

Chromatin immunoprecipitation (Myriam protocol). (Based on Bowler et al. Chromatin techniques for plant cells. Plant J. 2004.)

Chromatin immunoprecipitation combines immunoprecipitation of chromatin fragments and polymerase chain reaction to map sites of protein–DNA interaction *in vivo*. The ChIP technique has been successfully used in *Drosophila* and yeast to study histone modifications of eu/heterochromatin and localization of regulatory chromatin factors. The method relies on the rapid cross-linking of protein/DNA complexes within the nucleus of living cells, followed by chromatin isolation, its random shearing and immunoprecipitation with antibodies directed towards proteins of interest. The amount of co-immunoprecipitated DNA is analysed by PCR. The relative enrichment or depletion of a particular DNA fragment in the immunoprecipitated fraction reflects its *in vivo* association with the examined protein (Hecht and Grunstein, 1999; Orlando et al., 1997). In plants, ChIP was first used to map the subnuclear distribution of linker histone variants in *Arabidopsis thaliana* (Ascenzi and Gantt, 1999). Further studies in plants exploiting ChIP investigated the chromatin structure of the pea plastocyanin gene (*PetE*) by determination of the acetylation states of histones H3 and H4 and the nuclease accessibility of this gene (Chua et al., 2001). The protocols used for *Arabidopsis* as well as for pea were essentially adopted, with slight modifications, from either *Drosophila* (Orlando et al., 1997) or yeast (Hecht and Grunstein, 1999). The major difficulty encountered in plants was the limiting amount of material, as early protocols (Ascenzi and Gantt, 1999; Chua et al., 2001) required 100 g of *Arabidopsis* tissue for a single ChIP experiment. This problem was circumvented by recent studies that successfully combined various protocols (Gendrel et al., 2002; Johnson et al., 2002; Tariq et al., 2003). These methods used ChIP with antibodies directed against modified histone tails and allowed downscaling to 1–1.5 g of *Arabidopsis*. Here we describe a ChIP protocol adapted from Gendrel et al. (2002) with slight modifications.

Equipment and reagents

Miracloth

Formaldehyde (Fluka, Buchs SG, Switzerland)

Glycine 2 M

Double distilled autoclaved water

Vacuum chamber

Vortex

Rotator

Liquid nitrogen

Falcon tubes for 50 and 15 ml

Refrigerated centrifuge

Branson Sonifier 250 (Branson-sonifier, Frankfurt, Germany)

Protein-A beads, 0.5% BSA and Salmon sperm DNA. **(It would be perfect order: Sheared salmon sperm DNA/protein A agarose mix (Upstate Biotechnology, Lake Placid, NY, USA))**

1 M Tris–HCl, pH 6.5

5 M NaCl

0.5 M EDTA

Heating block at 65°C

Proteinase K (14 mg ml⁻¹; Boehringer Mannheim GmbH, Mannheim, Germany)

Novagen pellet paint (CN Bioscience, Darmstadt, Germany)

RNase A (DNase-free)

Phenol:chloroform:isoamylalcohol, 25:24:1

Chloroform

Ethanol

Extraction buffer 1	for 100 ml
0.4 M Sucrose	20 ml of 2 M stock
10 mM Tris-HCl, pH 8.0	1 ml of 1 M stock
5 mM β-ME	35 μl of 14.3 M stock
0.1 mM PMSF	50 μl of 0.2 M stock
Protease inhibitors	Protease inhibitors should be added immediately before use because they are quickly degraded
Aprotinin	100 μl of 1 mg ml ⁻¹ stock
Pepstatin A	100 μl of 1 mg ml ⁻¹
Leupeptin	100 μl of 1 mg ml ⁻¹
Antipain	100 μl of 1 mg ml ⁻¹
TPCK	100 μl of 3 mg ml ⁻¹
Benzamidine	100 μl of 1 mg ml ⁻¹
Extraction buffer 2	for 10 ml
0.25 M Sucrose	1.25 ml of 2 M stock
10 mM Tris-HCl, pH 8.0	100 μl of 1 M stock
10 mM MgCl ₂	100 μl of 1 M stock
1% Triton X-100	0.5 ml of 20% stock
5 mM β-ME	3.5 μl of 14.4 M stock
0.1 mM PMSF	5 μl of 0.2 M stock
Protease inhibitors	10 μl of each as in buffer 1
Extraction buffer 3	for 10 ml
1.7 M Sucrose	8.2 ml of 2 M stock
10 mM Tris-HCl, pH 8.0	100 μl of 1 M stock
0.15% Triton X-100	75 μl of 20% stock

2 mM MgCl ₂	20 μ l of 1 M stock
5 mM BME	3.5 μ l of 14.3 M stock
0.1 mM PMSF	5 μ l of 0.2 M stock
Protease inhibitors	as in buffer 1 and 2
Nuclei lysis buffer	for 5 ml
50 mM Tris-HCl, pH 8.0	0.25 ml of 1 M
10 mM EDTA	24 μ l of 0.5 M
1% SDS	0.25 ml of 20%
PMSF and protease inhibitors	as in buffer 1
ChIP dilution buffer	for 10 ml
1.1% Triton X-100	550 μ l of 20%
1.2 mM EDTA	24 μ l of 0.5 M
16.7 mM Tris-HCl, pH 8.0	167 μ l of 1 M
167 mM NaCl	334 μ l of 5 M
PMSF and protease inhibitors	as in buffer 1
Elution buffer	for 20 ml
1% SDS	1 ml of 20%
0.1 M NaHCO ₃	0.168 g
Low salt wash buffer	for 50 ml
150 mM NaCl	1.5 ml of 5 M
0.1% SDS	0.25 ml of 20%
1% Triton X-100	2.5 ml of 20%
2 mM EDTA	200 μ l of 0.5 M
20 mM Tris-HCl, pH 8.0	1 ml of 1 M
High salt wash buffer	for 50 ml
500 mM NaCl	5 ml of 5 M
0.1% SDS	0.25 ml of 20%
1% Triton X-100	2.5 ml of 20%
2 mM EDTA	200 μ l of 0.5 M
20 mM Tris-HCl, pH 8.0	1 ml 1 M
LiCl wash buffer	for 50 ml
0.25 M LiCl	3.125 ml of 4 M
1% NP-40	2.5 ml of 20%
1% sodium deoxycholate	0.5 g
1 mM EDTA	100 μ l of 0.5 M
10 mM Tris-HCl, pH 8.0	0.5 ml of 1 M

TE buffer	for 50 ml
10 mM Tris-HCl, pH 8.0	5 ml of 1 M
1 mM EDTA	100 μ l of 0.5 M

Experimental protocol

Note: All steps must be carried out at 4°C, unless stated otherwise.

Preparation of plant material.

1. Germinate *Arabidopsis* seeds on agar plates.
2. Harvest 2 g (different days after germination) of seedlings in a 50 ml Falcon tube.
3. Rinse seedlings twice with 40 ml of double distilled (dd) autoclaved water by gently shaking the tube (room temperature).

Formaldehyde cross-linking. 4. After thoroughly removing the water, submerge seedlings in 37 ml of 1% formaldehyde in cross-linking solution, extraction buffer 1 (room temperature) and vacuum infiltrate for 10 min.

5. Stop the cross-linking by addition of glycine to a final concentration of 0.125 M (2.5 ml of 2 M glycine in 37 ml of extraction buffer 1) and application of vacuum for additional 5 min. At this stage, seedlings should appear translucent.

6. Rinse seedlings twice with 40 ml cold dd autoclaved water.

7. Remove water as thoroughly as possible by placing seedlings on a paper towel before transferring to a new Falcon tube. At this stage cross-linked material can be either frozen in liquid nitrogen and stored at -80°C or processed further for chromatin isolation at step 8.

Isolation and sonication of chromatin.

8. Pre-cool mortar and pestle by filling with liquid nitrogen before placing seedlings in the rest of the liquid nitrogen and grinding them to a fine powder.

9. Resuspend the powder in 30 ml extraction buffer 1 (4°C) in a 50 ml Falcon tube.

10. Filter the solution through two layers of miracloth into a new 50 ml Falcon tube.

11. Spin the filtered solution for 20 min at 2100 **g** at 4°C (Freeling lab centrifuge)

12. Gently remove supernatant and resuspend the pellet in 1 ml of extraction buffer 2.

13. Transfer the solution to 1.5 ml Eppendorf tube. Leave it on ice 5-10 min.

14. Centrifuge at 12 000 **g** for 10 min at 4°C.

Made another wash if the pellet continues green.

OPTIONAL (I do not use to do it if the pellet is clean because you loose sample)

15. Remove the supernatant and resuspend pellet in 300 μ l of extraction buffer 3.

16. Overlay the resuspended pellet onto 300 μ l of extraction buffer 3 in a fresh Eppendorf tube.

17. Spin for 1 h at 16 000 *g* at 4°C.

18. Remove the supernatant and resuspend the chromatin pellet in 300 μ l of nuclei lysis buffer by vortexing and pipetting up and down (keep solution at 4°C). At this stage save a 1–2 μ l aliquot for later examination. Aliquots taken at this step as well as at step 21 (see below) must be treated as described in 'elution and reverse cross-linking' procedure (see below) before analysing on the gel.

19. Once resuspended, sonicate the chromatin solution for 20 sec, four times on 20% power 550 Sonic Dismembrator (2nd floor cold room), to shear DNA to approximately 0.5–2 kb DNA fragments.

20. Spin the sonicated chromatin suspension for 5 min at 4°C (16 000 *g*) to pellet debris. The sonicated chromatin solution can be frozen at –80°C or processed to step 20 for immunoprecipitation.

Immunoprecipitation.

21. Remove supernatant to a new tube. Use an aliquot of 1–2 μ l to check sonication efficiency by reverse cross-linking (follow from step 35) and electrophoretic determination of the average size of DNA fragments as compared with the aliquot from step 18.

22. Split the 200 μ l into two tubes with 100 μ l each (one for the Mock =negative control, and the other for the IP)

23. Add 900 μ l of ChIP dilution buffer to each tube. This dilutes the SDS to 0.1% SDS. **Take a sample of 20–40 μ l for the INPUT chromatin.**

24. Equilibrate Protein-A Sepharose CL-4B beads (Amersham Bioscience) in ChIP dilution Buffer (3Xwash). Antibodies (normally 1:200. It is the dilution that I use in the case of monoclonal FLAG Antibody) were prebound to Protein A beads by 2–4 h incubation at 4°C in ChIP Dilution Buffer with 0.5% BSA. Prior to use, the beads should be rinsed three times and resuspended in ChIP dilution buffer.

Remember prepare Protein –A beads for the the MOCK (same components without antibody)

25. Add to the 1ml diluted chromatin 30 μ l of Prot-A (already treated with BSA) and 2 μ g/ml of salmon sperm DNA (the ssDNA must be preheated 10 min at 95°C and then placed on ice for 5 min)

26. Incubate overnight at 4°C with gentle agitation (in the wheel).

27. Collect immunoprecipitate with 30 μ l of protein A agarose beads.

28. Prepare fresh elution buffer (1% SDS, 0.1 M NaHCO₃).

29. Pellet beads by centrifugation (2 min, 16 000 *g*) and wash them with gentle agitation for 10 min at 4°C each wash, using 1 ml of buffer per wash followed by pelleting the beads. Apply the following washes in the order listed below:

- (a) Low salt wash buffer (one wash).
- (b) High salt wash buffer (one wash).
- (c) LiCl wash buffer (one wash).
- (d) TE buffer (one washes).

After the final wash, remove TE thoroughly.

Elution and reverse cross-linking of chromatin.

30. Release bead-bound complexes by adding 250 μl of elution buffer (made fresh at step 30) to the pelleted beads.

31. Vortex briefly to mix and incubate at 65°C for 15 min with gentle agitation.

32. Spin beads and carefully transfer the supernatant (eluate) to a fresh tube and repeat elution of beads. Combine the two eluates.

33. Add 20 μl 5 M NaCl to the eluate to reverse the cross-links by an overnight incubation at 65°C.

34. Add 10 μl of 0.5 M EDTA, 20 μl 1 M Tris-HCl, pH 6.5, and 1.5 μl of 14 mg ml⁻¹ proteinase K to the eluate and incubate for 1 h at 45°C.

37. Extract DNA by phenol/chloroform/isoamyl alcohol (equal volume) and precipitate with 2 vol of ethanol 100% at -70°C for 2 h to overnight. Spin at 16000 rpm 20 min at 4°C to recover the DNA.

38. Dry briefly the pellet in the speed vac (5 min)

38. Resuspend the pellet in 25 μl of TE. The immunoprecipitated and purified DNA is then used in PCR reactions to amplify examined target sequence in relation to a reference sequence (internal control), preferably in a multiplex PCR in order to quantify the enrichment or depletion of target(s) as compared with the reference and mock control.

39. Use 1 μl for a 25 μl PCR reaction. The amount of recovered templates may vary between experiments depending upon efficiency of immunoprecipitation, thus PCR conditions, for example, number of cycles (I normally use 40 cycles PCR reaction) may need adaptation.