Detecting histone modifications in plants

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Abstract

Histone modifications play an essential role in chromatin-associated processes including gene regulation and epigenetic inheritance. It is therefore very important to quantitatively analyse histone modifications at both the single gene and whole genome level. Here, we describe a robust chromatin immunoprecipitation (ChIP) method for Arabidopsis, which could be adapted for other plant species. This method is compatible with multiple downstream applications including qPCR, tilling arrays and high-throughput sequencing.

Key Words: histone modification; epigenetics; chromatin immunoprecipitation (ChIP)

1. Introduction

In eukaryotes, DNA is wrapped around a histone octamer consisting of two copies of H2A, H2B, H3 and H4. Histone tails can be covalently modified at various amino acids (e.g. H3 lysine 4/9/27/36), and be of varying forms (e.g. mono/di/tri-methylation and acetylation) **(*1-2*)**. The modifications can be detected by immunoprecipitation (IP) with specific antibodies against the modification.

There are basically two types of chromatin IP, distinguished by different methods of chromatin conservation and DNA fragmentation prior to the IP. The most commonly used is X-ChIP, where the chromatin is cross-linked and then the DNA sheared by sonication. The other is native ChIP, where nuclei are extracted in their native form without cross-linking, and the DNA fragmented by nuclease digestion. We focus here on the X-ChIP procedure as it is a robust and non-biased technique favoured for analysis of histone modifications.

Several chromatin IP protocols have been described for *Arabidopsis* **(*3-5*)** and other plant species, e.g. maize **(*6*)** and tomato **(*7*)**. Here, we describe in detail a method used extensively in our work on *FLC* epigenetic silencing. It has been streamlined by incorporating new products, for example use of Chelex-100 resin instead of high salt reverse cross-linking **(*8*)** and optimised for use with a large number of samples where quantitative data is required.

2. Materials

Stock solutions (Section 2.1) are prepared and stored at room temperature. Buffers used in the experiment are prepared fresh on the day and kept at 4 ^{o}C, unless otherwise stated. β-mercaptoethanol and protease inhibitors are added to the solution just prior to use.

2.1 Stock solutions

1. PBS buffer (10x): NaCl 1.3 M, Na_2HPO_4 30 mM, NaH_2PO_4, pH 7.4
2. Sucrose, 2 M
3. Tris-HCl, 1 M pH8
4. $MgCl_2$, 1 M
5. Triton X-100, 10% (w:v)
6. EDTA, 0.5 M pH8
7. NaCl, 5 M
8. SDS, 10 % (w:v)
9. LiCl, 4M
10. NP-40, 10 % (w:v)

2.2 Chromatin extraction and DNA fragmentation

1. Cross-linking solution: 1% (v:v) formaldehyde (Simga) in phosphate-buffered saline (PBS) buffer, freshly prepared at room temperature.
2. Quenching solution: 2 M glycine, freshly dissolved in ddH_2O at room temperature.
3. Extraction buffer 1: 0.4 M sucrose, 10 mM Tris-HCl, pH 8.0, 5 mM β-mercaptoethanol, protease inhibitor cocktail (cOmplete, Roche).
4. Extraction buffer 2: 0.25 M sucrose, 10 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 1% (v/v) Triton X-100, 5 mM β-mercaptoethanol, protease inhibitor cocktail (cOmplete, Roche).
5. Extraction buffer 3: 1.7 M sucrose, 10 mM Tris-HCl, pH 8.0, 0.15% (v/v) Triton X-100, 2mM $MgCl_2$, 5 mM β-mercaptoethanol, protease inhibitor cocktail (cOmplete, Roche).
6. Nuclei lysis buffer: 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% (w/v) SDS, protease inhibitor cocktail (cOmplete, Roche).

2.3 Immunoprecipitation

1. ChIP dilution buffer: 1.1% (v/v) Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, protease inhibitor cocktail (cOmplete, Roche).

2. Antibodies against specific histone modifications **(*9*)** and no antibody control. We use rabbit polyclonal antibody anti-H3K27me3 (Millipore, 07-449, recently discontinued and replaced by 17-622) to detect H3K27me3, and rabbit polyclonal antibody anti-H3 (Abcam, ab1791) to detect H3 (*see* **Note 1**). For these rabbit polyclonal antibodies, rabbit IgG (Millipore) is used as the mock control.
3. Dynabeads protein A (Invitrogen) and magnet (*see* **Note 2**).
4. Low salt wash buffer: 150 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0.
5. High salt wash buffer: 500 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.0.
6. LiCl wash buffer: 0.25 M LiCl, 1% (v/v) NP-40, 1% (w/v) sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0.
7. TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
8. Elution: 10% (w:v) Chelex 100 resin (BioRad).
9. Proteinase K solution: 20 mg/ml Proteinase K (Roche) in water, stored at -20 ^{o}C.

2.4 DNA clean-up

1. Phenol/chloroform solution, mixture of phenol, chloroform, isoamyl alcohol in the ratio of 25:24:1 (v/v/v) (Sigma)
2. NaAc, 3 M pH 5.2.
3. GlycoBlue (Ambion)
4. Ethanol, absolute and 75 % (v:v).

2.5 Quantitative PCR (qPCR)

SYBR Green qPCR mix (Roche) and LightCycler 480 II Instrument (Roche), or an alternative quantification system.

3. Methods

The work flow for ChIP is illustrated in Figure 1. The whole procedure can be performed within two days, Day 1 for formaldehyde cross-linking, chromatin extraction, DNA fragmentation and immunoprecipitation, and Day 2 for immunoprecipitation washes and quantification. There are possible pausing points, for example after formaldehyde cross-linking and after DNA fragmentation, as mentioned in respective steps below.

3.1 Plant growth and formaldehyde cross-linking

1. Plant materials are grown on media or compost under desirable conditions. If grown on nutrient media, we recommend Murashige and Skoog (MS) minus glucose as this limits bacterial growth.
2. Harvest 1-2 g plant materials in a 50 ml Falcon tube (*see* **Note 3**).
3. Rinse plant material twice in ddH_2O and remove water throughout after the second rinse.
4. Submerge plant materials in 37 ml of cross-linking solution. Stuff the tube with nylon mesh to keep plants immersed in the buffer. Vacuum infiltrate at room temperature for three times, 5 min each time and release vacuum in between to allow buffer penetrating plant tissues. Shake desiccator slightly to remove air bubbles. At this stage, seedlings should appear 'water-soaked' or translucent (*see* **Note 4**)
5. Stop the cross-linking by addition of glycine quenching solution to a final concentration of 0.125 M (2.5 ml of 2 M stock into 37 ml of cross-linking buffer) and application of vacuum for additional 5 min.
6. Rinse off formaldehyde with 40 ml ddH_2O for at least three times.
7. Remove water as thoroughly as possible by placing seedlings on a paper towel before freezing in liquid nitrogen. At this stage cross-linked material can be either stored at -80 ^oC for up to several months or processed further for chromatin extraction.

3.2 Chromatin Extraction and DNA Fragmentation

All steps in this and the following Section 3.3 are carried out at 4 ^oC. Refrigerator centrifuges and motors are pre-cooled to 4 ^oC.

1. Grind plant materials in liquid nitrogen to a fine powder.
2. Resuspend the powder in 30 ml extraction buffer 1 in a 50 ml Falcon tube. Vortex immediately to mix and incubate for 5 min or until solution is homogenous. Make sure there is the volume of the buffer is more than five times more than that of the powder.
3. Filter the solution through a double layer of Miracloth into a new, ice-cold 50 ml Falcon tube. Repeat until solution is clear.
4. Spin the filtered solution for 20 min at 2880 g at 4 ^oC.
5. Gently remove supernatant and resuspend the pellet in 1 ml of extraction buffer 2.
6. Transfer the solution to 1.5 ml Eppendorf tube.
7. Centrifuge at 12000 g for 10 min at 4 ^oC.

8. Remove the supernatant and resuspend pellet in 300 µl of extraction buffer 3.
9. Overlay the resuspended pellet onto 900 µl of extraction buffer 3 in a fresh 1.5 ml Eppendorf tube.
10. Spin for 45 min to 1 h at 16000 g.
11. Remove the supernatant and resuspend the chromatin pellet in a DNA LoBind tube (Eppendorf) with 320 µl of nuclei lysis buffer by pipetting up and down. From this point on, DNA LoBind tubes or similar products are used to reduce sample-to-surface binding.
    Check point: save a 10 µl aliquot representing 'unsheared' chromatin) for later examination.
12. Once resuspended, sonicate the chromatin solution for 3 times, 5 min each time (30 sec on/off intervals) at the 'Low' setting using BioRupter (Diagenode) according to manufacturer's instructions (*see* **Note 5**). In between runs, cool the water bath by adding more ice and correcting the water level if necessary. This fragments DNA into manageable sizes, usually ranging from 200 – 800 bp and centering at 500 bp (*see Check point* below).
13. The sonicated chromatin solution can be frozen in liquid nitrogen and stored at -80 ^{o}C or processed further for immunoprecipitation.
14. Spin the sonicated chromatin suspension for 5 min at 16000 g to pellet debris.
    Check point: save a 10 µl aliquot representing 'sheared' chromatin, together with 'unsheared' chromatin' taken from step 11, to assess sonication efficiency (see ***Note 6****, Figure 2).*

3.3 Immunoprecipitation (IP)

1. Prepare Dynabeads proein A magnetic beads. Use 15 µl beads per IP, one tube for the mock control, one tube for each antibody. Use 4 µg of anti-H3k27me3 antibody per H3K27me3 IP and 3 µg of anti-H3 antibody per H3 IP (*see* **Note 7**).
2. Wash beads three times in ChIP dilution buffer. Let beads attach to the magnet and discard buffer. Add antibody and incubate in 50 µl ChIP dilution buffer for 1 h (*see* **Note 8**).
3. Wash prepared antibody coated beads three times with ChIP dilution buffer.
4. Take 10% volume of the chromatin solution (usually 30 µl) as input control. The input control samples need to be processed for DNA recovery as described in Section 3.4.
5. Transfer the remaining chromatin solution (270 µl) into a Falcon tube and dilute 10 times with ChIP dilution buffer, to dilute the 1% SDS to 0.1%.

6. Add 900 µl diluted chromatin solution to each tube with antibody coated beads, mock control, H3K27me3 IP and H3 IP respectively, and incubate rotating overnight or at least 2.5 hrs.
7. Apply following washes, 1 ml of wash buffer per IP sample (*see* **Note 9**). Attach beads to magnet after each wash to collect beads and discard supernatant.
    a. Low salt wash buffer, 2 washes, 5min each
    b. High salt wash buffer, 1 wash, 5min
    c. LiCl wash buffer, 1 wash, 5 min
    d. TE buffer, 2 washes, 5 min each
8. During the last TE wash, transfer the beads to a new LoBind tube to further lower background (optional).
9. After the final TE wash, remove TE buffer thoroughly. Carry out the following step immediately.

3.4 DNA Recovery and Clean-up

1. Elute immune complexes by adding 100 µl 10% Chelex resin and incubating at 95 ^{o}C, 1300 rpm for 10 min. Input samples, taken from step 4 Section 3.3, need to be treated the same way from this step onwards to recover DNA (*see* **Note 10**).
2. Cool on ice and collect contents by brief spin. Add 2 µl Proteinase K solution and incubate at 50 ^{o}C for 30 min to digest proteins.
3. Boil for another 10 min at 95 ^{o}C, 1300 rpm.
4. Brief spin to collect contents. Add ddH_2O to the sample to make up the volume to 500 µl. Spin and transfer supernatant to a new LoBind tube.
5. Use phenol-chloroform extraction to remove protein (*see* **Note 11**). Precipitate DNA with 1/10 v NaAc, 2 µl GlycoBlue and 2v absolute ethanol. Spin at top speed to pellet DNA and wash with 75% ethanol. Pellet DNA again after each wash by centrifugation at top speed for 3 min. Air dry DNA pellet and resuspend in ddH_2O for qPCR. Input samples can be further diluted 2-10 times to obtain similar concentration of DNA as the IP samples. DNA resuspension volume can be adjusted according to the subsequent assays.

3.5 Quantification and normalisation

The ChIPped DNA is quantified in qPCR reactions to examine enrichment of target sequences. Primers are designed using Primer3Plus **(*10*)** to cover sequences of interests and controls. Raw data from qPCR reactions are analysed using the 2^{-}

[Delta][Delta]C_T analysis method **(*11*)**. Data are represented as fold enrichment to the mock control (usually used for protein-DNA binding but not histone modifications), percentage of input, *(ChIP H3K27me3/Input) *100%*, or enrichment per nucleosome, *(ChIP H3K27me3/ChIP H3) *100%* (Figure 3a).

The enrichment of a certain histone modification at a specific locus can be expressed in relation to a reference sequence, an internal control. This normalisation is particularly relevant when comparing across different treatments and experiments, in which case variations between experiments need to be taken into account. The normalisation can be calculated as

*(Target sequence H3K27me3 / control sequence H3K27me3) *100%,*

or

*[(Target sequence H3K27me3/H3) / (control sequence H3K27me3/H3)] *100%*

(Figure 3b).

In the case of H3K27me3, as it's been mapped across the *Arabidopsis* genome **(*12*)**, loci constantly covered with high levels of H3K27me3 can be used as positive internal controls, e.g. *SHOOT MERISTEMLESS* (*STM*), or *AGAMOUS* (*AG*) **(*13*)**. Loci with low levels of H3K27me3 can be used as a negative internal control, e.g. *ACTIN* **(*14*)**.

4. Notes

1. There are commercially available, ChIP tested antibodies against histone modifications **(*9*)**. New antibodies need to be tested for ChIP with controls. There is no guarantee that antibodies which work in other applications, for example western blotting, would definitely work in ChIP.
2. Magnetic beads (for example Dynabeads from Invitrogen) have advantages over agarose beads. Magnetic beads are collected gently by placing in a magnetic field, with no columns or centrifugations involved. There is no bad volume remaining after each wash, which helps reduce background.
3. Mature plant tissue contains a high proportion of vacuole to cytoplasm, extensive secondary thickening and complex cell walls. These properties could significantly reduce efficiency of nuclei extraction so we recommend using young tissue if possible.
4. This is one of the key steps in ChIP. When working with a different type of tissue or plant species, cross-linking should be optimised to best preserve

chromatin structure and yet not to make the subsequent reverse cross-linking too difficult. Optimisation of cross-linking was described by Haring *et al*. (2007) **(*6*)**. In brief, after cross-linking, extract chromatin as described in Section 3.2 steps 1-11, take an aliquot (e.g. 10 µl) to extract free DNA using phenol-chloroform solution. Take another aliquot and reverse cross-linking as described in Section 3.4 to recover DNA. Run both samples on agarose gel to check cross-linking and reverse cross-linking efficiency. Be aware that there will still be free DNA with harsh cross-linking conditions; this 'open' chromatin with a loss of nucleosome is typically at genomic loci that are actively transcribed **(*15*)**.

5. The purpose of sonication is to shear DNA into manageable sizes. This can be achieved using water bath, e.g. BioRupter (Diagenode), or probe based sonication device. Sample needs to be kept ice cold, and rested on ice in between pulses. At this stage, chromatin solution can be viscous. Ensure there are no air bubbles in the samples. If using a probe based sonicator, the amplitude setting and duration are subjective and may vary between each application.
6. The 'sheared' and 'unsheared' chromatin samples are processed to recover DNA as described in Section 3.4 'DNA Recovery and Clean-up', treat with RNase to remove RNA and run on 1.5% agarose gel to check sonication efficiency. DNA fragmentation needs to be optimised when first time working on a different biological sample.
7. The amount of antibody used in one assay is usually recommended by its manufacturer, typically 1-10 µg. Concentrations of antibody in each batch may vary.
8. Binding reaction volume can be adjusted according to manufacturer's instructions. Excessive antibodies are washed away during the subsequent washes with ChIP dilution buffer.
9. In our experience these wash steps generally work fine for histone ChIP. If concern rises regarding weaker antibody-antigen binding and loss of ChIP signal, some steps, for example the high salt wash, can be skipped or salt concentration can be reduced. In contrast, if background level is high, wash steps can be repeated to reduce non-specific binding. For histone ChIP, due to its abundance, the signal-to-noise ratio is usually more satisfactory, compared to transcription factor ChIPs.
10. Chelex-100 resin is a quick way to reverse cross-linking **(*8*)**. Alternatively, it can be achieved by elution of bead-bound complexes using elution buffer (1%

[w/v] SDS, 0.1 M $NaHCO_3$, incubation at 65 ^oC for 15 min), followed by high salt (addition of NaCl to a final concentration of 0.2 M) reverse cross-linking at 65 ^oC for at least 4 hrs.

11. Protein in the sample is digested by proteinase K in previous steps. However we find that traces of protein left in the sample, especially in input samples, may interfere with ChIP quantification. Protein can be further removed from the sample by phenol-chloroform extraction. In the previous step, ddH_2O is added to the sample to increase the volume so that it is easy to take up the supernatant. An alternative, non-toxic method is to use resin that collects protein, for example StrataClean resin (Stratagene). ChIPped DNA is then precipitated and resuspended in a desirable volume. It can also be purified using commercially available PCR clean-up kits that collect both single-stranded and double-stranded DNA.

5. References

1. Margueron, R., and Reinberg, D. (2010) Chromatin structure and the inheritance of epigenetic information, *Nat Rev Genet* ***11***, 285-296.
2. Turner, B. M. (2007) Defining an epigenetic code, *Nat Cell Biol* ***9***, 2-6.
3. Lippman, Z., May, B., Yordan, C., Singer, T., and Martienssen, R. (2003) Distinct mechanisms determine transposon inheritance and methylation via small interfering RNA and histone modification, *PLoS Biol* ***1***, E67.
4. Bastow, R., Mylne, J. S., Lister, C., Lippman, Z., Martienssen, R. A., and Dean, C. (2004) Vernalization requires epigenetic silencing of *FLC* by histone methylation, *Nature* ***427***, 164-167.
5. De Lucia, F., Crevillen, P., Jones, A. M., Greb, T., and Dean, C. (2008) A PHD-Polycomb repressive complex 2 triggers the epigenetic silencing of *FLC* during vernalization, *Proc Natl Acad Sci U S A* ***105***, 16831-16836.
6. Haring, M., Offermann, S., Danker, T., Horst, I., Peterhansel, C., and Stam, M. (2007) Chromatin immunoprecipitation: optimization, quantitative analysis and data normalization, *Plant Methods* ***3***, 11.
7. Ricardi, M., Gonzalez, R., and Iusem, N. (2010) Protocol: fine-tuning of a Chromatin Immunoprecipitation (ChIP) protocol in tomato, *Plant Methods* ***6***, 11.
8. Nelson, J. D., Denisenko, O., Sova, P., and Bomsztyk, K. (2006) Fast chromatin immunoprecipitation assay, *Nucleic Acids Research* ***34***, e2.
9. Egelhofer, T. A., Minoda, A., Klugman, S., Lee, K., Kolasinska-Zwierz, P., Alekseyenko, A. A., Cheung, M.-S., Day, D. S., Gadel, S., Gorchakov, A. A., Gu, T., Kharchenko, P. V., Kuan, S., Latorre, I., Linder-Basso, D., Luu, Y., Ngo, Q., Perry, M., Rechtsteiner, A., Riddle, N. C., Schwartz, Y. B., Shanower, G. A., Vielle, A., Ahringer, J., Elgin, S. C. R., Kuroda, M. I., Pirrotta, V., Ren, B., Strome, S., Park, P. J., Karpen, G. H., Hawkins, R. D., and Lieb, J. D. (2010) An assessment of histone-modification antibody quality, *Nat Struct Mol Biol* ***advance online publication***.
10. Rozen, S., and Skaletsky, H. (2000) Primer3 on the WWW for general users and for biologist programmers., *Methods Mol Biol* ***132***, 365-386.

11. Livak, K. J., and Schmittgen, T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-[Delta][Delta]C_T method, *Methods* ***25***, 402-408.
12. Zhang, X., Clarenz, O., Cokus, S., Bernatavichute, Y. V., Pellegrini, M., Goodrich, J., and Jacobsen, S. E. (2007) Whole-genome analysis of histone H3 lysine 27 trimethylation in *Arabidopsis*, *PLoS Biol* ***5***, e129.
13. Finnegan, E. J., and Dennis, E. S. (2007) Vernalization-induced trimethylation of histone H3 lysine 27 at *FLC* is not maintained in mitotically quiescent cells, *Curr Biol* ***17***, 1978-1983.
14. Yu, X., and Michaels, S. D. The *Arabidopsis* Paf1c complex component CDC73 Participates in the modification of *FLOWERING LOCUS C* chromatin, *Plant Physiol.* ***153***, 1074-1084.
15. Giresi, P. G., Kim, J., McDaniell, R. M., Iyer, V. R., and Lieb, J. D. (2007) FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) isolates active regulatory elements from human chromatin, *Genome Research* ***17***, 877-885.

Figure captions

Figure 1 Work flow of chromatin immunoprecipitation (ChIP).

Figure 2 DNA fragmentation by sonication. a, before sonication; b, after sonication, using BioRupter (Diagenode) at LOW setting for 3 pulses of 5 min each, laddered using HyperLadder I (Bioline). Arrows indicate DNA.

Figure 3 Detection of H3K27me3 on *Arabidopsis* seedlings. a, H3K27me3 in vernalized seedlings. Data are presented as (ChIP H3K27me3/ChIP H3) *100%. *FLC* distal promoter quantified using primers 5'-atccagaaaagggcaaggag-3' and 5'-cgaatcgattgggtgaatg-3'; *FLC* locus quantified using primers 5'-ctttttcatgggcaggatca-3' and 5'-tgacatttgatcccacaagc-3'; *SHOOT MERISTEMLESS* (*STM*) locus quantified using 5'-gcccatcatgacatcacatc-3' and 5'-gggaactactttgttggtggtg-3'. b, H3K27me3/H3 on *FLC* locus (primers 5'-ctttttcatgggcaggatca-3' and 5'-tgacatttgatcccacaagc-3'), before (non-vernalized, NV), during (at the end of 8 weeks vernalization treatment, 8w0) and after vernalization (7 days post vernalization, 8w7). Data are calculated as [(*FLC* ChIP H3K27me3/H3) / (control sequence STM ChIP H3K27me3/H3)] *100%.