Chapter 19

Electrophoretic Mobility Shift Assay for Characterizing RNA–Protein Interaction

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Abstract

Electrophoretic mobility shift assay, or EMSA, is a well-established technique for separating macromolecules under native conditions based on a combination of shape, size, and charge. The use of EMSA can provide both general and specific information concerning the interaction between two macromolecules such as RNA and protein. Here we present a protocol for the practical use of EMSA to assess protein-RNA interactions and ribonucleoprotein (RNP) assembly. The conceptual framework of the assay is discussed along with a step-by-step procedure for the binding of archaeal ribosomal protein L7Ae to a box C/D sRNA. Potential pitfalls and common mistakes to avoid are emphasized with technical tips and a notes section. This protocol provides a starting point for the design and implementation of EMSA in studying a wide variety of RNP complexes.

Key words: EMSA, gel-shift, RNA–protein interaction, RNP assembly, radiolabeled RNA.

1. Introduction

During the course of research, it often becomes necessary to characterize the interaction between a protein and an RNA. Many methods are available for the analysis of protein–RNA interactions. Each approach depends upon the particular question being asked. Electrophoretic mobility shift assays (EMSA), commonly referred to as gel shift or band shift assays, provide a sensitive, straightforward, and low cost analysis of protein–RNA interactions. Here we will focus on using gel-shifts to observe the interaction between an in vitro synthesized box C/D sRNA and

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recombinant ribosomal protein L7Ae from *Methanocaldococcus jannaschii*.

Gel electrophoresis is based upon the principle that charged biological molecules will migrate through a gel or porous matrix in an electric field toward the opposite charge (1, 2). Polyacrylamide gels are the standard matrix for EMSA, giving a good balance between band resolution and broad separation ranges. Because EMSA is gel electrophoresis under native, non-denaturing conditions with a buffer of near neutral pH and low ionic strength, macromolecules are separated based not only on their size and charge but also on their shape. For example, an elongated or odd shaped protein or RNA will typically run slower than a more compact, globular protein or RNA with otherwise identical molecular weight and charge. For this reason it is not possible to use molecular weight standards in native gel electrophoresis to accurately estimate protein, RNA, or ribonucleoprotein (RNP) size. Native conditions are necessary to maintain stable non-covalent interactions between protein and RNA in an electric field. The RNA, being uniformly negatively charged, will migrate toward the cathode. RNAs bound by protein will typically migrate slower through the gel due to the increased size of the RNP complex, thus causing a "shift" in the RNA band observed on the gel.

The benefits of gel-shifts over other techniques for analyzing RNA–protein interactions include sensitivity, simple setup, relatively low cost in time and materials, and a limited requirement for knowledge of the RNA–protein interaction under investigation (3). The assay only requires knowing, with some degree of precision, what the DNA or RNA is that the protein binds to and having a relatively pure form of the nucleic acid. The protein can be recombinant or a purified fraction from an extract, but an extract itself may suffice, especially if antibodies are available for the protein of interest. Only minute amounts of radiolabeled RNA and small quantities of the protein are required since the RNA is usually limiting in the reaction and the reaction volume must be small enough to load on a gel. In general, the size and absolute purity of the nucleic acid or protein is not a concern, unlike in other methods, as long as their interaction causes an observable shift in the migration of the RNA or DNA through the gel (3). Furthermore, once the basic gel-running apparatus has been setup and reagents have been prepared, multiple gel shifts can be run simultaneously and the results easily known within the day of the experiment.

EMSA is a technique often used early in characterizing RNA–protein interaction, providing the information necessary to move on to more specific experiments. On the other hand, it can be used to ask very specific questions about an RNA–protein interaction, such as through systematic mutation of the RNA or

protein followed by a series of gel-shifts to assay binding. Combined with other biochemical, biophysical, or genetic approaches, EMSA is an exceptionally useful and informative tool. Although gel-shifts are simple in concept, they can sometimes pose difficult technical problems or generate puzzling results. In this chapter, we walk through an established experimental protocol showing real results and their interpretation, noting common mistakes to watch out for and tips to ensure high-quality data. A special notes section takes much of the guesswork and troubleshooting out of the method. The protocol shown here involves three parts: (1) preparation of radioactively labeled RNA, (2) a binding reaction that combines radiolabeled RNA and protein, and (3) separation of unbound RNA from protein-bound RNA by native polyacrylamide gel electrophoresis (PAGE).

The binding of ribosomal protein L7Ae to the sR8 RNA, a box C/D sRNA containing two K-turn motifs, is well-characterized and commonly used in our laboratory to train new students in the art of EMSA. Both protein and RNA genes have been cloned in our laboratory from the archaeal thermophile *M. jannaschii* (4, 5). Purification of L7Ae as a recombinant His(6X)-tagged protein is straightforward and the sR8 RNA can be quickly synthesized using an in vitro T7 RNA polymerase transcription kit (6). While the specific binding of L7Ae to a K-turn RNA has now been extensively studied with biophysical techniques, such as X-ray crystallography, fluorescence resonance energy transfer (FRET), and circular dichroism (7–11), it was originally characterized and continues to be investigated by EMSA (5, 11–14). In Archaea, L7Ae specifically recognizes a K-turn motif in the large ribosomal subunit as well as k-turns of the box C/D and box H/ACA sRNAs. For the box C/D sRNAs, L7Ae binds a terminal K-turn motif, called the box C/D, and an internal K-turn motif called the box C′/D′ (5). The box C/D sRNAs direct 2′-O-methylation of specific nucleotides through complementary base-pairing with target RNA substrates (*see* **Fig. 19.1a**). The initial in vitro binding of L7Ae is required for the subsequent binding of two other core proteins, Nop56/58 and fibrillarin, to generate an enzymatically active box C/D sRNP (5, 13) (*see* **Fig. 19.1b**). The bound core proteins are the catalytic engine of the RNP and are guided to the correct target RNA substrates by the RNA guide sequence.

2. Materials

2.1. General Methods

1. Redistilled phenol equilibrated in Tris-HCl, pH 8.0.
2. Chloroform:isoamyl alcohol (24:1).

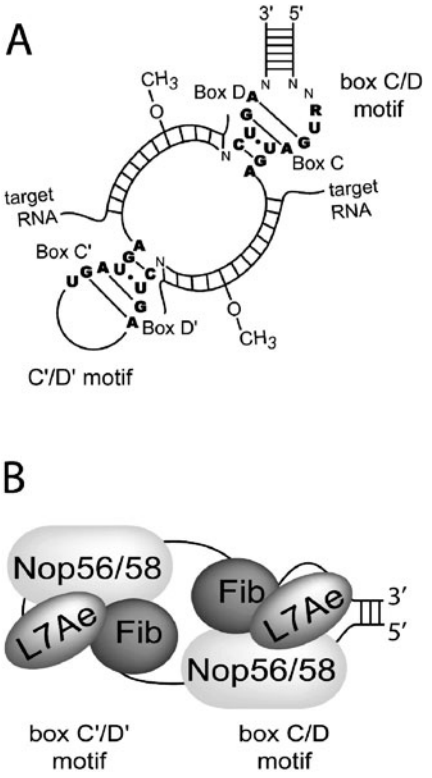

Fig. 19.1. Structure and function of the archaeal box C/D sRNP. **a** Secondary structure of archaeal box C/D sRNA base-paired to target RNA substrates. The conserved box C/D and box C′/D′ motif sequences are indicated. Guide regions base-pair with complementary target RNA substrates to guide site-specific 2′-O-methylation. **b** Three core proteins bind the archaeal box C/D sRNP to assemble in vitro an enzymatically active RNP. L7Ae initiates assembly by specifically recognizing and binding the terminal box C/D motif and internal box C′/D′ motif. Nop56/58 and fibrillarin core proteins then bind at each RNP.

3. RNase-free distilled/deionized water (ddH_2O).
4. 3 M sodium acetate solution, pH 5.2.
5. 100% ethanol.
6. 70% ethanol.

2.2. Preparation of Radiolabeled RNA

1. Calf intestinal phosphatase (CIP) and 10× CIP buffer: 0.5 M Tris-HCl pH 9.0, 100 mM $MgCl_2$, 10 mM $ZnCl_2$, 0.1 M spermidine-HCl.
2. Polynucleotide kinase (PNK) and 10× PNK buffer: 0.5 M Tris-HCl pH 7.6, 70 mM $MgCl_2$, 50 mM dithiothreitol (DTT).
3. [γ-^{32}P] adenosine triphosphate (ATP).
4. G-25 sephadex and minispin columns (Amersham Pharmacia).

5. TE buffer:10 mM Tris-HCl, pH 7.5, 1 mM EDTA.
6. 19:1 acrylamide:bisacrylamide.
7. 10× TBE: 0.89 M Tris base, 0.89 M boric acid, 20 mM EDTA.
8. Urea, molecular biology grade.
9. 10% ammonium persulfate (APS), prepared fresh.
10. N,N,N,N'-Tetramethyl-ethylenediamine (TEMED).
11. Gel loading buffer: 80% formamide, 1× TBE, 10 mM EDTA.
12. Bromophenol blue and xylene cyanol dyes.
13. Clear plastic wrap (Saran^{TM} wrap).
14. Black India ink.
15. RNA elution buffer: 0.3 M sodium acetate, 5 mM EDTA, 10 mM Tris-HCl, pH 7.4, 0.1% SDS.
16. Phosphorimager cassette or X-ray film (for visualizing radioactivity).
17. 0.45-μm syringe filter.

2.3. EMSA to Characterize RNA–Protein Interaction

1. Buffer D: 20 mM HEPES, pH 7.0, 0.1 M NaCl, 3 mM $MgCl_2$, 0.4 mM EDTA, 1 mM DTT, 20% glycerol.
2. 10× binding buffer: 0.1 M HEPES, pH 7.0, 1 M NaCl.
3. 10× phosphate dye: 25 mM potassium phosphate, pH 7.0, 25% sucrose, 0.1 mg/mL bromophenol blue.
4. 10× phosphate buffer: 0.25 M potassium phosphate, pH 7.0.
5. Glycerol, molecular biology grade.
6. 3MM Whatman filter paper.

3. Methods

3.1. General Methods

3.1.1. RNase-Free Technique

1. Use baked glassware and certified RNase-free or DEPC-treated plastic ware.
2. Wear gloves at all times. RNases from skin are the most common form of contamination.
3. Never reuse tips or tubes. Discard if you are unsure whether it has been contaminated.
4. Keep work surfaces clean and free of dust. Clean automatic pipettors regularly.

5. All reagents and buffers should be certified RNase-free from the manufacturer or prepared with RNase free chemicals and RNase-free water (ddH_2O). Everything that will touch the RNA must be free of RNases, especially protein solutions.
6. Solutions of RNA should be handled appropriately. Store dry or aqueous stocks at –20°C or colder. Do not expose RNA solutions to high concentrations of divalent metal ions, high pH (>9.0), or elevated temperatures for extended periods of time.

3.1.2. Phenol/Chloroform Extraction of RNA Solutions (Removal of Protein)

1. To an RNA solution, add 1 volume of phenol (*see* **Note 1**). Mix vigorously.
2. Separate aqueous and phenol layers by centrifugation at 10,000×*g* for 3 min.
3. Carefully transfer the top aqueous phase into a fresh tube with a pipette (*see* **Note 2**).
4. Add 1 volume of water to the phenol layer and repeat mixing and centrifugation.
5. Pool the first and second aqueous layers and add 1 volume of chloroform. Mix vigorously. Centrifuge at 10,000×*g* for 3 min.
6. Carefully transfer the top aqueous phase into a fresh tube with a pipette.
7. Precipitate the aqueous RNA solution.

3.1.3. Precipitation of RNA Solutions

1. To an aqueous RNA solution, add 1/10 volume of 3 M sodium acetate, pH 5.2.
2. Add ice-cold 100% ethanol to a final volume of 70% (a general rule of two volumes is sufficient), invert to mix, and incubate at –20°C for > 1 h (*see* **Note 3**).
3. Pellet precipitated RNA by centrifugation at >10,000×*g* for 20 min at room temperature. Carefully aspirate the ethanol solution.
4. Wash the pellet with one volume of ice-cold 70% ethanol by inverting tube several times. Immediately centrifuge at >10,000×*g* for 5 min. Carefully aspirate the ethanol.
5. Dry the pellet by lyophilization (using a "speed-vac") or laying the tube on its side in a hood.
6. Resuspend the pellet in ddH_2O and quantitate by absorbance at 260 nm (*see* **Note 4**).

3.2. Preparation of Radiolabeled RNA

RNA is most commonly "body-labeled," where the RNA transcript contains radioactive nucleotides within its sequence, or "end-labeled," where a radioactive nucleotide or phosphate is

placed at the end of the RNA sequence. Here we use 5′-end labeling, which requires that the RNA does not have a 5′-phosphate (*see* **Note 5**).

3.2.1. Dephosphorylation of RNA with Calf Intestinal Phosphatase (CIP)

1. Mix the reaction components below in a 1.5-mL microfuge tube:

   20 μg RNA

   20 μL 10× CIP buffer

   10 μL CIP (1 U/μL)

   ddH_2O to 200 μL
2. Incubate at 37°C for 45 min. Phenol/chloroform extract the reaction and precipitate the RNA.
4. Resuspend the dried pellet in 30 μL ddH_2O and quantitate by absorbance at 260 nm.

3.2.2. 5′-End Labeling with T4 Polynucleotide Kinase (PNK)

1. Mix the reaction components below in a 1.5-mL microfuge tube:

   50–80 pmol CIP-treated RNA (1–2 μg)

   2.5 μL 10× PNK buffer

   8–10 μL [γ-^{32}P] ATP (1 μCi/μL)

   1 μL PNK (20 U/μL)

   ddH_2O to 25 μL
2. Incubate at 37°C for 1.5 h. Add 25 μL of ddH_2O then phenol/chloroform extract.

CAUTION: Work behind a shield and use proper technique when handling radioactivity.

3.2.3. Purification of 5′-End Labeled RNA

Two methods are available for purification of radiolabeled RNA. The phenol/chloroform-extracted RNA can be filtered through size exclusion resin to remove free radioactive nucleotides and salts or purified by denaturing gel electrophoresis. Although more time consuming, gel purification is recommended for gel shifts of the highest quality. Gel purification is desirable if the starting RNA was not initially purified or degradation occurs during the labeling process. Simply label twice as much RNA and scale up the labeling reaction proportionately if you plan to gel purify your RNA.

3.2.3.1. Removing Unincorporated [γ-^{32}P]ATP by Size Exclusion

1. Filter phenol/chloroform-extracted RNA (50 μL) by centrifugation through a 2-cm bed of G-25 size exclusion resin packed in a mini-spin column (Amersham Pharmacia) (*see* **Note 6**). Spin at low speed (<1,000×*g*) for 3 min.
2. Check radioactivity by Cerenkov counting in a scintillation counter (*see* **Note 7**). Do not use scintillation fluid. Place 1

μL of eluate in a 0.5-mL microfuge tube in a scintillation vial for counting.

3. Record date and radioactive counts and store at –20°C. The half-life of ^{32}P is 14.2 days.

3.2.3.2. Gel Purification of Radiolabeled RNA

1. Prepare a 40 mL solution containing 6% acrylamide (19:1 acrylamide:bisacrylamide), 1× TBE and 7 M urea (*see* **Note 8**).
2. Add 10% APS (8 μL/mL) and TEMED (1 μL/mL). Mix by inverting.
3. Pour into assembled gel apparatus (15 × 17 × 1.5 cm) and position a comb in the top of the gel.
4. Allow the gel to polymerize for 20 min. Remove the comb; rinse out the wells with 1× TBE, and pre-run the gel with 1× TBE running buffer for ~20 min at 40–45 mA (*see* **Note 9**).
5. Add 1/2 volume (25 μL) of gel loading buffer to the extracted and 5′-end labeled RNA.
6. Boil the RNA sample for 3–5 min. Cool to room temperature.
7. Load the sample. In a separate lane load 20 μL gel loading dye (gel loading buffer +0.1 mg/mL bromophenol blue and xylene cyanol) (*see* **Table 19.1** for dye migration distances).

Table 19.1
Migration of dyes in EMSA

Acrylamide (19:1) (%)	Bromophenol blue (lower) dye band	Xylene cyanol (upper) dye band
5	35 nucleotides	130 nucleotides
6	26 nucleotides	105 nucleotides
8	19 nucleotides	75 nucleotides
10	12 nucleotides	55 nucleotides

8. Run gel at 40–45 mA (*see* **Note 9**). Run time is from 1 to 2 h. Use the dye bands as an approximation for where the RNA's migration position is in the gel (*see* **Table 19.1**).
9. Separate the glass plates with a wedge so that the gel sticks to one plate.
10. Cover the exposed gel with clear plastic wrap (Saran^{TM} wrap).
11. Place three small drops of radioactive dye (1 μL [γ-^{32}P]ATP in 30 μL black india ink) on the Saran^{TM} wrap at three corners of the gel and let them air dry.

12. Cover the dried drops with clear tape and place a phosphorimager cassette on top.
13. Expose for 5–10 min. Scan cassette in phosphorimager and place print out of gel under the glass plate (*see* **Note 10**). Align the dots and cut out the radioactive RNA band with a heat-treated razor blade.
14. Crush the gel slice into a fine paste in a 1.5-mL microfuge tube using a 1-mL pipette tip that has been sealed at the tip with a heat source.
15. Add 500 μL of RNA elution buffer. Rock at room temperature for 45 min (*see* **Note 11**).
16. Recover the eluted RNA by spinning at 10,000×*g* for 2 min. Filter the elution (solution on top of the gel bits) through a 0.45 μm syringe filter into a new 1.5-mL microfuge tube.
17. Repeat the elution with 300 μL of RNA elution buffer. Spin again and filter through the same syringe to pool with the previous elution. Split the elution into two tubes (400 μL each) and ethanol precipitate (omit addition of sodium acetate).
18. Check the radioactivity and handle as in **Section 3.2.3.1** above.

3.3. EMSA to Characterize RNA–Protein Interaction

3.3.1. RNA–Protein Binding Reactions

1. Add the components indicated in **Table 19.2** to a microfuge tube at room temperature in the order shown:
   a. Mix 10× binding buffer with the tRNA (*see* **Note 12**) and ddH_2O.
   b. Add sR8 RNA (*see* **Note 13**).
   c. Add buffer D and L7Ae protein (*see* **Note 14**).
2. Mix the reaction gently and incubate at 70°C for 8 min (*see* **Note 15**). Cool to room temperature, spin down to remove any precipitation. Transfer the reaction to a new tube, add 2 μL of 10× phosphate dye, and mix gently.

3.3.2. Resolving RNA–Protein Complexes by Native Page

3.3.2.1. Preparing the Native Polyacrylamide Gel

1. Prepare a 40-mL solution containing 6% acrylamide (19:1 acrylamide:bisacrylamide), 1× phosphate buffer, and 2% glycerol (*see* **Note 16**).

Table 19.2
Reaction components and set up for titration of L7Ae with sR8 RNA

	[serial dilution]	1×	20 kcpm/μL 0.2 pmol/μL	10 mg/mL	10×			
Rxn #	**L7Ae (μL)**	**Buffer D (μL)**	**sR8 (μL)**	**tRNA (μL)**	**Binding buffer (μL)**	**ddH_2O (μL)**	**Final volume (μL)**	**Final [L7](nM)**
1	1	10	1	1	2	5	20	0
2	1	9	1	2	2	5	20	20
3	1	9	1	2	2	5	20	50
4	1	9	1	2	2	5	20	100
5	1	9	1	2	2	5	20	200
6	1	9	1	2	2	5	20	300
7	1	9	1	2	2	5	20	400
8	1	9	1	2	2	5	20	600
9	1	9	1	2	2	5	20	800
10	1	9	1	2	2	5	20	1,000

2. Add 10% APS (8 μL/mL) and TEMED (1 μL/mL) and mix by inverting.
3. Pour into an assembled gel apparatus (16 × 18 × 1.5 cm) and position a 15-well comb in the top of the gel (*see* **Note 17**).
4. Allow the gel to polymerize for 20 min. Remove the comb and place the gel in a Hoefer SE600 apparatus (or comparable apparatus) filled with 3.5 L of 1× phosphate puffer (*see* **Note 18**). Add 0.5 L of buffer to the top tank and rinse out the wells with a glass Pasteur pipette.

3.3.2.2. Running the Gel

1. Load samples into the wells. Turn on cold water and stir bar to circulate buffer (*see* **Note 19**).
2. Run the gel at 150 V until the bromophenol blue band has migrated about 2/3 of the way through the gel (1.5–2 h).

3.3.2.3. Visualizing the Gel

1. Separate the glass plates so that the gel sticks to one plate.
2. Cut two pieces of 3MM Whatman filter paper slightly larger than the gel. Press filter paper against the gel. Peel the paper off the plate. The gel will stick to the paper (*see* **Note 20**).
3. Cover the exposed side of the gel with clear plastic wrap and dry in a gel dryer under vacuum at 80°C for 1 h.
4. When dry, discard the back piece of filter paper (it absorbed excess liquid and radioactivity). Expose the gel to a phosphorimager cassette overnight or to X-ray film for 2–4 h.

3.4. Analysis of EMSA Results

3.4.1. Titration of L7Ae onto sR8 RNA

Representative results from the above protocol are shown in **Fig. 19.2a**. The first gel lane on the left is RNA-only. L7Ae is then titrated in subsequent lanes. Increasing concentrations of L7Ae induced a shift in the RNA band, indicating slower migration of the RNA due to protein binding. Each discrete shift represents a unique RNP. The sR8 box C/D sRNA has two K-turns, which are both recognized by the L7Ae protein. Thus L7Ae binds the RNA twice. The first shift in RNA migration quickly becomes shifted again, suggesting cooperative binding. Indeed, quantification of these bands using ImageQuant software (Molecular Dynamics) revealed a sigmoidal binding curve, indicating cooperativity (*see* **Fig. 19.2b**). Since there are two binding events, the binding constants cannot be readily distinguished. However, using RNA constructs containing only one of the L7Ae binding sites allowed us to determine the binding constant for each (5).

3.4.2. Further Applications of EMSA

A number of variations to the protocol presented here allow for analysis of multiple proteins binding to an RNA species.

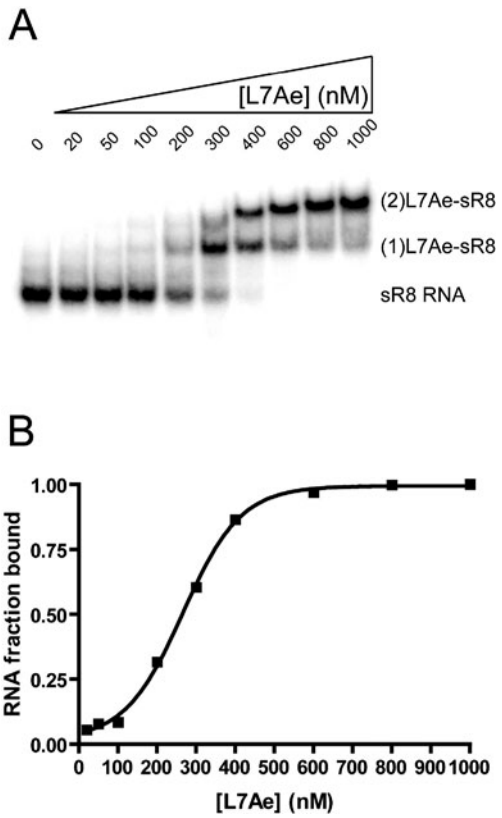

Fig. 19.2. EMSA of L7Ae binding to sR8 box C/D sRNA. **a** Titration of L7Ae with a fixed concentration of radiolabeled sR8 RNA (*see* **Table 19.2b**). L7Ae binds to the RNA twice, first forming an RNP with one L7Ae protein [(1)L7Ae-sR8] and two L7Ae proteins [(2)L7Ae-sR8]. Quantification of L7Ae binding to sR8 RNA. ImageQuant software was used to quantify the intensity of RNA and RNP bands. Band intensity was normalized and plotted using Prism 4 software (Graph Pad) as the fraction of RNA bound versus the concentration of L7Ae. The *sigmoidal curve* indicates the cooperative binding of two L7Ae proteins to sR8 RNA.

RNA-binding specificity, or demonstrating the presence of sequence-specific RNA-binding proteins in an extract (3). As an example of multicomponent RNP assembly, two other core proteins, Nop56/58 and fibrillarin, bind to form an enzymatically active box C/D sRNP in vitro. These interactions and their consequence on RNP assembly and structure have been investigated by our lab and others using EMSA (5, 11–14).

The specificity of protein binding can be determined using a variety of competitor RNAs in the binding reactions, such as specific RNA mutants. These experiments involve either radiolabeling the mutant RNA or titrating excess unlabeled mutant RNA into reactions with labeled wild-type RNA at fixed protein concentrations, often called a competition gel-shift assay. For the protocol presented here, we have included an excess of non-specific

tRNA in the reactions to ensure that the shifts in RNA migration are due to specific L7Ae binding. This is an important factor since many RNA-binding proteins bind RNA non-specifically at higher concentrations.

To determine the identity of the RNA-binding protein when an extract or partially purified protein sample is used, antibodies that bind suspected proteins can be included in the binding reaction. Most often, if the antibody binds a protein that is part of the RNP, then an additional upward shift of the RNP band is observed on the gel due to formation of an antibody–RNP complex. These assays are called supershift assays. However, antibodies can also block RNA binding of the protein, resulting in a slower migrating complex or a reduction in RNA binding and band shifting.

Several experimental variables can be modified to optimize binding for a particular RNA–protein interaction. These include altering the ionic strength (salt components) or pH of the binding reaction, or adding non-ionic detergents and different carrier RNAs or proteins. The protocol shown here for L7Ae and sR8 RNA has been optimized. The buffers used are mild and a good starting point for independent investigations of other protein–RNA interactions. Electrophoresis conditions can also be modified, such as the acrylamide percentage, ratio of acrylamide to bisacrylamide, and crosslinking percentage. Likewise, different running and gel buffers can be used. Common buffers are TBE, Tris-acetate-EDTA (TAE), or Tris-glycine buffers. In this protocol, we used phosphate buffer, which is a more universal buffer and provides optimal resolution for assembly of the archaeal box C/D sRNP. Occasionally, it may be useful to include salts, like magnesium, in the running buffer, although this requires re-circulation between the top and bottom tanks during electrophoresis to prevent salt deposition on the electrodes. Variations in other physical parameters may also be useful. For instance, the archaeal box C/D sRNP requires elevated temperatures for efficient protein binding. We recently made use of low temperature binding and resolution of reactions in a cold room to determine which steps in the assembly pathway were dependent on temperature and therefore RNA or protein conformational dynamics (11).

4. Notes

1. Use caution when working with phenol. Ideally, phenol should be redistilled. Equilibrate in 0.1 M, pH 8.0, Tris-HCl buffer prior to use. Addition of 0.2%

β-mercaptoethanol will prevent oxidation and extend shelf life. Store redistilled and equilibrated phenol at –20°C and working stocks at 4°C. Discard phenol after 6 months at –20°C or if it becomes discolored (most often a pink hue).

2. The phenol and aqueous layers can become inverted, such as with solutions of very high salt or sucrose concentration. Adding a small amount of water prior to or after extraction will determine which layer is aqueous. Be careful not to collect phenol with the aqueous phase. For best results, leave some of the aqueous phase on top of the phenol to ensure that phenol is not also collected. If phenol is collected with the aqueous phase, it is removed in the chloroform extraction step. Chloroform that is carried over in the last step of phenol/chloroform extraction is removed during RNA precipitation.
3. Very dilute RNA solutions often do not precipitate completely. In the protocols presented here, this is typically not a problem. However, if a problem arises, incubation at –20°C overnight (16 h) can increase precipitation efficiency. Also, small amounts of a carrier can be added to aid precipitation, such as glycogen or a non-specific RNA that is safe to have in your reactions later (we often use tRNA from *Escherichia coli* or yeast).
4. Use Beer's Law to calculate the RNA concentration from absorbance at 260 nm (if the extinction coefficient is known) or multiply the OD value at 260 nm by the general conversion factor of 40 μg/OD/mL for RNA. This will yield a value of μg/mL RNA.
5. If your RNA is synthetic, then it does not have a 5′ phosphate and this step should be omitted. If the RNA was transcribed in vitro or purified from an extract, you will need to dephosphorylate.
6. G-25 resin should be swollen and equilibrated in TE buffer prior to use. A swinging bucket rotor will provide a cleaner separation and more efficient removal of free [γ-^{32}P]ATP.
7. Do not use scintillation fluid for Cerenkov counting. Place 1 μL of eluate in a 0.5-mL microfuge tube in a scintillation vial for counting.
8. For your particular RNA, you may choose the gel percentage based on the size of the RNA to be resolved (*see* **Table 19.1**). We keep a stock of 20% acrylamide in 1× TBE, 7 M urea in our lab and dilute it with a stock of 1× TBE, 7 M urea to give the desired final acrylamide percentage. This circumvents the task of preparing fresh every time, which is unnecessary and time consuming. Keep stocks in the dark at room temperature and discard after 1 month.

9. Pre-running the gel is necessary to heat it up. It should be very warm or hot to the touch. The heat and the urea in the gel help to keep the RNA denatured. As a general rule, electrophorese the RNA until it has migrated approximately 2/3 of the way through the gel (*see* **Table 19.1**). Monitor the gel running apparatus closely so that the gel does not overheat. The glass plates should be warm or hot to the touch during the run, but not unbearable. If the plates get too hot they will crack. If samples "smile," where the middle samples run faster than the outer samples, this indicates uneven heating of the gel. Too much smiling should be avoided by reducing the current going through the gel.
10. We do not adjust the size of the phosphorimager picture, just the contrast if necessary, then print. As an alternative, the gel can be exposed to X-ray film for 5–15 min in the dark room and the developed film can be placed under the glass gel plate.
11. Alternatively, the elution step can be performed by rocking overnight at 4°C.
12. The storage and dilution buffer for L7Ae is buffer D. Always add protein solution directly to the reaction last and not to the sides of the tube.
13. tRNA serves as a non-specific RNA in these reactions at excess molar concentrations. Its presence ensures that only specific RNA binding is observed and provides better RNP resolution on the gel.
14. Radiolabeled RNA should be mixed with non-radiolabeled RNA to make a stock with 20,000 cpm's and 0.2 pmol of RNA per μL. 20,000 cpm's per reaction is usually sufficient; however, more or less may be necessary to optimize visualization of the radioactive RNA bands.
15. *M. jannaschii* is a thermophile and efficient binding of the L7Ae protein to sR8 box C/D sRNA requires elevated temperatures. Most RNA-binding proteins bind optimally in a range from 4 to 37°C. Frequently, a carrier protein, such as bovine serum albumin (BSA) is added in excess to keep protein levels at a relatively constant concentration, prevent protein precipitation, and provide clearer RNP resolution. BSA is not included in these reactions due to aggregation and precipitation that can occur under the extreme heat conditions employed for binding. The final reaction contains 20 mM HEPES, pH 7.0, 0.15 M NaCl, 0.5 mM DTT, 0.2 mM EDTA, 10% glycerol, 1.5 mM $MgCl_2$, 1 mg/mL tRNA.
16. Glycerol is an important component of the gel. Do not omit. During drying of the gel prior to visualization,

glycerol prevents the gel from shrinking and cracking, which can make a gel unusable for publication. Glycerol is also thought to aid in RNP resolution.

17. Prior to assembling glass plates with spacers for pouring the gel, the plates should be clean and free of detergent. Glass plates can be wiped or rinsed with ethanol before assembly to remove dust. Glass plates can also be baked, although a thorough washing is often sufficient to remove contaminating RNases.
18. 1× phosphate buffer is sensitive to fungal and bacterial growth and should be prepared fresh before use from a 10× stock. Running buffer can be reused up to several times if it is re-circulated between the top and bottom tanks during or after electrophoresis. However, reuse of buffer is not recommended.
19. Use care when loading the gel so as not to mix sample with running buffer. Start by placing the tip of the pipette at the bottom of the gel and slowly filling the well. When the tip is almost empty, pull it up to the top of the well to finish. Avoid blowing bubbles out of the pipette tip, which will push the sample out of the well and dilute it. Remember, native gels do not have a "stacking layer" so compact loading is important to form sharp bands in the gel. The gel must be kept cool for optimal resolution. Cold running tap water is usually sufficient. Do not allow the temperature of the gel to rise more than a few degrees above room temperature.
20. If the gel percentage is high (>14%), the gel may not stick to the filter paper. In this case, place plastic wrap on top of the gel, flip the gel and plat over, and peel the plate away from the gel. The gel should stick to the plastic wrap. The filter paper can then be placed on top of the gel for drying. For low percentage gels (4%) the gel can very easily lose shape, making the bands in the gel wavy after drying and visualization. Use caution in transferring the gel from the plate to the filter paper. Squirting ddH_2O onto the gel will help if the gel will not adhere to one plate.

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