

1. Cross-linking and Cell Harvesting

- 1.1. Start with two confluent 150 cm² dishes (1x10⁷ - 5x10⁷ cells per dish). Cross-link proteins to DNA by adding formaldehyde drop-wise directly to the media for a final concentration of 0.75% and rotate gently at room temperature (RT) for 10 min.*
- 1.2. Add glycine to a final concentration of 125 mM to the media and incubate with shaking for 5 min at RT.
- 1.3. Rinse cells 2 x with 10 ml cold PBS.
- 1.4. Scrape cells into 5 ml cold PBS and transfer into 50 ml tube.
- 1.5. Add 3ml PBS to dishes and transfer the remainder of the cells to the 50 ml tube.
- 1.6. Centrifuge for 5 min at 1,000 g.
- 1.7. Carefully aspirate off supernatant and resuspend pellet in FA Lysis Buffer (750 µl per 1x10⁷ cells).

* When using suspension cells, start with 1x10⁷ - 5x10⁷ cells and treat with both 0.75% formaldehyde and glycine as described above. Pellet cells by centrifugation (5 mins at 1,000 g). Wash 3 x with cold PBS and resuspend pellet in FA Lysis Buffer (750 µl per 1x10⁷ cells). Proceed to Step 2.1.



2. Sonication

- 2.1. Sonicate lysate to shear DNA to an average fragment size of 500 to 1000 bp. This will need optimizing as different cell lines require different sonication times. Follow the fragment size on a 1.5% agarose gel.
- 2.2. Centrifuge for 30 secs, 4°C, 8,000 g and transfer supernatant to new tube.**
- 2.3. Remove 50 µl of each sonicated sample. This sample is the INPUT and this is used for obtaining the DNA concentration (see Step 5).

**The lysed cells can be snap frozen in liquid nitrogen after and stored at -70°C for up to 2 months. Avoid multiple freeze-thawing.



3. Immunoprecipitation

- 3.1. Use approximately 25 µg of protein per IP. Protein concentration can be calculated using the Bradford assay. Dilute each sample 1:10 with RIPA Buffer. You will need one sample for the specific antibody, and one sample for the beads only control.
- 3.2. Add the primary antibody to all samples except the beads-only control. The amount of antibody to be added has to be determined empirically, 1-10 µg of antibody per 25 µg of protein often works well.
- 3.3. Add 20 µl of protein A/G beads (pre-absorbed with sonicated single stranded herring sperm DNA at 1.5 µg / 20 µl beads***) to all samples and IP overnight with rotation at 4°C.
- 3.4. Centrifuge the protein A/G beads for 1min at 2,000g and remove the supernatant.
- 3.5. Wash beads 3 x with 1ml wash buffer (centrifuge as above).
- 3.6. Wash beads 1 x with 1ml final wash buffer (centrifuge as above).

***Preparation of protein A/G beads with single stranded herring sperm DNA



- Mix an equal volume of Protein A and Protein G beads and wash 3 X in RIPA Buffer.
- Aspirate RIPA Buffer and add single stranded herring sperm DNA to 75 ng/μl beads and BSA to a final concentration of 0.1 μg/μl beads. Add RIPA Buffer to twice the bead volume and incubate for 30 min with rotation at 4°C.
- Wash once with RIPA Buffer and add RIPA Buffer to twice the bead volume.

4. Elution and reverse cross-link

- 4.1. Elute DNA by adding 120μl of Elution Buffer to the protein A/G beads and rotate for 15 min at 30°C.
- 4.2. Spin down and transfer the supernatant into fresh tube.**
- 4.3. The INPUTs can be included at this stage. Add 80μl of elution buffer (when using the DNA purification kit) or 400 μl TBS (when using phenol:chloroform) to each INPUT sample. Add either 2 μl RNase A (0.5 mg/ml) when purifying DNA using PCR purification kit or 5 μl of proteinase K (20 mg/ml) when purifying DNA using Phenol:Chlorophorm to the eluates (INPUTs and IP material) and heat at 65°C for 4-5hrs (or overnight).****
- 4.4 a. The DNA is then purified using a PCR purification kit following the manufacturer's instructions.
- 4.4 b. Alternatively the DNA can be Phenol:Chlorophorm extracted and ethanol precipitated in presence of 10μl glycogen (5 mg/ml) and taken up in 100 μl H₂O.
- 4.5. Store samples at -20°C or proceed with detection method (PCR, microarray, etc).*****
- 4.6 PCR is used to quantify DNA levels of specific loci. This is analyzed semi-quantitatively (analyses of PCR end-product by agarose gel) using primers which can be designed using the URL below.

http://biotools.umassmed.edu/bioapps/primer3_www.cgi

Alternatively, DNA levels are quantitatively measured by real-time PCR. Primers and probes are often designed using software provided with the real-time PCR apparatus.



**** The samples can be frozen and stored at -20°C after these steps.

***** The INPUT DNA is purified as described and the concentration should be calculated. Transfer 5 μl of the purified DNA in a tube containing 995 μl TE to give a 200-fold dilution and read the OD₂₆₀. The concentration of DNA in μg/ml is OD₂₆₀ X 10,000. This is used to calculate how much input DNA was included in the immunoprecipitation, and the sample values altered accordingly.

Solutions

FA lysis buffer

50 mM HEPES-KOH pH7.5
 140 mM NaCl
 1 mM EDTA pH8
 1% Triton X-100
 0.1% Sodium Deoxycholate
 0.1% SDS
 Protease Inhibitors (add fresh each time)

RIPA buffer

50 mM Tris-HCl pH8
 150 mM NaCl
 2 mM EDTA pH8
 1% NP-40
 0.5% Sodium Deoxycholate
 0.1% SDS
 Protease Inhibitors (add fresh each time)

Wash buffer

0.1% SDS
1% Triton X-100
2 mM EDTA pH8
150 mM NaCl
20 mM Tris-HCl pH8

Final wash buffer

0.1% SDS
1% Triton X-100
2 mM EDTA pH8
500 mM NaCl
20 mM Tris-HCl pH8

Elution buffer

1% SDS
100mM NaHCO₃

Proteinase K

Dissolve in H₂O at 20 mg/ml, store at -20°C