

RNA Pol II Immobilized Template Assay

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Template Preparation

- Wash Dynabeads M-280 Streptavidin (Dyna) 2X in 400ul TE (pH 7.5) + 1M NaCl.
- Resuspend beads in TE (pH 7.5) + 1M NaCl and 0.1% NP-40 to a final concentration of 10mg/ml and 0.003% NP-40.
- Add 8.6ng promoter DNA/ug beads to tube along with a quarter of that volume of 5M NaCl.
- Incubate for 30' @ room temp. while shaking at low speed in vortexer (make sure beads don't sink to bottom during incubation).
- Wash beads 1X in 400ul TE (pH 7.5) + 1M NaCl.
- Resuspend beads in 1ul Blocking Buffer/1ug beads.
- Incubate 15' @ room temperature. Occasionally, vortex beads briefly at LOW setting to make sure beads do not settle.
- Wash beads 3X, 400ul 1X Transcription Buffer.
- Resuspend beads in 1X Transcription Buffer to a final concentration of 10mg/ml. Use templates the same day — do not store. Keep on ice until ready to use.

PIC Assembly Reactions

This assay is performed using 100ul reactions.

Set up the following reaction mix:

Mix 1X Mix

- 1X Txn. Buffer 20ul 5X txn. Buffer
- 12mM phosphocreatine 12ul phosphocreatine (64 mg/ml in TE, pH 7.5)
- 2.5mM DTT 2.52ul 0.1M DTT
- 400ng creatine phosphokinase 0.4ul creatine phosphokinase (2.0mg/ml in HA + 0.1M KOAc)
- 0.05% NP-40 0.5ul 10% NP-40
- H2O 4.58ul H2O

40 ul total volume

Make 40ul aliquots of Mix.

- To each aliquot, add 1X Txn. Buffer to give a FINAL (after step 9) volume of 100ul (NOTE: if you are using the same amount of the same nuclear extract for each reaction, you do not have to make aliquots).
- Add nuclear extract to each aliquot (usually ~ 360ug/rxn.).
- Incubate rxns. for 10' @ room temp.
- Spin down rxns. @ 9K, 2', 4° C. Transfer supernatants to new tubes (add any recombinant proteins after this step).
- Add 1ug HaeIII digested E. coli DNA to each reaction.
- Add 120ng of Gal4-AH or 96ng of Gal4-VP16 per 5ul immobilized template to immobilized template. Incubate 10' @ room temp. with occasional gentle vortexing to prevent beads from settling.
- To each nuclear extract mix add 5ul immobilized template (+ volume of activator).
- Incubate rxns. 40' @ room temp. with occasional gentle vortexing to prevent beads from settling.
- Wash templates 3X, 400ul 1X Txn. Wash Buffer.
- Elute templates from the beads by digesting with 60 units PstI per 100ul rxn. for 30' @ 37° C.
- Transfer digest to new tube and add appropriate amount of 4X NuPAGE sample buffer. Store @ -20° C.

Transcription Reactions

These follow the above protocol, except that they are done as 50ul rxns. In addition, 20 units of RNase inhibitor are added to each reaction along with the HaeIII digested E. coli DNA. Transcription can be done with either washed or unwashed complexes. For unwashed complexes, add NTPs to a final concentration of 400uM after step 9 (you can omit steps 10 — 13). Then proceed as with a regular transcription reaction. For washed complexes, add NTPs after step 11 (omit steps 12 and 13). Washed complexes give only a single round of transcription.

Transcription is stopped by adding 360ul of Stop Mix per reaction. The reactions are then spun down and the supernatants transferred to new tubes to remove the beads. Only after this should the phenol/chloroform extractions be performed as described in the transcription protocol.

Primer extensions performed with these samples need to include actinomycin C1 at a final concentration of 15ug/ml.

NOTES:

Washes are performed using a pulled pasteur pipette. You add the wash buffer, vortex briefly at a low setting, do a quick spin in the centrifuge, and place the tube in the magnet. At most steps you can immediately remove the supernatant after this. However, when washing after the blocking step, you should leave the tube in the magnet for ~1min., since the blocking solution is quite viscous.

Reagents

Blocking Buffer

1X txn. buffer (pH 7.6)
60mg/ml casein
5mg/ml poly vinyl pyrrolidone
2.5mM DTT
pH to 8.0 using KOH

Store @ -20° C in 1ml aliquots

5x Acetate transcription buffer

500 mM Potassium Acetate, pH 7.6
100 mM HEPES, pH 7.6
5 mM EDTA
25 mM MgOAc
Store @ -20° C

1X Transcription Wash Buffer

1X txn. buffer (pH 7.6)
0.05% NP-40
2.5mM DTT
Make up just before use

Actinomycin C1: Stock of 5mg/ml in EtOH

Hae III digested E. coli DNA

Prepare genomic DNA from a non-transformed strain according to instructions in Promega Wizard Genomic DNA Purification Kit, except incubate in 6ul RNase Solution for 60 min. per reaction (can also use 4mg/ml RNase A). I usually do 12 reactions (12ml) at one time.

After rehydration, digest DNA with 0.5 units HaeIII/ug DNA overnight @ 37° C.

Phenol:Chloroform (2:1) extract DNA.
EtOH precipitate.

Resuspend in TE and quantitate. The concentration to aim for is ~1mg/ml. Store @ -20° C. (I also run it out on an agarose gel to compare it with an old prep)

Last modified 1/11/07