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NBP2-29497 Protocol

ChIP Protocol

To perform ChIP, it is necessary to shear chromatin into 200-1000 bp fragments. The following protocol assumes that optimized shearing conditions have already been determined. If they have not, please proceed to Appendix A (Shearing Optimization Protocol) for a complete protocol. We have successfully used this protocol with mammalian cells including HeLa, 293HEK, Raw, NIH 3T3, MCF7, and Ramos cells.

DAY 1
Before You Begin

4 5 x10^6 mammalian cells on a 10 cm tissue culture plate.
Cells should be treated as necessary to insure the transcriptional activation of the gene of interest.
1% Formaldehyde Solution at RT.
Add 270 ul of 37% formaldehyde to 10 ml cell culture media. It is important to use high quality formaldehyde.
10X Glycine at RT.
Ice for incubation of cells and PBS.
During the procedure high temperatures will reverse the formaldehyde cross-linking. Care must be taken at each step to keep the cells and reagents on ice whenever indicated in the protocol.
1X PBS: Add 3 ml of 10X PBS to 27 ml ddH2O.
SDS Lysis Buffer at RT.
100X Protease Inhibitor Cocktail (PIC) on ice.
100X PMSF on ice.
Optimize conditions to shear your cross-linked DNA ~200-1000 bp.

CROSS-LINK
NOTE: Cross-linking procedure may be omitted when using antibodies to Histone proteins, which are tightly bound to DNA. Protein properties must be determined empirically.
A. ADHERENT CELLS
1. For adherent cells, carefully aspirate cell culture media from the 10 cm plate containing cells.
2. Add 10 ml of 1% Formaldehyde Solution to plate.
This step serves to fix the cells, binding the protein/DNA complex in the nucleus.
3. Incubate at 37 degrees C for 10 min.
4. Add 1 ml of 10X Glycine to plate and gently swirl to mix.
This step serves to stop the fixing process, to ensure chromatin do not over fix.
5. Incubate at RT for 5 min.
6. Aspirate medium. Remove as much medium as possible without disturbing cells.
7. Wash cells 2x with 10 ml of ice cold 1X PBS.
8. Add 1 ml ice cold 1X PBS (supplemented with 10 ul 100X PMSF and 10 ul 100X PIC).
9. Scrape cells into a centrifuge tube.
10. Centrifuge at 1200 rpm (~3000 x g) at 4 degrees C for 5 min.
11. Carefully discard supernatant, leaving behind pelleted cells.
Cell pellet can be stored at -80 degrees C for several months.

B. SUSPENSION CELLS

1. For suspension cells, centrifuge cells at 1200 rpm for 5 min and carefully aspirate cell culture media.
2. Add 10 ml of 1% Formaldehyde Solution to the tube.
This step serves to fix the cells, binding the protein/DNA complex in the nucleus.
3. Incubate at 37 degrees C for 10 min.
4. Add 1ml of 10X Glycine to tube and gently swirl to mix.
This step serves to stop the fixing process, to ensure chromatin do not over fix.
5. Incubate at RT for 5 min.
6. Centrifuge at 1200 rpm for 5 min. Remove as much medium as possible without disturbing the cell pellet.
7. Wash cells 2x with 10 ml of ice cold 1X PBS.
8. Add 1 ml ice cold PBS (supplemented with 10 ul 100X PMSF and 10 ul 100X PIC).
9. Centrifuge at 12,000 rpm (~7000 x g) at 4 degrees C for 5 min.
10. Carefully discard supernatant, leaving behind pelleted cells.
Cell pellet can be stored at -80 degrees C for several months.
11. Continue with Lyse Protocol.

LYSE
12. Resuspend pellet in 1 ml SDS Lysis Buffer (supplemented with 10 ul 100X PMSF and 10 ul 100X PIC).
13. Incubate on ice for 10 min.
Optional: remove one 5 ul aliquot of cell lysate for agarose gel analysis of unsheared DNA.

SONICATE
14. Sonicate cell lysate on ice according to optimized conditions determined in Appendix A.
Keep cell lysate on ice during sonication.
15. Centrifuge at 15,000 rpm (~12,000 x g) at 4 degrees C for 10 min to pellet the insoluble material.
Optional: remove one 25 ul aliquot to determine DNA concentration and shearing efficiency.
16. Transfer supernatant to a fresh centrifuge tube.
At this time you should have ~1 ml of supernatant, enough for approximately five IPs (200 ul per IP).
Sheared sonicated material can be stored at -80 degrees C for several months.

PRECLEAR

Before You Begin

100X PIC on ice
Determine number of IPs (remember to include an appropriate antibody control).
Each IP requires that the supernatant be diluted 5-10 fold with ChIP Dilution Buffer containing PIC.
For example: One IP requires 200 ul supernatant diluted with 790 ul ChIP Dilution Buffer and 10 ul PIC.
17. For each IP you wish to run, aliquot 800 ul of ChIP Dilution Buffer containing PIC.
18. Add 200 ul of the supernatant (from Step 16) to each tube.
19. Add 75 ul of Salmon Sperm DNA/Protein A/G Agarose to each tube.
This step serves to remove molecules that can nonspecifically bind to the Protein G Agarose.
20. Rotate for 30 min at 4 degrees C.
21. Briefly centrifuge Salmon Sperm DNA/Protein A/G Agarose at 1200 rpm (~3000 x g) for 1 min.
It is not necessary to centrifuge Protein A/G Agarose beads at high g forces. If you are having trouble pelleting, please see the FAQ section.

22. Collect the supernatant by aliquoting into a fresh microfuge tube.
23. Aliquot a 10 ul sample of the supernatant into a fresh microfuge tube and store at 4 degrees C for use in Step 31 below.
This "Input" sample should be used as negative antibody control during PCR analysis on Day 3.

IMMUNOPRECIPITATE

Before You Begin
Immunoprecipitating antibody (user-provided): The amount of antibody will vary and needs to be empirically determined.
24. Add the immunoprecipitating antibody to the supernatant fraction.
For user-provided antibody, the appropriate amount of antibody (usually between 1-10 ug per tube) needs to be empirically determined.
For the positive control, add 1.0 ug per tube of RNA Polymerase II anti-body.
25. Rotate overnight at 4 degrees C.
Depending on the empirical properties of your antibody and target gene, this step can possibly be shortened.

DAY 2

Before You Begin
100X PIC and 100X PMSF on ice (optional addition to wash buffers, researcher determined).
1M $NaHCO_3$ at RT.
65 degrees C water bath.
Prepare 500 ul Elution Buffer (1% SDS, 0.1M $NaHCO_3$) per sample (and for Input tube from Step 23) as follows: 25 ul 20% SDS, 50 ul 1M $NaHCO_3$ and 25 ul ddH_2O.
Wash Buffer A, B, C and D on ice.

COLLECT
26. Add 60 ul of Salmon Sperm DNA/Protein A/G Agarose for 1 hr at 4 degrees C with rotation.
This step serves to collect the Antibody/Antigen/DNA complex.
27. Pellet Protein A/G Agarose by brief centrifugation 1200 rpm (~3000 x g) for 1 min and discard the supernatant fraction.

WASH
28. Wash the Protein A/G Agarose/Antibody/Chromatin complex on a rotating platform as follows:
Resuspend the beads in 1 ml cold Wash Buffer A.
Incubate for 3-5 min on a rotating platform.
Briefly centrifuge at 1200 rpm (~3000 x g) for 1 min and discard the supernatant fraction.
Repeat wash with 1 ml cold Wash Buffer B.
Repeat wash with 1 ml cold Wash Buffer C.
Repeat wash 2x with 1 ml cold Wash Buffer D.

ELUTE
29. Add 250 ul of the Elution Buffer to each Protein A/G Agarose/ Antibody/Chromatin complex.
30. Add 500 ul of the Elution Buffer to the Input tube (from Step 23) and set aside at RT until Step 35.
31. Mix by gently flicking tubes.
32. Incubate at RT for 15 min.
33. Pellet the Protein A/G Agarose/Antibody/Chromatin complex by brief centrifugation at 1200 rpm (~3000 x g) for 1 min and collect supernatant.
34. Repeat the elution steps for the Protein A/G Agarose/Antibody/ Chromatin complex a second time and combine eluates (total volume ~500 ul).

REVERSE CROSS-LINK*

35. Add 20 ul 5M NaCl and incubate at 65 degrees C for 4 hr to overnight.
This step serves to reverse the DNA/Protein cross-links.
36. Centrifuge for 30 s to collect liquid from sides of tubes and then add 1 ul of RNaseA (Cat # KC-130) and incubate for 30 min at 37 degrees C.
37. Add 10 ul 0.5M EDTA, 20 ul 1M Tris-HCl, pH 6.5 and 2 ul 10 mg/ml Proteinase K and incubate for 1 hr at 45 degrees C.

DAY 3

DNA PURIFICATION

38. Purify DNA for analysis using the Phenol/Chloroform Extraction Protocol detailed in Appendix B.
Alternatively, DNA purification columns can be used for purification.

PCR ANALYSIS AND PCR OF CONTROLS

Accurate PCR analysis of ChIP DNA requires that the PCR is stopped during the linear amplification phase. The appropriate number of PCR cycles for each experiment must be determined empirically.
1. Prepare the appropriate number of thermocycling tubes (0.2 ml) for sample analysis.
Recommended PCR Samples:
Human GAPDH Primers
Negative Control Primers
Input Sample
No DNA Sample
2. Add 10 ng of DNA and the appropriate amount of reagents to each thermocycling tube on ice, as indicated in the ReadyMixTM Taq PCR Reaction Mix.
3. Run the following PCR reaction program in your thermocycler.
Initial Denaturation 94 degrees C 3 min
Denature 94 degrees C 20 s
Anneal 59 degrees C 30 s 35 Cycles
Extension 72 degrees C 30 s
Final Extension 72 degrees C 5 min
4. Remove 10 ul of each PCR reaction for analysis on a 2% agarose gel electrophoresis with a 100 bp DNA Ladder.