Video Article

Chromatin Immunoprecipitation (ChIP) of Histone Modifications from *Saccharomyces cerevisiae*

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Abstract

Histone post-translational modifications (PTMs), such as acetylation, methylation and phosphorylation, are dynamically regulated by a series of enzymes that add or remove these marks in response to signals received by the cell. These PTMS are key contributors to the regulation of processes such as gene expression control and DNA repair. Chromatin immunoprecipitation (chIP) has been an instrumental approach for dissecting the abundance and localization of many histone PTMs throughout the genome in response to diverse perturbations to the cell. Here, a versatile method for performing chIP of post-translationally modified histones from the budding yeast *Saccharomyces cerevisiae (S. cerevisiae)* is described. This method relies on crosslinking of proteins and DNA using formaldehyde treatment of yeast cultures, generation of yeast lysates by bead beating, solubilization of chromatin fragments by micrococcal nuclease, and immunoprecipitation of histone-DNA complexes. DNA associated with the histone mark of interest is purified and subjected to quantitative PCR analysis to evaluate its enrichment at multiple loci throughout the genome. Representative experiments probing the localization of the histone marks H3K4me2 and H4K16ac in wildtype and mutant yeast are discussed to demonstrate data analysis and interpretation. This method is suitable for a variety of histone PTMs and can be performed with different mutant strains or in the presence of diverse environmental stresses, making it an excellent tool for investigating changes in chromatin dynamics under different conditions.

Video Link

The video component of this article can be found at https://www.jove.com/video/57080/

Introduction

The dynamic post-translational modification (PTM) of histones is a key regulatory mechanism for many DNA-templated processes, including transcription, replication and DNA repair[1,2]. The ability to determine the abundance and precise localization of modified histones concomitant with these processes is therefore critical to understanding their regulation under different conditions in the cell. The development of chromatin immunoprecipitation (chIP) as a method largely stemmed from biochemical studies of the interactions of proteins with DNA, particularly *in vitro* methods using chemical crosslinkers, coupled with the need to evaluate the dynamic nature of protein-DNA interactions *in vivo* and at specific regions of the genome[3,4,5]. The advancement of quantitative PCR (qPCR) and sequencing technologies has also expanded the ability to perform chIP experiments with quantitative comparisons and across whole genomes, making it a powerful tool for dissecting DNA-protein interactions at multiple levels.

Currently, chIP is a required method for any research group interested in chromatin-mediated regulation of the genome as there are no comparable methods for directly interrogating the physical link between a modified histone and a specific genomic locus *in vivo*. Although variations of this method using next generation sequencing to map histone modifications throughout the genome[6,7] are available, these approaches may address different scientific questions and their scale, cost and technical resources may be limiting for some research groups. Additionally, targeted chIP-qPCR is necessary to complement these approaches by providing methods to both optimize the chIP protocol prior to sequencing and to validate results from the epigenomic datasets. Mass spectrometry based approaches for identifying the full complement of histone marks associated with genomic regions have also emerged[8,9,10,11], however, these approaches have some limitations regarding which regions of the genome can be probed and they require technical expertise and instrumentation that will not be available to all research groups. Therefore, chIP remains a foundational method for analyzing the abundance and distribution of histone modifications under diverse conditions for all research groups interested in epigenetics, chromatin and the regulation of genomic functions.

Here, we describe a method for chIP using the budding yeast model *Saccharomyces cerevisiae (S. cerevisiae)* to investigate the distribution of histone PTMs at chromatin. This approach relies on a number of core components of chIP protocols developed in yeast and also applied to diverse model systems[12,13]. Interactions between modified histones and DNA in the cell are preserved by crosslinking with formaldehyde. Following lysate preparation, chromatin fragments are solubilized into uniformly-sized fragments by digestion with micrococcal nuclease. Immunoprecipitation of the modified histones is performed with either commercial or lab-generated antibodies and any associated DNA is

isolated and analyzed for enrichment at particular genomic regions using qPCR (**Figure 1**). For many histone modifications, the quantity of DNA obtained from this protocol is sufficient for testing more than 25 different genomic loci by qPCR.

This chIP method is highly versatile for monitoring the distribution of a single histone modification across multiple mutant strains or environmental conditions, or for testing multiple histone modifications in wildtype cells at a number of genomic loci. Furthermore, numerous components of the protocol are easily adjustable to optimize detection of either highly- or lowly-abundant histone marks. Finally, performing chIP of modified histones in budding yeast provides the opportunity to use key controls for antibody specificity that are largely unavailable in other systems. Namely, yeast strains can be generated that carry point mutations in histone residues that are targeted for modification, and, in some cases, there is only a single enzyme that catalyzes modification on a particular histone residue (*e.g.* histone lysine methyltransferases). Therefore, chIP can be performed in either the histone mutant or enzyme deletion strains to assay the extent to which non-specific binding of the antibody may be occurring and generating false positive results. This control is particularly valuable for newly-developed antibodies, and may even be used to validate antibody specificity for conserved histone modifications prior to their use in other systems. This approach complements other methods to test antibody specificity that distinguish among different modification states (such as mono-, di- and tri-methylation), including probing arrays of modified peptides and performing western blots of histones or nucleosomes with defined modifications. Overall, chIP in budding yeast is a powerful method for assessing the dynamics of histone PTMs throughout the genome and dissecting the mechanisms governing their regulation.

Protocol

1. Pre-bind antibody to magnetic beads

1. Mix the protein A/G magnetic beads well and transfer 20 µL of magnetic beads per one immunoprecipitation (IP) sample to a 1.5 mL tube.
   NOTE: When pipetting beads, use wide-bore, low-retention tips.
2. Place the tube with beads into a magnetic stand and allow beads to collect on side of the tube. Remove the supernatant.
3. Wash the beads 3 times with 1 mL of cold Tris-buffered saline (TBS) (50 mM Tris-HCl pH 7.5, 150 mM NaCl).
4. Add 200 µL of TBS to beads and appropriate amount of antibody. Rotate at 8 rpm overnight at 4 °C.
   Note: For many histone PTM antibodies, 1-3 µg of antibody per IP is sufficient. However, titration experiments are recommended to determine appropriate concentrations. Recommended concentrations for well-characterized, commercial histone PTM antibodies are often accurate and can be found in published reports or product literature.
5. Place the beads in a magnetic stand and remove the supernatant. Wash them with 1 mL of TBS.
6. Resuspend the beads in 20 µL of TBS per IP sample plus an additional 5 µL (in case of pipetting error) of TBS.
   NOTE: The beads can be stored short-term at 4 °C until use.

2. Grow yeast cells

1. Inoculate 10 mL of YPD (10% yeast extract, 20% peptone and 20% dextrose) with a single colony of the appropriate strain and grow it at 30 °C overnight in a shaking incubator at 220 rpm.
2. Measure the optical density at 600 nm (OD_{600}) of the yeast culture using a spectrophotometer. Dilute the culture to an OD_{600} = 0.2 in 100 mL YPD in a new flask.
3. Grow the cells at 30 °C in a shaking incubator at 220 rpm until they reach mid-log phase (OD_{600} = 0.6-0.8).

3. *In vivo* crosslinking of proteins to DNA

1. When cultures reach mid-log phase, add 2.7 mL of 37% formaldehyde directly to the medium, for a final concentration of 1% formaldehyde. Transfer the flask to a shaker at 25 °C (or room temperature) and shake it at 50 rpm for 15 min.
2. Add 5 mL of 2.5 M glycine to the medium and continue shaking at 50 rpm at room temperature for 5 min to quench the formaldehyde.
3. Transfer the cells to centrifuge bottles and spin the cells at 5800 x g for 5 min at 4 °C. Decant supernatant.
   1. Wash the cells by resuspending them in 45 mL of cold TBS and transfer the suspension into a 50 mL conical tube. Spin the tube at 2800 x g for 3 min at 4 °C. Remove the supernatant.
   2. Wash the cells in 10 mL of cold chIP Lysis Buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% nonidet octyl phenoxypolyethoxylethanol (NP-40), stored at 4 °C).
   3. Spin the cells at 2800 x g for 3 min at 4 °C. Remove the supernatant.
      NOTE: Cells can be frozen with liquid nitrogen and stored at -80 °C until further processing.

4. Make yeast lysates

1. Resuspend the cells in 1 mL of cold ChIP Lysis Buffer with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1:1000 dilution of the yeast protease inhibitor cocktail. Transfer the suspension to a 2.0 mL screw cap tube containing 200 µL of glass beads.
2. Transfer the cells to a bead beating apparatus for high speed agitation at 4 °C and bead beat 6 times for 30 s each. Keep the cells on ice for at least 1 min between each bead beating.
3. Transfer the lysate to a 1.5 mL tube using gel loading tips. Centrifuge the lysate at 15500 x g for 20 min at 4 °C.
4. Discard the supernatant and resuspend the pellet in 250 µL of MNase Digestion Buffer (10 mM Tris-HCl pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 21 mM $CaCl_2$, 1% NP-40, stored at 4 °C) by gently pipetting up and down.
5. Add 2.5 µL of MNase to the reactions. Mix them gently by inverting 4-6 times and immediately incubate it in a 37 °C water bath for 20 min.
   Note: Digest conditions should be optimized when a new stock of MNase is used. See step 10 for the protocol.
6. Immediately place the tubes on ice to stop the reaction and add 5 µL of 0.5 M EDTA for a final concentration of 10 mM EDTA. Mix them gently by inversion.

7. Centrifuge the reaction at 15500 x g for 15 min at 4 °C and transfer the supernatant to a new 1.5mL tube.
   NOTE: Soluble (digested) chromatin will be in the supernatant. The pellet contains anything undigested and insoluble cell debris.

5. Immunoprecipitate (IP) modified histones

1. Determine the protein concentration of each MNase-released chromatin fraction using a Bradford assay. Add 1 mL of Bradford reagent to each of 8 disposable cuvettes plus 1 additional cuvette for each protein sample to be tested.
   1. Prepare a stock solution of 2 mg/mL bovine serum albumin (BSA).
   2. Add the appropriate volume to achieve the following concentrations of BSA in 1 mL of Bradford reagent: 0 µg/mL, 0.5 µg/mL, 1 µg/mL, 2 µg/mL, 4 µg/mL, 6 µg/mL, 8 µg/mL and 10 µg/mL. Add 2 µL of the MNase-released chromatin fraction to the additional cuvettes.
   3. Cover the top of each cuvette with a small piece of parafilm and invert the cuvettes 4-6 times to mix the solution well. Incubate the cuvettes at room temperature for 15 min.
   4. Using a visible light spectrophotometer, measure the absorbance at 595 nm for the standard curve and experimental samples.
      Note: Use the cuvette without BSA (0 µg/mL) to blank the spectrophotometer prior to taking the first measurement.
   5. Plot a standard curve using the absorbance values for the different concentrations of BSA on software associated with the spectrophotometer or spreadsheet software. Based on the standard curve and the dilution factor used (1:500), use the absorbance values to calculate the protein concentration for each of the chromatin fraction samples.
2. For each IP, add 50 µg of total protein from the MNase-released chromatin fraction to ChIP Lysis Buffer to reach a final volume of 1 mL.
   NOTE: If setting up more than one IP per lysate, make a master mix of the lysate and ChIP Lysis Buffer and aliquot 1 mL to individual tubes for the IP.
3. Remove 100 µL from the IP mix to process as the input sample. Freeze the input sample at -20 °C until step 7.1.
   NOTE: Any remaining chromatin sample can also be frozen at -20 °C and used for a subsequent IP if desired.
4. Add 20 µL of magnetic Protein A/G beads pre-bound with antibody to each IP sample. Rotate the sample at 8 rpm for 3 h (standard) or overnight (if preferred) at 4 °C.

6. Wash IPs and elute histone-DNA complexes

1. Place the tubes in a magnetic stand and let beads collect on the side of the tube. Remove the supernatant using a pipette. Wash the beads successively with 1 mL of each of the buffers below.
   1. Perform each wash by rotating at 8 rpm for 5 min at 4 °C, placing beads in a magnetic stand, removing supernatant, and adding next buffer: 2 x - ChIP Lysis Buffer, 2 x - High Salt Wash Buffer (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% NP-40, stored at 4 °C), 1 x - LiCl/detergent Wash Buffer (10 mM Tris-HCl pH 8.0, 250 mM LiCl, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate, stored at 4 °C), 1 x - TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA, stored at 4 °C), and 1 x - TE .
   2. With last TE wash, transfer beads to a new tube using 0.5 mL of TE to transfer most of the beads, then another 0.5 mL of TE to wash the tube and transfer remaining beads.
2. Add 250 µL of freshly made ChIP Elution Buffer (1% SDS, 1 mM NaHCO$_3$). Vortex the solution briefly. Rotate the samples at 8 rpm for 15 min at room temperature.
3. Place tube in a magnetic stand and collect the beads. Carefully transfer the supernatant to a new tube and avoid the beads.
   NOTE: The eluate contains immunoprecipitated proteins and associated DNA.
4. Add another 250 µL of ChIP Elution Buffer to beads. Rotate the samples at 8 rpm for 15 min at room temperature.
5. Carefully transfer the supernatant to the tube containing the first elution. If necessary, remove a smaller volume (240 µL) for all IPs to avoid disturbing the beads.

7. Reverse protein-DNA crosslinks

1. Thaw the input samples and add 400 µL of ChIP Elution Buffer to each input.
2. To both input and IP samples, add 20 µL of 5 M NaCl, 5 µL of 20 mg/mL glycogen, and 12.5 µL of 20 mg/mL Proteinase K. Mix well by inverting or flicking tubes. Incubate the samples in a 65 °C water bath overnight.

8. Purify and concentrate DNA

1. Add 10 µL of 10mg/mL RNase A to the input and IP samples. Incubate the samples in a 37 °C water bath for 30 min.
2. Add 600 µL of phenol:chloroform:isoamyl alcohol (25:24:1 PCI) to the aqueous solution and mix them well. Spin them at 15500 x g for 5 min at room temperature.
   1. Remove the aqueous layer to a new tube. Add 600 µL of PCI and mix them well. Spin the tube at 15500 x g for 5 min at room temperature. Transfer the aqueous layer to a new tube.
      Caution: Work in the fume hood and wear appropriate personal protective equipment when working with PCI. Dispose liquid and solid waste as instructed by institutional guidelines.
3. Add 0.1x volume of 3M sodium acetate and 1 mL of cold 100% ethanol to precipitate the DNA. Invert the tube 4-6 times to mix the solution well. Incubate at -20 °C overnight.
   1. Spin the tube at 15500 x g for 20 min at 4 °C. Carefully remove the supernatant with a pipette.
   2. Wash the pellet in 1 mL of cold 70% ethanol. Spin it at 15500 x g for 10 min at 4 °C. Carefully remove the supernatant with a pipette.

3. Air-dry the pellets in the hood for approximately 20 min (until completely dry). Resuspend IP samples in 50 µL of nuclease-free water and resuspend input samples in 100 µL of nuclease-free water. Store the DNA samples at -20 °C until performing qPCR.

9. Quantitative PCR (qPCR) to detect enriched genomic regions

1. Make a qPCR master mix for each set of primers. One reaction contains 5 µL of 2x qPCR mix, 0.25 µL of 20 µM forward primer, and 0.25 µL of 20 µM reverse primer.
   NOTE: Proper primer design and testing for qPCR experiments are described elsewhere[14,15,16].
2. Make DNA master mixes for each DNA sample. One reaction contains 0.5 µL of DNA and 4.0 µL of nuclease-free water.
3. Add 5.5 µL of the appropriate primer master mix and 4.5 µL of the appropriate DNA master mix to each well on a 384-well qPCR plate.
   Note: Start with adding 5.5 µL of the primer mix to each well. Spin the plate at 200 x g for 1 min at room temperature before adding 4.5 µL of the DNA mix.
4. Adhere the seal to the plate and spin at 200 x g for 1 min at room temperature.
5. Perform qPCR using a real-time qPCR system with the following conditions: 95 °C for 3 min; 40 cycles of 95 °C for 10 s, 55 °C for 30 s; melting curve 65 °C to 95 °C with 0.5 °C increments, 5 s.
6. For each primer pair, calculate the percent input of the IP sample relative to the 5% input sample used in the qPCR. Use the means of the technical PCR triplicates to perform the calculations.
   NOTE: If substantial variation is observed in the PCR triplicates, it is best to repeat the qPCR with attention to master mix composition, pipetting errors and cross-contamination between wells in the 384-well plate.
   1. Adjust the Cq values for the input to 100% by subtracting the number of cycles representing the dilution factor from the raw Cq values.
      NOTE: Five percent input represents a dilution factor of 20-fold, which equals 4.32 cycles ($\log_2 20 = 4.32$).
   2. Calculate the percent input as $2^{(Cq_{input} - Cq_{IP})}$ multiplied by 100.

# 10. Determine MNase digest conditions (recommended prior to first full chIP experiment)

1. Follow steps 2.1 through 4.4 of the preceding protocol. Scale up the protocol to generate enough lysate for multiple samples of MNase digestion of the chromatin-containing pellet. At step 4.4, resuspend the pellets in 1.25 mL of MNase Digestion Buffer. Aliquot the samples to 5 tubes so that each contains 250 µL of the chromatin pellet resuspended in MNase Digestion Buffer.
2. Add 0, 1.5, 2.5 or 5 µL of MNase to each tube. Mix them gently by inverting 4-6 times and immediately incubate the tubes in a 37 °C water bath for 20 min.
3. Stop reactions by immediately placing tubes on ice and adding 5 µL of 0.5 M EDTA for a 10 mM final concentration. Mix the solution gently by inversion.
4. Centrifuge the tubes at 15500 x g for 15 min at 4 °C and transfer the supernatant to a new 1.5 mL tube.
5. Add SDS to 1% final concentration (25 µL of 10% stock solution) and $NaHCO_3$ to 0.1 M final concentration (25 µL of 1M stock solution) to the samples in the 1.5 mL tubes.
6. Add 20 µL of 5M NaCl, 5 µL of glycogen, and 12.5 µL of 20mg/mL proteinase K to the samples in the 1.5mL tubes. Incubate them at 65 °C overnight.
7. Add 10 µL of 10mg/mL RNaseA. Incubate at 37 °C for 30 min.
8. Add 300 µL of PCI to the aqueous solution and mix them well. Spin at 15500 x g for 5 min at room temperature.
   1. Remove aqueous layer to a new tube. Add 300 µL of PCI and mix well. Spin at 15500 x g for 5 min at room temperature. Transfer aqueous layer to a new tube.
9. Add 0.1x volume of 3 M sodium acetate (usually ~30 µL) and 1 mL of cold 100% ethanol to precipitate the DNA. Invert the tube 4-6 times to mix well. Incubate the tube at -80 °C for 1 h or -20 °C overnight.
   1. Spin the tube at 15500 x g for 20 min at 4°C. Carefully remove supernatant with a pipette.
   2. Wash the pellets in 1 mL of cold 70% ethanol. Spin at 15500 x g for 10 min at 4 °C.
   3. Let the pellets air-dry in the hood approximately 20 min (or until completely dry). Resuspend the pellets in 30 µL of nuclease-free water.
10. Add the DNA loading dye and run 20 µL on a 2% agarose gel to visualize digested DNA.

Representative Results

One key component of this protocol is optimizing the concentration of micrococcal nuclease (MNase) used to digest the chromatin into soluble fragments, as outlined in Step 10. This is critical for obtaining high resolution data regarding the distribution of histone modifications at genomic regions of interest. An MNase titration should be performed to determine the most suitable concentration to achieve primarily mono-nucleosomes with a smaller amount of di-nucleosomes in the soluble chromatin fraction. This can be visualized by extracting the DNA following MNase digestion of the chromatin-containing pellet and performing agarose gel electrophoresis (**Figure 2**). In the preparation of MNase used here, 2.5 µL of enzyme at 20 units/µL produced predominantly mono-nucleosomes, and therefore this amount was used for the chIP.

Two sets of representative results are shown for this chIP procedure (**Figure 3**). In the first example, a histone methyl mark is evaluated in either wildtype cells or cells lacking the methyltransferase which catalyzes this mark. Specifically, chIP was performed with an antibody against H3K4me2, as well as against histone H3 as a control, in wildtype and *set1Δ* cells. H3K4me2 primarily localizes just downstream of the transcription start site at the 5' end of coding regions and, in the absence of Set1, H3K4me2 cannot be detected in cells[17,18,19]. The *set1Δ* strain therefore serves as a control to indicate the amount of background signal that may be attributed to non-specific binding of other histone marks or DNA to the antibody. In this case, the wildtype strain showed clear enrichment for H3K4me2 at the 5' end of two genes known to be direct targets of Set1, *PMA1* and *ERG11*[20,21], whereas no signal was observed in *set1Δ* cells (**Figure 3A**). As expected, there is no enrichment of the H3K4me2 mark at telomere (TEL) *07L*. The expression of the middle sporulation gene *SPR3* has also been reported to be regulated by Set1[22,23], however the enrichment of H3K4me2 at the 5' end of the *SPR3* ORF is most similar to the levels observed at *TEL07L*, as compared to either *PMA1* or *ERG11*. Evaluating the localization of this methyl mark indicates that Set1-mediated regulation of *SPR3* is likely to occur by a different mechanism than at *PMA1* or *ERG11*, as previously observed[22].

In the second example, chIP of acetylated H4K16 is shown relative to histone H4 in wildtype cells and cells lacking the histone deacetylase Sir2, which removes H4K16ac marks. H4K16ac is depleted in the heterochromatic-like chromatin close to the telomere, and enriched in euchromatin more distal from the telomere[24,25], as demonstrated for *TEL07L* and *TEL15L* (**Figure 3B**). Additionally, the loss of Sir2 causes an increase in H4K16ac at specific telomeric and subtelomeric regions, although no change is observed at the control gene *PMA1*, where Sir2 is not known to localize. While these data show a clear increase in H4K16ac levels in *sir2Δ* cells, the detected change in H4K16ac, which is not expected to be as substantial as the change in H3K4me2 in wildtype versus *set1Δ* cells, may be obscured if chIP parameters are not properly optimized. Recommendations for optimization are described in the Discussion.
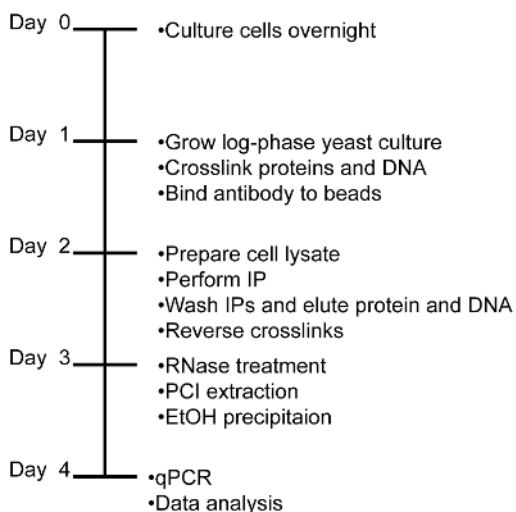

Figure 1. Timeline of chIP procedure. The typical schedule for the chIP protocol is shown. Possible variations are indicated in the text. Please click here to view a larger version of this figure.
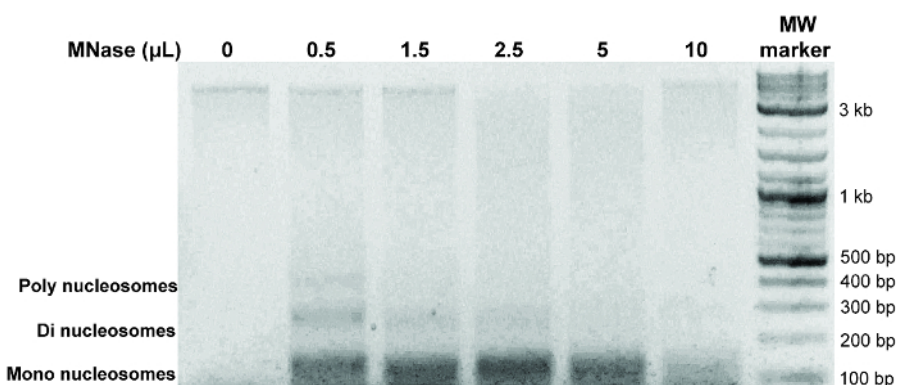
Figure 2. Test MNase digestion for solubilization of mono-nucleosomes. Agarose gel electrophoresis of DNA isolated following digestion of the chromatin pellet with varying amounts of MNase (20 units/µL). DNA from mononucleosomes migrates at approximately 150 bp, from dinucleosomes at approximately 300bp and DNA from polynucleosomes is found at increasingly higher molecular weights. Please click here to view a larger version of this figure.
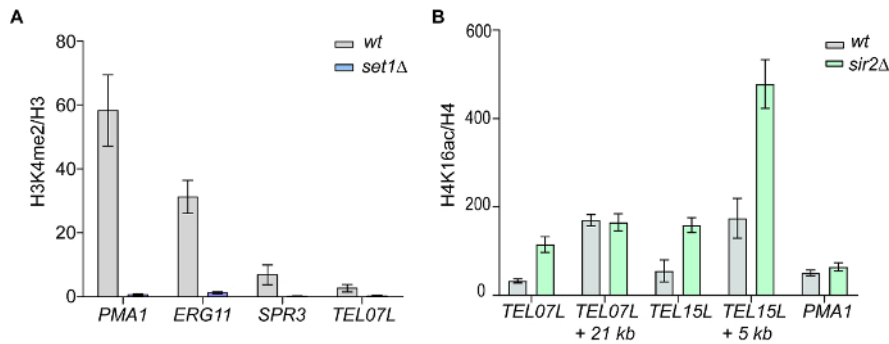
Figure 3. ChIPs of H3K4me2 and H4K16ac in wildtype and mutant yeast strains.(A) ChIP using antibodies against H3K4me2 and H3 was performed in wildtype and *set1Δ* cells. Percent input was calculated for each primer pair for both the H3K4me2 and H3 IPs and the ratio is graphed to indicate the relative enrichment of H3K4me2 at the indicated loci. The primers for *PMA1*, *ERG11* and *SPR3* generate amplicons at the 5' end of each ORF, whereas the *TEL07L* primer pair is adjacent to the telomere on the left arm of Chromosome 7. The mean of three biological replicates is shown and the error bars represent standard error of the mean. (**B**) ChIP was performed with antibodies recognizing H4K16ac and H4 in wildtype and *sir2Δ* cells and plotted as described for **Figure 3A**. Primer pairs amplify sequences adjacent to telomeres (*TEL07L* and *TEL15L*) and downstream within previously defined euchromatic regions of each subtelomere (at 21 kb and 5 kb distal from the telomere, respectively). Please click here to view a larger version of this figure.

Strain Number	Genotype	Reference
yEG230	MATα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0	Ref. 23
yEG223	MATα sir2Δ::NATMX	This study
yEG232	MATa set1Δ::KANMX	Ref. 23

Table 1. Yeast strains used in this study.

Oligo Number	Location	Sequence
oEG141	TELVIIL F	AGCCCGAGCCTGTACTAAAT
oEG142	TELVIIL R	CAAAAGAAACTTTTCATGGCA
oEG153	5'PMA1 F	TCAGCTCATCAGCCAACTCAAG
oEG154	5'PMA1 R	CGTCGACACCGTGATTAGATTG
oEG220	TELVIIL + 21KB F	AAACAATGGGACCCTTCTGA
oEG221	TELVIIL + 21KB R	AACACCTTGCAAAACACAGG
oEG280	TELXVL F	ATCCTGCAATTGGGCCACTAT
oEG281	TELXVL R	AGCGGAAGGCATATTAACGT
oEG282	TELXVL + 5KB F	AGGCGATGTAATCTCACCAA
oEG283	TELXVL + 5KB R	CATTCACACATCCTGCTACCA
oEG568	ERG11 F	CCTCTTATTCCGTCGGTGAA
oEG569	ERG11 R	TGTGTCTACCACCACCGAAA
oEG963	SPR3 F	TCTGGATTCGCTGAGGAAGT
oEG964	SPR3 R	TTTCAGTTCAGGGCTTTTCG

Table 2. Primers used in this study.

Discussion

The procedure described here allows for the efficient recovery of DNA associated with modified histones in yeast cells by immunoprecipitation. This is followed by qPCR using primers which amplify regions of interest to determine local enrichment or depletion of specific histone modifications. Despite being developed as a method almost 20 years ago, chIP remains the defining assay for investigating histone modification status at different genomic regions and under diverse conditions. Although chIP coupled to next generation sequencing technologies can interrogate histone modifications on a genome-wide scale[6,7], the costs and required data analysis of these approaches may limit their use in some lab settings. Furthermore, these epigenomic experiments are often followed by chIP coupled to qPCR to validate observations of histone modification status at some genomic loci. There are few comparable methods that provide the utility of chIP in physically linking a histone mark (or other protein) to a specific location within the genome and analyzing the dynamics of this interaction under different environmental or biological conditions. Although there has been recent success in isolating chromatin from defined genomic regions and identifying the local histone marks using mass spectrometry[8,9,10,11], these methods pose technical challenges and their utility across diverse lab types may be limited by adequate access to mass spectrometry instrumentation and data analysis resources. The protocol described here is technically and

economically accessible to diverse lab settings, with the primary possible hurdle being access to a qPCR machine. ChIP remains the gold-standard for defining genomic localization of modified histones in yeast and other systems.

This is a versatile protocol with a number of steps that can be optimized to suit different experimental conditions, including simultaneously testing multiple mutants or environmental conditions. Alternatively, sufficient chromatin is usually generated from one lysate to perform multiple IPs with up to at least four different antibodies. This protocol can also be used for chIP of non-histone chromatin proteins that are epitope-tagged, or for which antibodies have been generated. If this protocol is to be adapted for chIP of non-histone proteins, parameters including fixation time, chromatin concentration in the IP and antibody concentration are often critical to obtaining enrichment of the targeted protein over background. Most commonly, it is useful to increase the fixation time with formaldehyde to between 45 and 60 min and to increase the amount of chromatin used in the IP (up to 200 μg of total protein).

There are a number of potential variables that contribute to the quality and reproducibility of histone modification chIP experiments. Although a sub-optimal experiment may often yield positive results when comparing stark differences (for example, the levels of H3K4me2 in wildtype or *set1Δ* cells, as shown in **Figure 3A**), more subtle differences are likely to be masked in an improperly performed experiment (such as the differing levels of H4K16ac at *TEL07L* in wildtype or *sir2Δ* cells, as shown in **Figure 3B**). Experimental parameters to consider include the quantity of yeast cells used, fixation time, concentration of the chromatin fraction used in the IP, fragment size of the digested DNA-protein complexes and the antibody quality and concentration. One limitation of this approach is that optimization of experimental parameters is generally required for each histone modification being tested and the mutant strains and/or conditions under investigation. There may be subtle differences in histone mark levels or localization under different conditions, and the ability to detect these differences is dependent on the parameters described above. We discuss some of the key considerations for developing the most sensitive experimental approaches below.

As described in the protocol, it is recommended to test multiple concentrations of MNase to determine the optimal concentration prior to performing a full chIP experiment. Ensuring that the DNA within the IP is adequately fragmented is key to obtaining high resolution chIP results. Even though the amplicon size of the qPCR products may be small (generally less than 100 bp), if the fragment size of the DNA is substantially larger, the qPCR may falsely indicate enrichment at a specific locus when, in actuality, the histone mark may be localized hundreds of basepairs away. While this protocol relies on MNase to solubilize the chromatin into mono- and di-nucleosomes, other chIP protocols also commonly use sonication to shear chromatin[6,7]. Sonication is also a reliable method for solubilizing chromatin fragments, although the shear sizes tend to be less uniform and it can be more difficult to achieve consistent results between experiments. Although sonication may be preferable for chIP of some non-histone proteins, the use of MNase to digest chromatin into primarily mono-nucleosomes can improve the resolution of histone modification chIP experiments.

One requirement for a successful chIP experiment is an antibody that uniquely and with high affinity recognizes the histone modification of interest. The primary limitation of chIP as a method for histone modification detection is its reliance on a high-quality, validated antibody; without such a reagent, results obtained from chIP are not likely to be biologically-relevant. There are a number of commercially-available antibodies against histone modifications found in budding yeast, although the quality and validity of the antibodies is variable. Efforts to validate these antibodies have produced useful resources for determining the best available antibody for a given experiment[26]. It is recommended that antibodies used for chIP are validated with a diversity of methods for their specificity, including by probing arrays of modified and unmodified histone peptides, performing western blots of whole cell extracts and purified histones, and in chIP experiments with proper controls, such as strains with a modifying enzyme deleted or carrying point mutations in the modified histone residue. For new antibodies that recognize uncharacterized marks or lab-generated antibodies, these specificity experiments are critical to determining whether the antibody may be a valid reagent for chIP. In addition to validating the specificity of the antibody, performing an antibody titration for the IP from a wildtype strain and a negative control strain is often useful for determining the amount of antibody required (relative to a set chromatin concentration) to detect accurate differences in enrichment between strains or regions of the genome. Furthermore, if some data regarding genomic distribution patterns of the mark are known, positive and negative control primer sets should be included in all qPCR experiments to validate any individual experiment and to simplify comparisons between experiments.

Overall, this work describes a foundational method for researchers interested in interrogating histone modification status at any genomic region in budding yeast. The versatility of this protocol and its applicability to diverse experimental questions makes it an extremely useful tool for dissecting chromatin dynamics at the molecular level.

Disclosures

The authors have nothing to disclose.

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