

Native ChIP

by Ethan Ford (version 1/26/12)

Prepare nuclei

1. Grow three 10 cm ES cell plates (see note #1 and #2).
2. Wash each plate with 10 ml of PBS.
3. Add 1 ml 1x Trypsin/PBS to each plate and incubate until cells detach.
Trypsinize a longer than you would for normal cell passaging. It is important to be able to disassociate the ES cell colonies into single cells.
4. Add 10 ml of DMEM+10% FBS to first plate and dissociate cells by pipetting, transfer cells to the second plate and then transfer cells to third plate. Pipet cells until they are in a single cell suspension. Finally, transfer all cells to 15 ml tube.
5. Spin down at 3,000 rpm for 5 min.
6. Remove all supernatant and resuspend in 2 ml N-ChIP Buffer I. Note: cells must be in single cell suspension.
7. Add 2 ml N-ChIP Buffer II. Mix and incubate on ice for 10 min.
8. Add 8 ml of N-ChIP Buffer III to new 15 ml tube and layer nuclei from step 7 on top of the Buffer III sucrose cushion.
9. Spin at 3,000 rpm for 30 min.
10. Aspirate supernatant from top being sure that none of the upper layer is carried over to next step.
11. Resuspend the nuclei pellet in 1 ml MNase digestion buffer.

Digest chromatin with micrococcal nuclease

12. Add 1 μ l micrococcal nuclease (2,000 gel units/ μ l - NEB) and incubate at 37°C for 7 min (see note #3).
13. Add 5 μ l 0.5 M EDTA, pH 8.0 and 5 μ l 0.5 M EGTA and place on ice.

Recover soluble chromatin

14. Transfer to a 1.5 ml tubes and spin in microfuge (full speed) for 10 min.
15. Transfer supernatant to a new 1.5 ml tube and label S1 (for first soluble fraction). It is very important not to take any of the pellet, so it is better to leave some of the supernatant behind. This tube contains the low molecular weight chromatin and ideally is a mix of mono-, di- and tri- nucleosomes.
16. Resuspend the pellet in 1 ml N-ChIP Dialysis Buffer

Dialysis protocol:

1. Fill beaker with 1 liter of ice cold Dialysis Buffer and keep on ice.
2. Cut an approximately 10 cm piece of dialysis tubing and hold under running water until membrane becomes soft and you can open it up by rubbing. Rise with deionized water.
3. Fasten a dialysis clip on one end of the tubing.

4. Transfer the resuspended pellet into the tubing.
5. Close the tubing with a second dialysis clip being careful to keep the entire sample between the two clips. A small amount of air closed in with the sample is o.k.
6. Place in tubing in beaker with the Dialysis Buffer and a magnetic stir bar.
7. Let stir in cold room overnight.
8. Cut any extra tubing off the one end and carefully open the dialysis clip on that side.
9. Transfer the dialyzed sample to a new 1.5 ml tube.
10. Spin in microfuge at full speed for 10 min.
11. Transfer supernatant to a new 1.5 ml tube and label S2 (for second soluble fraction). This contains higher molecular weight chromatin.
12. Resuspend the pellet in 1 ml Dialysis Buffer and transfer to a new 1.5 ml tube labeled P.

17. Analyze digested chromatin on 2.2% agarose gel (see instruction below).

Immunoprecipitation

18. Transfer 50 µl of the S1 and S2 chromatin to new 1.5 ml tubes, add 2.5 µl 10% SDS to each and store at 4°C for the input control.
19. Aliquot the remaining chromatin equally into 1.5 ml tubes – one for each antibody and IgG control (see note 4).
20. Add 1 ml ChIP Incubation Buffer
21. Add the appropriate antibodies to the tubes.
22. Incubate overnight at 4° C.
23. Equilibrate Protein G Dynabeads. Prepare one 1.5 ml tube for each immunoprecipitation with 1 ml ChIP Incubation Buffer and appropriate amount of Protein G-Dyna beads to each tube. Mix by inverting and spin at 3k rpm in microfuge briefly to get liquid off top of tube. Place in magnetic rack and remove all liquid. Note: Protein-G beads are a significant source of background so it is best to use as few as possible. The stated binding capacity is 200 ng antibody/µl.
24. Transfer the chromatin to the tubes containing the washed beads.
25. Incubate 2 hours at 4° C on tube rotator in cold room.
26. Wash 2 times in Washing Buffer A.
27. Wash 2 times in Washing Buffer B.
28. Wash 2 times in Washing Buffer C.
29. Resuspend beads in: 48 µl Proteinase K digestion buffer
2 µl 10 mg/ml Proteinase K
30. Add 2 µl 10 mg/ml Proteinase K to input sample.
31. Process Input and IP samples in parallel in the following steps.
32. Incubate 30 minutes at 50° C.
33. Place tubes in magnetic rack for 3 minutes.

34. Transfer supernatant to new 0.2 ml PCR tubes.

Purify DNA with AMPure Beads

35. Add 50 µl of well mixed Ampure XP beads and 75 µl 20 % PEG₈₀₀₀ 1.25 M NaCl (see note 4).
36. Mix by pipetting up and down several times.
37. Incubate at room temperature for 15 min.
38. Place on magnetic rack for 5 min.
39. Remove and discard the supernatant. When removing supernatant do so very slowly with pipetman being careful not to take any beads.
40. Keep sample in magnetic rack and add 200 µl of freshly prepared 80% ethanol.
41. Incubate for 30 seconds. Remove and discard ALL the supernatant.
42. Repeat steps 40 and 41 one more time.
43. Let the beads dry at room temperature for 2 minutes.
44. Add 47 µl TE/10 and pipet up and down several times until pellet beads are completely resuspended.
45. Incubate at room temperature for 2 minutes.
46. Place in magnetic rack for 5 minutes.
47. Transfer 45 µl of the supernatant to a new 1.5 ml tube.

Post-ChIP analysis

48. Use 4 µl of eluted DNA for with QuantIT HS DNA Assay Kit (Invotrogen)
49. Run 5 µl of input DNA on a 2.2% agarose gel.
50. Use 2.5 µl DNA for qPCR.
51. Use 10 ng for ChIP-seq library preparation.

Analysis of chromatin fragmentation/Preparation of Input Chromatin

1. Transfer 50 µl of chromatin fractions S1, S2 and P to three 0.2 ml PCR tubes.
2. Add 2.5 µl SDS and 1 µl Proteinase K 10 mg/ml.
3. Incubate at 50°C for 30 minutes.
4. Add 50 µl of well mixed Ampure XP beads and 75 µl 20 % PEG₈₀₀₀ 1.25 M NaCl (see note 4).
5. Mix by pipetting up and down several times.
6. Incubate at room temperature for 15 min.
7. Place on magnetic rack for 5 min.
8. Remove and discard the supernatant. When removing supernatant do so very slowly with pipetman being careful not to take any beads.
9. Keep sample in magnetic rack and add 200 µl of freshly prepared 80% ethanol.
10. Incubate for 30 seconds. Remove and discard ALL the supernatant.
11. Repeat steps 9 and 10 one more time.
12. Let the beads dry at room temperature for 2 minutes.

13. Add 47 μ l TE/10 and pipet up and down several times until pellet beads are completely resuspended.
14. Incubate at room temperature for 2 minutes.
15. Place in magnetic rack for 5 minutes.
16. Transfer 45 μ l of the supernatant to a new 1.5 ml tube.
17. Run 3 μ l on a 2.2% agarose gel.
18. Keep remaining sample for input control.

Notes:

- 1) The protocol can easily be scaled up or down and works with both tissues and tissue culture cells.
- 2) The entire protocol except for the trypsinization and digestion with micrococcal nuclease must be performed with ice-cold solutions and every effort should be made to keep the samples ice cold.
- 3) The amount of micrococcal nuclease and the digestion time must be determined empirically for each cell type. Once calibrated for a cell type it is important to keep the cell number the same from experiment to experiment as the amount of material affects the rate of digestion.
- 4) There are couple options at this point.
 - a) Combine the S1 and S2 chromatin fractions.
 - b) Perform the immunoprecipitations separately with each fraction.
 - c) Only use the S1 or S2 fraction. I usually just use the S1 fraction so as to avoid the overnight dialysis step. However, PCR efficiency can be greatly reduced with mono-nucleosomal DNA especially if you are looking at a region with a positioned nucleosome and your primer set must be designed accordingly.
 - d) Another good option is to use the S1 fraction for ChIP-seq and the S2 fraction for ChIP-PCR. The mononucleosomal DNA in the S1 fraction is a good size for constructing libraries with and the larger DNA in the S2 fraction is good for ChIP-PCR.
- 5) AMPure XP guidelines:
 - a) Make sure that you achieve a homogenous solution of the beads and PEG by pipetting up and down a sufficient number of times.
 - b) When removing the supernatant after the binding step, I remove all but 5 μ l with a P200 pipetman and then go back and carefully remove the last 5 μ l with a P20 pipetman unless I have a lot of samples to process, then I just remove it all in one step with the P200.
 - c) When washing with ethanol the beads stick to the wall of the tube better so you can remove the supernatant more quickly and less carefully. However, I make sure I remove all traces of ethanol by going through each tube a second time.
 - d) At the elution step, when transferring the eluted DNA to a new tube it is important not to accidentally carryover any beads. I carefully look inside the tube as I am pipetting up the eluted DNA to make sure I don't accidentally take up and beads.

Then I look at the eluted DNA in the pipet tip see if I can see any beads. If there are, simply pipet the beads back into the tube you took them from and wait a few minutes for the sample to separate again in the magnetic rack. At this step it is important to leave at least 1.5 µl behind, as it is not possible to remove all the liquid and not take any beads.

6) When studying histone modifications such as acetylation and phosphorylation it is important to add deacetylase and phosphatase inhibitors respectively to all the solutions. 5 mM sodium butyrate is recommended as a deacetylase inhibitor.

7) DTT and protease inhibitors should be added to solutions immediately before use and any unused buffer should be discarded.

Protocol adapted with minor modifications from:

I strongly recommend you download the protocol from the below link.

Umlauf D, Goto Y and Feil R (2003) Site-specific analysis of histone methylation, *Methods Mol. Biol*, **287**: 99-120. <http://www.epigenesys.eu/>

Buffers

N-ChIP Buffer I

0.3M Sucrose
60mM KCl
15mM NaCl
5mM MgCl₂
0.1mM EGTA
15mM Tris-HCl, pH 7.5
+0.5mM DTT and protease inhibitors (see note 7)

N-ChIP Buffer II

0.3M sucrose
60 mM KCl
15mM NaCl
5mM MgCl₂
0.1mM EGTA
15mM Tris-HCl, pH 7.5
0.4% NP-40
+0.5mM DTT and protease inhibitors (see note 7)

N-ChIP Buffer III

1.2M sucrose
60 mM KCl
15mM NaCl
5mM MgCl₂
0.1mM EGTA
15 mM Tris-HCl, pH 7.5
+0.5mM DTT and protease inhibitors (see note 7)

MNase Digestion Buffer

0.32M sucrose

50mM Tris-HCl, pH 7.5

4mM MgCl₂

1mM CaCl₂

+protease inhibitors (see note 7)

Dialysis Buffer

1mM Tris-HCl, pH 7.5

0.2mM EDTA

ChIP Incubation Buffer

50mM NaCl

50mM Tris-HCl, pH 7.5

5mM EDTA

+protease inhibitors (see note 7)

Wash Buffer A

50mM Tris-HCl, pH 7.5

10mM EDTA

75mM NaCl

Wash Buffer B

50mM Tris-HCl, pH 7.5

10mM EDTA

125mM NaCl

Wash Buffer C

50mM Tris-HCl, pH 7.5

10mM EDTA

175mM NaCl