

# Agarose Gel Separation/Isolation of RNA-Protein Complexes

UNIT 27.1

## ELECTROPHORETIC ISOLATION OF RNA-PROTEIN COMPLEXES

BASIC  
PROTOCOL

Several methods are commonly used for detecting or separating RNA-protein complexes, including density-gradient sedimentation (Grabowski et al., 1985), vertical native polyacrylamide gels (Konarska and Sharp, 1986), and gel filtration (Reed, 1990). Native horizontal agarose minigels can be used for this purpose as well. Among the advantages of the agarose gel method are its simplicity, resolving power, and ability to detect complexes that are disrupted by other techniques. In addition, the use of low-melting-point agarose for fractionation readily allows for identification of the RNA species in each complex detected on the native gel.

### Materials

DNA encoding desired sequence for RNA transcription (UNITS 1.5-1.7)

Reaction mixture (e.g., HeLa cell nuclear extracts, UNIT 27.3)

10× sample loading dye or 10× heparin loading dye (see recipes)

Low-melting-point agarose (Life Technologies)

0.5× TBE electrophoresis buffer (APPENDIX 2)

Gel-fixing solution: 10% acetic acid/10% methanol

Additional reagents and equipment for preparing <sup>32</sup>P-labeled RNA transcripts (UNIT 27.3), electrophoresis using agarose minigels (UNIT 2.5A), and autoradiography (APPENDIX 3A)

**NOTE:** Extreme caution should be taken to avoid RNase contamination. The experimenter should always wear gloves, and all the tubes and tips that come into contact with the sample should be certified RNase free; however, DEPC treatment (UNIT 4.1) of solutions and apparatus is not necessary.

### Assembly of RNA-protein complexes

1. Prepare a <sup>32</sup>P-labeled RNA transcript from DNA encoding the desired sequence as described (UNIT 27.3).
2. Incubate the <sup>32</sup>P-labeled RNA transcript of interest in a 25- $\mu$ l reaction mixture under conditions that allow formation of RNA-protein complexes.

*For example, spliceosomal complexes are assembled by incubation in HeLa cell nuclear extracts under splicing conditions (UNIT 27.3).*

3. Gently mix (do not vortex) the samples with 2.5  $\mu$ l of 10× sample loading dye or 10× heparin loading dye and place on ice.

### Electrophorese RNA-protein complexes on agarose gels

4. Prepare a 7  $\times$  8-cm horizontal low-melting-point-agarose minigel in 0.5× TBE electrophoresis buffer in a horizontal agarose minigel apparatus (UNIT 2.5A).

*A thickness of ~0.5 cm is recommended (i.e., 25 ml melted agarose for a 7  $\times$  8-cm gel).*

*The optimal gel percentage should be determined for the RNA-protein complex of interest—e.g., 1.5% agarose is used for resolving spliceosomal complexes.*

5. Load 10  $\mu$ l RNA-protein complex sample (step 2) in each well.

*The remaining portion of each sample can be used for analysis of the RNA on a denaturing polyacrylamide gel (UNIT 2.12).*

RNA-Protein  
Interactions

27.1.1

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*Current Protocols in Molecular Biology* (2003) 27.1.1-27.1.5

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Supplement 63

6. Run the gel at 70 V in 0.5× TBE at either room temperature or 4°C.

*The resolution varies depending on temperature and must be determined for each complex. The length of time for running the gel must also be determined empirically for each type of RNA-protein complex. For the spliceosome, the 1.5% gel is run until the bromphenol blue dye reaches the bottom.*

#### **Detect RNA-protein complexes**

7. Incubate the agarose gel in gel-fixing solution for 15 min.
8. Dry the gel ~1 hr under vacuum at 80°C.
9. Visualize the complexes using a phosphorimager (*APPENDIX 3A*).

*For instructions regarding elution of the RNA from the gel, which can be important if multiple complexes are observed on the gel, see Support Protocol.*

### **SUPPORT PROTOCOL**

#### **ELUTION OF RNA FROM GEL-FIXED RNA-PROTEIN COMPLEXES**

If several RNA-protein complexes are detected on the native gel, it may be important to determine the identity of the RNA species in each complex. For example, in the case of the spliceosome, complexes can be resolved that contain the unspliced pre-mRNA, the excised intron, or the spliced mRNA. To do this, follow the subsequent procedure.

#### **Materials**

Gel containing bands of interest (see Basic Protocol)

2× PK buffer (see recipe)

Proteinase K

RNase-free phenol, pH 5.2

20 mg/ml glycogen, molecular-biology grade

100% ethanol

Formamide loading dye (see recipe)

Additional reagents and equipment for autoradiography (*APPENDIX 3A*)

1. Excise the bands of interest from the gel using a clean razor blade. Melt the bands by heating 5 min at 70°C.
2. Mix with an equal volume of 2× PK buffer. Add proteinase K to a final concentration of 0.25 mg/ml. Mix and incubate 10 min at 37°C.
3. Extract the samples with an equal volume of RNase-free phenol, pH 5.2. Add 1/10 vol of 20 mg/ml molecular-biology-grade glycogen (2 mg/ml final) to the aqueous phase, mix, and then add 3 vol of 100% ethanol to precipitate RNA.
4. Add 10 µl formamide loading dye to the RNA pellets and incubate 5 min at 70°C.
5. Run the RNA samples on a denaturing polyacrylamide gel (*UNIT 2.12*). Visualize the RNA species by autoradiography (*APPENDIX 3A*).

#### **REAGENTS AND SOLUTIONS**

*Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.*

#### **Formamide loading dye**

80% formamide

10 mM EDTA

0.1% (w/v) bromphenol blue

0.1% (w/v) xylene cyanol

Store up to 1 year at -20°C

### **Heparin loading dye, 10×**

Prepare the following in 1× TBE electrophoresis buffer (*APPENDIX 2*):

- 6.5 mg/ml heparin sulfate
  - 40% (v/v) glycerol
  - 0.5% (w/v) bromphenol blue
  - 0.5% (w/v) xylene cyanol
- Store up to one month at  $-20^{\circ}\text{C}$

### **PK buffer, 2×**

- 20 mM Tris-Cl, pH 8.0 (*APPENDIX 2*)
  - 300 mM NaCl
  - 25 mM EDTA
  - 2% (w/v) SDS
- Store up to one year at  $-20^{\circ}\text{C}$

### **Sample loading dye, 10×**

Prepare the following in 1× TBE electrophoresis buffer (*APPENDIX 2*):

- 40% (v/v) glycerol
  - 0.5% (w/v) bromphenol blue
  - 0.5% (w/v) xylene cyanol
- Store up to 1 year at  $-20^{\circ}\text{C}$

## **COMMENTARY**

### **Background Information**

Electrophoresis of RNA-protein complexes on horizontal native agarose minigels was developed as a simple and rapid method for the separation of spliceosomal complexes (Das et al., 2000). In addition, the method allowed for the detection of spliceosomal complexes that are too labile to be detected by any previously developed method (Chau and Reed, 1999; Luo and Reed, 1999; Das et al., 2000). The ATP-independent E complex was identified as the earliest discrete functional spliceosomal complex using gel filtration, but could not be detected by any of the native gel systems (Reed, 1990; Michaud and Reed, 1991). However, the E complex is readily detected on native agarose minigels. The advantage of the agarose gel over gel filtration for studies of the E complex is that multiple samples can be analyzed side-by-side on the same gel. The A, B, and C spliceosomal complexes assemble sequentially after the E complex and require ATP for their assembly. In the absence of heparin, these complexes comigrate as a large complex on agarose minigels, but can be resolved when heparin loading dye is used (Das and Reed, 1999). The yeast and mammalian A, B, and C complexes, as well as the yeast E complex, can be resolved on native polyacrylamide gels (Konarska et al., 1985; Konarska and Sharp, 1986; Pikielny et al., 1986; Seraphin and Rosbash, 1989). However, these gels are more difficult to pour than the

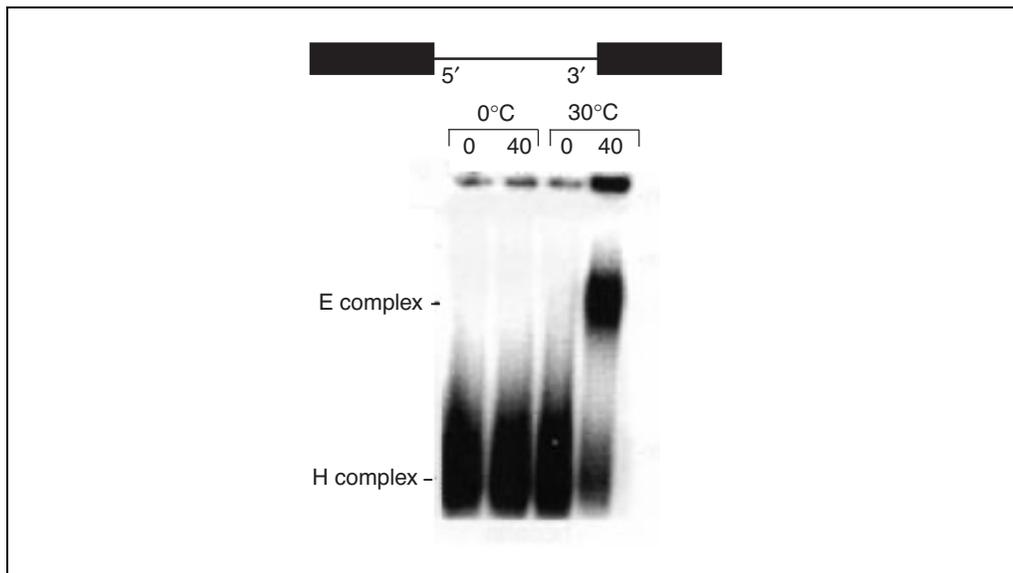
agarose gels and often do not allow as high a level of resolution. In addition, it is much simpler to cut out complexes and identify the RNA species in the complexes using the low-melting-point native agarose gels. Finally, the spliced mRNP complex, which promotes export of mRNA, was identified as a large heparin-sensitive complex on native agarose gels (Luo and Reed, 1999). This complex, like the E complex, can be detected by gel filtration, but not by any other native-gel method. Using the spliceosomal complexes as a precedent, the native low-melting-point-agarose gels may be a good choice for analysis of other types of RNA-protein complexes.

### **Critical Parameters**

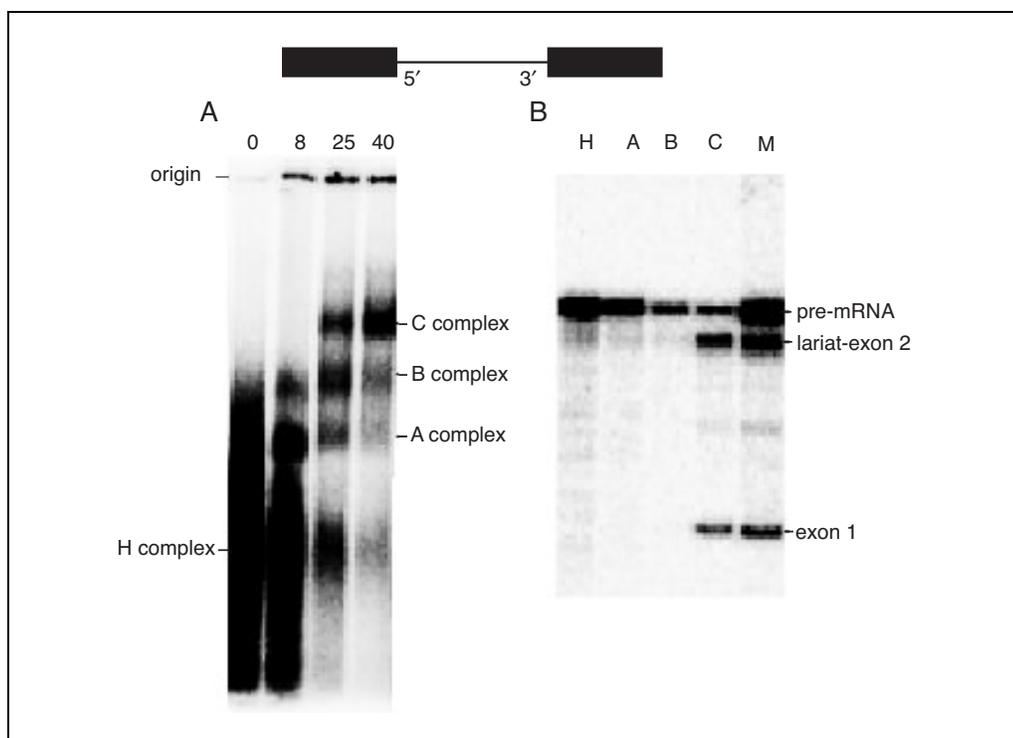
There are several parameters that can be tested to obtain the highest possible resolution of RNA-protein complexes by native agarose gel electrophoresis. The percentage of the gel, the length of time the gel is run, the voltage for running the gel, the sample concentration, and the temperature for running the gel can all be varied to optimize the results.

### **Troubleshooting**

With some RNA-protein complexes, a portion of the sample becomes stuck in the well of the gel. This problem can sometimes be overcome by running the gel 30 min at low voltage and then increasing the voltage for the rest of



**Figure 27.1.1** Detection of the earliest functional spliceosomal complex (the E complex), on a 1.5% native agarose minigel. E complex assembly requires incubation at 30°C and assembles in the absence of ATP. <sup>32</sup>P-labeled pre-mRNA containing one intron was incubated on ice or at 30°C for the times indicated (i.e., 0 or 40 min) in HeLa cell nuclear extracts in the absence of ATP. Aliquots of samples were gently mixed with sample loading dye (no heparin) and then loaded on the gel. The positions of the E complex and the nonspecific H complex are shown.



**Figure 27.1.2** Resolution of the ATP-dependent spliceosomal complexes on a 2% native agarose minigel. <sup>32</sup>P-labeled pre-mRNA containing an AG to GG mutation at the 3' splice site (which allows assembly of the A, B, and C spliceosomal complexes, but not splicing) was incubated in nuclear extract in the presence of ATP. **(A)** At the time points indicated (i.e., 0, 8, 25, or 40 min), heparin loading dye was added to the samples before loading on a 2% agarose gel. The positions of spliceosomal complexes and nonspecific H complex are shown. **(B)** The RNA species in each complex—i.e., H (nonspecific complex), A, B, or C—was identified by excising the complexes from the agarose gel, preparing RNA, and fractionating it on an 8% denaturing gel. An aliquot of a splicing reaction incubated for 40 min was run as a marker (M). The pre-mRNA and splicing intermediates (lariat-exon 2 and exon 1) are indicated.

the running time. The sample or a portion of it may also become stuck in the well if the reaction is too concentrated. Try diluting the reaction 5- to 10-fold with 0.5× TBE electrophoresis buffer. Another strategy for eliminating the material in the well is to use the heparin loading dye. Previous studies using native polyacrylamide gels have shown that heparin dissociates nonspecific interactions and allows samples to enter the well completely. The disadvantage of heparin treatment is that it disrupts many RNA-protein complexes. The heparin can be titrated to find an optimal amount that allows sample entry without disrupting the complex of interest.

### Anticipated Results

Two figures are shown to illustrate results obtained on native agarose gels when samples are treated without (Fig. 27.1.1) or with (Fig. 27.1.2) heparin.

### Time Considerations

Preparation of <sup>32</sup>P-labeled RNA takes ~1 to 2 hr. Assembly of the RNA-protein complex varies according to the complex of interest. Separation of large RNA-protein complexes on a native agarose gel takes ~3 to 4 hr. Fixing and drying the gel takes 1.5 hr. Exposure on the phosphor imager cassette can take from 30 min to overnight depending on the efficiency of complex assembly and the specific activity of the radiolabeled RNA.

### Literature Cited

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