Polymerase III in vitro Transcription

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For the following reactions, use appropriate shielding and dispose of radioactive waste properly!

A 20 microliter transcription reaction contains:

- 4.0 ul 5X Pol III transcription buffer
- 0.2 ul 0.1 M DTT
- 0.2 ul alpha amanatin (1 mg/ml)
- 0.2 ul RNAse Inhibitor (Amersham, 20 U)
- 2.0 ul NTP stock
- 0.5 ul alpha 32P GTP
- 140 ng plasmid template (pSK LEU3, pCH6), [use 280 ng 5S pUC template]
- H2O to 20 microliters final volume.

Transcription Protocol:

For a Typical transcription reaction, make an NTP mix containing:

- 2.0 ul NTP stock
- 0.5 ul alpha 32P GTP
- DNA template
- H20 to a final volume of 3 microliters

Make a second mix containing all other transcription components except protein:

- 4.0 ul 5X Pol III transcription buffer
- 0.2 ul 0.1 M DTT
- 0.2 ul alpha amanatin (1 mg/ml)
- 0.2 ul RNAse Inhibitor
- H20 to a final volume of 16 microliters less the maximum volume of protein to be added.

Aliquot the second mix to all reaction tubes and incubate on ice.

Add the protein extract (typically 20-40 micrograms of a whole cell extract) and any other proteins to be added. Add extract dialysis buffer (or protein dilution buffer) to bring all reactions to the same volume.

Add 3 ul of the NTP mix to each reaction, mix by flicking the tube 5-6 times, and immediately incubate at 30 degrees (the reactions will work well between room temp and 32 degrees).

After 30 min., stop the reaction with 180 microliters stop solution.

Extract once with phenol/CHCl3 (2/1). ETOH precipitate with 20 ul 3M NaOAc and 600 ul ETOH.

Freeze 10 min. Spin 10 min. Remove sup. with a drawn out pasteur pipette. Add 200 ul 100% ETOH and spin 3 min. Remove sup. again.

Dry pellets but do not overdry. Thoroughly resuspend pellets in Formamide dye solution containing 0.1% SDS.

Heat 1 min. 90 degrees and put on ice. Load to 0.4 mm thick urea acrylamide gel (don't forget to clean the wells of the gel just before loading). Typically, run 300V, 60 min until the bromphenyl blue is 3/4 of the way to the bottom.

Dry gel 20-30 min. Typically expose ~3 hr or overnight without a screen.

Solutions needed for Pol III transcription:

5X Pol III Buffer (store frozen) (1 ml):

10% glycerol	200 ul 50% glycerol
100 mM HEPES, 7.9	100 ul 1M HEPES
400 mM KCl	400 ul 1M KCl
25 mM MgCl2	25 ul 1 M MgCl2
5 mM EDTA	20 ul 0.25 M EDTA
255 ul H20	

NTP Stock (store frozen —70 deg):
5 mM each ATP, CTP, UTP
0.5 mM GTP

7.3 ml Stop mix (make fresh):

0.1 M NaOAc	250 ul 3M NaOAc
10 mM EDTA	300 ul 0.25 M EDTA
6.4 ml H20	
0.5% SDS	375 ul 10% SDS
5 ug/ml tRNA	1.2 ul tRNA (30 ug/ml)

Extract Dialysis Buffer (store frozen —70 deg):

20 mM HEPES, 7.9
100 mM KCl
5 mM MgCl2
1 mM EDTA
20% glycerol
2 mM DTT

Protein dilution buffer (5 ml) store -70 deg:

20 mM Tris 8.0	100 ul 1 M Tris 8.0
1 mM DTT	50 ul 0.1 M DTT
10% glycerol	1 ml 50% glycerol
150 mM KCl	0.75 ml 1 M KCl
1 mM EDTA	20 ul 0.25 M EDTA
50 ug/ml BSA	2.5 ul 100 mg/ml BSA

H2O to 5 ml total volume

20 ml Acrylamide Urea Gel:

3 ml 40/2% sequencing acrylamide
2 ml 10X TBE
8.5 g Urea
8 ml H20
microwave ~10 sec and stir to dissolve.
Degas

Add 175 ul 10% ammonium persulfate

Remove 1 ml acrylamide and add 1 ul TEMED. Pour plug at bottom of gel. Let polymerize a few minutes.

To the remaining acrylamide, add 18 microliters TEMED and pour gel. Gel can stored O/N, if kept wet and sealed at room temp.