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Improvement of DNA adenylation using T4 DNA ligase with a template strand and a strategically mismatched acceptor strand

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Abstract

DNA with a 5′-adenylpyrophosphoryl cap (5′-adenylated DNA; AppDNA) is an activated form of DNA that is the biochemical intermediate of the reactions catalyzed by DNA ligase, RNA ligase, polynucleotide kinase, and other nucleic acid modifying enzymes. 5′-Adenylated DNA is also useful for in vitro selection experiments. Efficient preparation of 5′-adenylated DNA is therefore desirable for several biochemical applications. Here we have developed a DNA adenylation procedure that uses T4 DNA ligase and is more reliable than a previously reported approach that used the 5′-phosphorylated donor DNA substrate to be adenylated, a DNA template, and ATP but no acceptor strand. Our improved DNA adenylation procedure uses the above components as well as an acceptor strand that has a strategically chosen C–T acceptor–template mismatch directly adjacent to the adenylation site. This mismatch permits adenylation of the donor DNA substrate but largely suppresses subsequent ligation of the donor with the acceptor, as assayed on nine different DNA substrates that collectively have all four DNA nucleotides represented at each of the first two positions. The new DNA adenylation procedure is successful using either laboratory-prepared or commercial T4 DNA ligase and works well on the preparative (2 nmol) scale for all nine of the test DNA substrates.
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1. Introduction

5′-Adenylated DNA (5′-AppDNA; Fig. 1A) is the biochemical intermediate of nucleic acid modifying enzymes such as DNA ligase [1–4] and RNA ligase [5,6]. This activated form of DNA is desirable for its potential utility as a DNA substrate in various reactions catalyzed by ribozymes and deoxyribozymes that are identified by in vitro selection (e.g., Refs. [7,8]). In addition, we envision the use of 5′-adenylated DNA as a building block in our efforts with double-stranded DNA as a macromolecular conformational constraint [9–11]. Chiuman and Li reported a procedure for 5′-adenylation of DNA oligonucleotides using T4 DNA ligase (Fig. 1B) [12]. They used a template DNA strand along with the DNA oligonucleotide substrate

that was to be adenylated; the latter strand is termed the "donor" because it donates the 5′-phosphate to which an AMP moiety is transferred. By omitting the "acceptor" strand that normally becomes ligated to the donor by DNA ligase or other ligase enzymes, the adenylated oligonucleotide was synthesized as the final product in high yield.

Unfortunately, we have not been uniformly successful in applying the reported DNA adenylation method [12] to a wide range of DNA oligonucleotide substrates. While in our hands some DNA donor substrates are adenylated efficiently, others react only poorly, and we have generally been unable to predict the outcome for any new substrate sequence. Moreover, in some cases a particular donor substrate that previously had been adenylated efficiently fails to react well, despite all efforts to be consistent in reaction setup. Therefore, we sought to improve the adenylation procedure itself. We noted a report by Testa and coworkers

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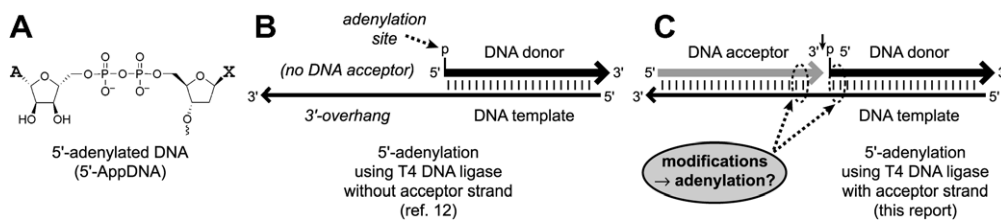 *E-mail address:* scott@scs.uiuc.edu (S.K. Silverman).

Fig. 1. 5′-Adenylated DNA and its preparation from a 5′-phosphorylated DNA donor substrate. (A) Structure of 5′-AppDNA. (B) Preparation of 5′-AppDNA using T4 DNA ligase and a template with a 3′-overhang but no acceptor strand, as reported by Chiuman and Li [12]. (C) The preparative approach investigated in this report, in which a DNA acceptor is included but strategic chemical modifications (i.e., functional group or nucleotide changes) are examined near the adenylation site. If the modifications suppress adenylation, then no reaction of the DNA donor substrate is observed. If the modifications permit adenylation but suppress subsequent ligation (joining of acceptor and donor strands at the site marked with the small arrowhead), then 5′-AppDNA is successfully made. If the modifications permit both adenylation and ligation, then the DNA acceptor becomes ligated to the DNA donor.

[13], in which these investigators sought to develop a "universal template" sequence for T4 DNA ligase. Their approach was to determine if certain Watson–Crick mismatches between template and acceptor (or template and donor) near the acceptor–donor ligation site nevertheless permit efficient ligation for a wide range of substrates. In some cases, they observed considerable accumulation of the adenylated donor strand (e.g., see Fig. 2 of Ref. [13]). Although incomplete ligation in this fashion was an undesired outcome in their study, we were interested to determine if one or more strategic chemical modifications of the template or acceptor strand (at either the functional group or nucleotide level) could be exploited to improve the DNA adenylation procedure with T4 DNA ligase (Fig. 1C). Here we report that a nontrivial modification of the Chiuman and Li DNA 5′-adenylation approach [12] by including an acceptor strand that has a single strategic mismatch with the template strand significantly expands the range of DNA substrates that may be successfully and reliably adenylated using T4 DNA ligase.

2. Materials and methods

2.1. Oligonucleotides

DNA oligonucleotides were prepared at IDT (Coralville, IA). The sequences of the donor DNA substrates (designated DNA1–DNA9) are given in Table 1. Two different template oligonucleotides were used for each donor DNA. The short template was 5′-X_nTATAG-3′, where X_n represents the sequence complementary to the donor. The long templates were complementary to both the donor and acceptor DNAs, producing a 23-nt overhang when the acceptor DNA was not included. Mismatches were tested by combining the appropriate donor, acceptor, and template oligonucleotides. The acceptor oligonucleotide used for final optimization studies was 5′-GAATTCTAATACGACTCACTAT<u>C</u>-3′, where <u>C</u> forms a mismatch with <u>T</u> in the template oligonucleotide (5′-X_n<u>T</u>ATAGTGAGTCGTATTAGAATTC-3′, where X_n represents the sequence complementary to the donor). Changes to the

Table 1
Donor DNA sequences used in this study (DNA1–DNA9)[a]

Name	Sequence
DNA1	5′-GGAAGAGATGGCGACGG-3′
DNA2	5′-AGAAGAGATGGCGACGG-3′
DNA3	5′-TGAAGAGATGGCGACGG-3′
DNA4	5′-CGAAGAGATGGCGACGG-3′
DNA5	5′-GCAAGACACGGTG-3′
DNA6	5′-GTCGGGTGGCATTCT-3′
DNA7	5′-CTCGGGTGGCATTCT-3′
DNA8	5′-GAGGAGAGCAATAGTAA-3′
DNA9	5′-GCTTCTCTACCGCTGCC-3′

[a] DNA2–DNA4 are related to DNA1 by changing only the 5′-terminal nucleotide. Similarly, DNA7 is related to DNA6 by changing only the 5′-terminal nucleotide. DNA5, DNA8, and DNA9 have no consistent homology to the other six sequences or to each other. DNA8 and DNA9 are derived from DNA1 by making systematic transitions or transversions for all but the 5′-terminal nucleotide.

sequences of the acceptor and template are indicated in the figures. Donor and acceptor oligonucleotides were purified by denaturing PAGE with running buffer 1× TBE (89 mM each Tris and boric acid and 2 mM EDTA, pH 8.3) as described previously [14,15]. Template DNA oligonucleotides were either purified by denaturing PAGE or extracted with phenol/chloroform and precipitated with ethanol.

2.2. Expression and purification of T4 DNA ligase

The 3H1 plasmid encoding C-terminal His$_6$-tagged T4 DNA ligase was kindly provided by Scott Strobel (Yale University). Approximately 25 ng of the plasmid was used to transform BL21(DE3)pLysS cells (Stratagene) according to the manufacturer's protocol. Individual colonies were used to inoculate four starter cultures each containing final concentrations of 5 mM glucose, 50 µg/mL ampicillin, and 100 µg/mL chloramphenicol in 5 mL of LB media. After overnight incubation at 37 °C with shaking at 220 rpm, each starter culture was used to inoculate an induction culture consisting of 750 mL 2×YT media supplemented with 19 mL 1 M potassium phosphate (pH 7.8), 3.8 mL 1 M glucose, 0.75 mL 50 mg/mL ampicillin, and 1.5 mL 50 mg/mL chloramphenicol. The four induction cultures were grown

at 37 °C with shaking to an OD_{600} of 0.4, and then shifted to 24 °C until the OD_{600} was ~0.9. Each culture was induced by adding 75 µL 1 M IPTG and incubated for 90 min at 24 °C with shaking. Cells were pelleted by centrifugation at 6000 rpm (5400g) for 5 min at 4 °C. The four cell pellets were stored at −20 °C prior to cell lysis and protein purification.

Each cell pellet was resuspended in 9.5 mL of Buffer A [700 mM NaCl, 50 mM HEPES (pH 7.2), 5 mM β-mercaptoethanol, 10% glycerol, and 1 mM imidazole (pH 7.4)] by pipetting and was brought to a final volume of 25 mL with Buffer A. The four pellets were processed in parallel and combined prior to lysis by sonication. Cell debris was pelleted by centrifugation at 11500 rpm (20000g) for 30 min at 4 °C and discarded. To the ~40 mL supernatant was added 3 mL of Ni–NTA agarose (Qiagen), and the resulting suspension was incubated at 4 °C for 30 min with gentle rocking on a nutator. Imidazole was added to a final concentration of 1 mM from a 1 M stock at pH 7.4, and the suspension was incubated at 4 °C for 30 min with rocking. The agarose was pelleted by centrifugation at 2000 rpm (700g) for 5 min at 4 °C and the supernatant was decanted. The agarose was washed three times by incubating the beads in 40 mL of Buffer A at 4 °C for 30 min with gentle rocking, pelleting the beads by centrifugation, and decanting the supernatant. A fourth incubation of the beads in 40 mL of Buffer A at 4 °C with gentle rocking was performed overnight. The beads were pelleted by centrifugation and the supernant was decanted. The beads were resuspended in 8 mL of Buffer B [700 mM NaCl, 50 mM HEPES (pH 7.2), 5 mM β-mercaptoethanol, 10% glycerol, and 10 mM imidazole (pH 7.4)] and loaded into a column (0.8 cm diameter × 4 cm height). The column was washed with 10 mL of Buffer B. T4 DNA ligase was eluted by washing the column with 8 mL of Buffer C [700 mM NaCl, 50 mM HEPES (pH 7.2), 5 mM β-mercaptoethanol, 10% glycerol, and 50 mM imidazole (pH 7.4)], followed by washing with 12 mL of Buffer D [700 mM NaCl, 50 mM HEPES (pH 7.2), 5 mM β-mercaptoethanol, 10% glycerol and 100 mM imidazole (pH 7.4)]. The eluent was collected in 1 mL fractions. Fractions containing T4 DNA ligase were identified by SDS–PAGE. Pooled fractions were dialyzed (10,000 MWCO) against 2 L of storage buffer [10 mM HEPES (pH 7.2), 50 mM KCl, 0.1 mM EDTA, 1 mM DTT and 50% glycerol] overnight at 4 °C with slow stirring. The concentration of the stock solution of T4 DNA ligase was 4.7 µg/µL as determined by Bradford assay [16].

2.3. Adenylation of DNA substrates under conditions used by Chiuman and Li (Fig. 2)

Analytical-scale adenylation assays were performed using the previously reported incubation conditions [12]. A sample containing a trace amount (<0.5 pmol) of 5′-^{32}P-radiolabeled DNA substrate, 25 pmol unradiolabeled 5′-monophosphorylated DNA substrate, and 50 pmol DNA template in 6 µL of 5 mM Tris (pH 7.5), 15 mM NaCl, and 0.1 mM EDTA was annealed by heating at 95 °C for 3 min and cooling on ice for 5 min. The solution was brought to 10 µL total volume containing 1× T4 DNA ligase buffer [50 mM Tris–HCl (pH 7.8), 10 mM $MgCl_2$, 20 mM NaCl, and 0.5 mM ATP], 0.1 mg/mL BSA and 1 µL T4 DNA ligase (1 U/µL; Fermentas). Final concentrations were 2.5 µM DNA substrate, 5 µM DNA template, and 0.1 U/µL T4 DNA ligase. The samples were incubated at 37 °C for 16 h. At appropriate time points, 2 µL was removed and quenched into 8 µL of stop solution (80% formamide, 1× TB, 50 mM EDTA, and 0.25% each bromophenol blue and xylene cyanol, where 1× TB contains 89 mM each Tris and boric acid at pH 8.3). Time points were 0, 6, and 16 h. Products were separated by 20% denaturing PAGE and imaged with a PhosphorImager.

The above assays incorporated two slight changes relative to the method of Ref. [12], which (1) used annealing conditions of 90 °C in water for 1 min followed by cooling at room temperature, and (2) used DNA substrate concentrations of 0.2–1 µM with 1.0 equivalents of DNA template. Either change could potentially contribute to the low adenylation activities that we observed using the method of Ref. [12]. Therefore, we tested both annealing conditions and also low (0.2, 1 µM) and high (2.5 µM) DNA substrate concentrations in side-by-side fashion for DNA substrates DNA1 and DNA6. In all cases the adenylation yields were comparable for the same DNA substrate (data not shown), indicating that these slight changes are not responsible for the low adenylation yields that we observed using the method of Ref. [12].

2.4. Initial analytical-scale adenylation assays (Figs. 3–6)

A sample containing 1–2 pmol 5′-^{32}P-radiolabeled DNA substrate, 50 pmol unradiolabeled 5′-monophosphorylated DNA substrate, 100 pmol DNA template, and (when included) 200 pmol DNA acceptor in 14 µL of 5 mM Tris (pH 7.5), 15 mM NaCl, and 0.1 mM EDTA was annealed by heating at 95 °C for 3 min and cooling on ice for 5 min. The solution was brought to 20 µL total volume containing 1× T4 DNA ligase buffer [40 mM Tris–HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT], 6 mM ATP, and 0.3 µL His_6-T4 DNA ligase (4.7 µg/µL as determined above). Final concentrations were 2.5 µM DNA substrate, 5 µM DNA template, 10 µM DNA acceptor (when included), and ~70 ng/µL His_6-T4 DNA ligase. Reactions were incubated at 37 °C for 24 h (Fig. 3); 25 °C for 3 h (Fig. 4); or 25 °C for 2 h (Figs. 5 and 6). At appropriate time points, 3 µL was removed and quenched into 7 µL of stop solution. Products were separated by 20% denaturing PAGE and imaged with a PhosphorImager. The 2′-methoxy-2′-deoxy-modified acceptor was 5′-GAATTCTAATACGACTCACTAT<u>A</u>-3′, where the <u>A</u> was 2′-methoxy. The 2′,3′-dideoxy-modified acceptor was 5′-GAATTCTAATACGACTCACTAT<u>C</u>-3′, where the <u>C</u> was dideoxy-C (the template nucleotide was

accordingly changed from T to G to retain Watson–Crick complementarity).

2.5. Analytical-scale adenylation reactions using the C–T acceptor–template mismatch and laboratory-prepared enzyme (Fig. 7)

A sample containing 2 pmol 5′-³²P-radiolabeled DNA substrate, 50 pmol unradiolabeled 5′-monophosphorylated DNA substrate, 100 pmol DNA template, and 200 pmol DNA acceptor (only for Fig. 7A) in 14 μL of 5 mM Tris (pH 7.5), 15 mM NaCl, and 0.1 mM EDTA was annealed by heating at 95 °C for 3 min and cooling on ice for 5 min. The solution was brought to 20 μL total volume containing 1× T4 DNA ligase buffer [40 mM Tris–HCl (pH 7.8), 10 mM MgCl$_2$, 10 mM DTT], 6 mM ATP, and 3 μL His$_6$-T4 DNA ligase (4.7 μg/μL as determined above). Final concentrations were 2.5 μM DNA substrate, 5 μM DNA template, 10 μM DNA acceptor (when included), and 70 ng/μL His$_6$-T4 DNA ligase. Samples were incubated at 25 °C for 2 h. At appropriate time points, 3 μL was removed and quenched into 7 μL of stop solution. Products were separated by 20% denaturing PAGE and imaged with a PhosphorImager.

2.6. Optimized preparative-scale adenylation reactions using the C–T acceptor–template mismatch (Fig. 8)

One of two closely related procedures was used. For DNA1–DNA3, a sample containing 2 nmol 5′-monophosphorylated DNA substrate, 2.2 nmol DNA template, and 2.4 nmol DNA acceptor in 280 μL of 5 mM Tris (pH 7.5), 15 mM NaCl, and 0.1 mM EDTA was annealed by heating at 95 °C for 3 min and cooling at 25 °C for 20 min. The solution was brought to 400 μL total volume containing 1× T4 DNA ligase buffer [40 mM Tris–HCl (pH 7.8), 10 mM MgCl$_2$, 10 mM DTT], 6 mM ATP, and 24 μL His$_6$-T4 DNA ligase (4.7 μg/μL as determined above). Final concentrations were 5.0 μM DNA substrate, 5.5 μM DNA template, 6.0 μM DNA acceptor and ∼300 ng/μL His$_6$-T4 DNA ligase. For DNA4–DNA9, the same procedure was followed except the annealing volume was 140 μL and the final sample volume was 200 μL; final concentrations were 10 μM DNA substrate, 11 μM DNA template, 12 μM DNA acceptor and ∼300 ng/μL His$_6$-T4 DNA ligase (i.e., only 12 μL of T4 DNA ligase was added). The two-fold more concentrated conditions used for DN4–DNA9 led to lower yields when tested with DNA1–DNA3. For DNA1–DNA9, samples were incubated at 25 °C for 2 h, extracted with phenol/chloroform, precipitated with ethanol, and separated by 20% denaturing PAGE (imaging by handheld UV viewer over fluorescent TLC plate). Adenylated DNA products were extracted from the gel and precipitated with ethanol as described previously [14,15]. The identities of the adenylated donor DNA oligonucleotides were confirmed by MALDI-TOF mass spectrometry. The observed (calculated) mass values for 5′-AppDNA were as follows: DNA1, 5757 (5758); DNA2, 5744 (5742);

DNA3, 5732 (5733); DNA4, 5717 (5718); DNA5, 4417 (4418); DNA6, 5023 (5024); DNA7, 4984 (4984); DNA8, 5727 (5725); DNA9, 5483 (5482). Mass spectra were obtained on an Applied Biosystems Voyager instrument in the Mass Spectrometry Laboratory of the University of Illinois School of Chemical Sciences.

2.7. Optimized analytical-scale adenylation reactions using the C–T acceptor–template mismatch and commercial enzyme (Fig. 9)

A sample containing a trace amount of 5′-³²P-radiolabeled DNA substrate, 25 pmol unradiolabeled 5′-monophosphorylated DNA substrate, 50 pmol DNA template, and 100 pmol DNA acceptor in 7 μL of 5 mM Tris (pH 7.5), 15 mM NaCl, and 0.1 mM EDTA was annealed by heating at 95 °C for 3 min and cooling on ice for 5 min. The solution was brought to 10 μL total volume containing 1× T4 DNA ligase buffer [40 mM Tris–HCl (pH 7.8), 10 mM MgCl$_2$, 10 mM DTT], 6 or 20 mM ATP, and 1.0 μL 0.04 U/μL T4 DNA ligase containing 1 mg/mL BSA. The diluted enzyme stock was prepared by diluting 1 U/μL T4 DNA ligase (Fermentas) with water and 10 mg/mL BSA to a final 10× concentration of 0.04 U/μL T4 DNA ligase and 1 mg/mL BSA. Final reaction concentrations were 2.5 μM DNA substrate, 5 μM DNA template, 10 μM DNA acceptor, 0.1 mg/mL BSA, 6 or 20 mM ATP, and 0.004 U/μL T4 DNA ligase. Samples were incubated at 25 °C. At appropriate time points, 1.5 μL was removed and quenched into 8 μL of stop solution. Products were separated by 20% denaturing PAGE and imaged with a PhosphorImager.

3. Results

3.1. Evaluation of DNA 5′-adenylation procedures with a panel of DNA substrates

In the DNA 5′-adenylation method of Chiuman and Li [12], the donor and template strands are included, but the acceptor strand is omitted (Fig. 1B). We chosen nine particular DNA sequences, denoted DNA1–DNA9 (Table 1), for evaluation of adenylation procedures. Collectively, these nine sequences have all four DNA nucleotides represented at both the first and second positions, as well as several different nucleotides represented at all other positions. Therefore, we anticipated that the adenylation results with this set of DNA oligonucleotides should encompass the outcome for essentially any arbitrary oligonucleotide that one might wish to adenylate. We evaluated the approach of Chiuman and Li with donor substrates DNA1–DNA9 using T4 DNA ligase from a commercial supplier. Although some adenylation was observed in all cases, the yields were mostly poor (Fig. 2; seven out of nine donor substrates had <35% adenylation yield). These findings established that the Chiuman and Li method is indeed
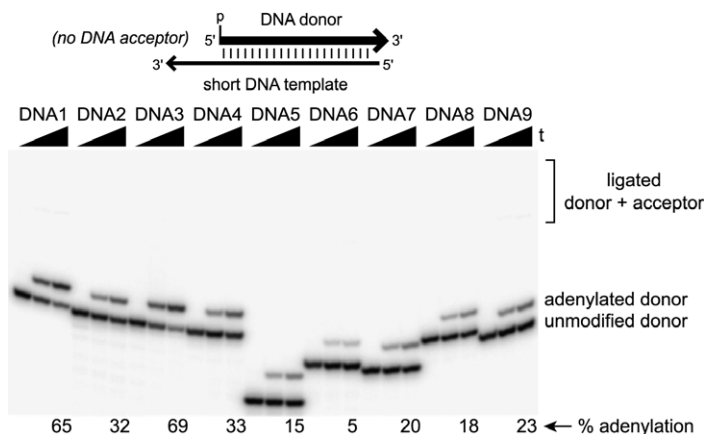
Fig. 2. DNA adenylation procedure of Chiuman and Li (no acceptor strand) [12] using DNA1–DNA9 as substrate and commercial T4 DNA ligase at 0.1 U/μL. Although some adenylation was observed in all cases, for most DNA substrates the yield was low. All reactions used the incubation conditions as reported [12], except with 0.1 mg/mL BSA in each final sample. Reactions using the reported 1 mg/mL BSA gave slightly lower yields (data not shown). $t = 0$, 6, 16 h at 37 °C.

problematic for certain DNA sequences, at least in our hands.

3.2. Atomic-level chemical modifications of the acceptor strand do not enhance adenylation

To begin our assessment of potential improvements to the Chiuman and Li method via inclusion of an acceptor strand, we examined whether atomic-level changes to the acceptor strand could still permit adenylation of the donor strand while suppressing subsequent ligation. A systematic basis for testing these and other changes to the adenylation procedure was provided by choosing two particular DNA substrates for all of the initial assays. The first substrate, DNA1, is a 17-mer that is adenylated well by the Chiuman and Li approach [12]. In our hands DNA1 is adenylated in good yield in the absence of an acceptor (Fig. 2), although

we do not observe the nearly quantitative yields previously reported [12], and on some occasions the adenylation yield is irreproducibly poor for no apparent reason (data not shown). The second substrate, DNA6, is a 15-mer for which we have had even more difficulty performing adenylation, generally obtaining a very poor yield (e.g., only 5% in Fig. 2). For these and subsequent assays we used His6-tagged T4 DNA ligase that was prepared in our laboratory by overexpression in *Escherichia coli* (see Section 2) rather than the commercial enzyme, although we later verified the applicability of our improved procedure with commercial enzyme.

Using the two test substrates, DNA1 and DNA6, we systematically examined the effect of including an acceptor strand that has either a 2′,3′-dideoxy modification or a 2′-methoxy-2′-deoxy modification (Fig. 3). As expected, including an unmodified acceptor strand led to very rapid
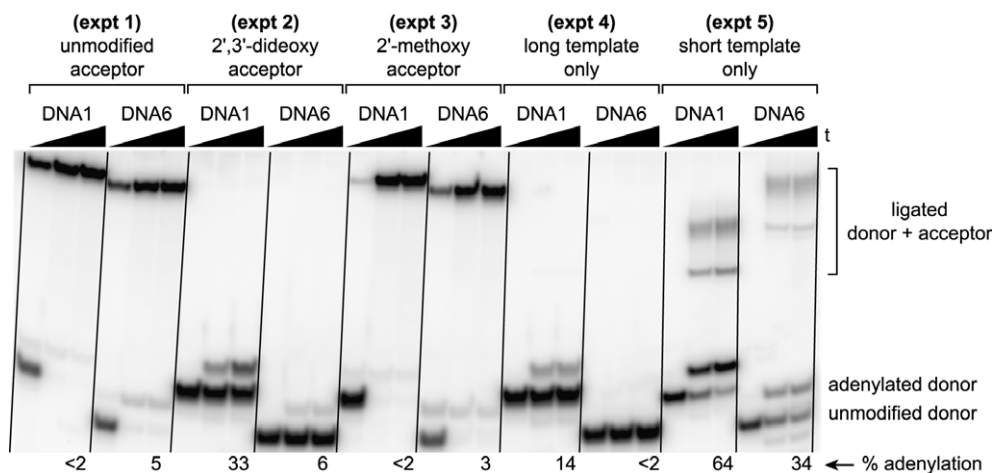

Fig. 3. Testing chemically modified acceptors for improvement to the DNA adenylation procedure. In each Experiments 1–3, both donor substrates DNA1 and DNA6 were tested using a template and an 23-nt acceptor that had at its 3′-terminus no modification (Experiment 1), a 2′,3′-dideoxy modification (Experiment 2), or a 2′-methoxy modification (Experiment 3). Experiments 4–5 used only a template that was either long (23-nt overhang; Experiment 4) or short (5-nt overhang; Experiment 5) and no acceptor. $t = 0$, 10, 24 h at 37 °C.

ligation (Experiment 1), and the 2′-methoxy modification of the acceptor did not suppress this ligation (Experiment 3). The 2′,3′-dideoxy acceptor inherently cannot become ligated, but this modification also largely prevented adenylation (Experiment 2). We concluded that no simple—i.e., readily available—chemical modification to the acceptor strand can achieve the desired effect of allowing adenylation while suppressing the subsequent ligation. We note that 3′-phosphate is also not anticipated to be useful based on the Chiuman and Li's data [12], although we did not test this directly.

We also tested variation of the template strand length in the absence of any acceptor. In the experiments of Chiuman and Li, template length did not appear to affect the adenylation outcome; the 3′-overhang of the template (i.e., the number of single-stranded nucleotides beyond the region of template paired with the donor) could be 1–11 nt with high adenylation yield in their small number of test cases [12]. Unexpectedly, in our hands the longer template strand (23 versus 5 nt of 3′-overhang) was much less effective at adenylation for both DNA1 and DNA6; compare Experiment 4 with Experiment 5 in Fig. 3. This provided additional encouragement for us to improve the overall procedure, so that a more reliable DNA adenylation method can be established.

3.3. Testing mismatches between template and donor or between template and both donor and acceptor

The data from Testa and coworkers towards development of a universal T4 DNA ligase template [13] suggested that we should investigate mismatches involving the template nucleotides on either side of the acceptor–donor ligation junction. At the outset of our investigation, we had no way to predict whether such mismatches would most effectively promote adenylation when they involve nucleotides of the donor strand, the acceptor strand, or both. Using the "difficult" DNA6 adenylation substrate as donor strand, we tested mismatches involving the first donor nucleotide either alone or additionally involving the first acceptor nucleotide (Fig. 4). When a G–A or G–G mismatch or a G–T wobble pair involving the first donor nucleotide was present, the yield of adenylated intermediate was quite low, and nearly complete ligation was observed. In contrast, when a G–A mismatch involving the first donor nucleotide was combined with an A–A mismatch involving the first acceptor nucleotide, essentially no reaction at all was observed. This set of experiments provided no support to pursue any strategy that is based on a mismatch involving the first donor nucleotide, either alone or in combination with a mismatch at the acceptor position.

3.4. Testing mismatches between template and acceptor

We turned to investigating mismatches between the template and acceptor strands, while leaving the donor sub-
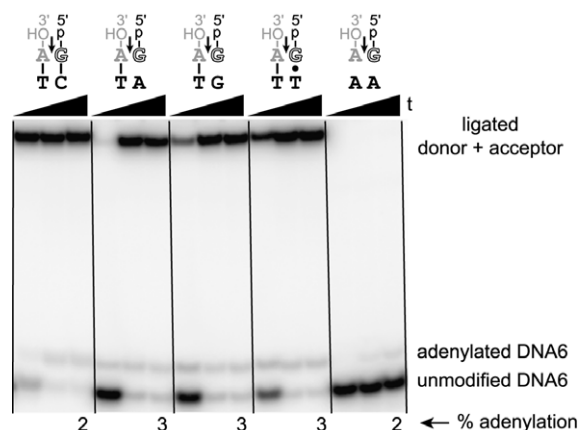

Fig. 4. Testing Watson–Crick mismatches at the first donor nucleotide position with or without an additional mismatch at the first acceptor position. In each assay, the nucleotides directly at the adenylation site are shown. The 5′-terminal pG of the donor substrate (DNA6) is in outline font. The 3′-terminal A of the acceptor is adjacent, with the adenylation (or ligation) site marked with an arrow. The template strand is on the bottom. $t = 0$, 1.5, 3 h at 25 °C.

strate fully Watson–Crick base paired with the template. A preliminary set of experiments suggested that mismatches involving the first acceptor nucleotide should be pursued in greater detail, because >50% adenylation— sometimes with appreciable subsequent ligation—was observed (data not shown). We arbitrarily chose a G–G mismatch involving the first acceptor nucleotide (which gave ⩾70% adenylation; see Fig. 5) for an experiment in which the ATP concentration was optimized. We surveyed adenylation of the DNA6 donor substrate using [ATP] from 20 μM to 20 mM. Between 500 μM and 10 mM ATP the adenylation yield was high (⩾60%), whereas the yield was lower outside of this range. We chose 6 mM ATP as our favored concentration because [ATP] values at the high end of the successful 500 μM to 10 mM range were more effective at suppressing undesired ligation of the adenylated substrate.

Testa and coworkers found that changes at the second position (counting from the 3′-end) of the acceptor strand could influence T4 DNA ligase activity [13]. Therefore, we first examined how such changes impact adenylation of DNA6 (Fig. 5). Again using a G–G mismatch involving the first acceptor nucleotide, we examined all four Watson–Crick base pair combinations at the second acceptor position, and we also examined the two possible orientations of a G–T wobble pair. Each of the four Watson–Crick base pairs at the second acceptor position led to good adenylation with the DNA6 donor substrate, with a variable amount of subsequent ligation (see upper four data sets in the plots of Fig. 5). Use of the T–A acceptor–template base pair led to the highest adenylation yield via having the smallest amount of subsequent ligation product, and a C–G base pair was nearly as successful, whereas A–T and especially G–C led to more ligation product. In contrast, the two wobble combinations both greatly suppressed adenylation, with T–G worse than G–T. We con-
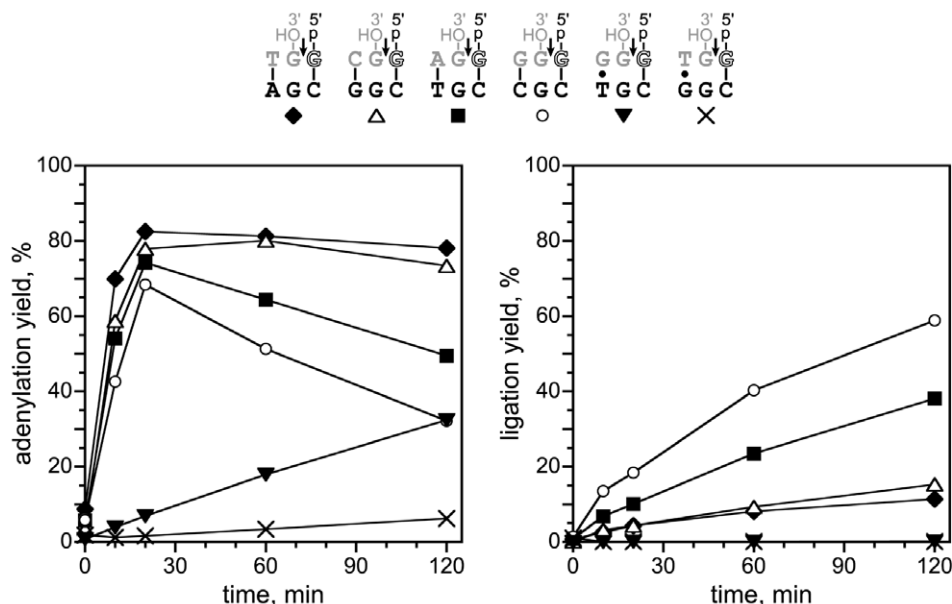
Fig. 5. Testing Watson–Crick and wobble pairs at the second acceptor nucleotide position (DNA6 as donor substrate, 25 °C). The tested combinations are shown at the top in a format similar to Fig. 4. In all cases, the first acceptor nucleotide position is a G–G mismatch; the second acceptor nucleotide position is one of the four Watson–Crick base pairs or one of the two wobble pairs.
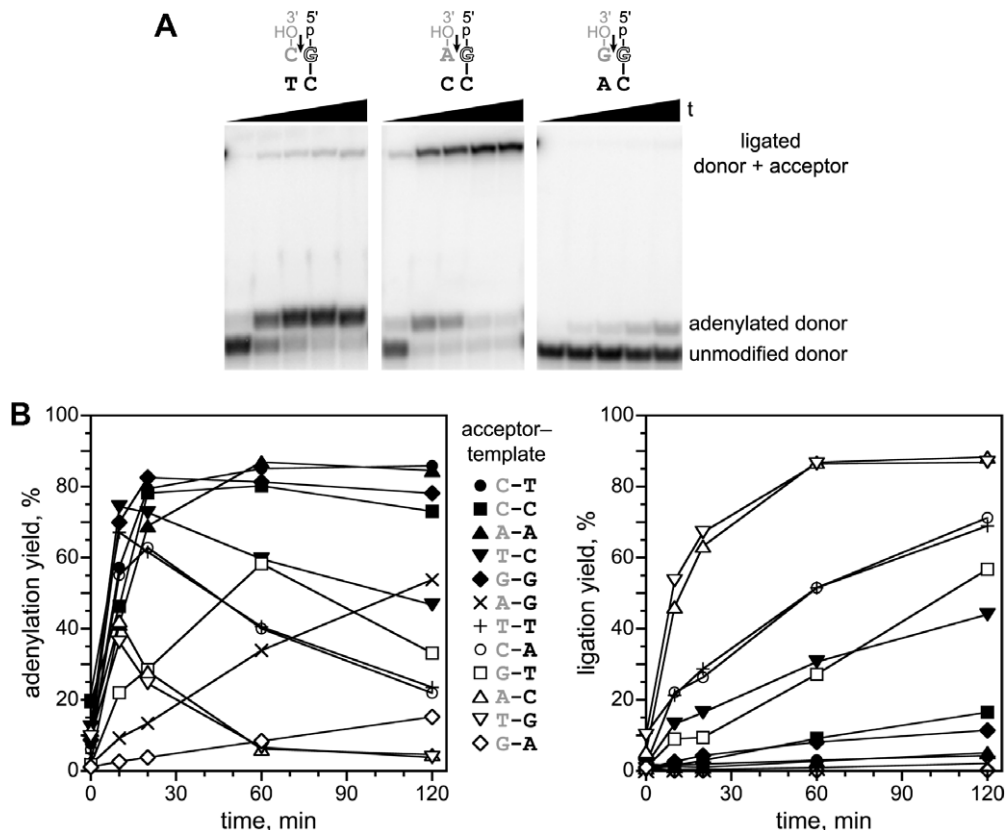

Fig. 6. Testing all twelve non-Watson–Crick combinations at the first acceptor nucleotide position (DNA6 as donor substrate, 25 °C). (A) PAGE images for three combinations that represent the spectrum of observations. (B) Data for all twelve combinations, showing that a C–T acceptor–template mismatch is optimal.

cluded that a Watson–Crick base pair is required at the second position of the acceptor for high adenylation yield, and furthermore that the T–A base pair is optimal at this position (although C–G would also work well).

Finally, we performed a comprehensive test of all twelve non-Watson–Crick nucleotide combinations at the first position of the acceptor strand (obviously, each of the four Watson–Crick combinations would lead to

efficient ligation; cf. Experiment 1 of Fig. 3). Using the DNA6 donor substrate, we were intrigued to find a sensitive dependence of both adenylation and ligation yields on the identities of the nucleotides at the first position of both acceptor and template. The three experimental outcomes shown in Fig. 6A represent the range of observations. The C–T acceptor–template combination led to high adenylation yield with very little subsequent ligation. The A–C combination led to good adenylation but rapid subsequent ligation, such that it would be experimentally challenging to stop the reaction at the adenylated interme-diate (the G–T combination provides another example, with adenylation rate slower than for A–C; Fig. 6B). Finally, the G–A combination led to very poor adenyla-tion with almost no formation of ligated product. From the data for all 12 non-Watson–Crick combinations (Fig. 6B), we concluded that the C–T acceptor–template combination is optimal, although several other combina-tions (e.g., C–C, A–A) would also be good choices. We used the C–T acceptor–template combination for our final experiments, which involved the full panel of donor sub-strates for adenylation.
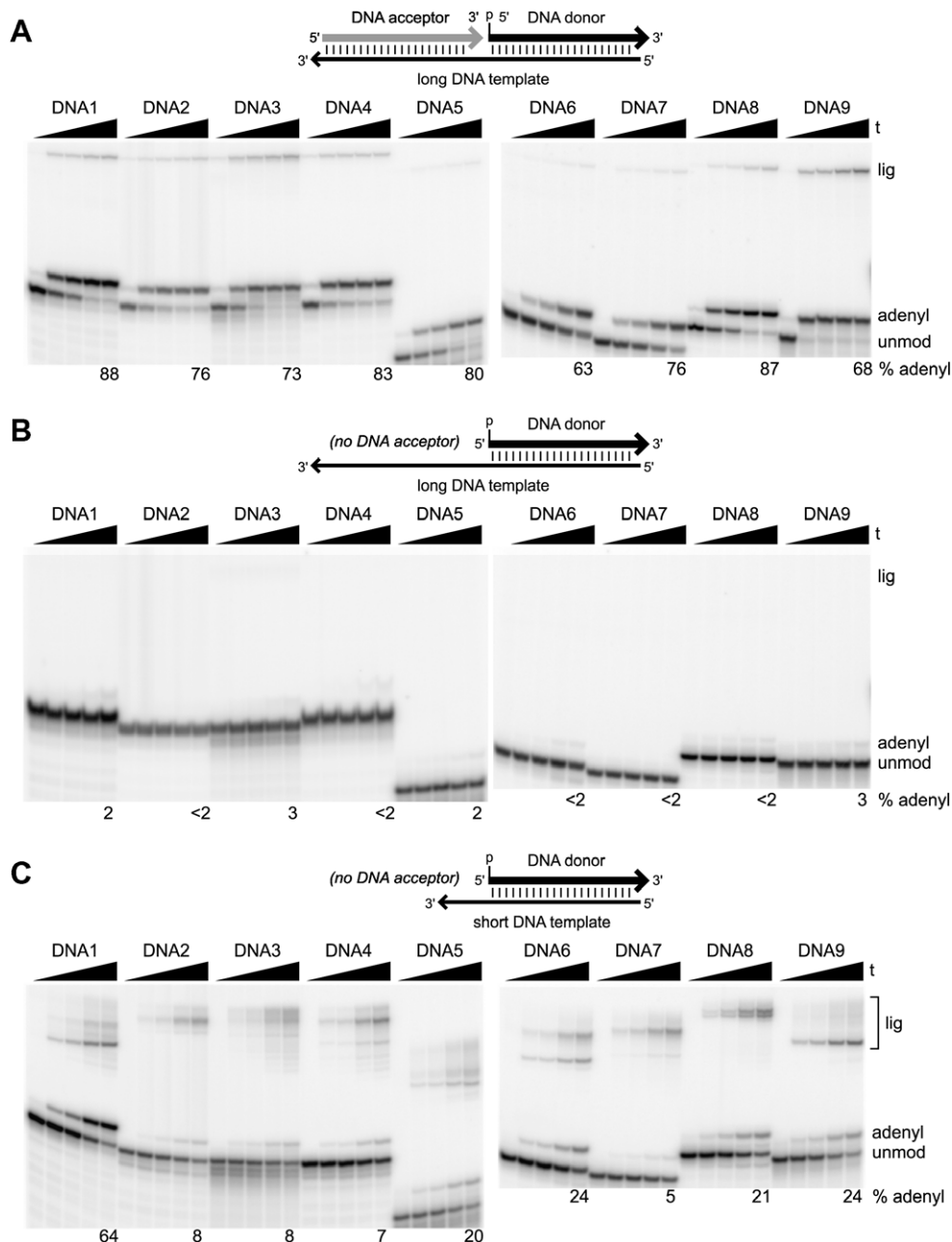

Fig. 7. Analytical-scale adenylation assays with DNA1–DNA9. (A) Using the optimized approach with an acceptor strand that has a C–T acceptor–template mismatch. (B) Using the same long template (23-nt overhang) as in (A), but omitting the acceptor strand. (C) Using a short template (5-nt overhang) and omitting the acceptor strand. $t = 0, 10, 20, 60, 120$ min at 25 °C.

3.5. Applying the improved adenylation method to a wide range of DNA substrates

With a tentatively improved method now developed using only DNA1 and DNA6, we expanded our focus to the full panel of nine DNA substrates, DNA1–DNA9. Small-scale adenylation assays were performed in three ways for each DNA oligonucleotide: (1) Using the new C–T acceptor–template combination that was developed above; (2) Using the same relatively long template strand as in (1) but omitting the acceptor; and (3) Using a short template with only five overhanging nucleotides. The results are shown in Fig. 7. Using the C–T acceptor–template combination, in all nine cases a high yield of adenylated DNA was observed, with at most a modest amount of undesired ligation (Fig. 7A; all nine >65% adenylation yield, and five out of nine >75%). In sharp contrast, using only the long template led to extremely poor adenylation (Fig. 7B; <5%), and using only the short template also led to poor adenylation but better than that observed with only the long template (Fig. 7C; all except DNA1 <25%). Therefore, the new strategy using the C–T acceptor–template combination is clearly superior to the previous accep-

tor-free approach [12] for general DNA adenylation using T4 DNA ligase.

The assays in Figs. 2 and 7C differ in that the former experiment used the incubation conditions from Chiuman and Li [12] with the commercial T4 DNA ligase, whereas the latter experiment used our optimized incubation conditions and laboratory-prepared enzyme. The adenylation yields were not uniformly higher using either set of conditions. Therefore, neither the incubation conditions nor the enzyme source is responsible for the difficulties we observe in DNA adenylation using the Chiuman and Li approach. We have no immediate explanation for the dependence of adenylation yield on the overhang length of the template strand when the acceptor strand is absent, as revealed most clearly upon comparison of Fig. 7B with C. In any case, for both tested overhang lengths the adenylation yields are generally low without the acceptor strand.

3.6. Preparative DNA adenylation using the improved procedure

To check that our new approach (which includes the mismatched acceptor strand and the long template) is suc-
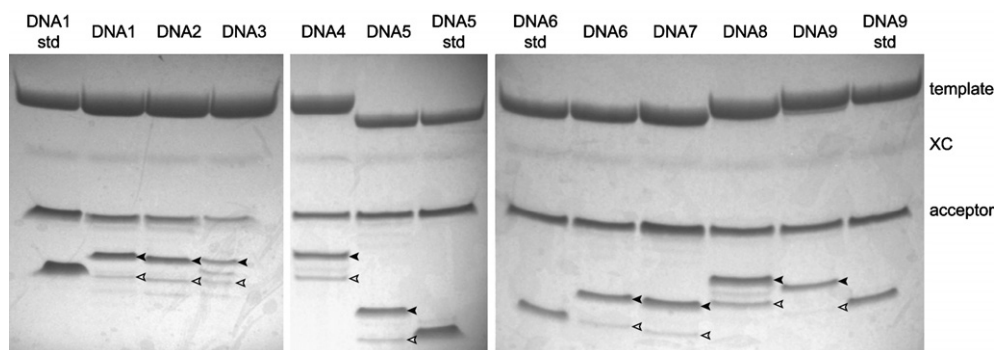

Fig. 8. Preparative-scale adenylation assays with DNA1–DNA9 and the optimized approach using an acceptor strand that has a C–T acceptor–template mismatch. The standard lanes used the indicated donor DNA substrates but omitted the ligase enzyme. See Section 2 for details. Because the template was the same length as donor and acceptor combined, any ligated donor + acceptor would co-migrate with the template. The open arrowheads mark the unadenylated donor substrates, and the filled arrowheads mark the adenylated donor substrates. XC = xylene cyanol dye. In some cases (e.g., DNA3 and DNA8), a small amount of nonspecific degradation is evident; nevertheless, the yield of adenylated DNA is clearly high.
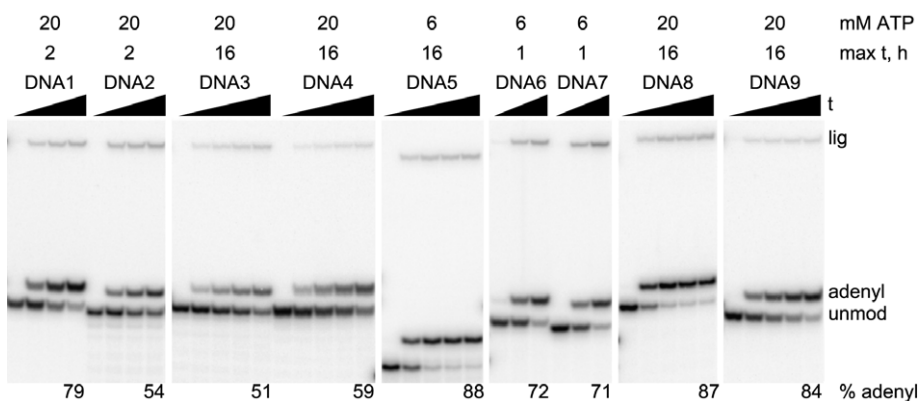

Fig. 9. Analytical-scale tests of newly developed adenylation procedure (C–T acceptor–template mismatch) using DNA1–DNA9 as substrate and commercial T4 DNA ligase at 0.004 U/μL. The ATP concentration (6 or 20 mM) and incubation time (1–16 h) were optimized for each DNA substrate, but these are the only two variables that needed to be optimized. Time points were either $t = 0$, 20 min, 1 h, 2 h; $t = 0$, 1, 3, 6, 16 h; or $t = 0$, 20, 60 min (the maximum time point is indicated above each set of lanes). Compare this figure to Fig. 6A, where the laboratory-prepared enzyme was used.

cessful preparatively, we adenylated each of the nine DNA oligonucleotide substrates DNA1–DNA9 on the 2 nmol scale. In all cases the adenylation reactions were successful (Fig. 8); the yield after PAGE separation, extraction, and precipitation was 0.4–1.0 nmol of 5′-adenylated DNA. The identities of all adenylated oligonucleotides were confirmed by MALDI-TOF mass spectrometry (see Section 2).

3.7. Validation of the new adenylation approach using commercial T4 DNA ligase

Many investigators would likely use a commercial source of the T4 DNA ligase enzyme rather than a laboratory-prepared sample (as was used in Figs. 7 and 8). Therefore, we verified that our new approach with the C–T mismatched acceptor strand can be used for successful DNA adenylation with the commercial enzyme. Preliminary experiments established that a much lower concentration of commercial enzyme is necessary for successful adenylation using our new approach in comparison with the method of Chiuman and Li (0.004 U/μL versus 0.1 U/μL, respectively; a 100-fold difference). Because of this relatively low protein enzyme concentration, we included BSA at 1× concentration of 0.1 mg/mL to provide the best adenylation yield (data not shown). We then demonstrated that the commercial T4 DNA ligase enzyme can successfully be used along with our new approach for adenylation of each of DNA1–DNA9 (Fig. 9; all nine oligonucleotides showed >50% adenylation yield, and six out of nine >70%). For each DNA substrate, a set of incubation conditions and a particular time point could readily be identified at which high adenylation yield could be obtained using the commercial enzyme. The two key variables are the ATP concentration and the incubation time. Depending on DNA sequence, the optimal ATP concentration was either 6 or 20 mM (the higher ATP concentration suppressed ligation following adenylation), and the optimal incubation time was 1–16 h.

4. Discussion

The straightforward availability of 5′-adenylated DNA will facilitate both biochemical investigations of nucleic acid modifying enzymes and in vitro selection experiments that require 5′-adenylated DNA substrates. Although the previously reported 5′-adenylation procedure is successful for certain DNA sequences [12], we have found that it does not work well for many substrates (Fig. 2). Furthermore, its idiosyncratic nature—i.e., in our hands we sometimes obtain a poor adenylation yield even with a DNA substrate that we efficiently adenylated on a prior occasion—is frustrating and suggests that the procedure is balanced on the edge of failure, with the key variable(s) that determine success versus failure not readily identified. These observations provided our motivation to pursue an improved DNA adenylation approach by including an appropriately modified acceptor
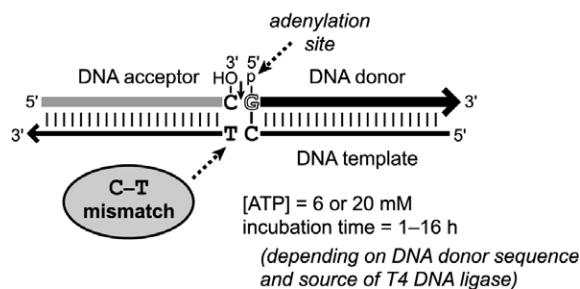

Fig. 10. Optimized strategy for preparation of 5′-adenylated DNA from a 5′-phosphorylated DNA donor substrate, template strand, and acceptor strand with a C–T acceptor–template mismatch. Note that the adenylation site (G in the figure) can be any nucleotide as long as the corresponding nucleotide in the template strand is its Watson–Crick complement.

strand in addition to the template strand. The initial experiments (Figs. 3–5) established that the first acceptor nucleotide position is key, and the remaining experiments (Figs. 6–8) showed that a C–T acceptor–template mismatch is optimal for successful adenylation of all nine of the test substrates (DNA1–DNA9) on both the analytical and preparative scales. Anyone seeking to use our new approach for adenylation will need to optimize the incubation conditions and time depending on enzyme source (Figs. 7A and 9), but this would be true even for the previously published procedure [12], and we have defined a rather narrow set of variables (specifically ATP concentration, ligase concentration, and incubation time) that must be explored to find a successful combination. For clarity and ease of reference, the final improved adenylation strategy is depicted in Fig. 10.

It is perhaps surprising that the adenylation and ligation behavior of T4 DNA ligase depends so sensitively on the identity of the acceptor–template mismatch at the first acceptor position (Fig. 6). This finding likely reflects subtle interactions among the DNA strands and the ligase during the enzyme-catalyzed reaction pathway; more studies are needed to understand these observations. We did not perform the time-consuming and comprehensive mismatch survey experiment of Fig. 6 for any DNA donor substrate other than DNA6, so we cannot know for sure that each acceptor–template mismatch leads to identical behavior with all possible DNA substrates. Nevertheless, the C–T mismatch that was identified as optimal using the DNA6 substrate (Fig. 6) was subsequently shown to support a high adenylation yield for all eight of the other DNA substrates on both the analytical and preparative scales (Figs. 7A and 8). This achieved our practical goal of improving the original procedure [12] and developing a high-yielding, simple, and reliable strategy for 5′-adenylation of essentially any DNA sequence.

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