abcam

Dual cross-linking ChIP protocol (Dual-X-ChIP)

Detailed procedure for dual cross-linking ChIP

Overview

ChIP is a powerful tool which uses isolated chromatin, and antibodies to the antigen of interest to determine whether a target binds to a specific DNA sequence or to map the distribution across the genome (ChIP-seq). This protocol provides specific details of how ChIP can be performed on cells using a dual cross-linking method to efficiently bind transcription factors to DNA within your chromatin sample. This type of double cross-linking is very effective when you are using ChIP to observe the binding pattern of transcription factors bound directly to DNA or even those found in DNA binding complexes not bound directly to DNA.

1. Cross-linking and cell harvesting

Both formaldehyde and EGS (ethylene glycol bis (succinimidyl succinate) are used in this protocol to dual 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 with EGS for 20–30 min, combined with a 10-minute formaldehyde treatment. 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 cm^2 dishes ($1x10^7$–$5x10^7$ cells per dish). Cross-link proteins to DNA by adding 20 mL ice cold PBS to each flask with EGS (300 mM) to final concentration of 1.5 mM, swirl gently at room temperature for 30 min.

2. Add formaldehyde (37%) to each flask to final 0.75% (for histone projects) or 1% (for transcription factor projects). Swirl gently at room temperature for 10 min (this timing will need to be optimized for different cell types). This should be carried out in hood as formaldehyde is harmful.

3. Add 1.5 mL of 2.5 M glycine (125 mM final) to the media and incubate with shaking for 5mins to quench formaldehyde. This process should still be processed in hood.

4. Rinse cells twice with 10 mL cold PBS.

5. Add 5 mL of cold PBS, scrape dishes thoroughly with a cell scraper and transfer into 50 mL tube.

6. Add 3 mL PBS to dishes, scrape again, and transfer the remainder of the cells to the 50 mL tube.

7. Centrifuge for 5 min, 4°C, 1,000 x g.

8. Carefully aspirate off supernatant and resuspend the pellet in ChIP Lysis Buffer (750 µL per $1x10^7$ cells) and incubate for 10 min on ice.

When using suspension cells, start with 1x10^7- 5x10^7 cells and treat with both 0.75% formaldehyde and glycine as described above (step 1). Pellet cells by centrifugation (5 mins, 1,000 g). Wash 3 times with cold PBS and resuspend pellet in ChIP Lysis Buffer (750 µL per 1x10^7 cells). Proceed to Step 2.

2. Sonication

1. Sonicate lysate to shear DNA to an average fragment size of 200–1000 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 step 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 for 10 min, 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, to determine DNA concentration and fragment size.

The sonicated chromatin can be snap frozen in liquid nitrogen and stored at -80°C for up to 3 months. Avoid multiple freeze-thaws.

3. Determination of DNA concentration and fragment size

1. The sonicated chromatin samples can be used to calculate the DNA concentration for subsequent IPs and measure DNA fragment size. Add 70 µL of elution buffer to the 50 µL of chromatin.

2. Add 4.8 µL of 5 M NaCl and 2 µL RNase A (10 mg/mL) and incubate while shaking at 65°C overnight.

3. Add 2 µL proteinase K (20 mg/mL) and incubate while shaking at 60°C for 1 h.

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.

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.

4. Purify DNA using a PCR purification kit or phenol:chloroform extraction. #

5. 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. Run purified DNA in a 1.5% agarose gel with a 100 bp DNA marker to determine fragment size.

4. Immunoprecipitation

1. Use the chromatin prepared from Step 2. Approximately 25 µg of DNA per IP is recommended. Dilute each sample 1:10 with RIPA Buffer. You will need one sample for the specific antibody and one sample for the control (beads only). Remove 50 µL of chromatin to serve as your input sample and store at -20°C until further use.

2. Add primary antibody to all samples except the beads-only control and rotate at 4°C for 1 hour. 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. Preparation of protein A/G beads: If using both Protein A and Protein G beads, mix an equal volume of Protein A and Protein G beads and wash three times in RIPA Buffer. Aspirate RIPA Buffer and add 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.

4. Add 60 µL of blocked protein A/G beads to all samples and IP overnight with rotation at 4°C.

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.

1. Centrifuge the immunoprecipitated samples for 1 min at 2,000 x g and remove the supernatant.

2. Perform the following washes: three times in wash buffer. After each wash, centrifuge for 1 min at 2,000 x g and remove the supernatant.

If high background is observed additional washes or washes with buffers with higher salt concentrations (up to 500mM NaCl) 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 µL of Elution Buffer to the protein A/G beads and vortex slowly for 15 min at 30°C.

2. Centrifuge for 1 min at 2,000 x g and transfer the supernatant into a fresh tube.

3. Add 4.8 µL of 5 M NaCl and 2 µL RNase A (10 mg/mL) and incubate while shaking at 65°C overnight.

4. Add 2 µL proteinase K (20 mg/mL) and incubate while shaking at 60°C for 1 h.

5. The DNA can be purified using a PCR purification kit or phenol:chloroform extraction.

6. 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.

A selection of pre-designed primers and probes are also available on our website.

Please use our troubleshooting tips to optimize the protocol.

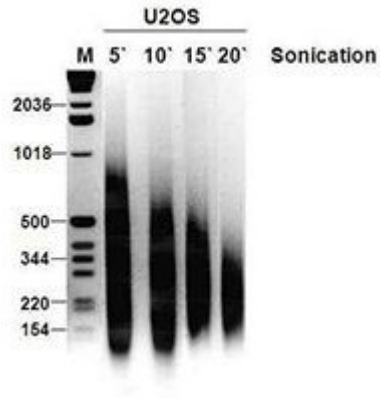

Figure 1. U2OS cells were sonicated for 5, 10, 15 and 20 min. The fragment size decreases during the time course. The optimal fragment size is observed at 15 min. NOTE; sonicating for too long will disrupt nucleosome-DNA interactions therefore the band size should not be smaller than 200bp.

Immunoglobulin isotypes

Species	Immunoglobulin isotype	Protein A	Protein G
Human	IgG1	+++	+++
	IgG2	+++	+++
	IgG3	-	+++
	IgG4	+++	+++
	IgM	Use anti human IgM	Use anti human IgM
	IgE	-	+
	IgA	-	+
Mouse	IgG1	-	+
	IgG2a	+++	+++
	IgG2b	++	++
	IgG3	+	+
	IgM	Use anti human IgM	Use anti human 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 immunoglobin isotypes.

Solutions

ChIP 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
20 mM Tris-HCl pH 8.0
150 mM NaCl

TE Buffer
10 mM Tris pH 8.0
1 mM EDTA

Elution Buffer
1% SDS
100mM NaHCO3