Chromatin Immunoprecipitation (ChIP) Protocol

Fixation and Chromatin Cross-Linking

Cultured Cells

In this protocol, we will be using one 150 cm^2 flask of HeLa cells (~1 x 10^7 cells) as an example. Start with cells that are 80% confluent. Other cell types may require optimization due to different chromatin yield and target protein abundance.

1. Add formaldehyde to a final concentration of 1% in growth medium. Incubate for 10 minutes at room temperature.

2. Add enough glycine to each flask to reach a final concentration of 125 mM in the media, and thereby quench the formaldehyde and stop the crosslinking. Incubate for 5 minutes at room temperature.

3. Remove all medium and wash with 20 mL of ice cold 1X PBS.
4. Repeat step 4.

5. Remove PBS and add 4 mL of ice cold 1X PBS with appropriate protease inhibitors. Scrape cells and transfer to a 15 mL conical tube.

6. Spin cells at 4° C for 5 minutes at 800 x g.

7. Discard supernatant and resuspend cell pellet in 400 µL of RIPA lysis buffer (containing protease inhibitors) per 1 x 10^7 cells (enough for 10 IPs). Transfer resuspended cells to a microcentrifuge tube and incubate at 4° C for 15 minutes.

Tissue Preparation

Freshly dissected or frozen tissue can be used, but it is important to work quickly on ice to prevent degradation of your target protein and its chromatin associations. Although your total chromatin yield will vary depending on tissue type, 25-50 mg of tissue is a good starting point.

1. Place the tissue in a small dish placed on top of ice to keep the tissue cold. Mince the tissue into small pieces (~2 mm) using two razor or scalpel blades.

2. Move the tissue pieces into a conical tube containing 10 volumes of ice cold PBS (or tissue culture medium) plus appropriate protease

inhibitors (1 ml of PBS for every 100 mg of tissue).

3. Fix the tissue by adding enough formaldehyde to reach of final concentration of 1%. Fix the tissue for 10-20 minutes.

4. Add enough glycine to each flask to reach a final concentration of 125 mM in the media, and thereby quench the formaldehyde and stop the crosslinking. Incubate for 5 minutes at room temperature.

5. Centrifuge the sample 4° C for 5 minutes at 800 x g.

6. Remove the supernatant and wash the cell pellet with 10 volumes of ice cold PBS plus protease inhibitors.

7. Centrifuge the sample 4° C for 5 minutes at 800 x g.

8. Remove the supernatant and wash the cell pellet with 10 volumes of ice cold PBS plus protease inhibitors.

9. The tissue can now be homogenized by any of various techniques such as dounce, rotor-stator, bead based, or ultrasonic homogenizers.

10. Centrifuge the homogenized sample 4° C for 5 minutes at 800 x g.

11. Discard supernatant and resuspend cell pellet in 400 µL of RIPA lysis buffer (containing protease inhibitors) for every 25 mg of tissue originally used. Transfer resuspended cells to a microcentrifuge tube and incubate at 4° C for 15 minutes.

DNA Shearing by Sonication

1. Sonicate cross-linked DNA to fragment sizes of 100-1000 base pairs.

    1. **Important:** Conditions for shearing must be empirically derived and optimized before proceeding to the following phases. Conditions vary depending on equipment, cell type, cell density, and cross-linking efficiency.

    2. Keep samples ice cold to prevent denaturing of chromatin.

    3. Keep sonicator tip close to the bottom of each tube and limit samples to a maximum of 400 µL to prevent foaming of samples, as foaming decreases efficiency.

4. A time-course of multiple short sonications is preferable to fewer extended treatments due to heat buildup and subsequent denaturing of samples. For example, six 15 second pulses at 50% output with a 60 second ice rest in between pulses works well for us.

2. Centrifuge sheared samples at 4° C for 5 minutes at 12,500 x g to remove debris. Remove supernatant and transfer to a new tube. Discard pellet. Sample can now be moved into ten 40 µL aliquots, each of which is sufficient for a single IP. Set aside one 40 µL aliquot for the input control, which will not go through the IP process. Although it is preferable to proceed directly to the following phases, sheared chromatin can now be frozen at -80° C for up to 1 month.

    1. Optional: Test the efficiency of the shearing by preparing an aliquot to run on a 1.5% agarose gel.

        1. Dilute a 40 µL aliquot with 160 µL of IP Elution Buffer and 8 µL of 5M NaCl.

        2. Incubate at 95° C for 15 minutes to reverse cross- linking.

        3. Optional: Cleanup DNA by purification with either a commercially available column kit or by phenol/chloroform extraction.

        4. Run 10 µL of sample on the 1.5% agarose gel with loading buffer and DNA binding dye alongside a 100 bp ladder. Verify fragment size.

Chromatin Immunoprecipitation

1. Dilute each IP sample 1:10 by adding 40 µL of sheared chromatin to 360 µL IP dilution buffer, along with protease inhibitors. Save your undiluted input sample at 4° C for the reverse crosslinking phase.

2. Add your antibody of interest to each of your samples. It is also recommended to run the separate following controls:

    1. Positive Control Antibody: An antibody known to work well in ChIP with your primer set.
    2. No Antibody Negative Control: A separate sample from step 1 that does not have any antibody added.

3. Incubate tubes with rotation overnight at 4° C.

4. For each IP, you will use 25 µL of fully vortex suspended Protein A/G Magnetic Bead slurry. Beads must first be washed by adding 25 µL of suspended beads to 1 mL of IP dilution buffer and vortexed. Pellet beads with separator and discard supernatant. **Important**: Do not let the beads dry out in any step!

5. Add each sample to 25 µL of washed beads and incubate tubes with rotation for one hour at 4° C.

6. Pellet magnetic beads with a magnetic separator and remove the supernatant. Add 500 µL cold low salt buffer wash buffer and wash for 5 minutes with rotation at 4° C. Pellet magnetic beads with magnetic separator and discard supernatant.

7. Add 500 µL cold high salt wash buffer and wash for 5 minutes with rotation at 4° C. Pellet beads with separator and discard supernatant.

8. Add 500 µL cold LiCl buffer and wash for 5 minutes with rotation at 4° C. Pellet beads with separator and discard supernatant.

9. Add 500 µL cold TE buffer and wash for 5 minutes with rotation at 4° C. Pellet beads with separator and discard supernatant.

10. Elute complex by adding 200 µL IP elution Buffer and rotate at room temperature for 15 minutes. Pellet beads with separator and discard beads, keeping the supernatant.

Reverse Cross-linking

For each IP sample, add 8 µL of 5M NaCl. For the 40 µL input control that did not go through the preceeding IP steps, add 160 µL of IP Elution Buffer and 8 µL of 5M NaCl.

1. Incubate at 95° C for 15 minutes.

    1. Optional: Some lysates may need extended reverse cross-linking and a protein degradation step. If you find your total DNA yield to be low, perform the following:

        1. After completion of Step 1, add 2 µL of the provided Proteinase K and incubate at 62° C for at least 2 hours (or overnight).

2. Incubate at 95° C for 10 minutes to deactivate the Proteinase K.

DNA Purification

1. DNA can now be purified with either a commercially available column kit or by phenol/chloroform extraction.

DNA PCR Amplification

Purified DNA can now be measured by PCR. Quantitative real-time PCR is the preferable method of amplification due to its sensitivity. The method described below uses a 2X SYBR green reaction mix containing all necessary components (dNTPs, DNA polymerase, buffers). It is recommended to run each PCR reaction in triplicate for each sample. Samples to be assayed include: immunoprecipitated sample from the antibody of interest, the positive control sample (if a positive control anitobdy was used), the no antibody negative control sample, and your purified input control. Forward and reverse primers are also needed for each region of interest that will be amplified. Relative background of the no antibody negative control will also be measured. Each sample will use 2 µL of purified DNA for its template.

1. For the input control fraction only, dilute the template to 1% of the original concentration (1:100 dilution). All other samples are left undiluted.

2. It is best to first create a PCR master mix for each primer set and dispense the mix into each reaction well first, adding the template last. In the case of your positive control primer set master mix, each reaction will contain the following:

    1. 7 µL of DNase free water
    2. 1 µL of 10 µM primers (final concentration 0.5 µM)
    3. 10 µL of 2x SYBR reaction mix
    4. 2 µL of purified DNA template (added directly to wells)

3. Perform PCR according to manufacturer's recommendations for the SYBR reaction mix.

Data Analysis

Data can now be analyzed by taking the cycle threshold (Ct) values from the qRT-PCR assay. All samples will be adjusted to a signal relative to the total input fraction.

1. Example
    1. First, find out what the 1% input control fraction would be at 100%. Ex. The raw Ct of the 1% input fraction=30. Adjust Ct from 1% to 100% by subtracting 6.64 cycles (log_2 of 100); 30 - 6.64 = 23.4 = 100% input Ct.

    2. Next, look at the Ct from the antibody of interest sample. Let's say this value is a Ct=28.

    3. Now, normalize the signal from the antibody of interest to the total input. In this example, signal relative to input would be 2^(23.4-28) = 0.04 = 4%. From this, it can be shown that 4% of all the available DNA in the sample (specific to your primer sequence) was bound to the protein of interest.

    4. Finally, look at the background signal levels to make sure that they are significantly less than the antibody precipitated samples. Measure the signal relative to input of the no antibody control. If the raw Ct from this reaction was 36, the signal relative to input would be 2^(23.4-36) = 0.01%. From this it can be said the background is significantly less compared to the antibody of interest (4% vs 0.01%).

Buffers

- IP Dilution Buffer: 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCL (pH8.1), 167 mM NaCl
- Low Salt Wash Buffer: 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl.
- High Salt Wash Buffer: 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl.
- LiCl Wash Buffer: 250mM LiCl, 1% NP-40, 1% Na-deoxycholate, 1mM EDTA, 10mM Tris, pH 8.1.
- TE Buffer: 10mM Tris-HCL pH 8.1, 1 mM EDTA
- IP Elution buffer: 1% SDS, 100mM NaHCO3