

# Chapter 2

## Measuring Dynamic Changes in Histone Modifications and Nucleosome Density during Activated Transcription in Budding Yeast

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### Abstract

Chromatin immunoprecipitation is widely utilized to determine the *in vivo* binding of factors that regulate transcription. This procedure entails formaldehyde-mediated cross-linking of proteins and isolation of soluble chromatin followed by shearing. The fragmented chromatin is subjected to immunoprecipitation using antibodies against the protein of interest and the associated DNA is identified using quantitative PCR. Since histones are posttranslationally modified during transcription, this technique can be effectively used to determine the changes in histone modifications that occur during transcription. In this paper, we describe a detailed methodology to determine changes in histone modifications in budding yeast that takes into account reductions in nucleosome.

**Key words:** Activated transcription, RNA polymerase II, Histone modifications, Acetylation, Gcn4, Gal4, *Saccharomyces cerevisiae*, Histone acetyltransferase, Histone deacetylase complexes

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### 1. Introduction

The packaging of the eukaryotic genome into chromatin imposes a physical barrier for all DNA-dependent processes, including gene transcription. During transcription activation, the barrier imposed by nucleosomes, the fundamental unit of chromatin is relieved through sliding, repositioning, or evicting the nucleosomes that occlude regulatory DNA sequences (1). Similarly, histones are transiently evicted from the coding regions to facilitate transcription elongation by RNA Polymerase II (Pol II) (2–5). Acetylation of lysine residues in the amino-termini of histones is a major determinant of nucleosome occupancies in the promoter region and across the coding sequences of genes during transcription activation (5–7). Histone acetylation is

dynamically regulated by histone acetyltransferase (HAT) and histone deacetylase complexes (HDACs) (5, 7–11). Additionally, the recruitment and function of these complexes, especially in coding regions is regulated by phosphorylation of the C-terminal domain (CTD) of Rpb1, the largest subunit of Pol II, and methylation of histone H3 (7, 12–15).

Genome-wide studies have revealed that histone occupancies at promoters are lower as compared to their corresponding coding regions (16, 17), where the occupancy of histones is inversely correlated with the rate of transcription (16). In the budding yeast *Saccharomyces cerevisiae*, activated transcription is associated with cotranscriptional loss of nucleosomes and this loss is more prominent at promoter regions. Transcription activation by Gcn4 or Gal4 is accompanied by a massive loss of nucleosomes from the promoters of their regulated genes, and the extent of histones lost from adjacent coding regions depends on both the rate of transcription and histone acetylation (5, 7, 9, 16). Our recent studies show that histone acetylation modulates histone occupancy in coding regions (5, 7). Deletion of *GCN5* or a mutation in *ESAI*, encoding the catalytic subunits of HAT complexes SAGA and NuA4, respectively, severely impairs histone acetylation and reduces histone eviction from the coding regions, and this reduced histone eviction is accompanied by lower transcription, which involves reductions in both the rate and processivity of Pol II during elongation (5, 9). Conversely, deletion of HDACs is accompanied by increased histone acetylation and greater histone eviction in the coding regions (7, 9). Thus, it appears that histone loss from the coding regions during transcription activation is regulated through modulation of histone acetylation by multiple HATs and HDACs and influences the elongation phase of transcription.

Chromatin immunoprecipitation (ChIP) has been extensively used to detect histone modifications that are associated with transcribed genes, as well as to detect the complexes that regulate histone modifications (8, 18). In this chapter, we provide a detailed experimental procedure for ChIP to detect both histone acetylation as well as recruitment of complexes that regulate modification and chromatin remodeling in *S. cerevisiae*. In this procedure, outlined in Fig. 1, the protein–protein and protein–DNA interactions are stabilized through use of formaldehyde (HCHO) as a cross-linking agent. The chromatin is then sheared into small pieces (~300–500 base pairs) and is subjected to immunoprecipitation using antibodies recognizing histones, a specific histone modification or a protein of interest that may be present as part of a larger complex. The detailed methodology provided is for the detection of histone modifications present at *ARG1*, a Gcn4-regulated gene, or at the Gal4-regulated gene *GALI* under activating conditions.

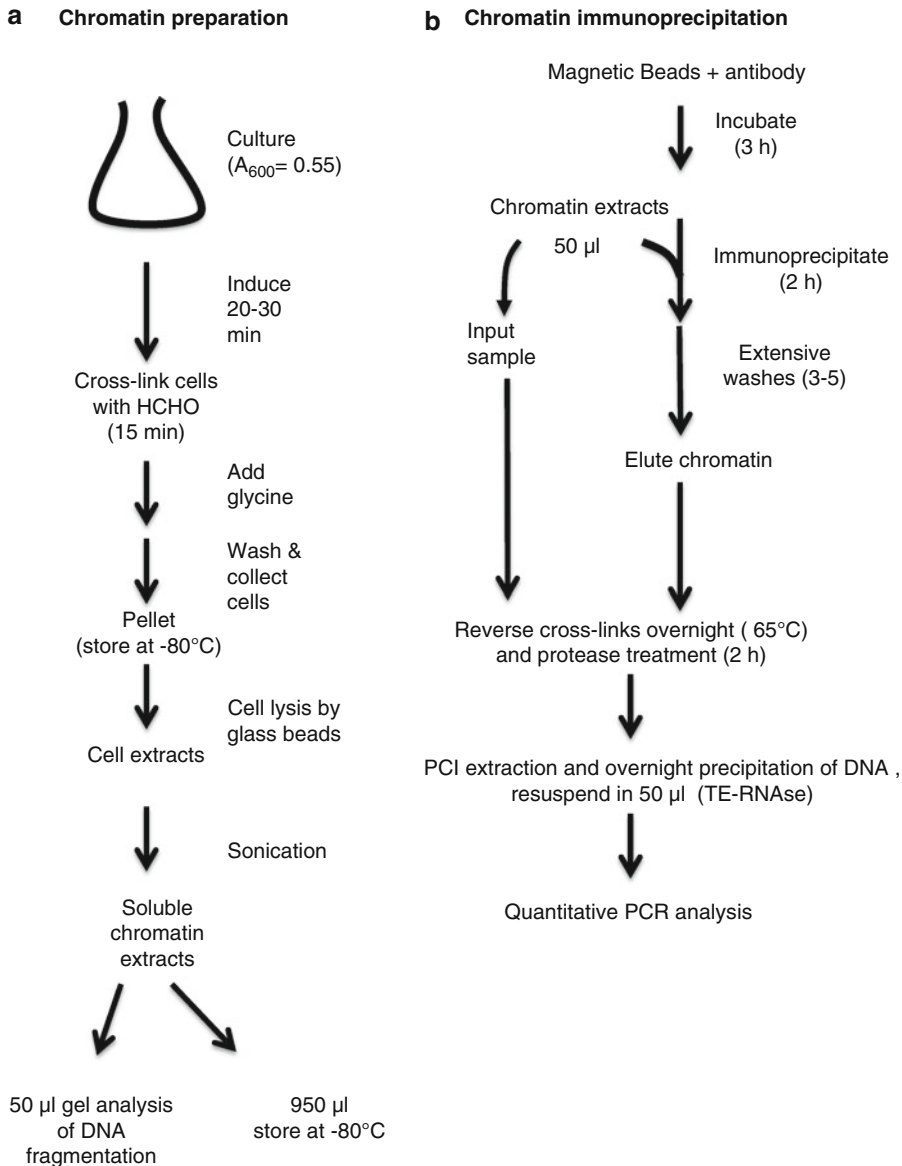


Fig. 1. Schematic diagram of entire ChIP protocol: (a) chromatin preparation and (b) chromatin immunoprecipitation.

## 2. Materials

### 2.1. Media

1. YPD: Bacto-yeast extract (1%), Bacto-peptone (2%), dextrose (2%).
2. YPR: Bacto-yeast extract (1%), Bacto-peptone (2%), raffinose (2%).

3. Synthetic complete (SC): For 1 L of SC media, add 2 g of amino acid mix, 2.25 g of yeast nitrogen base without ammonium sulfate and amino acids and 5 g of ammonium sulfate in 900 mL of distilled water. Autoclave the media and add 50 mL of 40% dextrose solution (filter sterilized) for a final volume of 1 L.
4. 20% galactose solution, sterilized (for induction of Gal4 targets).

## **2.2. Reagents, Solutions, and Other Materials**

1. Sulfometuron methyl (SM): 5 mg/mL in dimethyl sulfoxide (DMSO) (for induction of Gcn4 targets).
2. Formaldehyde (37% solution).
3. Cross-linking solution: 1 mM EDTA, 100 mM NaCl, 70 mM HEPES-KOH (pH 7.5). This can be stored at room temperature for up to 6 months. 37% formaldehyde is added to the required amount of the above solution to a final concentration of 11%.
4. Glycine stop solution: 2.5 M glycine (molecular biology grade) in distilled water heated to 70°C and stirred until completely dissolved. Filter-sterilize and store at room temperature.
5. FA lysis buffer\*: 50 mM HEPES-KOH (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 1% Triton X-100 in water. Filter-sterilize and store at room temperature, for up to 6 months.  
\*Before use, take the required amount of FA-lysis buffer and add the following protease inhibitors to the indicated final concentrations: PMSF (1 mM; from a 100 mM stock solution in isopropanol), leupeptin (1 µg/mL; stock 10,000 µg/mL) pepstatin A (1 µg/mL; stock 1,000 µg/mL), aprotinin (10 µg/mL; stock 10,000 µg/mL).
6. Acid-washed glass beads (0.4–0.6 mm).
7. 26 gauge needle.
8. Wash buffer II: 50 mM HEPES-KOH (pH 7.5), 500 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycholate, 1% Triton X-100.
9. Wash-buffer III: 10 mM Tris-Cl (pH 8.0), 250 mM lithium chloride, 1 mM EDTA, 0.5% sodium deoxycholate, 0.5% NP-40 substitute (Igepal CA-630; see Note 2 in Chapter 3).
10. Elution buffer: 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 1% sodium dodecylsulfate (SDS).
11. Elution wash buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.7% SDS.
12. Proteinase K, 10 mg/mL.
13. Phenol:chloroform:isoamyl alcohol (25:24:1, v/v).
14. Chloroform:isoamyl alcohol (24:1, v/v).
15. Glycogen, 20 mg/mL.
16. 4 M LiCl.

17. 100% ethanol.
18. Phosphate-buffered saline 1×: 137 mM NaCl, 2.7 mM KCl, 100 mM Na<sub>2</sub>HPO<sub>4</sub> and 2 mM KH<sub>2</sub>PO<sub>4</sub>.
19. PBS/BSA: 0.5% bovine serum albumin (BSA) in 1× PBS.
20. RNase A, 10 mg/ml.
21. 6× ChIP dye solution: 4 mg bromophenol blue in 100 mL of 15% Ficoll solution prepared in 1× TBE (89 mM Tris–borate, 89 mM Boric acid, 2 mM EDTA).
22. 6% TBE-polyacrylamide gel: Use 1.0 mm 12 or 15 well combs.
23. Magnetic beads: Pan anti-mouse IgG and sheep anti-rabbit IgG Dynabeads or equivalent.
24. 1× TBS: 50 mM Tris–HCl pH 7.5, 150 mM NaCl.
25. ChIP antibodies: Rabbit polyclonal anti-H3 (0.7 µL; ab1791: Abcam), rabbit anti-acetyl histone H4 (0.5 µL; 06-866, Upstate Biotechnology), rabbit monoclonal anti-trimethyl (Lys4) histone H3 (1.0 µL; 05-745: Upstate Biotechnology), anti-acetylhistone H3 (0.7 µL; 06-599; Upstate Biotechnology), anti-Gal4p antibodies (1.0 µL SC577X; Santa Cruz Biotechnology), mouse monoclonal anti-Rpb3 (1.0 µL; Neoclone), and anti-phospho-Ser5 Rpb1 (1.0 µL; H14; Covance). If other sources are used for specific antibodies, it will be necessary to determine experimentally the optimal amount to use for ChIP.
26. PCR master-mix: (prepared on ice, 13 µL per reaction). Combine 6.32 µL water with 1.5 µL each of 10× PCR buffer (generally provided with hot-start Taq polymerase) and 15 mM MgCl<sub>2</sub>, 1.5 µL of each of the specific and internal control primer sets (including forward and reverse primers; see Table 1 for a list of primers used for ChIP at the *ARG1* and *GALI* promoters together with control primers), 0.3 µL of dNTPs (10 mM) and hot-start Taq polymerase (added last), and 0.08 µL of [ $\alpha$ -<sup>33</sup>P]-dATP.
27. PCR tubes (0.2 mL thin-walled strip tubes).
28. Thermal cycler.
29. Whatman 3 MM paper.
30. Ethidium bromide, 5 µg/mL.

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### 3. Methods

#### 3.1. Cell Culture and Transcription Induction

1. Cell culture: Inoculate a single colony of wild-type (WT) cells in 5 mL of SC media lacking isoleucine and valine (SC<sup>ILV</sup>) (for experiments involving induction of genes by Gcn4;

**Table 1**  
**Primers employed for ChIP analysis to study *ARG1* and *GAL1***

Gene	Location (relative to ATG)	Sequence
<i>POL1</i>	+2,477/+2,707 (ORF)	5'-GACAAAATGAAGAAAATGCTGATGCACC-3' 5'-TAATAACCTTGGTAAAAACACCCTG-3'
<i>TEL VI-R</i>	+51/+307	5'-GCTGAGT <sup>T</sup> TAACGGTGATTATT-3' 5'-CCAGTCCTCATTTCCATCAAT-3'
<i>ARG1</i>	-376/-213 (UAS) -197/-51 TATA +23/+186 (5' ORF) +1,091/+1,258 (3' ORF)	5'-ACGGCTCTCCAGTCATTTAT-3' 5'-GCAGTCATCAATCTGATCCA-3' 5'-TAATCTGAGCAGTTGCGAGA-3' 5'-ATGTTCTTATCGCTGCACA-3' 5'-TGGCTTATTCTGGTGGTTTAG-3' 5'-ATCCACACAAACGAACTTGCA-3' 5'-TTCTGGGCAGATCTACAAAGA-3' 5'-AAGTCAACTCTTCACCTTTGG-3'
<i>GAL1</i>	-408/-261 (UAS) -185/-57 (TATA) +422/+567 (MID/5' ORF) +1,233/1,355 (3' ORF)	5'-TGTTCCGGAGCAGTGCGGCGC-3' 5'-ACGCTTAACTGCTCATTGCT-3' 5'-GGTTATGCAGCTTTTCCATT-3' 5'-CGAATCAAATTAACAACCATAGGA-3' 5'-CCAGTTGGTACATCACCTCA-3' 5'-ATCCTTCTGTGTCGGACTGG-3' 5'-CGTTCATCAAGGCACCAAAT-3' 5'-TCAGAGGGCTAAGCATGTGT-3'

or appropriate media depending on experiment) and grow the culture overnight (O/N) with shaking at 30°C to late log phase or to saturation. Dilute the O/N culture in 100 mL of the same medium such that the starting absorbance at 600 nm ( $A_{600}$ ) is ~0.1 and grow the cells at 30°C in an incubator shaker to  $A_{600}$  of 0.5–0.55 (see Note 1).

- (1) Induction of Gcn4 target genes: Just before the culture reaches the desired  $A_{600}$ , thaw an aliquot of sulfometuron methyl (SM). When the culture reaches an  $A_{600}$  of 0.55, add 12  $\mu$ L of SM solution to each flask and quickly place the flasks back into the incubator shaker for an additional 20–30 min (see Notes 2 and 3).
- (2) Induction of Gal4 regulated genes: Grow cells in YP-raffinose (YPR) to  $A_{600}$  of 0.55 and induce transcription for 20–30 min by adding galactose to a final concentration of 2% (10 mL of 20% galactose in 100 mL culture).
- Add 11 mL of freshly prepared 11% formaldehyde solution by mixing 37% HCHO and cross-linking solution to 100 mL of induced culture (see Note 4). Mix gently by swirling the flask and incubate for 15 min at room temperature with intermittent mixing every 4 min.

4. Add 15 mL of 2.5 M glycine to quench the formaldehyde and mix gently (see Note 5).
5. Subsequent steps are carried out at 4°C or on ice with ice-cold solutions unless stated otherwise. Transfer the culture to 50 mL tubes (2 per 100 mL culture) and collect the cells by centrifugation at  $1,500\times g$  for 5 min.
6. Decant the culture medium and wash two times with  $1\times$  TBS and collect the cells by centrifugation as in step 5. Decant the solution and add 1 mL of TBS, resuspend and transfer the cells from the  $2\times 50$  mL tubes into one 2 mL microcentrifuge (Eppendorf) tube.
7. Pulse-spin cells in a refrigerated microfuge to collect the pellet, which can be stored at  $-80^{\circ}\text{C}$  until further use.

### **3.2. Preparation of Soluble Chromatin**

1. Resuspend the cell pellet in 500  $\mu\text{L}$  of FA lysis buffer with protease inhibitors and add  $\sim 500$   $\mu\text{L}$  of acid-washed glass beads (see Note 6). Place the tubes in a vortex mixer (e.g., Vortex Genie) and vortex for 40 min in a cold room. It is important to check the tubes for any leakage during this process. A large amount of frothing occurs at this step.
2. Place the tubes in an ice-bucket and carefully puncture both the cap and bottom of the tube using a 26 G red-hot needle. Place the tube in an un-capped 15 mL Falcon tube (save the cap) and spin at  $300\times g$  for 1 min to collect cell lysate. Add an additional 500  $\mu\text{L}$  of FA-lysis buffer to the tube and collect the lysate by centrifugation at  $300\times g$  for 1 min. Discard the Eppendorf tube and place the 15 mL Falcon tube on ice and cap it.
3. Sonicate the cell lysate to shear the chromatin into  $\sim 300$  bp fragments. The proper settings on a particular sonicator must be experimentally determined (see Note 7). For example, the Branson 450 sonifier fitted with the tapered tip (1/8 in., cat # 101 148 062) is set at 1.8 output with 60% duty cycle. Using these settings, sonicate the samples 10 times, for 30 s with 30 s intervals on ice. To prevent overheating of samples, the tube is placed in a glass beaker filled with ice during the entire sonication step.
4. After sonication, transfer the lysate to a 1.5 mL Eppendorf tube and spin at  $16,000\times g$  for 30 min in a refrigerated microcentrifuge. Carefully remove the supernatant and transfer to a new 1.5 mL tube. Snap freeze by placing the tube in dry ice and store at  $-80^{\circ}\text{C}$  if not required immediately.

### **3.3. ChIP and Reversal of Cross-Linking**

1. Prepare antibody-bead conjugate. We use anti-mouse, anti-rabbit, and anti-IgM-conjugated magnetic beads (Dynabeads, Invitrogen). The particular type of magnetic beads employed depends on the specificity of the antibody (see Note 8).

For ChIP of acetylated H3, place 40  $\mu\text{L}$  of bead suspension in each 1.5 mL tube and spin for  $\sim 8$  s in a refrigerated microcentrifuge to pellet the beads. Place the tubes in a magnetic stand and remove the supernatant using suction. Wash beads twice with 1 mL of PBS containing BSA (5 mg/mL; PBS-BSA). Resuspend beads in 200  $\mu\text{L}$  of PBS-BSA and add 0.7  $\mu\text{L}$  of rabbit anti-acetyl histone H3 antibody and rotate the tubes in a cold room for 3 h. The antibody-coated beads can be prepared for any number of ChIPs.

2. Remove unbound antibody by washing beads twice with 1 mL of PBS-BSA. Resuspend beads in mixture of 30  $\mu\text{L}$  of PBS-BSA, 20  $\mu\text{L}$  of FA-lysis buffer and add 50  $\mu\text{L}$  of thawed chromatin extract, reserving an identical chromatin aliquot as an “input sample (In)” on ice. It is important that the extracts are thawed on ice. Rotate the tubes (beads and extracts) for 2 h in a cold room. Overnight binding often increases the background and thus should be avoided; although for low affinity antibodies, overnight binding may provide more complete immunoprecipitations.
3. Collect the beads by “pulse-spin” ( $\sim 8$  s) centrifugation and resuspend the beads in 1 mL PBS-BSA. Agitate the tubes vigorously by flicking the bottom of the tube (4–5 times), collect the pellet by centrifugation and aspirate the buffer under vacuum. Repeat the washes with 1 mL each of FA-lysis buffer and wash buffer I and II (once when using anti-rabbit antibody-conjugated beads and twice for anti-mouse antibody conjugated beads). Wash gently with 1 mL  $1\times$  TE, discard the supernatant, and pulse-spin again to remove any remaining TE.
4. To elute DNA from the immune complexes, add 100  $\mu\text{L}$  of elution buffer, vortex the tubes and incubate at  $65^\circ\text{C}$  in a water bath for 15 min. Collect the beads by centrifugation and transfer the supernatant to a new 1.5 mL tube. Add 150  $\mu\text{L}$  of elution wash buffer to the beads, vortex, and incubate for 10 min at  $65^\circ\text{C}$ . Collect the supernatant and combine it with the elution buffer eluate. This is marked “IP” sample.
5. Add 200  $\mu\text{L}$  of elution wash buffer to input tubes and leave both IP and In tubes at  $65^\circ\text{C}$  overnight to reverse the cross-linking.
6. On the next day, add 5  $\mu\text{L}$  of proteinase K to each tube and incubate in a  $37^\circ\text{C}$  water bath for 2 h to degrade the chromatin proteins.
7. The samples (IP and In) are then processed for DNA isolation, as follows.
8. Extract DNA from the samples using 250  $\mu\text{L}$  of phenol:chloroform:isoamyl alcohol (PCI) (25:24:1, v/v), vortexing for 15 s, centrifuging at  $16,000\times g$  for 10 min and

transferring the upper aqueous phase to a fresh 1.5 mL Eppendorf tube. Residual DNA is recovered from the chromatin samples by adding 130  $\mu$ L of water to the PCI and repeating the extraction procedure just described. The aqueous phases from the two extractions are combined, and further extracted sequentially with 380  $\mu$ L each of PCI and chloroform:isoamyl alcohol (24:1, v/v), exactly as above. To the final aqueous extract, add 1.0  $\mu$ L of glycogen and 50  $\mu$ L of 4 M LiCl. Fill the tube with 100% ethanol and incubate overnight at  $-80^{\circ}\text{C}$  to precipitate the DNA.

9. Collect the precipitated DNA by centrifugation at  $16,000\times g$  for 30 min, decant the supernatant by inverting the tube, add 70% ethanol and centrifuge at  $16,000\times g$  for 5 min. Decant the supernatant by inverting the tube and gently blotting with paper towel to remove the residual liquid. Dry the DNA pellet in a Speed-vac ( $\sim 5$  min) and resuspend in 30–50  $\mu$ L of TE containing RNase. DNA can be stored at  $-20^{\circ}\text{C}$  for PCR analysis at a later time.

### **3.4. PCR Analysis of Precipitated DNA**

1. The concentration of the DNA sequence of interest in the precipitated DNA is determined by PCR analysis. The primer sets are designed to amplify  $\sim 150$  bp segments. A primer set designed to amplify  $\sim 250$  bp is included in the reaction as an internal control (see Notes 9 and 10).
2. Thaw all reaction components on ice and dilute the In sample (1:300) in distilled water. Mix all samples well using a vortex mixer. For each reaction, use 2  $\mu$ L each of IP and diluted In samples.
3. Dispense 2  $\mu$ L of IP samples in triplicate and In samples in duplicate into PCR tubes.
4. Add 13  $\mu$ L PCR Master Mix to tubes containing IP and In samples, and pulse-spin in a microcentrifuge using adaptors for PCR-strips to collect the reaction mixture at the bottom of the tube.
5. The samples are amplified using the following settings: Initial denaturation at  $94^{\circ}\text{C}$  for 4 min, followed by 25 cycles of  $94^{\circ}\text{C}$  for 30 s,  $52^{\circ}\text{C}$  for 30 s,  $65^{\circ}\text{C}$  for 1 min, and a final extension for 5 min at  $65^{\circ}\text{C}$ .
6. After completion of PCR, add 3  $\mu$ L of  $6\times$  ChIP loading dye and briefly spin the tubes to collect the reaction mixture at the bottom of the tube.
7. Separate PCR products on a 6% TBE-polyacrylamide gel by electrophoresis at 100 V until the dye reaches the bottom of the gel. This allows for sufficient separation of the  $\sim 250$  bp control amplicon from the  $\sim 150$  bp experimental amplicon. The gel is transferred to a small tray containing a solution of

ethidium bromide (5  $\mu\text{g/mL}$ ) in water and incubated for 2 min. The gel is then transferred on a plastic sheet and DNA fragments are visualized under UV illumination. After ensuring that the reaction worked, the gel is trimmed such that 1–2 cm on either side of the band are included. After trimming, place a Whatman sheet of 3 MM paper about the size of the trimmed gel over the gel. Take the “gel-Whatman-plastic sheet” sandwich and flip it over so that the plastic sheet is facing up and carefully remove the plastic sheet. The gel is dried on a gel dryer for 45 min. The dried gels are wrapped in a plastic sheet and exposed to a phosphorscreen overnight.

8. The next day, the radioactivity in each DNA band is quantified using a phosphorimager. First, the ratio of intensity of the experimental to the control band is calculated for both IP and input samples. The resulting  $\text{IP}_{\text{exp}}/\text{IP}_{\text{con}}$  ratio is divided by the corresponding  $\text{In}_{\text{exp}}/\text{In}_{\text{con}}$  ratio to obtain the “occupancy” of the experimental over the control sequences in the IP versus In DNA samples, which is equated with the occupancy of the experimental sequences by the protein immunoprecipitated from chromatin. The occupancy values are averaged and standard error mean is calculated from ChIP experiments conducted in duplicate using two replicate cultures (four independent ChIPs in total) and PCRs in triplicate. Mean occupancy values of unity indicate no sequence-specific association of the protein of interest with the experimental sequences in chromatin. Although dependent on the magnitude of the SEM relative to the mean, occupancy values of two or more are generally statistically significant. The fold occupancy differs over a wide range for different chromatin-binding proteins. An activator like Gcn4 or Pol II that directly contacts DNA can exhibit occupancies of 10 or more for highly expressed genes. Coactivators typically exhibit occupancies between 2 and 10 (see Note 11).

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## 4. Notes

1. It is best to start early in the morning to allow sufficient time for cell growth and subsequent processing steps, which include induction (20–30 min), cross-linking (15 min), and washing of the cells (30 min). These steps usually take about 2 h after the cultures have reached the required  $A_{600}$  of 0.5–0.55. We have noticed that cultures grown beyond  $A_{600}$  of 0.6 frequently produce high nonspecific background signals (high  $\text{IP}_{\text{con}}$  values) and thus display decreased occupancies of the proteins of interest in chromatin.
2. We have noticed that optimal results are obtained for cells induced for 20–30 min.

3. After induction, all steps should be carried out as quickly as possible.
4. The 11% formaldehyde solution should be prepared fresh, just before the induction is complete.
5. We find that the addition of glycine does not completely inhibit HCHO action. It is therefore advisable to proceed to the next step immediately after the addition of glycine.
6. To prepare acid washed glass beads, take about 500 mL of glass beads in a 1,000 mL glass beaker. Carefully pour concentrated hydrochloric acid (in a fume hood) into the beaker to cover the glass beads. Cover the beaker with a glass plate (not with aluminum foil) and let it sit overnight. The following day, carefully decant the acid into a bottle and wash the beads with distilled water (500 mL each wash) at least five times. The beads must be washed with running distilled water until the pH is neutralized.
7. Efficient and complete fragmentation of the chromatin is crucial for obtaining a high degree of resolution and for preventing artifactual results. Optimal conditions for sonication must be empirically determined for each sonicator. We use a 450 Branson sonicator and a probe with a 1/8 in. tapered tip. The output knob is set to 1.8 with a 60% duty cycle. Care should be taken to prevent frothing of the sample during sonication, by keeping the tip of the probe submerged in the lysate and preventing any contact with the tube. It is important to check the extent of sonication by determining the length distribution of the sheared DNA fragments. One quick method is to reverse cross-linking ~50  $\mu$ L of chromatin sample, extracting the DNA with phenol: chloroform: isoamyl alcohol (PCI), resolving the fragments by agarose gel electrophoresis, and staining the DNA with ethidium bromide. Additionally, we generally analyze Gcn4 occupancies in the 5' end of the coding sequences in addition to the UAS of *ARG1*. Since Gcn4 is recruited exclusively to the UAS, significant Gcn4 occupancy of the 5' ORF is indicative of inefficient sonication. Alternately, antibodies against TBP or other promoter-restricted general transcription factors can be used to determine the efficiency of fragmentation.
8. The antibody used for ChIP depends on the histone modification or the chromatin protein under investigation. The beads utilized in ChIP depend on the species in which the antibody was raised as well as the isotype of the antibody. For example, if the histone antibodies are generated in rabbit, choose beads coated with anti-rabbit IgG. If the antibody belongs to an isotype other than IgG, beads coated with the relevant isotype should be used. Thus, the mouse monoclonal antibody against serine 5 phosphorylated Rpb1 (ser5P), the largest subunit of Pol II, belongs to the IgM isotype, hence beads coated with anti-IgM should be used for ChIP analysis of ser5P-Pol II occupancy.

9. We find that Platinum Taq (Invitrogen) gives the best results and linearity. However, other hot start polymerases can be standardized empirically.
10. The primers, with a  $T_m$  of 55°C, are designed to amplify a ~150 bp region, and the primer sets are selected to anneal with sequences separated by at least 300–500 bp in the DNA sequence of interest. The primer concentration should be experimentally tested to determine the linear range of the PCR reaction. This is determined as follows. The DNA is purified from the chromatin extracts, diluted 1:300, and PCR reactions are set up with varying amounts of DNA (1, 2, 4, and 6  $\mu$ L) and using 1–6  $\mu$ M of primer. The primer concentration that reveals a linear increase in  $In_{exp}$  band intensity with constant  $In_{exp}/In_{cont}$  ratios is chosen to conduct ChIP PCRs. These conditions should be established for each primer set to insure that the yield of the amplicon obtained in the PCR reaction is proportional to the amount of IP or In sample over the complete range of IP and In DNA concentrations represented in the samples used in the experiments. This condition must be fulfilled to achieve quantitative measurements of the concentrations of experimental and control DNA sequences present in the In and IP samples. Real-time PCR can be employed as an alternative means of quantification of DNA concentrations in the In and IP samples. We generally use sequences from the coding sequences of the *POL1* gene, or an intergenic region of chromosome V as an internal control, to determine the occupancy of factors that are recruited to coding regions. Alternately, the right arm of telomere VI (TELVIR) is used as an internal control for measuring changes in histone acetylation. This region is hypoacetylated and hence small changes in histone acetylation can be detected using TELVIR as an internal control.
11. Transcription activation is accompanied by varying levels of histone eviction at the promoters and coding regions of a transcribed gene. The absolute histone acetylation levels do not measure accurate changes in histone acetylation that occur during activated transcription across the gene of interest. The occupancy ratios ( $IP_{exp}/IP_{TELVIR}$  to  $In_{exp}/In_{TELVIR}$ ) for acetylated H3 and H4 should therefore be normalized to ratios obtained for total histone (H3 or H4) occupancy from the same chromatin sample. Thus, the changes in histone acetylation are presented as the fold change in acetylation relative to total H3 or H4. We have found that a “pan”-antibody against H3 provides the most reliable measurements of histone occupancy and, thus, we generally calculate the fold change in acetylation of H4 relative to total H3.

For analyzing the effects of HAT mutants (*gcn5 $\Delta$*  or *esa1-ts*), the histone acetylation is measured by calculating the  $IP_{exp}/In_{exp}$

ratios rather than occupancy values as defined above. This change in analysis is necessary because it is almost impossible to identify a control sequence for which  $IP_{\text{cont}}/In_{\text{con}}$  ratio is not substantially reduced by these HAT mutations. In such cases, it is necessary to carry out a large number of replicate immunoprecipitations, at least 4–6, to minimize variations in the amount of DNA that coimmunoprecipitates nonspecifically.

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