Cross-linking Chromatin immunoprecipitation (ChIP) Protocol

-Instruction

ChIP is a technique for studying the interactions between proteins with DNA as it is in nature. It depends on the specific antibody reactions with antigen, so which can truly reflect the combination of protein factors and genomic DNA in vivo. The target protein was cross-linked together with DNA. Then DNA is broken into small fragments by sonication or enzymatic hydrolysis. So we can pull down the predicted DNA fractions by the specific reactions between antibody and antigen. This process specifically enriched DNA fragments bound by target proteins. Finally, we can purify the DNA from the complex and validate the DNA following PCR or qPCR protocol.

-Solution and reagents

ChIP lysis buffer: 1% TritonX-100, 0.1% NaDOC, 0.1% SDS, 1mM EDTA (pH8.0), 140mM NaCl, 50mM Tris-HCl (pH 8.0)

RIPA buffer: 1% NP-40, 0.5%NaDOC, 0.1% SDS, 2mM EDTA (pH8.0), 150mM NaCl, 50mM Tris-HCl (pH 8.0)

Low salt wash buffer: 1% TritonX-100, 0.1% SDS, 2mM EDTA (pH8.0), 150mM NaCl, 20mM Tris-HCl (pH8.0)

High salt wash buffer: 1% TritonX-100, 0.1% SDS, 2mM EDTA (pH8.0), 500mM NaCl, 20mM Tris-HCl (pH 8.0)

LiCl buffer: 0.25M LiCl, 1% NP-40, 1% NaDOC, 1mM EDTA, 10mM Tris-HCl (pH 8.0)

TE buffer: 1mM EDTA, 10mM Tris-HCl (pH 8.0)

Elution buffer: 1% SDS, 100mM $NaHCO_3$

-Sample preparation

Cross-linking and lysis—for transcription factor and cofactors

Cells culture in 10cm culture dishes with 20mL culture medium. When the density of cells reaches to 80%-90%, it can be used to a ChIP assay. The number of cells differs from the target protein which you are research on. You can refer to the form as follow:

Protein	Number of cells(for a ChIP reaction)	Protein target abundance
Histone protein RNA polymerase II	10^4	high
Transcription factor	10^5-10^6	medium

Cofactor	10^7 or more	low

To ensure a better result, we recommend more than 4×10^6 cells for histone protein and RNA polymerase II, while transcription factor or cofactor need more cells. 1% formaldehyde is used as crosslinking agent to crosslink proteins and DNA. This process is time dependence. So we need to optimize the process for different cells. Crosslinking deficiency result a false negative result, while excessive crosslinking may cover epitope and result a false positive result. We recommend crosslink the sample foe 10min. The Crosslinking reaction can be reversed by glycine.

1. Take out the culture dishes and add 550μl 37% formaldehyde into 20ml culture medium. Mix the medium up to 1% final concentration of formaldehyde.
2. Place the culture dishes at RT for 10min. The process is the cross-link between protein and DNA and the time shouldn't too long, which will result in a false positive result.
3. Add 125mM glycine solution to terminate the cross-link reaction. Gently mix the solution up.
4. Place the culture dishes at RT for 5min.
5. Remove culture medium and wash the cells with ice PBS for 3 times.
6. Add 1ml ice PBS, Which contains protein inhibitors, into the dishes and scrap the cells from the dishes quickly.
7. Wash the bottom of dishes by PBS for twice with proper volume. Aspirate the PBS into the tube in step 6.
8. Centrifuge for 3min, 4℃ at 2500rmp to collect the pellets.
9. Add proper volume ChIP lysis buffer (1ml for 2×10^7 cells) to suspend the cells and lysate the cells on ice for 15min.
10. Sonicate the cells to broken the DNA and the ideal fractions of DNA is 200-1000bp. The condition of sonicate is differ from type of cells and the instrument you used. For each kind of cells, you have to explore the corresponding sonication conditions.
11. Centrifuge for 10min, 4℃ at 12000rmp..

Sonicate

The sonicate process is also time dependence. The ideal fragment of DNA is 200-1000bp, and it need to optimize for different cell lines.

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2. Centrifuge for 10min, 4℃ at 12000rmp. And separate the supernatant into a new tube.
3. Get 50μl supernatant for an agarose gel analysis to validate the effect of sonication.

Determination of DNA fragment

1. Add 70μl elution buffer to the 50ul chromatin.
2. Add 4.8μl 5M NaCl and 2μl 10mg/ml RNase A to incubate at 65℃ overnight. The purpose is to remove the interference of RNA.
3. Add 2μl 20mg/ml proteinase K to incubate at 60℃ for 1h. The aim is to break the reactions between protein and DNA and may be helpful to DNA purification.
4. Use a DNA purification kit to enrich the DNA or preform phenol-chloroform extraction and ethanol precipitation method.
5. 2% agarose gel is used to detect the sonication efficiency.

-Immunoprecipitation

1. Take 500μl chromatin (dilute by RIPA buffer) which contains DNA from 1×10^7 cells into a tube and take 50μl chromatin as a input group.
2. Before the immunoprecipitation, you have to design four groups of experiments:
   a. Experience group: add 2-5μg specific primary antibody into 500μl chromatin and place the tube on a rotary mixer at 4℃ overnight to form antigen-antibody complex.
   b. Input group: all process is same as experience group except for the immunoprecipitation reaction.
   c. Negative control: all process is same as experience group and instead the antibody with normal rabbit IgG.
   d. Positive control: all process is same as experience group and instead the antibody with a histone H3 or RNA polymerase II antibody
3. Add 50μl magnetic beads into the complex solution at 4℃ for 2-4h.
4. Attach the beads to magnet and remove the supernatant.

-Wash the beads

1. Wash the beads with filtered tips
   a. 2×1ml Low salt wash buffer
   b. 2×1ml High salt wash buffer
   c. 2×1ml LiCl buffer

d. 2× 1ml TE buffer

Wash the beads with the buffer above for twice in turn. Pipet 3-8 times by tips and rotate for 10min each.

2. Remove the wash buffer by magnet and collect the precipitation complex.

-Reverse crosslinking

1. Add 120μl Elution buffer into the precipitation complex and gently mix up at RT for 15min on rotator.
2. Repeat the process once again.
3. Collect the supernatant by magnet into a new tube.
4. Add 9.6μl 5M NaCl and 2μl 10mg/ml RNase into all groups and water bath at 65℃ overnight to reverse crosslinking.
5. Add 2μl 20mg/ml protein K incubate at 60℃ for 1h.

-DNA purification

You can extract DNA by a purification kit according to the instructions or preform phenol-chloroform extraction and ethanol precipitation method.

-Detection

Perform a PCR or Real time PCR assay to detect the predict DNA which may react with the target protein.