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(This protocol is the same as the one followed by Imke but modified (underlined) slightly for ChIP on HeLa-TDS stable cell lines expressing GFP tagged splicing factors)

Chromatin Immunoprecipitation Protocol for Mammalian Cells

1. Grow cells to confluency on four 14 cm dishes. This should give you cell material for 4 IPs. $\sim 10^8$ cells per IP
2. Cross-linking: Add formaldehyde (37% solution, J.T. Baker # 7040 or Merck), directly to culture medium to a final concentration of 1%, mix and incubate for 10 minutes at RT.
3. Wash: Aspirate medium thoroughly. Wash cells twice using cold PBS. Keep cells on ice.
4. Scrape cells into 50 ml PBS containing protease inhibitors (PI) 1/100 (20ul of 25X solution of Roche complete Protease Inhibitor Cocktail tablets) in a Falcon tube.
5. Pellet cells for 5 min at 3500 rpm at 4°C. Warm SDS Lysis Buffer to room temperature to dissolve precipitated SDS.
6. Resuspend cell pellet in 1 ml of SDS Lysis Buffer to which 1X PI has been added and incubate for 10 minutes on ice in a 15 ml falcon.
7. Sonicate lysate to shear DNA to lengths between 200 and 1000 basepairs being sure to keep samples on ice/ethanol bath.
We use the Branson sonicator at 30 % amplitude, 14 x 10s impulses, 20 s pauses. These conditions vary between cell types and sonicators!!!! You need to optimize the sonication conditions first in order to know the resolution of your assay.
8. Centrifuge lysate for 10 minutes at 14,000 rpm (20000g) at 4°C, and make 200 μ l aliquots into 2 ml-microcentrifuge tubes. Remember to freeze 50 μ l aliquot as input. You can freeze the extracts at -80°C .
9. Dilute the sonicated extract 10 fold in ChIP Dilution Buffer + PI (1 x final) by adding 1800 μ l ChIP Dilution Buffer+PI to the 200 μ l sonicated extracts for a final volume of 2ml.
10. To reduce nonspecific background, pre-clear the 2 ml diluted cell extract with 80 μ l of sepharose beads (Sigma # CL-4B-200) for at least 60 min at 4°C with rotation.
11. Pellet sepharose for 1 min at 2000 rpm and collect the supernatant fraction
12. Add the immunoprecipitating antibody to the 2 ml supernatant fraction in a new tube and precipitate overnight at 4°C with rotation.
The amount of antibody varies a lot depending on the protein to be precipitated, antibody efficiency etc... You have to determine the best antibody concentration empirically. For a negative control, perform an IgG (unspecific antibody) IP. I incubate the unspecific control over night with the other IPs, but other people use only beads or incubate the antibody for just one hour, I guess to reduce background. I use 12ug per IMMUNOPRECIPITATION of anti-GFP (MPI-CBG)
13. Add 60 μ l of blocked GammaBinding G sepharose beads (Pharmacia Biotech,# 17-0885-01) for one hour at 4°C with rotation to collect the antibody/protein complex.
To be sure to add the same amount of beads to every IP, cut the tips at the end.

14. Pellet beads by gentle centrifugation (1000 rpm at 4°C, 1 min). Carefully remove the supernatant that contains unbound, non-specific DNA. Transfer the beads into a new 1.5ml tube (cut the tips!). Wash the bead/antibody/protein complex for 4 min on a rotating platform with 1 ml of each of the buffers listed in the order as given below:
 - 1x Low Salt Immune Complex Wash Buffer
 - 1x High Salt Immune Complex Wash Buffer
 - 1x LiCl Immune Complex Wash Buffer
 - 1x TE

I wash at room temperature, but use cold buffers and a cold centrifuge. To be on the safe side, 1/100 PI (Roche) can be added to the wash buffers and/or the washes can be performed at 4°C.

15. Freshly prepare Elution Buffer.

16. Elute the protein complex from the antibody by adding 250 µl elution buffer to the pelleted bead/antibody/protein complex from step 14 above. Vortex briefly to mix and incubate at room temperature for 15 min with rotation. Spin down beads, and carefully transfer the supernatant fraction (eluate) to a 1,5 ml tube and repeat elution. Combine eluates (total volume = 500 µl).

17. Take 50 µl of the frozen Input (from step 8) and dilute it 10 x with ChIP Dilution Buffer (add 450 µl) and uncrosslink it with the other samples. This sample is considered to be your input/starting material for all the IPs done with this extract and is used in the PCR later.

18. Add 20 µl 5M NaCl to the combined eluates (500 µl) and input and reverse protein-DNA crosslinks by heating at 65°C for 6 hours. I don't have a good experience freezing samples at this point and never do it.

18. Add 10 µl of 0.5 M EDTA, 20 µl 1 M Tris-HCl, pH 6.5 and 2 µl of 10 mg/ml Proteinase K to the combined eluates and incubate for one hour at 45°C.

19. Recover DNA by using either the Qiagen PCR purification kit OR 1x phenol/chloroform extraction, 1x chloroform extraction, overnight precipitation at -20°C with 2 volumes ethanol and 1/3 volume Amm. Acetate (7,5 M) + 20 µg glycogen to visualize the DNA pellet. Wash pellets with 70% ethanol and air dry.
For me Phenol- chloroform gave a better DNA yield, lower Cts but lesser enrichment so I stick to using the PCR purification kit.

20. Resuspend pellets in an appropriate buffer (TE) + 0,1 mg/ml Rnase A.

For Real Time PCR, I dissolve the DNA in 70 µl of EB buffer. The volume can be changed as long as you stick to the same for all IPs that are to be compared.

21. Perform Real Time or conventional PCR to prove that the precipitated protein was bound to a specific DNA segment.

For Real Time PCR, I use 4 µl of undiluted DNA. For conventional PCR, a dilution series is necessary to be sure that you amplify in the linear range (for example, take 1, 3, and 10 µl). Always include the input/starting material to check for different primer pair efficiencies and DNA amount, if you want to compare ChIPs from different extracts. Also include always the negative control (IgG IP or beads only) to be sure that your amplified signal is above background.

Buffers and Reagents

SDS Lysis Buffer

1% SDS
10 mM EDTA
50 mM Tris-HCl, pH 8.1
add protease inhibitors before use

ChIP Dilution Buffer

0.01% SDS
1.1% Triton X-100
1.2 mM EDTA
16.7 mM Tris-HCl, pH 8.1
167 mM NaCl
add protease inhibitors before use

Low Salt Immune Complex Wash Buffer

0.1% SDS
1% Triton X-100
2 mM EDTA
20 mM Tris-HCl, pH 8.1
150 mM NaCl

High Salt Immune Complex Wash Buffer

0.1% SDS
1% Triton X-100
2 mM EDTA
20 mM Tris-HCl, pH 8.1
500 mM NaCl

LiCl Immune Complex Wash Buffer

0.25 M LiCl
1% NP-40
1% deoxycholic acid (sodium salt)
1 mM EDTA
10 mM Tris-HCl, pH 8.1

Elution Buffer

1% SDS
0.1M NaHCO₃

Blocked GammaBinding G sepharose beads

1 ml beads (50% gel slurry. If not, dilute appropriately in 1XPBS+20% ethanol)
0.2 mg salmon sperm DNA
0.5 mg BSA

TE

10mM Tris pH 8.1 and 1mM EDTA

25X Protease inhibitor cocktail (Roche) 1 tablet in 2ml H₂O