Synthetic Biology Hot Paper

DOI: 10.1002/ange.201303225

# Rapid, Isothermal DNA Self-Replication Induced by a Destabilizing Lesion**

*Abu Kausar, Catherine J. Mitran, Yimeng Li, and Julianne M. Gibbs-Davis**

One process that chemists have aimed to incorporate into artificial systems is self-replication.[1] Synthetic self-replicating systems use self-assembly or molecular recognition to initiate and propagate replication, which also gives insights into the origins of life.[1] For these synthetic processes to be functional as biomimetic models, they should demonstrate sigmoidal amplification at a constant temperature.[1c,e] However, synthetic replication processes based on biomolecules (like DNA) are plagued by product inhibition and competing background reactions, preventing these systems from exhibiting the sigmoidal growth characteristic of self-replication.[1e,2] One way that product inhibition has been avoided and exponential amplification achieved involved using stepwise procedures, whereby the composition of the system was physically modulated to facilitate replication.[3] This mechanical intervention, once every catalytic cycle, is similar to the need for thermal cycling in DNA replication strategies like the polymerase chain reaction, where heating is required to destabilize the product complex and turn over the reaction.[4]

For a system to be autonomous, the replication process must proceed with only one external intervention, the initial introduction of the target. Recently, Lincoln and Joyce reported the development of an autonomous, non-enzymatic self-replicating RNA system using a catalytic 100-base RNAzyme that exhibited sigmoidal replication.[5] The catalytic activity of the RNAzyme allowed it to ligate two fragments that contained regions complementary to the RNAzyme as well as fragments of the catalytically active domain. After ligation, this new catalytically active RNA strand ligated another set of fragment RNA to generate a copy of the original RNAzyme. Lincoln and Joyce found that the most active system contained a wobble G-U pair between the RNAzyme and the fragments, which destabilized the product complex allowing for sigmoidal amplification with a doubling rate of once per hour. However, for this ligation chain reaction to operate a majority of the sequence had to be conserved to maintain the catalytic properties necessary for ligation.[5]

Our aim has been to develop a general isothermal DNA-amplification system based on a ligase chain reaction (LCR),[6] similar to strategies for DNA[2a,c,d] and RNA[2b,5] amplification in non-enzymatic systems, as shown in the previous example, which would be useful in understanding the requirements for prebiotic oligonucleotide replication and have potential in DNA-based diagnostics of infectious diseases.[7] Herein, we report a rapid sigmoidal amplification of DNA while circumventing the need for destabilizing enzymes or mechanical or thermal intervention using a destabilizing abasic lesion. The simplicity and generality of our method suggests that this strategy will be accessible to many laboratories. Moreover, although an enzyme is used to ligate the two strands in our strategy, turnover is a consequence of the destabilized DNA duplex. Hence, this work can aid the development of non-enzymatic nucleic acid self-replication systems using chemical-ligation methods.[2b–d,8]

Similar to the RNAzyme system, cross-catalytic replication of DNA strands involves two coupled catalytic cycles where the product strand of one cycle is the template for the other cycle (Figure 1).[1c,2c] In the first step of our isothermal

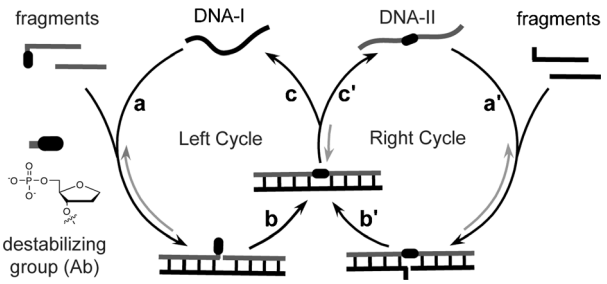


***Figure 1.*** Cross-catalytic amplification of **DNA-I** using an abasic destabilizing group in an LCR. Left cycle: A **DNA-I** strand templates the formation of **DNA-II** (see text for details). Right cycle: The **DNA-II** formed in situ catalyzes the formation of a new **DNA-I** strand, which feeds back into the left cycle.

LCR, a sequence (**DNA-I**) initiates the formation of its complement by hybridizing with two complementary fragments, one of which contains a destabilizing abasic group in place of the complementary nucleotide at the 5′phosphate terminus, to form a nicked duplex (Figure 1a). After ligation of the two fragments by T4 DNA ligase a new strand results, **DNA-II**, that is complementary to **DNA-I** with the exception of the central abasic nucleotide (Figure 1b). Owing to the destabilizing effects of the abasic group on the product duplex, the product **DNA-II** will dissociate from **DNA-I** at

[*] A. Kausar, C. J. Mitran, Y. Li, Prof. J. M. Gibbs-Davis
Department of Chemistry, University of Alberta
Edmonton, AB, T6G 2G2 (Canada)
E-mail: gibbsdavis@ualberta.ca
Homepage: http://www.chem.ualberta.ca/~gibbsdavis

[**] We thank Gareth Lambkin and the Biological Services lab in the Department of Chemistry (U. Alberta) for help and the available equipment. We acknowledge the Natural Sciences and Engineering Research Council of Canada-Discovery and Research Tools and Instrument Program for funding and the Canada Foundation for Innovation-Leaders Opportunity fund for infrastructure for this project.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201303225.

the same temperature in which it was formed (Figure 1c). The presence of a large excess of fragments prevents the reannealing of **DNA-I** and **DNA-II. DNA-I** is then free to serve as a template for the formation of more **DNA-II** destabilizing strands. This approach for using destabilizing groups to achieve turnover in DNA-templated ligations was first shown by Lynn and co-workers[9] and has now been demonstrated in several non-enzymatic single-cycle systems.[8b,10]

To achieve amplification of **DNA-I**, however, another catalytic cycle must be coupled to this first ligation process (Figure 1, right cycle). In this cycle, the **DNA-II** that was generated is complementary to two other fragments that are present leading to hybridization and the formation of another nicked duplex (Figure 1a′). This nicked duplex is ligated, and then dissociates once again owing to the destabilizing abasic site (Figure 1b′,c′). The ligated product of the right cycle contains a sequence identical to **DNA-I** but with the addition of a fluorescent label (**F-DNA-I**). Our previous work indicated that 1′,2′-dideoxyribose-5′-phosphate, a model abasic lesion (Ab), was an ideal destabilizing group that promoted isothermal replication and was compatible with ligation by T4 DNA ligase.[11] Unfortunately, our first efforts at cross-catalytic DNA self-replication by destabilization were extremely slow, requiring 20 hours to achieve 31 replication cycles when one Weiss unit of enzyme[12] was used per 15 μL reaction.[11]

Our previous work indicated that the concentration of the enzyme had a large impact on the turnover frequency for single-cycle experiments involving various destabilizing groups.[11] Therefore, to increase the replication rate we examined higher concentrations of T4 DNA ligase based on 2000 cohesive end units (or approximately seven Weiss units) per 15 μL experiment.[13] With this commercially available high concentration of enzyme, we monitored the kinetics of the replication process for a reaction mixture containing all four fragments (1.4 μM and 2.8 μM, fluorescently labeled and unlabeled, respectively) and either 14 nM or 0 nM **DNA-I** at time zero (Figure 2a; Supporting Information, Figure S1). Remarkably, self-replication occurred rapidly and consumed all of the fragments for the reaction initiated with 14 nM **DNA-I**, as determined by gel electrophoresis (Figure 2a, solid circles). The amount of **F-DNA-I** generated represented a 100-fold increase within 40 minutes, significantly faster than the reaction with low ligase concentration (31 copies in 20 hours). In contrast, decoupling the right cycle from the left cycle led to much slower **DNA-II** strand production and linear growth consistent with cross-catalysis being responsible for the rapid amplification (Figure 2b; Figure S2).

In our cross-catalytic system, we also observed that the reaction without any initial **DNA-I** exhibited sigmoidal growth after an induction period of about 40 minutes (Figure 2a, open circles), in contrast to the single-cycle experiment, where no reaction occurred without some initial **DNA-I** (Figure 2b, open circles). We reasoned that small quantities of **DNA-I** or **DNA-II** were synthesized in the cross-catalytic system through pseudo-blunt-end ligation of the fragments, as the fragments present in each cycle were complementary to those in the opposite cycle. Such blunt-end ligation is known

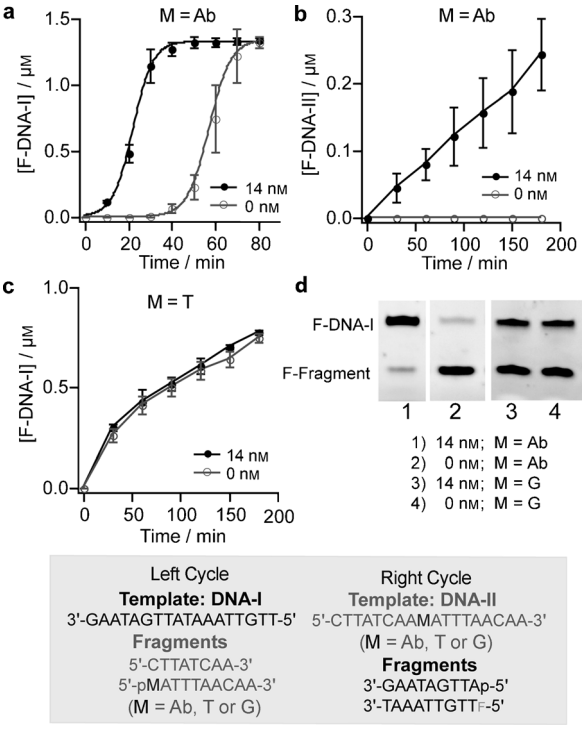


***Figure 2.*** a) Cross-catalytic replication of **F-DNA-I** at 30°C initiated by 14 nM or 0 nM **DNA-I** using destabilizing fragments. b) Single (left) cycle amplification at 30°C initiated by 14 nM and 0 nM **DNA-I** using destabilizing fragments, one with a fluorescein, leading to the formation of the fluorescently labeled complement (**F-DNA-II**). c) Production of **F-DNA-I** at 30°C initiated by 14 nM or 0 nM **DNA-I** using native DNA fragments. d) Fluorescent images of polyacrylamide gels after electrophoretic separation. **F-DNA-I** formed at 30°C: after 40 min (1,2) or 180 min (3,4). Gray box: P = phosphate and $T_F$ = fluorescein-modified thymine.

to occur in the presence of high concentrations of T4 DNA ligase.[14] (This reaction would be pseudo-blunt ended as there is a non-complementary one-base overhang, where the adenine is across from the abasic group.) The small quantities of **F-DNA-I** or **DNA-II** generated in this slow background process could then trigger cross-catalytic replication. Fitting the sigmoidal logistic growth function $f(t) = a/(1+be^{-ct})$ to the data plotted in Figure 2a allowed us to quantify the rate of replication, where $a$ is the maximum concentration of **F-DNA-I** formed, $b$ is the extent of sigmoidicity, and $c$ is the exponential rate.[5] We observed that the rates were the same within error for the target-initiated and background-triggered reactions ($0.22 \pm 0.05$ min$^{-1}$ versus $0.20 \pm 0.01$ min$^{-1}$, respectively), which indicated that the exponential growth came from the cross-catalysis and not the blunt-end reaction.

To determine the importance of the destabilizing group, we also examined replication in a native DNA system that contained the complementary thymidine rather than the abasic group (Figure 2c; Figure S3). The **DNA-I**-initiated and background-triggered reactions exhibited the same kinetic profile, which did not have the sigmoidal shape characteristic of cross-catalytic replication (Figure 2c). In this case, the background reaction was quite facile because it involved ligation of a one-base overhang between the native DNA

fragments (Figure 2, gray box, M = T). This native DNA ligation system failed to exhibit sigmoidal amplification at any temperature tested, indicating that the destabilizing group was essential to overcome product inhibition in the replication of an 18-base sequence (Figure S4).

Next, we attempted to use a mismatched base-pair (A:G) rather than the abasic group to destabilize the product duplex and facilitate turnover (Figure 2, gray box, M = G). However, this mismatched system also did not exhibit a significant difference between the template-initiated and background-triggered reactions (Figure 2d and Figure S5). Thermal denaturation experiments indicated that the mismatch did not destabilize the product duplex as much as the presence of the abasic group, which explained the lack of cross-catalysis in the mismatched system (Figure S6). Specifically, the native **DNA-II**:**DNA-I** product duplex exhibited a dissociation, or melting, temperature ($T_m$) of 49.2 °C, whereas the abasic-containing product duplex had a $T_m$ of 34.0 °C. In contrast, the mismatch-containing product duplex had a $T_m$ of 41.1 °C, consistent with less destabilization (Table S2).

To test whether the presence of one abasic group for an 18 bp replicating system was a general method for self-replication, we prepared destabilizing fragments complementary to a sequence associated with the hepatitis B virus,[15] which has a higher G:C content than our original **DNA-I**:**DNA-II** system (Figure 3). Despite the increase in the stability of the corresponding **H-DNA-I**:**H-DNA-II** product duplex, we still observed facile self-replication by simply increasing the replication temperature from 30 °C to 34 °C (Figure 3a; Figure S7). The exponential rate determined for this system was slightly slower than that of the **DNA-I** sequence ($0.10 \pm 0.02$ min$^{-1}$, corresponding to a doubling rate of seven minutes). Temperature variation studies indicated that the ideal replication temperature was 10 °C below the $T_m$ of the product duplex and 8 °C above the $T_m$ of the fragment:template nicked duplexes for the **H-DNA-I** system ($T_m$ values correspond to 1.3 μM per sequence; Figure 3b; Figures S8,S9). For the **DNA-I** system the same relative temperature dependence was observed with the optimal replication temperature lying 4 °C below the $T_m$ of the product duplex and approximately 15 °C above the melting temperature of the corresponding fragment:template nicked duplexes (Figure 3b; Figure S10). We conclude that the ideal temperature was great enough to facilitate product duplex dissociation yet still resulted in the formation of some nicked duplexes. Importantly, the more concentrated T4 DNA ligase compensated for the small amount of nicked duplexes present at this temperature.

To verify that the template was primarily free during replication, not bound in the product duplex, the reaction order was determined from the rate law: initial rate $\propto k[\text{template}]_0^p$, where $k$ is the rate constant for the templated reaction, $[\text{template}]_0$ is the initial template concentration, and $p$ is the reaction order of the template.[1e,16] We observed a linear dependence of the initial rate on the initial **DNA-I** concentration, indicating that $p$ was close to one, which is a hallmark of exponential replicating systems (Figure S11).[16] A reaction order of one rather than 0.5 also suggested that the template strand present was primarily single-stranded or in the nicked rather than product duplex, which supported that product inhibition has been minimized in our system.[1e]

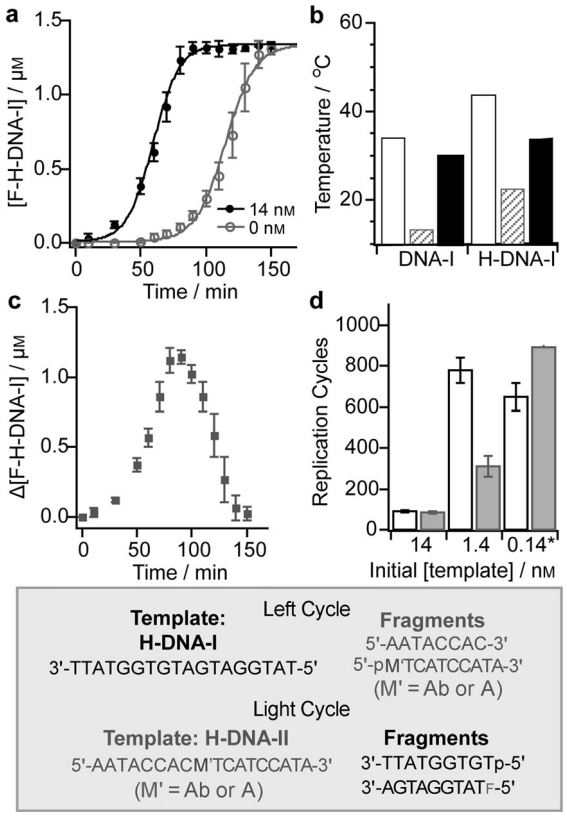


***Figure 3.*** a) Cross-catalytic replication of **F-H-DNA-I** at 34 °C initiated by 14 nM or 0 nM **H-DNA-I** using destabilizing fragments. b) Melting temperatures ($T_m$) of the product (white) and nicked (striped) duplexes for the **DNA-I** and **H-DNA-I** systems compared with the optimized replication temperature (black). c) Difference in the concentration of **F-H-DNA-I** formed between the reaction initiated with 14 nM and 0 nM **H-DNA-I**. d) Number of template-initiated replication cycles as a function of initial template concentration (**DNA-I** = white; **H-DNA-I** = gray). * = The fragment concentrations were used at a fourfold dilution compared with the typical replication conditions.

To quantify the amount of self-replication, we measured the difference in fluorescent template formed between the target-initiated and background-triggered reactions (ΔF-H-DNA-I, Figure 3c); the maximum difference was then divided by the initial template concentration to yield the net number of target-initiated self-replication cycles. For example, the 14 nM **H-DNA-I** template-initiated reaction exhibited a maximum ΔF-H-DNA-I value of $1.18 \pm 0.04$ μM, which corresponded to $85 \pm 3$ replication cycles (Figure 3d). Reactions initiated with 1.4 nM template led to net replication cycles of $780 \pm 40$ and $310 \pm 50$ for the **DNA-I** and **H-DNA-I** systems, respectively (Figure 3d).

To further increase the number of self-replication cycles required, we initiate the reaction with less template, but at template concentrations less than 1.4 nM the replication profile was the same as the background-triggered reaction. Therefore, we sought to delay the background reaction. We found that decreasing the fragment concentration fourfold decreased the rate of the background blunt-end reaction,

  

more so than its effect on the template-initiated reaction (Figure S12–S14; Table S3,S4). Consequently the time window increased allowing us to initiate the reaction with 0.14 nM template and observe $650 \pm 70$ and $920 \pm 50$ self-replication cycles for the **DNA-I** and **H-DNA-I** systems, respectively (Figure 3d, 0.14 nM). For the **H-DNA-I** system, we were also able to achieve an even lower detection limit and observed $6000 \pm 600$ replication cycles with 14 pM initial **H-DNA-I** template.

It seemed likely that further reduction of the fragment concentration would increase the observed replication cycles for our system. However, at fragment concentrations below 0.35 μM the replication process was difficult to monitor by fluorescent imaging after PAGE separation. Consequently, we attempted a step-wise serial ligation strategy, whereby **DNA-I** (140 fM) was combined with a low concentration of fragments (0.14 μM or tenfold more dilute than typical conditions). We also performed a control reaction lacking any initial **DNA-I**. After three hours, aliquots from the **DNA-I**-initiated and control experiment were added to two separate vessels containing the typical concentration of fragments and more T4 DNA ligase. After approximately 40 minutes, we observed a difference in **F-DNA-I** concentration (ΔF-DNA-I) of $0.10 \pm 0.03$ μM between the **DNA-I**-initiated and the control (background) reaction, which corresponded to $2.7 \pm 0.9$ million replication cycles for the former in less than four hours (Figure S15; Table S3). Although the sensitivity achieved is quite promising, other parameters like the sequence length and composition as well as the reaction conditions need to be optimized for use in biodiagnostics.

In conclusion, we have shown that replacing one nucleotide with an abasic group in an 18-base sequence can alter the recognition properties of DNA allowing it to self-replicate in a ligase chain reaction. This general and simple strategy hints at the possibility that early replicating systems combined molecular recognition with destabilizing interactions to achieve turnover without temperature variation. This idea of structural destabilization being important in early replicating systems has also been proposed and demonstrated for the polymerization, rather than ligation, of non-enzymatically synthesized oligonucleotides, although these methods have yet to be extended to cross-catalytic replication.[1f,17] Regarding the abasic modification, it is the most common lesion formed in DNA, because it can spontaneously occur through hydrolysis at the anomeric position of deoxyribose.[18] In RNA, abasic sites are less common owing to their slower formation.[19] Nevertheless, it is reasonable to expect this lesion must have coexisted with the first replicating nucleotides. Finally, the strong dependence of the replication rates on the concentration of the enzyme reveals that the ligation step must be very rapid to achieve appreciable replication. Future work will examine fidelity and selection in this lesion-induced replication process.

## Experimental Section

Detailed information regarding DNA synthesis, determination of reaction yields based on gel electrophoresis, and determination of net replication cycles is given in the Supporting Information. For ligation experiments, fragments and template were first combined in water in a 600 μL mini-centrifuge tube to a final volume of 10.00 μL, at 1.5 times their final concentration, and incubated at the desired reaction temperature while preparing the ligase master mix. Typically, T4 DNA ligase (8.00 μL) was mixed with ligation buffer (12.00 μL, 10× concentrated) and water (20.00 μL). A portion of this master mix (5.00 μL) was immediately added to each DNA mixture resulting in 2000 cohesive end units per 15 μL reaction, and the reactions were placed in a covered thermal incubator. To stop ligation, aliquots (3.00 μL) were removed at different times and immediately combined with aqueous EDTA (1 μL, 0.5 M).

Received: April 17, 2013
Published online: August 6, 2013

**Keywords:** autonomous replication · DNA replication · isothermal DNA amplification · oligonucleotides · origins of life

[1] a) L. E. Orgel, *Nature* **1992**, *358*, 203–209; b) N. Paul, G. F. Joyce, *Curr. Opin. Chem. Biol.* **2004**, *8*, 634–639; c) V. Patzke, G. von Kiedrowski, *Arkivoc* **2007**, 293–310; d) S. A. Benner, F. Chen, Z. Yang, *Chemical Synthetic Biology*, Wiley, Hoboken, **2011**, pp. 69–106; e) O. Taran, G. von Kiedrowski in *Chemical Synthetic Biology* (Eds.: P. L. Luisi, C. Chiarabelli), Wiley, Hoboken, **2011**, pp. 289–319; f) J. W. Szostak, *J. Syst. Chem.* **2012**, *3*:2; g) G. F. Joyce, *PLoS Biol.* **2012**, *10*, e1001323.

[2] a) G. von Kiedrowski, *Angew. Chem.* **1986**, *98*, 932–934; *Angew. Chem. Int. Ed. Engl.* **1986**, *25*, 932–935; b) W. S. Zielinski, L. E. Orgel, *Nature* **1987**, *327*, 346–347; c) D. Sievers, G. von Kiedrowski, *Nature* **1994**, *369*, 221–224; d) G. von Kiedrowski, B. Wlotzka, J. Helbing, M. Matzen, S. Jordan, *Angew. Chem.* **1991**, *103*, 456–459; *Angew. Chem. Int. Ed. Engl.* **1991**, *30*, 423–426.

[3] A. Luther, R. Brandsch, G. von Kiedrowski, *Nature* **1998**, *396*, 245–248.

[4] R. K. Saiki, D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, H. A. Erlich, *Science* **1988**, *239*, 487–491.

[5] T. A. Lincoln, G. F. Joyce, *Science* **2009**, *323*, 1229–1232.

[6] S. C. Andras, J. B. Power, E. C. Cocking, M. R. Davey, *Mol. Biotechnol.* **2001**, *19*, 29–44.

[7] a) P. Gill, A. Ghaemi, *Nucleosides Nucleotides Nucleic Acids* **2008**, *27*, 224–243; b) M. Parida, S. Sannarangaiah, P. K. Dash, P. V. L. Rao, K. Morita, *Rev. Med. Virol.* **2008**, *18*, 407–421.

[8] a) J. Ye, Y. Gat, D. G. Lynn, *Angew. Chem.* **2000**, *112*, 3787–3789; *Angew. Chem. Int. Ed.* **2000**, *39*, 3641–3643; b) T. N. Grossmann, A. Strohbach, O. Seitz, *ChemBioChem* **2008**, *9*, 2185–2192; c) J. P. Schrum, A. Ricardo, M. Krishnamurthy, J. C. Blain, J. W. Szostak, *J. Am. Chem. Soc.* **2009**, *131*, 14560–14570.

[9] a) Z.-Y. J. Zhan, D. G. Lynn, *J. Am. Chem. Soc.* **1997**, *119*, 12420–12421; b) P. Luo, J. C. Leitzel, Z.-Y. J. Zhan, D. G. Lynn, *J. Am. Chem. Soc.* **1998**, *120*, 3019–3031.

[10] A. P. Silverman, E. T. Kool, *Chem. Rev.* **2006**, *106*, 3775–3789.

[11] A. Kausar, R. D. McKay, J. Lam, R. S. Bhogal, A. Y. Tang, J. M. Gibbs-Davis, *Angew. Chem.* **2011**, *123*, 9084–9088; *Angew. Chem. Int. Ed.* **2011**, *50*, 8922–8926.

[12] B. Weiss, A. Jacquemin-Sablon, T. R. Live, G. C. Fareed, C. C. Richardson, *J. Biol. Chem.* **1968**, *243*, 4543–4555.

[13] A cohesive end unit is the amount of enzyme needed to catalyze the ligation of 50% of 0.12 M lambda DNA in 20 μL in 30 minutes at 16 °C. One Weiss unit is equal to approximately 300 cohesive end units.

[14] G. J. S. Lohman, S. Tabor, N. M. Nichols, *Curr. Protoc. Mol. Biol.* **2011**, *Chapter 3*, 3.14.11–13.14.17.

  

[15] A. Abe, K. Inoue, T. Tanaka, J. Kato, N. Kajiyama, R. Kawaguchi, S. Tanaka, M. Yoshiba, M. Kohara, *J. Clin. Microbiol.* **1999**, *37*, 2899 – 2903.
[16] M. Levy, A. D. Ellington, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 6416 – 6421.
[17] a) X. Li, A. F. Hernandez, M. A. Grover, N. V. Hud, D. G. Lynn, *Heterocycles* **2011**, *82*, 1477; b) X. Li, Z.-Y. J. Zhan, R. Knipe, D. G. Lynn, *J. Am. Chem. Soc.* **2002**, *124*, 746 – 747.
[18] L. A. Loeb, B. D. Preston, *Annu. Rev. Genet.* **1986**, *20*, 201 – 230.
[19] a) N. K. Kochetkov, E. I. Budovskii in *Organic Chemistry of Nucleic Acids* (Eds.: N. K. Kochetkov, E. I. Budovskii), Plenum, New York, **1972**, pp. 425 – 448; b) P. A. Küpfer, C. J. Leumann, *Nucleic Acids Res.* **2007**, *35*, 58 – 68.

  