PUBLISHED IN ASSOCIATION WITH
COLD SPRING HARBOR LABORATORY PRESS

Profiling histone modification patterns in plants using genomic tiling microarrays

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In eukaryotes, the processes of transcription, replication and homologous recombination occur in the context of chromatin. Although tightly packed, this structure is highly dynamic, being modified through the action of many enzymatic activities that reorganize nucleosomes; covalently modify histones through acetylation, phosphorylation, methylation and so on; exchange histones with variants; and in many eukaryotes, including plants and mammals, methylate DNA residues[1]. Transcriptional activity is usually associated with hyperacetylation of histones tails as well as with di- or trimethylation of Lys4 in histone H3. Conversely, silent chromatin typically correlates with histone hypoacetylation and di- or trimethylation of histone H3 Lys9 (refs. 2,3). Because of its small genome, *Arabidopsis thaliana* serves as a powerful system for understanding the role of various histone modifications in a complex organism, notably in association with DNA methylation and in relation to the epigenetic inheritance of silent chromatin[4]. Here we describe a chromatin immunoprecipitation (ChIP) protocol developed for *A. thaliana* that permits, in combination with hybridization to genomic tiling microarrays, the mapping of histone modifications with high resolution along large genomic regions[5]. After cross-linking, chromatin is immunoprecipitated using antibodies directed against specific histone modifications. DNA recovered from the precipitate is amplified, labeled, hybridized to microarrays and compared to total DNA. This protocol has been used successfully to map histone H3 methylated at Lys4 or Lys9 across a 1.5-Mb region[5] and should have broad applications in plants and other organisms. A protocol that outlines the profiling of DNA methylation patterns at similar high resolution has also been developed[6].

MATERIALS

REAGENTS

Antibody to be used for immunoprecipitation (for example, anti–dimethyl-H3(Lys9) or anti–dimethyl-H3(Lys4); Upstate Biotech or Abcam)

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)

Protein A–agarose beads (Roche)

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)

EQUIPMENT

Mortar and pestle

Miracloth (Millipore)

Sonicator (Sanyo Soniprep 150)

Vacuum chamber

Harvesting and formaldehyde cross-linking

PROCEDURE

1| Harvest between 1.5 and 2 g of *A. thaliana* tissue (flowers, leaves or seedlings) after growth on soil or *in vitro* in a 50-ml Falcon 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 reversing 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 an 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 ml	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 0.2M	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.

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 Falcon tube. To promote resuspension, reverse 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 two layers of Miracloth into a fresh 50-ml Falcon tube placed 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 resuspension 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:

Nuclei lysis buffer		**ChIP dilution buffer**	
1 M Tris-HCl (pH 8)	0.5 ml (50 mM final)	20% Triton X-100	1.1 ml (1.1% final)
0.5 M EDTA	200 µl (10 mM final)	0.5 M EDTA	48 µl (1.2 mM final)
20% SDS	0.5 ml (1% final)	1 M Tris-HCl (pH 8)	334 µl (16.7 mM final)
PI (Complete Mini tablet)	1 tablet	5 M NaCl	668 µl (167 mM final)
Water	to 10 ml	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.

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.
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%. If the SDS concentration is greater than 0.1%, it is likely that the antibody will become denatured.

19| Divide the chromatin solution for each sample equally among three tubes. To each tube add 40 µl of protein A–agarose beads 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, each with a different antibody, and one negative control. The sample can be further divided if more antibodies are to be used. 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 5 µl of the desired antibody to two of the three tubes; the third tube without antibody is the 'no antibody control' (it is very important to include this negative control). Incubate the three tubes at 4 °C for at least 5 h or overnight with gentle agitation or rotation.
Concentrations may vary among antibodies and should be determined empirically. Typically, a 1:200 dilution of the antibody is a good starting point.

22| Add 50 µl of protein A–agarose beads to each tube and collect the immune complexes by incubation at 4 °C for at least 1 h with gentle agitation or rotation.
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 3,800*g* 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.

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 reversing the tubes every 3 min. Centrifuge the beads at 3,800*g* 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 5M NaCl and incubate at 65 °C for at least 6 h to overnight.

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| Extract the DNA with phenol/chloroform (1:1, vol/vol) and recover by precipitation with ethanol in the presence of 0.3 M sodium acetate (pH 5.2) and 2 µl glycogen carrier (10 mg/ml). Wash the DNA pellets with 70% ethanol and resuspend each pellet in 16 µl of sterile distilled water.

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.

TROUBLESHOOTING TABLE

PROBLEM	SOLUTION
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.

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, the protein A–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.

COMMENTS

Chromatin immunoprecipitation is a powerful method that allows the identification of DNA sequences associated with specific proteins in an *in vivo* context[8]. Numerous protocols have been described for yeast and animal systems and, more recently, for plants. Our protocol is an adaptation of that originally described in ref. 9 and is well suited for working with all kinds of starting material such as seedlings, leaves and inflorescences. Thus far, we have used it to map several histone modifications[5,9,10] as well as histone variants in *A. thaliana* (A. Bulski and V.C., unpublished data). Recently, our protocol or similar ones have been used to map transcription factor binding sites by PCR amplification in several plant species (see, for instance, refs. 11–13), indicating the generality of the method for mapping DNA-protein associations *in vivo*. Because of the inherent incompleteness of the cross-linking step[8], however, one should bear in mind that transient interactions may be difficult to detect with this technique.

SOURCE

This protocol was provided directly by the authors listed on the title page. For further details on standard procedures, see Sambrook, J. & Russell, D.W., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, 2001). Details of microarray hybridization are given in Bowtell, D. & Sambrook, J. (eds.) *DNA Microarrays: A Molecular Cloning Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, USA, 2003).

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