COUPLED IN VITRO TRANSCRIPTION / TRANSLATION

Prepare the DNA template:

1. Prepare plasmid DNA containing the protein-coding sequences downstream of a bacteriophage promoter by CsCl or Qiagen or PEG-NaCl methods as the quality of the DNA is crucial for the in vitro transcription step.

2. Digest 10 μg of plasmid DNA with a restriction enzyme that cuts downstream of the termination codon. The digest should be complete, for mHSF1 and mHSF2 in pGEM vectors SmaI is very convenient.

3. Phenol extract the DNA and ethanol precipitate it. Dissolve the DNA in 50 μl of TE.

Preparation of mRNA template:

4. Set up the in vitro transcription reaction as follows-

8 μl water
5 μl DNA (about 1 μg)
5 μl 5X ribonucleoside triphosphate mix
2.5 μl 10X SP6/T7 RNA polymerase buffer
2.5 μl 10 mM spermidine in the case of SP6 polymerase only, when using T7 polymerase substitute with water
1 μl RNasin (30-60U)
1 μl SP6/T7 RNA polymerase (5-20U)

incubate for 60 min at : 37^{o}C when using T7 RNA polymerase and
40^{o}C when using SP6 RNA polymerase

(5X ribonucleoside mix: 5mM rATP, rUTP, rCTP and CAP structure analog.
0.5mM rGTP.)

5. Add 25 μl of phenol (the usual TE buffered phenol) vortex and immediately spin for 4 min at high speed. The aqueous phase is then transferred to a tube containing 6 μl of 10 M ammonium acetate. Ethanol precipitate the RNA by adding 75 μl of ice-cold ethanol and incubate in dry-ice methanol bath for 10 min or at -20^{o}C overnight. Spin at high speed for 20 min to obtain the RNA pellet. Wash the pellet with ethanol and let the pellet air dry for about a minute.

6. Resuspend the RNA in 24 μl of water, add 6 μl of 10 M ammonium acetate, mix and reprecipitate the RNA. Dissolve the RNA in 10 μl of TE.

The quality of the RNA can be checked by running it on an agarose gel.

Prepare the protein by in vitro translation:
(Basically from the Promega instructions.)

7. Allow the solutions in the translation kit to thaw slowly on ice. Once thawed the retic lysate should be aliquoted in tubes each containing 35 μl of lysate. This is done to prevent the lysate from undergoing multiple freeze thaw cycles.

8. Heat the RNA at 65^{o}C for 10 min.
Set up the reaction as follows:

17.5 μl nuclease treated retic lysate
3.5 μl water
0.5 μl RNasin
0.5 μl 1 mM amino acid mix - meth
1 μl RNA solution from above
2 μl ^{35}S-methionine

Alternatively cold methionine can be used - use 2 μl of 1 mM L-methionine. I usually set up the reaction as mentioned above but before adding methionine, I take 2 μl of the reaction and add to it 0.5 μl of ^{35}S methionine. To the bulk of the reaction mix then I add 2 μl of cold methionine. This enables me to use the 2.5 μl side reaction for checking the quality of translation by fluorography. The reason for using cold methionine for translation is that labeled translation products cannot be used for a conventional gel shift assay.

8. Incubate at 30 ^{o}C for one hour.

9. Analyze the products of the translation by one dimensional SDS-PAGE, include ^{14}C labeled protein molecular weight standards. Visualize the labeled proteins by fluorography.

<u>References</u>:

In vitro transcription: Krieg, P. and Melton, D. (1984) Nuc. Acids Res. **12**, 7057.

In vitro translation: Pelham, H.R.B. and Jackson, R.J. (1976) Eur. J. Biochem. **67**, 247.