

RNase Protection Assay for RNA Modification Detection

References: *Current Protocols*, Chapter 4.7.1
Sambrook, Fritsch and Maniatis, Chapter 7.71
Segal and Eichler, 1991, *JBC* 266(36):24385-9.

Buffers:

Denaturation Buffer: 2 Parts freshly thawed formamide (40% final)
1 Part 5X Denaturation Buffer
2 Parts sterile ddH₂O

5X Denaturation Buffer: 100 mls
200 mM PIPES, pH 6.4 6.93 g (pH with HCl)
2 M NaCl 11.69 g
5 mM EDTA 0.19 g (Do not add from stock) •• Treat with DEPC ••

Digestion Buffer: 1 ml from Stocks
300 mM NaCl 60 µl of 5M NaCl
10 mM Tris-HCl, pH 7.5 10 µl of 1 M Tris-Cl, pH 7.5
5 mM EDTA 10 µl of 0.5 M EDTA
920 µl ddH₂O

Freshly add: RNase A to 20 µg/ml (1/500 of 10 mg/ml stock; Sigma R-5000)
RNase T₁ to 3 µg/ml (1/500 of 1.5 mg/ml stock; Sigma R-1003)

Formamide Loading Buffer: 4 Parts freshly thawed formamide
1 Part 5X Loading Buffer

5X Loading Buffer:
50 mM EDTA, pH 8.0
5 mg/ml Bromphenol Blue •• Treat with DEPC ••

Yeast tRNA: 10 mg/ml in sterile ddH₂O

Procedure:

See instructions for labelling of yeast pre-rRNA with [³H]-methyl-methionine.

1. Dissolve one [³H]-rRNA pellet (from ~7 A₆₀₀ units of culture) in 50 to 200 µl of Denaturation Buffer. The standard reaction volume is 50 µl. 200 µl will provide enough for four separate 50 µl reactions. More than 4 reactions from one RNA pellet may give a faint signal. Repipet sample to dissolve; heat briefly to 37°C if necessary. Formamide can hinder dissolution of pellet, so pellets may be dissolved in aqueous medium followed by addition of formamide to 40% final.

- Use a new box of tips. Wear washed gloves. Take precautions against RNase contamination of sample.

2. Plan to add ~300 pmoles of DNA oligonucleotide probe per RNA pellet. If one RNA pellet is in 50 µl, then add 1 µl of 300 pmol/µl probe solution. If the RNA pellet was dissolved in 200 µl, then add 4 µl of 75 pmol/µl probe to 200 µl of Denaturation Buffer. Then, divide solution into 4 tubes with 51 µl each.

- We assume 30 µg RNA per sample pellet is ~30 pmoles total RNA. Probe is calculated to be present at ~10-fold excess.

3. Heat to 85°C for 15 minutes.
4. Transfer mixture rapidly to prewarmed hybridization temperatures (for our 34-mer probe, 60°C is good). Incubate 4 hours and cool to room temperature (RT), but do not leave at RT for long.
5. Add 250 µl Digestion Buffer containing RNases. Incubate at 30°C for 30 minutes.
6. Add 16 µl of 10% SDS. Vortex briefly. Add 4 µl of 25 mg/ml Proteinase K (aliquots at -70°C) and incubate at 37°C for 15 minutes. Thaw Proteinase K aliquots only once and discard excess. Cool to RT.
7. Add 1 µl of 10 mg/ml yeast tRNA. Mix.
8. Add 300 µl Phenol:CHCl₃:IAA. Vortex vigorously for 30 seconds. Spin in microfuge at RT for 5 minutes. Transfer upper phase to new tube (don't take any of the interface layer).
9. Add 600 µl (at least 2 volumes) of 100% ethanol and vortex. Sample must be completely mixed.
10. Store solution at -20°C for 30 minutes (can go overnight).
11. Spin in microfuge at 4°C for 20 minutes at top speed.
12. Wash pellet with 70% ethanol. Spin again for 10 minutes.
13. Wrap tube top with parafilm. Poke holes with needle. Speed-Vac *only for a few minutes until dry*. Over-dried pellets will not dissolve easily.
14. Resuspend pellet in 5 µl formamide loading buffer. Heat for 5 minutes at 95°C. For this, heat a beaker of water to boiling in microwave, cool to 95°C, and float samples in covered beaker. Plunge into ice bath.
15. Pre-run a 12% polyacrylamide/7 M urea gel for 30 minutes before washing out lanes and loading samples. Run at 17 W, constant power.
16. Fluorography with En³Hance:
 - A. Fix gel in three 15 minute washes of 1X gel fixer (40% MeOH, 10% AcCOOH). Use ~10X gel volume for each wash.
 - B. Immerse in En³Hance for 45 minutes. Agitate gently.
 - C. Precipitate for 30 minutes in slowly running dH₂O. Adjust flow of dH₂O to allow running water to make contact with both sides of gel.
17. Dry gel. Place gel on plastic wrap, front side down. Place wet 3MM paper on gel. Turn over and dry.