Site-specific incorporation of nitroxide spin-labels into 2′-positions of nucleic acids

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A protocol is described for the incorporation of nitroxide spin-labels into specific 2′-sites within nucleic acids. This labeling strategy facilitates the investigation of nucleic acid structure and dynamics using electron paramagnetic resonance (EPR) spectroscopy and macromolecular complex formation using paramagnetic relaxation enhancement NMR spectroscopy. A spin-labeling reagent, 4-isocyanato TEMPO, which can be prepared in one facile step or obtained commercially, is used for postsynthetic modification of site-specifically 2′-amino-modified nucleic acids. This spin-labeling protocol has been applied primarily to RNA, but is also applicable to DNA. Subsequently, EPR spectroscopic analysis of the spin-labeled nucleic acids allows for the measurements of distances, solvent accessibilities and conformation dynamics. Using the spin-labeling strategy described here, spin-labeled samples can be prepared in 2–4 d.

INTRODUCTION

Electron paramagnetic resonance (EPR) spectroscopy has emerged as a valuable technique for the investigation of macromolecular structure and dynamics[1–3]. Employing EPR spectroscopy, information can be obtained regarding the distances within a macromolecule or complex as well as solvent accessibility and dynamics at individual sites. Such information is often difficult to obtain by other means, and highly complementary to the information obtained by high-resolution structural methods such as NMR spectroscopy or x-ray crystallography, as well as other low-resolution techniques such as fluorescence spectroscopy. To date, EPR spectroscopy has been highly successful in the study of membrane proteins[3]. Site-specifically spin-labeled proteins can be obtained through either 'site-directed spin-labeling' that combines site-directed mutagenesis and postsynthetic spin-labeling[1] or incorporation of the rigid spin-probe TOAC (2,2,6,6-tetramethylpiperidine-1-oxyl-amino-4-carboxylic acid) via solid-phase peptide synthesis[4].

Over the past several decades, many functional RNA molecules have been discovered, including viral RNAs, ribozymes, aptamers and riboswitches. During this time, several biophysical methods have been developed for obtaining information about the structure and dynamics of these functional RNAs[2]. We developed a general method for the incorporation of EPR spin probes into the internal positions of RNA[5], and have employed this method to extensively characterize the interactions of the HIV-1 TAR RNA with metal ions[6,7], small molecule inhibitors[8] and peptides that mimic the Tat protein[6,9], which is a biological partner of the TAR RNA. We have also used this method to spin-label DNA both at a terminal (T.E.E. and S.Th.S., unpublished data) and an internal nucleotide[5], but have not studied the effect of the modification on DNA duplex stability and structure. Furthermore, DeRose and co-workers[10] and our group[11] have applied this labeling strategy to study the folding and catalysis of the hammerhead ribozyme (**Fig. 1**). This labeling strategy has also been used in combination with pulsed electron–electron double resonance spectroscopy to measure nanometer distances in RNA[12]. These studies provide the fundamental groundwork for future EPR studies on various functional RNAs, which are made possible by the facile RNA spin-labeling protocol presented here. In addition, this spin-labeling methodology can be used to prepare samples for paramagnetic relaxation enhancement NMR spectroscopy in which the unpaired electron induces fast relaxation of neighboring resonances, allowing for the determination of local contacts[13].

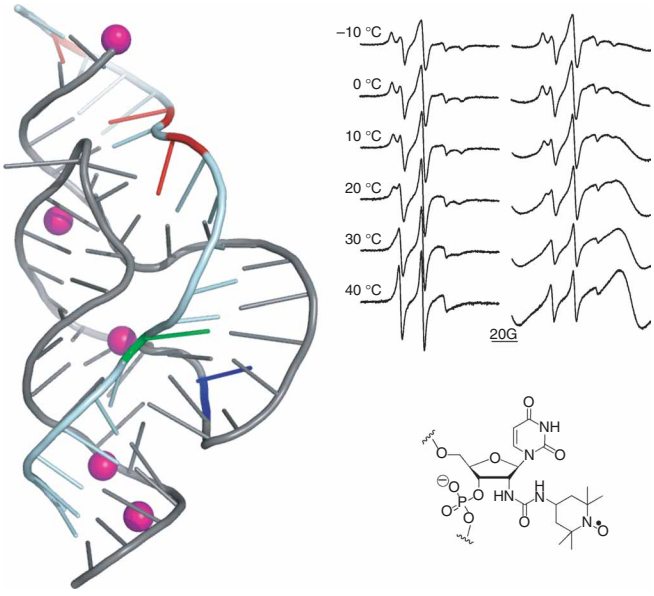

Figure 1 | Electron paramagnetic resonance (EPR) spectroscopy of 2′-spin-labeled hammerhead ribozymes obtained using the spin-labeling strategy presented here. Nucleotides spin-labeled by DeRose and co-workers[10] (nucleotides depicted in red) and in our laboratory[11] (blue) are mapped onto a cartoon representation of a hammerhead ribozyme crystal structure (PDB ID 2OEU[29]) solved in the presence of Mn^{2+} (magenta spheres). The site of cleavage is shown in green, while the ribozyme strand is shown in dark gray, and the substrate strand is shown in light blue–gray. The structure of a 2′-spin-labeled nucleotide is shown in the lower right-hand corner. The EPR spectra of a minimal hammerhead ribozyme spin-labeled at U7 were recorded as a function of temperature in the presence of Mg^{2+} (left column) or Mn^{2+} (right column) in PNE buffer[11]. The EPR spectra are reprinted with permission from ref. 11. Copyright (2005) American Chemical Society.

Preparation of 2′-ureido TEMPO-labeled nucleic acid samples can be accomplished in two simple steps, both of which are accessible to a wide range of scientists (**Fig. 2**). We have extensively used this method to label RNA, and therefore we will describe the procedure for RNA. The first step is the preparation of the spin-labeling reagent, 4-isocyanato TEMPO (**2**), which has recently become commercially available. The second step is the reaction of compound **2** with 2′-amino-modified RNAs that can be purchased from several different vendors who offer custom oligonucleotide synthesis. The main advantages of this approach are the ease (one or two simple reactions), efficiency (typically greater than 95% spin-labeling) and speed of preparation (2–4 d) of spin-labeled samples. Because the ribose ring is the site of modification, this spin-label can, in principle, be incorporated into any position within the RNA; however, only nucleic acids containing 2′-amino-modified pyrimidines (or 2′-amino-modified phosphoramidites for in-house oligonucleotide synthesis) are currently commercially available. The 2′-ureido spin-label modification does not significantly disrupt the RNA structure[5], especially in comparison with 2′-amido-modified RNAs[14]. Another advantage of this approach is that the 2′-ureido linkage provides moderate restriction on the motions of the probe[5]. Ideally, the spin-label would be attached rigidly to the nucleotide[15]; however, rigid spin probes are not currently available for incorporation into RNA. Finally, this method is complementary to other site-specific spin-labeling strategies for RNA, in which the spin-label is attached at the 5′- (refs. 16,17) or 3′- (ref. 18) ends of RNA, the phosphate backbone[19], the ribose ring[20], the nucleobase 4-thiouridine[21–24] or via an acetylene linkage to the 5-position of pyrimidines[25,26] or the 2-position of adenine[26]. Although not directly related to the purpose of this protocol, which is the incorporation of EPR spin-labels into oligoribonucleotides, spin-labels have also been attached to the ribose positions of nucleosides[27].

Experimental design

This protocol describes the preparation of site-specifically 2′-spin-labeled RNA in two steps from commercially available materials for the characterization of RNA structure and dynamics by EPR spectroscopy. The two steps used for RNA spin-labeling were described in our early publications[5,6]. The first step is the synthesis of the spin-labeling reagent, 4-isocyanato TEMPO (**2**), from 4-amino TEMPO (**1**) and diphosgene (**Fig. 2**). Recently, the spin-labeling reagent **2** has become commercially available, thus expediting the labeling process.

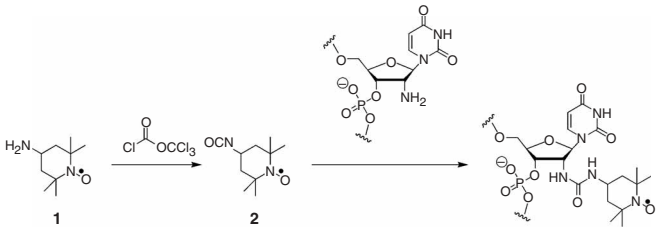

Figure 2 | Preparation of the spin-labeling reagent, 4-isocyanato TEMPO (**2**), and the subsequent coupling to 2′-amino-modified oligonucleotides to yield 2′-ureido-linked spin-labeled nucleic acids.

The second step involves postsynthetic labeling of 2′-amino-modified RNA, which does not need to be purified beforehand, with the spin-labeling reagent **2** and the purification of the spin-labeled RNA. We recommend performing this spin-labeling reaction on an analytical scale before preparatory scale RNA spin-labeling, in order to verify that the labeling reaction works. Amounts of the reagents for the analytical scale reactions are listed in parenthesis at the relevant juncture past Step 18 of the PROCEDURE (see below). In addition, any time past Step 24, we recommend monitoring the extent of the labeling reaction and the purity of the RNA product through analysis by denaturing gel electrophoresis or HPLC (see Step 40). This analysis can be run in parallel with the analysis of analytical spin-labeling reaction mixtures. We have used this method to spin-label RNAs up to 38 nt in length[11], although it may be possible to spin-label longer RNAs and purify them by this methodology.

Depending on the length of the desired RNA, it may be necessary to ligate a short spin-labeled RNA to a longer RNA, prepared by either solid-phase synthesis or *in vitro* transcription. For a recent report of RNA ligation, see Hobartner *et al.*[28]; we have had success with the conditions similar to those described therein (T.E.E. and S.Th.S., unpublished data). EPR spectroscopy of the spin-labeled RNA is subsequently described with suggestions for maximizing the sensitivity of changes in dynamics.

MATERIALS

REAGENTS

- 2′-Amino-modified RNA (see REAGENT SETUP)
- 4-Amino-2,2,6,6-tetramethylpiperidine-1-oxyl (4-amino TEMPO, **1**; Sigma-Aldrich, cat. no. 163945) **! CAUTION** Do not proceed with the present protocol if the purchased 4-amino TEMPO appears to be contaminated with water. **▲ CRITICAL** Please confirm purity of **1** by checking that it is a solid at 4 °C. If 4-amino TEMPO is a liquid at this temperature, this may indicate that the sample contains water that will interfere with the formation of the isocyanate. For instructions as to how to purify **1** before its use in the present protocol, please see **Table 1**.
- Methylene chloride, anhydrous (CH_2Cl_2; Sigma, cat. no. 07-2230)
- Diphosgene (Trichloromethyl chloroformate; Sigma, cat. no. 23261) **! CAUTION** Hazardous and should be handled in a well-ventilated area, such as a fume hood. See material safety data sheet. We recommend storing the solution under argon in a desiccated container when not in use. While measuring the desired volume of diphosgene with a glass syringe, we recommend a transfer under a positive pressure of argon. Alternatively, the solution can be covered with an inverted funnel attached to a flow of argon. Subsequently, this syringe needs to be thoroughly cleaned and dried or the metal plunger will erode rapidly.
- Rock salt
- Ammonium chloride (NH_4Cl; Sigma, cat. no. A9434)
- Sodium hydroxide (NaOH; Sigma, cat. no. 71692)
- Sodium sulfate (Na_2SO_4; Fisher, cat. no. 71959)
- 4-isocyanato TEMPO (OPTIONAL, see above) (compound **2**, 4-isocyanato-2,2,6,6-tetramethyl-1-piperidinyloxy; CAS 88418-69-3; Toronto Research Chemicals, cat. no. I80700) **! CAUTION** Harmful.
- Dimethylformamide (DMF; Sigma, cat. no. 227056, Sure/Seal, anhydrous, amine-free)
- Boric acid (Sigma, cat. no. B6768)
- Formamide (Sigma, cat. no. F9037)
- Chloroform ($CHCl_3$; Sigma, cat. no. C2432)
- Sterile water
- Acetic acid (Sigma, cat. no. 45726)
- Ethanol (Sigma, cat. no. 277649)
- Urea (Sigma, cat. no. U5378)
- Triethylamine (Et_3N; Sigma, cat. no. 17924)

- Acetonitrile (CH_3CN; Sigma, cat. no. 00687)
- Monosodium phosphate (NaH_2PO_4; Sigma, cat. no. S3139)
- Disodium phosphate (Na_2HPO_4; Sigma, cat. no. S3264)
- Na_2EDTA (Sigma, cat. no. E0399)
- Toluidine blue O (Sigma, cat. no. T-3260)
- Sucrose (Sigma, cat. no. 84097)
- Sodium acetate (Sigma, cat. no. 71183) (see REAGENT SETUP)
- *N,N,N′,N′*-Tetramethylethylenediamine (OPTIONAL, see Step 40 of the PROCEDURE) (TMEDA, Sigma, cat. no. T7024)
- 40% Acrylamide solution (OPTIONAL, see Step 40 of the PROCEDURE) (acrylamide:bis-acrylamide, 29:1; Fisher, cat. no. BP1408)
- Ammonium persulfate (OPTIONAL, see Step 40 of the PROCEDURE) (Sigma, cat. no. A9164)
- Borate buffer (see REAGENT SETUP)
- Tris–HCl (see REAGENT SETUP)
- PNE buffer (see REAGENT SETUP)
- Sucrose/PNE buffer (see REAGENT SETUP)
- 10× TBE buffer (see REAGENT SETUP)
- Denaturing gel (see REAGENT SETUP)

EQUIPMENT

- 10-ml Round bottom flasks
- Rubber septa
- Separatory funnel
- Stir plate
- 1.5-ml microtubes (Fisher Scientific, cat. no. 05-408-129)
- 0.1-ml Hamilton glass syringe (Fisher Scientific, cat. no. 509537237)
- 0.8 × 1.0 mm^2 Quartz capillary tubes (VitroCom, Inc)
- Vacuum centrifuge (DNA Speed Vac DNA110; Savant)
- Varian ProStar two-pump HPLC system with 260-nm wavelength UV monitor
- Varian Microsorb-MV analytical reverse phase column (OPTIONAL, see Step 40 of the PROCEDURE) (300 Å, C18, 4.6 × 250 mm^2, 5-µm column)
- Dionex DNAPac PA-100 4 × 250 mm^2 analytical column (Dionex) (OPTIONAL, see Step 40 of the PROCEDURE)
- Sep-Pak column (Waters)
- X-band EPR spectometer (Bruker EMX with a TE102 cavity)

REAGENT SETUP

2′-Amino-modified RNA can be obtained either through in-house solid-phase chemical synthesis prepared with commercially available modified 2′-amino phosphoramidites (Chemgenes, cat. no. ANP-9403 for 2′-amino uridine phosphoramidite or cat. no. ANP-5322 for 2′-amino cytosine phosphoramidine) or from a custom RNA synthesis commercial vendor. We have obtained 2′-amino-modified RNAs both in-house and through commercial vendors, and have observed the best results with the oligomers supplied by Dharmacon (a subsidiary of Fisher Scientific). Dharmacon offers 2′-amino-modified uridine- and cytidine-containing custom RNAs and may prepare 2′-amino-modified adenosine-containing custom RNAs upon request.

70 mM Borate buffer, pH 8.6 Dissolve 433 mg boric acid in 80 ml sterile water. Adjust pH of the solution with approximately 50 mg NaOH to 8.6, and then adjust final volume to 100 ml with sterile water. Filter using a 0.22-µm sterile filter.

3.0 M Sodium acetate, pH 4.6 Dissolve 17.2 ml acetic acid in 60 ml sterile water. Adjust pH of solution to 4.6 with approximately 6 g NaOH, and then adjust the final volume to 100 ml with sterile water. Filter using a 0.22-µm sterile filter.

50 mM Et_3NHOAc, pH 7.0 Dissolve 5.07 g Et_3N in 800 ml sterile water. Adjust the pH of the solution to 7.0 with approximately 6 g acetic acid, and then adjust the final volume to 1 l with sterile water. Filter using a 0.22-µm sterile filter. (OPTIONAL, see Step 40 of the PROCEDURE.)

25 mM Tris–HCl, pH 8.0 Dissolve 3.03 g Tris in 800 ml sterile water. Adjust the pH of the solution to 8.0 with HCl, and then adjust the final volume to 1 l with sterile water. Filter using a 0.22-µm sterile filter. (OPTIONAL, see Step 40 of the PROCEDURE.)

PNE buffer (100 mM NaCl, 10 mM sodium phosphate, 0.1 mM EDTA, pH 7.0) Dissolve 584 mg NaCl, 60 mg NaH_2PO_4, 71 mg Na_2HPO_4 and 3.7 mg Na_2EDTA in 80 ml water. Adjust the pH of the solution to 7.0 with NaH_2PO_4, and then the final volume to 100 ml. Filter using a 0.22-µm sterile filter.

20% (wt/vol) sucrose/PNE buffer Dissolve 10 g sucrose in PNE buffer to 50 ml total volume.

10× TBE buffer Dissolve 108 g Tris, 55 g boric acid and 7.4 g Na_2EDTA in sterile water to a total volume of 1 l. (OPTIONAL, see Step 40 of the PROCEDURE.)

15% Denaturing gel Dissolve 48 g urea, 10 ml 10× TBE buffer, 38 ml 40% bisacrylamide to 100 ml total volume by adding 8 ml sterile water. The gel matrix should be polymerized with 50 µl of 50% aqueous ammonium persulfate and 150 µl TMEDA. (OPTIONAL, see Step 40 of the PROCEDURE.)

EQUIPMENT SETUP

Dry all glassware to be used for the synthesis of spin-labeling reagent 2 in a 160 °C oven overnight.

PROCEDURE

Preparation of spin-labeling reagent 2 ● TIMING 3 h

1| Weigh 198 mg (1.15 mmol) 4-amino TEMPO (**1**) into a 10-ml round bottom flask.

▲ CRITICAL STEP All glassware should be dried and the reaction should be carried out under an inert atmosphere of nitrogen or (preferably) argon.

? TROUBLESHOOTING

2| Dissolve **1** in 1.5 ml anhydrous CH_2Cl_2 and stopper the reaction with a rubber septum. The chamber should be flushed with a positive flow of an inert gas, such as nitrogen, or preferably argon for several minutes using a gas line coupled with a syringe as well as a second syringe needle, open to the air for venting. The venting needle and then the gas line can be removed after several minutes of flow.

3| Pre-cool the solution from Step 2 in a rock salt/ice-water bath at –8 °C for 20 min.

4| Separately, in a 10 ml round bottom flask with a rubber septum, pre-cool to –8 °C a solution of 25 µl (0.21 mmol) diphosgene in 1.5 ml CH_2Cl_2.

5| Using a cannula, rapidly (approximately 5–10 s) add the solution of **1** under a positive pressure of nitrogen as described earlier to the stirred solution of diphosgene. A cannula is a long double-ended syringe needle that is inserted through the septa of both flasks. The cannula is inserted into the liquid to be transferred and the (nitrogen) pressure in that flask is increased relative to the other flask, thereby pushing the liquid from one flask to the other via the cannula.

6| Remove the cooling bath and allow the reaction to stir for 2 min.

7| Dilute the crude reaction mixture to a total volume of 20 ml using CH_2Cl_2.

8| Transfer the reaction mixture to a separatory funnel and wash the organic layer sequentially with NH_4Cl (1 M aq, 4 × 20 ml) and NaOH (1 M aq, 20 ml).

9| Add Na_2SO_4 sufficiently to dry the organic solution (approximately 0.2 g), swirl the flask occasionally for 5 min and remove the salt by filtration.

10| Remove the solvent *in vacuo* at room temperature (~22 °C) using rotary evaporation to yield the product **2** as a peach-colored solid.
▲ **CRITICAL STEP** Aside from NMR analysis[5], the color and consistency of the isocyanate product is the best indicator of the quality of the spin-labeling reagent. If it appears plum colored (i.e., dark colored) rather than peach colored (i.e., light colored) or amorphous rather than solid, the reaction was most likely unsuccessful. For ^{1}H-NMR analysis, the unpaired electron broadens the width of the resonances, making proper integration difficult; nevertheless, the chemical shift of the C4 proton clearly changes to approximately δ 3.7 upon conversion of the amine to the isocyanate.

11| Dissolve the isocyanate product **2** in anhydrous CH_2Cl_2 to 1 mg per 100 µl and store in a desiccated chamber at –20 °C. A screw-capped glass jar containing a drying agent, such as Ca_2SO_4, can be used for this purpose.
■ **PAUSE POINT** Once stored at –20 °C, the isocyanate will be stable for at least 1–2 months in CH_2Cl_2. However, storing **2** in DMF at –20 °C is not recommended due to rapid hydrolysis of **2**. Please note that compound **2** can now be purchased (see above).
? TROUBLESHOOTING

Spin-labeling of 2′-amino-modified RNA ● TIMING 4–8 h
12| If necessary, remove the groups protecting the 2′-hydroxyl moiety of the RNA according to protocol provided by vendor.

13| Dissolve the crude (i.e., not yet gel- or HPLC-purified), deprotected 2′-amino-containing RNA (e.g., one quarter of the total amount of RNA obtained from a 1 µmol synthesis for a preparatory scale reaction) in 50 µl sterile water in a microtube.

14| To convert the RNA ammonium salts from the chemical synthesis into the corresponding sodium salts, precipitate the RNA by adding to its solution 5 µl sodium acetate 3.0 M at pH 4.6 and 0.3 ml cold (–20 °C) absolute ethanol. Store the resulting mixture at –20 °C for at least 4 h or at –80 °C for 30 min.
▲ **CRITICAL STEP** It is important to exchange the ammonium cations from the RNA deprotection with sodium so that the ammonia does not react with isocyanate **2**.

15| Centrifuge the sample at 10,000*g* for 15 min at 4 °C to obtain an RNA pellet.

16| Remove the aqueous ethanol supernatant.

17| Wash the pellet carefully two times by adding to it and decanting 50 µl cold (–20 °C), 70% aqueous ethanol.

18| Dry the pellet *in vacuo* in a vacuum centrifuge or air dry for 10 min.
▲ **CRITICAL STEP** As anticipated in the EXPERIMENTAL DESIGN, we recommend performing the spin-labeling reaction on an analytical scale before preparatory scale RNA spin-labeling to verify that the labeling reaction works. Amounts for analytical scale reactions are listed in parenthesis in the following steps. In addition, we recommend UV/Vis quantification of the 2′-amino-modified RNA and analysis by denaturing gel electrophoresis (Step 40) or HPLC to gauge its purity. This analysis can be run in parallel with the analysis of analytical spin-labeling reaction mixtures.

19| Dissolve the RNA in 100 µl of 70 mM borate buffer, pH 8.6 (RNA concentration approximately 2 mM). (For analytical scale reactions, use 2.5 µl of approximately 2 mM RNA in 70 mM borate buffer, pH 8.6.)

20| Cool the 2′-amino-containing RNA solution in a salt/ice-water bath (–8 °C) in a cold room (4 °C) for 20 min.

21| Add 60 µl cold (0 °C) formamide, and cool mixture to –8 °C for 20 min. (For analytical scale reactions, use 1.5 µl formamide.)

22| While the above reaction is cooling, prepare a 1 mg (5 µmol) sample of **2** by removing the CH_2Cl_2 from an aliquot of the solution prepared in Step 11 *in vacuo* (e.g., use a vacuum centrifuge) at room temperature.

23| Dissolve **2** in 67.6 µl anhydrous DMF, for a final concentration of 75 mM in **2**, and cool to –8 °C.
▲ CRITICAL STEP It is critical to use anhydrous, amine-free DMF due to the high reactivity of isocyanates, such as compound **2**, toward water and especially free amines, such as ammonia or methylamine, which are the contaminants in low-grade DMF solutions.

24| Add 40 µl of the solution of **2** in DMF from Step 23 to the RNA-containing solution. This yields a 200 µl solution with final concentrations of approximately 1 mM 2′-amino RNA, 15 mM isocyanate **2**; 50% aqueous borate buffer, 30% formamide, 20% DMF, vol/vol/vol. (For analytical scale reactions, use 1.0 µl of 75 mM **2** in DMF to make 5 µl total volume.)

25| Incubate the reaction mixture for 30–60 min.

26| Treat the oligoribonucleotide solution with a second aliquot of 40 µl isocyanate **2** (75 mM in DMF), freshly prepared as described in Steps 15 and 16. (For analytical scale reactions, use 1.0 µl of 75 mM **2** in DMF.)

27| Incubate for 30–60 min.

28| Treat the oligoribonucleotide solution with a third 40 µl aliquot of isocyanate **2** (75 mM in DMF), freshly prepared as described in Steps 15 and 16. (For analytical scale reactions, use 1.0 µl of 75 mM **2** in DMF.)

29| Incubate for 30–60 min.

30| Remove the reaction mixture from the rock salt/ice-water bath and wash the approximately 280 µl solution two times with $CHCl_3$ (300 µl) at room temperature. (For analytical scale reactions, move to Step 37.)

31| Precipitate the RNA by adding 30 µl 3.0 M sodium acetate at pH 4.6 and 1.3 ml cold (–20 °C) absolute ethanol. Store the resulting mixture at –20 °C for at least 4 h or at –80 °C for 30 min.

32| Centrifuge the sample at 10,000*g* for 15 min at 4 °C to obtain an RNA pellet.

33| Remove the aqueous ethanol supernatant.

34| Wash the pellet carefully two times by adding and decanting 50 µl cold (–20 °C) of 70% aqueous ethanol.

35| Dry the pellet *in vacuo* in a vacuum centrifuge for 10 min.

36| Dissolve the pellet in sterile water (50 µl).
■ PAUSE POINT The spin-labeled RNA can now be safely stored at –20 °C for several weeks until purification.
? TROUBLESHOOTING

Monitoring the extent of the spin-labeling reaction ● TIMING **5 h**
37| Remove a 1.0 µl aliquot of the reaction mixture. This step and those that follow (Steps 38–40) can take place at any time after Step 24 through Step 30. We find it useful to perform this analysis during and/or after the second and third additions to determine the extent of reaction and whether or not additional (i.e., a third, fourth) isocyanate aliquots should be added.

38| Dilute the 1.0-µl aliquot of the reaction mixture from Step 37 with 19 µl water in a microtube.

39| Remove the excess labeling reagent by washing the aqueous sample two times by adding 75 µl $CHCl_3$, vortexing the sample, centrifuging at 10,000*g* for 3 min, and, finally, removing the organic layer.

40| Analyze the diluted reaction mixture from Step 38 above by reverse-phase HPLC (Method A below), by denaturing polyacrylamide gel electrophoresis (PAGE) (Method B), or by ion exchange (IE) chromatography (Method C).
▲ CRITICAL STEP We suggest using reverse-phase HPLC (Method A) to analyze shorter oligonucleotides (up to 25 nt) because it is quickest and thus the most convenient approach. Denaturing PAGE (Method B) is, however, best for longer RNAs. In this approach, the unlabeled 2′-amino-modified RNA sample should be analyzed beforehand to determine the retention time as well as the purity of original sample. We recommend preparing also a third sample for denaturing PAGE analysis by mixing an aliquot from the reaction after Step 39 with a sample of the 2′-amino-modified RNA to verify that the product has a different retention time from the starting RNA. IE chromatography may be used in lieu of denaturing PAGE or reverse-phase chromatography if these techniques are not available.

(A) Reverse phase HPLC

(i) Using an analytical column (C18, 4.6 × 250 mm^2, 5-µm column) at 1.5 ml min^{-1}, implement an analytical HPLC program with the following specifications:
solvent A: 50 mM Et_3NHOAc (pH 7.0)
solvent B: 70% CH_3CN/30% 50 mM Et_3NHOAc (pH 7.0)

15-min linear gradient from 0 to 23% B
5-min linear gradient to 100% B
Isocratic for 10 min
3-min linear gradient to initial conditions
15-min equilibrium time between runs

(B) Denaturing PAGE

(i) Purify the spin-labeled RNA by 15% denaturing gel electrophoresis (20-cm gel, 400 V for 4 h, using 1.0× TBE running buffer) and detect the bands by UV shadow visualization and/or toluidine blue staining.

(C) IE chromatography

(i) Carry out analytical IE-HPLC on a Dionex DNAPac PA-100 4 × 250 mm^2 analytical column heated at 50 °C by a column warmer. Please note that separation will not be achieved without heating the column. Solvent gradients for analytical IE-HPLC can be run at 1.0 ml min^{-1}, implementing a program with the following specifications:
solvent A: 25 mM Tris · HCl, pH 8.0
solvent B: 1.0 M NaCl, 25 mM Tris–HCl, pH 8.0
35-min linear gradient from 10% B to 80% B
5-min linear gradient to 10% B

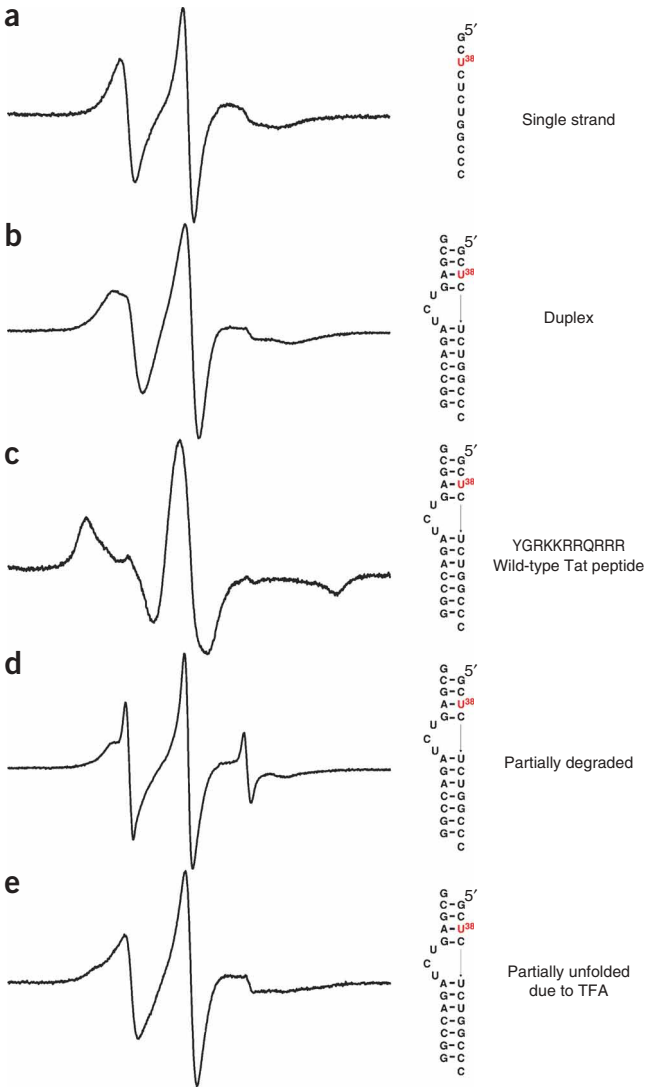

Figure 3 | Electron paramagnetic resonance (EPR) spectra of 2′-spin-labeled RNA. (**a**) A single-stranded, spin-labeled RNA. (**b**) TAR RNA containing a 2′-spin-label at nucleotide U38, located in a duplex region (U38 TAR RNA). (**c**) U38 TAR RNA in complex with the wild-type Tat peptide YGRKKRRQRRR. (**d**) U38 TAR RNA sample, which has been partially degraded. (**e**) U38 TAR RNA, which has been partially denatured due to the presence of residual trifluoroacetic acid from peptide synthesis.

Purification of 2′-spin-labeled RNA by denaturing PAGE

● TIMING **1–3 d**

41| Dilute the 50 µl solution from Step 36 with 150 µl 8 M aqueous urea.

? TROUBLESHOOTING

42| Purify the RNA by 15–20% denaturing PAGE. Use a 20-cm gel for short oligonucleotides (i.e., up to 20 nt in length) and run the gel for 6–20 h at 400–600 V or until the RNA has traveled three quarters the length of the gel. Use 40-cm gel for longer oligos, up to 50 nt in length, running gel for up to 72 h at 600 V or less time if higher voltage, for example, 1,200–1,800 V, can be used. If equipped with a temperature sensor for the glass plates, we recommend running the gel at room temperature and setting an upper temperature limit of 42 °C to ensure that the plates do not crack.

▲ CRITICAL STEP Before the preparatory scale purification, we recommend running an analytical gel containing both a sample of unlabeled 2′-amino RNA as a control and the spin-labeling reaction mixture to determine how far one must run the gel to obtain resolution of the labeled and unlabeled samples. Also, it may be useful to run a mixture of an unmodified sample with a reaction mixture to aid in determining component separation. It is important to keep in mind that bands in the preparatory scale gel will be considerably thicker than in the analytical gel, and thus adequate resolution should be observed first on an analytical gel.

43| Visualize the bands by UV shadowing and excise the spin-labeled RNA band from the gel.

44| Crush the gel slices.

■ PAUSE POINT Soak the gel fragments into sterile water overnight at 4 °C on a rotator.

45| Desalt the solubilized RNA (see Step 44) via Sep-Pak chromatography as described by the manufacturer's manual.

■ PAUSE POINT Store the spin-labeled RNA in sterile water at approximately 1 mM concentration (approximately 150 µl or as desired) at –20 °C. RNA stored in this manner should remain highly pure without degradation for many months.

Analysis of 2′-spin-labeled RNA by EPR spectroscopy ● TIMING **1–4 h**

46| Evaporate the solvents from the 2′-spin-labeled RNA solution and from other desired components (small molecules, metal ions, peptide, etc.) *in vacuo* using rotary evaporation at room temperature.

47| Suspend the residues from Step 46 in 10 µl of 20% sucrose/PNE buffer or PNE buffer alone (50–200 µM RNA), and anneal the RNA in the presence of the other components as desired or via thermal cycler: (i) −65 °C for 2 min, (ii) −42 °C for 5 min, (iii) −22 °C for 15 min.

48| Transfer the EPR sample into a 0.8 × 1.0 mm^2 quartz capillary tube for EPR spectroscopy and store at 4 °C in between experiments.

49| Acquire EPR spectrum of RNA sample using an X-band EPR spectometer (9.3 GHz) and appropriate experimental parameters such as 3355 *g* center field, 100 kHz frequency modulation, 1.0 G modulation amplitude, 8 mW power, 20.48 ms time constant, 40.96 ms conversion, 1,024 points, sweep time 42 s, 110 G sweep width, and an appropriate number of scans to give reasonable signal-to-noise ratio, which can range from 10 to 300 scans depending on the spectral width. The EPR spectrum of a single-stranded RNA in 20% sucrose/PNE should demonstrate higher mobility (narrower spectrum) than a corresponding duplex RNA under the same conditions (**Fig. 3**).

? TROUBLESHOOTING

● **TIMING**

Steps 1–11: 3 h
Steps 12–36: 4–8 h
Steps 37–40: 5 h
teps 41–45: 1–3 d
Steps 46–49: 1–4 h

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Steps	Problem	Possible reasons	Solution
1	Amine **1** is a liquid at 4 °C	The sample may contain water, which will interfere with the formation of the isocyanate	Compound **1** can be dried using the following protocol: Dissolve 5 g of **1** in diethyl ether (20 ml). Dry the resulting mixture by stirring over granular KOH (2 g) for 1 h. Filter the solution and dry the residue under vacuum overnight
1–11	Isocyanate **2** is a plum-colored, amorphous solid, that is, it is not pure	The reaction was allowed to proceed too long	After 2 min of reaction time (Step 6), move quickly to the extraction work up
12–36	Labeling efficiency is low	Spin-labeling isocyanate **2** has hydrolyzed	Make sure that spin-labeling compound **2** is freshly prepared in anhydrous, amine-free dimethylformamide immediately before the reaction with 2′-amino RNA. Furthermore, it is important that compound **2** is stored in anhydrous CH_2Cl_2 in a desiccated chamber at −20 °C
12–36	Labeling efficiency is low	2′-Trifluoroacetamido protecting group from solid-phase synthesis has not been entirely removed from the RNA sample by vendor	To investigate the extent of 2′-deprotection by vendor, digest the RNA with phosphodiesterase and alkaline phosphotase and analyze by HPLC[5] as described in Step 34. If the protecting group has not been completely removed, this will reveal an additional peak corresponding to the 2′-trifluoroacetamido nucleoside. If observed, contact the RNA vendor
37–41	Cannot resolve 2′-amino (nonreacted) RNA from 2′-spin-labeled product	Ineffective resolving power	For denaturing polyacrylamide gel electrophoresis (PAGE), use a high concentration of acrylamide (15–23%) and 40 cm or longer gels

TABLE 1 | Troubleshooting table (continued).

Steps	Problem	Possible reasons	Solution
			should be used to increase the resolution. Be sure to run RNA at least 80% of the way down the gel. For HPLC, using a column warmer can increase resolving power
46–49	EPR spectrum of product has free spin contaminant with fast mobility [similar spectral features (narrow lines) to that observed for a partially degraded sample, **Fig. 3d**]	Failure to remove hydrolysis products of **2**	A free spin contaminant can sometimes be observed, even after denaturing PAGE analysis. Perform additional aqueous $CHCl_3$ extractions followed by ethanol precipitation. Alternatively, purify the RNA by size exclusion chromatography
46–49	Sample precipitates	Sample forms aggregate after annealing	Anneal RNA in 7.0-μl buffer in the absence of other components (divalent metal ions, small molecules, peptides, etc.) and add the other components in 3.0-μl buffer after annealing
46–49	For samples with peptides bound to RNA, mobility of sample becomes rapid (**Fig. 3e**)	RNA has become denatured due to residual trifluoroacetic acid (TFA) from peptide synthesis	Residual TFA should be removed from peptide sample by lyophilization of peptide sample to dryness, repeatedly if necessary

ANTICIPATED RESULTS

Production of the spin-labeling compound **2** typically occurred in 30% yield (approximately 70 mg, 90% theoretical yield based on stoichiometry of the starting amine). The stoichiometry of the isocyanate reaction is six molecules of **1** added to one molecule of diphosgene (1.1 equivalents are used in Step 4) yield two molecules of **2** and four molecules of the HCl salt of **1** that precipitates during the reaction. The extent of spin-labeling should be greater than 90% and is often 95–98%. Yields for preparatory scale production of spin-labeled RNA typically range from 100 to 170 nmol for one quarter of the total RNA obtained from a 1.0 μmol synthesis, depending on the length and quality of the RNA synthesis.

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