Preface

The transfer of hereditary information from genes to proteins is one of the essential processes in all living organisms on our planet. Some genes are expressed without modulation throughout the life of a cell, while many others require various degrees of control to precisely balance cellular metabolism with environmental conditions. For many years, researchers attributed this regulatory function to protein molecules, which can direct gene expression at multiple levels, in response to various input signals, and with different degrees of selectivity. Even when the control of gene expression was achieved via direct interactions between proteins and mRNAs, the active role was routinely assigned to proteins, while RNAs were considered merely as recipient molecules. The discovery of RNA interference and multiple bacterial regulatory RNAs caused a shift from the perception of proteins as the predominant regulators of gene expression to the acknowledgement of the importance of RNAs in many regulatory circuits. Such a viewpoint received strong support several years ago after the discovery of riboswitches and related RNA sensors – mRNA regions capable of alternating their conformations in response to the presence of cellular metabolites and other physical or chemical cues. These classes of RNA pass on cellular and environmental information directly to transcription or translation machinery without the assistance of proteins.

The riboswitches are commonly defined as evolutionarily conserved mRNA regions capable of specific binding to metabolite molecules, and, as a result, adopting a particular RNA conformation that modulates gene expression. This definition restricts riboswitches to mRNAs responding to small organic molecules, and this volume is primarily focused on the techniques used for the identification and characterization of such RNAs. However, the meaning of the term riboswitch can be broadened to incorporate classes of RNA that undergo conformational transitions in response to other stimuli in order to control the expression of genes. Therefore, several contributions to the book are devoted to the mRNAs which adopt complexly folded conformations and directly sense environmental signals or recognize molecules other than metabolites. Among the many RNA-based regulatory systems, there is a special place for mRNAs which sense the regulatory signal delivered by a specific RNA. These RNAs participate in diverse regulatory mechanisms, overviewed in the book, and often require in vivo techniques, described in two chapters, for the elucidation of their function.

This volume includes comprehensive and up-to-date coverage of various methods used to study riboswitches and other RNAs involved in gene expression control. Although some protocols utilize the intrinsic properties of metabolite molecules and can be applied exclusively to elucidate the function of metabolite-binding RNAs, a majority of the methods originate from rigorously tested procedures previously used for the characterization of various RNA molecules and RNA–ligand complexes.

Several chapters in this book describe classical and emerging biochemical techniques, such as chemical and enzymatic RNA synthesis, RNA structure probing, and footprinting. The latter two techniques, powerful and fast methods of gaining preliminary structural information, have become routine procedures in a number of laboratories. Nevertheless,

many other researchers hesitate to utilize these techniques to the fullest extent due to difficulties in the reproducibility and interpretation of the results. The expert contributions to this volume will guide one through all the obstacles in these techniques and will ultimately convert troublesome applications into facile and readily reproducible protocols.

Modern biophysical methods also represent a major part of the current volume. Several chapters include the description of cutting-edge technologies used to study riboswitch structures and their folding. These contributions cover a wide range of protocols from X-ray and NMR experiments to single-molecule fluorescent studies and isothermal titration calorimetry. Biophysical techniques are complemented by SELEX and related protocols, which allow for a transition from in vitro to in vivo experiments. In vivo methods, however, are not limited by the elucidation of the function of engineered riboswitches. Microbiologists, cell biologists and geneticists can appreciate the chapters focusing on various aspects of the identification and characterization of riboswitches and regulatory RNAs in living cells. One chapter is devoted to probably the most intriguing and demanding aspects of riboswitch research: computerized searches for riboswitches in genomic sequences, which could be helpful in the prediction of the alternative secondary and tertiary riboswitch structures, and in the identification of the candidate molecules that partner with riboswitches.

Research success in any given field is critically dependent upon advances in experimental methodologies. This volume summarizes and illustrates key methods that depict the remarkable recent progress in the riboswitch field and other areas of RNA biology. The wide scope of this book is a feature that will undoubtedly appeal to researchers with different backgrounds, working in various areas of modern biology ranging from evolutionary biology to pharmacology and biotechnology. Moreover, the protocols presented in the book can be successfully utilized by researchers with different levels of expertise, both beginners, who wish only to try the "RNA kitchen", and experts, who would like to expand their RNA methodology.

I thank the authors who contributed to this volume and whose work will hopefully help readers find new stimulating approaches suited for application in their experiments.

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Chapter 2

Enzymatic Ligation Strategies for the Preparation of Purine Riboswitches with Site-Specific Chemical Modifications

Renate Rieder, Claudia Höbartner, and Ronald Micura

Summary

One of the most versatile riboswitch classes refers to purine nucleoside metabolism. In the cell, purine riboswitches of the respective mRNAs either act at the transcriptional or translational level and off- or on-regulate genes upon binding to their dedicated ligands. Biophysical studies on ligand-induced folding of these RNA domains in vitro contribute to understanding their regulation mechanisms in vivo. For such studies, in particular, for approaches using fluorescence spectroscopy, the preparation of large RNAs with site-specific chemical modifications is required. Here, we describe a strategy for the preparation of riboswitch aptamers and aptamers adjoined to their expression platforms by chemical synthesis and enzymatic ligation. The modular design enables fast access to a large number of purine riboswitch derivatives with the modification of interest at any strand position. We exemplarily provide a detailed protocol for the preparation of adenosine deaminase (*add*) A-riboswitch variants with 2-aminopurine (AP) modifications at the 40-nmol scale.

Key words: Ligation, Riboswitch, Aminopurine, RNA modification

1. Introduction

Purine riboswitches consist of a highly conserved aptamer domain of 60–70 nucleotides and an adjoining expression platform of about the same size. While the aptamer sequences are highly conserved, sequences of the expression platform are more diverse to fulfill the specific requirements that arise from different functional determinants, such as transcriptional vs. translational regulation or gene repression vs. gene activation *(1–12)*. The general construction scheme of purine riboswitches enables a modular strategy for the efficient preparation of riboswitch variants with site-specific

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chemical modifications by ligation of chemically synthesized RNA fragments. The two prime limits that are encountered in this approach are size limitation with respect to chemically synthesized RNA strands *(13)* and the requirement of high-yielding ligation sites *(14)*. Both issues render such an undertaking methodologically challenging; for the class of purine riboswitches, we offer a satisfying solution described below.

In case of purine aptamer domains, two efficient ligation sites have been identified (**Fig. 1a**) *(15)*. One site resides in loop L2 between nucleotides 35 and 36 (numbering referring to **ref.** *12*;

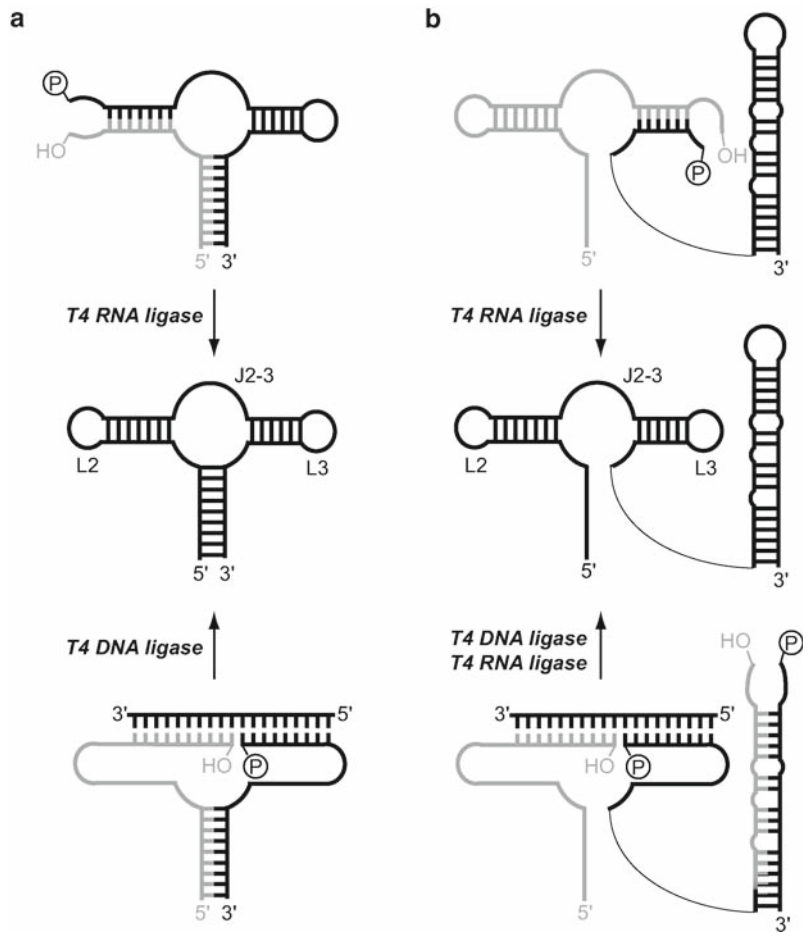

Fig. 1. Ligation strategies for the assembly of the aptamer domain and the full-length *add* A-riboswitch. The aptamer domain (**a**) can be prepared via single-stranded ligation by T4 RNA ligase (*top*) or via splinted ligation by T4 DNA ligase (*bottom*). For the full-length domain (**b**) two RNA fragments can be ligated by T4 RNA ligase (*top*). Alternatively, the use of T4 DNA ligase *and* T4 RNA ligase allows the simultaneous ligation of three fragments to the full-length domain (*bottom*). In the ligation reaction, the free 3′ hydroxyl group of the acceptor strand (*light gray*) is joined to the 5′ monophosphate of the donor strand (*black*). When three RNA fragments are ligated, the middle fragment acts as a donor at its 5′ monophosphate (*black*) and as an acceptor at its 3′ hydroxyl (*light gray*).

see also **Fig. 2a**) and enables ligation of a ~50 nt donor strand and a ~20 nt acceptor strand by employment of T4 RNA ligase (**Fig. 1a**, top). The alternative ligation site resides in junction J2–3 between nucleotides 52 and 53 (**Fig. 1a**, bottom) and requires a ~40 nt acceptor strand that is ligated to a ~30 nt donor strand by T4 DNA ligase via a 23 nt DNA splint. Especially when RNA fragments are obtained from commercial suppliers, the latter ligation site is more convenient with regard to readily available RNA lengths; custom synthesis limits are often less than 40 nt at 1 μmol or larger scales.

For full-length purine riboswitch domains, two pathways have been explored. One possibility is ligation of two strands, a ~50 nt acceptor and a ~60 nt donor strand. The site of ligation is positioned in loop L3 between nucleotides 64 and 65, and thus enables use of T4 RNA ligase (**Fig. 1b**, top) for the enzymatic reaction. The more flexible concept, however, is to join three shorter strands *(12)*. Thereby, one ligation site refers to the site optimized for aptamer preparation alone and requires T4 DNA ligase together with a DNA splint. The second site is located in the loop of the repressor element and fulfils all criteria for efficient T4 RNA ligation (**Fig. 1b**, bottom).

The enzymatic preparations described here are typically performed with 40–60 nmol of each ligation fragment in equimolar ratio. For instance, the aptamer domain of *add* A-riboswitch is ligated at 60-nmol scale by either T4 RNA ligase or T4 DNA ligase (**Fig. 1a**) and isolated with yields higher than 50% *after* purification by anion-exchange chromatography (36 nmol; ~820 μg; ~30 OD_{260}) *(12, 15)*. Yields of the corresponding full-length riboswitch domains, following the three-strand ligation approach at 40-nmol scale with simultaneous usage of T4 RNA ligase and T4 DNA ligase (**Fig. 1b**, bottom), are 40% *after* purification by anion-exchange chromatography (15 nmol; ~580 μg; ~20 OD_{260}; see protocol below). Yields of the full-length domain of *add* A-riboswitch following the two-strand ligation are slightly lower (**Fig. 1b** , top).

The following protocol provides all necessary details for efficient ligation of full-length purine riboswitch domains based on three individual RNA strands, exemplified for the *add* A-riboswitch (**Fig. 2a**). Analysis of the ligation reaction and purification of the ligation products are performed by anion-exchange chromatography (**Fig. 2b**). For characterization of the HPLC-purified riboswitch domains, liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) is applied (**Fig. 2c**). The flexibility of the modular concept is illustrated in **Fig. 3**. Variable combinations of the three-strand blocks rapidly enable access to a large number of 2-aminopurine modified full-length riboswitch domains with different 5′ and 3′ flanking sequences to assess their influence on the ligand-induced folding process.

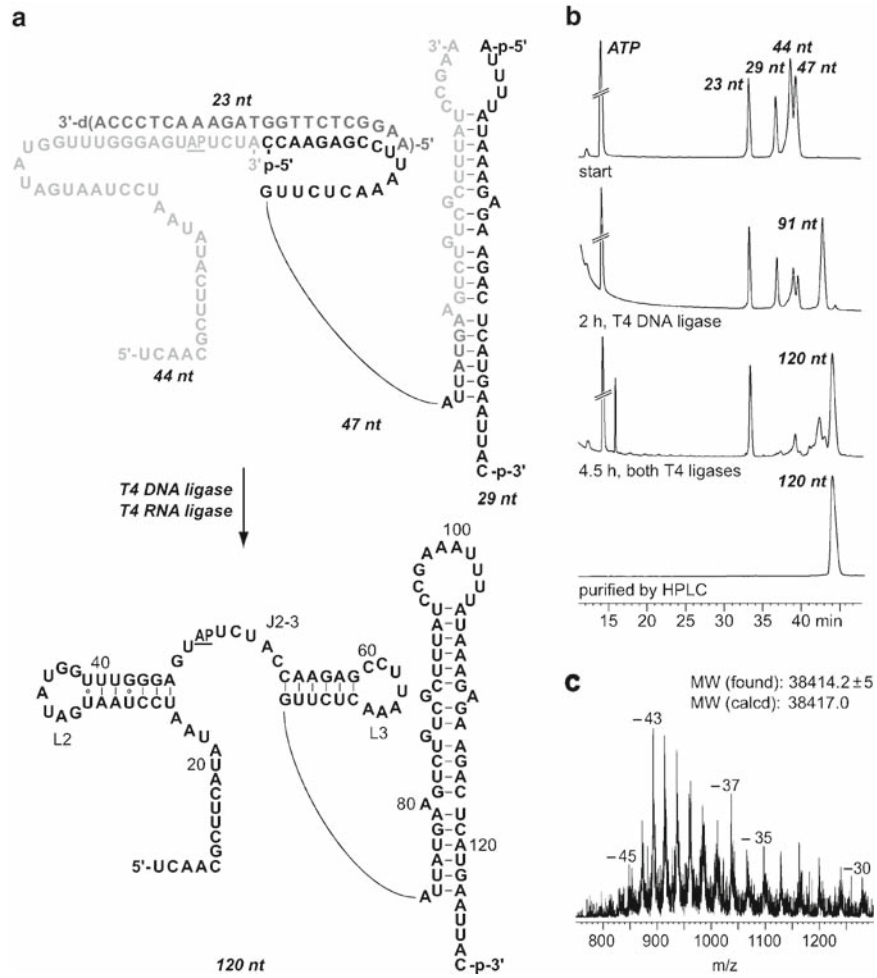

Fig. 2. (**a**) Preparation of the 2-aminopurine (AP)-modified full-length *add* A-riboswitch by the three-strand ligation strategy using T4 DNA ligase *and* T4 RNA ligase (compare Fig. 1b, bottom). (**b**) Anion-exchange HPLC analysis of the one-pot ligation reaction. The individual traces show the sample composition before addition of ligase (start), after addition of T4 DNA ligase (2 h reaction time; accumulation of 91 nt intermediate), and after addition of T4 RNA ligase (4.5 h reaction time; formation of 120 nt ligation product). The bottom trace shows the HPLC-purified ligation product that was further characterized by LC-ESI-MS (**c**).

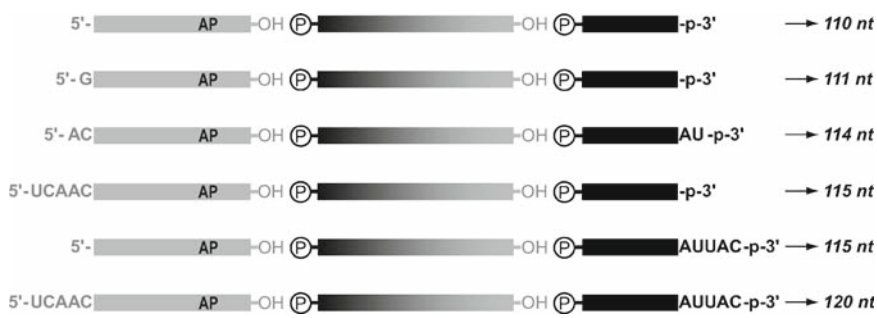

Fig. 3. Modular concept for the construction of 2-aminopurine (AP)-modified full-length riboswitch domains with varying 5′ and 3′ ends. The two-step ligation of three fragments allows a high flexibility in arranging a small number of different RNA fragments (modules) to a large number of riboswitch variants. For instance, a single central module is used for ligation to one out of four different 5′-modules and one out of three different 3′-modules. Out of 12 possible combinations, the synthesis of six riboswitch domains ranging from 110 to 120 nt has been experimentally verified.

2. Materials

2.1. Ligation Reaction

1. *RNA strands, DNA splint oligonucleotide*. We prepared the modified and unmodified RNA and the splint by solid-phase synthesis, but they can also be purchased from commercial suppliers. We recommend HPLC purification of the oligo(ribo) nucleotides.
2. 10 × Ligation buffer (Fermentas, Hanover, MD): 400 mM Tris–HCl, 100 mM $MgCl_2$, 100 mM dithiothreitol (DTT), 5 mM ATP, pH 7.8 at 25°C.
3. 50% (w/v) Polyethylene glycol (PEG) 4,000 solution (Fermentas).
4. T4 DNA ligase, 5 U/mL (Fermentas), in storage solution: 20 mM Tris–HCl, pH 7.5, 1 mM DTT, 50 mM KCl, 0.1 mM EDTA, 50% (v/v) glycerol.
5. T4 RNA ligase, 10 U/mL (Fermentas), in storage solution: 10 mM Tris–HCl, pH 7.5, 1 mM DTT, 50 mM KCl, 0.1 mM EDTA, 50% (v/v) glycerol.

2.2. Purification

1. A phenol/chloroform/isoamyl alcohol (25/24/1, v/v/v) solution is extracted three times with water and stored under a water layer at 4°C (*see* **Notes 1** and **2**).
2. Chloroform/isoamyl alcohol (24/1, v/v) solution.
3. HPLC system.
4. Eluant A: 25 mM Tris–HCl, pH 8.0, 6 M urea. Eluant B: 25 mM Tris–HCl, pH 8.0, 0.5 M $NaClO_4$, 6 M urea. Prepare the eluants from a stock solution of 250 mM Tris–HCl, pH 8.0. Filtration of the eluants through a cellulose acetate filter, 0.2 μm pore size (Sartorius, Goettingen, Germany), is strongly recommended.
5. Anion exchange column DNAPac PA-100 or 200, 4 × 250 mm, (Dionex, Sunnyvale, CA).
6. Anion exchange column DNAPac PA-100, 9 × 250 mm (Dionex).
7. Sep-Pak Plus C18 Environmental cartridges (Waters, Milford, MA).
8. 0.15 M triethylamine bicarbonate buffer $(Et_3NH)HCO_3$: Prepare 1 M Et_3N in water and pass CO_2 into the solution until the pH reaches about 8. Store at 4°C. Dilute to 0.15 M just before use.
9. HPLC-grade acetonitrile (CH_3CN) (Acros Organics, Geel, Belgium), 50% CH_3CN in H_2O.

2.3. Mass Spectrometry

1. 20 mM EDTA solution. Dissolve ethylenediaminetetraacetic acid disodium salt dihydrate in water; the pH does not need to be adjusted.
2. LC-ESI-MS system. We recommend the use of a Finnigan LCQ Advantage ion trap instrumentation (Thermo Fisher Scientific, Waltham, MA) connected to an Ettan micro HPLC system (GE Healthcare, Buckinghamshire, UK) with –4 kV applied to the spray needle.
3. XTerra MS C18, particle size 2.5 µm, 1 × 50 mm (Waters).
4. *Eluant A*: 8.6 mM Et_3N, 100 mM 1, 1, 1, 3, 3, 3-hexafluoroisopropanol, pH 8.3. Prior to the addition of 1, 1, 1, 3, 3, 3-hexafluoroisopropanol, dissolve triethylamine in water. Store at 4°C. Eluant B: methanol, HPLC grade (Acros Organics).

3. Methods

3.1. General Aspects on the Design of Ligation Sites for Purine Riboswitch Domains

3.1.1. Requirements for the 5′ and 3′ Ends of the RNA Fragments

T4 ligases catalyze the formation of a phosphodiester linkage between the 5′ monophosphate of a donor oligonucleotide and the free 3′ hydroxyl of an acceptor oligonucleotide with the assistance of exogenous ATP. To avoid undesired by-products during ligation, it is advisable to use a donor that is blocked at the 3′ end (e.g., phosphorylated) and an acceptor that has a free 5′ hydroxyl group in addition to the 3′-OH. If three RNA fragments are ligated consecutively (*see* **Fig. 2a**), the middle RNA fragment acts as donor and simultaneously as acceptor and therefore requires a monophosphate at the 5′ end and a free hydroxyl at the 3′ end (*see also* **Fig. 3**). Such a fragment is prone to circularization and to competitive ligation to any unblocked 3′ termini. It should be embedded in a preligation complex that adapts a defined and stable secondary structure that resembles the structure of the intended ligation product. To achieve such a design, the ligation sites must be selected according to the individual substrate characteristics and preferences outlined under **Subheading 3.1.3**.

3.1.2. Length of RNA Strands

To meet no restriction for positioning of chemical modifications within the riboswitch target, we choose a ligation design that relies on all RNA fragments synthesized by solid-phase synthesis. This means that the individual strands are preferably shorter than 45 nt and none is larger than 60–65 nt. The respective 3′ and 5′ phosphate groups are directly introduced by solid-phase synthesis (*see* **Fig. 2a**).

3.1.3. T4 RNA versus T4 DNA Ligation Site

T4 RNA ligase and T4 DNA ligase differ in their substrate specificity. T4 RNA ligase prefers single-stranded RNA; in particular,

the 5′ phosphate should be accessible for the enzyme without restrictions *(16)*. In contrast, T4 DNA ligase recognizes a nicked, double-stranded substrate, which is generated by the addition of a splint oligonucleotide to the two RNA molecules. Importantly, only perfectly aligned junctions get ligated *(17)*.

The efficiency of T4 RNA ligation is influenced by the ribonucleoside at the 3′ terminus of the acceptor (A > C ≥ G > U) and the ribonucleoside at the 5′ terminus of the donor (pC > pU ≈ pA > pG). The yield of ligation is even more dependent on structural premises that are given by the RNA secondary structure. T4 RNA ligation is most advantageous if the ligation site mimics its natural substrate, the 7 nt tRNA anticodon loop. In this sense, and with respect to purine riboswitches investigated here, nicks in loops L2, L3, and in the loop of the repressor or terminator element are highly appropriate for usage of T4 RNA ligase (**Fig. 1**).

Different to T4 RNA ligase, T4 DNA ligase is rather unaffected by the nature of the terminal ribonucleoside. As long as the junction is without gaps or mispaired nucleotides, no further structural requirements are needed. Significantly, the size and the nature of the splint are crucial. The interaction of the splint and RNA has to be potent enough to break up intramolecular base pairing within the RNA substrate that interferes with intermolecular base pairing to form the preligation complex.

In our experience, T4 RNA ligase is preferable over T4 DNA ligase whenever possible because of lower amounts of enzyme needed, easier product purification, and therefore higher isolated yields.

3.1.4. Ligation Conditions

Different conditions are needed for T4 RNA ligation compared to T4 DNA ligation. For riboswitch aptamer sequences, we observed that high RNA concentrations (40 μM each strand) and low temperature (21°C) are optimal for T4 RNA ligation, while lower concentration (10 μM each strand) and higher temperatures (37°C) are recommended for T4 DNA ligation. When both ligases are used in a one-pot ligation of three RNA fragments (**Fig. 2**), it is important to carefully optimize the ligation conditions with regard to RNA and ligase concentrations, buffer composition, and reaction temperature (*see* **Note 3**).

3.2. Ligation of Three Purine Riboswitch Fragments Using T4 DNA Ligase and T4 RNA Ligase

1. Mix 40 nmol containing aliquots from aqueous stock solutions of the three RNA strands and the splint oligonucleotide. Adjust the volume to 2,940 μL by the addition of water.
2. Heat to 90°C for 3 min. Then cool to room temperature for 20 min.
3. Add 400 μL of 10× ligation buffer and 400 μL of PEG 4,000 solution. Add 200 μL of T4 DNA ligase in storage solution.

4. Incubate the reaction solution at 33°C for 2 h.
5. Add 60 µL of T4 RNA ligase in storage solution and incubate for another 2.5 h at 33°C.

3.3. Purification of Ligation Product by Anion-Exchange HPLC

1. Extract the reaction mixture twice with 4 mL phenol/chloroform/isoamyl alcohol, and once with 4 mL chloroform/isoamyl alcohol. Keep the organic layers separated.
2. Wash the organic layers stepwise with 4 mL water (twice) and combine with the extracted reaction mixture (*see* **Note 4**).
3. Concentrate to a volume of ~1 mL to make the stock solution of crude oligonucleotide (*see* **Note 5**).
4. Analyze the crude product by anion-exchange chromatography. Use 10–20 µL of the stock solution.

We recommend the use of Dionex DNAPac PA-100 or 200 columns (4 × 250 mm) at 80°C. Flow rate: 1 mL/min; gradient: 0–60% eluant B in eluant A within 45 min; UV detection at 260 nm.

5. Purify the ligation product by anion-exchange chromatography. Use 100–150 µL portions of stock solution to avoid overloading of the column. Collect and combine product-containing fractions (*see* **Note 6**).

We recommend the use of Dionex DNAPac PA-100 columns (9 × 250 mm) at 80°C. Flow rate: 2 mL/min; gradient: approximately 30–50% eluant B in eluant A within 20 min; UV detection at 275 nm.

6. Dilute product-containing fractions 1 to 1 with 0.15 M $(Et_3NH)HCO_3$ buffer.
7. Pretreat the C18 cartridge consecutively with 20 mL CH_3CN, 30 mL CH_3CN/H_2O, 30 mL H_2O, and 30 mL 0.15 M $(Et_3NH)HCO_3$.
8. Load the product on the cartridge.
9. Wash with 10 mL 0.15 M $(Et_3NH)HCO_3$ buffer, and then twice with 10 mL H_2O.
10. Elute product with 40 mL H_2O/CH_3CN (1/1) and evaporate to dryness.
11. Dissolve RNA in 1 mL H_2O (*see* **Note 7**).
12. Quantify isolated product by UV spectroscopy (A260).

3.4. Characterization of Ligation Product by Mass Spectrometry

1. Dry an aliquot of the RNA solution containing 250 pmol of RNA and redissolve it in 20 µL of 20 mM EDTA solution.
2. Analyze the RNA by LC-ESI-MS in the negative ion mode. Inject 10–20 µL.
   In our setup, the ion trap instrumentation is connected to a micro-HPLC system equipped with XTerra MS C18 column.

The samples are analyzed at 21°C; flow rate: 30 µL/min; gradient: 0–100% eluant B in eluant A within 30 min; UV detection at 254 nm.

4. Notes

1. Unless denoted otherwise, chemicals are available at Sigma-Aldrich, St. Louis, MO.
2. Only HPLC-grade water should be used in the protocols.
3. For optimization of ligation conditions, we recommend ligation on a small scale (using 400 pmol of each RNA fragment) and analysis by HPLC.
4. Stepwise back-extraction of the organic layers is required to prevent phenol accumulation in the product-containing aqueous layer.
5. Do not evaporate to dryness. Difficulties in redissolving the reaction mixture in 1 mL may occur. Mostly, pellets can be dissolved in additional water.
6. Store fractions at 4°C until they are desalted all together.
7. Store at –20°C. For prolonged storage, lyophilize the stock solution.

Acknowledgments

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