ChIP Protocol

Wende/Fong/Duncan
Kelly Lab
Revised May 29, 2007

DAY ONE (for cells):

Crosslink and cell harvest (C_2C_{12} myotubes):

1. 2X10 cm plates per condition.
2. Wash plates with PBS (2x10ml).
3. Refresh media 10 ml per plate.
4. Crosslink by adding 108λ 37% formaldehyde to each plate.
5. Incubate 10' @ RT on platform shaker.
6. Stop reaction by adding 505λ 2.5 M glycine.
7. Incubate 5' @ RT on platform shaker.
8. Rinse w/ 5 ml ice cold PBS (2X).
9. Trypsin/EDTA 1 ml/plate 5' @ 37°C.
10. Add 1 ml PBS; 20% FCS.
    a. 4ml PBS + 1ml FCS
11. Scrape & transfer to 15 ml conical.
12. Rinse 2 x 1ml/plate ice cold PBS.
13. Pellet 8' 1000rpm (IEC) 4°C. 240xg
14. Wash pellet in 1 ml PBS containing 50 μg/ml PMSF.
    a. 4ml + 12λ 100mM PMSF
15. Pellet 8' 1000rpm (IEC) 4°C. 600 x G

Cell lysis:

1. Chill 1 ml Cell Lysis Buffer (CLB) on ice for 5'.
2. Aspirate PBS.
3. Gently resuspend pellet in CLB w/ 1.5x packed cell volume
4. Incubate on ice 10'.
5. Pellet 5' 1500 rpm (IEC) 4°C. 600xg
6. Aspirate CLB.

Nuclei lysis:

1. Chill 4 ml Nuclei Lysis Buffer (NLB) on ice for 3minutes; longer may precipitate SDS.
2. Vigorously resuspend pellet in 1 ml NLB.
3. Incubate 2 hr @ 4°C.

Sonication:

1. Chill 13 ml IP Dilution Buffer (IDP) on ice for 5'.
2. Transfer lysed nuclei to 15 ml Falcon 2096 conical tube.
3. Add 800λ IDP.
4. Sonicate on ice.
   a. 8 repeats
   b. 30 bursts each
   c. Output 10%
   d. Duty 30%
   e. 1 minute rest between repeats
5. Transfer to microcentrifuge tubes (2ml).
6. Clear insoluble material 16,000 rpm (IEC) 10min @ 4°C microcentrifuge 16Kxg
7. Transfer supernatant to 15ml conical.
8. Dilute to required volume keeping NLB:IDP @ 1:4.
   a. Vol. = 900λ * Condition + 300λ Input
   b. Add ~3.0ml IDP

DAY ONE (for minced tissue):

Solutions and Buffers to prepare the day of the experiment:

bracketed volumes are for 2 groups of 3 hearts
double-check volumes needed per experiment!

1. Prepare the following at RT:
   Butyric Acid (0.5M) [1 mL]
   PMSF (0.1M) [1 mL]
   25X Complete (25X) [4 mL, two tablets]
2. Make the appropriate additions of above solutions to:
   Cell Lysis Buffer [10mL]
   Nuclear Lysis Buffer [5mL]
   IP Dilution Buffer [10mL]
   Keep at RT until protocol directs to put on ice.
3. Prepare PBS + Protease Inhibitors:
   50 mL 1X PBS
   150 uL 0.1M PMSF
   2.0 mL 25X Complete

Tissue Harvest and Crosslinking:

1. Harvest hearts, trimming off atria. Use one adult heart per Ab condition.
2. In a 10cm cell culture dish, mince tissue on ice.
3. Add 10mL of 1X DMEM (4.5g/L glucose) to the dish; keep on ice while other hearts are harvested and minced.
4. Crosslink by adding 270uL of 37% formaldehyde [in the hood!] to each plate.
5. Incubate for 10 minutes at RT on orbital shaker, speed ~50.
6. Stop the reaction by adding 505uL of 2.5M glycine.
7. Incubate for 5 minutes at RT on orbital shaker, speed ~50.
8. Wash tissue 2X with 10mL 1X PBS + protease inhibitors.
   -pipet off as much PBS as possible
9. Scrape and transfer tissue to a 15mL conical using a metal spatula.
10. Rinse dish 1X with 1mL 1X PBS + protease inhibitors and transfer to conical, try to rinse all tissue down to the bottom of the conical.
11. Pellet tissue: spin in IEC 1000rpm for 8 minutes at 4°.
12. Wash each pellet in 1mL 1X PBS + protease inhibitors, try to rinse all tissue down to the bottom of the conical.
13. Pellet tissue: spin in IEC 1000rpm for 8 minutes at 4°.

Cell Lysis:

1. Chill Cell Lysis Buffer (CLB) on ice for 5 minutes.
2. Aspirate off PBS.
3. Gently re-suspend tissue pellet in 1.5X packed volume [~1.5 mL] of tissue by pipetting, using a 1000uL tip with the end cut off.
4. Incubate on ice for 10 minutes.
5. Pellet: spin in IEC 1500rpm for 5 minutes at 4°.
6. Aspirate off CLB.

Nuclei Lysis:

1. Chill Nuclei Lysis Buffer (NLB) on ice for 3 minutes (do not go longer or SDS may ppt).
2. Re-suspend each pellet in 0.75 mL NLB by pipetting, using a 1000uL tip with the end cut off.
3. Incubate at 4° for 2 hours (in cold room with gentle rocking). You will still have visible tissue at the end of this incubation.

Sonication:

1. Chill IP Dilution Buffer (IDB) on ice for 5 minutes.
2. Add 600uL of IDB to each lysed nuclei sample.
3. Sonicate on ice:
   40% Duty, Output 5, 10 repeats of 15 seconds.
   Avoid foaming!
4. Transfer samples to 2X 1.5 mL microfuge tubes.
5. Clear insoluble material: spin 15,000 rpm for 10 minutes at 4°.
6. Transfer supernatant to 15 mL conical (recombine samples here)
8. Dilute to required volume, keeping NLB:IDB at 1:4.
   To 0.75 mL NLB + 0.6 mL IDB
   Add 2.4 mL IDB
9. Transfer a 50 uL aliquot of each sample to a 1.5 mL tube (for reverse cross-link).
10. Proceed to Pre-clearing and Immunoprecipitation or snap freeze both aliquots in N_2(l) and store at -80°.

THE FOLLOWING IS THE SAME FOR CELLS OR TISSUE:

Pre-clear:

1. Add 50µl preimmune rabbit serum.
2. Mix @ 4°C for 10min.
3. Add 200µl protein A-Sepharose, 50% slurry.
4. Mix 10min @ 4°C.

Immunoprecipitation:

1. Pellet mixture.
   a. 2000 rpm (IEC) microfuge 2min @ 4°C
2. Aliquot supernatant
   a. 180λ for input (set @ 4°C until reverse cross-link)
   b. 900λ for each condition
3. Add antibody (varies considerably).
4. Mix o/n @ 4°C. **END OF DAY 1**
5. Clear insoluble material 16,000 rpm (IEC) microcentrifuge 10min @ 4°C 16Kxg
6. Pipette 60µl Protein-A sepharose/ reaction.
7. Transfer samples to sepharose mix >2 hrs @ 4°C

Washing and Elution

1. Pellet 4000 rpm (IEC) microcentrifuge 2min @ RT-remove to ice **IMMEDIATELY!**
    a. All washes done with 400λ ice-cold buffers.
    b. Wash 4x IP wash buffer #1 (20ml)
    c. Wash 1x IP wash buffer #2 (5ml)
    d. Wash 3x TE buffer (15ml)
2. Pellet 4000xg microcentrifuge 2' @ RT remove to ice **IMMEDIATELY**!
3. Let sit 1' and remove supernatant.

Elution

4. Add 150µl RT elution buffer. (5ml)
5. Vortex #3 for 15'.
6. Pellet 4500xg 2' @ RT.
7. Transfer supernatant to new tube.
8. Repeat above and combine elutions
    a. Transfer 140λ after each.

Reverse crosslinking:

1. Add to IP sample:
    a. 1λ 20 mg/ml RNase A and 18λ 5M NaCl
2. Add to input sample:
    a. 1λ 20 mg/ml RNase A and 11.5λ 5M NaCl
3. Incubate @ 67°C ≥ o/n. **END OF DAY 2**
4. Add 3λ 20 mg/ml Proteinase K; gently mix.
5. Incubate @ 45°C for 3 hr.

Nucleic acid purification:

1. Add 120λ TE to input sample; 20λ to IP.
2. Add 1λ 10mg/ml tRNA.
3. Extract w/ 300λ 1:1 Phenol/Chloroform
    a. Vortex 10-15"
    b. Spin full speed 5' RT.
4. Repeat Phenol/Chloroform extraction.
5. Extract w/ 300λ Chloroform
6. Final transfer 300λ.
7. Add 30λ 3M NaOAc (pH 5.2), 0.5λ 10 mg/ml tRNA, 0.5λ 10 mg/ml glycogen.
8. Add 770λ 100% EtOH (~2.5X), invert to mix.
9. Incubate 15' @ -80°C then -20°C o/n. **END OF DAY 3**
10. Spin full speed 20' @ 4°C.

11. Wash pellet 800λ 70% cold EtOH.
12. Spin full speed 20’ @ 4°C.
13. Air dry pellet 5-10’ @ RT.
14. Dissolve IP in 30λ & input in 66.7λ TE, pH 8.0.
15. Incubate 5’ 37°C, vortex, pulse spin.
16. Store samples @ -20°C. **END OF DAY 4**

NOTE: Please refer to above protocols (variations exist for most of these steps, specific to cell-type, etc.) and may be altered (incubation times, stopping points, etc.).