In vitro Transcription: RNApII and III

NOTE 1: This document contains **3 x allied protocols:** Making IVT grade whole cell extracts (WCEs) (**section A**), and performing RNApII (**section B**) and RNApIII (**section C**) assays. All require significant optimization but are worth the effort. It is strongly advised to normalize RNApII activity (either between multiple preparations of the same strain or WT Vs. mutants) by RNApIII activity rather than amount of protein.

A. Preparation of IVT-grade WCEs

NOTE 2: Making 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 3**) 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 3: 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°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.

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. (<u>If isolating from a 4L culture you will likely need to split the sample between 2 x 50ml falcons</u>). 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 4:** 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 5:** Keep the aliquots reasonably small: multiple freeze-thaw cycles kill Tx activity.

B. IVT - RNApII

NOTE 6: The product of these reactions is RNA so avoid RNAse contamination: use DEPC-ddH_2O (see **Northern Protocol**), clean, dedicated glassware, and wear clean gloves.

NOTE 7: Both the RNApII (**Section B**) and RNApIII (**Section C**) protocols look very complicated but are actually quite straightforward. It's generally easier to do both at once (see **Note 1**): see MKeogh notebook **pH10.89**. It is possible to do the reactions, precipitate the products, and resolve, blot and expose the gels in one day (albeit a long one).

B.1 Assemble **RNApII Mix A** on ice (10µl mix A / Tx Reaction)

25mM HEPES-KOH pH7.6	(0.36µl of 1M stock)
100mM KAc pH7.6	(0.24µl of 5M stock)
10mM MgAc pH7.8	(0.15µl of 1M stock)
10U RNasin (Promega)	(0.25µl)
10mM phosphocreatine	(Sigma; 0.6µl of 0.5M in 20mM Tris-Ac pH7.8)
1U creatine kinase	(Sigma C-3755; in ***Creatine kinase buffer***; 0.33µl)
Vector (titrate 100 - 250ng): see **NOTE 8**	
ddH_2O	to 10µl final

NOTE 8: The lab has many potential templates to hand (** is my preferred). Prepare templates as CsCl grade supercoiled DNA (Qiagen DNA not suitable: it doesn't enrich for supercoiled), so have fun with that. The non-promoter templates and the one without a GAL4-binding site (see **NOTE 10**) are used as controls. It will be necessary to titrate the amount of template DNA from batch to batch. Note that all these templates are G-less (**eg.** Sawadogo & Roeder (1985) *PNAS* **82:**4394) which aids detection immensely. When the Tx reaction is incubated in the presence of ATP, CTP and radio-UTP, transcription terminates at the first G-encountered. Addition of the chain-terminator 3'-o-methyl-GTP (see **B.2** and **NOTE 14**) and digestion of any G-containing RNAs with $RNAseT_1$ (see **NOTE 11**) also aids background.

	GAL4-BS	**Cyc1Prom**
pUC18-$(C_2AT)_{19}$ (SB650) ~ 400 bp G-less	-	-
p$(C_2AT)_{19}$ (F48) ~ 400 bp G-less	-	-
p1xGal^{G-} (SB970) ~ 400 bp G-less	1	-
pJJ460 (F59/b262) ~ 250, 277bp G-less	-	+
** pGAL4C^{G-} (F348) ~ 350, 375bp G-less	1	+

pUC18-G5cyc1^{G-} (SB649) ~ 250, 277bp G-less	5	+

B.2 Assemble **RNApII NTP mix** on ice (3µl / Tx reaction) (see **NOTE 9**)

0.3µl 100 x rNTP mix (50mM rATP, rCTP; 1mM UTP)
0.5µl {α-^{32}P} UTP (NEN-Life Science products)
0.5µl 50mM 3'-o-methyl-GTP
ddH_2O to 3µl

NOTE 9: Ribonucleotide 5' Triphosphates (Roche / Boehringer)

rATP (# 1140 965) 100mM (400µl / 40µM) $79
rCTP (# 1140 922) 100mM (400µl / 40µM) $79
rGTP (# 1140 957) 100mM (400µl / 40µM) $79
rUTP (# 1140 949) 100mM (400µl / 40µM) $79
3'-o-methyl-GTP 5' triphosphate (#27-4675, Amersham Biosciences)

See also **Note 13** – attempts to compromise Tx elongation …

B.3 On ice add (per tube):

10µl RNApII Mix A
1µl Gal4-VP16 activator (titrate 0 -> 100ng: see **NOTE 10**)
1 – 16µl WCE (titrate with **TxbºB**): usual range 50-200µg WCE / rxn

Incubate RTº 5m. Add 3µl RNApII NTP mix (**final reaction volume: 30µl**). Incubate 25ºC, 45m.

NOTE 10: Detailed protocols for the production of recombinant GAL4-VP16 activator are described elsewhere (http://mckeogh.googlepages.com/protocols).

B.4 During incubation prepare **RNAse T_1** in **Stop solution** (200µl per reaction: see **NOTE 11**). Terminate by adding to the IVT reaction and incubate 25ºC, 20m.

NOTE 11: RNase T_1 (E.C. 3.1.27.3) is an endonuclease that specifically cleaves 3' of G residues. As such it cuts non-specifically transcribed RNAs and leaves the G-less cassettes untouched: this cleans up the background considerably.
Re. RNase T_1 enzymology: optimal pH 7.5, strongly inhibited by 1mM Ag^{2+}, Zn^{2+}, Cu^{2+}, Hg^{2+}; stimulated by histidine and EDTA, possibly by their chelation of inhibitor cations; stable to 100ºC, 10m @ pH6; increasingly unstable pH>9, fairly stable to acid.

Suppliers: Worthington >3x10^5 U/mg in 2.8M NH_4SO_4; LS03519, 10^5U
Sigma R-1003: 10^6 U, $41.50

RNAse T_1 in **Stop Solution**
10mM Tris.HCL pH 7.5
300mM NaCL
5mM EDTA
add 50U/ml RNAse T_1 (Sigma R-1003: 10^6 U, $41.50) **(Stock 500U/µl)**

B.5 Add 13µl 10% SDS and 5µl 10µg/µl Proteinase K per tube and incubate 37°C, 20m.

B.6 Add 2µl 10µg/µl tRNA and 84µl 10M NH_4OAc per tube. Vortex to mix and add 200µl PCI (25:24:1 PhOH:$CHCl_3$:IsoamylAlcohol). Vortex vigorously and centrifuge (14K, 10m, 25°C). Transfer the upper layer to a new ependorff and add 200µl $CHCl_3$. Vortex vigorously and centrifuge (14K, 10m, 25°C). Transfer the upper layer to a new ependorff (**care:** the $CHCl_3$ tries to follow) and add 2.5 vol EtOH (-20°C). Precipitate 60m, –80°C; **can stop here and –80°C O/N.**

B.7 Collect by centrifugation (14K, 10m, 25°C) and wash pellets with 70% EtOH. Remove supernatant and dissolve pellets in 10µl ***gel loading buffer***. Incubate 94°C 5m and put on ice. Collect by flash-spin centrifugation and ensure pellet is completely dissolved.

B.8 Prepare a 6% 0.4mm **(!! THIN !!)** denaturing urea-acrylamide gel (see **NOTE 12**) (**NB. Prerun the gel 150V, 30m; flush wells before loading the samples**). Run (eg. 16 x 16cm: 300V, 90m) until XC reaches the bottom. Dry gels onto 3MM blotting paper (Whatman) and expose to a phosphoimager plate or X-ray film. Typically see three products for pGAL4CG-: lowest band is specific transcript, the larger are read-though.

> **NOTE 12:** Small SDS-PAGE gel size works well: scale volumes and voltages accordingly. Mixes for 6% and 8% acrylamide gels are given in the buffers section.
>
> **NOTE 13:** It is recommended to include a labelled DNA ladder to aid sizing of the transcripts. One possible is the ØX174 Hinf digest (Promega) which contains a lot of small and mid-length DNA fragments. P^{32} label using standard methods. **Remember that the RNA is single stranded** ….
>
> **NOTE 14:** We have tried to compromise Tx elongation *in vitro* to identify required factors (**with mixed success**). This includes the addition of Sodium Citrate (0–15mM), modifying the Mg^{2+} concentration (5-20mM) and titrating 3' nucleotide analogs to induce pauses (see mkeogh notebook pH8.52ish): these act as chain terminators and must be removed to allow elongation to proceed.
>
> **eg.** 3'-0-Me-ATP (1µM $200; IBA Gmbh, Germany; www.iba-go.de)
> Make stock to 10mM with 100µl ddH_2O. Titrate up to 10% of ATP levels (5mM) in the reaction (add to the RNApII NTP mix in **Step B.2**).
>
> Thus for 3µl:
>
> | | | |
> |---|---|---|
> | 0% | 0mM | 0µl |
> | 1% | 0.05mM | 0.015µl |
> | 2.5% | 0.125mM | 0.038µl |
> | 5% | 0.25mM | 0.075µl |
> | 10% | 0.5mM | 0.15µl |

C. IVT - RNApII

NOTE 15: Easier and more reproducible reactions: a more robust enzyme? Template is pTZ1 (F83), encoding a Tyr tRNA. Qiagen grade DNA is fine.

C.1 Assemble **RNApIII Mix A** on ice (10µl mix A / Tx Reaction) – mix is similar (but not identical) to that used in RNApII IVT (**Step B.1**).

25mM HEPES-KOH pH7.6	(0.36µl of 1M stock)
100mM KAc pH7.8	(0.24µl of 5M stock)
10mM MgAc pH7.8	(0.15µl of 1M stock)
10U RNasin (Promega)	(0.25µl)
10mM phosphocreatine	(Sigma; 0.6µl of 0.5M in 20mM Tris-Ac pH7.8)
1U creatine kinase	(Sigma; in ***Creatine kinase buffer***; 0.33µl)
10µg/ml α-Amanitin	(0.3µl of 1mg/ml ddH_2O stock; **CARE: v.toxic**)
pTZ1 template (100ng; titrate if desired)	
ddH_2O	to 10µl final

C.2 Assemble **RNApIII NTP mix** on ice (3µl / Tx reaction)

0.3µl 100 x rNTP mix (50mM rATP, rCTP; 1mM UTP)
0.5µl {α-^{32}P} UTP (NEN-Life Science products)
0.3µl 50mM rGTP
ddH_2O to 3µl

C.3 On ice add (per tube):

10µl RNApIII Mix A
1 – 16µl WCE (titrate with **Txb°B**): usual range 25-100µg WCE / rxn

Incubate RT° 5m. Add 3µl RNApIII NTP mix (**final reaction volume: 30µl**). Incubate 25°C, 45m.

C.4 Terminate by adding 200µl **Stop Solution** (see **NOTE 11;** NO RNAse T_1) per RNApIII IVT reaction. Add 13µl 10% SDS and 5µl 10µg/µl Proteinase K per tube and incubate 37°C, 20m.

C.5 Add 2µl 10µg/µl tRNA and 84µl 10M NH_4OAc per tube. Vortex to mix and add 200µl PCI (25:24:1 PhOH:$CHCl_3$:IsoamylAlcohol). Vortex vigorously and centrifuge (14K, 10m, 25°C). Transfer the upper layer to a new ependorff and add 200µl $CHCl_3$. Vortex vigorously and centrifuge (14K, 10m, 25°C). Transfer the upper layer to a new ependorff (**care:** the $CHCl_3$ tries to follow) and add 2.5 vol EtOH (-20°C). Precipitate 60m, –80°C; **can stop here and –80°C O/N.**

B.6 Collect by centrifugation (14K, 10m, 25°C) and wash pellets with 70% EtOH. Remove supernatant and dissolve pellets in 10µl ***gel loading buffer***. Incubate 94°C 5m and put on ice. Collect by flash-spin centrifugation and ensure pellet is completely dissolved.

B.7 Prepare an 8% 0.4mm (**!! thin !!**) denaturing urea-acrylamide gel (see **NOTE 12**) (**NB. Pre-run the gel 150V for 30m; flush wells before loading the samples**). Run (eg. 16 x 16cm: 300V, 90m) until XC reaches the bottom. Dry gels onto 3MM blotting paper (Whatman) and expose to a phosphoimager plate or X-ray film. Typically see two products: the Tyr tRNA contains an intron.

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)

Creatin Kinase Buffer

20mM HEPES-KOH pH 7.6
50% Glycerol
100mM KAc
0.1mM EDTA
Add Creatine Kinase (Sigma) to 20mg/ml and **store 100µl aliquots at –80°C**

Stop Solution (+/- RNAse T_1 as indicated)

10mM Tris.HCL pH 7.5
300mM NaCL
5mM EDTA
50U/ml RNAse T_1 (Sigma R-1003: 10^6 U, $41.50) **(Stock 500U/µl)**

Sequencing loading buffer

50% Formamide
1mM EDTA
0.1% SDS
0.1% Xylene Cyanol FF
0.1% Bromophenol Blue

Denaturing Urea-Acrylamide gel mix (SequaGel system - http://nationaldiagnostics.com)

Volumes given are per 50mls – scale accordingly.

		6% (60-150nt)	**8% (40-100nt)**
Concentrate	237.5g acrylamide, 12.5g methylene bis-acrylamide, 7.5M urea	12ml	16ml
Diluent	7.5M Urea	33ml	29ml
Buffer	10xTBE (0.89M Tris-Borate, 20mM EDTA pH 8.3), 7.5M urea	5ml	5ml
10% APS		400µl	400µl
TEMED		20µl	20µl

For a manuscript methods section:

In vitro transcription assays

Assays were performed as described previously (Keogh et al. 2002) with minor modifications. 4L of each strain was grown overnight to mid log phase ($OD_{\lambda 600} \approx 1.2$), centrifuged and washed twice with 500mls ice-cold ddH_2O. All manipulations from this point on were performed on ice at 4°C and all buffers contained protease inhibitors as standard. The yeast pellet (≈ 5g/L) was resuspended in 2 mls Transcription Buffer A (200mM Tris pH 7.9, 390mM NH_4SO_4, 10mM $MgSO_4$, 1mM EDTA, 20% Glycerol, 2mM DTT) per gram wet weight and subjected to glass bead disruption. The lysate was collected and centrifuged at 9000rpm for 20 minutes. The supernatant was collected, 1/7 Volume of 4M potassium acetate (pH 7.6) was added dropwise and the resulting mix rotated at 4°C for 30 minutes. The mixture was centrifuged in a Beckman 45Ti rotor at 40000rpm for 90 minutes and the supernatant carefully collected

avoiding the pellet and lipid layer. An equal volume of saturated 4M NH_4SO_4 (pH7) was added dropwise with mixing, the mix placed on a rotator for 30 minutes and centrifuged at 20000 rpm for 20 minutes. The supernatant was discarded and the pellet resuspended in 50µl Tx b^{o}B (20mM HEPES-KOH pH 7.5, 10mM magnesium acetate, 150mM potassium acetate, 10mM EGTA, 20% glycerol, 5mM DTT) / gram original wet weight. The resulting solution was dialyzed against three changes of 200 volumes Tx b^{o}B for four hours until the conductivity of the lysate was similar to the dialysis buffer. Lysates (30 - 60 mg/ml) were stored in aliquots at -80^{o}C.

RNApII transcription reactions were assembled on ice, containing; 12µl mix A (25mM HEPES-KOH pH7.8, 10% glycerol, 100mM KAcetate pH7.8, 10mM MgAcetate pH7.8, 5mM EGTA, 2.5mM DTT, 10mM phosphocreatine (Sigma), 1U creatine kinase (Sigma), 10U RNasin (Promega)), 3µl NTP mix (5mM ATP, CTP, 100µM UTP, 0.25µl {α-^{32}P} UTP (NEN-Life Science products), and 5mM 3'-o-methyl-GTP (Amersham Pharmacia Biotech)), 100ng plasmid template (pCYC-GAL4 CG$^-$), whole cell extract (as indicated) and GAL4-VP16 activator (as indicated) to 30µl per reaction. Further additions were as described in each case. Reactions were allowed to proceed at 25^{o}C for 45 - 60 minutes, and terminated by the addition of 200µl of RNAse T_1 buffer (10mM Tris.HCL pH 7.5, 300mM NaCL, 5mM EDTA, 50U/ml RNAse T_1), followed by incubation at room temperature for 15 minutes. SDS was then added to 0.5% and proteinase K to 100µg/ml followed by incubation for 20 minutes at 37^{o}C. 10µg tRNA was then added and the reactions phenol-chloroform extracted and ethanol precipitated. RNA was resuspended in 10µl 0.1% SDS in 50% formamide and resolved on a 6% 0.4mm denaturing urea-acrylamide gel. Gels were dried onto gel blot paper (Whatman) and exposed to X-ray film for autoradiography.

RNApIII transcription reactions were as above with minor differences. Reactions were assembled on ice, containing; 12µl mix A (25mM HEPES-KOH pH7.8, 10% glycerol, 100mM KAcetate pH7.8, 10mM MgAcetate pH7.8, 5mM EGTA, 2.5mM DTT, 10mM phosphocreatine (Sigma), 1U creatine kinase (Sigma), 10U RNasin (Promega), 10µg/ml α-Amanitin), 3µl dNTP mix (5mM ATP, GTP, CTP, 100µM UTP, 0.25µl {α-^{32}P} UTP (NEN-Life Science products), 100ng pTZ1 plasmid template encoding a Tyr tRNA and whole cell extract (25-100µg) to 30µl per reaction. Reactions were allowed to proceed at 25^{o}C for 30 minutes and terminated by the addition of 200µl Stop buffer (10mM Tris.HCL pH 7.5, 300mM NaCL, 5mM EDTA). SDS was then added to 0.5% and proteinase K to 100µg/ml followed by incubation for 20 minutes at 37^{o}C. 10µg tRNA was then added and the reactions phenol-chloroform extracted and ethanol precipitated. RNA was resuspended in 10µl 0.1% SDS in 50% formamide and resolved on an 8% 0.4mm denaturing urea-acrylamide gel. Gels were dried onto gel blot paper (Whatman) and exposed to X-ray film for autoradiography.