A. Embryo Collection and Fixation for Chromatin IPs

1. Wash down the embryos and transfer into largest sieves (112µm/355µm/710µm – bottom to top)
2. Dechorionate embryos for 2.5 min in 400 ml 3% NaOCl (50% bleach) at RT in a beaker with stirring.
3. Transfer the embryos into pre-weighed sieve (small one, pre-weighed) and wash them well with tap water. Remove as much liquid as possible by blotting the sieve with a paper towel.
4. Calculate the weight of the embryos and transfer to ca. 50 ml PBT in a stirred 150ml cylinder with a funnel (place sieve up side down over the funnel and wash with PBT)
5. Stir the embryos and aliquot the equivalent volume of 1.5 g of embryos into 50 ml falcon tubes.
6. Add PBS/Triton to 50 ml and wash the embryos. Let the embryos settle (without centrifugation). Discard ca 40ml of PBT.
7. Place a sufficient number of Nitex membranes (approximately 5x5 cm each) onto a pile of tissue paper and pipette respective volume of embryo suspension onto Nytex membrane (corresponding to ca. 1.5 g of embryos). Fold the membrane over to cover the embryos and blot dry gently with a paper towel.
8. Transfer each membrane into a separate 50 ml Falcon tube with 10 ml cross-linking solution + 30 ml heptane (9.5ml Cross-linking solution + 485 µl 37% Formaldehyde + 30ml Heptane),
9. Shake off the embryos, recover the membrane and shake the tube vigorously at room temperature (20-25 °C) for 15 min. CAUTION: n-heptane is highly flammable and toxic to aquatic organisms. Formaldehyde is a potent mutagen and an expected human carcinogen
   CRITICAL: This step cross-links proteins to chromatin as well as to other proteins. The time required for this step needs to be kept constant between repeated collections. Importantly, as some proteins are more easily crosslinked to chromatin than others, the formaldehyde concentration / length of cross-linking reaction might require optimization for different proteins of interest.
10. Pellet the embryos in each tube by centrifugation at 500xg for 1 min
11. Replace the supernatant with 30 ml PBS/Glycin/Triton and shake vigorously at room temperature for at least 1 min to stop the cross-linking reaction. *CAUTION: The supernatant contains n-heptane and formaldehyde.*
12. Pellet the embryos by centrifugation at 500xg for 1 min
13. Carefully decant the supernatant and wash the pellet with 50 ml ice-cold PBT.
14. Pellet the embryos by centrifugation at 500xg for 1 min, decant the supernatant and resuspend the embryos in approximately 10 ml PBT per tube.
15. Transfer the embryos onto separate Nitex membranes as in step 7, fold the membrane over to cover the embryos and blot them dry with a paper towel.
16. Transfer a small number of embryos (100-200) from any of the membranes into a microfuge tube containing 0.5 ml heptane and 0.5 ml methanol. Shake vigorously to devitellinise the embryos, let them settle and then remove as much liquid as possible. Wash the embryos with methanol twice and store at –20 °C. This sample from the collection is set aside to evaluate if the collected embryos are at the correct developmental stage
    CAUTION: Methanol is toxic and highly flammable.
17. Transfer the remaining aliquots of dry cross-linked embryos from the Nitex membranes (step 15) into separate 12ml cryotubes and snap-freeze in liquid nitrogen.
18. Store embryos at -80C

PBT	PBS with 0.1% Triton
cross-linking solution	50 mM Hepes, 1 mM EDTA, 0.5 mM EGTA, 100 mM NaCl, pH 8 (make 500 ml)
PBS/glycine/triton	125 mM glycine + 0.1% Triton in PBS

B. Chromatin prep

1. Thaw embryos quickly and resuspend them in 15ml ice-cold PBT + 1x protease inhibitors.
2. Dounce 20x with loose pestle on ice.
3. Centrifuge at 400g for 1min and transfer the supernatant to a fresh tube.
4. Centrifuge at 1100g for 10min at 4°C and discard the supernatant.
5. Resuspend pellet in 15ml ice-cold Cell lysis buffer + 1x protease inhibitors.
6. Dounce 20x with tight pestle.
7. Transfer into two 15ml polystyrene falcon tubes.
8. Centrifuge at 2000g for 4min at 4°C to pellet the nuclei. Discard the supernatant. (Nuclei can be frozen in liquid nitrogen and stored at -80°C at this point).
9. Resuspend the nuclei in 1ml of ice-cold Nuclear Lysis Buffer + 1x protease inhibitors and incubate for 20min at RT.
10. Add 1ml ice-cold Nuclear Lysis Buffer + 1x protease inhibitors to each tube.
11. Sonicate using the bioruptor pico sonicator water bath (12 cycles 30s on/30s off, high-energy settings for app. 250bp chromatin) with sonication beads.
12. Transfer the chromatin to two 1.5ml Eppendorf tubes and centrifuge at 14000rpm for 10min at 4°C.
13. Pool the supernatants to ensure a homogenous sample and transfer sheared fixed chromatin aliquots (e.g. 200µl) to 1.5ml low-binding tubes. Put aside a 50µl aliquot for quality control. Flash freeze in liquid nitrogen and store at -80°C.

Solutions

PBT	PBS with 0.1% Triton + protease inhibitors
Cell lysis buffer	5mM HEPES, pH 8.0, 85mM KCl, 0.5% NP40 + protease inhibitors Autoclave without NP40 and then add appropriate amount of NP40 from 10% stock.
Nuclear Lysis Buffer	50mM HEPES, pH 8.0, 10mM EDTA, 0.5% N-Laurylsarcosine (add after filtration) + protease inhibitors
Protease inhibitors	For a 50x stock, dissolve one tablet of cOmplete (Roche) in 1 ml of nuclease-free water. Stock can be stored at -20°C for up to 1 month. Only thaw it one time and trash it afterwards.

Chromatin quality control

1. Add 50 µl of 100 µg/ml of RNase A in TE and incubate ay 37ºC for 30min.
2. Add 5 µl SDS to a final concentration of 0.5% from a 10% stock and incubate with 2,5 µl of proteinase K (0.5mg/ml final) at 37ºC overnight.
3. Transfer the sample to 65ºC for 6 hours to reverse the cross-links.
4. Adjust the sample to 200µl with TE buffer and extract the DNA with phenol-chloroform.
5. Determine the concentration of purified DNA with NanoDrop and run 0.5-1µg on a 1.5% TAE agarose gel to determine the size distribution of the sheared DNA fragments.

C. Chromatin IP

Day 1: Beads preabsorption and antibody addition

1. Thaw an aliquot of chromatin and transfer 5-50µg to a fresh low-binding tube. *(5ug in our case)*
2. Adjust the volume to 500µl with ice-cold TE buffer containing 2x protease inhibitors.
3. Add 400µl of ice-cold IP dilution buffer.
4. Take out 10 µL as 1% input and store this sample at 4°C
5. Add the appropriate antibody (or prebleed for a mock reaction) to each tube. Incubate overnight at 4°C with gentle mixing on a rotating wheel.

Day 2: Purification of immunocomplexes

For each precipitation (including the mock reactions and always one or two extra reactions) wash 25 µL ProteinA+ProteinG magnetic bead mix with 1ml RIPA buffer on a rotating wheel at 4ºC for 5 min. Place the tubes on a magnetic rack and resuspend in 100ul of fresh RIPA buffer.

1. Add 100µl of bead suspension to each chromatin sample and incubate at 4ºC for 3 hours on rotating wheel.
2. Place the tubes on a magnetic rack. Rinse once with ice-cold RIPA buffer and collect the beads again.
3. Wash complexes with 1ml of the following buffers (after each wash collect the beads on a magnetic rack):
   - 1x RIPA buffer, 10 minutes
   - 4x RIPA-500 buffer, 10 minutes
   - 1x LiCl buffer, 10 minutes
   - 2x TE buffer, 10 minutes

   Critical: the number and length of washes determines the yield and purity of the final sample and may be subject to an optimization for different antibodies.
4. Resuspend the beads in 50 µl TE buffer. From here on, also include the 1% quality control sample (see step 4 in Day1). Adjust its volume to 50 µl with TE.
5. Add 50µg/ml RNase A to all samples (from 100 µg /ml stock), and incubate for 30 minutes at 37°C.
6. Adjust the samples to 0.5% SDS (5µl 10% SDS), 0.5 mg/ml proteinase K (2.5µl of 20mg/ml) and incubate at 37°C overnight

Day 3: Reverse crosslinking and DNA purification

1. Incubate the samples at 65°C for at least 6 hrs to reverse the formaldehyde crosslinks (and to elute the antibody/antigen complexes)
2. Collect the beads on a magnetic rack, transfer the supernatant to a new tube.
3. Phenol chloroform extract DNA using 2ml Phase Lock tubes:
   1. Spin 2ml Eppendorf Phase Lock Tubes for 3 sec at 14,000rpm
   2. Add 100 µl TE to each reaction to bring the volume to 200µL
   3. Add 300 µl Phenol:Chloroform:Isoamylalcohol (25:24:1) to each sample
   4. Transfer to Phase lock tubes, mix by pipetting (do not vortex) and spin for 5 min at 14000rpm
   5. Add 300µl chloroform, mix by pipetting (without touching the gel layer) and spin for 5 minutes at 14000rpm
   6. Transfer the upper phase to fresh tube (ca. 200-250 µl)
4. Precipitate DNA:
   1. Add 10µl glycogen (5mg/ml) as carrier
   2. Add 20µl 3M NaOAc pH5.3
   3. Add 550µl 100% EtOH

4. Precipitate ≥1h at –80°C (better overnight)
5. Spin for 30 min at 14,000 rpm, 4°C
6. Wash with 500µl 70% EtOH
7. Spin for 10 min at 14,000rpm, 4°C
8. Air-dry the pellet for about 5 min
9. Resuspend in 30 µl TE
10. Determine concentration using QuBit (if the sample will be used for library preparation)
11. Store at –20°C.
12. Use 2 µl of eluate to check for enrichment of specific fragments by real-time PCR.

Solutions

IP dilution buffer	0.35M NaCl, 2.5% Triton X-100, 0.25% SDS, 0.25% Na deoxycholate
RIPA	140mM NaCl , 10mM Tris-HCl pH8.0, 1mM EDTA, 1% Triton X-100, 0.1% SDS, 0.1% Na deoxycholate
RIPA-500 buffer	as RIPA, but 500mM NaCl instead of 140mM
LiCl buffer	250mM LiCl, 10mM Tris-HCl pH8.0, 1mM EDTA, 0.5% NP-40, 0.5% Na deoxycholate
TE buffer	10mM Tris-HCl pH 8.0, 1mM EDTA
Protease inhibitors	For a 50x stock, dissolve one tablet of cOmplete (Roche) in 1 ml of nuclease-free water. Stock can be stored at -20°C for up to 1 month. Only thaw it one time and trash it afterwards.

D. Library Preperation for Illumina sequencers

Use the following Kit and follow the manufacturer's manual:

NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® E7645S

You can try to measure the concentration of your ChIP before library prep with the Qubit DNA HS Kit. If the concentration is too low to measure, you should use everything for the library prep. If you can measure the concentration, start for 4 ug of DNA.

Run your finished libraries on the Bioanalyzer using a DNA HS Chip.
You should see one peak around 350-500 bp.
Make sure you don't have primer dimer contamination. If you do, make another 1.4x SPRI bead cleanup.

Multiplex libraries in an equimolar ratio and submit libraries for sequencing.