Chromatin immunoprecipitation protocol for *Saccharomyces cerevisiae*

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Introduction

ChIP (chromatin immunoprecipitation) is a powerful tool that allows one to determine whether and where a protein or protein modification is associated with chromatin in vivo. The technique starts with a formaldehyde treatment of cells to crosslink protein-protein and protein-DNA complexes. After cross-linking, the cells are lysed and crude extracts are sonicated to shear the DNA. Proteins together with crosslinked DNA are immunoprecipitated. Protein-DNA crosslinks in the immunoprecipitate and input (non-immunoprecipitated whole cell extract) are then reversed and the DNA fragments are purified. Real-time quantitative PCR can then be used to amplify the region where either a protein or protein modification is present. DNA fragments of this genomic locus should be enriched in the immunoprecipitate compared to that in the input (which represents all portions of the genome). This protocol has been successfully used in the Gasser lab to study proteins at replication forks and chromosomal DNA double-strand breaks (DSBs) in budding yeast (*Saccharomyces cerevisiae*) (Cobb *et al*., 2003; van Attikum *et al*., 2004).

Procedure

Preculture of yeast cells for ChIP of proteins at DSB

We have used strains with JKM179 background (Lee *et al*., 1998) to study binding of proteins at DSBs. This strain expresses the HO endonuclease from a galactose-inducible promoter (HO is repressed when glucose is present in the medium). HO induces a unique DSBs at the MAT locus. This break is usually repaired by recombination. However, in this strain the donor loci for repair were deleted in order to prevent repair by recombination. This allows us to monitor protein binding at the HO-induced DSB.

1. Inoculate YPAD medium with strain JKM179 or one of its derivatives;
2. Incubate and rotate at 180-210 rpm overnight at 30°C;
3. Dilute the overnight culture in YPLGg medium (volume should be consistent with number of time points, use 100ml/timepoint);
4. Incubate and rotate at 180-210 rpm overnight at 30°C until OD600 = ~0.3-0.4;
5. Pour 100ml culture into a new flask and add glucose to a final concentration of 2%;

6. Add galactose (Fluka) to a final concentration of 2% to the rest of the overnight culture
7. Incubate and rotate at 180-210 rpm at 30°C;
8. At each desired time point take 100ml culture and pour it into a new flask for formaldehyde cross-linking (usually samples are taken at 0.5, 1, 2 and 4 hours for cells grown in galactose and at 2 hours for cells grown on glucose) (see comment 1).

Preculture of yeast cells for ChIP of proteins at replication forks

1. Inoculate YPAD medium with strain of interest;
2. Incubate and rotate at 180-210 rpm overnight at 30°C;
3. Dilute the overnight culture to $1x10^6$ cells/ml in YPAD medium (volume should be consistent with number of time points, use 50ml/timepoint);
4. Incubate and rotate at 180-210 rpm at 0°C until $5x10^6$ cells/ml;
5. Spin down the cells at 3000 rpm (1620 *g*) at room temperature for 3 minutes;
6. Remove supernatant and re-suspend cells in YPAD pH5;
7. Spin down the cells at 3000 rpm (1620 *g*) at room temperature for 3 minutes;
8. Remove supernatant and re-suspend cells in YPAD pH5;
9. Add alpha-factor (see note 1) and incubate and rotate at 180-210 rpm at 30°C for 1.5 hours to arrest cells in G1;
10. Spin down the cells at 3000 rpm (1620 *g*) at room temperature for 3 minutes;
11. Remove supernatant and re-suspend cells in YPAD;
12. Spin down the cells at 3000 rpm (or 1620 *g*) at room temperature for 3 minutes;
13. Remove supernatant and re-suspend cells in YPAD without or with 200mM hydroxyurea;
14. Incubate and rotate at 180-210 rpm, at 16°C when cultures do not contain hydroxyurea, and at 30°C when cultures contain hydroxyurea;
15. At each desired time point take 50ml culture and pour it into a new flask for formaldehyde cross-linking (samples are taken at 0 (cells in G1), 10, 20, 40 and 60 minutes after release into S-phase).

Day 1

Formaldehyde cross-linking (see comment 2)

1. Add 1.5ml 36% formaldehyde to 50ml of yeast cell culture (1% final);
2. Shake slowly at 100 rpm at 30°C for 15 minutes;
3. Add 2.5ml 2.5M glycine;
4. Shake slowly at 100 rpm at 30°C for 5 minutes;
5. Spin down the cells at room temperature at 3000 rpm (1620 *g*) for 3 minutes;
6. Remove supernatant and wash cells with 25ml 1x PBS;
7. Spin down the cells at room temperature at 3000 rpm (1620 *g*) for 3 minutes;
8. Remove supernatant and re-suspend cells in 1ml 1x PBS;
9. Transfer cells to a 2ml tube with screw cap;
10. Spin down cells at room temperature at 10000 rpm (9500 *g*) for 2 minutes and remove supernatant;
11. Freeze pellet at -80°C.

Day 2

Preparation of beads covered with antibodies

1. Spin down 2x40µl (80µl) dynabeads (see note 2) per sample, place tube in magnetic stand and remove supernatant;
2. Wash dynabeads with 0.5ml 1x PBS-BSA (5mg/ml) by constant rotation for 30 minutes at 4°C;
3. Spin down dynabeads, place tube in magnetic stand and remove supernatant;
4. Respend dynabeads in 0.5ml 1x PBS-BSA (5mg/ml);

5. Add ~5 µg antibody to 40µl dynabeads, do not add antibody to the other 40µl dynabeads;
6. Rotate for 2 hours at 4°C;
7. Spin down dynabeads, place tube in magnetic stand and remove supernatant;
8. Wash dynabeads with wash buffer by rotation for 5 minutes at 4°C;
9. Repeat the last two steps;
10. Re-suspend each portion of dynabeads in 40µl 1x PBS-BSA (5mg/ml).

Cell extracts (WCE) (see comment 3)

1. Add ~400µl Zirconia/Silica (Biospec Products, Inc.) beads to the cell pellet;
2. Add 600µl lysis buffer;
3. Beadbeat 3x1 minute (with 1 minute time intervals) at maximum at 4°C (Mini beadbeater-8, Biospec Products, Inc.);
4. Make a hole in the bottom of the tube using a needle;
5. Place the tube in a 3ml polypropylene tube (12x55mm, Semadin) and put these in a 12ml falcon tube;
6. Centrifuge at 2000 rpm (380 *g*) at 4°C for 5 minutes to recover the extract;
7. Add 600µl lysis buffer to recoverd extract and re-suspend the pellet;
8. Sonicate 4x20 seconds: 1x on 2.5, 2x on 3.5 and 1x micro, 20 seconds each (Sonic Dismembrator 550, Fischer Scientific);
9. Transfer the extract into a pre-chilled eppendorf tube;
10. Centrifuge at 7000 rpm (4650 *g*) at 4°C for 2 minutes;
11. Transfer supernatant to new pre-chilled eppendorf tube;
12. Save 25µl aliquot (INput) in 2ml safe capslock eppendorf tubes (in view of phenol extraction), freeze at -20°C till crosslinking reversal step (see note 3).

Immunoprecipitation

1. Divide the cell extract over two eppendorf tubes;
2. Add 40µl dynabeads with antibody to one halve of the extracts;
3. Add 40µl dynabeads without antibody to the other halve of the extracts;
4. Rotate for 2 hours at 4°C;
5. Spin down dynabeads, place tube in magnetic stand, remove supernatant, re-suspend dynabeads in 600µl lysis buffer;
6. Mix beads for 5 minutes at 4°C;
7. Repeat the last two steps;
8. Mix dynabeads for 5 minutes at 4°C;
9. Spin down dynabeads, place tube in magnetic stand, remove supernatant, re-suspend dynabeads in 600µl wash buffer;
10. Mix dynabeads for 5 minutes at 4°C;
11. Spin down dynabeads, place tube in magnetic stand, remove supernatant, re-suspend dynabeads in 600µl TE buffer;
12. Mix dynabeads for 1 minute at 4°C;
13. Spin down dynabeads, place tube in magnetic stand, remove supernatant;
14. Re-suspend dynabeads in 120 µl TE/1%SDS buffer;
15. Mix dynabeads for 10 minutes at 65°C;
16. Centrifuge 5 seconds at 13000 rpm and place tube in magnetic stand;
17. Transfer supernatant to 2 ml safe capslock eppendorf tubes (see note 3);
18. Add 130µl and 100µl TE/1%SDS buffer to IP and IN samples, respectively;
19. Submerge the tubes in water and incubate overnight at 65°C.

Day 3

Recovery of DNA (reverse cross-linking) (see comment 4)

1. Add 240µl and 370µl TE buffer to IP and IN samples, respectively
2. Add 20µl proteinase K (10mg/ml, Eurobio);
3. Incubate for 2 hours at 37°C;
4. Add 50µl 5M LiCl to IP and IN samples;

5. Add 400µl phenol-chloroform to IP and IN samples and vortex;
6. Centrifuge for 5 minutes at 13000 rpm;
7. Transfer supernatant to new eppendorf tubes;
8. Add 2µl glycogen (20mg/ml, Roche Molecular Diagnostics)
9. Add 0.7 volume of isopropanol;
10. Incubate 30 minutes at -80°C;
11. Centrifuge 30 minutes at 13000 rpm at 4°C;
12. Remove supernatant and add 0.5 ml 70% ethanol;
13. Centrifuge 30 minutes at 13000 rpm at 4°C;
14. Remove supernatant;
15. Dry DNA under vacuum;
16. Re-suspend DNA in 30µl water.

Quantitative real-time PCR

Input and immunoprecipitated DNA are analysed by real-time PCR using a 7000 Sequence Detector System (Applied Biosystems). Regions from an HO-induced DSB, a replication fork or a control region (SMC2 gene) are amplified using TaqManTM PCR. This involves a double-dye oligonucleotide (TaqManTM probe, can be obtained from Eurogentec) that has a fluorescent reporter dye (FAMTM) and a quencher (TAMRATM) at its 5' and 3' ends, respectively, and primers that anneal upstream and downstream of the probe, respectively. During the amplification process the probe is cleaved by the 5' -> 3' exonuclease activity of the Taq DNA polymerase. This removes the fluorophore from the probe and separates it from the quencher. As a consequence the fluorophore starts to fluoresce. This fluorescence can be measured, and the level is directly proportional to the amount of DNA amplified during the PCR reaction. Primers and probes are designed using the Primer Express software (Applied Biosystems) and tested for comparable and linear amplification of specific and control fragments from a genomic DNA template using TaqManTM Universal PCR master mix (Applied Biosystems).

Absolute fold enrichment for proteins and protein modifications at regions near an HO-induced DSB can be calculated as follows. For each timepoint the signal from a region near the DSB is normalized to that from the SMC2 control locus on chromosome VI in immunoprecipitated and input DNA samples. Then, for each timepoint and region near the DSB the normalized signals from immunoprecipitated DNA are normalized to those from the normalized input DNA, because end-resection could have reduced the available DNA template. Finally, for each timepoint the normalized signals from immunoprecipitations with antibody are normalized to those from immunoprecipitations without antibody.

Absolute fold enrichment for proteins and protein modifications at regions near a replication fork can be calculated in a slightly different way. For each time point the signals from a region near the replication fork in the immunoprecipitations with and without antibody, respectively, are normalized to those from the input DNA. Finally, for each time point the normalized signals from immunoprecipitations with antibody are normalized to those from immunoprecipitations without antibody.

Materials & Reagents

Lysis buffer	50mM	Hepes pH 7.5
	140mM	NaCl
	1mM	EDTA
	1%	Triton X-100
	0.1%	Na-deoxycholate
		Protease inhibitors

Wash buffer	100mM Tris pH 8 250mM LiCl 0.5% NP-40 0.5% Na-deoxycholate 1mM EDTA
Protease inhibitors	1% Trasylol 1mM PMSF 2µg/ml Antipain 300µg/ml Benzamidin 0.5µg/ml Leupeptin 1µg/ml PepsatinA 20µg/ml TPCK 10µg/ml TLCK
LiCl buffer	5M LiCl 50mM Tris pH 8
YPLGg medium	1% Yeast extract 2% Bactopeptone 2% Lactic acid 3% Glycerol 0.05% Glucose pH 6.6
YPAD medium	1% Yeast extract 2% Bactopeptone 0.0025% Adenine 2% Glucose

Author's notes

1. The optimal concentration of alpha-factor (Product nr. PE050103, Lipal Biochemicals) for efficient arrest in G1 and release from G1-arrest after removal of alpha-factor, depends on the strain background and should be determined empirically.
2. We have successfully used Sheep Anti-Mouse IgG (Product nr. 110.31, Dynal Biotech ASA) and Sheep Anti-Rabbit IgG (Product nr. 1120.04, Dynal Biotech ASA) Dynabeads for ChIP.
3. 10µl aliqouts may be saved for Western analysis.

Reviewer's comments

1. The HO endonuclease can also be induced by adding galactose directly into cell cultures that have been pre-grown in raffinose-containing media.) **Reply by authors**: In principle this should work. We have never tried this because the galactose promoter, which drives HO expression, may be leaky on raffinose and therefore HO-induced double-strand breaks may be induced.
2. The optimal crosslinking time varies from few minutes to few hours, and should be determined empirically. Addition of protein-protein crosslinkers may improve signals for some targets.
3. Cell breakage can also be achieved using a vortex shaker, such as Eppendorf model 5432 at 4°C for 40 minutes). **Reply by authors**: True, but cell breakage has to be analysed for every experiment as this method probably gives less reproducible results in terms of lysis efficiency.

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4. After reversal of crosslinks, DNA can also be recovered without Protease K treatment using commercial DNA recovery kits, such as the Qiagen PCR cleanup kit.

References

Cobb, J.A., Bjergbaek, L., Shimada, K., Frei, C. and Gasser, S.M. (2003) DNA polymerase stabilization at stalled replication forks requires Mec1 and the RecQ helicase Sgs1, *EMBO Journal*, **22**: 4325-4336.

Lee, S.E., Moore, J.K., Holmes, A., Umezu, K., Kolodner, R.D. and Haber, J.E. (1998) Saccharomyces Ku70, mre11/rad50 and RPA proteins regulate adaptation to G2/M arrest after DNA damage, *Cell*, **94**: 399-409.

van Attikum, H., Fritsch, O., Hohn, B. and Gasser, S.M. (2004) Recruitment of the INO80 complex by H2A phosphorylation links ATP-dependent chromatin remodeling with DNA double-strand break repair, *Cell*, **119**: 777-788.