

## Separation of *Drosophila* RNA Silencing Complexes by Native Gel Electrophoresis

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### 1. Introduction

Large, multicomponent complexes mediate many stages of eukaryotic gene expression, from transcription to translation. Despite their size, these complexes and their precursors can often be resolved and analyzed by native gel electrophoresis. Although other techniques exist for large-scale separations, native gels require very little sample, making them superior for analytical applications. We have developed a native gel system for rapidly and reliably separating complexes that form on short interfering RNAs (siRNAs) (**1**). This approach has facilitated our analysis of RNA silencing complexes and holds promise for additional mechanistic studies, particularly those that are limited by sample size [such as experiments with mutant lysates (**2**)]. In this chapter, we describe the procedures involved in reproducibly detecting *Drosophila* siRNA/protein complexes and discuss significant issues as well as techniques for optimization.

### 2. Materials

1. Oligonucleotide siRNAs (Dharmacon or IDT).
2. T4 Polynucleotide kinase (New England Biolabs).
3. [ $\gamma$ -<sup>32</sup>P]ATP (MP Biomedicals).
4. 1X Annealing buffer: 30 mM HEPES, pH 7.5, 100 mM potassium acetate, 2 mM magnesium acetate.
5. 1X Lysis buffer: 1X annealing buffer with 5 mM dithiothreitol (DTT) and 1 mg/mL Pefabloc SC (Boehringer Mannheim).
6. 20% 19 : 1 Acrylamide : bisacrylamide (Bio-Rad).
7. Elution buffer: 0.5 M sodium acetate, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0, 0.1% sodium dodecyl sulfate (SDS).
8. 40% Acrylamide (Bio-Rad).
9. 2% Bisacrylamide (Bio-Rad).

From: *Methods in Molecular Biology*, vol. 309: *RNA Silencing: Methods and Protocols*  
Edited by: G. G. Carmichael © Humana Press Inc., Totowa, NJ

10. Ammonium persulfate (Fisher).
11. TEMED (Bio-Rad).
12. 2X TBE: 178 mM Tris base, 178 mM boric acid, 4 mM EDTA, (final pH 8.3).
13. Glycerol (Fisher).
14. Creatine phosphate (Sigma).
15. Creatine kinase (Calbiochem).
16. 100 mM ATP (Amersham).
17. Dithiothreitol (Sigma).
18. *Drosophila* embryo extract.
19. Native gel dye: 2 mM Tris-HCl, pH 7.4, 30 mg/mL Ficoll-400, 0.04% bromophenol blue.
20. Heparin mix: 60 mM potassium phosphate, 3 mM magnesium chloride, 3% PEG<sub>8000</sub>, 8% glycerol, 4 mg/mL heparin.
21. BioMax XAR film (Kodak).
22. RNA guard ribonuclease inhibitor (Amersham).
23. 5X RNA; mix: 125 mM creatine phosphate, 5 mM ATP, 25 mM dithiothreitol.

### 3. Methods

#### 3.1. siRNA Preparation

##### 3.1.1. siRNA Labeling

Synthetic single-stranded siRNAs can be labeled either at their 3' or 5' ends, using poly(A) polymerase (Amersham) with [ $\gamma$ -<sup>32</sup>P]cordycepin 5'-triphosphate (New England Nuclear) or T4 polynucleotide kinase (PNK) with [ $\gamma$ -<sup>32</sup>P]ATP, respectively, using the manufacturer's instructions (*see Note 1*). Isolate the labeled siRNAs in a 15% (19:1 acrylamide:bisacrylamide) denaturing gel run in 1X TBE and recover them from the gel by gentle agitation at room temperature in 400  $\mu$ L of elution buffer.

Approximately 75–85% of the siRNA will elute from the gel in 2 h. After transferring the aqueous phase to a fresh tube, precipitate the siRNAs by adding 1.1 mL of absolute ethanol, chilling the mixture for at least 20 min at  $-20^{\circ}\text{C}$ , and spinning in a centrifuge at  $4^{\circ}\text{C}$  for 15 min at 16,000g. Wash the pelleted siRNAs with 70% ethanol and quantify by Cerenkov counting.

##### 3.1.2. siRNA Annealing

Anneal the siRNAs by combining an equimolar amount of each strand in 1X annealing buffer, heating to  $95^{\circ}\text{C}$  for 2 min and cooling at  $37^{\circ}\text{C}$  for 1 h (*see Note 2*). A typical annealing mixture contains at least 0.5  $\mu\text{M}$  RNA in a 10- $\mu\text{L}$  volume. The siRNAs should be stored at  $-20^{\circ}\text{C}$  at this concentration and diluted in 1X annealing buffer to approx 7500 cpm/ $\mu\text{L}$  just prior to use (*see Note 3*).

#### 3.2. Native Gel Preparation

1. To prepare the 4% (40 : 1 acrylamide : bisacrylamide) native gel, combine 13.8 mL distilled water ( $\text{dH}_2\text{O}$ ), 20 mL 2X TBE, 4 mL 40% acrylamide, and 2 mL 2% bisacrylamide, and mix well.

2. Add 180  $\mu\text{L}$  10% ammonium persulfate and 43.3  $\mu\text{L}$  TEMED, and mix. This recipe produces a solution adequate for a 0.8-mm  $\times$  17-cm  $\times$  23-cm gel. The combs we use produce wells that are approx 0.6 cm wide and are inserted into the gel to a depth of roughly 1 cm.
3. Pour an excess amount of gel solution on top of the comb after inserting it into the gel to facilitate polymerization of the wells. It might be necessary to raise the top end of the gel (approx 25°) to prevent the solution from leaking out of the top. The gel will polymerize in approx 20 min.
4. After the gel polymerizes, assemble the gel apparatus and fill the chamber with 1X TBE.
5. Remove the comb and, using a razor blade, cut off any excess polymerized material at the top of the gel. The gel should be chilled at 4°C until the temperature of the buffer drops to 6°C (see **Note 4**).
6. Pre-run the gel for at least 30 min at constant power (10 W, which corresponds to approx 360 V using a gel of the dimensions noted above).

### 3.3. RNAi Reactions

#### 3.3.1. Lysate Preparation

1. Prepare *Drosophila* embryo lysates as previously described (3). Briefly, collect 0–2 h embryos in a mesh-lined basket and treat them with 50% bleach for 4 min.
2. After rinsing away the bleach with  $\text{H}_2\text{O}$ , blot the embryos on Kimwipes until blotting no longer leaves water on the Kimwipes.
3. Carefully transfer the embryos to a preweighed microcentrifuge tube and weigh.
4. Add 1  $\mu\text{L}$  of 1X lysis buffer per milligram of embryos and homogenize (on ice) using a pestle.
5. Spin the lysate at 16,000g at 4°C for 25 min to pellet the cellular debris.
6. Collect the lysate, avoiding the insoluble material, and spin for 5 min. Repeat as necessary until the lysate is free of insoluble material.
7. Flash-freeze the lysate in liquid  $\text{N}_2$  and store at  $-80^\circ\text{C}$ .

#### 3.3.2. Sample Preparation and Analysis

1. A typical RNAi reaction mixture supplemented with glycerol can be used for native gel analysis. However, the conditions can be modified to optimize complex formation (see **Note 5**). We observe consistent, robust complex formation using the following mixture: 0.75  $\mu\text{L}$  of *Drosophila* embryo extract, 1.75  $\mu\text{L}$  of 1X lysis buffer, 1  $\mu\text{L}$  of 5X RNAi mix, 0.375  $\mu\text{L}$  of 0.2  $\mu\text{g/mL}$  creatine kinase, 0.125  $\mu\text{L}$  of RNAGuard, 0.5  $\mu\text{L}$  of glycerol, and 0.5  $\mu\text{L}$  of siRNA duplex (see **Subheading 3.1.2**). This mixture can be prepared in larger volumes and separated into 5  $\mu\text{L}$  aliquots. Incubate the 5  $\mu\text{L}$  reaction mixtures at 25°C for 30–60 min (see **Note 5**) and transfer the tubes to ice.
2. Add 1  $\mu\text{L}$  of heparin mixture, mix well by pipetting up and down, and spin quickly at 4°C.
3. Immediately load onto a prechilled, prerun native gel (see **Section 3.2**).
4. Load the native gel dye into an unused well.
5. Run the gel at constant power (10 W) until the bromophenol blue dye migrates 11 cm from the bottom of the well. The total run time should be approx 1.5 h.

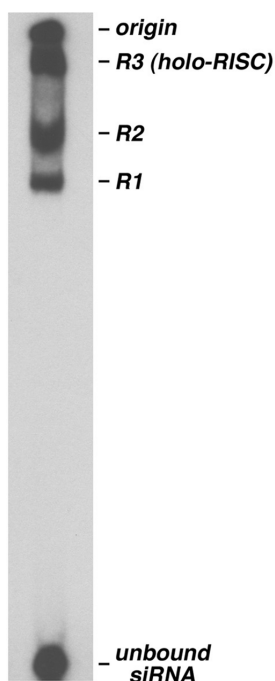


Fig. 1. Native polyacrylamide gel analysis of complexes formed on a radiolabeled siRNA duplex in *Drosophila* embryo lysate. The name of each complex (**I**) is given on the right, (see **Note 6**).

6. Transfer the gel to Whatman paper, dry in a gel-drying apparatus, and expose to film (with an intensifying screen) at  $-80^{\circ}\text{C}$  or to a phosphorimager screen overnight. A typical autoradiograph of a native gel lane is shown in **Fig. 1**. We initially named the three complexes R1, R2, and R3, in order of decreasing mobility (**I**). The R2 complex has a somewhat variable mobility, but it always migrates between R1 and R3. R1 and R2 are assembly intermediates, whereas R3 is a large (approx 80S) functional form of RISC (**I**) (see **Note 6**).

#### 4. Notes

1. We have used both methods and prefer 5'-end labeling because the reagents are comparatively inexpensive. Furthermore, T4 PNK can be used to label siRNAs that already have a 5'-phosphate—prior treatment with a phosphatase is not required. Although an initial report indicated that siRNA 5'-phosphate groups are rapidly removed in *Drosophila* embryo lysates (**4**), later work (**5**) indicated that this is not a consistent phenomenon.
2. For most applications, only one strand of the siRNA duplex needs to be labeled. One should be mindful, however, of the asymmetry rules that determine which RNA strand is incorporated into RISC (**5,6**).

3. It may be necessary in some circumstances to control the exact molar amount of siRNA added to a reaction. In these cases, adjust your dilution protocol accordingly. When this level of control is not required, the dilution protocol described will provide a robust signal in an overnight exposure to film (using an intensifying screen), provided that the label has not decayed much beyond two half-lives.
4. It is critical that the gel be prechilled in order to see all of the complexes (particularly R2, which is less stable than the other complexes). It takes about 2 h to chill 1 L of buffer from room temperature to approx 6°C in a 4°C cold room. This amount of time can be significantly reduced by adding frozen 1X TBE to the buffer.
5. A number of factors contribute to consistent and robust complex formation. These factors include incubation time, total protein concentration in the lysate, gel buffer conditions (temperature), and sample volume. Because the complexes appear to be linked in a pathway (*I*), longer incubation times will favor formation of higher-order complexes (R3, holo-RISC). Likewise, higher protein concentrations will favor holo-RISC formation (presumably by providing more of a limiting factor, although too much protein disrupts complex formation for unknown reasons). We recommend performing an extract titration and time-course to determine the conditions most suitable for analysis. Once the optimal protein concentrations are determined, it would be wise to normalize all extract preparations to match the one determined to be optimal. As noted above, R2 is more stable at lower temperatures. It is also more consistently observed in 5-μL reaction mixtures compared to 10-μL mixtures. This may be due to a surface tension effect, as the mixture spreads to cover the bottom of the microcentrifuge tube at lower volumes.
6. Zamore and co-workers independently developed an agarose native gel system for similar purposes (*7*) and observed two complexes, A and R, which may resemble R2 and R3, respectively. They also detected a complex (B) that was not apparent in our polyacrylamide gels; conversely, they did not observe a complex that was clearly analogous to R1 (*7*).

## Acknowledgments

We are grateful to Richard Carthew and members of his laboratory for help with *Drosophila* manipulations and for advice and discussions. This work was supported by an NIH Biophysics Training Grant (J. W. Pham), a Burroughs Wellcome Fund New Investigator Award in the Basic Pharmacological Sciences (E. J. Sontheimer), and NIH grant GM068743-01 (R. W. Carthew and E. J. Sontheimer).

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RNA Silencing

Methods and Protocols

Carmichael, G. (Ed.)

2005, X, 338 p. 74 illus., Hardcover

ISBN: 978-1-58829-436-4

A product of Humana Press