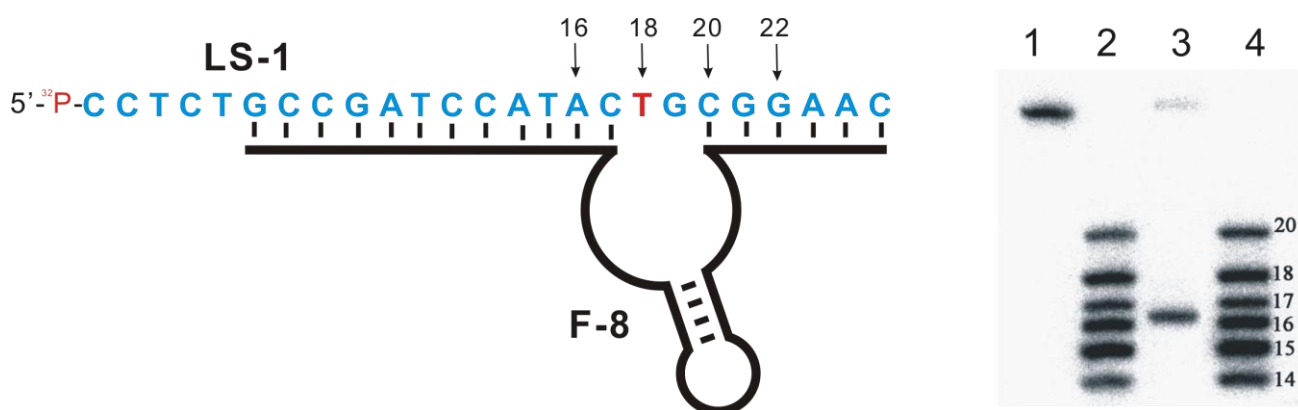


Figure S5.1: PAGE gel (20%) analysis of cleaving reaction catalyzed by **F-8**. Lane 1: 5'-³²P labeled DNA substrate LS-1; Lanes 2 and 4: 5'-³²P labeled DNA markers with different lengths; lane 3: 5'-³²P labeled DNA substrate was cleaved by **F-8** under standard conditions (50 mM HEPES pH 7.4, 400 mM NaCl, 100 mM KCl, 10 mM MgCl₂, 7.5 mM MnCl₂, 50 μM CuCl₂) at 37 °C for 12 h. Cleaved left-hand fragment has slightly faster mobility than corresponding 17nt marker maybe because of the presence of phosphate group at the 3' end.

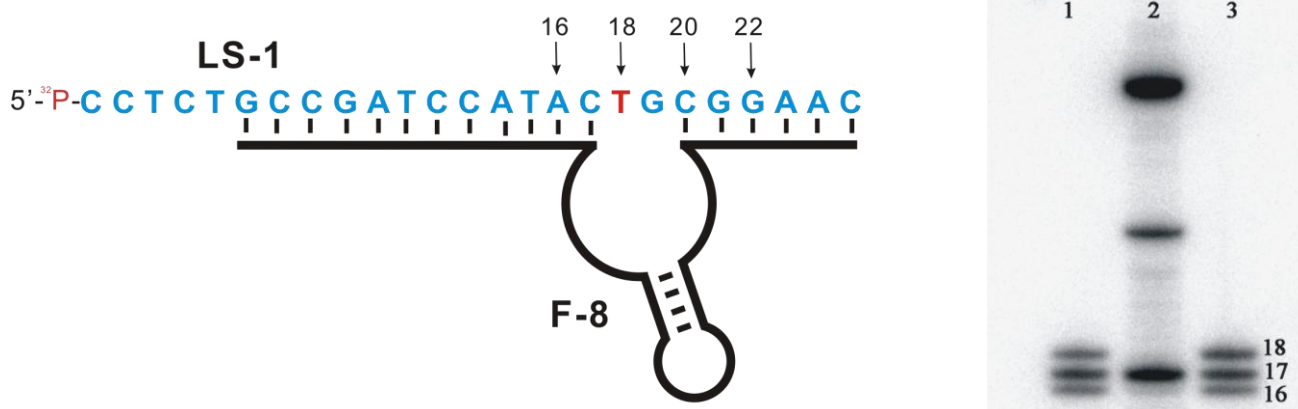


Figure S5.2. Treatment of cleaved product with FastAP™ alkaline phosphatase. Lanes 1 and 3 were 5'-³²P-radiolabeled synthetic oligonucleotides with different lengths. Lane 2: left-hand cleavage product was firstly treated with FastAP™ enzyme (37 °C for 30 min) and labeled again with PNK and [γ-³²P]ATP. As shown in Lane 2, the left-hand cleavage product has the same migration rate as the 17nt synthetic DNA marker. Comparing **Figure S5.1** and **Figure S5.2**, we speculate that the left-hand cleavage product is a 17-nucleotide oligonucleotide with 3'-terminal phosphate group.

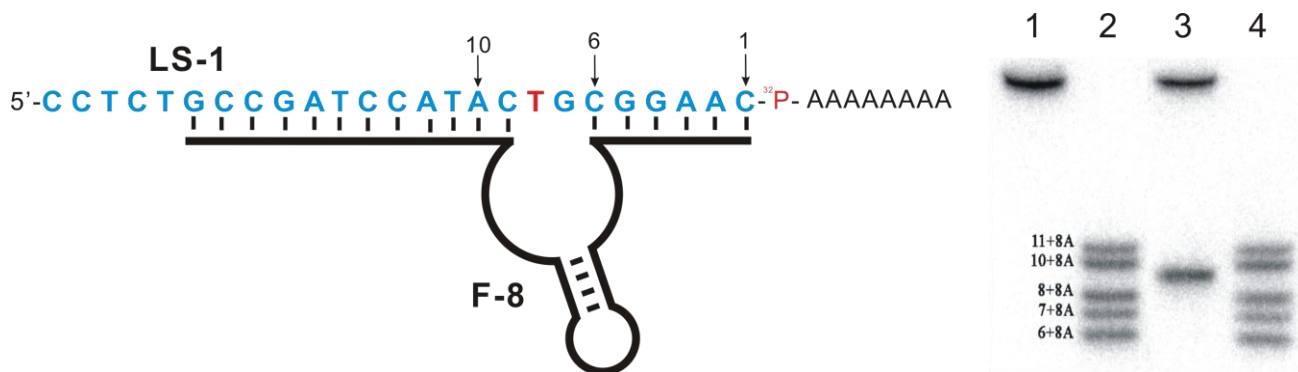


Figure S5.3: PAGE gel (20%) analysis and mass spectrometry to assign right-hand cleaved fragment. Substrate **LS-1+8A** was prepared by ligating a labeled oligonucleotide (5'-³²P-AAAAAAAA-3') with **LS-1** using a DNA splint and T4 DNA ligase. Lane 1: 3'-³²P Labeled DNA substrate **LS-1+8A**. Lanes 2 and 4: 3'-³²P-radiolabeled labeled DNA markers with different lengths. As shown in lane 3: The 3'-³²P Labeled DNA substrate **LS-1+8A** was cleaved by **F-8**. As shown in lane 3, the right cleavage product has slightly faster than a synthetic DNA marker 10+8A. This result indicates the cleavage product could be the 10+8A oligonucleotide with a 5'-terminal phosphate or modified phosphate residue. Cleavage assays were under standard single-turnover conditions. (50 mM HEPES pH 7.4, 400 mM NaCl, 100 mM KCl, 10 mM MgCl₂, 7.5 mM MnCl₂, 50 μM CuCl₂) at 37 °C

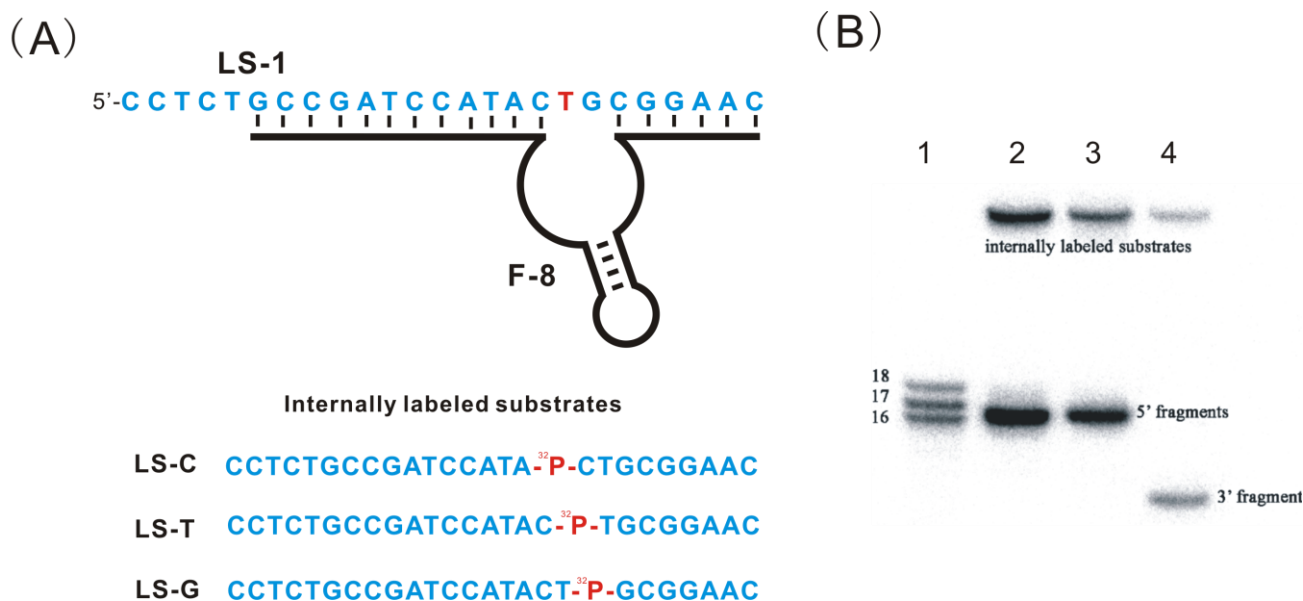


Figure S5.4. The PAGE gel analysis of cleavage site of deoxyribozyme **F-8** by using internally labeled substrates. (A) Three internally labeled substrates were prepared using T4 DNA ligase and were purified by denaturing 10% PAGE prior to use. (B) **F-8**-mediated cleavage of various internally 5'-³²P-radiolabeled substrates. Lane 1: DNA markers (loaded with 5'-³²P-radiolabeled synthetic oligonucleotides with different lengths), Lanes 2-4, cleavage of **LS-C**, **LS-T** and **LS-G**, respectively. The **LS-C** and **LS-T** substrates were cleaved to obtain ³²P labeled left-hand fragment (Lane 2 and 3), while **LS-G** to get right-hand fragment (Lane 4). Cleavage assays were under standard single-turnover conditions. (50 mM HEPES pH 7.4, 400 mM NaCl, 100 mM KCl, 10 mM MgCl₂, 7.5 mM MnCl₂, 50 μM CuCl₂) at 37 °C

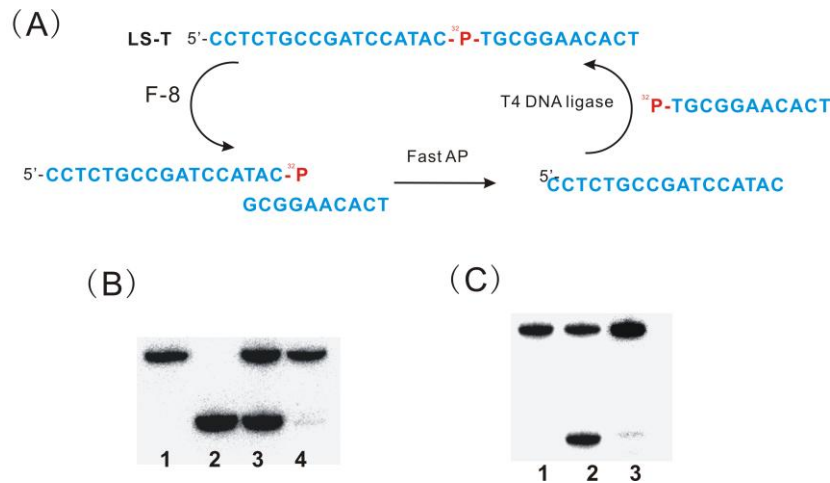


Figure S5.5. Dephosphorylation with FastAP™ enzyme and ligation assays with T4 DNA ligase to confirm 3'-terminal of left-hand cleavage product by deoxyribozyme **F-8**. A) Cleavage reactions were carried out by using internally labeled substrate **LS-T**. Then, the left-hand product was treated with FastAP™ (37 °C for 30 min) and ligated with 5'-³²P-TGCGGAACACT-3' to get **LS-T** again (25 °C for 6 h). B) Dephosphorylation with FastAP™ enzyme. Lane 1: internally-labeled **LS-T**; Lane 2: 5'-³²P-labeled 17-nt DNA marker; Lane 3: cleavage of **LS-T** by **F-8**. Lane 4: treatment of cleavage product in lane 3 with with FastAP™ enzyme; C) Ligation reaction with T4 DNA ligase. Lane 1: internally-labeled **LS-T**; Lane 2: cleavage of **LS-T** by **F-8**. Lane 3: the cleaved product after treatment with FastAP™ was ligated with a synthetic 11-nucleotide oligonucleotide (5'-³²P-TGCGGAACACT-3'). Cleavage assays were under standard single-turnover conditions. (50 mM HEPES pH 7.4, 400 mM NaCl, 100 mM KCl, 10 mM MgCl₂, 7.5 mM MnCl₂, 50 μM CuCl₂) at 37 °C. These results demonstrated that left-hand cleaved product carries an unmodified phosphate group at its 3' terminus.

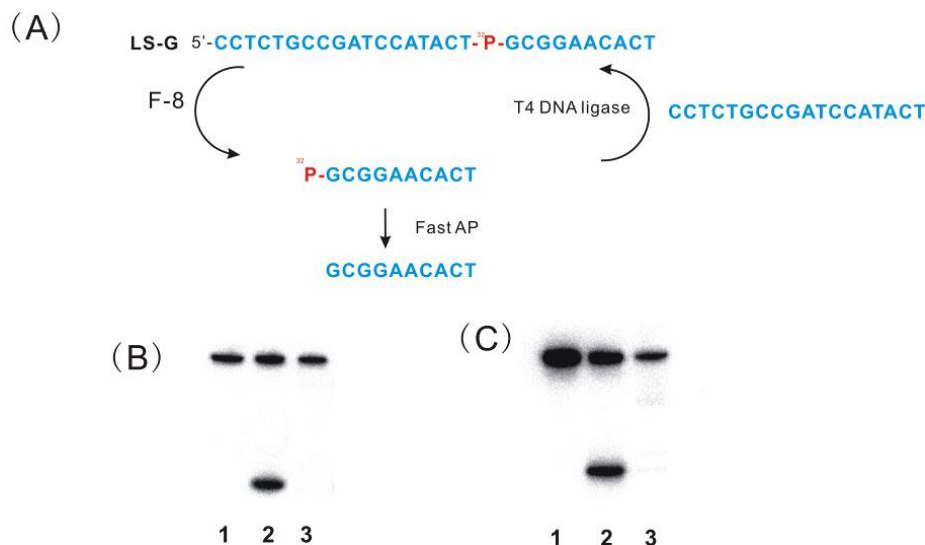
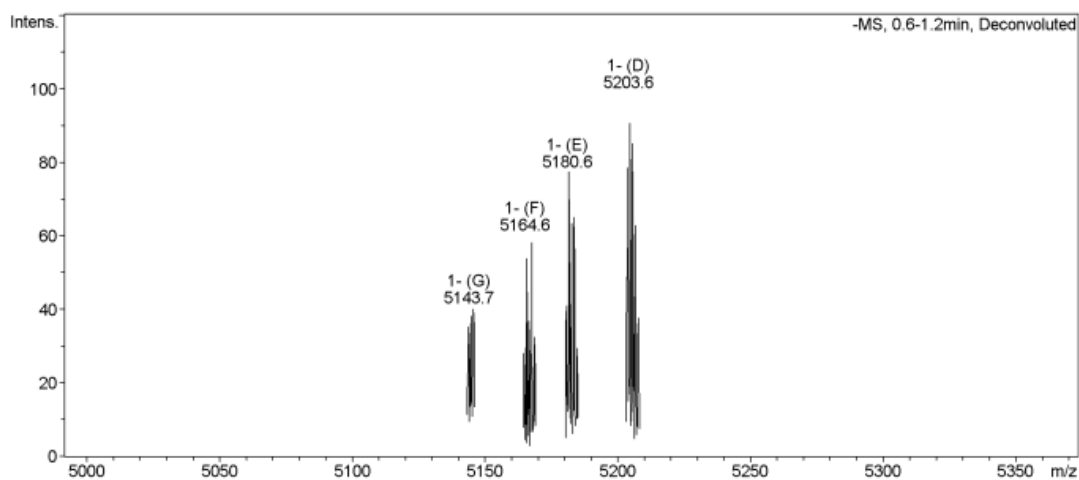


Figure S5.6. Dephosphorylation with FastAP™ enzyme and ligation assays with T4 DNA ligase to assign 5'-terminal of right-hand cleaved product by deoxyribozyme **F-8**. A) Cleavage reactions were carried out by using internally labeled substrate **LS-G**. Then, the right-hand product was treated with FastAP™ and ligated with 5'-CCT CTG CCG ATC CAT ACT-3' to get **LS-G** again. B) Dephosphorylation with FastAP™ enzyme. Lane 1: internally-labeled **LS-G**; Lane 2: cleavage of **LS-G** by **F-8**; Lane 3: treatment of cleavage product in lane 3 with FastAP™ enzyme. C) Ligation reaction with T4 DNA ligase. Lane 1: internally-labeled **LS-G**; Lane 2: cleavage of **LS-G** by **F-8**; Lane 3: the cleaved product in Lane2 was ligated with a synthetic 11-nucleotide oligonucleotide(5'-CCT CTG CCG ATC CAT ACT-3'). These results demonstrated that right-hand cleaved product carries an unmodified phosphate group at its 5' terminus. Cleavage assays were under standard single-turnover conditions.

Acquisition Parameter

Source Type	ESI	Ion Polarity	Negative	Set Nebulizer	0.8 Bar
Focus	Active	Set Capillary	3500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	3000 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Source

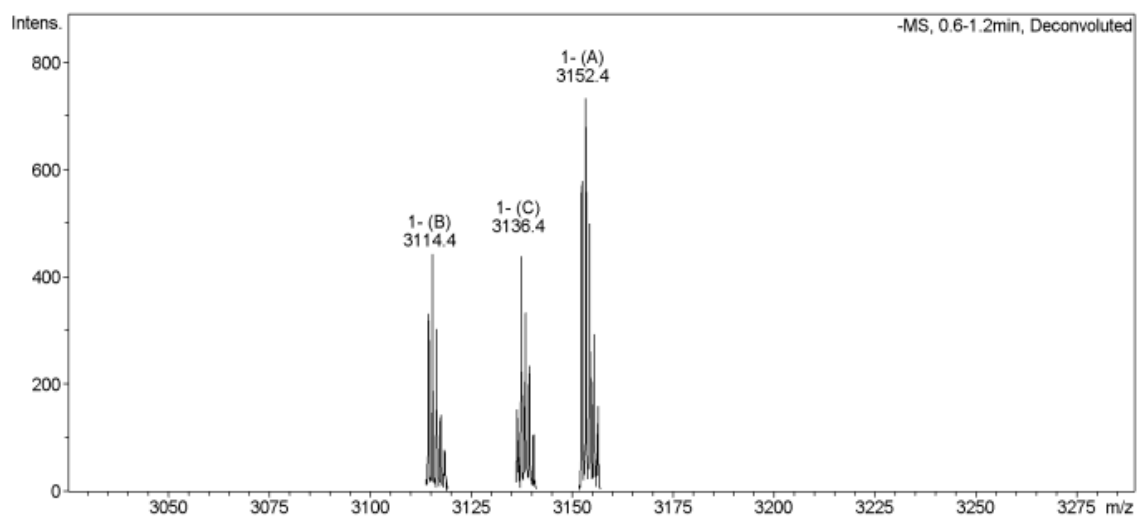


Meas. m/z	#	Formul a	Score	m/z	err [ppm]	Mean err [ppm]	mSigma a	rdb	e ⁻ Conf	N-Rule
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Mass spectrometry analysis of the cleavage products (Left-hand) which separated from HPLC.(100 mM TEAA, pH 7.0, HPLC gradient 5-40% acetonitrile/20 min at 0.8 mL/min, 50 °C.)

Acquisition Parameter

Source Type	ESI	Ion Polarity	Negative	Set Nebulizer	0.8 Bar
Focus	Active	Set Capillary	3500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	3000 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Source



Meas. m/z	#	Formul a	Score	m/z	err [ppm]	Mean err [ppm]	mSigma a	rdb	e ⁻ Conf	N-Rule
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Mass spectrometry analysis of the cleavage products (Right-hand) which separated from HPLC. (100 mM TEAA, pH 7.0, HPLC gradient 5-40% acetonitrile/20 min at 0.8 mL/min, 50 °C.)

deoxyribozyme	substrate	mass (L,calcd.)	mass (L found)	mass (R,calcd.)	mass (R found)
F-8	LS-1	5142.8	5143.7	3114.5	3114.4

Table S5.7. Mass spectrometry analysis of the cleavage products formed by deoxyribozyme **F-8** using substrate **LS-1**. L and R in the table respectively represent left-hand (calcd for $[M-H]^-$ 5142.8, found 5143.7; $[M-2H+Na]^-$ 5164.8, found 5164.6; $[M-2H+K]^-$ 5180.7, found 5180.6) and right-hand cleavage products (calcd for $[M-H]^-$ 3114.5, found 3114.4; $[M-2H+Na]^-$ 3136.4, found 3136.4; $[M-2H+K]^-$ 3152.4, found 3152.4.). The data are consistent with denaturing PAGE indicating the cleavage leads to 3'-phosphate and 5'-phosphate termini.

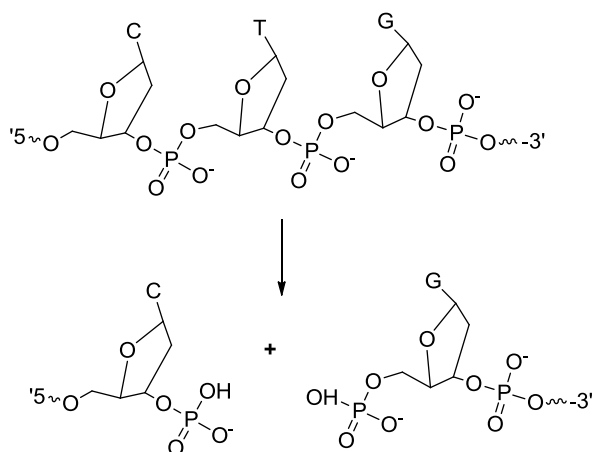


Figure S5.8. Chemical structure of cleavage product formed by deoxyribozyme **F-8**.

6. Metal ions requirements for deoxyribozyme F-8.

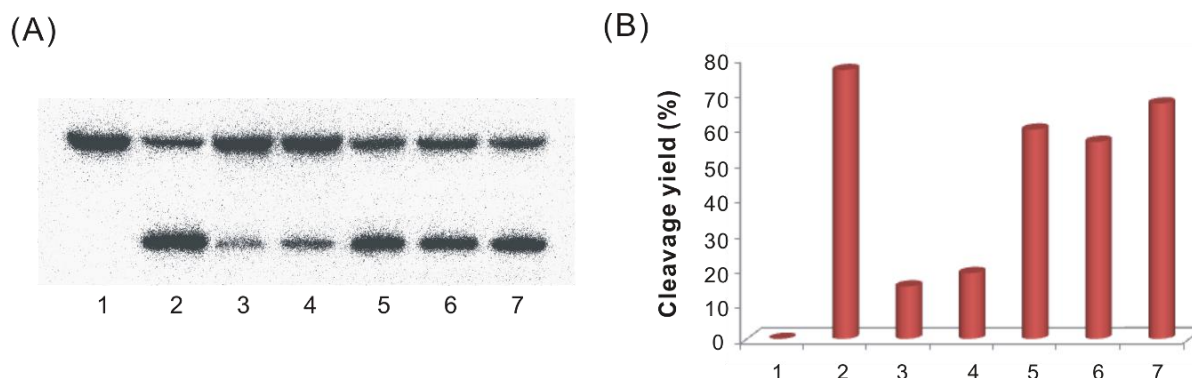


Figure S6.1. Metal ions requirements for the deoxyribozyme F-8. (A) Denaturing PAGE image. Lane 1: 5'-³²P-radiolabeled substrate; Lane 2: Cleaving reaction by F-8 was carried out in buffer (50 mM HEPES, pH 7.4, 400 mM NaCl, 100 mM KCl, 10 mM MgCl₂, 7.5 mM MnCl₂, 50 μM CuCl₂, 25 °C); Lane 3: minus Cu²⁺; Lane 4: minus Mn²⁺; Lane 5: minus Na⁺ and K⁺; Lane 6: minus Mg²⁺; Lane 7: minus Na⁺, K⁺ and Mg²⁺; (B): Quantization of cleavage yield to corresponding reactions of A.

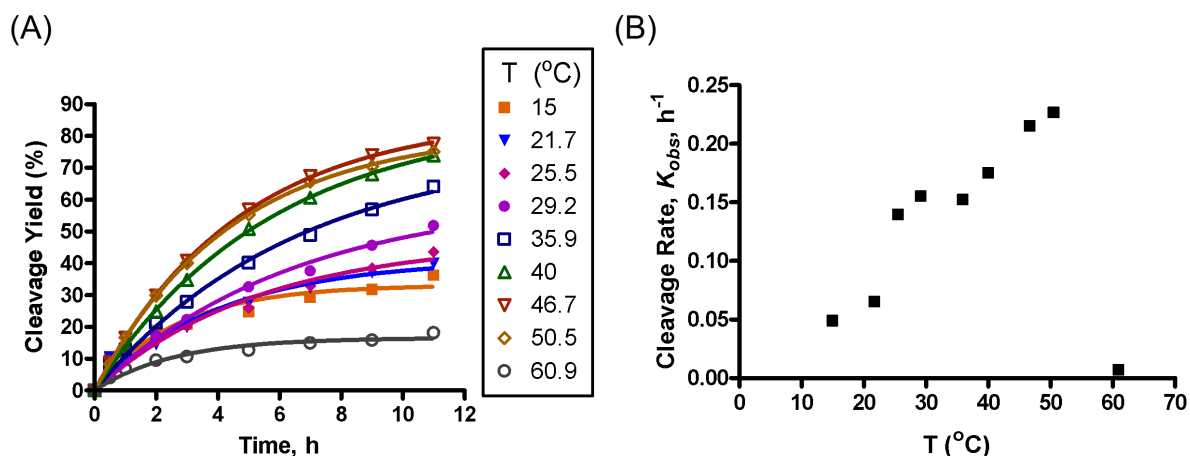


Figure S6.2: Temperature dependence. Assays were performed under the standard reaction conditions (50 mM HEPES, pH 7.4, 400 mM NaCl, 100 mM KCl, 10 mM MgCl₂, 7.5 mM MnCl₂, 50 μM CuCl₂) at different temperatures. Kinetic characterizations for temperature dependence were shown in (A) and (B). The rate constant increases nearly linearly with temperature and reaches a maximum at 50 °C before decreasing at higher temperatures. The optimum temperature is higher than many other DNAzymes, which shows broader temperature tolerance.

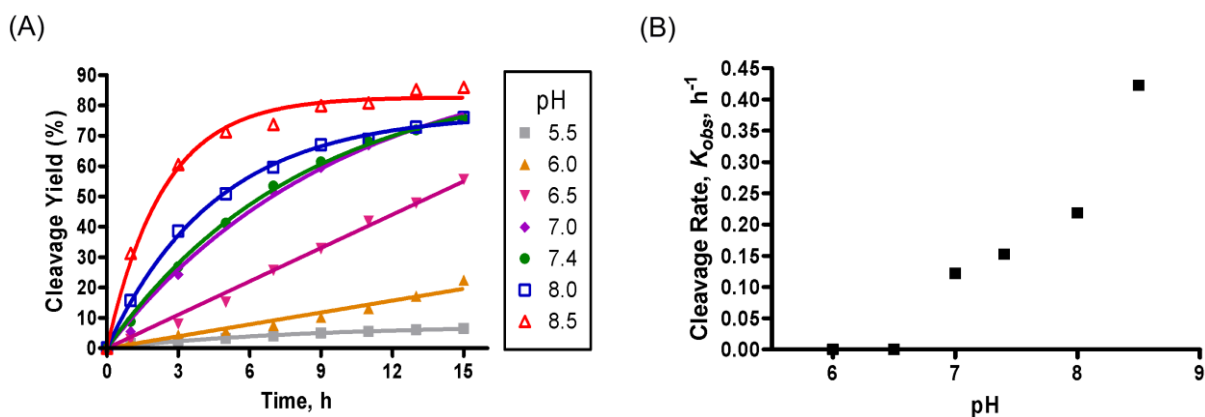


Figure S6.3: pH dependence. Assays were performed under the standard reaction conditions (50 mM HEPES pH 7.4, 400 mM NaCl, 100 mM KCl, 10 mM MgCl₂, 7.5 mM MnCl₂, 50 μ M CuCl₂, 37 °C) with different pH (ranging from 5.5 to 8.5). Above pH value 8.5, physical precipitation was clearly evident. Kinetic characterizations for pH dependence were shown in (A) and (B). We selected a physiological pH value 7.4 for following requirements assays.

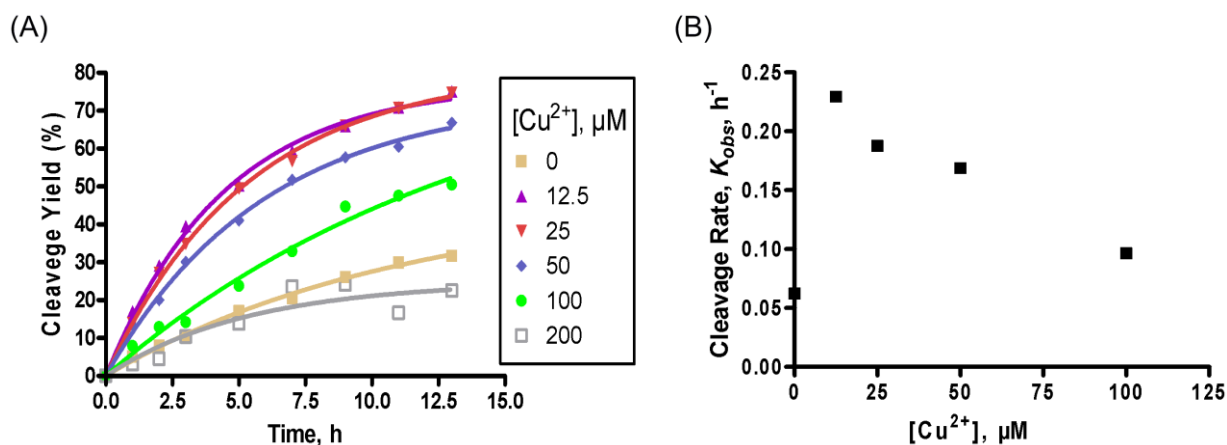


Figure S6.4. Cu²⁺ dependence. Assays were performed under the standard reaction conditions (50 mM HEPES, pH 7.4, 400 mM NaCl, 100 mM KCl, 10 mM MgCl₂, 7.5 mM MnCl₂, pH 7.4, 37 °C) with different Cu²⁺ concentrations (ranging from 0 to 200 μ M). Kinetic characterizations were shown in (A) and (B). The rate of DNA cleavage by F-8(8) is highly dependent on the concentration of Cu²⁺. At a low concentration of Cu²⁺ (about 12 μ M), the cleavage activity reached maximum and at higher concentrations it was inhibited.

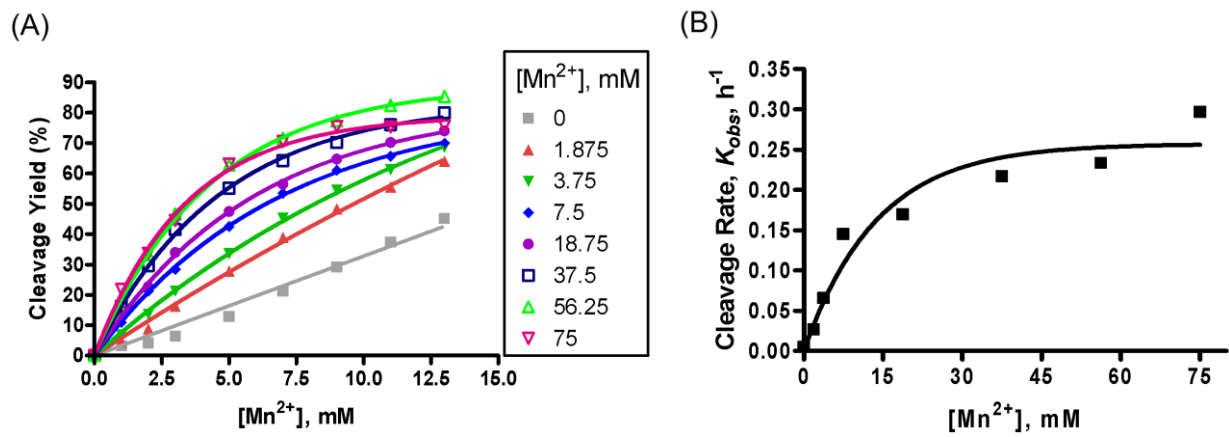


Figure S6.5. Mn^{2+} dependence. Assays were performed under the standard reaction conditions (50 mM HEPES, pH 7.4, 400 mM NaCl, 100 mM KCl, 10 mM $MgCl_2$, 12.5 μM $CuCl_2$, pH 7.4, 37 $^{\circ}C$) with different Mn^{2+} concentrations (ranging from 0 to 75 mM). Within a certain range, the cleavage rate increased with the Mn^{2+} concentration and displays maximal activity near 40 mM.

7. Secondary structure characterization of deoxyribozyme F-8.

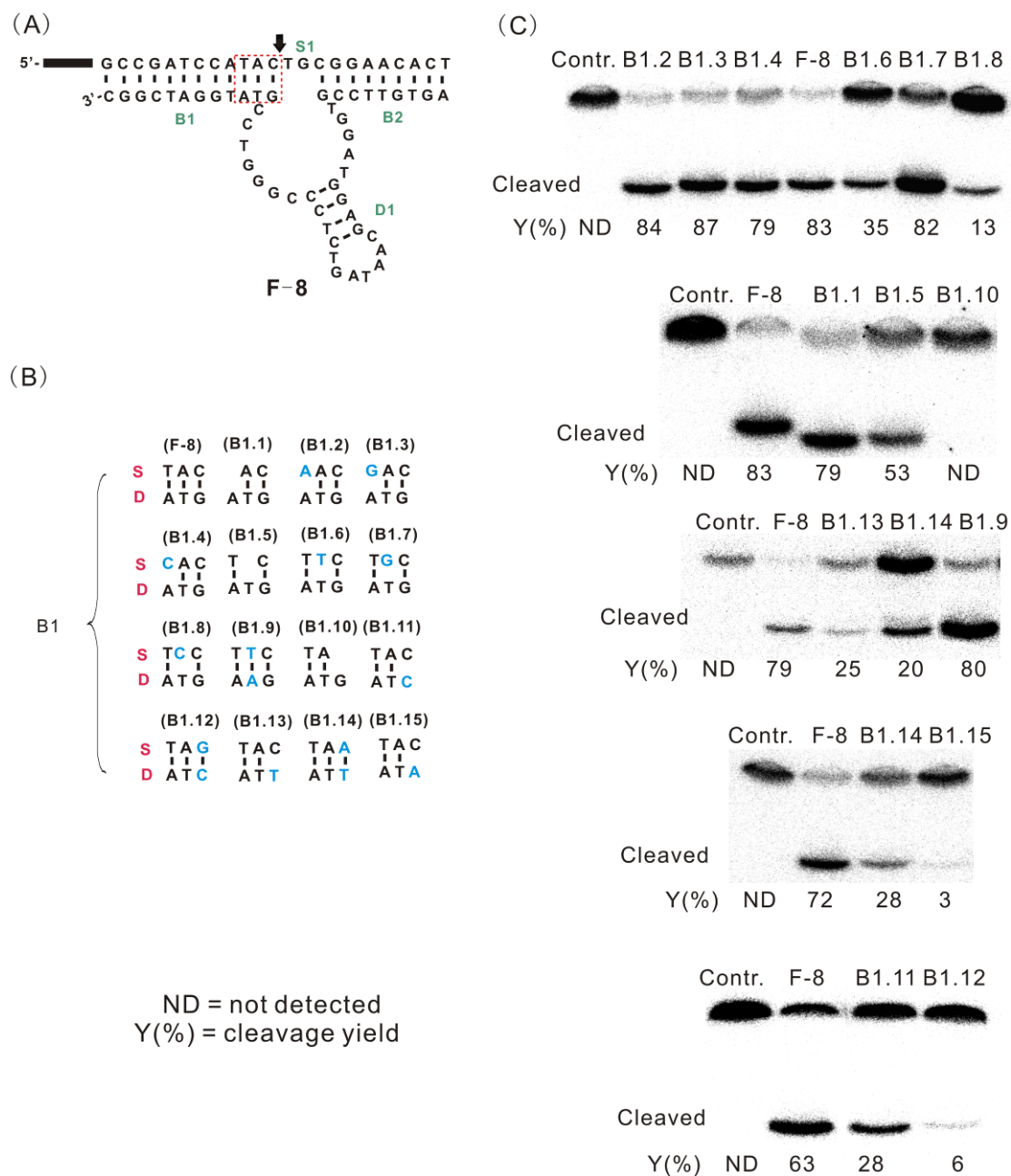


Figure S7.1. Secondary structure of deoxyribozyme **F-8**. (A) A proposed structural mode of deoxyribozyme **F-8** containing three short duplexes (B1, B2, and D1) and a single-strand region (S1). (B) Investigating the importance of left-hand binding arm B1 to the function of the deoxyribozyme **F-8**. The binding arm was replaced either with mismatch pairs or with co-varied base pairs. (C) Denaturing PAGE images corresponding to the reaction in B. Cleavage assays were under standard single-turnover conditions.

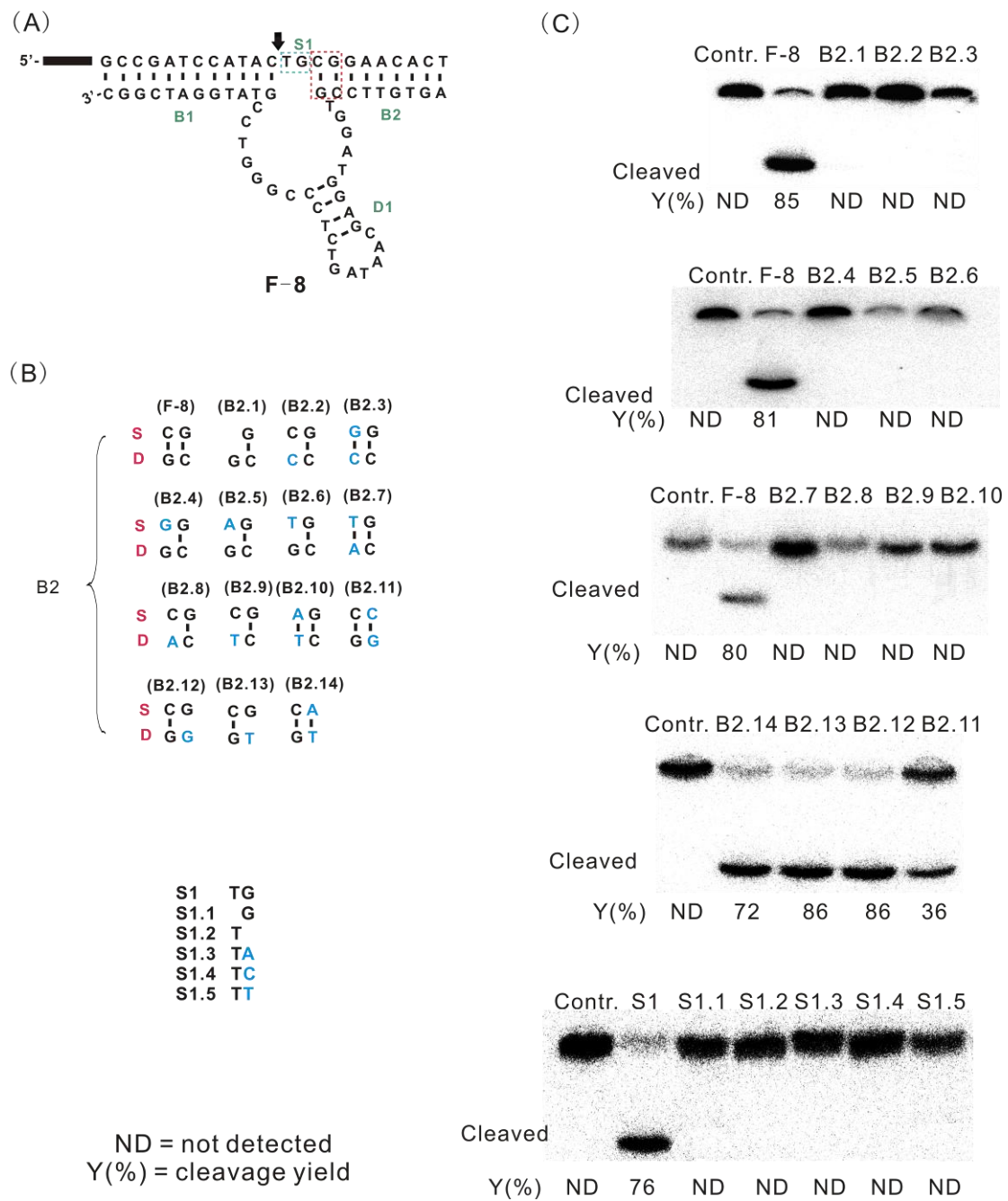


Figure S7.2. Secondary structure characterization of deoxyribozyme **F-8**. (A) A proposed structural mode of deoxyribozyme **F-8**. (B) Investigating the importance of B2 and S1 to the function. The right binding arm and single-strand S1 were replaced either with mismatch pairs or with covaried base pairs. (C) Denaturing PAGE images corresponding to (B). Cleavage assays were under standard single-turnover conditions.

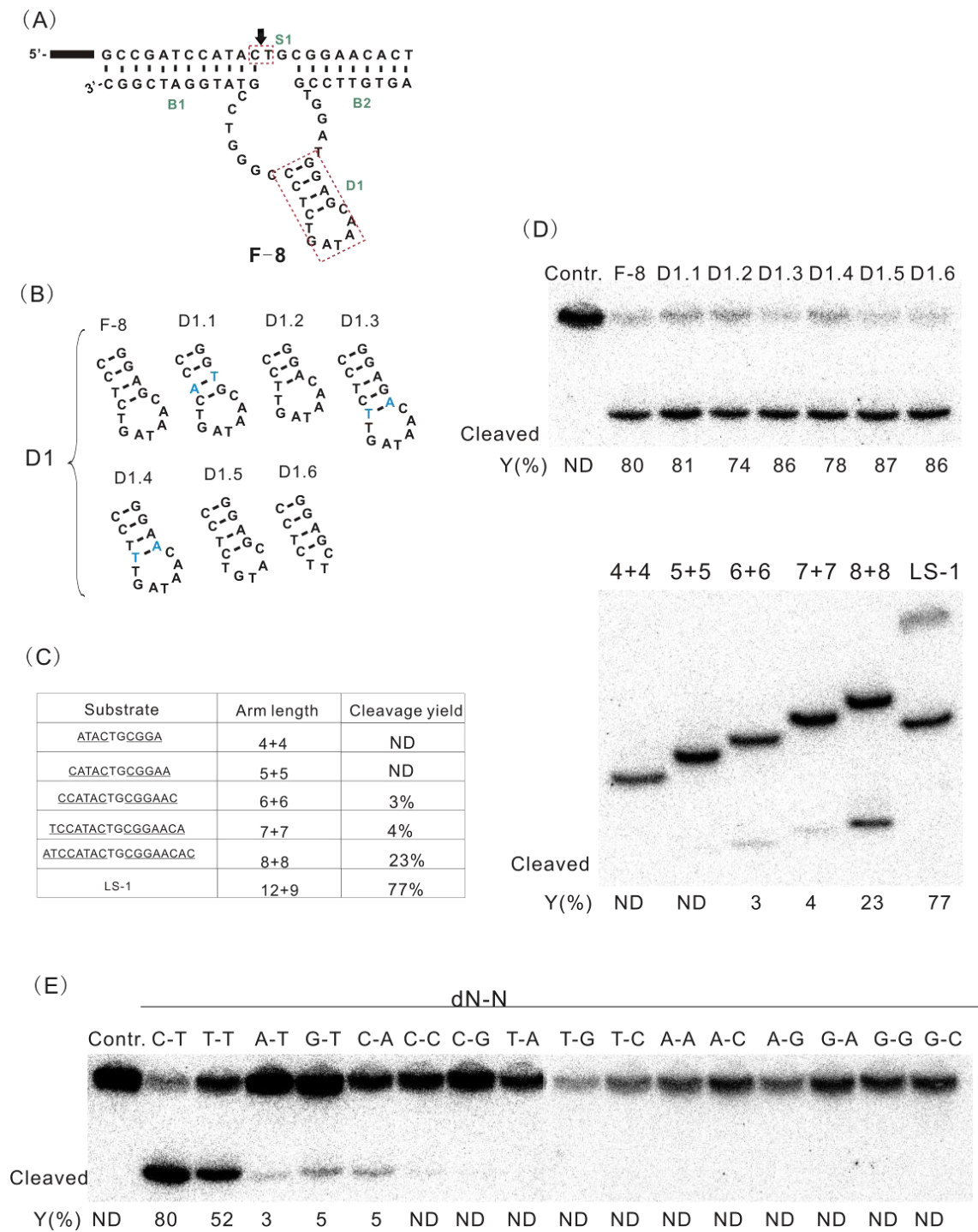


Figure S7.3. Secondary structure characterization of deoxyribozyme **F-8**. (A) A proposed structural mode of deoxyribozyme **F-8**. (B) Investigating the importance of D1 to the function. The importance of D1 was researched by replacing either with mutagenesis or with decreased length of base pairs and loop. (C) Analysis of the effect of varying substrate-recognition domain length. (D) Denaturing PAGE images corresponding to B and C. (E) Dinucleotide junction specificities at the cleavage site of **F-8**. Cleavage assays were under standard single-turnover conditions.

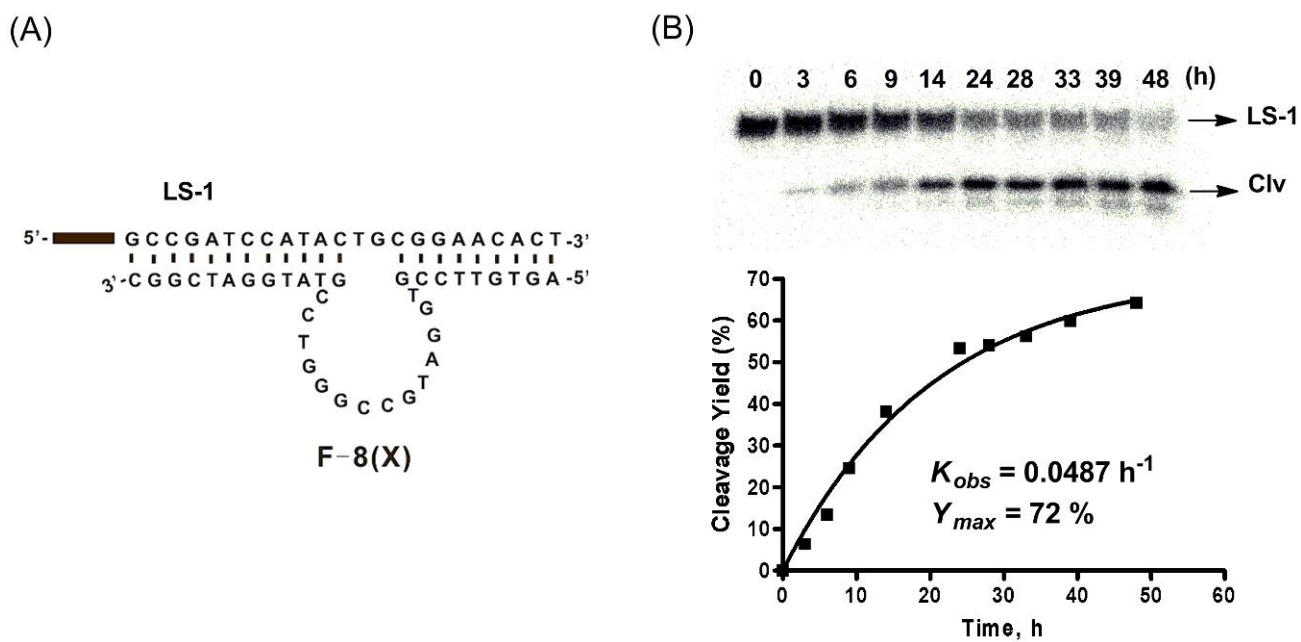


Figure S7.4. Kinetic characterizations for the deoxyribozyme **F-8(X)**. Time courses experiments were performed under standard single-turnover reaction conditions. (A) Secondary structure characterization of deoxyribozyme **F-8(X)**. (B) The cleavage yields were plotted over time and curve fitted using GraphPad software Prism 4.0. Insets are corresponding denaturing PAGE images. Rate constants (k_{obs}) and maximum cleavage yields (Y_{max}) are reported on the curve.

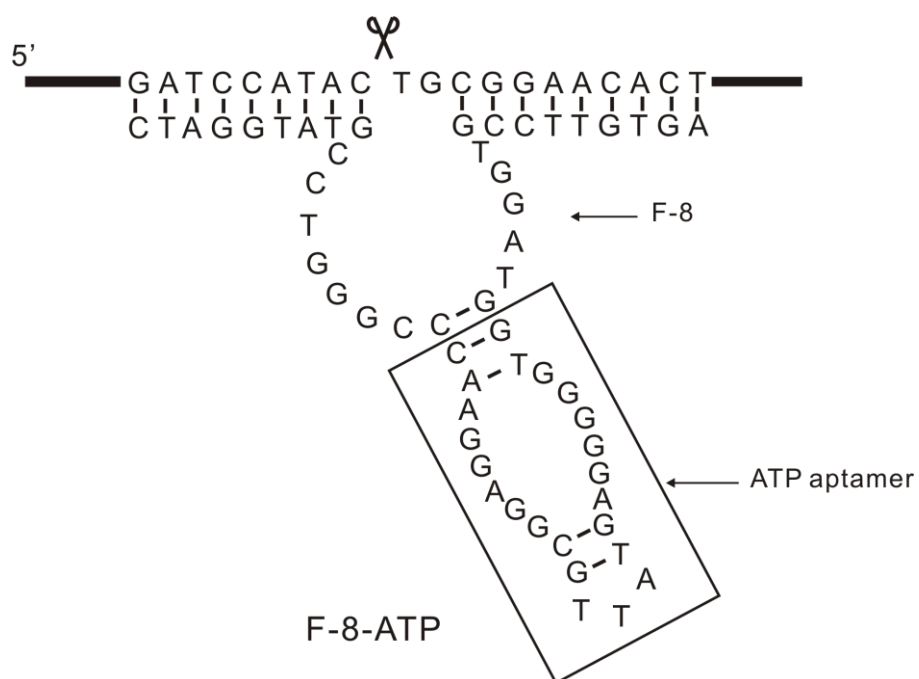


Figure S7.5. Sequence and structure of allosteric DNAzyme **F-8-ATP**, which contain the catalytic core of **F-8** DNAzyme and the ATP aptamer (the framed region)..

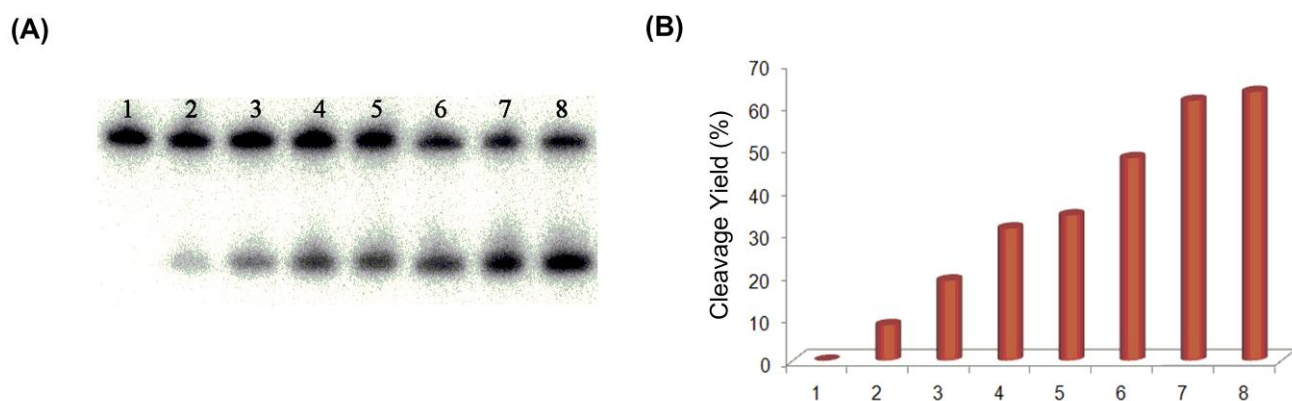


Figure S7.6. (A) PAGE gel analysis of the cleavage assays. Cleavage reactions were carried out with **F-8-ATP** and 50 μM H_2O_2 for 4 min in the presence/absence of ATP under the standard cleavage conditions. Lane 1, DNA control, Lane 2, cleavage of **F-8-ATP** in the absence of additional ATP; lanes 3-8 cleavage in the presence of additional ATP 0.01, 0.025, 0.05, 0.1, 0.5, 2.5 mmol respectively. (B) Quantization of % cleaved DNA corresponding to A.

8. Chemical Mechanism of the DNA Enzymes.

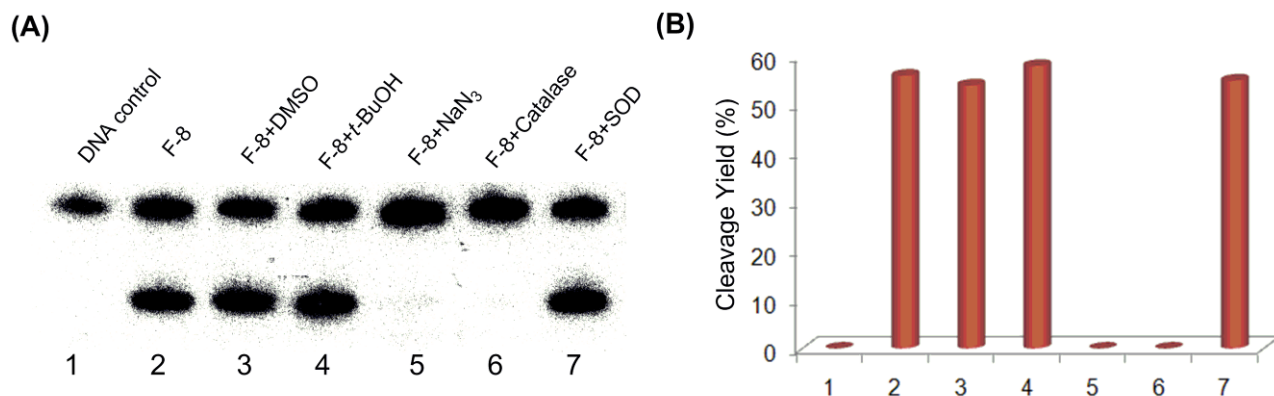


Figure S8.1. Effect of ROS scavengers on the cleavage reaction of deoxyribozyme **F-8** under the standard reaction conditions. A) lane 1: 5'-³²P labeled DNA substrate; lanes 2: cleavage of **F-8** in the absence of additional scavengers. Lanes 3-7: cleavage of **F-8** in the presence of additional scavengers 0.1 M DMSO, 0.1 M *t*-BuOH, 0.1 M NaN₃, 10 U catalase, 10 U SOD respectively; B) Quantitation of % cleaved DNA of corresponding reactions in A.

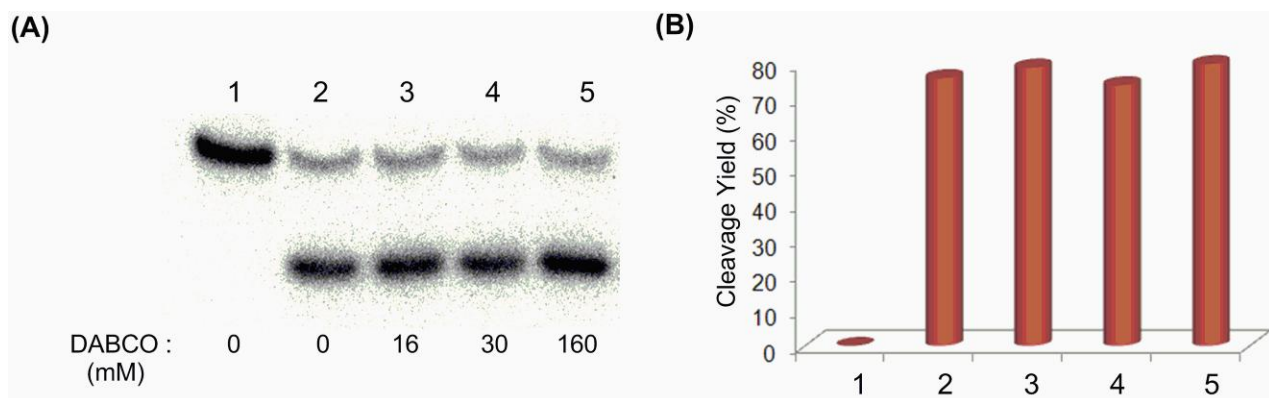


Figure S8.2. Effect of singlet oxygen scavenger DABCO on the cleavage reaction of deoxyribozyme **F-8** under the standard reaction conditions. A) Lane 1, 5'-³²P labeled DNA substrate; Lanes 2: cleavage of **F-8** in the absence of additional scavengers. Lanes 3-5: cleavage of **F-8** in the presence of additional 0.016-0.16 M DABCO respectively; B) Quantitation of % cleaved DNA of corresponding reactions in A.

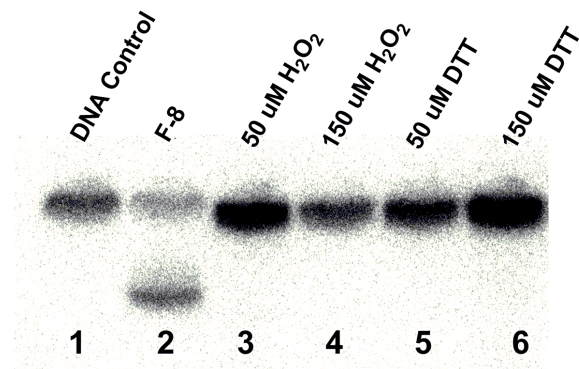


Figure S8.3. Control experiments ($\text{H}_2\text{O}_2/\text{DTT}$) on the DNA cleavage reaction. Lane 1, $5'$ - ^{32}P labeled DNA substrate; Lane 2: DNA Cleavage by **F-8** under the standard reaction conditions; Lanes 3-4: DNA cleavage in the presence of 50-150 μM H_2O_2 alone; Lanes 5-6: DNA cleavage in the presence of 50-150 μM DTT alone.

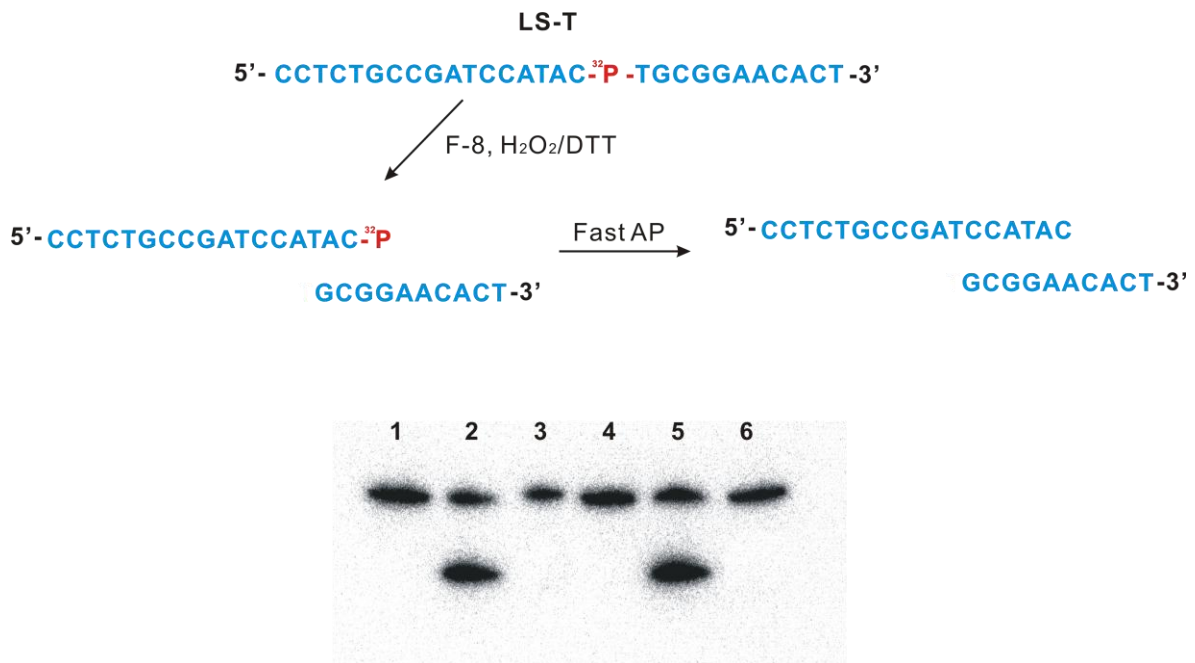


Figure S8.4. Dephosphorylation with FastAP™ enzyme assays to confirm 3'-terminal of left-hand cleavage product by **F-8** and $\text{H}_2\text{O}_2/\text{DTT}$. Cleavage reactions were carried out by using internally labeled substrate **LS-T**. Then, the left-hand product was treated with FastAP™ (37 °C for 30 min). Lane 1: internally-labeled **LS-T**; Lane 2: cleavage of **LS-T** by **F-8** and 100 μM H_2O_2 (for 20 min) under the standard reaction conditions. Lane 3: treatment of cleavage product in lane 2 with with FastAP™ enzyme; Lane 4: internally-labeled **LS-T**; Lane 5: cleavage of **LS-T** by **F-8** and 100 μM DTT (for 20 min) under the standard reaction conditions. Lane 6: treatment of cleavage product in lane 5 with with FastAP™ enzyme. Cleavage assays were under standard single-turnover conditions. (50 mM HEPES pH 7.4, 400 mM NaCl, 100 mM KCl, 10 mM MgCl_2 , 7.5 mM MnCl_2 , 50 μM CuCl_2) at 37 °C. These results demonstrated that left-hand cleaved product carries an unmodified phosphate group at its 3' terminus.

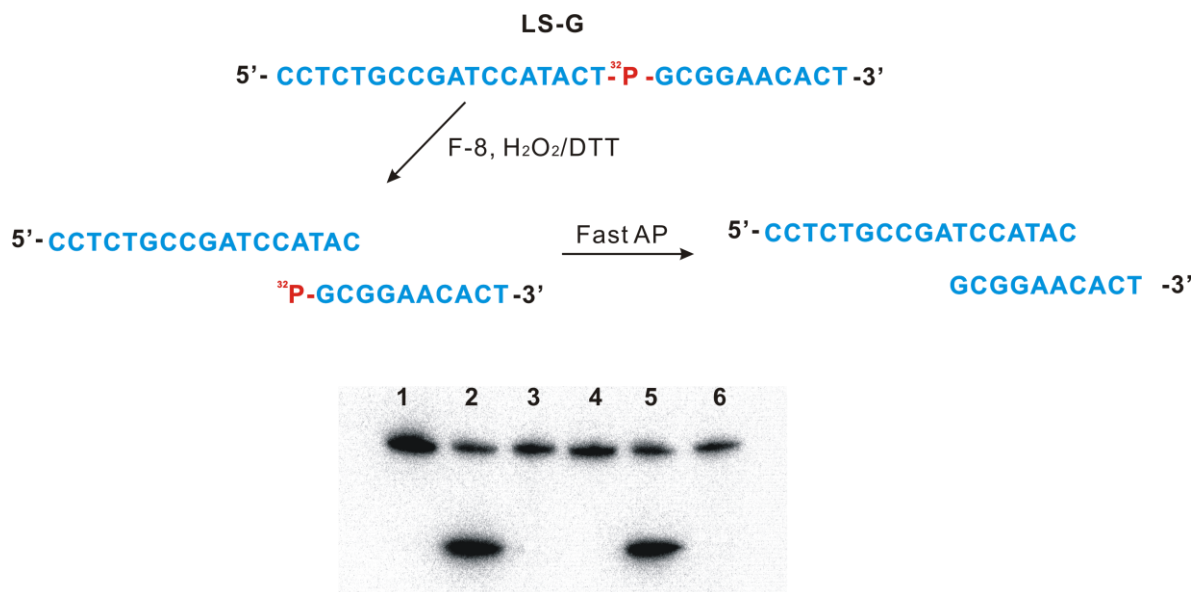


Figure S8.5. Dephosphorylation with FastAP™ enzyme assays to confirm 5'-terminal of right-hand cleavage product by **F-8** and H₂O₂/DTT. Cleavage reactions were carried out by using internally labeled substrate **LS-G**. Then, the left-hand product was treated with FastAP™ (37 °C for 30 min). Lane 1: internally-labeled **LS-G**; Lane 2: cleavage of **LS-G** by **F-8** and 100 μM H₂O₂ (for 20 min) under the standard reaction conditions. Lane 3: treatment of cleavage product in lane 2 with with FastAP™ enzyme; Lane 4: internally-labeled **LS-G**; Lane 5: cleavage of **LS-G** by **F-8** and 100 μM DTT (for 20 min) under the standard reaction conditions. Lane 6: treatment of cleavage product in lane 5 with with FastAP™ enzyme. Cleavage assays were under standard single-turnover conditions. (50 mM HEPES pH 7.4, 400 mM NaCl, 100 mM KCl, 10 mM MgCl₂, 7.5 mM MnCl₂, 50 μM CuCl₂) at 37 °C. These results demonstrated that left-hand cleaved product carries an unmodified phosphate group at its 3' terminus.

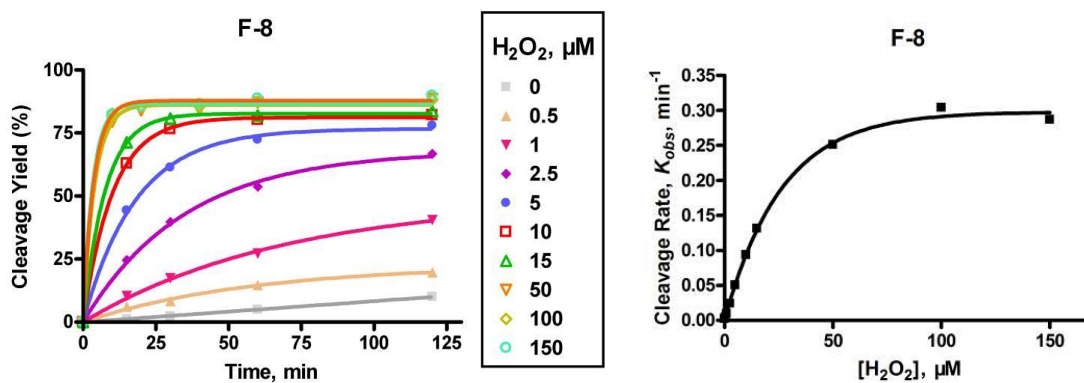


Figure S8.6. Single-turnover experiments to validate the participation of H₂O₂ in the DNA cleavage reactions catalyzed by **F-8**. Assays were performed under the standard reaction conditions (50mM HEPES, 400mM NaCl, 100mM KCl, 10 mM MgCl₂, 7.5 mM MnCl₂, 50 μM CuCl₂, pH 7.4, 37 °C), except for the variation of the H₂O₂ concentration (ranging from 0 to 150 μM). Kinetic characterizations were shown respectively. In the presence of 50 μM H₂O₂, the cleavage rate for **F-8** was increased rapidly with maximal $k_{obs} \sim 0.25$ min⁻¹ (~107-fold) for **F-8**.

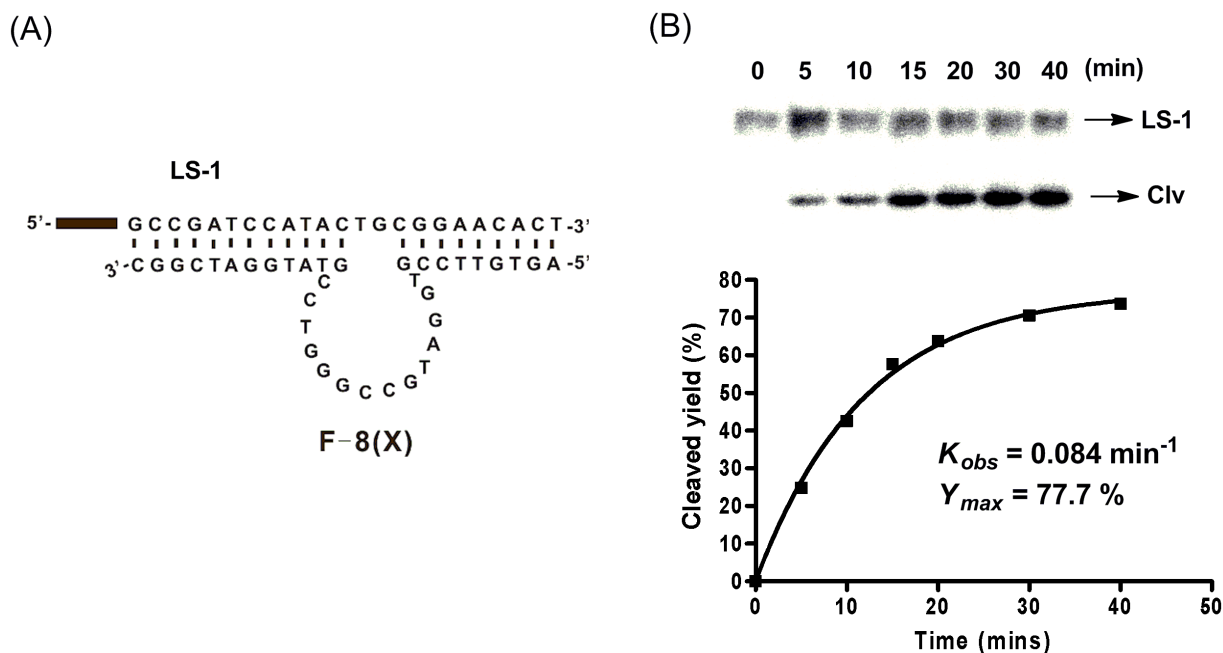


Figure S8.7: Kinetic plots of shortest deoxyribozyme **F-8(X)** in the presence of H_2O_2 . Time course experiment was performed under single-turnover conditions in the presence of $50 \mu\text{M H}_2\text{O}_2$. The total cleaved yield of substrate was plotted over time and curve fitted under single-turnover kinetic. Rate constant (k_{obs}) and maximum cleavage yields (Y_{max}) is reported on the curve. In the presence of $50 \mu\text{M H}_2\text{O}_2$, it also can rapidly enhance catalytic rate (~ 103 -fold).

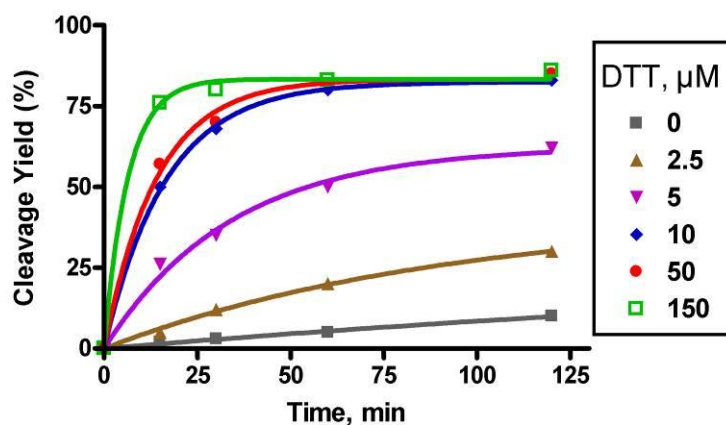
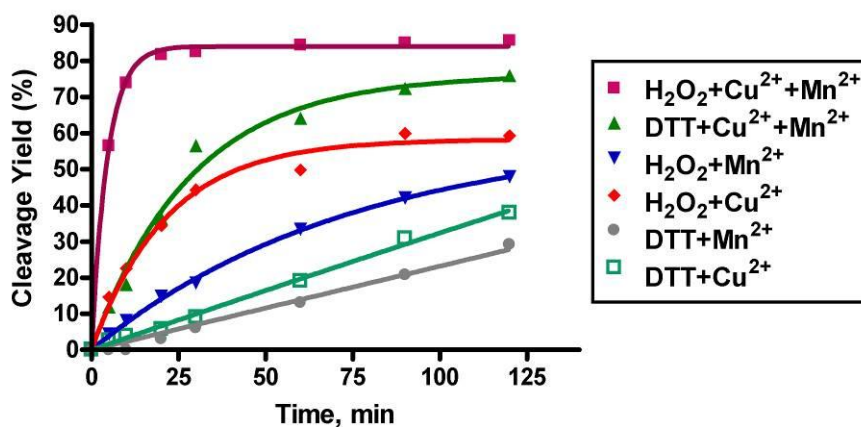
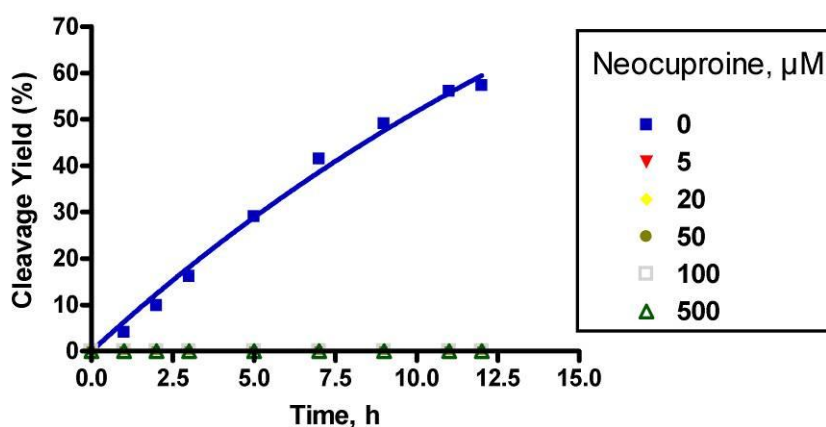


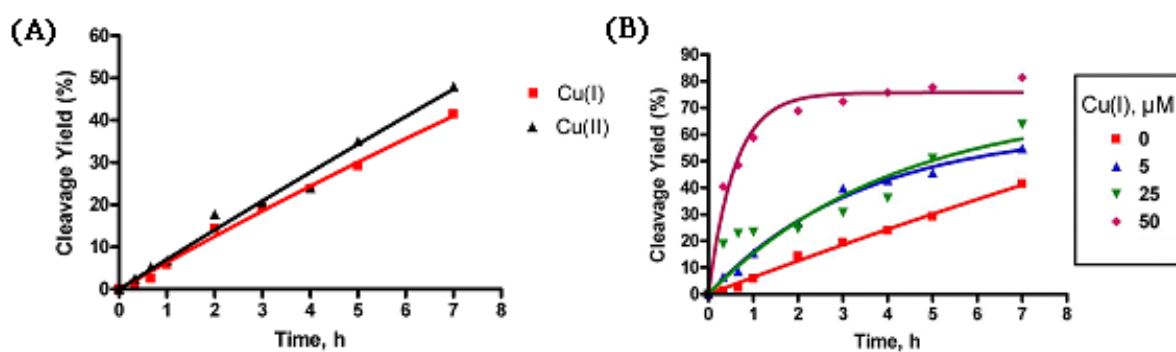
Figure S8.8. Single-turnover experiments to validate the participation of DTT in the DNA cleavage reactions catalyzed by **F-8**. Assays were performed under the standard reaction conditions (50 mM HEPES , 400 mM NaCl , 100 mM KCl , 10 mM MgCl_2 , 7.5 mM MnCl_2 , $50 \mu\text{M CuCl}_2$, $\text{pH } 7.4$, $37 \text{ }^\circ\text{C}$), except for the variation of the DTT concentration (ranging from 0 to $150 \mu\text{M}$).



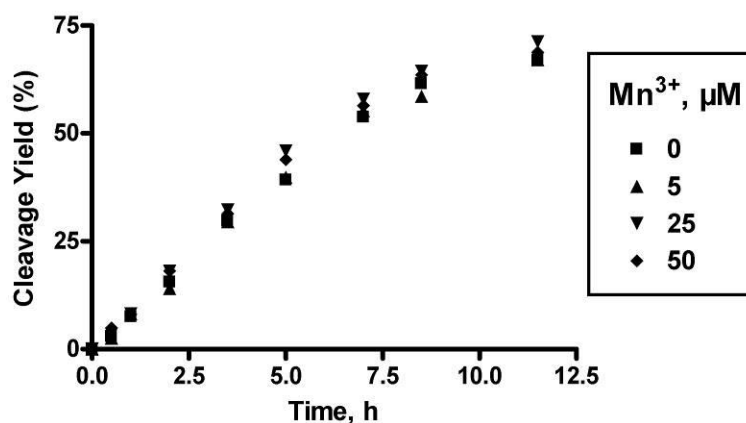
FigureS8.9. The effect of DTT and H₂O₂ either in addition to Mn²⁺ and Cu²⁺ or in place of one of the two metal ions were investigated. Assays were performed under the standard reaction conditions (50 mM HEPES, 400 mM NaCl, 100 mM KCl, 10 mM MgCl₂, 7.5 mM MnCl₂, 50 μM CuCl₂, pH 7.4, 37 °C).



FigureS8.10. Single-turnover experiments to validate the presence of Cu(I) chelator neocuproine in DNA cleavage by **F-8**. A 1 mM stock solution of neocuproine in ethanol was prepared. Assays were performed under the standard incubation conditions supplemented with neocuproine (5-500 μM final ethanol concentration 10 % v/v). The assay without any added neocuproine but with 10 % ethanol revealed a modest decrease in rate and yield due solely to the ethanol, which is required for neocuproine solubility. Inclusion of neocuproine was detrimental to catalytic function. We could not find any cleavage products.



FigureS8.11. Single-turnover experiments to validate the presence of Cu(I) in DNA cleavage by **F-8**. A 1 mM stock solution of tetrakis (acetonitrile) copper(I) hexafluorophosphate in acetonitrile was prepared. (A) Cleavage reactions were performed under the incubation conditions (50 mM HEPES pH 7.4, 400 mM NaCl, 100 mM KCl, 10mM MgCl₂ and 7.5 mM MnCl₂) supplemented with Cu(I) (red) or Cu(II) (black) (final acetonitrile concentration 5 % v/v). (B) Assays were performed under the standard incubation conditions (50 mM HEPES pH 7.4, 400 mM NaCl, 100 mM KCl, 10mM MgCl₂, 7.5 mM MnCl₂, 50 μM CuCl₂) supplemented with Cu(I) (0-50 μM final acetonitrile concentration 5 % v/v). The assay without any added Cu(I) but with 5 % acetonitrile revealed a modest decrease in rate and yield (red line).



FigureS8.12. Single-turnover experiments to validate the absence of Mn(III) in DNA cleavage by **F-8**. A 1 mM stock solution of Mn(OAc)₃•2H₂O in ethanol was prepared. Assays were performed under the standard incubation conditions supplemented with Mn(III) (5-50 μM final ethanol concentration 5% v/v). The assay without any added Mn(III) but with 5 % ethanol revealed a modest decrease in rate and yield.

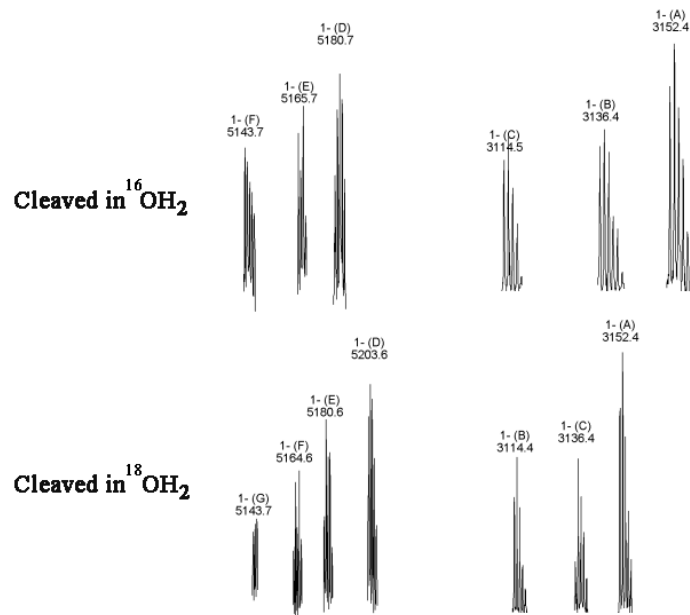
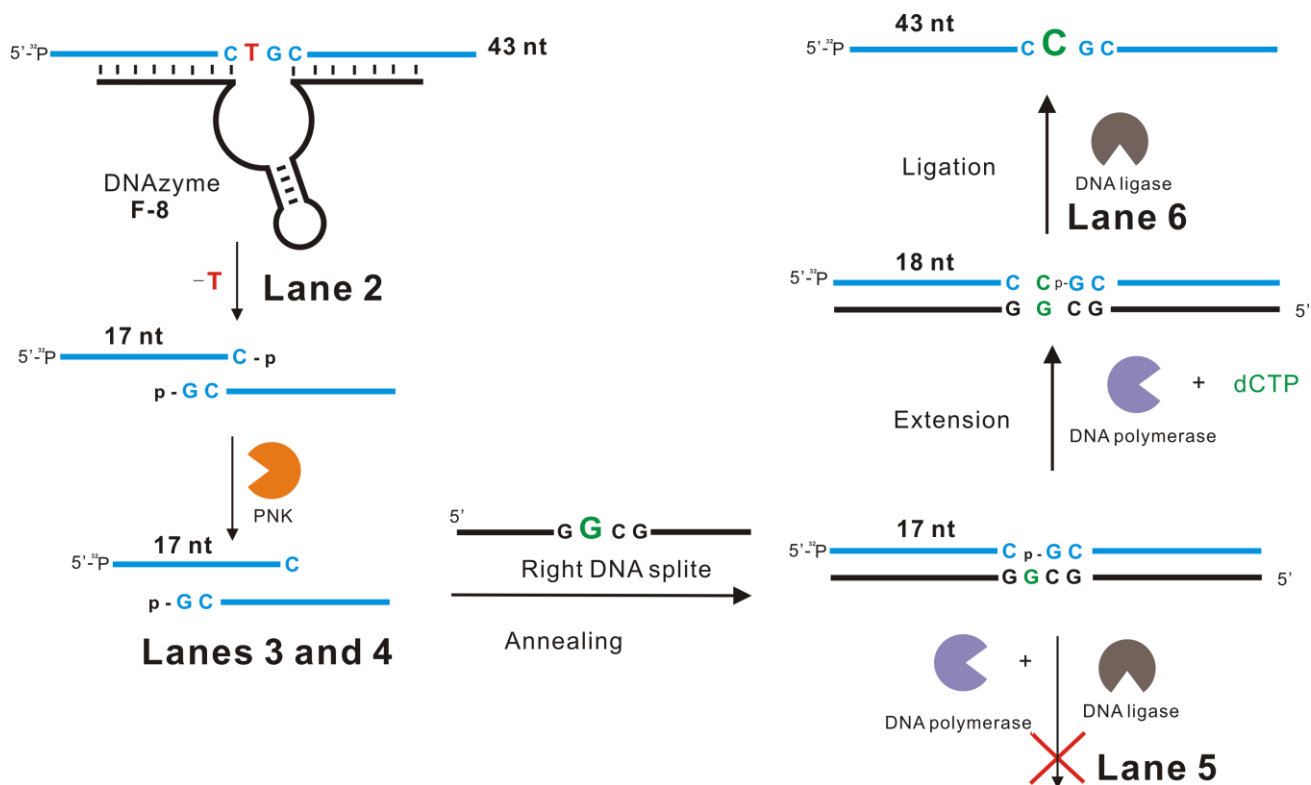


Figure S8.13. Cleaving reactions catalyzed by **F-8** in ^{18}O -water or in natural-abundance. In (^{16}O) water, calcd for left-hand product $[\text{M}-\text{H}]^-$ 5142.8, found 5143.7; $[\text{M}-2\text{H}+\text{Na}]^-$ 5164.8, found 5165.7; $[\text{M}-2\text{H}+\text{K}]^-$ 5180.7, found 5180.7. Calcd for right-hand product $[\text{M}-\text{H}]^-$ 3114.5, found 3114.5; $[\text{M}-2\text{H}+\text{Na}]^-$ 3136.4, found 3136.4; $[\text{M}-2\text{H}+\text{K}]^-$ 3152.4, found 3152.4. In ^{18}O -water, calcd for left-hand product $[\text{M}-\text{H}]^-$ 5142.8, found 5143.7; $[\text{M}-2\text{H}+\text{Na}]^-$ 5164.8, found 5164.6; $[\text{M}-2\text{H}+\text{K}]^-$ 5180.7, found 5180.6. Calcd for right-hand product $[\text{M}-\text{H}]^-$ 3114.5, found 3114.4; $[\text{M}-2\text{H}+\text{Na}]^-$ 3136.4, found 3136.4; $[\text{M}-2\text{H}+\text{K}]^-$ 3152.4, found 3152.4. These results indicate that the oxygen atom in the cleaved 5'-or 3'-phosphate group is not come from water, which exclude the hydrolysis mechanism.



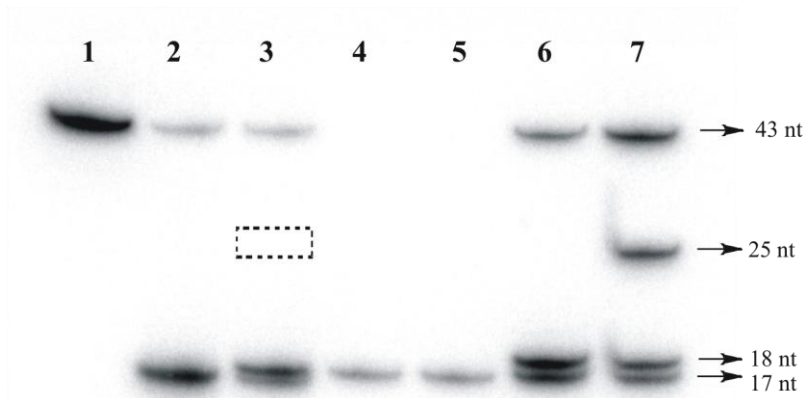


Figure S8.14. Control experiments of conversion of a single deoxybonucleotide T in a DNA strand to a deoxyribonucleotide C using a combination of DNAzyme F-8 and natural enzymes. 20% PAGE analysis of the various stages of the single-nucleotide excision repair process. Lane 1, the original 43-nt 5'-³²P-labelled DNA substrate. Lane 2, cleavage products generated by **F-8**. Lane 3, the cleavage products after dephosphorylation with PNK. The dotted box indicates the expected position of the downstream cleavage product, which was invisible on the autoradiogram since it contained no ³²P. Lane 4, recovered the processed DNA fragments. Lane 5, extension and ligation reactions in the absence of dCTP. Lane 6, the reaction products obtained after purifying the cleavage fragments, annealing them with right DNA splint, extending them by a C (18 nt) using DNA polymerase, and finally ligating to generate full-length substrate (43 nt). Lane 7, synthetic DNA markers. Cleavage assays were under standard single-turnover conditions (about 1 μM deoxyribozyme combined with 10 nM substrate).



Figure S8.15. PAGE gel analysis of the cleavage assay of repaired oligonucleotide. Lane 1, the original 43-nt 5'-³²P-labelled DNA substrate; Lane 2, cleavage products generated by **F-8**; Lane 3, recovered base repair oligonucleotide in Figure S8.14, lane 6; Lane 4, repaired oligonucleotide cleavage by F-8.