# Plant Chromatin Immunoprecipitation and ChIP/Chop

Sasha Preuss, Richard Lawrence and Craig Pikaard, Biology Department, Washington University at St. Louis, MO 63130, USA.

This protocol is adapted from:

Gendrel, A. V., Lippman, Z., Yordan, C., Colot, V. & Martienssen, R. A. (2002) Science 297, 1871-1873

Nelson, J.D., Denisenko, O., Sova, P., and Bomsztyk, K. 2006. Fast chromatin immunoprecipitation assay. Nucleic Acids Res 34(1): e2.

---

1. Grow 2 large petri plates (150 mm) of Arabidopsis seedlings (several hundred/plate) on sterile MS-agar, 4g/L phytogel, 1% sucrose, pH 5.2 8-12 days.

2. Harvest 3 grams of seedlings from each plate into a separate 50 ml conical centrifuge tube.

3. Rinse seedlings gently with 40 ml deionized (Milli-Q) water; repeat the rinse a second time.

4. Add 37 ml of 1.0% formaldehyde

5. Stuff the top of each 50 ml conical tube (containing the formaldehyde-soaked seedlings) with nylon mesh to keep the seedlings immersed in the liquid during vacuum infiltration (and to aid later decanting and rinsing steps). Also poke holes in the cap of the conical tube and then screw the cap onto the tube.

6. Vacuum infiltrate the seedlings for 10 minutes in a desiccator attached to a vacuum pump. You should pull a vacuum in excess of 25 inches mercury

7. Quench the crosslinking by adding 2.5 ml of 2M glycine ([Final] = 0.125M) and vacuum infiltrate for an additional 5 minutes.

8. Rinse the seedlings 2 times with Milli-Q (deionized) water, then remove as much water as possible.

9. Grind seedlings in liquid Nitrogen to a fine powder using a mortar and pestle.

10. Add the powder to 30 ml of Extraction Buffer 1 in a 50 ml conical tube, vortex, place on ice.

11. Filter solution through 2 layers of Miracloth, into a 30mL oak ridge tube.

12. Centrifuge solution 20 minutes @ 4000rpm (1940 x g) at 4 ˚C in a Beckman

JA-20 rotor.

13. Remove supernatant and resuspend pellet in 1 ml extraction buffer 2 at 4 ˚C. (transfer to 1.5ml eppendorf tube)

14. Centrifuge at top speed in microfuge (~14,000 x g), 10 minutes at 4 ˚C.

15. Remove supernatant and resuspend pellet in 300 ul extraction buffer 3 at 4 ˚C.

16. In a clean eppendorf tube, add 300ul extraction buffer 3. Layer the resuspended pellet from step 15 on top of this 300 ul cushion.

17. Spin solution 1 hour @ top speed in microfuge at 4 ˚C.

18. Remove supernatant and resuspend chromatin pellet in 500 ul of nuclear lysis buffer (keep on ice). Pool all resuspended chromatin samples of the same genotype or chemical treatment.

19. Sonicate chromatin solution on ice 4 times, 10 seconds each @ 40% duty cycle, power setting 2 on a Branson sonifier (Kranz lab)—pause 1 min. between each 10 sec pulse.

20. Centrifuge the chromatin sample in a microfuge 10 minutes, top speed (14,000 x g) at 4˚C. Transfer supernatant to new tube and spin 10 minutes, top speed at 4˚C. Remove supernatant (crude chromatin) to new tube. The chromatin can be frozen at –80˚C at this point.

21. Transfer two 100 ul aliquots of chromatin to separate siliconized microfuge tubes. Add 900 ul of ChIP dilution buffer containing 1mM PMSF and 1:100 Sigma-Aldrich plant protease inhibitors (added just before use) to each tube. If you are not using the sigma plant protease inhibitor cocktail see the end of this document for concentrations. *Using siliconized tubes significantly reduces carry over of non-antibody bound chromatin*.

22. Equilibrate Protein A agarose beads blocked with salmon sperm DNA (Upstate Biotechnology) by rinsing 3x in 1 ml of ChIP dilution buffer.

23. Preclear each chromatin sample by adding 40 ul of equilibrated Protein A beads to each sample and mixing on a rotating mixer at 4 ˚C for 1 hour.

24. Spin samples of chromatin and beads at 0.8 RPM, 2 min at 4 ˚C to pellet beads.

25. Combine two 1000 ul samples and split into 3 tubes (e.g. for mock, H3K4me, and H3K9me IPs) of 600ul and one of 60ul (10 % input) using siliconized microcentrifuge tubes. Add desired antibodies to each tube except one (this is the mock control). Typical antibodies and amounts are:
anti- H3 K9 dimethyl (Abcam) = 10 ul
anti- H3 K4 trimethyl (Abcam) = 10 ul

26. Incubate chromatin plus antibodies on a rotating mixer wheel, 4 hours to overnight at 4 ˚C (4 hours works fine – no reason to go longer unless you need a break).

27. Capture immune complexes by adding 40ul of Protein A agarose beads (equilibrated in ChIP dilution buffer) and rotating at 4 ˚C for 1 hour.

28. Pellet beads by centrifuging 0.8 RPM, 2 min. at 4 ˚C.

29. Wash with 1 ml of each of the following wash buffers 5 min. at 4 ˚C each:
Low Salt Wash Buffer
High Salt Wash Buffer
LiCl Wash Buffer
TE (2 times)

30. Remove residual TE

31. add 100ul 10% Chelex resin (10g/100ml water, purchased from Biorad), and vortex. Chelex binds ions that are required for the action of nucleases, protecting the DNA during boiling to reverse the formaldehyde crosslinks. The use of Chelex improves the yield of the IP'ed DNA significantly.

32. reverse protein DNA crosslinks by boiling 10 minutes, allow to cool to room temp.

33. Add 1 ul of 20mg/ml Proteinase K, incubate 30 min at 50˚C

34. boil 10 minutes

35. centrifuge 5 minutes at full speed, at room temperature. Collect supernatant into new tube

36. add 100ul TE to the pellet, vortex, centrifuge. Collect and combine both supernatants in a single tube

37. For amplification of ribosomal RNA genes, we typically use 2.5 ul of DNA in a PCR reaction amplified for 26 cycles

**Chip-Chop PCR to assay methylation density of ChIPed DNA**

Take DNA from the ChIP reaction and digest with McrBC (concentration and time need to be empirically determined).

| | |
|---|---|
| 10x NEB buffer 2 | 2ul |
| 100x BSA | 0.2ul |
| 100x GTP | 0.2ul (Note: GTP is sensitive to freeze thaw cycles). |
| DNA | 5 ul |
| McrBC | 1 ul |
| Water | to 20 ul |

Be sure to also do a no McrBC control

Incubate 3 hrs at 37˚C, kill reaction for 15 minutes at 65˚C
Use 2.5 ul of DNA in PCR reaction, typically 28 cycles for rRNA genes

**plant ChIP solutions**

Extraction Buffer 1

| | for 100 ml: | for 250 ml: |
|---|---|---|
| 0.4M sucrose | 20 ml | 2M 50ml |
| 10mM Tris-HCl pH 8 | 1 ml 1M | 2.5ml |
| 5mM BME (2 (beta)-mercaptoethanol) | 35 µl 14.3M | 87.5 ml |
| 1mM PMSF | 1ml 0.1M | 2.5ml |

+ Pis (protease inhibitors)

**Extraction Buffer 2**

| | for 10 ml: |
|---|---|
| 0.25M sucrose | 1.25 ml 2M |
| 10mM Tris-HCl pH 8 | 100 µl 1M |
| 10mM MgCl2 | 100 µl 1M |
| 1% Triton X-100 | 100ul 100% |
| 5mM BME | 3.5 µl 14.3M |
| 1mM PMSF | 100ul .1M |

+ PIs
H20 to volume

**Extraction Buffer 3**

| | for 10 ml: |
|---|---|
| 1.7M sucrose | 8.5 ml 2M |
| 10mM Tris-HCl pH 8 | 100 µl 1M |
| 0.15% Triton X-100 | 15ul 100% |
| 2mM MgCl2 | 20 µl 1M |
| 5mM BME | 3.5 µl 14.3M |
| 1mM PMSF | 100ul .1M |

+ PIs
H20 to volume

**Nuclei Lysis Buffer**

| | for 5 ml: |
|---|---|
| 50mM Tris-HCl ph 8 | 0.25 ml 1M |
| 10mM EDTA | 100 µl 0.5M |
| 1% SDS | 0.5 ml 10% |
| 1mM PMSF | 50ul .1M |

+ PIs H20 to volume

**ChIP Dilution Buffer**

| | for 10 ml: |
|---|---|
| 1.1% Triton X-100 | 110 µl 100% |
| 1.2mM EDTA | 24 µl 0.5 M |
| 16.7mM Tris-HCl pH 8 | 167 µl 1M |
| 167mM NaCl | 334 µl 5M |

H20 to volume

**Low Salt Wash Buffer**

| | for 50 ml: |
|---|---|
| 150mM NaCl | 1.5 ml 5M |
| 0.2% SDS | 1ml 10% |
| 0.5% Triton X-100 | .25 ml 100% |
| 2mM EDTA | 200 µl 0.5M |
| 20mM Tris-HCl pH 8 | 1 ml 1M |
| H20 to volume | |

**High Salt Wash Buffer**

| | for 50 ml: |
|---|---|
| 500mM NaC | 5ml 5M |
| 0.2% SDS | 1 ml 10% |
| 0.5% Triton X-100 | .25 ml 100% |
| 2mM EDTA | 200 µl 0.5M |
| 20mM Tris-HCl pH 8 | 1 ml 1M |
| H20 to volume | |

**LiCl Wash Buffer:**

| | for 50 ml: |
|---|---|
| 0.25M LiCl | 3.125 ml 4M |
| 0.5% NP-40 | 0.25 ml 100% |
| 0.5% deoxycholate sodium salt | 0.25 g |
| 1mM EDTA | 100 µl 0.5M |
| 10mM Tris-HCl pH 8 | 0.5 ml 1M |
| H20 to volume | |

**TE Buffer:**

| | for 50 ml: |
|---|---|
| 10mM Tris-HCl pH 8 | 0.5 ml 1M |
| 1mM EDTA | 100 µl 0.5M |
| H20 to volume | |

**Protease Inhibitors**

we use sigma plant protease inhibitor cocktail at 1:100 dilution, you may also combine the components yourself

| | per 10ml: |
|---|---|
| Antipain 2.5ug/ml | 2.5ul of 1mg/ml |
| Bestatin .5ug/ml | 5ul of 1mg/ml |
| Leupeptin .5ug/ml | 1ul of 5mg/ml |
| Pepstatin A 4ug/ml (in MetOH) | 40ul of 1mg/ml |