Chromatin Immunoprecipitation (ChIP) Analysis for Protein-DNA Interactions

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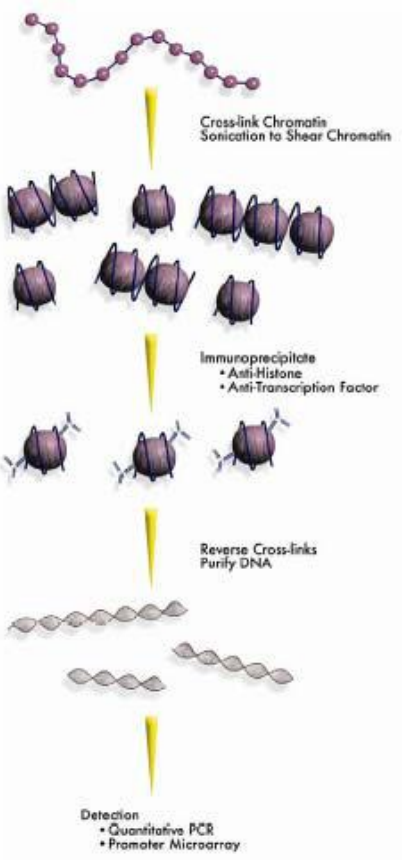
General Background and Considerations



ChIP is a widely used method to identify specific proteins associated with a region of the genome, or in reverse, to identify regions of the genome associated with specific proteins. These proteins can be isoforms of histones modified at a particular amino acid or other chromatin associated proteins. When employed with antibodies that recognize histone modifications, ChIP can be used to "measure" the amount of the modification. An example of this would include measurement of the amount of histone H3 acetylation associated with a specific gene promoter region under various conditions that might alter expression of the gene. Histones are not the only proteins that can be studied using this technique. Much of the recent interest has been in analyzing transcription factor distribution throughout the genome or at specific loci.

When performing ChIP, cells are first fixed with formaldehyde to crosslink proteins to DNA and then chromatin is harvested from the cells and subjected to an immunoselection process, which requires the use of specific antibodies. Any DNA sequences cross-linked to the protein of interest will co-precipitate as part of the chromatin complex. After the immunoselection of chromatin fragments and purification of associated DNA, the detection of specific DNA sequences is performed. If the DNA which will be detected is associated with the protein or histone modification being examined, the relative representation of that DNA sequence will be increased (or enriched) by the immunoprecipitation process.

Generally, standard PCR is performed to identify the DNA sequence (the gene or region of the genome) associated with the protein of interest. The relative abundance of a specific DNA sequence isolated via the protein-specific immunoselection is compared to DNA obtained when using an unrelated antibody control. DNA fragments are run on gels to facilitate quantitation of the PCR products. A much more accurate alternative to standard PCR is real time quantitative PCR (RT-qPCR). Cloning of sequences from a ChIP experiment is also possible, to create libraries of fragments that are enriched for those that interact with a particular protein. The combination of chromatin IP with microarray applications (ChIP on chip) is a novel technique that is becoming more popular, allowing the generation of genome-wide maps of protein-DNA interactions or histone modifications.

References:

*Das PM, et al., Biotechniques **37**: 961-969, 2004*
*Luo, R.X., et al., Cell **92:** 463-473, 1998.*
*Braunstein, M., et al., Mol. Cell. Biol. **16**: 4349-4356, 1996.*
*Manabe, I., et al., J. Clin. Invest. **107**: 823-834, 2001.*
*Cervoni, N., & Szyf, M., J. Biol. Chem. **276:** 40778-40787, 2001.*

Materials and Reagents

hiTE Buffer: 50mM Tris-HCl, 10mM EDTA, pH 7.5.

PBS: 10mM Na_2HPO_4 (dibasic, anhydrous), 2mM KH_2PO_4 (monobasic, anhydrous), pH 7.4, 150mM NaCl, 10mM sodium phosphate.

Formaldehyde: from Fisher Scientific

ChIP Lysis Buffer: 50mM HEPES/KOH pH 7.5, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate. Add protease inhibitors prior to use.

High Salt ChIP Wash Buffer: 50mM HEPES, pH 7.5 by KOH, **500mM NaCl**, 1mM EDTA, 1% Triton X-100,0.1% SDS, 0.1% sodium deoxycholate. Add protease inhibitors prior to use.

Protease Inhibitor Cocktail: ***Complete*** Protease Inhibitor Cocktail tablets (Roche Biochemicals). One tablet per 10ml of PBS or Lysis Buffer.

ChIP Elution Buffer: **hiTE Buffer** containing 1% SDS.

Protein A/G agarose: Sepharose 4B Protein G beads (GE Health Amersham Pharmacia).

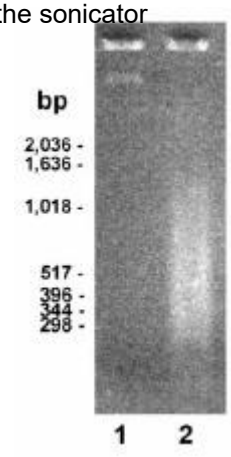
Glycine: 2.5M Glycine.

Sonicator: Fisher Scientific F60 Model

5M NaCl

Part I. Optimization of DNA Shearing

One of the important parameters for ChIP assay is to establish optimal conditions required for shearing cross-linked DNA to 200-1000 base pairs in length. Optimal conditions required for shearing cross-linked DNA to 200-1000 base pairs in length depend on the cell type, cell concentration per lysis buffer and the sonicator equipment, including the power settings and number of pulses.



We are using **Fisher's F60 Sonicator** and our experience shows DNA is sheared to the appropriate length with **12-second pulses** x **4-5** (i.e., keep 1~2min. between pulses; make sure samples are on ice all times) at **80%** of maximum power (i.e., **Setting = 14**). Once sonication conditions have been optimized, keep cell number consistent for subsequent experiments. You're strongly encouraged to optimize the DNA shearing condition using the following **two** methods. Method #1 is simplistic and should be initially used to obtain the preliminary settings for further testing described in Method #2. If you have already obtained some of the sonication conditions, you can directly proceed with Method #2.

Be sure to keep the sample on ice at all times as the sonication generates heat which will denature the DNA and proteins. Check the size of sonicated DNA by gel electrophoresis after reversion of cross-links.

Method #1: Direct use of purified genomic DNA.

1. Add 1~2ug of purified genomic DNA into the 2-5ml microfuge tubes (the # of tubes will depend on how many sonication conditions you want to test). Bring up the volume in each tube to 200ul with ddH2O, Keep samples on ice.
2. Perform sonication (Fisher's **F60** model) by changing either the power settings (e.g., full scale = 20; 80% = 16; and 70% = 14) and/or the number of 10 to 15-second pulses (usually between 3 to 5 times). Please keep samples on ice all time; wait for 1-2 min. between pulses to avoid rapid heating up of the samples.
3. Transfer the sonicated DNA samples to a fresh set of 1.7ml tubes. Perform ethanol precipitation using our regular lab protocol.
4. Resuspend the DNA samples in 10-20ul of ddH2O. Load onto the1.2% ~ 1.5% agarose minigel and run it for 40-60min at 60-70 volts. *Ideally, the center of the DNA smear should migrate along the 500bp-position*.

Method #2: Use of the cross-linked cells.

Note: *Whenever possible, place samples on ice.*

1. Plate C3H10 cells in **one T-75 flask** (in **20ml** complete medium) at 70% confluency at 37C 5% CO2 incubator. It should reach 80-90% confluency overnight, yielding ~1 x 10^7 cells.
2. Crosslink proteins to DNA by adding **540ul** of 37% Formaldehyde directly to the 20ml cells culture medium (at a final concentration of 1% Formaldehyde). Incubate the flask room temperature for 5-10 minutes.
3. Add **1.0ml** of 2.5M Glycine (final concentration 125 mM) to the medium for 10min, at room temperature to quench the formaldehyde.
4. Aspirate medium, removing as much medium as possible. Wash cells using 5 ml of ice cold PBS containing protease inhibitors (i.e., we are using Roche's Complete PI cocktail tablets). ***Note: Add protease inhibitors to PBS just prior to use***.
5. Add **2ml cold** PBS, and scrape cells into a 50-ml conical tube.
6. Pellet cells for 3000 rpm, 5 minutes at 4ºC. Remove PBS and add 2.0 ml **ChIP Lysis Buffer** containing protease inhibitors to lyse cells for 30 min on ice.
7. Prepare multiple 200µl aliquots for sonication. *Note: The 200 ul of ChIP Lysis Buffer is per 2 X 10^6 cells; if more cells are used, the resuspended cell pellet should be divided into 200µl aliquots so that each 200µl aliquot contains ~1 X 10^6 cells*.
8. Sonicate lysate to shear DNA to lengths between 200 and 1000bp being sure to keep samples ice cold (e.g., 80% power ×12sec×4times, between pulses incubate on ice for 1-2min).
9. Recover DNA by phenol/chloroform extraction, ethanol precipitation, wash ×2. Run samples (e.g., 20ul per sample) in 1.5% agarose gel to visualize shearing efficiency.

Part II. Chromatin Immunoprecipitation Protocol

Note: Numerous controls can be set up. Most common ones are ***treated*** (e.g., AdWnt3A) *vs.* ***untreated*** (e.g., AdGFP), and/or ***gene-specific antibody*** (e.g., anti-β-catenin) *vs.* ***non-specific antiserum*** (e.g., a control IgG). Additionally, PCR reactions can be carried out to detect control genomic loci (e.g., GAPDH promoter region).

A. *In Vivo* Crosslinking and Lysis

Prior to starting this section:

- *Obtain ice for incubation of PBS (see Step 3) and for incubating culture dish (see Step 6).*
- *Prepare 1X PBS and put on ice. This will be used for washes and needs to be ice cold.*
- *Warm SDS Lysis Buffer to room temperature to ensure SDS is in solution before proceeding with cell lysis*.

1. Plate C3H10 T1/2 cells in T-75 flasks at 70-80% confluency.
2. Infect cells with an optimal titer of **AdWnt3A** or **AdGFP** in T-75 flasks containing **20ml** of growth media. For C3H10T1/2 cells, there are approximately 1 x **10^7** cells per T-75 flask. This will generate a preparation of chromatin that can be used for up to **5** separate immunoprecipitations per flask.
3. At 36 to 45hrs after infection, add **540µl** of 37% Formaldehyde to **20ml** of growth media (Final concentration is ~1%) to crosslink and gently swirl the flask to mix.
4. Incubate at room temperature for 10 minutes.
5. Meanwhile, remove **10m**l of ice cold 1X PBS to a separate tube for every T-75 flask and add one tablet of the Complete PI tablet. Put on ice.

6. Add **1.0ml** of 2.5M Glycine (final concentration 125 mM) to each T-75 flask to quench excessive Formaldehyde.
7. Swirl to mix and incubate at room temperature for 5 minutes.
8. Aspirate medium as completely as possible, being careful not to disturb the cells.
9. Add **10ml** of cold 1X PBS to wash cells.
10. Remove PBS. Add **1.0ml** cold PBS containing Protease Inhibitor Cocktail. Scrape cells from each flask into a microfuge tube. Spin at 700xg at 4°C for <5 min. to pellet cells.
11. During spin, prepare **~10ml** of **ChIP Lysis Buffer** containing Complete PI Inhibitors Cocktail .
12. Resuspend cell pellet in **1.0ml** of **ChIP Lysis Buffer** with PI cocktail. Cell Density is important for reliable cell lysis. Adjust accordingly if different cell concentrations are desired as the ratio of lysis buffer to cell (for every $1x10^7$ C3H10T1/2 cells,1.0ml of Lysis Buffer is recommended for this protocol).
13. Aliquot between **200µl** per microfuge tube. Lysate can be frozen at -80°C at this step.
14. If optimal conditions for sonication have already been determined, proceed to Section B.

B. Sonication to Shear DNA

Prior to starting this section:
Optimal conditions required for shearing cross-linked DNA to 200-1000bps in length need to be determined as described in Part I.

1. If desired, remove 5µl of cell lysate from Section A, **Step 13** for agarose gel analysis of unsheared DNA. If lysate from Section A, **Step 13** was previously frozen, thaw on ice.
2. Sonicate cell lysate on ice using the conditions optimized in **Part 1**, **Method #2**. Sheared cross-linked chromatin can be stored at -80°C for up to a few months.
3. Spin at 12,000-15,000 x g at 4°C for 10 minutes to remove insoluble material, transfer supernatant into new sterile microtubes in 100µl aliquots. Each **100µl** aliquot contains 2 x 10^6 cell equivalents of lysate which is enough for one immunoprecipitation.

C. Immunoprecipitation (IP) of Crosslinked Protein/DNA

1. Prepare enough **ChIP Lysis Buffer** containing protease inhibitors for the number of desired immunoprecipitations and store on ice.
   - Each IP requires the addition of 900µl of **ChIP Lysis Buffer with PIs**.
   - Samples include Wnt3A vs. GFP-treated; anti-β-catenin vs. mouse IgG control. In some cases, no antibody/IgG controls can also be set up.
2. Prepare one microfuge tube containing 100µl of sheared crosslinked chromatin (Section B, step 3) for the number of desired immunoprecipitations and put on ice. If lysate has been previously frozen, thaw on ice.
   - Alternatively, if multiple immunoprecipitations will be performed from the same lysate preparation, place the entire volume for the number of desired immunoprecipitations in one large tube that will be able to accommodate a volume of 1.1ml for each IP.
   - Each 100µl will contain ~2 x 10^6 cell equivalents of chromatin.
3. Add 400µl of **ChIP Lysis Buffer** into each tube containing 100µl of cell lysate/chromatin.

4. Add 60µl of pre-washed Protein G Agarose for each IP.
   • The Protein G Agarose is **50% slurry**, which should be washed in **ChIP Lysis Buffer** containing PI cocktails twice. Gently mix by inversion before removing.
   • This step serves to "preclear" the chromatin, *i.e.*, to remove proteins or DNA that may bind nonspecifically to the Protein G agarose.
5. Incubate for 1 hour at 4°C with rotation.
6. Pellet agarose by brief centrifugation (3000-5000 x g for 1 minute).
   • Do not spin Protein G Agarose beads at high speeds. Applying excessive g-force may crush or deform the beads and cause them to pellet inconsistently.
7. Remove **10µl** (1%) of the supernatant as **Input** and save at 4°C until **Section D, step 1**.
   • If different chromatin preparations are being carried together through this protocol, remove 1% of the chromatin as Input from each.
8. Collect the supernatant by aliquoting 400-500 µl into fresh microfuge tubes.
9. Add the immunoprecipitating antibody to the supernatant fraction:
   • For **anti-β-catenin**, add **1.0-10µg** of antibody per tube.
   • For the negative control, Normal Mouse IgG, add 1.0µg of antibody per tube.
10. Incubate at 4°C for 2~4 hours with rotation.
    • It may be possible to reduce the incubation time of the IP. This depends on many factors (antibody, gene target, cell type, etc.) and will have to be tested empirically.
11. Add **100µl** of Protein G Agarose (**pre-washed with ChIP Lysis Buffer**) for 1 hour at 4°C with rotation.
    • This serves to collect the antibody/antigen/DNA complex.
12. Pellet Protein G Agarose by brief centrifugation (3000-5000 x g for 1 minute) and remove the supernatant fraction.
13. Wash the Protein G Agarose-antibody/chromatin complex by resuspending the beads in 1.0ml each of the cold buffers containing **PI cocktail** in the order listed below and incubating for 3-5 minutes on a rotating platform followed by brief centrifugation (3000-5000 x g for 1 minute) and careful removal of the supernatant fraction: **a) ChIP Lysis Buffer** wash 5min × 2 times, at RT
    b) High Salt ChIP Lysis Buffer (w/0.5M NaCl) wash 5min × 2 times, at RT
    c) ChIP Lysis Buffer wash 5min × 1 times, at RT
    d) hiTE buffer wash 5min × 1 times, at RT

D. Elution of Protein/DNA Complexes

Prior to starting this section:

• Bring **ChIP Elution Buffer** to room temperature. A precipitate may be observed but will go into solution once room temperature is achieved.
• Set water bath to 65°C for use in **Section E**.

1. Make **ChIP Elution Buffer** for all IP tubes and all Input tubes (see **Section C**, **step 7**).
2. For **Input** tubes (see **Section C**, **step 7**), add **200µl** of **Elution Buffer** and set aside at room temperature until **Section E**.
3. Add **100µl** of **Elution Buffer** to each tube containing the antibody/agarose complex. Mix by flicking tube gently.
4. Incubate at room temperature for 15 minutes.

5. Pellet agarose by brief centrifugation (3000-5000 x g for 1 minute) and collect supernatant into new microfuge tubes.
6. Repeat steps 4-6 and combine eluates (**total volume = 200µl**).

E. Reverse Crosslinks of Protein/DNA Complexes to Free DNA

1. To all tubes (IPs and Inputs) add 8µl of **5M NaCl** and incubate at **65°C** for 4-5 hours (or overnight) to reverse the DNA – Protein crosslinks (***Note: This step can also be carried out in PCR heating blocks***). After this step the sample can be stored at -20°C and the protocol continued the next day.
2. **Optional**: to all tubes, add 1µl of RNase A and incubate for 30 minutes at 37°C.
3. **Optional**: add 4µl 0.5M EDTA, 8µl 1M Tris-HCl and 1µl Proteinase K and incubate at 45°C for 1-2 hours.

F. DNA Purification and Quantitative Real-Time PCR Analysis

1. To each uncrosslinked sample (~200µl), add 100µl of 7.5M (NH4)2OAc and 250µl of PC-8. Perform PC-8 extraction as **our regular lab protocol**. Repeat PC-8 extraction(s) if necessary.
2. To each sample, add 5µl seeDNA co-precipitate to each sample and mix well. Add 700µl cold 100% ethanol. Spin samples (in 1.7ml Eppendorf tubes) at top speed for 5 min. Wash pellets with 70% ethanol twice. Air-dry the pellets.
3. Dissolve samples in 100µl ddH2O. Samples are ready for being used for real-time PCR /regular PCR analysis or Kept at -20C or -80C.
4. Perform real-time PCR /regular PCR use **our regular lab protocols**.