

Chromatin Immunoprecipitation Assays

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Summary

Chromatin immunoprecipitation (ChIP) is a powerful tool to study protein–DNA interaction and is widely used in many fields to study proteins associated with chromatin, such as histone and its isoforms and transcription factors, across a defined DNA domain. Here, we show the step-by-step methods currently used in our lab to immunoprecipitate the formaldehyde crosslinked chromatin and further analyze the immunoprecipitated DNA by semiquantitative PCR.

Key Words: Chromatin; immunoprecipitation; semiquantitative PCR.

1. Introduction

Chromatin immunoprecipitation (ChIP) assay refers to a procedure used to determine whether a given protein binds to a specific DNA sequence *in vivo*; in so doing, it allows one to determine the chromatin architecture of specific DNA sequences. Two approaches that differ primarily in how the chromatin is prepared have been employed. The first uses native chromatin prepared by standard micrococcal nuclease digestion of nuclei and is referred to as nChIP (1,2). This method is used for the study of proteins that bind to DNA with high affinity, such as histones and their modified isoforms. The second method uses crosslinked chromatin prepared by adding formaldehyde to cells or exposing the cells to ultraviolet (UV) irradiation; the chromatin is then fragmented to small sizes by sonication (3–6). This procedure is referred to as xChIP. This method is the only option when one is interested in proteins that bind to DNA with lower affinity, including most of the nonhistone proteins.

Among various crosslinking agents, formaldehyde (HCHO) is the most commonly used. It was shown that formaldehyde efficiently crosslinks protein–DNA, protein–RNA, and protein–protein *in vivo* by interacting between the amino and imino groups of lysine, arginine, and histidine and those of DNA

bases (7,8). Furthermore, the chromatin structure is faithfully preserved by HCHO treatment, and the crosslinks can be readily reversed under mild conditions (3). This technique was first applied to study DNA binding proteins (6), and then its applications were broadened to include analysis of general transcription factors and protein complexes associated with chromatin remodeling and high-resolution mapping (9,10). Recent advances include combining the ChIP assay with DNA microarray or cloning techniques to identify novel target genes or the DNA binding site of selected proteins in the global genome environments (11,12). **Figure 1** outlines the main procedures of the xChIP assay. Briefly, living cells are first fixed by HCHO, and the crosslinked chromatin is then sheared and solubilized by sonication. This is followed by the selective immunoprecipitation of protein–DNA complexes utilizing specific protein antibodies. The crosslinks are then reversed, and the immunoprecipitated DNA is analyzed.

2. Materials

1. Cells to be tested.
2. Medium and supplements appropriate for the cells to be studied.
3. 37% Formaldehyde.
4. 2.5 M Glycine.
5. IB buffers: 10 mM Tris-HCl, pH 8.0, 3 mM CaCl₂, 2 mM MgCl₂, 1% NP-40.
6. Phosphate-buffered saline (PBS): 8 g NaCl, 0.2 g KCl, 1.15 g Na₂HPO₄ · 7H₂O, 0.2 g KH₂PO₄.
7. ChIP lysis buffer: 50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate.
8. Complete protease inhibitor cocktails.
9. Branson 450 Sonifier.
10. Antibodies against proteins of interest.
11. Salmon sperm DNA/Protein-A agarose beads.
12. ChIP lysis buffer (high salt): lysis buffer containing 500 mM NaCl.
13. LiCl/detergent solution: 10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA.
14. TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0.
15. DNase-free RNase A.
16. ChIP elution buffer: 1% sodium dodecyl sulfate (SDS), 0.1 M NaHCO₃ (needs to be made fresh).
17. 10X Proteinase K buffer: 0.1 M Tris-HCl, pH 7.8, 50 mM EDTA, 5% sodium dodecyl sulfate (SDS).
18. 20 µg/µL Proteinase K in H₂O.
19. Ethanol.
20. Phenol/chloroform/isoamyl alcohol (25:24:1).
21. 3 M NaAc, pH 4.8.
22. Primers to amplify genes of interest.

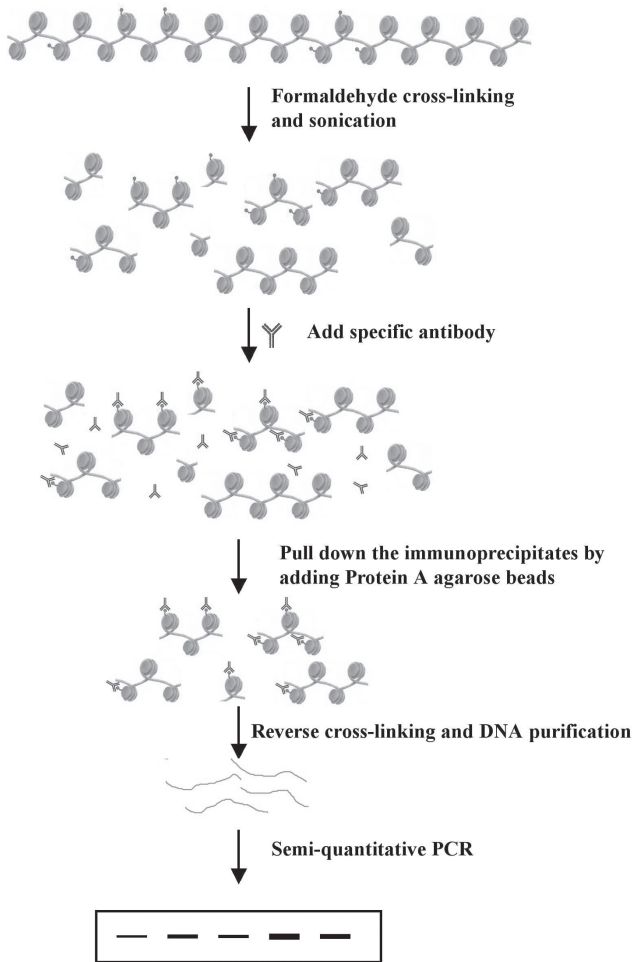


Fig. 1. Schematic procedures of chromatin immunoprecipitation (ChIP) assay. PCR, polymerase chain reaction.

23. 2.5 mM dNTP: 2.5 mM each deoxynucleoside triphosphate, dATP, dTTP, dGTP, and dCTP.
24. [α - 32 P]dATP or [α - 32 P]dCTP.
25. *Taq* DNA polymerase.
26. 10X *Taq* buffer: 100 mM Tris-HCl, pH 8.3, 15 mM $MgCl_2$, 500 mM KCl.
27. Thermocycler.
28. Agarose gel electrophoresis apparatus.
29. Ethidium bromide.
30. 6X DNA sample buffer: 0.25% bromophenol blue and 40% sucrose in water.

31. Vertical electrophoresis apparatus.
32. 30% Acrylamide/*bis*-acrylamide (37.5:1) solution.
33. 1 L: 5X TBE: 54 g Tris base, 27.5 g boric acid, and 20 mL 0.5 M EDTA (pH 8.0).
34. 10% Ammonium persulfate.
35. TEMED (*N,N,N,N*-tetramethylethylenediamine).
36. Kodak X-ray film or PhosphorImager system.

3. Methods

The methods described below outline (1) the preparation of soluble chromatin extract, (2) immunoprecipitation of crosslinked chromatin and (3) analysis of immunoprecipitated DNA by semiquantitative polymerase chain reaction (PCR).

3.1. Preparation of Soluble Chromatin Extract

3.1.1. Formaldehyde Crosslinking Protein–DNA Complexes *In Vivo*

1. Grow cells in appropriate medium and supplements.
 - a. Collect cells by trypsinization followed by low-speed centrifugation.
 - b. Resuspend 2.5×10^8 cells in 30 mL media (without serum) in a 50-mL conical tube.
 - c. In a fume hood, add 0.81 mL of 37% formaldehyde solution directly to the cell suspension to a final concentration of 1%.
 - d. Incubate the mixture at room temperature for 5–15 min, with occasionally shaking (*see* **Notes 1** and **2**).
 - e. The cell numbers used here are enough for at least four immunoprecipitation.
2. Add 1.5 mL of 2.5 M glycine to a fixed cell suspension, arriving at a final concentration of 0.125 M to quench the crosslinks, and then incubate at room temperature for 5 min, with occasional shaking.
3. Centrifuge cells (5 min at 2000 rpm) and discard the supernatant. Wash the cells twice with 20–30 mL of ice-cold PBS, collect cells by centrifugation, and discard the supernatant. Maintain cells on ice (If you are collecting many samples, they may be frozen at -80°C).

3.1.2. Solubilization of Chromatin by Sonication

1. Resuspend cell pellet gently with 1 mL of ChIP lysis buffer supplemented with complete protease inhibitor cocktails and incubate on ice for 30 min. Transfer lysate to round-bottomed 3.5-mL Nunc cryotube vials (*see* **Note 3**). Put cryotube vials in glass beakers filled with crushed ice.
2. Using a Branson 450 Sonifier with a standard tip set at 40–50% output, 90% duty cycle; sonicate extracts for 8 s six times. In between pulses, let samples sit on ice for at least 2 min. This should shear chromatin to a final average size of 500–1000 bp (*see* **Fig. 2** and **Note 4**).
3. Centrifuge samples at the maximum speed for 15 min at 4°C . Transfer supernatant to a fresh 1.5-mL microcentrifuge tube and centrifuge samples again for 15

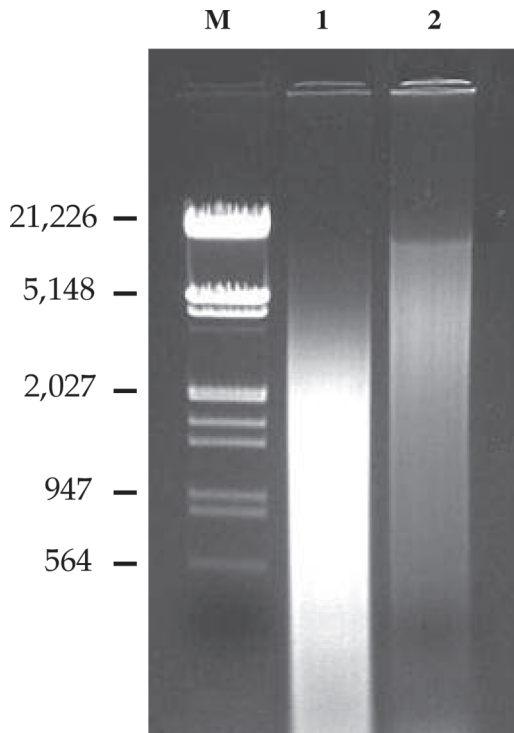


Fig. 2. The average size of the sheared DNA is dependent on the extent of crosslinking. *M* molecular weight markers; lane 1, chromatin with a bulk size of 1 kb; lane 2, the chromatin DNA has an average size of 2–5 kb, and a large size of chromatin DNA (20 kb) is noticed, indicating the chromatin is overcrosslinked.

min at the maximum speed at 4°C. Collect the supernatant, which now contains the chromatin extract.

4. Add 80 μ L salmon sperm DNA/protein A agarose beads to the cell lysate to preclear the chromatin extract, and incubate on a rotation wheel for 60 min at 4°C. Centrifuge samples at 8000 rpm for 2 min, and then transfer the supernatant to a fresh tube.

3.2. Immunoprecipitation and Purification of Crosslinked Chromatin

3.2.1. Immunoprecipitation of Crosslinked Chromatin

1. For quantifying the chromatin DNA, a fraction of the chromatin extract, usually 20–50 μ L, was used to purify DNA using methods described in **Subheading 3.2.2.** or a DNA purification kit. The chromatin used for immunoprecipitation is standardized using the respective amount of DNA.
2. Add the primary antibody against the protein of interest to 300–400 μ g chromatin extract (as DNA). Bring the volume to 500 μ L with cold ChIP lysis buffer

supplemented with protease inhibitor cocktails. Rotate the reaction at 4°C for 4 h to overnight (see **Notes 5** and **6**).

3. Equilibrate the salmon sperm DNA/protein A beads (see **Note 7**) with ChIP lysis buffer. Rotate at 4°C for 1 h.
4. Add 30 μ L bed salmon sperm DNA/vol protein A agarose beads. Incubate on a rotating wheel for 1–3 h at 4°C. Centrifuge sample for 2 min at 8000 rpm at 4°C.
5. Keep 50 μ L of supernatant, bring the volume to 500 μ L with TE, add 20 μ L 5 M NaCl, and discard the remainder. This is the unbound fraction. (If needed, a portion of 10% of that used for immunoprecipitation is saved as input fraction.) This will be needed as the input control for subsequent analyses.
6. Add 1 mL ChIP lysis buffer supplemented with the protease inhibitor cocktails to the beads to wash immunoprecipitates, incubate on ice for 5 min, with occasional inversion, and then centrifuge at 8000 rpm for 2 min. Discard the supernatant, and repeat this procedure once.
7. Wash the beads with 1 mL ChIP lysis buffer (high salt) supplemented with the protease inhibitor cocktails, and repeat once.
8. Wash the beads with 1 mL LiCl/detergent solution supplemented with protease inhibitor cocktails, and repeat once.
9. Add 1 mL TE to the beads and repeat incubation and centrifugation.
10. Suspend the beads in 200 μ L TE, pH 8.0, containing 20 μ g of DNase-free RNase A. Incubate at 37°C for 30 min. Add 1 mL TE, mix, and collect the beads.
11. Add 250 μ L ChIP elution buffer, mix, and incubate at room temperature for 15 min. Vortex occasionally. Centrifuge briefly, transfer eluate to fresh tube and wash beads with 250 μ L ChIP elution buffer again. Pool the supernatant.

3.2.2. Purification of Immunoprecipitated DNA (IP DNA)

1. Add 20 μ L 5 M NaCl to the 500 μ L eluent. Incubate the eluent and the input or unbound fraction for at least 6 h at 65°C to reverse crosslinks.
2. The immunoprecipitates are removed from the solution with the addition of 1 mL ethanol, followed by incubation at –70°C for at least half an hour. Centrifuge at the maximum speed for 15 min, and wash the pellet with cold 70% ethanol. Centrifuge again, discard the supernatant, and air-dry the pellet.
3. Dissolve the pellet in 178 μ L TE. Add 20 μ L 10X proteinase K buffer and 2 μ L 20 μ g/ μ L proteinase K. Incubate at 50°C for 30 min.
4. Extract with 200 μ L 25:24:1 phenol/chloroform/isoamyl alcohol. Vortex vigorously for 1 min. Separate phases by centrifugation at the maximum speed for 5 min at room temperature. Repeat phenol/chloroform/isoamyl alcohol extraction once and chloroform extraction once. Back extract the organic phases sequentially with 150 μ L TE. Pool the two aqueous solutions.
5. Add 35 μ L 3 M NaAc, pH 4.8, 1 mL 100% ethanol. Precipitate the DNA by centrifugation and wash with 70% ethanol as described.
6. Resuspend IP DNA in 50 μ L TE, and input or unbound DNA in 100 μ L TE, and store at –20°C.

3.3. Semiquantitative PCR of IP DNA

IP DNA can be further analyzed by quantitative PCR, semiquantitative PCR, real-time PCR, or slot-blot hybridization, and other techniques. The steps described below are semiquantitative PCR (*see Note 8*). The final reaction volumes all are 50 μ L.

3.3.1. Optimizing the PCR Conditions

1. Quantify the input or unbound DNA concentration using spectrometer.
2. Add 0.1–0.5 μ g DNA into each 0.5-mL thin-walled PCR tube. The amount of DNA depends on the sensitivity of individual primers and genes amplified; we use higher amounts of DNA for the initial experiment.
3. Add 5 μ L 10X *Taq* buffer, 2 μ L 2.5 mM dNTP, and 2.5 μ *Taq* DNA polymerase.
4. Incubate the samples in a thermocycler. Amplification parameters depend greatly on the primers and the thermocycler used; adjustment of the parameters might be necessary, and typically 25–30 cycles are used.
5. Separate PCR products using agarose gel electrophoresis and visualize with ethidium bromide.
6. If regular 10X *Taq* buffer doesn't work, a PCR Optimizer Kit (Invitrogen) can be used to choose optimal buffer according to the manufacturer's protocol.

3.3.2. Semiquantitative PCR

1. Serial dilute input or unbound DNA, starting from 0.1 μ g/10 μ L. Add 10 μ L to each PCR tube.
2. Add appropriate amount (add $[n+1] \times$ each components for n samples) PCR buffer, primers, 2.5 mM dNTP, [α - 32 P]dATP or [α - 32 P]dCTP, *Taq* DNA polymerase and H₂O to a 1.5-mL Eppendorf tube, mix well, and separate equal amounts (40 μ L) of the mixture into each PCR tube.
3. Incubate the samples in a thermocycler using the conditions decided on above. Separate the PCR products as described in **Subheading 3.3.3**.
4. Analyze the IP DNA in the same fashion, using 2.5–5 μ L as a template. Make sure the signal falls into a linear dose–response range. If not, reduce the template or cycle number. Once the conditions are established, it is not necessary to amplify serial diluted input DNA every time for semiquantitative PCR, but an input DNA control is still needed for normalizing the signal.

3.3.3. Separation of PCR Products Using Native Polyacrylamide Gels

Most commercial vertical electrophoresis devices can be used; we use Bio-Rad Mini-PROTEIN II electrophoresis apparatus.

1. Prepare the glass plates and spacers according to the manufacturer's protocol.
2. Mix 3 mL 5X TBE buffer, 4 mL 30% acrylamide/*bis*-acrylamide (37.5:1) solution, 7.85 mL H₂O, 150 μ L fresh made 10% ammonium persulfate, and 6 μ L TEMED. This gel mixture is enough for casting two 1.5-mm-thick 8% polyacry-

lamide gels using Mini-PROTEIN II electrophoresis apparatus. The components of the mixture can be adjusted according to the different electrophoresis devices and gel concentrations.

3. Fill the space between the two glass plates to the top with the gel mixture, and insert the appropriate comb; we usually use a 10-well comb.
4. Allow the acrylamide to polymerize for at least 60 min at room temperature.
5. Attach the gels to the electrophoresis tank, fill with 1X TBE buffer, and carefully remove the comb.
6. Add 10 μ L 6X DNA sample buffer to the PCR products, load 10 μ L of the sample mixture, and run the gels at 100 V until the marker dyes have migrated the desired distance.
7. Move one glass plate, check that the gel remains attached to the other glass plate, and put a dry 3MM filter paper over the gel; the paper should be bigger than the gel. Detach the gel from the glass by lifting the paper; the gel should stick to the paper evenly. Expose to Kodak X-ray film at -70°C or screen of the PhosphorImager at room temperature. According to our experience, drying the gel is not necessary. Examples of ChIP assay results are given in **Fig. 3**.

4. Notes

1. The extent of crosslinking is critical and depends on the protein of interest. The conditions for crosslinking should be optimized. The concentration of formaldehyde, the length of crosslinking or the temperature of crosslinking should be adjusted for different cell types. Insufficient crosslinking may lead to incomplete fixation, and the average size of DNA fragments is less than 500 bp. Overcrosslinking may result in a substantial loss of material and prevent the production of small chromatin fragments, even by prolonged sonication (**13,14**).
2. A nuclei isolation step can be included before crosslinking to keep the cytoplasmic proteins from interfering. This can be done by resuspending the cells in IB buffers and incubating them on ice for 15 min. For specific experiments, such as exposure of cells to hypoxia, crosslinking should be done as soon as the cells are moved out from the hypoxia culture conditions. Nuclei isolation can still be done after fixation, but a longer incubation time is needed.
3. The sonicator needs to be calibrated to yield the final desired average length of DNA. It is important to adjust the tip immersion depth. Violent motion on the surface and foaming results in a loss of energy. Small volumes of cell suspension in a 1.5 mL Eppendorf tube foam very easily; use a microtip if small volumes of samples are being used. We used a cell suspension of more than 1 mL in a 3.5-mL round-bottomed Nunc Cryotube vials, which reduced the possibility of foaming and possible sample loss. Variables such as processing time and output control settings can all be adjusted to produce optimal results. The addition of microglass beads (0.1–0.5 mm diameter) may improve the shearing efficiency. A ratio of one part glass beads to three to five parts liquid is recommended.
4. The size of the DNA fragments may be critical for high-resolution analysis. If the aim of the experiment is to show binding of a protein to a particular site, or a

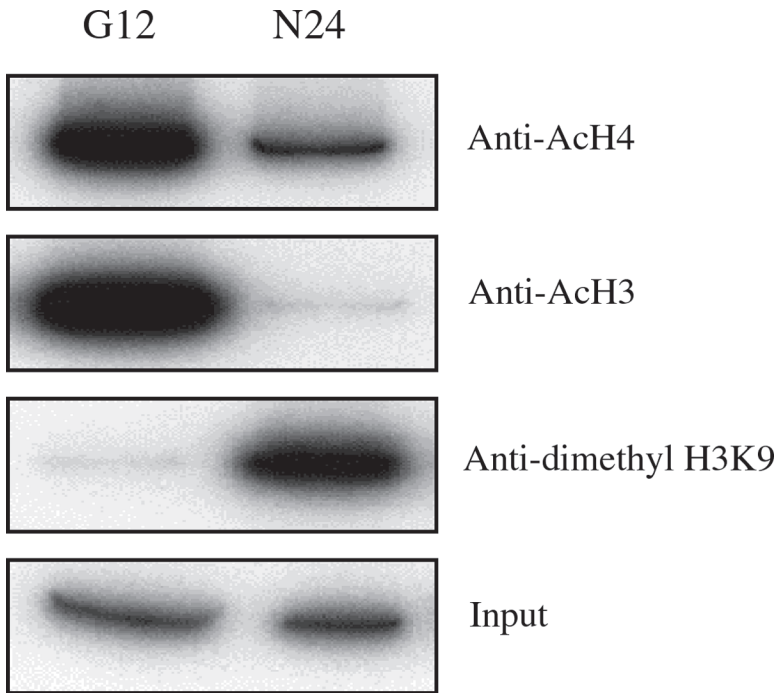


Fig. 3. Crosslinked chromatin of G12 and N24 (a derivate of G12 with an Ni silenced *gpt* gene) cells was immunoprecipitated with antiacetylated H3, antiacetylated H4, and antidimethyl H3K9 antibodies (Upstate Biotechnology). Immunoprecipitated DNA was analyzed by semiquantitative PCR using primers specific for the *gpt* gene (17). Input shows the equal amount of chromatin used. The cycle conditions were 95°C 5 min, then 94°C 30 s, 55°C 30 s, 72°C 45 s for 30 cycles.

specific modification of histone in a particular site, fine tuning of the extent of crosslinking and sonication variables may be required. Another way is to prepare nucleosomes using micrococcal nuclease after mild crosslinking (15,16) or to perform nChIP if the target of interest is histone or its isoforms (1,2). Otherwise, optimizing this parameter is not as important.

5. Preliminary immunoprecipitation experiments should be performed to determine the appropriate amount of antibody to be used. Excess antibodies results in higher overall DNA yields. Normally, 5 µg of antibodies will produce enough IP DNA from 300–400 µg chromatin (as DNA), but a lower specificity of antibody lowers the relative enrichment of IP DNA specifically.
6. It is very important to set a control to avoid nonspecific binding. Affinity-purified antibodies can reduce the amount of non-specific binding, but a mock IP (IP without antibody) is still needed. If polyclonal antisera are used, a control using unimmunized sera from the same species should be included. It is necessary to

include a control using non-crosslinked chromatin extract to avoid nonchromatin proteins and free DNA binding. This control allows one to monitor whether the high salt wash is efficient.

7. Antibodies from different species and different antibody subtypes have different binding properties with beads. When monoclonal antibodies are used, protein A beads are not efficient to bind the antibodies, then protein A/G beads or other specific beads (such as GammaBind [Pharmacia]) should be used.
8. It is essential to test the sensitivity and efficiency of the PCR before analyzing the immunoprecipitated DNA. The signal should be proportional to the amount of template DNA. It is recommended that ^{32}P be added in the reaction, because much less template DNA is then needed and the amount of the PCR product falls more easily into the linear dose–response range. Furthermore, multiple genes can be analyzed simultaneously.

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