

Pre-mRNA Splicing Protocols (Lamond Lab)

www.lamondlab.com

Transcription for Biotinylated pre-mRNA (120 ul reaction)

12 ul 10x Transcription buffer
18 ul 100 mM DTT
18 ul 5 mM rNTP's
12 ul 10 mM GpppG
3.6 ul RNasin (10,000 units)
3.6 ul Biotin-16-UTP (250 nmol)
6 ug Plasmid DNA
7.5 ul alpha 32P GTP
2.4 ul T3 RNA Polymerase (17U/ul)
dH2O

Incubate reaction 1 hr. 30C. Purify over G-50 spin column. Phenol/chloroform extract 1x. EtOH precipitation with Na acetate. Wash 70% EtOH. Resuspend in 50 ul dH2O.

Purification of Splicing Complexes

Thaw aliquots of HeLa nuclear extract on ice (will need 4 ml for this prep). Spin 14K for 10 min. and use supernatant.

Prepare master mix:

- 1.1 ml 20mM DTT
- 1.1 ml 20 mM MgCl₂
- 1.1 ml 15/50 mM ATP/CrPO₄
- 150 ul RNasin
- 3. 45 ml dH₂O
- 10 ul 32P pre-mRNA biotinylated

Set up 10 x 1.5 ml eppendorf tubes. To each tube add 0.4 ml HeLa nuclear extract and 0.7 ml master mix.

Incubate reaction 1 hr. 30C. Pool reactions and load into 10 ml superloop for the FPLC.

Purify spliceosome over a S-500 gel filtration column (column buffer: 20 mM TrisHCl pH 7.8, 60 mM KCl, 2.5 mM EDTA pH 8.0, 0.1% Triton X-100), collecting 2 ml fractions.

Pool 10 fractions containing the peak of the S complex in a 50 ml Falcon tube and store at 4C while preparing beads.

At this point, add 30 ul 1M DTT and 10 ul RNasin to the pooled fractions.

Preparing Streptavidin-Agarose Beads:

Take 1 ml of strep. bead slurry (0.5 ml beads) and spin 4000 rpm for 3 min.

Remove liquid and add 1 ml preblocking buffer (20 mM Hepes pH 7.9, 0.3 M KCl, 0.01% NP-40, 50 ug/ml glycogen, 0.5 mg/ml BSA, 50 ug/ml yeast tRNA).

Incubate 4C for 15 min. on an end-over-end rotator. Spin 4000 rpm and remove buffer. Wash 4 times with 1ml wash buffer (20 mM Hepes pH 7.9, 50 mM KCl, 0.1% NP-40). Remove last of wash buffer and beads are now ready to mix with the pooled splicing complex fractions.

Affinity Selection:

Incubate pooled splicing complex fractions/strep. bead mixture on an end-over-end rotator 4C for 4 hrs.

Spin 1500 rpm for 4 min. and discard supernatant. Wash beads 3 times with 10 ml wash buffer (20 mM TrisHCl pH 7.5, 100 mM NaCl). Add 1 ml wash buffer to beads to resuspend them and transfer to a 1.5 or 2 ml eppendorf tube. Spin 2000 rpm for 20 sec. and discard supernatant. Add 0.3 ml elution buffer (2% SDS, 20 mM TrisHCl pH 7.5, 20 mM DTT) to beads and incubate 4C on the rotator for 5 min. Spin 2000 rpm for 20 sec. and transfer supernatant to a new tube. Repeat this 2 more times to remove all traces of beads. Heat eluted proteins 65C for 10 min. Precipitate eluted proteins overnight 4C with 1 ml MetOH and 12 ug glycogen. Spin 14K 15 min. and remove MetOH. Resuspend pellet in 50 ul elution buffer.