

RNase Protection Assay

Prepare RPA probe

In vitro RXN	20 μ L	
DNA (see note)		note: use ~1 μ g linearized 5KB plasmid
5X TXN buffer	4 μ L	txn buffer contains Spermidine, assemble rxn at RT
100mM DTT	2 μ L	
10X NTP mix	2 μ L	10X = 5mM ACG & vary UTP for probe specific activity.
RNase OUT	1 μ L	Invitrogen 10777-019
T7 RNA pol	1 μ L	Promega P207b
[α - ³² P] UTP	5 μ L	10mCi/mL. 3000Ci/mmol
H ₂ O	to 20 μ L	

Incubate in vitro transcription reaction 60min @ 37°C.

Add 2 μ L (2U) DNase I (Promega M601A) and incubate 15min @ 37°C.

Precipitate transcription reaction.

Add 10 μ g yeast tRNA, 100 μ L 2.5M NH₄OAc, & 375 μ L 100% EtOH. Incubate on ice 15min, and then centrifuge 15min @ 4°C.

Resuspend pellet in 10 μ L formamide loading buffer, heat @ 95°C 2min and load on denaturing acrylamide gel (see denaturing gel electrophoresis protocol).

Run until Bromophenol blue is 1/2 to 2/3 down the gel.

Cut full length probe from gel and elute into 400 μ L elution buffer [2M NH₄OAc, 1% SDS, 25 μ g/mL tRNA] for 2-4h @ 42-65°C while rotating. Precipitate eluate w/ 1mL 100% EtOH. Incubate on ice 15min, and then centrifuge 15min @ 4°C.

Resuspend probe in 50 μ L hybridization buffer [80% formamide, 40mM PIPES (pH 6.6), 400mM NaCl, 1mM EDTA]. Count 1 μ L in liquid scintillation counter.

Hybridize probe to sample RNA

Speed vac 1-10 μ g sample RNA (precipitate if not stored in H₂O). Prepare two tubes with 10 μ g tRNA as controls. Resuspend in 30 μ L hybridization buffer containing 1-5 $\times 10^5$ cpm of each probe. (Number of cpm must be determined by titration of sample RNA to guarantee probe is in excess.)

Incubate 5min @ 95°C to denature RNA and immediately transfer to desired hybridization temperature and incubate overnight. Hybridization temp will range from 30-60°C, a good starting point is 42°C.

Digest with Ribonuclease

Add 350 μ L of RNase digest buffer [10mM Tris (pH 7.4), 300mM NaCl, 5mM EDTA, RNaseA/T1 (ambion AM2286)]. RNase concentration must be empirically determined. (start with 1:100 dilution) Digest for 60min @ 30°C.

Add 20 μ L 10% SDS, and 50 μ g Proteinase K. Incubate 15min @ 37°C.

Extract once with 400 μ L phenol/chloroform/ isoamyl alcohol, vortex 15s, and spin 5min @ RT. Remove aqueous (top) layer and ethanol precipitate with 1mL 100% EtOH, and 10 μ g yeast tRNA.

Dry pellet and dissolve in 5 μ L formamide loading buffer. Incubate @ 95°C, 3min, followed by ice. Run on denaturing urea gel.