ChIP-chip protocol adapted for the mod-ENCODE project

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**Formaldehyde Cross-linking of Chromatin from any Drosophila tissue (Embryos, Larvae, Pupae, Adult flies)**

- **Solutions and Materials**

  **Formaldehyde**, 37% or 20 % from Tusimis

  **Glycine solution** 2.5 M

  **N-lauroylsarcosine** (Sigma L-9150): 10% solution in bi-distilled water. Store at room temperature.

  **Buffer A1 and Lysis buffer:**

  These 2 solutions can be prepared in large volume and stored at R.T as «Basic». The final reagents are added just before the experiment in smaller volume (usually 50 ml) and kept at 4°C for up to one week.

  **EDTA-free protease inhibitors cocktail** (complete, EDTA-free, Roche, Cat No. 1 873 580, use following manufacturer's instructions) herein referenced as «Tablets». You can dissolve 1 Tablet in 50 ml of solution for 1X or create a stock 25X solution in 2 ml of water.

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  **Buffer A1**:
  - **Basic**: 60 mM KCl, 15 mM NaCl, , 15 mM HEPES (pH7.6).

    Add the following to the Basic right before the experiment on ice. The volumes are given for a final volume of 50 ml:
    - 4 mM MgCl$_2$ => 200 µl of 1M
    - 0.5 mM DTT => 25 µl of 1M
    - 0.5% Triton X-100 => 2.5 ml of 10%
    - Finally dissolve 1 Tablet of inhibitors

    [Optional] 10 mM Sodium butyrate => 500 µl of 1M. This reagent can be added if planning to ChIP against histones modifications since butyric acid has been associated with the ability to inhibit the function of histone deacetylase enzymes.]

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  **Lysis Buffer**:
  - **Basic**: 140 mM NaCl, 15 mM HEPES pH 7.6, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate.

    Add the following to the Basic right before the experiment on ice. The volumes are given for a final volume of 50 ml:
    - 1% Triton X-100 => 5 ml of 10%
    - 0.5 mM DTT => 25 µl of 1M
    - Finally dissolve 1 Tablet of inhibitors

    0.05% SDS => 125 µl of 20% SDS
    **IMPORTANT**: prepare 1 Falcon tube of Lysis buffer with SDS and another without.

    [Optional] 10 mM Sodium butyrate => 500 µl of 1M]

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  **Sonifier Bioruptor from Diagenode**: follow manufacturer's instruction for proper using
**Protocol**

1. Homogenize the material (about 150-200 mg of embryos, pupae, adults, larvae, sufficient for four-five independent immuno-precipitations) in 5 ml of buffer A1 + 1.8% formaldehyde (290 µl of 37% solution) at room temperature using first a Potter homogenizer and then a Douncer with type A pestle (3 strokes). Wait 15' (total time starting from beginning of homogenization). Add glycin solution to 225 mM (540 µl of 2.5 M solution for 6 ml of cross-linked mixture), mix and incubate 5'. Put on ice. Keep on ice unless specified.

2. Transfer the homogenate to a 15 ml tube. Centrifuge 5 min, at 4000 g at 4°C. Discard supernatant. Add 3 ml of buffer A1, resuspend pellet and spin down the same way. Repeat washing step 3 times.

3. Wash once in 3 ml of lysis buffer without SDS. Spin down, 5 min at 4000 g.

4. Resuspend cross-linked material in 0.5 ml of lysis buffer and add SDS to 0.1% (2.5 µl) and N-lauroylsarcosine to 0.5% (12.5 µl). Incubate 10 min at 4°C in a rotating wheel.

5. Sonicate in Polypropylene 15ml Falcon 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 a cold room or by a cold water pump.

6. Rotate 10'. Transfer to eppendorf tubes and centrifuge 5', room temperature at maximum speed. Transfer supernatant to the new tube. Add another 0.5 ml of lysis buffer to the pellet, rotate for 10 min. Repeat centrifugation and combine supernatants. Centrifuge the combined supernatant 2x10 min at max speed. The supernatant from this stage will be referred as chromatin extract. Chromatin can be stored at –80°C for several months at this stage. Add sodium azide to 0.02% for storage.

7. [Optional: Purification of chromatin. Put chromatin extract in Centricon YM-100 column (4212), centrifuge 3x40' (or more) at 1000 g while adding lysis buffer. At least 3 vol. of lysis buffer should pass through column. Bring the final volume of chromatin extract to 1 ml by lysis buffer + protease inhibitors.]
Chromatin Immunoprecipitation

- **Solutions and Materials**

  **Lysis buffer:**
  (see above)

  5mg/ml RNaseA (DNase free)

  **PAS suspension:**
  100 mg of CL-4B (Amersham, 17-0780-01) PAS should be resuspended in 1 ml of lysis buffer + protease inhibitors + 0.1 mg/ml BSA for 50 %v suspension. Wash in lysis buffer 2-3 times, overall equilibration time 1h. Store up to one week at 4 C.

  **TE:**
  10 mM Tris-HCl pH 8.0, 1 mM EDTA

  **Elution buffer1:**
  10 mM EDTA, 1% SDS, 50 mM Tris-Cl pH 8.

  **Elution buffer2:**
  TE+0.67% SDS.

  **LiCl 4M**
• **Protocol**

1. To an amount of chromatin corresponding to 150 mg of biological material, suspended in a final volume of chromatin extract of 1 ml (in lysis buffer + protease inhibitors), add 100 µl of PAS suspension for preincubation. Incubate several hours or overnight at 4°C, then remove PAS. Crosslinked chromatin at this stage can be stored several days at 4°C or frozen at –70°C.

2. **[Optional : Check for amount of DNA]** From the 1 ml solution above, take a 100 µl aliquot, Add proteinase K up to 100 µg/ml and SDS to 1%, incubate 6h at 60°C, then 20 min at 70°C, add RNAse to 50 µg/ml and incubate additional 2h at 37°C. Phenol-chlorophorm extract, ethanol precipitate. Run on an agarose gel to check amount and size of DNA.

3. Separate chromatin sample into 4x250 µl aliquots (one aliquot is enough for one IP but the amount may be decreased further). Immunoprecipitate chromatin by adding the antibody (Ab) of interest (amount of Ab should be determined empirically, usually the same concentration than for a successful IF experiment). Keep a control sample without Ab IP, named "Mock".

4. Incubate 4 h at 4°C on a head to head rotating wheel, then add 50 µl of PAS suspension and incubate 4 h or overnight. Spin down PAS and proceed to washes.

5. Wash PAS 4x with lysis buffer, followed by 2x with TE (without protease inhibitors). Each wash is for 5 min at 4°C, using 1 ml of solution.

6. Elution of precipitated material. Spin down PAS. Add 100 µl of elution buffer, mix and incubate 10 min at 65°C. Spin down PAS and transfer supernatant to new tube. Add 150 µl of elution buffer 2 to PAS, mix, centrifuge at full speed and transfer eluate to a tube together with the eluate from the first centrifugation. The combined material is the "chromatin precipitate" (approx. 250 µl).

7. Incubate precipitate 6 h (or overnight) at 65 C to reverse cross-links. Add 250 µl of Proteinase K solution, incubate at 50°C 2-3 h.

8. Add 55 µl of 4M LiCl and 500 µl of phenol-chlorophorm. Mix and microfuge at full speed at RT. Transfer aqueous phase to a new tube and precipitate with 1 ml of 100% ethanol. Wash with 750 µl of 70% ethanol. Spin down and dry precipitate.

9. Dissolve in 25 µl of water. This is the chromatin immunoprecipitate or “Native ChIP” sample.
**Ligation-mediated PCR**

- **Solutions and Materials**

**Proteinase K solution (250 µl):**
- 0.5 µl of 20 mg/ml glycogen solution
- 5 µl of 20 mg/ml proteinase K stock
- 244.5 µl TE

**Polynucleotide Kinase (New England Biolabs) 10,000 Units/ml**

**Klenow Fragment polymerase (Promega) 5,000 Units/ml**

**T4 DNA ligase (New England Biolabs) 400,000 Units/ml with its supplied buffer**

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**10 µM linker DNA:**
Two oligonucleotides must be annealed:
(i) a 24-mer of sequence 5´-AGA AGC TTG AAT TCG AGC AGT CAG (phosphorylated at 5´-end);
(ii) a 20-mer of sequence 5´-CTG CTC GAA TTC AAG CTT CT.

To produce the linker mix:
- 20 µl of 100 µM 24-mer Phospho-primer
- 20 µl of 100 µM 20-mer primer
- 160 µl of TE
- incubate in a PCR machine using the following program:
  - 5 min at 70°C (remove secondary structures)
  - 5 min at 55°C (annealing)
- let cool down slowly (0.01 °C/sec) to 25°C, incubate 2 h at this temperature and then cool down (0.01 °C/sec) to 4°C. This can be stored at -20°C and make 10 µM aliquots.

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**dNTP 2mM Mix**
From each dNTP (Promega, PCR grade, 100mM) prepare a 2mM Mix.
- 20 µl of each dNTP
- 920 µl H₂O
- Make 10X 100 µl aliquots and store at -20°C.

**Taq polymerase (Promega) 5,000 Units/ml**

**Qiagen QIAquick PCR purification kit (Cat No. 28104)**
Protocol

1. Add 3 µl of T4 ligase buffer and 1 µl (10U) of Polynucleotide Kinase (PNK). Incubate at 37°C for 30'.

2. Inactivate PNK at 68°C 20'. Cool down to 37°C. Add 1 µl of 2 mM dNTP mix, 1 µl of Klenow fragment (5 U), incubate 30' at 37°C, then inactivate at 75°C 10' and cool slowly to 4°C.

3. Take 9 µl of the reaction mixture (store the rest at −20°C for further experiments), add 1 µl of 10 mM ATP, 1 µl 10 µM linker and 4U of T4 DNA ligase. Incubate overnight at 4°C.

4. The liglation mixture is directly used for PCR amplification (add to tube with ligation reaction 44 µl of bi-distilled H2O (PCR grade), 8 µl Taq polymerase buffer, 10 µl of 2 mM dNTP mixture, 5 µl of 25 mM solution of MgCl2, 0.5 µl Taq polymerase, 1.5 µl of X-Chip 20-mer primer). PCR amplification scheme is as follows. 1 cycle of : 2 min at 94°C; 34 cycles of : 1 min at 94°C / 1 min at 55°C / 3 min at 72°C; 1 cycle of : 1 min at 94°C / 1 min at 55°C / 10 min at 72°C.

5. PCR products are purified on Qiagen QIAquick columns (following manufacturer's instructions). Final elution of the DNA is in 50 µl of Qiagen "EB buffer" (10 mM Tris-Cl, pH 8.5). The concentration of the eluted DNA is determined by spectrophotometer (ideally a Nanodrop). The size and yield of DNA is further checked by Agarose gel electrophoresis.

6. This is the ChIP sample. It can be used for labeling before hybridization on microarrays. For assay by qPCR, use the native IP as a template.
References
