

# Simple and efficient synthesis of 5' pre-adenylated DNA using thermostable RNA ligase

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## ABSTRACT

**We report a simple method of enzymatic synthesis of pre-adenylated DNA linkers/adapters for next-generation sequencing using thermostable RNA ligase from *Methanobacterium thermoautotrophicum* (MthRnl). Using RNA ligase for the reaction instead of the existing chemical or T4 DNA ligase-based methods allows quantitative conversion of 5'-phosphorylated single-stranded DNA (ssDNA) to the adenylated form. The MthRnl adenylation reaction is specific for ATP and either ssDNA or RNA. In the presence of Mg<sup>+2</sup>, the reaction has a pH optimum of 6.0–6.5. Unlike reactions that use T4 DNA ligase, this protocol does not require synthesis of a template strand for adenylation. The high yield of the reaction simplifies isolation and purification of the adenylated product. Conducting the adenylation reaction at the elevated temperature (65°C) reduces structural constraints, while increased ATP concentrations allow quantitative adenylation of DNA with a 3'-unprotected end.**

## INTRODUCTION

The increasing demand for 5'-adenylated DNA linkers/adapters correlates with the rapid progress in high-throughput next-generation sequencing (NGS) of small RNAs. The construction of cDNA libraries for NGS requires attachment of 5' and 3' sequencing platform-specific adapters for downstream amplification. For the first-strand cDNA synthesis, a 3' adapter is ligated to the 3'-end of the RNA to introduce a sequence for annealing the primer used by reverse transcriptase. This protocol required dephosphorylation at the 5'-end of RNA prior to ligation to prevent self-circularization. In addition, self-ligation of the 3' DNA adapter is blocked by modification at the 3'-terminus. In protocols that ligate an adapter to the 5'-end of the RNA, prior to reverse transcription, the RNA must be re-phosphorylated to allow

adapter ligation. Improved protocols for cDNA library construction use pre-adenylated single-stranded DNA (ssDNA) (AppDNA), as a substrate in a 3' adapter ligation reaction with no ATP and either T4 RNA ligase 1 (1) or a truncated version of T4 RNA ligase 2 (2). The use of the adenylated linkers removes the need to dephosphorylate the RNA prior to ligation and prevents unwanted ligation products.

Current methods for synthesis of AppDNA include either chemical synthesis or enzymatic synthesis using T4 DNA ligase. The commonly used chemical method is a two-step reaction including activation of AMP followed by coupling of adenosine 5'-phosphorimidazolide to a 5'-phosphorylated DNA oligonucleotide in solution or during solid phase oligonucleotide synthesis (3,4). This chemical method does not result in quantitative conversion of the phosphorylated substrate to the adenylated product. Additional purification steps are required to separate two closely related DNAs differing by only 1 nt. This results in lower yields and higher cost of adenylated DNA oligonucleotides.

Adenylated high-energy AppDNA or AppRNA are intermediates of the enzymatic ligation reaction and usually do not accumulate during ligation. Current enzymatic methods for ssDNA adenylation use interrupted double-stranded DNA ligation with T4 DNA ligase, which includes a DNA template complementary to a phosphorylated ssDNA donor but no DNA acceptor (5,6). While the enzymatic approach can produce conversion of substrate to product with high yield, it is not always reliable. Another improved approach includes additional acceptor template-mismatched substrates for the T4 DNA ligase, adjacent to the ligation site (7). The use of T4 DNA ligase for adenylation requires the synthesis of a template or mismatched acceptor and template for each DNA linker, annealing, adenylation and then purification of the product away from the template strand.

Template-independent RNA ligases that use single-stranded nucleic acids prefer RNA but can also ligate DNA to itself or to RNA. These properties make them a potentially useful choice for the ssDNA adenylation

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reaction. RNA ligases are found in all phylogenetic domains. Regardless of their physiological activities, RNA ligases are ATP dependent and can be divided into two classes on the basis of RNA substrate specificity, Rnl1 and Rnl2. Rnl1 family ligases repair single-stranded breaks in tRNA. This family include T4 RNA ligase 1 (8) and a thermostable RNA ligase 1 from *Thermus scitoductus* bacteriophage TS2126 (CircLigase<sup>TM</sup>) (9). T4 RNA ligase 2 is a typical example of Rnl2 family, which can seal nicks in duplex RNAs (10). Archaeal RNA ligase from the thermophilic archaeon *Methanobacterium thermoautotrophicum* (MthRnl) was found by sequence homology resembling T4 RNA ligase 2, but specific to circularization of RNA and ssDNA (11).

Accumulation of adenylated short oligo(dT)<sub>n</sub> was observed in reactions with T4 RNA ligase 1 (12). T4 RNA ligase 1 was also reported to accumulate the intermediate AppRNA product when using annealed complementary blocking DNA (13). Some archaeal RNA ligases accumulate AppRNA when an excess of ATP is used in the reaction i.e. (11,14). The substrate binding specificity of RNA ligases, in general, is more relaxed for donor substrates than acceptors (15) and some can efficiently ligate (circularize) ssDNA (9). To our knowledge, however, no method has been demonstrated that allows scalable high yield adenylation of 5'-phosphorylated ssDNA using RNA ligase.

## MATERIALS AND METHODS

### Enzymes

Thermostable RNA ligase, MthRnl, previously described in (11), was from New England Biolabs, Ipswich, MA (5' DNA Adenylation kit). According to the manufacturer, the enzyme was purified to >95% purity by SDS-PAGE, free from ribo-, exo-, endo-nucleases and phosphatase activities and nucleic acids. Depending on the preparation, MthRnl was purified in almost quantitatively (> 97%) adenylated form or as a free apoenzyme. The state of enzyme adenylation was analyzed by mass-spectrometry using ESI-TOF 6210 (Agilent Technology) and by testing for DNA adenylation in the absence of ATP. While we did not see any difference in activity between these two forms, adenylated form of MthRnl was used in this study, except for protein adenylation assays. T4 RNA ligases 1 (T4Rnl1), T4 RNA ligase 2 (T4Rnl2) and T4 RNA ligase 2 truncated (T4Rnl2tr) were from NEB. CircLigase<sup>TM</sup> was from Epicentre Biotechnologies.

### Nucleic acids

Oligonucleotides used in this study were synthesized at Integrated DNA Technologies:

pAGT GAA TTC GAG CTC GGT ACC CGG TGG  
ATC CTC TAG AGT CGA CCT GCA GG  
(pDNA50)  
pTCG TAT GCC GTC TTC TGC TTG-NH<sub>2</sub>  
(pDNA21-NH<sub>2</sub>)

pTCG TAT GCC GTC TTC TGC TTG-3bioTEG  
(pDNA21-3bioTEG)  
pCTA TAG AAA CCC ACG CAA AGC CC-ddC  
(pDNA23-ddC)  
pCTG TAG GCA CCA TCA AT-NH<sub>2</sub> (pDNA17c-NH<sub>2</sub>)  
pATG TAG GCA CCA TCA AT-NH<sub>2</sub> (pDNA17a-NH<sub>2</sub>)  
pTTG TAG GCA CCA TCA AT-NH<sub>2</sub> (pDNA17t-NH<sub>2</sub>)  
pGTG TAG GCA CCA TCA AT-NH<sub>2</sub> (pDNA17g-NH<sub>2</sub>)  
FAM-rCrUrG rArUrG rArArA rCrCrC rArCrG  
rCrArA rArGrC rCrC(FAM-RNA23 acceptor)  
rCrUrA rUrAC rArArC rCrUrA rCrUrA rCrCrU  
rCrArA rA (RNA22 acceptor).  
Pre-adenylated DNA linkers were synthesized at NEB using MthRnl and method described in this study:  
rAppCTG TAG GCA CCA TCA AT-NH<sub>2</sub> (AppDNA17c-NH<sub>2</sub>)  
rAppTCG TAT GCC GTC TTC TGC TTG-NH<sub>2</sub>  
(AppDNA21-NH<sub>2</sub>).

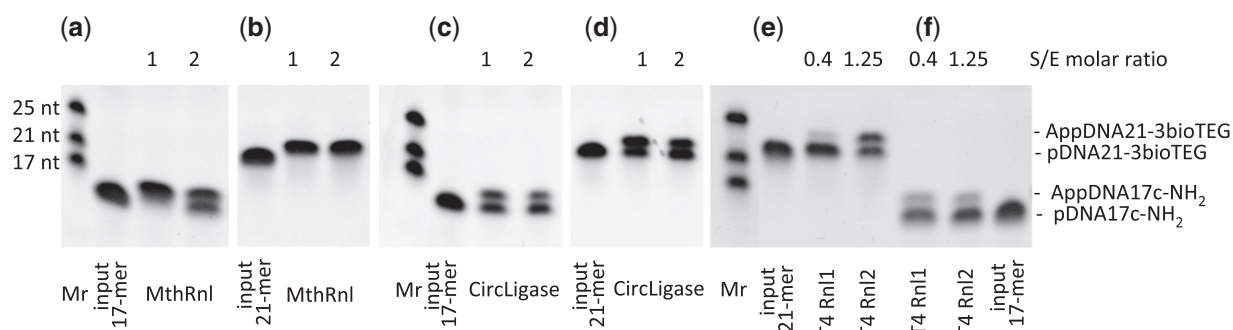
### Reaction conditions

Standard ssDNA adenylation reactions were performed in a reaction mixture (10 µl total) containing 50 mM sodium acetate, pH 6.0 buffer, 5 pmol of 3'-blocked, 5'-phosphorylated ssDNA oligonucleotide, 100 µM ATP, 10 mM MgCl<sub>2</sub>, 5 mM DTT, 0.1 mM EDTA, 5 pmol (230 ng) of MthRnl (monomer). Assays were performed in 200 µl PCR tubes using an S1000 Thermal Cycler (BioRad) at 65°C for 60 min, followed by inactivation of the enzyme at 85°C for 5 min. After addition of 5 µl formamide loading buffer, the reaction mixture was separated on 15% Urea-TBE denaturing polyacrylamide minigels (Invitrogen), stained with SYBR Gold (Invitrogen) and visualized using AlphaImager HP (Alpha Innotech). All parameters that are different from the standard reaction conditions are indicated in figure legends.

DNA circularization assays were performed in a standard reaction containing excess of pre-adenylated MthRnl, 25 pmol (monomer) and 5 pmol of 5'-phosphorylated ssDNA with free 3'-end (pDNA50) and variable concentrations of ATP as indicated in figure legends. The circular form of ligated DNA was confirmed by resistance to Exonuclease I (NEB) digest, and the linear adenylated form by ESI-MS analysis and by functional assay with T4Rnl2tr (described below).

For preparative DNA adenylation, 30 µM of DNA substrate and 15–30 µM of enzyme monomer were used in reactions as described above in corresponding volume at 65°C for 2 h and 5 pmol DNA aliquots were gel analyzed. The remainder of the reactions were treated with Proteinase K (NEB), extracted with phenol-chloroform-isopropanol (25:24:1), chloroform-isopropanol (49:1) and ethanol precipitated.

DNA adenylation using T4 Rnl1, T4 Rnl2 or CircLigase was performed using 3'-blocked, 5'-phosphorylated DNA oligonucleotides pDNA17c-NH<sub>2</sub> and pDNA21-3bioTEG for 1 h in conditions recommended by the manufacturers for ligation reactions.



**Figure 1.** Comparison of different ligases in the efficiency of ssDNA adenylation. The RNA ligases used were MthRnl, CirLigase<sup>TM</sup>, T4Rnl1 and T4Rnl2 with two substrates, pDNA17-NH<sub>2</sub> (a, c and f) and pDNA21-3bioTEG (b, d and e) and analyzed by a 15% urea-PAGE as described in 'Materials and Methods' section. Single-stranded RNA size markers (Mr) are included for reference. 'Input' lanes are reactions without ligase. Above each gel, the molar ratio (S/E) of substrate to enzyme for each reaction is given. The molarity of MthRnl was calculated based on the molecular weight of a monomer.

The non-adenylated form of MthRnl was used to study enzyme adenylation. The reactions conditions were as described for DNA adenylation with 10 pmol of MthRnl (460 ng), 50  $\mu$ M ATP, no DNA and 1  $\mu$ Ci of  $\alpha$ -[<sup>32</sup>P]-ATP (Perkin-Elmer) for 30 min. Reaction products were separated on 10–20% Tris-Glycine SDS polyacrylamide minigels (Invitrogen), visualized with Coomassie blue stain and after drying exposed to PhosphoStorage Screen (BioRad) and scanned on a Typhoon 9400 Imager (GE). Alternatively, protein bands were isolated and radioactivity counted with a Liquid Scintillation Analyzer 2100TR (Packard).

For pH optimization bis-Tris Propane-HCl buffer, pH adjusted at 25°C, was used. However, the real pH of the reaction should be recalculated for the corresponding temperature using  $\Delta pK_a/^\circ C = -0.016$ . At the optimal reaction temperature, 65°C, the shift is  $-0.62$  pH. Sodium acetate buffer used in standard condition does not shift the pH with temperature.

For functional assays, ligation of preparatively adenylated DNA with an RNA acceptor using truncated T4 RNA ligase 2 (NEB) without ATP were performed according to the manufacturer's suggested protocol. Ten microliter ligation reactions containing 5 pmol of the RNA acceptor, 7 pmol AppDNA17c-NH<sub>2</sub> in 10 mM Tris-HCl pH 7.5 buffer, 10 mM MgCl<sub>2</sub>, 1 mM DTT and 200 U of truncated T4 RNA ligase 2 were incubated for 2 h at 25°C. Reactions were stopped by adding 5  $\mu$ l formamide loading buffer, heat inactivated at 95°C for 3 min and the products were separated, stained and visualized as described for the DNA adenylation above.

## RESULTS AND DISCUSSION

### Screening RNA ligases for DNA adenylation

The goal of efficient synthesis of 5'-adenylated DNA prompted us to develop a simple, one-step protocol using RNA ligase. Initial screening of commercially available RNA ligases, T4 RNA ligase 1, T4 RNA ligase 2, CirLigase<sup>TM</sup> and the recently introduced thermophilic RNA ligase from *M. thermoautotrophicum* (MthRnl) produced promising results (Figure 1). Reactions were

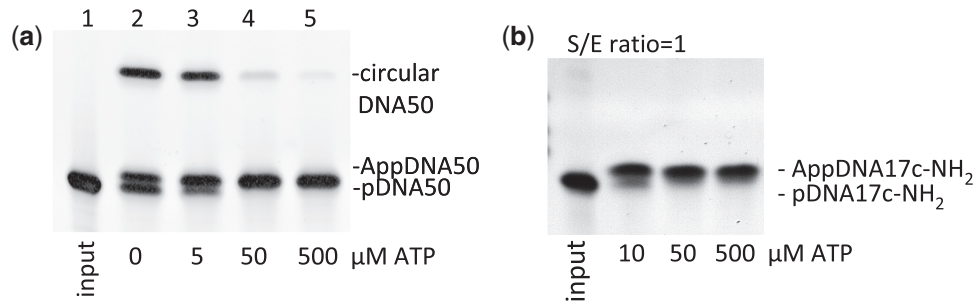
performed using conditions recommended by the manufacturers with two different substrates. In general, reactions were carried out with an equimolar or lower ratio of substrate to enzyme (S/E). All tested RNA ligases showed some DNA adenylation activity, which resulted in the accumulation of AppDNA that ran 1 nt slower than the substrate on denaturing polyacrylamide gels. Only MthRnl was capable of driving all of the substrate into product (Figure 1a and b). Mass spectroscopy confirmed the identity of the products. The molecular weight of AppDNA was increased by 329 Da in comparison to pDNA substrate, which corresponds to a molecular weight of AMP minus H<sub>2</sub>O. Additional confirmation was made by functional assay in ligation reaction with truncated T4 RNA ligase2 (see below, Figure 6).

### Protocol optimization

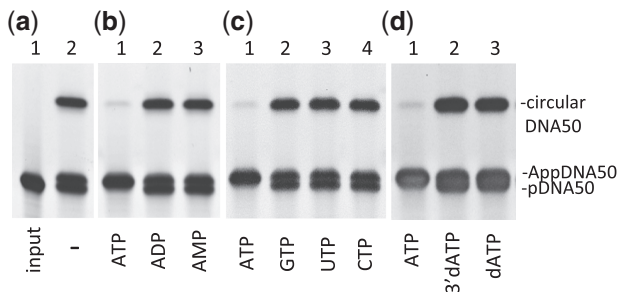
Further optimization of the DNA adenylation method was performed using MthRnl. MthRnl is a thermophilic ligase that catalyzes the intramolecular ligation of RNA (circularization) and is also able, with lower efficiency, to circularize ssDNA as described previously (11). MthRnl is moderately thermostable (optimum 60–65°C), ATP dependent and requires Mg<sup>2+</sup> or Mn<sup>2+</sup> for activity. The enzyme is a homodimer in solution and dimerization of MthRnl is required for ligase activity (11). For simplicity, we use the monomer molecular weight for calculation of the substrate/enzyme molar ratios in the reaction.

ATP inhibits RNA circularization by MthRnl, yielding the intermediate 5'-adenylated product, AppRNA (11). Competition between ATP and AppRNA for the same binding site may account for this behavior. Adenylated ligase is unable to bind AppRNA (15).

The adenylation and ligation of DNA are also influenced by ATP concentration. At high concentrations of ATP adenylation of DNA is enhanced but DNA ligation is inhibited. As shown in Figure 2a, when the concentration of ATP was increased to 50  $\mu$ M, the AppDNA product accumulated and DNA ligation was almost completely inhibited. In the 5'-adenylation of DNA with a 3' protected end, AppDNA formation also increased with increasing ATP concentration and reached saturation near 50  $\mu$ M (Figure 2b). DNA ligation is more



**Figure 2.** ATP dependence of DNA adenylation by MthRnI. (a) Reaction of preadenylated MthRnI (25 pmol of monomer) with 5 pmol of 50-nt long 5'-phosphorylated ssDNA with unprotected 3'-end (pDNA50) at various concentrations of ATP as indicated. (b) Optimal concentration of ATP in DNA linker adenylation was determined by reaction of 5 pmol of 5'-phosphorylated ssDNA with protected 3'-end (pDNA17c-NH<sub>2</sub>) for 1 h with various concentration of ATP, as indicated, under standard reaction conditions. The reaction products were analyzed by 15% urea-PAGE.

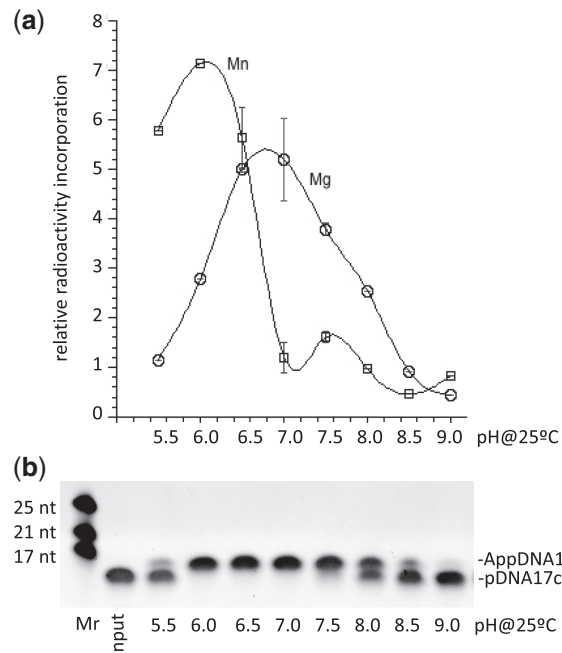


**Figure 3.** Effect of mononucleotides on ssDNA adenylation and circularization by preadenylated MthRnI (a-d). All reactions except 'input' in (a) contained excess (25 pmol) of pre-adenylated enzyme and 5 pmol pDNA50, which has an unprotected 3'-end. (a) Lane 2 contains no ATP. (b) 500 μM ATP (lane 1) was substituted with 500 μM of ADP (lane 2) or AMP (lane 3). (c) 500 μM ATP (lane 1) was substituted with 500 μM of GTP (lane 2), UTP (lane 3) and CTP (lane 4). (d) 500 μM ATP (lane 1) was substituted with 500 μM of cordycepin triphosphate (3'-dATP, lane 2) and dATP (lane 3). Reactions were performed for 1 h at 65°C and analyzed by 15% urea-PAGE as described in 'Materials and Methods' section.

sensitive to inhibition by ATP than ligation of RNA. The midpoint of ATP inhibition for RNA circularization is 50 μM (11).

It is possible that in the absence of an RNA acceptor, the ligase could use a second molecule of ATP as an alternative acceptor. This would create a 1 nt longer byproduct, pppApDNA, which could not be used for subsequent ligation. However, this does not appear to be the case because (i) the adenylated DNA product is efficiently used for ligation (Figure 6), (ii) The inhibition of ligation with pre-adenylated enzyme is specific for ATP while other potential acceptor mononucleotides do not have this property. When 500 μM of ATP was substituted in reaction with 500 μM of ADP, AMP, UTP, CTP, GTP, dATP or 3'-dATP (Figure 3b-d, respectively), no inhibition of circularization was observed, (iii) When radioactive  $\gamma$ -[<sup>32</sup>P]ATP was used in the reaction, only a trace amount of radioactivity was incorporated into the product (data not shown).

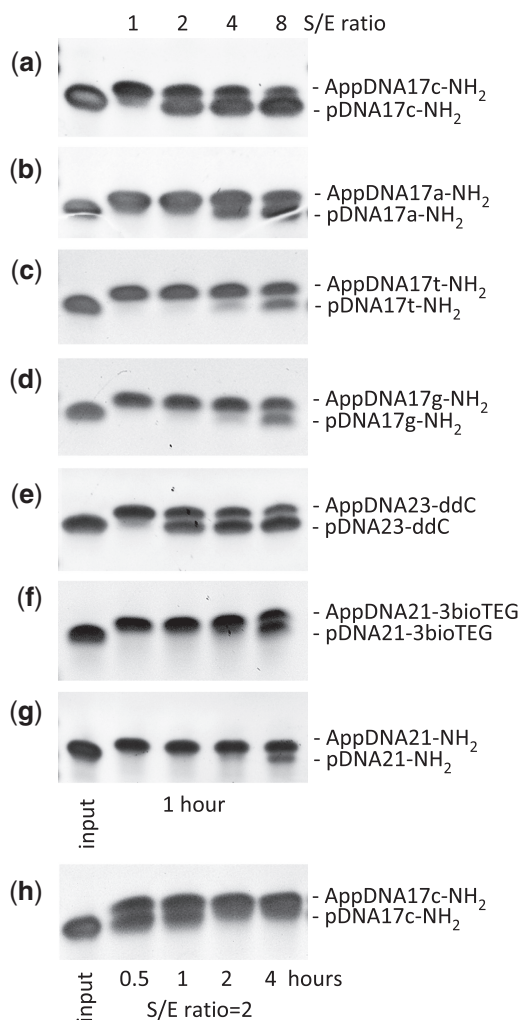
A model for efficient ATP inhibition of DNA ligation is likely the same as the one described above for RNA ligation. After AppDNA dissociates from RNA ligase its rebinding will be blocked if the enzyme is also adenylated.



**Figure 4.** pH dependence of adenylation of MthRnI and ssDNA. (a) Adenylation of non-adenylated MthRnI in the presence of Mg<sup>+2</sup> or Mn<sup>+2</sup>, ATP and  $\alpha$ -[<sup>32</sup>P]ATP with no DNA substrate. The products were analyzed by an SDS-PAGE and radioactive bands quantified as described in 'Materials and Methods' section. (b) pH dependence of pDNA17c-NH<sub>2</sub> adenylation by MthRnI under reaction conditions as described in 'Material and Methods' section in bis-Tris propane-HCl buffer. The products were analyzed by 15% urea-PAGE. The size markers at the left of the gel (Mr) are single-stranded RNA.

This ATP blockage of ligation allows efficient adenylation of ssDNA even when a free 3' OH is available for ligation. We concluded that the range of ATP concentrations 100–500 μM is sufficient for DNA adenylation even without protection of the 3'-end, and does not produce unwanted ligation products.

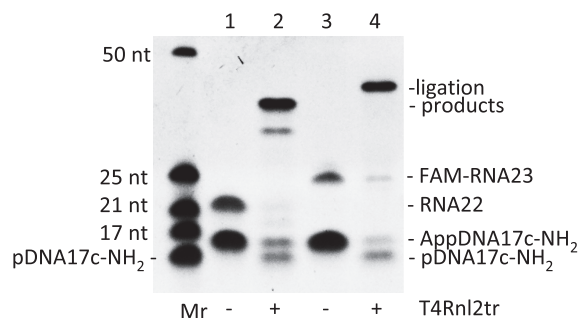
In the presence of Mg<sup>+2</sup>, pH optima for both MthRnI enzyme and DNA adenylations are around pH 6.5–7.0 (@25°C) (Figure 4a and b). When Mg<sup>+2</sup> was substituted by Mn<sup>+2</sup>, pH optima are shifted to 5.5–6.0 (@25°C) (Figure 4a; data not shown for DNA adenylation). DNA adenylation reaction reached saturation pH with 10 mM Mg<sup>+2</sup> and 5 mM Mn<sup>+2</sup> at corresponding pH with



**Figure 5.** Effect of substrate sequence and 3'-modifications on the efficiency of ssDNA adenylation. Each 1 h reaction had 5 pmol of the indicated substrate and serially diluted amounts of enzyme under standard reaction conditions as described in 'Materials and Methods' section and analyzed by 15% urea-PAGE (a-g). The molarity of the enzyme was calculated based on the molecular weight of the monomer of MthRnl. The activity of MthRnl in the reaction over time (h) was assayed as described for (a) using an S/E of 2 with different incubation times as indicated.

similar efficiency, in contrast to Step 3 DNA ligation, where  $Mn^{+2}$  is more active (data not shown). In addition, at 65°C  $Mn^{+2}$  is less stable and more prone to oxidation. We choose 10 mM  $Mg^{+2}$  and pH 6.0 at 65°C (see 'Materials and Methods' section for calculation of pH shift with temperature) for our experiments.

Our initial data for DNA adenylation by MthRnl, shown in Figure 1, revealed some variation in reaction efficiency depending on which substrate was used. Adenylation of the 17-nt long pDNA17c-NH<sub>2</sub>, with 3'-amino block (panel a), was slower than the 21-nt long pDNA21-3bioTEG, with a 3'-biotinylated triethylene glycol (3bio-TEG) modification and a different sequence (panel b). To determine the substrate parameters affecting the efficiency of DNA adenylation, we analyzed a range of 5'-phosphorylated ssDNA oligonucleotides of different length, sequence and 3'-modifications. These oligos were



**Figure 6.** Ligation of adenylated DNA linker made with MthRnl to RNA. AppDNA17c-NH<sub>2</sub> was ligated to RNA22 (lane 2) and FAM-RNA23 (lane 4) using T4 RNA ligase 2 truncated without ATP as described in 'Materials and Methods' section. Lanes 1 and 3 are controls without ligase. Single-stranded RNA size markers (Mr) are included for reference. The products were analyzed by a 15% urea-PAGE.

tested at different ratios of enzyme to substrate. First, we replaced 5'-cytosine in pDNA17c-NH<sub>2</sub> with adenine, thymine or guanine. As shown in Figure 5, MthRnl is least active with 5'-cytosine, requiring an equimolar amount of enzyme to complete the reaction (panel a). Similar activity was observed with another 5' cytosine-terminated oligonucleotide, pDNA23-ddC (panel e). However, the difference in adenylation of other three substrates was slightly better, within a dilution factor of two to four (panels b-d). Similar activity was observed with other oligonucleotides (panels f and g). DNA length, within the tested range of 17-50 nt, was not a factor in the efficiency of adenylation. Potentially a 3'-modified substrate end could be bound to an acceptor binding site and sterically affect 5'-adenylation, preventing binding of ATP or DNA donor. However, comparison of adenylation of two different 3'-modifications, amino and 3bio-TEG, with identical sequences, pDNA21-NH<sub>2</sub> and pDNA21-3bioTEG, showed no differences in activity (panels f and g). To rule out that the differences in DNA adenylation are not due to DNA secondary structure but rather enzyme specificity, self-complementarity and hairpin formation of the oligonucleotides were calculated at the reaction conditions used for adenylation using OligoCalc and mFold calculators (16,17). At low ionic strength and 65°C, no significant secondary structures were found.

In standard reaction condition at 65°C MthRnl was active for at least 2 h as demonstrated in Figure 5h. Doubling of incubation time to 2 h using pDNA17c-NH<sub>2</sub> with the substrate in a 2-fold excess (S/E = 2) still resulted in complete adenylation of the oligo (panel h). This is the same adenylation observed in a 1 h reaction with an equal ratio of enzyme and substrate, (S/E = 1) (Figure 5a). After 2 h of incubation at 65°C with a substrate, activity of the enzyme gradually diminished over the next few hours (data not shown).

The adenylated oligonucleotides, produced by preparative scale MthRnl adenylation, were tested for their ability to ligate to an RNA acceptor using truncated T4 RNA ligase 2 without ATP (Figure 6). T4 RNA ligase 2 (truncated) is defective in self-adenylation and readily accepts pre-adenylated substrate for ligation (18).

Adenylated DNA, AppDNA17c-NH<sub>2</sub>, preparatively produced with MthRnl, was used for ligation with two different RNA acceptors. In both reactions, ligation products were observed (lanes 2 and 4). During ligation reaction, some deadenylation of pre-adenylated DNA occurs as expected due to reversibility of Step 2 of ligation reaction in the absence of ATP. This product runs ~1 nt faster than pre-adenylated substrate. Partial degradation of RNA22 during ligation may be responsible for smaller ligation product in lane 2. The same result was achieved with AppDNA21-NH<sub>2</sub> (data not shown). This result demonstrates that pre-adenylated DNA, synthesized with MthRnl, is a functional substrate for truncated T4 RNA ligase 2 in the absence of ATP.

This one-step quantitative conversion of ssDNA to adenylated DNA using thermostable RNA ligase, MthRnl, greatly simplifies existing chemical and enzymatic methods. In summary, the range of DNA concentrations 0.5–30 μM was successfully tested. To achieve quantitative DNA adenylation, single turnover reaction condition with substrate to enzyme ratio 1:1 should be used. The temperature 60–65°C is optimal for activation of enzyme and its stability. pH optimum range adjusted to 65°C is 6.0–6.5. Make sure ATP is not a limiting factor in preparative adenylation. In this case and in adenylation of DNA with non-protected 3'-ends concentration of ATP should be increased to 0.5–1.0 mM. A significant benefit of this method is that high yield of the reaction and lack of a template strand eliminates the need for additional purification. The method requires only basic lab equipment and is easily scalable to micromolar level. It reduces cost and adds flexibility in designing custom adenylated DNA oligonucleotides for various applications.

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*Conflict of interest statement.* L. A. M. and A. M. Z. are employees of New England Biolabs, a company that sells Mth RNA ligase and other proteins for RNA and DNA research.

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