Chapter 10

Efficient Splinted Ligation of Synthetic RNA Using RNA Ligase

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Abstract

RNA ligation allows the creation of large RNA molecules from smaller pieces. This can be useful in a number of contexts: to generate molecules that are larger than can be directly synthesized; to incorporate site-specific changes or RNA modifications within a large RNA in order to facilitate functional and structural studies; to isotopically label segments of large RNAs for NMR structural studies; and to construct libraries of mutant RNAs in which one region is extensively mutagenized or modified. The impediment to widespread use of RNA ligation is the low and variable efficiency of standard ligation strategies, which frequently preclude joining more than two pieces of RNA together.

We describe a method using RNA ligase (Rligation), rather than DNA ligase (Dligation), in a splint-mediated ligation reaction that joins RNA molecules with high efficiency. RNA ligase recognizes single-stranded RNA ends, which are held in proximity to one another by the splint. Monitoring the reaction is easily accomplished by denaturing gel electrophoresis and ethidium bromide staining. Using this technique, it is possible to generate a wide range of modified RNAs from synthetic oligoribonucleotides.

Key words RNA ligation, Rligation, T4 RNA ligase, Oligoribonucleotide, Synthetic RNA, 2′ ACE, RNA modifications, RNA library

1 Introduction

1.1 Applications of RNA Ligation

RNA ligation is an essential method to generate site-specifically mutated, modified, or labeled RNA molecules, as direct synthesis of RNA oligonucleotides is limited to lengths of approximately 100 nucleotides (<50–75 nt for modified oligonucleotides). Chemical synthesis of RNA permits the incorporation of modified residues either internally at the base or sugar phosphate backbone or at either end of the RNA. The only limitation to modification is the availability of the reactive phosphoramidite. Modified residues containing reactive groups can also be incorporated into synthetic RNA to allow for post-synthetic labeling of the RNA when direct incorporation of a label is not possible.

The ligation of several modified RNA oligonucleotides, or modified RNA oligonucleotides with in vitro transcribed RNAs,

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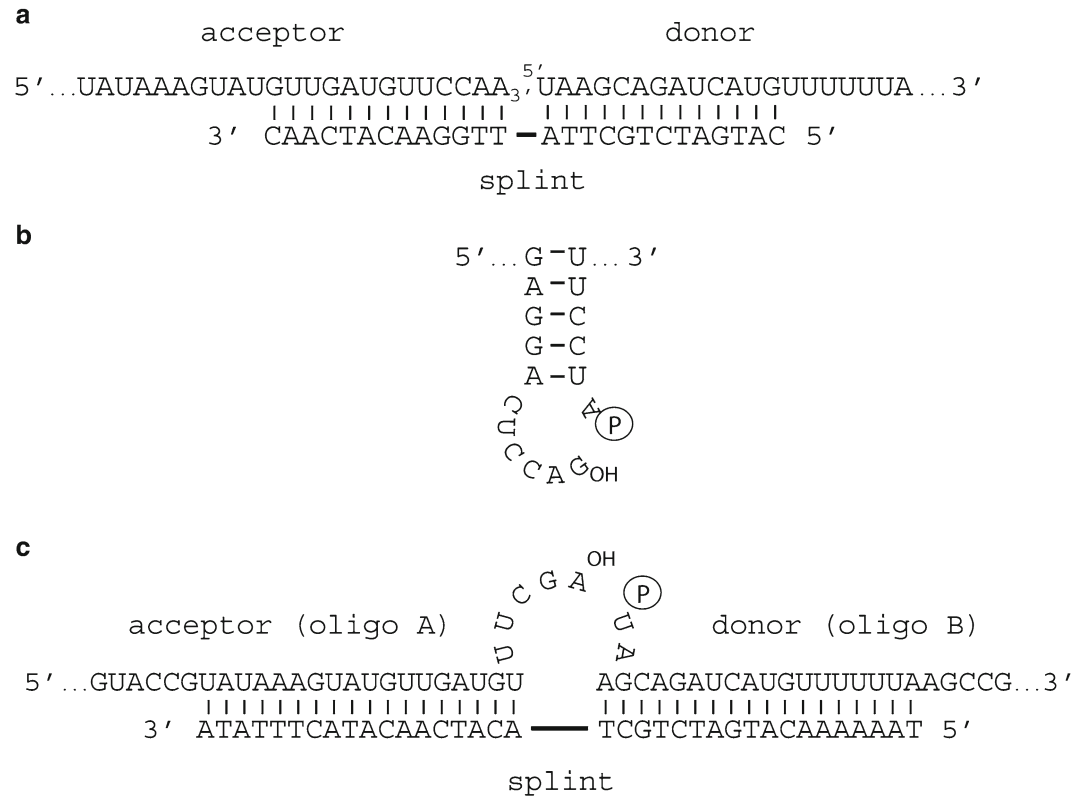

Fig. 1 Substrates and splint designs for ligases. (**a**) Dligation design with splint completely complementary to RNA acceptor and donor. (**b**) The tRNA anticodon loop that is the natural substrate for T4 RNA ligase. (**c**) Rligation design with splint not complementary to ends of RNA acceptor and donor. Circled "P" is the 5′ phosphate, and "OH" is the 3′ hydroxyl that is used in the ligation reaction. 5′ and 3′ ends of each RNA molecule are indicated. The heavy line in the splints simply denotes the continuous DNA backbone (i.e., there are no missing nucleotides)

has led to new insights into the structure and function of large RNAs. For example, to investigate the function of post-transcriptionally modified nucleotides in U2 snRNA in pre-mRNA splicing, Dönmez et al. ligated modified oligonucleotides together to generate full-length U2 with modifications at specific positions [1]. Similarly, RNA ligation has been used to incorporate fluorescent dyes at specific positions of pre-messenger RNA to monitor spliceosome assembly and splicing [2]. Furthermore, the NMR structure of a 100 kDa RNA (310 nt, the internal ribosomal entry site of the hepatitis C virus) was determined after using T4 RNA ligase to generate segmentally ^{15}N-labeled RNA [3].

1.2 Dligation

The most common method for ligating RNA uses T4 DNA ligase on RNA fragments that are held together by a DNA splint (Fig. 1a; [4]). The splint is completely complementary to the ends of the RNA fragments, thereby mimicking the nicked, double-stranded

DNA that is the natural substrate for DNA ligase. The design of a splinted ligation using DNA ligase is relatively straightforward—the ligation junction can be anywhere in the RNA molecule with no sequence restriction. The main requirement is simply the presence of a 5′-monophosphate on the downstream RNA (the donor) and a free 3′-hydroxyl on the upstream RNA (the acceptor, Fig. 1a). Circularization of the donor RNA, often the most important source of unwanted products, is insignificant due to DNA ligase's strict requirement for double-stranded substrate.

Despite the apparent geometric fidelity of this arrangement, RNA turns out to be only a mediocre substrate for DNA ligase, resulting in the need for high concentrations of ligase (often stoichiometric), which can become cost prohibitive when large amounts of product are required. Due to the inefficiency of DNA ligase on an RNA/DNA substrate, long ligation times are required, which increase the chance of RNA degradation. Unproductive hybridization intermediates between RNAs and the DNA splint also contribute to low ligation yields. Ligation efficiency can be increased in some cases by using very long DNA splints to reduce the number of unproductive hybrid complexes formed [5].

1.3 Rligation

To overcome these problems, T4 RNA ligase can be used instead of T4 DNA ligase, but this brings its own set of challenges. The natural substrate for T4 RNA ligase is the anticodon loop of tRNA (Fig. 1b; [6]), but RNA ligase will readily join any single-stranded RNA with a 5′-phosphate and a 3′-hydroxyl. Consequently, 5′-phosphorylated RNA is rapidly circularized by RNA ligase, necessitating a mechanism for conferring specificity to the reaction. The use of a DNA splint can be effective but requires a different design from the splints used with DNA ligase [7, 8].

Since RNA ligase requires single-stranded RNA substrates, the splint must hold the RNA fragments in proximity to one another while allowing the ends the flexibility to reach into the enzyme active site (Fig. 1c). In practice, this is achieved by making the splint complementary to the 3′ end of the phosphate *acceptor* (A) oligonucleotide except at the last 4–8 nucleotides. Similarly, complementarity to the phosphate *donor* (B) oligonucleotide starts with the 3rd nucleotide from the 5′ end. The resulting free ends of 4–8 nucleotides at the 3′ end of A and two nucleotides at the 5′ end of B closely match the 5 and 2 nucleotide single-stranded ends of tRNA prior to ligation.

The presence of the splint is frequently sufficient to sequester the 5′ end of the B oligonucleotide, thereby preventing circularization, but the 2′ ACE protecting groups introduced by 5′-silyl-2′-acetoxy ethyl orthoester solid-phase synthesis chemistry further inhibit side reactions [8]. Chemical protecting groups at the 2′ position can sterically reduce or eliminate reaction at the 3′-OH. By selectively deprotecting only the acceptor RNA fragment,

phosphorylating only the donor fragment, and using an appropriate splint, high reaction yields and specificity can be achieved.

When one or more of the RNAs to be ligated are made enzymatically using T7 RNA polymerase, a different approach is necessary to prevent unwanted products. To prevent circularization or concatamerization of the donor molecule, a blocked 3′ end is necessary, either with a 2′,3′-cyclic phosphate, a 3′-phosphate, or a dideoxy residue. On the acceptor molecule, a 5′-OH will prevent the formation of these unwanted products.

1.4 Outline of Method

The RNA ligation described here involves a design stage followed by the actual ligation reactions. First, break points are chosen in the full-length molecule to facilitate incorporation of modifications and to minimize the total number of ligation steps. Second, splint sequences are chosen for each oligonucleotide junction. Finally, the two oligonucleotides and the corresponding splint are mixed in the appropriate molar ratio with RNA ligase. Reaction products are analyzed, and additional ligation steps can be carried out iteratively.

2 Materials

As RNA is highly sensitive to nucleases, and as nucleases are ubiquitous in the environment, it is important to take all possible precautions to avoid nuclease contamination: purchase nuclease-free lab supplies (e.g., pipette tips, microcentrifuge tubes), use ultrapure water to make all buffers and solutions , and filter solutions through high protein-binding nitrocellulose filters.

2.1 Ligation Reaction

1. RNA oligonucleotides: Make a stock solution of the B oligonucleotide by resuspending to a final concentration of 500 μM in water. Check the concentration by measuring the A260 on a spectrophotometer. Do not resuspend the A oligonucleotide in water.
2. DNA (splint) oligonucleotides: Make a stock solution of the splint oligonucleotide by resuspending to a final concentration of 200 μM in water. Check the concentration by measuring the A260 on a spectrophotometer.
3. 2′ ACE deprotection buffer: 100 mM acetate adjusted to pH 3.8 with TEMED.
4. 10× T4 RNA ligase buffer: 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP.
5. T4 RNA ligase.

2.2 Analysis

1. Vertical gel system; glass gel plates, approximately 16 × 14 cm (*see* **Note 1**).
2. 20× Tris–Borate–EDTA (TBE) gel running buffer: 1.8 M Tris base, 1.8 M boric acid, 25 mM EDTA.

3. 7 M urea/12 % acrylamide: Mix 4.5 mL of 40 % (19:1) acrylamide, 750 μL 20× TBE, 6.3 g urea, and 5 mL water in a 50 mL glass beaker. Add a stir bar and stir on a magnetic stir plate until the urea has dissolved completely. Bring the volume to 15 mL with water (*see* **Note 2**).
4. Ammonium persulfate: 10 % solution in water (*see* **Note 3**).
5. *N,N,N′,N′*-tetramethylethylenediamine (TEMED).
6. Urea sample buffer: 4.2 g urea, 500 μL 20× TBE, 20 μL 0.5 M EDTA, 2.5 mg xylene cyanol, 2.5 mg bromophenol blue, water to 10 mL. Filter sterilize.
7. Ethidium Bromide: 10 mg/mL in water.
8. UV gel documentation system.

2.3 Product Purification

1. Formamide.
2. Fluor-coated thin layer chromatography plate.
3. Handheld UV light.
4. Disposable 1.5 mL microcentrifuge tube pestle.
5. DTR gel filtration cartridge (Edge BioSystems).
6. 20 mg/mL glycogen.
7. 3 M NaOAc.
8. 70 and 100 % EtOH.

3 Methods

3.1 Oligonucleotide Design

As RNA secondary structure may interfere with base pairing to the splint, it is helpful to place ligation junctions in single-stranded or loop regions. The use of small loops (left and right arrows, Fig. 2) as ligation junctions has not been extensively examined, but since the natural substrate for RNA ligase is such a loop, it is likely that they would work well. Under favorable conditions, e.g., with an extensive stem stabilizing the arrangement, a splint is not necessary [3, 9]. Large loops and single-stranded regions provide the other likely locations for junctions (Fig. 2, middle arrow, bottom, and top arrow).

Examine the predicted secondary structure of the desired, full-length RNA to identify loops that can serve as the junctions between RNA fragments (e.g., using mfold, [10]). In order to achieve the highest ligation yields possible, one should consider the sequence requirements of T4 RNA ligase when designing a ligation scheme. RNA ligase has been shown to have a slight preference for pyrimidines over purines at the 5′-terminal position of the donor, and ligation efficiency is highest if the last two nucleotides of the acceptor are not uridine—adenosine is best, followed by guanosine and cytidine [7, 11, 12]. Although we have found little or no difference in product yields based on sequence specificity

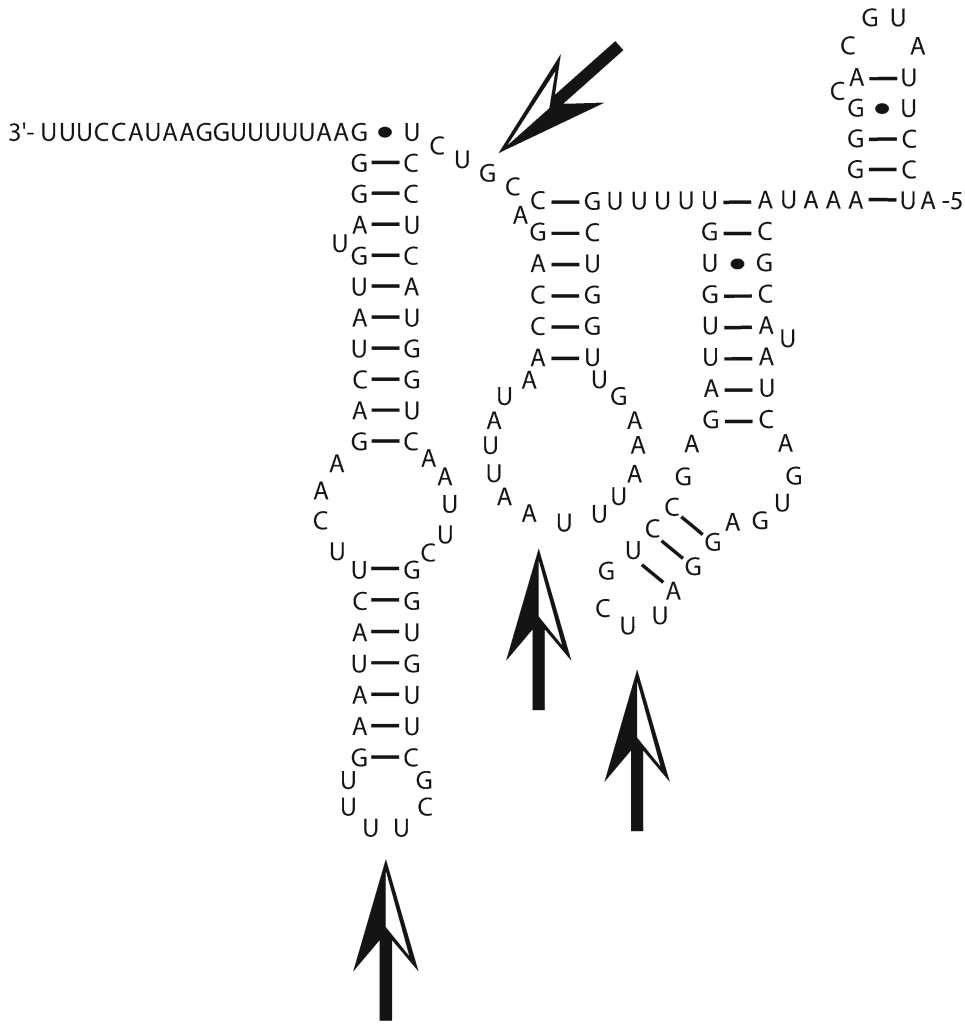

Fig. 2 Example of how to choose optimal ligation junctions for Rligation. *Bottom arrows* indicate loops, and *top arrow* indicates single-stranded region between secondary structure elements

in either the donor or acceptor substrates, it would be prudent to avoid junctions that contain uridines at the acceptor 3′-terminus whenever possible [8]. In addition, although the tRNA substrate of T4 RNA ligase has a single-stranded region of 5 nucleotides on the acceptor and 2 nucleotides on the donor, optimum lengths may vary for other sequences. The length of the donor loop seems to be most sensitive to changes in length, with ligation efficiency decreasing for lengths other than 1 or 2 nucleotides [8].

Current oligonucleotide synthesis limits RNA fragment length to approximately 100 nucleotides, but the limit is generally lower if modified nucleotides are included within the fragment, with the efficiency dropping off dramatically after approximately 50 nt [13]. Design all RNA molecules except the first acceptor (most 5′

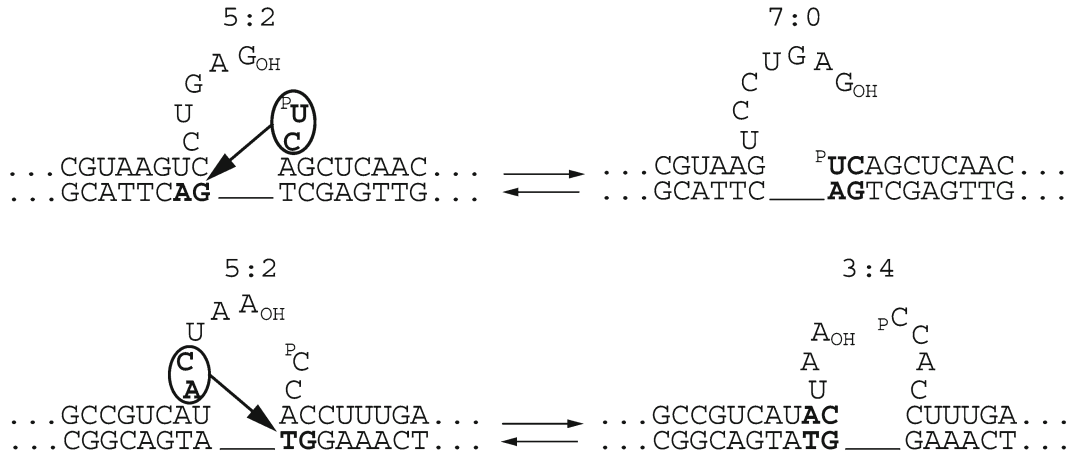

Fig. 3 Example of base-pairing ambiguity between splint and RNA acceptor and donor. In the top example, the 5′ end of the donor can base-pair to the splint, resulting in a 7:0 junction that is a poor substrate for RNA ligase. Similarly, in the bottom example, base pairing of the acceptor strand to the splint can extend further than desired, again resulting in suboptimal ligation efficiency

molecule) with a 5′-phosphate. This guarantees the fidelity of the ligation junction, as only full-length donor molecules will be ligated (any truncated products produced during synthesis are chemically capped and will not contain the necessary phosphate at the 5′ end).

3.2 Splint Design

Design the splint to have approximately equal binding to the A and B oligonucleotides. 18 nucleotides of complementarity work well, but it is important to calculate the T_m for each half to ensure they are comparable. T_ms of at least 40 °C yield good results. Close attention should be paid to ensure that the first two nucleotides of the donor molecule are not complementary to the last two nucleotides in the acceptor-binding region of the splint, which could result in the loss of the single-stranded linker on the donor. Similarly, if the first nucleotides in the acceptor single-stranded loop were complementary to the first nucleotides in the donor-binding region of the splint, then the number of single-stranded nucleotides on the acceptor side of the loop would decrease, while the number on the donor side would increase (Fig. 3).

3.3 Ligation

1. For a two-piece ligation, deprotect oligonucleotide A by resuspending in 400 µL 2′-ACE deprotection buffer. Pipet up and down to completely dissolve the RNA pellet, vortex 10 s, and then spin 10 s. Heat 30 min at 60 °C. Dry down in a SpeedVac at 55 °C. This will take over an hour. Resuspend in water to make a 500 µM stock solution. Determine the concentration by UV spectroscopy.
2. Prepare the polyacrylamide gel: To 15 mL of 12 % acrylamide/7 M urea, add 150 µL 10 % ammonium persulfate and

15 μL TEMED in a fume hood. Swirl to mix. Quickly fill the gel cassette with this acrylamide mixture, being careful to avoid and/or remove any bubbles. Insert the comb, ensuring that the acrylamide reaches all the way to the top of the notched plate. Leave to polymerize at ambient temperature for at least 1 h (*see* **Note 4**).

3. From the concentrated oligonucleotide stocks, make up 20 μM dilutions for the initial small-scale analysis.
4. Anneal the oligonucleotides by mixing them in a 1:1.5:2 molar ratio of A/splint/B. Oligonucleotide concentrations between 1 and 10 μM, depending on the size of the oligonucleotide, are usually sufficient for post-ligation visualization with EtBr (*see* **Note 5**). For example, for a 20 μL ligation reaction, mix 2 μL of 20 μM oligonucleotide A, 3 μL of 20 μM splint, and 4 μL of 20 μM oligonucleotide B in a 0.2 mL PCR tube. Add 2 μL 10× T4 RNA ligase buffer and 8.5 μL of water. Heat at 65 °C for 3 min, followed by 5 min at 25 °C in a thermocycler (*see* **Notes 6–8**).
5. Add 0.5 μL of 10 U/μL T4 RNA ligase. Mix thoroughly by flicking the bottom of the tube (do not vortex), and pulse briefly in a minicentrifuge to ensure the reaction mixture is at the bottom of the tube. Incubate at 37 °C.
6. The reaction can be completed within 5 min, but the appropriate incubation time must be determined empirically for each oligonucleotide/splint combination. From the 20 μL reaction above, remove 5 μL aliquots at 0, 5, 30, and 60 min.

3.4 Analysis

1. Stop the reactions by addition of 100 μL deprotection buffer, heat 30 min at 65 °C, and evaporate to dryness in a SpeedVac (*see* **Notes 9** and **10**).
2. While the sample is drying, pre-run the gel for 15–30 min at 400 V in 1× TBE. Before running, remove the comb and use a needle and syringe to rinse out each well with running buffer. Flush out any bubbles from the bottom of the gel.
3. Resuspend the sample in 10 μL urea sample buffer, and heat for 3 min at 65 °C. While the samples are heating, stop the gel and again rinse out each well. Load heated RNA samples in the prepared polyacrylamide gel.
4. Run the gel at 400 V until the smallest RNA in the sample is about 75 % of the way to the bottom of the gel, as judged by the positions of the bromophenol blue and xylene cyanol dyes—approximately 15 and 40 nt, respectively (see Sambrook [14] for dye migration in denaturing polyacrylamide gels of various percentages).

5. Remove the spacers and then the siliconized glass plate and carefully peel the gel off of the uncoated glass plate into a shallow dish containing 0.5 μg/mL ethidium bromide in water. Shake gently for 15–30 min.
6. Wearing gloves, lift gel out of the staining solution, rinse briefly in water, and place in a gel documentation apparatus equipped with a UV light. Quantitate ligation efficiency based on the amount of the limiting oligonucleotide (A) that has shifted into the A/B product. If efficiency is poor, modify the reaction conditions as outlined in the troubleshooting section.

3.5 Preparation

To produce ligated RNA products on a preparative scale, for subsequent use, scale up the reaction and purify the products as follows:

1. Combine 1–10 nmol of each RNA and DNA oligonucleotide in a minimal reaction volume with RNA ligase buffer (*see* **Note 11**). Denature and anneal as above. Add 5–10 U of RNA ligase/nmol phosphorylated 5′ ends (*see* **Note 12**).
2. Incubate the optimal length of time at 37 °C, as determined above in the small-scale reactions.
3. Stop by addition of an equal volume of formamide (NOT sample buffer with dyes), and then heat 3 min at 65 °C before loading on a pre-run gel, as above. Ensure that the wells are large enough to accommodate the entire sample volume in as few lanes as possible. Load a few μL of urea sample buffer in an empty lane to use as a marker.
4. Run the gel at 400 V until the smallest RNA is 75 % of the way down the gel.
5. Remove the top gel plate and cover the gel with plastic wrap. Flip the gel over and carefully release the gel from the bottom plate using one of the spacers. Fold the plastic wrap over the gel and place on a fluor-coated TLC plate. Using a handheld UV lamp on the short wavelength setting (254 nm), quickly identify the location in the gel of the desired RNA product by looking for the shadow cast by the RNA. Draw a box around the product on the plastic wrap using a permanent marker.
6. Move the wrapped gel onto a clean, scratch-proof surface. Using a sterile scalpel, cut the marked fragment of gel away from the remainder, peel off the plastic wrap, cut into several pieces if necessary, and place in a 1.5 mL microcentrifuge tube(s) (*see* **Note 13**).
7. Crush the gel slice using a disposable pestle. Add 400 μL water and crush some more. Elute the RNA from the gel fragment by heating at 70 °C for 10 min.

8. While heating, spin the DTR cartridge 3 min at 850 × *g*. Discard the flowthrough.
9. Pulse gel solution briefly in a microcentrifuge and then load entire slurry onto the pre-spun DTR cartridge. Spin in microcentrifuge 3 min at 850 × *g* to remove the acrylamide from the eluted RNA.
10. Precipitate the RNA by adding 15 μg glycogen, one tenth volume of 3 M NaOAc, and 2.5 volumes of ice-cold 100 % EtOH. Vortex to mix. Spin 30 min at max speed at 4 °C in microcentrifuge. Aspirate the supernatant, wash the pellet with cold 70 % EtOH, and spin again for 5 min. Aspirate the supernatant and allow the pellet to air-dry for 5 min.
11. If this fragment is to be used in a subsequent ligation step, deprotect as described above, combining all tubes from the gel elution into one (*see* **Note 14**).
12. Resuspend in a small volume of water (i.e., 20 μL), and determine the concentration by UV spectrophotometry.

3.6 Simultaneous Ligations

It is possible to carry out two or more ligation reactions simultaneously, but each combination of reactions must be independently optimized. For a three-way ligation of oligonucleotides A, B, and C, the most probable competing reaction is circularization of the B oligonucleotide, which is the only one that is both phosphorylated and deprotected. Consequently, the splints and other oligonucleotides must be used in excess to sequester the ends of B and minimize its auto-ligation.

1. Order oligonucleotides B and C with 5′ phosphate groups. Deprotect oligonucleotides A and B as described above in ligation **step 1**.
2. A good starting point is to set up the ligation as in **step 4** (small scale), with final concentrations of 2.0 μM oligonucleotide A, 1.5 μM splint 1 (for AB junction), 1.0 μM oligonucleotide B, 1.5 μM splint 2 (for BC junction), and 2.0 μM oligonucleotide C. Anneal and ligate as described above. Ratios of oligonucleotides and splints may need to be modified to obtain better yields of product ABC.
3. To assess the degree to which circularization of B is limiting product yield, carry out a control ligation with only oligonucleotide B. Run this on the gel next to the products of the ABC ligation to ascertain whether a substantial fraction of B in the three-way ligation is circularizing. If so, increase the concentration of the two splints.

3.7 Troubleshooting

When ligation efficiency is not satisfactory:

1. Alter the ratios of RNA oligonucleotides to splint. Other things that might improve product yield include increased ligation time, increase/decrease in ligation temperature, or cycling several times between 65 °C and the ligation temperature. Up to 25 % PEG 8000 can be added to the annealing reaction to increase molecular crowding, and 10–20 % DMSO can be added to disrupt RNA secondary structure. Check the annealed reactions on a native gel to see if the RNA is forming a complex with the splint. If most of the RNA is in the complex, proceed with the ligation.
2. Redesign the ligation junction in the RNA—change the placement of the junction and/or the number of single-stranded nucleotides in one or both sides of the loop. Increased splint length can disrupt secondary structure. Incorporation of the nucleotide purine into the middle of the splint can prevent a stable base-pairing interaction between donor- and acceptor-binding regions of the splint, thereby maintaining the desired single-stranded loop when it is not feasible to change the junction site (*see* Fig. 3).

4 Notes

1. Glass plates separate most reliably when only one of them is siliconized. Clean both plates carefully with dish soap and rinse extensively with deionized water. Allow to dry on a rack. Wipe with 70 % ethanol, apply nonstick solution (e.g., Gel Repel, Aardvark Science) according to the manufacturer's directions to the notched plate only, and buff again with 70 % ethanol. Mark the other side of the plate with a permanent marker. Repeat when notched plate no longer separates cleanly from the gel.
2. Make an acrylamide gel in the 8–15 % range depending on the sizes of the RNAs to be ligated.
3. Ammonium persulfate solution can be stored at 4 °C for up to 1 month.
4. The gel can be stored at 4 °C overnight. Place a wet paper towel over the comb and wrap the gel in plastic wrap prior to storage.
5. Approximately 200 ng of each oligonucleotide gives a strong band when stained with EtBr.
6. The molar ratios of oligonucleotides are designed to maximize incorporation of oligonucleotide A into the final product while minimizing side reactions. The splint is used at a molar excess relative to oligonucleotide A to ensure that all of oligonucleotide A is associated with splint and can therefore participate

in the desired reaction. Similarly, an excess of oligonucleotide B is used to ensure that all of the A/splint complexes can react productively with oligonucleotide B.

7. Annealing in the absence of buffer results in partial deprotection of oligonucleotide B, which increases the rate of side reactions such as circularization.
8. Anneal in the smallest reasonable volume to increase efficiency of RNA/oligonucleotide binding.
9. 2′ ACE-protected RNAs bind ethidium bromide poorly and are consequently difficult or impossible to observe by ethidium bromide staining. Oligonucleotides must therefore be deprotected prior to analysis or, alternatively, detected by another method, e.g., silver stain or Stains-All (Sigma). For an initial, quick screening of the ligation, one can forego deprotection, stopping the ligation instead by adding 10 μL of urea sample buffer, heating 3 min at 65 °C, and placing on ice. Oligonucleotide B will not be visible on the gel, but if the ligation was successful, there will be an obvious decrease in the amount of oligonucleotide A, as well as the appearance of the slower mobility A/B ligated product.
10. If the DNA splint is too close to the same size as one of the RNA reactants or products, it may be necessary to degrade the splint enzymatically prior to visualizing the RNA. To do this, add 1 unit of RNase-free DNase I (Invitrogen) at the end of the ligation reaction, and incubate a further 15 min at 37 °C. Stop the reaction and analyze the products as above. It is useful to run a control sample without DNase to confirm that the DNA oligonucleotide has indeed been degraded.
11. In order to purify the RNA by UV shadowing, at least 0.1 OD_{260} unit of ligated product is required.
12. 1 U of RNA ligase is defined as the amount of enzyme required to convert 1 nmol of 5′-[^{32}P]rA_{16} into a phosphatase-resistant form in 30 min at 37 °C (NEB).
13. Place a maximum of 0.1 g of gel in each tube.
14. If using 2′ ACE-protected oligonucleotides, the ligation reactions proceed most efficiently when performed in a 5′ to 3′ direction. For example, if three oligonucleotides, A, B, and C, are to be ligated, first ligate A and B, deprotect the AB product, and then ligate AB to C.
15. Post-synthetic modification of oligonucleotides prior to ligation may require deprotection. If oligonucleotide A needs to be deprotected before ligation, the overall yield of the ligation may be reduced due to circularization of A. Increasing the ratio of splint/A can decrease the likelihood of this unwanted side reaction (*see* Subheading 3.6).

Acknowledgments

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