Chapter 4

Chromatin Immunoprecipitation (ChIP) Assay in *Candida albicans*

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Abstract

Chromatin immunoprecipitation (ChIP) is a widely used technique which can determine the in vivo association of a specific protein on a particular DNA locus in the genome. In this method cross-linked chromatin is sheared and immunoprecipitated with antibodies raised against a target protein of interest. The end result of this process is the enrichment of DNA fragments associated with the desired protein. Thus, interactions between proteins and genomic loci in cellular context can be determined by this technique. Here, we are describing a ChIP protocol that is optimized for *Candida albicans*. The protocol requires 4–5 days for completion of the assay and has been used to produce robust ChIP results for diverse proteins in this organism and its related species including *Candida dubliniensis* and *Candida tropicalis*.

Key words Protein–DNA interaction, Antibody, Affinity purification, *Candida dubliniensis*, *Candida tropicalis*, Polymerase chain reaction (PCR), Microarray, Next-generation sequencing

1 Introduction

Chromatin immunoprecipitation (ChIP) is a useful technique that has become indispensable over the last decade for studying the in vivo interactions of proteins with specific regions of a genome. The first ChIP assay was developed for analyzing the binding of RNA polymerase II over transcribed and poised genes in *Escherichia coli* and *Drosophila* [1–3]. Over the years, this technique has found its use in diverse cellular processes ranging from the identification of transcription factor binding sites to post-translational modifications (PTMs) of histones associated with expressed or silent chromatin [4]. The basic principle behind this technique involves the use of a small, easily diffusible and reversible cross-linking agent (like formaldehyde) for capturing in vivo protein–DNA interactions. Following cross-linking, the protein bound DNA is sheared by sonication or digested by enzymes to generate suitably smaller

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fragments which are then immunoprecipitated (IP) with antibodies specific to the protein of interest. During immunoprecipitation, the DNA sequences associated with the protein are preferentially pulled down from the sheared chromatin, thereby giving a realistic approximation of the genomic regions associated with the protein. Following elution of protein–DNA complexes from Protein-A or Protein-G agarose resins, chromatin is de-cross-linked, treated with proteases, and DNA fragments are purified. The technique described above is commonly known as the X-ChIP (Fig. 1). On the other hand, in native ChIP (N-ChIP), proteins remain in the native state, i.e., they are not cross-linked to DNA by formaldehyde. In the N-ChIP technique, shearing of chromatin is performed by micrococcal nuclease (MNase) digestion that fragments chromatin in mononucleosomes as compared to the random fragmentation obtained by sonication. The N-ChIP protocol is suitable for proteins that can remain stably associated during chromatin processing and immunoprecipitation (such as histones) [5]. There are several methods of analysis by which the enriched protein binding sites on the genome can be detected. In the initial studies the IP DNA was immobilized onto a nitrocellulose membrane and hybridized with a radioactive probe from the region of interest to detect binding [3]. However, because of its convenience and to facilitate fast and accurate detection, polymerase chain reaction (PCR), both in its conventional semi-quantitative end-point form [6] and the more sophisticated quantitative real-time PCR (qPCR) [7], is more commonly used for determining enrichment of proteins at specific genomic regions. Subsequently, the applicability of ChIP assays widened significantly as genome-wide association of proteins can be studied by hybridizing ChIP DNA to a DNA microarray (ChIP on chip or ChIP-chip) [8], or can be sequenced by the next-generation sequencing technique (ChIP-sequencing or ChIP-seq) [9, 10].

In this chapter, we describe a ChIP protocol that is followed in our laboratory to study in vivo protein–DNA interactions in the pathogenic budding yeast *Candida albicans* [11–15]. Given the prominence of *C. albicans* as a prolific opportunistic pathogen [16, 17], understanding the global distribution of proteins such as transcription factors and histone PTMs is important for constructing regulatory networks controlling virulence and associated attributes in this organism. Apart from that, *C. albicans* is important as an alternative model system for studying the evolutionary transitions in the mechanism of basic biological processes such as cell division due to the atypical architecture of well-known chromatin landmarks such as centromeres [11, 12]. Thus, an optimized ChIP protocol for *C. albicans* will provide a robust tool for studying these relevant problems.

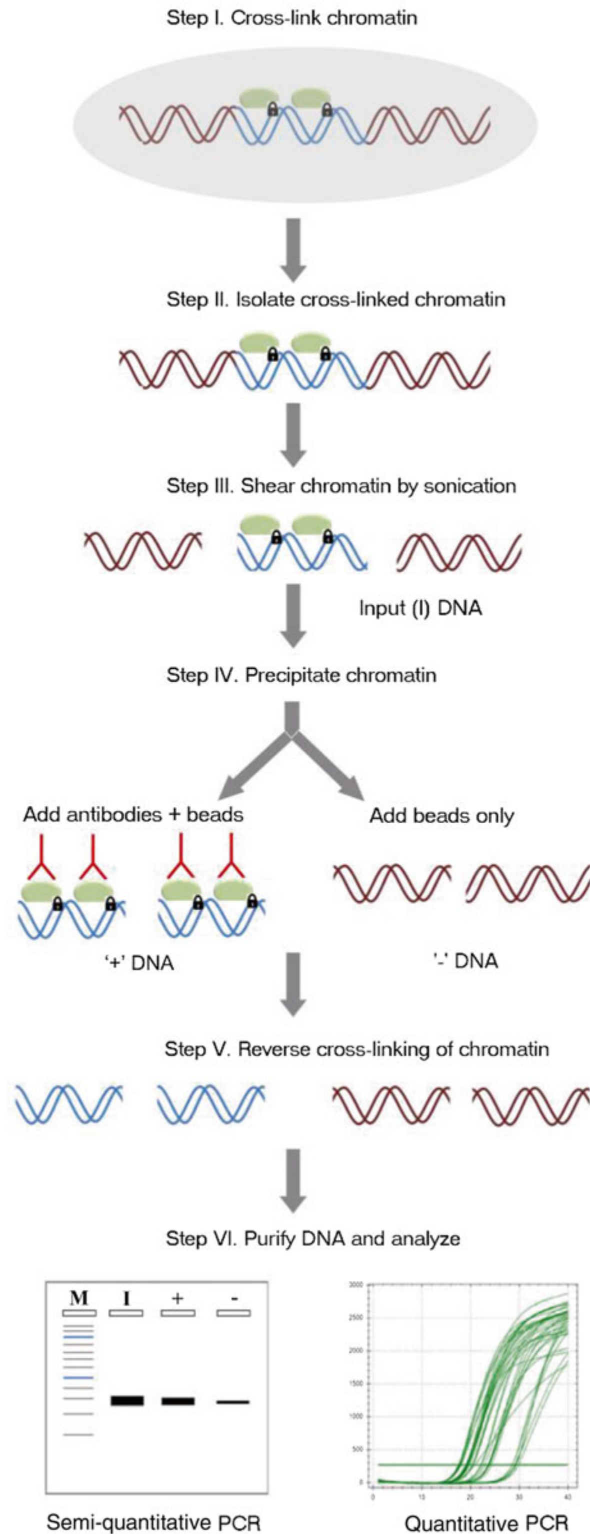

Fig. 1 Schematic of major steps of the chromatin immunoprecipitation assay

2 Materials

Prepare all buffers and other solutions using autoclaved double distilled water and analytical grade reagents. Prepare and store all the solutions at room temperature unless stated otherwise. Strictly follow the appropriate disposal regulations when disposing waste materials.

2.1 Reagents

1. Growth media.
2. Formaldehyde (37 %) (*see* **Note 1**).
3. 2.5 M glycine.
4. 10 % Na-dodecyl sulfate (SDS) (*see* **Note 2**).
5. Lyticase (Sigma Cat. No. L2524).
6. Protease Inhibitor Cocktail (Sigma Cat. No. P8215).
7. Antibodies to the protein of interest (ChIP grade).
8. Protein-A Sepharose beads (Sigma Cat. No. P9424) (*see* **Note 3**).
9. RNase A (Sigma Cat. No. R4875).
10. Proteinase K (Thermo Scientific Cat. No. EO0492).
11. Phenol:chloroform:isoamyl alcohol (Tris saturated) (25:24:1) (Sigma Cat. No. P2069) (*see* **Note 4**).
12. Ethanol.
13. 4 M LiCl.
14. Agarose.
15. PCR reagents.
16. Autoclaved distilled water.

2.2 Buffers

1. Resuspension buffer: 0.1 mM Tris–HCl (pH 9.4), 10 mM Dithiothreitol (Sigma Cat. No. D0632). Make the buffer just before use.
2. Spheroplasting buffer: 20 mM Na-HEPES (pH 7.4), 1.2 M Sorbitol.
3. Post-spheroplasting buffer: 20 mM Na-PIPES (pH 6.8), 1.2 M Sorbitol, 1 mM $MgCl_2$.
4. PBS (1×): 2.68 mM KCl, 1.47 mM KH_2PO_4, 136.89 mM NaCl, 8.10 mM Na_2HPO_4.
5. Wash buffer I: 0.25 % Triton X-100, 10 mM EDTA (pH 8.0), 0.5 mM EGTA, 10 mM Na-HEPES (pH 6.5).
6. Wash buffer II: 200 mM NaCl, 1 mM EDTA (pH 8.0), 0.5 mM EGTA, 10 mM Na-HEPES (pH 6.5).
7. Extraction buffer I: 50 mM HEPES-KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA (pH 8.0), 1 % Triton X-100, and 0.1 %

Na-deoxycholate. Mix well to dissolve detergent, filter sterilize, and store at 4 °C.

8. Extraction buffer II: 50 mM HEPES-KOH (pH 7.5), 500 mM NaCl, 1 mM EDTA (pH 8.0), 1 % Triton X-100, and 0.1 % Na-deoxycholate. Mix well to dissolve detergent, filter sterilize, and store at room temperature.
9. LiCl wash buffer: 10 mM Tris–HCl (pH 8.0), 250 mM LiCl, 1 mM Igepal CA-630, 0.5 % Na-deoxycholate, and 1 mM EDTA (pH 8.0). Mix well to dissolve detergent, filter sterilize, and store at room temperature.
10. Elution buffer I: 50 mM Tris–HCl (pH 8.0), 10 mM EDTA (pH 8.0), 1 % SDS.
11. Elution buffer II: 10 mM Tris–HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.67 % SDS.
12. IP Dilution buffer: 167 mM Tris–HCl (pH 8.0), 167 mM NaCl, 1.1 mM EDTA (pH 8.0), 1.1 % Triton X-100.
13. TE buffer (1×): 10 mM Tris–HCl (pH 8.0), 1 mM EDTA (pH 8.0).
14. TAE buffer (1×): 40 mM Tris, 20 mM acetic acid, 1 mM EDTA (pH 8.0).
15. Protein-A Sepharose beads suspension: Transfer 1 ml of Protein-A Sepharose beads (shipped as ethanol slurry) into a 15 ml disposable conical tube using a cut-off tip. Wash beads twice in ten volumes of 1× TE (pH 8.0) stored at 4 °C. Briefly, add 10 ml of 1× TE (pH 8.0) and gently rotate on a rotator mixer at 4 °C for 10 min. Spin down beads at 4 °C, 2750×*g*, 10 min. Aspirate supernatant. Repeat wash and spin. Aspirate supernatant. Estimate pellet volume and add an equal volume of 1× TE (pH 8.0) to generate 50 % slurry. Store the beads at 4 °C.

2.3 Equipment

1. Conical flasks.
2. Centrifuge (benchtop).
3. Gel electrophoresis apparatus.
4. Shaker-incubators.
5. Microcentrifuge.
6. Micropipettor with tips.
7. PCR thermocycler.
8. Rotator.
9. Sonicator.
10. Spectrophotometer.
11. Tubes (conical, 15 ml, 50 ml).
12. Tubes (microcentrifuge, 1.5 ml).
13. Vortex mixer.

3 Method

Inoculate an isolated colony of *C. albicans* from a freshly streaked plate into 5 ml of suitable growth media. Grow in a rotating shaker-incubator at 30 °C (or desired temperature), 180 rpm overnight. Next day, inoculate 100 ml media with adequate preculture volume necessary to bring culture to 1.000 OD_{600} units by 5–6 h. Grow in a rotating shaker-incubator at 30 °C (or desired temperature), 180 rpm for 5–6 h (*see* **Note 5**).

3.1 Cross-Linking and Chromatin Preparation

1. Harvest cells at OD_{600} = 1.000 (~2 × 10^7 cells/ml)
2. Fix cells with 2.7 ml of 37 % formaldehyde solution per 100 ml of culture for 15 min at room temperature with occasional gentle swirling (*see* **Note 6**). Henceforth, all the reagent volumes are indicated for a 100 ml ChIP experiment.
3. Quench the cross-linking reaction with 5.4 ml of 2.5 M glycine and incubate at room temperature for 5 min. Store the flask in an ice bath prior to centrifugation (if doing more than one cross-linking at a time). Spin down cells in pre-chilled falcon tubes at 4 °C, 2750 × *g* for 10 min. Pour off the supernatant into a formaldehyde waste bottle (*see* **Note 7** and Table 1).
4. Wash cell pellet with 10 ml of ice-cold resuspension buffer. Incubate on a rotating shaker at 30 °C, 150 rpm for 15 min. Pour resuspended cells into a 50 ml conical tube and spin down cells at room temperature, 2750 × *g* for 10 min. Pour off supernatant.
5. Wash cells with 20 ml of spheroplasting buffer. Spin down cells at room temperature, 2750 × *g* for 10 min. Resuspend pellet in 5 ml of spheroplasting buffer. Remove 50 μl to 1 ml of 5 % SDS (0 min time point) and read OD_{800}.
6. Spheroplast cells by adding 50–70 μl of lyticase (10 mg/ml) to the tube. Incubate the cells at 30 °C, 65 rpm for ~45 min to obtain >90 % spheroplasts (*see* **Note 8** and Table 1).
7. Next, add 20 ml of post-spheroplasting buffer to the tube and spin at room temperature, 2750 × *g* for 5 min. Keep spheroplasts on ice hereafter.
8. Wash the spheroplasts sequentially with 15 ml of ice-cold 1× PBS, 15 ml of buffer I, and 15 ml of buffer II. Pellet cells at 4 °C, 2750 × *g* for 5 min, between each wash.
9. Resuspend the pellet in 2 ml of ice-cold extraction buffer I and add 10 μl of protease inhibitor cocktail to the solution. Distribute 330 μl of the lysate, thus prepared, to 1.5 ml Eppendorf tubes. Keep the tubes on ice (*see* **Note 9**).

Table 1
Troubleshooting tips for ChIP protocol

Problem	Possible reason	Solution
1. Yield of input DNA is low	1. Too few cells 2. Loss of spheroplasts during washing	1. Proper cell number (as mentioned in the protocol) should be monitored while harvesting the cells 2. Supernatant should be decanted carefully while washing the spheroplasts or reduce the time of spheroplasting (if the pellet is loose)
2. Chromatin fragments are too large (>900 bp)	1. Cells were over cross-linked 2. Foaming during sonication 3. Insufficient sonication 4. Improper spheroplasting	1. Cross-linking time has to be reduced 2. If using a probe sonicator, the sonicator tip should be kept centered and away from the bottom of the tube 3. Number of cycles of sonication has to be increased 4. Ensure proper spheroplasting by monitoring at OD_{800} during lyticase treatment and also confirm by microscopic observation
3. No specific signal/enrichment is observed in IP	1. Protein of interest did not cross-link efficiently to chromatin 2. Sheared DNA fragments are too large or too small 3. Antibodies did not efficiently bind to protein from cross-linked chromatin 4. Protein epitope recognized by antibody is masked by cross-linking 5. Loss of signal during beads washing 6. Protein of interest is expressed in vivo at a low level	1. Increase the time of formaldehyde cross-linking 2. Adjust the sonication time to get the DNA fragment size between 200 and 700 bp 3. Antibody specificity can be indirectly gauged by western blot and immunoprecipitation. However, it may be necessary to generate another batch of antibodies (preferably polyclonal) 4. This is a problem specific to monoclonal/peptide antibodies. It may be necessary to generate polyclonal antibodies against the protein Cross-linking time needs to be optimized so that over cross-linking is prevented 5. Washing stringency to be reduced with longer washing times being avoided 6. Expression of the protein of interest can be artificially increased under regulatory promoter such as *PCK1* [20] and *TDH3* [21]
4. High background signal in negative control	1. Nonspecific binding of protein to the IP beads 2. Genomic or plasmid DNA contamination in the buffers or PCR reagents	1. Pre-clear lysates with beads before adding antibodies [22] 2. Perform control PCR without chromatin DNA to check for contamination

10. Sonicate lysate to shear DNA. Sonication can be performed with 12 rounds of 10 s bursts at 30 % amplitude using a sonicator (Sonics Vibra-Cell). Keep samples on ice at all times and wait 1 min between bursts. Alternatively, sonication can be done in a bath sonicator such as Bioruptor (Diagenode) using 16 rounds of 15 s on and 15 s off cycle at "High" amplitude setting. After every four cycles, change the water of the bath to maintain 4 °C ambient temperature. These conditions will generate an average chromatin size between 200 and 700 bp (*see* **Notes 10** and **11**, Fig. 2a, and Table 1).
11. Spin down cell debris in a microcentrifuge at 4 °C, 19200 × *g* for 15 min. Collect the supernatant (*see* **Note 12**).

3.2 Chromatin Immunoprecipitation

1. Input (I): Remove 500 μl of cleared lysate to a 15 ml disposable conical tube as input (I). Add 1.5 ml elution buffer I, and incubate at 65 °C overnight to reverse cross-links.
2. Immunoprecipitate (IP): Divide remaining lysate in half between two 15 ml disposable conical tubes. Add 5.7 volumes IP dilution buffer. Leave one as "without antibody" or "beads only" control ("–"). To the "with antibody" ("+") sample add the requisite antibody (typically 5–10 μg is sufficient). Incubate on a rotator at 4 °C, overnight.

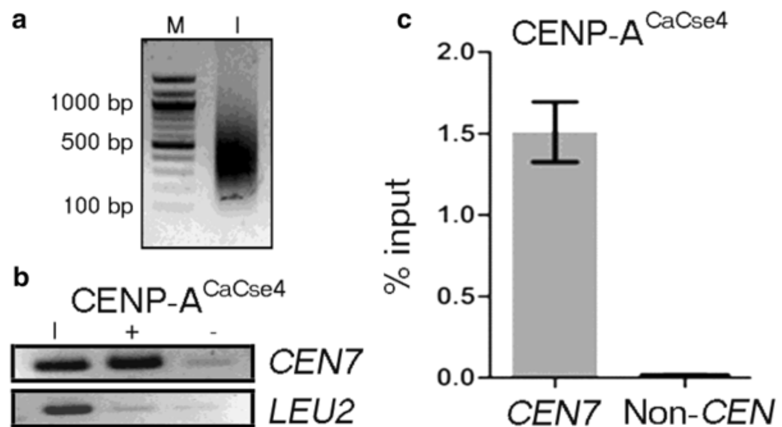

Fig. 2 Analysis of ChIP DNA. (**a**) A 2 % agarose gel image showing sheared and purified input (I) DNA of *C. albicans* along with 100 bp ladder (M). The number of sonication cycles used in this experiment was 16 (Bioruptor, Diagenode). (**b**) The ChIP-PCR profile of centromere 7 (*CEN7*) region of *C. albicans* showing the enrichment of CENP-A^{CaCse4}. Input (I), total chromatin; +, precipitation with antibodies (anti-CENP-A^{CaCse4} - Prot A); –, precipitation without antibodies. The *LEU2* locus serves as a noncentromeric negative control region. (**c**) The quantitative real-time PCR (qPCR) profile of centromere region of *C. albicans* showing enrichment of CENP-A^{CaCse4} at the centromere (unpublished)

3.3 Immunoprecipitate (IP) Capture

1. Next day, add 50 μl of washed Protein-A-linked Sepharose beads (50 % slurry in 1× TE pH 8.0) per ml of IP to each IP reaction. Incubate on a rotator at 4 °C for 10–12 h (*see* **Note 13**).

3.4 IP Washes and Elution

1. Spin down captured complex/beads at room temperature, 2750 × *g* for 3 min, and aspirate supernatant.
2. Wash beads sequentially using the following protocol: Twice for 5 min each in 12.5 ml extraction buffer I, once for 5 min in 12.5 ml extraction buffer II, once for 5 min in 12.5 ml LiCl wash buffer, and twice for 5 min in 12.5 ml 1× TE (pH 8.0). Incubate tubes on a rotator at room temperature for 5 min between each wash. After each wash spin down beads at room temperature, 2750 × *g* for 3 min, and aspirate the supernatant carefully with a micropipette (*see* **Note 14**).
3. Elute IP in 1/10 volume of elution buffer I. Incubate the tubes at 65 °C for 15 min. Spin down beads at room temperature, 2750 × *g* for 3 min and transfer eluate to a fresh 15 ml tube.
4. Elute a second time with 1/6.7 volume of elution buffer II. Incubate at 65 °C for 5 min. Spin down beads at room temperature, 2750 × *g* for 3 min and pool eluates I and II. Incubate at 65 °C overnight to reverse cross-links (*see* **Note 15**).

3.5 IP DNA Purification

1. Add 1.56 ml of 1× TE (pH 8.0) and 5 μl RNase A (10 mg/ml) to the de-cross-linked eluate. Incubate at 37 °C for 3 h.
2. Add 46 μl of Proteinase K (20 mg/ml). Incubate at 37 °C for 3 h.
3. Add 405 μl of 4 M LiCl and 4.05 ml phenol:chloroform:isoamyl alcohol and vortex for 10 s. Spin down phenol extraction at room temperature, 2750 × *g* for 5 min. Pool aqueous layer and distribute 500 μl into 1.5 ml Eppendorf tubes. Add 1 ml of ice-cold 100 % ethanol and precipitate DNA at −20 °C, overnight (*see* **Note 16**).

3.6 Input (I) DNA Purification

1. To the input, add 665 μl of 1× TE (pH 8.0) and 5 μl RNase A (10 mg/ml). Incubate at 37 °C for 1 h.
2. Add 25 μl Proteinase K (20 mg/ml). Incubate at 37 °C for 4 h.
3. Add 230 μl of 4 M LiCl and 2.3 ml of phenol:chloroform:isoamyl alcohol and vortex for 10 s.
4. Pool aqueous layer and distribute 500 μl into 1.5 ml Eppendorf tubes.
5. Add 1 ml of 100 % ethanol per tube and vortex. Precipitate DNA at room temperature for ≥15 min.
6. Spin down the precipitate in a microcentrifuge at room temperature, 19200 × *g* for 20 min.

7. Wash DNA pellet with 1 ml of 70 % ethanol. Spin down the precipitate in a microcentrifuge at room temperature, 19200 × *g* for 5 min.
8. Dry the pellet in speedvac for 10 min.
9. Resuspend DNA pellet in 50 μl of 1× TE (pH 8.0) and store at 4 °C.
10. Check size of sheared input DNA: Run 5 μl input DNA on a 1 % agarose 1× TAE gel at 100 V for 35 min. Include 0.5 μg 100 bp DNA ladder. Use 0.5× bromophenol blue tracking dye. The average fragment size of the sheared DNA should be ideally between 200 and 700 bp in range (Fig. 2a) (*see* **Note 17** and Table 1).

3.7 IP DNA Precipitation

1. Spin ethanol-precipitated IP DNA in microcentrifuge at 4 °C, 19200 × *g* for 30 min. Wash each DNA pellet with 1 ml of ice-cold 70 % ethanol and spin down in a microcentrifuge at 4 °C, 19200 × *g* for 15 min.
2. Dry pellet in speedvac for 10 min.
3. Resuspend DNA in 100 μl 1× TE (pH 8.0) by pipetting up and down and transferring until the last tube is completed. Store at 4 °C (short term) or at −20 °C (long term).

3.8 PCR

1. Set up 25 μl reactions with three units of Taq DNA polymerase and 50 pmol of each primer. Initially try 2 μl of IP material (both +DNA and −DNA samples) and 1/2000 of input (I) (2 μl of 1:40 dilution).
2. Amplify with a customized PCR program.
3. Electrophorese on 2 % agarose 1× TAE gels and visualize bands after staining with ethidium bromide (*see* **Note 18**) (Fig. 2b). Digitally photograph the gels and determine densitometry values with Quantity One software. The relative enrichment can be computed as (+DNA) – (−DNA)/I DNA of the test (e.g., centromere) divided by (+DNA) – (−DNA)/I DNA of the control (e.g., noncentromeric locus) (*see* **Note 19** and Table 1).
4. Alternatively perform quantitative real-time PCR reaction (qPCR) with the IP and input DNA (Fig. 2c). The qPCR enrichment is usually determined by the percent input method (% input) [18]. In brief, the C_t values for input (I) are adjusted for the dilution factor and then the percent of the input chromatin immunoprecipitated by antibodies is calculated as $100 \times 2^{(\text{Adjusted Input } C_t - \text{IP } C_t)}$ (*see* **Note 20**).

3.9 Controls

1. Negative control: Use a "without antibody" sample as a negative control to determine the background of the assay. Alternatively, for an epitope-tagged protein, ChIP DNA

obtained from an untagged strain can be used as a negative control. ChIP DNA obtained by using a control immunoglobulin (nonspecific antibodies of similar isotype or pre-immune serum) can also be used as a negative control.

2. Positive and negative control loci: A genomic locus where the protein of interest is known to bind and a locus where it does not bind can be used as positive and negative control loci respectively for conventional semi-quantitative PCR as well as qPCR analysis. These controls confirm the specificity of the protein binding.
3. Non-template control: A non-template control should be included in the PCR or qPCR reactions for checking PCR contamination.
4. Positive control antibodies: ChIP with well-established ChIP-grade antibodies against proteins such as histones provides a positive control to test whether all the steps of the protocol are working. However, it does not ensure the success of another ChIP experiment being performed.

4 Notes

1. Formaldehyde is flammable. It is highly toxic on inhalation, swallowing, or in contact with skin. Formaldehyde should be used with adequate safety measures such as protective gloves, clothing, and glasses in a sufficiently ventilated area. The formaldehyde waste should be disposed of as per regulations for hazardous waste.
2. SDS causes irritation on contact with eye and skin. It causes respiratory tract irritation on inhalation, nausea and vomiting on ingestion. SDS should be used with adequate safety measures, such as protective gloves, clothing, and glasses and sufficient ventilation.
3. Alternatively Protein-G sepharose beads can be used, depending on the subtype of the primary antibody.
4. Old phenol:chloroform:isoamyl alcohol solution or solutions with low pH causes DNA degradation. Phenol is toxic and causes burns on the skin. It is toxic when swallowed. Further, it is irritating to the eyes, respiratory system, and skin. The phenol:chloroform:isoamyl alcohol solution should be used with adequate safety measures, such as protective gloves, clothing, and glasses and sufficient ventilation. The phenol:chloroform:isoamyl alcohol waste should be disposed of as per regulations for hazardous waste.

5. The culture should not be harvested beyond 1 OD_{600} (mid-log phase) as cultures grown beyond this density tend to produce lower ChIP DNA yield.
6. Cross-linking time has to be optimized for each protein analyzed. For example, 15 min cross-linking time is sufficient for histones whereas for proteins that transiently bind to the DNA or associate with the DNA through another protein/protein complex, the cross-linking time can be extended to 90 min or more. Over cross-linking can interfere with the chromatin shearing and lead to denaturation of the protein or even can cross-link nonspecific DNA–protein interaction. Insufficient cross-linking leads to reduced recovery of regions associated with the protein of interest.
7. Be careful while decanting the supernatant as the pellet may be loose. After pelleting at this step, the cell pellet can be stored at –20 °C for a few days, alternatively.
8. The time for spheroplasting varies between different strains in *C. albicans* and also depends on the morphological states (yeast, pseudohyphae, and hyphae). Thus, the time and the amount of lyticase to be used for spheroplasting have to be optimized for the above conditions. It has also been observed that the spheroplasting efficiency varies with the batch of lyticase used. Over-spheroplasting results in a very loose pellet leading to a loss of chromatin in subsequent washes. Insufficient spheroplasting can reduce the efficiency of chromatin shearing.
9. Use cut micropipette tips to resuspend the spheroplasts gently by pipetting up and down. Be careful while decanting the supernatant between each wash so as not to lose spheroplasts. Take care that the pellet is dissolved uniformly in the extraction buffer.
10. The sonication time has to be optimized for different strains and morphological states being studied. The sonication efficiency is also reduced by over cross-linking and improper spheroplasting. The size of the sheared chromatin obtained should be checked during optimization, as discussed later.
11. Avoid foaming and heating of the samples during sonication as it can result in reduction in sonication efficiency and degradation of chromatin, respectively.
12. The cleared lysate can be stored at 4 °C for a few days.
13. Make sure that the bead slurry is completely resuspended before adding to the IP. Cut off the ends of regular micropipette tips and use them to transfer beads.
14. Be careful not to aspirate any beads while taking the supernatant.
15. The minimum time for reverse cross-linking is 6 h. Extending the de-cross-linking step overnight or up to 15 h does not cause a significant damage to the DNA.

16. Glycogen can be used during IP DNA precipitation for increasing the yield of IP DNA. Alternatively, DNA can be purified by QIAGEN DNA purification kits.
17. The size of the sheared chromatin is to be optimized with the desired spacing and length of the PCR probes. For example, an average fragment size of 400 bp is optimum for probes spaced ~1 kb apart and an amplicon length of 200–300 bp.
18. Ethidium bromide is a potent mutagen and can be absorbed through skin. It is an irritant to the eyes, skin, mucous membranes, and upper respiratory tract. Ethidium bromide should be used with adequate safety measures, such as protective gloves, clothing, and glasses and sufficient ventilation. The ethidium bromide waste should be disposed of as per regulations for hazardous waste.
19. For increasing the reliability, replicates of the PCR reaction can be run with serial dilutions of input and IP DNAs in order to ensure that the reactions are in a linear range of detection. Further, a combination of test and control primers can be used for amplification so as to obtain test and control bands in the same agarose lane.
20. The amount of both IP and input DNAs has to be standardized for qPCR experiment. The C_t value between 20 and 25 cycles is most desirable. At least three technical replicates of each qPCR reaction are required to ensure accuracy of the results.

5 Applications

The ChIP technique is a robust and reliable method to study in vivo binding patterns of proteins. The applicability of this technique is enhanced dramatically by inclusion of ChIP-chip and ChIP-seq assays. In the ChIP-chip technique, a reference and test IP DNA is purified, amplified by ligation-mediated PCR (LM-PCR), and differentially labeled with fluorescent dyes. Next, they are hybridized to a DNA tiling array comprised of oligonucleotide probes covering the entire genome, individual chromosomes or specific genomic loci. The relative fluorescent intensity of the test versus reference channels gives the degree of enrichment of the protein of interest. The end result of this process is the generation of high-resolution genome-wide maps of protein occupancy. However, with the development of large-scale next-generation sequencing techniques, ChIP-seq has largely replaced ChIP-chip as the method of choice for genome-wide studies of protein–DNA interactions. In this method, the IP DNA is purified, processed, and analyzed by massively parallel DNA sequencing [10]. The major advantages of ChIP-seq over ChIP-chip are higher resolution

and coverage. The high-resolution genome-wide profiles of protein binding generated by ChIP-chip and ChIP-seq analyses help in delineating expression and regulatory domains on chromatin. These techniques are also indispensable for finding genome-wide binding of evolutionarily conserved proteins in relatively less worked out organisms, whose whole genome has been sequenced. A detailed protocol of ChIP-seq assay for *C. albicans* has been described elsewhere [15].

Further, the protocol described here has also been used for performing ChIP and ChIP-seq in the related *Candida* species viz. *Candida dubliniensis* [19, 15] and *Candida tropicalis* (unpublished). Thus, this procedure can be used as a standard ChIP protocol for many *Candida* sp.

6 Limitations

Despite their immense utility, ChIP and its associated techniques have several limitations. First, success of this assay depends primarily on the choice of the antibodies. Although, several antibodies can perform well in applications such as western blot, they often fail to recognize the presented epitope in a cross-linked chromatin. Further, under in vivo conditions, they can also show cross-reactivity with other chromatin proteins. These problems manifest themselves in the form of low signal to noise ratio in the output data. Second, the resolution of the traditional ChIP assays is not enhanced enough to identify precise binding sites of a protein. Although, the resolution is significantly increased in ChIP-chip and ChIP-seq assays, the enhanced sensitivity leads to increased probability of obtaining false positive hits. Therefore, whenever possible, the ChIP-seq and ChIP-chip hits should be validated by conventional PCR. Finally, binding of proteins to a genomic locus by ChIP does not, by itself, demonstrate the functional significance of the binding. Thus, additional experiments must be performed to determine the implications of the binding.

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