Chapter 16

Chromatin Immunoprecipitation for Detecting Epigenetic Marks on Plant Nucleosomes

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Abstract

Due to high resolution and reproducibility, chromatin immunoprecipitation (ChIP) has been used as a standard tool to investigate epigenetic marks including modified histones and specific histone variants (e.g., centromere-specific histone H3, CENH3) in this decade. Here, I describe a sensitive and low-background ChIP protocol for a wide range of plant species.

Key words Chromatin immunoprecipitation (ChIP), Epigenetic marks, Histone modification, Histone variants, CENH3

1 Introduction

Chromatin immunoprecipitation (ChIP) consists of several steps including fixation, isolation of nucleosomes, and immunoprecipitation of the nucleosomes using specific antibodies. Then the precipitated nucleosomes have been analyzed by several different ways including blot hybridization, hybridization on DNA chips (ChIP-chip), real-time quantitative polymerase chain reaction (ChIP-qPCR), or sequencing using next-generation sequencer (ChIP-seq). In the primitive ChIP analyses using chromatins from plant species, precipitated DNA had been analyzed by blot hybridization, but the sensitivity was not so high [1–5]. The current detection methods, ChIP-qPCR and ChIP-seq, are sensitive enough to analyze single-copy DNA sequences even in the plant species having large genomes [6–9]. This increased sensitivity has made ChIP more common in plant science, but there are some difficulties including how to isolate good chromatins from different species and tissues, how to select good antibodies, and how to reduce ChIP background. Here, I describe a ChIP protocol including some special knacks to solve the above problems.

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2 Materials

Prepare all solutions using ultrapure water (e.g., milli-Q water) and analytical grade reagents, and store all reagents at room temperature (unless indicated specially).

2.1 Fixation

1. 10× Phosphate-buffered saline (PBS): Add 800 ml water to a 1 l beaker, and solve 80.0 g NaCl, 2.0 g KCl, 14.4 g Na_2HPO_4, and 2.4 g KH_2PO_4. Adjust pH to 7.4 with 1 M HCl, and make up to 1 l with water.
2. Fixation buffer: Add 100 ml of 1× PBS in a 200 ml glass bottle. Add 1 g paraformaldehyde in the bottle and, incubate at 80 °C to solve paraformaldehyde. Add 200 μl of Triton-X100, and store at 4 °C. Use within a week.
3. A vacuum pump.
4. 1 M Glycine solution: Add 80 ml water to a 100 ml beaker. Solve 7.5 g glycine and make up to 100 ml with water.
5. Paper towels.
6. Liquid nitrogen.

2.2 Isolation of Nucleosome

1. A mortar and pestle.
2. 1 M Tris–HCl pH 7.5: Add 800 ml water to a 1 l beaker, and add 121.1 g Tris base and 60 ml 12 M HCl. Adjust pH to 7.5 with 1 M HCl, and make up to 1 l with water.
3. 1 M $CaCl_2$ solution: Add 80 ml water to a 100 ml beaker. Solve 11.1 g $CaCl_2$. Make up to 100 ml with water.
4. 1 M $MgCl_2$ solution: Add 80 ml water to a 100 ml beaker. Solve 20.3 g $MgCl_2 \cdot 6H_2O$, and make up to 100 ml with water.
5. Protease inhibitor solution: Available as tablets (e.g., cOmplete™ ULTRA tablets, Sigma-Aldrich, St. Louis, MO, USA). Solve one tablet to 1 ml water, and store at −20 °C.
6. Phenylmethanesulfonyl fluoride (PMSF) solution: Solve 17 mg phenylmethanesulfonyl fluoride into 10 ml isopropanol.
7. TBS with protease inhibitors: Add 800 ml water to a 1 l beaker, and add 10 ml 1 M Tris–HCl pH 7.5, 3 ml 1 M $CaCl_2$, and 2 ml 1 M $MgCl_2$, and make up to 1 l with water. Add 1 ml/l protease inhibitor solution and 1 ml/l PMSF solution before use.
8. Miracloth (Merck Millipore, Billerica, MA, USA).
9. 12% (w/v) Sucrose in TBS with protease inhibitors: Add 80 ml TBS with protease inhibitors to a 100 ml beaker. Solve 12.0 g sucrose, and make up to 100 ml with TBS with protease inhibitors.
10. MNase digestion buffer: Add 80 ml water to a 100 ml beaker. Add 5 ml 1 M Tris–HCl pH 7.5, 100 μl 1 M $CaCl_2$, and 400 μl 1 M $MgCl_2$ into the water. Make up to 100 ml with water.

11. 1 U/μl MNase solution: Add 50 μl water in a bottle containing 50 U nuclease, micrococcal (Sigma-Aldrich, #N5386-50UN). Mix gently to solve all powder in the bottle. Transfer the solution into a 1.5 ml tube. Store at –20 ° C.
12. 0.1 U/μl MNase solution: Dilute 1 U/μl MNase solution ten times by water. Store at –20 ° C.
13. 0.5 M EDTA (pH 8.0): Add 800 ml water to a 1 l beaker. Add 186.1 g disodium ethylenediaminetetraacetate $2H_2O$ and 20.0 g NaOH to the water. Adjust pH to 8.0 with NaOH. Make up to 1 l with water.
14. Lysis buffer: Add 100 μl 1 M Tris–HCl to 100 ml water. Add 1 ml/l protease inhibitor solution and 1 ml/l PMSF solution before use.

2.3 Chromatin Immunoprecipitation

1. 1 M NaCl: Add 80 ml water to a 100 ml beaker. Solve 5.8 g NaCl into the water. Make up to 100 ml with water.
2. Incubation buffer: Add 80 ml water to a 100 ml beaker. Add 2 ml 1 M Tris–HCl pH 7.5, 1 ml 0.5 M DETA pH 8.0, and 5 ml 1 M NaCl into the water. Make up to 100 ml with water. Add 1 ml/l protease inhibitor solution and 1 ml/l PMSF solution before use.
3. Normal sera of rabbit or mouse (e.g., MP bio, Santa Ana, CA, USA #55989).
4. Anti-histone H3 antibody (e.g., Abcam, Cambridge, UK #ab1791) or anti-histone H4 antibody (e.g., medical and biological laboratories, Nagoya, JAPAN #MABI0400).
5. Antibodies for your interested modifications.
6. Dynabeads® Protein A or G (Thermo Fisher Scientific, Waltham, MA, USA).
7. Magnetic tube stands.
8. Wash buffer: Add 80 ml water to a 100 ml beaker. Add 5 ml 1 M Tris–HCl pH 7.5, 2 ml 0.5 M DETA pH 8.0, and 5 ml 1 M NaCl, and make up to 100 ml with water.
9. 10% (w/v) SDS solution: Add 80 ml water to a 100 ml beaker. Solve 10.0 g sodium dodecyl sulfate (SDS), and make up to 100 ml with water.
10. Elution buffer: Add 80 ml water to a 100 ml beaker. Add 2 ml 1 M Tris–HCl pH 7.5, 1 ml 0.5 M DETA pH 8.0, 5 ml 1 M NaCl, and 10 ml 10% (w/v) SDS, and make up to 100 ml with water.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Fixation

This fixation steps are not necessary for native-ChIP (*see* **Note 1**).

1. Soak 1–10 g fresh plant materials (*see* **Note 2**) in 10–100 ml the fixation buffer.
2. Vacuum (−50 kPa) for 1 min and release it to infiltrate the fixation buffer.
3. Stir it for 10 min.
4. Add 1 M glycine (one-quarter volume of the fixation buffer) to stop the fixation.
5. Stir it for 5 min.
6. Remove the buffer.
7. Add 1× PBS (two volume of the fixation buffer).
8. Stir it for 5 min at 4 °C.
9. Repeat **steps 6–8** twice (three times in total).
10. Dry briefly the materials by blotting with paper towels.
11. Freeze in liquid nitrogen.
12. Store at −80 °C.

3.2 Isolation of Nucleosome

1. Grind the frozen plant material to a fine powder using a mortar and pestle in liquid nitrogen.
2. Suspend the powder in 100 ml of TBS with protease inhibitors.
3. Filter the suspension through Miracloth into 50 ml tubes.
4. Spin the tube at 600 × *g* for 10 min at 4 °C.
5. Remove supernatant by decantation.
6. Suspend the pellet in 50 ml of 12% (w/v) sucrose in TBS with protease inhibitors.
7. Spin the tube at 600 × *g* for 10 min at 4 °C.
8. Remove the supernatant by decantation.
9. Suspend the pellet in 3 ml of MNase digestion buffer (*see* **Note 3**).
10. Transfer the suspension into three separate 1.5 ml tubes.
11. Add 0.5, 1, and 3 μl of MNase solution to the three tubes, respectively (*see* **Note 4**).
12. Incubate the tubes at 37 °C for 10 min.
13. Add 15 μl of 0.5 M EDTA to stop digestion.
14. Spin the tubes at 6000 × *g* for 1 min.
15. Transfer supernatants into new 1.5 ml tubes.
16. Keep the supernatants on ice.
17. Suspend the pellet at **step 14** in 1 ml of lysis buffer.
18. Mix these suspensions by vortex mixer for 1 h at 4 °C.
19. Spin the tubes at 6000 × *g* for 1 min.

20. Transfer supernatants into new 1.5 ml tubes.
21. Keep the supernatants on ice.
22. Pick up 50 μl each of the supernatants at **steps 16** and **21**.
23. Incubate the picked supernatants at 65 °C for at least 4 h to remove the cross-link (*see* **Note 5**).
24. Purify DNA from the suspension by phenol/chloroform extraction and ethanol precipitation or DNA purification kits (e.g., Qiagen).
25. Check amounts and sizes of the nucleosome by electrophoresis with 2% (w/v) agarose gel using a half of the purified DNA.

3.3 Immunoprecipitation of Nucleosomes

1. Transfer supernatants at **steps 16** and/or **21** of Subheading 3.2 containing optimum size of nucleosomes for your purpose (*see* **Note 6**) into a 15 ml tube.
2. Add the same volume of incubation buffer.
3. Transfer equal volume of the mixture into a set of 1.5 ml tubes (*see* **Note 7**).
4. Add 2–5 μl of normal sera or antibodies per tube (*see* **Note 8**).
5. Mix these at 4 °C overnight.
6. Add 10 μl of equilibrated Dynabeads Protein A or G per tube (*see* **Note 9**).
7. Mix these for 30 min (*see* **Note 10**).
8. Capture the beads by magnet, and remove supernatant.
9. Add 1 ml of wash buffer.
10. Mix these for 10 min.
11. Capture the beads by magnet, and remove supernatant.
12. Repeat **steps 9–11** twice (three times in total).
13. Suspend the beads in 200 μl of wash buffer.
14. Transfer the suspensions into new 1.5 ml tubes (*see* **Note 11**).
15. Capture the beads by magnet, and remove supernatant.
16. Suspend the beads in 100 μl of elution buffer.
17. Incubate at 65 °C for 4–16 h to remove the cross-link (*see* **Note 5**).
18. Mix these by vortex mixer for 1 min.
19. Capture the beads by magnet, and transfer the supernatants into new 1.5 ml tubes.
20. Purify DNA from the supernatants by phenol/chloroform extraction and ethanol precipitation with glycogen or DNA purification kits (e.g., Qiagen).
21. Solve the purified DNA into 20 μl each of TE.
22. Store 4at −20 °C.
23. Use 0.5 μl/tube of the DNA solutions for qPCR.

4 Notes

1. In general, the native ChIP (ChIP without fixation) does not include fixation artifacts (e.g., misfixed unrelated chromatins). Since bindings between histones and DNA in nucleosomes are strong, only intact nucleosomes precipitate in the native ChIP. Though epigenetic marks (including methylations and acetylations of histones and specific histone variants) are detectable in the native ChIP, some modifications are unstable and not easily detected in the native ChIP. If you would encounter such difficulties perform fixation before ChIP. Also, if your target is not involved in nucleosomes (e.g., transcription factors), fixation is necessary.
2. Amounts of plant materials for ChIP are related to many different factors (genome sizes of the plants, abundance of target sequences, efficiencies of antibodies, detection methods, etc.). For example, theoretically, one hundred times more nucleosomes are required for onion (genome size = 16 Gb) than *Arabidopsis thaliana* (genome size = 160 Mb). Use 1–10 g of the plant materials for pilot experiments.
3. For isolating nucleosomes, there are two different ways, MNase digestion and sonication. In general, MNase digestion is milder than sonication, because sonication possibly damages the nucleosome complex. The MNase digestion produces distinct ladder patterns composed of mono- to the integer polymer nucleosomes in electrophoreses, whereas sonication usually produces unclear and smear pattern (Fig. 1). Especially, only MNase digestion can produce monomers without linker DNA. However, MNase may have selectivity for DNA sequences.
4. Since nucleosomes of Poaceae species are more sensitive than other species, use 0.1 U/μl MNase solution for Poaceae species, but 1 U/μl MNase solution for others. Digestion efficiency is affected by purity of the nucleosome suspension, so prepare three different concentrations of the enzyme to get optimum nucleosomes for your ChIP (*see* **Note 6**).
5. For the native ChIP, this step is not necessary.
6. Nucleosomes containing more than monomer are easier to precipitate than monomeric nucleosomes, but these make ChIP results unclear. For example, ChIP using only monomeric nucleosomes clearly determines status of the position about 200 bp resolutions, and the resolutions are low (600 bp for dimer and 1 kb for trimer) in ChIP using longer nucleosomes (Fig. 2). On the other hand, qPCR using DNA from monomeric nucleosomes are more difficult than using DNA from longer nucleosomes (Fig. 3). For example, when you

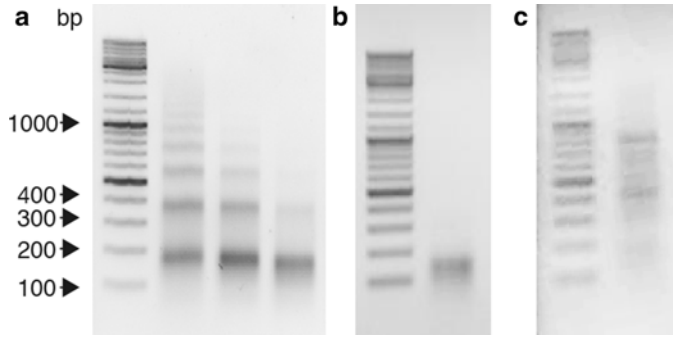

Fig. 1 Quality and quantity check of nucleosomes by electrophoresis. Inverted images of electrophoreses of DNA from isolated nucleosomes using 2% (w/v) agarose gel. (**a**) A serial dilution of MNase digestions. (**b**) DNA from mono-nucleosomes produced by an MNase digestion. (**c**) DNA from nucleosomes produced by a sonication

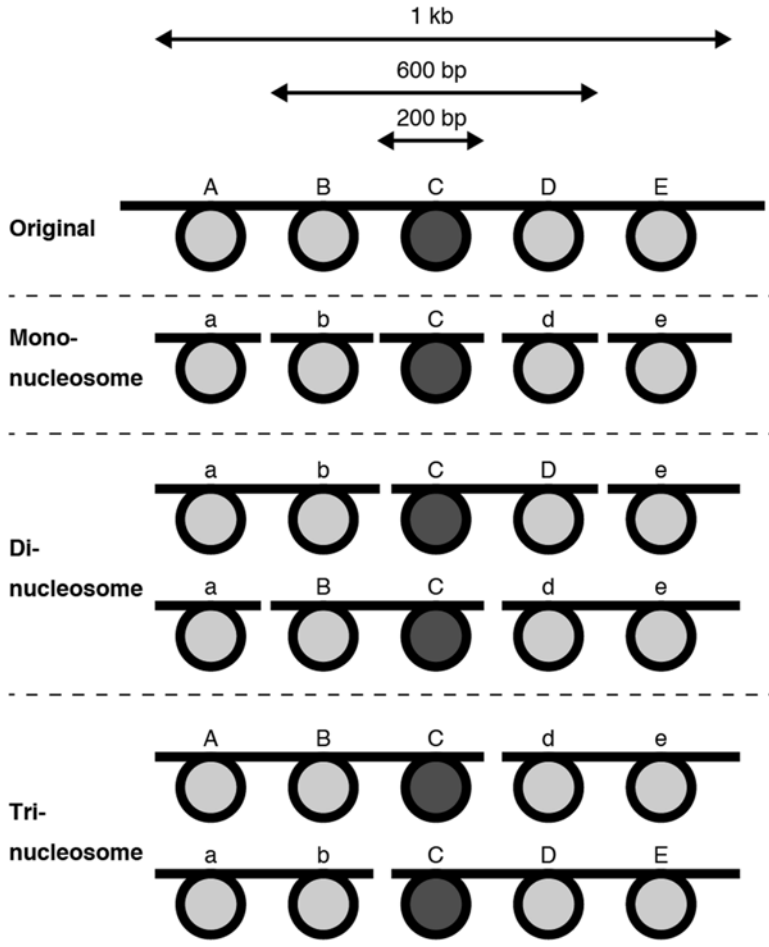

Fig. 2 Resolutions of ChIP-qPCR with mono-, di-, and tri-nucleosomes. PCR sites are indicated on fragments alphabetically. *Capital* and *lowercase letters* indicate PCR positive and negative, respectively. A nucleosome with *dark color* has a target epigenetic mark. In ChIP-qPCR with mono-nucleosomes, only the site C is positive, and there is no false positive. In ChIP-qPCR with di-nucleosomes, the sites B and D are false positive. In ChIP-qPCR with tri-nucleosomes, the sites A, B, D, and E are false positive

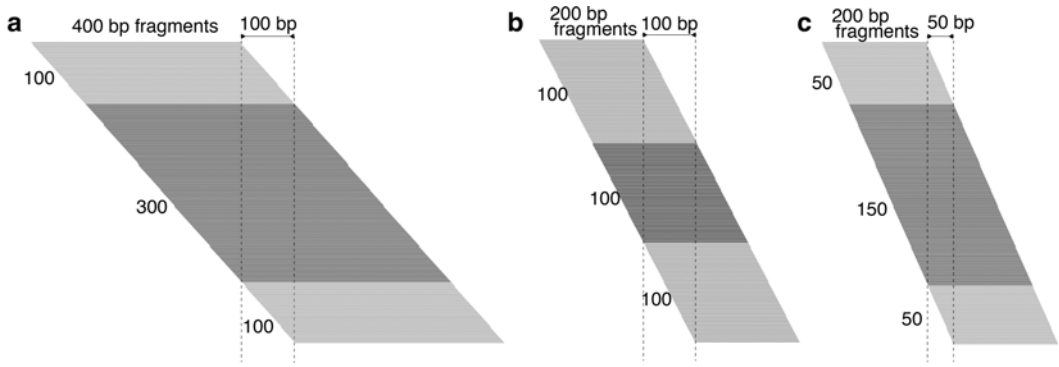

Fig. 3 Usable template contents in DNA pools from mono- and di-nucleosomes. *Arrowheads* indicate primer sites. DNA fragments containing both or one of the primers are indicated as *black lines* or *gray lines*, respectively. (**a**) A 100 bp PCR product and DNA pool from di-nucleosomes. (**b**) A 100 bp PCR product and DNA pool from mono-nucleosomes. (**c**) A 50 bp PCR product and DNA pool from mono-nucleosomes

amplify a 100 bp PCR product using a DNA pool containing 400 bp fragments from di-nucleosome, 60% of the fragments are estimated to act as templates (Fig. 3a). However, when you amplify corresponding to a 100 bp PCR product using a DNA pool containing 200 bp fragments from mono-nucleosome, only 33% of the fragments containing the region act as templates (Fig. 3b). To get similar sensitivity for the case using a DNA pool containing 400 bp fragments and 100 bp PCR products, you should design a primer set to amplify 50 bp fragment for the mono-nucleosome (Fig. 3c).

7. Prepare at least three tubes for negative control, positive control, and antibodies of your interest for statistic tests.
8. Use normal sera of rabbit or mouse as a negative control, and anti-histone H3 or anti-histone H4 antibodies as a positive control for modified histones. The amounts of antibodies depend on quality and concentration of the antibodies and amount of target modifications on nucleosomes. Use 2–5 μl each of antibodies for pilot experiments. I strongly recommend to check the quality of antibodies by immunostaining before use, because the quality of antibodies is dependent on the product and lot. *Arabidopsis* nuclei are a good material for checking the qualities of antibodies against modified histones (Fig. 4).
9. The magnetic beads drastically reduce the ChIP background compared with the agarose or sepharose beads, probably because the magnetic beads have less nonspecific binding to nucleosomes and DNA. Since binding specificities of protein A and G to the antibodies from different animals are different, select beads optimum for the antibodies. Transfer enough

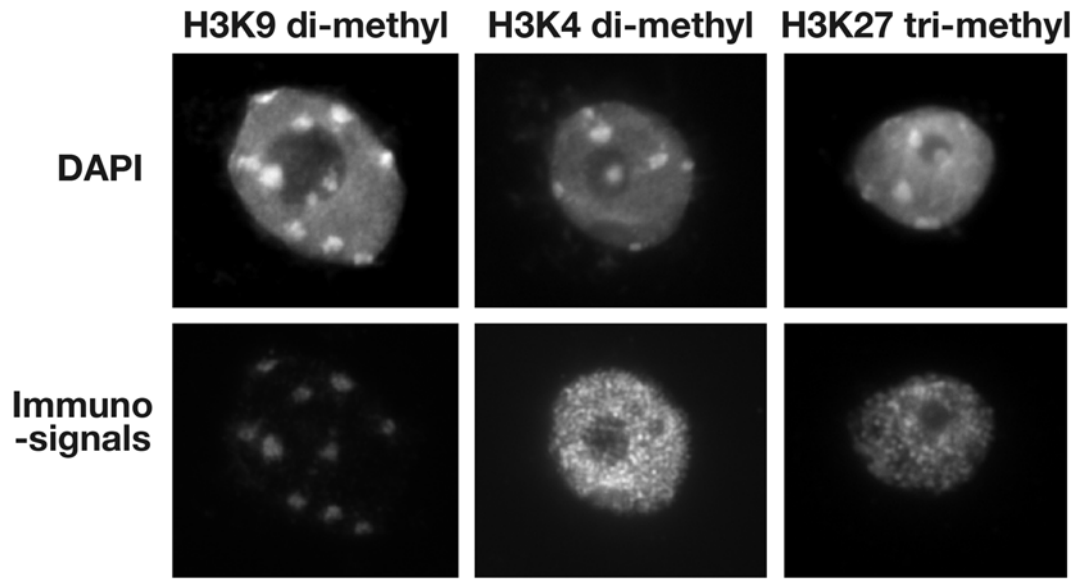

Fig. 4 Typical images of immunostaining for *Arabidopsis* nuclei using anti-modified histone antibodies. Basically, heterochromatic modifications (e.g., H3K9 di- or tri-methyl) are observed on chromocenters in interphase nuclei, but euchromatic modifications (e.g., H3K4 di- or tri-methyl) are observed out of chromocenters in interphase nuclei. However, a heterochromatic modification specific for open reading frames of inactive genes, H3K27 tri-methyl, shows similar pattern to the euchromatic modification on the nuclei

amount (10 μl/tube) of protein A or G Dynabeads into a 1.5 ml tube, and wash the beads three times using 1 ml of incubation buffer. Then, suspend the equilibrated beads into the original volume of Incubation buffer.

10. Do not precipitate the beads through the incubation. It reduces binding efficiency of the bead to antibodies.
11. This is a very important step to avoid contamination of non-specifically bounded nucleosomes on tube wall.

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