Engineering DNA Turnover

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Tuning DNA Stability To Achieve Turnover in Template for an Enzymatic Ligation Reaction**

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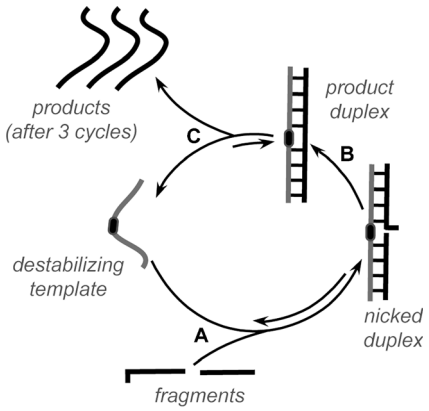
In the last decade, DNA-based systems have been developed that are capable of performing sophisticated functions initiated by molecular recognition. Key examples are the DNA walkers where directional motion or load pick-up, transfer, and release are achieved with molecular and spatial selectivity.[1,2] Attempts to develop autonomous DNA self-replicating systems, however, without specific sequence requirements,[3] have in recent years lagged behind.[4–6] As one of the hallmarks of organisms is their ability to amplify information and materials through biocatalysis and self-replication,[6,7] the development of truly biomimetic systems capable of integrated functions requires incorporating amplification into self-assembly and nanotechnology.[8] Not only do replicating DNA systems provide tools for DNA-based nanotechnology[8] and insights into the origins of life,[6,7,9] but they also can be used to isothermally amplify signal in DNA detection, which can simplify the requirements for point-of-care diagnostics.[10]

One method for introducing amplification into DNA-based systems involves generating turnover in DNA-templated processes.[11] To achieve turnover, the DNA template that facilitates the reaction of two complementary fragment strands (Scheme 1, steps A and B) must dissociate from the product after it has formed (Scheme 1, step C). For ligation reactions, however, turnover is minimal under isothermal conditions owing to the enhanced affinity of the template for the ligated product.[11] Consequently, many detection strategies have focused on template-triggered scission and transfer reactions rather than ligations to avoid this product inhibition.[11] One method for introducing turnover into ligation reactions exploits the sensitivity of DNA to destabilizing modifications present in the middle of a duplex.[5,12,13] By ligating strands at such a destabilizing site, one can modulate the stability of the hybridization complex without changing the temperature or any other reaction condition.[14] If the stabilities of the complexes before and after ligation are properly balanced, isothermal turnover should be achieved.



Scheme 1. Isothermal turnover in DNA-templated ligation reactions using destabilizing templates.

Herein we report a general strategy for achieving turnover in template for ligations using T4 DNA ligase. Utilizing modified DNA template strands, the product duplex is destabilized after ligation. As a result, the template strand is released, freeing it to template more ligation reactions. Introducing turnover into simple, enzymatic ligation reactions provides an avenue for amplifying DNA-based assemblies constructed by enzymatic ligation.[15–17] Isothermal ligation strategies also have potential applications in cross-catalytic replication of DNA, which represents a general method for amplifying any DNA sequence.[6,7]

Two of the most successful examples of isothermal turnover in nonenzymatic, chemical ligation systems were reported by Kool and Seitz, which proved useful in DNA and mRNA detection.[11,13] In the Kool system, destabilization was introduced in a ligating fragment strand by adding an alkyl group between the terminal nucleotide and the electrophilic end.[18] After ligation with a nucleophilic fragment using target DNA as a template, the resulting alkyl bridge caused the product duplex to dissociate. With this example in mind we synthesized DNA templates containing short alkyl chains in place of a complementary nucleotide (Figure 1). We also investigated a model abasic DNA lesion known to destabilize DNA duplexes (Figure 1, **Ab**).[19] Previously, PNA analogues of abasic groups have been demonstrated by Seitz to avoid product inhibition[20] and improve selectivity[21] in chemical ligation systems using PNA. Here, the abasic group results in a template that is missing a base but still contains the canonical phosphate-sugar backbone.

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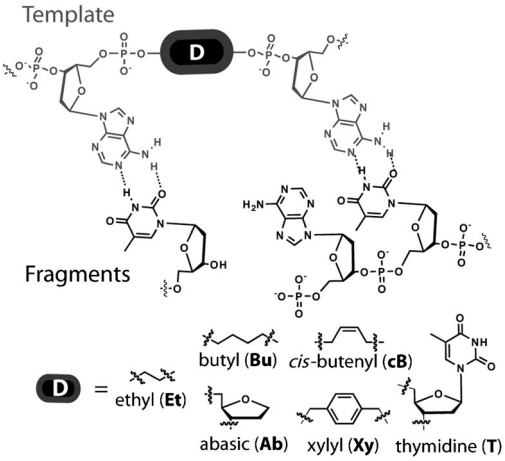


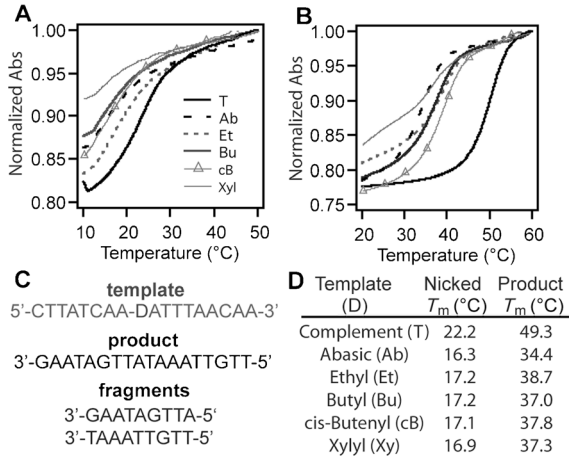
Figure 1. The nicked site prior to ligation. The destabilizing templates contain a modification (**D**) in place of a thymidine. The perfect template contains the complementary thymidine (**T**).

Achieving the greatest turnover requires that the product duplex be less stable than the nicked duplex (Scheme 1). However, because of multivalency[22] the product duplex is invariably more stable, even with the destabilizing modification. As a result, a more reasonable goal is to introduce a modification that renders the product and nicked duplexes closer in stability, so that a temperature can be found where both can form but remain labile. To determine whether the duplex stabilities of our destabilizing templates were optimal, we monitored their thermal dissociation behavior. The temperature at which half the duplex has dissociated is the melting temperature (T_m), providing a way to compare duplex stabilities.

The thermal dissociation curves are shown for the nicked duplexes (template + two fragments) and the product duplexes (template + product) in Figure 2A and B, respectively. The 18-base sequence used in these experiments and the position of the nicked site and destabilizing group are given in Figure 2C. To illustrate the extent of destabilization, we compared these results with the behavior of a perfectly complementary system (Figure 2A and B, black solid traces, where **D** = thymidine). For all of the destabilizing templates, the decrease in T_m between the nicked and corresponding product duplexes was 18–22 °C. In contrast, the T_m difference was 27 °C for the natural DNA system (Figure 2D). The smaller ΔT_m for the destabilized templates suggested that a temperature might be found where hybridization of the nicked duplex (Scheme 1, step A) and dissociation of the product duplex (Scheme 1, step C) were both possible. It still remained to be seen, however, whether the T4 DNA ligase, known to ligate mismatched DNA,[23] would tolerate non-natural modifications on the DNA template strand.

In our ligation experiments, the template was mixed with the complementary reactive strands (containing a 3′-OH and 5′-phosphate at the ligation site) and 1 unit of T4 DNA ligase for each 15-μL reaction.[24] We labeled the 5′ end of one of the fragments with fluorescein to follow ligation by fluorescent imaging after separation by denaturing polyacrylamide gel electrophoresis (PAGE). The gel image in Figure 3A illustrates the ligation mixtures with one equivalent of template after 20 h of reaction time, where a product band was evident for all of the destabilizing templates. This result is significant since it proves that T4 DNA ligase tolerates nonnatural modifications to the DNA template near the site of ligation. It is important to note that ligation with all of the destabilizing templates was hampered when the ligation site was opposite the 5′ end rather than the 3′ end of the destabilizing group (see Supporting Information, Figure S1).

To determine whether these destabilizing templates could turn-over in the reaction, we next monitored the ligation reaction with substoichiometric amounts of template. At



Template (D)	Nicked T_m (°C)	Product T_m (°C)
Complement (T)	22.2	49.3
Abasic (Ab)	16.3	34.4
Ethyl (Et)	17.2	38.7
Butyl (Bu)	17.2	37.0
cis-Butenyl (cB)	17.1	37.8
Xylyl (Xy)	16.9	37.3

Figure 2. The thermal dissociation profiles of A) nicked duplexes (template:fragments) and B) product duplexes (template:product). [DNA] = 1.3 μM per strand (pH 7.0, 20 mM PBS, 10 mM $MgCl_2$). C) Sequence of strands where D is thymidine or a destabilizing group. D) Table of dissociation (melting) temperatures (T_m).

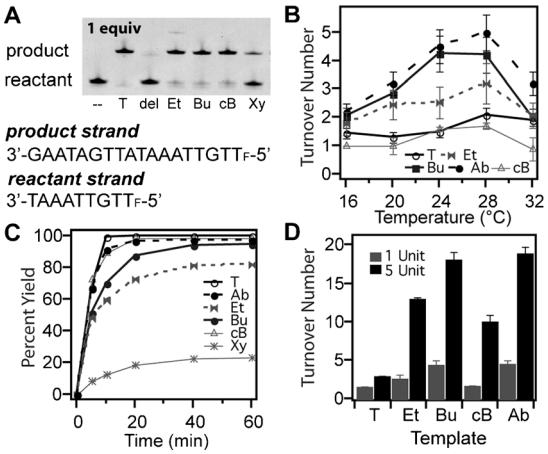


Figure 3. Template labels: – no template; **T** thymidine; **del** deletion; **Et** ethyl; **Bu** butyl; **cB** *cis*-butenyl; **Xy** xylyl; **Ab** abasic. A) Fluorescent images of denaturing polyacrylamide gels for ligation mixtures using fluorescein-labeled thymine ($\mathbf{T_F}$) with 1 equiv template. B) Turnover number (TON) versus temperature for ligations with 0.01 equiv template. C) Percent yield versus time with 1 equiv template. D) TON versus enzyme concentration (1 unit vs 5 unit) with 0.01 equiv template at 24 °C. Conditions unless otherwise noted: 1 equiv (1.4 μM) $\mathbf{T_F}$-labeled reactant, 1 unit T4 DNA ligase, 20 h, 16 °C.

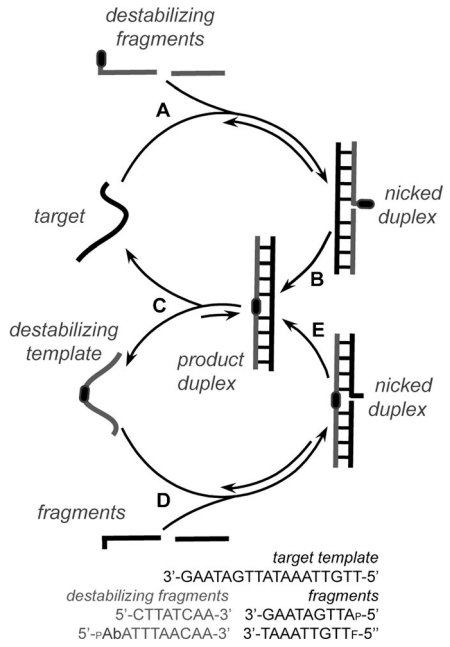
20 °C, when 0.01 equivalents of complementary template (**T**) were used only 1.3 % of the fluorescent fragment strand was ligated, indicating that the dissociation of the perfect product duplex was unfavorable. In contrast, using the same amount of an abasic template (**Ab**) led to 3.2 % of the ligated product. From the ratio of [product]/[template] the turnover number (TON) was calculated. As shown in Figure 3B, the degree of turnover for the perfect template (**T**) was between 1 and 2 for all reaction temperatures using 0.01 equivalents of template. In contrast, the turnover was greater than 2 for several of the destabilizing templates. The only inactive templates that yielded little or no ligated product under these substoichiometric conditions were the xylyl template (**Xy**) and a template containing a deletion of the thymidine (**del**, data not shown). With the *cis*-butenyl template, turnovers were between 0.9 and 1.7, which indicated that this rigid linkage did not promote catalytic behavior.

For all of the active destabilizing templates, the highest TON was observed at 28 °C, well above the melting temperature of their corresponding nicked duplexes (Figure 3B). Specifically, the highest TON observed at this enzyme concentration was 5.0 for the abasic template (Figure 3B, **Ab**). To explain the temperature trends, we propose that the decrease in nicked duplex stability at 28 °C is compensated for by the higher rate of ligation or dissociation of the product duplex. At temperatures higher than 28 °C, however, the decrease in TON suggests that the formation of the nicked duplex becomes unfavorable. Importantly, at all temperatures, no background ligation was observed in the absence of template (Figure 3A, –). The enzyme requirement that the DNA be double-stranded eliminated any background reaction illustrating a major advantage to this approach. Moreover, these results also suggest that employing a catalyst which favors double-stranded DNA might be a way to avoid non-templated background in chemical ligations.[5]

To see how the destabilizing template influenced the rate of ligation, we measured the yield versus time in ligations using one equivalent of template. As shown in Figure 3C, within 10 min ligation is complete when natural DNA template (**T**) is used.[25] Most of the destabilizing templates are only a little slower with the **Ab** and **cB** template requiring 20 min, and the other templates requiring less than 40 min.[26] Comparing our results with those of chemical ligation methods that demonstrate lower rates of ligation[18,27] indicates that faster ligation methods lead to greater turnover, which is consistent with previous reports on isothermal chemical ligation methods.[20,21]

In the experiments described above we used the typical ligase concentration for ligating nicked duplexes of 1 unit enzyme per equivalent of fluorescent fragment strand (1.4 μM, 15 μL). To see whether increasing enzyme concentration would increase the amount of turnover, ligation reactions using concentrated enzyme (5 units per reaction) were performed with the same amount of template (0.01 equivalents). At higher enzyme concentration, the perfect DNA template (**T**) still exhibited a TON close to one. In contrast, the **Bu** and **Ab** templates generated 18 product strands per template (Figure 3D), which is 5-fold higher than the maximum turnover number of 3.5 previously reported by the Seitz group using similar probe (1.2 μM) and template (12 nM) concentrations.[20] Although both Kool[18] and Seitz[20] report higher TONs (91.6 and 226, respectively) these were only achieved by increasing the fragment:template ratio to 10000, which facilitates dissociation of the product duplex. This required that they use HPLC or radio-imaging with PAGE, which allowed them to reliably distinguish 0.0916 % or 0.226 % of product forming from reactions with non-negligible background. In contrast, our approach has the advantage of no background reaction when template is absent. Moreover, T4 DNA ligase, standard DNA ligating groups, and the commercially available abasic destabilizing modifications should prove more accessible to nonsynthetic labs than these chemical methods.

This methodology for using catalytic amounts of template to amplify a DNA-ligated material made by T4 DNA ligase can be applied in smart, DNA-based systems. For example, an environmental stimulus can be used to release destabilizing template causing the amplification of a DNA material, like DNA-ligated gold nanoparticle aggregates.[16] If, however, one wanted to use this principle of destabilization for native DNA detection, another complementary ligation cycle must be included. Scheme 2 illustrates a cross-catalytic replication strategy that can be initiated by a native DNA strand representing a target sequence. In this set of experiments, the destabilizing template is formed in situ by a ligation reaction templated by the natural DNA target (Scheme 2, steps A and B). As a result of ligation, the same product duplex is formed as in the previous cycle. Consequently, the product:template duplex should be destabilized leading to the release of the original target and the newly formed destabilizing template (Scheme 2, step C). This destabilizing template can now generate a copy of the original target template (Scheme 2, steps D and E), which goes on to catalyze the formation of more destabilizing templates. As the product of



Scheme 2. Cross-catalytic cycles with destabilizing fragments.

each cycle is a template for the other, significant amplification of the original target should ensue.

To verify that we could observe cross-catalysis, we combined the fragment strands listed in Scheme 2, including a fluorescent modified fragment corresponding to the bottom cycle (T_F, fluorescein-modified thymine). We then introduced a target DNA sequence which, although active in the top cycle, should have no effect on the fluorescein-labeled fragment. Therefore, the formation of any fluorescent ligated product would signify cross-catalysis. Previous reports suggested that T4 DNA ligase would ligate a destabilizing fragment terminated with a 5′-phosphate abasic group.[28] Indeed, as shown in Figure 4 target-initiated cross catalysis

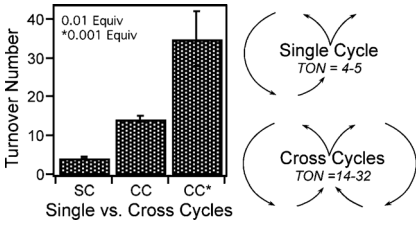


Figure 4. SC: Single-catalyst cycle using an abasic destabilizing template and fragments. CC: Cross-catalytic cycle using target as template and fragments, one of which contained an abasic group. Conditions: 0.01 equiv template (14 nM); *0.001 equiv (1.4 nM); 1 unit T4 DNA ligase; 24 °C; 20 h.

occurred for the **Ab**-substituted system. Not only did we observe self-replication in this two-cycle system, but the exhibited cross-catalytic TON (defined as [product]/[initial template]) was greater than that for the single catalytic cycle (TON 14 vs 4.5; Figure 4, CC vs SC, respectively). Decreasing the number of equivalents of template (CC*) further increased the number of cross-catalytic turnovers, indicating that dissociation is favored as the fragment:template ratio becomes larger.[18,20] The highest cross-catalytic turnover of 32 corresponds to the target undergoing on average 32 cycles of self-replication (Figure 4, CC*). As a point of reference, our system exhibits the same number of self-replication cycles as shown by Albagli et al. in one of the few reported cross-catalytic chemical ligation systems applicable to DNA amplification and detection.[29] In their system, however, temperature cycling was used to achieve turnover, whereas our system requires no mechanical or thermal intervention.

For the cross-catalytic reactions using 1 unit of ligase and the 5′-phosphate abasic fragment, we observed a small level of background (0.5% and 3.7% product at 20 and 24 °C, respectively, with no template present).[30] In contrast, the 5′-phosphate thymidine fragment exhibited a large amount of background (25% ligation product at 20 °C) (see Supporting Information, Figure S2). The reason for the high background in the natural system is based on our probe design, which leads to a one-base overhang of thymidine on one fragment and deoxyadenosine on the other. Although this overhang exists in our **Ab**-modified system, replacing the thymidine with the abasic group prevents hydrogen bonding with the adenine thus minimizing nonspecific joining of the fragments. Consequently, very little background ligation is observed.

In conclusion, we have demonstrated that DNA templates for enzymatic ligation reactions can turn-over in the ligation cycle by introducing a destabilizing modification into the template strand. This work complements previous studies illustrating how destabilizing fragments can be used to facilitate turnover.[11,13] Additionally, the turnover numbers observed in our system are higher than the best isothermal chemical ligation strategies at similar strand lengths, concentrations, and ratios.[11,13] We have also taken this idea a step further by adding in another cycle that leads to a self-replicating DNA system using destabilization to overcome product inhibition. The success of the **Ab** templates in both cycles is especially promising as this phosphoramidite is commercially available allowing simple access to templates capable of turnover. Not only will this strategy enable advances in nanotechnology in the replication of DNA materials, but it should also be useful in isothermal target amplification for the broad field of DNA diagnostics. Finally, with simple destabilizing groups and rapid ligation methods, turnover and target-initiated self-replication are now possible, which should reinvigorate interest in autonomous DNA replication.[4–6] We are currently working on expanding this destabilization approach to other rapid ligation systems using nonenzymatic strategies.

Experimental Section

Preparation of DNA strands: DNA was synthesized on an ABI 392 solid-phase synthesizer using Glen Research reagents. Strands were purified by Glen-Pak DNA Purification cartridges (cat. 60-5200-01) according to the DMT-On protocol. Standard nucleotide phosphoramidite and the following were used: Chemical Phosphorylation Reagent II (cat. 10-1901-90), Fluorescein-dT Phosphoramidite (cat. 10-1056-95), and dSpacer CE Phosphoramidite (cat 10-1914-90) for the abasic (**Ab**) template and fragments. All other destabilizing templates were prepared from the corresponding protected diols with solid-phase synthesis. OligoCalc (http://www.basic.northwestern.edu/biotools/oligocalc.html) was used to determine the extinction coefficients where the destabilizing templates' absorptivity was assumed to be **Ac**, with **D** = a deletion.

Strands:

Ac	3′-AACAATTTA-**D**-AACTATTC-5′; **D** = T or destabilizing group
A	3′-GAATAGTT-A-TAAATTGTT-5′
C	3′-GAATAGTTA-5′
$\mathbf{C_P}$	3′-GAATAGTTA$_{\text{Phosphate}}$-5′
B	3′-TAAATTGTT-5′
$\mathbf{B_F}$	3′-TAAATTGTT$_{\text{Fluorescein}}$-5′
Cc	3′-AACTATTC-5′
$\mathbf{Bc_P}$	3′-AACAATTTAT$_{\text{Phosphate}}$-5′
$\mathbf{BcAb_P}$	3′-AACAATTTA(*Ab*)$_{\text{Phosphate}}$-5′

Thermal dissociation experiments: 1.3 nmol of each DNA sequence (**Ac** and **A** for the product duplex experiments and **Ac**, **B**, and **C** for the nicked duplex experiments) were combined in PBS buffer (1.0 mL, 10 mM $MgCl_2$, 20 mM pBS, pH 7.0) and hybridized for ca. 15 min. While stirring at 100 rpm, absorbance readings at 260 nm were taken from 10 to 60 °C at 1 °C intervals, with 1 min hold time.

Ligation experiments. Strand amounts: Single-cycle reactions: $\mathbf{B_F}$ 1 equiv; $\mathbf{C_P}$ 2 equiv; template (**Ac**) 1 or 0.01 equiv. Cross-cycle reactions: $\mathbf{B_F}$ 1 equiv; $\mathbf{C_P}$ 2 equiv; **A** 0.01 or 0.001 equiv; **Cc** 2 equiv; and 2 equiv of either strand $\mathbf{Bc_P}$ or $\mathbf{BcAb_P}$. In a typical ligation, where 1 equiv = 21 pmol, the appropriate amounts of DNA fragments and template were first combined in water in a 400 μL minicentrifuge tube to reach a final volume of 10 μL and incubated at the desired reaction temperature. While several of these DNA solutions incubated, in a separate mini-centrifuge tube, T4 DNA ligase (8 μL) at lower concentration (1 unit μL^{-1}, Invitrogen cat. 15224-017) or higher concentration (5 unit μL^{-1}, Invitrogen cat. 46300-018) was mixed with ligation buffer (24 μL, 5× concentrated) and water (8 μL). A portion of this ligase mixture (5 μL) was immediately added to each of the DNA solutions (final [DNA] = 1.4 μM for each equivalent). The reactions were then placed in a covered thermal incubator for 20 h unless otherwise noted. To stop ligation, $EDTA_{(aq)}$ (1 μL, 0.5 M) was added for every unit of enzyme present. For the kinetic experiments, aliquots (3 μL) were removed from the bulk ligation mixture at various reaction times and placed in a separate microcentrifuge tube containing $EDTA_{(aq)}$ (1 μL, 0.5 M). Samples were stored at 4 °C until analyzed by 15 % denaturing PAGE.

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[24] One unit is the amount of enzyme needed to catalyze the conversion of 1 nmol of ^{32}P-labeled pyrophosphate into ATP in 20 min at 37 °C (Invitrogen).
[25] The rate of ligation for perfect template should first be measured to verify the enzyme activity. If the enzyme is less active due to improper storage or handeling, no turnover is observed.
[26] We attribute the leveling off of the signal for the ethyl and butyl templates to subtle differences in the extinction coefficients used to calculate the DNA concentration. The leveling off of the xylyl suggests that most of the xylyl template is in a conformation that does not allow for hybridization or ligation.
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[30] The more concentrated enzyme (5 units per 15 μL reaction), capable of ligating blunt ends, displayed very high background under identical reaction conditions.

