NMR Spectroscopy of Large Functional RNAs: From Sample Preparation to Low-Gamma Detection

Robbin Schnieders,[1,2] Bozana Knezic,[1,2] Heidi Zetzsche,[1,2] Alexey Sudakov,[1,2] Tobias Matzel,[1] Christian Richter,[1] Martin Hengesbach,[1] Harald Schwalbe,[1] and Boris Fürtig[1,3]

[1]Institute for Organic Chemistry and Chemical Biology, Center for Biomolecular Magnetic Resonance (BMRZ), Johann Wolfgang Goethe-University, Germany
[2]These authors contributed equally to this work.
[3]Corresponding author: *fuertig@nmr.uni-frankfurt.de*

NMR spectroscopy is a potent method for the structural and biophysical characterization of RNAs. The application of NMR spectroscopy is restricted in RNA size and most often requires isotope-labeled or even selectively labeled RNAs. Additionally, new NMR pulse sequences, such as the heteronuclear-detected NMR experiments, are introduced. We herein provide detailed protocols for the preparation of isotope-labeled RNA for NMR spectroscopy via in vitro transcription. This protocol covers all steps, from the preparation of DNA template to the transcription of milligram RNA quantities. Moreover, we present a protocol for a chemo-enzymatic approach to introduce a single modified nucleotide at any position of any RNA. Regarding NMR methodology, we share protocols for the implementation of a suite of heteronuclear-detected NMR experiments including ^{13}C-detected experiments for ribose assignment and amino groups, the CN-spin filter heteronuclear single quantum coherence (HSQC) for imino groups and the ^{15}N-detected band-selective excitation short transient transverse-relaxation-optimized spectroscopy (BEST-TROSY) experiment. © 2020 The Authors.

Basic Protocol 1: Preparation of isotope-labeled RNA samples with in vitro transcription using T7 RNAP, DEAE chromatography, and RP-HPLC purification

Alternate Protocol 1: Purification of isotope-labeled RNA from in vitro transcription with preparative PAGE

Alternate Protocol 2: Purification of isotope-labeled RNA samples from in vitro transcription via centrifugal concentration

Support Protocol 1: Preparation of DNA template from plasmid

Support Protocol 2: Preparation of PCR DNA as template

Support Protocol 3: Preparation of T7 RNA Polymerase (T7 RNAP)

Support Protocol 4: Preparation of yeast inorganic pyrophosphatase (YIPP)

Basic Protocol 2: Preparation of site-specific labeled RNAs using a chemo-enzymatic synthesis

Support Protocol 5: Synthesis of modified nucleoside 3′,5′-bisphosphates

Support Protocol 6: Preparation of T4 RNA Ligase 2

Support Protocol 7: Setup of NMR spectrometer for heteronuclear-detected NMR experiments

Support Protocol 8: IPAP and DIPAP for homonuclear decoupling

Basic Protocol 3: ^{13}C-detected 3D (H)CC-TOCSY, (H)CPC, and (H)CPC-CCH-TOCSY experiments for ribose assignment

Basic Protocol 4: ^{13}C-detected 2D CN-spin filter HSQC experiment

CURRENT PROTOCOLS
A Wiley Brand

Basic Protocol 5: ^{13}C-detected C(N)H-HDQC experiment for the detection of amino groups
Support Protocol 9: ^{13}C-detected CN-HSQC experiment for amino groups
Basic Protocol 6: ^{13}C-detected "amino"-NOESY experiment
Basic Protocol 7: ^{15}N-detected BEST-TROSY experiment

Keywords: heteronuclear detection • isotope labeling • NMR • RNA • large functional RNAs

INTRODUCTION

RNAs are macromolecules that play indispensable roles in the biological processes of all living organisms. Besides the well-known RNAs that are involved in coding and decoding of genes (mRNAs, tRNAs, and rRNAs), there is a plethora of functional, diverse classes of RNAs that are essential in regulation and expression, such as riboswitches (Winkler, Nahvi, & Breaker, 2002) and RNA thermometers (Altuvia, Kornitzer, Teff, & Oppenheim, 1989), to only name a few. To exert their biological function, RNAs have to adopt certain defined conformations described by distinct secondary and tertiary structures.

Among other techniques, NMR spectroscopy is one of the most powerful methods for studying RNA's structure and conformational dynamics in solution. This statement is illustrated by the fact that ~40% of all current RNA structures were determined by NMR techniques (Berman et al., 2000). Besides structural information, information on dynamics (Dethoff, Petzold, Chugh, Casiano-negroni, & Al-hashimi, 2012), the interactions with other RNAs (Davis et al., 2005), proteins (Carlomagno, 2014), ions (Butcher, Allain, & Feigon, 2000), and small ligands (Reining et al., 2013) can be characterized by NMR spectroscopy. However, for RNAs, the NMR technique currently sets a size limitation to molecules of up to ~150 nucleotides (nt) using site-selective labeling strategies (Alvarado et al., 2014).

For all NMR studies, the preparation of milligram quantities of RNA in an isotope-labeled form is a prerequisite. Isotope labeling can include ^{15}N-only, ^{15}N,^{13}C as well as ^{15}N,^{13}C,^{2}H, which are NMR active but non-radioactive isotopes enriched above their natural abundance of 0.3%, 1%, and 0.01%, respectively. While the incorporation of ^{13}C,^{15}N isotopes has little if any effect on RNA sample stability, ^{2}H incorporation can change for example the thermal stability of an RNA of interest (Katz, Crespi, & Finkel, 1964). For the synthesis of isotope-labeled RNAs, both biochemical and chemical methods have been developed in the past. However, due to the restricted access to isotope-labeled building blocks required for chemical solid phase synthesis, biochemical synthesis that relies on the enzymatic in vitro transcription with DNA-dependent RNA polymerases has become the method of choice in many laboratories, also due to easier setup requirements.

The exact workflow of the biochemical synthesis of an RNA of interest is variable depending on the RNA to be investigated and the aim of the NMR spectroscopic study. In any case the synthesis may include the preparation of a set of required enzymes in

house, the design and synthesis of a proper DNA template, and finally the purification of the RNA in order to make an appropriate NMR sample. When the characterization of an RNA that is >100 nt is planned, selective or segmental labeling strategies may be favorable due to fewer signals that are more readily identifiable. This labeling can be, for example, conducted enzymatically with ligation-based approaches as was demonstrated for segmentally labeled RNA (Duss, Lukavsky, & Allain, 2012; Tzakos, Easton, & Lukavsky, 2007).

NMR experiments that are based on the excitation and detection of [1]H-nuclei (in the following referred to as "protons") represent the current experimental gold standard for detailed NMR spectroscopic characterizations. This decision of using proton excitation and detection experiments results from the high gyromagnetic ratio and natural abundance of the [1]H-isotope that concomitantly lead to a high sensitivity. On the other side, a low chemical shift dispersion due to the chemical similarity in building blocks, the small number of protons in the nucleobases of RNA as well as a rapid solvent exchange of, for example, imino protons, put limitations to proton-based NMR spectroscopy. Heteronuclear-detected NMR experiments represent valuable alternatives to overcome these restrictions as they exhibit a higher chemical shift dispersion and are not participating in the exchange processes mentioned.

Within this protocol, we provide a detailed guide on the preparation of isotope-labeled RNA for NMR studies by in vitro transcription with T7 RNA polymerase (Guillerez, Lopez, Proux, Launay, & Dreyfus, 2005). Because this process is very elaborate, we omit description of some standard procedures (e.g., how to conduct a denaturing polyacrylamide gel electrophoresis) at the same level of detail that is provided for the rest of our protocols. This is indicated in the appropriate place with references to other Current Protocols in Nucleic Acid Chemistry protocols that describe these standard methods in a detailed manner. Our description within this protocol covers the synthesis of the DNA template (Support Protocols 1 and 2), the optimization of the transcription reaction (Basic Protocol 1), the purification of the RNA (Basic Protocol 1, Alternate Protocols 1 and 2) as well as the expression and purification of several required enzymes within this process (Support Protocols 3 and 4). Furthermore, we provide a general guide on the ligation-based chemo-enzymatic synthesis of an RNA that is labeled or modified at a single position (Basic Protocol 2; Keyhani, Goldau, Blümler, Heckel, & Schwalbe, 2018). Here, the chemical synthesis of the required 3′,5′-nucleoside bisphosphate (Support Protocol 5), the ligation reactions with T4 RNA Ligases 1 and 2 as well as the expression and purification of T4 RNA Ligase 2 (Support Protocol 6) are described. Moreover, we provide protocols for carrying out heteronuclear-detected NMR experiments in general (Support Protocols 7 and 8) and describe how to set-up ^{13}C-detected NMR experiments for the ribose assignment, namely (H)CC-total correlation spectroscopy (TOCSY), (H)CPC and (H)CPC-HCC-TOCSY experiments (Basic Protocol 3; Richter et al., 2010). Furthermore, a guide to set up a so-called CN-spin filter heteronuclear single quantum coherence (HSQC) experiment for the determination of the status of hydrogen bonding is provided (Basic Protocol 4; Fürtig et al., 2016). For the characterization of amino groups in RNA, we provide a protocol for the ^{13}C-detected C(N)H-heteronuclear double-quantum correlation (HDQC) experiment (Basic Protocol 5, Support Protocol 9) as well as the "amino"-nuclear Overhauser effect spectroscopy (NOESY) experiment (Basic Protocol 6; Schnieders et al., 2019). With the first set of experiments, all amino resonances can be detected as sharp NMR signals. The latter experiment brings amino groups in direct structural context and yields correlations that are not accessible with [1]H-detected experiments. Lastly, the application of the ^{15}N-detected band-selective excitation short transient transverse-relaxation-optimized spectroscopy (BEST-TROSY) experiment for the imino groups is described (Basic Protocol 7; Schnieders et al., 2017).

STRATEGIC PLANNING

When approaching the synthesis and purification of a new RNA of interest for NMR spectroscopic analysis, several important decisions have to be made regarding construct design, labeling, purification strategy, and buffer composition. In this section we will address the different strategic options and will give a small guide on how to plan your RNA synthesis based on your specific requirements.

RNase-free working conditions

In contrast to other biomolecules, working with RNA poses its own challenges when it comes to sample handling. To protect against RNA viruses, humans have evolved a multitude of RNases, RNA degrading enzymes that are expressed on the skin and hairs. Therefore, additional precautions have to be taken to avoid RNase contamination that would subsequently lead to damage or degradation of the RNA sample produced in the laboratory. Those measures apply to personal lab behavior as well as chemicals, solutions, and labware used during RNA handling.

Most importantly, wear gloves at all times to meticulously avoid bringing your skin in direct or indirect contact with an RNA sample. Use consumables and chemicals that are certified to be RNase free by the manufacturer as much as possible. If no RNase-free option is commercially available, a few other protective measures can be taken to avoid RNase contamination. For liquids one can filter solutions prior to use with a cutoff of 1 to 2 kDa or add ribonuclease inhibitor (e.g., RNasin® by Promega) directly to the reaction mix. Instruments or labware can be treated with diethyl dicarbonate (DEPC) solution, and heat-proof glassware can also be heated to 200°C for 2 hr.

Construct design

Because in vitro transcription primarily utilizes T7 RNA polymerase (T7 RNAP) for RNA synthesis, one has to consider two crucial aspects for construct design. First, with the predominantly used (class III) T7 promoter sequence, the polymerase requires at least two guanosine residues as initiating nucleotides in order to transcribe with proper efficiency and yields are dramatically reduced in presence of other starting nucleotides.

Second, T7 RNAP tends to add one or sometimes even a few additional nucleotides at the 3′ end during run-off transcription, which leads to transcript inhomogeneity (Milligan, Groebe, Witherell, & Uhlenbeck, 1987; Milligan & Uhlenbeck, 1989). If this affects your synthesis yield or product homogeneity, both of these difficulties can be avoided by incorporating self-cleaving ribozymes 5′ and 3′ to your RNA sequence of interest (Schürer, Lang, Schuster, & Mörl, 2002). Moreover, a flanking ribozyme can also stabilize the transcription of an otherwise very short RNA product (≤10 nucleotides) that might be subject to abortive transcription initiation. Regarding a subsequent purification via reversed-phase HPLC (RP-HPLC), it is advisable to choose ribozymes that differ significantly in length from your RNA of interest (length difference >15 nt). For a more detailed explanation on the features of possible ribozyme cassettes and how to incorporate them into your sequence we recommend chapter 2 of The Handbook of RNA Biochemistry (Mörl & Hartmann, 2008).

As illustrated in Figure 1, one can either use a linearized DNA plasmid (Support Protocol 1) or polymerase chain reaction (PCR) product (Support Protocol 2) as DNA template for in vitro transcription.

If the RNA transcription cassette is plasmid encoded, it should include a suitable restriction site at the 3′ end to produce run-off transcripts when the linearized plasmid is transcribed. Because DNA plasmid preparation requires cloning, amplification in bacterial cells, plasmid purification, and linearization, it is rather time consuming, while PCR is
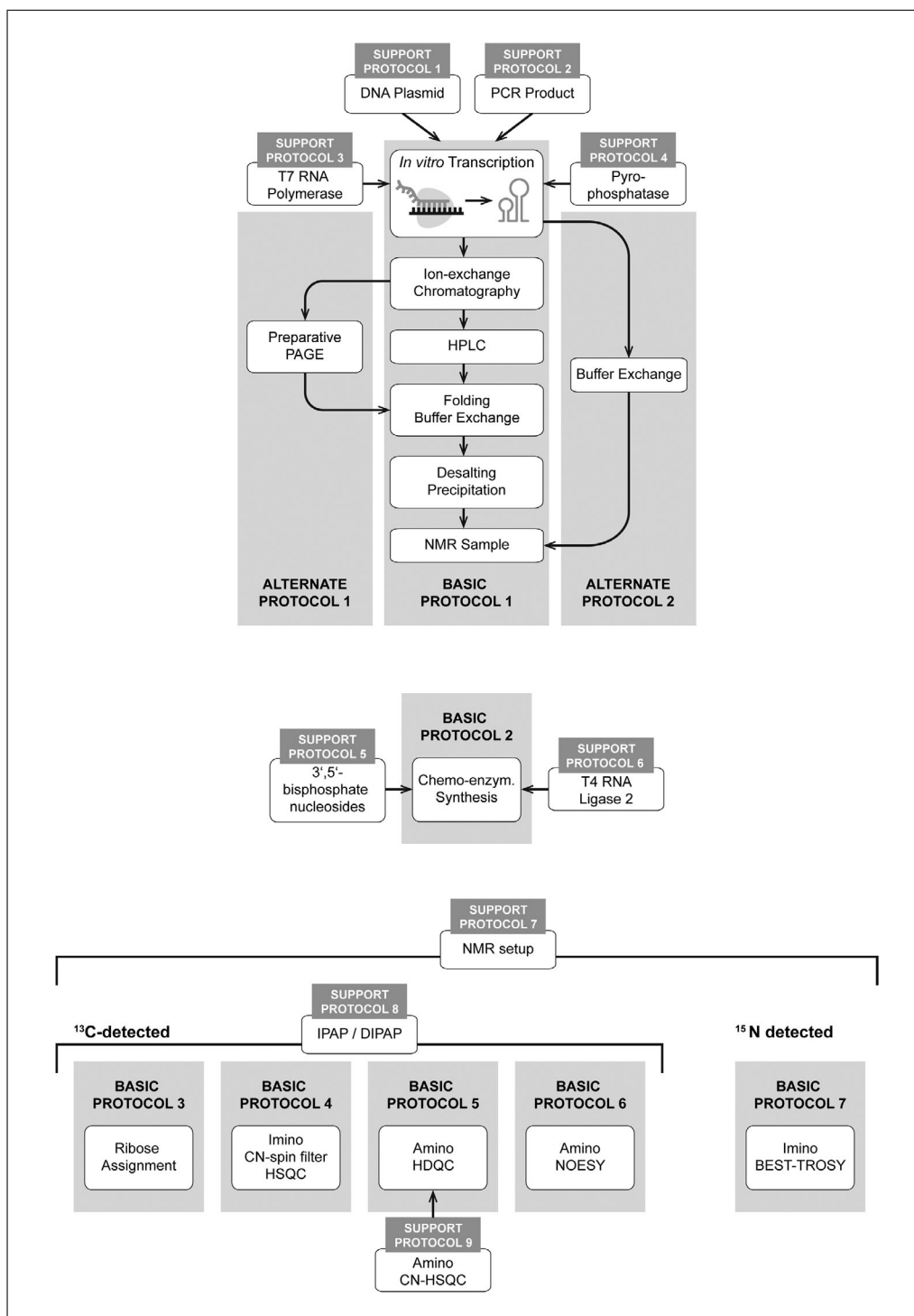
Figure 1 Schematic outline for the preparation of isotope-labeled RNA samples for NMR purposes including alternate purification pathways and sets of heteronuclear NMR experiments. All Basic, Alternate, and Support Protocols illustrated in this scheme are described within the scope of this publication.

the considerably faster option. Nonetheless, we sometimes experience a higher transcription efficiency when using plasmid DNA over PCR amplified templates, which might be attributed to the polymerases' ability to bind the DNA upstream of the T7 promoter and then slide along the strand until it reaches the promoter sequence. In the case of a PCR product this sequential scanning of the T7 RNAP is not possible, so only more direct encounters with the T7 promoter lead to transcription.

Isotope labeling

The labeling strategy for an RNA depends strongly on the objective of the study. For initial assessment of secondary structures via imino proton pattern or NOESY-based assignment of small RNAs, an unlabeled high-concentration sample (>250 μM) is often already sufficient. Moreover, the use of commercially available isotope-labeled rNTPs for NMR-scale RNA production can be rather expensive and should therefore only be considered when transcription and purification protocols are established, and scientific questions cannot be addressed with ^{1}H experiments.

With increasing RNA size, even proton-based assignments become more difficult due to spectral overlap. Here, more sophisticated heteronuclear NMR experiments can provide additional information on the nucleotide identity. ^{15}N labeling of guanine and uracil residues for example will advance base-specific assignment of resonances through the use of ^{15}N HSQC experiments, while ^{13}C labeled nucleotides can aid in resolving resonances from aromatic and sugar protons via ^{13}C HSQC experiments. Especially with RNAs of increasing length, it is advisable to selectively label only one or two types of nucleotides at a time to dramatically decrease signal overlap. In the case of more advanced structural analyses, full ^{13}C and ^{15}N labeling, sometimes combined with deuteration, is indispensable.

Furthermore, if your project requires a position-selective rather than a uniform isotope labeling strategy, we recommend utilizing the chemo-enzymatic synthesis approach described in Basic Protocol 2. This method not only allows for an incorporation of a single labeled nucleotide into an otherwise unlabeled RNA but also gives rise to RNA constructs that combine differently labeled segments into one strand.

RNA purification

For purification of the RNA to generate NMR samples of sufficient concentration and purity, three major routes are available.

Basic Protocol 1 describes a widely used chromatographic approach, where anion exchange fast protein liquid chromatography (FPLC) and "ion-pair" RP-HPLC are used to successively remove the T7 RNAP, DNA template, residual rNTPs and RNA byproducts including ribozymes from the RNA of interest. After the chromatographic steps, the RNA is freeze dried and desalted before being precipitated with LiClO$_4$/acetone. Finally, the RNA is folded into ideally one homogenous conformation and transferred into a suitable NMR buffer. Note that refolding conditions may vary with the RNA of interest and therefore have to be determined individually. This purification strategy is applicable to RNAs within a wide range of sizes and structures.

If it is not possible to completely separate the ribozymes from your RNA of interest via RP-HPLC or if an HPLC instrument is not available, we recommend switching to a preparative polyacrylamide gel electrophoresis approach, as shown in Alternate Protocol 1. Here, the RNAs are separated in a large-scale denaturing gel electrophoresis and the RNA of interest is extracted from the gel matrix afterwards.

The quickest purification route is described in Alternate Protocol 2, where merely components of low molecular weight, such as Mg(OAc)$_2$ and residual rNTPs, are removed from the reaction mix and the buffer is exchanged by repeated washing cycles using centrifugal concentrators (Helmling et al., 2015). In this protocol, the conformation adopted by the RNA during transcription is largely maintained, because no denaturing purification steps are performed. Nonetheless, this method should only be utilized when the transcription produces a single RNA product, as byproduct RNAs like ribozymes are not separated from the RNA of interest. In this case, 3′-end homogeneity of the RNA is

achieved through the use of 2′-methoxy modified primers during PCR. A PCR product carrying the 2′-methoxy modification at the 3′ end has shown to significantly increase product homogeneity for run-off transcription (Helmling et al., 2015). We recommend using this purification strategy for high throughput RNA structure analysis rather than titration experiments, because the presence of remaining T7 RNAP might interfere with ligand binding.

NMR buffer composition

RNA NMR samples are usually prepared in a buffer containing as few protons as possible (to avoid using deuterated buffer agents) and exhibiting a slightly acidic pH value (to guarantee long term stability of the sample and to reduce solvent exchange of labile protons). Typically, we use the following buffer composition as a starting point: 50 mM KCl, 25 mM K_2HPO_4/KH_2PO_4, and 5% (v/v) D_2O at pH 6.2. Depending on the nature of your experiments and the investigated RNA, bivalent cations such as Mg^{2+} can be added to stabilize the RNA's structure. As a reference substance for ^1H spectra we recommend sodium trimethylsilylpropanesulfonate (DSS), as its proton chemical shift is neither temperature nor pH dependent. Further adjustments in salt and buffer composition or pH might be required for individual studies, especially if the RNA is investigated as a part of an RNA protein complex.

PREPARATION OF ISOTOPE-LABELED RNA SAMPLES WITH IN VITRO TRANSCRIPTION USING T7 RNAP, DEAE CHROMATOGRAPHY, AND RP-HPLC PURIFICATION

The analysis of RNAs via NMR spectroscopy requires preparation of a sample with sufficient amount of pure RNA. Typically, samples between 50 and 500 μM in 300 μl buffer volume are used, but higher sample concentrations can be advantageous provided correct folding conditions can be established. In the following, we describe a standard protocol for the preparation of isotope-labeled RNA for NMR applications using the T7 RNAP. This polymerase can be purchased or prepared in house; the respective instructions for the preparation are described in Support Protocol 3. In a first step, the DNA template from which the RNA will be transcribed is amplified. For the amplification two methods are available. The first requires a DNA plasmid, which is amplified in cells, linearized, and subsequently purified (Support Protocol 1). A second approach uses a DNA template produced by solid phase synthesis in combination with a PCR using Phusion® High-Fidelity DNA polymerase (Support Protocol 2). After DNA amplification, test-transcriptions are used to optimize reaction conditions to obtain the highest yield of the target RNA. Under these conditions the preparative transcription reaction can be performed and purified via diethylaminoethanol (DEAE) and RP-HPLC chromatography. Alternative procedures for the purification are shown in Alternate Protocols 1 and *2*.

Materials

    Double distilled water (ddH$_2$O)
    DNA template (see Support Protocols 1 and 2)
    T7 RNA polymerase (T7 RNAP; see Support Protocol 3)
    Yeast inorganic pyrophosphatase (YIPP; see Support Protocol 4)
    500 mM tris/glutamate buffer, pH 8.1 (glutamic acid; Merck, cat. no. G1149)
    Spermidine (Merck, cat. no. 85558)
    Magnesium acetate (Mg(OAc)$_2$; Carl Roth, cat. no. 0275.1)
    Isotope-labeled rNTPs (Silantes/Eurisotop)
    DTT (Carl Roth, cat. no. 6908.4)
    Dimethyl sulfoxide (DMSO; Carl Roth, cat. no. A994.2)
    Denaturing RNA loading buffer (see recipe)

Denaturing PAGE gel solution (see recipe)

Denaturing PAGE running buffer ($1\times$ TBE; see recipe)

Diethylaminoethyl-Sepharose (DEAE-Sepharose®; GE Healthcare, cat. no. 17-0709-01)

3 M sodium acetate (NaOAc), pH 5.5 (Carl Roth, cat. no. 6773.2)

0.1% (v/v) diethyl pyrocarbonate (DEPC; Carl Roth, cat. no. K028.2)

Absolute ethanol (Merck, cat. no. 32205-2.5L-M)

HPLC buffer A (50 mM potassium phosphate, pH 5.9, 2 mM tetrabutylammonium hydrogen sulfate)

HPLC buffer B (HPLC buffer A plus 60% acetonitrile; acetonitrile, Thermo Fisher Scientific, cat. no. A-0627/17)

2% (w/v) lithium perchlorate ($LiClO_4$)/acetone ($LiClO_4$, Acros Organics, cat. no. 194711000)

40% (v/v) glycerol (native RNA loading buffer; Carl Roth, cat. no. 3783.1)

Native PAGE gel solution (see recipe)

Native PAGE running buffer ($1\times$ TA; see recipe)

NMR buffer (see recipe)

Magnesium chloride ($MgCl_2$; Carl Roth, cat. no. HN03.2)

Deuterium oxide (D_2O; Deutero, cat. no. 00507)

3-(Trimethylsilyl)propane-1-sulfonate, sodium salt (DSS)

Incubator

Shaking incubator

PAGE casting chamber and glass plates (Biometra multigel from Analytik Jena Company)

Heat block

Glass column for DEAE-Sepharose ® (e.g., Econo)

UV/Vis spectrophotometer (NanoDrop One/One; Thermo Fisher Scientific)

Freezer ($-20°C/-80°C$)

Centrifuge (Megafuge 8R; Thermo Fisher Scientific)

Lyophilizer/centrifugal vacuum concentrator (Alpha 2-4, Christ; vacuum concentrator plus, Eppendorf)

Shaker

HPLC device (Elite LaChrom; VWR-Hitachi)

HPLC column (e.g., PerfectSil™ RP18, pore size: 300 Å, particle size: 5 μm, $10\times$ 250 mm)

Thin layer chromatography (TLC) plate (ALUGRAM® Xtra SIL G/UV; Macherey-Nagel)

Ultraviolet (UV) lamp, 245 nm (Hanau Fluotest; Heraeus)

Centrifugal concentrator (e.g., Vivaspin™, Sartorius™)

NMR tube (Deutero, Shigemi)

1. Work RNase free. Work on ice.

2. Prepare DNA template for in vitro transcription via plasmid amplification (see Support Protocol 1) or PCR (see Support Protocol 2).

3. Prepare T7 RNAP according to Support Protocol 3.

4. Prepare YIPP according to Support Protocol 4.

5. Prepare all stock solutions for the transcription reactions including all optimization reactions (see steps 7-12).

   The following concentrations for the stock solutions might be helpful: 500 mM tris/glutamate buffer, 200 mM spermidine, 300 mM Mg(OAc)$_2$, up to 100 mM rNTP mix, and 1 M DTT.
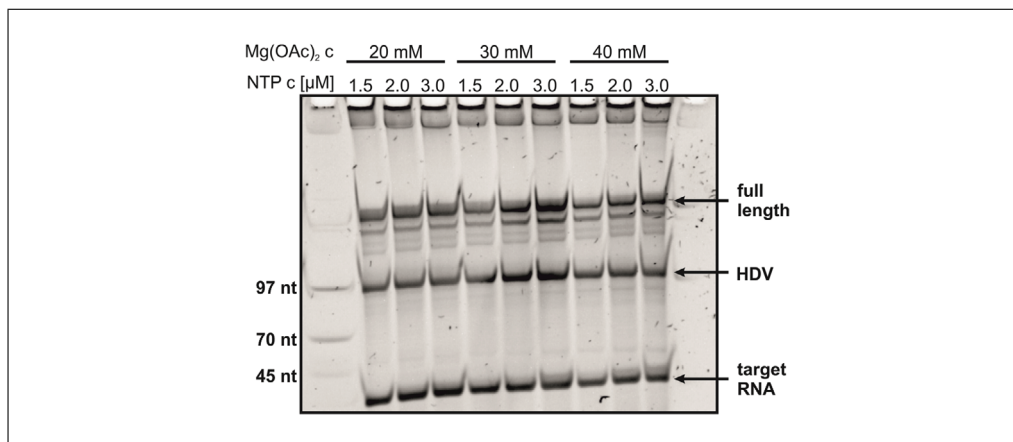
Figure 2 12% denatured PAGE of Mg(OAc)$_2$ and rNTP transcription optimization. Sample 2 with 20 mM Mg(OAc)$_2$ and 2.0 mM per rNTP shows the highest amount of product with the smallest possible amount of reactants.

In vitro transcription

6. Check all test-transcriptions with an analytical denaturing PAGE (denat. PAGE).

   The exact composition for a denat. PAGE solution has to be adjusted for each sample. One should take into account the length of the target RNA and its ribozymes (if present) and the required separation efficiency. For RNAs shorter than 20 nt, 20% denat. PAGE may be used. For up to 50 nt RNAs, a 15% denat. PAGE may be used. For RNAs longer than 50 nt, 8%-10% denat. PAGE may be used. For further instructions see Current Protocols article: Andrus & Kuimelis, 2000.

7. Optimize transcription reaction (see step 13) on a test scale and start with optimization of the rNTP concentration relative to the Mg(OAc)$_2$ concentration (Fig. 2).

   For test-transcriptions, a volume of 50 µl is sufficient. Use unlabeled rNTPs for the optimization. You may optimize the rNTP composition according to the ratio of nucleotides in the transcribed RNA sequence. Concentrations might be in a range of 2-5 mM per rNTP and 5-60 mM Mg(OAc)$_2$. Final Mg(OAc)$_2$ concentration further depends on the type of DNA template (PCR product or plasmid). If the RNA contains a ribozyme, you will have to choose a higher Mg(OAc)$_2$ concentration to assist the cleavage. The aim of this optimization is a clean product and a maximum yield of target RNA with a reasonable amount of byproduct, which can be removed subsequently.

8. Optimize amount of DNA via test-transcriptions.

   Use 4%-10% (v/v) of PCR product. It can be used directly after the PCR without any purification steps if the product is homogeneous or potential byproducts are not transcribed. When using plasmid DNA, optimize 25-200 ng/µl.

9. Perform test-transcriptions with up to 20% (v/v) DMSO.

   DMSO can increase the homogeneity of the target RNA but has to be tested in advance. We observe a positive effect primarily for transcriptions with PCR DNA templates.

10. Optimize incubation time at 37°C via a test-transcription.

    Prepare one test-transcription and take samples for analysis in suitable intervals. The incubation time can differ greatly with different RNA constructs. You may incubate 4-24 hr.

11. Optimize tris/glutamate buffer concentration, if required. Use 100-200 mM tris/glutamate buffer.

12. Perform a test-transcription with the optimized parameters and isotope-labeled rNTPs.

Table 1 Reaction Composition for a Transcription Reaction

Reagent	Quantity
ddH$_2$O	Add up to 10-25 ml
Tris/glutamate buffer (pH 8.1)	100-200 mM
Spermidine	2 mM
Mg(OAc)$_2$	5-60 mM
rNTPs	2-5 mM
DTT	20 mM
DNA	As optimized
DMSO	Up to 20%
YIPP	9.6 µg/ml
T7 RNA polymerase	20 µg/ml

13. Perform preparative transcription with the optimized conditions in a 50-ml reaction tube according to the pipetting scheme outlined in Table 1.

    If using a PCR DNA template, a transcription volume of 10 ml is sufficient. If using plasmid DNA template, usually a volume of 25 ml is required. Add the DNA template to the mixture in the order shown in Table 1 as a high concentration of Mg^{2+} may lead to DNA precipitation. You may add 9.6 µg/ml YIPP after 1-2 hr of incubation. Add the enzymes last.

14. Incubate transcription reaction at 37°C and maximum 120 rpm as long as optimized.

15. Check whether target RNA has been transcribed with a denat. PAGE.

Anion exchange chromatography

16. Remove enzymes, DNA template, free rNTPs, and excess salt with DEAE chromatography using the following conditions:

    a. Pour ~10 ml DEAE sepharose resin into an empty chromatography column and let resin settle completely. Drain supernatant EtOH from DEAE column and wash with 20 ml ddH$_2$O.

    b. For inactivation of possible RNase contaminations, wash column with 50 ml 0.1% (v/v) DEPC and then incubate with 0.1% (v/v) DEPC overnight.

    c. Drain the 0.1% (v/v) DEPC solution and wash column with 100 ml hot (~60° to 90°C) ddH$_2$O and equilibrate with 50 ml 0.1 M NaOAc.

    Try to avoid disturbing the column resin by carefully pouring the water along the inner wall of the column. For each washing step, make sure resin is settled completely before starting elution.

    d. Centrifuge transcription reaction (15 min, 4,000 × g, 4°C) and transfer supernatant onto the column.

    Make sure that the resin is settled completely before starting elution.

    Optional: Wash salt pellet with 5 ml H$_2$O and centrifuge again for 15 min before pooling both supernatants.

    e. Collect flowthrough in 10 ml fractions. Load and elute successively with 50 ml of 0.6 M, 1.0 M, 2.0 M, and 3.0 M NaOAc solution.

    Expect RNA to elute at around 2 M NaOAc.

Table 2 RP-HPLC Gradient for RNAs With Up to 40 Nucleotides

Time (min)	HPLC buffer A (%)	HPLC buffer B (%)
0	100	0
5	67	33
30	63	37
45	0	100

Table 3 RP-HPLC Gradient for RNAs With Up to 160 Nucleotides

Time (min)	HPLC buffer A (%)	HPLC buffer (%)
0	100	0
5	55	45
30	50	50
45	0	100

17. Use UV/vis absorption spectroscopy to identify fractions with a high absorption at 260 nm and check the corresponding fractions on an analytical denat. PAGE to determine RNA containing fractions.

18. Dilute salt concentration of the product fractions with ddH$_2$O to 0.3-0.6 M NaOAc.

    Lower NaOAc concentration ensures that salt stays in solution during precipitation.

19. Add four volumes of ice-cold absolute ethanol to the product fractions.

20. Incubate at −80°C overnight.

21. Centrifuge at 10,000 × g and 4°C for 30-60 min.

    Determine the maximum speed tolerated by the sample tube.

22. Remove supernatant and keep it for further precipitation.

    Sometimes, the RNA does not precipitate quantitatively. Repeat steps 20-22 twice with the supernatant to ensure no RNA remains in the solution. Incubate supernatant from the precipitation for 3-6 hr at −80°C.

23. Air dry pellet or use a vacuum concentrator for 2 min and reconstitute RNA pellet in ∼1 ml ddH$_2$O. An OD$_{260}$ of ∼100 is suitable.

24. Purify RNA sample via HPLC. Suitable gradients for different RNA lengths we use in our labs are shown in Tables 2 and 3. In this case an ion-pair RP HPLC with the PerfectSil RP18 column is used at 60°C, which fits for RNA lengths of up to 180 nt. This column is equilibrated with HPLC buffer A for 10-15 min and a flow rate of 5 ml/min is usually used. A different device or another column may require a slightly modified gradient.

    If a new RNA construct is purified, an analytical run should be conducted to determine a suitable gradient. The gradient depends not only on the RNA length but also on its structure, sequence, and potential impurities.

25. Remove HPLC buffer with a lyophilizer or centrifugal vacuum concentrator.

26. Reconstitute pellet in 1 ml ddH$_2$O.

27. Add five volumes of 2% (w/v) LiClO$_4$/acetone and incubate 2 hr at −20°C.

28. Centrifuge at 10,000 × g and 4°C for 30-60 min.

You may centrifuge with higher speed if possible. Stick to the speed limit specified in the device manual of the reaction tube.

29. Remove supernatant and reconstitute pellet in ddH$_2$O or NMR buffer depending on the folding method.

    If you use a 20-ml centrifugal concentrator, you may reconstitute in a volume of ∼10 ml.

Folding and buffer exchange

The folding protocol might need to be adapted for each individual RNA construct. Therefore, some common folding pathways are introduced here. The RNA can be folded in water or in NMR buffer as well as at a high or low RNA concentration. Take into account that high salt and RNA concentrations can induce dimerization. Do not freeze the sample after folding.

30. Check for a homogenous fold via a native PAGE. The loaded sample has to have a concentration of an NMR sample (>100 μM). Load 500 nmol RNA. Use UV shadowing to reveal the bands.

    Do not use urea or EDTA for native PAGE. Furthermore, keep in mind the temperature, as the RNA should not be exposed to a higher temperature than 25°C. Therefore, the power should not exceed 1 W and a cooling system should be applied if possible.

31. Optimize folding conditions for the target RNA if they are unknown and check via a native PAGE.

    For slow folding, denature the RNA (3-5 min, 95°C) and leave the sample on the switched off heat block until it reaches room temperature. For fast folding, place the sample directly on ice after denaturing. After denaturing, the RNA can be folded by rapid dilution with five- to ten-fold ice-cold ddH$_2$O or ice-cold NMR buffer. This optimization can be performed at this point in ddH$_2$O or in step 36 in NMR buffer.

32. Fold RNA sample into a single conformation. Folding can either occur now or in step 40.

    Do not freeze the sample after folding.

33. Prepare a centrifugal concentrator as described in the device manual.

    Choose a molecular weight (MW) cutoff, which is about a third of the size of the target RNA. This way the RNA construct will not pass through the filter.

34. Transfer RNA sample into NMR buffer using the centrifugal concentrator and centrifuge at the recommended speed.

    Stick to the speed limit specified by the manufacturer otherwise the membrane could be damaged. The temperature inside the centrifuge should be in a range of 4°-25°C, depending on type and stability of the target RNA. If a 20-ml centrifugal concentrator is used, centrifuge until the volume is reduced to 1 ml.

35. Refill centrifugal concentrator with NMR buffer and mix it carefully with a pipet to not damage the concentrator membrane. Then, centrifuge it to obtain one-tenth of the volume.

    Mixing the sample prevents the RNA from aggregating because the concentration increases gradually towards the membrane. Further, it can lead to faster repetition cycles.

36. Repeat step 35 three to ten times to remove remaining salts.

    The overall number of repetition cycles depends strongly on the RNA construct, HPLC buffer conditions, and the RNA purity required for later experiments. Some RNAs tend to aggregate during buffer exchange. They have to be recovered after each cycle and should undergo the buffer exchange cycle described in step 35 at least six times.
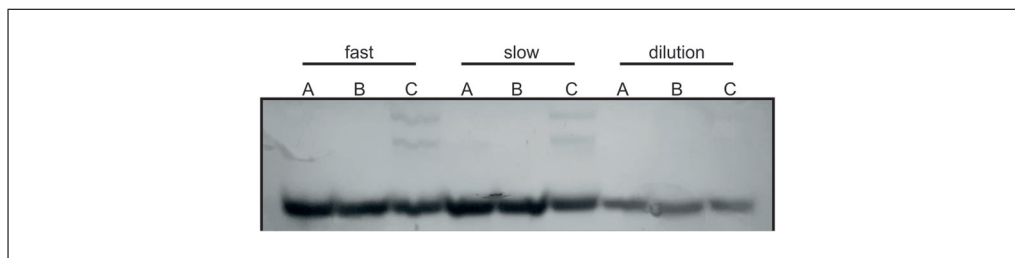
Figure 3 10% native PAGE of 3 different RNAs (A, B, C) with 3 different folding conditions. The RNAs have a similar length and are present in NMR buffer. The fast folding condition implies direct cooling on ice after denaturing. The slow folding condition implies cooling on a heating block. For the dilution method, the denatured RNA was diluted with 10-fold ice-cold NMR buffer and concentrated with a centrifugal concentrator. For RNAs A and B, the folding conditions result in one homogeneous conformation indicated by one band. RNA C adopts two other but minor conformations.

37. Centrifuge after the last iteration to reduce the volume to 200-300 μl.

38. Remove RNA from the centrifugal concentrator via a pipet.

    RNAs can stick to the membrane and therefore have to be removed vigorously with a pipet. It is recommended that the RNA solution is pipetted along the inside of the membrane and that the membrane is washed the same way with a small additional amount of NMR buffer (~50 μl) after removal.

39. Determine RNA concentration via UV/vis absorption spectroscopy.

    Take into account $MgCl_2$, D_2O, and DSS which may be added afterwards and will dilute the sample. We recommend an RNA concentration of at least 300 μM.

40. Fold RNA unless it was folded before and check the final fold via a native PAGE (see step 30 and Fig. 3).

    If the RNA tends to aggregate during buffer exchange, it should be folded afterwards.

41. Add $MgCl_2$ if required, unless it was added before.

    Mg^{2+} ions may stabilize the RNA structure. Each novel RNA construct should be titrated with $MgCl_2$. Therefore, add $MgCl_2$ stepwise to the RNA (1 mM) and measure a ^1H-1D NMR spectrum after each step, to detect differences in the RNA structure and stability. You may add $MgCl_2$ to the RNA sample after folding because the denaturation step should be done in the absence of Mg^{2+} ions. $MgCl_2$ may also be added to the NMR buffer that is used for the buffer exchange (see steps 35 and 36).

42. Add 5% D_2O and 100 μM DSS to the RNA sample.

    If the DSS stock solution is prepared in D_2O, check if additional D_2O has to be added. Otherwise prepare a highly concentrated DSS stock solution in NMR buffer.

43. Wash the Shigemi tube with ddH_2O and incubate it with a 0.1% (v/v) DEPC solution overnight. Repeatedly wash tube with ddH_2O (at least five times or more) and dry it via a lyophilizer or drying oven.

44. Fill the Shigemi tube with the NMR sample (optimal filling height: 300 μl) and place the insert above the sample so that no air bubbles are trapped between the insert and the sample.

    It can be helpful to push the insert with high velocity and pressure into the tube. The lower part of the insert should be surrounded by sample solution.

45. Seal the Shigemi tube with sealing film (e.g., Parafilm) and label the tube. Store sample at 4°C.

    Sealing stabilizes the insert.

46. Perform essential NMR experiments (e.g., ^{1}H 1D, ^{1}H 2D NOESY, ^{1}H,^{15}N-HSQC) for the assignment of the RNA. For basic NMR experiments for RNA, we refer to the review article: NMR spectroscopy of RNA (Fürtig, Richter, Wöhnert, & Schwalbe, 2003). For more sophisticated heteronuclear experiments see Basic Protocols 3-7.

PURIFICATION OF ISOTOPE-LABELED RNA FROM IN VITRO TRANSCRIPTION WITH PREPARATIVE PAGE

Alternate Protocol 1 describes a second approach for the purification of isotope-labeled RNAs from in vitro transcription. This method utilizes preparative PAGE instead of RP-HPLC and is therefore an alternative if no HPLC device is available or if the device does not separate the target RNA product sufficiently from byproducts (Current Protocols article: Hengesbach et al., 2008; Petrov, Wu, Puglisi, & Puglisi, 2013). This protocol starts after the successful in vitro transcription of the target RNA and yields a pure NMR sample. This method is not suitable if the NMR sample has to be free of any acrylamide impurities, which might be eluted together with RNA from the preparative PAGE. If acrylamide affects the results, an HPLC run to separate it from the RNA is inevitable.

Additional Materials (see also Basic Protocol 1)

Double distilled water (ddH$_2$O)
Diethylaminoethyl-Sepharose (DEAE-Sepharose®; GE Healthcare, cat. no. 17-0709-01)
3 M sodium acetate (NaOAc), pH 5.5 (Carl Roth, cat. no. 6773.2)
0.1% (v/v) diethyl pyrocarbonate (DEPC; Carl Roth, cat. no. K028.2)
Denaturing PAGE gel solution, 8%-15% (see recipe)
Formamide (denaturing RNA loading buffer for preparative PAGE; Merck, cat. no. 47671)
Denaturing PAGE running buffer (1× TBE; see recipe)
Dye-containing loading buffer (see recipe)
Elution buffer: 0.3 M sodium acetate (NaOAc), pH 5.5
Absolute ethanol (Merck, cat. no. 32205-2.5L-M)

UV/Vis spectrophotometer (NanoDrop One/One; Thermo Fisher Scientific)
NAP^{TM} column (GE Healthcare)
Glass column for DEAE-Sepharose® (e.g., Econo)
Centrifugal concentrator (e.g., Vivaspin^{TM}, Sartorius^{TM})
Sterile filters, 0.2 μm (Carl Roth)
PAGE casting chamber and glass plates (Biometra multigel; Analytik Jena Company)
Heat block
Sterile surface
Thin layer chromatography (TLC) plate (ALUGRAM® Xtra SIL G/UV; Macherey-Nagel)
UV lamp, 245 nm (Hanau Fluotest; Heraeus)
Sterile scalpel
Syringe
Shaker
Freezer (−20°C/−80°C)
Centrifuge (Megafuge 8R; Thermo Fisher Scientific)
Centrifugal vacuum concentrator (Vacuum concentrator plus; Eppendorf)

NOTE: This protocol starts with the purification of a previously transcribed RNA. For the in vitro transcription, refer to Basic Protocol 1, steps 1-15.

1. Work RNase free.

2. Transcribe RNA as described in Basic Protocol 1, steps 1-15.

3. Determine amount of RNA and sample volume which has to be purified and check which PAGE size fits the RNA sample. Take into account the additional formamide (see step 9 below) and the required separation efficiency, e.g., the size difference between product bands.

   A midi PAGE (~20-cm migration distance) is usually sufficient for the amount of RNA obtained from a 15-ml transcription if no byproducts occur, whereas a maxi PAGE (~50-cm migration distance) is suitable if the transcription was conducted in a 25-ml scale or if a greater separation efficiency is required. This case occurs if more byproduct is produced or if the ribozymes have a similar length as the target RNA product.

4. Remove free rNTPs and excess salt from the transcription reaction either via DEAE chromatography according to Basic Protocol 1, a NAP column, or via a centrifugal concentrator.

   You may precipitate RNA to remove DMSO and to reduce the volume if you use an NAP column.

5. Prepare TBE buffer as needed for your PAGE casting device.

   Make sure there is enough buffer to refill the PAGE casting device in case of leakage.

6. Prepare 8%-15% PAGE gel solution and filter with sterile filters (0.2 μm).

   Determine a suitable acrylamide percentage; higher concentrations may be more suitable for smaller constructs. Lower concentrations generally increase the elution yield. Use appropriate beakers. About two times the PAGE gel solution volume should be sufficient. The end volume of the gel solution depends on your PAGE device. In case of a midi gel with a migration distance of ~20 cm, 70 ml PAGE gel solution could be sufficient. For a maxi gel with a migration distance of ~50 cm, 250 ml PAGE gel solution may be prepared. Make sure there is an excess of gel solution in case of leakage; in some cases, a fast refill or selective injection of tetramethylethylenediamine (TEMED) may help out.

7. Set PAGE glass plates and check for leakage.

   Check for equal thickness of spacers and combs. The PAGE device and the glass plates have to be RNase free.

8. Add ammonium persulfate (APS) and TEMED to the PAGE gel solution to start polymerization immediately before pouring.

   Adjust APS and TEMED to the desired volume and consider the time needed for casting the gel. 0.1% (w/v) APS and 0.1% (v/v) TEMED should be used for midi PAGE gels. For maxi PAGE gels use about 0.06% (w/v) APS and 0.06% (v/v) TEMED. Polymerization time is 10-20 min.

9. Add at least 0.5 volumes of formamide to your sample and denature RNA 3-5 min at 95°C.

   Check if the end volume of the sample fits into the gel pockets (see step 3). Do not use any dyes.

10. Set gel into the PAGE device. If using a maxi gel, pre-run gel for 30 min.

    Rinse wells thoroughly with running buffer directly after setting the gel and prior to loading the gel to prevent sedimentation of urea.

11. Add 20 μl dye-containing loading buffer in one spare gel pocket to determine gel-running progress.

12. Load denatured RNA sample into free gel pockets.

If you have just one type of comb at hand (with multiple pockets), you may destroy the pockets gently, to extend the loading volume for the sample. Use a sterile needle or spatula.

13. Run preparative PAGE.

    Running parameters depend on the type of PAGE and the size of the target RNA. For midi PAGEs, set ~30 W and for maxi PAGEs, 50 W is sufficient. Check for PAGE running buffer leakage after 15-30 min. If required and available, start the fan after 30 min to prevent the sample from swirling up. Other types of cooling may be started from the beginning. Running time depends strongly on the length of your target RNA. Therefore, follow the dye running progress.

14. Remove gel from glass plates and place it onto a sterile surface.

    Make sure UV radiation passes through the sterile surface. Sterile plastic bags may be used. Cut the bag with a sterile scalpel and place the gel on the inside.

15. Place a fluorescent TLC plate underneath the gel.

    The plate should be wrapped with a clear and clean plastic film in case you cut through the sterile surface.

16. Check for a UV shadow caused by the RNA using a UV lamp (245 nm). Identify desired RNA band and mark band with a sterile scalpel.

    Be careful and illuminate the gel only briefly with the UV lamp to avoid UV damage of the RNA. A picture of the mini PAGE of the sample may help to find the target RNA band faster.

17. Cut the desired band out of the gel and then cut into small pieces.

18. Mix gel pieces with 10-20 ml elution buffer and press the mix through a syringe.

    This process increases the gel surface for better elution.

19. Shake gel pieces at 4°-25°C overnight to elute RNA into the elution buffer.

    Optionally freeze and thaw gel slices to disrupt the gel matrix and to increase the elution efficiency of the RNA. After the overnight elution, incubation at 65°C for 15 min and additional shaking for 30 min at 1,300 rpm can help to increase the amount of eluted RNA.

20. Filter elution buffer with a sterile filter (0.2 μm) and keep gel pieces for further elution.

    In some cases, the elution of RNA is difficult and it may not be completed overnight. Therefore, add fresh 0.3 M NaOAc to the separated gel pieces and repeat steps 19 and 20.

21. Add two-and-a-half to four volumes of ice-cold absolute ethanol to precipitate RNA and place mixture at −20°C overnight.

    Incubation at −80°C for 2 hr may also be sufficient.

22. Centrifuge at 10,000 × g and 4°C for 30-60 min.

    You may centrifuge at higher speed if possible. Stick to the speed limit specified in the device manual of the reaction tube.

23. Remove supernatant and keep it for further precipitation.

    In some cases, the RNA does not precipitate entirely. In this case, incubation and centrifugation may be repeated.

24. Air dry pellet or dry via a vacuum concentrator for 2 min and reconstitute it in ddH$_2$O.

Run an analytical denat. PAGE to check the purity of the target RNA.

25. Proceed with folding, buffer exchange, and NMR sample preparation as described in Basic Protocol 1, steps 30-46.

PURIFICATION OF ISOTOPE-LABELED RNA SAMPLES FROM IN VITRO TRANSCRIPTION VIA CENTRIFUGAL CONCENTRATION

This Alternate Protocol 2 describes a fast purification method for isotope-labeled RNAs from in vitro transcription using a centrifugal concentrator (see Fig. 4). It is the quickest purification method among the purification strategies described within this protocol. However, this method should only be applied when the transcription produces a single RNA product, as byproduct RNAs or other transcribed RNAs such as ribozymes are not separated. Therefore, it is important to generate 3′-end homogeneity of the transcribed RNA by using PCR DNA templates with 2′-*O*-methyl-modifications at the last two nucleotides of the 5′ end (Helmling et al., 2015; Kao, Zheng, & Rüdisser, 1999; Support Protocol 2). We also advise that this purification procedure not be used for NMR titration experiments, because remaining enzymes in solution might interfere with ligand binding.

Materials

Transcribed RNA (see Basic Protocol 1, steps 1-15)
Double distilled water (ddH$_2$O)
500 mM tris/glutamate buffer, pH 8.1 (glutamic acid; Merck, cat. no. G1149)
NMR buffer (see recipe)
Denaturing RNA loading buffer (see recipe)
Denaturing PAGE gel solution (see recipe)
Denaturing PAGE running buffer (1× TBE; see recipe)
Centrifugal concentrator (e.g., Vivaspin™, Sartorius™)
Centrifuge (Megafuge 8R; Thermo Fisher Scientific)
UV/Vis spectrophotometer (NanoDrop One/One; Thermo Fisher Scientific)
PAGE casting chamber and glass plates (Biometra multigel; Analytik Jena Company)
Heat block

1. Prepare centrifugal concentrator as described in the device manual.

Choose a MW cutoff, which will be about a third of the size of your target RNA. This way the RNA will not pass through the filter.
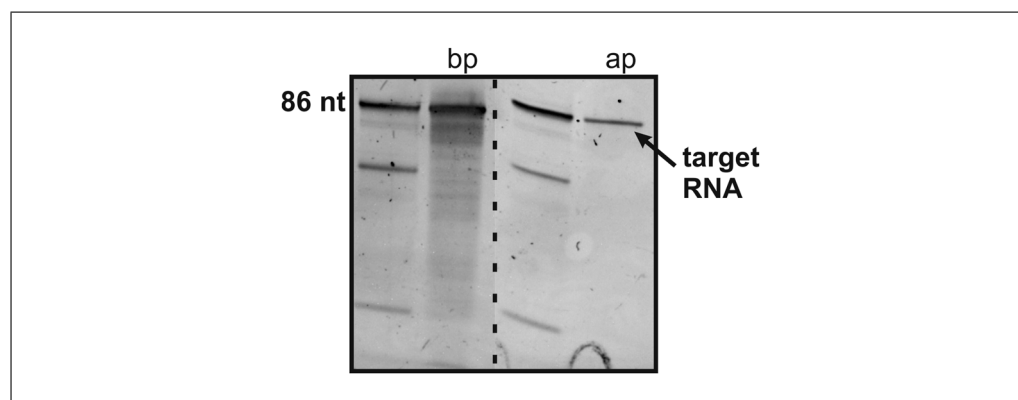

Figure 4 10% denaturing PAGE of the target RNA before purification (bp) and after purification (ap) with a centrifugal concentrator.

2. Remove precipitated magnesium pyrophosphate by centrifuging the transcription mixture at 3,000 × *g* for 5 min at room temperature. Then, transfer supernatant to the centrifugal concentrator.

3. Wash transcription mixture with 5 ml tris/glutamate buffer (pH 8.1) to remove remaining pyrophosphate.

4. Concentrate solution to 1.0-0.3 ml and wash with 60-160 ml NMR buffer depending on the transcription scale.

   Mix sample carefully between the steps with a pipet, without touching the membrane. Stick to the speed limit specified by the manufacturer otherwise the membrane could be damaged. The temperature inside the centrifuge should be in a range of 4°-25°C, depending on type and stability of the target RNA. Higher temperature will increase the flowthrough.

5. Analyze flowthrough and the solution above the membrane (target RNA) by UV/vis absorption spectroscopy and analytical denaturing PAGE to ensure that no RNA has passed through the membrane of the centrifugal concentrator.

6. In the last washing step, concentrate solution to 200-300 μl and remove RNA from the centrifugal concentrator with a pipet.

   The RNA can stick to the membrane and therefore has to be removed vigorously with a pipet. It is recommended that the RNA solution is pipetted along the inside of the membrane and that the membrane is washed with a small additional amount of NMR buffer (∼50 μl) after removal.

7. Determine RNA concentration via UV/vis absorption spectroscopy.

   Take into account $MgCl_2$, D_2O, and DSS which will be added afterwards and will dilute the sample.

8. Proceed with folding and NMR sample preparation as described in Basic Protocol 1, steps 30-46.

SUPPORT PROTOCOL 1

PREPARATION OF DNA TEMPLATE FROM PLASMID

Every DNA-dependent RNA polymerase used in an in vitro transcription requires a DNA template from which the sequence can be read and an RNA strand can be synthesized. A commonly used template is a linearized DNA plasmid encoding the RNA transcription cassette. With the linearization directly downstream of the transcription cassette no transcription terminator is required but instead the polymerase will create run-off transcripts (Diaz, Rong, McAllister, & Durbin, 1996). In this Support Protocol 1, we provide step-by-step instructions on how to prepare a plasmid DNA template.

Materials

Double distilled water (ddH$_2$O)
Competent DH5α *Escherichia coli* cells (e.g., New England Biolabs)
SOC medium (supplied with competent cells for plasmid transformation)
LB-agar plates with 100 μg/ml ampicillin
LB medium (see recipe)
100 mg/ml ampicillin
50% glycerol (for glycerol stock; see step 6)
Plasmid DNA purification kit (e.g., Macherey Nagel)
Restriction enzyme (e.g., New England BioLabs)
10× CutSmart® reaction buffer (New England Biolabs, cat. no. B7204S; supplied with enzyme)
Agarose gel solution (see recipe)

DNA loading buffer (e.g., Gel Loading Dye: Purple, New England Biolabs or see recipe)

DNA running buffer (1× TAE; see recipe)

Phenol-chloroform-isoamyl alcohol (PCI) solution (e.g., ROTI®, cat. no. A156.1)

Chloroform (Thermo Fisher Scientific, cat. no. 10293850)

3 M sodium acetate (NaOAc), pH 5.5

Absolute ethanol (Merck, cat. no. 32205-2.5L-M)

2-Propanol (VWR Chemicals, cat. no. 20842.330)

Shaking incubator

UV/Vis spectrophotometer (NanoDrop One/One; Thermo Fisher Scientific)

Centrifuge (Megafuge 8R; Thermo Fisher Scientific)

Agarose gel casting chamber and glass plates

Freezer (−20°C/−80°C)

Transformation and amplification

This protocol assumes the previous successful insertion of the RNA transcription cassette into a suitable bacterial plasmid. Any plasmid with a (class III) T7 promoter sequence and ampicillin resistance should be suitable.

1. Perform plasmid transformation into competent DH5α *E. coli* cells according to manufacturer instructions.

2. Prepare 5 ml, 50 ml, and 1 L LB medium with 100 µg/ml ampicillin in appropriate flasks.

   Add ampicillin directly prior to use.

3. Pick an isolated clone from the LB-agar plate (e.g., with a sterile pipet tip) and transfer into the 5-ml culture flask.

   You can also inoculate with a previously prepared glycerol stock. For that, either briefly thaw the glycerol stock and add 20-50 µl to your 5-ml culture or transfer part of the frozen glycerol stock directly using, e.g., a sterile pipet tip.

4. Grow cells ∼4-6 hr at 37°C and 120 rpm before transferring the 5-ml starter culture into 50 ml starter culture. Incubate the 50-ml culture at 37°C and 120 rpm overnight.

5. Measure OD_{600} of the starter culture and transfer the appropriate volume into the main culture to adjust the initial OD_{600} to ∼0.1.

6. Incubate the main culture another 6-10 hr at 37°C and 120 rpm.

   For long-term storage and fast inoculation, we recommend preparing a glycerol stock. Therefore, during the exponential phase of cell growth, take 0.5 ml cell culture and mix it thoroughly with 0.5 ml 50% glycerol. Freeze the cell suspension in liquid nitrogen and store at −80°C. Use the glycerol stock for future inoculations.

7. Centrifuge culture medium at 4,000 × *g* for 15 min and 4°C to harvest cells.

8. Isolate plasmid DNA according to the instructions of a plasmid DNA purification kit.

Linearization

9. Perform a restriction digestion on an analytical scale of 50 µl. For this, prepare a reaction mix according to the protocol in Table 4.

   While the total volume of enzyme needed depends on the concentration set by the manufacturer, make sure that the enzyme volume does not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol. If necessary, increase the volume of the reaction mix.

Table 4 Pipetting Scheme for the Linearization of Plasmid DNA

Reagent	Quantity
Restriction enzyme	10 units
10× Cutsmart reaction buffer	5 µl (1×)
DNA plasmid	1 µg
ddH$_2$O	Add up to 50 µl

10. Mix all reaction components with the enzyme being the last component to be added.

11. Incubate digestion reaction 1 hr at the temperature optimal for the restriction enzyme.

    Check enzyme specifications; usually it is 25°C or 37°C.

12. Verify linearization efficiency with a gel electrophoresis using a 1% agarose gel. Apply ~150 ng of DNA per sample.

13. After successful test linearization, prepare a preparative scale digestion. Calculate the amount of enzyme required to digest the total amount of plasmid (e.g., 1 U/µg DNA). Consult the manufacturer specifications and protocols of your individual enzyme if necessary.

    The amount of enzyme used for a preparative digestion can be significantly reduced when the incubation time is extended. For an incubation time of 8 or 16 hr, only 0.25 or 0.13 U per µg DNA are required, respectively. Check the manufacturer information of your restriction enzyme to see whether it is suitable for an extended digestion.

    The enzyme volume should not exceed 10% of the total reaction volume to prevent star activity due to excess glycerol.

Phenol-chloroform-isoamyl alcohol extraction

14. Add one volume of PCI solution to the digestion reaction solution and mix vigorously before centrifuging for 10 min at 9,000 × g at 4°C.

15. Carefully transfer aqueous phase (top phase) into a new tube and repeat extraction again with one volume of fresh PCI. Transfer aqueous phase again into a new tube.

16. Add one volume of chloroform to the aqueous phase. Mix thoroughly and centrifuge for 10 min at 9,000 × g. Carefully transfer aqueous phase (top phase) into a new tube.

    In this chloroform step, residual traces of phenol are removed.

17. Add one-tenth volume of 3 M NaOAc to the aqueous phase, mix, and precipitate with 0.7 volumes of 2-propanol or 2.5 volumes of absolute ethanol. Incubate at −20°C for 1 hr.

18. Centrifuge 30 min at 9,000 × g and 4°C, carefully decant supernatant, and wash pellet with 5 ml 70% ethanol, before centrifuging again for 15 min at 4°C and 9.000 × g.

19. Decant supernatant and air dry pellet 5-10 min. Resolve DNA in ~500-2,000 µl ddH$_2$O.

20. Determine concentration via UV/vis absorption at 260 nm and perform an agarose gel electrophoresis with linearized plasmid and undigested plasmid as a control.

    See Figure 5 for reference. The linearized plasmid should run differently (usually higher) in the agarose gel than the supercoiled plasmid.
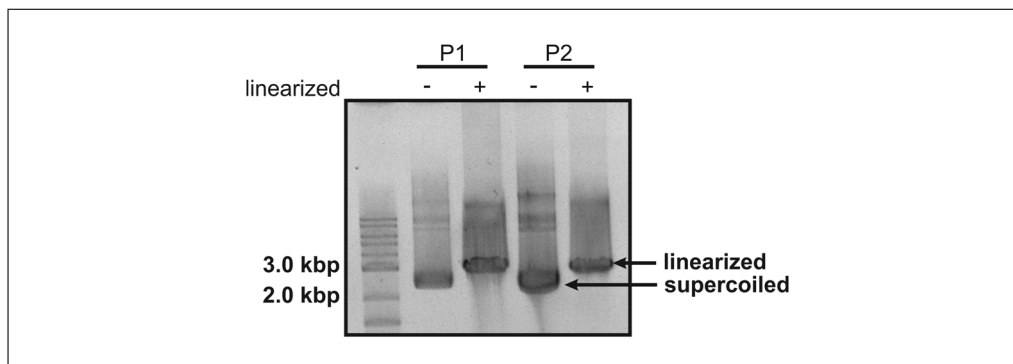
Figure 5 1% agarose gel of the linearization of two plasmids P1 (2,929 bp) and P2 (2,872 bp). For both plasmids the typical band shift from supercoiled to linear conformation can be observed. Note that only linearized DNA accurately matches the band height of a reference ladder.

PREPARATION OF PCR DNA AS TEMPLATE

PCR is a fast and convenient method for the amplification of DNAs (Mullis et al., 1986). The template DNA for this method is usually purchased or synthesized in house by solid phase synthesis. The correct design of this template DNA is described in Strategic Planning. If 3′ homogeneity is required, using 2′-*O*-methyl modified reverse primers is an alternative solution to the application of ribozymes. The methoxy group at the 5′ end of the PCR template DNA stops the transcription because it destabilizes the complex between the polymerase and the template, preventing non-templated RNA synthesis (Kao et al., 1999).

For a maximum amount of pure PCR product, reaction conditions such as the primer, template, and $MgCl_2$ concentrations have to be optimized. Furthermore, the annealing temperature has to be optimized in order to obtain a high yield of product DNA. All optimizations and the procedure for a successful PCR are described within this support protocol.

Materials

Double distilled water (ddH$_2$O)
PCR template DNA (Eurofins Genomics)
Forward DNA primer (Eurofins Genomics)
Reverse DNA primer (Eurofins Genomics)
Phusion® High-Fidelity DNA Polymerase (New England Biolabs, cat. no. M0530)
Phusion high-fidelity (HF)/GC buffer (New England Biolabs, cat. no. B0518S/B0519S)
dNTPs (New England Biolabs, cat. no. N0447L)
Native PAGE gel solution for DNA (see recipe)
Agarose gel solution (see recipe)
DNA loading buffer (e.g., Gel Loading Dye: Purple, New England Biolabs or see recipe)
DNA running buffer (1× TAE; see recipe)
Dimethyl sulfoxide (DMSO)

Thermocycler (Biometra Tone; Analytik Jena Company)
Agarose gel casting chamber and glass plates
PAGE casting chamber and glass plates (Biometra multigel; Analytik Jena Company)
Freezer (−20°C/−80°C)

1. Design PCR template DNA (see Strategic Planning) and the primers. Purchase constructs or prepare them via solid phase synthesis (e.g., Eurofins Genomics).
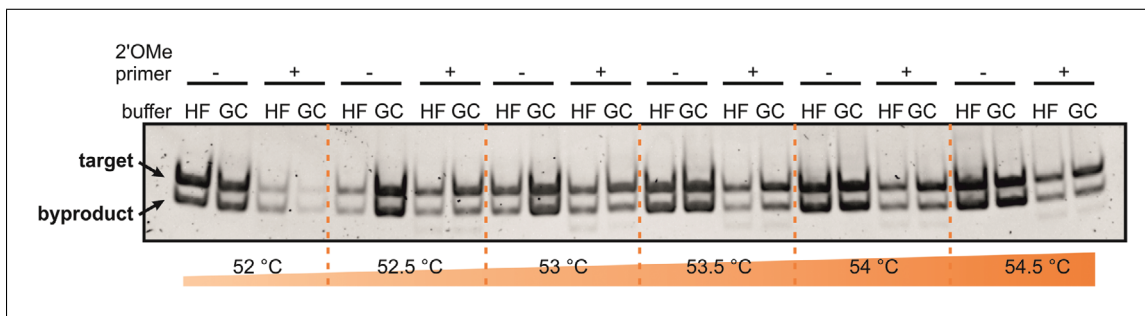
Figure 6 6% native PAGE for DNA of PCR annealing temperature, primer, and buffer optimization. Methoxy primer yields less product. At higher annealing temperatures the amount of product increases. The effect of GC buffer instead of HF buffer is negligible.

Table 5 PCR Program for the Amplification of a Synthetic DNA Construct

Step	Temperature	Time
1. Denaturing	98°C	2 min
2. Annealing	50°-60°C	20 s
3. Elongation	72°C	15 s
4. Denaturing	98°C	10 s
5. Go to step 2.		Repeat 20-30 times

The primers need to have a similar melting temperature. Furthermore, they should have a length of 10-20 nt. Keep in mind that the primer sequence could potentially appear repeatedly in the target DNA sequence leading to mispriming and unwanted byproducts.

2. Reconstitute lyophilized PCR template DNA with ddH$_2$O to obtain a concentration of 100 µM.

   Prepare a diluted DNA stock solution with a concentration of 1 µM, to avoid repeated thaw and freeze cycles of the original stock solution for each PCR experiment.

3. Reconstitute lyophilized primers with ddH$_2$O to obtain a concentration of 100 µM.

4. Check the following optimization steps on a native PAGE for DNA or an agarose gel.

   A native PAGE for DNA will reveal sharper bands than an agarose gel. However, for larger DNA constructs (>1 kbp) an agarose gel is more suitable, as the DNA migrates faster through the gel matrix.

5. Optimize annealing temperature and the number of cycles (Fig. 6). Optimize elongation time according to the template length. An exemplary program is shown in Table 5.

   3°C above the primer melting temperature is a good choice for an initial annealing temperature. Optimize in a range of ±10°C around the primer melting temperatures. For the number of cycles, use up to 30 cycles.

6. Optimize primer concentrations to maximize the yield of target DNA (Fig. 7). Try 0.5-1.0 µM for each primer.

7. Optimize PCR template DNA concentration to maximize the yield of target DNA and to avoid additional byproduct (see Troubleshooting). Try 1-3 ng/µl template.

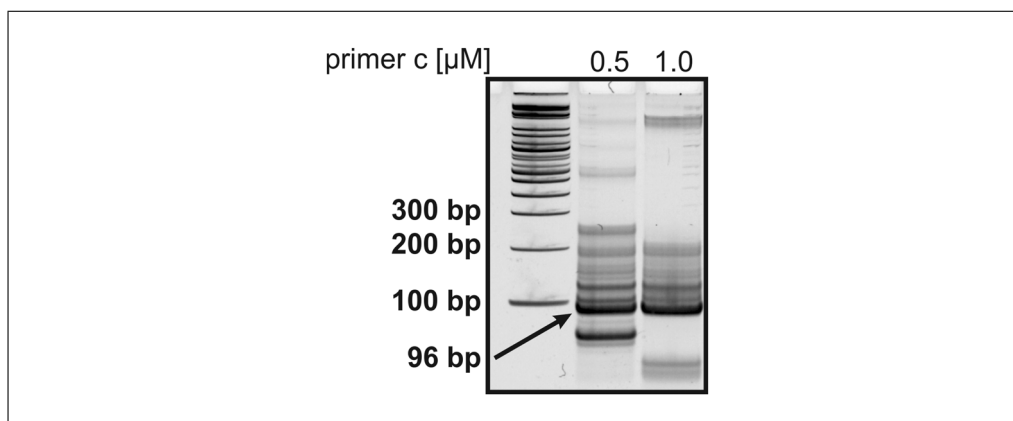8. Optimize DMSO concentration in case of byproducts to facilitate the binding of the primers. Use up to 3% DMSO.

Figure 7 10% native PAGE for DNA of the PCR primer optimization. The sample with 1.0 µM primer concentration yields less byproducts. Therefore, this condition is to be chosen.

Table 6 Pipetting Scheme for a PCR

Reagent	Quantity
ddH$_2$O	To target volume
HF/GC buffer	1×
PCR template DNA	1-3 ng/µl
Forward primer	0.5-1.0 µM
Reverse primer	0.5-1.0 µM
dNTP mix	200 µM
DMSO (optional)	Up to 3%
Phusion DNAP	1 U/50 µl PCR

If using DMSO, reduce the annealing temperature (∼1°-2°C) as DMSO decreases the oligonucleotide melting temperature. An additional annealing temperature optimization with DMSO can be conducted.

9. Determine amount of DNA required for the preparative transcription reaction.

10. Set a master mix with all reagents in a 2-ml reaction tube as shown in the pipetting scheme (Table 6) and distribute to PCR reaction tubes. Each tube should contain 50 µl reaction mixture.

11. Perform a PCR with the optimized conditions.

12. Combine samples from the individual PCR reaction tubes and run an analytical agarose gel electrophoresis or a native PAGE for DNA to confirm a successful reaction.

13. Use PCR product directly for transcription without any purification steps in case of single pure product.

   If byproducts are formed but are not transcribed, the PCR product can be used for further transcription without purification. Store at −20°C if not used directly.

PREPARATION OF T7 RNA POLYMERASE (T7 RNAP)

Although T7 RNAP is commercially available, purchasing the amount required for an in vitro RNA transcription in NMR scale can be quite expensive. This support protocol provides a cost-saving and straightforward alternative to the commercial product. Furthermore, we recommend using the P266L mutant of T7 RNAP since it significantly

improves in vitro transcription, particularly from templates carrying unfavorable initial sequences (Guillerez et al., 2005).

Materials

BL21 DE3 *E. coli* cells carrying pBH161 plasmid (plasmid was a gift from M. Dreyfus, CNRS, Paris, France; cells supplied by New England Biolabs)
LB or TB medium (see recipe)
100 mg/ml ampicillin
Antifoam Y-30 emulsion (e.g., MilliporeSigma)
1 M IPTG
T7 RNAP buffer A (see recipe)
T7 RNAP buffer B (see recipe)
SDS stacking and resolving gel (see recipe)
SDS running buffer (see recipe)
SDS sample buffer (see recipe)
Coomassie staining solution (see recipe)
T7 RNAP buffer C (see recipe)
Glycerol (Carl Roth, cat. no. 3783.1)

Shaking incubator
UV/Vis spectrophotometer (NanoDrop One/One; Thermo Fisher Scientific)
Centrifuge (Megafuge 8R; Thermo Fisher Scientific)
High-pressure homogenization (e.g., Microfluidizer® M-110 P; Microfluidics)
FPLC system
5-ml Ni-NTA affinity column (e.g., GE HisTrap HP 5 ml column)
SDS casting chamber and glass plates (XCell SureLock Mini-Cell Electrophoresis System)
Preparative size exclusion column (e.g., GE HiLoad 26/600 Superdex 200 pg gel filtration column)
Freezer ($-20°$C/$-80°$C)

Expression and cell harvest

1. Prepare 50 ml starter culture and 1 L main culture containing LB or TB medium with 100 μg/ml ampicillin.

   Although LB medium is more commonly used in bacterial cell cultures, TB medium can yield an up to three times higher cell density, which results in an increased amount of expressed protein. Based on previous expressions, 1 L TB medium yields between 5-10 mg of purified protein.

   Add ampicillin directly prior to use.

2. Inoculate starter culture with a glycerol stock of BL21 DE3 *E. coli* cells containing the pBH161 plasmid and incubate at 37°C and 120 rpm overnight.

3. Measure OD_{600} of the starter culture and transfer the appropriate volume into the main culture to adjust initial OD_{600} to $\sim$0.1.

   If you are planning to add antifoam emulsion to the main culture to prevent foam formation, consider that antifoam will increase the optical density of the culture medium and should be added to the main culture prior to any blank measurements.

4. Incubate main culture at 37°C and 120 rpm until OD_{600} of 0.6-0.8 for LB medium or 1-2 for TB medium is reached.

   Due to the higher maximum cell density in TB medium, the ideal induction point within the exponential growth is at a higher OD_{600} value. OD_{600} of 0.6-0.8 is usually reached within 3-4 hr; OD_{600} of 1-2 within 4-5 hr.

5. Add IPTG to a final concentration of 0.5 mM to induce protein expression.

6. Grow cells at 37°C until OD_{600} of 10-15 is reached. This usually takes 3 hr more.

7. Centrifuge culture medium at 4,000 × g and 4°C for 15 min to harvest cell pellet.

Purification

Perform all purification steps at 4°C.

8. Resuspend cell pellet in T7 RNAP buffer A (∼25 ml lysate per 1 L cell culture).

9. Lyse cell suspension via high-pressure homogenization.

   Set the system pressure to 15,000 psi. After washing the homogenizer with 300-400 ml water followed by 200 ml T7 RNAP buffer A, the lysate is cycled through the system five to six times while constantly keeping the coil beaker ice cooled. Be aware that those volumes apply to the Microfluidizer® and may change with the different models or homogenization methods. For more details, refer to the manufacturer's protocols.

10. Centrifuge lysate at >35,000 × g for 30 min at 4°C.

11. Decant supernatant and filter with 0.8-μm pore size if precipitates are visible.

12. Equilibrate a 5-ml Ni-NTA column with ten column volumes (CV) T7 RNAP buffer A.

    For flow rates and pressure limits, use settings in accordance with the column specifications.

13. Load protein solution onto the column.

14. Wash column with ten CV T7 RNAP buffer A.

15. Wash column with ten CV 5% T7 RNAP buffer B.

16. Wash column with ten CV 10% T7 RNAP buffer B.

17. Apply a gradient of 100% T7 RNAP buffer B over 100 ml and collect eluate in fractions of 5 ml (see Fig. 8A for reference).

18. Run a 12% SDS-PAGE of all fractions and pool fractions that contain T7 RNAP (see Fig. 9A for reference).

19. Equilibrate a gel filtration column with 1.5 CV 2.5× T7 RNAP buffer C.

20. Load T7 RNAP onto the size exclusion column.

    The maximum volume to be loaded depends on the column size and specifications. Check the manufacturer's recommendations to ensure optimal separation. If necessary, reduce the volume of the enzyme solution via centrifugal concentration.

21. Elute with one CV of 2.5× T7 RNAP buffer C and collect eluate in fractions of 5 ml (see Fig. 8B for reference).

22. Run a 12% SDS-PAGE of all fractions and pool fractions that contain T7 RNAP (see Fig. 9B for reference).

23. Check selected fractions for RNase contamination by incubating an analytical amount of each fraction with ∼15 pg of RNA of choice overnight at room temperature or for 4 hr at 37°C. Also include a control sample without protein. Check on a denaturing PAGE if the RNA is still intact. The RNA band should be distinct and sharp. A smeared RNA band or fragmentation into smaller RNAs is an
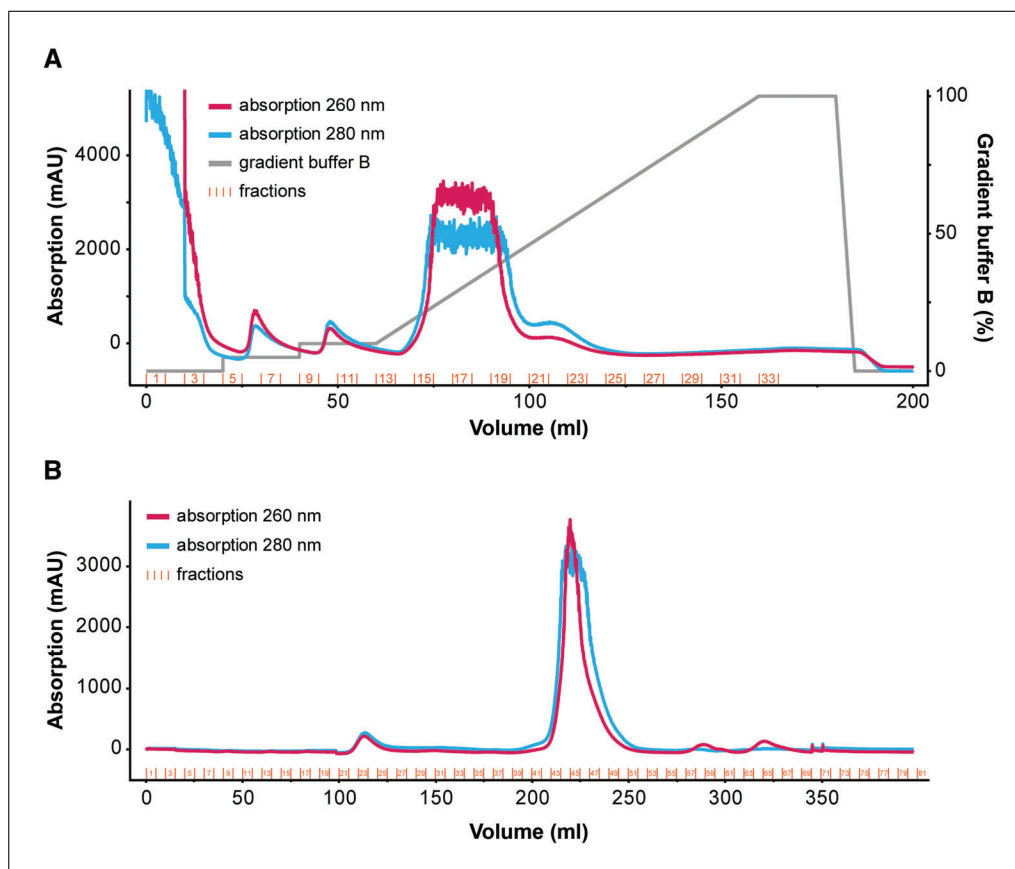
Figure 8 Chromatograms for the purification of the T7 RNAP by (**A**) Ni-NTA chromatography and (**B**) size exclusion chromatography. The absorptions at 260 nm (pink) and 280 nm (blue) as well as the gradient (gray) are given. The fractions were collected in 5 ml volumes (red).
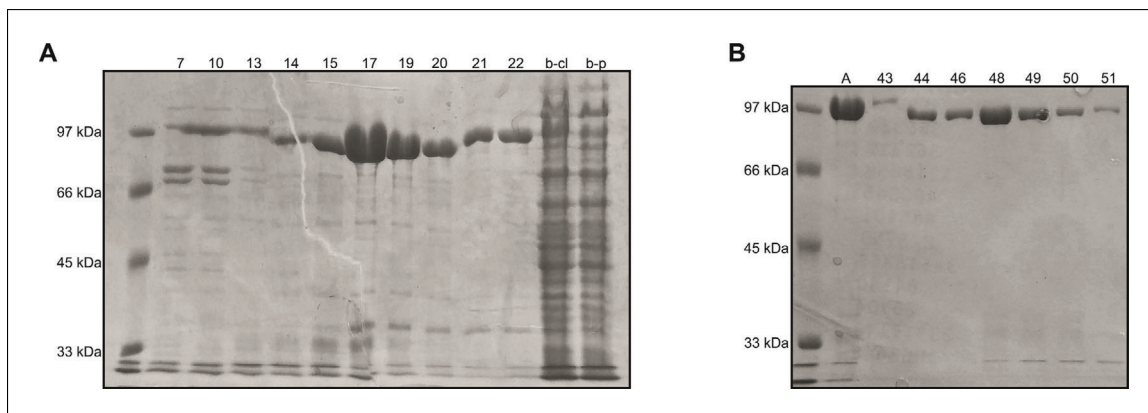

Figure 9 12% Coomassie-stained SDS-PAGE with fractions of (**A**) Ni-NTA column purification and (**B**) size exclusion purification. Fractions potentially containing T7 RNAP were analyzed. Using a low molecular weight marker (left-most lane) the T7 RNAP can be identified as the upper band close to the 97 kDa marker fragment. 3 µl of protein solution were applied, respectively. Samples b-cl and b-p were taken before cell lysis (b-cl) and before purification (b-p), respectively. Based on this PAGE, fractions 15-20 were pooled. (**B**) Sample A was taken before size exclusion purification. Based on this PAGE, fractions 44-49 were pooled.

indication of RNase digestion. Pool protein that does not show any signs of RNase activity.

24. Adjust protein concentration to 6 mg/ml with $2.5\times$ T7 RNAP buffer C.

25. Add glycerol to a final concentration of 60% (final protein concentration: 2.4 mg/ml; $1\times$ T7 RNAP buffer C), aliquot, and store the enzyme at –80°C.

PREPARATION OF YEAST INORGANIC PYROPHOSPHATASE (YIPP)

The RNA yield of a transcription is strongly dependent on the Mg^{2+} ion concentration in the reaction mixture. The formation of pyrophosphate leads to a precipitation of magnesium pyrophosphate concomitantly decreasing the concentration of Mg^{2+} ions in the solution. Because the folding of ribozymes and the activity of the T7 RNAP is dependent on Mg^{2+} ions, their concentration should be kept stable during the transcription (Karlsson, Baronti, & Petzold, 2020). To achieve this stability, 9.6 µg/ml YIPP can be used to decompose pyrophosphate to monophosphate, thereby preventing the removal of magnesium by precipitation (Kunitz, 1952).

Materials

pET-21a(+)-IPP1-His plasmid (plasmid was a gift from Sebastian Maerkl; Addgene, plasmid #118978, see Lavickova & Maerkl, 2019)
LB medium (see recipe)
100 mg/ml ampicillin
BL21(DE3) Competent *E. coli* cells (New England Biolabs)
1 M IPTG
YIPP buffer A (see recipe)
YIPP buffer B (see recipe)
SDS stacking and resolving gel (see recipe)
SDS running buffer (see recipe)
SDS sample buffer (see recipe)
Coomassie staining solution (see recipe)
YIPP buffer C (see recipe)
YIPP buffer D (see recipe)
Glycerol (Carl Roth, cat. no. 3783.1)

Shaking incubator
UV/Vis spectrophotometer (NanoDrop One/One; Thermo Fisher Scientific)
Centrifuge (Megafuge 8R; Thermo Fisher Scientific)
High-pressure homogenization (e.g; Microfluidizer® M-110 P; Microfluidics)
FPLC system
5-ml Ni-NTA affinity column (e.g., GE HisTrap HP 5 ml column)
SDS casting chamber and glass plates (XCell SureLock Mini-Cell Electrophoresis System)
Preparative size exclusion column (e.g., GE HiLoad 26/600 Superdex 75 pg gel filtration column)
Freezer (−20°C/−80°C)

Expression and cell harvest

Based on previous expressions, 1 L LB medium yields between 35-40 mg of purified protein.

1. Prepare 50 ml starter culture and 1 L main culture containing 2× LB medium and 100 µg/ml ampicillin.

    Add ampicillin directly prior to use.

2. Inoculate starter culture with a glycerol stock of BL21 DE3 *E. coli* cells containing pET-21a(+)-IPP1-His plasmid and incubate at 37°C and 160 rpm overnight.

3. Measure OD_{600} of the starter culture and transfer the appropriate volume into the main culture to adjust initial OD_{600} to ∼0.1.

4. Incubate main culture at 37°C and 130 rpm until OD_{600} of 0.6-0.8 is reached.

OD$_{600}$ of 0.6-0.8 is usually reached within 3-4 hr.

5. Add a final concentration of 1 mM IPTG to induce protein expression.

6. Grow cells for another 5 hr at 37°C.

7. Centrifuge culture medium at 4,000 × g for 30 min and 4°C to harvest cell pellet.

Purification

To maintain the thermostability of the enzyme, it is recommended that all purification steps are performed at 4°C.

8. Resuspend cell pellet in YIPP buffer A (∼25 ml lysate per 1 L cell culture).

9. Lyse cell suspension via high-pressure homogenization.

   Set the system pressure to 15,000 psi. After washing the homogenizer with 300-400 ml water followed by 200 ml YIPP buffer A, the lysate is cycled through the system five to six times while constantly keeping the coil beaker ice cooled. Be aware that those volumes apply to the Microfluidizer® and may change with the different models or homogenization methods. For more details, refer to the manufacturer's protocols.

   Centrifuge lysate at >35,000 × g for 30 min.

10. Decant supernatant and filter with 0.8-μm pore size if precipitates are visible.

11. Equilibrate a 5-ml Ni-NTA column with ten CV of YIPP buffer A.

12. Load protein solution onto the column.

13. Wash column with ten CV of YIPP buffer A.

14. Elute with a gradient of YIPP buffer B (0%–100% over 50 min) and collect eluate in fractions of 5 ml (see Support Protocol 3, Fig. 8A for reference).

15. Run a 15% SDS-PAGE of all fractions and pool fractions that contain YIPP (33.2 kDa; see Support Protocol 3, Fig. 9A for reference).

    The following size exclusion step is not compulsory but recommended.

16. Equilibrate size exclusion column with 1.5 CV of YIPP buffer C.

17. Load YIPP solution onto the size exclusion column.

    The maximum volume to be loaded depends on the column's size and specifications. Check the manufacturer's recommendations to ensure optimal separation. If necessary, reduce the volume of the enzyme solution via centrifugal concentration.

18. Elute with one CV of YIPP buffer C and collect eluate in fractions of 5 ml (see Support Protocol 3, Fig. 8B for reference).

19. Run a 15% SDS-PAGE of all fractions and pool fractions that contain YIPP (see Support Protocol 3, Fig. 9B for reference).

20. Check YIPP-containing fractions for RNase contamination by incubating an analytical amount of each fraction with ∼15 pg of RNA of choice overnight at room temperature or for 4 hr at 37°C. Also include a control sample without protein. Check on a denaturing PAGE if the RNA is still intact. The RNA band should be distinct and sharp. A smeared RNA band or fragmentation into smaller RNAs is an indication of RNase digestion. Pool protein that does not show any signs of RNase activity.

21. Dialyze combined fractions twice (1 hr each) against 1 L of 2× YIPP buffer D.

22. Adjust concentration to ∼2.4 mg/ml using centrifugal concentration.

23. Add 50% (v/v) glycerol to reach a final stock concentration of 1.2 mg/ml.

24. Aliquot and store the enzyme at –20°C.

PREPARATION OF SITE-SPECIFIC LABELED RNAS USING A CHEMO-ENZYMATIC SYNTHESIS

The NMR spectroscopic characterization of increasingly long RNAs is hampered by an increased resonance overlap. This problem can potentially be avoided by site-specific labeling methods. However, the most commonly used method for these purposes, solid phase synthesis, is limited to ∼50 nt (Jud & Micura, 2017). Herein, we provide a protocol for the chemo-enzymatic synthesis of RNAs that contain a single modified/labeled nucleotide at a desired position without any size limitation. By the site-specific incorporation of ^{13}C, ^{15}N labeled or unnatural modified nucleosides, such as ^{19}F or ^{13}C-methoxy groups, the signal abundance is dramatically reduced and spectra with single signals can be obtained (Keyhani et al., 2018). Using this method, it is even possible to incorporate modified nucleosides carrying sterically demanding modifications such as photoremovable protecting groups (e.g., ortho-nitrophenylethyl) or photoswitches (e.g., azobenzene).

The site-specific incorporation of a modified nucleotide into an RNA involves three enzymatic steps (enzymatic pathway in Fig. 10). The first step is a 3′ extension of the acceptor RNA strand (RNA 1) with the modified nucleoside 3′,5′-bisphosphate (for synthesis see Support Protocol 5) using T4 RNA Ligase 1 (T4 Rnl1). Further extensions are blocked by the phosphate group at the 3′ position of the modified nucleotide. The ligation mixture can be either purified by RP-HPLC or the non-ligated RNA can be oxidized by NaIO$_4$ to remove it from the enzymatic pathway. The phosphate group at the 3′ position is removed by a phosphatase and the RNA is (splint) ligated with the 5′-phosphorylated donor RNA strand (RNA 2) in presence of a DNA splint by T4 RNA Ligase 2 (T4 Rnl2; for enzyme preparation see Support Protocol 6). The RNA is purified, e.g., by RP-HPLC. The enzymes can be purchased commercially, however, we recommend preparing T4 Rnl2 in house due to the significant cost factor and observed higher ligation efficiency as described in Support Protocol 6.

Materials

Double distilled water (ddH$_2$O)
3′,5′-Bisphosphate nucleoside (see Support Protocol 5)
Acceptor RNA (RNA 1) with an OH group at the 3′ end (see Basic Protocol 1)
Donor RNA (RNA 2) with a phosphate group at the 5′ end (Eurofins Genomics or see Basic Protocol 1)
DNA splint (Eurofins Genomics)
T4 Polynucleotide Kinase (T4 PNK; New England Biolabs, cat. no. M0201S)
T4 RNA Ligase 1 (New England Biolabs, cat. no. M0204S)
T4 RNA Ligase 1 buffer (supplied with the T4 RNA Ligase 1)
Phosphatase and respective buffer (e.g., shrimp alkaline phosphatase; e.g New England Biolabs, cat. no. M0371S)
10× CutSmart® reaction buffer (New England Biolabs, cat. no. B7204S; supplied with shrimp alkaline phosphatase)
T4 RNA Ligase 2 (see Support Protocol 6; New England Biolabs, cat. no. M0239S)
T4 DNA Ligase buffer (New England Biolabs, cat. no. B0202S)
DMSO (Carl Roth, cat. no. A994.2)
ATP (MilliporeSigma, cat. no. A6419-1G)
Phenol-chloroform-isoamyl alcohol (PCI) solution (e.g., ROTI®, cat. no. A156.1)
Chloroform (Thermo Fisher Scientific, cat. no. 10293850)
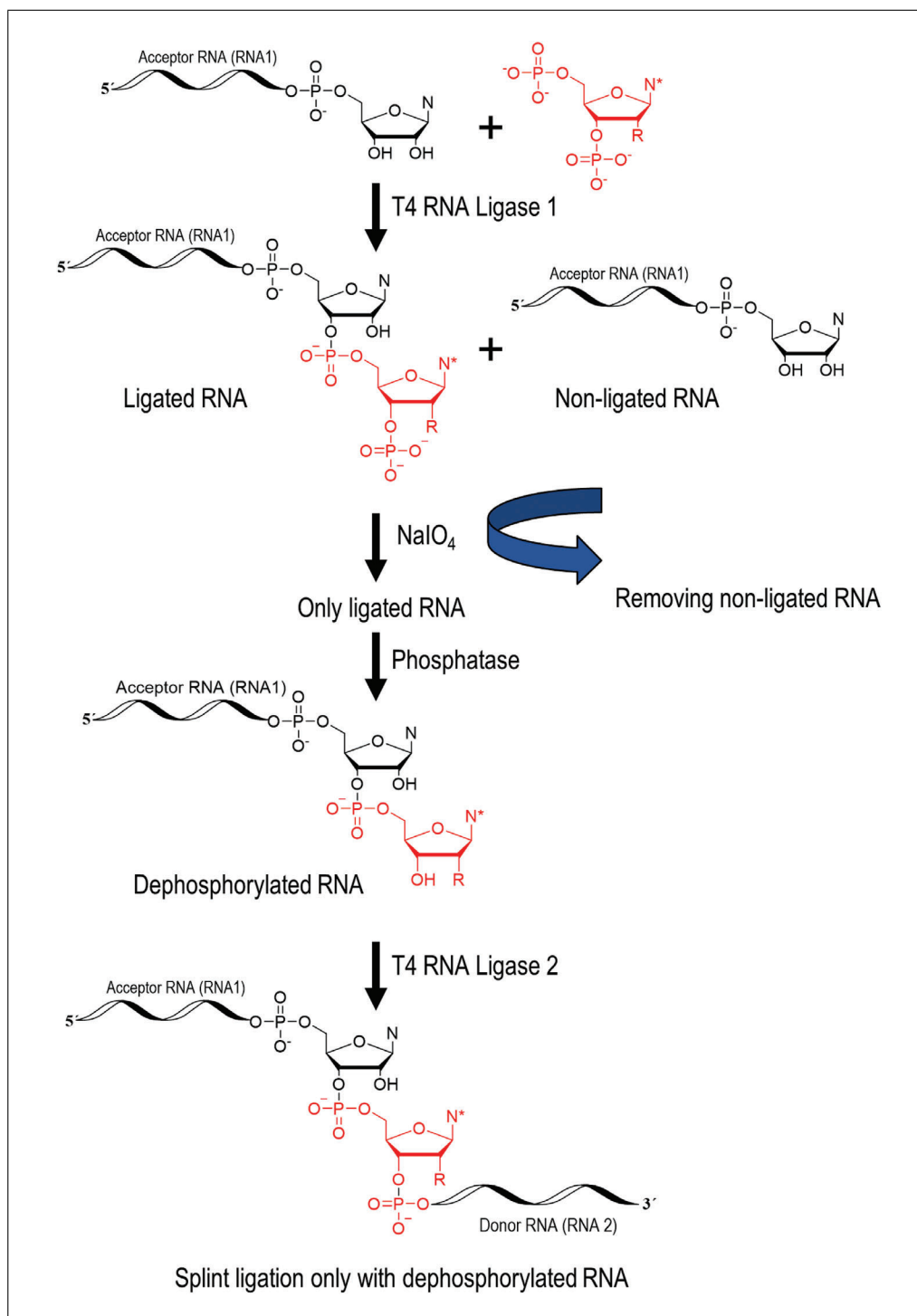Sodium periodate (NaIO$_4$; Carl Roth, cat. no. 2603.1)

Figure 10 Schematic overview of the enzymatic site-specific incorporation of modified nucleotides employing periodate oxidation. Through the oxidation, the non-ligated RNA (after ligation) is removed from the enzymatic pathway. No byproducts remain after the splinted ligation. The acceptor RNA strand (RNA 1) requires an OH group at the 3′ end and the donor RNA strand (RNA 2) requires a phosphate group at the 5′ end for the chemo-enzymatic incorporation.

50% (v/v) ethylene glycol (Grüssing, cat. no. 103081000U)
3 M sodium acetate (NaOAc), pH 5.5 (Carl Roth, cat. no. 6773.2)
Absolute ethanol (Merck, cat. no. 32205-2.5L-M)
Denaturing RNA loading buffer (see recipe)
Denaturing PAGE gel solution (see recipe)
Denaturing PAGE running buffer (1× TBE; see recipe)

DNase and appropriate buffer (e.g., Turbo™ DNase, Thermo Fisher Scientific, cat. no. AM2238)

Hexafluoro-2-propanol (HFIP; Fluorochem, cat. no. 003409)

Methanol (Thermo Fisher Scientific, cat. no. M/4000/17)

Materials for preparative PAGE (see Alternate Protocol 1)

Shaking incubator

Centrifuge (Megafuge 8R; Thermo Fisher Scientific)

Centrifugal concentrator (e.g., Vivaspin™, Sartorius™)

Freezer (−20°C/−80°C)

Lyophilizer/centrifugal vacuum concentrator (Alpha 2-4, Christ; vacuum concentrator plus, Eppendorf)

UV/Vis spectrophotometer (NanoDrop One/One; Thermo Fisher Scientific)

PAGE casting chamber and glass plates (Biometra multigel; Analytik Jena Company)

Heat block

HPLC device (Elite LaChrom; VWR-Hitachi)

HPLC column (e.g., XBridge Peptide BEH C18: 300 Å, 3.5 μm, 4.6 × 250 mm)

1. Prepare a modified nucleoside 3′,5′-bisphosphate as described in Support Protocol 5.

2. Prepare acceptor RNA strand (RNA 1) and donor RNA strand (RNA 2) as described in Basic Protocol 1, steps 1-27 or purchase them commercially.

   The acceptor RNA strand requires an OH group at the 3′ end and the donor RNA strand requires a phosphate group at the 5′ end for the chemo-enzymatic incorporation. There are no length limits for the acceptor or donor RNAs, although in the case of a very short fragment (<10 nt) and a very long fragment (>150 nt), RP-HPLC separation will be challenging.

3. Prepare DNA splint for the last enzymatic step or purchase it commercially.

   The DNA splint should have a length of at least 40 base pairs. Both sides need an overlap of at least 10 nt each. The overlap does not need to be equal on both sides. The melting temperature should exceed 40°C to ensure proper splint annealing.

4. Perform a 5′-phosphorylation of the donor RNA strand (RNA 2), e.g., with T4 polynucleotide kinase (T4 PNK). Follow the manufacturers protocol.

   As an alternative, a short (<30 nt) 5′-phosphorylated RNA 2 strand can be purchased commercially.

5. We recommend purchase of T4 RNA Ligase 1 (T4 Rnl1) and the phosphatase commercially and preparation of T4 RNA Ligase 2 (T4 Rnl2) in house as described in Support Protocol 6.

3′ extension of the acceptor RNA (RNA 1)

6. Prepare ligation mixture that includes all components from Table 7. Incubate ligation mixture at 37°C and 300 rpm overnight. In addition, prepare a negative control without T4 Rnl1.

   The negative control is necessary to track whether the non-ligated RNA is removed from the enzymatic pathway during the oxidation (step 9). Therefore, it will be treated as all the other samples in the following steps and will be referred to as "step 1 negative control" over the course of this protocol. The ligation efficiency depends on the type of the modification. Scaling up the reaction may lead to a decreased ligation efficiency. It is recommended that several batches are prepared in parallel instead of one large batch.

7. Perform PCI extraction to remove T4 Rnl1.

Table 7 Pipetting Scheme for the 3′ Extension of RNA 1

Reagent	Quantity
Acceptor RNA (RNA 1)	50 μM
3′,5′-Bisphosphate nucleoside	200 μM
T4 RNA Ligase buffer	1×
DMSO	20%
T4 RNA Ligase 1	10 units per nmol RNA
ATP	1 mM
ddH$_2$O	Add up to 50-200 μl

Table 8 Pipetting Scheme for Oxidation of Non-ligated RNA

Reagent	Quantity
RNA	30 μM
NaIO$_4$	50 mM
ddH$_2$O	Add up to 150-200 μl

We recommend using a 5 Prime Phase Lock Gel from Quantabio for a better phase separation, especially for volumes <150 μl. For a 5 Prime Phase Lock Gel extraction, follow the manufacturer's manual.

As an alternative, the ligation mixture can be purified by RP-HPLC (see step 31). In this case, the oxidation in step 9 is not required; continue with step 16. Take into account that the length of both RNAs differs in one nucleotide. For a long (>30 nucleotides) acceptor RNA (RNA 1), RP-HPLC purification will not be possible.

8. Add one volume of PCI solution to the reaction solution and mix vigorously. Centrifuge 10 min at 10,000 × *g* and 4°C.

9. Carefully transfer aqueous phase (upper phase) into a new tube and repeat extraction with one volume of fresh PCI solution.

    Two extractions are typically sufficient.

10. Add one volume of chloroform to the aqueous phase (to remove phenol traces) and mix thoroughly. Centrifuge 10 min at 10,000 × *g* and 4°C. Carefully transfer aqueous phase (upper phase) into a fresh reaction tube.

11. Prepare centrifugal concentrator as described in the device manual.

    Choose a MW cutoff which is about a third of the size of your target RNA. This way the RNA will not pass through the membrane.

12. Transfer RNA in the centrifugal concentrator and centrifuge at the recommended speed to remove ligation buffer.

    ATP and DTT in the ligation buffer will prevent the oxidation by NaIO$_4$. Repeated ethanol precipitations (approximately three) could also be sufficient to remove the ligation buffer.

13. Perform oxidation with NaIO$_4$ to remove non-ligated RNA from the enzymatic pathway. Oxidize the "step 1 negative control." The pipetting scheme for the oxidation is demonstrated in Table 8. Incubate reaction 1 hr and 300 rpm at room temperature (23°C).

    NaIO$_4$ is sensitive to light. Therefore, it is recommended that the reaction mixture is protected from light, e.g., with aluminum foil.

Table 9 Pipetting Scheme for Dephosphorylation of Ligated RNA

Reagent	Quantity
Ligated RNA	20-40 μM
CutSmart® buffer	1×
rSAP	1 unit for 500 pmol RNA
ddH$_2$O	Add up to 50-100 μl

14. Quench oxidation reaction by adding 0.5 volumes of 50% (v/v) ethylene glycol. Incubate mixture 5 min at 300 rpm.

15. Add NaOAc solution (pH 5.5) to the aqueous phase to a final concentration of 0.3 M. Add 2.5 volumes of cold absolute ethanol and incubate at least 6 hr at −20°C.

16. Centrifuge at 8,000 × g and 4°C for 40 min. Remove supernatant and keep it for further precipitation.

    Sometimes the RNA does not precipitate quantitatively.

17. Air dry the pellet (15-30 min) or/and use a vacuum concentrator for 2 min.

18. Reconstitute RNA pellet in 50-200 μl ddH$_2$O.

19. Analyze RNA by UV/vis absorption spectroscopy and analytical denaturing PAGE.

    Take into account that the UV/vis absorption may be higher due to leftover ATP and nucleoside 3′,5′-bisphosphate, which may not have been entirely removed in step 3.

Dephosphorylation of the ligated RNA

20. For the dephosphorylation with, e.g., quick calf intestinal alkaline phosphatase (CIP), Antarctic phosphatase (AnP), or shrimp alkaline phosphatase (rSAP), see the respective phosphatase manufacturer's manual. Dephosphorylate "step 1 negative control" as well.

    We recommend the following protocol with the pipetting scheme given in Table 9. Incubate at 37°C for a minimum of 6 hr. The dephosphorylation should lead to a nearly quantitative turn over. Scale up of the reaction may lead to a decrease in ligation efficiency. Therefore, it is advisable to prepare several batches in parallel instead of one large batch.

21. Perform PCI extraction to remove phosphatase as described in steps 3-6.

    Heat deactivation is not recommended, as the high cation concentration can lead to RNA degradation.

22. Perform an ethanol precipitation as described in steps 11-13.

23. Reconstitute RNA pellet in 50-100 μl ddH$_2$O.

24. Analyze RNA by UV/vis absorption spectroscopy and analytical denaturing PAGE.

Splinted ligation of the dephosphorylated RNA with donor RNA (RNA 2)

25. Prepare mixture for the splinted ligation according to Table 10. Splint ligate the "step 1 negative control" as well. Additionally, prepare a negative control without T4 Rnl2.

    Optimization of the ratio of the acceptor, donor RNA, and DNA splint as well as their concentrations in the reaction mixture is recommended. Scale up of the reaction may lead to a decrease in ligation efficiency and thus any scale up has to be tested as well. We recommend preparing several batches in parallel instead of one large batch. Our tests showed ligation efficiency is higher with T4 DNA Ligase buffer than T4 Rnl2 buffer.

Table 10 Pipetting Scheme for Splinted Ligation of Dephosphorylated RNA and Donor RNA (RNA 2)

Reagent	Quantity
Dephosphorylated RNA	1-8 μM
Donor RNA (RNA 2)	1-8 μM
DNA Splint	1-8 μM
T4 DNA ligase buffer	1×
ddH$_2$O	Add up to 50-1,000 μl
Ratio for optimization (Dephosphorylated RNA/Donor RNA/DNA Splint)	1:1:1, 1:2:1, 2:1:2, 1:1:2

Table 11 Gradient for RP-HPLC Purification of Splint Ligated RNA

Time (min)	HFIP buffer (%)	Methanol (%)
0	95	5
2	95	5
30	54.5	45.5
32	0	100

26. Heat sample to 80°C for 4 min.

27. Cool sample to 37°C over 10 min.

28. Incubate 15 min at 37°C and 300 rpm.

29. Add 1% (v/v) in-house produced T4 Rnl2 (2 mg/ml) and incubate reaction for another 2-3 hr at 37°C and 300 rpm.

30. Perform PCI extraction as described in steps 3-6.

31. Perform ethanol precipitation as described in steps 11-13.

32. Reconstitute RNA pellet in 20-200 μl ddH$_2$O.

33. Remove DNA splint with a DNase, e.g., Turbo^{TM} DNase. Follow the manufacturer's manual.

    Very long incubation time (e.g., overnight) may lead to RNA degradation.

34. Analyze RNA by analytical denat. PAGE. Apply an RNA sample with RNA 1 and RNA 2 to the denat. PAGE as a reference.

    The "step 1 negative control" and the negative control without T4 Rnl2 should not show any product bands on the denat. PAGE. In case there is a product band, this is indicative of either an incomplete DNase digestion or a failed oxidation with NaIO$_4$.

35. Purify product RNA by RP-HPLC at 60°C.

    We use an XBridge Peptide BEH C18 (300 Å, 3.5 μm, 4.6 × 250 mm) column from Waters (Figs. 11 and 12). RP-HPLC gradient is described in Table 11.

    As an alternative to RP-HPLC, the product RNA can be purified by preparative PAGE (see Alternate Protocol 1).

36. Remove RP-HPLC buffer via lyophilizer or centrifugal vacuum concentrator.

37. Reconstitute pellet in 20-200 μl ddH$_2$O.

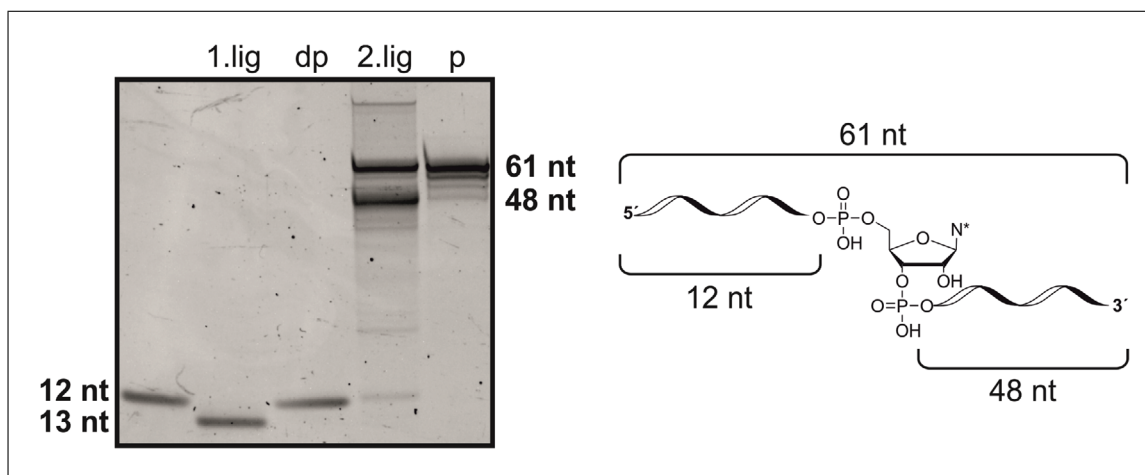38. Analyze fractions by analytical denaturing PAGE.

Figure 11   20% denaturing PAGE shows the first ligation step (1.lig), the dephosphorylation of the RNA (dp), the second splint ligation step (2.lig), and the purified target RNA (p). The 13-mer 1.lig RNA moves faster in the gel electrophoresis because of the additional negative charge of the 3′-end phosphate group.
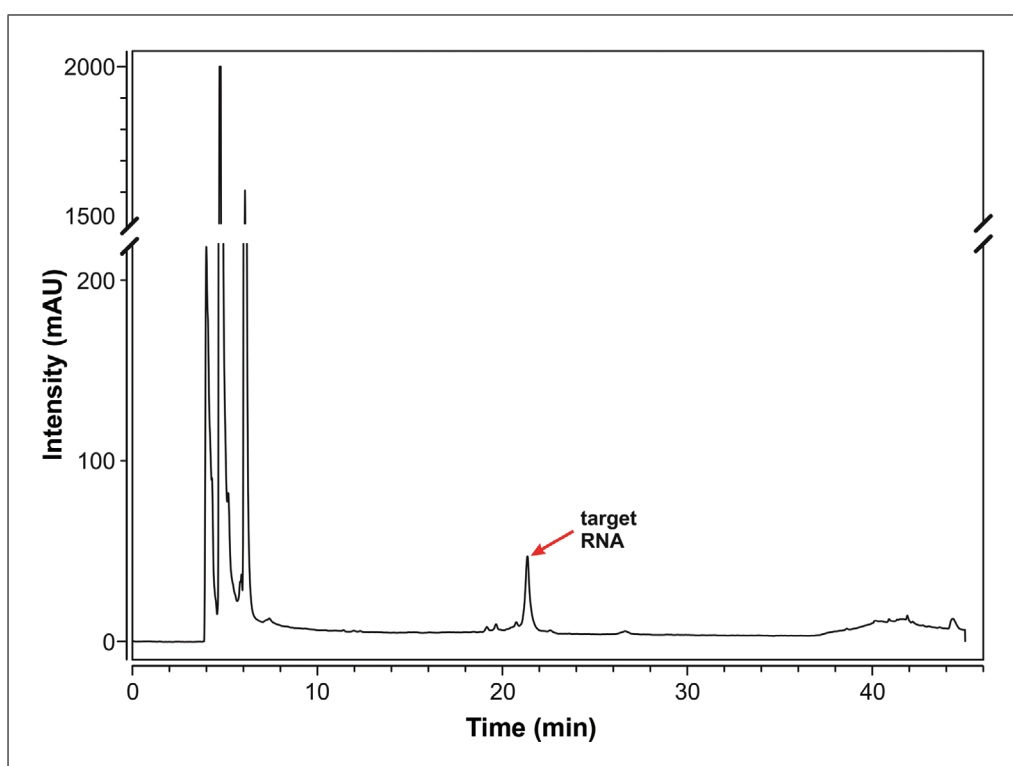

Figure 12   Chromatogram of the preparative RP-HPLC purification of a splint ligated RNA, which was prepared via the chemo-enzymatic synthesis approach.

39. Combine fractions containing clean product.

40. Fold RNA into a single conformation and prepare NMR sample as described in Basic Protocol 1, steps 30-46.

SYNTHESIS OF MODIFIED NUCLEOSIDE 3′,5′-BISPHOSPHATES

The modified nucleosides require a phosphate group at the 3′,5′ positions for site-specific incorporation into the RNA as described in Basic Protocol 2. This support protocol describes the synthesis of a modified nucleoside 3′,5′-bisphosphate (Barrio et al., 1978; Fig. 13).

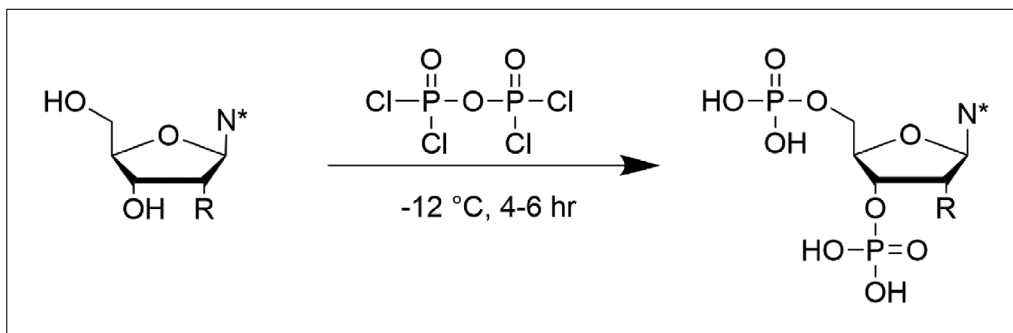*SUPPORT PROTOCOL 5*

Schnieders et al.

Figure 13 Synthesis of modified nucleoside 3′,5′-bisphosphates. N* describes a modified nucleobase (e.g., ^{13}C, ^{15}N labeled nucleobase, photoremovable protecting groups) and R describes modifications at the 2′-position (e.g., fluoro, methoxy group).

Materials

Modified nucleoside (see Basic Protocol 2)
Inert gas (e.g., argon)
0.1% (v/v) diethyl pyrocarbonate (DEPC; Carl Roth, cat. no. K028.2)
Triethylamine (VWR Chemicals, cat. no. 28745.296)
Triethylammonium bicarbonate buffer (TEAB), 1.0 M, pH 8.0
Triethylammonium bicarbonate buffer (TEAB), 0.4 M, pH 8.0
Diphosphoryl chloride (MilliporeSigma, cat. no. 381829-5ML)
Double distilled water (ddH$_2$O)
2-Propanol (VWR Chemicals, cat. no. 20842.330)
25% ammonia solution (NH$_3$; VWR Chemicals, cat. no. 1133.1000)

Schlenk tube
Immersion chiller (FT902; Julabo)
Thin layer chromatography (TLC; ALUGRAM® Xtra SIL G/UV; Macherey-Nagel)
UV lamp, 245 and 365 nm (UVGL-58; Analytik Jena Company)
Lyophilizer/centrifugal vacuum concentrator (Alpha 2-4, Christ; vacuum concentrator plus, Eppendorf)
HPLC device (Elite LaChrom, VWR-Hitachi)
HPLC column (e.g., MZ Aqua Perfect, 100 Å, 5 μm; 4.6 × 250 mm: analytical; 10 × 250 mm: preparative)
UV/Vis spectrophotometer (NanoDrop One/One; Thermo Fisher Scientific)
Freezer (−20°C/−80°C)

1. Perform synthesis under a protective atmosphere for exclusion of water (e.g., argon).

   Diphosphoryl chloride hydrolyzes upon contact with water.

2. Prepare 1 M TEAB buffer. Adjust pH to 8.0 with dry ice.

   Incubate pH electrode in 0.1% (v/v) DEPC solution for 30 min prior to usage to avoid RNase contamination. Wash electrode with ddH$_2$O. Wait for the dry ice to dissolve, then check the pH. Work under a fume hood.

3. Prepare 0.4 M TEAB buffer. Adjust pH to 8.0 with dry ice.

4. Cool modified nucleoside to −12°C in a Schlenk tube.

   You may start with an amount of 100 mg.

5. Add ten equivalents of diphosphoryl chloride and stir 4-6 hr at −12°C.
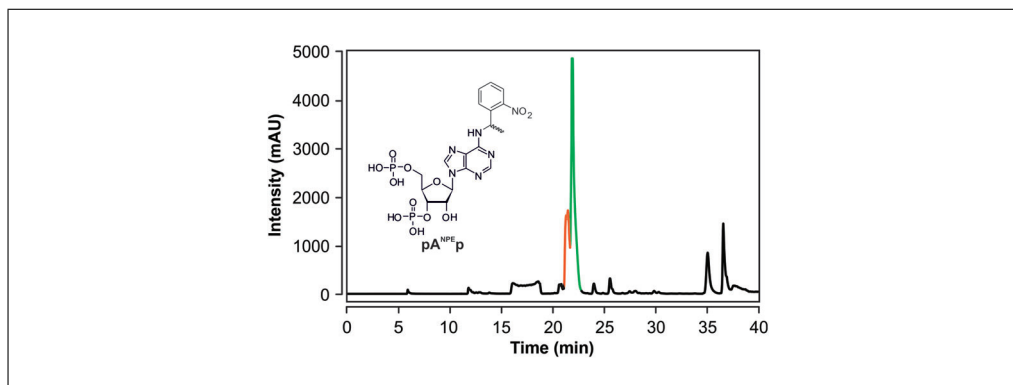
6. Check reaction progress by TLC.

Figure 14 RP-HPLC chromatogram of the purification of ortho-nitrophenylethyl photocaged adenosine 3′,5′-bisphosphate (pA^{NPE}p). The green signal indicates the desired product while the orange signal shows a monophosphorylated species. The picture was adapted from Keyhani et al., 2018.

Table 12 Gradient for RP-HPLC Purification of Modified Nucleoside 3′,5′-Bisphosphate

Time (min)	0.4 M TEAB (%)	Acetonitrile (%)
0	100	0
10	100	0
40	70	30
50	50	50

Use 6:3:1 2-propanol/NH$_3$/H$_2$O as the eluent. The reactant migrates usually faster compared to the product. The product has an R_f value of ~0.1-0.3.

7. After complete conversion of the starting material (3-6 hr), slowly add ice (two to three pieces) to the reaction mixture.

    In this step, the remaining diphosphoryl chloride is hydrolyzed. The ice cools the exothermic reaction, when 1.0 TEAB (pH 8) buffer is added.

8. Adjust pH to 6.5 (use pH paper) with pre-chilled 1.0 M TEAB buffer (pH 8.0).

    Pay attention during the addition of 1.0 M TEAB buffer (pH 8.0) because strong gas formation occurs. In case the reaction mixture is not neutralized after addition of 20 ml of 1 M (pH 8) TEAB, add small volumes (e.g., 0.5 ml) stepwise of a 1:1 water/triethylamine solution.

9. Reduce reaction volume (3-5 ml for a 100-mg scale) with a centrifugal vacuum concentrator at 4°C.

    In case of precipitation, add water to reconstitute precipitate. A smaller reaction volume facilitates the RP-HPLC purification.

10. Purify with RP-HPLC at room temperature.

    We use a MZ Aqua Perfect (100 Å, 5 μm, 4.6 × 250 mm: analytical, 10 × 250 mm: preparative) column (Fig. 14). The gradient for the purification is shown in Table 12.

    Possible byproducts of the synthesis are mono- or triphosphates, which may not be separated by RP-HPLC.

11. Determine fractions containing product by mass spectrometry and pool them.

12. Remove RP-HPLC buffer (triethylamine) via co-evaporation and reconstitute pellet in 10 ml ddH$_2$O.

    You may use a lyophilizer or centrifugal concentrator.

13. Repeat step 12 seven to ten times with an analytical amount of the substance that is analyzed via NMR spectroscopy. Ligation yield is not affected by triethylamine. To remove triethylamine, three to five cycles of co-evaporation are sufficient.

   The removal of triethylamine is necessary for the NMR spectroscopic characterization.

14. Determine concentration with UV/vis absorption spectroscopy.

15. Characterize product by mass spectrometry and NMR spectroscopy (^{1}H-1D, ^{13}C-1D, ^{31}P-1D, ^{1}H,^{1}H-correlation spectroscopy [COSY], ^{1}H,^{13}C-HSQC, ^{1}H,^{31}P-HMBC).

   Byproducts like mono- or triphosphate nucleosides will not be accepted by the T4 RNA Ligase 1. Therefore, a complete separation is not required. The yield depends on the type and position of the modification. The ^{31}P-1D signals are usually at ~0 ppm.

16. Store product at −20°C in the absence of light.

PREPARATION OF T4 RNA LIGASE 2

T4 RNA Ligase 2 (T4 Rnl2; Ho & Shuman, 2002) is used for Basic Protocol 2 and is commercially available. If purchasing, make sure the ligase has 2′,3′cycP esterase activity. However, purchasing the ligase is very costly. Therefore, this straightforward protocol provides an alternative for an in-house preparation of the enzyme. Our tests showed that the in-house produced T4 Rnl2 is even more efficient than the purchased T4 Rnl2 (Fig. 15).

Materials

   LB medium (see recipe)
   100 mg/ml ampicillin
   BL21(DE3) Competent *E. coli* cells (New England BioLabs)
   pET-RNL2 plasmid (plasmid was a gift from S. Shuman, The Sloan-Kettering Institute, New York)
   Antifoam Y-30 emulsion (e.g., MilliporeSigma)
   1 M IPTG
   T4 RNA Ligase 2 (Rnl2) buffer A (see recipe)
   T4 RNA Ligase 2 (Rnl2) buffer B (see recipe)
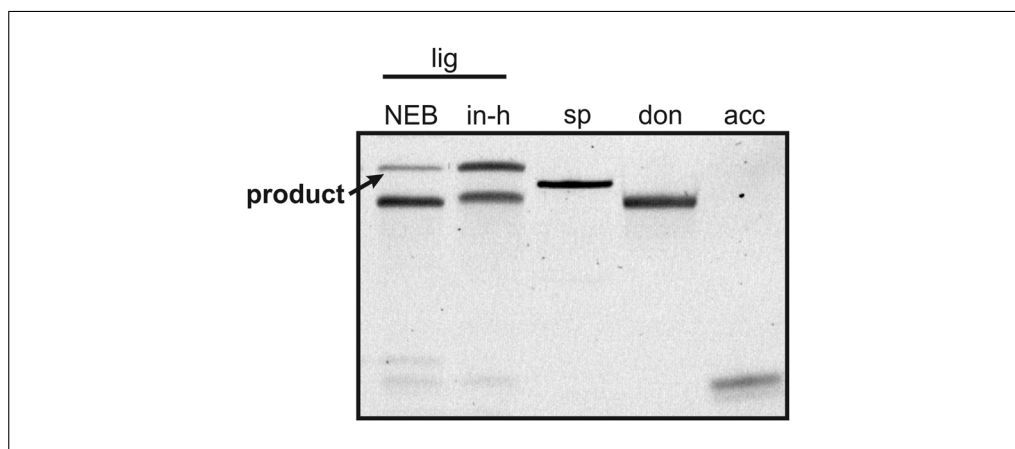   T7 RNAP buffer A (see recipe)

Figure 15 15% denaturing PAGE of two ligation reactions. The first lane shows the result of a splinted ligation reaction performed with a T4 Rnl2 purchased from New England Biolabs (NEB). The second lane shows the result of a splinted ligation reaction performed with an in-house produced T4 RNA Ligase 2 (in-h). The last three lanes show splint DNA (sp), donor RNA (don), and acceptor RNA (acc).

SDS stacking and resolving gel (see recipe)
SDS running buffer (see recipe)
SDS sample buffer (see recipe)
Coomassie staining solution (see recipe)
T4 RNA Ligase 2 (Rnl2) buffer C (see recipe)
Glycerol (Carl Roth, cat. no. 3783.1)

Shaking incubator
UV/Vis spectrophotometer (NanoDrop One/One; Thermo Fisher Scientific)
Centrifuge (Megafuge 8R; Thermo Fisher Scientific)
High-pressure homogenization (e.g., Microfluidizer® M-110 P; Microfluidics)
FPLC system
5-ml Ni-NTA affinity column (e.g., GE HisTrap HP, 5-ml column)
SDS casting chamber and glass plates (XCell SureLock Mini-Cell Electrophoresis
    System)
Preparative size exclusion column (e.g., GE HiLoad 26/600 Superdex 200 pg)
Freezer ($-20°C/-80°C$)

Expression and cell harvest

1. Prepare 200 ml starter culture and 1 L main culture containing LB medium and 100 µg/ml ampicillin.

    Add ampicillin directly prior to use.

2. Inoculate starter culture with a glycerol stock of BL21 DE3 *E. coli* cells containing the pET-RNL2 plasmid and incubate at 37°C and 160 rpm overnight.

3. Measure OD_{600} of the starter culture and transfer the appropriate volume into the main culture to adjust the initial OD_{600} to ∼0.1.

4. Incubate main culture at 37°C and 130 rpm until OD_{600} of 0.8 is reached.

    If you are planning to add antifoam emulsion to the main culture to prevent foam formation, consider that antifoam will increase the optical density of the culture medium and should be added to the main culture prior to any blank measurements.

5. Add IPTG to a final concentration of 1 mM to induce protein expression.

6. Grow cells at 37°C until OD_{600} of 2 is reached. This usually takes 6 hr in total.

7. Centrifuge culture medium at 4,000 × *g* and 4°C for 15 min to harvest cell pellet.

Purification

Perform all purification steps at 4°C.

8. Resuspend cell pellet in T4 RNA Ligase 2 buffer A (∼25 ml lysate per 1 L cell culture).

9. Lyse cell suspension via high-pressure homogenization.

    Set the system pressure to 15,000 psi. After washing the homogenizer with 300-400 ml water followed by 200 ml T7 RNAP buffer A, the lysate is cycled through the system five to six times while constantly keeping the coil beaker ice cooled. Be aware that those volumes apply to the Microfluidizer® and may change with the different models or homogenization methods. For more details, refer to the manufacturer's protocols.

10. Centrifuge lysate at 35,000 × *g* and 4°C for 30 min.

11. Decant and filter supernatant.

12. Equilibrate a 5-ml Ni-NTA column with ten CV of T4 RNA Ligase 2 buffer A.

For flow rates and pressure limits, use settings in accordance with the column specifications.

13. Load protein solution onto the column.

14. Wash column with ten CV of T4 Rnl2 buffer A.

15. Wash column with ten CV of 4% T4 Rnl2 buffer B.

16. Elute with a gradient of T4 Rnl2 buffer B (0% to 100% over 50 min) and collect eluate in fractions of 2.5 ml.

17. Run a 12% SDS-PAGE of all fractions and pool fractions that contain T4 Rnl2 (42 kDa).

18. Equilibrate a size exclusion column with 1.1 CV of T4 Rnl2 buffer C.

19. Load T4 Rnl2 solution onto the size exclusion column.

    The maximum volume to be loaded depends on the column size and specifications. Check the manufacturer's recommendations to ensure optimal separation. If necessary, reduce the volume of the enzyme solution via centrifugal concentration.

20. Elute with 1.1 CV of T4 Rnl2 buffer C and collect eluate in fractions of 5 ml.

21. Run a 12% SDS-PAGE of all fractions and pick fractions that contain T4 Rnl2.

22. Check selected fractions for RNase contamination by incubating an analytical amount of each fraction with ~15 pg of RNA of choice overnight at room temperature or for 4 hr at 37°C. Also include a control sample without protein. Check on a denaturing PAGE if the RNA is still intact. The RNA band should be distinct and sharp. A smeared RNA band or fragmentation into smaller RNAs is an indication of RNase digestion. Pool protein that does not show any signs of RNase activity. Adjust concentration to 4 mg/ml using a centrifugal concentrator.

23. Add 50% glycerol for a final stock concentration of 2.0 mg/ml.

24. Aliquot and store the enzyme T4 Rnl2 at −20°C.

Heteronuclear-detected NMR experiments for RNA

The following protocols describe the implementation of several ^{13}C- and ^{15}N-detected NMR experiments for RNA. These include experiments for chemical shift assignment of ^{1}H and ^{13}C atoms of the ribose ring, the CN-spin filter HSQC experiment for imino and the C(N)H-HDQC as well as the "amino"-NOESY for amino groups. The selection is complemented with a protocol for execution of the ^{15}N-detected BEST-TROSY experiment. For all experiments, Topspin parameter sets are provided, which were generated at 600 MHz and need to be adapted using the command "paracon" at the respective spectrometer. Also note that the provided pulse sequences are not necessarily compatible with the "getprosol" command.

SETUP OF NMR SPECTROMETER FOR HETERONUCLEAR-DETECTED NMR EXPERIMENTS

This support protocol describes the general procedure, which should be conducted in advance every time when the acquisition of heteronuclear-detected NMR experiments is planned. Consequently, it is to be applied before the experiments described in Basic Protocols 3-7 are conducted.

Materials

≥0.5 mM RNA sample (300 µl) of interest (see Basic Protocols 1 and 2; Alternate Protocols 1 and 2)

NMR spectrometer (equipped preferably with a z-axis gradient ^{13}C, ^{15}N [^{1}H]-TXO cryogenic probe; alternatively, a z-axis gradient ^{1}H, [^{13}C, ^{15}N]-TCI cryogenic probe)

1. Set NMR spectrometer to the desired temperature, equip NMR tube with a spinner, place NMR sample in the spectrometer, and let it equilibrate for ~10 min.

   We typically conduct heteronuclear-detected NMR experiments at 298 K.

2. Conduct the following steps in order to set up the NMR spectrometer.

   Note that these steps should be conducted thoroughly as sensitivity is a limiting factor with heteronuclear-detected NMR experiments.

   a. Locking: Lock spectrometer frequency to either 95% H_2O and 5% D_2O or 100% D_2O depending on the solvent of your NMR sample.
   b. Tuning and matching: All channels should be tuned and matched from high to low gyromagnetic ratio for the recommended probes in this protocol (^{1}H, ^{13}C, ^{2}H, ^{15}N). Recheck all channels if the necessary adjustments were large. For optimal sensitivity, tuning and matching should be on point for the channel, where the direct detection takes place (usually ^{13}C).
   c. Shimming: Import a recent shim file, which matches your sample conditions including type of NMR tube, salt concentration, and temperature. Conduct automatic shimming until the quality of the shim is considered good (line width of, e.g., DSS ~1 Hz).
   d. Determine ^{1}H 90° pulses.

   We usually determine the ^{1}H pulse automatically or in a simple one-pulse 1D experiment by conducting a 360° pulse and varying the pulse length until the remaining signal is at the zero crossing. Set the ^{1}H carrier frequency on the water resonance (4.7 ppm).

   e. Determine ^{13}C and ^{15}N 90° pulses and set the carrier frequency on-resonant.

   The determination of the correct 90° pulse length for ^{13}C and ^{15}N is based on SOFAST HMQC (Schanda & Brutscher, 2005). Read in parameter set pulse_calib13C with pulse sequence decp90_sfhmqc.ric (^{13}C) or parameter set pulse_calib15N with pulse sequence decp90f3_sfhmqc.ric (^{15}N). Both sequences are modified from Bruker pulse library. Enter the previously determined ^{1}H 90° pulse and a default pulse for ^{13}C/^{15}N. Set constant 11 (cnst11) to 1 and record the experiment with an appropriate number of transients (e.g., 8). Processing should give you a 1D spectrum with signal. Set the phase correction 0 order manually or with the command apk0. This value should not be changed during the further calibration procedure. Set cnst11 to 2 and repeat the experiment. Here, all signals should be at the zero-crossing point. Change the length of the ^{13}C/^{15}N 90° pulse until the leftover signal is minimal. With newer hardware (Bruker AVIII and higher), this decoupler X-nuclei pulses, determined by the method described above, can be transferred to the observe channel. For older hardware, the 90° pulse needs to be checked in ^{13}C/^{15}N 1D experiments. The shape pulse can also be calculated from these values, either directly in the pulse sequence or via the PROSOL table (see Bruker manual).

3. Check the quality of your NMR sample and the shim by recording reference experiments like ^{1}H-1D and ^{13}C/^{15}N-HSQC experiments.

IPAP AND DIPAP FOR HOMONUCLEAR DECOUPLING

As usually uniformly ^{13}C-labeled RNAs are used for carbon-direct detected NMR experiments, methods to decouple homonuclear $^1J_{CC}$ scalar couplings are needed. This can be achieved using in-phase anti-phase (IPAP; Hammarstroem & Otting, 1994; Ottiger, Delaglio, & Bax, 1998) or S3E (Meissner, Duus, & Sørensen, 1997) schemes as well as selective decoupling sequences during acquisition or maximum entropy reconstruction (Hoch, Stern, & Mobli, 2011; Serber et al., 2000). As we usually work with IPAP

and double IPAP (DIPAP) sequences, we will describe the general procedure on how to set them up in the following protocol. This Support Protocol is thus relevant for Basic Protocols 3-6.

Materials

>0.5 mM RNA sample (300 µl) of interest (see Basic Protocols 1 and 2; Alternate Protocols 1 and 2)

NMR spectrometer equipped preferably with a *z*-axis gradient ^{13}C, ^{15}N [^{1}H]-TXO cryogenic probe; alternatively, a *z*-axis gradient ^{1}H, [^{13}C, ^{15}N]-TCI cryogenic probe

1. Before starting measurements, the following information is needed:

   a. Are one/two (IPAP/DIPAP) sequences for homonuclear decoupling needed? This depends on the number of carbon atoms which need to be decoupled and whether their chemical shifts and coupling constants are different from each other. For an overview, see Fiala & Sklenár, 2007.
   b. What is the size of the $^1J_{CC}$ coupling constants (Fiala & Sklenár, 2007)?
   c. What is the chemical shift of the carbon nucleus that is detected ($^{13}C_{on}$) and what is/are the chemical shift(s) of the one(s) which is/are decoupled ($^{13}C_{off}/^{13}C_{off}$; Fiala & Sklenár, 2007)?

   Based on this information, the IPAP parameters will be determined in step 4.

2. If not already the case, include IPAP/DIPAP sequence in the pulse sequence of interest either during the last insensitive nuclei enhancement by polarization transfer (INEPT) step (IPAP, Fig. 16A; DIPAP, Fig. 16C) or as a separate element.

3. Set up NMR spectrometer according to Support Protocol 7.

4. Choose pulse shapes, carrier frequencies, and pulse length for on- and off-resonant carbon pulses. Those parameters are summarized in Table 13 (IPAP) and Table 14 (DIPAP) for all possible carbon atoms in RNA.

   Note that pulse lengths and offsets need to be adjusted according to the magnetic field strength, when measuring at a field strength different from 800 MHz.

5. Choose delays Δ and T, where $\Delta = 1/2^1J_{CX}$ and $T = 1/4^1J_{CC}$ (Tables 13 and 14).

   If there are different $^1J_{CC}$ coupling constants present in nucleotides of interest, e.g., $^1J_{C4C5}(C) = 55$ Hz and $^1J_{C6C5}(A) = 75$ Hz, the IPAP time can be calculated using an intermediate coupling constant (e.g., T = 3.9 ms in Table 13). Note that this can only be applied if coupling constants and the chemical shifts of $^{13}C_{on}$ and $^{13}C_{off}$ are in the same range.

6. Record the experiment of interest. Conduct the experiment with double/quadruple of the desired points and half/quarter of the desired transients for IPAP/DIPAP, respectively.

   The reason for this is that the experiments are recorded in an interleaved manner, which divides the number of points by two/four while processing doubles/quadruples the signal intensity.

7. Processing: As experiments with IPAP/DIPAP are recorded in an interleaved manner and the decoupling is based on a combination of in-phase and anti-phase doublets/quadruplets (Fig. 16B and D), the in-phase (IP) and anti-phase (AP) spectra need to be split first. After splitting, they need to be processed and combined. Shift the resulting spectrum by $0.5 \times {}^1J_{CC}$ to the correct chemical shift.

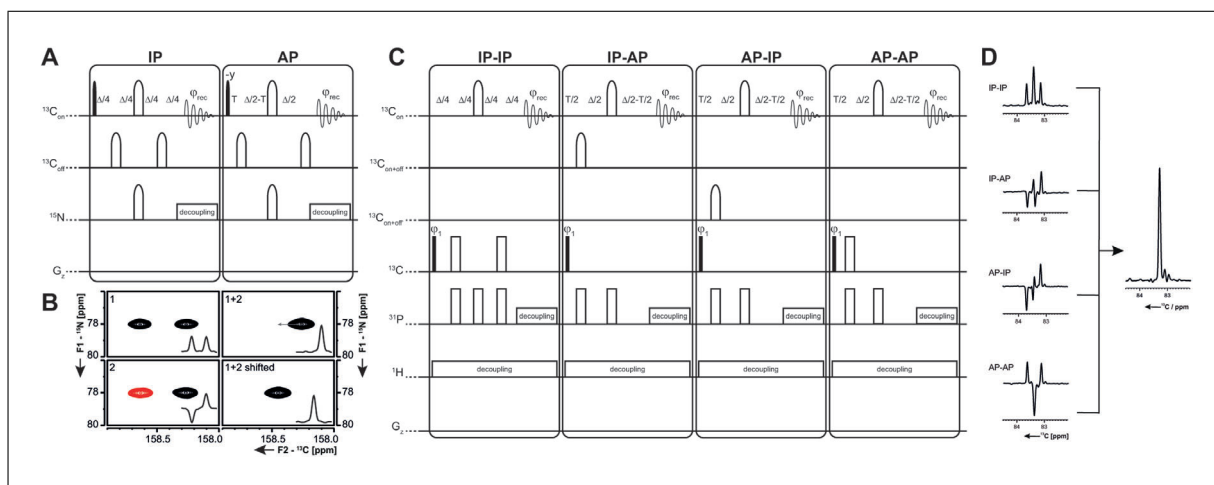**Figure 16** (**A**) Pulse scheme for an IPAP decoupling on the example of a CN-transfer as present in a CN-HSQC experiment. Semi-elliptic narrow filled shapes indicate selective 90° pulses (Q5, on-resonant) and selective 180° pulses are represented by semi-elliptic unfilled shapes (^{13}C Q3 on- and off-resonant, ^{15}N Reburp; Geen & Freeman, 1991). If not indicated otherwise, pulses are applied along the *x*-axis. Delays are $\Delta = 1/2J_{CN}$ and $T = 1/4J_{CC}$. The receiver phase φ_{rec} has to be set individually depending on the phase cycling in the respective experiment. Decoupling for ^{15}N nuclei in this case can be achieved using state-of-the-art decoupling schemes like asynchronous (Shaka, Barker, & Freeman, 1985). (**B**) IPAP decoupling exemplary for a CN-HSQC experiment with 1 IP- (upper left) and 2 AP-spectrum (lower left), 1+2 the summation of IP- and AP-spectrum (upper right) and 1+2 shifted by 0.5*J_{CC} (lower right). 1D traces are included in each spectrum. (**C**) Pulse scheme for a DIPAP decoupling during a CP-transfer. Narrow filled bars represent rectangular 90° pulses while wide-open bars illustrate rectangular 180° pulses. Semi-elliptic unfilled shapes represent selective 180° pulses. If not indicated otherwise, pulses are applied along the *x*-axis. Delays are $\Delta = 1/2J_{CP}$, $T = 1/4J_{CC}$ while in this case the $^1J_{CC}$ coupling constants to the two neighboring carbon atoms are of the same size. Pulse phases are $\varphi_1{}^{IPIP} = 4(x), 4(-x)$, $\varphi_1{}^{IPAP} = 4(y), 4(-y)$, $\varphi_1{}^{IPAP} = 4(y), 4(-y)$, $\varphi_1{}^{APIP} = 4(y), 4(-y)$, and $\varphi_1{}^{APAP} = 4(x), 4(-x)$. The receiver phase φ_{rec} has to be set individually depending on the phase cycling in the respective experiment but is identical for the different sub-spectra (IP-IP, IP-AP, AP-IP, and AP-AP). Decoupling in this case for ^{31}P and ^{1}H nuclei can be achieved using state-of-the-art decoupling schemes like asynchronous GARP4 sequences (Shaka et al., 1985) and WALTZ sequences (Zhou et al., 2007), respectively. (**D**) Illustration of DIPAP decoupling for a ^{13}C,^{15}N-labeled UTP sample. Linear combination of all four spectra gives rise to the virtually decoupled signal on the right. IPAP, in-phase anti-phase; HSQC, heteronuclear single quantum coherence; DIPAP, double in-phase anti-phase.

Note that depending on the software, which is used for processing, there are programs available that do this automatically (e.g., splitcomb in Topspin). If multiple residues with different $^1J_{CC}$ coupling constants are recorded in one experiment, process them differently using the corresponding $^1J_{CC}$ coupling constant. For atoms where no homonuclear $^1J_{CC}$ coupling is present, split the spectra, phase them correctly (AP spectrum is $-90°$ phase shifted relative to the IP spectrum), and combine them.

^{13}C-DETECTED 3D (H)CC-TOCSY, (H)CPC, AND (H)CPC-CCH-TOCSY EXPERIMENTS FOR RIBOSE ASSIGNMENT

NMR spectroscopy of large RNAs but also proteins is often difficult as the signal-to-noise ratio (S/N) deteriorates with larger molecular weight of the RNA of interest, which is caused by the following main points. With an increased molecular weight the number of signals increases, which concomitantly leads to resonance overlap. For RNA, this resonance overlap is particularly pronounced for ^{1}H-nuclei due to a poor chemical shift dispersion. Furthermore, an increased transverse relaxation rate leads to signal broadening, which is especially prominent for ^{1}H-nuclei as well. When conducting ^{13}C-detected NMR experiments these problems can be substantially reduced. ^{13}C nuclei feature a higher chemical shift dispersion and at the same time exhibit more favorable relaxation properties. To exploit these properties also in the assignment of the ribose moiety, where resonance overlap is particularly problematic, a set of experiments was developed by Richter et al., (2010). Here, we will provide a detailed guide on how to set up the

Table 13 Parameters for IPAP Sequences in Different Nucleobases and in Ribose (C1′ and C5′)[a]

	Observed carbon	Decoupled carbon	$^{13}C_{on}$	^{13}C selective pulse 180°	^{13}C selective pulse 90°	$^{13}C_{off}$ offset	Delay T
A	C6[b]	C5	158 ppm	Q3 400 μs	Q5 600 μs	−8,000 Hz	3.3 ms
	C4[c]	C5	150 ppm	Q3 750 μs	Q5 900 μs	−6,400 Hz	4.1 ms
G	C6[d]	C5	157 ppm	Q3 380 μs	Q5 560 μs	−8,600 Hz	2.8 ms
	C4[c]	C5	150 ppm	Q3 750 μs	Q5 900 μs	−6,400 Hz	4.1 ms
C	C6[c]	C5	140 ppm	Q3 750 μs	-	−9,000 Hz	3.8 ms
	C4[b]	C5	167 ppm	Q3 400 μs	Q5 600 μs	−14,400 Hz	4.5 ms
U	C6[c]	C5	140 ppm	Q3 750 μs	-	−8,000 Hz	3.8 ms
	C4[c]	C5	167 ppm	Q3 750 μs	Q5 940 μs	−13,400 Hz	3.9 ms
Ribose	C1′[e]	C2′	90 ppm	Q3 1500 μs	-	−4,000 Hz	6.3 ms
	C5′[e]	C4′	82 ppm	Reburp 2,000 μs (C3′, C4′)	-	−1,000 Hz (C3′, C4′)	6.3 ms
				Reburp 4,900 μs (C4′, C5′)		0 Hz/−4,000 Hz (C4′/C5′)	
A and C	C6 and C4[b]	C5 and C5	162 ppm	Q3 400 μs	Q5 600 μs	−11,000 Hz	3.9 ms

[a]Pulse lengths, shapes, offsets, and IPAP time T were taken from literature references listed below. All parameters are calculated for an 800 MHz NMR spectrometer.
[b]Schnieders et al., 2019.
[c]Fiala & Sklenár, 2007.
[d]Fürtig et al., 2016.
[e]Richter et al., 2010.

Table 14 Parameters for DIPAP Sequence for Detection on C4′ and C5 (for G)[a]

	Observed carbon	Decoupled carbon	$^{13}C_{on}$	^{13}C selective pulse 180°	^{13}C selective pulse 90°	$^{13}C_{off/off}$ offset	Delay T
G	C5	C4 and C6	119 ppm	Q3.surbop 500 μs (C5)	Q5 1,000 μs (C5)	−11,500 Hz (C6)	7.7 ms (C5-C6)
				Q3.surbop 900 μs (C6)		4,600 Hz (C4+C5)	5.7 ms (C5-C4)
				Reburp 600 μs (C4+C5)			
Ribose	C4′	C5′ and C3′	82 ppm	Reburp 4,900 μs (C4′)	-	−1,000 Hz (C3′,C4′)	6.3 ms
				Reburp 2,000 μs (C3′,C4′)		0 Hz/−4,000 Hz (C4′/C5′)	
				Reburp 4,900 μs (C4′,C5′)			

[a]Pulse lengths, shapes, offsets, and IPAP time T were taken from Richter et al., 2010.

^{13}C-detected (H)CC-TOCSY, (H)CPC, and (H)CPC-CCH-TOCSY experiments (coherence transfer pathway in Fig. 17).

Materials

≥0.5 mM uniformly ^{13}C-labeled RNA (see Basic Protocol 1)
NMR buffer (see recipe)
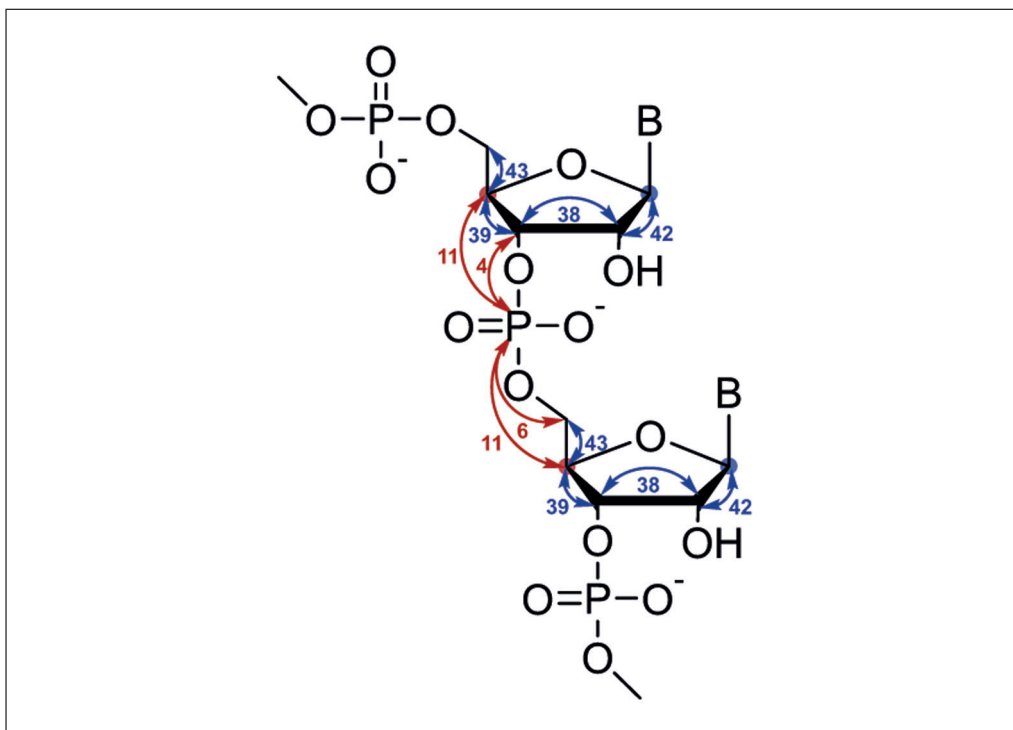Other RNA specific requirements like Mg^{2+} ions or small molecule ligands

Figure 17 Coherence transfer pathway for the (H)CC-TOCSY (blue) and the (H)CPC experiment (red). Coupling constants, which are used in the transfers are given. The figure has been adapted from Schnieders, Keyhani, Schwalbe, & Fürtig, 2020.

NMR tube (e.g., Shigemi)

NMR spectrometer equipped preferably with a *z*-axis gradient ^{13}C, ^{15}N [^{1}H]-TXO cryogenic probe; alternatively a *z*-axis gradient ^{1}H, [^{13}C, ^{15}N]-TCI cryogenic probe

1. Set up the NMR spectrometer according to Support Protocol 7.

2. Record a ^{1}H,^{13}C-HSQC spectrum of the ribose region as a reference spectrum.

3D (H)CC-TOCSY experiment

3. Load parameter set c_3D-hCC-TOCSY-H1′C1′ with pulse sequence c_HC1ccflopsy.ric.

4. Use IPAP for homonuclear decoupling according to Support Protocol 8, applying the pulse lengths listed in Table 13.

5. Adjust TOCSY transfer length depending on the desired correlations.

   For the observation of C1′-C5′ use 15 ms and for C1′ and C2′ only use 3 ms.

6. Choose carrier frequencies according to the resonance distribution.

   Set the ^{1}H carrier frequency to the water resonance frequency (4.7 ppm). Set the ^{13}C carrier frequency to the middle of the desired carbon correlations. Set cnst21 to the C1′ middle frequency (e.g., 90 ppm), cnst22 to the C2′ middle frequency (e.g., 70 ppm), and cnst23 to the middle frequency of all desired carbon correlations (equals carrier frequency). Set the ^{15}N carrier frequency to 160 ppm.

7. Adjust number of transients and points and test the experiment by recording the first increment. Check whether the S/N is sufficient. If not, adjust number of transients.

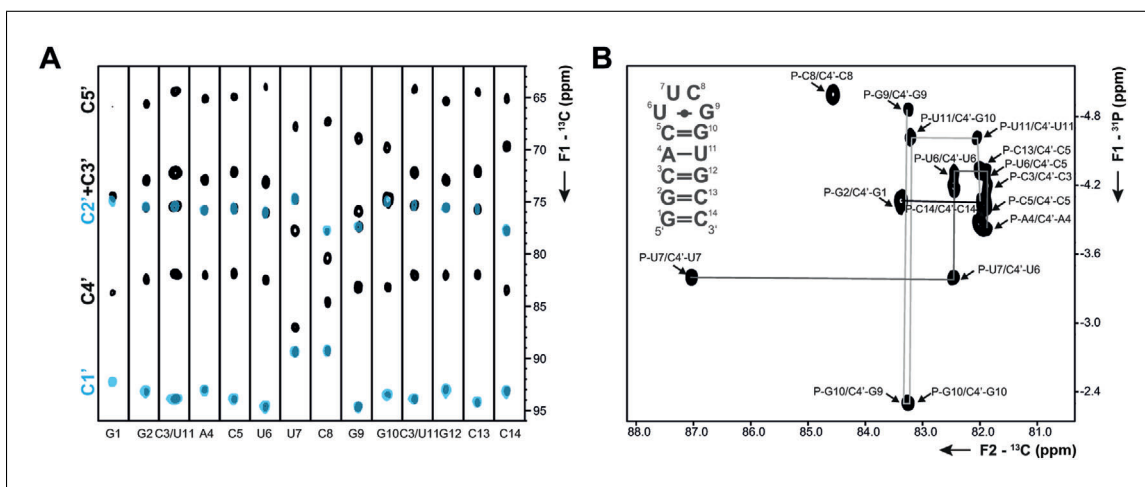8. If available, you may apply non-uniform sampling (NUS).

Figure 18 (**A**) 2D planes of the 3D (H)CC-TOCSY spectrum for the different nucleotides of a 14-nt RNA with UUCG tetraloop with long (black) and short (blue) TOCSY mixing time. (**B**) 2D (H)CPC spectrum of a 14-nt RNA with UUCG tetraloop. A walk from the phosphor nuclei to the C4′ atoms of the neighboring nucleotides is indicated. The figure has been adapted from Schnieders et al., 2020. TOCSY, total correlation spectroscopy.

9. When the experiment is completed, process spectrum as described in Support Protocol 8 (sample data: Fig. 18A).

2D (H)CPC experiment

10. Include the non-standard selective pulse for C4′ and C5′, which is used for the DIPAP step (see Supporting Information), to the list of shapes.

11. Test pulse in a 1D experiment by loading parameter set C13PGF2igF3se2d with pulse sequence zgpgf2igf3se2d.t1.

12. Load parameter set c_2D-hCPC with pulse sequence c_HCPC.t14.

13. Choose carrier frequencies according to the resonance distribution.

    Set the ^{1}H carrier frequency to the water resonance frequency (4.0 ppm). Set the ^{13}C carrier frequency to the middle of the C4′ resonances (e.g., 83 ppm). Set the ^{31}P carrier frequency to the middle of the phosphor resonances (e.g., −1 ppm).

14. Set cnst2 to 166 Hz ($^1J_{CH}$ coupling).

15. For DIPAP, choose shaped pulses for sp21 (Reburp.1000), sp28 (Reburp.1000), and sp29 (reburp_c4c5bs_2.1000) as well as pulse lengths for p23 (2 ms, 600 MHz) and p25 (6.5 ms, 600 MHz) according to the magnetic field strength.

16. Adjust number of transients and points and test the experiment by recording the first increment. Check whether the S/N is sufficient. If not, adjust number of transients.

17. If available, you may apply non-uniform sampling (NUS).

18. Processing: Process spectrum as described in Support Protocol 8 (sample data: Fig. 18B).

2D (H)CPC-CCH-TOCSY experiment

19. Load parameter set c_3D-hCC-TOCSY-H1′C1′ with pulse sequence c_HC1cc flopsy.

20. Set TOCSY mixing time to 32 ms.

21. Set cnst2 to 166 Hz ($^1J_{CH}$ coupling).

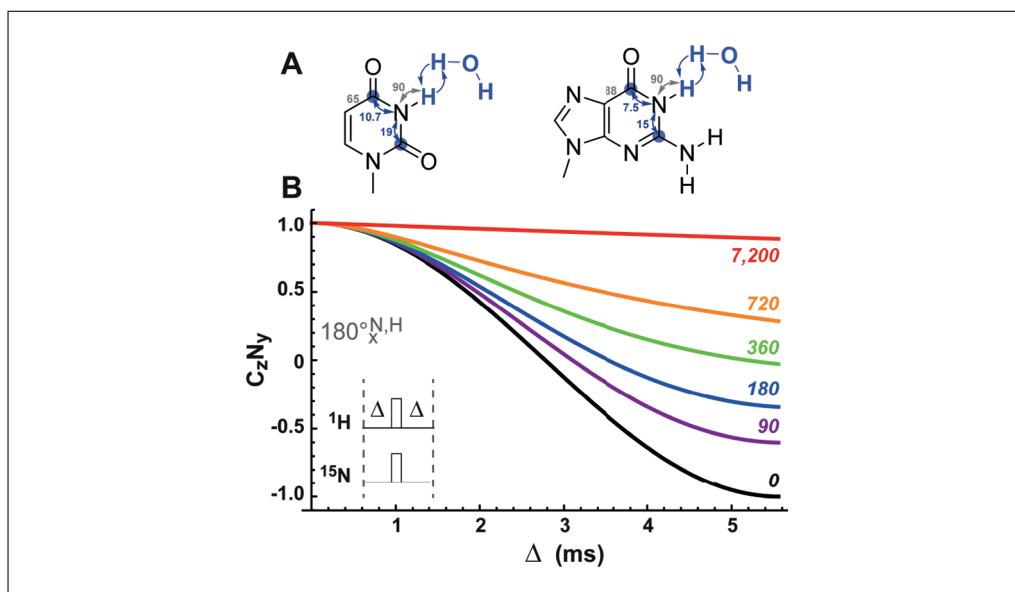22. Set carrier frequencies according to the resonance distribution.

Figure 19 (**A**) Schematic coherence transfer pathways for the ^{13}C-detected 2D CN-spin filter HSQC experiment in Us (left) and Gs (right). The sizes of the $^1J_{CN}$, $^1J_{NH}$, and $^1J_{CC}$ coupling constants are given (Fiala & Sklenár, 2007). Coupling constants that are only indirectly used for signal intensity modulation or for decoupling are depicted in gray. The carbon nuclei where the detection happens are marked with a circle. (**B**) Simulated C_zN_y coherence in dependence of the transfer time D during the NH spin filter element for different imino proton exchange rates, ranging from 0 s^{-1} (black) to 7,200 s^{-1} (red). The figure has been adapted from Fürtig et al., 2016 and Schnieders et al., 2020. HSQC, heteronuclear single quantum coherence.

> *Set the ^1H carrier frequency to the water resonance frequency (4.0 ppm). Set the ^{13}C carrier frequency to the middle of the C1′ resonances (e.g., 90 ppm). Set the ^{31}P carrier frequency to the middle of the phosphor resonances (e.g., −1 ppm).*

23. For IPAP, choose Q3 shapes for sp24 (C1′ selective) and sp27 (C2′ selective) as well as pulse lengths for p12 (2 ms, 600 MHz) according to the magnetic field strength.

24. Adjust number of transients and points and test the experiment by recording the first increment. Check whether the S/N is sufficient. If not, adjust number of transients.

25. If available, you may apply non-uniform sampling (NUS).

26. Processing: Process spectrum as described in Support Protocol 8.

^{13}C-DETECTED 2D CN-SPIN FILTER HSQC EXPERIMENT

NMR spectroscopy of RNA usually focuses on the imino proton resonance as a reporter for base pairing. The reason is that it is only observable when protected through a hydrogen bond. Otherwise, it rapidly exchanges with solvent water and the resonance is broadened beyond detectability. While this allows quick secondary structure determination, any information about flexible regions of the RNA is not available. With the ^{13}C-detected CN-spin filter HSQC experiment, however, those flexible regions are accessible as here the detection happens on carbon (Fürtig et al., 2016). Moreover, the status of hydrogen bonding of any imino-proton-carrying nucleobase (G or U) can be determined as signal intensities are modulated depending on the imino proton exchange rate (Fig. 19B). The schematic coherence transfer in Figure 19A shows that this experiment is composed of a CN-transfer step that is succeeded by an NH spin filter element. The differences in signal intensity result from a modulation of the observed scalar $^1J_{NH}$ coupling on the imino proton exchange rate. If the proton exchange is slow, the $^1J_{NH}$ coupling can evolve during the spin filter element. However, if the imino proton exchange with solvent water
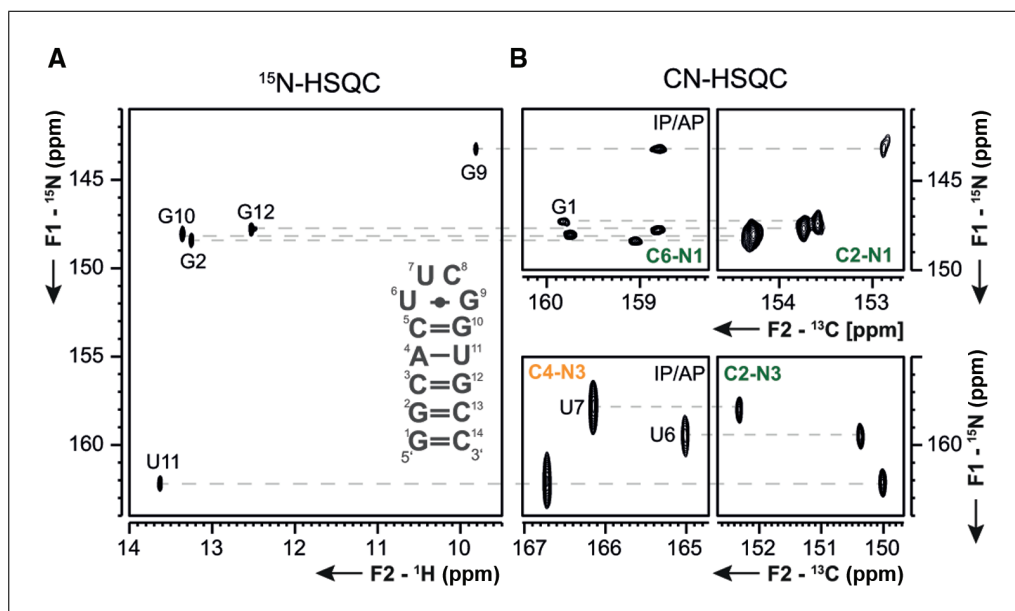
Figure 20 (**A**) ^{15}N-HSQC spectrum of the imino region of a uniformly ^{13}C,[15]N-labeled 14-nt long hairpin RNA with UUCG tetraloop. (**B**) ^{13}C-detected CN-HSQC for both correlations in Gs (upper panels) and Us (lower panels). $^1J_{CN}$ coupling constants utilized for the CN-transfer were 17 Hz (yellow) and 27 Hz (green). Horizontal gray dashed lines mark resonances with correlations to the same nitrogen atoms (N3 for U and N1 for G). Correlations, which cannot be observed in the ^{1}H-detected ^{15}N-HSQC experiment, are labeled in the CN-HSQC spectra (G1, U6, and U7). The figure has been adapted from Fürtig et al., 2016 and Schnieders et al., 2020. HSQC, heteronuclear single quantum coherence.

is fast, scalar relaxation of the second kind leads to a decoupling of the scalar coupling which results to no effect on the signal intensity (Fig. 19B).

Materials

≥0.5 mM RNA sample with ^{13}C,[15]N-labeled imino group containing nucleotide of interest (G or U) or uniformly ^{13}C,[15]N-labeled RNA (see Basic Protocol 1)
NMR buffer (see recipe)
Other RNA specific requirements like Mg^{2+} ions or small molecule ligands

NMR tube (e.g., Shigemi)
NMR spectrometer equipped preferably with a *z*-axis gradient ^{13}C, [15]N [^{1}H]-TXO cryogenic probe; alternatively, a *z*-axis gradient ^{1}H, [^{13}C, ^{15}N]-TCI cryogenic probe

1. Set up NMR spectrometer according to Support Protocol 7.

2. Record a ^{1}H,^{15}N-HSQC spectrum of the imino region for comparison (Fig. 20A).

3. Record ^{13}C-detected CN-HSQC spectra in order to optimize the CN-transfer time, which depends on the desired correlations for G (C2N1 or C6N1) or U (C2N3 or C4N3).

   Calculate with different $^1J_{CN}$ coupling constants (e.g., 15–30 Hz) to vary the CN-transfer time. It is sufficient to acquire the first increment with a high number of transients (e.g., 1,024) without using IPAP sequences for homonuclear decoupling. Transfer times for the different correlations that work best in our hands are given in Table 15.

   Note that in principle both correlations in uridines and guanosines are possible; we however advise working with the C2N3 correlation in uridines and the C2N1 correlation in guanosines as the $^1J_{CN}$ scalar coupling constants are larger and IPAP schemes for homonuclear CC-decoupling are not needed.

Table 15 Experimentally Determined $^1J_{CN}$ Coupling Constants Which Are Used to Calculate CN-Transfer Time in the CN-HSQC Experiment and Suggested Carrier Frequencies for ^{13}C and ^{15}N for Different Correlations[a]

	G		U	
	C2N1	C6N1	C2N3	C4N3
$^1J_{CN}$	27 Hz	27 Hz	27 Hz	17 Hz
^{13}C carrier	156 ppm	161 ppm	154 ppm	169 ppm
^{15}N carrier	147 ppm		160 ppm	

[a]Note that chemical shifts are referenced.

4. Set up 2D CN-HSQC experiment using the optimized transfer time. Use IPAP sequences for homonuclear decoupling if needed (G-C6N1 and U-C4N3) and implement them according to Support Protocol 8. Set carrier frequencies and spectral windows according to the correlations of interest (Table 13). Check whether the number of transients is sufficient in the first increment of the CN-HSQC experiment. Compare spectra with a standard $^1H,^{15}N$-HSQC spectrum of the imino region (Fig. 20A and B).

5. Load parameter set C_CON_SQXF or C_CON_IASQXF (IPAP) with the pulse sequences c_con_sqxf and c_con_iasqxf, respectively. Set the delay for the CN-transfer as optimized in the CN-HSQC experiment. If necessary, use IPAP sequences as conducted for the CN-HSQC experiment or according to Support Protocol 8. Use a $^1J_{NH}$ coupling constant of 90 Hz to calculate spin filter delay ($1/^1J_{NH}$).

   Set carrier frequencies based on the CN-HSQC spectrum according to the correlations of interest (Table 15). Check in the first increment whether the S/N is sufficiently large.

6. Processing: If IPAP schemes were used, process spectra according to Support Protocol 8. If this was not the case, simple 2D processing can be applied.

7. Data interpretation: Based on the signal intensities in this 2D experiment, the status of hydrogen bonding can be estimated.

   If the correlations to the imino nitrogen atoms appear inverted in sign with respect to other non-imino CN-correlations, the exchange of the imino proton is slow. This is for example the case for U11 in the 14-nt UUCG hairpin RNA (see Fig. 21). If the imino proton is not protected from hydrogen exchange, the signal intensities are not affected, and the correlation appears with the same sign as other non-imino CN-correlations (e.g., U7 Fig. 21). Signals, which are close to zero crossing correspond to imino protons that are partly protected from solvent exchange either through a weak hydrogen bond or due to steric hindrance (e.g., U6 Fig. 21). If the exact imino proton exchange rate is to be determined, the CN-spin filter HSQC experiment can be modified to a pseudo 3D experiment according to Fürtig et al., 2016.

^{13}C-DETECTED C(N)H-HDQC EXPERIMENT FOR THE DETECTION OF AMINO GROUPS

Amino groups are important functional groups in RNA and are known to be involved in many different kinds of interactions due to their hydrogen bonding potential. However, NMR spectroscopy of RNA often sets a focus on the characterization of the imino protons as their resonances are well dispersed and allow a fast secondary structure determination. The resonances of amino groups on the other hand exhibit—especially for guanosines and adenosines—very broad lines, which are often beyond detectability. The reason for this is a partially restricted rotation around the $C-NH_2$ bond that is regularly in the intermediate exchange regime on the NMR time scale (Mueller, Legault, & Pardi,
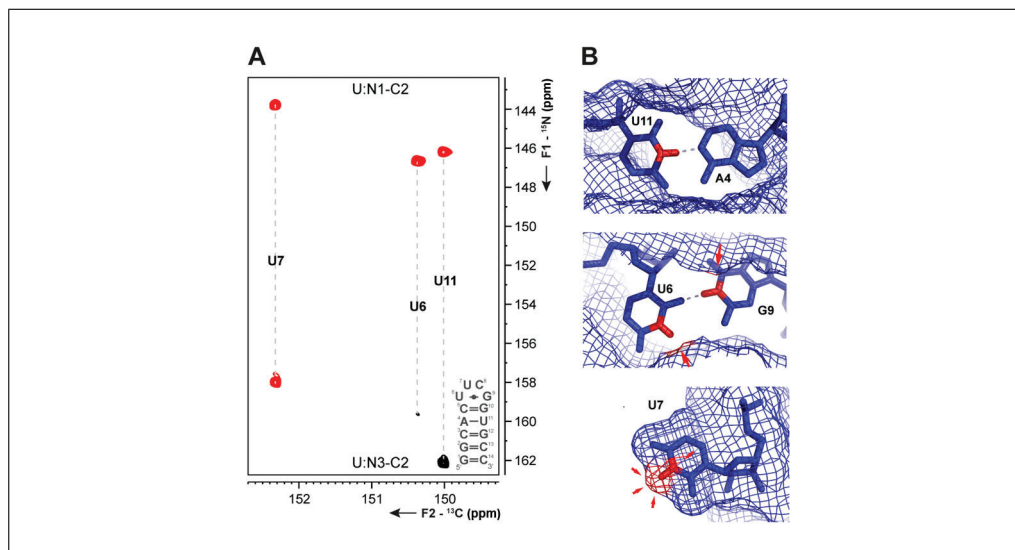
Figure 21 (**A**) ^{13}C-detected CN-spin filter HSQC spectrum for the uridines of a 14-nt long hairpin RNA with UUCG tetraloop (Nozinovic, Fürtig, Jonker, Richter, & Schwalbe, 2010). The experiment was recorded with 440 scans per increment and spectral widths of 33 ppm (512 complex points) and 34 ppm (64 complex points) in the direct and indirect dimensions, respectively. The CN-transfer delay was set to match a $^{1}J_{CN}$ coupling constant of 27 Hz. Carrier frequencies were 4.7 ppm, 160 ppm, and 157 ppm for ^{1}H, ^{13}C, and ^{15}N, respectively. With an inter-scan delay of 2 s the experiment was recorded for 36 hr. (**B**) Structural interactions of the uridines U11 (AU Watson-Crick base pair), U6 (sheared GU base pair), and U7 (solvent exposed) of the 14-nt RNA. The figure has been taken from Schnieders et al., 2020. HSQC, heteronuclear single quantum coherence.

1995). By moving away from ^{1}H-direct detection to ^{13}C-detected NMR experiments and by evolving ^{1}H double quantum (DQ) coherence, the effect of the rotation on the signal line shapes is diminished in the C(N)H-HDQC experiment (Fig. 22A; Schnieders et al., 2019). With this experiment, one is able to detect a full set of sharp amino resonances for all of the amino-group bearing nucleotides. The experiment combines a CN-HSQC with an additional NH transfer (magnetization transfer, Fig. 22B) where ^{1}H-double quantum coherence is selected and evolved in the indirect dimension. As described in the Support Protocol 8, homonuclear $^{1}J_{CC}$ coupling in adenosines and cytidines is decoupled using IPAP sequences. This protocol will explain step by step how to successfully set this experiment up and what to consider.

Materials

≥0.5 mM RNA sample with ^{13}C,^{15}N-labeled amino group containing nucleotide of interest (G, C, or A) or uniformly ^{13}C,^{15}N-labeled RNA (see Basic Protocol 1)
NMR buffer (see recipe)
Other RNA specific requirements like Mg^{2+} ions or small molecule ligands

NMR tube (e.g., Shigemi)
NMR spectrometer equipped preferably with a *z*-axis gradient ^{13}C, ^{15}N [^{1}H]-TXO cryogenic probe; alternatively, a *z*-axis gradient ^{1}H, [^{13}C, ^{15}N]-TCI cryogenic probe

1. Prepare NMR spectrometer according to Support Protocol 7.

2. Record a ^{1}H,^{15}N-HSQC spectrum of the amino region to get information about resonance distribution and quality of the sample (Fig. 23A). The recommended pulse scheme includes a CPMG-like element for the HN transfer (Mueller et al., 1995; Mulder, Spronk, Slijper, Kaptein, & Boelens, 1996).
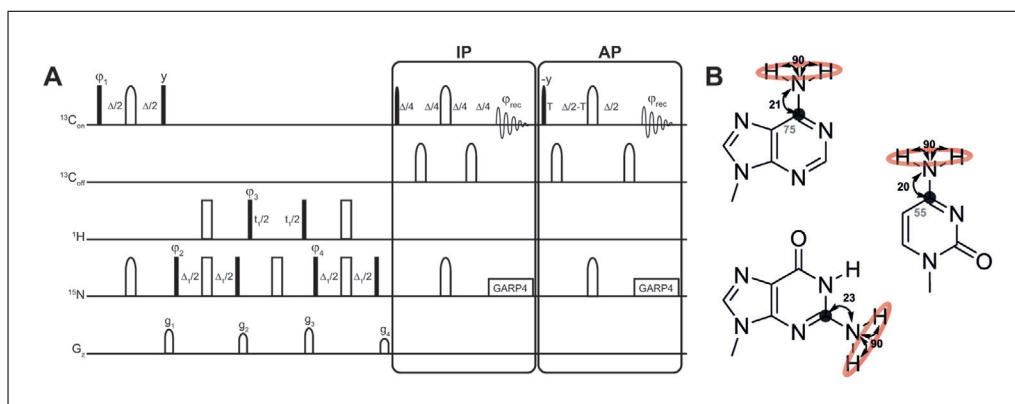
Figure 22 (**A**) Pulse scheme of the ^{13}C-detected C(N)H-HDQC experiment with IPAP sequence for homonuclear CC-decoupling in adenosines and cytidines. Rectangular 90° and 180° pulses are indicated by narrow filled and wide-open bars, respectively. Semi-elliptic narrow filled bars represent selective 90° pulses (^{13}C Q5 600 μs at 800 MHz). Selective 180° pulses (^{13}C Q3 400 μs, on- or off-resonant, and ^{15}N Reburp [Geen & Freeman, 1991] 1.2 ms at 800 MHz) and gradients are shown as semi-elliptic wide-open shapes. The default phase is x. ^{15}N nuclei are decoupled during acquisition using asynchronous GARP4 sequences (Shaka et al., 1985). Gradient pulses were applied for 1 ms with a smoothed square amplitude (SMSQ10.100) and 100% gradient strength corresponds to 53 G/cm. The t_1-time was incremented with half of the dwell time. Frequency discrimination in the indirect dimension φ_3 was cycled with 45°. Pulse phases, delays, and gradient strengths are as follows: $\varphi_1 = x, -x$, $\varphi_2 = (4)x, (4)(-x)$, $\varphi_3 = (2)x, (2)y, (2)(-x), (2)(-y)$, $\varphi_4 = (8)x$, $(8)(-x)$, $\varphi_{rec} = x, (2)(-x), x, -x, (2)x, (2)(-x), (2)x, -x, x, (2)(-x), x$, $\Delta = 1/2^1J_{CN} = 19.23$ ms, $\Delta_1 = 1/2^1J_{NH} = 4.17$ ms, T = $1/4^1J_{CC} = 4.5$ ms (cytidine) or 3.3 ms (adenosine), 50% (g_1), 60% (g_2), 48% (g_3), and 35% (g_4). (**B**) Schematic magnetization transfer pathway of the C(N)H-HDQC experiment for A (top), G (bottom), and C (middle). The size of the $^1J_{CN}$ and $^1J_{NH}$ scalar couplings is indicated. Coupling constants, which are used for IPAP decoupling schemes are depicted in gray. The carbon where detection happens is marked with a circle. The rotation of the protons around the CN bond is indicated with a red elliptic line. Both of the schemes were adapted from Schnieders et al., 2019, 2020. HDQC, heteronuclear double-quantum correlation; IPAP, in-phase anti-phase.

For uniformly labeled RNAs, use carrier frequencies of 4.7 ppm (^1H), 150 ppm (^{13}C), and 85 ppm (^{15}N) while covering a spectral window of ~45 ppm in the ^{15}N-dimension. Calculate the INEPT delay with a $^1J_{NH}$ coupling constant of $^1J_{NH} = 90$ Hz. The number of required transients depends on the sample concentration and should be set appropriately (we use 8 scans for a 980 μM, 34-nt long RNA).

3. Record a ^{13}C-detected 2D CN-HSQC spectrum of the amino region as described in Support Protocol 9, to optimize the CN-transfer step and the IPAP for homonuclear CC-decoupling.

4. Set up the ^{13}C-detected C(N)H-HDQC (parameter set C_CNH_DQSP/C_CNH_IADQSP [IPAP], pulse sequence c_cnh_dqsp/c_cnh_iadqsp) experiment with optimized CN-transfer and selective pulses from the CN-HSQC experiment (Support Protocol 9).

   Choose the ^1H carrier frequency and the spectral window according to the signal distribution in the $^1H,^{15}N$-HSQC spectrum. Note that signals are coming to resonance at the mean ^1H chemical shift when setting the spectral window. Calculate the NH-transfer time from a relaxation optimized coupling constant of $^1J_{NH} = 110$ Hz and note that overall transfer time is $1/2J_{NH}$. Be sure to record the experiment with a sufficient number of transients and check for sufficient S/N in the first increment. Also keep in mind that if IPAP is used, the effective resolution will be half as high.

5. Process your spectra separately for C, A, and G according to Support Protocol 8.

6. Evaluate HDQC spectra (Fig. 23B, C, and D) in combination with the CN-HSQC (Fiala & Sklenář, 2007) and ^{15}N-HSQC spectra. For completion of resonance assignment, HNCO type experiments (for G), the TROSY relayed HCCH-COSY
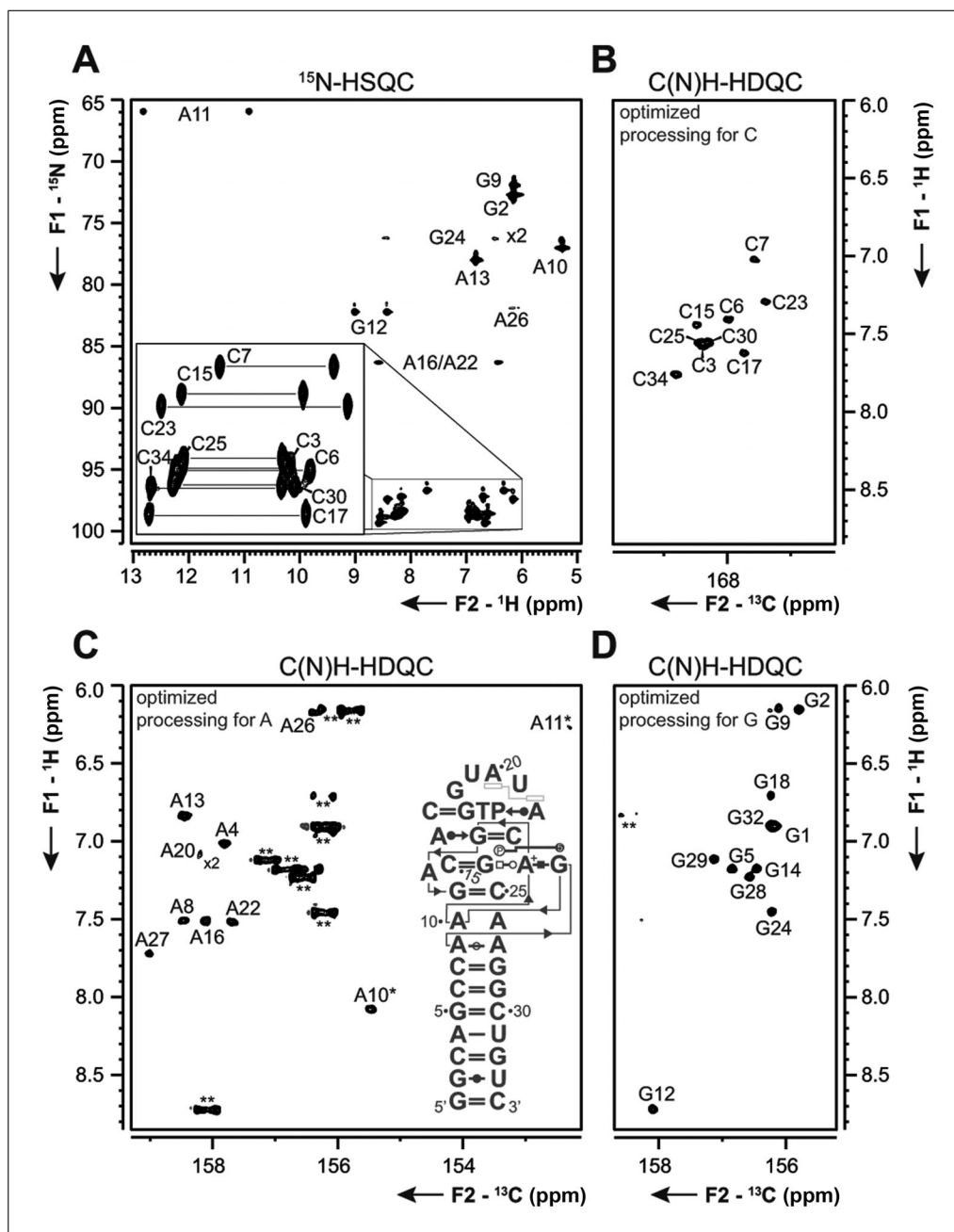
Figure 23 (**A**) ^{15}N-HSQC spectrum of the amino region of a 34-nt GTP class II aptamer (Wolter et al., 2016, 2017). The experiment was recorded with 8 scans per increment and spectral widths of 24 ppm (2,048 complex points) and 40 ppm (128 complex points) in the direct and indirect dimensions, respectively. Carrier frequencies were 4.7 ppm, 150 ppm, and 85 ppm for ^{1}H, ^{13}C, and ^{15}N, respectively. With an inter-scan delay of 1 s the experiment was recorded for 40 min. (**B**, **C**, and **D**) C(N)H-HDQC spectra for the 34-nt GTP class II aptamer (secondary structure in part C) with optimized processing for C, A, and G, respectively. The spectra originate from the same experiment and only differ in their processing. The experiment was recorded with 80 scans per increment and spectral widths of 50 ppm (1,024 complex points) and 5 ppm (160 complex points) in the ^{13}C and ^{1}H dimensions, respectively. Carrier frequencies were 154 ppm (^{13}C), 7.1 ppm (^{1}H), and 90 ppm (^{15}N). With an inter-scan delay of 2.5 s, the experiment was recorded for 19 hr. B was processed using a $^1J_{CC}$ coupling constant of 55 Hz and in C the $^1J_{CC}$ coupling constant was 75 Hz. D was processed by splitting IP and AP spectra, phase shifting the latter (AP) by −90°, and adding them up. Signals marked with * are folded in the indirect dimension and resonances marked with ** appear in the spectra C and D as pseudo doublets and arise due to processing from guanosines and adenosines, respectively. The figure was taken from Schnieders et al., 2019. HSQC, heteronuclear single-quantum correlation; HDQC, heteronuclear double-quantum correlation; IP, in-phase; AP, anti-phase.

experiment (for A; Simon, Zanier, & Sattler, 2001), ^{1}H,^{1}H-NOESY experiments (mainly for C), and non-selective CN-HSQC experiments with different CN-transfer times can be acquired.

^{13}C-DETECTED CN-HSQC EXPERIMENT FOR AMINO GROUPS

In order to optimize parameters for the carbon direct-detected C(N)H-HDQC experiment and to assign resonances in this experiment, an amino selective CN-HSQC experiment should be conducted. Parameters which are to be optimized are the CN-transfer time and the IPAP sequence depending on the nucleotide type of interest (G, A, or C).

Materials

≥0.5 mM RNA sample with ^{13}C,^{15}N-labeled amino group containing nucleotide of interest (G, C, or A) or uniformly ^{13}C,^{15}N-labeled RNA (see Basic Protocol 1)
NMR buffer (see recipe)
Other RNA specific requirements like Mg^{2+} ions or small molecule ligands

NMR tube (e.g., Shigemi)
NMR spectrometer equipped preferably with a *z*-axis gradient ^{13}C, ^{15}N [^{1}H]-TXO cryogenic probe; alternatively, a *z*-axis gradient ^{1}H, [^{13}C, ^{15}N]-TCI cryogenic probe

1. Prepare NMR spectrometer according to Support Protocol 7.

2. Load parameter set C_CON_SQSP_NH2 with pulse program c_con_sqsp_NH2.

   This pulse sequence does not contain an IPAP scheme for homonuclear CC-decoupling and is only used for fast optimization of the CN-transfer time.

3. Choose selective 180° pulses.

   We use Q3 shapes for ^{13}C and Reburp shapes for ^{15}N. Set pulse lengths so that the desired band widths are reached. In our hands, 400 μs (^{13}C, Q3) and 1,200 μs (^{15}N, Reburp) work well at 800 MHz.

4. Optimize CN-transfer. In theory, the time for the CN-transfer equals 1/2J_{CN}.

   With coupling constants of around 21 Hz for the different nucleotide types (G = 23 Hz, C = 21 Hz, and A = 20 Hz; Fiala & Sklenár, 2007), this transfer time would be 23.8 ms long. However, transverse relaxation leads to a constant decrease of the signal and therefore, depending on the rotational correlation time of the RNA, the time with maximum transfer efficiency is often shorter and needs to be determined experimentally (Fig. 24). Furthermore, transfer efficiencies also vary between the different amino group carrying nucleotides.

   To determine the time with the maximum transfer efficiency, record the first increment of this 2D CN-HSQC experiment with a high number of transients (e.g., 1,024) for several transfer times (e.g., 12-40 ms). Comparison of integrals within the different regions then yields the optimized transfer times for the respective nucleotide type (Fig. 24). Optimized transfer times that we determined for a 14-nt RNA are depicted in Table 16.

   If the experiment is conducted for all nucleotide types at the same time, an average transfer time has to be chosen. In our hands, best results were achieved when working with an INEPT-transfer time, which is optimized for adenosines (19.2 ms), as these resonances tend to exhibit the lowest S/N.

5. Load parameter set C_CON_IASQSP_NH2 with pulse sequence c_con_iasqsp_NH2 to include the IPAP sequence for homonuclear CC-decoupling in adenosines and cytidines according to Support Protocol 8. As the $^1J_{CC}$ coupling constants differ in A and C from 75 to 55 Hz, respectively, best results are achieved when their
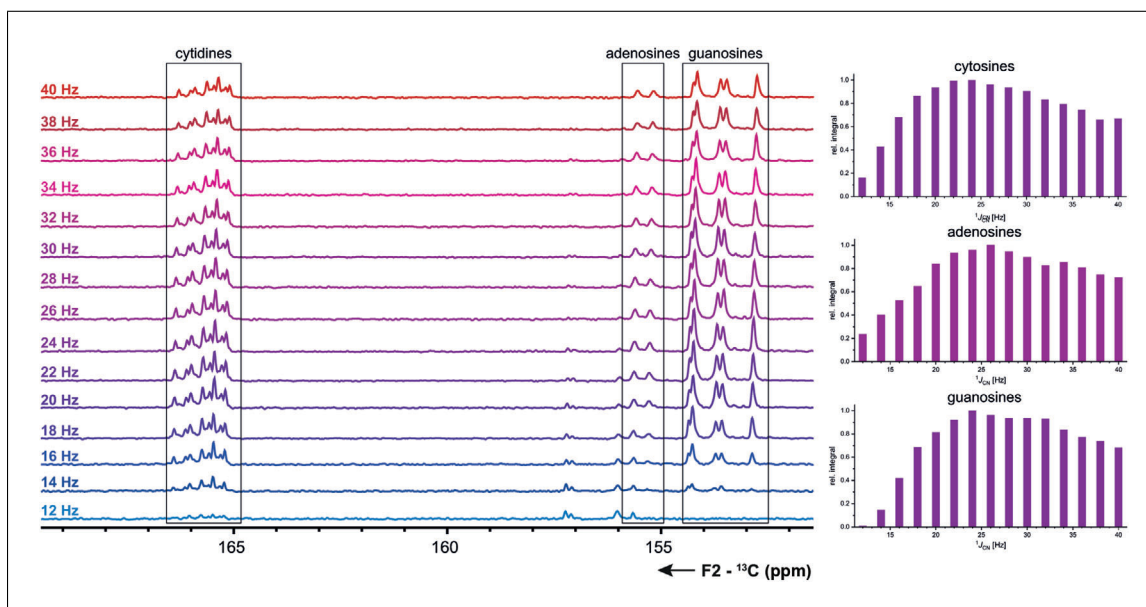
Figure 24 Optimization of the CN-transfer delay in an amino-selective CN-HSQC experiment calculating with different $^1J_{CN}$ coupling constants (12–40 Hz). Regions where cytidines, adenosines, and guanosines come to resonance are marked. The respective regions were integrated to determine the time with maximum transfer efficiency (right). Optimal transfer times are 26, 26, and 24 Hz for cytosines, guanosines, and adenosines, respectively. HSQC, heteronuclear single-quantum correlation.

Table 16 Experimentally Derived CN-INEPT Transfer Times for Guanosines, Cytidines, and Adenosines of a 34-Nucleotide Long RNA

	Guanosines	Cytidines	Adenosines
$1/2\,^1J_{CN}$	20.8 ms	19.2 ms	19.2 ms

correlations are recorded in separate experiments. However, it is also possible to use an IPAP delay with an intermediate $^1J_{CC}$ coupling constant of 65 Hz (see also Table 13 in Support Protocol 8). Use the 180° selective pulses as in the non-IPAP version. We use Q5 shapes for the selective 90° carbon pulse and in our hands 600 μs works well at 800 MHz. Set offset for the off-resonant ^{13}C-pulse (Table 13 in Support Protocol 8). Here, we use −11,000 Hz at 800 MHz.

6. Process spectra as described in Support Protocol 8 while using the correct $^1J_{CC}$ coupling constants for C and A even if an intermediate IPAP delay was used. If no homonuclear coupling is present as is the case for G, spectra have to be processed differently as described in Support Protocol 8 (Fig. 25).

***BASIC
PROTOCOL 6***

^{13}C-DETECTED "AMINO"-NOESY EXPERIMENT

Due to the inherently low proton density, structural characterization of RNA often suffers from a low number of inter-residual long-range nuclear Overhauser effect (NOE) contacts. Imino and amino protons are often involved in exchange processes that do not allow their detection, which in turn reduces the number of available NOE contacts even further. In amino groups, this exchange process is a restricted rotation around the C-NH$_2$ bond, which is often in an intermediate exchange regime on the NMR timescale. This broadens the signals beyond detectability as soon as the amino proton coherence is in the transverse plane. The "amino"-NOESY experiment partly removes this effect by moving away from ^{1}H-detection towards ^{13}C direct detection and by spin-locking the ^{1}H coherence in a HN-TOCSY transfer (Fig. 26). With this experiment, additional NOE contacts can be determined which are not accessible using any other conventional NOESY experiment.
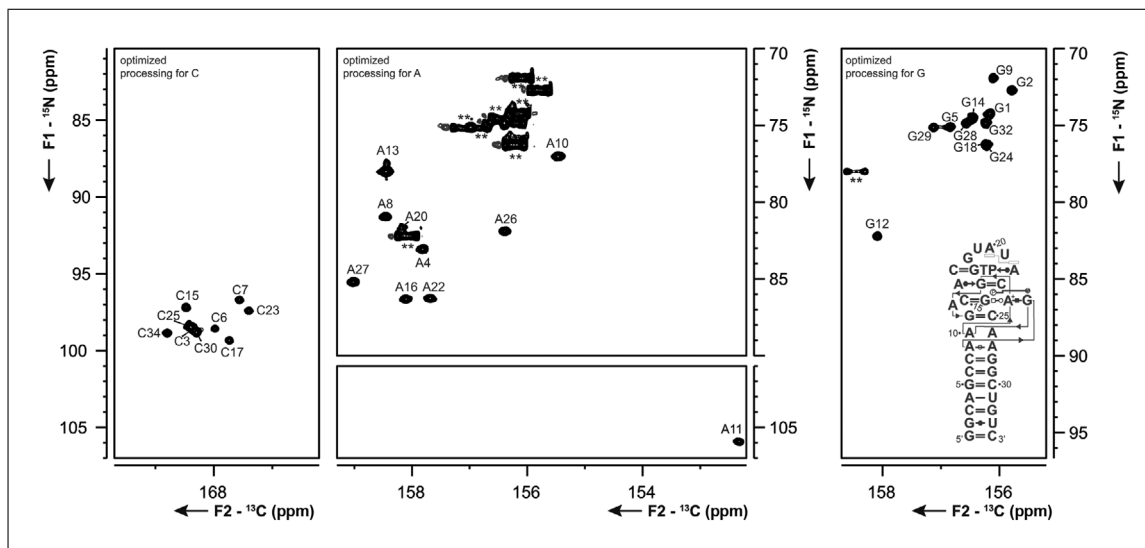
Schnieders et al.

Figure 25 ^{13}C-detected CN-HSQC spectra of the 980 μM uniformly ^{13}C,^{15}N-labeled 34-nt GTP class II aptamer RNA (right; Wolter et al., 2016, 2017). The spectra originate from the same experiment and are processed differently for cytidines (left), for adenosines (middle), and guanosines, respectively. Signals marked with ** in the spectra for adenosines and guanosines appear from processing and belong to guanosines and adenosines, respectively. The experiment was conducted with 48 scans per increment and with spectral widths of 50 ppm (1,024 complex points) in the direct dimension and 43 ppm (128 complex points) in the indirect dimension. Carrier frequencies were 160 ppm (^{13}C), 4.7 ppm (^{1}H), and 86.5 ppm (^{15}N), respectively. With an inter-scan delay of 2.5 s the overall experimental time was 9 hr 15 min. The figure was taken from Schnieders et al., 2019. HSQC, heteronuclear single-quantum correlation.
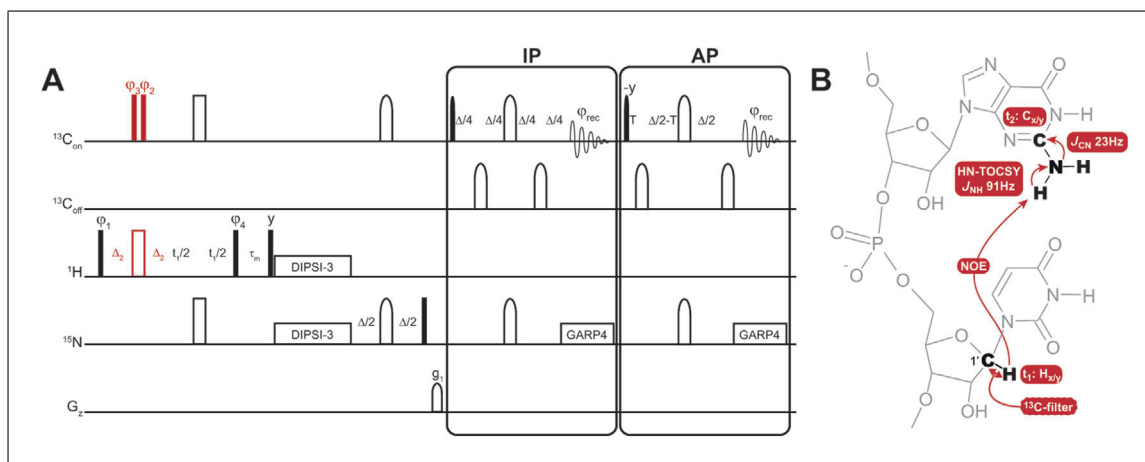

Figure 26 (**A**) Pulse scheme of the "amino"-NOESY experiment with optional ^{13}C filter (red) and IPAP scheme for homonuclear decoupling. Narrow filled bars represent 90° pulses, rectangular 180° pulses are shown as wide open bars. All pulse lengths are at 800 MHz. Gradients and selective 180° pulses (^{13}C Q3 400 μs, on- or off-resonant and ^{15}N Reburp [Geen & Freeman, 1991] 1.2 ms) are shown as semi-elliptic unfilled shapes. Semi-elliptic narrow filled shapes represent selective 90° pulses (^{13}C Q5 600 μs, on resonant). The default phase is *x*. ^{15}N nuclei were decoupled using asynchronous GARP4 sequences (Shaka et al., 1985) during acquisition. Gradient pulses were applied for 1 ms with smoothed square amplitude (SMSQ10.100) and 100% gradient strength corresponds to 53 G/cm. Pulse phases, delays, and gradient strengths are as follows: $\varphi_1 = x, -x$, $\varphi_2 = (4)x, (4)(-x)$, $\varphi_3 = (2)x, (2)(-x)$, $\varphi_4 = (8)x, (8)(-x)$, $\varphi_{rec} = x, (2)(-x), x, -x, (2)x, (2)(-x), (2)x, -x, x, (2)(-x), x, -x, (2)x, -x, x, (2)(-x), (2) x, (2)(-x), x, -x, (2)x, -x$, $\Delta = 1/2^1J_{CN} = 19.23$ ms, $\Delta_2 = 1/^1J_{CH} = 6.25$ ms, $t_m = 150$ ms, $T = 1/4^1J_{CC} = 4.5$ ms (cytidine) or 3.3 ms (adenosine) and 35% (g_1). The DIPSI-3 scheme was applied for 7.8 ms at 3.6 kHz. (**B**) Schematic magnetization transfer pathway for the "amino"-NOESY experiment with optional ^{13}C filter. Scalar coupling constants and the detected coherences are annotated. If not stated otherwise the transfer method is INEPT. The figure has been adapted from Schnieders et al., 2019. NOESY, nuclear Overhauser effect spectroscopy; IPAP, in-phase anti-phase; INEPT, insensitive nuclei enhancement by polarization transfer.

Especially, inter-residual H1′-to-amino-group contacts are of high interest and can improve RNA structure calculations where a limited number of NOE contacts is available. We will describe how to set up two variants of this "amino"-NOESY experiment, one with and one without a ^{13}C filter, in this basic protocol. The experiment with ^{13}C filter only selects for NOE contacts to ^{13}C-bound protons, while the other one is not selective.

Materials

≥0.5 mM RNA sample with ^{13}C,^{15}N-labeled amino group containing nucleotide of interest (G, C, or A) or uniformly ^{13}C,^{15}N-labeled RNA (see Basic Protocol 1)
NMR buffer (see recipe)
Other RNA specific requirements like Mg^{2+} ions or small molecule ligands

NMR tube (e.g., Shigemi)
NMR spectrometer equipped preferably with a *z*-axis gradient ^{13}C, ^{15}N [^{1}H]-TXO cryogenic probe; alternatively a *z*-axis gradient ^{1}H, [^{13}C, ^{15}N]-TCI cryogenic probe

1. Conduct steps 1-3 from Basic Protocol 5.

2. Load parameter set C_NOESYIASQSPCPXF with pulse sequence c_noesyiasqspcp or if only contacts to ^{13}C-bound protons are desired, load parameter set C_NOESYIASQSPCPXF with pulse sequence c_noesyiasqspcpxf.

3. Set selective pulses, CN-transfer delay, and IPAP delay as determined in the CN-HSQC experiment.

4. Set NOESY mixing time (D8).

   We typically use mixing times around 150 ms for this experiment.

5. Set HN-TOCSY pulses.

   Here, we use TOCSY pulses with medium selectivity (72 µs at 800 MHz).

6. Set carrier frequency and spectral window in the indirect dimension depending on the NOE contacts of interest.

   If no ^{13}C filter is applied, imino protons have to be covered by the spectral window. In our hands a carrier frequency of 8.7 ppm with a spectral window of 12 ppm in the ^{1}H dimension worked well, but those parameters should be adapted to the RNA sample of interest. If the ^{13}C filter is included, the spectral window can be reduced to only cover aromatic and ribose protons, e.g., 5.5 ppm spectral window with a carrier frequency of 6 ppm.

7. Set number of transients and number of points.

   Be careful to conduct the experiment with a sufficient number of transients because this experiment has a limited sensitivity. We use, for example, 128 transients for the standard "amino"-NOESY experiment and 256 transients for the one with ^{13}C filter for a 960 µM, 34-nt long RNA. Resolution in the ^{1}H dimension should be as high as possible. We conducted the experiments with a resolution of ∼40 Hz.

8. Start measurement and check whether the S/N is sufficient after a couple of increments.

9. Process spectra according to Support Protocol 8.

10. Assign NOESY spectrum (Fig. 27) using CN-HSQC, ^{15}N-HSQC, ^{1}H,^{1}H-NOESY, and HNCO-type spectra.

11. Include the newly determined NOE contacts in a structure calculation.

    Note that signal intensities do not only depend on the proton-proton distances but also on transfer efficiencies and the rotational frequency around the C-NH$_2$ bond. Therefore,
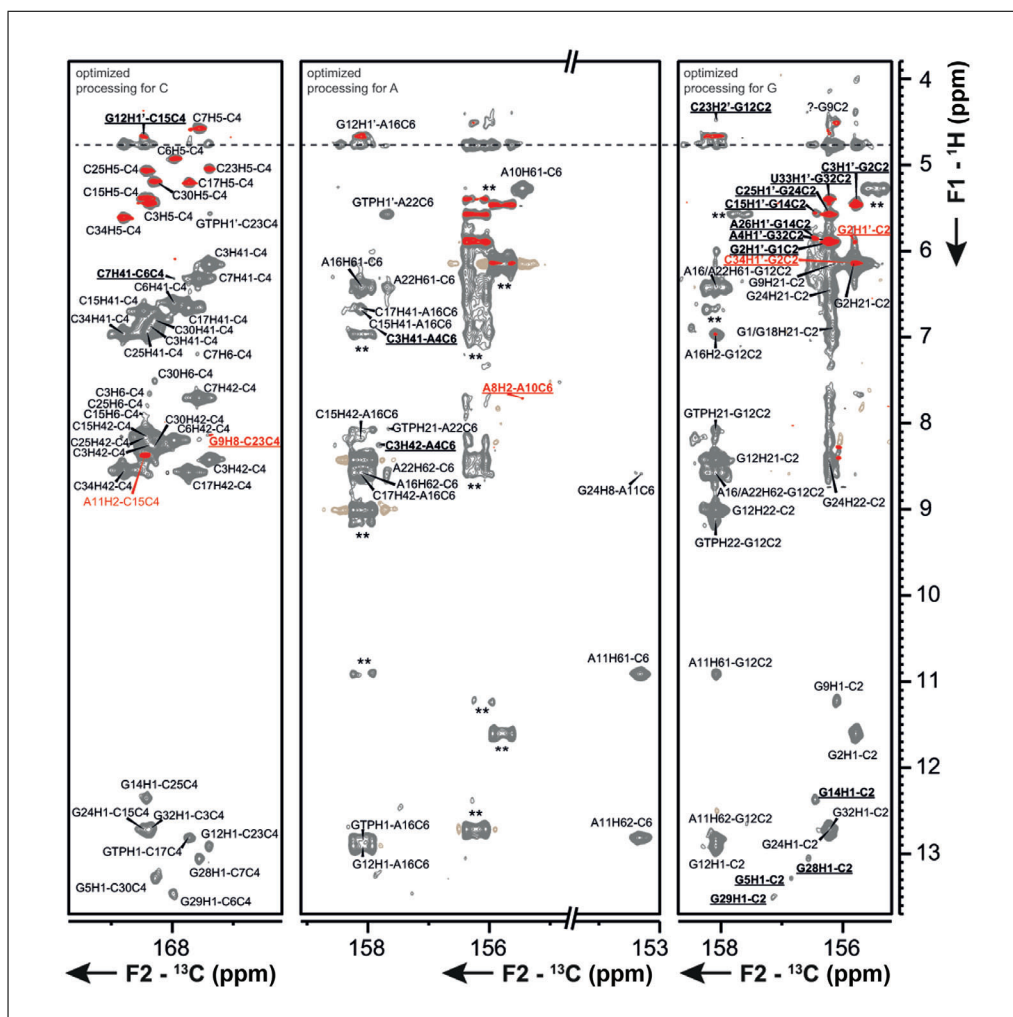
Figure 27 Overlay of "amino"-NOESY spectra of the 34-nt GTP class II aptamer (Wolter et al., 2016, 2017) with (red) and without (gray) ^{13}C filter. Newly obtained NOE contacts are written in bold and are underlined. NOE contacts, which are obtained from the "amino"-NOESY with ^{13}C filter are written in red. Signals marked with ** appear in the spectra with optimized processing for adenosines and guanosines as pseudo doublets due to processing and arise from guanosines and adenosines, respectively. The dashed line marks the water resonance. The "amino"-NOESY experiment without ^{13}C filter (gray) was recorded with 128 scans per increment and spectral widths of 70 ppm (1,024 complex points) and 12 ppm (448 complex points) in the direct and indirect dimensions, respectively. Carrier frequencies were 160 ppm (^{13}C), 8.65 ppm (^{1}H), and 85 ppm (^{15}N). With an inter-scan delay of 1.2 s the experiment was recorded for 2 days. The "amino"-NOESY experiment with ^{13}C filter (red) was recorded with 256 scans per increment and spectral widths of 70 ppm (1,024 complex points) and 5.4 ppm (208 complex points) in the direct and indirect dimensions, respectively. Carrier frequencies were 160 ppm (^{13}C), 6 ppm (^{1}H), and 85 ppm (^{15}N). With an inter-scan delay of 1 s the experiment was recorded for 1 day 15 hr. The spectra for C, A, and G are processed differently according to their $^1J_{CC}$ coupling constants. The figure was taken from Schnieders et al., 2019. NOESY, nuclear Overhauser effect spectroscopy.

> *NOE contacts can only be determined qualitatively. We assumed that the maximum distance is 6.5 Å and thus included the contacts with this upper distance limit in the structure calculation.*

^{15}N-DETECTED BEST-TROSY EXPERIMENT

Despite the disadvantage in loss of sensitivity, ^{15}N-direct detection can become interesting due to the favorable relaxation behavior of ^{15}N-nuclei when it comes to molecules with a large rotational correlation time (Fig. 28). Therefore, several ^{15}N-detected HN correlation experiments were tested on RNA and the effect of the molecular size on
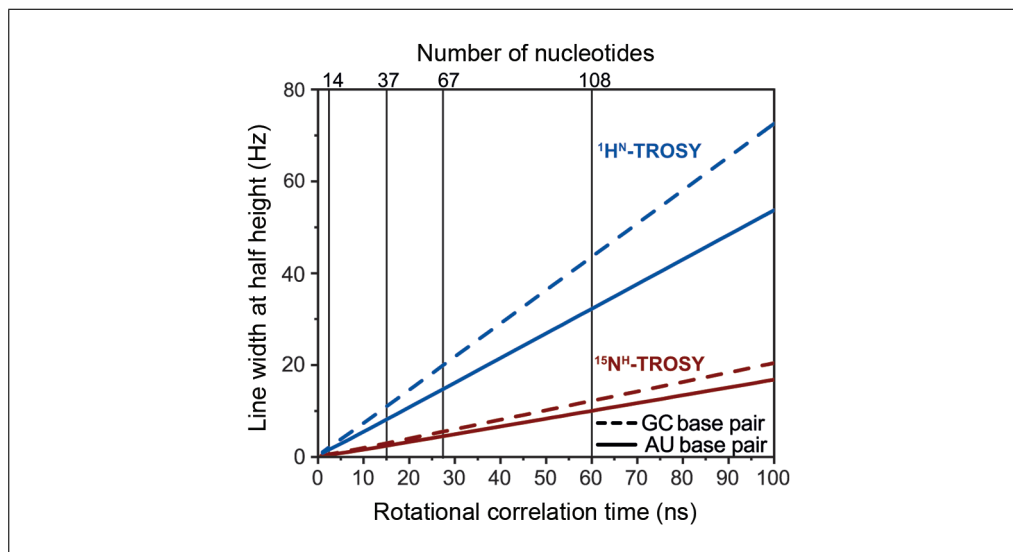
Figure 28 Simulation of the peak width at half height in dependence of the rotational correlation time for $^1H^N$-TROSY and $^{15}N^H$-TROSY components. Calculations were conducted separately for GC and AU Watson-Crick base pairs. Rotational correlation times for a 14-nt (pdb 2KOC; Nozinovic et al., 2010), a 37-nt (2LHP; Ziegeler, Cevec, Richter, & Schwalbe, 2012), a 67-nt (2NC1; Imai, Kumar, Hellen, Souza, & Wagner, 2016), and a 108-nt (2NBX; Imai et al., 2016) long RNA were determined from pdb structures using HYDRONMR (de la Torre, Huertas, & Carrasco, 2000). TROSY, transverse relaxation optimized spectroscopy.

sensitivity, resolution, and relaxation behavior was investigated. The most sensitive ^{15}N-detected HN correlation experiment, the ^{15}N-detected BEST-TROSY experiment, is described in this basic protocol.

Materials

> ≥0.5 mM RNA sample with ^{15}N-labeled imino group containing nucleotide of
> interest (G or U) or uniformly ^{15}N-/ $^{13}C,^{15}N$-labeled RNA (see Basic Protocol 1)
> NMR buffer (see recipe)
> Other RNA specific requirements like Mg^{2+} ions or small molecule ligands

> NMR tube (e.g., Shigemi)
> NMR spectrometer equipped preferably with a z-axis gradient ^{13}C, ^{15}N [^1H]-TXO
> cryogenic probe; alternatively, a z-axis gradient ^1H, [^{13}C, ^{15}N]-TCI cryogenic
> probe

1. Prepare spectrometer according to Support Protocol 7.

2. Record a ^1H-detected ^1H,^{15}N-BEST-TROSY reference spectrum in order to check the quality of the NMR sample and to determine carrier frequencies and spectral windows for the ^{15}N-detected BEST-TROSY experiment.

3. Load parameter set N_HNBTROSY_F2IG_TS21 with pulse sequence n_hnbtrosy_f2ig_ts21.

4. If the sample is not ^{13}C-labeled, leave out the carbon decoupling.

5. Set shapes and lengths for the selective pulses.

   We use Reburp, Eburp2, and Eburp2.tr shapes (Geen & Freeman, 1991) for sp26, sp28, and sp29, respectively. Pulse lengths of 1,050 μs (Reburp) and 1,275 μs (Eburp2) work well in our hands at 800 MHz.

6. Set carrier frequencies and spectral windows as determined in the ^1H-detected BEST-TROSY experiment. Note that the resolution in the direct dimension should be as high as possible due to the very sharp lines in the ^{15}N-dimension.
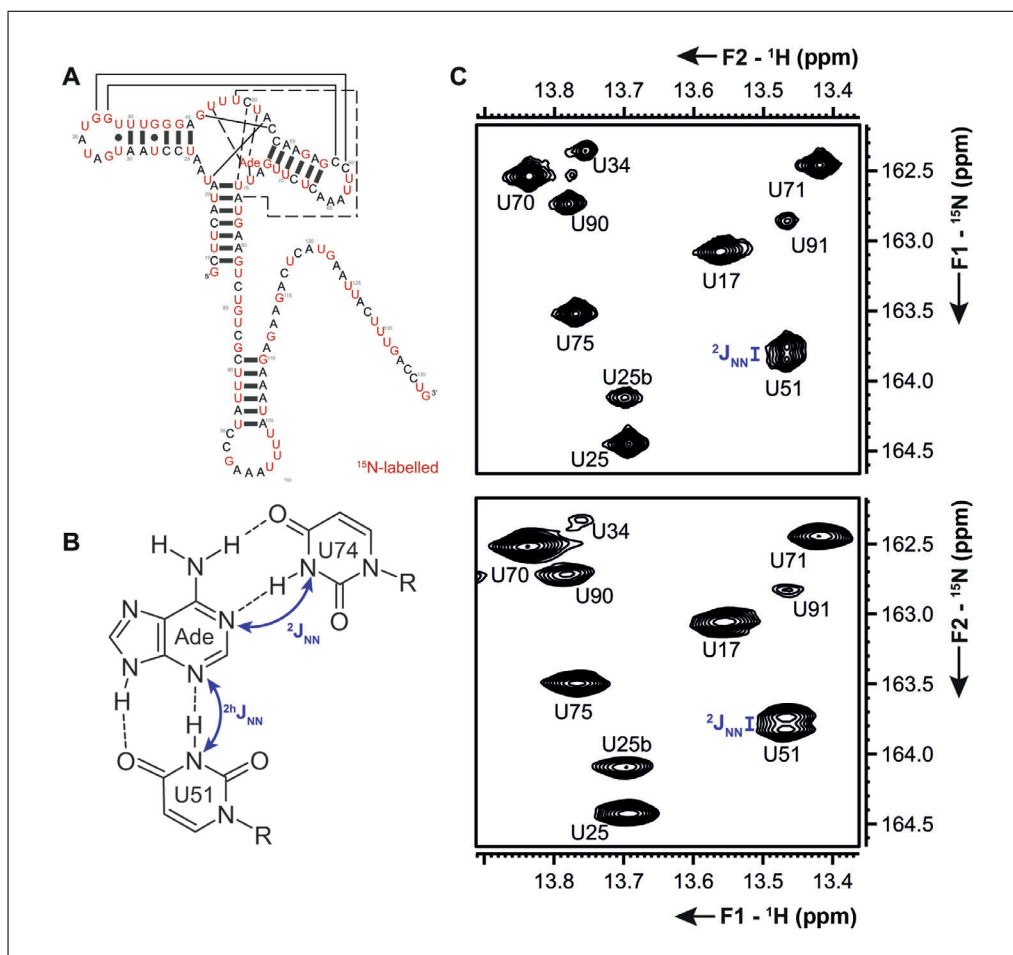
Figure 29 (**A**) Secondary structure of the 127-nt adenine-sensing riboswitch in the ligand-bound holo conformation (Reining et al., 2013). (**B**) Schematic representation of the binding pocket with residues U51 and U74 (Serganov et al., 2004). The $^{2h}J_{NN}$ couplings across the hydrogen bonds are marked. (**C**) Extract from a ^{1}H-detected (top) and a ^{15}N-detected (bottom) BEST-TROSY spectrum of the 127-nt RNA in the presence of 5 mM Mg^{2+} and 2 equivalents of adenine at 298 K. The spectra were recorded on 800 MHz NMR spectrometers, one equipped with a TCI probe for the ^{1}H-detected experiment and the other one equipped with a TXO probe for the ^{15}N-detected BEST-TROSY experiment. Carrier frequencies for the ^{1}H-detected BEST-TROSY experiment were 4.7 ppm (^{1}H), 101 ppm (^{13}C), and 153 ppm (^{15}N), respectively. The spectral widths were 25 ppm (^{1}H) and 28 ppm (^{15}N), respectively. Rectangular pulses on ^{1}H, ^{13}C, and ^{15}N were applied with field strengths of 21.7, 22.3, and 7.6 kHz, respectively. The experiment was recorded with 64 number of transients and acquisition times of 0.3 s in the direct and 0.3 s in the indirect dimension. With a relaxation delay of 0.5 s, the duration of the experiment was 20 hr 30 min. The ^{15}N-detected BEST-TROSY experiment was conducted with carrier frequencies of 155 ppm, 120 ppm, and 12.175 ppm for ^{15}N, ^{13}C, and ^{1}H, respectively. The spectral widths were 35 ppm in the direct and 5.2 ppm in the indirect dimension. Rectangular pulses were carried out using field strength of 7.3 kHz (^{15}N), 21.9 kHz (^{13}C), and 21.3 kHz (^{1}H), respectively. The number of transients per increment were 576 and the relaxation delay was 0.2 s. The experiment was recorded for 20 hr with acquisition times of 0.3 s in the ^{15}N and 0.03 s in the ^{1}H-dimension. The figure was taken from Schnieders et al., 2017, 2020. BEST-TROSY, band-selective excitation short transient transverse-relaxation-optimized spectroscopy.

7. Set number of transients.

   We used, for example, 576 transients for a 0.5 mM, 127-nt long RNA adapting three conformations.

8. Start experiment and check whether the S/N is sufficient in the first increment (Fig. 29).

REAGENTS AND SOLUTIONS

Autoclave water prior to preparation of the respective solution. Sterile filter all solutions and buffers.

Agarose gel solution

ddH$_2$O
1× TAE
1%-1.5% (w/v) agarose
Store at room temperature for up to 1 year.

About 0.5 L is sufficient for one gel.

Coomassie staining solution

10% (v/v) ethanol
5% (v/v) acetic acid
0.0025% (w/v) Coomassie brilliant blue G250
0.0025% (w/v) Coomassie brilliant blue R250
Store at room temperature for up to 1 year.

1 L is sufficient for 10-20 gels.

DNA loading buffer, 5×

ddH$_2$O
40% (v/v) glycerol
0.1% bromophenol blue
0.1% xylene cyanol
Store at room temperature for up to 1 year.

1 ml is sufficient for 100-200 gel samples.

DNA running buffer (1× TAE)

ddH$_2$O
40 mM Tris base
20 mM acetic acid
10 mM EDTA
Mix and adjust pH to 8.0.
Store at 4°C for up to 1 year.
About 0.5 L is sufficient for one gel.

Denaturing PAGE gel solution

ddH$_2$O
1× TBE
8%-20% (v/v) acrylamide (Carl Roth, cat. no. A124.2)
7 M urea
To start polymerization add:
0.1% (w/v) ammonium persulfate (APS)
0.1% (v/v) tetramethylethylenediamine (TEMED; Carl Roth, cat.no. 2367.2)
Store at 4°C for up to 1 year.

About 10 ml is sufficient for one gel.

Denaturing PAGE running buffer (1× TBE)

ddH$_2$O
91 mM Tris base (Thermo Fisher Scientific, cat. no. BP1525)
2.7 mM EDTA (Carl Roth, cat.no. CN06.3)

89 mM boric acid (Merck, cat.no. B0394)
Store at 4°C for up to 1 year.

About 0.5 L is sufficient for one PAGE.

Denaturing RNA loading buffer

90% (v/v) formamide (Merck, cat. no. 47671)
10% (v/v) 10× TBE
0.1% bromophenol blue
0.1% xylene cyanol
Store at room temperature for up to 1 year.

1 ml is sufficient for 100-200 gel samples.

LB medium

5 g/L yeast extract
10 g/L tryptone
5 g/L NaCl
Autoclave 15 min at 121°C to sterilize. Allow to cool before making additions, such as antibiotics, if desired.
Store at room temperature for up to 1 year without antibiotics.

Native PAGE gel solution

ddH$_2$O
1× TA
10% (v/v) acrylamide
To start polymerization add:
0.1% (w/v) ammonium persulfate (APS)
0.1% (v/v) tetramethylethylenediamine (TEMED)
Store at 4°C for up to 1 year.
About 10 ml is sufficient for one gel.

Native PAGE gel solution for DNA

ddH$_2$O
1× TAE
8%-15% (v/v) acrylamide
To start polymerization add:
0.1% (w/v) ammonium persulfate (APS)
0.1% (v/v) tetramethylethylenediamine (TEMED)
Store at 4°C for up to 1 year.

10 ml is sufficient for one gel.

Native PAGE running buffer (1× TA)

ddH$_2$O
50 mM tris acetate
100 mM sodium acetate
pH 8.2
Store at 4°C for up to 1 year.

About 0.5 L is sufficient for one PAGE.

NMR buffer

ddH$_2$O
25 mM potassium phosphate buffer (K$_2$HPO$_4$/KH$_2$PO$_4$; Carl Roth, cat. no. P749.2/3904.1)

50 mM KCl (optional)
MgCl$_2$ (depending on folding protocol; optional)
pH 6.2
Store at room temperature for up to 1 year.

20-100 ml is sufficient for one RNA.

SDS resolving gel

8%-15% (v/v) acrylamide/bisacrylamide (29:1)
0.375 M Tris·HCl
0.1% (w/v) APS
0.1%(v/v) TEMED
Mix and adjust pH to 8.8
Store at 4°C for up to 1 year.

About 10 ml is sufficient for one gel.

SDS running buffer

25 mM Tris
250 mM glycine
1% (v/v) SDS
Store at room temperature for up to 1 year.

0.5 L is sufficient for one gel.

SDS sample buffer, 2×

0.25 M Tris·HCl
4% (w/v) SDS
20% (v/v) glycerol
10% (v/v) 2-mercaptoethanol
0.25% (w/v) bromphenol blue
Mix and adjust pH to 6.8
Store at −20°C for up to 1 year.

1 ml is sufficient for 100-200 gel samples.

SDS stacking gel

5% (v/v) acrylamide/bisacrylamide (29:1)
0.25 M Tris·HCl
0.1% (w/v) APS
0.1%(v/v) TEMED
Mix and adjust pH to 6.8
Store at 4°C for up to 1 year.

About 10 ml is sufficient for one gel.

T4 RNA Ligase 2 (Rnl2) buffer A

250 mM NaCl
50 mM Tris·HCl
10% (w/v) sucrose
0.1% (v/v) Triton X-100
Protease inhibitor cocktail (EDTA free)
Mix and adjust pH to 7.5.
Filter and degas. Add 1 mM DTT (reducing agent) directly prior to use; 1 L is
   sufficient for one purification.
Store at room temperature for up to 2 weeks.

T4 RNA Ligase 2 (Rnl2) buffer B

250 mM NaCl
50 mM Tris·HCl
10% (v/v) glycerol
500 mM imidazole
0.05% (v/v) Triton X-100
Mix and adjust pH to 8.
Filter and degas. Add 1 mM DTT (reducing agent) directly prior to use; 0.5 L is
    sufficient for one purification.
Store at room temperature for up to 2 weeks.

T4 RNA Ligase 2 (Rnl2) buffer C

100 mM KCl
20 mM Tris·HCl
70 mM $(NH_4)_2SO_4$
0.2 mM EDTA
Mix and adjust pH to 7.7.
Filter and degas; 1 L is sufficient for one purification.
Store at room temperature for up to 2 weeks.

T7 RNAP buffer A

400 mM NaCl
50 mM Tris·HCl
20 mM imidazole
Protease inhibitor cocktail (EDTA free)
Mix and adjust pH to 8.1.
Filter and degas.
Store at 4°C for 6 months.
Add 5 mM 2-mercaptoethanol (reducing agent) directly prior to use; 2 L is
    sufficient for one purification.

T7 RNAP buffer B

400 mM NaCl
50 mM Tris·HCl
500 mM imidazole
Protease inhibitor cocktail (EDTA free)
Mix and adjust pH to 8.1. Filter and degas.
Store at 4°C for 6 months.
Add 5 mM 2-mercaptoethanol (reducing agent) directly prior to use; 1 L is
    sufficient for one purification.

T7 RNAP buffer C

150 mM NaCl
20 mM Na_2HPO_4/NaH_2PO_4
1 mM EDTA
Mix and adjust pH to 7.7. Filter and degas.
Store at 4°C for 6 months.
Add 5 mM DTT (reducing agent) directly prior to use; 2 L is sufficient for one
    purification.

TB medium

24 g/L yeast extract
12 g/L tryptone
4 ml/L glycerol

17 mM KH$_2$PO$_4$

72 mM K$_2$HPO$_4$

Mix and adjust pH to pH 7.4.

Autoclave 15 min at 121°C to sterilize. Allow to cool before making additions, such as antibiotics, if desired.

Store at room temperature for up to 1 year without antibiotics.

YIPP buffer A

50 mM Tris·HCl

300 mM NaCl

10 mM imidazole

Mix and adjust pH to 8.0.

Store at 4°C for 6 months.

Filter and degas. Add 1 mM DTT (reducing agent) directly prior to use; 1 L is sufficient for one purification.

YIPP buffer B

50 mM Tris·HCl

300 mM NaCl

500 mM imidazole

Mix and adjust pH to 8.0.

Store at 4°C for 6 months.

Filter and degas. Add 1 mM DTT (reducing agent) directly prior to use; 0.5 L is sufficient for one purification.

YIPP buffer C

25 mM K$_2$HPO$_4$/KH$_2$PO$_4$ (e.g., Carl Roth, cat. no. P749.2/3904.1)

150 mM KCl

Mix and adjust pH to 7.2.

Store at room temperature for up to 1 year.

Filter and degas. Add 1 mM DTT (reducing agent) directly prior to use; 1 L is sufficient for one purification.

YIPP buffer D

50 mM Tris·HCl

100 mM KCl

0.1 mM EDTA

50% (v/v) glycerol

Mix and adjust pH to 8.0.

Store at room temperature for up to 2 weeks.

Filter and degas. Add 1 mM DTT (reducing agent) directly prior to use; 2 L is sufficient for one purification.

COMMENTARY

Background Information

Because this protocol covers a wide range of different methods from sample preparation to NMR experiments, the different historical backgrounds will be briefly summarized in the following sections.

Preparation of ^{13}C,^{15}N-labeled RNA

^{13}C,^{15}N-isotope labeling is usually inevitable for NMR structure determination and often also required if functional studies are conducted. There are two methods, which are used for these purposes, namely solid phase synthesis and in vitro transcription using T7 RNA polymerase. Solid phase synthesis requires phosphoramidites and was first conducted to yield an isotope-labeled RNA in 1994 using ^{13}C-labeled building blocks (Quant et al., 1994). As this method is limited in molecular size and needs a costly apparatus,

the in vitro transcription with T7 RNA polymerase represents a commonly used alternative. This enzymatic method can be conducted using ^{13}C,^{15}N-labeled rNTPs, which was first reported in 1992 (Batey, Inada, Kujawinski, Puglisi, & Williamson, 1992; Nikonowicz et al., 1992). The isotope-labeled rNTPs are commercially available nowadays.

Synthesis of selectively labeled RNA

Due to the poor chemical diversity in building blocks, the increased amount of resonances and the signal broadening, NMR spectra of RNAs >50 nt usually exhibit severe resonance overlap. To avoid this, methods to segmentally or selectively label RNAs are being developed either using solid phase synthesis or enzymatic methods. As reported in 1996, the segmental deuterium labeling of an RNA via solid phase synthesis was crucial for the assignment of the respective NMR spectra (Földesi, Yamakage, Nilsson, Maltseva, & Chattopadhyaya, 1996). However, the size limitation brought along with this approach can be negotiated and/or avoided by applying enzymatic methods. Segmental isotopic labeling with T4 DNA ligase was first performed in 1996 and yielded a partially ^{15}N-labeled RNA (Xu, Lapham, & Crothers, 1996). To improve this approach to an extent where the site specific labeling or modification of an RNA is possible, in 2018 a chemo-enzymatic pathway was used to implement ^{13}C,^{15}N-labeled nucleosides as well as nucleosides provided with photoactivatable groups and azobenzene (Keyhani et al., 2018).

Heteronuclear-detected NMR experiments for RNA

Heteronuclear-detected NMR experiments are nowadays possible as there has been a constant development in cryogenic probes (Kovacs, Moskau, & Spraul, 2005) as well as probes optimized for heteronuclear detection schemes. The era of heteronuclear-detected multidimensional NMR experiments for RNA started in 2007 with two almost simultaneously published articles by Farès, Amata, and Carlomagno (2007) and by Fiala and Sklenár (2007). Both works develop NMR experiments for the chemical shift assignment of the quaternary carbons in the nucleobases of RNA. In ^{1}H-detected NMR experiments these resonances are only accessible employing many transfer steps, which in turn reduces sensitivity, particularly for larger RNA molecules. Farès et al. (2007) developed a set of ^{1}H-excited and ^{13}C-detected NMR experiments with reduced transfer times for the carbon chemical shift assignment of the nucleobase. Furthermore, they revealed a correlation of several nucleobase carbon chemical shifts to the structural context (base pairing, stacking) of the respective nucleotide. Fiala and Sklenár (2007) developed a set of experiments for the measurement of J_{CC} coupling constants. Furthermore, they presented a ^{13}C-excited and ^{13}C-detected ^{13}C,^{15}N-HSQC experiment that can be used for an almost complete resonance assignment of the nucleobase through a correlation of the C-N fragments, while completely evading long transfer times. Therefore, this experiment is commonly applied for the nucleobase assignment of RNA (Wolter et al., 2019).

Critical Parameters

Basic Protocol 1: Preparation of isotope-labeled RNA

Probably the most important parameter in the production of RNA is to work with very clean equipment and reagents to avoid RNase contamination. If possible, sterile products should be used. Wherever this is not feasible, reagents, consumables, and flasks should be autoclaved or treated with 0.1% DEPC solution. Along the same lines, all home-made or purchased enzymes should not only be tested for functionality but also for RNase contamination on a test scale prior to usage. However, degradation is not only a problem during the sample preparation but also afterwards. Thus, RNAs should be stored at low temperatures (4°C) and checked for degradation in the case of long storage times. Furthermore, when the preparation of an isotope-labeled RNA is planned, the scale-up of the transcription reaction should first be conducted using unlabeled rNTPs to check whether the final yield is sufficient.

Basic Protocol 2: Preparation of site-specific labeled RNAs

In addition to the critical parameters listed above for Basic Protocol 1, the synthesis of 3′,5′-bisphosphate nucleosides for Basic Protocol 2 requires the corresponding labeled or modified starting material. Here the purity and integrity of each reagent should be checked prior to use via NMR measurements and mass spectra. Furthermore, the laboratory should be equipped for working under a protective atmosphere as the synthesis of the nucleoside 3′,5′-bisphosphate has to be performed with the exclusion of water.

Table 17 Troubleshooting for Support Protocol 2

Problem	Possible reasons	Solution
Product smear on the analytical gel/no clean band on the analytical gel	Too much template used Many side products	Use less template or dilute template (1:10; 1:100; 1:1000) Use fewer cycles
Low product yield	Specificity of primer binding is low GC rich sequence DNA is too long or too complex	Optimize annealing temperature Optimize MgCl$_2$ concentration (purchased PCR buffer may contain MgCl$_2$ already) Try GC buffer for GC rich sequences, long templates, or complex structures
No product at all	PCR does not work	Check primer and template sequences Check PCR program for the cycler and order of steps

Basic Protocols 3–7: Heteronuclear-detected NMR experiments on RNA

As the major drawback in heteronuclear-detected NMR experiments is the low sensitivity, it is very important to conduct the experiments under the best possible conditions. A parameter which can be easily controlled is the RNA concentration, which should be as high as possible, as the S/N scales directly with the sample concentration. In addition, the NMR spectrometer that is used for heteronuclear detection should at least be equipped with a cryogenic probe and best with a cryogenic probe optimized for heteronuclear detection. Along the same lines, the setup of the spectrometer should be always performed thoroughly, as the sensitivity drops with inaccurate settings.

Troubleshooting

For troubleshooting the protocols contained in this article, see Table 17 (Support Protocol 2), Table 18 (Support Protocols 3, 4, and 6), Table 19 (Alternate Protocol 2), Table 20 (Basic Protocol 2), Table 21 (Support Protocol 5), and Table 22 (Basic Protocols 3, 4, 5, 6, and 7).

Understanding Results

Basic Protocol 1

The analysis of the test transcriptions demonstrates which condition is ideal for the RNA to be transcribed at. The respective PAGE gel should show a band with the size of the target RNA and optimization should lead to a maximum of band intensity. It is favorable to optimize the byproducts to a low band intensity albeit it is not essential in case of eventual purification by HPLC or preparative PAGE (Basic Protocol 1 and Alternate Protocol 1). The transcription conditions have to result in a sharp band for the target RNA without any byproducts if the fast purification via centrifugal concentrator is used (Alternate Protocol 2). The preparative transcription and purification should finally yield 60 to 300 nmol in about 300 μl, depending on the type of labeling and the RNA sequence.

Support Protocol 1

The concentration of extracted DNA plasmid can be easily quantified via UV/vis absorption at 260 nm. Because the isolation procedure of plasmid DNA from bacterial cells can be performed with a highly optimized commercially available kit, the expected yields can range from 2 to 8 mg DNA per liter LB medium. Variability in the yield can occur based on the copy number of the specific plasmid used and the total amount of cells grown. The efficiency of the restriction digestion, which is analyzed visually via agarose gel, can be expected to give quantitative amounts of linearized plasmid. With careful execution, only ∼5% to 10% of DNA will be lost in the subsequent extraction.

Support Protocol 2

The exact amount of DNA after a successful preparative PCR is not determinable because the DNA must not be purified for further transcription reactions. But if necessary, it is possible to estimate the amount of DNA via a native PAGE. One may do so by adding a standard DNA ladder to the PAGE. An average amount of DNA amplified by preparative PCR is 1 nmol (about 60 μg of a 100-bp DNA). A native PAGE further will show if the PCR results in pure target product or if

Table 18 Troubleshooting for Support Protocols 3, 4, and 6

Problem	Possible reasons	Solution
Cells grow slowly/poorly	Wrong medium composition Initial OD significantly lower than 0.1 Cryostock cells dead Cold medium might slow cell growth Wrong plasmid/antibiotic Insufficient aeration	Check recipe Check OD at beginning Test expression on small scale (5 ml) and new transformation, if necessary Preheat medium to expression temperature Check plasmid (sequencing) Increase shaking, add antifoaming agent
Low protein yield despite good cell growth	No protein expression Protein degradation/export Incomplete cell lysis	Check expression vector/IPTG stock, check aeration or foaming of medium Lower expression time/temperature Extend homogenization cycles
No protein after column (Ni-NTA or SEC)	Protein did not bind to column Wrong buffer composition (buffers swapped)	Regenerate column, check flowthrough Check recipe/prepare new buffers
Bad separation on column or protein band in flowthrough	Column binding capacity exceeded Column material old/used Equilibration step not done thoroughly Broken column	Use column with larger bed volume Regenerate column Perform all equilibration steps as described in the protocol Regenerate column/use a new one
Protein band runs high in SDS gels	Aggregation/dimerization	Check if reducing agent was added to buffer Ensure temperature is at 4°C
Protein inactive/unstable after storage	Wrong pH or salt concentration/no glycerol in storage buffer Protein was stored too long at room temperature	Check recipe/measure pH Store at −20°C

Table 19 Troubleshooting for Alternate Protocol 2

Problem	Possible reasons	Solution
Slow flowthrough per centrifugation step	High RNA concentration	Increase temperature to 15°C during centrifugation and mix solution over membrane with a pipet In case of further problems see Troubleshooting in the device manual

byproducts are present. The PCR should be optimized to yield a maximum band intensity of target product and a minimum band intensity of byproduct. In this case, the byproduct does not have to be removed if it is not transcribed or if RP-HPLC or a preparative PAGE are applied subsequently.

Support Protocol 3

With a protein yield of ~4 to 8 mg of pure T7 RNA polymerase per liter medium, this protocol provides an easy and cost-effective alternative to the commercially available enzyme. Enzyme concentration can be measured via UV/vis absorption at 280 nm and enzyme activity should be tested in analytical scale transcriptions. Even after 1.5 hr of transcription incubation, an analytical RNA PAGE should show significant amounts of synthesized RNA.

Support Protocol 4

The protein purification protocol described here provides the possibility to express and purify highly active YIPP for in vitro transcription of RNA. Expected yields are 35

Table 20 Troubleshooting for Basic Protocol 2

Problem	Possible reasons	Solution
Ligation efficiency is low	Too low/high ATP concentration Low 3′,5′-bisphosphate concentration T4 RNA Ligase 1 is inactive	Adjust ATP concentration to 1 mM Use larger excess of 3′,5′-bisphosphate Verify ligase activity; purchase a new T4 RNA Ligase 1 stock, if necessary
Oxidation with $NaIO_4$ does not work	DTT was not completely removed $NaIO_4$ is inactive	Remove DTT by centrifugal concentrator Prepare a fresh $NaIO_4$ solution from a new stock
RNA degradation during splint ligation with T4 RNA Ligase 2	Degradation is construct dependent RNase contamination	Check solutions and components for an RNase contamination
RNA degradation during DNase digestion	RNA digestion by DNase	Shorter incubation time, RNase contamination of DNase stock

Table 21 Troubleshooting for Support Protocol 5

Problem	Possible reasons	Solution
3′,5′-bisphosphate product is not detectable in mass spectrum after HPLC purification	Byproducts are not separated completely The reaction temperature was not constant Reaction apparatus was not dry Diphosphoryl chloride is hydrolyzed	Perform a test ligation, only 3′,5′-bisphosphate nucleosides will be incorporated by T4 RNA Ligase 1 Make sure reaction temperature is $-12°C \pm 2°C$ Heat reaction apparatus (drying oven) Use a new stock of diphosphoryl chloride or try a larger excess (15 equivalents)

Table 22 Troubleshooting for Basic Protocols 3-7

Problem	Possible reasons	Solution
IPAP decoupling is not working	Pulse length incorrect Carrier frequency offset incorrect Delay incorrect Processing	Check with Table 13, calculate band width of excitation Recheck chemical shifts of off- and on-resonant ^{13}C nuclei. Do pulses hit all desired nuclei? Recheck $^1J_{CC}$ coupling; if that is correct, measure $^1J_{CC}$ coupling in a coupled ^{13}C-1D Sometimes AP spectrum needs scaling with regard to IP spectrum; change scaling factor and see whether the result improves
Insufficient S/N	NMR spectrometer not sensitive enough for ^{13}C detection General settings incorrect Experiment settings incorrect RNA concentration not sufficient	Change to a spectrometer with higher sensitivity for ^{13}C nuclei Check general set up and run a CN-HSQC experiment to test the sensitivity Recheck all delays and pulses; record the first increment with a high number of transients (e.g., 2,048) and check for signal Increase RNA concentration, if possible; otherwise increase number of transients and if this is not feasible, the experiment might not be sensitive enough for the current experimental set up

to 40 mg of protein per 1 liter LB medium, which is enough for 29 to 33 ml of ready to use stock solution. The purity of the protein can be analyzed via SDS-PAGE or analytical FPLC. The protein activity can be tested via small scale in vitro transcription with transcription buffer as a reference. In the case of an active protein, the transcription reaction should be a clear solution in contrast to the cloudiness of the reference sample.

Basic Protocol 2

After the ligation with a modified nucleotide, the small acceptor RNA (RNA 1; <17 nt) will move faster during the gel electrophoresis because of the additional negative charge of the 3′-end phosphate group. A dephosphorylated RNA will move more slowly during the gel electrophoresis because of the additional nucleotide without a 3′-end phosphate group. For a larger acceptor RNA (>17 nt) no difference will be visible in the gel electrophoresis. The splint ligation with the donor RNA (RNA 2) will show a significantly larger RNA product, which should be detectable via gel electrophoresis.

Support Protocol 5

The yield depends on the type and position of the modification. The modified nucleoside 3′,5′-bisphosphate is characterized by mass spectrometry and NMR spectroscopy. Byproducts like 2′,5′-bisphosphate, mono- or triphosphate nucleosides often cannot be separated via HPLC. In case of characterization difficulties, a test ligation should be performed with T4 RNA Ligase 1 and a short acceptor RNA (<17 nt). A positive ligation result will indicate that the synthesis was successful. Byproducts will not be accepted by the T4 RNA Ligase 1.

Support Protocol 8

The implementation of an IPAP/DIPAP sequence should always lead to a completely decoupled singlet signal of the desired carbon atom.

Basic Protocol 3

The (H)CC-TOCSY experiment yields a spectrum in which the C1′ atoms are correlated to all other carbon atoms of the same ribose ring. This information can be used for a sequential assignment in the (H)CPC and (H)CPC-CCH-TOCSY experiments. In the (H)CPC spectrum, the ^{31}P nucleus is correlated to C5′ and C4′ in 3′ as well as to C3′ and C4′ in the 5′ direction. The (H)CPC-CCH-TOCSY experiment yields correlations between the ^{31}P nucleus and both of the C1′ atoms of the adjacent ribose moieties. Thus, by combining the information obtained in the three experiments, a full resonance assignment of the ribose carbon atoms can be obtained.

Basic Protocol 4

Using the CN-spinfilter HSQC information, the status of hydrogen bonding can be obtained. This information can be extracted from the signal intensities of the imino nitrogen resonances in the C-N correlated spectrum. In case of hydrogen bonding, the sign of the corresponding resonance is inverted as opposed to the remaining resonances. If the imino proton is not involved in hydrogen bonding, the resonance stays unperturbed. Consequently, it might happen that resonances of nucleobases in rather unstable hydrogen bonding do not give rise to a signal as they are at the zero crossing.

Basic Protocol 5

Using the C(N)H-HDQC experiment, a C-H correlated spectrum should be obtained. This spectrum should yield a sharp resonance for each guanosine, adenosine, and cytidine in the sequence. The experiment usually works most reliably for guanosines.

Support Protocol 9

In the amino-selective CN-HSQC spectrum, C-N correlations of all amino groups are obtained. If a correlation is not visible using this experiment (for possible reasons see Troubleshooting), neither will it be in the C(N)H-HDQC spectrum.

Basic Protocol 6

In the C-H correlated "amino"-NOESY spectrum, contacts to spatially close protons or ^{13}C-bound protons (filtered version) and amino groups are detected. In the version without carbon filter, broad single quantum diagonal peaks might appear. In helical parts of an RNA, inter-residual correlations between guanosine amino groups and preceding as well as cross-strand H1′ protons are expected. Correlations might be used in structure calculations as upper distance restraints (6.5 Å).

Basic Protocol 7

The ^{15}N-detected BEST-TROSY experiment yields analogous information as compared to its ^{1}H-detected counterpart. Thus, one resonance for each imino proton, which is sufficiently protected from fast solvent exchange, is expected.

Table 23 Required Time for Execution of Different Protocols

Basic Protocol 1	10 days
Alternate Protocol 1	3 days
Alternate Protocol 2	1-2 days
Support Protocol 1	4-5 days
Support Protocol 2	1 day
Support Protocol 3	3-4 days
Support Protocol 4	5-7 days
Basic Protocol 2	8-9 days
Support Protocol 5	7-10 days
Support Protocol 6	5-7 days
Support Protocol 7	1-2 hr
Support Protocol 8	Depends on the experiment in which the IPAP sequence is embedded
Basic Protocol 3	1 day ((H)CC-TOCSY), $\sim$ 1.5 day ((H)CPC), $\sim$2.5 days ((H)CPC-CCH-TOCSY)
Basic Protocol 4	Depends on sample concentration and RNA length (e.g., for 500 μM 14-nt RNA $\sim$1.5 days per experiment)
Basic Protocol 5	Depends on sample concentration and RNA length (e.g., for 500 μM 14-nt RNA $\sim$20 hr)
Support Protocol 9	$\sim$30 min per 1D, depending on sample concentration and RNA length (e.g., for 980 μM 34-nt RNA $\sim$10 hr per experiment)
Basic Protocol 6	Depends on sample concentration and RNA length (e.g., for 980 μM 34-nt RNA $\sim$1 day and 15 hr per experiment)
Basic Protocol 7	Depends on sample concentration and RNA length (e.g., for 500 μM 14-nt RNA $\sim$12 hr per experiment)

Time Considerations

See Table 23 for times required to perform the protocols included in this article.

Acknowledgments

Author Contributions

Robbin Schnieders: Conceptualization; data curation; formal analysis; resources; visualization; writing-original draft; writing-review & editing. **Bozana Knezic:** Conceptualization; data curation; visualization; writing-original draft; writing-review & editing. **Heidi Zetzsche:** Conceptualization; data curation; visualization; writing-original draft; writing-review & editing. **Alexey Sudakov:** Conceptualization; data curation; visualization; writing-original draft; writing-review & editing. **Tobias Matzel:** Conceptualization; data curation; visualization; writing-original draft; writing-review & editing. **Christian Richter:** Conceptualization; data curation; resources; visualization; writing-original draft; writing-review & editing. **Martin Hengesbach:** Conceptualization; data curation; funding acquisition; methodology; writing-original draft; writing-review & editing. **Harald Schwalbe:** Conceptualization; funding acquisition; methodology; project administration; writing-review & editing. **Boris Fürtig:** Conceptualization; data curation; funding acquisition; methodology; resources; visualization; writing-original draft; writing-review & editing.

Literature Cited

Altuvia, S., Kornitzer, D., Teff, D., & Oppenheim, A. B. (1989). Alternative mRNA structures of the c111 gene of bacteriophage lambda determine the rate of its translation initiation. *Journal of Molecular Biology*, *210*, 265–280. doi: 10.1016/0022-2836(89)90329-X.

Alvarado, L. J., Leblanc, R. M., Longhini, A. P., Keane, S. C., Jain, N., Yildiz, Z. F., … Dayie, T. K. (2014). Regio-selective chemical-enzymatic synthesis of pyrimidine nucleotides facilitates RNA structure and dynamics studies. *ChemBioChem*, *15*, 1573–1577. doi: 10.1002/cbic.201402130.

Andrus, A., & Kuimelis, R. G. (2000). Polyacrylamide gel electrophoresis (PAGE) of synthetic nucleic acids. *Current Protocols in Nucleic Acid Chemistry*, 10.4.1–10.4.10. doi:10.1002/0471142700.nc1004s01.

Barrio, J. R., Barrio, M., del, C. G., Leonard, N. J., England, T. E., & Uhlenbeck, O. C. (1978). Synthesis of modified nucleoside 3′,5′-bisphosphates and their incorporation into oligoribonucleotides with T4 RNA ligase. *Biochemistry*, *17*(11), 2077–2081. doi: 10.1021/bi00604a009.

Batey, R. T., Inada, M., Kujawinski, E., Puglisi, J. D., & Williamson, J. R. (1992). Preparation of isotopically labeled ribonucleotides for multidimensional NMR spectroscopy of RNA. *Nucleic Acids Research*, *20*(17), 4515–4523. doi: 10.1093/nar/20.17.4515.

Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., … Bourne, P. E. (2000). The protein data bank. *Nucleic Acids Research*, *28*(1), 235–242. doi: 10.1093/nar/28.1.235.

Butcher, S. E., Allain, F. H. T., & Feigon, J. (2000). Determination of metal ion binding sites within the hairpin ribozyme domains by NMR. *Biochemistry*, *39*(9), 2174–2182. doi: 10.1021/bi9923454.

Carlomagno, T. (2014). Present and future of NMR for RNA–protein complexes : A perspective of integrated structural biology. *Journal of Magnetic Resonance*, *241*, 126–136. doi: 10.1016/j.jmr.2013.10.007.

Davis, J. H., Tonelli, M., Scott, L. G., Jaeger, L., Williamson, J. R., & Butcher, S. E. (2005). RNA helical packing in solution: NMR structure of a 30 kDa GAAA tetraloop-receptor complex. *Journal of Molecular Biology*, *351*(2), 371–382. doi: 10.1016/j.jmb.2005.05.069.

de la Torre, J. G., Huertas, M. L., & Carrasco, B. (2000). HYDRONMR: Prediction of NMR relaxation of globular proteins from atomic-level structures and hydrodynamic calculations. *Journal of Magnetic Resonance*, *147*, 138–146. doi: 10.1006/jmre.2000.2170.

Dethoff, E. A., Petzold, K., Chugh, J., Casiano-negroni, A., & Al-hashimi, H. M. (2012). Visualizing transient low-populated structures of RNA. *Nature*, *491*(7426), 724–728. doi: 10.1038/nature11498.

Diaz, G. A., Rong, M., McAllister, W. T., & Durbin, R. K. (1996). The stability of abortively cycling T7 RNA polymerase complexes depends upon template conformation. *Biochemistry*, *35*(33), 10837–10843. doi: 10.1021/bi960488+.

Duss, O., Lukavsky, P. J., & Allain, F. H. T. (2012). isotope labeling and segmental labeling of larger RNAs for NMR structural studies. *Isotope Labeling in Biomolecular NMR, Advances in Experimental Medicine and Biology*, *992*, 121–142. doi: 10.1007/978-94-007-4954-2.

Farès, C., Amata, I., & Carlomagno, T. (2007). ^{13}C-detection in RNA bases: Revealing structure-chemical shift relationships. *Journal of the American Chemical Society*, *129*(17), 15814–15823. doi: 10.1021/ja0727417.

Fiala, R., & Sklenár, V. (2007). ^{13}C-detected NMR experiments for measuring chemical shifts and coupling constants in nucleic acid bases. *Journal of Biomolecular NMR*, *39*(2), 153–163. doi: 10.1007/s10858-007-9184-4.

Földesi, A., Yamakage, S. I., Nilsson, F. P. R., Maltseva, T. V., & Chattopadhyaya, J. (1996). The use of non-uniform deuterium labelling ['NMR-window'] to study the NMR structure of a 21 mer RNA hairpin. *Nucleic Acids Research*, *24*(7), 1187–1194. doi: 10.1093/nar/24.7.1187.

Fürtig, B., Richter, C., Wöhnert, J., & Schwalbe, H. (2003). NMR spectroscopy of RNA. *ChemBioChem*, *4*(10), 936–962. doi: 10.1002/cbic.200300700.

Fürtig, B., Schnieders, R., Richter, C., Zetzsche, H., Keyhani, S., Helmling, C., … Schwalbe, H. (2016). Direct ^{13}C-detected NMR experiments for mapping and characterization of hydrogen bonds in RNA. *Journal of Biomolecular NMR*, *64*, 207–221. doi: 10.1007/s10858-016-0021-5.

Geen, H., & Freeman, R. (1991). Band-selective radiofrequency pulses. *Journal of Magnetic Resonance*, *93*(1), 93–141.

Guillerez, J., Lopez, P. J., Proux, F., Launay, H., & Dreyfus, M. (2005). A mutation in T7 RNA polymerase that facilitates promoter clearance. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(17), 5958–5963. doi: 10.1073/pnas.0407141102.

Hammarstroem, A., & Otting, G. (1994). Improved spectral resolution in ^{1}H NMR spectroscopy by homonuclear semiselective shaped pulse decoupling during acquisition. *Journal of the American Chemical Society*, *116*, 8847–8848. doi: 10.1021/ja00098a070.

Helmling, C., Keyhani, S., Sochor, F., Fürtig, B., Hengesbach, M., & Schwalbe, H. (2015). Rapid NMR screening of RNA secondary structure and binding. *Journal of Biomolecular NMR*, *63*, 67–76. doi: 10.1007/s10858-015-9967-y.

Hengesbach, M., Kobitski, A., Voigts-Hoffmann, F., Frauer, C., Nienhaus, G. U., & Helm, M. (2008). RNA intramolecular dynamics by single-molecule FRET. *Current Protocols in Nucleic Acid Chemistry*, *34*, 11.12.1–11.12.22. doi: 10.1002/0471142700.nc1112s34.

Ho, C. K., & Shuman, S. (2002). Bacteriophage T4 RNA ligase 2 (gp24.1) exemplifies a family of RNA ligases found in all phylogenetic domains. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(20), 12709–12714. doi: 10.1073/pnas.192184699.

Hoch, J. C., Stern, A. S., & Mobli, M. (2011). Maximum entropy reconstruction. *Encyclopedia of Magnetic Resonance*, *2*(4). doi: 10.1002/9780470034590.emrstm0299.pub2 .

Imai, S., Kumar, P., Hellen, C. U. T., Souza, V. M. D., & Wagner, G. (2016). An accurately pre-organized IRES RNA structure enables eIF4G capture for initiation of viral translation. *Nature Structural and Molecular Biology*, *23*(9), 859–864. doi: 10.1038/nsmb.3280.

Jud, L., & Micura, R. (2017). An unconventional acid-labile nucleobase protection concept for guanosine phosphoramidites in RNA solid-phase synthesis. *Chemistry - A European Journal*, *23*, 3406–3413. doi: 10.1002/chem.201605056.

Kao, C., Zheng, M., & Rüdisser, S. (1999). A simple and efficient method to reduce nontemplated nucleotide addition at the 3′ terminus of RNAs transcribed by T7 RNA polymerase. *RNA*, *5*(9), 1268–1272. doi: 10.1017/S1355838299991033.

Karlsson, H., Baronti, L., & Petzold, K. (2020). A robust and versatile method for production and purification of large-scale RNA samples for structural biology. *RNA*, *26*(8), rna.075697.120. doi: 10.1261/rna.075697.120.

Katz, J. J., Crespi, H. L., & Finkel, A. J. (1964). Isotope effects in fully deuterated hexoses, proteins and nucleic acids. *Pure Applied Chemistry*, *8*, 471–481. doi: 10.1351/pac196408030471.

Keyhani, S., Goldau, T., Blümler, A., Heckel, A., & Schwalbe, H. (2018). Chemo-enzymatic synthesis of position-specifically modified RNA for biophysical studies including light control and NMR spectroscopy. *Angewandte Chemie - International Edition*, *57*, 12017–12021. doi: 10.1002/anie.201807125.

Kovacs, H., Moskau, D., & Spraul, M. (2005). Cryogenically cooled probes — A leap in NMR technology. *Progress in Nuclear Magnetic Resonance Spectroscopy*, *46*, 131–155. doi: 10.1016/j.pnmrs.2005.03.001.

Kunitz, M. (1952). Crystalline inorganic pyrophosphatase isolated. *The Journal of General Physiology*, *35*(3), 423–450. doi: 10.1085/jgp.35.3.423.

Lavickova, B., & Maerkl, S. J. (2019). A simple, robust, and low-cost method to produce the PURE cell-free system. *ACS Synthetic Biology*, *8*, 455–462. doi: 10.1021/acssynbio.8b00427.

Meissner, A., Duus, J. Ø., & Sørensen, O. W. (1997). Spin-state-selective excitation. Application for E. COSY-type measurement of J HH coupling constants. *Journal of Magnetic Resonance*, *128*, 92–97. doi: 10.1006/jmre.1997.1213.

Milligan, J. F., Groebe, D. R., Witherell, G. W., & Uhlenbeck, O. C. (1987). Oligonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Research*, *15*(21), 8783–8798. doi: 10.1093/nar/15.21.8783.

Milligan, J. F., & Uhlenbeck, O. C. (1989). Synthesis of small RNAs using T7 RNA polymerase. *Methods in Enzymology*, *180*(C), 51–62. doi: 10.1016/0076-6879(89)80091-6.

Mörl, M., & Hartmann, R. K. (2008). Production of RNAs with homogeneous 5′ and 3′ ends. In R. K. Hartmann, A. Bindereif, A. Schön, & E. Westhof (Eds.), *Handbook of RNA Biochemistry*. Weinheim, Germany: Wiley-VCH. doi: 10.1002/9783527619504.ch2.

Mueller, L., Legault, P., & Pardi, A. (1995). Improved RNA structure determination by detection of NOE contacts to exchange-broadened amino protons. *Journal of the American Chemical Society*, *117*(45), 11043–11048. doi: 10.1021/ja00150a001.

Mulder, F. A. A., Spronk, C. A. E. M., Slijper, M., Kaptein, R., & Boelens, R. (1996). Improved HSQC experiments for the observation of exchange broadened signals. *Journal of Biomolecular NMR*, *8*, 223–228. doi: 10.1007/BF00211169.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., & Erlich, H. (1986). Specific enzymatic amplification of DNA in vitro: The polymerase chain reaction. *Cold Spring Harbor Symposia on Quantitative Biology*, *51*, 263–273. doi: 10.1101/SQB.1986.051.01.032.

Nikonowicz, E. P., Sirr, A., Legault, P., Jucker, F. M., Baer, L. M., & Pardi, A. (1992). Preparation of ^{13}C and ^{15}N labelled RNAs for heteronuclear multi-dimensional NMR studies. *Nucleic Acids Research*, *20*(17), 4507–4513. doi: 10.1093/nar/20.17.4507.

Nozinovic, S., Fürtig, B., Jonker, H. R. A., Richter, C., & Schwalbe, H. (2010). High-resolution NMR structure of an RNA model system: The 14-mer cUUCGg tetraloop hairpin RNA. *Nucleic Acids Research*, *38*(2), 683–694. doi: 10.1093/nar/gkp956.

Ottiger, M., Delaglio, F., & Bax, A. (1998). Measurement of J and dipolar couplings from simplified two-dimensional NMR spectra. *Journal of Magnetic Resonance*, *131*, 373–378. doi: 10.1006/jmre.1998.1361.

Petrov, A., Wu, T., Puglisi, E. V., & Puglisi, J. D. (2013). RNA purification by preparative polyacrylamide gel electrophoresis. In J. Lorsch (Ed.), *Methods in Enzymology* (Vol. *530*, pp. 315-330). Amsterdam: Elsevier. doi: 10.1016/B978-0-12-420037-1.00017-8.

Quant, S., Wechselberger, R. W., Wolter, M. A., Wörner, K.-H., Schell, P., Engels, J. W., … Schwalbe, H. (1994). Chemical synthesis of 13C-labelled monomers for the solid-phase and template controlled enzymatic synthesis of DNA and RNA oligomers. *Tetrahedron Letters*, *35*(36), 6649–6652. doi: 10.1016/S0040-4039(00)73458-7.

Reining, A., Nozinovic, S., Schlepckow, K., Buhr, F., Fürtig, B., & Schwalbe, H. (2013).

Three-state mechanism couples ligand and temperature sensing in riboswitches. *Nature*, *499*(7458), 355–359. doi: 10.1038/nature12 378.

Richter, C., Kovacs, H., Buck, J., Wacker, A., Fürtig, B., Bermel, W., & Schwalbe, H. (2010). ^{13}C-direct detected NMR experiments for the sequential J-based resonance assignment of RNA oligonucleotides. *Journal of Biomolecular NMR*, *47*(4), 259–269. doi: 10.1007/s10858-010-9429-5.

Schanda, P., & Brutscher, B. (2005). Very fast two-dimensional NMR spectroscopy for real-time investigation of dynamic events in proteins on the time scale of seconds. *Journal of the American Chemical Society*, *127*(22), 8014–8015. doi: 10.1021/ja051306e.

Schnieders, R., Keyhani, S., Schwalbe, H., & Fürtig, B. (2020). More than proton detection—new avenues for NMR spectroscopy of RNA. *Chemistry - A European Journal*, *26*(1), 102–113. doi: 10.1002/chem.201903355.

Schnieders, R., Richter, C., Warhaut, S., De Jesus, V., Keyhani, S., Duchardt-Ferner, E., … Fürtig, B. (2017). Evaluation of ^{15}N-detected H–N correlation experiments on increasingly large RNAs. *Journal of Biomolecular NMR*, *69*(1), 31–44. doi: 10.1007/s10858-017-0132-7.

Schnieders, R., Wolter, A. C., Richter, C., Wöhnert, J., Schwalbe, H., & Fürtig, B. (2019). Novel ^{13}C-detected NMR experiments for the precise detection of RNA structure. *Angewandte Chemie - International Edition*, *58*, 9140–9144. doi: 10.1002/anie.201904057.

Schürer, H., Lang, K., Schuster, J., & Mörl, M. (2002). A universal method to produce in vitro transcripts with homogeneous 3′ ends. *Nucleic Acids Research*, *30*(12), e56. doi: 10.1093/nar/gnf055.

Serber, Z., Richter, C., Moskau, D., Bo, J., Gerfin, T., Marek, D., … Dötsch, V. (2000). New carbon-detected protein NMR experiments using cryoprobes. *Journal of the American Chemical Society*, *122*(7), 3554–3555. doi: 10.1021/ja991371m.

Serganov, A., Yuan, Y., Pikovskaya, O., Polonskaia, A., Malinina, L., Phan, A. T., … Patel, D. J. (2004). Structural basis for discriminative regulation of gene expression by adenine- and guanine-sensing mRNAs. *Chemistry and Biology*, *11*, 1729–1741. doi: 10.1016/j.chembiol.2004.11.018.

Shaka, A. J., Barker, P. B., & Freeman, R. (1985). Computer-optimized decoupling scheme for wideband applications and low-level operation. *Journal of Magnetic Resonance*, *64*(3), 547–552. doi: 10.1016/0022-2364(85)90122-2.

Simon, B., Zanier, K., & Sattler, M. (2001). A TROSY relayed HCCH-COSY experiment for correlating adenine H2/H8 resonances in uniformly ^{13}C-labeled RNA molecules. *Journal of Biomolecular NMR*, *20*, 173–176. doi: 10.1023/A:1011214914452.

Tzakos, A. G., Easton, L. E., & Lukavsky, P. J. (2007). Preparation of large RNA oligonucleotides with complementary isotope-labeled segments for NMR structural studies. *Nature Protocols*, *2*(9), 2139–2147. doi: 10.1038/nprot.2007.306.

Winkler, W., Nahvi, A., & Breaker, R. R. (2002). Thiamine derivatives bind messenger RNAs directly to regulate bacterial gene expression. *Nature*, *419*, 952–956. doi: 10.1038/nature01145.

Wolter, A. C., Duchardt-Ferner, E., Nasiri, A. H., Hantke, K., Wunderlich, C. H., Kreutz, C., & Wöhnert, J. (2016). NMR resonance assignments for the class II GTP binding RNA aptamer in complex with GTP. *Biomolecular NMR Assignments*, *10*, 101–105. doi: 10.1007/s12104-015-9646-7.

Wolter, A. C., Pianu, A., Kremser, J., Strebitzer, E., Schnieders, R., Fürtig, B., … Wöhnert, J. (2019). NMR resonance assignments for the GTP-binding RNA aptamer 9-12 in complex with GTP. *Biomolecular NMR Assignments*, *13*, 281–286. doi: 10.1007/s12104-019-09892-z.

Wolter, A. C., Weickhmann, A. K., Nasiri, A. H., Hantke, K., Ohlenschläger, O., Wunderlich, C. H., … Wöhnert, J. (2017). A stably protonated adenine nucleotide with a highly shifted pKa value stabilizes the tertiary structure of a GTP-binding RNA aptamer. *Angewandte Chemie - International Edition*, *56*, 401–404. doi: 10.1002/anie.201609184.

Xu, J., Lapham, J., & Crothers, D. M. (1996). Determining RNA solution structure by segmental isotopic labeling and NMR: Application to Caenorhabditis elegans spliced leader RNA 1. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(1), 44–48. doi: 10.1073/pnas.93.1.44.

Zhou, Z., Kümmerle, R., Qiu, X., Redwine, D., Cong, R., Taha, A., … Winniford, B. (2007). A new decoupling method for accurate quantification of polyethylene copolymer composition and triad sequence distribution with ^{13}C NMR. *Journal of Magnetic Resonance (San Diego, Calif. : 1997)*, *187*(2), 225–233. doi: 10.1016/j.jmr.2007.05.005.

Ziegeler, M., Cevec, M., Richter, C., & Schwalbe, H. (2012). NMR studies of HAR1 RNA secondary structures reveal conformational dynamics in the human RNA. *ChemBioChem*, *13*, 2100–2112. doi: 10.1002/cbic.201200401.