Electronic Supplementary Material (ESI) for Chemical Science.

Supporting Information for

Generation of artificial sequence-specific nucleases via preassembled inert-template

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Table S1. Sequences of DNA or RNA used in this work

Strand name	Sequence (from 5' to 3')[a]	Melting temperature(°C)[b]
S-DNA-1	A*A*A*A*A*A*A*A*A*A*A*G*G*T*G*C*A*T*C*T*A*G*T*G*C*A*G*A*T*A	60.8
Mid-labeled-S-DNA-1	A*A*A*A*A*A*A*A*A*A*A*G*G*T*G*C*A*T*C*T(-FAM)*A*G*T*G*C*A*G*A*T*A	60.8
Target-1	5'FAM-TAT CTG CAC TAG ATG CAC CTT-3'BHQ-1	61.2
c-Target-1	AAG GTG CAT CTA GTG CAG ATA	61.2
Unlabeled Target-1#	TAT CTG CAC TAG ATG CAC CTT	61.5
S-DNA-1-1 mismatch	A*A*A*A*A*A*A*A*A*A*A*G*G*T*G*C*A*T*C*T*A***C***T*G*C*A*G*A*T*A*	60.8
S-DNA-1-2 mismatch	A*A*A*A*A*A*A*A*A*A*A*G*G*T*G*C*A*T*C*T*A***C***T***C***C*A*G*A*T*A*	60.0
S-DNA-1-3 mismatch	A*A*A*A*A*A*A*A*A*A*A*G*G*T*G*C*A*T*C*T***C******C***T***C***C*A*G*A*T*A*	62.3
S-DNA-1-4 mismatch	A*A*A*A*A*A*A*A*A*A*A*G*G*T***C***C*A*T*C*T***C******C***T***C***C*A*G*A*T*A*	61.5
S-DNA-1-5mismatch	A*A*A*A*A*A*A*A*A*A*A*G*G*T***C***C***C***T*C*T***C******C***T***C***C*A*G*A*T*A*	63.1
S-DNA-2	A*A*A*A*A*A*A*A*A*G*G*T*T*G*A*G*T*A*G*A*G*C*C* A*C*A*T	58.4
Target-2	5′FAM-ATG TGG CTC TAC TCA ACC-3′BHQ-1	58.6
Target-3	AAA AAA AAA AT(-FAM)AT CTG CACT AG ATG CAC CTT(-BHQ-1)A AAA AAA AAA	61.2
c-Target-3	TTT TTT TTT TAA GGT GCA TCT AGT GCA GAT ATT TTT TTT TT	61.2
59-bp dsDNA	(+)ACT ACG AAT TCA AGA TCA TAT CTG CAC TAG ATG CAC CTT ACT CAG TCA ATG CAA TAC CA (-)TG GTA TTG CAT TGA CTG AGT AAG GTG CAT CTA GTG CAG ATA TGA TCT TGA ATT CGT AGT	75.4
S-RNA strand	A*U*A*C*A*G*C*U*A*A*G*G*U*G*C*A*U*C*U*A*G*U*G*C*A*G*A*A*U*A	60.1
Target RNA strand	5'FAM-UAUCUGCACUAGAUGCACCUU-3'BHQ-1	60.5
MB-1	5'FAM-CGGAAGACAGATGCTAAGTGCTTGACCTTCCG-3'Dabcyl	57.7
MB-2	5'FAM-CCT GGC ACT CCA CAG TCA CAT ACT GCC AGG-3'Dabcyl	69.4

MB-3	5'FAM-CGT CGC TGG GTA GGG CGG GTT GGG GCG ACG-3'Dabcyl	66
ssDNA-1	5'FAM-ATG TGG CTC TAC ATC AAC C-3'BHQ-1	59.4
ssDNA-2	5'FAM-GAT CCT GGC GTA GGC AAG AGT-3'BHQ-1	65.8
dsDNA	5'FAM-ACT ACT GGA GTA TGT GTC TGT GGA GA-3'BHQ-1 TGA TGA CCT CAT ACA CAG ACA CCT CT	66.1
DNA strand	AAAAAAAAAAAGGTGCATCTAGTGCAGATA	61.2
S-DNA-18-nt	A*A*A*A*A*A*A*T*T*A*A*G*G*T*G*C*A*T*C*T*A*G*T*G*C*A*G*T*A*A*	58.8
S-DNA-15-nt	A*A*A*A*A*A*A*T*T*G*T*G*C*A*T*C*T*A*G*T*G*C*A*G*T*A*A*C*A*T*	53.1
S-DNA-14-nt	A*A*A*A*A*A*A*T*T*A*G*G*T*G*C*A*T*C*T*A*G*T*G*A*T*A*T*A*A*C*	46.1
S-DNA-13-nt	A*A*A*A*A*A*A*T*T*G*G*T*G*C*A*T*C*T*A*G*T*G*A*T*A*T*A*A*C*A*	42.3
S-DNA-12-nt	A*A*A*A*A*A*A*T*T*G*T*G*C*A*T*C*T*A*G*T*G*A*T*A*T*A*A*C*A*T*	35.2[c]
S-DNA-9-nt	A*A*A*A*A*A*A*T*T*C*A*T*C*T*A*G*T*G*A*T*A*T*A*A*T*A*A*C*A*T*	23.1[d]
S-DNA-14-nt mismatch	A*A*A*A*A*A*A*T*T*A*G*G*T*G*C****C****T*C*T*A*G*T*G*A*T*A*T*A*A*C*	46.3
Unrelated RNA strand	UUGUACUACACAAAAGUACUG	46.4
S-DNA-LNA	A*A*A*A*A*A*A*A*A*A*A*G*G*T*G****C****A*T*****C****T*A*G*T*G*C*A*G*A*T*A	70.2

[a] The complementary parts are underlined. The mismatch bases are shown in bold and italics. The phosphorothioated nucleotides (at 3' side) are indicated with an asterisk after the nucleotides. FAM is fluorescein. Dabcyl is 4-((4-(dimethylamino)phenyl)azo)-benzoyl. BHQ1 is Black Hole Quencher 1. In S-DNA-LNA, the nucleotides shown in bold are LNA nucleotides.

[b] For those sequences with underlined regions, the melting temperatures were measured experimentally by using a DNA intercalating dye (LC green) for their hybrids with the sequence only complementary to the underlined parts; For other sequences without the underlined regions, the melting temperatures were measured experimentally for their hybrids with the totally complementary sequences.

[c-d] These two melting temperatures were predicted by Oligoanalyzer as the two duplexes were too short. Their melting temperatures could not be precisely determined by the above LC green method.

Materials and methods

Materials

DNase I and RNase A were purchased from Sangon Co. (China). DNase buffer was purchased from New England Biolabs (MA, USA). DNA strands were synthesized and purified by HPLC (Sangon Co., China). RNA strands were synthesized and purified by Genepharma (China). The sequences of all the DNA and RNA strands that have been studied in this work are summarized in Table S1. GelSafe Dye was from Yuanpinghao Bio. (Beijing, China).

Preparation of the DNase I/S-DNA complex.

1 μL of 5 mg/mL DNase I was mixed with 1.45 nmol of S-DNA in 50 μL of Buffer-1 (50 mM sodium phosphate, 0.15 M NaCl, 1 mM EDTA, pH 7.2) and incubated at 37ºC for 1 h. The resultant solution was ultrafiltrated using ultrafiltration tubes (Sartorius, China) with Buffer 2 (50 mM sodium phosphate, 0.15 M NaCl, pH 7.2) for two times with a cutoff at 30 KDa to remove the excess free S-DNA strands. The first ultrafiltration was performed at 4ºC, 5000 g, and the second ultrafiltration at 4ºC, 3000 g.

Preparation of the RNase A/S-RNA complex.

1 μL of 2 mg/mL RNase A was mixed and incubated with 1.55 nmol of S-RNA strand in 50 μL of Buffer-1 at 37ºC for 1 h. The following steps were the same as above procedures.

Fluorescence measurement

To a 200 μL PCR tube, 3 μL of the DNase I/S-DNA complex solution was mixed with 1 μL of target strands or reference strands (50 μmol) in 46 μL of DNase Buffer. The solution was immediately put into a Rotor-Gene Q 5plex Instrument (QIAGEN, Hilden, Germany) for fluorescence measurement at 37ºC. Fluorescence intensity was recorded once a cycle (5 s per cycle) for 240 cycles with gain level of 7. The excitation and emission wavelengths were set to 470 nm and 510 nm, respectively. The rate of fluorescence increase was determined by the slope of the linear portion of the time curve.

For the reaction of normal DNase I with different DNA strands, 1 μL of 50 ng/μL DNase I was mixed with1μL of target strands or reference strands (50 μmol) in a 50-μL DNase Buffer solution. Then the fluorescence intensity of the solution was measured in the same manner as described above. The gain level was set at 3.

Agarose gel electrophoresis analysis

Agarose gel electrophoresis was carried out using 2.5% agarose gel at 120 V for 30 min in a 0.5×TBE buffer (90 mMTris, 90 mM boric acid, 2 mM EDTA; pH 8.0). 9 μL of samples solution and 1.8 μL of loading buffer were added to each well. After separation, the gels containing DNA or RNA were stained using GelSafe Dye and visualized at a wavelength of 595 nm using a Tanon 1600 gel imaging system (Tanon, China). For the experimental results shown in Fig. 3c, the gel was not stained and the signals were produced by FAM labeled on the target RNA strand and detected at 535 nm.

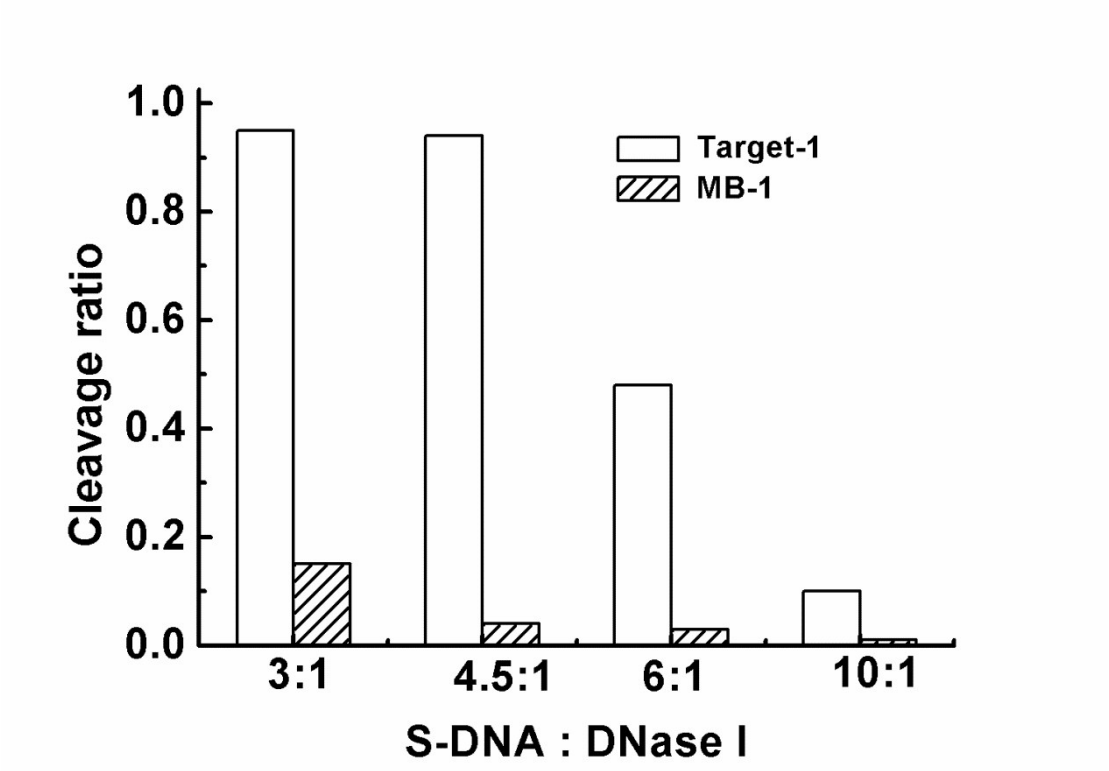

Figure S1. Cleavage ratios of Target-1 and unrelated strand (MB-1) by the DNase I/S-DNA-1 complex prepared at different S-DNA-1 to DNase I molar ratios. The concentration of DNase I is fixed at 0.1 mg/mL and the incubation time is 1 h. The cleavage ratio was defined as the ratio of ΔF to ΔF_T and determined based on the data shown in Fig. 1b. ΔF_T denoted the total increase of the fluorescence intensity of Target-1 or MB-1 and was obtained by subtracting its initial background fluorescence intensity from its maximum fluorescence intensity when completely degraded by normal DNase I (the plateau value in Fig 1b). ΔF denoted the increase of the fluorescence intensity of Target-1 or MB-1 after 15-min degradation by the DNase I/S-DNA-1 complex and was obtained by subtracting the initial background fluorescence intensity from that observed at the end-point of the reaction (900 s).

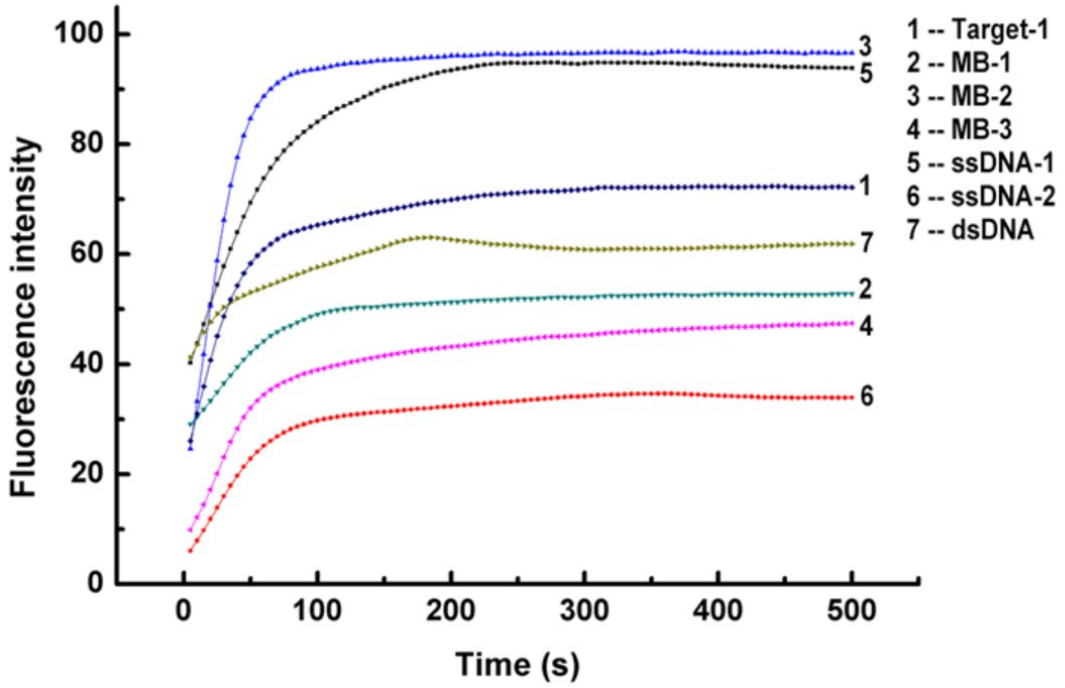

Figure S2. Degradation of Target-1 and six unrelated sequences by normal DNase I.

The cleavage was finished within 100 s.

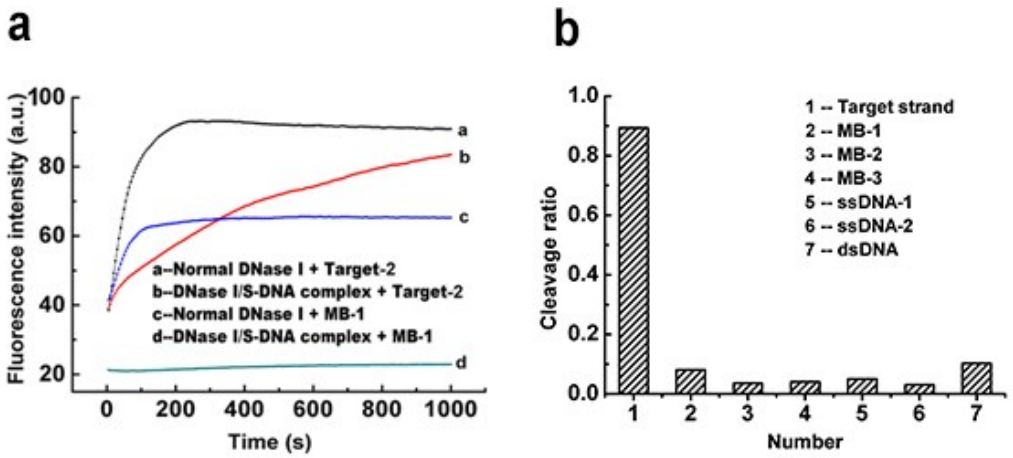

Figure S3. The DNase I/S-DNA-2 complex showed sequence specificity toward Target-2 (a), and the specificity is uniformly high against different unrelated strands (b).

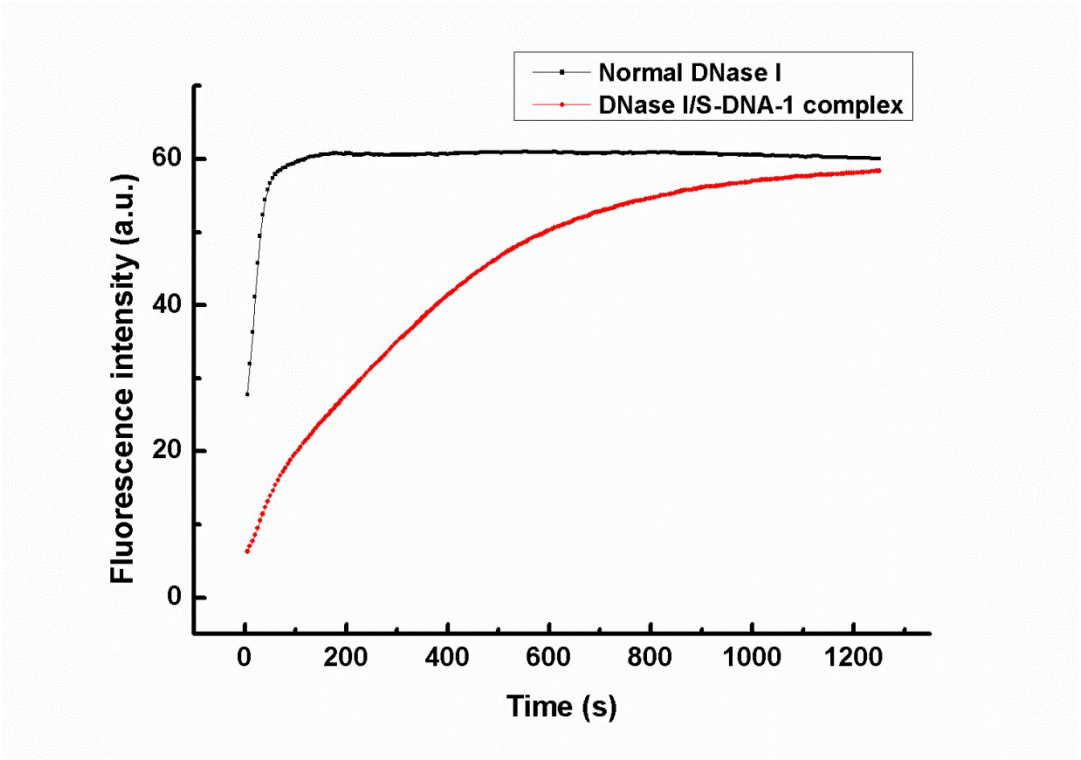

Figure S4. Degradation of longer target strands (Target-3) by the DNase I/S-DNA-1 complex. The cleavage rate is similar to that of the shorter target strands (Target-1).

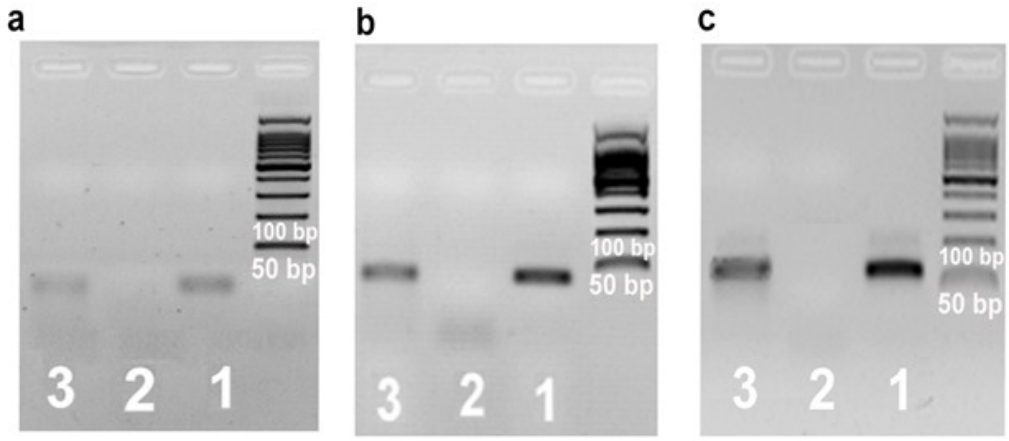

Figure S5. Gel electrophoresis results of DNase I/S-DNA-1 complex cleaving (a) 21-bp dsDNA (Target-1/c-Target-1 duplex), (b) 41-bp dsDNA (Target-3/c-Target-3 complex), and (c) 59-bp dsDNA (containing the 21-bp sequence of Target-1/c-Target-1 duplex). Lane 1: dsDNA. Lane 2: dsDNA + normal DNase I. Lane 3: dsDNA + DNase I/S-DNA-1 complex. All the cleavage reactions lasted for 15 min.

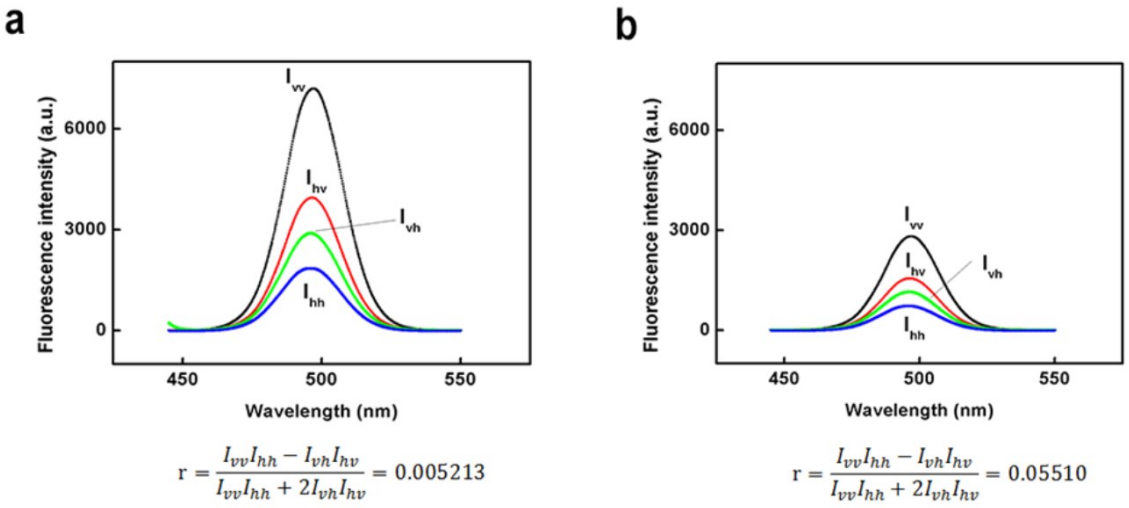

Figure S6. Fluorescence anisotropy results for verification of the formation of DNase I/S-DNA complex. (a) Mid-labeled-S-DNA-1 mixed with DNase I. The fluorescence anisotropy of the solution was measured immediately after the Mid-labeled-S-DNA-1 was mixed with DNase I. (b) DNase I/Mid-labeled-S-DNA-1 complex. The fluorescence anisotropy of (b) increased by about 10 folds in comparison with that of (a), indicating strong binding between DNase I and Mid-labeled-S-DNA-1 after incubation. I_{vv} corresponds to vertically polarized excitation and vertically polarized emission. I_{vh} corresponds to vertically polarized excitation and horizontally polarized emission. I_{hh} corresponds to horizontally polarized excitation and horizontally polarized emission. I_{hv} corresponds to horizontally polarized excitation and vertically polarized emission.

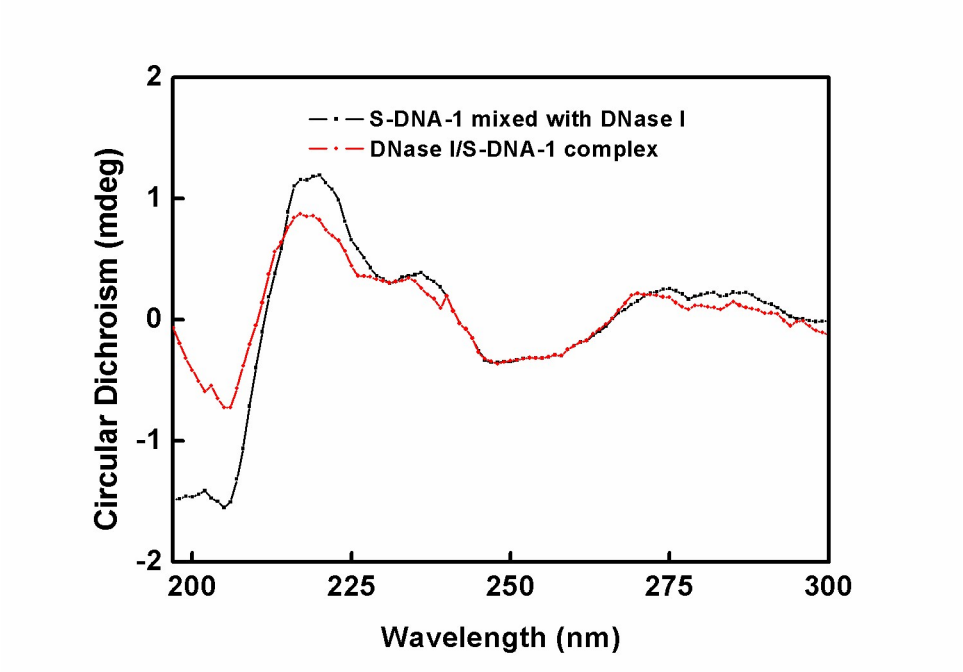

Figure S7. CD spectra results for verification of the formation of DNase I/S-DNA complex. The two curves showed significant difference at ~220 nm, indicating that the secondary structure of S-DNA after incubation with DNase I for 1 h was more compressed than that of S-DNA freshly mixed with DNase I[1]. These results clearly proved that S-DNA was tightly bound to DNase I and confined in a limited space.

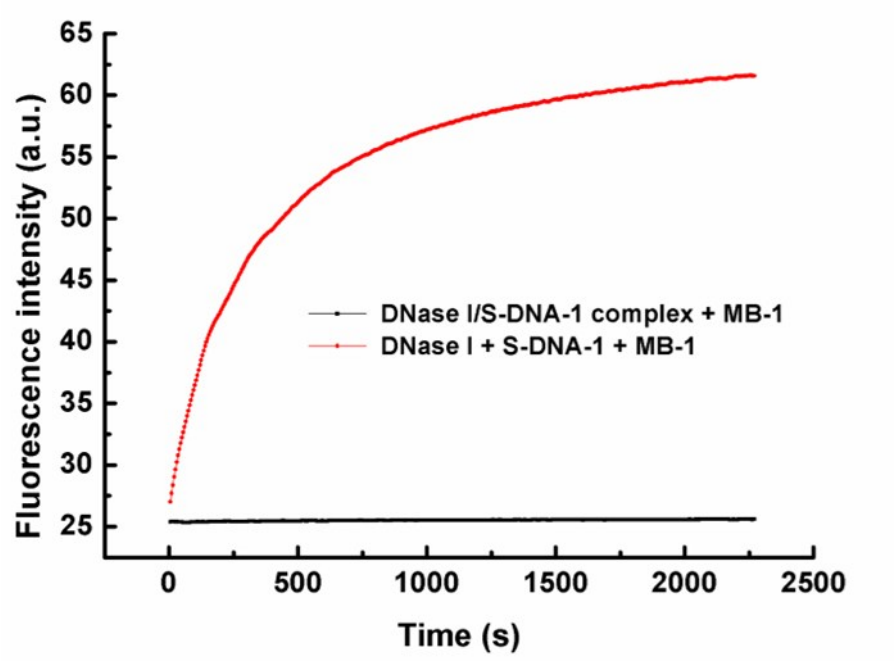

Figure S8. Measurement of the dissociation constant of DNase I/S-DNA complex. In Buffer I, 0.1 mg/mL DNase I and S-DNA-1 at a molar ratio of 1:4.5 was incubated at 37°C for 1 h. The residual activity of the product solution (corresponding to the remaining amount of free DNase I) was measured by MB-1. The total amount of DNase I was measured by first incubating the free enzyme in the absence of S-DNA-1 under the same conditions as above and then reacting the product solution with MB-1 with the addition of S-DNA-1.

The cleavage rate of MB-1 by the total amount of DNase I was observed to be 508-fold of that by the residual DNase I in the product solution. The amount of resultant DNase I/S-DNA-1 complex was obtained by subtracting the residual amount of free DNase I from the total amount of DNase I. So the dissociation constant of DNase I/S-DNA-1 complex can be calculated according to the following equation,

$$K_d = \frac{[DNase\ I][S-DNA-1]}{[DNase\ I/S-DNA-1]}$$

which was found to be 22 nM.

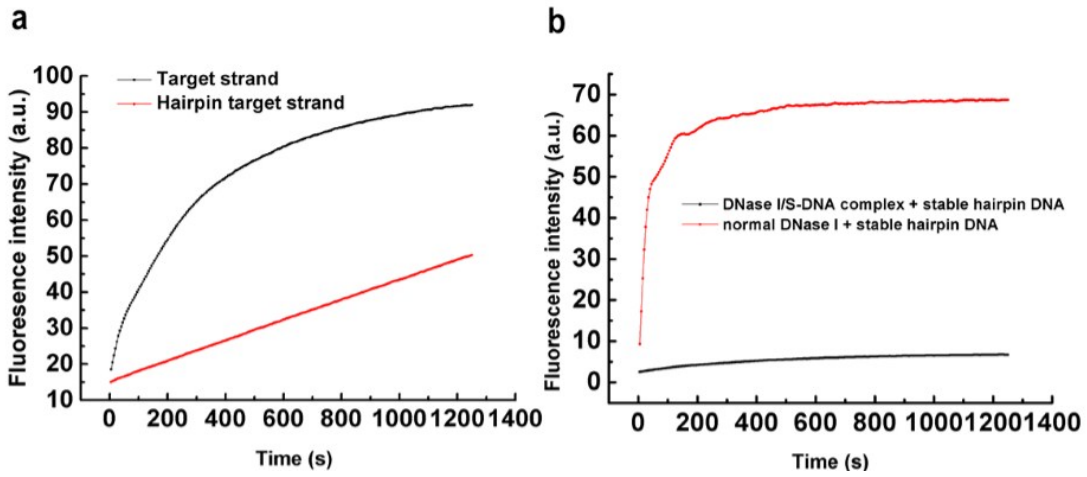

Figure S9. (a) Cleavage of a hairpin DNA containing the sequence of Target-1 by the DNase I/S-DNA-1 complex. The free Target-1 strand was also measured for comparison. The sequence of the hairpin target strand is 5'FAM-CGG CCG TAT CTG CAC TAG ATG CAC CTT CGG CCG-3' Dabcyl (the underlined part was the same as the squence of Target-1). (b) Cleavage of a stable hairpin DNA target by the DNase I/S-DNA complex. The sequence of the target hairpin DNA is 5'FAM-CGCCGC GAA GCGGCG-3'Dabcyl, and the sequence of the template S-DNA is 5'-AAAAAAAAA CGCCGC TTC GCGGCG-3'. Since both the target DNA and the template S-DNA may be folded into stable hairpin structures by themselves, it is very difficult for them to hybridize with each other. Thus the cleavage rate of the target DNA is very slow.

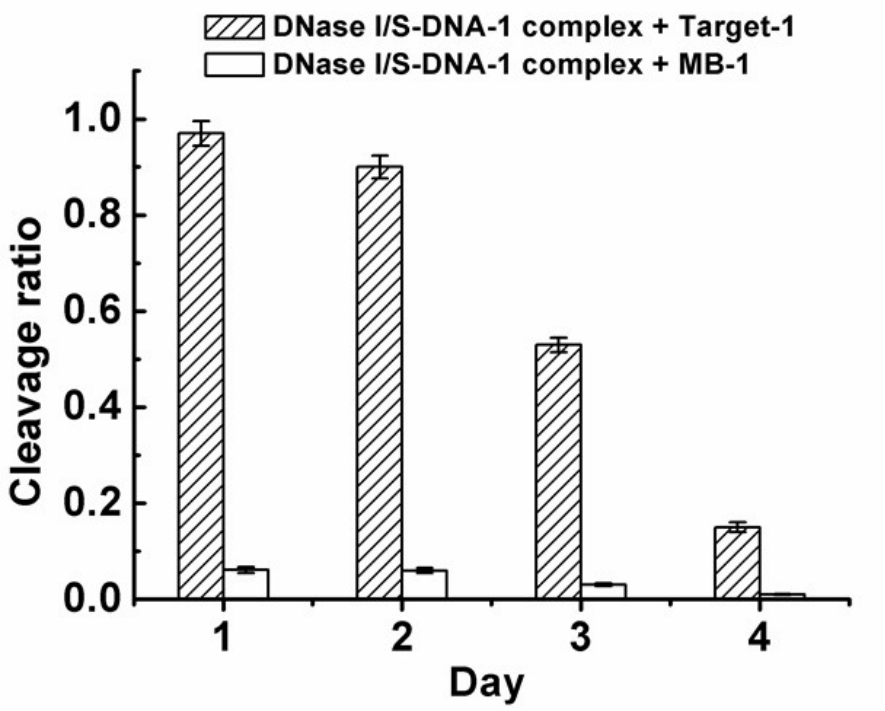

Figure S10. The sequence specificity of DNase I/S-DNA-1 complex after storage at 4ºC for 4 days. The cleavage ratio is determined by comparison of the cleavage rate of the same DNA strand by the DNase I/S-DNA-1 complex to that by normal DNase I. The DNase I/S-DNA-1 complex could be stably stored at 4ºC for two days without significant loss of its specific cleavage activity.

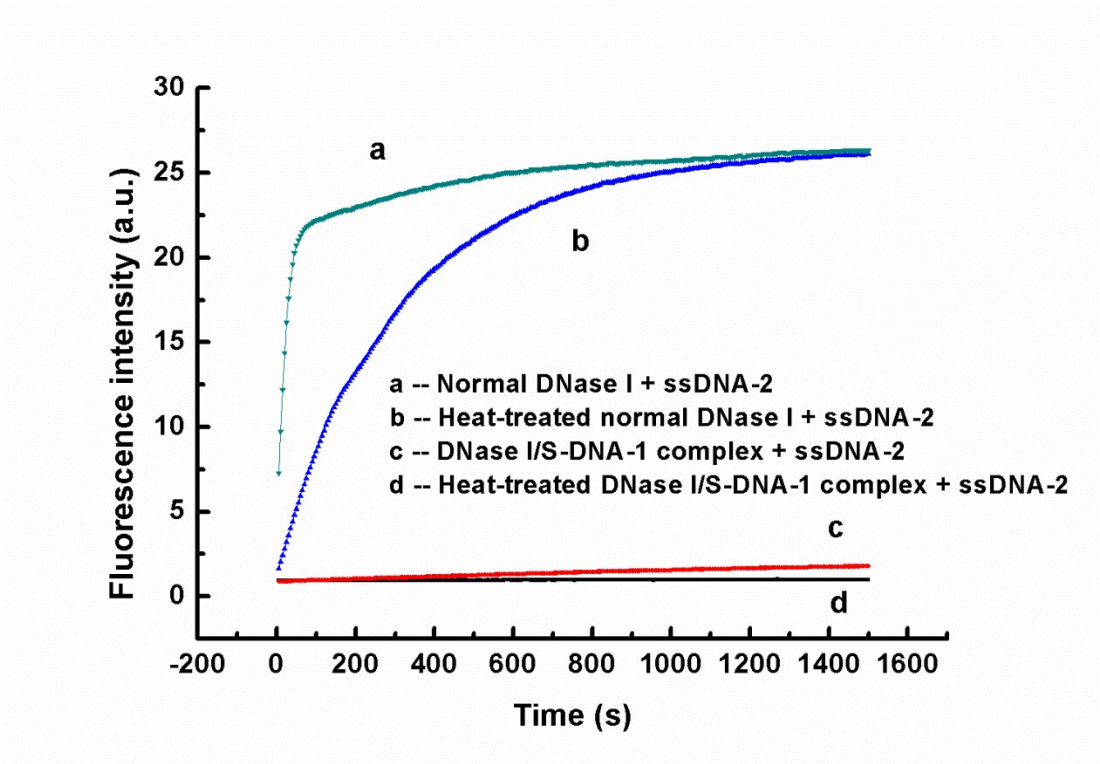

Figure S11. The stability of DNase I/S-DNA complex after heat treatment at 50ºC for 10 min. The normal DNase I was also tested in the same manner for comparison. ssDNA-2 was used as a probe to monitor the variation of the enzyme activities before and after the heat treatment. Since even free DNase I lost most of its activity at 53°C after 10 min, temperature higher than 50°C was not further tested for the DNase I/S-DNA complex.

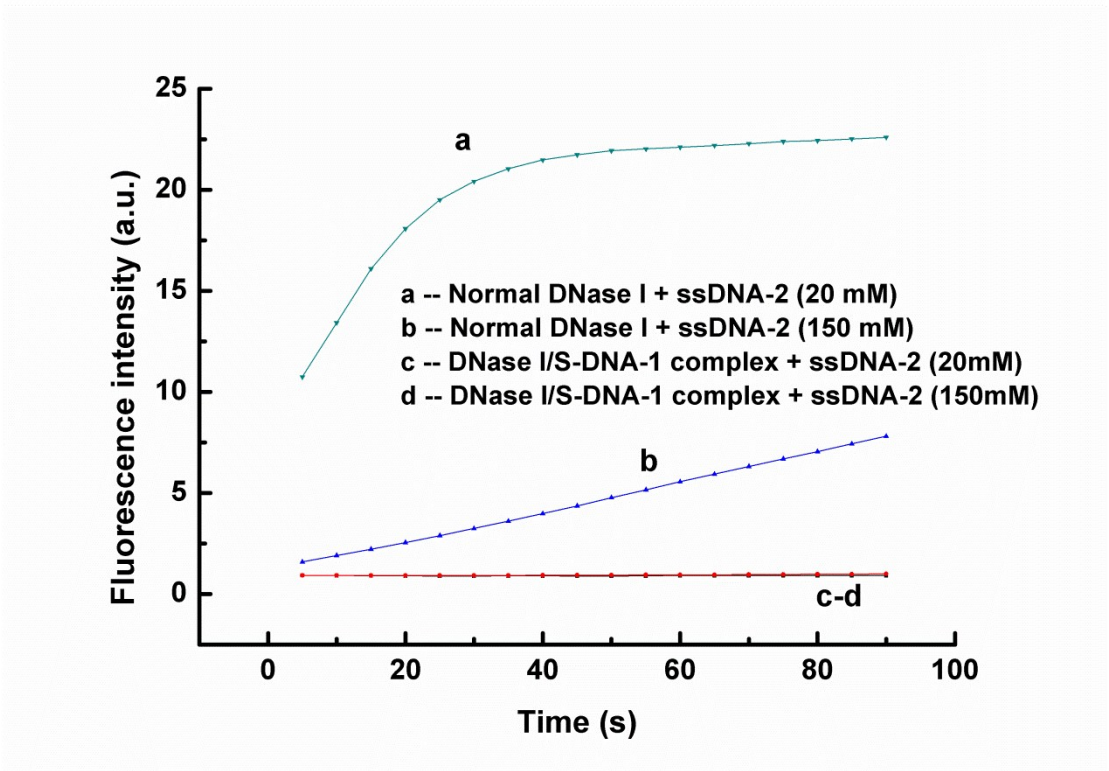

Figure S12. The stability of DNase I/S-DNA complex at normal (20 mM) and high (150 mM) salt concentrations. The normal DNase I was also tested in the same manner for comparison. ssDNA-2 was used as a probe to monitor the variation of the enzyme activities under different conditions. Since even free DNase I lost most of its activity at salt concentration of 150 mM, higher salt concentration was not further tested for the DNase I/S-DNA complex.

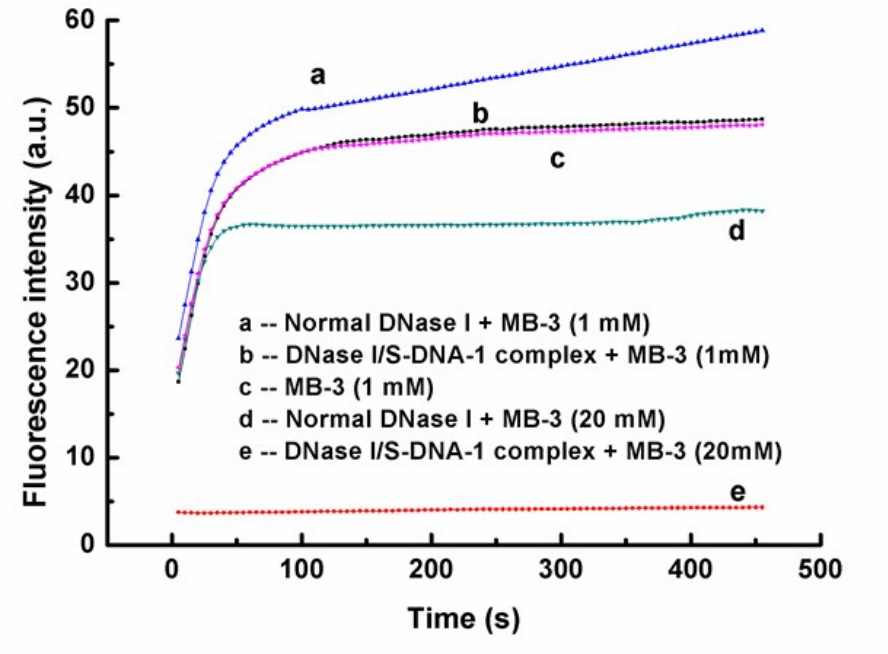

Figure S13. The stability of DNase I/S-DNA complex at normal (20 mM) and low (5 mM) salt concentrations. The normal DNase I was also tested in the same manner for comparison. MB-3 was used as a probe to monitor the variation of the enzyme activities under different conditions. At low salt concentration (1 mM), MB-3 itself shows a notable background fluorescence (Curve c), most likely because the stem part tends to stretch out at low salt concentrations. Since even free DNase I lost most of its activity at salt concentrations lower than 1 mM, it was not further tested for the DNase I/S-DNA complex.

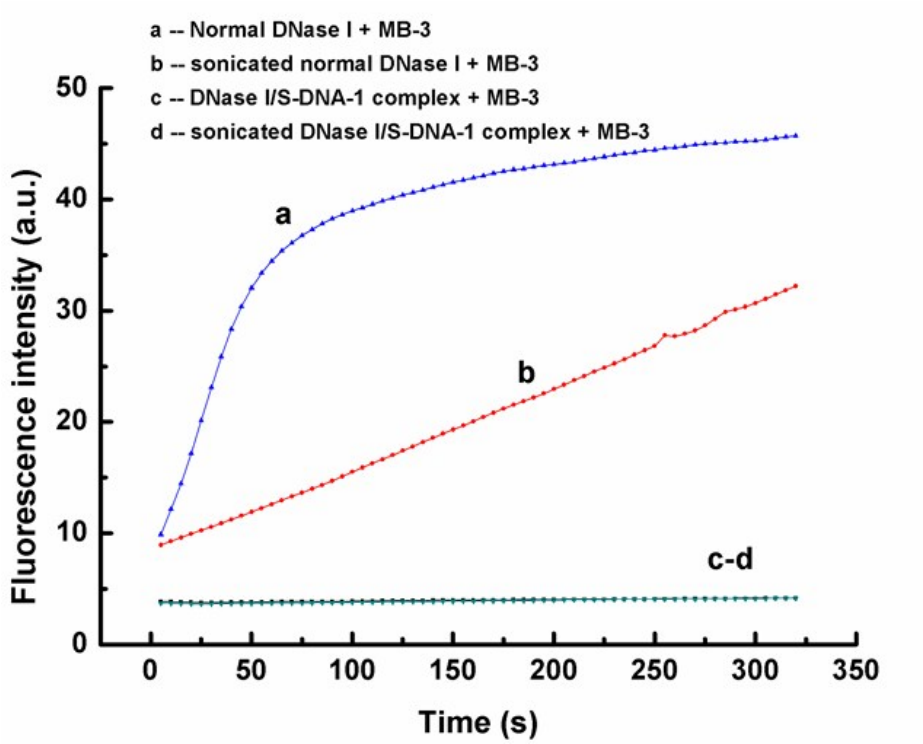

Figure S14. The stability of DNase I/S-DNA complex after ultrasonication treatment at room temperature for 50 min (150 W cm^{-2} and 40 kHz). The normal DNase I was also tested in the same manner for comparison. MB-3 was used as a probe to monitor the variation of the enzyme activities under different conditions. Further longer sonication time was not tested since even the free DNase I would lose most of its activity after sonication for more than 50 min.

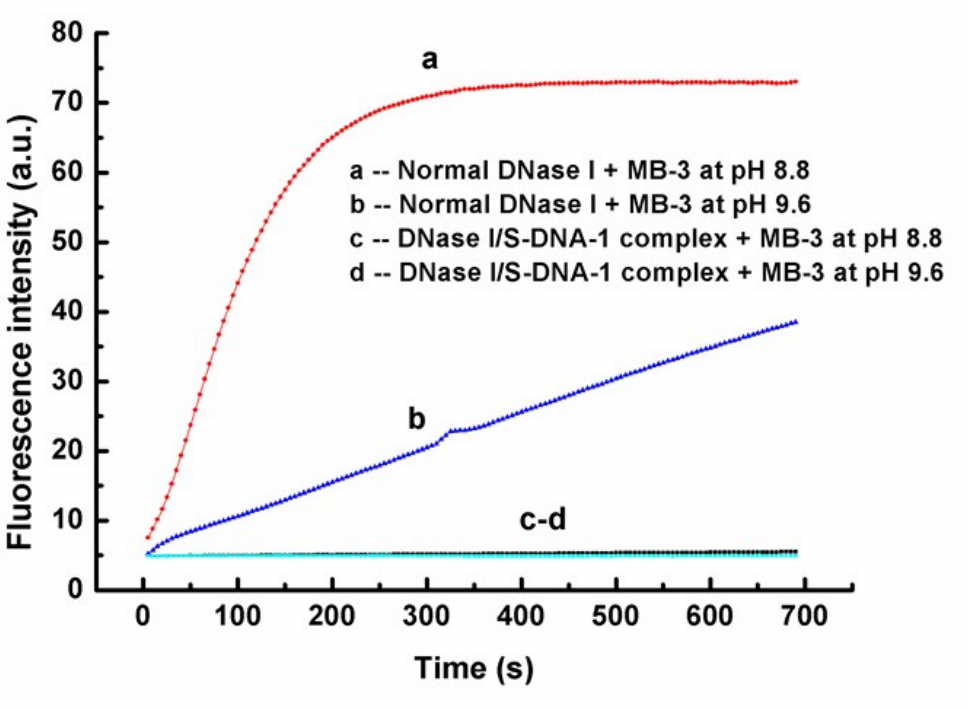

Figure S15. The stability of DNase I/S-DNA complex at higher pH (8.8 and 9.6). The normal DNase I was also tested in the same manner for comparison. MB-3 was used as a probe to monitor the variation of the enzyme activities under different conditions. As can be seen that even free DNase I could not work efficiently at pH 9.6, this should also be the limiting condition for the DNase I/S-DNA complex.

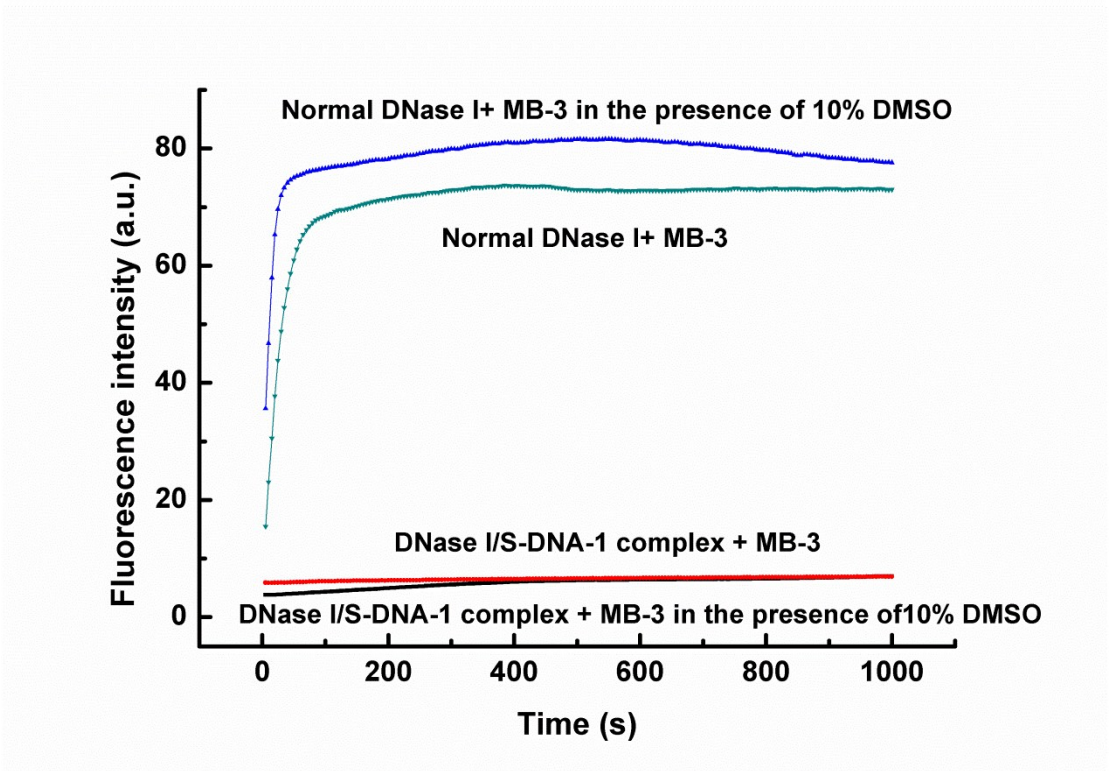

Figure S16. The stability of DNase I/S-DNA complex in the presence of 10% DMSO. The normal DNase I was also tested in the same manner for comparison. MB-3 was used as a probe to monitor the variation of the enzyme activities under different conditions.

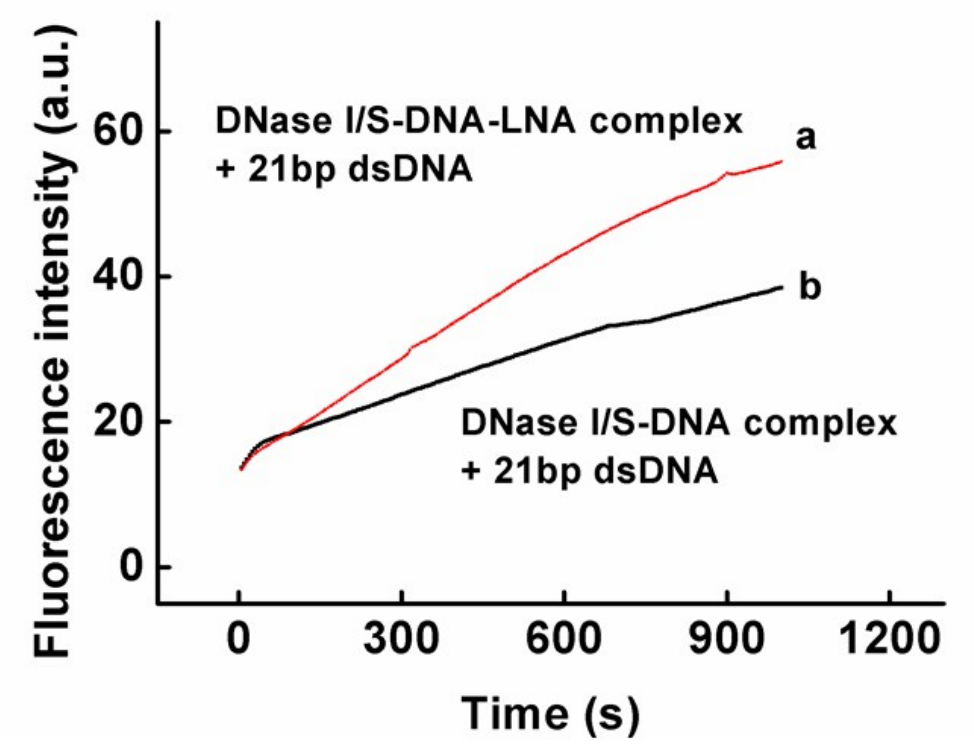

Figure S17. Enhancement of the cleavage rate of targeted dsDNA by using DNase I/S-DNA-LNA complex. The S-DNA-LNA template contains two locked nucleic acid (LNA) nucleotides (see Table S1).

Discussion on the stability of DNase I/S-DNA-1 complex in organic solvents

In the presence of 10% ethanol, digestion of the reference probe MB-3 was clearly observed, which increased by about 8 folds in comparison with that in normal buffer. By contrast, no significant variations of the activity and specificity were observed for the complex in the presence of 10% DMSO. As a control, we also tested the digestion rate of the same probe by normal DNase I in the presence of 10% ethanol, which was observed to increase by only about 2 folds as compared to that than in the normal buffer (Fig. 2f). These data suggested that the DNase I/S-DNA complex might have partially dissociated in 10% ethanol and released part of the activity of free DNase I. This is most likely because of disruption of the hydration layer of proteins by ethanol which has a low dielectric constant (25.3) and thereby loosen the conformational structure of the complex[2,3]. By contrast, DMSO has the highest dielectric constant (47.2) among those commonly used organic solvents, which explains why DMSO cannot loosen the DNase I/S-DNA complex.

References

1. Kypr, J., et al. (2009). "Circular dichroism and conformational polymorphism of DNA." Nucleic Acids Research 37(6): 1713-1725.

2. Zaks, A. and A. M. Klibanov (1988). "The Effect of Water on Enzyme Action in Organic Media." Journal of Biological Chemistry 263(17): 8017-8021.

3. Klibanov, A. M. (1997). "Why are enzymes less active in organic solvents than in water?" Trends in Biotechnology 15(3): 97-101.