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Identification of Transcription Factor–DNA Interactions Using Chromatin Immunoprecipitation Assays

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Abstract

Expression of almost every gene is regulated at the transcription level. Therefore, transcriptional factor Transcription factors, consequently, have marked effects on the fate of a cell by establishing the gene expression patterns that determine biological processes. In the auditory and vestibular systems, transcription factors have been found to be responsible for development, cell growth, and apoptosis. It is vital to identify the transcription factor target genes and the mechanisms by which transcription factors control and guide gene expression and regulation pathways. Compared with earlier methods devised to study transcription factor–DNA interactions, the advantage of the chromatin immunoprecipitation (ChIP) assay is that the interaction of a transcription factor with its target genes is captured in the native context of chromatin in living cells. Therefore, ChIP base assays are powerful tools to identify the direct interaction of transcription factors and their target genes *in vivo*. More importantly, ChIP assays have been used in combination with molecular biology techniques, such as PCR and real time PCR, gene cloning, and DNA microarrays, to determine the interaction of transcription factor–DNA from a few potential individual targets to genome-wide surveys.

Keywords

Transcription factor; target gene; protein–DNA interaction; inner ear; gene expression; chromatin immunoprecipitation; microarray

1. Introduction

The ChIP assay is a very powerful technique used to identify regions of a genome associated with specific proteins including but not limited to transcription factors within their native chromatin context (1). In this technique, intact cells are fixed with formaldehyde to cross-link proteins with their associated DNA. Then, the cells are lysed and chromatin is isolated from the nuclei. After fragmentation by sonication, chromatin fragments, containing the protein of interest and their associated DNA, are selectively precipitated using a specific antibody. Eventually, the sequence identities of the precipitated DNA fragments are determined. ChIP has been coupled with many commonly used molecular biology techniques such as PCR and real-time PCR, gene cloning, and DNA microarray to elucidate DNA–protein interactions (2–4). In a standard ChIP procedure, specific DNA sequences are examined by PCR using gene-specific primers. In this case, one must first presume that a specific promoter might be bound by the protein of interest in order to be able to design promoter-specific primers and/or probes. Therefore, this protocol can only be used to identify known target genes for a given protein. Fortunately, techniques have also been developed to identify unknown DNA sequences interacting with known proteins without requiring prior knowledge of such interactions. ChIP cloning is effective for isolating individual target genes, in which the precipitated DNA

fragments in a ChIP assay are cloned and subsequently sequenced. In contrast, a combination of ChIP and DNA microarray (ChIP-chip) enables the genome-wide surveys of transcription factor target genes (5,6).

2. Materials

2.1. Reagents

1. Phosphate Buffered Saline (PBS): 1. 06 mM KH_2PO_4 , 155. 17 mM NaCl, 2. 97 mM Na_2HPO_4 in ddH₂O, pH 7.4.
2. 100X Protease inhibitor cocktail (cat no. P8340, Sigma-Aldrich, St. Louis, MO.): 104 mM AEBSF [4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride], 0.085 mM aprotinin, 1.53 mM bestatin hydrochloride, 1.40 mM E-64 [N-(trans-Epoxysuccinyl)-L-leucine 4-guanidinobutylamide], 1.90 mM leupeptin hemisulfate salt, 4.22 mM pepstatin in DMSO. Divide into 10 μL aliquots and store at -20°C .
3. 100X Phenylmethanesulfonyl Fluoride (PMSF) solution: Make a 100 mM solution of PMSF in isopropanol. Divide into 10 μL aliquots and store at -20°C . Add PMSF immediately before use since PMSF has a short half-life of ~ 30 min in aqueous solutions.
4. PBS/protease inhibitors (pH 7.4): 1 mM of PMSF and 1X protease inhibitor cocktail in PBS, pH 7.4.
5. 37% Formaldehyde solution (Sigma-Aldrich).
6. 1.25 M Glycine solution in PBS, pH 7.4.
7. Swelling buffer: 5 mM piperazine-N, N'-bis[2-ethanesulfonic acid] (PIPES), pH 8.0, 85 mM KCl, 1% (Octylphenoxy) polyethoxyethanol (IGEPAL[®] CA-630, Sigma-Aldrich); before use, add 1 mM of PMSF and 1X protease inhibitor cocktail.
8. Nuclei lysis buffer: 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS; before use, add 1 mM of PMSF and 1X protease inhibitor cocktail.
9. 5 M NaCl in ddH₂O.
10. 10 mg/mL Proteinase K.
11. 10 mg/mL DNase-free RNase A.
12. Qiaquick PCR Purification kit (cat. no. 28104, Qiagen, Valencia, CA.).
13. Protein A/G Plus Agarose (cat. no. sc-2003, Santa Cruz Biotechnology, Inc., Santa Cruz, CA).
14. IP dilution buffer: 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl; before use, add 1 mM of PMSF and 1X protease inhibitor cocktail.
15. IP washing buffer A: 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl, pH 8.0, 150 mM NaCl; before use, add 1 mM of PMSF and 1X protease inhibitor cocktail.
16. IP washing buffer B: 2 mM EDTA, 0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl, pH 8.0, 500 mM NaCl; before use, add 1 mM of PMSF and 1X protease inhibitor cocktail.
17. IP washing buffer C: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1% Igepal[®], 1% sodium deoxycholate; before use, add 1 mM of PMSF and 1X protease inhibitor cocktail.

18. TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0.
19. IP elution buffer: 50 mM NaHCO₃, 1% SDS; before use, add 1 mM of PMSF and 1X protease inhibitor cocktail.
20. 10 mM Tris-HCl buffer, pH 8.0.
21. 3 M sodium acetate, pH 5.2.
22. Phenol, saturated with Tris-HCl, pH 8.0.
23. Chloroform
24. 100% ethanol and 80% ethanol made in ddH₂O.
25. IgG from rabbit serum (cat no. I5006, Sigma-Aldrich).
26. RNA polymerase II 8WG16 monoclonal antibody (cat. no. MMS-126R, Covance, Princeton, NJ).
27. Liquid nitrogen.

2.2. Equipment

1. Medimachine[™] (BD Biosciences, San Jose, CA) disaggregation system.
2. 50 µL Medicon (BD Biosciences) disposable polyethylene chambers.
3. Eppendorf microcentrifuge 5417R (Eppendorf of North America, Westbury, NY).
4. Eppendorf multipurpose centrifuge 5804 R (Eppendorf of North America).
5. Rotating wheel/platform for mixing.
6. Water bath or heat platform.
7. Spectrophotometer.
8. Thermocycler.

2.3. Other Supplies

1. 2 mL Kontes dounce tissue grinder (VWR International, West Chester, PA).
2. 15 mL polystyrene graduated tubes.
3. 18-gauge blunt needle and 1 mL syringe.
4. Cell scraper.

3. Methods

3.1. Preparation of Cross-Linked Cells

3.1.1. Inner Ear Tissue

1. Dissect and collect cochlear tissue (~100 mg) from the mouse inner ear (*see* Note 1).
2. Snap freeze the tissue in liquid nitrogen and store at −80 °C for chromatin preparation the next day.

¹We typically collect tissues the day before chromatin purification and store the tissue in a −80 °C freezer after it is snap frozen in liquid nitrogen. We typically prepare chromatin from 100 mg of cochlear tissues (~20 mice) and use the chromatin equivalent of 30 mg starting tissue per antibody in every ChIP. The exact amount of tissue has to be determined empirically, depending upon the abundance of a specific transcription factor in the tissue, the quality of the antibody used, and the cross-linking conditions.

3. Thaw tissue sample on ice.
4. Wash tissue once with 1 mL of ice cold PBS. Centrifuge for 1 min at 500 g. Save tissue pellet and discard supernatant.
5. Cut tissue to ~2 mm small pieces and resuspend in 1 mL of PBS/protease inhibitors (*see* Note 2).
6. Cross-link proteins to DNA by adding 27 μ L of 37% formaldehyde to the sample and incubate for 15 min at room temperature with shaking (*see* Note 3).
7. Stop cross-linking by adding 115 μ L of 1.25 M glycine solution to the reaction and incubate for 5 min at room temperature with shaking.
8. Centrifuge tissue sample at 500 g for 1 min at 4 °C and discard the supernatant.
9. Wash tissue once with 1 mL of ice cold PBS/protease inhibitors (*see* Note 4).
10. Resuspend tissue in 1 mL of PBS/protease inhibitors. Transfer the sample to a Medicone and grind the tissue for 2 min using a Medimachine to disaggregate the tissue. Collect cells from Medicone using an 18 gauge blunt needle and a 1 mL syringe (*see* Note 5).
11. Check cell suspension by microscope then centrifuge tube at 500 g for 1 min at 4 °C to collect the cells. The cell pellet can be stored at –80 °C for several months.

3.1.2. Adherent Cells

1. Grow $\sim 3 \times 10^7$ cells in four 75 cm² culture flasks (*see* Note 6).
2. Replace medium with 20 mL of PBS and add 37% formaldehyde directly to flasks to a final concentration of 1%. Incubate cells for 15 min at room temperature with rotation (*see* Note 3).
3. Stop cross-linking by adding 1.25 M glycine to a final concentration of 0.125 M and incubate for 5 min with rotation.
4. Wash cells twice with 20 mL of PBS (*see* Note 4).
5. Scrape cells from culture flasks and transfer to a 15 mL conical tube (*see* Note 7).
6. Rinse cells once with 5 mL of PBS/protease inhibitors (*see* Note 2).
7. Collect cross-linked cells by centrifuging for 5 min at 500 g at 4 °C and discard the supernatant. Cell pellet can be stored at –80 °C for several months.

²PMSF has a short half-life in aqueous solutions. A stock solution of 100 mM in isopropanol should be made and stored at –20 °C and diluted in the buffer immediately before use.

³In general, cross-linking of DNA and proteins is required for non-histone proteins, such as transcription factors, since they have a weaker association with DNA compared with histones. The aim of cross-linking is to stabilize the association of proteins with DNA at the binding site of chromatin. One percent formaldehyde is commonly used for cross-linking in cells/tissues and 15 min of incubation at room temperature works the best in our experiments. Optimal conditions for formaldehyde cross-linking can be determined using a time-course experiment. Excess cross-linking (>30 min) may lead to difficulties in the chromatin fragmenting to the desired size and to a reduction in antigen availability, thereby producing unsatisfactory results in the ChIP assay (7).

⁴Wash tissue or cells thoroughly and try to remove as much solution containing formaldehyde as possible.

⁵We use 50 μ L Medicones and a Medimachine from Becton Dickinson to obtain a single cell suspension.

⁶Use $\sim 1 \times 10^7$ cells per antibody per ChIP. Fewer cells can result in a lower signal-to-noise ratio. Gene expression should be properly induced and cell cultures should be healthy at the time of cross-linking.

⁷A brief incubation with trypsin may help in the release of nuclei from cells that do not swell well. It is important to wash trypsinized cells thoroughly with ice cold PBS before continuing with the rest of the protocol.

3.1.3. Suspension Cells

1. Grow $\sim 3 \times 10^7$ cells and collect cells by centrifugation for 1 min at 500 g. Resuspend cells in 20 mL of PBS in a 50 mL tube.
2. Add 37% formaldehyde directly to cell suspension to a final concentration of 1%. Incubate cells for 15 min at room temperature with rotation (*see* Note 3).
3. Stop cross-linking by adding 1.25 M glycine to the reaction to a final concentration of 0.125 M and incubate for 5 min at room temperature with rotation.
4. Wash cells twice with 20 mL of PBS (*see* Note 4).
5. Resuspend cells in 5 mL of PBS/protease inhibitors and transfer cells to a 15 mL conical tube (*see* Note 2).
6. Collect cross-linked cells by centrifuging for 5 min at 500 g at 4 °C and discard supernatant. Cell pellet can be stored at –80 °C for several months.

3.2. Preparation of Sonicated Chromatin

3.2.1. Chromatin Isolation and Sonication

1. Resuspend cell pellet obtained previously from one of the tissue types (*see* **Sections 3.1.1, 3.1.2, or 3.1.3**) in 1 mL of swelling buffer in a 1.5 mL tube. Incubate on ice for 15 min swirling occasionally to resuspend cells.
2. Dounce cells using a 2 mL B dounce to release nuclei and then centrifuge at 2500 g for 5 min at 4 °C to pellet nuclei (*see* Note 8).
3. Discard supernatant and resuspend nuclei in 1 mL of nuclei lysis buffer. Incubate on ice for 15 min.
4. Transfer the sample to a 15 mL polystyrene conical tube and sonicate cross-linked chromatin to an average size of 200–1000 bp (*see* Note 9).
5. Transfer the sample to a 1.5 mL centrifuge tube and centrifuge at the maximal speed of a microfuge for 15 min at 4 °C.
6. Transfer supernatant to a new 1.5 mL tube and keep it on ice. This supernatant is cross-linked sheared chromatin ready for the ChIP experiment (*see* Note 10). However, before performing ChIP, the size and the concentration of sonicated DNA must be checked (*see* Note 9). Use 10 μ L of this sample to check the quality of the DNA, following the protocol provided in **Section 3.2.2**.

⁸The quality of the nuclei preparation is improved by including an extra step of douncing the cells in swelling solution, which reduces contamination from cytoplasm. The background in ChIP experiments significantly decreases when using chromatin purified from these nuclei.

⁹It is critical to shear chromatin to ~500 bp fragments to pinpoint the DNA binding site of the protein of interest. In general, the larger the fragment size, the more difficult it is to map the exact location of a protein binding site. Optimal conditions for sonication need to be determined empirically to ensure that the majority of chromatin fragments are between ~200–1000 bp. Factors affecting sonication include the volume of the sample, depth of the sonication probe, sonication strength, the duration of sonication, and the condition of cross-linking. One should try to perform sonications under the same conditions to achieve consistent results. We use twelve pulses at 20 s each with a 60 s rest interval at the maximum power of a Bioruptor™ (Wolf Laboratories Limited, Pocklington, York, UK) sonicator. In addition, it is very important to keep samples cold during the entire process.

¹⁰Fresh chromatin usually gives the best ChIP results, even though sonicated chromatin can be stored at –80 °C for a short period of time. In this protocol, sheared chromatin is prepared in nuclear lysis buffer containing 1% SDS, which can be stored at 4 °C for 24–48 h before use without apparent loss of ChIP efficiency.

3.2.2. Quality Check of Sonicated DNA

1. Reverse cross-linking: Mix 10 μ L of chromatin sample obtained from **step 6 of Section 3.2.1** with 40 μ L of IP elution buffer. Add 2 μ L of 5 M NaCl and boil for 15 min. Cool to room temperature (*see* Note 11).
2. Add 1 μ L of 10 mg/mL DNase-free RNase A and incubate for 5 min at room temperature.
3. Add 1 μ L of 10 mg/mL Proteinase K and incubate at 55 °C for 30 min.
4. Purify sonicated DNA fragments using a Qiaquick PCR Purification kit following the manufacturer's instructions and elute DNA in 50 μ L of ddH₂O.
5. Determine concentration of DNA sample using a spectrophotometer.
6. Load ~2–3 μ g of purified sheared DNA on a 1% agarose gel. The sonicated DNA fragments should be a smear between ~200–1000 bp.

3.3. Preclear and Immunoprecipitation (IP)

1. Add 20 μ L of Protein A/G Plus-agarose beads into each sonicated chromatin sample (~1 mL) and rotate for 2 h at 4 °C.
2. Spin at 3000 g for 5 min at 4 °C and transfer supernatant into a fresh tube. This supernatant is precleared soluble chromatin.
3. Divide the sample into 300 μ L aliquots (each aliquot is equivalent to 30 mg of starting tissue and is good for one ChIP experiment) and save 30 μ L of chromatin for the preparation of a DNA sample that is 10% of the total input (*see* Note 12).
4. Add 10 μ g of primary antibody to each 300 μ L aliquot and rotate for 2 h in a cold room (*see* Notes 13 and ¹⁴).
5. Add 40 μ L of Protein A/G Plus-agarose beads per IP sample and incubate overnight with constant rotation at 4 °C.
6. Centrifuge at 3000 g for 5 min at 4 °C and discard the supernatant.
7. Wash beads twice with 1 mL of IP washing buffer A (*see* Note 15).
8. Wash beads twice with 1 mL of IP washing buffer B.
9. Wash beads twice with 1 mL of IP washing buffer C.

¹¹To achieve the best result of reversing cross-linking for ChIP, incubate the sample in 0.2 M NaCl from 4 h to overnight at 65 °C. Incomplete reversal of cross-links will result in loss of material. To check the sonication efficiency, however, a short protocol of boiling for 15 min is sufficient.

¹²This input sample is equivalent to 10% of the starting genomic DNA for each ChIP assay. It is very useful during post-IP DNA analysis as a control for PCR effectiveness and quantitation of different samples.

¹³Antibodies are required in ChIP experiments to capture chromatin regions that contain the protein of interest and their associated DNA. The quality of antibodies is crucial for the recovery of these DNA fragments. The best antibodies are those with high specificity and high affinity, recognizing the epitope after protein-DNA cross-linking. In general, polyclonal antibodies are preferred over monoclonal antibodies to overcome epitope masking. The optimal amount of a specific antibody for the maximum binding of the protein of interest can be determined by performing pilot experiments using increasing amounts of the antibody combined with the cell lysate. However, 10 μ g of primary antibody for chromatin obtained from ~30 mg of tissue or ~10⁷ cells is a good starting concentration for most antibodies.

¹⁴It is crucial to include proper controls. This protocol is designed to prepare enough sheared chromatin sufficient for three ChIP reactions. Typically, we run an experimental ChIP reaction in parallel with a negative control and a positive control. In the negative control reaction, normal rabbit IgG is used as the primary antibody to eliminate any false positive that may be derived from the system. In the positive control, an RNA polymerase II antibody is used to ensure that each step of the ChIP experiment is working.

¹⁵Serial washings with low-salt and high-salt buffers, lithium chloride buffer, and Tris-EDTA buffer are essential to remove non-specific binding. For each wash, resuspend beads in 200 μ L of ice cold buffer by gently tapping the tube and then adding the remaining 800 μ L of solution. Rotate for 10 min and collect the beads by centrifuging at 1000 g for 3 min at 4 °C. Orient the cap hinge of tubes on the outside of the rotor to mark the location of the pellet and then pipette out the wash solution carefully.

10. Wash beads once with 1 mL of TE buffer.
11. Elute antibody/protein/DNA complexes from protein A/G agarose beads. Add 100 μ L of IP elution buffer into each sample, resuspend beads, and incubate for 30 min at room temperature with rotation. Spin down beads and carefully transfer supernatant to fresh tubes. Repeat elution once and combine the two eluates for each sample.
12. Centrifuge samples at the maximal speed of a microfuge for 5 min at 4 °C to remove any remaining beads and transfer supernatant to fresh tubes.

3.4. Cross-Linking Reversal and DNA Purification

1. Add 4 μ L of 5 M NaCl to the chromatin solution (200 μ L) to a final concentration of 0.2 M. In parallel, thaw 10% of the total input sample obtained from **step 3 of Section 3.3** (30 μ L) and supplement with 170 μ L of IP elution buffer. Add 4 μ L of 5 M NaCl to the input sample as well. Heat samples 65 °C from 4 h to overnight to reverse the cross-linking (*see Note 11*) and then cool to room temperature.
2. Add 1 μ L of 10 mg/mL DNase-free RNase A and incubate for 30 min at 37 °C.
3. Add 2 μ L of 10 mg/mL Proteinase K and incubate for 2 h at 55 °C and then cool samples to room temperature (*see Note 16*).
4. Add 200 μ L of phenol to each sample and shake thoroughly by hand. Spin at the maximal speed of a microfuge for 5 min. Transfer the upper aqueous layer to fresh tubes.
5. Add 200 μ L of chloroform and shake thoroughly by hand. Spin at the maximal speed of a microfuge for 5 min. Transfer the upper aqueous layer to fresh tubes. Repeat this step once and then transfer the upper aqueous layer to fresh tubes. Combine the two samples.
6. Add 20 μ L of 3 M sodium acetate (pH 5.2) and 400 μ L of absolute ethanol, and mix. Incubate samples for 15 min at –80 °C.
7. Spin down at the maximal speed of a microfuge for 15 min at 4 °C. Discard the supernatant.
8. Wash pellet with 500 μ L of 80% ethanol and spin down at the maximal speed of a microfuge for 2 min at 4 °C. Discard the supernatant. Repeat this step once.
9. Vacuum dry the pellet and dissolve each sample in 100 μ L of 10 mM Tris-Cl buffer (pH 8.0).
10. Determine the concentration of the DNA sample using a spectrophotometer.

3.5. Post- Immunoprecipitation Analysis

Some commonly used molecular biology techniques such as PCR and real-time PCR, gene cloning, and DNA microarrays have been used to analyze the precipitated DNA fragments obtained in the ChIP assay. The detailed protocols for these analyses are out of the realm of this chapter. Here, we will only discuss the general strategies of using these methods in post-ChIP DNA analysis.

3.5.1. PCR Amplification—Real-time PCR is the preferred method for DNA quantification over the Southern slot blot, since ChIP usually yields very small amounts of DNA. The association of a specific DNA sequence with a certain transcription factor is determined by the

¹⁶Insufficient proteinase K treatment may cause poor results in the DNA analysis experiments, especially in a ChIP-chip assay.

level of the DNA sequence enrichment in the precipitated DNA compared to the non-precipitated control sample. To get meaningful data, the following control reactions should be included:

1. For PCR effectiveness, use the total input DNA (**step 3 of Section 3.3**) after the cross-links have been reversed, at various dilutions (1%, 2%, etc.).
2. The mock ChIP reaction, which is performed the same way as the experimental ChIP reaction, except in the absence of primary antibody.
3. Most importantly, an internal control specific to a chromatin region that is not enriched in the ChIP reaction has to be included to verify that the observed enrichment of DNA sequences is genuine.

3.5.2. ChIP Cloning—In this technique, the precipitated DNA fragments in a ChIP assay are first blunted using T4 DNA polymerase and then cloned into a vector. The identities of the cloned DNA fragments are determined by subsequent sequencing and DNA sequence data base search. The main advantage of this technique is that it allows the isolation of individual target genes, regardless of whether the target is known or unknown. However, some drawbacks of this technique should be noted. First, nonspecific sequences have an equal chance of being selected in ChIP cloning. To decrease the amount of nonspecific DNA, the precipitated chromatin sample may be re-immunoprecipitated, which usually results in a lower amount of precipitated DNA. PCR amplification has been used to overcome this problem, but it can cause experimental bias as well. It is recommended to pool DNA samples from multiple immunoprecipitation reactions to achieve a better resolution during the analysis of the protein-DNA interaction in ChIP cloning. Second, the size of DNA fragments is critical for ChIP cloning. To reduce the number of non-specific clones, the chromatin should be sonicated to ~1–2 kb fragments and only clones containing DNA fragments of 500 bp or larger should be analyzed (7). This technique can be used to detect a small number of transcription factor target genes.

3.5.3. ChIP-Chip Assay—In this method, the DNA samples are fluorescent-labeled after ChIP and then analyzed on DNA microarray chips. Two color array platforms are commonly used for this purpose. The enriched DNA is usually labeled with a fluorescent molecule, such as Cy5 or Alexa 647, and the input genomic DNA is labeled with a different fluorescent dye, such as Cy3 or Alexa 555. The two probes are then combined and hybridized to a single DNA microarray. The enrichment levels of DNA fragments can be determined while using the input genomic DNA sample as a reference. Ideally, to achieve a comprehensive and unbiased survey of protein-DNA interactions, the DNA microarrays used in ChIP-chip assays should contain elements that represent the entire genome. This method was used successfully in yeast systems to create high-resolution genome-wide maps of DNA-protein interactions *in vivo* (8,9). However, given the high complexity of mammalian genomes, it is technically challenging to design suitable microarrays. Alternatively, CpG island microarrays have been used to investigate genome-wide transcription-DNA (10,11).

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