Chromatin Immunoprecipitation Protocol

Protocol For ChIP

(For scientific research purposes only, not for clinical diagnosis!)

Day 1:

1. Formaldehyde cross-linking and ultrasonic disruption of cells

1.1 For cells: measure the volume of the medium, and add 37% formaldehyde until a final concentration of 1% is reached.

1.2 Incubate for 10 minutes at 37 ℃

1.3 Terminate the cross-linking: Block the reaction with 500 µl Glycine 2.5 M (final concentration 125 mM). Incubate for 5 minutes at room temperature.

1.4 Discard the medium and wash the cells 3 times with ice cold PBS;

1.5 Using a cell scraper scrape off the cells in PBS, centrifuge at 2000g/min for 5min, and then discard the supernatant;

1.6 Breaking the cells in IP Lysis Buffer supplemented with protease inhibitor(the volume of IP Lysate Buffer depends on the amount of cells).

1.7 To facilitate the cell membrane breaking，the cells were repeatedly blown with a pipette (or shaken on a vortex mixer);

1.8 Ultrasonic Crushing: Ningbo Xinzhi JY 92-IIN, 2# probe, 30% power, 4.5s ultrasound, 10.5s interval,5min, and 20 times. During the ultrasonic process, please be careful to keep the sample in the ice bath, avoid ultrasonic bubbles.

2. Re-clear samples, and IP overnight

2.1 After ultrasonic breaking, centrifuge at 12000rpm, 4℃ for 10min. Remove supernatant into a fresh tube. Transfer 90µL lysis solution for Input， store the others at -80 °C.

2.2 Take 40 µ l ultrasonic crushing product as input, add 10 µ L 5 * loading buffer, heat-denaturate it and perform WB detection to confirm that the sample contains the target protein. The remaining 50 µ l product was added with 2.5 µ l protease K and 2 µL 5M NaCl (the final concentration of NaCl was 0.2M), and the solution was decrosslinked at 55°C overnight;

2.3 Measure the nucleic acid concentration .Take part of the sample for PCR amplification, and then run agarose electrophoresis to detect the effect of ultrasonic ， and confirm whether the sample contains the target DNA;

2.4 After confirming the Input result, take 100 µl of the ultrasonically crushed product , add 900 µl of ChIP Dilution Buffer containing 1 mM-PMSF and 20 µl of 50×PIC (cooktail). Add 60 µl of Protein A+G Agarose/Salmon Sperm DNA for each sample. Incubate for 1 hours at 4ºC in a rotating wheel.

2.5 Incubate for 10 minutes at 4ºC ， centrifuge at 4000 rpm for 5 minutes;

2.6 Transfer the sample into two 1.5mL EP tubes.For the IgG tubes,add 1µg of IgG（of the same species）. For the IPs, use 2 µg of antibody per tube. Incubate overnight at 4ºC in a rotating wheel.

Day 2：

3. Wash samples, elute, and reverse crosslink.

3.1 Add 200µl Protein A+G Agarose/Salmon Sperm DNA to each tubes . Rotate for 2h at 4 °C to pull down immuno complexes.

3.2 Let all tubes standing at 4℃ for 10min.Centrifuge at 4000 rpm for 1 minutes. Discard the supernatant.

3.3 Wash the precipitate complex with the following solution in turn. Cleaning steps: add the solution, turn it upside down slightly, centrifuge at 4000rpm for 3min, and remove the supernatant.

Washing solution: 1x low salt immune complex wash buffer.
1x high salt immune complex wash buffer.
1x LiCl immune complex wash buffer.
2x TE buffer.

The samples can be used for Western Blot analysis. Add 30µl 1X loading buffer to 30µl beads,then denature them for WB.

3.4 Freshly prepare elution buffer（0.5gSDS+0.42gNaHCO3+50ml Purified Water）. Add 200 µL of elusion buffer to Elute protein;

3.5 Add 20µl 5 M NaCl and 10µl proteinase K to the eluents. And heat all samples at 55 °C over night to reverse the crosslinking.

Day 3：

4. Purify DNA with Spin Column

4.1 Add 200 µl Buffer GB to the sample, mix thoroughly by vortex, and incubate at 70°C for 10min to yield a homogeneous solution. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the tube.

4.2 Add 200µl ethanol (96-100%) to the sample, and mix thoroughly by vortex for 15 s. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the tube.

4.3 Transfer the mixture from step 2 into the Spin Column CB3 (in a 2 ml collection tube) and centrifuge at 12,000 rpm(~13,400 × g) for 30s. Discard flow-through and place the spin column into the collection tube.

4.4 Add 500 µl Buffer GD(Ensure ethanol(96-100%) has been added) to Spin Column CB3, \ and centrifuge at 12,000 rpm(~13,400 × g) for 30s, then discard the flow-through and place the spin column into the collection tube

4.5 Add 700µl Buffer PW (Ensure ethanol (96-100%) has been added) to Spin Column CB3, and centrifuge at 12,000 rpm (~13,400 × g)for 30s. Discard the flow-through and place the spin column into the collection tube.

4.6 Add 500µl Buffer PW to Spin Column CB3, and centrifuge at 12,000 rpm (~13,400 × g)for 30s. Discard the flow-through and place the spin column into the collection tube.

4.7 Centrifuge at 12,000 rpm(~13,400 × g) for 2 min,and place it at room temperature for 10min to dry themembrane completely.

4.8 Transfer the Spin Column CB3 in a new clean 1.5 ml microcentrifuge tube, and add 50-200 µl Buffer TE directly to the center of thememembrane. Incubate at room temperature(15-25°C) for 2min, and then centrifuge for 2min at 12,000 rpm(~13,400 × g).

5. DNA Analysis（Real-Time PCR or DNA Sequencing）

5.1 Real-Time PCR

Using JASPAR or other database to predict the transcription factor binding sites. Design the primers according to the sites. Make a mixture in 200µl microcentrifuge tube:

2× qPCR Mix 10µl

2.5µM primer 2µl

Template 2µl

ddH2O 6µl1

Run reaction

Pre-incubation 95℃，10min

Amplification: 40 cycles of 95℃，15s→60℃，60s

Melting curve 60℃→95℃，the temperature grows 0.3℃ every 15s.

Run PCR reaction on a 2.5% TAE gel and document.

5.2 DNA Sequencing

Collect DNA, measuring the concentration,and then send to a sequencing company.