

An efficient chromatin immunoprecipitation (ChIP) protocol for studying histone modifications in *Arabidopsis* plants

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Chromatin immunoprecipitation (ChIP) is a powerful tool for the characterization of covalent histone modifications and DNA–histone interactions *in vivo*. The procedure includes DNA–histone cross-linking in chromatin, shearing DNA into smaller fragments, immunoprecipitation with antibodies against the histone modifications of interest, followed by PCR identification of associated DNA sequences. In this protocol, we describe a simplified and optimized version of ChIP assay by reducing the number of experimental steps and isolation solutions and shortening preparation times. We include a nuclear isolation step before chromatin shearing, which provides a good yield of high-quality DNA resulting in at least 15 µg of DNA from each immunoprecipitated sample (from 0.2 to 0.4 g of starting tissue material) sufficient to test ≥25 genes of interest. This simpler and cost-efficient protocol has been applied for histone-modification studies of various *Arabidopsis thaliana* tissues and is easy to adapt for other systems as well.

INTRODUCTION

Cells of a multicellular organism are genetically similar but structurally and functionally different because of the differential expression of their genes. Over the past two decades, increasing evidence has suggested a role for epigenetic mechanisms in the control of developmental, adaptive and signaling processes in various biological systems. The epigenetic concept refers to the study of heritable changes in gene function that occur without alterations of DNA sequences. A combination of DNA methylation and various histone modifications, causing changes in chromatin structure, are among the best-studied gene repressive and activating epigenetic mechanisms¹.

As DNA–protein interactions are very important for chromatin structure and for cellular processes controlled by DNA accessibility, developing methods and techniques to study protein–DNA interactions have been central to the studies of DNA replication, recombination, DNA repair and transcription. For example, electrophoretic mobility shift assay (EMSA)^{2,3}, reporter gene assays^{4,5}, DNA microarrays⁶, mass spectrometry (MS)^{7,8}, yeast one-hybrid (Y1H)^{9,10} and chromatin immunoprecipitation (ChIP)^{11–13} are among the widely used current tools for analyzing DNA–protein interactions. Each of these methods has its advantages, scope of applicability but, also, limitations. For example, EMSA provides the ability to resolve complexes of different stoichiometry or conformation but the method requires the use of radioactively labeled material and may be expensive and time-consuming; with reporter gene assays, it is critical that expression of the reporter gene does not disturb the metabolism of the transformed cells and that the gene of interest is not endogenously expressed by the target cells as this may create background false signals; microarray and MS techniques are promising new approaches but they are still very costly and require an extensive support network beyond the expertise of a single investigator; an advantage of Y1H is that the detection of protein–DNA interactions takes place *in vivo* inside the nucleus providing correct folding and modifications of the prey

proteins. However, this technique is less reliable for DNA–protein interactions in heterologous (non-yeast) systems and displays a tendency for high levels of false-positive interactions.

The ChIP procedure consists of discrete steps including *in vivo* cross-linking of proteins to DNA with formaldehyde, isolation of the chromatin complex, shearing DNA along with bound proteins into small fragments of ~500 bp (200–1,000 bp) by ultrasound, immunoprecipitation with antibodies specific to the DNA-bound proteins, release of the co-precipitated DNA and, finally, PCR amplification with specific primers to determine whether DNA sequences of interest were precipitated. The latter steps involve comparison of the intensity of PCR signals from the precipitated template with positive and negative controls. In an alternative method, the precipitated DNA may be used for hybridization on DNA microarray chips (ChIP-on-chip method) to detect global interactions at multiple genetic loci^{14,15}.

ChIP assays are powerful tools for revealing specific DNA–protein interactions that, nonetheless, have inherent limitations. Thus, a requirement for highly specific antibodies against the protein of interest, or a particular modification, is of critical importance. False-negative signals may originate from inefficient antibody (Ab) binding or epitope disruption during the cross-linking process; on the other hand, formaldehyde may fix transiently, or even nonspecifically, adjacent proteins to result in false-positive signals. As a result, it is recommended that calibration curves be built after the cross-linking and before the precipitation with the Ab to determine the optimal amounts of chromatin to be used in each experiment and to ensure equivalent amounts of starting material¹⁶; serially diluted chromatin samples are used to define the point when detectable bands would be amplified from tested chromatin templates (immunoprecipitated with the antibodies of interest), while controls (mock ChIPed chromatin templates) would be below concentrations capable of amplifying visible bands. In general, ChIP procedures are delicate and require a few

TABLE 1 | Similarities and differences between available ChIP protocols.

	Other protocols ^{25–28}	Our protocol
Cross-linking and extraction Buffer I	0.4 M sucrose, 10 mM Tris–HCl (pH 8), 10 mM MgCl ₂ , 5 mM β-ME, 0.1 mM PMSF, 2 PI complete tablet, 1% formaldehyde solution ^{25,26}	0.4 M sucrose, 10 mM Tris–HCl (pH 8), 1 mM PMSF, 1 mM EDTA, 1% formaldehyde ²⁸
Extraction buffer II ^{25,26}	0.25 M sucrose, 10 mM Tris–HCl (pH 8), 10 mM MgCl ₂ , 5 mM β-ME, 0.1 mM PMSF, 1% Triton X-100, 2 PI complete tablet	
Extraction buffer III ^{25,26}	1.7 M sucrose, 10 mM Tris–HCl (pH 8), 2 mM MgCl ₂ , 5 mM β-ME, 0.1 mM PMSF, 0.15% Triton X-100, 2 PI complete tablet	
Nuclei isolation buffer		0.25 M sucrose, 15 mM PIPES (pH 6.8), 5 mM MgCl ₂ , 60 mM KCl, 15 mM NaCl, 1 mM CaCl ₂ , 0.9% Triton X-100, 2 μg ml ^{−1} pepstain A, 2 μg ml ^{−1} aprotinin
Nuclei lysis buffer	50 mM Tris–HCl (pH 8), 10 mM EDTA, 1% SDS, 1 PI (complete mini tablet) ^{25–27}	50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1% SDS, 0.1% Na deoxycholate, 1% Triton X-100, 1 μg ml ^{−1} pepstain A, 1 μg ml ^{−1} aprotinin ²⁸
ChIP dilution buffer	16.7 mM Tris–HCl (pH 8), 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl ^{25–28}	Use nuclei lysis buffer
Washing and elution buffers	Similar washing and elution buffers are used by all protocols	
Elution of immunoprecipitated complexes	Performed at 65 °C for 15 min two times ^{25–28}	Performed at room temperature for two times with gentle rotation, first one 15 min and the second one 30 min
DNA precipitation	DNA precipitation at −20 °C for 3 h or overnight	DNA precipitation at −80 °C for 1 h
DNA yield	Not reported	Starting with 0.2–0.4 g of raw tissue, at least 15 μg DNA is obtained

rounds of pilot runs before one begins to ‘feel’ them. A limitation of the ChIP assay is that cross-linked DNA may be difficult to digest during subsequent enzymatic studies.

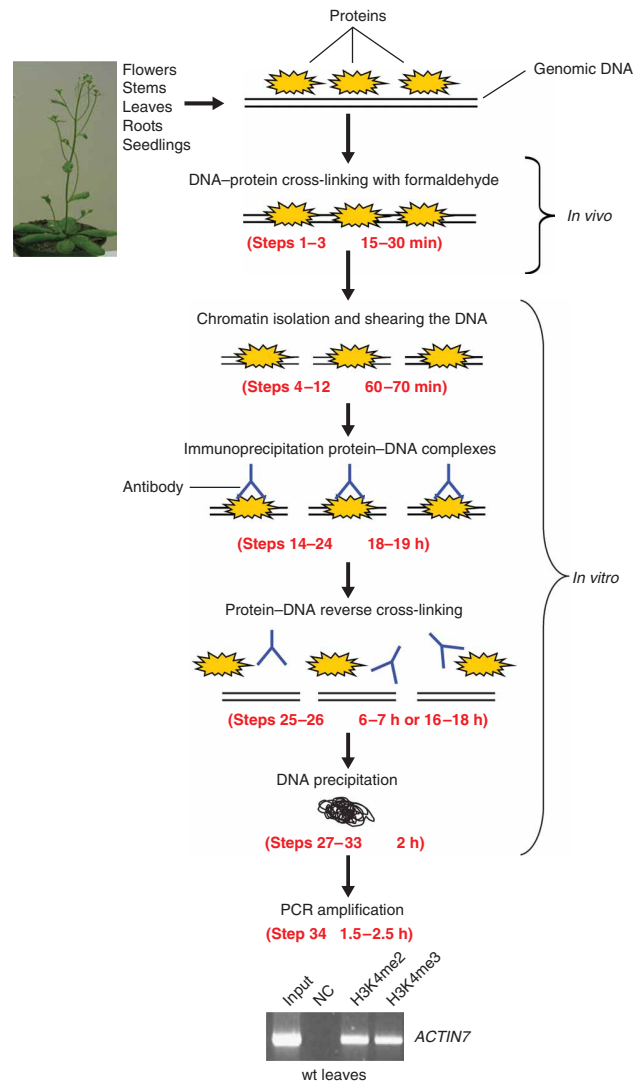
Initially, protocols for ChIP assays were developed and described for mammalian¹⁷, yeast¹⁸ and *Drosophila*¹⁹ chromatin. It is important to point out that structural anatomical difference between plant and animal cells, such as rigid cell walls, high levels of cellulose and lignin and large vacuoles, posed limitations for direct application of ChIP protocols used with animal cells. Consequently, vacuum infiltration has been introduced to ensure penetration of the DNA–protein cross-linking solution into plant cells²⁰. Furthermore, the presence of large vacuoles in mature plant cells (occupying >90% of the volume)^{21,22} results in a relatively low yield of nuclei per gram of used tissue. Moreover, vacuoles are known to be a source of abundant proteolytic activities and hence require special attention during isolation procedures and use of healthy fresh plant tissues²³. It is obvious that ChIP protocols developed for animal systems could not be employed directly for ChIP assays in plants. As a result, plant-specific ChIP protocols have been developed and used for analyzing a large number of epigenetically controlled genes in plants^{20,24–31}.

We have carefully examined available protocols and selectively integrated sections of published procedures, modified implemented approaches and introduced a new nuclei-isolation step to propose a simplified, efficient ChIP protocol for studying histone modifications and associated activities in *Arabidopsis*^{16,32–34}. Differences with published protocols include a single step and one-buffer solution for each cross-linking and nuclei isolation as opposed to three or

four different solutions for cross-linking and chromatin extraction^{25–29}, as well as reduced preparation time and costs (**Table 1**). The nuclei-isolation step (**Fig. 1** and **Table 1**) is important for the purity and yield of DNA obtained at the end. By comparison, in our hands, the use of a published method for isolating cross-linked chromatin from crude cross-linked tissues^{25–29} resulted in lower DNA template purity and yield, particularly from root and leaf tissues. Rigid cell walls, large vacuoles and paucity of nuclei in these cells, apparently, contributed to this outcome. Introduction of a nuclei-isolation step resolved this problem to a large extent. We achieved elution of cross-linked DNA–proteins complexes at room temperature (22–25 °C) also, instead of at 65 °C, and avoided purification of immunoprecipitated DNA using costly commercial spin columns^{29,33,34}. Based on previously described protocols^{20,28}, we have tested and determined that formaldehyde cross-linking in the presence of sucrose, Tris–HCl and EDTA is much more efficient resulting in better protein–DNA cross-linking than formaldehyde solution alone^{25,26}. Apparently, the change in osmotic pressure, in and out of the plant cells, produced by sucrose and sodium salts makes formaldehyde penetration more efficient.

This protocol is fast, reliable and has been a preferred tool in our efforts to map histone–DNA associations in *Arabidopsis* (**Fig. 1**). It has been used for analyzing histone-modification profiles including H3K4me2, H3K4me3, H3K9me2, H3K27me2 and H3K27me3 of a large number of tissue-specific, developmentally regulated and facultatively expressed (house-keeping) genes in their respective transcribed and silent states^{16,32–34}. Furthermore, using antibodies against specific amino acid modifications, like di- and trimethylated

Figure 1 | Principal steps and duration of chromatin immunoprecipitation protocol. Harvest the plant tissues followed by cross-linking the protein–DNA with formaldehyde. Isolate the chromatin and shear the DNA along with the bound protein by sonication. Immunoprecipitate the DNA–protein complex using specific antibodies. Reverse the cross-linking of DNA–protein complexes to release the DNA and digest the proteins. Finally, DNA precipitation and PCR amplification are carried out. Antibodies specific against di- and trimethylated Lys 4 of histone H3 were used to immunoprecipitate chromatin samples extracted from *Arabidopsis* wild-type (wt) leaves. *ACTIN7* is a constitutively expressed gene that carries di- and trimethylated Lys 4 of histone H3 and was used as control for the quality of the templates. Input: positive control (genomic DNA), NC: negative control (without Ab), H3K4me2: Histone H3 Lys 4 dimethylation and H3K4me3: Histone H3 Lys4 trimethylation.



transition to flowering^{37–41}. Analyzing *Arabidopsis* genes by the ChIP approach in the *clf* background, we have determined nucleosomal H3K27me3 profiles generated by the methylating activity of CLF³³.

Lys 4 of histone H3 (H3K4me2, H3K4me3) and trimethylated Lys 27 of histone H3 (H3K27me3) in wild-type and in *atx1*, *atx2*, *clf* mutant backgrounds, we were able to establish the contribution and the biological relevance of activities encoded by the *Arabidopsis* homologs of *Trithorax* (*ATX1* and *ATX2*) and of *Enhancer-of-zeste* (*CURLY LEAF*, *CLF*)^{16,32–34}. In particular, our ability to discriminate between H3K4me2 and H3K4me3 modifications at specific gene loci has allowed us to define *ATX2* as a histone Lys dimethyltransferase, thus providing the first example of separated di- and trimethylating activities in *Arabidopsis*³⁴. In addition, using the described ChIP method, we have demonstrated that despite having highly conserved coding sequences, the two duplicated paralogs, *ATX1* and *ATX2*, have diverged their biochemical activities remarkably³⁴. Using antibodies specific against *ATX1* and the ChIP approach described here, we have successfully identified nucleosomes targeted directly by the modifying activity of *ATX1*^{16,32–34}.

During the following procedure, we use the study of the nucleosomal patterns of the *FLOWERING LOCUS T* (*FT*) gene in wild-type and in *clf* backgrounds as an example of how to follow and use this protocol. The *FT* gene belongs to a family of genes characterized by a PEBP (phosphatidylethanolamine binding protein) domain and plays a key role as an activator of floral transition^{35,36}. Using the proposed ChIP protocol, we demonstrate a role for *CLF* in depositing H3K27me3 marks on *FT* nucleosomes and their correlation with *FT* expression during the transition to flowering. The Polycomb-group protein, *CLF*, is a known repressor of expression of floral homeotic genes in vegetative tissues and of genes promoting

MATERIALS

REAGENTS

- Sucrose (Sigma, cat. no. 84097)
- Formaldehyde 37% (Sigma, cat. no. F8775) **! CAUTION** Very toxic if inhaled, ingested or absorbed through skin.
- Cheesecloth miniwipes (Fisher, cat. no. 06-665-28)
- EDTA disodium salt (Sigma, cat. no. E5134) (see REAGENT SETUP)
- SDS (Sigma, cat. no. L6026) **! CAUTION** Toxic, avoid contact with body parts.
- Tris base (Sigma, cat. no. 08656)
- Glycine (Sigma, cat. no. G7403)
- Triton X-100 (Sigma, cat. no. T8787)
- Surfact-Amps NP-40 (Pierce, cat. no. 28324)
- Ethanol (Sigma, cat. no. 459828)
- Phenol/chloroform/isoamyl alcohol (25:24:1) (Pierce, cat. no. 17908) **! CAUTION** Dangerous if inhaled, absorbed through skin or swallowed. Causes respiratory tract, eye and skin burns.
- Sodium acetate (Sigma, cat. no. S2889)
- Sodium chloride (Sigma, cat. no. S7653)

- Sodium bicarbonate (Sigma, cat. no. S6297)
- Sodium deoxycholate (deoxycholic acid, sodium salt) (Sigma, cat. no. D6750)
- HEPES (N-(2-hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid); Sigma, cat. no. H-4034)
- PIPES (piperazine-N,N'-bis 2-ethanesulfonic acid) (Sigma, cat. no. P-1851)
- Potassium chloride (Sigma, cat. no. P9541)
- Calcium chloride (Sigma, cat. no. C2661)
- Magnesium chloride (Sigma, cat. no. M8266)
- Lithium chloride (Sigma, cat. no. L4408) **! CAUTION** Harmful if swallowed or inhaled.
- Plant material (*Arabidopsis* seedlings, leaves, stems or flowers)
- Ultrapure water, such as MilliQ (Millipore)
- Proteinase K (20 mg ml⁻¹) (Fermentas, cat. no. EO0491)
- Protease inhibitor: PMSF (Sigma, cat. no. P7626) (see REAGENT SETUP)
- Protease inhibitor: pepstatin A (Sigma, cat. no. P5318) (see REAGENT SETUP)
- Protease inhibitor: aprotinin (Sigma, cat. no. A3428) (see REAGENT SETUP)
- Anti-dimethyl-histone H3 (Lys 4) (Upstate, cat. no. 07-030)
- Anti-trimethyl-histone H3 (Lys 4) (Upstate, cat. no. 07-473)



- Anti-trimethyl-histone H3 (Lys 27) (Upstate, cat. no. 07-449)
- Salmon sperm DNA/protein A agarose (Upstate, cat. no. 16-157) (see REAGENT SETUP)
- Glycogen (20 mg ml⁻¹) (Fermentas, cat. no. R0561)
- ACTIN7 forward 5'-GGTGAGGATATTCAGCCACTTGTCTG-3' (Integrated DNA Technologies)
- ACTIN7 reverse 5'-TGTGAGATCCCGACCCGCAAGATC-3' (Integrated DNA Technologies)
- FT forward 5'-TCAATCACTCTAAAGGTTACTTATGGC-3' (Integrated DNA Technologies)
- FT reverse 5'-AAACAATATAAACACGACACGATGAAT-3' (Integrated DNA Technologies)
- DNA sample buffer 6× (Fermentas)

EQUIPMENT

- Sonicator (Model 100 Sonic Dismembrator; Fisher Scientific)
- Vacuum chamber (Model 7049-00; Cole Palmer Instrument)
- Sorvall centrifuge
- Refrigerated microcentrifuge
- Vortexer
- Rotating platform
- Heat block
- Freezers (-80 and -20 °C)
- Falcon tubes (50 ml)
- Eppendorf tubes (1.5 and 2 ml)
- 50-ml Sorvall centrifuge tubes

REAGENT SETUP

Cross-linking buffer 0.4 M sucrose, 10 mM Tris-HCl pH 8, 1 mM PMSF, 1 mM EDTA and 1% formaldehyde. ▲ **CRITICAL** Use high-quality formaldehyde and make fresh buffer before each experiment. Presence of sucrose in the cross-linking buffer increases the efficiency of the DNA-protein cross-linking. The addition of the protease inhibitor (PMSF) should be done directly before using the buffer.

Nuclei isolation buffer 0.25 M sucrose, 15 mM PIPES pH 6.8, 5 mM MgCl₂, 60 mM KCl, 15 mM NaCl, 1 mM CaCl₂, 0.9 % Triton X-100, 1 mM PMSF, 2 μg ml⁻¹ pepstatin A and 2 μg ml⁻¹ aprotinin. ▲ **CRITICAL** Prepare fresh and keep at 4 °C. The addition of the protease inhibitors PMSF, pepstatin A and aprotinin should be done directly before using the buffer.

PROCEDURE

Cross-linking ● TIMING 15–30 min

1| Harvest *Arabidopsis* plant tissues in 50-ml Falcon tubes: use 2 g of flowers, 4 g of leaves, 4 g of stems grown on soil or 4 g of seedlings grown in Petri dishes containing MS medium.

2| Add 37 ml cross-linking buffer to each sample and apply vacuum for 10 min at room temperature for cross-linking.

▲ **CRITICAL STEP** Ensure that the vacuum infiltration is working well. The plant tissues should look translucent after cross-linking, because formaldehyde penetrates plant cell wall during vacuum infiltration. The cross-linking time and the formaldehyde concentration are very important; otherwise, they can affect chromatin shearing efficiency, reversal of cross-linking and DNA precipitation. For example, short time of vacuum infiltration can lead to inefficient cross-linking, whereas long time can cause excessive cross-linking.

? TROUBLESHOOTING

3| Stop the cross-linking reaction by adding 2.5 ml of 2 M Glycine (final concentration of 100 mM) and by placing the tissue under the vacuum infiltration for an additional 5 min at room temperature.

Chromatin isolation and sonication ● TIMING 60–70 min

4| Wash plant tissues three times in sterile deionized water, remove the water as much as possible by blotting the tissues between paper towels and quick-freeze in liquid nitrogen.

5| Grind the tissue to a fine powder using prechilled mortars and pestles and ensure that the samples do not thaw during grinding.

6| Resuspend each sample in 25 ml of cold nuclei isolation buffer.

7| Vortex briefly to mix and keep the samples on ice until complete homogenization is achieved (15–30 min).

8| Filter the homogenized slurry through four layers of cheesecloth and centrifuge the filtrate at 11,000g for 20 min using Sorvall SA-600 rotor at 4 °C. You will see a tight white pellet at the bottom of the tube with an overlay of chlorophyll.

? TROUBLESHOOTING

Nuclei lysis buffer 50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% SDS, 0.1% sodium deoxycholate and 1% Triton X-100, 1 μg ml⁻¹ pepstatin A and 1 μg ml⁻¹ aprotinin. ▲ **CRITICAL** Prepare fresh and keep at 4 °C. The addition of the protease inhibitors pepstatin A and aprotinin should be done directly before using the buffer.

Elution buffer 0.5% SDS and 0.1 M NaHCO₃. ▲ **CRITICAL** Prepare fresh and keep at room temperature to avoid SDS precipitation.

Low salt wash buffer 150 mM NaCl, 20 mM Tris-HCl pH 8, 0.2% SDS, 0.5% Triton X-100 and 2 mM EDTA. ▲ **CRITICAL** Prepare fresh and keep at 4 °C.

High salt wash buffer 500 mM NaCl, 20 mM Tris-HCl pH 8, 0.2% SDS, 0.5% Triton X-100 and 2 mM EDTA. ▲ **CRITICAL** Prepare fresh and keep at 4 °C.

LiCl wash buffer 0.25 M LiCl, 1% sodium deoxycholate, 10 mM Tris-HCl pH 8, 1% NP-40 and 1 mM EDTA. ▲ **CRITICAL** Prepare fresh and keep at 4 °C.

TE buffer 1 mM EDTA and 10 mM Tris-HCl pH 8. ▲ **CRITICAL** Prepare fresh and keep at 4 °C.

PMSF Prepare a 200 mM stock solution in isopropanol. Make aliquots and store at -20 °C.

Pepstatin A Prepare a 1 mg ml⁻¹ stock solution in methanol. Make aliquots and store at -20 °C.

Aprotinin Prepare 1 mg ml⁻¹ stock solution in water. Make aliquots and store at -20 °C.

Pre-equilibrated salmon sperm DNA/protein A agarose beads Pre-equilibrate the protein A agarose beads with lysis buffer as follows: take 50 μl salmon sperm DNA/protein A agarose beads for each sample, centrifuge at 3,800g for 2 min at 4 °C to pellet the agarose beads, discard supernatant, add 50 μl lysis buffer, mix for 2 min at 4 °C with gentle rotation, centrifuge at 3,800g for 2 min at 4 °C, repeat. Resuspend protein agarose A beads with 50 μl lysis buffer.

EDTA 50 mM (stock 1 M pH 8).

Ethidium bromide solution Dilute to 10 mg ml⁻¹ in ddH₂O.

Running buffer Tris-acetate-EDTA (TAE) 1×.

TAE 50× stock 242 g Tris base, 57.1 ml acetic acid, 100 ml of 0.5 M EDTA. Add ddH₂O to 1 l and adjust pH to 8.5.

DNA sample buffer 6× (Fermentas; 10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol, 60 mM EDTA).

1% (wt/vol) Agarose gel 1 g Agarose/100 ml 1× TAE, containing 5 μl ethidium bromide solution.

9| Discard the supernatants and resuspend the pellet (nuclei) in 2 ml of cold nuclei lysis buffer.

▲ **CRITICAL STEP** Set aside 5 µl from each sample for the comparison of extracted chromatin with the sonicated chromatin samples (Step 12) by gel electrophoresis (Step 13).

10| Divide the samples into four aliquots of 500 µl each in Eppendorf tubes. Shear the DNA into ~500 bp (200–1,000 bp) fragments by sonicating five times for 15 s at power 6. Keep the samples on ice for 1 min at each interval.

▲ **CRITICAL STEP** Do not allow the sample to foam or warm, as this may affect sonication efficiency. Keep the tip of the sonicator probe not more than a few millimeters from the bottom of the tube to avoid foaming. Sonication leads to warming of the sample, so keep the sample on ice during sonication.

11| Centrifuge samples for 10 min at 13,800g at 4 °C to pellet debris.

12| Collect samples by combining the supernatants (sonicated chromatin) from all the four tubes of the same sample.

▲ **CRITICAL STEP** Set aside 5 µl from each sample to compare with the aliquot from Step 9.

■ **PAUSE POINT** Sonicated chromatin can be frozen at –80 °C for 3 months without significant loss of chromatin quality or can be used directly for immunoprecipitation.

Agarose gel electrophoresis of sheared DNA ● **TIMING** 40–50 min

13| To test the sonication efficiency, mix DNA samples (from Steps 9 and 12) with 6× DNA sample buffer (5:1) and load in the wells of a 1% agarose gel containing 5 µl ethidium bromide solution (see REAGENT SETUP). Perform electrophoresis at 5 V cm^{–1} or 100–120 V for at least 30 min (depending on the unit and the size of DNA fragment being resolved). Visualize resolved DNA fragments under UV transillumination (260–340 nm range) and document. A smear from 200 to 1,000 bp, but concentrated ~500 bp should be observed in the sonicated samples.

? **TROUBLESHOOTING**

Immunoprecipitation ● **TIMING** 18–19 h

14| Take a 100 µl aliquot from the sonicated chromatin sample (from Step 12) and dilute tenfold with lysis buffer.

15| Preclear the diluted sonicated chromatin by adding 50 µl salmon sperm DNA/protein A agarose beads (use pre-equilibrated slurry; see REAGENT SETUP) for 1 h at 4 °C with gentle rotation.

▲ **CRITICAL STEP** Prepare one 100 µl aliquot (as described in Steps 14 and 15) for each Ab to be tested; an additional aliquot of the same sample is included to be treated in the same way as the rest of the aliquots but without Ab to serve as negative control.

16| Centrifuge the samples at 3,800g for 2 min at 4 °C to pellet the agarose beads.

▲ **CRITICAL STEP** The centrifuge speed in Steps 16, 19, 20, 22 and 23 must be no > 3,800g in a microcentrifuge to avoid damaging of agarose beads.

17| Add 5 µl of the appropriate Ab to the supernatant and incubate at least 5 h to overnight at 4 °C with gentle rotation.

18| Add 60–75 µl salmon sperm DNA/protein A agarose beads (pre-equilibrated slurry; see REAGENT SETUP) and continue the incubation for 2 h at 4 °C.

19| Centrifuge samples at 3,800g for 2 min at 4 °C to collect the agarose beads and the chromatin.

20| Wash the agarose beads for 5 min each time with gentle rotation at 4 °C with 1 ml of each of the following buffers and centrifuge for 3,800g for 2 min at 4 °C: one time with low salt wash buffer, one time with high salt wash buffer, one time with LiCl wash buffer and two times with TE buffer.

21| Elute the immuno-complexes from the agarose beads by adding 250 µl of freshly prepared elution buffer by incubating at room temperature for 15 min with gentle rotation.

▲ **CRITICAL STEP** Elution buffer should be freshly prepared; for example, prepare it during the agarose beads washing time. Do not vortex the agarose beads to avoid damaging of agarose beads

22| Centrifuge the samples at 3,800g for 2 min at 4 °C and transfer the supernatant into a new Eppendorf tube.

23| Repeat the elution step by adding another 250 µl of the freshly prepared elution buffer to the beads from Step 22 and incubating at room temperature for 30 min with gentle rotation. Centrifuge the samples at 3,800g for 2 min at 4 °C.

24| Combine the two eluates (from Steps 22 and 23; total eluate is 500 µl). At the same time, add 450 µl elution buffer to 50 µl sonicated chromatin (from Step 12) to serve as an input control (positive control).

Reverse cross-linking and protein digestion ● TIMING 5–6 h (or overnight)

25| Add 20 µl of 5 M NaCl to each tube (Step 24) and incubate at 65 °C for at least 4 h to overnight to reverse cross-linking.

26| Add the following solutions to each tube: 10 µl of 0.5 M EDTA, 20 µl of 1 M Tris-HCl pH 6.5, 1 µl proteinase K (20 mg ml⁻¹) and incubate for 1.5 h at 45 °C to digest the proteins.

DNA precipitation ● TIMING 2 h (or overnight)

27| Add equal volume (550 µl) of phenol/chloroform/isoamyl alcohol to each tube and vortex briefly.

28| Centrifuge the samples in a microcentrifuge at 13,800g for 15 min at 4 °C and transfer the supernatant (~500 µl) into 2-ml Eppendorf tube.

29| Add the following solutions to each tube: 2.5 volume of 100% EtOH, 1/10 volume of 3 M sodium acetate pH 5.2, 4 µl glycogen (20 mg ml⁻¹) and incubate for 1 h at -80 °C to precipitate the DNA.

30| Centrifuge the samples at 13,800g for 15 min at 4 °C.

31| Discard the supernatant and wash the pellet with 500 µl of 70% EtOH (vol/vol) and centrifuge again at 13,800g for 10 min at 4 °C.

32| Discard the supernatant and dry the pellet at room temperature.

? TROUBLESHOOTING

33| Dissolve the DNA in 50 µl TE and store at -80 °C.

■ **PAUSE POINT** Store the DNA at -80 °C and use it within 3 months. After this period you will observe significant loss of DNA quality.

? TROUBLESHOOTING

PCR and data analysis ● TIMING 1.5–2.5 h

34| Proceed to PCRs using gene specific primers. In our example of the *FT* gene, PCRs were carried out in a final volume of 25 µl containing: 2 µl DNA template (25 ng µl⁻¹), 2.5 µl PCR buffer (10×), 5 µl MgCl₂ (17.5 mM), 1 µl dNTPs (10 mM), 1 µl primers (forward and reverse, 50 ng µl⁻¹), 0.4 µl *Taq* polymerase (Invitrogen, 5 U µl⁻¹) and 13 µl ddH₂O sterile. The PCR products were amplified with the following condition: 95 °C for 3 min, 38 cycles of 94 °C for 30 s, 50 °C for 30 s, 72 °C for 1 min and 72 °C for 10 min. *ACTIN7* gene (AT5G09810) is used as control carrying the Histone 3 Lys 4 methylation mark as shown in Figure 2.

? TROUBLESHOOTING

● TIMING

Day 1

Steps 1–3: requires only 20 min.

Steps 4–12: intermittent manipulations; actual hands-on time is estimated to be 1.5–2 h. Pause point after Step 12.

Step 13: requires 40–50 min. Estimated time depends on electrophoresis unit.

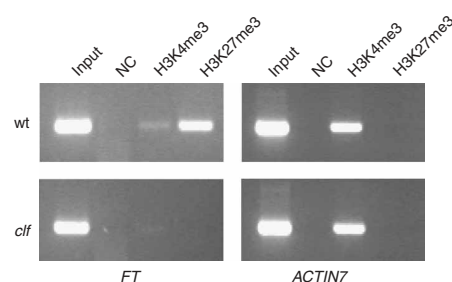
Steps 14–17: total time 1.5 h to 6.5 h. Immune complex formation is performed for at least 5 h to overnight.

Day 2

Steps 18–24: intermittent manipulations; actual hands-on time is estimated to be 4 h.

Steps 25 and 26: estimated time 5.5 h. Actual hands-on time is estimated to be 10 min. There are 5.5 h of incubation time.

Figure 2 | Histone H3 Lys 27 methylation profiles of Flowering Locus T (*FT*) nucleosomes in *clf* mutant background. ChIP assays with antibodies specific against trimethylated Lys 4 of histone H3 and trimethylated Lys 27 of histone H3 using *Arabidopsis* wild-type (wt) and *clf* mutant leaves after flowering. Each immunoprecipitation experiment is independently performed, as biological replicates. *ACTIN7* is a constitutively expressed gene that carries trimethylated Lys 4 of histone H3 and was used as control for the quality of the templates; the same amounts are used to amplify *FT* (AT1G65480). Input: positive control (genomic DNA), NC: negative control (without Ab), H3K4me3: Histone H3 Lys 4 trimethylation and H3K27me3: Histone H3 Lys 27 trimethylation.



PROTOCOL

Day 2–3

Steps 27–33: intermittent manipulations, ~2 h if DNA precipitation is carried out at –80 °C.

Pause point after Step 33.

Step 34: total time 1.5–2.5 h to overnight. PCR performed on day 2 and left overnight in thermocycler.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Steps	Problem	Possible reasons	Solution
2	Nontranslucent tissue	Too much amount of tissue	Make sure that you take the appropriate amount of tissues
		Vacuum infiltration does not work	Make sure that your vacuum is working well
8	No visible white nuclei pellet	Sometimes, white pellet does not form	It may happen but it does not mean that the chromatin extraction did not work. Our advice is to proceed to the next step
13	No difference between the sonicated and non-sonicated samples after running the gel	Sonication is not enough, sonication did not work well or proteins affect the DNA running in the gel	Adjust the sonication condition based on the sonicator available in your lab. Sometimes, it is better to do this comparison after reversal of cross-linking because proteins bound to DNA may affect the DNA running in the gel
32 and 33	No DNA yield or lower than expected	Ab is not working properly or Ab may not be specific	Use high affinity purified Ab
		The cross-linking time is too long	Reduce the cross-linking time; prolonged cross-linking might result in difficult cross-linking reversal and a tight DNA–protein complex that would be hard to separate
34	No PCR signal	Insufficient number of PCR cycles	Increase reasonably the number of cycles until obtaining a visible band
		PCR did not work	Always use positive (sonicated chromatin sample reverse cross-linked) and negative (samples treated in the same way as in the immunoprecipitated samples but without Ab) controls
			Check the annealing temperature

ANTICIPATED RESULTS

A good ChIP protocol may be defined as one applicable for a broad variety of plant tissues, yielding high quality of end DNA in satisfactory amounts, and generating least amounts of false signals upon amplification. Duration of the preparation procedures and cost efficiency are additional features that make a protocol attractive to researchers. The method described is a simplified and improved version of several procedures^{20,24–29}. Using this protocol, we obtain good-quality DNA and at least 15 µg of DNA from 0.2 to 0.4 g of raw tissue for each immunoprecipitated sample. This amount of DNA is sufficient to carry out ~25 PCRs and results for tested genes can be obtained in 2–3 d. This protocol has been used to study histone modifications in flower, stem, leaf and seedling tissues^{16,32–34}. We expect that it can be easily adopted for uses in other systems as well as to test different histone modifications or DNA–protein interactions.

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