www.rockland.com
tech@rockland.com
+1 484.791.3823

Protocol

Chromatin Immunoprecipitation (ChIP)

Chromatin Immunoprecipitation (ChIP) assays are used to evaluate the association of proteins with specific DNA regions. The technique involves cross-linking 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.

The step-by-step protocol is described for cultured cells grown in 150 mm dishes, containing 2–5 x 107 cells per dish. Modifications of this protocol1-3 compared to those generally found in the literature are as follows:

- After cross-linking of the cells with formaldehyde, nuclei are prepared by incubation and dounce homogenization of the cells in swelling buffer. In our experience, the inclusion of this step significantly reduces background.

- In many widely used ChIP protocols, after cross-linking, 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 increases the recovery for some antibodies. 1% SDS appears to improve sonication efficiency.

Reagents Required

Product	Preparation	Item No.
Dulbecco's Modified Eagle Medium (DMEM)	Supplement with 10% FBS.	
10% Formaldehyde		
Glycine (1.375 M)		
PBS (Phosphate Buffered Saline)	Use 10X PBS, pH 7.2 (0.2 M Potassium Phosphate, 1.5 M NaCl). Dilute appropriate volume to 1X with molecular biology-grade water.	MB-017 / MB-029-1000
PMSF (Phenylmethansulfonylfluorid)		
0.4% Trypan Blue		

Swelling Buffer	25 mM 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	50 mM 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)	
Sonicated λDNA		
Bovine Serum Albumin (BSA)		BSA-10
Protein A or Protein G Sepharose™		PA50-00-0002 / PG50-00-0002
Wash Buffer A **Note:** This is the same as Sonication Buffer but contains 500 mM NaCl	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)	
Wash Buffer B	20 mM Tris, pH 8.0 1 mM EDTA 250 mM LiCl 0.5% NP-40 0.5% Na-deoxycholate 0.5 mM PMSF Protease inhibitor cocktail (Roche)	MB-005
TE Buffer	Use 1X TE pH 8.0 or dilute appropriate volume of 10X TE pH 8.0.	MB-040 / MB-007
Elution Buffer	50 mM Tris, pH 8.0 1 mM EDTA 1% SDS 50 mM $NaHCO_3$	MB-005
NaCl	Prepare 4 M stock.	
Phenol:Chloroform:Isoamyl Alcohol		
Chloroform:Isoamyl Alcohol		
Glycogen	Prepare 20 mg/mL stock.	
Na-Acetate	Prepare 3 M stock.	

Ethanol	
Tris HCl pH 7.6	Prepare from 1.0 M Tris HCl pH 7.6.

Procedure

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 minutes.
   Note: Cross-linking time influences the ChIP efficiency. For instance, for ChIPing histone modifications 10 minutes cross-linking is perfect, while for ChIPing transcription factors longer cross-linking times can be employed (up to 30 minutes). We normally incubate 30 minutes.
3. Add 3 mL glycine 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/ 0.5 mM PMSF. Centrifuge at 1000 rpm for 5 minutes in cold centrifuge.
   Note: You may not want to use toxic PMSF or any other protease inhibitors at this step.
6. Resuspend pellet in at least 10 volume 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).
   Note: We wash cross-linked cells first with 30 ml cold PBS and then with a buffer similar to the Swelling Buffer.
7. Centrifuge at 2,000 rpm for 5 minutes.
8. Resuspend pellet (nuclei) in 5–10 mL Sonication Buffer.
   Note: The volume depends on the number of nuclei. Use a volume to obtain approximately 2–3 x 10^6 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).
   Note: We sonicate 12 times for 10 seconds with intervals of 30 seconds while holding tube on ice-ethanol (-16°C). The sample must stay 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 DNA should be evaluated by agarose gel electrophoresis after de-crosslinking and phenol extractions. Typically, short sonication results in high recoveries (%ChIP⁄input) but low resolution, while longer sonication times result in lower recovery but higher resolution. Gel images can be not sufficient and conclusive. ChIP experiment will provide better clues about specific signal-to-background ratio.
10. Centrifuge at 14,000 rpm for 15 minutes.
11. Take the supernatant and centrifuge again at 14,000 rpm for 15 minutes.
    Note: We centrifuge 5 minutes once.
12. Take the supernatant (this contains the crude soluble chromatin) and add sonicated λDNA (to 1 µg/mL final concentration) and BSA (to 1 mg/mL final concentration).
    Note: 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. A 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.

Note: Before the pre-clearing step, the columns should be washed 3 times 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 pre-clearing step is not necessary. We skip it because it makes no difference for single gene ChIP as for our ChIP-on-chip experiments.

14. Centrifuge samples at 2000 rpm for 5 minutes and remove the supernatant. This is the precleared soluble chromatin).
    Note: At this point, the samples can be frozen to -80°C. The use of fresh chromatin increases the recovery, although cross-linked chromatin can be stored at -80°C. Storage longer than 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.
    Note: Optimal ratio can be ~2–4 µg of antibody against transcriptional factors per 1–2 million cells. A 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 6,000 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 times with 1 mL Wash Buffer A.
22. Wash 2 times with 1 mL Wash Buffer B.
23. Wash 2 times with 1 mL TE Buffer.
24. Add 200 µL Elution Buffer to the beads and incubate at 65°C for 10 minutes. Centrifuge at 14,000 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).
    Note: 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
    Note: 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.
    Note: We do not perform steps 27–29.
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.
30. Extract 2 times with phenol:chloroform:isoamyl alcohol and once with chloroform:isoamyl alcohol.
    Note: 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.
    Note: We add 10 µg of glycogen.
32. Vortex and leave to precipitate -20°C overnight.

33. Centrifuge at 14,000 rpm 30 minutes. Wash 1 time with 80% EtOH. Speedvac. We wash with 70% ethanol, then carefully remove it and leave tubes open for few minutes so no ethanol is left.
34. Resuspend IP and input samples in 100 µL 10 mM Tris (pH 7.6). Proceed to PCR analysis.

References

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