ChIP with nAb

Background

Chromatin Immunoprecipitation (ChIP) is a technique employed when investigating intracellular DNA-protein interactions. This method is useful for examining DNA binding proteins that interact with specific DNA regions (transcription factors, promoters, etc.). ChIP reactions are broken down into three key steps:

1. Crosslinking between DNA and binding proteins.
2. Shearing of DNA into fragments.
3. Immunoprecipitation of DNA-protein complexes.

A number of DNA binding proteins are expressed at very low levels, placing a greater degree of importance on the immunoprecipitation step. This step is generally performed with immobilized antibodies against the specific protein or an affinity tag.

nAbs are highly specific antibodies that enables complete sample pulldown. They display a high degree of stability, permitting their use in a variety of cell lysates and buffer conditions. All nAbs are available pre-conjugated to agarose resin and are ready for direct use in ChIP reactions.

Sample Protocol

The following protocol is for a ChIP reaction using GFP-nAb for immunoprecipitation of a GFP-tagged chromatin-associated protein (e.g. a transcription factor or part of a DNA-binding complex).

Materials

- GFP-nAb Agarose Resin
- nAb Agarose Control
- Cell lysis buffer for ChIP (cold) [5 mM PIPES (pH 8.0), 85 mM KCl, 0.5% Nonidet P-40 (NP-40), Store at 4°C.]
- Cells (50-100 million per experiment)
- Dilution buffer [16.7 mM Tris-Cl (pH 8.0), 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, 5% Triton X-100, Store at 4°C]
- EDTA
- Elution buffer for ChIP [50 mM Tris-Cl (pH 8.0), 10 mM EDTA, 1% SDS, Formaldehyde (37%), Glycine (1.375 M), Growth medium, Store at RT]
- High-salt wash buffer for ChIP [50 mM HEPES (pH 7.9), 500 mM NaCl, 1 mM EDTA, 0.1% SDS, 5% Triton X-100, 0.1% deoxycholate, Store at 4°C]
- Micrococcal nuclease (MNase)
- Micrococcal nuclease digestion buffer (MNase digestion buffer) [10 mM Tris-Cl (pH 7.4), 15 mM, NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, Store at 4ºC]
- Nuclei lysis buffer for ChIP [50 mM Tris-Cl (pH 8.0), 10 mM EDTA, 1% SDS, Store at RT]
- PCR or real-time PCR primers
- Phenol:chloroform (pH 8.0)
- Phosphate-buffered saline (PBS) (1X)
- Protease inhibitors (Roche)
- Proteinase K (20 µg/uL)

Equipment

- Cell scraper
- Centrifuge (benchtop)
- Dishes or flasks (tissue culture)
- Gel electrophoresis apparatus
- Gel-loading tips (narrow, for aspiration; see Step 20)
- Ice
- Incubators preset to 55°C, 65°C
- Microcentrifuge
- Micropipettor with tips
- PCR apparatus
- Rotator
- Sonicator
- Spectrophotometer
- Spin column
- Tubes (conical, 15-mL)
- Tubes (microcentrifuge, 1.5-mL)

Protocol

Cross-linking and Chromatin Preparation

Begin with ~50-100 million cells per experimental condition. The time requirement for this portion of the method is ~2 h.

Prior to performing this protocol, prepare cells expressing a GFP-tagged chromatin-interacting protein of interest, and an identical batch of non-transfected cells as a control

1. Add 27 µL of 37% formaldehyde per milliliter of cell culture (in growth medium on tissue culture plates or in tissue culture flasks) while slowly shaking the cells for 10 min at room temperature.
The final concentration of formaldehyde is 1%.
2. Quench cross-linking by adding 100 µL of 1.375 M glycine per milliliter of culture.
3. Treat the cells as follows:

    For adherent cells

    i. Remove the growth medium and dispose of it appropriately.

    ii. Wash cells twice with 10 mL of cold 1X PBS, and then scrape the cells into 1X PBS in a 15-mL conical tube.

    iii. Pellet the cells at 1500 rpm for 10 min at 4°C.

    For nonadherent cells

    i. Transfer the cells to a 15-mL conical tube.

    ii. Pellet the cells at 1500 rpm for 10 min at 4°C.

    iii. Wash the cells twice with 10 mL of cold 1X PBS.

4. Resuspend the cell pellet in 10 mL of cell lysis buffer for ChIP (cold) and incubate for 10 min on ice.
5. Pellet the cell nuclei at 1000 rpm in a benchtop centrifuge for 10 min at 4°C. Aspirate the supernatant carefully.
6. (Optional) Resuspend the cell nuclei in 500 µL of MNase digestion buffer. Add MNase and incubate for 1 h on ice. Stop the digest by adding EDTA to a final concentration of 50 mM.

    The amount of MNase will need to be optimized according to final chromatin size in Step 8. A range of 50-300 U is a convenient starting point.

7. Resuspend the nuclear pellet in 1 mL of nuclei lysis buffer for ChIP supplemented with protease inhibitors and incubate for 10 min on ice.

8. Proceed with sonication. Ensure that the samples do not foam and are kept as cold as possible.
    Sonication conditions will need to be optimized. Typically, six 15-sec pulses followed by 45-sec rest periods at output 6.0 have been found to work. It is helpful to analyze each sample for proper sonication. For this analysis, reverse the cross-links and purify a small volume of chromatin as described below, and then analyze on an agarose gel. DNA should ideally be between 300 and 1000 bp in size.
9. Transfer chromatin to a 1.5-mL microcentrifuge tube and freeze at −80°C for storage.

Chromatin Preclearing and Immunoprecipitation

Note: to save time and avoid sample loss due to pipetting error, all steps involving nAb™ Binding Control Agarose and GFP-nAb™ Agarose Resin can also be performed using microcentrifuge spin columns as supplied with the GFP-nAb™ Spin Kit.

10. Thaw the chromatin on ice. Centrifuge samples at high speed in a microcentrifuge for 15 min at 4°C to pellet the precipitated SDS. Transfer the supernatant to a fresh microcentrifuge tube.
    At this step, it is helpful to aliquot chromatin for storage at −80°C.
11. Measure the DNA concentration (A_{260}) of the chromatin using a spectrophotometer. Use nuclei lysis buffer for ChIP devoid of SDS as a blank.
    Typically, the chromatin concentration should be >750 ng/µL. The A_{260}/A_{280} *ratio should be ~1.4-1.6.*
12. Aliquot 100 µg of chromatin per antibody to be used into microcentrifuge tubes.
    It is important to have an irrelevant antibody such as α-glutathione S*-transferase as a control.*
13. Dilute the chromatin to a final volume of 500 µL with dilution buffer supplemented with protease inhibitors.
14. To preclear the chromatin, add 50 µL of nAb™ Binding Control Agarose to the chromatin and rotate for 1 h at 4°C.
    Use large-orifice tips or cut off the end of regular micropipettor tips for transfer of beads.
15. Centrifuge the chromatin samples at 1000 x g for 2 min at 4°C. Transfer the supernatants to fresh microcentrifuge tubes.
16. Add 10 to 50 µl of GFP-nAb™ Agarose Resin to the chromatin samples and rotate for 1 hour at 4°C.
    10µl of GFP-nAb agarose slurry will bind approximately 5µg of GFP.

IP Washes and Elution
This portion of the method requires ~2 h, followed by an overnight incubation.

17. Centrifuge the samples at 1000 x g for 2 min at 4°C.
18. Collect 25 µL from the supernatant of the control sample as a 5% input. Keep this sample on ice for later use.
19. Carefully aspirate the supernatants of all samples.
    Be careful not to aspirate any beads. Narrow gel-loading tips are useful for careful aspiration.
20. Add 1 mL of high-salt wash buffer for ChIP to all samples and rotate for 10 min at room temperature.
21. Centrifuge the samples at 1000 x g for 2 min at room temperature.
22. Carefully aspirate the supernatants and add 1 mL of high-salt wash buffer for ChIP. Rotate for 10 min at room temperature.
23. Repeat Steps 21-22 twice for a total of four high-salt washes.
24. Aspirate supernatants and wash twice with TE as above.
25. Resuspend beads and input samples in 300 µL of elution buffer for ChIP supplemented with 1 µL of proteinase K (20 µg/µL), and incubate samples for 2 h at 55°C.
    Do not resuspend beads by pipetting. Instead, gently flick or vortex samples.
26. To reverse cross-links, incubate overnight at 65°C.

DNA Purification
The time required for DNA purification is ~1 h.

27. Centrifuge the samples at full speed for 5 min at room temperature.
28. Transfer supernatants to fresh microcentrifuge tubes.
At this point, DNA can be purified by spin columns or standard phenol:chloroform extraction. If phenol:chloroform extraction is performed, yeast tRNA or glycogen carrier should be added.
29. Resuspend DNA in 50 µL of H_2O or TE buffer and analyze by PCR or real-time PCR.

Adapted from *Transcriptional Regulation in Eukaryotes: Concepts, Strategies, and Techniques*, 2nd edition, by Michael F. Carey, Craig L. Peterson, and Stephen T. Smale. CSHL Press, Cold Spring Harbor, NY, USA, 2009