

Arabidopsis Chromatin Immunoprecipitation Protocol

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1. Sow ~200 surface sterilized *Arabidopsis* seeds on each 150 mm x 15 mm petri plate containing 1/2X MS medium without sucrose. Stratify at 4°C for 2-3 days. Grow seedlings for 9 days at 22°C in 16 hour daylight.
2. Soak seedlings in each plate with 50ml 10 μ M dexamethasone (DEX, Cat #D1756, Sigma, St. Louis, MO) or mock for 4 hours with gentle agitation on an orbital shaker. Before the end of the DEX treatment, prepare enough 1% formaldehyde for cross-linking in step 5.
Note: Prepare 20 mM DEX stock solution in 100% ethanol. The working solution is diluted with 1/2 X MS liquid medium plus 1% sucrose. "Mock" consists of an equal volume of ethanol in MS liquid.
3. Harvest seedlings by scraping them from the plate with gloved fingers and collect each plate in one 50ml conical tube.
4. Add 40 ml deionized water to each tube and mix gently to rinse seedlings in room temperature. Cover each tube with miracloth and pour out water. Repeat the rinse 2 more times.
5. Pour 37 ml 1% formaldehyde directly into each tube. Use miracloth to keep seedlings submerged, then vacuum infiltrate for 10 minutes at room temperature to crosslink protein and DNA. During vacuum infiltration, formaldehyde solution should boil.
Note: This step is time sensitive. Infiltration should start immediately after addition of formaldehyde and should not last more than 10 minutes.
6. Quench cross-linking by adding 2.5 ml of 2M glycine (Final = 0.125 M) into each tube with a 5 ml pipette and pipette up and down to mix well. Immediately continue vacuum infiltration for an additional 5 min.
Note: Try to add glycine as fast as possible to avoid over-crosslinking. Cut a small opening on miracloth and stick the pipette tip directly into tubes to add glycine. There is no need to remove the miracloth cover.
7. Pour off formaldehyde and immediately add 40 ml deionized water. Pour seedlings into a Buchner Funnel with filter paper. Vacuum dry the seedlings. Add an additional 40 ml deionized H₂O to rinse the seedlings.
8. Transfer dry seedlings into a new conical tube. Freeze in liquid nitrogen. Seedlings can be stored at -80°C for several weeks. (Dry ice may be used to freeze seedlings if doing more than 4 samples.)
9. Grind seedlings in liquid nitrogen to a fine powder with a chilled mortar and pestle.
10. Add powder to 30 ml of Extraction Buffer 1 in a 50 ml conical tube on ice. Vortex, place on ice.
11. Filter solution through 2 layers of miracloth in a plastic funnel into a new 50ml conical tube.
12. Centrifuge solution for 20 min. at 4000 rpm, 4°C.
13. Pour out supernatant and resuspend pellet in 1ml Extraction Buffer 2 by pipetting up and down gently with a cut 1000 μ l tip. Transfer into a 2 ml microfuge tube.
14. Microcentrifuge at top speed (~14000xg) 10 min at 4°C.

15. Remove supernatant with a pipette and resuspend pellet in 300 μ l Extraction Buffer 3
Note: Pellet is slightly green. Resuspension is difficult due to viscosity. AVOID introducing bubbles! Stir pellet gently with a cut 200 μ l tip and resuspend by gently pipetting up and down. Avoid froth formation to prevent protein degradation in subsequent steps.
16. In a clean 1.5 ml tube, add 300 μ l Extract Buffer 3. Layer resuspended pellet from step 15 on top of the 300 μ l “cushion” using a cut 200 μ l tip.
17. Microfuge solution 1 hour at top speed at 4°C to pellet nuclei.
18. Pipette to remove supernatant and resuspend the nuclei pellet in 500 μ l of ice cold Nuclei Lysis Buffer. (Keep on ice.)
19. Sonicate chromatin solution on ice 4 times: 12 seconds each pulse at 40% duty and power 6 (~20% output). Pause 1 min between each 12 second pulse. The sonicator we use is made by Heat System-Ultrasonics (Farmingdale, NY) model W-375 with a microtip size of 1/8” diameter. Appropriate sonication conditions will need to be determined empirically for your device. Average fragment size should be ~600 bp.
20. Microcentrifuge the chromatin sample 10 min. at top speed at 4°C.
21. Transfer supernatant to a new tube to remove debris and spin again for 10 min. at top speed at 4°C. Transfer supernatant (crude chromatin) to a new tube.
Note: Chromatin may be frozen at –80°C at this point.
22. Prepare Protein A Agarose beads (Cat # 20333, Pierce, Rockford, IL):
Pipette 560 μ l 50% slurry into each of 4 tubes (enough to pre-clear and IP 8 samples). Spin down at top speed at 4°C for 8 seconds. Discard supernatant. Add 1 ml ChIP Dilution Buffer to each tube to equilibrate. Wash slurry with ChIP Dilution Buffer and spin down at top speed at 4°C for 8 seconds. Repeat 3 times. After the last wash, resuspend the beads with an equal volume of ChIP Dilution Buffer as the Protein A bead volume. (1/2 volume of the original 50% slurry = 280 μ l, so the final equilibrated slurry volume should be about 560 μ l in each tube.)
23. Transfer 3 150 μ l aliquots of chromatin (step 21) to separate microcentrifuge tubes. Add 1350 μ l of ChIP Dilution Buffer to each tube (This 10X dilution dilutes the SDS in the Nuclei Lysis Buffer to 0.1%.)
24. Pre-clear each chromatin sample by adding 45 μ l of equilibrated Protein A slurry to each sample and rotate at 4°C for 1 hour on a Mini Labroller (Cat #H5500, Labnet International, Edison, NJ).
25. Spin down Protein A beads at top speed at 4°C for 8 seconds. Combine the three supernatants, mix, and divide into 5 tubes of 600 μ l each. Set aside one 60 μ l aliquot as 10% Input.
26. Add 10 μ l (2 μ g) anti-GR P-20 antibody (Cat #SC1002, Santa Cruz Biotechnology, Santa Cruz, CA) to 4 tubes for IP and sterile water to one tube for a control.
27. Incubate chromatin plus antibodies by rotating on the Labroller, overnight at 4°C.
28. Collect immune complexes by adding 45 μ l of Protein A Agarose beads to each tube and rotating at 4°C for 1 hour.
29. Microfuge at top speed at 4°C for 8 seconds to pellet beads.

30. Wash beads sequentially with 1 ml of each of the following wash buffers by rotating for 5 min. at 4°C then microfuging at 4°C at top speed for 8 seconds to pellet beads.
 Low Salt Wash Buffer
 High Salt Wash Buffer
 LiCl Wash Buffer
 TE (2 times)
31. Add 250µl of Elution Buffer to pelleted beads. Vortex. Incubate at 65°C for 15 min. with agitation (use the Labroller in a 65°C incubator). Pellet beads by microfuging at top speed, room temperature, for 8 seconds. Collect supernatant with a cut 200µl tip to fresh tube. Repeat elution step and combine the two eluates.
32. Bring the volume of 10% Input (step 25) to 500 µl with Elution Buffer. Add 20 µl 5M NaCl to the eluate and reverse protein-DNA crosslink at 65°C overnight (without agitation).
33. Add 10 µl of 0.5M EDTA, 20µl Tris-Cl pH 6.5 and 1.5 µl of 20 mg/ml proteinase K to the eluate and incubate 1hour at 45°C.
34. Recover DNA by adding an equal volume (500 µl) of 1:1 phenol:chloroform to the eluate, vortex, and spin at top speed for 6 minutes at room temperature. Transfer aqueous phase (upper layer) with a cut 200 µl tip into a 2 ml tube.
35. Precipitate DNA by adding 1µl 15 mg/ml glycogen, 1/10 volume 3M sodium acetate, and 2.5 volumes 100% ethanol. Mix well and store the at -20°C for 30 min.
36. Spin at top speed for 30 min at 4°C.
37. Wash pellet with 70% ethanol and air dry
38. Resuspend DNA pellet in 50 µl TE. Use 1µl for PCR. For Input, resuspend DNA pellets in 750µl TE and use 1µl for PCR.

***Arabidopsis* ChIP solutions**

Notes about Protease Inhibitors (PIs)

1. We use two types of protease inhibitors, PMSF (Cat#71110, EMD Chemicals, Gibbstown, NJ), and Pepstatin A.

1) Complete protease inhibitor cocktail tablet (Cat#11697489001, Roche Diagnostics, Indianapolis, IN). Use 1 tablet for 50 ml solution.

2) HaltTM Protease inhibitor cocktail (Cat#78410, Pierce, Rockford, IL)

Because these two PI cocktails do not contain Pepstatin A, we use Pepstatin A (Cat#11359053001, Roche Diagnostics, Indianapolis, IN).

2. For all solutions with PMSF and PIs, prepare the stock solution without the inhibitors. Take an appropriate volume of stock (volume depends on the number of samples in the experiment) and add a proportional amount of PMSF and PIs into the stock just before use. When making the stock solution, the volume of PMSF and PIs may be ignored.

Extraction Buffer 1

0.4M sucrose	for 100 ml: 20 ml 2M
10 mM Tris-HCl pH 8	1 ml 1M
5 mM BME (2/ β -mercaptoethanol)	35 μ l 14.3M
1 mM PMSF	1ml 0.1M
PIs (protease inhibitors)	2 tablets (from Roche)
Pepstatin A 4 μ g/ml	400 μ l 1mg/ml
	H ₂ O to volume

Extraction Buffer 2

0.25 M sucrose	for 10 ml: 1.25 ml 2M
10 mM Tris-HCl pH 8	100 μ l 1M
10 mM MgCl ₂	100 μ l 1M
1% Triton X-100	100 μ l 100%
5 mM BME	3.5 μ l 14.3M
1mM PMSF	100 μ l 0.1M
PIs	100 μ l 100X (from Pierce)
Pepstatin A 4 μ g/ml	40 μ l 1mg/ml
	H ₂ O to volume

Extraction Buffer 3

1.7 M sucrose	for 10 ml: 8.5 ml 2M
10 mM Tris-HCl pH 8	100 μ l 1M
0.15% Triton X-100	15 μ l 100%
2 mM MgCl ₂	20 μ l 1M
5 mM BME	3.5 μ l 14.3M
1 mM PMSF	100 μ l 0.1M
PIs	100 μ l 100X (from Pierce)
Pepstatin A 4 μ g/ml	40 μ l 1mg/ml
	H ₂ O to final volume

Nuclei Lysis Buffer

50 mM Tris-HCl pH 8
10 mM EDTA
1% SDS
1 mM PMSF
PIs
Pepstatin A 4 μ g/ml

for 5 ml:

0.25 ml 1M
100 μ l 0.5M
0.5 ml 10%
50 μ l 0.1M
50 μ l 100X (from Pierce)
20 μ l 1mg/ml
H₂O to volume

ChIP Dilution Buffer

1.1% Triton X-100
1.2 mM EDTA
16.7 mM Tris-HCl pH 8
167 mM NaCl
1 mM PMSF
PIs
Pepstatin A 4 μ g/ml

for 10 ml:

110 μ l 100%
24 μ l 0.5M
167 μ l 1M
334 μ l 5M
100 μ l 0.1M
100 μ l 100X (from Pierce)
40 μ l 1mg/ml
H₂O to volume

Low Salt Wash Buffer

150 mM NaCl
0.2% SDS
0.5% Triton X-100
2 mM EDTA
20 mM Tris-HCl pH 8

for 50 ml:

1.5 ml 5M
1 ml 10%
0.25 ml 100%
200 μ l 0.5M
1 ml 1M
H₂O to volume

High Salt Wash Buffer

500 mM NaCl
0.2% SDS
0.5% Triton X-100
2 mM EDTA
20 mM Tris-HCl pH 8

for 50 ml:

5ml 5M
1 ml 10%
0.25 ml 100%
200 μ l 0.5M
1 ml 1M
H₂O to volume

LiCl Wash Buffer:

0.25 M LiCl
0.5% NP-40
0.5% sodium deoxycholate
1 mM EDTA
10 mM Tris-HCl pH 8

for 50 ml:
3.125 ml 4M
0.25 ml 100%
0.25 g
100 μ l 0.5M
0.5 ml 1M
H₂O to volume

Protein A Bead Elution Buffer

1% SDS
0.1M NaHCO₃

for 100ml
10 ml 10%
0.84 g
H₂O to volume

TE Buffer:

10 mM Tris-HCl pH 8
1 mM EDTA

for 50 ml:
0.5 ml 1M
100 μ l 0.5M
H₂O to volume