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Chromatin Immunoprecipitation (ChIP) Protocol for *A. thaliana*

This document provides a protocol for a chromatin immunoprecipitation experiment in *A. thaliana* of a GFP-tagged protein of interest using the ChromoTek GFP-Trap.

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1. Introduction

Chromatin immunoprecipitation (ChIP) is a powerful method for the analysis of DNA-interacting proteins in an *in vivo* context. The presented chromatin immunoprecipitation protocol for *A. thaliana* tissue has was published by Christoph Schuster, Christophe Gaillochet and Jan U. Lohmann in Development (2015) 142, pages 3343-3350. It is modified from protocols developed by Anne-Valérie Gendrel, Zachary Lippman, Rob Martienssen & Vincent Colot in Nature Methods (2005), Vol 2, pages 213-218, and Zachary Lippman, Anne-Valérie Gendrel, Vincent Colot & Rob Martienssen in Nature Methods (2005), Vol 2, pages 219–224. The protocol can be applied for all kinds of *A. thaliana* starting material such as seedlings, leaves and inflorescences. Because of the inherent incompleteness of the cross-linking Step 3, however, one should bear in mind that transient interactions may be difficult to detect with this technique.

2. Materials

Reagents

- GFP-Trap® Agarose gta-20 (ChromoTek)
- *A. thaliana* tissue of choice
- Extraction buffers 1, 2 and 3 (prepared as described in Table 1 and pre-chilled to 4 °C)
- 37% formaldehyde, ACS grade (Sigma), diluted to 1% in water or extraction buffer 1
- 2 M glycine
- Glycogen carrier (10 mg/mL; Roche)
- High-salt wash buffer: 500 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8)
- LiCl wash buffer: 0.25 M LiCl, 1% NP-40 (or IGEPAL CA-360), 1% DOC, 1 mM EDTA, 10 mM Tris-HCl (pH 8)
- Low-salt wash buffer: 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8)
- β-mercaptoethanol (β-ME)
- 0.2 M phenylmethylsulfonyl fluoride (PMSF)
- Protease inhibitor tablets (PIs; Roche): Complete Mini (for small volume of buffer) or Complete (for large volumes of buffer)
- Binding control “blocked agarose beads”, bab-20 (ChromoTek)
- Proteinase K (10 mg/mL)
- Tris-EDTA (TE) buffer: 10 mM Tris-HCl (pH 8), 1 mM EDTA
- 1 M Tris-HCl (pH 6.5)
- Liquid nitrogen
- Triton X-100 (diluted to 20%)
- Ultrapure water, such as MilliQ (Millipore)
- MinElute Kit (Qiagen)

Equipment

- Mortar and pestle
- 90 µm and 50 µm nylon mesh
- Sonicator (Sanyo Soniprep 150)
- Vacuum chamber

ChIP Procedure

2.1. Harvesting and formaldehyde cross-linking

1. Harvest between 1.5 and 2 g of *A. thaliana* tissue (flowers, leaves or seedlings) of wild type col-0 and GFP-fusion transgenic line after growth on soil or *in vitro* in a 50 mL tube. *The protocol describes the preparation of one chromatin sample that could serve for two different immunoprecipitation reactions, as well as a negative control (see Step 19), depending on the amount of starting material and on the experiment planned. Prepare the appropriate number of samples to be used for additional immunoprecipitation reactions.*

2. Rinse the tissue twice with 40 mL of water by gently inverting the tube.

3. Remove as much water as possible from the tissue, add 37 mL of 1% formaldehyde and place the tissue under vacuum for 15 min at 15–25 °C.
   ▲Critical step
   ➡Troubleshooting

4. Stop the cross-linking by adding glycine to a final concentration of 0.125 M (add 2.5 mL of 2 M glycine to the tissue in 37 mL of 1% formaldehyde in Step 3). Place the tissue under vacuum for additional 5 min.

5. Rinse the tissue two or three times with 40 mL of water to remove all the formaldehyde. After the rinses, remove as much water as possible by blotting between paper towels if necessary.
   ■ **Pause Point** At this stage, the cross-linked tissue can be either frozen in liquid nitrogen and stored at –80 °C or used directly for chromatin extraction.
   ▲ **Critical step**

Table 1: Preparation of extraction buffers

Buffer	Reagent	Amount	Final concentration
Extraction Buffer 1	2 M sucrose	20 mL	0.4 M
	1 M Tris-HCl (pH 8)	1 mL	10 mM
	1 M $MgCl_2$	1 mL	10 mM
	14.3 M β-ME	35 µL	5 mM
	0.2 M PMSF	50 µL	0.1 mM
	PI (Complete tablet)	2 tablets	
	Water	to 100 mL	
Extraction Buffer 2	2 M sucrose	1.25 mL	0.25 M
	1 M Tris-HCl (pH 8)	100 µL	10 mM
	1 M $MgCl_2$	100 µL	10 mM
	20% Triton X-100	0.5 µL	1%
	14.3 M β-ME	3.5 µL	5 mM
	0.2 M PMSF	5 µL	0.1 mM
	PI (Complete Mini tablet)[a]	1 mL	
	Water	to 10 mL	
Extraction Buffer 3	2 M sucrose	8.5 mL	1.7 M
	1 M Tris-HCl (pH 8)	100 µL	10 mM
	20% Triton X-100	75 µL	0.15%
	1 M $MgCl_2$	20 µL	2 mM
	14.3 M β-ME	3.5 µL	5 mM
	0.2 M PMSF	5 µl 0.2 M	0.1 mM
	PI (Complete Mini tablet) [a]	1 mL	
	Water	to 10 mL	

[a] Dissolve one PI Mini tablet in 1 mL of water before adding to the buffer.

2.2. Chromatin extraction and sonication

6. Grind the tissue to a fine powder with a mortar and pestle in liquid nitrogen.
   Ensure that the tissue does not thaw during grinding.

7. Add the powder to 30 mL of extraction buffer 1 (prechilled to 4 °C) in a 50 mL tube. To promote resuspension, invert the tube and place it on ice for 5 min.

The volume of extraction buffer 1 can be adjusted relative to the amount of ground powder (typically a fivefold excess of buffer is used for each volume of packed powder).

8. Filter the solution through a 90 µm and 50 µm nylon mesh into a fresh 50-mL tube place on ice.
   Repeat once if necessary to remove additional debris.

9. Centrifuge the filtered solution at 3,000 g at 4 °C for 20 min.

10. Gently remove and discard the supernatant and resuspend the pellet thoroughly in 1 mL of extraction buffer 2 (4 °C).

11. Transfer the suspension to a 1.5 mL microcentrifuge tube and centrifuge at 12,000 g at 4 °C for 10 min.
    There should be a tight white pellet at this stage (nuclei and debris) along with an overlay of chlorophyll.
    ➡ **Troubleshooting**

12. Remove the supernatant and resuspend the pellet thoroughly in 300 µL of extraction buffer 3 (4 °C).
    Resuspension can be difficult, especially because extraction buffer 3 is quite thick, but try to avoid foaming. Gently vortexing the sample will assist the resuspension.

13. Add 300 µL of extraction buffer 3 (4 °C) into a fresh microcentrifuge tube. Carefully layer the resuspended pellet (300 µL solution from Step 12) over the extraction buffer 3 and centrifuge at 16,000 g at 4 °C for 1 h.

14. Prepare a fresh 10 mL of nuclei lysis buffer and 20 mL of ChIP dilution buffer by combining the following reagents for each buffer:

Buffer	Reagent	Amount	Final concentration
Nuclei lysis buffer	1 M Tris-HCl (pH 8)	0.5 mL	50 mM
	0.5 M EDTA	200 µL	10 mM
	20% SDS	0.5 mL	1%
	PI (Complete Mini tablet)	1 tablet	
	Water	to 10 mL	
ChIP dilution buffer	20% Triton X-100	1.1 mL	1.1%
	0.5 M EDTA	48 µL	1.2 mM
	1 M Tris-HCl (pH 8)	334 µL	16.7 mM
	5 M NaCl	668 µL	167 mM
	Water	to 20 mL	

15. Remove the supernatant (Step 13) and resuspend the chromatin pellet in 300 µL of nuclei lysis buffer by pipetting the solution up and down or by vortexing gently (keep the solution cold between vortexing).
    Resuspension can be difficult, but try to avoid foaming. Set aside 1–2 µL from each sample to compare the extracted chromatin with matched sonicated samples by gel electrophoresis.

16. Sonicate the resuspended chromatin solution five times for 15 s each at ~10% power (setting 2.5 on the sonicator, Sanyo Soniprep 150). Place the sample on ice for 1 min between each sonication treatment.
Be careful not to foam the solution or heat it too much while sonicating.
A bit of sample (~20–40 µL) is usually lost during sonication. It does not matter, but the extent of loss should be minimized.
■ **Pause Point** At this point, the chromatin solution can be frozen at –80 °C or used directly for immunoprecipitation.

2.3. Immunoprecipitation of chromatin

17. To pellet the debris, centrifuge the sonicated chromatin solution at 12,000 g at 4 °C for 5 min. Remove the supernatant to a clean tube and discard the pellet. Remove 10 µL from each sample into a clean tube and set aside at –20 °C to serve as the 'input DNA control' in Step 27.
Set aside 1–2 µL from each sample to compare with the aliquot from Step 15; assess the sonication efficiency by gel electrophoresis. Load the samples on a 1% agarose gel. You should see a smear with a peak of intensity between 500 bp and 1 kb.
➡ **Troubleshooting**

18. Measure the volume of the chromatin sample and add ChIP dilution buffer (prepared in Step 14) to bring the volume to 3 mL. Measure DNA conc. with Nanodrop and adjust all samples to lowest DNA conc.
The initial sample volume will be less than 300 µL because of loss from sonication and removal of debris by centrifugation. The dilution to 3 mL reduces the SDS concentration to 0.1%.

19. Divide the chromatin solution for each sample equally among two tubes. To each tube add 20 µL of binding control agarose beads (ChromoTek) and pre-clear the solution by incubating at 4 °C for 1 h with gentle agitation or rotation.
The division of the solution may depend on the amount of starting material and on the experiment planned. For example, dividing the sample into three parts provides for two immunoprecipitation reactions, and one negative control. The sample can be further divided if more immunoprecipitation reactions are to be performed. Alternatively, the remaining chromatin sample may be frozen at –20 °C for future use.
▲ **Critical step**

20. Centrifuge the chromatin solutions with beads at 12,000 g at 4 °C for 30 s and transfer the supernatants to fresh tubes. Discard the beads.

21. Add 20 µL GFP-Trap agarose beads (ChromoTek) to each tube. The negative control is wild type plants with GFP-Trap agarose beads. Incubate the two tubes at 4 °C overnight with rotation.

22. The buffers to be used in Step 25 should be placed on ice at this point.
▲ **Critical step**

23. Prepare fresh elution buffer by combining the following reagents:

20% SDS	1 mL
$NaHCO_3$	0.168 g
Water	to 20 mL

24. Recover the beads by centrifugation at no more than 3,800 g at 4 °C for 30 s to avoid damaging the beads; then remove the supernatant.
The supernatant may be saved to be used in place of the total DNA control, for a comparison with the immunoprecipitation sample, as described in ref. 7.

25. Wash the beads using the procedure and sequence of buffers below. Perform each wash at 4 °C with gentle agitation or rotation, using the appropriate buffer. After each wash recover the beads by centrifugation at 3,800g at 4 °C for 30 s.

Buffer	First wash	Second wash
(i) Low salt wash buffer	Quick	5 min
(ii) High salt wash buffer	Quick	5 min
(iii) LiCl wash buffer	Quick	5 min
(iv) TE buffer	Quick	5 min

After the last wash, carefully remove as much TE as possible from the beads.

2.4. Elution and reversal of cross-linking

26. To elute the immune complexes from the washed beads, add 250 µL of elution buffer (made fresh at Step 23) to each sample. Vortex briefly to mix and incubate at 65 °C for 15 min with gentle agitation or by inverting the tubes every 3 min. Centrifuge the beads at 3,800g at 15–25 °C for 2 min and carefully transfer each supernatant fraction (eluate) to a fresh tube. Repeat this step with 250 µL of elution buffer and combine the two eluates for each sample.

27. Add 20 µL of 5 M NaCl to each sample (combined eluate) and reverse the cross-linking by incubation at 65 °C for at least 6 h to overnight. Include also the 'input DNA control' (set aside in Step 17); add elution buffer to 500 µL and 20 µL 5 M NaCl and incubate at 65 °C for at least 6 h to overnight. Digest with 1 µL RNase A (10 mg/mL), 37 °C for 15 min.

28. To each sample of eluate and to the input DNA control, add the following:

0.5 M EDTA	10 µL
1 M Tris-HCl (pH 6.5)	20 µL
10 mg/mL proteinase K	2 µL

Incubate at 45 °C for 1 h.

29. Purify DNA with Qiagen MinElute Kit, add 2 x 20 µL Elution buffer for elution.

30. Remove 2 µL of each resuspended pellet and dilute tenfold in water. To analyze the success of the experiment, carry out amplification of 1–2 µL of the diluted DNA as a template, using PCR primer pairs that amplify sequences for which the histone modification status is known.
The purified DNA (we recommend using 7 µL of each sample) may be used to perform random amplification for microarray analysis as described in Steps 17–27 of the protocol for profiling of DNA methylation patterns[6]. The amplified product may be analyzed by hybridization to genomic tiling microarrays.

3. Troubleshooting ➡

Problem	Solution
General: Fixation time too long and epitope is not accessible anymore	Make sure that your IP-reagent still recognizes your protein of interest after cross-linking. Carry out a fixation time course for your cell line to determine the optimal fixation time for it and the epitope of interest. Cell lines and epitopes differ in both fixation efficiency and sensitivity to fixation reagents.
General: Overheating of sample	Keep sample on ice, reduce pulse duration, and extend periods between pulses.
General: Paraformaldehyde interferes with protein-protein interaction	Use methanol-free formaldehyde to avoid over-fixation.
General: Temperature too high during cross-linking	Watch cross-linking temperature. Fixation is diffusion dependent and therefore affected by temperature.
Step 3: The tissue does not look translucent, as if the infiltration did not work.	Make sure the appropriate amount of tissue is being used, because too much tissue can be a problem. Also ensure that you have a good vacuum and that your seal is secure.
Step 11: There is no visible white nuclei pellet at the bottom of the tube.	Sometimes a white pellet will not form, but this is not an indication that the chromatin extraction did not work. Proceed to the next step.
Step 17: In comparing the samples from Steps 15 and 17 by gel electrophoresis, it appears that the sonication step did not work.	The gel electrophoresis can also be done after reversal of the cross-linking (Step 27), because proteins bound to DNA can affect how the DNA runs on a gel. You should, however, always see a difference between a DNA smear from the sample that was sonicated and the one that was not. If the difference is not obvious, sonicate again once or twice for 15 s and slightly increase the power setting of the sonicator.

4. Critical Steps ▲

Step 3: Make sure that the infiltration worked. The tissue should look translucent or 'water-soaked' after cross-linking, because formaldehyde should penetrate the cell wall with vacuum infiltration. The length of time for infiltration is also very important. Too short a period of infiltration can lead to inefficient cross-linking, whereas too long a period can result in excessive cross-linking.

Step 5: It is essential to remove as much water as possible, as otherwise grinding the tissue in liquid nitrogen will be very difficult (the remaining water will be transformed to ice).

Steps 19 and 22: Before use, both the binding control agarose beads and GFP-Trap agarose beads should be rinsed three times and resuspended with ChIP dilution buffer to remove storage buffer from the beads. Cut off the end of a pipette tip to assist in measuring out the correct volume of beads. For example, add 40 µL of ChIP dilution buffer to 40 µL of beads and gently flick the tube to mix. Centrifuge quickly to recover the beads and then remove 40 µL of liquid. Repeat the wash twice.

5. ChromoTek GFP-Trap

ChromoTek GmbH pioneers a new class of immunologic research tools derived from single domain camel antibodies for cell biology and proteomics. ChromoTek is the inventor of GFP-Trap, a 1 pM affinity anti-GFP nanobody coupled to agarose beads, which is considered the gold standard for immunoprecipitation of GFP-fusion proteins.

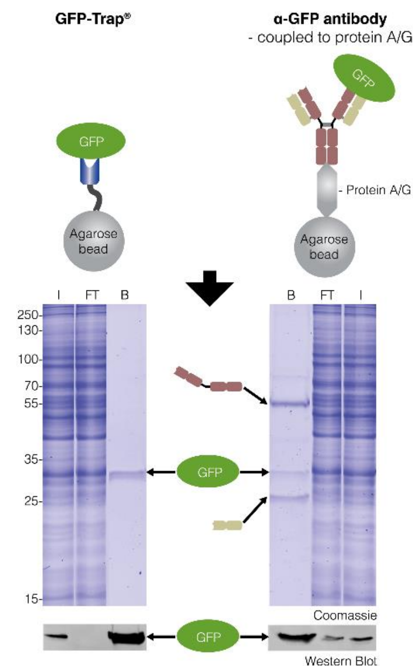

Figure 1: Immunoprecipitations of GFP from cells expressing GFP:
Input (I), non-bound (FT) and bound (B) fractions were separated by SDS-PAGE followed by Coomassie staining and Western Blotting. The bound fraction of the conventional GFP antibodies shows in addition to GFP contaminating heavy chain and light chain (right). The bound fraction of the GFP-Trap does not contain these contaminating polypeptide chains (left).
Note effective binding of GFP-fusion proteins: No GFP-fusions left in non-bound (FT) lane in Western Blot when using GFP-Trap (left).

6. Alternative Immunoprecipitation

The GFP-Nanobody of the GFP-Trap detects a 3-dimensional epitope of GFP. Sometimes when doing a ChIP experiment, the cross-linking may alter the GFP-fusion protein to an extend that the binding to the GFP-Trap may be compromised. In this case, we recommend using the ChromoTek polyclonal GFP-antibody [PABG1]: The GFP antibody is incubated with the cell lysate followed by addition of Protein A/G beads. Alternatively, the GFP antibody:Protein A/G complex is formed first and subsequently the cell lysate is added. Note that in this approach the antibody will co-elute with the GFP-fusion protein from the Protein A/G beads.

7. References

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