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Nonradioactive Methods for the Detection of RNA-Protein Interaction

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1. Introduction

RNA in biological systems is associated with proteins. Recent work in eukaryotes has identified common motifs present in families of RNA-binding proteins. Usually, RNA-binding proteins recognize both sequence and structure at their target sites. Therefore, identification of proteins that interact with a specific RNA sequence contributes to the understanding of biological processes. Thus, the genome of human immunodeficiency virus (HIV) encodes proteins, Tat, Rev and NC, that bind to specific viral RNA motives *(1,2)*. These interactions mediate different steps of virus replication i.e., transactivation of transcription, nuclear export of viral transcripts, or packaging of two RNA genomes into the mature virion. In addition, we recently reported that Nef, an accessory protein encoded by HIV-1, belongs to the family of RNA-binding proteins *(3)*. Using different-size variant proteins and point-mutated proteins, it was found that the amino terminal Arg-rich domain of Nef is involved in the RNA-binding activity. Nef proteins from HIV-2 and SIV (simian immunodeficiency virus) also showed RNA-binding capacity *(4)*.

In this chapter we describe methodologies for the direct detection of RNA-protein interactions using biotinylated riboprobes. The northwestern assay involves the interaction of labeled RNA with proteins immobilized on a membrane support. This assay allows the identification of the specific protein present in a crude extract that interacts with a given RNA molecule. The UV crosslinking and gel retardation assays mimic the in vivo binding reaction in solution conditions using purified proteins and RNA molecules. Gel retardation assay separates RNA-protein complexes from free RNA in a gel matrix. This technique is very useful for dissociation kinetics and ionic strength analysis of the binding reaction. A UV crosslinking assay is particularly appropriate for detecting labile protein-nucleic acid complexes, because the UV irradiation fixes the complex in amounts sufficient for further analysis by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE). All these techniques use biotinylated probes to detect RNA-protein complexes on blotted membranes. The advantages of these nonradioactive methods when compared to their radioactive counterparts are their safety, low cost, easy handling, and long-term storage of labeled riboprobes *(5,6)*.

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2. Materials

RNases are ubiquitous and heat stable; care should be taken to avoid nuclease contamination of either reagents and equipment. All solutions should be treated with diethylpyrocarbonate (DEPC), an inhibitor of RNases, or dissolved in DEPC-treated dH_2O (*see* **Note 1**). Gloves should be worn.

2.1. In Vitro Synthesis of Biotin-Labeled Riboprobes

1. 5 µg Linearized template DNA in RNase-free dH_2O.
2. 2.5 m*M* ATP, CTP, and GTP, 10 m*M* UTP (Amersham Pharmacia Biotech) and 10 m*M* biotin-21-UTP (Clontech, Palo Alto, CA).
3. 40 U T7 or SP6 RNA Polymerase, 50 U RNasin ribonuclease inhibitor and RQ1 RNase-free DNase (Promega, Madison, WI).
4. 5X Transcription buffer: 200 m*M* Tris-HCl, pH 7.5, 30 m*M* $MgCl_2$, 10 m*M* spermidine, 50 m*M* NaCl.
5. 100 m*M* DL-dithiothreitol (DTT).
6. Sephadex G-50 column (Pharmacia).
7. DEPC-treated dH_2O (*see* **Note 1**).

2.2. Northwestern Assay

1. 10X Ponceau S: 2% (w/v) ponceau S, 30% (v/v) trichloroacetic acid, 30% (v/v) sulfosalicylic acid.
2. Binding buffer: 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 50 m*M* NaCl, 0.1% (v/v) Triton-X-100, 1X Denhardt's solution (50X: 1% Ficoll (Pharmacia, Piscataway, NJ), 1% polyvinylpyrrolidone, and 1% bovine serum albumin [BSA]).
3. Transfer buffer: 50 m*M* Tris-HCl, pH 8.3, 380 m*M* glycine, and 20% (v/v) methanol.
4. Streptavidin-peroxidase (POD) conjugate at 500 U/mL (Boehringer Mannheim, Mannheim, Germany).
5. Solution A: 100 m*M* Tris-HCl, pH 8, 2 m*M* H_2O_2.
6. Solution B: 2.5 m*M* luminol, 75 µ*M* D(-)luciferin (Boehringer Mannheim). Prepare a solution of 2.5 m*M* luminol dissolved in 100 m*M* Tris-HCl, pH 8, (store at 4°C in dark bottle), and a stock solution of 35 m*M* D(-)luciferin dissolved in 100 m*M* Tris-HCl, pH 8, (store in 25 µL aliquots at –70°C protected from light). To prepare solution B, mix 10 mL 2.5 m*M* luminol and 22 µL 35 m*M* D(-)luciferin (*see* **Note 4**).
7. Nitrocellulose membrane (Bio-Rad, Hercules, CA).
8. SDS 15% (w/v) polyacrylamide gel *(7)*.
9. DEPC-treated dH_2O (*see* **Note 1**).

2.3. Gel Retardation Assay

1. 100 m*M* sodium phosphate, pH 7: mix 57.7 mL of 0.5 *M* Na_2HPO_4 with 42.3 mL 0.5 *M* NaH_2PO_4, add DEPC-treated dH_2O to 500 mL.
2. 99% Glycerol.
3. 250 m*M* HEPES, pH 7.5.
4. 100 m*M* KCl.
5. 20 U RNasin ribonuclease inhibitor.
6. 20X SSC: 3 *M* NaCl, 300 m*M* $Na_2Citrate{\cdot}2H_2O$, Adjust pH to 7 with HCl.
7. Nitrocellulose membrane.
8. Agarose (low melting point) dissolved in 10 m*M* sodium phosphate, pH 7, at 1% (w/v). To prepare 250 mL of agarose use a 1-L bottle and dissolve by heating in a microwave until the solution is completely clear.

9. Paper towels.
10. Whatman 3MM paper (Whatman, Maidstone, Kent, UK).
11. BSA.
12. Phosphate-buffered saline (PBS): 137 m*M* NaCl, 2.7 m*M* KCl, 4.3 m*M* $Na_2HPO_4 \cdot 7H_2O$, 1.4 m*M* KH_2PO_4.
13. Buffer P: PBS plus 0.05% (v/v) Tween 20.
14. Streptavidin-POD conjugate at 500 U/mL.
15. Solution A and B (*see* **Subheading 2.2., steps 5** and **6**).
16. Vacuum oven.
17. Loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol).
18. DEPC-treated dH_2O (*see* **Note 1**).

2.4. UV Crosslinking

1. UV lamp with 254 nm wavelength.
2. 96 Microwell plate.
3. 250 m*M* HEPES, pH 7.5.
4. 100 m*M* KCl.
5. Streptavidin-POD conjugate at 500 U/mL.
6. Solution A and B (*see* **Subheading 2.2., steps 5** and **6**).
7. RNase A at 1.5 µg/µL.
8. 5X Buffer sample: 29.2 mL 1 *M* Tris-HCl, pH 6.8, 22 mL 99% glycerol, 8.3 g sodium dodecyl sulfate (SDS), 7.75 g DTT, 50 mg bromophenol blue, to 100 mL with dH_2O.
9. SDS 15% (w/v) polyacrylamide gel *(7)*.
10. Buffer P: PBS plus 0.05% (v/v) Tween 20.
11. Transfer buffer (*see* **Subheading 2.2., step 3**).
12. DEPC-treated dH_2O (*see* **Note 1**).

3. Methods

3.1. In Vitro Synthesis of Biotin-Labeled Riboprobes

Time required: *see* **Note 3**.

1. Add the following components at room temperature in the order listed:

5X Transcription buffer	20 µL
100 m*M* DTT	10 µL
RNasin ribonuclease inhibitor	50 U
2.5 m*M* GTP, ATP, and CTP	20 µL
10 m*M* UTP	2.5 µL
10 m*M* biotin-21-UTP	2.5 µL
5 µg Linearized template DNA	
T7 or SP6 RNA polymerase	20 U
DEPC-treated dH_2O	to 100 µL

2. Mix and incubate for 45 min at 37°C.
3. Add again 20 U of T7 or SP6 RNA polymerase and incubate for a further 45 min at 37°C.
4. Add 1 U RQ1 RNase-free DNase per µg of template DNA. Incubate 30 min at 37°C.
5. The unincorporated nucleotides are eliminated from the reaction mixture by chromatography on a Sephadex G-50 column (*see* **Notes 2** and **11**).
6. Determine riboprobe concentration by measuring optical density at a wavelength of 260 nm. Concentration should be between 100–500 ng/µL (*see* **Note 9**).

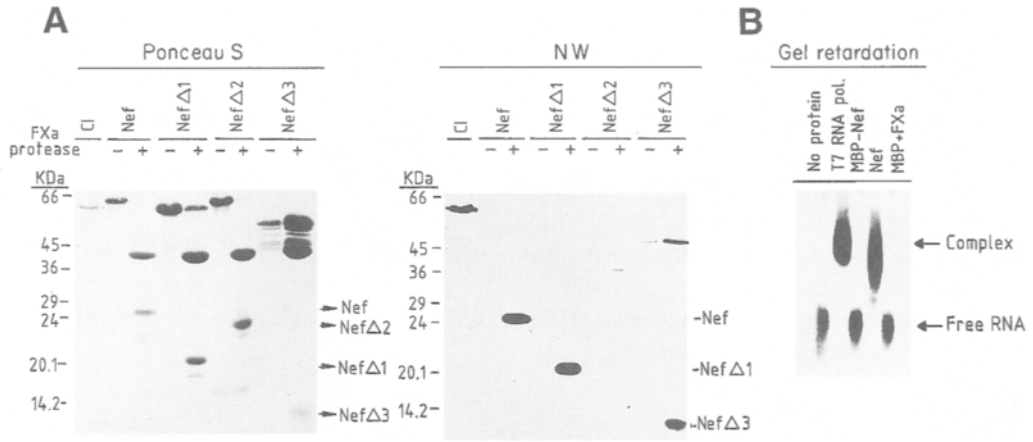

Fig. 1. A. Northwestern assay of HIV-1 nef proteins with biotinylated riboprobe. The left panel shows ponceau S staining of different proteins separated by SDS-PAGE and transferred to nitrocellulose membrane. After renaturation of the proteins in binding buffer, the membrane was incubated with 600 ng of biotinylated riboprobe (7830-8932 nucleotides of BH10 HIV-1 RNA), with streptavidin-POD peroxidase and then with detection solution. An autoradiography (2 min exposure) is shown in the right panel (NW). Potyvirus CI protein was used as a positive control. All proteins (except CI) were purified as fusion constructs with maltose binding protein (MBP, 41 KDa). MBP was separated from different nef proteins by Factor Xa protease digestion (+) or not (–). Nef (from HIV-1 bru) is the wild-type protein (27 KDa); nef Δ1 lacks 33 amino acids from the carboxy terminus (20 KDa), nef Δ2 lacks 22 amino acids from the amino terminus (23 KDa), and nef Δ3 lacks 104 amino acids from the carboxy terminus (13 KDa). B. Gel retardation assay with biotinylated riboprobe. Fifty nanograms of biotinylated riboprobe (7830–8932 nucleotides of BH10 HIV-1 RNA) were incubated with different proteins, loaded on the agarose gel, transferred by capillary action to nitrocellulose membrane, and incubated with detection solution. Autoradiography (1 min exposure) shows the biotinylated-free RNA or RNA-protein complexes. MBP-nef, nef, T7 RNA polymerase (positive control) and MBP + Factor Xa protease were tested for their ability to bind RNA; only nef and T7 RNA polymerase form complexes with RNA.

3.2. Northwestern Assay

Time required: *see* **Note 3**.

1. Protein samples (0.5–5 μg) are diluted 1:1 in 2X buffer sample (*see* **Note 7**) and run in a standard SDS-15% PAGE at 30 mA (*see* **Note 8**). Do not boil protein samples.
2. The gel is transferred to a nitrocellulose filter in transfer buffer at 200 mA for 16 h (*see* **Note 10**) *(8)*.
3. Stain the filter with ponceau S (*see* **Fig. 1A**, left panel) and mark the position of molecular weight marker with a glasswriter (*see* **Note 12**).
4. For renaturation of the proteins incubate the nitrocellulose filter in a RNase-free tray (*see* **Note 1.2**) containing binding buffer at room temperature with agitation on a platform shaker for 2 h (change binding buffer every 30 min).
5. Incubate the renatured proteins with the riboprobe at 20–50 ng riboprobe/ml binding buffer (usually 1–5 μL of transcription reaction in 10 mL of binding buffer) at room temperature for 1 h.

6. Wash the filter three times (2 min each) with binding buffer to remove the unbound riboprobe.
7. Incubate with streptavidin-POD conjugate, diluted 1:20,000 in binding buffer, for 30 min. Cover the dish with aluminum paper until **step 10**.
8. Wash three times (2 min each) with binding buffer.
9. Incubate for 1 min in detection solution (mix 10 mL of freshly made solution A with 10 mL of freshly made solution B; *see* **Subheading 2.2., steps 5** and **6**).
10. Air dry the filter, cover with a plastic sheet, and expose to X-ray films (**Fig. 1A**, right panel), usually for 30 s to 10 min (*see* **Note 5**).

3.3. Gel Retardation Assay

Time required: *see* **Note 3**.

1. Decontaminate all plasticware with H_2O_2 (*see* **Note 1.b.**).
2. Prepare 1% (w/v) agarose gel of appropriate length (approx 15 cm) and depth (approx 8 mm) and let solidify at 4°C. Use 10 m*M* sodium phosphate, pH 7, as electrophoresis buffer and prerun the agarose gel for 15 min at 4°C at 20 V ***(9)***.
3. Set up binding reaction in a microcentrifuge tube:

250 m*M* HEPES, pH 7.5	1.6 µL
100 m*M* KCl	2 µL
Biotinylated RNA	20–200 ng
RNasin ribonuclease inhibitor	20 U
Purified protein	0.5–2 µg (<5 µL)
DEPC treated dH_2O	to 20 µL

4. Mix and incubate at room temperature for 15 min.
5. Add 2.2 µL 99% glycerol to the reaction mixtures and load the samples. Load in an adjacent lane 5 µL of loading buffer. Run at 20 V for 16 h or until the bromophenol blue has migrated 80% of the gel length, at 4°C.
6. Incubate the agarose gel in 20X saline sodium citrate (SSC) for 1 h with agitation at room temperature.
7. Meanwhile, cut a piece of nitrocellulose filter to the gel size. Wet with dH_2O and soak in 20X SSC for 30 min.
8. Transfer the gel to the filter by capillary action ***(10)*** as follows. Place a piece of Whatman 3MM paper wetted with 20X SSC on a piece of glass to form a support that is longer and wider than the agarose gel. Place the support inside a large baking dish. Fill the dish with 20X SSC. Immerse the Whatman 3MM paper edges into the 20X SSC. Place the gel on the support, avoiding air bubbles. Surround the gel with Saran Wrap. Place the wet nitrocellulose membrane on top of the gel, avoiding air bubbles (mark position of the wells on the nitrocellulose membrane with a pencil or glasswriter). Place on top of nitrocellulose, in the order listed, four pieces of Whatman 3MM paper, 10 cm of paper towels, a glass plate, and a 500-g weight. Allow overnight capillary transfer.
9. Dry the nitrocellulose by baking for 2 h at 80°C in a vacuum oven.
10. Incubate the nitrocellulose filter in buffer P plus 3% (w/v) BSA for 30 min.
11. Wash once with buffer P for 1 min.
12. Incubate with streptavidin-POD conjugate dissolved 1:20,000 in buffer P for 30 min. Cover the dish with aluminum foil until **step 15**.
13. Wash with buffer P for 10 min. Repeat this step twice.
14. Incubate for 1 min in detection solution (mix 10 mL of freshly made solution A with 10 mL of freshly made solution B; *see* **Subheading 2.2., steps 5** and **6**).
15. Air dry in the dark, cover with plastic sheet, and expose the filter to X-ray films for 30–300 s (*see* **Fig. 1B** and **Note 5**).

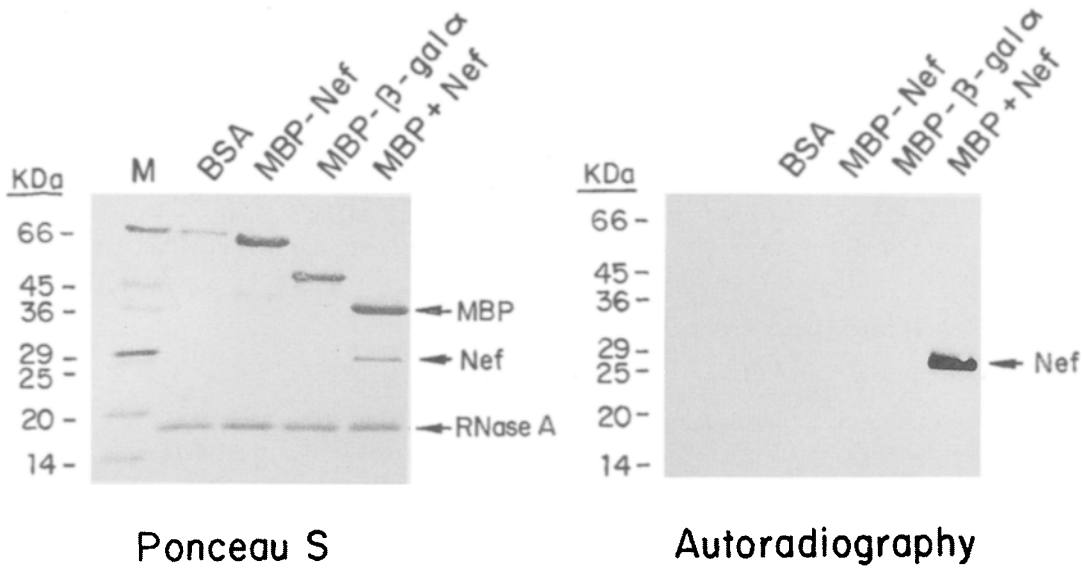

Fig. 2. UV crosslinking assay of HIV-1 nef protein using biotinylated riboprobe. Ponceau S staining (left panel) of the nitrocellulose membrane and autoradiography (right panel) of the membrane after treatment with detection solution. Eight hundred nanograms of biotinylated RNA (1-829 nucleotides of BH10 HIV-1) were used in each reaction. Autoradiography (3 min exposure) is shown. Only free nef protein interacts with RNA.

3.4. UV Crosslinking Assay

Time required: *see* **Note 3**.

1. Set up binding reaction in a microcentrifuge tube (*see* **Note 7**):

250 m*M* HEPES, pH 7.5	1.6 µL
100 m*M* KCl	2 µL
Biotinylated RNA	300–1500 ng
Purified protein	0.5–2 µg (<5 µL)
DEPC-treated dH_2O	to 20 µL

2. Mix and incubate at room temperature for 15 min.
3. Transfer the reaction to a 96-microwell plate and place the plate on ice during exposure to UV. Irradiate the reaction mixture for 10 min at 4°C using a 254-nm UV lamp placed 4 cm above the samples. Protect from irradiation outside the sample by covering the lamp with aluminum foil during this step (*see* **Note 6**).
4. Add to the reaction 1 µL of 1.5 µg/µL RNase A and incubate for 30 min at 37°C.
5. Add 5 µL 5X buffer sample and separate proteins by SDS-15%PAGE at 30 mA.
6. Transfer the gel to a nitrocellulose membrane at 200 mA in transfer buffer overnight.
7. Stain the proteins with 1X ponceau S (**Fig. 2**, left panel). Mark molecular weight standards on the nitrocellulose filter with a glasswriter (*see* **Note 12**).
8. Incubate the filter in buffer P plus 3% (w/v) BSA for 30 min.
9. Wash once with buffer P for 1 min.
10. Incubate with streptavidin-POD conjugate dissolved 1:40,000 in buffer P for 30 min. Cover the dish with aluminum foil until the last step.
11. Follow **Subheading 3.3., steps 13–15** (**Fig. 2**, right panel).

4. Notes

1. DEPC reacts rapidly with amines and cannot be used to treat solutions containing buffers such as Tris. DEPC can modify purine residues in RNA by carboxymethylation. DEPC is suspected to be a carcinogen.
   a. DEPC treatment: Add the DEPC to dH_2O or to the indicated solution to a concentration of 0.1% (v/v). Mix well and allow to stand overnight in a fumehood. Autoclave the liquids to eliminate the DEPC.
   b. Preparation of RNase-free plasticware: Wash the electrophoresis tanks, combs, and casting trays with 3% (v/v) H_2O_2 for 1 h and rinse with DEPC-treated dH_2O.
2. Do not use phenol extraction, as biotinylated probes are soluble in the phenol layer.
3. Duration of procedures:

a. In vitro synthesis of biotin labeled riboprobe:	1 d
Transcription	1.5 h
DNA digestion	30 min
Chromatography	15 min
b. Northwestern assay: 2 d	
SDS-PAGE (10 × 10 cm)	3 h
Transfer	overnight
Renaturation and detection of RNA	4 h
c. Gel retardation assay: 3 d	
Gel preparation:	4 h
Running the samples:	overnight
Transfer	1.5 h + overnight
Detection of RNA	4 h
d. UV crosslinking assay: 2 d	
Reaction, UV light and RNA digestion	1.5 h
Running the gel	3 h
Transfer	overnight
Detection of RNA	2 h

4. D(-)Luciferin solution sometimes does not become completely clear but works well nonetheless.
5. Usually 1 min exposure is enough to detect biotinylated RNA. Exposition can be extended for 3 h. However if the signal is strong, long exposures could decrease the band intensity.
6. Longer periods of irradiation will not improve the signal intensity. Protein degradation and/or oligomerization may occur if longer periods of irradiation are used.
7. Streptavidin-POD conjugate can interact with some proteins such as RNase A. Moreover, some proteins, such as propionyl-CoA carboxylase and pyruvate carboxylase, can interact with biotin. In the case of Northwestern and UV crosslinking assays, a negative control without biotinylated riboprobe is recommended.
8. Electrical currents greater than 30 mA can cause irreversible denaturation of the protein. A 10 × 10 cm SDS-PAGE at 30 mA requires 3 h electrophoresis.
9. Biotinylated transcripts can be quantified by serial dilution of 1 µL transcription reaction in 20X SSC spotted to nitrocellulose with a dot-blot apparatus (Bio-Rad). Filters are dried at 80°C for 2 h *in vacuo* and the biotinylated riboprobes are detected as in **Subheading 3.3., steps 10–15** is described.
10. Protein transfer from the gel to the nitrocellulose filter is performed wet, because semidry transfer causes heating of the samples and may inhibit subsequent renaturation of the transferred proteins *(11)*.
11. Alternatively, ethanol precipitation can be performed.

12. Ponceau S staining fades after renaturation and treatment of the nitrocellulose membrane with detection solution.
13. Potential problems:
    a. In vitro synthesis of biotin labeled riboprobes:
        i. Low amounts of riboprobe: Make sure that reaction components are at room temperature. Add larger amount of linearized plasmid dissolved in RNase free dH_2O to the reaction mixture and make sure that no NaCl is present in the template solution (NaCl concentration higher than 30 m*M* could inhibit RNA polymerase activity). Make sure that all solutions are RNase free.
        ii. Presence of transcripts longer than expected: DNA template should be completely digested with an enzyme that generates a protruding 5' terminus. If it is impossible to use such a restriction enzyme, the linear DNA template should be "blunt ended."
    b. Northwestern assay: If the riboprobe is correct, the problems could be:
        i. The positive control does not bind RNA: Solutions and/or labware contaminated with RNase is a possible cause. Treat all the solutions with DEPC and the plasticware with 3% (v/v) H_2O_2. Check that luminol, luciferin, and H_2O_2 are at a correct concentration and stored in the dark.
        ii. A nonspecific background signal is obtained: Reduce the riboprobe concentration or increase the duration of the washes.
    c. Gel retardation assay:
        i. Biotinylated RNA cannot be visualized: Incorrect transfer. Use a heavier weight for the transfer. The paper towels perhaps may not have been in contact with the transfer buffer.
        ii. Spots in autoradiography: Air bubbles between nitrocellulose and the agarose gel were present.
    d. UV crosslinking assay:
        i. The proteins disappear: Check the transfer buffer and current during the transfer procedure. There is excess UV light irradiation.
        ii. Band smearing: Incomplete RNase A digestion.
        iii. Low band intensity: Concentrate the riboprobe by ethanol precipitation to 1 µg/µL.

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