In vitro transcription with yeast nuclear extract

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Wear gloves throughout, use RNAse free solutions (either autoclaved or sterile filtered) and clean bench and pipetmen with 95% ethanol before use to eliminate any stray RNAses.

1. For a 25 microliter transcription reaction, add the following to a microfuge tube (when doing multiple reactions, it is easiest to make a larger mix and aliquot to individual reaction tubes):

- 5 microliters 5x transcription buffer
- 0.63 microliters 0.1 M DTT
- 3 microliters phospho creatine (64 mg/ml pH 7.5)
- 0.1 microliter creatine phospho kinase
- microliter NTP mix (10 mM each ATP, CTP, GTP, UTP
- 150 ng plasmid DNA template
- (0.125 microliters 10% NP40 can be added if desired but is not necessary)
- 10 units RNAse inhibitor (Amersham ~105 units/microliter)
- 24 ng Gal4-VP16 (0.2 microliters 0.12 mg/ml) or 30 ng Gal4-AH (0.3 microliters 0.1 mg/ml)
- H20 to a final volume of 20 microliters (the final volume can be more or less than 20 microliters depending on the amount of protein to be added).

(add RNAse inhibitor, creatine phosphokinase, and activator protein to the mix last after all other components have been added and mixed well)

2. After addition of all components, mix well and aliquot to individual tubes at room temperature. Set up the reaction so that the Gal4-activator has at least 10 min to bind the template before the nuclear extract is added.

3. Depending on the volume of extract to be added to each reaction, add buffer HA + 0.1 M potassium acetate to each tube so that the final volume of the reaction will be 25 microliters (e.g. if 2.5 microliters of extract will be added, add 2.5 microliters HA + 0.1 M KOAc). Mix well and quick spin tubes. This addition of HA + 0.1M can be omitted if the same volume of extract will be added to all tubes.

4. Add yeast transcription extract directly to reaction and mix well by gently tapping the tube 5-6 times. Incubate 30-45 min at room temp. (typically add between 60 - 120 micrograms yeast nuclear extract per reaction. The optimum amount depends on the extract used and the response is not always linear with amount of extract added).

5. After the 30-45 min incubation, add 180 microliters stop mix.

6. Extract 1x with phenol/chloroform (2/1). Carefully transfer the aqueous layer to new tubes.

7. Ethanol precipitate by adding 1/10 volume 3 M sodium acetate and 3x vol ethanol. Wash pellets with 80% ethanol. Dry in speedvac. Assay RNA synthesis using primer extension.

5x Acetate transcription buffer

500 mM Potassium Acetate, pH 7.6
100 mM HEPES, pH 7.6
5 mM EDTA
25 mM MgOAc
Creatine phospho kinase (sigma C-3755 165 units/mg)
2.0 mg/ml in HA + 0.1 M potassium acetate

Stored in 12 microliter aliquots at -70 degrees. The enzyme is stable to a few freeze thaw cycles if thawed on ice and quickly refrozen.
Phospho creatine (64 mg/ml)
128 mg phospho creatine
2 ml TE pH 7.5
add 4 microliters 1 M Acetic Acid
check pH with paper, should be ~ 7.5

Store in aliquots at -70 degrees. Stable to multiple freeze thaws.

Stop mix
167 microliters 3 M Na Acetate
200 microliters 0.25 M EDTA
250 microliters 10% SDS
85 micrograms tRNA
Add H2O to a final volume of 5 ml

HA + 0.1 M potassium acetate
20 mM HEPES 7.9
10% glycerol
1 mM EDTA
100 mM potassium acetate
1 mM DTT

This buffer can be made up with DTT and aliquoted and stored at -70 degrees.

Note: The following potassium glutamate buffer can also be used for vitro transcription instead of the above acetate buffer but the acetate buffer is recommended.

5x glutamate transcription buffer (5 ml)
250 microliters 1 M HEPES 7.6
1.25 ml 2 M potassium glutamate
250 microliters 1 M MgOAc
500 microliters 0.25 M EGTA pH 8
1.75 ml 50% glycerol
1 ml H2O

Gal4-AH:
The most recent prep is 0.75 mg/ml in HAZ + ~0.6 M KOAc. It is convenient to dilute a working stock to 0.1 mg/ml in HA + 0.15 M KOAc and store in aliquots at - 70 degrees. This is stable to multiple freeze thaws if handled as above for creatine phospho kinase.

Gal4-VP16:
The most recent prep is 0.5 mg/ml in HAZ + 0.15 M KOAc. It is convenient to dilute a working stock to 0.12 mg/ml in HA + 0.15 M KOAc. Store in aliquots at -70 degrees

Primer Extension Assay

1. Add 10 microliters primer annealing mix to each dried RNA sample. Vortex well to resuspend RNA.

2. Heat 1 min at 90 - 100 degrees. Quickly transfer to 48 degree water bath or heat block and incubate for 45 minutes.

3. Quick spin tubes in microfuge and add 20 microliters synthesis mix. quick spin and mix well by tapping tubes. Incubate at 37 degrees for 30 min.

4. Ethanol precipitate and wash pellets with 80% ethanol. Dry in speedvac.

5. Resuspend pellets in 3 microliters 40 micrograms/ml RNAse A (this step digests RNA which interferes with the running of the sequencing gel). Incubate 3 min at room temp.

6. Add 3 microliters sequencing gel loading buffer (formamide/EDTA plus bromophenyl blue and xylene cyanol) and mix well.

7. Heat 1 min at 90 - 100 degrees and immediately put on ice.

8. Load to 6% DNA sequencing gel and run till bromophenyl blue is 2-3 inches from the bottom of gel.

Primer Annealing mix (for 1 reaction (10 microliters))
2 microliters 5x annealing buffer
~0.15 microliters ^{32}P kinased lacI oligo (typically 1-5 x 105 cpm from 0.5 microgram kinased oligo resuspended in 100 microliters TE)
H2O to a final volume of 10 microliters

Synthesis mix (for 1 reaction (20 microliters))
4 microliters 5X synthesis buffer
0.3 microliters each 10 mM dNTP (dATP, dCTP, dTTP, dGTP)
14.3 microliters H2O
mix well and just before use, add 0.5 microliters MMLTV reverse transcriptase (100 units; Gibco-BRL or USB)

1 ml MMLTV Primer Annealing Buffer (5x)
25 microliters 1 M Tris 8.3
375 microliters 1 M KCl
20 microliters 0.25 M EDTA, pH 8
580 microliters H2O

1 ml MMLTV Synthesis Buffer (5x)
250 microliters 1 M Tris 8.3
375 microliters 1 M KCl

22.5 microliters 1 M $MgCl_2$
150 microliters 0.5 M DTT
202 microliters H_2O

store the above two 5x buffers at -20 degrees.