

Protocol for chromatin immunoprecipitations from *Drosophila* Embryos and analysis on genomic tiling microarrays

Modified from (Birch-Machin et al., 2005)

Embryo collection and fixation

Outline:

The aim of the method is to isolate fixed chromatin from *Drosophila* embryos.

Essentially the chromatin is fixed, then nuclei are purified, nuclei are lysed and the chromatin is fragmented by sonication. 1.5g of embryos are collected for each chromatin prep. This is usually enough to do at least 5 ChIP reactions.

Abbreviations:

SN =supernatant

min =min

1. Wash the embryos from the agar plates and transfer them into large sieves.
2. Dechorionate embryos for 2.5 min in 400 ml 3% NaOCl (50% bleach) at room temperature in a beaker; use a stirring bar to keep them in suspension.
3. Wash them embryos well with tap water and transfer them into pre-weighed medium sized sieves. Calculate the weight of the embryos and transfer all of them into 50 ml PBT (PBS with 0.1% Triton) in a stirred beaker; aliquot the equivalent volume of 1.5 g of embryos into 50 ml falcon tubes.
4. Add PBS/Triton to 50 ml and wash the embryos. Let the embryos settle (without centrifugation).
5. Discard supernatant (SN) and add 10 ml cross-linking solution (50 mM Hepes, 1 mM EDTA, 0.5 mM EGTA, 100 mM NaCl, 1.8 % formaldehyde, pH 8.0) and 30ml n-heptane.
6. Shake vigorously at room temperature for 15min.
7. Centrifuge 1min at 500g to pellet embryos and discard SN by carefully pipetting off as much fix/heptane as possible with 20 ml pipette.

8. Resuspend in 30ml PBS/glycine/triton (125 mM glycine + 0.1% Triton in PBS).
9. Shake embryos for 1 min and centrifuge again for 1 min at 500g.
10. Carefully decant the supernatant.
11. Remove SN, add 5 0ml ice-cold PBT and resuspend. Centrifuge as above, decant the supernatant.
12. Pour embryos onto Nytex membrane lying on top of paper tissues to blot the liquid. Try to get embryos as dry as possible.
13. Transfer a few embryos into an Eppendorf tube with 500 μ l MetOH and 500 μ l heptane. Shake vigorously, wash 2x with MetOH and store at -20°C for staging.
14. Transfer the remaining embryos into round-bottom 15 ml tubes (Falcon tubes **do not** survive freezing in liquid Nitrogen !)
15. Freeze embryos in liquid nitrogen and store at -80°C

The whole protocol takes roughly 60 minutes if all buffers and especially the fix are prepared in advance.

Chromatin prep from frozen fixed embryos with the bioruptor sonicator

1. Thaw the embryos quickly and resuspend them in 15 ml ice-cold PBT + protease inhibitors.
2. Dounce 20x with loose pestle on ice and transfer the lysate into a suitable centrifuge tube.
3. Centrifuge at 400g for 1min and transfer SN to a fresh centrifuge tube.
4. Centrifuge at 1100g for 10min at 4°C and discard SN.
5. Resuspend pellet in 15 ml ice-cold Cell lysis buffer (see below) + protease inhibitors.
6. Dounce 20x with the tight pestle.
7. Transfer two equal aliquots into 15ml falcon tubes.
8. Centrifuge at 2000g for 4min at 4°C to pellet the nuclei. Discard SN. (Nuclei can be frozen in liquid nitrogen and stored at -80°C at this point)

9. Resuspend in 1 ml of ice-cold Nuclear Lysis Buffer (see below) + protease inhibitors and incubate for 20min at RT
10. Add 1 ml ice-cold Nuclear Lysis Buffer + protease inhibitors
11. Sonicate in the bioruptor sonicator water bath (Diagenode, Liège, Belgium) in 15 ml falcon tubes 10x (15s on/ 15s off). Make sure that the water bath always contains a little bit of ice.
12. Transfer the chromatin to 1.5 ml-ependorf tubes and centrifuge at 20.000g for 10 min at 4°C.
13. Pool all supernatants and then transfer the sheared chromatin aliquots (e.g. 200 µl) to cryotubes and flash freeze in liquid nitrogen. Store at -80°C.

Buffers:**Cell lysis buffer**

= 5mM Hepes, pH 8, 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, 10mM EDTA·Na₂, 0.5% N-Laurylsarkosin + protease inhibitors

Protease inhibitors:

Leupeptin 1000x = 10 mg/ml in DMSO

Pepstatin 1000x = 10 mg/ml in DMSO

Aprotinin 1000x = 10 mg/ml in H₂O

PMSF 100x = 100mM in 2-propanol

Chromatin IP using Protein A beads

Always use siliconized tubes for the immune-precipitations !

RIPA : 140mM NaCl / 10mM Tris-HCl pH8,0 / 1mM EDTA / 1% TritonX100 / 0.1% SDS / 0.1% sodium deoxycholate, 1mM PMSF (on ice); add PMSF immediately before use from a 100mM stock in isopropanol). Add protease inhibitors for the precipitation but not to the wash buffers.

RIPA with 0.5 M NaCl : as above, but 500mM NaCl instead of 140mM

LiCl : 250mM LiCl / 10mM Tris-HCl pH 8,0 / 1mM EDTA / 0,5% NP-40 / 0.5% sodium deoxycholate

TE : 10mM Tris-HCl pH 8,0 / 1mM EDTA.

Dilution buffer: 4% glycerol / 10mM Tris-HCl pH 8,0 / 1mM EDTA / 0.5mM EGTA

Preabsorption + antibody addition, 1st day :

1. For each precipitation (also remember the mock reactions !) wash 25 μ l of 50% Protein A Sepharose (PAS) suspension (Protein A Sepharose CL4B, Sigma) once with 1ml RIPA buffer.
2. Spin down at 1000g, discard the supernatant and resuspend in 100 μ l 1x RIPA buffer per 25 μ l ProtA beads.
3. Thaw an aliquot of chromatin and bring to 500 μ l with dilution buffer.
4. Add 100 μ l 10% Triton X100, 100 μ l 1% Deoxycholate, 100 μ l 1% SDS, 100ul 1.4 M NaCl, 10 μ l 100mM PMSF.
5. Resuspend the beads prepared above by vortexing and add 100 μ l of the suspension.

6. Incubate for 1h at 4°C, then spin down for 2 min at 1000g at 4 °C. This acts as a preclearing step to reduce non-specific binding to protein A sepharose.
7. Transfer the chromatin to a new tube and take out 10 µl as (ca.) 1% input. Store this sample at 4 °C
8. Add 1-3 µl of the appropriate antibody (or prebleed) to each tube. Incubate overnight at 4°C with gentle mixing. **ALLWAYS INCLUDE A MOCK CONTROL.**
9. For each precipitation (also remember the mock reactions !) wash 25 µl of 50% Protein A Sepharose (PAS) suspension (Protein A Sepharose CL4B, Sigma) once with 1ml RIPA buffer + 1 mg/ml BSA and incubate with another 1 ml of RIPA buffer + 1 mg/ml BSA O/N at 4 °C

Purification of immuno-complexes, 2nd day :

10. Spin down the preblocked Protein A beads, discard the supernatant and resuspend in 100 µl of RIPA buffer per reaction.
11. Purify the immune-complexes by adding 100 µl of preblocked PAS solution and incubate for 3h at 4°C with gentle mixing.
12. Wash the beads for 10 min each with the following buffers:
 - Once with RIPA buffer
 - 4 times with RIPA buffer containing 0.5M NaCl
 - Once with LiCl buffer
 - Twice with TE.

Carry out all steps at 4°C using 1ml wash buffer and always spin at 1000g for 2 min to pellet PAS before removing the supernatant.

13. Resuspend the PAS complexes in 100 µl TE buffer.
14. Add 50 µg/ml RNase to all samples (from 1 mg/ml stock), and incubate for 30 min at 37°C.
15. Adjust the samples to 0.5% SDS, 0.5 mg/ml proteinase K and incubate at 37°C O/N

Proteinase K digestion, DNA purification, 3rd day :

16. Incubate the samples at 65°C for 6 hrs to reverse the formaldehyde crosslinks (and to elute the antibody/antigen complexes)
17. Phenol chloroform extract and precipitate the DNA, resuspend the pellet in 30 µl H₂O.
18. Store at -20°C.

Use 1-2 µl of the eluate to check for enrichment of specific fragments by real-time PCR.

Blunting of IP-ed DNA: Optional, though advisable

19. Use 7 µl from each IP/mock for each reaction – also include No-DNA control (H₂O)

Set up following blunting reaction, 37°C, 30 min in incubator

29,5	µl	H ₂ O
10	µl	5x T4 polymerase buffer
2,5	µl	dNTPs (each 2mM)
1	µl	<u>T4 DNA polymerase (Invitrogen)</u>
43µl	+ 7µl IP-material	

20. Stop the reaction by incubating 5 min in 65°C incubator
21. Purify DNA with the Qiagen Min-Elute kit, wash with buffer PE twice and elute with 20µl H₂O prewarmed to 65°C
22. Speedvac to =< 7µl, (add water to 7µl if necessary) and continue with the ligation of linkers.

Purification of genomic DNA used as a reference in hybridizations

1. It is advisable to prepare a sufficient amount of sheared genomic DNA for use as a reference in all experiments. It can be obtained easily from some of the frozen chromatin preps: Bring 200 μ l of an undiluted chromatin prep to 50 μ g/ml RNase (from 1 mg/ml stock) and incubate for 30min at 37°C.
2. Adjust the samples to 0.5% SDS, 1 mg/ml proteinase K and incubate at 37 °C O/N
3. Incubate the samples at 65°C for 6 hrs to reverse the formaldehyde crosslinks.
4. Phenol chloroform extract and precipitate the DNA, resuspend the pellet in 50 μ l TE. Estimate the concentration spectrometrically.
5. Store at -20°C.

Linker ligation + PCR amplification of chromatin IP eluates

Preparation of annealed linkers:

Order the following primers:

24mer **5' phosphorylated** AGA AGC TTG AAT TCG AGC AGT CAG

20mer CTG CTC GAA TTC AAG CTT CT

- Adjust to 10 μ M with TE.

Linkers are made by annealing two complementary oligos. Equal molar quantities of oligos are mixed and incubated in the PCR machine (0.5 ml tubes) using the following temperature steps:

- 95 °C for 5 min
- 70 °C for 5 min
- 55 °C for 5 min
- ramp: 0.1 °C/s to 4 °C

Store annealed linkers at -20°C . Prepare 1 μM diluted linker for the reaction.

Linker ligation:

1. Ligate the linker to 7 μl of the IPed material by adding 1 μl 10x Roche ligation buffer, 1 μl **1 μM** annealed linker and 1 μl (Roche) T4 DNA ligase (Roche). Incubate overnight at 16°C . Also include “no DNA control” !
2. Amplify the DNA directly without any purification, also include “no DNA control” ! :

PCR reaction A

<u>1x</u>		
10	μl	10x Amplitaq buffer (15 mM Mg^{2+})
10	μl	dNTPs (each 2mM)
1	μl	20mer (100 μM)
1	μl	Amplitaq Pol (5U/_1, Roche)
<u>68.0</u>	<u>μl</u>	<u>H_2O</u>
90 μl	+ 10 μl ligation	

using the following PCR setup:

Step	Time	Temperature
1	2	55°C
2	5	72°C
3	5	94°C
4	1	94°C
5	1	55°C
6	1	72°C
20 cycles of 4-7		
8	5	72°C
9	Hold	4°C

3. 20 cycles of PCR reaction A will not generate enough product to be visualized on an agarose gel. Repeat the PCR using 10 μl of unpurified PCR reaction A mix as a template for PCR reaction B using ca. 20 cycles

to generate enough DNA for a labeling reaction. Store the remainder of PCR reaction A as a stock for further amplifications. This two step amplification ensures that both reactions of 20 cycles stay within the linear range.

- The efficiency of the ligation and PCR reaction A can be assessed by running realtime PCR on a 1/100 dilution of the **unpurified** amplified material – conservation of enrichment should be observed.

5. **PCR reaction B**

<u>1x</u>		
10	μl	10x Amplitaq buffer (15 mM Mg ²⁺)
10	μl	dNTPs (each 2mM)
1	μl	20mer (100μM)
1	μl	Amplitaq Pol (5U/_1, Roche)
<u>68,0</u>	<u>μl</u>	<u>H₂O</u>
90μl		+ 10μl PCR reaction A

using the following PCR setup (same as before):

Step	Time	Temperature
1	2	55 °C
2	5	72 °C
3	5	94 °C
4	1	94 °C
5	1	55 °C
6	1	72 °C
20 cycles of 4-7		
8	5	72 °C
9	Hold	4°C

- If necessary repeat PCR reaction B until a sufficient amount of amplicon has been generated. Use the same number of amplification cycles for mock and ChIP reactions !
- Check 5μl on a 1% agarose gel; the product should be a smear with a peak around 500bp.

8. Purify the amplification products from linker DNA and nucleotides using Quiagen PCR purification columns according to the manufacturer's conditions. Elute with 30 μ l 65 °C buffer EB and carefully quantitate the PCR yields with the Nanodrop spectrometer. Expected yields vary for each precipitated protein: ca. 1-4 μ g

Quantitative real-time PCR verification of chromatin immunoprecipitation results

Before moving ahead with the microarray hybridisation re-check the enrichment of a positive control by quantitative real-time PCR, using a 1:100 dilution of PCR-A. Fold enrichments are normalized against a PCR reaction with a negative control primer pair (i.e. a sequence which should not be bound by the transcription factor), for both the IP and the mock reactions. The final enrichment fold is measured as the ratio of normalized ChIP/mock results for each assayed sequence.

Direct Klenow Labeling using BioPrime DNA Labling System

1. Bring **0.5** µg of PCR amplified DNA to a volume of 10.5 µl with H₂O and mix with 10 µl of 2.5x random prime reaction buffer (Invitrogen).

(2.5X Random Primers Solution:

125 mM Tris-HCl (pH 6.8),

12.5 mM MgCl₂,

25 mM 2-mercaptoethanol,

750 µg/ml oligodeoxyribonucleotide octamers)

2. To denature the DNA, incubate the sample at **100°C for 5 min** and place on ice.

3. Then add the following reagents:

- 2.5 µl of 10x UTP-mix (Bioprime kit)

- 1.5 µl Cy3 or Cy5-dUTP

- 0.5 µl Klenow polymerase

(40 U/µl, Invitrogen's Bioprime CGH kit, 18095-011)

4. Mix and incubate at **37°C for at least 4 hrs (up to O/N)** in the dark
5. Purification with Qiagen minElute columns: Use the columns according to the regular protocol in the manual but elute with 20 µl EB instead of 10 µl into a brown Eppendorf tube.
6. Quantify the DNA content and the specific activity with the Nanodrop spectrometer. Hybridize 1 µg of DNA per channel to genomic micorarrays.

- Combine the respective cy3 and cy5-labelled samples and speed-vac dry.

Hybridization of Microarrays in Corning hybridization chambers

- Resuspend the speed-vac dried labeled probe in **35 µl** of hybridization mix.

Hybridization Mix:

35 ml per slide working concentration	stock solution	1x	3x	5x	7x	9x	11x
3x SSC	20x SSC	6 µl	18 µl	30 µl	42 µl	54 µl	66 µl
1% SDS	10% SDS	4 µl	12 µl	20 µl	28 µl	36 µl	44 µl
5x Denhardt's	50x Denhardt's	4 µl	12 µl	20 µl	28 µl	36 µl	44 µl
0.8mg/ml polyA	10mg/ml polyA	3.2 µl	9.6 µl	16 µl	22.4 µl	28.8 µl	35.2 µl
50% Formamide	100% Formamide	20 µl	60 µl	100 µl	140 µl	180 µl	220 µl

- Pipet 15 µl 3x SSC into each humidifier well of the chamber and add an additional 10 µl 3xSSC to each side of the slide in the chamber, to provide humidity.
- Clean a suitable coverslip with compressed air to remove any particles and set it aside
- Boil the resuspended probe at 100°C in a heat block for 2 minutes, quick spin (10s at >10.000g), and let it cool down at RT for 10s.
- Pipet the probe onto the centre of the coverslip, avoid bubbles !
- Immediately lay down the slide, cDNA facing down, over the cover slip, again avoid bubbles !
- Put the slide into a hybridization chamber, attach the lid of the chamber, seal and submerge it in a 42°C water bath. Make sure the chamber is level. Hybridize for 12-16h in the dark.

Post-hybridisation Washing of the microarrays

8. Prepare wash solutions #1 (1X SSC / 0.03% SDS), #2 (0.2X SSC), and #3 (0.05X SSC)

	Wash 1	Wash2	Wash3
<i>dH2O</i>	237	248	250
<i>20X SSC</i>	12.5	2.5	0.63
<i>10% SDS</i>	0.75	--	--
<i>Final vol.:</i>	250 mL	250 mL	250 mL

9. Pour the wash solutions in suitable glass chambers fitted with slide holders.
10. Disassemble the hybridization chamber and quickly submerge each array in wash #1. (If the array is exposed to air while the cover slip starts to fall off, you may see high background fluorescent signal on the side of the array.)
11. Let the array sit in wash #1 until the cover slip slides off.
12. Gently plunge the slide rack up and down several times to wash the array; be sure not to scratch the array with the loose cover slip
13. Transfer each array to the slide rack in wash #2 using two forceps, dip the array very briefly onto lint-free paper towel in between. Wash the arrays again for 2 min.
14. Move each array from wash#2 to wash #3 with forceps. Wash a third time for 2 min. (It is critical to remove all SDS.)
15. Take the slide rack out of the washing solution and blot it very briefly on a lint free paper towel before centrifuging it on microtiter plate carriers (place paper towels below rack to absorb the liquid) for 5', 800 rpm.
16. Scan the array immediately.