

## SPLICING ASSAYS (20 ul volume)

2.0 ul 20 mM MgCl<sub>2</sub>  
2.0 ul ATP/CrPO<sub>4</sub>  
1.0 ul RNAsin  
1.0 ul 100 mM DTT  
1.0 ul [<sup>32</sup>P]pre-mRNA  
7.0 ul Nuclear Extract  
6.0 ul dH<sub>2</sub>O (or buffer)

Start reaction by adding the nuclear extract (2 hours at 30C)  
Quick 5 min spin (to get the solution off the sides)  
Add to tube containing 3 ul stop solution and 3.5 ul proteinase K/tRNA  
Spin to get solution off sides  
Heat at 45C for 45 min.

Add 210 ul of EtOH/NH<sub>4</sub>Acetate  
Precipitate on ice 15 min  
Spin 10 min  
Rinse with 500 ul of 70% EtOH  
Spin 5 min  
Speedivac 5 min  
Add loading buffer (5 ul formamide LB)  
Incubate 65C for 10 min  
Run on 10% PA (1:30)/8 M Urea gel in 1X TBE at 30W for 70 min.  
Run markers and pre-mRNA along with samples

Dry gel and expose overnight

## **Depletion of proteins prior to splicing**

First prepare protein A or protein G sepharose beads (Pharmacia) with the antibody of interest coupled to them. The beads are stored in 20% ethanol, so before use they must be washed several times in PBS or a Tris buffer. After the last wash bring the beads up in the same buffer as a 50% slurry, so that it's easier to aliquot them in specific amounts later. Add the antibody to the beads and shake or rotate for 1 hr. at 4C. Centrifuge 1 min at 13,000 g, remove the antibody solution, and wash the beads 2X with 20 mM Tris, pH 7.5 and 2X with 20 mM Tris pH 7.5, 0.5 M NaCl to remove any nonspecifically bound proteins. Wash again with 20 mM Tris pH 7.5 and store the antibody beads in this buffer at 4C (never freeze the beads). As a general rule, beads can be stored for 1-2 weeks.

If the antibody is affinity purified or monoclonal, measure the concentration and bind ~ 2 mg/ml antibody to the beads. Then try depleting your protein from a cell lysate or nuclear extract using increasing amounts of antibody beads. For example, prepare a batch of protein G sepharose with your antibody bound to it at 2 mg/ml. Then try to deplete from 50 ul of nuclear extract using either 5, 10, 15 or 20 ul of antibody beads. To keep the amount of beads a constant 20 ul, add 15, 10 and 5 ul of protein G sepharose to the first 3 conditions. On a blot, check the extract before depletion, the beads themselves, and the extract after depletion. After depleting, wash the beads as described above (2X no salt, 2X high salt, 1X no salt), add running buffer and run on a gel. This will tell show you what came down specifically on your antibody beads.

Once you have established a protocol for depleting your protein, a good control experiment is to specifically prevent the depletion of your protein by preabsorbing the antigen against the antibody prior to exposing the beads to the nuclear extract. If it's a protein antigen, use a 2-fold molar excess of the recombinant protein. If it's a peptide antigen, use a 10-fold molar excess of the peptide. Calculate the molarity of the antibody by the mg/ml amount you added to the beads, divided by 160,000 (approx. molecular weight of an antibody). Add the protein/peptide solution to the antibody beads and shake at 4C for 1 hr. Wash beads a few times with 20 mM Tris, pH 7.5, 150 mM NaCl.

If you have to use a polyclonal antiserum, try binding an equal amount of antiserum to beads (e.g. 50 ul antiserum to 50 ul beads). A good control is the same prep, using the preimmune serum from the animal, if you have it. Wash and store the beads as described above.

To deplete the extract, remove excess liquid from the beads and add the extract to the beads. Shake or rotate at 4C for at least 1 hr. (we occasionally do this overnight as well, so we can splice in the morning). Spin to pellet the beads and carefully remove the extract, making sure you don't take any beads with it. At this point you can give the extract another good spin, and don't use all of it for splicing in case there are still some beads at the bottom. The extract can now be used in splicing assays.

