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Chromatin Immunoprecipitation (ChIP) Assay*

Chromatin Immunoprecipitation (ChIP) assays are used to evaluate the association of proteins with specific DNA regions. The technique involves crosslinking of proteins with DNA, fragmentation and preparation of soluble chromatin followed by immunoprecipitation with an antibody recognizing the protein of interest. The segment of the genome associated with the protein is then identified by PCR amplification of the DNA in the immunoprecipitates. Here we describe a useful ChIP protocol as well as Re-ChIP, Alternative Crosslinking Method and Nucleosome ChIPs.

Reagents Required

1X TE, pH 8.0 (10 mM Tris HCl, 1.0 mM EDTA) DEPC TREATED. p/n MB-040. Store at RT up to one month.

10X PBS pH 7.2 (0.2 M Potassium Phosphate 1.5 M Sodium Chloride) p/n MB-008. Store at RT up to one month.

Procedure

Modifications of this protocol (Refs. 1-3) compared to those generally found in literature are as follows:

1. After crosslinking of the cells with formaldehyde nuclei are prepared by incubation and dounce homogenization of the cells in Swelling buffer. In our experience, inclusion of this step significantly reduces background.

2. In many widely used ChIP protocols, after crosslinking the cells are lysed in 1% SDS containing buffer. After sonication and before immunoprecipitation SDS is diluted 10 times. In our protocol the cells are lysed in 0.1% SDS-containing buffer and immunoprecipitation is performed in the same solution. The inclusion of at least 0.1% SDS and harsh washing conditions are necessary to reduce background due to non-specific sticking of chromatin to Protein-G-Sepharose beads. Performing sonication in a 0.1% SDS-containing buffer compromises shearing efficiency; therefore sonication conditions should be carefully controlled. On the other hand avoiding the use of 1% SDS for lysis, greatly enhanced the ChIP signals obtained with several antibodies.

   In our hands preparing chromatin using lysis buffer containing 1% SDS, followed by dialysis against the same buffer with lower SDS concentration clearly increases the recovery for some antibodies. 1% SDS appears to improve sonication efficiency.

The step-by-step protocol is described for cultured cells grown in 150 mm dishes, containing 2-5 x 10^7 cells per dish.

1. Replace medium with 27 ml DMEM/10% FBS.

2. Add 3 ml formaldehyde (from 10% stock) and mix immediately. Incubate at room temperature for 10 min.

   Cross-linking time influences the ChIP-efficiency. For instance, for ChIPing histone modifications 10 min cross-linking is perfect, while for ChIPing transcription factors longer cross-linking times can be employed (up to 30 min). We normally incubate 30 min.

3. Add 3 ml glycine (from 1.375 M stock) and mix immediately.

4. Place the plate on the top of ice and wash 3 times with 20 ml ice-cold PBS/ 0.5 mM PMSF.

5. Scrape cells in 20 ml ice-cold PBS/ 0.5mM PMSF. Centrifuge at 1000 rpm for 5 minutes in cold centrifuge.

   You may not want to use toxic PMSF or any other protease inhibitors at this step.

6. Resuspend pellet in at least 10 vol. Swelling buffer. Incubate in ice for 10 minutes. Dounce 10-20 times up-down. (Check nuclei in microscope by mixing an aliquot with equal volume of 0.4% Trypan blue).

   We wash cross-linked cells first with 30 ml cold PBS and then with buffer similar to the Swelling buffer.

7. Centrifuge at 2000 rpm for 5 minutes.

8. Resuspend pellet (nuclei) in 5-10 ml Sonication buffer.

The volume depends on the number of nuclei. Use a volume to obtain approximately 2-3x10^6 nuclei per ml.

We dilute cells in broad ranges: 106 cells per 30-300 µl. Branson250 sonicator works efficiently and almost independently of cell concentration.

9. Sonicate 9 times for 10-20 seconds at 80% setting (VibraCell Sonicator). Keep sample in ice and allow sample to cool in ice for 1 minute between each sonication (fragment size should be 200-1000 nt).

   We sonicate 12 times x10 seconds with intervals 30 seconds, hold tube on ice-ethanol (-16°C). We found very important that sample stays cold during sonication.

   This step has to be optimized for each cell type and instrument. Pilot experiments using different settings and times should be performed and after decrosslinking and phenol extractions the size of the DNA is evaluated by agarose gel electrophoresis.

   Typically, short sonication results in high recoveries (%ChIP/input) but low resolution, while longer sonication times result in lower recovery but higher resolution. Gel image can be not sufficient and conclusive. ChIP experiment will provide better clues about specific signal-to-background ratio.

10. Centrifuge at 14000 rpm for 15 minutes.

11. Take the supernatant and centrifuge again at 14000 rpm for 15 minutes.

    We centrifuge 5 minutes once.

12. Take the supernatant (this contains the crude Soluble Chromatin) and add sonicated DNA (to 1 µg/ml final concentration) and BSA (to 1 mg/ml final concentration).

    When performing ChIP-on-chip, such competitor DNA should be omitted because of the random amplification step involved. We try to avoid contamination with any non-relevant DNA.

    The concentration of the antibody should be empirically determined.

    Optimal ratio can be ~2-4 µg of antibody against transcriptional factors per 1-2 million cells. Different ratio was found for histone Abs.

13. Preclear the lysate by incubating by constant rotation with Protein-A or G Sepharose (use 40-50 µl Sepharose per ml lysate) for 2 hours in the cold room.

    Before the preclearing step the columns should be washed 3x with Sonication buffer and then preincubated for at least 4 hours with Sonication buffer containing 1 µg/ml sonicated λDNA + 1 mg/ml BSA. We get much nicer results (less background), if preclearing is done twice with new Protein-G Sepharose .

    We found preclearing step is not necessary. We skip it because it makes no differences as for single gene ChIP as for our ChIP-on-chip experiments.

14. Centrifuge samples at 2000 rpm for 5 minutes. Take the supernatant. This is the precleared Soluble Chromatin).

    At this point the samples can be frozen to -80°C .

    The use of fresh chromatin clearly increases the recovery, although cross-linked chromatin can be stored at -80°C. Storage longer that 1-2 months is not recommended. Chromatin prepared in lysis buffer containing 1% SDS can be stored at 4°C for 1-2 days until use.

15. Save a 50 µl (1/20th of amount used per IP) aliquot at -20°C. (for preparation of INPUT DNA)

16. Divide the sample into 1 ml aliquots in microcentrifuge tubes for IP.

17. Add 5 µg of antibody. Rotate in the cold room for 2 hours.

The concentration of the antibody should be empirically determined.

Optimal ratio can be ~2-4 µg of antibody against transcriptional factors per 1-2 million cells. Different ratio was found for histone Abs

18. Add 40 µl Protein-A or G Sepharose per IP (equilibrated as above) and incubate overnight by constant rotation in the cold room.

19. Centrifuge the beads at 6000 rpm for 3 minutes.

20. Wash 2 times with 1 ml Sonication buffer.

    Each wash includes 10 minute constant rotation of the tubes in the cold room.

21. Wash 2 x with 1 ml Wash buffer A.

22. Wash 2 x with 1 ml Wash buffer B.

23. Wash 2 x with 1 ml TE buffer.

24. Add 200 µl Elution buffer to the beads and incubate at 65°C for 10 minutes. Centrifuge at 14000 rpm for 1 minute. Transfer supernatant to a new tube and elute beads again. Combine eluates (400 µl final volume, adjust with Elution buffer if necessary).

    We elute once with 400 µl of Elution buffer, 30 minutes rotation at room temperature.

25. Add 21 µl NaCl (from 4 M stock). In parallel thaw the input sample (50 µl) and supplement with 350 µl Elution buffer. Add 21 µl NaCl (from 4M stock).

26. Incubate at 65°C for at least 5 hours.

    This is the de-crosslinking step, which can also be done overnight

    We de-crosslink 4 hours at 65°C, incubation o/n can be a problem.

27. Add 1 µl RNAse A (from 10 mg/ml, DNAse-free stock) and incubate at 37°C for 1 hour.

28. Add 4 µl EDTA (from 0.5 M stock) and 2 µl Proteinase K (from 10 mg/ml stock).

29. Incubate at 42°C for 2 hours.

    We do not perform steps 27-29.

30. Extract 2 times with phenol/chloroform/isoamylalcohol and once with chloroform/isoamylalcohol.

    We extract once each step.

31. Add 1 µl glycogen (from 20 mg/ml stock), 40 µl Na-acetate (from 3M stock) and 1 ml EtOH.

    We add 10 µg of glycogen.

32. Vortex and leave to precipitate -20°C overnight.

33. Centrifuge at 14000 rpm 30 minutes. Wash 1 x with 80% EtOH. Speedvac.

    We wash with 70% ethanol, then carefully remove it and leave tubes open for few minutes so no ethanol left. Speedvac with such a tiny pellet can be not desirable.

34. Resuspend IP and INPUT samples in 100 µl 10 mM Tris (pH 7.5). Proceed to PCR analysis.

Re-ChIP assay

Re-ChIP stands for sequential chromatin immunoprecipitations with two antibodies to study the simultaneous presence of two proteins, or different histone modifications in the genome sequence of interest (Refs. 2 and 5)

We perform elution with "Elution buffer" (contains 1% SDS). We found it crucial to immobilize antibodies on prot A/G beads to avoid leakage of antibody from first ChIP to the second ChIP. We incubate a fraction of eluate from the first ChIP with empty beads as an antibody leakage control.

1. After the step 24 of the standard protocol, incubate the beads with equal volume of 10 mM DTT for 30 minutes at 37°C.
2. Centrifuge at 14000 rpm for 1 minute and transfer the supernatant into a new tube.
3. Repeat step 1 and combine the eluates.
4. Dilute the eluted sample 40 times with Sonication buffer, keep 10% of the sample for input and proceed with step 18 of the standard protocol by adding the second antibody.

Alternative method for crosslinking

A general observation in ChIP assays is that the signals obtained for histones and DNA-binding factors are much stronger than those proteins that do not directly contact DNA but recruited via protein-protein interactions. There could be several explanations for this phenomenon. One of them is crosslinking efficiency by formaldehyde. In our experience ChIP signals for such proteins (e.g. CBP and some other cofactors) can be improved 2-4 times by using 3'-dithiobispropionimidate (DTBP from Pierce) in conjunction with formaldehyde. The protocol was adopted from Ref. 4 and is described for cells grown in 150 mm dish.

1. Wash cells 3x with ice-cold PBS (pH 8.0)
2. Prepare (freshly) 5 mM DTBP in ice cold PBS (pH 8.0) and add to plates sitting on ice. (To cover cells 20-25 ml of this solution is needed per 150 mm plate).
3. Incubate on ice for 30 minutes.
4. Wash cells twice with cold PBS (pH 8.0).
5. Add 20-25 ml ice-cold Quenching buffer (100 mM Tris pH 8.0, 150 mM NaCl) per plate.
6. Incubate on ice for 10 minutes.
7. Take the plates out from ice and wash 3 times with PBS at room temperature.
8. Add 27 ml PBS to each plate + 3 ml formaldehyde (from 10% stock). Mix well and incubate at room temperature for 10 minutes.
9. Add 3 ml Glycine (from 1.375 M stock). Mix well.
10. Wash 3 times with cold PBS/0.5mM PMSF.
11. Continue with step 5 of standard protocol.

Nucleosome ChIP (Ref. 5)

This method allows ChIP analysis at nucleosome resolution by taking advantage of the fact that micrococcal nuclease (MNase) can efficiently digest crosslinked chromatin. It is applicable to study histone modifications and factor occupancy on nucleosomes, but not if the factors are recruited to naked DNA sequence. It should be noted however that, if two neighboring nucleosomes are bridged via intermediary factors, they are not expected to be resolved. In order to

investigate individual nucleosomes, the sonication step of the standard protocol is replaced by micrococcal nuclease digestion of crosslinked chromatin. Complete digestion (i.e. to obtain mononucleosome-sized fragments) requires pure nuclei. While nuclei obtained by the standard protocol step 7 is often fine, we obtain more reproducible results by including the steps below.

1. After step 6 resuspend cells in at least 10 pellet volume of Sucrose buffer A and perform dounce homogenization (10 up and downs). Check nuclei by trypan blue staining.

2. Layer the nuclear suspension over an equal volume of Sucrose buffer B and centrifuge for 15 minutes at 3000 rpm. This is performed in 15 ml tubes, 5 ml nuclear suspension + 5ml Sucrose buffer B.

3. Remove stepwise the supernatant by 1 ml pipette and collect purified nuclei from the bottom of the gradient by resuspension in 1 ml Buffer NUC. Transfer into a clean tube.

4. Wash nuclei with Buffer NUC once and resuspend them in Buffer NUC, to obtain 0.2 OD260 per 5 µl sample. OD is measured by diluting 5 µl nuclei in 1 ml 1M NaOH.

5. Add CaCl2 to 3 mM final concentration and immediately add 100 units/ml micrococcal nuclease (MNase, Worthington).

   In most cases this amount of MNase is sufficient to obtain mononucleosome-sized fragments, but may need to be optimized for the particular experiment.

6. Incubate for 5 minutes at 37°C and stop reactions by the addition of an equal volume of 2x Sonication buffer X.

7. Vortex and check lysis in microscope. If not completely lysed perform one short (5 second) sonication. Centrifuge at 14000 rpm for 15 minutes, take the soluble chromatin containing supernatant and proceed to step 14.

   At the end of the procedure the purified DNA fragments are around 146 bp in size. In order to design primers for PCR analysis the positions of the nucleosomes should be determined. In our experience low resolution mapping by indirect end-labeling of partially digested chromatin with MNase is sufficient.

Materials & Reagents

Swelling buffer	• 25 M Hepes, pH 7.8 • 1.5 mM $MgCl_2$ • 10 mM KCl • 0.1% NP-40 • 1 mM DTT • 0.5 mM PMSF • Protease inhibitor cocktail (Roche)

Sonication buffer	• 50mM Hepes pH 7.9 • 140 mM NaCl • 1 mM EDTA • 1% Triton X-100 • 0.1% Na-deoxycholate • 0.1% SDS • 0.5 mM PMSF • Protease inhibitor cocktail (Roche)
Wash buffer A	• 50 mM Hepes pH 7.9 • 500 mM NaCl • 1 mM EDTA • 1% Triton X-100 • 0.1% Na-deoxycholate • 0.1% SDS • 0.5 mM PMSF • Protease inhibitor cocktail (Roche) This is the same as "Sonication buffer" but contains 500 mM NaCl.
Wash buffer B	• 20mM Tris, pH 8.0 • 1mM EDTA • 250mM LiCl • 0.5% NP-40 • 0.5% Na-deoxycholate • 0.5 mM PMSF • Protease inhibitor cocktail (Roche)

Elution buffer	• 50mM Tris, pH 8.0 • 1mM EDTA • 1% SDS • 50mM $NaHCO_3$
Sucrose buffer A	• 0.32mM sucrose • 15mM Hepes pH 7.9 • 60mM KCL • 2mM EDTA • 0.5mM EGTA • 0.5% BSA • 0.5mM spermidine • 0.15mM spermine • 0.5mM DT
Sucrose buffer B	• 0.32mM sucrose • 15mM Hepes pH 7.9 • 60mM KCL • 2mM EDTA • 0.5mM EGTA • 0.5mM spermidine • 0.15mM spermine • 0.5mM DTT This is the same as Sucrose buffer A without BSA + 30 % sucrose.

Buffer NUC	• 15mM Hepes pH 7.5 • 60mM KCL • 15mM NaCl • 0.34mM sucrose • 0.15mM mercaptoethanol • 0.15mM spermine • 0.5mM spermidine
2x Sonication buffer X	For Nucleosome ChIP • 90mM Hepes pH 7.9 • 220mM NaCl • 10mM EDTA • 2% Triton X 100 • 0.2% Na-deoxycholate • 0.2% SDS • 0.5mM PMSF • Protease inhibitor cocktail (Roche)

References

1. Soutoglou E, Talianidis I (2002) Coordination of PIC assembly and chromatin remodeling during differentiation-induced gene activation. *Science* **295**: 1901-1904

2. Hatzis P, Talianidis I (2002) Dynamics of enhancer-promoter communication during differentiation-induced gene activation. *Mol Cell* **10**: 1467-1477

3. Kouskouti A, Scheer E, Staub A, Tora L, Talianidis I (2004) Gene-Specific Modulation of TAF10 Function by SET9-Mediated Methylation. *Mol Cell*, **14**: 175-182

4. Fujita N, Wade PA (2004) Use of bifunctional cross-linking reagents in mapping genomic distribution of chromatin remodeling complexes. *Methods*, **33**: 81-85

5. Kouskouti A, Talianidis I (2005) Histone modifications defining active genes persist after transcriptional and mitotic inactivation. *EMBO J.*, **24**: 347-357.

*Modified from the method of A. Kouskouti and I. Kyrmizi (2005)