Protocol

RNase Footprinting to Map Sites of RNA–Protein Interactions

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The binding of a protein to an RNA sequence protects that the region of the RNA from ribonuclease (RNase) digestion; this protected region is known as the protein's "footprint." In this protocol, end-labeled RNAs with and without bound protein are digested with RNase, and the products of digestion are analyzed by gel electrophoresis on denaturing polyacrylamide gels. If the experiment is performed properly, a comparison of the banding patterns from the two samples will reveal the binding site of the protein. The binding site—or footprint—will be detected as a region without bands in the protein-bound sample. In the sample without bound protein, the bands should cover the entirety of the RNA molecule. To establish appropriate digestion conditions for the procedure (i.e., ≤ 1 cleavage event per molecule), it is necessary to titrate the amount of RNase under a range of time and temperature conditions. RNase I cleaves after every nucleotide of RNA and works well under many assay conditions, but other enzymes with different cleavage specificities can also be used. RNase VI is preferable when analyzing structured RNA; RNase A is preferable when using pyrimidine-rich RNAs; and RNase T1 is useful for G-rich RNAs. Choosing enzymes with preference for double-stranded (such as RNase VI) versus single-stranded (such as RNase I) RNA may be helpful. Often, a combination of nucleases is advantageous.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

Aurintricarboxylic acid (ATA; 5 mg/mL in 50 mM Tris [pH 7.5]; Sigma-Aldrich)
EDTA (0.2 M, pH 8.0)
Ethanol (100%)
Formamide gel-loading buffer<R>
GlycoBlue (10 mg/mL)
NaOH (1 N)
Phenol
Polyacrylamide gels (8%–10%) and electrophoresis reagents
Protein of interest and suitable binding buffer (see Steps 9 and 15)
RNA sample of interest (end-labeled and purified)

See 5'-End Labeling of RNA with [γ - 32 P]ATP and T4 Polynucleotide Kinase (Rio 2014b), 3'-End Labeling of RNA with [5'- 32 P]Cytidine 3',5'-Bis(phosphate) and T4 RNA Ligase 1 (Nilsen 2014b), or 3'-End Labeling of RNA with

Adapted from *RNA: A Laboratory Manual* by Donald C. Rio, Manuel Ares Jr, Gregory J. Hannon, and Timothy W. Nilsen. CSHL Press, Cold Spring Harbor, NY, USA, 2011.

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Yeast Poly(A) Polymerase and 3'-Deoxyadenosine 5'-[α -³²P]Triphosphate (Rio 2014a). Make sure that unincorporated nucleotides have been removed by gel purification or by using a spin column.

RNase I dilution buffer <R>
RNase of interest
RNase A
RNase I (Ambion)
RNase T1 (1 mg/mL in TE buffer [pH 7.5]; Sankyo)
RNase VI
SDS extraction buffer <R>
Sodium acetate (3 M, pH 5.2)
T1 sequencing buffer <R>
Tris-HCl (1 M, pH 8.0)
tRNA from *E. coli* (10 mg/mL)
Urea loading dye <R>

Equipment

Denaturing polyacrylamide gel electrophoresis system and power supply

Gel plates must be thoroughly cleaned with a solution of 5% SDS before use.

Dry ice
Gel dryer
Ice
SpeedVac
Water baths or heat blocks (set at the binding temperature [see Steps 9 and 15], at 30°C or 50°C [see Steps 1 and 2], and at 95°C)
Whatman 3 MM paper

METHOD

Preparing Markers

Create sequencing markers from the end-labeled RNA by partial T1 digestion (Step 1) or base hydrolysis (Step 2).

1. Prepare T1 markers as follows.
 - i. Incubate 100,000 cpm of end-labeled RNA in 10 μ L of T1 sequencing buffer for 10 min at 50°C.
 - ii. Stop the reaction with 2 μ L of ATA and 12 μ L of loading buffer.
2. Generate an alkaline ladder as follows.
 - i. Deliver 100,000 cpm of end-labeled RNA into 9 μ L of 2 mM EDTA (pH 8.0) containing 20 μ g of *E. coli* tRNA.
 - ii. Add 1 μ L of 1 N NaOH and incubate for 20 min at 30°C.
 - iii. Stop the reaction by adding 2 μ L of 1 M Tris-HCl (pH 8) and freeze on dry ice until solid. When required, add an equal volume of loading buffer and thaw to room temperature.

Establishing RNase Digestion Conditions

It is important to determine the nuclease concentration that yields a pattern of fragments that covers the entirety of the RNA (≤ 1 cleavage event per molecule). Titrations are performed on naked end-labeled RNA.

3. Resuspend an end-labeled RNA (5' or 3') in H₂O and adjust to $\sim 25,000$ cpm/ μ L.
4. Set up a series of series of dilutions of RNase I in 10 μ L of RNase I dilution buffer.
For example, for the dilution series, use 0, 0.3, 1.0, 3.0, and 0.9 units of RNase I.

5. To each enzyme dilution, add a mixture of 25,000–50,000 cpm end-labeled RNA with 10 μ g of *E. coli* tRNA.

The addition of “carrier” RNA to the digestions helps to keep the reactions reproducible and easier to control.

6. Incubate the reactions for 5 min at 4°C and at room temperature.
7. Stop the reactions by adding an equal volume of denaturing gel-loading buffer.
8. Analyze the extent of cleavage for each reaction by electrophoresis of the samples through an 8% denaturing polyacrylamide gel.

The optimal concentration will show unreacted substrate and a ladder of bands below it. Overdigestion will yield only small fragments. Choose the amount of enzyme that gives a pattern of bands representing cleavage after each nucleotide with some (~20%) of the transcript remaining uncleaved.

Footprinting with RNase I

These procedures are illustrated in Figure 1A.

9. Using the same amount of substrate as in Step 5, set up a series of binding reactions with increasing amounts of protein. Include a no-protein control.

Binding conditions can be established using a filter-binding assay (see [Filter-Binding Assay for Analysis of RNA–Protein Interactions](#) [Rio 2012]) or a gel-shift assay (see [Electrophoretic Mobility Shift Assays for RNA–Protein Complexes](#) [Rio 2014c]).

10. After binding is complete, add 10 μ g of tRNA and the amount of RNase I determined in Step 8. Incubate according to the appropriate RNase I conditions used in Step 6.

It may be necessary to try two to three enzyme concentrations (one above and one below the optimum).

FIGURE 1. (A) A “typical” footprinting experiment. (B) Example of RNase I footprinting. An end-labeled molecule containing a 5' splice site (shown schematically below the gel) was digested with RNase I either in the absence (lanes *minus*) or presence of U1 snRNP either containing (lanes *U1 snRNP*) or lacking (lanes *U1ΔA*) U1-specific protein U1A. A clear footprint that covers the 5' splice site region is observed.

11. Add 0.2 mL of SDS extraction buffer to stop the reactions.
RNase I is inactivated by SDS.
12. Recover the RNAs by phenol extraction and ethanol precipitation (adding 20 µg of GlycoBlue as carrier). Alternatively, add 2 volumes of gel-loading dye and analyze ~10,000 cpm on a denaturing polyacrylamide gel. Freeze the remainder at –80°C.
13. Analyze the footprints by polyacrylamide gel electrophoresis as in Step 8. Load an equal cpm of markers (from Steps 1 or 2) and sample (from Step 12) onto the gel. Carefully pour the bottom buffer out of the gel apparatus to avoid nuclease contamination, and clean the gel plates with a solution of 5% SDS.
Sample results are shown in Figure 1B. See Troubleshooting.

Footprinting with Other Nucleases

14. Titrate the selected enzyme(s) as described in Steps 3–8 until suitable cleavage patterns are observed on denaturing polyacrylamide gels.
Look for a uniform ladder with a “core” of undigested RNA at the top. Once the optimal times and enzyme concentrations are found, they should remain consistent and reproducible.
15. Set up a series of binding reactions as in Step 9.
Because RNase VI requires magnesium for cleavage, it must be included in the protein binding buffer.
16. Add 10 µg of carrier tRNA to each sample, and then add the amount of enzyme determined in Step 14. Incubate the reactions under the conditions determined in Step 14.
17. Add 3 µL of ATA and mix by pipetting up and down.
18. Dilute the reactions with 200 µL of SDS extraction buffer and add 0.3 M sodium acetate. Recover the RNA by phenol extraction and ethanol precipitation (adding 20 µg of GlycoBlue as carrier).
If the protein concentration is low and the buffer does not interfere with gels, the samples can be analyzed directly after addition of ATA without purification. Add an equal volume of urea loading dye and load 5-µL samples onto a gel. This is not advisable for samples containing RNase A.
19. Resuspend the RNA in 5 µL of H₂O and add 10 µL of formamide gel loading buffer. Load ~5-µL samples and carry out electrophoresis through a denaturing 8%–10% polyacrylamide gel.
20. Dry the gel and visualize the bands by phosphorimager or autoradiography.
See Troubleshooting.

TROUBLESHOOTING

Problem (Steps 13 and 20): The footprint observed after electrophoresis of the cleaved binding complex is fuzzy or unclear.

Solution: For footprinting to be successful, it is necessary that a substantial fraction (≥80%) of the target RNA be bound. If less RNA is bound, the unbound RNA, even in the protein-binding site, will be digested, thus obscuring the signal. Consider the following.

- In Steps 9 and 15, make sure that 100% of the RNA is bound in some reactions.
- Purify the binding complexes from Steps 9 or 15 by binding with antibody or tagged proteins and selecting with an affinity reagent. The nuclease cleavage reactions can be performed while the complex is bound to beads. If cleavage is performed in the presence of beads, it may be necessary to increase the time of digestion. Stop the reactions by adding 0.2 mL of SDS extraction buffer, remove the beads by centrifuging in a microcentrifuge for 5 sec, and continue with RNA recovery by phenol extraction as in Step 12.
- Reverse footprinting has a distinct advantage over conventional footprinting in that only a small fraction of the labeled RNA needs to be bound. See **Reverse Footprinting to Map Sites of RNA–Protein Interactions** (Nilsen 2014a).

RECIPES

Formamide Gel-Loading Buffer

Reagent	Quantity (for 10 mL)	Final concentration
Deionized formamide	9.5 mL	95%
Bromophenol blue	2.5 mg	0.025% (w/v)
Xylene cyanol FF	2.5 mg	0.025% (w/v)
EDTA (0.5 M, pH 8.0)	100 μ L	5 mM
H ₂ O	400 μ L	

Store in aliquots at -20°C .

RNase I Dilution Buffer

Reagent	Quantity (for 10 mL)	Final concentration
Tris (1 M, pH 8.0)	0.2 mL	20 mM
NaCl (5 M)	0.2 mL	100 mM
EDTA (0.5 M, pH 8.0)	20 μ L	1 mM
Glycerol (50%, w/v)	4 mL	20% (w/v)

Store indefinitely at 4°C

SDS Extraction Buffer

Reagent	Quantity (for 500 mL)	Final concentration
Tris-HCl (1 M, pH 7.5)	10 mL	20 mM
EDTA (0.5 M, pH 8.0)	1 mL	1 mM
SDS (10% w/v)	25 mL	0.5% (w/v)
H ₂ O	464 mL	—

Store indefinitely at room temperature.

T1 Sequencing Buffer

Reagent	Quantity (for 1 mL)	Final concentration
Sodium acetate (3 M, pH 5.3)	6.6 μ L	20 mM
Urea (molecular biology grade)	0.42 g	7 M
EDTA (0.5 M, pH 8.0)	2 μ L	1 mM
H ₂ O	adjust to 1 mL	

Store for 6 mo at room temperature.

Urea Loading Dye

Reagent	Quantity (for 10 mL)	Final concentration
Urea	4.8 g	8 M
Tris-HCl (1 M, pH 8.0)	200 μ L	20 mM
EDTA (0.25 M)	40 μ L	1 mM
Bromophenol blue/xylene cyanol (1%, w/v)	0.5 mL	0.05% (w/v)
ddH ₂ O	to 10 mL

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