FA-CROSSLINKING AND CHROMATIN IMMUNOPRECIPITATION (X-ChIP)

FA-treatment

- 250ml Flask containing 35ml Pc5/5 cells, 4 days post subculturing.
- Add 105µl pep25 elicitor from stock solution (100µg/ml in H_2O).
- Incubate 3h under gentle shaking.
- Add 1ml 37% formaldehyde (FA in 10% methanol) to a final concentration of 1%.
- Incubate for 7min (can be extended up to 20min).
- Quench reaction by addition of 4 ml glycin stock solution (1.0M) to 0.1M final concentration.
- Harvest cells by vacuum filtration, freeze in liquid N_2

Extraction of nuclei

- Grind cells to powder in liquid N_2.
- Transfer cell powder to 50ml Falcon tube, add approximately 30ml high glycerol buffer.
- Incubate suspension 20min at 4°C under gentle agitation (thereby releasing nuclei that are still entrapped in cell ghosts after grinding).
- Filter through 20µm and 70µm nylon mesh into 50ml Falcon tube, twirl mesh to press filtrate through filter.
- Increase volume to 40ml with HSB.
- Centrifuge at 4°C, 4K, 1h in Heraeus Varifuge.
- Gently resuspend nuclei in 25ml low glycerol buffer (LGB).
- Centrifuge at 4°C, 4K, 20 min in Heraeus Varifuge.
- Resuspend pellet in 0.5 to 1.0ml sonication buffer containing 0.5% SDS, and transfer to 15ml Falcon tube.
- Incubate at 4°C under agitation for 20min to denature the chromatin proteins (important to increase efficiency of the following sonication step).
- Dilute 1:2 with sonication buffer lacking SDS (reduces foaming during sonication, if foaming is still a problem reduce SDS content further).
- Sonicate on ice-ethanol bath in Branson sonicator at position 4, constant duty cycle, 4-5 times 30s pulses separated by 2min cooling on ice.
- Transfer to 2.0ml centrifugation tube, spin 15min full speed in a lab centrifuge at 4°C.
- Use 50µl of supernatant for pilot de-crosslinking and store remainder at -80°C (shock freeze in liquid N_2).

Pilot Extraction of free DNA from nuclei (process duplicates for best quantification)

Proteinase K treatment:

- To 50µl extract add 150µl sonication buffer containing 0.25% SDS.
- Add 5µl Proteinase K from 10mg/ml stock (200µg/ml final concentration).
- Incubate overnight at 37°C.
- Incubate tubes at 65°C for at least 6h.
- Extract with 200µl phenol/TE.
- Extract with 200µl phenol/chloroform.
- Add 1µl RNAseA (10mg/ml) and incubate 15min at room temperature.
- Add 20µl NaAcetate 3M, 500µl ethanol, 1µl glycogen stock (10mg/ml) and precipitate at –20°C overnight.
- Pellet DNA and wash with 70% ethanol.
- Dissolve in 100µl H_2O.
- Check size of fragments on agarose gel (20µl, also adding RNAse to the loading buffer). The bulk of the DNA (runs as a smear) should be around 1.0 kB. If DNA is too large, try re-sonication. Detection of discrete bands are indicative of RNA.
- Quantitate DNA in spectrophotometer without further dilution.
- Optional: quantitate the amount of PCR amplifiable DNA in appropriate dilution series (e.g. 1/500, 1/1000, 1/2000, 1/4000).
- If the results are satisfactory, proceed to next step.

Immunoprecipitation of Protein/DNA complexes

- Thaw extract on ice, centrifuge for10min (14K, 4°C), transfer supernatant to new tube avoiding precipitates that form during freezing.
- Mix 200µl sonicated chromatin with 300µl RIPAF-SDS (samples should be adjusted to equal amounts based on the pilot de-crosslinkings quantitation results).
- Add 3µl preimmune serum, 30µl ProteinA-coupled agarose beads (e.g. Pharmacia ProteinA 4 CLB).
- Incubate for 1h, 4°C, using rotation mixer.
- Centrifuge for 5min in a table centrifuge, full speed at 4°C.
- Transfer supernatant to new tube.
- Add 3µl specific antibody (or control antibodies), incubate for 4h on ice.
- Repeat centrifugation step as above, transfer supernatant to new tube
- Add 30µl washed Protein A beads.

    washing of beads:

    take x times 20 µl 50% slurry (per sample)
    wash 3 times with 1ml RIPAF
    add RIPAF to 30 µl

- Incubate on rotator for 1-2h in the cold room.
- Centrifuge for 5min in a table centrifuge, full speed, 4°C, transfer supernatant to new tubes (will be further processed for input samples and can be analysed by western blot)
- Wash beads 3 times with 1ml ice-cold RIPAF.

- Wash again with 800µl RIPAF and transfer beads and liquid to fresh tube (reduces background considerably).
- Repeat centrifugation, remove supernatant, re-spin and take off remainder of supernatant.
- Elute twice with 200µl glycin elution buffer and neutralize pooled samples by the addition of 100µl 1.0M Tris-HCl pH 9.7.

Extraction of free DNA from immunoprecipitates and input aliquots

Immunoprecipitates (IP) 400µl eluates
- Add 10µl SDS 10%.
- Add 5µl Proteinase K (10mg/ml).
- Incubate, extract and precipitate as described in the 'pilot extraction' using however twice the volume.
- Resuspend pellet in 100µl H_2O, and use 5µl per PCR reaction.

Input samples: 40µl supernatant from IP (process duplicates per extract).

- Add 160µl RIPAF.
- Add 5µl Proteinase K (10 mg/ml).
- Incubate, extract and precipitate as described in the 'pilot extraction'.
- Resuspend in 1000µl H_2O, (1/100 dilution as compared to IP sample). Use 5µl, 2.5µl, 1.25µl and 0.675µl as starting point per PCR reaction (depends on antibody and extract and may have to be diluted more).

Protein analysis following immunoprecipitation
- 100µl of IP eluate (remainder following DNA extraction) or 100µl supernatant from immunoprecipitation.
- Add 400µl acetone, precipitate overnight at -20°C.
- Centrifuge for 15 min in a table centrifuge at full speed at 4°C.
- Air dry pellets briefly, add 20µl SDS loading buffer with 10% 2-mercaptoethanol.
- Incubate 1h at 95°C to break the cross-links (not all proteins survives this treatment without degradation!).
- Analyse by standard SDS-PAGE and by western blotting.

Buffers and solutions

High glycerol buffer	Stock	200ml
20mM Hepes(NaOH) pH 7.4	0.5M	8ml
5mM MgCl2	1M	1ml
5mM KCl	3M	332µl
50mM saccharose	1M	10ml
70%glycerin	100%	140ml
1mM DTT	1M	200µl

1µg/ml leupeptin (optional)	1mg/ml	200µl
200µM PMSF (optional)	200mM	200µl

Low glycerol buffer = High glycerol buffer containing only 10% glycerol.

Sonication buffer	stock	40 ml
1mM EDTA pH 8.0	0.5 M	80 µl
10mM Tris.HCl pH 7.4	1 M	400 µl

Sonication buffers required at three different SDS concentrations:

1.) 0.5% SDS
2.) 0.25% SDS
3.) without SDS

RIPAF	stock	100ml
50mM Hepes(NaOH) pH 7.4	0.5M	10ml
140mM NaCl	5M	2.8ml
1mM EDTA	0.5 M	1.0ml
1.0% triton X-100	100 %	1.0ml
0.1% DOC	10 %	1.0ml
0.1% SDS	10 %	1.0ml

RIPAF solution is also required without SDS for sample dilution prior immunoprecipitation.

Note: Hepes should be used instead of Tris-HCl since Tris can be cross-linked by residual formaldehyde in the extracts. Titration of Hepes by KOH is not compatible with the SDS in the immunoprecipitation. Potassium forms precipitates with SDS which in turn trap protein resulting in high, unspecific background signals. NaOH is suitable for titrating Hepes buffer but the buffer should not older than one week.

Glycin elution buffer	Stock	100ml
0.1M glycin pH 2.5	1M	10ml
0.5M NaCl	5M	10ml
0.05% Tween20	100%	50µl

Glycin elution buffer: Prepare100ml batch and store 10 ml aliquots at -20°C.

Other stock solutions:

- Pep25 elicitor (100µg/ml).
- 37% formaldehyde in 10%methanol.

- Proteinase K (10mg/ml).
- SDS 10%.
- glycogen (10 mg/ml).