Immobilized Template Assay

This document contains **3 x allied protocols:** Making IVT-grade whole cell extracts (WCEs) **(A)**; making and immobilizing the biotinylated template **(B)**; and performing the immobilized template assays (**C**). The WCE isolation procedure is identical to Section A of the IVT protocol.

A. Preparation of WCEs

NOTE 1: Making IVT-grade extracts takes an entire day, and a long day at that. Collect cell pellets (**step A.2**) from the strains of interest and store at –80°C. Then isolate WCEs from four (or sometimes even six) at a time. Save time by preparing all buffers the day before and store overnight in the coldroom.

A.1 Grow 4L (see **NOTE 2**) of each strain overnight to mid log phase ($OD_{\lambda 600} \approx 1.2$). A handy rule of thumb on how to achieve this: on day 1 inoculate a large loopful from a fresh plate into 10ml YPD. Grow 5 hours at 30°C and around 5PM place 500µl of this culture per L fresh media. Incubate overnight at 30°C with agitation: you should be in the ballpark by 9AM next morning (timings approximate and for WT strain in YPD).

NOTE 2: In general I make IVT-WCEs from 4L cultures but have stepped it down to 2L (from 1L wasn't too impressed). If you step up (>4L) use an appropriately-sized bead-beater for lysis, although this significantly lengthens the time required for **Step A.3** since it has to be cleaned out and refilled between samples. Pack around with ice/salt mix, and lyse by 15 rounds mechanical disruption: 30s on, 90s off. When using a bead beater ensure to fill it completely to reduce bubbles during the lysis. Also make sure it's sealed correctly: I've lost a sample this way.

A.2 Collect cells by centrifugation in 500ml flasks (4K, 10m, 4°C). Wash with ddH_2O and transfer to 50ml falcons in ***Transcription Buffer A*** (**Txb^{0}A**). Pellet cells by centrifugation (6K, 10m, 4°C) and determine the wet wt of the pellet (estimated yield under conditions described in **Step A.1** is 5g/L). All manipulations from this point are performed on ice at 4°C and all buffers contain protease inhibitors as standard.

Alternative (Jessica Dermody): Collect a frozen pellet from a fermenter run (20g aliquots at –80°C). Crush to small pieces with a hammer (**CARE:** flying debris) and grind to a fine powder in a pre-chilled (**CARE:** liquid N_2) mortar and pestle. Resuspend in 1ml **Txb^{0}A** per gram wet weight. Divide into 2 nalgene 30ml tubes and add 4mls glass beads / tube. Lyse by 15 x cycles: 30s vortex (max speed), 30s on ice. Spin down beads / lysate in a punctured tubes (to remove beads) and clarify lysate by centrifugation (9K, 20m, 4°C). Transfer to a large

Ultra tube and add 1/9 vol 5M KAc dropwise with mixing. Spin 40K, 30m, 4°C and carefully transfer supernatant to a fresh tube. Go to **Step A.5**.

A.3 Thaw cell pellets and resuspend in 2mls **Txb°A** per gram wet weight. Add an equal volume of acid washed glass beads and chill on ice for 10mins. (If isolating from a 4L culture you will likely need to split the sample between 2 x 50ml falcons). Lyse by ten rounds mechanical disruption: 1 min vortex (max speed), 1 min on ice. Estimate % lysis by microscopy: want >80% but advance when you're comfortable.

A.4 Collect glass beads and debris by centrifugation (8K, 10m, 4°C) and transfer supernatant to fresh 50ml falcons on ice. Resuspend the slurry in half the volume **Txb°A** used in **step A.2** and collect by centrifugation as above. Pool the supernatant with that on ice and discard the slurry. Add 1/7 Volume of 4M potassium acetate (pH 7.6) dropwise (while vortexing gently) and rotate the mix at 4°C for 30 minutes.

A.5 Centrifuge in a Beckman 45Ti rotor (40K, 60m, 4°C) and collect the supernatant avoiding the pellet and lipid layer (**careful**: don't get greedy). Add an equal volume of saturated (4M) NH_4SO_4 (pH 7.0) dropwise with mixing (will cloud up as the proteins precipitate). Place the mix on a rotator for 30 minutes and centrifuge (20K, 20m, 4°C). Discard the supernatant and resuspend the protein pellet in 50µl ***Transcription Buffer B*** **(Txb°B) / 0mM KAc** per gram original wet weight.

NOTE 3: Resuspension will take about 10mins. Cut the end off a 1ml pipette tip and mix gently while dislodging the pellet off the centrifuge tube. **Avoid bubbles**.

A.6 Dialyze the resulting straw-colored solution against 3 x 1L **Tx b°B** (mM KAc as below: 60-90m each, **do not leave overnight)**.

Dialysis conditions: 90m against **Tx b°B / 50mM KAc**
60m against **Tx b°B / 75mM KAc**
60m against **Tx b°B / 150mM KAc (normal KAc conc)**

A.7 Measure the conductivity of the lysate periodically and compare to a KAc curve (0mM – 1M). End point is when close to the dialysis buffer (150mM KAc), so continue dialysis if necessary. Estimate concentration by Biorad Dc assay (**expect 30 - 60 mg/ml**) and store in aliquots at -80°C.

NOTE 4: Keep the aliquots reasonably small: multiple freeze-thaw cycles kill Tx activity.

B. Template creation

Below, how to biotinylate linear template DNA (two possible approaches) and immobilize onto beads. Protocol as described refers to template pGAL4CG- (F348/b1457: Cyc1 promoter and 1x Gal4 binding site upstream of a ~ 350, 375bp G-less cassette in a pUC18 backbone). Addition of Gal4-VP16 to assays will activate transcription (see: **IVT RNApII protocol**).

NOTE 5: It is recommended to simultaneously test a promoterless control [eg. $p(C_2AT)_{19}$ **(pUC13 backbone, F48)** or pUC18-$(C_2AT)_{19}$ **(pUC18 backbone, SB650**)] to determine the baseline level of non-specific binding.

As used in: Cho *et al* (1997) *Genes Dev* **11:**3319.

Method 1 : Klenow fill-in

B.1 Digest 100µg of pGAL4CG- with Ehe1 (NEB; blunt cutter) and AflIII (NEB). (alternatively sequentially cut with HindIII, fill-in with klenow and cut with AflIII). Either approach will excise a 1.1kB fragment from the pUC18 backbone. Gel-purify and Geneclean (or QiaexII, or equivalent) to clean up. **NB.** This is a large amount of DNA to purify, so plan accordingly: split the sample and check the binding capacities of the resins you plan to use.

B.2 Biotinylate at the AflIII site: (200µl rxn)

DNA	x µl
10x Klenow b^o	20µl
dGTP/dCTP/dTTP mix (10mM each)	4µl
Biotin-14-dATP (0.4mM) (Gibco 19524-016; 50nM)	20µl
Klenow	2µl
ddH_2O	up to 200µl

Mix on ice and incubate 30^{o}C, 3h or 25^{o}C overnight.

B.3 Remove unincorporated biotin-ATP to avoid competition at the immobilization step (**B.7**). Add 50µl TE pH 8.0, 30µl 3M NaOAc pH 5.2 and 625µl EtOH (-20^{o}C). –80^{o}C, 60m to precipitate and microfuge (12K, 10m, 25^{o}C). Remove supernatant and wash pellet with 70% EtOH (-20^{o}C). Pellet by centrifugation: 12K, 10m, 25^{o}C, resuspend in 80µl ddH_2O and store at –80^{o}C. Go to **Step B.7.**

NOTE 6: Confirm efficient biotinylation by mixing purified fragment with streptavidin and incubating 5m, RT^o. Resolve on an 1% Agarose EtBr gel relative to a lane with DNA only. Binding of streptavidin will cause a mobility shift. Multiple bands may be visible if you titrate the streptavidin: indicate monomer – tetramer binding to the biotin-label.

Method 2 : PCR-label **(**Preferred**)**

B.4 Order 5' biotinylated-oligomers specific to the pUC backbone of pGAL4CG- (F348/b1457: Cyc1 promoter and 1x Gal4 binding site upstream of a ~ 350, 375bp G-less cassette in a pUC18 backbone).

(oligo #374, 200µM) Biotin 520 (-70) 5'– ATg CTT CCg gCT CgT ATg TTg
(oligo #375, 200µM) Biotin 60 (-330) 5'– ACg gCT ACA gCT TgT CTg TAA gC

> **NOTE 7:** 520 and 660 refer to the sites where the oligos anneal, while –70 and –330 indicate the approximate distance from the vector polylinker. It is possible to label the templete at either end: (i) use #374 as a forward pair in concert with oligo #33 (Reverse primer; AgC ggA TAA CAA TTT CAC ACA ggA); or (ii) use #375 as a reverse pair in concert with oligo #32 (-40 primer; CgC CAg ggT TTT CCC AgT CAC gAC).

B.5 Amplify by PCR with the appropriate pairs (**NOTE 7**). Enzyme of choice is up to you, but I recommend Denville Taq-Pro which gives great yields. Make 500µl reaction mix and split to 10 x 50µl for the PCR:

10x TaqPro b^o	50µl
dTNP mix (2mM each)	50µl
$MgCl_2$ (50mM)	20µl
Primer F (2.5µM)	50µl
Primer R (2.5µM)	50µl
Template (pGAL4CG-)	10µl
TaqPro	5µl
ddH_2O	to 500µl

Mix on ice, flash spin and PCR amplify (35x cycles T_1-T_3):

T_1	94^{o}C, 45s
T_2	50^{o}C, 45s
T_3	72^{o}C, 2m
T_4	72^{o}C, 5m
T_5	4^{o}C, ∞

Confirm amplification and biotinylation (see **NOTE 6**) and estimate yield on a 1% Agarose / EtBr gel.

B.6 Remove free biotinylated primer to avoid competition in the immobilization step (**B.7**). Pool as appropriate (PCR products should be very clean: if not you have to gel-purify), add 1/10 vol 3M NaOAc pH 5.2 and 2.5 vol EtOH (-20^{o}C). –80^{o}C, 60m to precipitate and microfuge (12K, 10m, 25^{o}C). Remove supernatant and wash pellet with 70% EtOH (-20^{o}C). Pellet by centrifugation: 12K, 10m, 25^{o}C, resuspend in 80µl ddH_2O and store at –80^{o}C.

Immobilizing the template to magnetic beads

B.7 Wash 200µl Dynal beads (see **NOTE 8**) in 3 x 1ml ***buffer I***. Collect beads between washes by centrifugation. Resuspend beads to 200µl in *buffer I* and add 20pmole biotinylated DNA (10pmole / mg WCE). Rotate at RT° overnight.

> **NOTE 8:** Dynabeads M-280 Streptavidin (10mg/ml ≈ 6.7 x 10^8 beads/ml). While these beads are expensive they're the best we've tried: beads from other suppliers gave hideous backgrounds (see below). You will also need a powerful magnet for the wash steps (**eg.** magnetic stand Dynal MPC®-S (20 µl - 2 ml) **#120-20D**).

B.8 Wash beads 3 x 1ml ***buffer I***, then 3 x 1ml TE pH 8.0. Collect beads between washes by centrifugation. Resuspend beads to 80µl in TE pH 8.0 and store at 4°C until ready to use. You will need 3µl for an assay to be detected by western, 10µl for an immobilized template *in vitro* Tx assay.

C. Immobilized Template assays

> The biggest problem with these assays is high-background, caused by non-specific binding, either to the beads themselves or the DNA. Methods to reduce this include pre-blocking with BSA (Bovine Serum Albumin) or introducing an excess of competitor DNA (promoterless template or *E.coli* genomic DNA). You can also try adding Sarkosyl. Perhaps the best way of reducing background, however, is to use as little extract as possible: for this reason the greater the activity, the better the extracts. See also: **NOTE 5**.

C.1 (Optional, but recommended if possible): Pre-incubate the beads (3µl / reaction) with BSA (20µg / reaction) in 50µl ***simple transcription buffer*** (0.6ml PCR tube). Incubate 30m, RT° with rotation. Use the magnetic stand to separate the beads from the solution and remove the supernatant. The beads are now ready: no further washing is required. Cap the tube and place on ice until ready.

> Seong-Hoon numbers (per reaction): 50µg (5µl) Dynabeads / 0.43µg template (600bp). Templates always bound fresh (**Steps B.7 – B.8**) for each experiment.

C.2 Assemble in another tube a 50µl binding reaction in ***simple transcription buffer***: 20 – 200µg WCE (titrate), +/- 100ng Gal4-VP16, ≥3µg competitor DNA (titrate), 2U hexokinase and 0.5mM glucose (latter two deplete endogenous NTPs). Collect by centrifugation and incubate on ice 5 mins. Transfer to the magnetic bead tube and incubate for 45m at RT° with rotation.

C.3 Isolate the beads and transfer the supernatant (**Depleted**) to another tube on ice (save it). Wash the beads with 4 x 200µl ***wash buffer***. Remove the wash and resuspend beads in ≈10µl buffer of choice.

eg.1 For westerns: resuspend in 10µl wash buffer, add an equal volume ***2 x reducing sample loading buffer***. Incubate 95°C and load all on SDS-PAGE. Also load some input WCE and **Depleted** WCE.

eg.2 Can also try numerous enzymatic assays, such as (de)phosphorylation, (de)acetylation, guanylylation or transcription (*see RNApII IVT protocol for comparison*).

Buffers:

Stocks: (store all at 4°C**)**

1M HEPES.KOH (pH7.5)	1L
5M KAc (pH7.6)	2L
2M Tris.Ac (pH 7.6)	1L
Saturated (4M) NH_4SO_4 (pH 7.0)	500ml
50% Glycerol	4L

Additives: (all stored at –20°C)

1M DTT (1ml aliquots)
100mM PMSF in MeOH (100x)
1mg/ml Pepstatin A (1000x; 1ml aliquots in MeOH)
1mg/ml Aprotinin (1000x; 1ml Aliquots in ddH_2O)
1mg/ml Leupeptin (1000x; 1ml Aliquots in ddH_2O)
1mg/ml Antipain (1000x; 1ml Aliquots in ddH_2O)
1mg/ml benzamidine.HCL (1000x; 1ml Aliquots in ddH_2O)

Transcription Buffer A **(Txb°A)**

200mM Tris.HCl (pH 7.9)
20% Glycerol
390mM NH_4SO_4
10mM $MgSO_4$
1mM EDTA
1mM DTT
Protease inhibitors as standard (incl. 1mm PMSF)

Transcription Buffer B **(Txb°B)**

20mM HEPES-KOH pH 7.6
20% Glycerol
10mM MgAc
0 - 150mM KAc **(assume 150mM unless noted otherwise)**
10mM EGTA
5mM DTT
Protease inhibitors as standard (incl. 1mm PMSF)

Buffer I

2M NaCl
10mM Tris pH 7.4
0.01% NP-40

Simple Transcription Buffer

25mM HEPES-KOH pH 7.6
10% Glycerol
10mM MgAc
100 - 125mM KAc **(as noted)**
5mM EGTA
2.5mM DTT
Protease inhibitors as standard (incl. 1mm PMSF)

Wash Buffer

20mM HEPES-KOH pH 7.6
10% Glycerol
10mM MgAc
125mM KAc
2.5mM EGTA
1mM DTT
0.01% NP40
Protease inhibitors as standard (incl. 1mm PMSF)

2x reducing loading buffer

60mM Tris pH 6.8
2% SDS
10% Glycerol
0.2% Bromophenol Blue
100mM DTT

As previously published: Cho EJ *et al* (1997) *Genes Dev* **11:**3319.

Immobilized template assay

The CYC1 promoter-containing plasmid pGAL4CG was digested with *Hin*dIII and the single-stranded overhang filled in using Klenow fragment of DNA polymerase I (NEB). The DNA was then digested with *Afl*III and end-labeled with biotin-14-dATP (GIBCO/BRL). As a promoter-less control, *Sma*I-*Afl*III fragment of $p(C_2AT)_{19}$ was biotinylated by end-filling with Klenow at the *Afl*III site. The two fragments contain identical 400-nucleotide G-less cassettes, and the pGAL4CG additionally contains ~200 nucleotides carrying one GAL4-binding site and the CYC1 basal promoter region. The biotinylated DNA fragments from pGAL4CG or $p(C_2AT)_{19}$ were separated from vector DNA by agarose gel electrophoresis, and electroeluted from gel slices. The purified DNA fragments were bound to Streptavidin-linked magnetic beads (Dynabeads M280 Streptavidin, Dynal, Inc.) in buffer F (2 M NaCl, 10 mM Tris-HCl at pH 7.4, 0.01% NP-40). The DNA bound beads were washed several times in buffer F and subsequently in TE buffer (10 mM Tris-HCl at pH 7.9, 1 mM EDTA). Finally, the beads were preincubated with 50 µg/ml of BSA for 30 min and washed twice with transcription reaction buffer (25 mM HEPES-KOH at pH 7.6, 10 mM magnesium acetate, 5 mM EGTA, 2.5 mM DTT, 100 mM potassium acetate, 10% glycerol).

Whole cell extracts were prepared from YSB143 (*MAT***a,** *ura3-52, leu2-3,112, his3Δ200, sua7::LEU2,* [pRS313-SUA7]) as described in Woontner *et al*. (1991). Transcription initiation complexes were assembled on immobilized template DNA by incubation of 120µg of whole cell extract and 250ng of GAL4-VP16 with 3µl beads in 60µl transcription reaction buffer. Two units of hexokinase and 2 mM glucose were added to the mixture during the assembly reaction to deplete endogenous NTPs. After 40 min, 5 µCi of [α^{32}P]ATP, phosphocreatine, and creatine kinase (Sigma) were added to label the CTD of the RNA Pol II largest subunit. To inhibit phosphorylation of the CTD, kinase inhibitor H8 (Sigma) was added to a final concentration of 2 mM, where indicated. After 30 min of labeling, 1 µM unlabeled dATP was added to complete phosphorylation of the CTD and incubation continued for another 30 min. The transcription complexes were magnetically purified and thoroughly washed with simple transcription buffer containing 0.003% NP-40. Beads were resuspended in the enzyme-GMP complex assay buffer with 3 µM [-^{32}P]GTP, and incubated at 30°C to label the Ceg1 protein. The reaction was stopped after 30 min by addition of loading buffer for SDS-PAGE and electrophoresed on a 4%-20% gradient polyacrylamide gel. The proteins were analyzed by immunoblotting with polyclonal antibodies against TFIIB (anti-Sua7), TFIIE (anti-Tfa1), TFIID, (anti-TBP), TFIIH (anti-Tfb1, provided by W.J. Feaver and R.D. Kornberg), and mAb 8WG16 against the unphosphorylated Pol II CTD (Thompson et al. 1989). Autoradiography was used to detect phosphorylated Pol II and capping enzyme.