Protocol

RNA Structure Determination Using Nuclease Digestion

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Determining RNA structures (i.e., single- and double-strand regions) is often useful when assessing the potential for certain RNAs to interact with proteins or when determining whether RNAs that are dissimilar in sequence can form the same structure. A number of ribonucleases (RNases) have been used to map RNA structure, but many of these are no longer available. However, three commonly available RNA endonucleases (RNase T1, RNase I, and RNase V1) can provide a wealth of structural information. Cleavages of end-labeled RNA are initiated by one of the RNases (H₂O is used for mock-treated controls), terminated with aurintricarboxylic acid (a potent RNase inhibitor), and detected by electrophoresis on denaturing polyacrylamide gels. Because there are enzymes that can cleave only when the RNA is single stranded (e.g., RNase T1) or double stranded (e.g., RNase V1), it is possible to do parallel analyses.

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 article 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)

EDTA (0.2 M, pH 8.0)

Loading dye <R>

NaOH (1 N)

Reaction buffer A (denaturing) <R>

Reaction buffer A (native) <R>

RNases:

- RNase T1 (0.25 unit/μL)
- RNase I (12.5 units/μL)
- RNase V1 (6.25×10^{-3} units/μL)

RNase T1 cleaves 3' to an unpaired guanine; RNase I cleaves 3' to all bases with a slight preference for pyrimidines; and RNase V1 cleaves double-strand regions with a preference to either end of a helix. RNase V1 will also cleave single-strand stacked regions. RNase V1 cleavage requires MgCl₂, so a "denaturing" control (no MgCl₂) cannot be performed with this enzyme.

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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RNA to be probed (~ 0.2 – $1 \mu\text{g}$)

The RNA must be end-labeled (e.g., with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and OptiKinase); see Steps 1 and 5.

T1 sequencing buffer <R>

TE buffer

Tris–HCl (1 M, pH 8)

tRNA carrier (from *E. coli*; $10 \mu\text{g}/\mu\text{L}$)

Equipment

Denaturing polyacrylamide gel electrophoresis system and power supply to run an 8% gel

Dry ice

Gel dryer

Phosphorimager or autoradiography materials

Water baths (set to temperatures to accommodate specific reactions)

METHOD

After optimizing the conditions (reagents and times of incubation) for this procedure, assemble two reactions, one in “native” buffer (folded RNA) and one in “denaturing” buffer (unfolded RNA). See Figure 1.

1. Dilute end-labeled RNA with reaction buffer A (native or denaturing) to obtain 25,000 cpm in $25 \mu\text{L}$ for each reaction.
2. Preincubate the reaction for 10 min at 37°C .
3. Add RNase to one reaction at a time ($1 \mu\text{L}$ to $25 \mu\text{L}$).

Because the incubation times are short, perform one reaction at a time. Final concentrations will be $0.01 \text{ unit}/\mu\text{L}$ RNase T1, $0.5 \text{ unit}/\mu\text{L}$ RNase I, or $2.5 \times 10^{-4} \text{ units}/\mu\text{L}$ RNase V1.

4. Incubate the reactions for 5 sec at 37°C , quickly add $3 \mu\text{L}$ of ATA, and mix by pipetting up and down.
5. Add an equal volume of loading dye. Analyze on an 8% denaturing polyacrylamide gel with appropriate markers. Load equal cpm of markers and sample.

FIGURE 1. Schematic depiction of RNA secondary structure mapping. An RNA with three stem-loops is drawn. After digestion with single-strand-specific nucleases, a representation of expected results is shown. A reversed pattern of protected and sensitive regions would occur if the RNA were digested with a nuclease specific for double-strand regions.

Markers for nuclease probing are generated from the end-labeled RNA by partial RNase T1 digestion or base hydrolysis.

- For T1 digestion, incubate the RNA (100,000 cpm) in 10 μ L of T1 sequencing buffer for 10 min at 50°C. Stop the reaction with 2 μ L of ATA and 12 μ L of loading dye.
- To generate an alkaline ladder, use 100,000 cpm of end-labeled RNA in 9 μ L of 2 mM EDTA (pH 8.0) containing 20 μ g of E. coli tRNA. Add 1 μ L of 1 N NaOH and incubate for 20 min at 30°C. Stop the reaction by adding 2 μ L of 1 M Tris-HCl (pH 8) and freeze on dry ice until solid. Add an equal volume of loading dye and thaw to room temperature.

RECIPES

Loading Dye

Reagent	Quantity (for 10 mL)	Final concentration
Urea (ultrapure)	5.4 g	9 M
Xylene cyanol/ bromophenol blue (1%, w/v) (optional)	500 μ L	0.05% (w/v)

Add enough 10 $\times$ TBE electrophoresis buffer <R> (diluted to 1 $\times$) to reach the 10-mL mark in a 15-mL conical tube containing urea. Heat at 50°C until dissolved. Store at room temperature. Make sure urea is in solution before using.

Reaction Buffer A (Denaturing)

Reagent	Quantity (for 100 mL)	Final concentration
HEPES/KOH (1 M, pH 7.5)	1 mL	10 mM
EDTA (0.2 M, pH 8)	0.5 mL	1 mM
H ₂ O	98.5 mL	

Store indefinitely at room temperature.

Reaction Buffer A (Native)

Reagent	Quantity (for 100 mL)	Final concentration
HEPES/KOH (1 M, pH 7.5)	1 mL	10 mM
KCl (2 M)	5 mL	100 mM
MgCl ₂ (1 M)	0.5 mL	5 mM
H ₂ O	93.5 mL	

Store indefinitely at 4°C. Adjust MgCl₂ concentration between 5 and 25 mM for individual substrates.

TBE Electrophoresis Buffer (10 $\times$)

Reagent	Quantity (for 1 L)	Final concentration
Tris base	121.1 g	1 M
Boric acid	61.8 g	1 M
EDTA (disodium salt)	7.4 g	0.02 M

Prepare with RNase-free H₂O. Dilute 100 mL to 1 L to make gel running buffer. Store for up to 6 mo at room temperature.

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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.

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