In vitro transcription with yeast nuclear or whole cell extracts

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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 minimize RNAse contamination.

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

- 5.0 microliters 5x transcription buffer
- 0.63 microliters 0.1 M DTT
- 3.0 microliters creatine phosphate (64 mg/ml pH 7.5)
- 0.1 microliter creatine phospho kinase
- 1.0 microliter NTP mix (10 mM each ATP, CTP, GTP, UTP) *(see note below)*
- 150 ng plasmid DNA template
- *(optional) 0.125 microliters 10% NP40*
- 0.25 microliter (10 units) RNAse inhibitor (e.g., RNAseOut, Thermo, 40U/microliter)
- 24 ng Gal4-VP16 or Gcn4 (0.2 microliters 0.12 mg/ml); 30 ng Gal4-AH (0.3 microliters 0.1 mg/ml)
- H_20 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). For most experiments, omit the NTP mix to allow PICs to preform before adding NTPs.

2. After addition of all components, mix well and aliquot to individual tubes at room temperature. Set up the reaction so that the 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. If allowing PICs to preform, incubate 10 min on ice or RT. *(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).

Add 1 microliter NTP mix and Incubate 30-45 min at room temp (23 deg) if letting PICs preform, otherwise the reaction starts with the addition of extract if NTPs already added.

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

6. Phenol extract 1x with 140 microliters phenol/chloroform/Isoamyl alcohol (25/25/1). Carefully transfer the aqueous layer to new tubes.

7. Ethanol precipitate by adding 1/10 volume 3 M sodium acetate, pH 5.2 and 2.5x vol 100% ethanol; freeze ~ 10 min on dry ice or O/N @ -20 deg. Wash pellets with 80% ethanol. Dry in speedvac.

8. Assay RNA synthesis using primer extension (see below).

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 (Millipore C-3755; >150 units/mg).
Make working solution 2.0 mg/ml enzyme in HA + 0.1 M potassium acetate. Store in 12 microliter aliquots at -80 degrees. The enzyme is stable to a few freeze thaw cycles if thawed on ice and quickly refrozen.

Creatine phosphate (64 mg/ml)
128 mg creatine phosphate (Millipore 2380) in 2 ml TE pH 7.5

Adjust pH by adding ~4 microliters 1 M Acetic Acid. check pH with paper, should be ~ 7.5. If using the Creatine Phosphate disodium salt, likely no need to adjust pH.

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

Stop mix (adjust vol as necessary)
167 microliters 3 M Na Acetate
200 microliters 0.25 M EDTA
250 microliters 10% SDS
(optional) 85 micrograms tRNA (purified by phenol extraction and Ethanol ppt)
Add H2O to a final volume of 5 ml

HA + 0.1 M potassium acetate
20 mM HEPES 7.9 @ RT
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 for most studies.*

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 - 80 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 -80 deg.

Gcn4- full length:
The most recent prep is 1.2 micrograms/microliter. Dilute working solution to 0.12 micrograms/microliter in HA + 0.1. Store in aliquots at -80 deg.

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 degrees. Quickly transfer to 50 degree thermomixer (IR700-lacI primer), or 48 deg (if using shorter old lacI primer). Incubate 45 min with thermomixer lid on to minimize evaporation.

3. Quick spin tubes in microfuge and put in room temp. rack. Immediately 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 4 microliters 30 micrograms/ml RNAse A diluted in H2O (this step digests RNA from the extract which interferes with running of the sequencing gel). Incubate 3 min at room temp.

6. Add 6 microliters sequencing gel loading buffer

7. Heat 1.5 min at 90 degrees and immediately put on ice.

8. Load to 6% TBE/Urea gel and run till bromophenol blue is just near the bottom (or 2-3 inches from the bottom of gel if using a conventional long sequencing gel).

9. Visualize primer extension products on Odyssey scanner, putting gel directly on scanner (0 mM offset) or by phosphorimager if using ^{32}P labeled primer.

Primer Annealing mix (for 1 reaction (10 microliters))
2 microliters 5x annealing buffer
0.65 microliters 1 micromolar IR700-lacI primer
H2O to a final volume of 10 microliters

Alternative ^{32}P primer: 0.15 microliters ^{32}P kinased lacI oligo (typically 1-5 x 10^5 cpm from 0.5 microgram kinased oligo resuspended in 100 microliters TE).

Synthesis mix (for 1 reaction (20 microliters))
4 microliters 5X synthesis buffer
1.2 microliters 10 mM dNTP mix (dATP, dCTP, dTTP, dGTP)
14.3 microliters H2O
mix well
Add 0.5 microliters M-MLV reverse transcriptase (200 u/microliter) (e.g., Invitrogen)

1 ml M-MLV 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 M-MLV Synthesis Buffer (5x)
250 microliters 1 M Tris 8.3
375 microliters 1 M KCl
22.5 microliters 1 M MgCl2
150 microliters 0.5 M DTT
202 microliters H2O

store the above two 5x buffers at -20 or -80 degrees.

Formamide Gel Loading Buffer (for IR700 dye primers):
0.85 ml deionized formamide
25 microliters saturated solution of bromophenol blue
50 microliters EDTA

For 32P labeled primers:
Same as above, except can add 25 microliters Xylene Cyanol dye.

Novex TBE/Urea gels 6% (Thermo)
Max sample volume: 10 microliters
Clean wells before use to remove urea from wells
Run gel @ 300V for ~26 min.

Put gel directly on Odyssey scanner, 0 mm offset.