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Immunoprecipitation, Decross-linking, and DNA Extraction

In 1 collection

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ABSTRACT

A plethora of biological processes like gene transcription, DNA replication, DNA recombination, and chromosome segregation are mediated through protein–DNA interactions. A powerful method for investigating proteins within a native chromatin environment in the cell is chromatin immunoprecipitation (ChIP). Combined with the recent technological advancement in next generation sequencing, the ChIP assay can map the exact binding sites of a protein of interest across the entire genome. Here we describe a-step-by step protocol for ChIP followed by library preparation for ChIP-seq from yeast cells.

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COLLECTIONS

Analysis of the Chromosomal Localization of Yeast SMC Complexes by Chromatin Immunoprecipitation

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Analysis of the Chromosomal Localization of Yeast SMC Complexes by Chromatin Immunoprecipitation

GUIDELINES

Chromatin immunoprecipitation (ChIP) is broadly used to study chromatin dynamics. Changes in occupancy of chromosomal proteins at specific loci within the genome can be measured by using ChIP-qPCR. However, this technique is costly and time consuming with high variability per experiment. Alternatively, ChIP-seq can be used to measure differences in a protein's occupancy genome wide. Finally, calibrated ChIP-seq is essential when measuring changes in occupancy between different experimental samples.

Here we describe an optimized ChIP protocol for yeast SMC proteins that can be completed within 3 days for samples analyzed by qPCR and 4 days for samples to be further processed by calibrated deep sequencing. The protocol encompasses five distinct steps: cross-linking and cell harvesting; cell lysis and sonication; immunoprecipitation, decross-linking and DNA extraction and finally determination of the size and DNA concentration of sonicated samples. These five steps are outlined here.

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MATERIALS TEXT

Yeast Strains and Growth Material:

1. Haploid *S. cerevisiae* strains of w303 background we have used include: (a) no tag control (AM1176), (b) *SCC1-6HA* (AM1145), (c) *BRN1-6HA* (AM5708), (d) *SCC2-6HIS-3FLAG* (AM6006), and (e) *SCC1-6HA pMET3-CDC20* (AM1105) as previously described [9,10,11,12].
2. For studies of protein occupancy during meiosis we have used diploid *S. cerevisiae* strains of SK1 background including (a) *REC8-3HA ndt80*Δ (AM4015), as previously described [13] and (b) *REC8-6HIS-3FLAG* (AM11000).
3. Haploid *S. pombe* strains used for calibration are: (a) *RAD21-3HA* (spAM76), (b) *RAD21-6HA* (spAM635), (c) *RAD21-6HIS-3FLAG* (spAM1863), or (d) *CND2-6HA*(spAM1862).
4. YPDA media: 1% yeast extract, 2% peptone, 2% glucose.
5. YPG agar plates: 1% yeast extract, 2% peptone, 2.5% glycerol, 2% agar.
6. YPDA4% agar plates: 1% yeast extract, 2% peptone, 4% glucose, 2% agar.
7. BYTA media: 1% yeast extract, 2% Bacto tryptone, 1% potassium acetate, 50 mM potassium phthalate.
8. SPO media: 0.3% potassium acetate, pH 7.0.
9. YES media: 0.5% yeast extract, 3% glucose, 225 mg/L supplements.

Equipment and Reagents:

1. 37% formaldehyde solution for molecular biology.
2. 2.5 M glycine: Dissolve 93.8 g glycine in ddH_2O (may require gentle heating) and bring up to 500 ml with ddH_2O.
3. Diluent buffer: 0.143 M NaCl, 1.43 mM EDTA, 71.43 mM Hepes–KOH pH 7.5.
4. TBS buffer: 20 mM Tris–HCl pH 7.5, 150 mM NaCl.
5. 2× FA lysis buffer: 100 mM Hepes–KOH pH 7.5, 300 mM NaCl, 2 mM EDTA, 2% Triton X-100, 0.2% Na-deoxycholate.
6. FastPrep screw-cap tubes.
7. 100 mM PMSF.
8. Protease inhibitor tablets Complete EDTA free.
9. Zirconia/Silica beads 0.5 mm diameter.
10. FastPrep-24 5G Homogenizer.
11. Bioruptor Twin.
12. Dynabeads Protein G.
13. Magnetic rack.
14. ChIP Wash buffer 1—low salt: 1× FA lysis buffer, 0.1%SDS, 275 mM NaCl.
15. ChIP Wash buffer 2—high salt: 1× FA lysis buffer, 0.1%SDS, 500 mM NaCl.
16. ChIP Wash buffer 3: 10 mM Tris–HCl pH 8.0, 0.25 M LiCl, 1 mM EDTA, 0.5% NP-40. 0.5% Na-deoxycholate.
17. ChIP Wash buffer 4 (TE): 10 mM Tris–HCl pH 8.0, 1 mM EDTA.
18. Chelex 100 Resin.
19. 10 mg/ml Proteinase K
20. TES buffer: 50 mM Tris–HCl pH 7.5, 10 mM EDTA, 1% SDS.
21. Nuclease-free molecular biology grade water.
22. Filter tips.
23. Luna Universal Probe qPCR Master Mix.
24. LightCycler 480 Multiwell Plate 96.
25. LightCycler real-time PCR.
26. Qiagen purification kit.
27. LoBind DNA microcentrifuge tubes.
28. Quick blunting kit.
29. AMPure XP beads.
30. Klenow 3' to 5' exo minus.
31. Quick ligation kit (T4 DNA ligase).
32. NEXTflex DNA Barcodes—12 (Bioo Scientific; #NOVA-514102).
33. Phusion High-Fidelity DNA polymerase.
34. DynaMag-PCR magnet.
35. WizardSV Gel and PCR cleanup system.
36. Qubit dsDNA-HS Assay kit (Invitrogen).
37. Qubit Fluorometric Quantitation machine.
38. Agilent 2100 Bioanalyzer system.
39. High Sensitivity DNA Reagents kit (Agilent Technologies).
40. High Sensitivity DNA Chips (Agilent Technologies).
41. MiniSeq High throughput Reagent Kit (150-cycle) (Illumina).
42. Illumina Mini-seq.

SAFETY WARNINGS

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For hazard information and safety warnings, please refer to the SDS (Safety Data Sheet).

Formaldehyde and PMSF are toxic if inhaled, ingested or absorbed through the skin. Always wear a lab coat and gloves, and work in a chemical hood.

Immunoprecipitation, Decross-linking, and DNA Extraction 5m

1 5m

Prewash (*n* × 15) µl of Protein G Dynabeads (*n* = number of IP samples) in **1 mL ice-cold 1× FA buffer** containing 0.1% SDS, **1 Milimolar (mM) PMSF** and protease inhibitors with rotation for **00:05:00** .
Place the microcentrifuge tubes on a magnet and discard the supernatant. (1/3)

1.1 5m

Prewash (*n* × 15) µl of Protein G Dynabeads (*n* = number of IP samples) in **1 mL ice-cold 1× FA buffer** containing 0.1% SDS, **1 Milimolar (mM) PMSF** and protease inhibitors with rotation for **00:05:00** .
Place the microcentrifuge tubes on a magnet and discard the supernatant. (2/3)

1.2 5m

Prewash (*n* × 15) µl of Protein G Dynabeads (*n* = number of IP samples) in **1 mL ice-cold 1× FA buffer** containing 0.1% SDS, **1 Milimolar (mM) PMSF** and protease inhibitors with rotation for **00:05:00** .
Place the microcentrifuge tubes on a magnet and discard the supernatant. (3/3)

2

Add the antibody against the protein of interest to **1 mL chromatin extract** (step 13 in protocol "Cell Lysis and Sonication") and **15 µl prewashed Dynabeads** and mix.

The amount of SDS in immunoprecipitation can interfere with antibody binding efficiency therefore, lower amount of SDS can also be used. Either omit SDS in 2xFA buffer or reduce SDS added afterward to a final concentration of 0.05% before chromatin immunoprecipitation.

The amount of antibody added should be *in excess* of the protein being immunoprecipitated and should be determined empirically. We use the following amounts of antibodies per IP:

- **10 µl 9E10** (Tonbo Biosciences)
- **7.5 µl 12CA5** (Roche)
- **10 µl SV5-PK1** (Bio-Rad)
- **10 µl anti-GFP** (Roche)
- **5 µl M2 FLAG** (Sigma)

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No blocking and preclearing is required for the magnetic beads.

3 5m

Incubate on a rotating wheel at **4 °C** **Overnight** .

4

Place the tubes on the magnet and discard the supernatant. Perform the following washes, using 1 ml per sample of Wash buffer on a rotating wheel for 5 min at room temperature. Discard supernatants after each wash.

4.1 5m

Wash using **1 mL ChIP Wash buffer 1 - low salt** on a rotating wheel for **00:05:00** at **Room temperature** . Discard supernatant after wash.

4.2 5m

Wash using **1 mL ChIP Wash buffer 2 - high salt** on a rotating wheel for **00:05:00** at **Room temperature** . Discard supernatant after wash.

4.3 5m

Wash using **1 mL ChIP Wash buffer 3** on a rotating wheel for **00:05:00** at **Room temperature** . Discard supernatant after wash.

4.4 5m

Wash using **1 mL ChIP Wash buffer 4** on a rotating wheel for **00:05:00** at **Room temperature** . Discard supernatant after wash.

5

Following the final wash, place the samples on magnetic rack and discard the supernatant without disturbing the beads.

6 Move forward with:
1. reversal of cross-linking and isolation of DNA for qPCR, OR
2. ChIP-seq preparation after the final wash

Step 6 includes a Step case.

qPCR

ChIP-seq

step case

qPCR

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Use Chelex 100 as previously described [14].

7

Add **0.2 mL 10% slurry (wt/vol)** in sterile water Chelex-100 resin directly to the washed Dynabeads (IP sample) and to **10 µl thawed "Input" samples** (see step 14 from protocol "Cell Lysis and Sonication"). Keep the Chelex beads in suspension while pipetting.

8 10m

Briefly vortex samples to mix the slurry and boil for **00:10:00** .

9

Cool the tubes to **Room temperature** and quickly centrifuge condensate to the bottom of the tube at **400 x g** .
Proceed immediately to the next step.

> Variation in the amount of beads added can affect the specific signal–background ratio. Make sure to keep the slurry suspended while distributing.

10 30m

Add **2.5 µl Proteinase K (10 mg/ml)** to each sample and incubate on a heat block at **55 °C** for **00:30:00** with occasional mixing to resuspend beads.

11 10m

Boil the samples at **100 °C** for **00:10:00** to inactivate the Proteinase K.

12

Spin the tubes briefly at **400 x g** and carefully transfer approximately **120 µl DNA supernatant** , termed the IP sample in a new tube.

> Make sure not to transfer any Chelex resin as it can lead to loss of PCR signal.

13

Store samples at **-20 °C** . (PAUSE POINT).

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14 Purified DNA from step 12 can be used in qPCR, following the subsequent steps. We use Luna Universal Probe qPCR Master Mix in a 10 µl reaction in a 96-well plate and run the following program:

14.1 Set up the following reaction mix:

3 µl DNA Template (for Input use 1:300, for IP use 1:6 dilutions)

0.125 µl primer pair (20 µM each)

5 µl master mix

ddH_2O up to 10 µl final volume

14.2 Run the following program:

Step	Temperature	Time	Cycles
Initial denaturation	95 °C	1 min	
Denaturation	95 °C	15 sec	42 cycles
Extension	60 °C	30 sec	
Melt curve	according to Lightcycler 480 recommendations		

14.3

Perform data analysis using Microsoft Excel software using the following formulas:

Determine the ChIP enrichment (i.e., ChIP/Input value), by calculating ΔCt using the following formula:

$$\Delta Ct = Ct^{ChIP} - (Ct^{Input} - log_{primer efficiency}(Input dilution factor))$$

From this, the ChIP enrichment is calculated using:

$$ChIP/Input = (primer efficiency)^{(-\Delta Ct)}$$

where Ct values are mean threshold cycles of PCR performed in triplicate per DNA sample.

To increase accuracy and reproducibility, we advise the use of an electronic dispensing pipette for qPCR. For primer design we recommend the use of Prime3Plus software. In the general settings of the software, we use product size range 70–200 bp (optimal amplicon size ~120 bp), optimal primer size 20 bp in length and optimal primer Tm for use with Luna Universal Probe qPCR Master Mix should be designed to anneal at 60 °C, with optimal primer GC% between 50% and 60%. Once primers are designed determine their efficiency and specificity using genomic DNA prior to performing qPCR with ChIP DNA sample. Finally, also include a No Template Control (NTC) in the reaction, where no amplification and no melting curve should be generated.

15 To determine whether the amount of immunoprecipitated DNA is sufficient for library preparation for ChIP-seq, quantify **3 µl ChIP products and Input** using the Qubit HS kit.

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Most commonly, immunoprecipitated DNA yields are in the range of 2–10 ng.

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