

CROSS-LINKING CHROMATIN IMMUNOPRECIPITATION (X-ChIP) PROTOCOL

ChIP is a powerful tool that allows the specific identification of proteins or histone modifications to regions of the genome. Chromatin is isolated and antibodies to the antigen of interest are used to determine whether the target binds to a specific DNA sequence or to map the distribution across the genome (microarray or DNA sequencing). This can be performed both spatially and temporally. This protocol provides specific details of how a ChIP can be performed on cells.

1. Cross-linking and Cell Harvesting



Formaldehyde is used to cross-link the proteins to the DNA. Cross-linking is a time dependent procedure and optimization will be required. We would suggest cross-linking the samples for 2 - 30 min. Excessive cross-linking reduces antigen accessibility and sonication efficiency. Epitopes may also be masked. Glycine is added to quench the formaldehyde and terminates the cross-linking reaction.

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 to a final concentration of 0.75 % and rotate gently at room temperature (RT) for 10 min.
2. Add glycine to a final concentration of 125 mM to the media and incubate with shaking for 5 min at RT.
3. Rinse cells 2 x with 10 ml cold PBS.
4. Scrape cells into 5 ml cold PBS and transfer into 50 ml tube.
5. Add 3 ml PBS to dishes and transfer the remainder of the cells to the 50 ml tube.
6. Centrifuge for 5 min, 1,000 g.
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 (Section 1). Pellet cells by centrifugation (5 mins, 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

1. Sonicate lysate to shear DNA to an average fragment size of 500 - 1000 bp. This will need optimizing as different cell lines require different sonication times.



The cross-linked lysate should be sonicated over a time-course to identify optimal conditions. Samples should be removed over the time-course and DNA isolated as described in Section 3. The fragment size should be analyzed on a 1.5 % agarose gel as demonstrated in Figure 1.

2. After sonication, pellet cell debris by centrifugation 30 sec, 4 °C, 8,000 g. Transfer supernatant to a new tube. This chromatin preparation will be used for the immunoprecipitation (IP) in Step 4.
3. Remove 50 µl of each sonicated sample, this sample is the INPUT. This is used to quantify the DNA concentration (see Step 3) and as a control in the PCR.



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

3. Determination of DNA concentration

1. The INPUT samples are used to calculate the DNA concentration for subsequent IPs. The DNA is purified using either a PCR purification kit (add 70 µl of Elution Buffer and proceed to Step 3.2a) or phenol:chloroform (add 350 µl of Elution Buffer and proceed to Step 3.2b).
2. a. Add 2 µl RNase A (0.5 mg/ml). Heat with shaking at 65 °C for 4-5 hr (or overnight) to reverse the cross-links. DNA is purified using a PCR purification kit according to the manufacturer's instructions. The samples can be frozen and stored at -20 °C.



Samples are treated with RNase A as high levels of RNA will interfere with DNA purification when using the PCR purification kit. Yields can be severely reduced as the columns become saturated.

2. b. Add 5 µl proteinase K (20 mg/ml). Heat with shaking at 65 °C for 4-5 hr (or overnight) to reverse the cross-links. The DNA is phenol:chloroform extracted and ethanol precipitated in the presence of 10 µl glycogen (5 mg/ml). Resuspend in 100 µl H₂O. The samples can be frozen and stored at -20 °C.



Samples are treated with proteinase K, which cleaves peptide bonds adjacent to the carboxylic group of aliphatic and aromatic amino acids. Cross-links between proteins and DNA are disrupted which aids DNA purification.

3. To determine the DNA concentration, transfer 5 µl of the purified DNA into 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 the DNA concentration of the chromatin preparation.

4. Immunoprecipitation

1. Use the chromatin preparation from Step 2.2, an equivalent amount of approximately 25 µg of DNA per IP is recommended. Dilute each sample 1:10 with RIPA Buffer. You will need one sample for the beads-only control.
2. Add primary antibody to all samples except the beads-only control. The amount of antibody to be added should be determined empirically; 1-10 µg of antibody per 25 µg of DNA often works well.
3. Add 20 µl of protein A/G beads (pre-adsorbed with sonicated single stranded herring sperm DNA and BSA, see step 4.3a) to all samples and IP overnight with rotation at 4 °C.
3. a. Preparation of protein A/G beads with single stranded herring sperm DNA. If using both Protein A and Protein G beads, 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 a final concentration of 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 RT. Wash once with RIPA Buffer and add RIPA Buffer to twice the bead volume.



Protein A beads, protein G beads or a mix of both should be used. Table 1 shows the affinity of protein A and G beads to different Immunoglobulin isotypes.

4. Centrifuge the protein A/G beads for 1 min, 2,000 g and remove the supernatant.
5. Wash beads 3 x with 1 ml Wash Buffer. Centrifuge 1 min, 2,000 g and remove the supernatant.
6. Wash beads 1 x with 1 ml Final Wash Buffer. Centrifuge 1 min, 2,000 g and remove the supernatant.



If high background is observed additional washes may be needed. Alternatively, the sonicated chromatin may also be pre-cleared by incubating with the Protein A/G beads for 1 hr prior to Step 4.2. Any non-specific binding to the beads will be removed during this additional step. Transfer the supernatant (sonicated chromatin) to a new tube and incubate with the antibody and beads as described in Step 4.2 onwards.

5. Elution and reverse cross-links

1. Elute DNA by adding 120 μ l of Elution Buffer to the protein A/G beads and rotate for 15 min, 30 °C.
2. Centrifuge for 1 min, 2,000 g and transfer the supernatant into a fresh tube. The samples can be stored at -20 °C
3. The DNA can be purified using a PCR purification kit (proceed with Step 3.2a) or phenol:chloroform (add 280 μ l of Elution Buffer and proceed with Step 3.2b).
4. DNA levels are quantitatively measured by real-time PCR. Primers and probes are often designed using software provided with the real-time PCR apparatus. Alternatively an online design tool is used. [Click here](#)

A selection of pre-designed primers and probes are also available on our website. [Click here](#).

Please use our troubleshooting tips to optimize the protocol. [Click here](#).

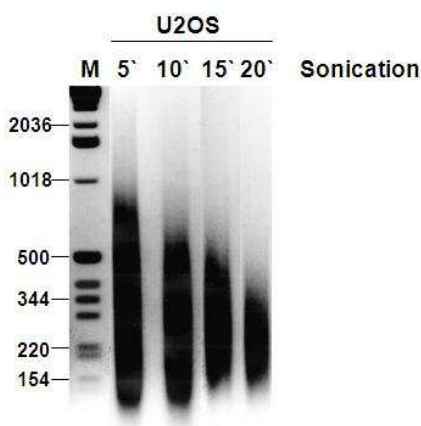


Figure 1. U2OS cells were sonicated for 5, 10, 15 and 20 min. The fragment size decreases during the time course. The optimal fragment size is observed at 15 min. NOTE; sonicating for too long will disrupt nucleosome-DNA interactions therefore the band size should not be smaller than 200bp.

Species Immunoglobulin Isotype	Protein A	Protein G
Human IgG1	+++	+++
IgG2	+++	+++
IgG3	-	+++
IgG4	+++	+++
IgM	Use anti Human IgM	
IgE	-	+
IgA	-	+
Mouse IgG1	+	+++
IgG2a	+++	+++
IgG2b	++	++
IgG3	+	+
IgM	Use anti Mouse IgM	
Rat IgG1	-	+
IgG2a	-	+++
IgG2b	-	++
IgG2c	+	++
Chicken All isotypes	-	++
Cow All isotypes	++	+++
Goat All isotypes	-	++
Guinea Pig All isotypes	+++	++
Hamster All isotypes	+	++
Horse All isotypes	++	+++
Pig All isotypes	+	++
Rabbit All isotypes	+++	++
Sheep All isotypes	-	++

Table 1. The affinity of protein A and G beads to different Immunoglobulin isotypes.

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