# In Vitro Transcription and S1 nuclease protection analysis of transcripts

References: McStay and Reeder (1986). Cell 47:913-920.

Procedure

1) Prepare a 2x reaction mix as follows (CAUTION: ALPHA-AMANITIN IS EXTREMELY TOXIC!!!):

| Mix composition | Recipe for 1ml |
|---|---|
| 30mM HEPES pH 7.9 | 30 ul of 1M stock |
| 80mM KCl | 40ul of 2M stock |
| 12mM MgCl2 | 12 ul of 1M stock |
| 1mM DTT | 1ul of 1M stock (stored frozen) |
| 200ug/ml alpha amanitin (toxic!) | 200ul of 1 mg/ml stock (in water, stored frozen) |
| 1mM each rATP, GTP,CTP, UTP | 10ul each of 100mM stocks (in 100mM HEPES pH7.9) |
| Water to 1ml. 677ul water | |

3)Transcription reactions

To sterile 1.5 ml microfuge tubes add:

20ul cell extract or column fraction (in 100mM KCl buffer)

1ul of template DNA (10-200ng depending on degree of purification)

-Mix by tapping, or vortex at moderate speed (about 5-6 on scale of 10)
-incubate 5 min at room temp
-add 20ul 2X reaction mix and mix by tapping or vortexing
-incubate at 25 degrees C for 1-3hours

4) Stopping the transcription reactions

Stop reactions by adding 360ul of stop buffer:

| Buffer composition | recipe for 50ml |
|---|---|
| 0.15M NaCl | 1.5 ml of 5M stock |
| 50mM Tris-HCl pH 8.0 | 2.5 ml of 1M stock |
| 250mM sodium acetate | 4.2 ml of 3M stock |
| 0.25% SDS | 1.25ml of10% stock |
| 6mM EDTA pH 8.0 | 0.6ml of 0.5M stock |

- extract the stopped reactions once with 400ul of 1:1 phenol:chloroform (chloroform is actually 24:1 chloroform:isoamyl alcohol)

-vortex 5 seconds, then spin at room temp. in microfuge for 3 min.

-remove aqeous to fresh tube containing 10-20ul of 5’ end-labeled DNA probe

-Add 800 µl of absolute ethanol, vortex to mix. Store –80C, 30' (add ammonium acetate to 2.5M; add 2.5 volumes absolute ethanol, mix, store on ice 5 min.

-Spin 15-20 minutes in microfuge at top speed.

-Pour off supernatant and wash pellets with 1 ml 70% EtOH. Mix by inversion, spin again 5 min.

-pour off supernatant and dry pellets.

-resuspend thoroughly the RNA/ probe pellet in 30ul formamide hybridization buffer:

| formamide S1 hybridization buffer | recipe for 1.5ml |
|---|---|
| 40mM PIPES pH 6.4 | 0.12ml 0.5M stock |
| 400mM NaCl | 0.12ml of 5M stock |
| 1mM EDTA | 3ul of 0.5M stock |
| 80% deionized formamide | 1.2ml of 100% |
| water | 57ul |

-cover hybridization with 50ul paraffin oil.

-Incubate hybridization at 90 degrees in dry-bath incubator for 15 minutes to denature probe.

-carry hot block to water bath set at proper hybridizing temperature (generally about 37-52 degrees, determined empirically, but depending on GC content and length of hybrid that can form). 37 degrees often works fine.

-quickly move tube from 90 degree hot block to 37 degree water bath.

-hybridize 2 hours to overnight.

-chill tubes on ice, or if maintaining stringency is important, pop open tubes in water bath and add S1 digestion mix.

-add to each tube 270 ul of ice-cold S1 digestion mix, containing about 125units S1 nuclease/ml (S1 is added, just before use, to a volume of S1 digestion buffer appropriate for the number of transcription reactions).

| S1 digestion buffer | recipe for 50ml |
|---|---|
| 5% glycerol | 3.1ml 80% stock |
| 1mM Zinc Sulfate | 50ul 1M stock |
| 30mM NaOAc (sodium acetate) | 0.5ml 3M stock pH 4.5 (pH is critical) |
| 50mM NaCl | 0.5ml 5M stock |
| sterile water | to 50ml |

-spin 5 seconds in microfuge to get S1 digestion mix through the paraffin oil layer.

-vortex briefly. Repeat spin to get thin layer of oil at top again.

-incubate 30min in 37 degree Celsius water bath.

-stop reactions by removing 280ul from the bottom of the tube (avoid paraffin oil) to a fresh tube containing:

10ul of 10%SDS
5ul of 0.5M EDTA (the EDTA chelates the Zinc, stopping enzymatic activity)

-vortex briefly to mix.

-add 30ul 7.5M Ammonium acetate and vortex

-add 1ml cold (kept in freezer) absolute ethanol to precipitate DNA/RNA hybrid and vortex. store -80, 30'.

-Spin 15 -20minutes at top speed in microfuge (room temperature is OK, 4 degrees won't hurt).

-carefully remove supernatant (pellets may not be visible) and discard in radioactive waste container.

-add 1ml cold (kept in freezer) 70% Ethanol, mix by inversion twice, and spin 5 minutes.

-carefully remove supernatant and dry pellets under vacuum (or in 65 degree water bath with tube caps open).

-resuspend pellets in 6-8 ul of formamide-dye (sequencing gel loading buffer), amended to contain NaOH to chew up RNA in the RNA/DNA hybrid:

Formamide-dye Loading Buffer

90% deionized formamide (deionized with 0.25g mixed bed resin per 10ml formamide)
10mM NaOH
1mM EDTA
0.1% (1mg/ml)Bromophenol blue
0.1% (1mg/ml) Xylene cyanol

-boil 3 min., chill on ice. Load on 6 or 8% Urea-PAGE (sequencing) gel beside end-labeled pBR322 Hpa II digested (or other) molecular weight markers.