Recombinant Gal4-VP16 activator

For *in vitro* Transcription (IVT) assays with RNApII

NOTE 1: For general notes on recombinant protein expression see the *E.coli* lab protocols section.

NOTE 2: Gal4-VP16 is a potent transcriptional activator (see: Sadowski *et al* (1988) *Nature* **335:**563). Contains the DNA-binding domain of yeast Gal4 fused to a highly acidic portion of the herpes simplex virus protein VP16. Potent activator when added to RNApII IVT reactions in which the template (**eg.** (pGAL4CG$^-$; **F348 / b1457**) contains a Gal4-binding site near the promoter.

See Keogh Notebook: **pH7-143**, **pH7-150, pH8-9.**

1. Transform expression plasmid pGAL4-VP16 (BE367 / b2911; **Amp^R**) (from Pat Nakatani) into *E.coli* strain BL21/DE3 (or BL21/DE3 pLysS). This fusion protein is HIS6 tagged internally (GAL4-his6-VP16).

2. Grow 100ml culture overnight at 37^{o}C (LB^{Amp}; LBA). Sub-innoculate next morning 1/100 into 2L LBA and grow to $OD_{600} \approx 0.6$ at 37^{o}C. Chill the cultures 15 minutes on ice and induce with 1mM IPTG at RT^o for two hours.

NOTE 3: Supplement culture media with 20μM $ZnSO_4$ (aids solubility). All steps from this point are performed at 4^{o}C, and all buffers contain 10μM $ZnSO_4$ and protease inhibitors as standard.

3. Harvest cells by centrifugation, wash pellet with ice-cold ddH_2O and transfer to a 50ml Falcon tubes in (can freeze pellets -80^{o}C at this point). Resuspend in 30mls ***buffer A*** (4^{o}C) and sonicate on ice (3 x 20s pulses 50% output, 40 duty cycle). Add Triton X-100 to 1% and mix by inversion.

4. Clarify by centrifugation (10K, 15m, 4^{o}C) and transfer the supernatant to a 50ml falcon tube on ice. Add 2ml Ni-NTA resin (Qiagen) (pre-equilibrate in ***buffer A***) and incubate at 4^{o}C overnight with gentle rolling.

5. Collect beads by centrifugation (1.5K, 5m, 4^{o}C) and wash in batch with 20mls ***buffer A*** and 20mls ***buffer E / 20mM Imidazole***. Collect after each resuspension by centrifugation and discard the supernatants (keep some to test by Western analysis). Transfer the beads to a disposable column and elute with ***buffer E / 200mM Imidazole***. Collect 500μl fractions.

6. Analyze fractions by Biorad Dc and SDS-PAGE **(GAL4-his6-VP16 ≈ 30 kD).** Estimate the cleanliness of the prep (how many bands visible on commassie staining SDS-PAGE) and yield per L.

7.1 Option 1: Pool positive fractions and dialyze against 2 x 1L ***buffer E / 0mM Imidazole*** (2 hrs per, 4°C). Compare concentration (by Biorad Dc) before and after dialysis (to ensure no holes in the membrane) and store in small aliquots at –80°C.

7.2 Option 2 (clean up further by FPLC: may not be necessary): Pool positive fractions and load onto a 1.5ml Mono-Q column (Biorad: Flow Rate (FR) 0.5ml/min). Wash with 5mls ***buffer E / 0mM Imidazole***. Apply a 20ml linear gradient of 100-700mM NaCl in ***buffer E / 0mM Imidazole*** (Gal4-VP16 elutes at ≈350mM NaCl). Pool positive fractions, estimate concentration by Biorad Dc and store in aliquots at -80°C.

> **NOTE 4:** It may be necessary to clean the Mono-Q column (if it hasn't been used in a while or the **back pressure looks suspiciously high**). If so reverse the flow and successively wash (FR: 0.5ml/min) with:
>
> - 500µl 2M NaCl
> - 5ml ddH_2O
> - 500µl 2M NaOH
> - 5ml ddH_2O
> - 500µl 1% TFA
> - 5ml ddH_2O
>
> Then reverse the flow to normal and equilibrate in ***buffer E / 0mM Imidazole***.

Buffers:

Buffer A -

10mM Tris.HCl (pH 8.0)
10% Glycerol
500mM NaCL
0.1% Tween 20
10mM Imidazole
10mM β-ME
10µM $ZnSO_4$
Protease inhibitors as standard (incl. 1mm PMSF)

Buffer E -

20mM HEPES.KOH (pH 7.5)
20% Glycerol
100mM NaCL
1mM EDTA
1mM DTT
10µM $ZnSO_4$
Protease inhibitors as standard (incl. 1mM PMSF)
Imidazole as indicated

For a manuscript methods section:

Recombinant GAL4-VP16 fusion protein: E. coli strain BL21/DE3 was transformed with pGAL4-VP16 (kind gift of Pat Nakatani). A 2L culture was grown at 37°C to $OD_{600} \approx 0.5$ and induced by the addition of 1mM IPTG. Cells were further cultured for two hours at room temperature in the presence of 20μM $ZnSO_4$. All steps from this point on were performed at 4°C and all buffers contained 10μM $ZnSO_4$ and protease inhibitors as standard. Lysates were prepared by sonication in lysis buffer (10mM Tris.Cl pH8.0, 500mM NaCl, 10% Glycerol, 10mM β-ME, 0.1% Tween-20, 10μM $ZnSO_4$, 10mM Imidazole, 1mM PMSF), Triton X-100 added to 0.1% and extracts clarified by centrifugation. The soluble extract was incubated at 4°C with gentle rolling overnight with 2ml Ni-NTA resin (Qiagen). The resin was applied to a column, washed with 15mls buffer E (20mM HEPES-KOH pH7.6, 100mM NaCl, 20% Glycerol, 1mM EDTA, 1mM DTT, 10μM $ZnSO_4$, 20mM Imidazole, 1mM PMSF) and eluted with buffer E / 200mM Imidazole. Positive fractions were pooled, applied to a 1.5ml Mono-Q column (Biorad) and washed with 5mls buffer E (no imidazole). A 20ml linear gradient of 100-700mM NaCl in buffer E was applied where GAL4-VP16 elutes at ≈ 350mM NaCl. Positive fractions were pooled and stored in aliquots at -80°C.