ChIP-seq Protocol for RNA-Binding Proteins

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ChIP-seq: Cells were grown according to the approved ENCODE cell culture protocols. Cells were fixed in 1% formaldehyde and resuspended in lysis buffer. Chromatin was sheared to 100-500 bp using a Branson Sonifier cell disrupter. Antibody and magnetic beads were coupled, then combined with solubilized chromatin. Target-chromatin complexes were pulled-down using magnet, washed and then eluted. After reverse cross-linking, RNase treatment, proteinase K treatment, immunoprecipitated DNA was extracted with phenol-chloroform, ethanol precipitated. One to ten nanograms of DNA were end-repaired, adapter-ligated and sequenced by Illumina Hiseq 2000/2500.

ChIP procedure:

1. **Preparing magnetic beads**
   i. Aliquot 25 µl of Pierce protein A/G magnetic beads or 100 µl of Dynal beads to each 1.5 ml microfuge tube. Set up 1 tube of beads per immunoprecipitation.
   ii. Wash beads twice with ChIP Dilution Buffer. Resuspend beads with 1 ml Blocking Buffer and block beads for at least 2 hours or overnight at 4 °C on a rotator.
   iii. Wash beads once with 1 ml of ChIP Dilution Buffer, once with 1 ml of 5 mg/ml BSA in PBS, resuspend beads in 250 µl of 5 mg/ml BSA in PBS and add 6-10 µg antibody. Incubate for at least 1 hour at room temperature or overnight at 4 °C on a rotator.
   iv. Wash beads twice with 0.5 ml of 5 mg/ml BSA in PBS.
   v. Resuspend each aliquot of beads in 50 µl of 5 mg/ml BSA in PBS.

ChIP Dilution Buffer

20 mM Tris-Cl, pH8.0
2 mM EDTA
150 mM NaCl
1% Triton X-100

Blocking Buffer 1 ml

ChIP Dilution Buffer	1 ml
Glycogen (20 mg/ml)	10 µl
BSA (20 mg/ml)	10 µl
tRNA (20 mg/ml)	10 µl

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2. **Formaldehyde crosslinking cells**

For adherent cells:

i. Use 1-2x10^7 cells for each immunoprecipitation.

ii. Quickly rinse the cells with PBS once and add fresh 1% Formaldehyde Solution in PBS. Swirl briefly.

iii. Incubate cells with Formaldehyde Solution for 20 min at room temperature with slow shaking.

iv. Add 1/10 volume of 1.375 M glycine and incubate for 15 min at room temperature with slow shaking to quench formaldehyde.

v. Rinse cells 3 times with cold PBS, scrape, transfer cells into 50 ml conical tubes and spin at 500*g* for 5 min at 4 °C. Discard supernatant and resuspend pellet in 2x10^7 cells per 1 ml PBS with gentle inversion.

vi. Aliquot 1-2x10^7 cells to individual 1.5 ml microfuge tube and spin at 500*g* for 5 min at 4 °C. Discard supernatants. (We recommend to use freshly crosslinked cell for further experiments.)

For suspension cells:

i. Use 1-2x10^7 cells for each immunoprecipitation.

ii. Harvest cells in 50 ml conical tubes, quickly wash cells with PBS once by spinning at 500*g* for 5 min at 4 °C and resupension. Discard supernatants.

iii. Resuspend cells with fresh 1% Formaldehyde Solution in PBS. Swirl briefly.

iv. Incubate cells with Formaldehyde Solution for 20 min at room temperature with slow shaking.

v. Add 1/10 volume of 1.375 M glycine and incubate for 15 min at room temperature with slow shaking to quench formaldehyde.

vi. Wash cells 3 times with cold PBS, and resuspend pellet in 2x10^7 cells per 1 ml PBS with gentle inversion.

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vii. Aliquot 1-2x10^7 cells to individual 1.5 ml microfuge tube and spin at 500*g* for 5 min at 4 °C. Discard supernatants. (We recommend to use freshly crosslinked cell for further experiments.)

3. **Cell sonication and immunoprecipition**
   i. Resuspend each pellet of 1-2x10^7 cells in 1.5 ml Cell Lysis Buffer and incubate on ice for 20 min with inversion every 4 min.
   ii. Spin at 3500 rpm for 5 min at 4 °C. Discard supernatant.
   iii. Wash pellet once with 1 ml of Cell Lysis Buffer. Discard supernatant.
   iv. Resuspend pellet with 500 µl of Nuclear Lysis buffer.
   v. Immerse tube in an ice-water bath to keep sample cool during sonication.
   vi. Position tube so the sonicator probe sits approximately 0.5 cm above the bottom of the tube. Make sure the probe is centered and does not contact the sides of the tube.
   vii. Sonicate suspension. [Branson Sonifier cell disruptor 185; output: 4; sonicate for 10 s for 7 times and wait 1 min in between to cool down]
   viii. Freeze sample at -20 or -80 °C, thaw on ice and spin at 13,200 rpm for 15 min at 4 °C to pellet SDS and debris.
   ix. Transfer the supernatant to a fresh microfuge tube.
   x. Adjust the cleaned nuclear extract to 1 ml with TE Buffer.
   xi. Add 300 µl of IP MIX to bring the volume of chromatin solution to 1.3 ml.
   xii. Save 100 µl of chromatin solution from each sample as input DNA. Store at –20 °C.

Cell Lysis Buffer

10 mM Tris-Cl, pH8.0
10 mM NaCl
0.5% NP-40
1X proteinase inhibitor cocktail

Nuclear Lysis buffer

50 mM Tris-Cl, pH8.0
10 mM EDTA, pH8.0
1% SDS
1X proteinase inhibitor cocktail

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IP MIX 300 µl
1% Triton X-100
0.1% DOC
1X proteinase inhibitor cocktail
TE Buffer up to 300 µl

4. **Immunoprecipition**
   i. Combine 1.2 ml chromatin solution with the beads and rotate overnight at 4 °C.

5. **Wash**
   i. Use DynaMag™-2 Magnet to precipitate the beads. Keep tubes on ice. Wash 3 times with TSE I Buffer, 3 times with TSE II Buffer and once with Wash buffer III. Remove buffer by aspiration.
   ii. Wash once with 1 ml of TE Buffer.
   iii. After removing TE Buffer, spin the tubes for 10 s at 1000 rpm and remove remaining liquid with a pipet.

TSE I Buffer

20 mM Tris-HCl, pH8.0
2 mM EDTA, pH8.0
150 mM NaCl
1% Triton X-100
0.1% SDS
1X proteinase inhibitor cocktail

TSE II Buffer

20 mM Tris-HCl, pH8.0
2 mM EDTA, pH8.0
500 mM NaCl
1% Triton X-100
0.1% SDS
1X proteinase inhibitor cocktail

Wash buffer III

10 mM Tris-HCl, pH8.0
1 mM EDTA, pH8.0
250 mM LiCl
1% NP-40
1% deoxycholate
1X proteinase inhibitor cocktail

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6. **Elution and decrosslinking**
   i. Resuspend beads with 170 µl of Elution Buffer and shake on thermomixer at 1200 rpm for 20 min.
   ii. Spin for 10 s at 1000 rpm and precipitate the beads by magnet and transfer liquid to a new tube.
   iii. Reverse crosslink immunoprecipitated sample and 50 µl input chromation in 120 µl Elution Buffer overnight at 65 °C on thermomixer.

Elution Buffer

10 mM Tris-Cl, pH8.0
1 mM EDTA
1% SDS

7. **Purification of DNA**
   i. Add 170 µl of TE Buffer to each tube to dilute SDS in Elution Buffer.
   ii. Add 7 µl of 10 mg/ml RNaseA (~0.2 mg/ml final concentration), mix by inverting the tube several times and incubate at 37 °C for 2 h.
   iii. Add 7 µl of 20 mg/ml Proteinase K and 1.5 µl of 20 mg/ml Glycogen, mix well and incubate at 37 °C for 2 h.
   iv. Extract twice with 350 µl phenol and once with 350 µl phenol/chloroform/isoamyl alcohol, vortex 20 s and separate phases with 2 ml Phaselock tube.
   v. Transfer aqueous layer to a new tube, add 1/10 volume of 3M NaAc and 750 ml EtOH. Incubate overnight at -20 °C.
   vi. Spin at 13,200 rpm for 15 min at 4 °C to pellet DNA. Wash pellet with 1 ml 75% EtOH and spin again at 13,200 rpm for 5 min at 4 °C.
   vii. Remove any remaining 75% EtOH, air dry pellet and resuspend in 25 µl of 10 mM Tris-HCl, pH 8.0.

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Library construction

We followed illumina ChIP-seq library preparation manual (Preparing Samples for ChIP Sequencing of DNA) to construct libraries for sequencing. And it can be download from the illumina website, https://support.illumina.com/content/dam/illumina-support/documents/myillumina/53e21a89-5a39-403c-a95f-87263b44e531/chip-seq_sample_prep_11257047_reva.pdf).