Formaldehyde Cross-linking of Chromatin from Drosophila

Protocol from modEncode IGSB University of Chicago originally written by Alex Crofts and Sasha Ostapenko and updated by Matt Kirkey.

1. Set centrifuge on fast temp to cool down to 4°C (sign out centrifuge).
2. Turn on sonicator, clean probes (sign out sonicator).
3. Prep A1 and Lysis buffers.
4. Collect material, wash with embryo wash buffer.
5. Add material to a Broeck-type homogenizers(Wheaton #357426). Embryo's can be collected directly in Douncer-type homogenizer(Wheaton #357544).
6. Add 6 mL A1 buffer to Potter homogenizers.
7. Add 500 uL of 20% formaldehyde (to 1.8%) and begin 15' timer.
8. Homogenize material (100-300mg/IP of embryos, larvae, pupae, adults) first in Broeck homogenizer (frosted) and then in Douncer (clear) with type A pestle (tight) at RT (6 strokes each).
9. Transfer homogenate to 15mL polystyrene tube. Mix by inverting every 5 minutes.
10. Once 15' timer is up, add 540 uL of 225 mM glycine solution, mix and incubate 5' at RT.
11. Put on ice and keep on ice unless specified.
12. Centrifuge 5' at 4000 rpm at 4°C. Discard supernatant.
13. Add 3mL of buffer A1. Resuspend the pellet and centrifuge for 5' at 4000 rpm and 4°C. Repeat this washing step 3 more times.
14. Wash once in 3 mL of lysis buffer **without SDS**. Centrifuge for 5' at 4000 rpm and 4°C. Discard supernatant.
15. Resuspend the cross-linked material in 0.5 mL of lysis buffer. Add SDS to 0.1% (2.5 uL of 20% SDS) and N-lauroylsarcosine to 0.5% (12.5 uL of 20% solution).
16. Incubate 10' at 4°C on rotating wheel.
17. Briefly spin down tubes and resuspend sample using pipette.
    a. Sonicate 15mL tubes according to Diagenode instructions. The settings are: *high power, 15 minutes, 30 seconds off, 30 seconds on.* The water bath should be kept cold either in cold room or by a cold water pump.
18. Wash probes after sonication.
19. Transfer to eppendorf tubes (tube #1) and rotate for 10' at 4°C on rotating wheel.
20. Centrifuge tubes for 3' at RT at max speed (15,000 rpm). Transfer supernatant to a new eppendorf tube (tube #2).
21. Add 0.5 mL of lysis buffer **with SDS** (2.5 uL of 20% SDS to 0.1%) to eppendorf tube with pellet (tube #1). Rotate for 10' at 4°C on rotating wheel.
22. Centrifuge tubes for 3' at RT at max speed (15,000 rpm). Combine supernatants in eppendorf tube (tube #2).

23. Centrifuge combined supernatant for 7' at RT at max speed (15,000 rpm). Transfer supernatant to a new eppendorf tube (tube #3). Supernatant from this stage will be referred to as chromatin extract.
24. Add sodium azide to a concentration of 0.02% for storage (2 uL)
25. Chromatin extracts can be stored at -80°C for **2 months** at this stage.

Materials:

10x Embryo Wash Buffer (EWB):

0.03% Triton X-100
68 mM NaCl

A1 Buffer

60 mM KCl, 15 mM NaCl, 15 mM HEPES pH 7.6,

For 1 liter A1 buffer:

952 mL MilliQ H_2O
30mL 2 M KCl
3 mL 5 M NaCl
15 mL 1 M HEPES (Cellgro #25-060-cl 1M)

Add the following on ice prior to the experiment, its good at 4°C for 1 week.
4mM MgCl2, 0.5% Triton X100,0.5 mM DTT, protease inhibitor.

Per 50ml A1 buffer, add:

47.5 mL A1 buffer basic
2.5 mL 10% Triton X100 (Fisher BP151500 500ml)
25 uL 1 M DTT (D-L-Dithiothreitol 1M Aqueous solution #64656310x 5ml)
200uL 1 M MgCl2
Dissolve 1 tablet of inhibitors (Roche #1873580 complete EDTA free)

Lysis Buffer

140 mM NaCl, 15 mM HEPES pH 7.6, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate.

For 1 liter lysis buffer:

944 mL MilliQ H_2O
28 mL 5M NaCl
15 mL 1M HEPES (Cellgro #25-060-cl 1M)
2 mL .5M EDTA (Boston Bioproducts #BM-150 Ph 8.0)
1 mL .5M EGTA (Boston Bioproducts #BM-151 Ph 8.0)
10 mL 10% Sodium Deoxycholate (Sigma 30970-25g)

Add the following on ice prior to the experiment, its good at 4°C for 1 week.
1% Triton X-100, 500 uM DTT, protease inhibitor.

Per 50ml lysis buffer, add:

45 mL lysis buffer
5 mL 10% Triton X-100 (Fisher BP151-500 500ml)
25 uL 1M DTT (DL-Dithiothreitol 1M Aqueous solution #646563-10x 5ml)
1 tablet of inhibitors (Roche #1873580 complete EDTA-free)

Formaldehyde Cross-linking of Chromatin from Tissue Collections:

Protocol from modEncode IGSB University of Chicago written by Alex Crofts and Sasha Ostapenko

1. Dissect tissue from larva in PBS and store in petri dish on ice.
2. Transfer to eppendorf tubes.
3. Spin down and remove supernatant
4. Resuspend tissue in A1 buffer.
5. Rotate for 5' at RT.
6. Spin down, remove supernatant.
7. Resuspend in 500uL of A1 + Formaldehyde (1.8%) fixative. Start timer.
8. Rotate twice at RT for 10'.
9. Add 54 uL of 2.5 M glycine (~225mM) and rotate at RT for 5'.
10. Spin down and remove supernatant.
11. Resuspend in 1mL LB. Rotate for 5'.
12. Spin down and remove supernatant.
13. Add 500uL Lysis buffer and SDS to a concentration of 0.1%.
14. Rotate 20' at 4°C.
15. Sonicate in 15 mL polystyrene tube according to Diagenode instructions.
16. Rotate for 10' at 4°C in eppendorf tubes.
17. Centrifuge tubes for 3' at max speed (15,000 rpm). Transfer supernatant to a new eppendorf tube.
18. Add sodium azide to 0.02% for storage.
19. Chromatin extracts can be stored at -80°C for 2 months at this stage.

Materials:

PBS:

137 mM NaCl
2.7 mM KCl
10 mM Na2HPO4
1.8 mM KH2PO4
1 mM CaCl2
0.5 mM MgCl2

A1 Buffer

60 mM KCl, 15 mM NaCl, 15 mM HEPES pH 7.6,

For 1 liter A1 buffer:

952 mL MilliQ H_2O
30mL 2 M KCl
3 mL 5 M NaCl
15 mL 1 M HEPES (Cellgro #25-060-cl 1M)

Add the following on ice prior to the experiment, its good at 4°C for 1 week.
4mM MgCl2, 0.5% Triton X100,0.5 mM DTT, protease inhibitor.

Per 50ml A1 buffer, add:

47.5 mL A1 buffer basic
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25 uL 1 M DTT (D-L-Dithiothreitol 1M Aqueous solution #64656310x 5ml)
200uL 1 M MgCl2
Dissolve 1 tablet of inhibitors (Roche #1873580 complete EDTA free)

Lysis Buffer

140 mM NaCl, 15 mM HEPES pH 7.6, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate.

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Add the following on ice prior to the experiment, its good at 4°C for 1 week.
1% Triton X-100, 500 uM DTT, protease inhibitor.

Per 50ml lysis buffer, add:

45 mL lysis buffer
5 mL 10% Triton X-100 (Fisher BP151-500 500ml)
25 uL 1M DTT (DL-Dithiothreitol 1M Aqueous solution #646563-10x 5ml)
1 tablet of inhibitors (Roche #05056489001 complete EDTA-free)

Chromatin Immunoprecipitation Protocol

Day 1:

1. Thaw chromatin extracts on ice over an hour. **Do not heat with hand.**
2. Meanwhile prepare beads (see below). Each chromatin extract will be cleared with 100ul of prepared beads, and 50ul of prepared beads will be used for each ChIP replicate. Beads are prepared and maintained in a 50/50 by volume mixture of beads and LB buffer.
3. Briefly, check thermofisher chart to determine which beads (A or G) to prepare and then determine the amount of prepared beads needed [(the # extracts to be cleared X 100) + (the number of IPs to use that bead type x 50) = final volume in uL of 50% beads 50% lysis buffer needed].
4. Blocking the beads: Once beads are prepared add 1ul thawed 100x BSA for every 100ul prepared beads. Rotate at 4°C for 1 hour.
5. Washing: After 1 hour of clearing, remove beads from rotator and put on ice. Quickly spin on bench-top centrifuge, about 5 seconds. Remove the supernatant and wash 3x with cold lysis buffer. Be sure to resuspend beads by flicking instead of pipetting so no beads are lost.
6. After the beads are washed, add 100ul of resuspended 50/50 bead mixture into each thawed chromatin extract sample on ice. Be sure to add the correct beads (a or g) to the correct sample (based on which antibody will be used on the sample for immunoprecipitation).
7. Rotate bead/chromatin samples for 4 hours at 4°C. In the meantime decide how many IPs will be made from each extraction sample. For modENCODE *Drosophila* work, each IP should contain between 80 and 120mg of biological material - at least 100mg per IP is desired. Referencing the mg recorded from the extraction source, you can estimate how much volume in each sample will correspond to approx. 100mg of material. Be sure to prepare an Input. The Input is 40-100mg of material that will be used as a control during sequencing later on. After 4 hour rotation, quickly spin down samples to pellet the beads in the extraction samples. Immediately put on ice.
8. Divide the supernatant into the labeled microcentrifuge tubes prepared earlier, avoiding the beads. Don't forget to make Input samples.
9. After the extraction samples have been divided into individual IPs in properly labeled tubes, add the X ug desired antibody to each IP (for modENCODE *Drosophila* work ~15ug of antibody was used per IP). Do not add any antibody to the INPUT samples.
10. Rotate IP samples w/ antibody overnight at 4°C. Leave the INPUT samples at 4°C.

Day 2:

11. The next day add 50ul prepared beads to the appropriate IP samples. Rotate samples at 4°C for 4 hours.
12. Set 2 heat blocks to 60 and 65 degrees. After 4 hours begin washing the beads. Spin samples at 4°C for 1 minute at 13,000rpm. Using a vacuum trap, remove supernatant avoiding the beads. Add 1 ml cold lysis buffer to each sample and rotate at 4°C for 5 minutes. Repeat 3 more times for a total of 4x lysis buffer washes.
13. After the last wash with lysis buffer, spin and remove supernatant with vacuum trap. Wash twice with cold TE buffer as described above. After the last TE buffer wash, spin down and remove supernatant.
14. Add 100ul elution buffer 1 to each bead sample and place in 65 degree heat block for 10 minutes
15. After 65 degree IP samples have been heated for 10 minutes, spin at room temp for 1 minute at 13,000rpm. Transfer supernatant to a new labeled tube leaving the beads.
16. To the beads, add 150ul elution buffer 2. Spin down for 1 minute at room temp at 13,000 rpm. Transfer supernatant to the same new labeled tube, creating a chromatin elution sample of approximately 250ul - now called the chromatin ImmunoPrecipitate, IP.
17. Place IP samples at 65C overnight to reverse crosslinks on a shaking heat block in order to prevent all the liquid from condensing at the top.
18. To the INPUTs add 0.5ul 20mg/ml Protienase K for every 100ul of input volume and 5ul of 20% SDS per 100 ul of input volume. Place INPUTS at 60C overnight on a shaking heat block in order to prevent all the liquid from condensing at the top.

Column purification Day3:

19. Remove samples from heat block, set heat block to 70°C
20. Add INPUTs to 70°C heat block and incubate for 20 min
21. Add 1.2 µl Proteinase K to each sample and incubate at 50°C for 2 hours
22. Add 0.12 µl RNaseA to each INPUT and incubate at 37°C for 2 hours
23. Follow the minElute protocol:
    a. Add 600 µl Buffer PB to each sample and INPUT
    b. Add entire sample (~750 µl) to a minElute column
    c. Centrifuge for 1 min at 13,000 rpm
    d. Discard flow-through and put column back into tube
    e. Add 750 µl Buffer PE to each column, centrifuge for 30 sec at 13,000 rpm
    f. Discard flow-through and put column back into tube, centrifuge at 13,000 rpm for 3 min.
    g. Add 13 µl EB to each column, incubate for 1 min, centrifuge for 1 min at 13,000 rpm
24. Store samples at -30°C.

Bead Preparation

Preparing Protein A Beads:

0.1g of Protein A beads should be used for every 1 mL of lysis buffer to achieve a 50% by volume even mixture of beads and buffer. 0.1g beads + 1 mL lysis buffer will total 1 mL 50/50 beads & buffer. Use .05g of beads and .5 mL lysis buffer for .5 mL total volume for example.

Tare a labeled microcentrifuge tube on a scale, then tap in protein A beads (don't use a scrapper - beads are too staticky). Weigh periodically until desired bead weight in the tube has been achieved. Then lay the tube on its side and tap until the beads are spread evenly across the side of the tube. While still on it's side add appropriate volume of lysis (or preferred) buffer starting at the back of the tube to ensure all of the beads become evenly hydrated. Then block the beads as described below. Be sure to keep beads on ice or at 4°C at all times.

Preparing Protein G Beads: Protein G beads are already in mixture with PBS + ethanol. Resuspend beads by gently shaking the bottle and remove slightly less than desired final volume of beads/buffer volume determined above. If you need a final bead volume of 500ul, remove 400ul bead suspension from the bottle, for example. This should give you approximately 250ul of beads. Once beads have settled, removed the ethanol above them and wash beads with lysis buffer 3 times to remove all of the ethanol. Resuspend beads in lysis buffer to make a 50/50 by volume suspension. Then perform the blocking step below.

Blocking: All beads must be blocked. After beads (either A or G) have been resuspended in lysis buffer add 1ul of thawed 100x BSA for every 100ul total bead suspension volume (5ul BSA for 500ul 50/50 mixture beads and buffer, for example). Rotate BSA + bead suspension at 4°C for one hour. After rotation, put on ice and allow beads to settle (or spin 1 minute 13,000 rpm at 4°C). Remove the BSA lysis buffer above the beads with a pipette. Add the same volume of lysis buffer as there are beads to wash. Flick to resuspend beads in the lysis buffer. Centrifuge briefly to settle the beads, and repeat lysis buffer wash 2 more times. **The beads are now considered "prepared".**

Prepared beads can be stored for as long as the protease inhibitors in the lysis buffer are good for.

TE buffer

10 mM Tris-HCl pH 8.0, 1 mM EDTA

for 50 mL of TE buffer:

500 uL of 1M Tris-HCl

100 uL of 0.5 M EDTA

Elution Buffer 1

10 mM EDTA, 1% SDS, 50 mM Tris-HCl pH 8

For 50mL Elution buffer 1:

1 mL of 0.5 M EDTA

2.5 mL of 20% SDS

2.5 mL of 1M Tris-HCl

Elution Buffer 2

29% TE, 0.67% SDS

For 1 liter lysis buffer:

Proteinase K Solution

0.5 uL of 20 mg/mL glycogen

5uL 20 mg/mL Proteinase K stock

244.5 uL TE

modENCODE NuGEN Library Generation Protocol

NuGEN protocol adapted for modENCODE by Matt Kirkey, December 2, 2013

END REPAIR

1. Remove the End Repair Buffer Mix (blue: ER1), End Repair Enzyme Mix (blue: ER2), and End Repair Enhancer (blue: ER3) from -20°C storage. Remove DMSO from -20°C storage and set aside.
2. Spin down ER2 and ER3 and place on ice.
3. Thaw ER1 at room temperature. Mix by vortexing, spin, and place on ice.
4. Obtain aliquot of nuclease free water and store at room temperature.
5. Vortex and spin down ChIP samples.
6. Add 5 uL to PCR tubes (1 uL for INPUTS, see NOTES).
7. Add 5 uL of nuclease free water to tubes (9 uL for INPUTS), bringing sample to 10uL.
8. Prepare a master mix according to the volumes (per sample) below.
9. Carefully mix by pipetting and place on ice.
10. Add 5 uL of the End Repair Master Mix to each sample tube.
11. Mix by pipetting, cap and spin tubes, and place on ice.
12. Place tubes on a pre-warmed thermal cycler programmed to run:
    a. 25° C → 30 minutes
    b. 70° C → 10 minutes
    c. hold at 4° C
13. Continue immediately with the Ligation protocol.

ER1	ER2	ER3
3.85 uL	0.55 uL	1.1 uL

NOTES:

- It is difficult to accurately quantify ChIP material, so we use a standard volume of ChIP material for our library generation.
- If ChIP DNA concentration is expected to be low, we recommend using a full 10 uL of ChIP material for library generation.
- Inputs normally have much higher DNA concentrations, so a smaller volume of starting material is acceptable.

LIGATION

1. Remove the Ligation Buffer Mix (yellow: L1), Ligation Adaptor Mixes (yellow: L2), and Ligation Enzyme Mix (yellow: L3) from -20°C storage.
2. Spin down L3 and place on ice.
3. Thaw L1 and L2 at room temperature. Mix by vortexing, spin, and place on ice.
4. Add 3 uL of appropriate L2 to each sample. Mix thoroughly by pipetting.
5. Prepare a master mix according to the volumes (per sample) below.
   NOTE: L1 is very viscous, pipette **slowly**.
6. Carefully mix by pipetting and place on ice.
7. Add 12 uL of Ligation Master Mix to each sample tube.
8. Mix by pipetting, cap and spin tubes, and place on ice. Proceed immediately with the incubation.
9. Place tubes on a pre-warmed thermal cycler programmed to run:
   a. 25° C → 30 minutes
   b. 70° C → 10 minutes
   c. hold at 4° C
   NOTE: Leave the thermal cycler lid open during ligation incubation.
10. Remove the tubes from the thermal cycler, spin to collect condensation, and place on ice.
11. Continue immediately with the Ligation Purification protocol.

Water	L1	L3
4.95 uL	6.6 uL	1.65 uL

LIGATION PURIFICATION

1. To bind, add 5 volumes of Buffer PB (150 uL) to each sample and pipette mix.
2. Transfer the sample to a MinElute column and centrifuge for 1 minute at 13,000 rpm.
3. Discard flow-through. Blot collection tube on clean paper towel.
4. To wash, add 750 uL of Buffer PE to the column and centrifuge for 30 seconds at 13,000 rpm.
5. Discard flow-through and place column back on the collection tube. Centrifuge the column for an additional 2.5 minutes at 13,000 rpm.
6. Place the column on a clean, labelled microcentrifuge tube.
7. To elute DNA, add 18.5 uL Buffer of EB to the center of the column membrane, incubate at RT for 1 minute, and then centrifuge for 1 minute at 13,000 rpm.
8. Add an additional 18.5 uL of Buffer EB to the center of the column membrane, incubate at RT for 1 minute, and then centrifuge for 1 minute at 13,000 rpm.
9. Be careful to aspirate any pelleted material.

LIBRARY AMPLIFICATION

1. Remove the Amplification Buffer Mix (red: P1), Amplification Primer Mix (red: P2) and Amplification Enzyme mix (red: P3) from -20°C storage. Obtain the DMSO (red: P4) set aside earlier at RT.
2. Spin down P3 and place on ice.
3. Thaw P1 and P2 at room temperature. Mix by vortexing, spin, and place on ice.
4. Prepare a master mix according to the volumes (per sample) below. Add P3 at the last moment.
5. Mix by pipetting and place on ice.
6. Add 44 uL of Amplification Master Mix to each sample tube.
7. Mix by pipetting, cap and spin tubes, and place on ice. Proceed immediately with the incubation.
8. Place tubes on a pre-warmed thermal cycler programmed to run:
    a. 72° C → 2 minutes
    b. 94° C → 30 seconds, 60° C → 30 seconds, 72° C → 1 minute (15 cycles)
    c. 72° C → 5 minutes
    d. hold at 4° C
9. Remove the tubes from the thermal cycler, spin to collect condensation, and place on ice.
10. Continue with the Amplified Library Purification protocol.

P1	P2	P4	P3
38.5 uL	4.4 uL	4.4 uL	1.1 uL

AMPLIFIED LIBRARY PURIFICATION

1. To bind, add 5 volumes of Buffer PB (400 uL) to each sample and pipette mix.
2. Transfer the sample to a MinElute column and centrifuge for 1 minute at 13,000 rpm.
3. Discard flow-through. Blot collection tube on clean paper towel.
4. To wash, add 750 uL of Buffer PE to the column and centrifuge for 30 seconds at 13,000 rpm.
5. Discard flow-through and place column back on the collection tube. Centrifuge the column for an additional 2.5 minutes at 13,000 rpm.
6. Place the column on a clean, labelled microcentrifuge tube.
7. To elute DNA, add 21 uL Buffer EB to the center of the column membrane, incubate at RT for 1 minute, and then centrifuge for 1 minute at 13,000 rpm.
8. Be careful to aspirate any pelleted material.
9. Store libraries at -20°C.

SPRI selection adapted for modENCODE

This protocol is designed to select a DNA library for the size range of ~200-800 bp. This range can be modified using different bead ratios.

Left-sided selection:

1. Add elution buffer (EB) to DNA libraries to bring total volume to 50 uL.
2. Add 42.5 uL of thoroughly resuspended SPRI magnetic beads to library.
3. Gently vortex to mix.
4. Let incubate at RT for 1 minute.
5. Place samples on magnetic stand.
6. Allow SPRI beads to settle to the magnet.
7. Remove the clear supernatant and discard.
8. With samples still on the stand, add 180 uL of 85% ethanol and incubate at RT for 30 seconds.
9. Remove the ethanol wash and discard.
10. Remove the samples from the magnetic stand.
11. Add 52 uL of EB.
12. Gently vortex to mix.
13. Let incubate at RT for 1 minute.
14. Place samples on magnetic stand.
15. Allow SPRI beads to settle to the magnet.
16. Carefully aspirate 50 uL of the supernatant containing the left-side selected library into a new 1.5 mL tube.

Right-sided selection:

1. Add 28 uL of thoroughly resuspended SPRI magnetic beads to left-side selected library.
2. Gently vortex to mix.
3. Let incubate at RT for 1 minute.
4. Place samples on magnetic stand.
5. Allow SPRI beads to settle to the magnet.
6. Carefully aspirate 75 uL of the supernatant containing the size selected library into a new 1.5 mL tube, discard tubes with beads.
7. Add 93 uL of thoroughly resuspended SPRI magnetic beads to the size selected library.
8. Gently vortex to mix.
9. Let incubate at RT for 1 minute.
10. Place samples on magnetic stand.
11. Allow SPRI beads to settle to the magnet.
12. Remove the clear supernatant and discard.
13. With samples still on the stand, add 180 uL of 85% ethanol and incubate at RT for 30 seconds.
14. Remove the ethanol wash and discard.
15. Remove the samples from the magnetic stand.
16. Add 25 uL of EB.
17. Gently vortex to mix.
18. Let incubate at RT for 1 minute.
19. Place samples on magnetic stand.
20. Allow SPRI beads to settle to the magnet.
21. Carefully aspirate 24 uL of the supernatant containing the size selected library into a new 1.5 mL tube.
22. Store size selected library at -80°C.