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**Characterization of protein-DNA association *in vivo* by  
chromatin immunoprecipitation**

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running title: Characterization of protein-DNA association by ChIP

## **Abstract**

Chromatin immunoprecipitation (ChIP) is one of the most powerful methods to identify and characterize the association of proteins with specific genomic regions in the context of intact cells. In this method, cells are first treated with formaldehyde to crosslink protein-protein and protein-DNA complexes *in situ*. Next, the crosslinked chromatin is sheared by sonication to generate small chromatin fragments, and the fragments associated with the protein of interest are immunoprecipitated using antibodies to the protein. Finally, protein-DNA crosslinks are reversed and the DNA is examined for the presence of particular sequences by quantitative polymerase chain reaction (PCR). Enrichment of specific sequences in the precipitate indicates that the sequences are associated with the protein of interest *in vivo*. The ChIP method described here is intended for studying protein-DNA association in the budding yeast *Saccharomyces cerevisiae*, but it can be easily implemented to other cell types, including fly, mammalian and plant cells.

**Key Words:** chromatin, cross-linking reagents, DNA, DNA-binding proteins, formaldehyde, immunoprecipitation, polymerase chain reaction, protein-DNA interactions, yeast.

## 1. Introduction

Chromatin immunoprecipitation (ChIP) is a highly powerful method to identify and characterize protein-DNA interactions *in vivo* within a natural chromatin environment. The basic ChIP method is remarkably versatile and has been used in a wide range of cell types, including yeast, fly, mammalian and plant cells [for examples, see ref. (1-8), (9-11), (12-18) and (19,20) respectively]. The ChIP method uses formaldehyde (molecular formula: HCHO) as a crosslinking agent to "freeze" protein-DNA interactions directly *in situ*, within intact cells, and to prevent dissociation and redistribution of proteins during chromatin extraction and immunoprecipitation (9,21). Formaldehyde is a membrane soluble and dipolar compound which reacts with amino and imino group of amino acids (side-chain of lysines, arginines and histidines) and of nucleic acids (e.g., adenines and cytosines). Formaldehyde produces both protein-protein, protein-DNA and protein-RNA crosslinks. Therefore, it can be used to study proteins that are not directly crosslinkable to DNA but interact with proteins that are themselves directly crosslinkable to DNA (22). Another key advantage of using formaldehyde is that the crosslinks are fully reversible (9), which simplifies subsequent characterization of the interacting molecules. Following fixation with formaldehyde, cells are lysed and the crosslinked chromatin is isolated and sonicated to produce short DNA fragments (400 bp average). Then the DNA fragments associated with the protein of interest are purified by selective immunoprecipitation with an antibody specific for the protein (either antibodies to the native protein or antibodies to the tag in case the protein was tagged). Finally, following reversal of crosslinks and purification of DNA, the pool of immunoprecipitated DNA fragments is examined for the presence of specific regions by quantitative polymerase chain reaction (PCR).

## 2. Material

### 2.1. Cell growth and formaldehyde crosslinking

1. Liquid media for yeast (23).
2. Formaldehyde, 37% aqueous solution.
3. 2.5 M glycine, autoclaved and stored at room temperature.
4. 20 mM Tris-HCl, pH 8.0, ice cold.
5. FA lysis buffer: 50 mM Hepes-KOH, pH7.5, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.1% (w/v) deoxycholic acid sodium salt, 0.1% (w/v) SDS, filter sterilized through 0.22- $\mu$ m filter and stored at 4°C (see **Note 1**).
6. Falcon 14-mL polypropylene round bottom tubes (Becton Dickinson).
7. 100 mM phenylmethylsulfonyl fluoride (PMSF) in isopropanol, stored up to 9 mo at room temperature. PMSF is a strong neurotoxin and should be used with extreme caution.

## **2.2. Preparation of crosslinked chromatin extracts**

1. Ice-cold FA lysis buffer.
2. 100 mM PMSF.
3. Protease Inhibitor Cocktail Tablets (Roche).
4. 0.5 mm zirconia/silica beads (BioSpec Products, Inc.).
5. Vortexer (e.g. Vortex-Genie 2, Scientific Industries).
6. Kimwipes.
7. Black (22 G) hypodermic needle.
8. 50-mL NALGENE Oak Ridge centrifuge tubes (Nalge Nunc International).
9. 15-mL COREX centrifuge tubes.
10. 2-mL microcentrifuge tube (Eppendorf).
11. Sonication device fitted with a microtip (e.g., Bronson Sonifier 250, Branson Ultrasonics Corporation).
12. 1.5-mL Safe-Lock tubes (Eppendorf).
13. 5X elution buffer: 125 mM Tris-HCl pH7.5, 25 mM EDTA, 2.5% (w/v) SDS. Filter through 0.22- $\mu$ m filter and store at room temperature.
14. 20 mg/mL Pronase in water (Roche).
15. TE buffer: 10 mM Tris-HCl pH8, 1 mM EDTA. Autoclave and store at room temperature.
16. Agarose and DNA gel electrophoresis equipment.

## **2.3. Immunoprecipitation**

1. 1.5-mL Costar low-binding microcentrifuge tubes (Corning Life Sciences).
2. Protein A Sepharose CL-4B gel (Amersham Biosciences), 25% slurry in PBS prepared from dry powder as recommended by the manufacturer. Store at 4°C.
3. PBS: 20 mM Na phosphate pH 7.0, 150 mM NaCl, 10 mM EDTA. Filter through 0.45- $\mu$ m filter, autoclave and store at room temperature.
4. 20 mg/mL Acetylated-Bovine Serum Albumin (Ac-BSA) (Sigma).
5. 5 mg/mL sonicated salmon sperm DNA (400 bp average size) in TE.
6. Rotating wheel.
7. Drawn-out Pasteur pipets.
8. FA-lysis/0.5 M NaCl buffer: 50 mM Hepes-KOH pH7.5, 500 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.1% (w/v) deoxycholic acid sodium salt, 0.1% (w/v) SDS. Filter sterilized through 0.22- $\mu$ m filter. Store at room temperature.

9. LiCl/detergent buffer: 10 mM Tris-HCl pH 8.0, 0.25 M LiCl, 1 mM EDTA, 0.5% (v/v) Nonidet P40, 0.5% (w/v) deoxycholic acid sodium salt. Filter sterilized through 0.22- $\mu$ m filter and store at room temperature.
10. Elution buffer: 25 mM Tris-HCl pH7.5, 5 mM EDTA, 0.5% (w/v) SDS. Filter sterilized through 0.22- $\mu$ m filter and store at room temperature.
11. 65°C water bath or heat block.
12. 1.5 ml Safe-Lock tubes (Eppendorf).

#### **2.4. Reversal of the crosslinks and purification of DNA**

1. 5X elution buffer.
3. 65°C air incubator or oven.
4. 10 mg/mL DNase-free RNase (Roche).
5. 25:24:1 (v/v/v) phenol/chloroforme/isoamyl alcohol.
6. Chloroforme.
7. 20 mg/mL glycogen, Molecular Biology grade (Roche).
8. 4 M LiCl, filter through 0.45- $\mu$ m filter and autoclaved.
9. 100% ethanol.

#### **2.5. Analysis of immunoprecipitated DNA by quantitative PCR**

1. Oligonucleotides: 24- to 26mers with approximately 40% GC content and similar melting temperature (around 60°C), designed to produce 150-300 bp fragments. Prepare 20  $\mu$ M working dilutions in water and store at -20°C.
2. HotStarTaq DNA polymerase with 10X reaction buffer (Qiagen).
3. High purity dNTP mix containing 2 mM dATP, dCTP, dGTP and dTTP each (Amersham Biosciences).
4. 10 mCi/mL [ $\alpha$ -P32]dATP (specific activity: 3,000 Ci/mmol).
5. Thin-walled 0.2-mL PCR tubes, DNase- and RNase-free.
6. Aerosol filter pipet tips (optional).
7. Thermal cycler with a heated cover.
8. Gel loading buffer: 50% glycerol (w/v), 10 mM EDTA, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol. Store at 4°C.
9. Apparatus and accessories for polyacrylamide gel electrophoresis.
10. 40% acrylamide-bisacrylamide solution, 37.5:1.
11. 10% (w/v) ammonium persulfate in water, store up to 2 wk at 4°C.
12. TEMED (N,N,N',N'-tetramethylethylenediamine).
13. 10X TBE (0.89 M Tris base, 0.89 M boric acid, 0.025 M EDTA, pH8.0).
14. Whatman 3MM chromatography paper.

15. Plastic wrap.
16. Gel-drying system.
17. Autoradiography films, intensifying screen and cassette.
18. PhosphorImaging equipment, e.g., PhosphorImager (Molecular Dynamics) or Storm system (Amersham Biosciences).

### 3. Method

The method described here is based on that developed by Strahl-Bolsinger *et al.* (5) and focuses more specifically on the budding yeast *Saccharomyces cerevisiae* (see **Note 2**). This section outlines (1) the growth and fixation with formaldehyde of yeast cells, (2) the extraction of the crosslinked chromatin, (3) the immunoprecipitation of the crosslinked chromatin, (4) the reversal of the crosslinks and purification of DNA, and (5) the analysis of DNA by quantitative PCR.

#### 3.1. Cell growth and formaldehyde fixation

##### 3.1.1. Cell growth

1. Inoculate 5 mL of appropriate liquid growth medium with a single colony picked from a fresh plate and allow to grow overnight at 30°C.
2. Dilute an aliquot into 100 mL of fresh liquid growth medium in a 500-mL flask so that the culture will have reached an optical density (OD) at 600 nm of 1-2 (ca. 2-3  $10^7$  cells/mL) the next morning (see **Note 3**).

##### 3.1.2. Formaldehyde fixation

1. When the culture has reached the desired density, add 2.8 mL of 37% formaldehyde (see **Note 4**) directly to the medium (final concentration = 1%). After adding formaldehyde, mix rapidly and leave the suspension at room temperature for 15 min with occasional shaking (every 5 min) (see **Note 5**).
2. Add 20 mL of 2.5 M glycine (final concentration = 0.4 M) to stop the crosslinking reaction, mix, and incubate for 5 min at room temperature.
3. Transfer the suspension into a 250-mL centrifuge bottle and pellet cells by centrifugation for 8 min at 10,000g at 4°C.
4. Discard the supernatant (see **Note 6**) and resuspend cells in 250 mL of cold 20 mM Tris-HCl, pH 8.0.
5. Centrifuge for 8 min at 10,000g at 4°C to pellet cells and discard the supernatant.
6. Resuspend cells in 5 mL of cold FA-lysis buffer and add 50  $\mu$ L of 100 mM PMSF (final concentration = 1mM) (see **Note 7**). Transfer the suspension into a 14-mL round-bottom polypropylene tube and pellet cells by centrifugation for 5 min at 2,500g at 4°C.

7. Discard the supernatant by aspiration with a Pasteur pipet connected to a vacuum aspirator and keep on ice. At this point cells can either be directly extracted or frozen and stored in a -80°C freezer for several weeks.

### **3.2. Extraction of the crosslinked chromatin**

Tubes should be kept on ice between the successive manipulations during extraction of crosslinked chromatin.

1. If cells were frozen, thaw on ice first.
2. Resuspend cells in 1 mL of ice-cold FA-lysis buffer containing 1 mM PMSF and 1X protease inhibitor cocktail.
3. Add 1.5 mL of 0.5 mm zirconia/silica beads (see **Note 8**).
4. To lyse cells, vortex vigorously twelve times for 1 min and place on wet ice for 1 min in between each time (see **Note 9**).
5. Add 4 mL of FA-lysis buffer containing 1 mM PMSF and 1X protease inhibitor cocktail.
6. To collect the suspension without the beads, wipe off ice and water from the outside of the tube with a Kimwipe, invert the tube, puncture the bottom with a hot black (22G) needle and insert the tube in a 50-mL Oak Ridge centrifuge tube. Centrifuge for 2 min at 1,000g at 4°C to bring the suspension down. Repeat centrifugation if part of the suspension has not been transferred to the 50-mL tube.
7. Transfer the whole extract in a 15 ml COREX tube and centrifuge for 20 min at 20,000g at 4°C to pellet the crosslinked chromatin (see **Note 10**).
8. Discard the supernatant and add 1.6 mL of cold FA-lysis buffer containing 1 mM PMSF and 1X protease inhibitor cocktail to wash the pellet. Break the pellet up by gently pipetting up and down with a 1-mL micropipettor.
9. Transfert the whole suspension into a 2-mL tube taking care that no material is left behind in the COREX tube. Spin at maximum speed in a microcentrifuge for 20 min at 4°C.
10. Discard the supernatant and add 1.6 mL of cold FA-lysis buffer containing 1 mM PMSF and 1X protease inhibitor cocktail. Break the pellet up as in **step 8** and place the tube on a holder in an ice-water bath.
11. Sonicate the suspension to yield DNA fragments in a size range between 100 and 1000 bp with an average of 400 bp. Use a sonication device fitted with a microtip. Place the microtip at the 0.5 mL graduation mark on the 2 mL tube and hold the tube firmly to prevent movement during sonication. Sonicate for short cycles (20 sec maximum) at the maximum microtip power setting. Place the tube in ice-water at least 5 min in between two sonication cycles to avoid excessive heating in the sample. If several samples are processed in turns, wash thoroughly the microtip with water and

ethanol in between samples to avoid cross-contamination. Every sonication device is different and the number of cycles required with a particular device to achieve the desired DNA fragment size should be determined in pilot experiment. Four to six cycles are generally sufficient to get DNA fragments with an average size of 400 bp (see **Note 11**).

12. Transfer the suspension in a 15-mL COREX tube and add 4 mL of cold FA-lysis buffer containing 1 mM PMSF and 1X protease inhibitor cocktail. Let stand on ice for 30-60 min.
13. Centrifuge for 20 min at 12,000g at 4°C to remove cell debris and insoluble components.
14. Transfer the supernatant (which contains the fragmented crosslinked chromatin) into a fresh 14-mL round-bottom polypropylene tube and discard the pellet. Save 100 µL to check the size of the DNA fragments and divide the rest into 0.5 mL aliquots. Freeze the aliquots in liquid nitrogen and store at -80°C (see **Note 12**).
15. To check the size of the DNA fragments, mix 100 µL of extract with 25 µL of 5X elution buffer in a 1.5-mL Safe-Lock tube, add 6 µL of 20 mg/mL Pronase, incubate 1 hr at 37°C, and proceed as described in **Subheading 3.4, Steps 2-8** to reverse crosslinks and purify DNA. Resuspend purified DNA in 50 µL of TE and analyze 20 µL by electrophoresis on a 1.5 % agarose gel and staining with ethidium bromide (see **Note 13**).

### **3.3. Immunoprecipitation**

A control immunoprecipitation with either pre-immune serum, immune serum depleted of the antibodies, peptide-block antibodies, or no antibody at all should be performed to determine the specificity of the immunoprecipitation (see **Note 14**).

#### **3.3.1. Coupling of antibodies to Protein A beads**

1. In a 1.5 mL low-binding microcentrifuge tube, combine the appropriate amount of antibody (amount sufficient to deplete at least 90% of the antigen from 500 µL of chromatin extract, see **Note 15**) with 60 µL of 25% protein A Sepharose bead slurry (see **Note 16**), 25 µL of 20 mg/mL Acetylated-Bovine Serum Albumin (Ac-BSA), and 4 µL of 5 mg/mL sonicated salmon sperm DNA. Bring the volume to 1 mL with ice-cold PBS.
2. Incubate for 4 hr to overnight at 4°C on a rotating wheel.
3. Microcentrifuge 5 s at maximum speed at room temperature.
4. Aspirate the supernatant (which contains unbound antibodies) using a drawn out Pasteur pipet connected to a vacuum aspirator.

5. Add 1 mL of ice-cold PBS and resuspend the beads by inverting the tube.
6. Repeat **step 3** and **4** to collect beads.
7. Add 25  $\mu$ L of 20 mg/mL Ac-BSA, 4  $\mu$ L of 5 mg/mL sonicated salmon sperm DNA, and mix by gently flicking the bottom of the tube. Keep on ice.

### 3.3.2. Immunoprecipitation

1. If chromatin extracts were frozen, thaw on ice first and spin in a microcentrifuge for 20 min at 16,000g at 4°C to clarify the extract from protein aggregates which may have formed during freezing and thawing.
2. Add 500  $\mu$ L of chromatin extract to the antibody-conjugated Protein A beads prepared before.
3. Incubate for 1-2 hr at room temperature on a rotating wheel (see **Note 17**).
4. Microcentrifuge 1 min at 1000g at room temperature to collect the beads and remove the supernatant by aspiration with a drawn out Pasteur pipet connected to a vacuum aspirator.
5. Add 1.4 mL of FA-lysis/0.5 M NaCl buffer and incubate for 5 min at room temperature on a rotating wheel (see **Note 18**).
6. Microcentrifuge 1 min at 1000g at room temperature and remove the supernatant as in **step 4** (see **Note 19**).
7. Repeat **step 5-6** twice.
8. Repeat **step 5-6** with 1.4 mL of LiCl/detergent buffer.
9. Repeat **step 5-6** with 1.4 mL of TE buffer.
10. Add 125  $\mu$ L of elution buffer, mix by vortexing, and incubate 20 min in a 65°C water bath or heat block to elute the immunoprecipitate from protein A beads.
11. Microcentrifuge 1 min at maximum speed at room temperature and transfer eluate to a 1.5-mL Safe-Lock tube.

### 3.4. Reversal of the crosslinks and purification of DNA

It is important to treat at the same time as the immunoprecipitate an aliquot of the chromatin extract (routinely 100  $\mu$ L, i.e., 20% of the volume used for the immunoprecipitation, adjusted to 25 mM Tris-HCl pH7.5, 5 mM EDTA, 0.5% (w/v) SDS with 25  $\mu$ L of 5X elution buffer). This will serve as the total (or input) control for later analysis.

1. Add 6  $\mu$ L of 20 mg/mL Pronase to the immunoprecipitate and the total chromatin aliquot, and incubate 1 h at 37°C to digest proteins.
2. Place samples in a air incubator or an oven at 70°C and incubate for at least 6 h up to overnight to reverse protein-DNA crosslinks (see **Note 20**).
3. Add 5  $\mu$ g of DNase-free RNase and incubate 30 min at 37°C.

4. Extract samples once with an equal volume of 25:24:1 phenol/chloroforme/isoamyl alcohol and once with an equal volume of chloroforme.
5. Add 20  $\mu\text{g}$  of glycogen and 1/10 volume of 4 M LiCl, and vortex. Add 2 volumes of 100% ethanol and vortex again. Incubate at least 2 h up to overnight at  $-20^{\circ}\text{C}$  to precipitate DNA (see **Note 21**).
6. Microcentrifuge at maximum speed for 20 min at  $4^{\circ}\text{C}$ .
7. Remove the supernatant, wash the pellet with 1 mL of 100% ethanol, and microcentrifuge at maximum speed for 5 min at  $4^{\circ}\text{C}$  (see **Note 22**).
8. Remove the supernatant and air dry the pellet for 10-15 min at room temperature.
9. Resuspend immunoprecipitate and total DNA in 200  $\mu\text{L}$  of TE buffer (see **Note 23**).

### **3.5. Analysis of DNA by quantitative PCR**

Our method of choice to detect the presence of specific genomic fragments in the immunoprecipitate DNA is PCR (see **Note 24** and **25**). The relative enrichment of a particular fragment in the immunoprecipitate DNA is calculated by dividing the amount of PCR product obtained from the immunoprecipitate by that obtained from the total input DNA. For accurate quantification, it is essential to make sure that calculation is based on PCR signals that are in the linear range of the amplification reaction. Indeed, the amount of product generated during amplification is proportional to the amount of template only during the logarithmic-linear phase of the reaction. Therefore, it is essential to process several dilutions of the immunoprecipitate and input DNA (two- or threefold serial dilutions in TE buffer) and check that the amount of PCR product does decrease proportionally with the amount of template.

It is equally important to perform appropriate PCR controls (i.e., with oligonucleotide primers specific for genomic regions which are not expected to associate, and therefore coimmunoprecipitate, with the protein of interest). These controls will serve to determine the background level of the whole ChIP experiment and to appreciate the significance of the enrichment obtained for the other regions.

#### **3.5.1. PCR analysis**

PCR amplifications are carried out in 15  $\mu\text{L}$  containing 1  $\mu\text{M}$  of each oligonucleotide primer, 0.2 mM of each dNTP, 0.1 mCi/mL of  $[\alpha\text{-P32}]\text{dATP}$  (specific activity, 3,000 Ci/mmol) and 0.75 units of HotStarTaq DNA polymerase (Qiagen) or equivalent *Taq* polymerase (see **Note 26**). To micropipet reagents and DNA samples, use preferentially aerosol filter tips to prevent PCR contaminations.

1. Prepare a common PCR premix containing the appropriate amount of  $\text{H}_2\text{O}$ , 10x PCR buffer, primers, dNTPs,  $[\alpha\text{-P32}]\text{dATP}$  and *Taq* polymerase (see **Note 27**). The minimal number of samples includes two or three different dilutions (two- or three-fold serial

- dilutions) of the immunoprecipitate and the input DNA. Adjust the final premix volume assuming that 5  $\mu$ L of DNA template will be added next.
2. Distribute 10  $\mu$ L of the premix into 0.2-mL PCR tubes at room temperature.
  3. Add the DNA template. The amount of template added is typically 5  $\mu$ L of 1:1 (undiluted), 1:2 and 1:4 dilutions of the immunoprecipitate DNA (which corresponds to 1/40, 1/80 and 1/160 of the immunoprecipitate assuming a sample volume of 200  $\mu$ L) and 5  $\mu$ L of 1:25, 1:50 and 1:100 dilutions of the input DNA sample (which corresponds to 1/5,000, 1/10,000 and 1/20,000 of the total input DNA assuming a sample volume of 200  $\mu$ L and reversal of 100  $\mu$ L of chromatin extract).
  4. Place the PCR tubes in a thermal cycler with a heated cover and start amplification using a cycling program consisting in a 2 min initial denaturation at 95°C (15 min if Qiagen HotStarTaq DNA polymerase is used) followed by 25 cycles with 30 sec at 94°C (denaturation), 30 sec at 55°C (annealing) and 60 sec at 72°C (elongation), and by a final extension step at 72°C for 5 min.

### 3.5.2. Gel electrophoresis and quantitation

PCR products are separated by vertical electrophoresis on nondenaturing polyacrylamide gels and visualized by film autoradiography. Intensity of radioactive PCR signals is quantitated by PhosphorImager analysis.

1. After PCR is completed, microcentrifuge tubes for a few seconds to bring condensation down.
2. Add 2  $\mu$ L of loading buffer and mix by vortexing.
3. Load 4  $\mu$ L on a 8% polyacrylamide/1X TBE gel (20cm X 20cm X 1mm, 25 wells) and run the gel at 10 Volt/cm, taking care to avoid excessive heating.
4. Stop migration when the bromophenol blue dye has reached two-thirds of the plate length. Discard the 1X TBE buffer present in the lower reservoir of the electrophoresis tank in a radioactive waste (this buffer may contain free [ $\alpha$ -P32]dATP).
5. Detach the plates from the electrophoresis apparatus and carefully pry them apart so that the gel is still attached to one plate.
6. Transfert the gel onto a piece of Whatman 3MM paper, place on a preheated gel dryer connected to a vacuum pump, cover with plastic wrap, and dry for 20 min at 80°C.
7. Expose the dried gel to an autoradiography film or to a storage phosphor screen for visualization and quantification of PCR products (see **Note 28 and 29**).

## 4. Notes

1. Solutions used for chromatin extraction and immunoprecipitation should be prepared from Molecular Biology grade reagents using distilled and deionized water. It is

convenient to prepare solutions from individual concentrated stock solutions (e.g., prepare FA lysis buffer from 0.5 M Hepes-KOH pH7.5, 5 M NaCl, 0.5 M EDTA pH8.0, 20% (v/v) Triton X-100, 10% (w/v) deoxycholic acid sodium salt, and 10% (w/v) SDS solutions). Solutions must be filtered through 0.22- or 0.45- $\mu$ m filter (respectively for sterilization and/or removal of particles) and, when appropriate, autoclaved. Do not autoclave solutions with HEPES or detergent.

2. Implementation of the ChIP method to cell types other than yeast will require adapt the steps of cell fixation and chromatin extraction to the particular characteristics of the cells used. For examples, *drosophila* and human cells do not have a cell wall as yeast cells do and therefore can be lysed without vortexing in the presence of glass beads [see ref. **(10,12,16)**]. Other steps (immunoprecipitation, reversal and DNA analysis) can be implemented without modifications.
3. Growth properties of strains depend on their genetic background. Important differences exist among strains. In addition, the growth rate of a strain depends on the composition of the growth medium. Therefore the size of the inoculate that will lead to the desired cell density the next morning should be determined in advance in pilot experiments.
4. Formaldehyde is toxic and should be manipulated with gloves and in a fume hood to prevent contact with skin and inhalation of noxious fumes. In addition, flasks should be kept covered (for example with aluminium foil) after addition of formaldehyde.
5. The optimal concentration of formaldehyde, incubation time and temperature at which the reaction is performed should be determined experimentally. Crosslinking times reported previously range from 10 min at room temperature to overnight at 4°C. The use of 1% formaldehyde at room temperature for 15 min represents standard conditions that were shown to work well with a number of proteins. However, these conditions should not be considered to be suitable for any proteins. The extend of crosslinking is one of the most important parameters for the succes of the ChIP experiment. Excessive crosslinking may result in poor cell breakage and poor chromatin fragmentation during extraction and sonication. Excessive crosslinking may also alters reactivity of the epitopes recognized by the antibodies, resulting in poor immunoprecipitation. On the other hand, suboptimal crosslinking may result in fewer crosslinks between the protein of interest and DNA, leading to weak enrichment of the genomic targets of the protein.
6. Formaldehyde-containing wastes must be disposed of as hazardous chemical waste. Do not discard them down the sink but keep them in appropriate bottles and consult your institution's safety office for the rules regarding storage and disposal.

7. PMSF is unstable in aqueous solution (half-life of 35 min at pH8.0) and should be added to buffers immediately before use.
8. 0.5-mm Acid-washed glass beads (Sigma) can be used instead of 0.5-mm zirconia/silica beads.
9. Cell-breakage efficiency should be greater than 90% to achieve maximum extraction. It is important to make sure that bead movement is not constricted during vortexing, which could otherwise result in poor and unequal cell lysis among samples. Eight samples can be processed in no longer than 48 min if two vortexers are used at the same time. It may be convenient to use a multivortexer or a BeadBeater (BioSpec Products, Inc.) if one wants to process more samples.
10. After centrifugation of the whole cell lysate at 20,000g, the crosslinked chromatin is found in the pellet with cell debris and potential unbroken cells. This characteristic of the crosslinked chromatin allows to separate it from soluble proteins that might interfere during immunoprecipitation.
11. Sonication is a crucial step because it promotes solubilization of the crosslinked chromatin and allows to separate it from cell debris and insoluble components. In addition, shorter DNA fragments allow to map DNA-protein interactions with higher resolution, and to define more accurately regions where proteins are recruited.
12. The amount of chromatin extract prepared from 100 mL of culture grown to an OD<sub>600</sub> of 1-2 is sufficient to carry out up to 10 immunoprecipitations. Chromatin extracts can be stored at -80°C for several months without damage. However, it is recommended to use fresh extracts in experiments studying proteins never assayed before.
13. It is important to check the size of the DNA fragments to confirm that they are in the expected range.
14. An alternative control in experiments using cells expressing epitope-tagged proteins (e.g., HA- or Myc-tagged proteins) is to perform a parallel immunoprecipitation (including antibodies) with chromatin extract prepared from untagged, isogenic cells.
15. It is recommended to perform preliminary experiments to determine the appropriate amount of antibodies to use. Chromatin extract should be subjected to immunoprecipitation with varying amounts of antibodies, and the supernatant after immunoprecipitation examined by immunoblotting for the presence of the antigen. It is important to remember that immunoprecipitation of proteins is less efficient in crosslinked extracts compared to noncrosslinked ones, probably owing to modification or masking of the epitopes, and it has been reported that epitopes of some proteins seem to become inaccessible when the protein gets crosslinked to the DNA (either

- directly or indirectly) [see ref. **(3)**]. This potential problem can be circumvented by using polyclonal antibodies (Pabs) rather than monoclonal ones.
16. Substitute protein G for protein A if the antibody is from a species or subclass that does not bind to protein A [see ref. **(24)** for protein A/G affinities for antibodies from various species and subclasses].
  17. Prolonged incubations (e.g., overnight in the cold room) tend to result in increased background (i.e., non specific precipitation of chromatin fragments) and should be avoided.
  18. It is not recommended to complete all the washes too quickly (i.e., without incubation time) because this may not allow enough time for proteins included within the antigen-antibody-protein A bead latticework to diffuse out of it. Instead, beads should be washed over approx 30 min (all washes included).
  19. Remove supernatant as completely as possible during washes to achieve the lowest background as possible. The Pasteur pipet should be moved progressively toward the bottom of the tube as the wash buffer is withdrawn until the very surface of the beads. Use gentle vacuum to avoid removing beads.
  20. It is preferable to use an air incubator or oven instead of an heat-block or water bath because it prevents concentration of the sample by condensation.
  21. Glycogen serves as a carrier to maximize DNA precipitation and helps to visualize the pellet after centrifugation.
  22. The DNA pellet from the immunoprecipate sample will be very small and nearly invisible. Therefore, the supernatant should be removed with extreme cautious to avoid any loss of material.
  23. Alternatively, the DNA can be purified using the QIAquick PCR purification kit (Qiagen). Follow the protocol described in the QIAquick spin handbook supplied with the kit. To elute DNA from the QIAquick column, add 200  $\mu$ l of TE buffer, let the column stand for 10 min at room temperature, and microcentrifuge at maximum speed for 1 min.
  24. Alternatively, coimmunoprecipitated DNA can be analyzed by slot blot or Southern hybridization [for examples, see ref. **(1,4,10)**]. For slot blot analysis, immunoprecipitated and total DNA samples are immobilized on a nylon membrane by slot blot, and the membrane is hybridized with P32--labelled probes for specific genes. For Southern analysis, immunoprecipitated DNA is radiolabelled and used as a probe against genomic DNA fragments separated by agarose gel electrophoresis and immobilized onto a hybridization membrane. Both methods were showed to give good

results but we favor PCR analysis because of high sensibility and specificity of the PCR method.

25. PCR, slot blot or Southern hybridization analyses can be carried out in any laboratory provided with standart equipment. Unfortunately, these analyses are restricted to studying interaction of proteins with a limited number of target fragments. Methods of global analysis using DNA microarrays have recently been developped to monitor protein-DNA interactions across the entire yeast genome **(25-27)**. In these methods, immunoprecipitate and input DNA fragments are amplified and differentially labelled using different fluorescent dyes, and then hybridized to microarray plates that contains the whole genome (or only intergenic regions in case of transcription factors that specifically target promoter region). This approache allows to localize DNA-bound proteins at the whole genome level.
26. It is highly convenient to use a *Taq* DNA polymerase which is inactive until PCR cycles start. This permits set-up at ambient temperature and reduces formation of nonspecific products and primer-dimers.
27. To minimize exposure to radiation, PCR reactions should be set up behind an appropriate shield and in a place designated for radioactive work. PCR samples must be in a shielded container during storage and transportation.
28. Exposition for 1 h at -80°C should be long enough to detect a signal when using an intensifying screen. After exposition, the film can be scanned and signals quantitated using an imaging software if the laboratory is not equiped for phosphorimager autoradiography.
29. A faster and more convenient way to quantitate the immunoprecipitate DNA is using real-time PCR if one has the necessary instrument (e.g., LightCycler from Roche or ABI PRISM 7700 from Applied Biosystems).

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