IN-VITRO TRANSCRIPTION
Protocol #2

I. Preparation of Nuclear Extracts

Solutions:

1. **Buffer A**: 10 mM HEPES (pH7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF.

2. **Buffer C** (Extraction Buffer): 50 mM Tris-HCl (pH 7.9), 0.42 M KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM DTT, 20 % glycerol, 10% sucrose.

3. **Buffer D** (TM 0.1): 50 mM Tris-HCl (pH 7.9), 0.1 M KCl, 12.5 mM $MgCl_2$, 1.0 mM EDTA 1 mM DTT, 1 mM PMSF, 20 % glycerol.

Procedure:

A. Pellet cells (6 - 8 x 10^8 about 4 l of HeLa cells at 4 x 10^5/ml) in 500 ml bottles, 5 min. at 3 K at 4°C.

B. Place bottles on ice and resuspend pellet in 10 ml ice-cold 1XPBS. Transfer to a chilled 30 ml Corex tube and pellet at 3 K again. Repeat once more with 10 ml 1X PBS.

C. Resuspend cells <u>gently</u> in 4 pelleted cell volumes (PCV) of Buffer A (it will be approximately 16 ml) and allow to swell on ice for 10 min.

D. Dounce cells 20 -30 strokes with 40 ml Dounce (Type B pestle) -- keep on ice and avoid aeration. Check lysis under microscope by diluting a few µl of nuclei 1:10 in Buffer A and placing some on a hemocytometer. 90 % lysis should be achieved -- nuclei are round and usually iridescent under the scope.

E. Pellet nuclei (4°C) at 3K for 5 min.

F. Extract nuclei by adding 4 PCV of Buffer C (≈10 -12 ml) and stir slowly for 30 min at 4°C. Best accomplished with beaker of ice on stir plate in cold room and micro stir bar -- gentle, no bubbles!

G. Transfer to 30 ml thick-walled plastic centrifuge tube and centrifuge at 15K for 30 min.

H. Transfer supernatant to 30 ml Corex tube and again while stirring gently at 4°C add 0.33 g/ml ammonium sulfate (enzyme grade from Schwarz-Mann) over the course of 1 - 2 hours. After all ammonium sulfate is added let it stir for another 1 - 1.5 hours.

I. Transfer to 30 ml thick-walled plastic centrifuge tube and centrifuge at 15K, 15 min.

J. Dissolve pellet in 0.05X the original supernatant volume -- about 500 -600 µl of Buffer D (TM 0.1M).

K. Dialyze against 2l of TM 0.1M overnight.
L. Centrifuge 5 min in eppyfuge.
M. Aliquot 25 µl/tube of extract into 500 µl eppy tubes while the tubes are on ice. Caps are closed tightly and the tubes are dropped into liq. N_2. Retrieve floating tubes with forceps carefully and put on dry ice -- then store at -70°C.

Note: The protein concentration should be ≈ 10 mg/ml. The final volume after dialysis is about 1 ml. For in-vitro transcription it is probably best to use a tube only once; however, gel shifts can be accomplished with samples that have been freeze-thawed several times.

II. In-Vitro Transcription Reaction

Solutions:

1. 2X Transcription Buffer: 24 mM HEPES (pH 7.9), 24% glycerol, 120 mM KCl, 16 mM $MgCl_2$, 2 mM DTT, 1 mM EDTA
2. Ribonucleotide mix: 7 mM rNTPs
3. Transcription Stop Mix: 20 mM EDTA, 0.2 M NaCl, 250 µg/ml tRNA
4. 0.5 M ammonium acetate dissolved in 100% ethanol
5. 0.3 M sodium acetate
6. 5X Hybridization Buffer: 1.25 M KCl, 10 mM Tris-HCl (pH 7.9), 1 mM EDTA
7. Primer Extension Buffer: 20 mM Tris-HCl (pH 8.0 at 23°C), 10 mM $MgCl_2$, 5 mM DTT, 10 µg/ml actinomycin D, 0.5 mM dNTPs (dATP, dGTP, dCTP, and dTTP)
8. RNasin (placental RNase inhibitor)

Procedure:

A. In a 1.5 ml eppy tube on ice add template DNA (250 - 500 ng), 12.5 µl of 2X transcription buffer, 2U/reaction RNasin, and 2 µl nuclear extract (e.g. 20 µg -- this usually works well if stock is concentrated, i.e. 10 µg/µl). The optimal DNA concentration is 20 µg/ml, so add DNA(s) so that the total does not exceed this if you want to avoid competition between plasmids. It is usually best to make a master mix minus the nuclear extract and aliquot out into each tube -- improves consistency.
B. Make final volume 21 µl with ddH_2O.
C. Incubate on ice for 15 min,
D. To initiate transcription add 4 µl of 7 mM rNTPs and place the reaction at 30°C for 90 min. If you're interested in maximizing the difference between control and

samples that have HSF added place the reactions at 37°C. This appears to lower basal level transcription without lowering the activated level substantially -- hence the fold increase is better (4 - 5 X).

E. Stop reaction with 4 volumes of stop mix.

F. Phenol/Chloroform extract 2X and place supernatant in a new tube after each extraction.

G. Precipitate with 2.5 volumes of 0.5 M ammonium acetate in 100% ethanol.

H. Resuspend pellet in 100 µl of 0.3 M NaOAc and precipitate with 3 volumes of 95% ethanol.

I. Resuspend RNA pellet in 16 µl of TE (pH 8.0). A pipette tube should be used to move droplet of TE around the side of the eppy tube to insure that all RNA (even that on the wall of the tube) is dissolved. Spin briefly to recover volume.

J. Place 8 µl of RNA in a clean microfuge tube (this is important!), add 2 µl of 5 X hybridization buffer and 25- 50 fmoles of kinase primer.

K. Place sealed tube at 85°C for 5 min. and then quickly (I use a floating rack so it is a one transfer step) transfer to an appropriate temperature water bath for 30 min hybridization . The CAT primer (used with many of our HSP70 constructs) should be placed at 55 - 57 °C. The primer for Δ50 HSEMAXI and Δ50VI -- Carl Wu's Drosophila HSP70 constructs are better done at 65-67°C.

L. Remove tubes to ice-water bath. Then place on ice and dilute with 23 µl of primer extension buffer.

M. Place tubes at 42°C for several minutes and then add 50 -200 units of Murine Molony Reverse Transcriptase (MMLV-RT, 200 U/µl, made by BRL). Seal tubes and let reaction proceed for 60 min.

N. Ethanol precipitate with 100 µl of 95% (place on dry ice).

O. Pellet cDNAs in eppy fuge 15 min.

P. Resuspend pellet in formamide buffer and run aliquot on 6%, 8.3 M urea gel as described for sequencing. For practical reasons a 1.5 mm gel poured in "gel-shift" plates is used.

Q. For size markers on gel-- pBR322 digested with Hpa II or Msp I (labeled with Klenow and dCTP) gives a good range of fragments from 622 bp to 9 bp.

Submitted by: Paul Kroeger 11/94