Adenylylation of small RNA sequencing adapters using the TS2126 RNA ligase I

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ABSTRACT

Many high-throughput small RNA next-generation sequencing protocols use 5′ preadenylylated DNA oligonucleotide adapters during cDNA library preparation. Preadenylylation of the DNA adapter’s 5′ end frees from ATP-dependence the ligation of the adapter to RNA collections, thereby avoiding ATP-dependent side reactions. However, preadenylylation of the DNA adapters can be costly and difficult. The currently available method for chemical adenylylation of DNA adapters is inefficient and uses techniques not typically practiced in laboratories profiling cellular RNA expression. An alternative enzymatic method using a commercial RNA ligase was recently introduced, but this enzyme works best as a stoichiometric adenylylating reagent rather than a catalyst and can therefore prove costly when several variant adapters are needed or during scale-up or high-throughput adenylylation procedures. Here, we describe a simple, scalable, and highly efficient method for the 5′ adenylylation of DNA oligonucleotides using the thermostable RNA ligase I from bacteriophage TS2126. Adapters with 3′ blocking groups are adenylylated at >95% yield at catalytic enzyme-to-adapter ratios and need not be gel purified before ligation to RNA acceptors. Experimental conditions are also reported that enable DNA adapters with free 3′ ends to be 5′ adenylylated at >90% efficiency.

Keywords: RNA-seq; sequencing adapters; small RNA; preadenylylation; 5′ adenylylation; RNA ligase

INTRODUCTION

The ligation of DNA oligonucleotide sequencing adapters to unknown RNA allows the RNA to be sequenced via its cDNA after reverse transcription. This approach is especially useful for small RNA and other nonpolyadenylated RNA. A key technological advance in this methodology was the use of preformed 5′ adenylylated adapters (AppDNA, where pp represents a 5′ to 5′ diphosphate linkage) to provide a binding site for the reverse transcriptase primer (Lau et al. 2001). (We note that the act of adding an adenylyl group can be described as either adenylation or adenylylation [Itzen et al. 2011].) Adapters modified in this way represent isolatable substrate intermediates that can enter the DNA- and RNA-ligase multistep reaction pathways just prior to formation of the new phosphodiester bond (Shuman 2009). Using the preadenylylated adapter frees the ligation reaction from ATP dependence, thereby avoiding the ATP-dependent circularization and multimerization of RNAs having 5′ phosphorylated ends (Hafner et al. 2008). Because many naturally occurring RNAs (e.g., pre-miRNA, mature miRNA, and various RNA degradation products) bear a 5′ phosphate, this advantage has made the use of preadenylylated sequencing adapters commonplace in small RNA sequencing applications.

Adenylylation at the 5′ end of synthetic DNA oligonucleotides may be carried out chemically to produce the preadenylylated form of the sequencing adapter (Unrau and Bartel 1998; Lau et al. 2001; Pfeffer et al. 2005; Hafner et al. 2008). This reaction requires the synthesis of a chemically activated form of the 5′ phosphoryl group on adenosine-5′-monophosphate, 5′-AMP (Lohrmann and Orgel 1978; Dai et al. 2009). Such reagents are moisture-sensitive and cannot be stored for very long. During coupling of the activated adenylyl to the 5′ end of an oligodeoxynucleotide, water reduces the coupling efficiency by competing with the DNA 5′ hydroxyl for the activated AMP. If one has access to a DNA synthesizer, the 5′ end can be adenylylated before the DNA is released from the synthesis support (Dai et al. 2009). Although kinetically slow, this method was shown to work well for oligomers smaller than 11 nt, but was observed to be less efficient for longer DNA oligonucleotides. Chemical
preadenylation is currently offered by commercial DNA suppliers, but for some laboratories the cost may be prohibitive. In both cases, in solution and on solid support, the instability of the coupling reagents requires that the synthesis of the activated 5′-AMP be repeated before each use. In our estimation, the number of laboratories in need of adenylylated DNA sequencing adapters has outpaced the adoption of chemical adenylylation techniques.

Adenylation may also be carried out enzymatically using DNA and RNA ligases. The ATP-dependent nucleic acid ligases share a three-step mechanism in which adenosine-5′-monophosphate is transferred from ATP to an enzyme lysine residue and then to the 5′-phosphate of the donor fragment to produce the 5′-adenylated oligonucleotide, which normally goes on to be ligated to the 3′ end of the acceptor in the third and final step (Shuman 2009). Early efforts to halt the ligation reactions after the second step, allowing the adenylylated intermediate to accumulate as the product, mainly used T4 DNA ligase. To stop the reaction after the second step, the fragment to be adenylylated (i.e., the phosphate donor oligonucleotide) was hybridized to a template strand but the acceptor oligonucleotide was either omitted (Chiuman and Li 2002; Vigneault et al. 2008) or included with optimized mismatches to the template near the ligation site (Wang and Silverman 2006; Patel et al. 2008). Although useful, these early attempts did not work well for all sequences and required the synthesis and subsequent removal of the template and mismatched acceptor DNA. In 2008, Ho and coworkers made the observation (Torchia et al. 2008) that 5′-phosphorylated single-stranded (ss) DNA and RNA could be efficiently adenylylated in the presence of ATP by an RNA ligase with Rnl2 sequence homology from the archaeabacterium *Methanobacterium thermoaerotrophicum* (MthRnl) (Ho and Shuman 2002). Without ATP supplementation, the third enzymatic step proceeded to circularize a significant amount of the oligonucleotide, but in the presence of ATP the ligase active site is maintained in the adenylylated state (EpA). The enzyme-bound adenosyl-5′-phosphate, favored by high ATP concentrations, appears to prevent the adenylylated 5′ end of the donor from simultaneously gaining access to the ligase active site, thereby inhibiting the third (i.e., ligation) step, which would in the studied case result in substrate circularization (Torchia et al. 2008; Zhelkovsky and McReynolds 2011). This ATP trapping effect appears to be general for ATP-dependent nucleic acid ligases (Uhlenbeek and Cameron 1977; Sugino et al. 1978; Ho and Shuman 2002; Yin et al. 2004; Torchia et al. 2008).

Importantly, MthRnl did not need the donor substrate to be hybridized to a template strand. This result was later optimized with the additional convenience of 3′ blocking groups on the donor oligonucleotide to ensure no circularization could occur (Zhelkovsky and McReynolds 2011). Chemical blocking groups at the 3′ end do not affect the use of the adapters as binding sites for the reverse transcriptase primer.

The MthRnl has simplified the enzymatic 5′ adenylylation of oligodeoxynucleotides. However, it appears to work best when used as a stoichiometric adenylylating reagent rather than as a catalyst, which may limit its usefulness in high-throughput and scale-up applications. Our laboratory has made frequent use of the thermostable Rnl1 from bacteriophage TS2126 (Blondal et al. 2005) in the synthesis of circularized oligonucleotide transcription templates (Seidl and Ryan 2011; Seidl et al. 2013; Lama et al. 2014). Here we report that recombinant TS2126 Rnl1 is also highly effective at converting oligonucleotide sequencing adapters to their 5′ adenylylated form. This enzyme provides a useful alternative to the MthRnl and has advantages under scale-up conditions because it is efficient when used catalytically at comparatively high adapter substrate-to-enzyme ratios.

**RESULTS AND DISCUSSION**

A commercial version of TS2126 Rnl1 was previously shown to fare poorly in an adenylylation comparison with commercial MthRnl (Zhelkovsky and McReynolds 2011), but its capabilities were not explored in depth. Using our recombinant version of TS2126 Rnl1 (Seidl and Ryan 2011), we tested the enzyme over a range of DNA substrate-to-enzyme ratios (S/E) from 0.5 to 4, and did so at two ATP concentrations (50 and 500 μM). The DNA adapter (adapter 1T; Table 1) was phosphorylated during solid-phase synthesis at the 5′ end and blocked at the 3′ end by an aminolinker blocking group to prevent any possibility of adapter circularization or concatamerization (Hafner et al. 2008). At 1 μM enzyme concentration, attempts to scale up the reaction by increasing the S/E ratio from 0.5 to 4 led as expected (Zhelkovsky and McReynolds 2011) to reduced adenylylation at both ATP concentrations (Fig. 1A). Because large amounts of TS2126 Rnl1 are easily made in bacteria (Seidl and Ryan 2011) (and see Materials and Methods), we tested among other variables increasing the concentration of the enzyme. At 5 μM TS2126 Rnl1, adenylylation efficiency increased at both ATP concentrations. At 10 μM enzyme the reaction proceeded to near completion over an S/E ratio of 0.5–10 at 500 μM ATP (gel not shown). To accurately measure the small amount of nonadenylated DNA adapter 1T remaining and to gauge day to day reproducibility, we included a trace of 32P-end-labeled DNA adapter 1T remaining to quantify the two forms in multiple independent experimental repeats, as shown in Figure 1B (see Materials and Methods). As the S/E ratio increased from 0.5 to 10, the average ligation efficiency dropped from 98% at an S/E of 1 to ~95% at an S/E of 10. These results show that under easily obtainable conditions, the use of the enzyme as a rechargeable catalyst, rather than as a stoichiometric reagent delivering one adenylate, as MthRnl does, is feasible.

Adapter 1T contains a 5′ T residue. To learn whether the identity of the 5′ nucleotide influences the efficiency of adenylylation, we varied the 5′ terminal nucleotide of this adapter (adapters 1A, 1C and 1G; Table 1). At the highest S/E ratios, only the 5′ G residue caused a decrease in efficiency, and
this effect was small (Fig. 2A). Thus, any nucleotide may be used at the 5' end without significant loss of adenylylation efficiency, but in cases where the highest efficiency is required, a 5' C should be avoided. Two other, unrelated DNA adapters, adapters 2 and 3 (Table 1), worked as well as the adapter 1 series (Fig. 2B). Based on this representative set of adapters, there appears to be no obvious sequence restriction in the adenylylation reaction catalyzed by TS2126 Rnl1.

We compared TS2126 Rnl1 with the commercially available MthRnl enzyme at 10 µM each enzyme, but under otherwise favorable adenylylation conditions for both. As previously described (Zhelkovsky and McReynolds 2011), the MthRnl worked well at the stoichiometric S/E of 1, but so too did TS2126 Rnl1 (Fig. 3A, panels 1 and 2). Thus, although previously unrecognized, the TS2126 Rnl1 can work just as efficiently as the MthRnl enzyme under low S/E ratios as long as the enzyme is used at relatively high concentration. However, under conditions where the enzyme (10 µM), ATP (500 µM), and S/E (5–10) were all used at levels that would be convenient for larger scale adenylylation, the TS2126 Rnl1 showed superior conversion efficiency (Fig. 3A, panel 1 versus 2 and 3). The commercially available enzyme CircLigase (Epicentre) is reported to be identical to the TS2126 Rnl1 (Zhelkovsky and McReynolds 2011). We tested this enzyme under our storage buffer, possibly Triton X-100, which cannot be easily removed, may slightly decrease the adenylylation efficiency at the comparatively high enzyme concentrations we use for optimal adenylylation.

To verify that the reaction products we observe in these experiments were indeed the adenylylated form of the oligonucleotides, we confirmed that a 5' phosphate was required for product formation (Fig. 4A) and that other nucleotide triphosphates (NTPs) could not substitute for ATP (Fig. 4B). Omitting ATP led to incomplete adapter adenylylation at an S/E of 1. The AMP transferred under ATP-free conditions (Fig. 4B). It is therefore likely that our TS2126 Rnl1 preparation and CircLigase are similarly efficient under scale-up adenylylation conditions, but components in the CircLigase storage buffer, possibly Triton X-100, which cannot be easily removed, may slightly decrease the adenylylation efficiency at the comparatively high enzyme concentrations we use for optimal adenylylation.

To verify that the reaction products we observe in these experiments were indeed the adenylylated form of the oligonucleotides, we confirmed that a 5' phosphate was required for product formation (Fig. 4A) and that other nucleotide triphosphates (NTPs) could not substitute for ATP (Fig. 4B). Omitting ATP led to incomplete adapter adenylylation at an S/E of 1. The AMP transferred under ATP-free conditions must originate from the adenylylated form of the purified recombinant protein (i.e., the EpA form). The ~40% conversion observed in Figure 4B, lane 2, allows us to estimate that ~40% of the TS2126 Rnl1 made by our procedure is in the adenylylated form. Among the nucleotides tested,

![FIGURE 1. DNA adapter 5' adenylylation using TS2126 RNA ligase 1 (TS2126 Rnl1).](https://example.com/fig1)

**TABLE 1.** Adapter and acceptor sequences used for adenylylation and ligation reactions

| Name | Sequence | Size (nt) |
|------|----------|----------|
| Adapter 1T | 5’-pTCACCTCGATGCCGCTCTCTGTG-TNH2 | 26 |
| Adapter 1A | 5’-pACACCTCGATGCCGCTCTCTGTG-TNH2 | 26 |
| Adapter 1C | 5’-pCCACCTCGATGCCGCTCTCTGTG-TNH2 | 26 |
| Adapter 1G | 5’-pGCACCTCGATGCCGCTCTCTGTG-TNH2 | 26 |
| Adapter 1T-3’OH | 5’-pTCACCTCGATGCCGCTCTCTGTG | 26 |
| Adapter 2 | 5’-pTCTAGAGGACCACCATAT-RNH2 | 17 |
| Adapter 3 | 5’-pTCACCTCGATGCCGCTCTCTGTG-RNH2 | 26 |
| Acceptor 1 | 5’-ACCGAATTCTCTACTArArArA | 18 |
| Acceptor 2 | 5’-rGrGrUArGrGrGrGrUrUrArArCrGrA | 19 |

Underlined base indicates the different base identity at 5' end. R-NH2, 3' blocking group.
Last, we investigated whether a 3′ blocking group on the adapter is in fact necessary to avoid self-ligation to the circular form during adenylation with TS2126 Rnl1. Adapter 1T was remade with a free 3′ end in place of a blocking group (adapter 1T-3′OH, Table 1). Substrate 1T-3′OH was then incubated under our scale-up adenylation conditions with increasing ATP concentrations (Fig. 6). High ATP concentrations favor the trapping of the adenylylated donor, as described above. At low (50 μM) ATP, where the adenylation step is reportedly slow compared with the ligation step (Blondal et al. 2005), the substrate was ligated to the circular form without the accumulation of any adenylylated product (lane 4). Increasing ATP 10-fold led to approximately equal amounts of adenylylated and circularized substrate (lane 5), whereas an additional 10-fold increase in ATP resulted in a 92% yield of the adenylylated unblocked oligonucleotide (lane 7). Concentrations of ATP in this range were previously reported for the TS2126 Rnl1 (Blondal et al. 2005), but the assay used did not distinguish between the adenylylated

**FIGURE 2.** TS2126 Rnl1 adenylation is generally efficient and sequence-independent. (A) Adenylation yield analysis under reaction conditions identical to those in Figure 1B as a function of the identity of the 5′ nucleotide. Adapter 1A has a 5′ A, adapter 1C has a 5′ C, and adapter 1G has a 5′ G. The 5′ end of the adapter is labeled with 32p phosphate. Adenylation percentages were estimated using the Phosphorimager Molecular Dynamics ImageQuant software. (B) Adenylation reactions for two other adapters of varying lengths, adapter 2 and adapter 3, using 5 μM TS2126 Rnl1, 500 μM ATP, and S/E of 1. For comparison, the result for adapter 1T under the same conditions can be found in Figure 1A, lane 7, lower panel. Denaturing polyacrylamide gels with Stains-All visualization.

| Storage Buf. | TS | Epi | Epi |
|--------------|----|-----|-----|
| 10 μM Rnl | (-) | (+) | (+) |
| 0.5 mM ATP, S/E = 10 | AppDNA | pDNA | pDNA |
| Adenylation % | δδδδδδδδ | δδδδδδδδ | δδδδδδδδ |

**FIGURE 3.** An adenylation efficiency comparison between TS2126 Rnl1, MthRnl, and CircLigase. (A) The three enzymes were compared for their ability to adenylylate adapter 1T at increasing S/E ratios using 10 μM enzyme and either 0.1 or 0.5 mM ATP. (B) The effect of CircLigase enzyme storage buffer components on adenylation efficiency of adapter 1T. TS, TS2126 Rnl1 or its storage buffer components used at 1× during adenylation; Epi, Epicentre CircLigase or its storage buffer components used at 1× during adenylation. The reduced amount of adenylation in lane 3 compared with lane 2 is due to CircLigase buffer components. The A CircLigase and B exposures have lane 4 in common.
and circularized forms, as we do here. The highest ATP concentration used in that report, 10 mM, resulted in complete inhibition of the enzyme’s activity, presumably through divalent cation chelation by ATP. Our observations at 10 mM ATP also resulted in lower enzymatic activity, although we can specify the product distribution (≈20% adenylylation) that results from this putative chelation effect (lane 8).

Blondal et al. found that the rate of substrate adenylylation was much slower than the rate of ligation, and concluded from this observation that the adenylylated-donor–enzyme complex cannot dissociate from TS2126 Rnl1 until ligation has taken place. Our results show that this does not hold true at intermediate ATP concentrations. Using TS2126 Rnl1 at a catalytic (10-fold lower) concentration compared with a DNA substrate with a free 3′ end, we were able to adenylylate 92% of the substrate, indicating that the adenylylated DNA dissociated from the ligase to allow turnover. Overall, we show by optimizing the ATP concentration that an unblocked DNA adapter can be adenylylated at >90% efficiency, with only 6% lost to circularization. We note also that under the conditions found here, the amount of dimer products resulting from at least one intermolecular ligation remained <2%.

Although we show that TS2126 Rnl1 can be used to adenylylate unblocked DNA substrates, for those who wish only to preadenylylate a 3′ adapter for small RNA-seq applications, it is advantageous to block the 3′ end during adapter synthesis to completely prevent adapter circularization and concatemerization. Interestingly, bacterial RNA 3′ phosphate cyclase A (RtcA), an enzyme unrelated to the nucleic acid ligases, was recently found to adenylylate the 5′ end of RNA and DNA oligonucleotides. Because it is not a ligase, it does not require blocking of the 3′ end to prevent subsequent oligonucleotide circularization and concatenation (Chakravarty and Shuman 2011). Like TS2126 Rnl1, in the presence of ATP RtcA catalyzes multiple rounds of 5′ adenylylation via a covalent enzyme–AMP intermediate. Although its performance under scale-up conditions has not been reported, RtcA seems to provide another alternative to small-scale enzymatic adenylylation by MthRnl, especially for applications where a free 3′OH is needed for subsequent manipulation.

In conclusion, we have found that, contrary to previously published work, TS2126 Rnl1 can be an effective DNA 5′ adenylylating enzyme. Our method provides a simple and effective alternative for the covalent attachment of 3′ RT primer binding site adapters to RNA for the construction of small RNA-seq libraries and other applications. Our key finding is that TS2126 Rnl1 leads to nearly quantitative adapter adenylylation when the enzyme is used at concentrations in the 5–10 μM range. In the presence of ATP, the enzyme is repeatedly charged with AMP and turns over so that it can be used catalytically, rather than as a stoichiometric adenylylating reagent. We also found that the commercially available CircLigase, reported to be the same enzyme as TS2126 Rnl1, is a similarly effective adenylylating enzyme; although when used at optimal concentrations, enzyme storage buffer components may decrease its adenylylation activity. Adapters need not be blocked at the 3′ end, but for maximum yield they should be made with a 3′ blocking group. The method we describe here should prove useful to those needing to preadenylylate a variety of sequencing adapters, or large amounts of a single adapter, and suggests a new use for the commercial CircLigase enzyme.

**FIGURE 4.** Requirements for DNA adapter adenylylation by TS2126 Rnl1. (A) Adenylylation reactions of adapter 2 by TS2126 Rnl1 with and without 5′ phosphorylation. Reaction conditions were the same as in Figure 2B. (B) ATP is required for the TS2126 Rnl1 adenylylation reaction. ATP-independent product results from single-turnover transfer of AMP from TS2126 Rnl1 adenylylated during bacterial expression. Adapter 1T and TS2126 Rnl1, 5 μM. All NTPs, 500 μM.

**FIGURE 5.** DNA adapters adenylylated using TS2126 Rnl1 are ligated to small RNA acceptors with high efficiency. (A) Ligation between adapter 2 adenylylated with TS2126 Rnl1 and the chimeric 5′-DNA/RNA-3′ acceptor 1 using the T4Rnl2trK227Q ligase. The expected product size is 35 nt. (B) Ligation of adapters 1T and 2, after adenylylation with TS2126 Rnl1, to RNA acceptor 2. (C) DNA adapters adenylylated with TS2126 Rnl1 do not require gel purification prior to ligation to an RNA acceptor. Ligation efficiency comparison of adapter 1T adenylylated using TS2126 Rnl1 with (lane 3) or without (lane 2) gel purification prior to T4Rnl2trK227Q ligation to acceptor 2. RNA acceptor to DNA adapter molar ratio was 1:2 in all ligations. M, ss DNA size markers; T4Rnl2trK227Q, T4 RNA ligase 2, truncated K227Q mutant from NEB.
otic acid adapter lacking a 3' and 3' and acceptor 2 have previously been used in small RNA experiments were dephosphorylated with calf intestinal alkaline phosphatase (Promega) and then re-5'-phosphorylated with [γ-32P]-ATP using T4 polynucleotide kinase according to the manufacturer's instructions. Adapters 1T and 3 and acceptor 2 have previously been used in small RNA cDNA library construction (Williams et al. 2013).

FIGURE 6. TS2126 Rnl1 adenylylation of a 5' labeled DNA oligonucleotide adapter lacking a 3' blocking group. ATP concentration was increased in order to trap the adenylylated adapter and suppress self-ligation. Of note, 5 mM ATP led to successful adenylylation with minimal self-ligation (lane 7). Estimates of product distributions are shown. Rounding off led to sums >100% in some lanes.

MATERIALS AND METHODS

Adapter and acceptor oligonucleotides

Custom acceptors and 5' phosphorylated adapters (Table 1) were purchased from Integrated DNA Technologies (IDT) and gel purified using denaturing polyacrylamide gel electrophoresis (DPAGE). End-labeled adapters used at trace levels in adenylylation quantification experiments were dephosphorylated with calf intestinal alkaline phosphatase (Promega) and then re-5'-phosphorylated with [γ-32P]-ATP using T4 polynucleotide kinase according to the manufacturer's (New England Biolabs, NEB) instructions. Adapters 1T and 3 and acceptor 2 have previously been used in small RNA cDNA library construction (Williams et al. 2013).

TS2126 Rnl1 expression, purification, and quantification

TS2126 RNA ligase 1 (Rnl1) was expressed and purified as previously described (Seidl and Ryan 2011) except that cells were lysed by sonication and the bead-bound ligase was washed with a more stringent buffer (10 mM Tris–HCl pH 8.0, 0.5 M NaCl, 50 mM Imidazole). In a representative batch of TS2126 Rnl1 expression and purification, 400 mL of bacterial culture yielded 4.16 mg of purified protein in 3.2 mL buffer after dialysis, a final concentration of 1.3 mg/mL. This amount is equivalent to 93 nmol based on the monomer molecular weight of 44870 Da. Using a substrate:enzyme (S/E) ratio of 10, which has an average adenylylation yield of ≥95%, this amount of enzyme would be sufficient for the adenylylation of 884 nmol of sequencing adapters. MthRnl was purchased from NEB. The concentration of both enzymes was determined by (i) Bradford (Bio-Rad cat. #500-0006) and (ii) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie staining and comparison to bovine serum albumin (BSA) standards. Molar concentration estimates were based on monomer molecular weights.

Adenylylation reactions

Adenylylation reactions using MthRnl were done according to the manufacturer’s protocol except that the enzyme concentration was increased to 10 µM, reaction time was doubled to 2 h, and in one reaction the ATP concentration was increased from 100 to 500 µM for comparison with TS2126 Rnl1. TS2126 Rnl1 adenylylation reactions were done at pH 7.5 in 20 µL containing 50 mM MOPS, 10 mM KCl, 5 mM MgCl2, 1 mM DTT, 2.5 mM MnCl2, and the specified ATP/ligase/adapter concentrations for 2 h at 60°C followed by 5 min of inactivation at 80°C. Nucleic acids were isolated using phenol:chloroform:isoamyl alcohol (PCI) and ethanol precipitation. DNA products were resolved by 17% denaturing PAGE (DPAGE) and visualized by Stains-All (Sigma-Aldrich) or in the case of radioactive examples by use of a Molecular Dynamics phosphorimager (Seidl et al. 2013). Gel-purified adenylylated adapters used for RNA ligation assays were obtained through a preparative adenylylation reaction using 10/50/500 µM of TS2126 Rnl1/adapter/ATP, respectively, in 20 µL. The adenylylated products were gel purified (except as indicated in Fig. 5) by 0.75 mm 20% DPAGE and located by UV shadowing. Products were excised and soaked overnight in extraction buffer (0.5 M ammonium acetate, 20 mM magnesium chloride, 1 mM EDTA, 0.2% SDS), followed by PCI extraction and ethanol precipitation (e.g., 86% isolated yield for adapter 1T). Product quantification for adenylylation efficiency analysis in Figures 1B, 2A, and 3A,B was done using Molecular Dynamics Phosphorimagery and MD ImageQuant software. Adenylylation efficiency (i.e., percent yield) is defined as [adenylylated/(adenylylated + unadenylylated)] × 100.

Acceptor RNA ligation reactions

Ligation reactions (10 µL) contained 1 µM acceptor RNA or DNA/RNA chimera, 2 µM adenylylated adapter, 1× T4 Rnl buffer (NEB), 200 U T4Rnl2trK227Q (NEB), and 20% PEG 8000, and were incubated overnight at 16°C.

ACKNOWLEDGMENTS

This work was supported by the National Institute of General Medical Sciences (NIH/NIGMS) under grant number 1SC1GM083754 (to K.R.). Support from the U.S. Army Research Laboratory and U.S. Army Research Office under grant number W911NF-13-1-0148 is also gratefully acknowledged. Additional infrastructure support at the City College of New York was provided by the National Center for Research Resources (2G12RR03060-26A1) and the National Institute on Minority Health and Health Disparities (8G12MD007603-27).

Received March 6, 2015; accepted June 25, 2015.

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