

ChIP and DamID

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Chromatin immunopurification (ChIP) and DNA adenine methyltransferase identification (DamID) microarray:

ChIP

- ChIP for fly genomic DNA using Affymetrix arrays ([jump](#))
- ChIP for fly genomic DNA using Nimblegen arrays ([jump](#))

DamID

- DamID with Affymetrix microarrays ([jump](#))
- DamID with Nimblegen microarrays ([jump](#))

Nimblegen microarray hybridisation

- Nimblegen processing of ChIP or DamID samples on 2.1M microarrays ([jump](#))

Affymetrix microarray hybridisation

- Affymetrix processing of ChIP or DamID DNA GeneChip Drosophila tiling 2.0R arrays ([jump](#))

ChIP for fly genomic DNA using Affymetrix arrays

Outline

The protocol described here has been published by Sandmann et al. (2006) Nat Protoc 1(6): 2839-55 ([abstract](#)), with modifications by Nich Phochanukul and Jelena Aleksic.

Before you start:

The PCR amplification step varies according to the microarray format that will be used, so first decide whether you are going to use [Affymetrix](#) or [Nimblegen](#) microarrays and then follow the appropriate protocol.

This protocol produces 4 ml of crosslinked chromatin, from 1.5 g of crosslinked embryos, which is enough for ~10 IP reactions. Biological replicates are required for microarrays, and 3 is recommended for reliable results, so note that you will have to collect 4.5 g of embryos in total 3 x 1.5 g (one for each biological replicate). For experiments where collecting enough embryos is difficult, downscaled protocols exist the main difference is that a different size of Douncer / homogeniser would be used, as the quantity of embryos is important for the homogenisation to work well using a large Douncer.

1. [Equipment and reagents](#)
2. [Prepare solutions](#)
3. [Embryo collection and crosslinking](#)
4. [Sonication](#)
5. [Chromatin quality control](#)
6. [Immunopurification and decrosslinking](#)
7. [Amplification](#)

1. Equipment and reagents

- SB2 Fixed Speed Rotator, set at 60 °C incline (Stuart)
- Rotary shaker Rotatest R100/TW (Luckham)
- Avanti J-E Centrifuge with Rotor J.S-5.3 (Beckman Coulter)
- Micro 20 centrifuge (Hettich)
- Hot block (Grant, QBT2)
- Water bath
- PCR Machine (Dyad Thermal Cycler)
- Speed vac (Savant)
- Spectrophotometer (Nanadrop, ND-100)
- Gel electrophoresis equipment
- Bioruptor sonicator water batch (Diageode)
- 15 ml dounce homogeniser, Wheaton (VWR; Cat. No. 432-1272)
- Liquid nitrogen
- 15 ml Falcon tubes
- 50 ml Falcon tubes
- Commercial bleach
- n-Heptane (Sigma; Cat. No. H9629)
- Methanol (Merck; Cat. No. 106009)
- Protease inhibitor cocktail tablets (Roche; Cat. No. 04693124001)
- 2 ml phase-lock heavy gel tubes (Eppendorf; Cat. No. 0032-005-152)
- RNase A (Qiagen; Cat. No. 1006693)
- Proteinase K (Roche, Cat. No. 745723)
- Phenol:chloroform:isoamylalcohol (Ambion; Cat. No. 9732)
- Chloroform (Sigma; Cat. No. C2432)
- Glycogen (Roche; Cat. No. 901393)
- Salmon Sperm DNA/Protein A Agarose (Millipore; Cat. No. 16-157)
- Bovine serum albumin (BSA) (Sigma; Cat. No. A7906)
- beta-galactosidase antibody (Abcam; Cat. No. ab616)
- T4 DNA polymerase (Promega; Cat. No. M4211)
- T4 DNA ligase (Invitrogen; Cat. No. 15224-041)

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- Taq DNA polymerase, 5U/μl (Roche; Cat. No. 11146173001)
- 100mM Deoxynucleotide set (Sigma; Cat. No. DNTP-100A)
- QIAquick PCR purification kit (Qiagen; Cat. No. 28104)
- 24-mer oligonucleotide: 5'-AGA AGC TTG AAT TCG AGC AGT CAG-3', 5' phosphorylated
- 20-mer oligonucleotide: 5'-CTG CTC GAA TTC AAG CTT CT-3'
- Sodium dodecyl sulphate, SDS (BDH; Cat. No. 444464T)
- Triton X-100 (Sigma; Cat. No. T8787)
- Sodium chloride (Sigma; Cat. No. S3014)
- Potassium chloride (Sigma; Cat. No. P9541)
- Disodium hydrogen orthophosphate (Fluka BioChemika; Cat. No. 71643)
- Sodium dihydrogen orthophosphate (BDH; Cat. No. 444433M)
- Sodium acetate (BDH; Cat. No. 102364Q)
- Ethylenediaminetetraacetic acid disodium salt dihydrate, EDTA (Sigma; Cat. No. E5134)
- Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid, EGTA (Sigma; Cta. No. E4378)
- Formaldehyde (36.5-38%) (Sigma; Cat. No. F8775)
- HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Sigma; Cat. No. H3375)
- Glycine (BDH; Cat. No. 101194M)
- IGEPAL CA-630 (v/v) (Sigma; Cat. No. I8896)
- N-lauroylsarcosine sodium salt (Sigma; Cat. No. L9150)
- Tris hydrochloride (Sigma; Cat. No. T5941)
- Deoxycholate (xxx; Cat. No.)
- Sodium deoxycholate (Sigma; Cat. No. D6750)
- Lithium chloride (Sigma; Cat. No. L9650)
- NP-40 (Fluka, BioChemika; Cat. No. 74385)
- Aminoallyl-UTP-sodium salt (Sigma; Cat. No. A-5660)

2. Prepare Solutions

10 x PBS, pH 7.4:

- 80 g Sodium chloride
- 2 g Potassium chloride
- 14.4 g Disodium hydrogen orthophosphate
- 2.4 g Sodium dihydrogen orthophosphate
- Make to a total volume of 1 litre

PBT:

- 0.1% Triton X-100 (v/v) in PBS

Crosslinking solution:

- 1 mM EDTA
- 0.5 mM EGTA
- 100 mM NaCl
- 1.8%, formaldehyde (v/v)
- 50 mM HEPES, pH 8.0

Note: this solution including formaldehyde cannot be stored for longer periods. Make a stock solution excluding formaldehyde, then add the formaldehyde to an aliquot of the stock solution immediately before use.

Stop solution:

- 125 mM glycine; 0.1% Triton X-100 (v/v) in PBS

Cell lysis buffer:

- 85 mM KCl
- 0.5% IGEPAL CA-630 (v/v)

- 5 mM HEPES, pH 8.0

Nuclear lysis buffer:

- 10 mM EDTA
- 0.5% N-lauroylsarcosine (w/v)
- 50 mM HEPES, pH 8.0

TE buffer:

- 1 mM EDTA
- 10 mM Tris-HCl, pH 8.0

12% sodium deoxycholate:

- Dissolve 2.4 g sodium deoxycholate in 20 ml water

RIPA buffer:

- 1 mM EDTA pH 8
- 10 mM Tris-HCl pH 8
- 140 mM NaCl
- 0.1% SDS
- 0.1% sodium deoxycholate
- 1% Triton X-100

RIPA500 buffer:

- 1 mM EDTA pH 8
- 10 mM Tris-HCl pH 8
- 500 mM NaCl
- 0.1% SDS
- 0.1% sodium deoxycholate
- 1% Triton X-100

LiCl buffer:

- 250 mM LiCl
- 0.5% sodium deoxycholate
- 1 mM EDTA
- 10 mM Tris-HCl pH 8.0
- 0.5% NP-40

Annealed linkers:

- 24-mer oligonucleotide: AGA AGC TTG AAT TCG AGC AGT CAG, 5' phosphorylated
- 20-mer oligonucleotide: CTG CTC GAA TTC AAG CTT CT

Resuspend each oligonucleotide in water to a final concentration of 100 μ M. Mix equal amounts of 24- and 20-mer oligonucleotides in a 1.5 ml reaction tube. Incubate the tube in a beaker with approximately 400 ml boiling water for 5 minutes, move the beaker from the heat plate and let the water slowly cool to room temperature. Annealed linkers can be stored at -20 °C for years. Dilute an aliquot to 1 μ M before use in a ligation reaction. Alternatively, a slow temperature ramp in a thermocycler can be used to generate double-stranded linkers.

3. Embryo collection and crosslinking

Overview

The aim of this stage is to collect enough correctly staged crosslinked embryos. The embryos can be collected over time, and stored at -80 °C until a sufficient quantity is available for carrying on with the experiment. 1.5 g is required for each biological replicate, and it is recommended to perform 3 biological replicates for each ChIP experiment, so 4.5 g in total is required (each 1.5 g of embryos should be kept separate from the other two at all times, to ensure you have independent biological replicates).

Timing: 1 hour

Protocol

Unless indicated otherwise, centrifugation and incubation steps are performed at room temperature (20-25 °C).

1. Collect large amounts (1.5 g total required) of staged wild-type or transgenic embryos from population cages and dechorionate them in 50% commercial bleach at room temperature for 5 minutes.
2. Transfer the embryos into sieves and wash them well with tap water. Resuspend them in 10 ml PBT, in a 50ml Falcon tube (transfer into PBT using a brush or a small metal spatula).
3. Pellet the embryos by centrifugation at 500g for 1 minute, and carefully take out and discard the supernatant without disturbing the pellet.
4. Add 10 ml crosslinking solution + 30 ml heptane, and shake the tube vigorously at room temperature (20-25 °C) for 20 minutes using a Luckham Rotatest R100/TW rotary shaker at the maximum rotation speed setting. The time required for this step should be kept constant between repeated collections. Importantly, as some proteins are more easily crosslinked to chromatin than others, the formaldehyde concentration/length of crosslinking reaction might require optimization for different proteins of interest.
5. Pellet the embryos in each tube by centrifugation at 500g for 1 minute, replace the supernatant with 30 ml stop solution and shake vigorously by hand at room temperature for at least 1 minute to stop the crosslinking reaction.
6. Pellet the embryos by centrifugation at 500g for 1 minute, carefully decant the supernatant and wash the pellet with 50 ml PBT.
7. Pellet the embryos by centrifugation at 500g for 1 minute, decant the supernatant and resuspend the embryos in approximately 10 ml PBT per tube.
8. Transfer the embryos into a 1.5 ml tube (*Tip: cut off the tip of a 1 ml pipette tip, leave the embryos to settle, then discarding the supernatant*).
9. QC point: 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 devitellinize the embryos, let them settle and then remove as much liquid as possible. Wash the embryos with methanol twice and store at in the freezer at -20 °C. This sample from the collection is set aside to evaluate if the collected embryos are at the correct developmental stage.
10. Centrifuge the embryos (~500g for 1 minute) so that they settle at the bottom of the tube, and take off the supernatant (ideally, the embryos should be dry). Weigh the embryos (*Tip: use an empty 1.5ml tube to zero the scales*).
11. Freeze the embryos in liquid nitrogen. (*Tip: proceed straight to the next step if you have 1.5 g of embryos the freezing step is there to help collect enough embryos over time*).

PAUSE POINT Crosslinked embryo collections can be stored at -80 °C for at least 1 year.

4. Sonication

Overview

This part of the protocol involves using a Dounce homogeniser to disrupt the cell and nuclear membranes in order to extract the chromatin. The chromatin is then sonicated using a Bioruptor, in order to fragment it. Each 1.5 g aliquot of crosslinked embryos provides sufficient material for at least ten immunoprecipitations. Note that the homogenisation step is sensitive to the quantity and volume of embryos present, so a smaller homogeniser should be used if the protocol is being downscaled.

Timing: 1.5 hours

Protocol

1. Thaw each 1.5 g embryo sample at room temperature, resuspend them in 15 ml cold PBT supplemented with protease inhibitors, and transfer the suspension into a Dounce homogenizer.
2. Homogenize each 1.5 g aliquot of embryos in a Dounce homogenizer on ice by applying 20 strokes with the loose-fitting pestle.
3. Transfer the lysate into a suitable centrifuge tube and centrifuge at 400g at 4 °C for 1 minute to precipitate the vitelline membranes and large debris.
4. Decant the supernatant into a fresh centrifuge tube and centrifuge at 1,100g at 4 °C for 10 minutes. Decant and discard the supernatant.
5. Resuspend the cell pellet in 15 ml cold cell lysis buffer supplemented with protease inhibitors.
6. Homogenize the cells in a 15 ml Dounce homogenizer on ice by applying 20 strokes with the tight-fitting pestle. Split the sample into two approximately 8 ml aliquots in two separate 15 ml Falcon tubes.
7. Centrifuge the samples at 2,000g at 4 °C for 4 minutes to pellet the nuclei. Discard the supernatant.

PAUSE POINT Nuclei can be frozen in liquid nitrogen and can be stored for at least 1 year at -80 °C.

8. Resuspend each pellet in 1 ml of cold nuclear lysis buffer supplemented with protease inhibitors and incubate at room temperature for 20 minutes.
9. Add 1 ml of cold nuclear lysis buffer supplemented with protease inhibitors to each sample, and sonicate using a precooled Bioruptor sonicator water bath (15 seconds on / 15 seconds off cycles, low-energy settings, for 5 minutes). Ensure that the water bath remains cold by adding small amounts of ice in between sonication cycles.
10. Transfer the chromatin into 1.5 ml Eppendorf tubes and centrifuge at 20,000g at 4 °C for 10 minutes.
11. Pool the supernatants to ensure a homogenous sample, remove 50 µl for quality assessment and freeze the remaining chromatin in 400 µl aliquots in liquid nitrogen.

PAUSE POINT The samples can be stored at -80 °C for at least 1 year.

4. Chromatin quality control

Overview

The purpose of this stage is to verify that the chromatin has been extracted and fragmented to the expected sizes, before proceeding to the immunopurification step.

Timing: 2 days, includes overnight incubation

Protocol

1. To determine the yield and average fragment length of the chromatin preparations, dilute the 50 µl of chromatin set aside after sonication with 50 µl buffer TE.
2. Add RNase to 50 µg/ml, and incubate at 37 °C for 30 minutes.
3. Add 5 µl of 10% SDS, 2.5 µl proteinase K (20 mg/ml), then incubate at 37 °C overnight. (*Tip: perform the incubations in a water bath, as this ensures that the samples are heated evenly*).
4. Incubate at 65 °C for 6 hours.
5. Pre-spin the phase-lock tubes at 15,000g for 20-30 seconds.
6. Working inside the fume cupboard, add 300 µl Phenol:Chloroform:Isoamylalcohol pH 8.0 (use the lower phase and do not shake the bottle). Apply the sample to the phase-lock tube, shake vigorously for 15 seconds (do not vortex!), centrifuge at 15,000g for 5 minutes.
7. Add 300 µl of chloroform, shake vigorously, centrifuge at 15,000g for 5 minutes.
8. Carefully transfer the aqueous upper phase to new tubes (~100 µl).
9. Add 1.25 µl of glycogen (20mg/ml stock), 25 µl of 3M sodium acetate pH5.2 and 550 µl of 100% ethanol.
10. Mix thoroughly, and incubate at -80 °C for at least 30 minutes.
11. Centrifuge at 15,000g for 30 minutes at 4 °C to precipitate DNA.
12. Wash the pellet once with 1 ml of 70% ethanol, centrifuge at 15,000g for 10 minutes at 4 °C.

- Air-dry the pellet and resuspend in 50 µl milliQ H₂O. Determine the concentration of sheared DNA spectrometrically and inspect its size distribution by gel electrophoresis using a 1% agarose gel. The Bioruptor settings used in this protocol give rise to an average fragment length of approximately 500 bp. Check if the samples have been sheared to comparable fragments lengths within the desired range.

5. Immunopurification and decrosslinking

Overview

Polyclonal antibodies should be precleared with methanol-stained embryos to increase their specificity (this step is not necessary for commercially available monoclonal antibodies). The sample is precleared with the Salmon Sperm DNA/Protein A Agarose beads (from now on referred to as ProtA Agarose beads in the text), to remove any particles that would unspecifically bind to them, and then incubated with the antibodies overnight. The ProtA Agarose beads to be used with the sample later in the protocol are pre-blocked with BSA overnight, to reduce non-specific binding of chromatin.

Day 1: (~6 hours total)

- Preclear antibody (~3 hours)
- Preclearing ProtA Agarose beads, sample preclearing, adding antibodies (~3 hours)

Day 2: (~7.5 hours total)

- Adding ProtA Agarose beads, followed by an incubation (3 hours)
- Wash steps (3 hours)
- Prepare sample for decrosslinking (1 hour)

Day 3 (~8 hours total):

- Move sample to hot block, then incubate (6 hours)
- Crosslink reversal and DNA purification (~2 hours)

Protocol

DAY 1

Pre-clearing the antibody

This step is not necessary for commercially available purified antibodies please skip to step 7, and use the "control mix" quantities for both the antibody of interest and the control antibody.

1. Wash 50-100 µl of methanol-fixed embryos in PBT (this separates the embryos).
2. Wash 3x 15 minutes in 1 ml PBS.
3. Make the following premix (in the given order):
 - ◆ 160 µl TE buffer
 - ◆ 40 µl 10% Triton-X 100 (final: 1%)
 - ◆ 40 µl 12% deoxycholate (final: 0.1%)
 - ◆ 40 µl 1% SDS (final: 0.1%)
 - ◆ 40 µl 1.4 M NaCl (final: 0.14M)
4. Add sample antibody to the premix above. (Quantities vary depending on the antibody, 0.5-3 µg of the appropriate antibody is normally sufficient).
5. Incubate antibody with 50-100 µl methanol-fixed embryos at 4 °C for 2-4 hours.
6. Pre-cool TE buffer (2 ml) in the fridge for later.

Adding ProtA Agarose beads and sample preclearing

7. Defrost the 400 µl sonicated chromatin samples on ice.
8. Pool 25 µl of 50% ProtA Agarose beads (salmon sperm DNA/ProtA agarose) suspension per precipitation (including the controls), and wash the beads once with 1 ml RIPA buffer.

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9. Pellet the ProtA Agarose beads by centrifugation at 1,000g for 2 minutes, discard the supernatant, and resuspend in 100 µl RIPA buffer per 25 µl ProtA beads.
10. Take the defrosted 400 µl aliquot of chromatin, centrifuge at maximum speed for 1 minute at 4 °C to precipitate the SDS, then **keep the supernatant** and discard the precipitate. (*There should be a large white precipitate at the bottom of the tube*)
11. Split into 200 µl reactions, and adjust the volume with cold TE buffer to 300 µl for the sample antibody reaction and 500 µl for the control reaction. *When using commercially available purified antibodies, use the "control mix" quantities for both the antibody of interest and the control antibody.*
12. Add the solutions below to the **samples**, in the order given:
 - ◆ 60 µl 10% Triton-X100 (final: 1%)
 - ◆ 60 µl 12% deoxycholate (final: 0.1%)
 - ◆ 60 µl 1% SDS (final: 0.1%)
 - ◆ 60 µl 1.4 M NaCl (final: 0.14 M)Add the solutions below to the **controls**, in the order given:
 - ◆ 100 µl 10% Triton-X100 (final: 1%)
 - ◆ 100 µl 12% deoxycholate (final: 0.1%)
 - ◆ 100 µl 1% SDS (final: 0.1%)
 - ◆ 100 µl 1.4 M NaCl (final: 0.14 M)
13. Resuspend the ProtA Agarose beads from step 9, and add 100 µl to each sample. Incubate on a Stuart SB2 Fixed Speed rotating wheel (set at 60 ° incline) at 4 °C for 1 hour.
14. Pellet the beads by centrifugation at 1,000g, at 4 °C for 2 minutes. (*Removes chromatin nonspecifically binding to ProtA Sepharose*)
15. Transfer the supernatant to a new tube, avoiding any bead carryover. Retain 10 µl of the sample in a separate tube and store at 4 °C until later (*Tip: use instead 6.4 µl with 3.6 µl TE buffer for the sample antibody if you haven't used a commercial antibody.*).

Preabsorption and antibody addition

16. Take the 20 mg/ml BSA stock solution out of the freezer to defrost.
17. Add 5 µl of the beta-galactosidase antibody to the control sample, and 360 µl of the precleared antibody premix to main reaction sample. Incubate at 4 °C overnight on a rotation wheel.
18. For each precipitation, pool 25 µl of 50% ProtA suspension and wash with 1ml RIPA buffer supplemented with 1 mg/ml BSA. Pellet the beads by centrifugation at 1,000g for 2 minutes, and incubate in 1 ml RIPA buffer supplemented with 1 mg/ml BSA on a Stuart SB2 Fixed Speed rotating wheel at 4 °C overnight.

DAY 2

Purification of immunocomplexes

19. Centrifuge the preblocked ProtA Agarose beads at 1,000g for 2 minutes, discard the supernatant, and resuspend the beads in 100 µl RIPA buffer per reaction.
20. Precool some RIPA buffer at 4 °C for later.
21. Add 100 µl of ProtA Agarose bead suspension to each 1 ml chromatin sample, and incubate on a rotating wheel at 4 °C for 3 hours.
22. Pellet the beads by centrifugation at 1,000g for 2 minutes, discard the supernatant, and rinse the beads once with 1 ml cold RIPA buffer.
23. Pellet again at 1,000g for 2 minutes, then wash with 1 ml of each of the following solutions and incubate at 4 °C on a rotating wheel for 10 minutes each time:
 - ◆ 1x with RIPA buffer
 - ◆ 4x with RIPA500
 - ◆ 1x with LiCl buffer
 - ◆ 2x with TE buffer
24. Resuspend the beads in 100 µl TE buffer supplemented with 50 µg/ml RNase A, and incubate at 37 °C for 30 minutes. From this point on, also include the 10 µl input chromatin samples increase the volume to 100 µl by adding 90 µl TE buffer, then add RNase and include with the other samples.
25. Add 5 µl of 10% SDS, 2.5 µl proteinase K (20 mg/ml), then incubate at 37 °C overnight. *Tip: Perform the incubations in a water bath, as this way the samples are heated more evenly.*

DAY 3

Crosslink reversal and DNA purification

26. Incubate at 65 °C for 6 hours.
27. Pre-spin the phase-lock tubes at 15,000g for 20-30 seconds.
28. Working inside the fume cupboard, add 300 µl Phenol:Chloroform:Isoamylalcohol pH 8.0 (use the lower phase and do not shake the bottle).
29. Apply the sample to the phase-lock tube, shake vigorously for 15 seconds (do not vortex!), centrifuge at 15,000g for 5 minutes.
30. Add 300 µl of chloroform, shake vigorously, centrifuge at 15,000g for 5 minutes.
31. Carefully transfer the aqueous upper phase to new tubes (~100 µl left).
32. Add 1.25 µl of glycogen (20 mg/ml stock), 25 µl of 3M sodium acetate pH5.2, and 550 µl of 100% ethanol.
33. Mix thoroughly, and incubate at -80 °C for at least 30 minutes.
34. Centrifuge at 15,000g for 30 minutes at 4 °C to precipitate DNA.
35. Wash the pellet once with 1 ml of 70% ethanol, centrifuge at 15,000g for 10 minutes at 4 °C.
36. Air-dry the pellet and resuspend in 30 µl miliQ H₂O. Store at -20 °C.

6. Amplification

Overview

Day 1

- Generation of blunt ends (~1h)
- Purification and linker ligation (~1h, then overnight)

Day 2

- PCR 1 (~2.5h)
- PCR 2 (~2.5h)

Final QC steps

- Run gel (~1.5h)
- Purify PCR product (~1h)

Protocol

DAY 1

Generating blunt ends

1. Set up the reaction below in PCR tubes and incubate at 37 °C for 30 minutes. Include a "no DNA" sample containing water as a contamination control.
 - ◆ 7 µl Sample DNA
 - ◆ 5 µl 10x T4 polymerase buffer
 - ◆ 2.5µl 2mM dNTPs
 - ◆ 1 µl T4 DNA polymerase (5 U/µl)
 - ◆ 34.5 µl Water
2. Purify using the Qiagen Qiaquick PCR purification kit.
3. Dry using the speed-vac, then resuspend in 6 µl water.

Ligation

4. In the same tube, set up the following reaction. Add another "no DNA" control at this step. Vortex the buffer to remove of any precipitation.
 - ◆ 6 µl DNA sample from step 3
 - ◆ 2 µl 5x ligation buffer
 - ◆ 1 µl Annealed linkers (1 µM)

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- ◆ 1 µl T4 DNA ligase (5 U/µl)
 - ◆ Water up 10 µl total.
5. Incubate at 16 °C overnight.

DAY 2

LM-PCR

The protocol requires to perform two PCR amplifications to obtain enough DNA for the labeling reactions. The reason for this is to stay in the linear range during the amplification.

IMPORTANT: If you are planning on using Nimblegen arrays please refer to the ChIP for fly genomic DNA using Nimblegen arrays for the following steps!

6. Set up the PCR reaction below in the same tube.
- ◆ 10 µl Ligation from step 5
 - ◆ 10 µl 10x TaqDNA buffer (15 mM Mg²⁺)
 - ◆ 10 µl 2mM dNTPs
 - ◆ 1 µl TaqDNA polymerase (5 U/µl)
 - ◆ 1 µl 100 µM 20-mer oligo
 - ◆ Water up to 68 µl total.
7. Run PCR program:
- ◆ 55 °C 2 min
 - ◆ 72 °C 5 min
 - ◆ 94 °C 5 min
 - ◆ 94 °C 1 min
 - ◆ 55 °C 1 min
 - ◆ 72 °C 1 min
 - ◆ Repeat steps 4-6 for 19 cycles
 - ◆ 72 °C 5 min
 - ◆ 4 °C hold.
8. Take 10 µl of above PCR reaction. Make sure you use the dNTP/dUTP mix in the following PCR!
- ◆ 10 µl PCR product from step 7
 - ◆ 10 µl 10x TaqDNA buffer (15 mM Mg²⁺)
 - ◆ 2 µl dNTP/dUTP mix for Affy (10mM dCTP,10mM dATP,10mM dGTP, 8mM dTTP, 2mM dUTP)
 - ◆ 1 µl TaqDNA polymerase (5 U/µl)
 - ◆ 1 µl 100 µM 20-mer oligo
 - ◆ Water up to 68 µl total.
9. Run PCR program:
- ◆ 55 °C 2 min
 - ◆ 72 °C 5 min
 - ◆ 94 °C 5 min
 - ◆ 94 °C 1 min
 - ◆ 55 °C 1 min
 - ◆ 72 °C 1 min
 - ◆ Repeat steps 4-6 for 19 cycles
 - ◆ 72 °C 5 min
 - ◆ 4 °C hold.

Quality control

10. Analyse 5 µl of the final PCR product on a 1% agarose gel to see if the sample has been amplified.
11. Purify using the QiaQuick PCR purification kit.
12. Measure concentration using a Nanodrop.

For hybridisation to Affymetrix GeneChip Drosophila Tiling 2.0R arrays 7.5 µg amplified DNA is required for each sample and control. Continue with [Affymetrix Processing of ChIP or DamID DNA to GeneChip Drosophila Tiling 2.0R Array](#) protocol.

ChIP for fly genomic DNA using Nimblegen arrays

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- 2 ml phase-lock heavy gel tubes (Eppendorf; Cat. No. 0032-005-152)
- RNase A (Qiagen; Cat. No. 1006693)
- Proteinase K (Roche, Cat. No. 745723)
- Phenol:chloroform:isoamylalcohol (Ambion; Cat. No. 9732)
- Chloroform (Sigma; Cat. No. C2432)
- Glycogen (Roche; Cat. No. 901393)
- Salmon Sperm DNA/Protein A Agarose (Millipore; Cat. No. 16-157)
- Bovine serum albumin (BSA) (Sigma; Cat. No. A7906)
- beta-galactosidase antibody (Abcam; Cat. No. ab616)
- T4 DNA polymerase (Promega; Cat. No. M4211)
- T4 DNA ligase (Invitrogen; Cat. No. 15224-041)

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- Taq DNA polymerase, 5U/μl (Roche; Cat. No. 11146173001)
- 100mM Deoxynucleotide set (Sigma; Cat. No. DNTP-100A)
- QIAquick PCR purification kit (Qiagen; Cat. No. 28104)
- 24-mer oligonucleotide: 5'-AGA AGC TTG AAT TCG AGC AGT CAG-3', 5' phosphorylated
- 20-mer oligonucleotide: 5'-CTG CTC GAA TTC AAG CTT CT-3'
- Sodium dodecyl sulphate, SDS (BDH; Cat. No. 444464T)
- Triton X-100 (Sigma; Cat. No. T8787)
- Sodium chloride (Sigma; Cat. No. S3014)
- Potassium chloride (Sigma; Cat. No. P9541)
- Disodium hydrogen orthophosphate (Fluka BioChemika; Cat. No. 71643)
- Sodium dihydrogen orthophosphate (BDH; Cat. No. 444433M)
- Sodium acetate (BDH; Cat. No. 102364Q)
- Ethylenediaminetetraacetic acid disodium salt dihydrate, EDTA (Sigma; Cat. No. E5134)
- Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid, EGTA (Sigma; Cta. No. E4378)
- Formaldehyde (36.5-38%) (Sigma; Cat. No. F8775)
- HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Sigma; Cat. No. H3375)
- Glycine (BDH; Cat. No. 101194M)
- IGEPAL CA-630 (v/v) (Sigma; Cat. No. I8896)
- N-lauroylsarcosine sodium salt (Sigma; Cat. No. L9150)
- Tris hydrochloride (Sigma; Cat. No. T5941)
- Deoxycholate (xxx; Cat. No.)
- Sodium deoxycholate (Sigma; Cat. No. D6750)
- Lithium chloride (Sigma; Cat. No. L9650)
- NP-40 (Fluka, BioChemika; Cat. No. 74385)

2. Prepare Solutions

10 x PBS, pH 7.4:

- 80 g Sodium chloride
- 2 g Potassium chloride
- 14.4 g Disodium hydrogen orthophosphate
- 2.4 g Sodium dihydrogen orthophosphate
- Make to a total volume of 1 litre

PBT:

- 0.1% Triton X-100 (v/v) in PBS

Crosslinking solution:

- 1 mM EDTA
- 0.5 mM EGTA
- 100 mM NaCl
- 1.8%, formaldehyde (v/v)
- 50 mM HEPES, pH 8.0

Note: this solution including formaldehyde cannot be stored for longer periods. Make a stock solution excluding formaldehyde, then add the formaldehyde to an aliquot of the stock solution immediately before use.

Stop solution:

- 125 mM glycine; 0.1% Triton X-100 (v/v) in PBS

Cell lysis buffer:

- 85 mM KCl
- 0.5% IGEPAL CA-630 (v/v)
- 5 mM HEPES, pH 8.0

Nuclear lysis buffer:

- 10 mM EDTA
- 0.5% N-lauroylsarcosine (w/v)
- 50 mM HEPES, pH 8.0

TE buffer:

- 1 mM EDTA
- 10 mM Tris-HCl, pH 8.0

12% sodium deoxycholate:

- Dissolve 2.4 g sodium deoxycholate in 20 ml water

RIPA buffer:

- 1 mM EDTA pH 8
- 10 mM Tris-HCl pH 8
- 140 mM NaCl
- 0.1% SDS
- 0.1% sodium deoxycholate
- 1% Triton X-100

RIPA500 buffer:

- 1 mM EDTA pH 8
- 10 mM Tris-HCl pH 8
- 500 mM NaCl
- 0.1% SDS
- 0.1% sodium deoxycholate
- 1% Triton X-100

LiCl buffer:

- 250 mM LiCl
- 0.5% sodium deoxycholate
- 1 mM EDTA
- 10 mM Tris-HCl pH 8.0
- 0.5% NP-40

Annealed linkers:

- 24-mer oligonucleotide: AGA AGC TTG AAT TCG AGC AGT CAG, 5' phosphorylated
- 20-mer oligonucleotide: CTG CTC GAA TTC AAG CTT CT

Resuspend each oligonucleotide in water to a final concentration of 100 μ M. Mix equal amounts of 24- and 20-mer oligonucleotides in a 1.5 ml reaction tube. Incubate the tube in a beaker with approximately 400 ml boiling water for 5 minutes, move the beaker from the heat plate and let the water slowly cool to room temperature. Annealed linkers can be stored at -20 °C for years. Dilute an aliquot to 1 μ M before use in a ligation reaction. Alternatively, a slow temperature ramp in a thermocycler can be used to generate double-stranded linkers.

3. Embryo collection and crosslinking

Overview

The aim of this stage is to collect enough correctly staged crosslinked embryos. The embryos can be collected over time, and stored at -80 °C until a sufficient quantity is available for carrying on with the experiment. 1.5 g is required for each biological replicate, and it is recommended to perform 3 biological

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replicates for each ChIP experiment, so 4.5 g in total is required (each 1.5 g of embryos should be kept separate from the other two at all times, to ensure you have independent biological replicates).

Timing: 1 hour

Protocol

Unless indicated otherwise, centrifugation and incubation steps are performed at room temperature (20-25 °C).

1. Collect large amounts (1.5 g total required) of staged wild-type or transgenic embryos from population cages and dechorionate them in 50% commercial bleach at room temperature for 5 minutes.
2. Transfer the embryos into sieves and wash them well with tap water. Resuspend them in 10 ml PBT, in a 50ml Falcon tube (transfer into PBT using a brush or a small metal spatula).
3. Pellet the embryos by centrifugation at 500g for 1 minute, and carefully take out and discard the supernatant without disturbing the pellet.
4. Add 10 ml crosslinking solution + 30 ml heptane, and shake the tube vigorously at room temperature (20-25 °C) for 20 minutes using a Luckham Rotatest R100/TW rotary shaker at the maximum rotation speed setting. The time required for this step should be kept constant between repeated collections. Importantly, as some proteins are more easily crosslinked to chromatin than others, the formaldehyde concentration/length of crosslinking reaction might require optimization for different proteins of interest.
5. Pellet the embryos in each tube by centrifugation at 500g for 1 minute, replace the supernatant with 30 ml stop solution and shake vigorously by hand at room temperature for at least 1 minute to stop the crosslinking reaction.
6. Pellet the embryos by centrifugation at 500g for 1 minute, carefully decant the supernatant and wash the pellet with 50 ml PBT.
7. Pellet the embryos by centrifugation at 500g for 1 minute, decant the supernatant and resuspend the embryos in approximately 10 ml PBT per tube.
8. Transfer the embryos into a 1.5 ml tube (*Tip: cut off the tip of a 1 ml pipette tip, leave the embryos to settle, then discarding the supernatant*).
9. QC point: 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 devitellinize the embryos, let them settle and then remove as much liquid as possible. Wash the embryos with methanol twice and store in the freezer at -20 °C. This sample from the collection is set aside to evaluate if the collected embryos are at the correct developmental stage.
10. Centrifuge the embryos (~500g for 1 minute) so that they settle at the bottom of the tube, and take off the supernatant (ideally, the embryos should be dry). Weigh the embryos (*Tip: use an empty 1.5ml tube to zero the scales*).
11. Freeze the embryos in liquid nitrogen. (*Tip: proceed straight to the next step if you have 1.5 g of embryos the freezing step is there to help collect enough embryos over time*).

PAUSE POINT Crosslinked embryo collections can be stored at -80 °C for at least 1 year.

4. Sonication

Overview

This part of the protocol involves using a Dounce homogeniser to disrupt the cell and nuclear membranes in order to extract the chromatin. The chromatin is then sonicated using a Bioruptor, in order to fragment it. Each 1.5 g aliquot of crosslinked embryos provides sufficient material for at least ten immunoprecipitations. Note that the homogenisation step is sensitive to the quantity and volume of embryos present, so a smaller homogeniser should be used if the protocol is being downscaled.

Timing: 1.5 hours

Protocol

1. Thaw each 1.5 g embryo sample at room temperature, resuspend them in 15 ml cold PBT supplemented with protease inhibitors, and transfer the suspension into a Dounce homogenizer.
2. Homogenize each 1.5 g aliquot of embryos in a Dounce homogenizer on ice by applying 20 strokes with the loose-fitting pestle.
3. Transfer the lysate into a suitable centrifuge tube and centrifuge at 400g at 4 °C for 1 minute to precipitate the vitelline membranes and large debris.
4. Decant the supernatant into a fresh centrifuge tube and centrifuge at 1,100g at 4 °C for 10 minutes. Decant and discard the supernatant.
5. Resuspend the cell pellet in 15 ml cold cell lysis buffer supplemented with protease inhibitors.
6. Homogenize the cells in a 15 ml Dounce homogenizer on ice by applying 20 strokes with the tight-fitting pestle. Split the sample into two approximately 8 ml aliquots in two separate 15 ml Falcon tubes.
7. Centrifuge the samples at 2,000g at 4 °C for 4 minutes to pellet the nuclei. Discard the supernatant.

PAUSE POINT Nuclei can be frozen in liquid nitrogen and can be stored for at least 1 year at -80 °C.

8. Resuspend each pellet in 1 ml of cold nuclear lysis buffer supplemented with protease inhibitors and incubate at room temperature for 20 minutes.
9. Add 1 ml of cold nuclear lysis buffer supplemented with protease inhibitors to each sample, and sonicate using a precooled Bioruptor sonicator water bath (15 seconds on / 15 seconds off cycles, low-energy settings, for 5 minutes). Ensure that the water bath remains cold by adding small amounts of ice in between sonication cycles.
10. Transfer the chromatin into 1.5 ml Eppendorf tubes and centrifuge at 20,000g at 4 °C for 10 minutes.
11. Pool the supernatants to ensure a homogenous sample, remove 50 µl for quality assessment and freeze the remaining chromatin in 400 µl aliquots in liquid nitrogen.

PAUSE POINT The samples can be stored at -80 °C for at least 1 year.

4. Chromatin quality control

Overview

The purpose of this stage is to verify that the chromatin has been extracted and fragmented to the expected sizes, before proceeding to the immunopurification step.

Timing: 2 days, includes overnight incubation

Protocol

1. To determine the yield and average fragment length of the chromatin preparations, dilute the 50 µl of chromatin set aside after sonication with 50 µl buffer TE.
2. Add RNase to 50 µg/ml, and incubate at 37 °C for 30 minutes.
3. Add 5 µl of 10% SDS, 2.5 µl proteinase K (20 mg/ml), then incubate at 37 °C overnight. (*Tip: perform the incubations in a water bath, as this ensures that the samples are heated evenly*).
4. Incubate at 65 °C for 6 hours.
5. Pre-spin the phase-lock tubes at 15,000g for 20-30 seconds.
6. Working inside the fume cupboard, add 300 µl Phenol:Chloroform:Isoamylalcohol pH 8.0 (use the lower phase and do not shake the bottle). Apply the sample to the phase-lock tube, shake vigorously for 15 seconds (do not vortex!), centrifuge at 15,000g for 5 minutes.
7. Add 300 µl of chloroform, shake vigorously, centrifuge at 15,000g for 5 minutes.
8. Carefully transfer the aqueous upper phase to new tubes (~100 µl).
9. Add 1.25 µl of glycogen (20mg/ml stock), 25 µl of 3M sodium acetate pH5.2 and 550 µl of 100% ethanol.
10. Mix thoroughly, and incubate at -80 °C for at least 30 minutes.
11. Centrifuge at 15,000g for 30 minutes at 4 °C to precipitate DNA.
12. Wash the pellet once with 1 ml of 70% ethanol, centrifuge at 15,000g for 10 minutes at 4 °C.
13. Air-dry the pellet and resuspend in 50 µl miliQ H₂O. Determine the concentration of sheared DNA spectrometrically and inspect its size distribution by gel electrophoresis using a 1% agarose gel. The

Bioruptor settings used in this protocol give rise to an average fragment length of approximately 500 bp. Check if the samples have been sheared to comparable fragments lengths within the desired range.

5. Immunopurification and decrosslinking

Overview

Polyclonal antibodies should be precleared with methanol-stained embryos to increase their specificity (this step is not necessary for commercially available monoclonal antibodies). The sample is precleared with Salmon Sperm DNA/Protein A Agarose beads (from now on referred to as ProtA Agarose beads in the text), to remove any particles that would unspecifically bind to them, and then incubated with the antibodies overnight. The ProtA Agarose beads to be used with the sample later in the protocol are pre-blocked with BSA overnight, to reduce non-specific binding of chromatin.

Day 1: (~6 hours total)

- Preclear antibody (~3 hours)
- Preclearing ProtA Agarose beads, sample preclearing, adding antibodies (~3 hours)

Day 2: (~7.5 hours total)

- Adding ProtA Agarose beads, followed by an incubation (3 hours)
- Wash steps (3 hours)
- Prepare sample for decrosslinking (1 hour)

Day 3 (~8 hours total):

- Move sample to hot block, then incubate (6 hours)
- Crosslink reversal and DNA purification (~2 hours)

Protocol

DAY 1

Pre-clearing the antibody

This step is not necessary for commercially available purified antibodies please skip to step 7, and use the "control mix" quantities for both the antibody of interest and the control antibody.

1. Wash 50-100 μ l of methanol-fixed embryos in PBT (this separates the embryos).
2. Wash 3x 15 minutes in 1 ml PBS.
3. Make the following premix (in the given order):
 - ◆ 160 μ l TE buffer
 - ◆ 40 μ l 10% Triton-X 100 (final: 1%)
 - ◆ 40 μ l 12% deoxycholate (final: 0.1%)
 - ◆ 40 μ l 1% SDS (final: 0.1%)
 - ◆ 40 μ l 1.4 M NaCl (final: 0.14M)
4. Add sample antibody to the premix above. (Quantities vary depending on the antibody, 0.5-3 μ g of the appropriate antibody is normally sufficient).
5. Incubate antibody with 50-100 μ l methanol-fixed embryos at 4 °C for 2-4 hours.
6. Pre-cool TE buffer (2 ml) in the fridge for later.

Adding ProtA Agarose beads and sample preclearing

7. Defrost the 400 μ l sonicated chromatin samples on ice.
8. Pool 25 μ l of 50% ProtA Agarose beads (salmon sperm DNA/ProtA agarose) suspension per precipitation (including the controls), and wash the beads once with 1 ml RIPA buffer.
9. Pellet the ProtA Agarose beads by centrifugation at 1,000g for 2 minutes, discard the supernatant, and resuspend in 100 μ l RIPA buffer per 25 μ l ProtA beads.

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10. Take the defrosted 400 μ l aliquot of chromatin, centrifuge at maximum speed for 1 minute at 4 °C to precipitate the SDS, then **keep the supernatant** and discard the precipitate. (*There should be a large white precipitate at the bottom of the tube*)
11. Split into 200 μ l reactions, and adjust the volume with cold TE buffer to 300 μ l for the sample antibody reaction and 500 μ l for the control reaction. *When using commercially available purified antibodies, use the "control mix" quantities for both the antibody of interest and the control antibody.*
12. Add the solutions below to the **samples**, in the order given:
 - ◆ 60 μ l 10% Triton-X100 (final: 1%)
 - ◆ 60 μ l 12% deoxycholate (final: 0.1%)
 - ◆ 60 μ l 1% SDS (final: 0.1%)
 - ◆ 60 μ l 1.4 M NaCl (final: 0.14 M)Add the solutions below to the **controls**, in the order given:
 - ◆ 100 μ l 10% Triton-X100 (final: 1%)
 - ◆ 100 μ l 12% deoxycholate (final: 0.1%)
 - ◆ 100 μ l 1% SDS (final: 0.1%)
 - ◆ 100 μ l 1.4 M NaCl (final: 0.14 M)
13. Resuspend the ProtA Agarose beads from step 9, and add 100 μ l to each sample. Incubate on a Stuart SB2 Fixed Speed rotating wheel (set at 60 ° incline) at 4 °C for 1 hour.
14. Pellet the beads by centrifugation at 1,000g, at 4 °C for 2 minutes. (*Removes chromatin nonspecifically binding to ProtA Sepharose*)
15. Transfer the supernatant to a new tube, avoiding any bead carryover. Retain 10 μ l of the sample in a separate tube and store at 4 °C until later (*Tip: use instead 6.4 μ l with 3.6 μ l TE buffer for the sample antibody if you haven't used a commercial antibody.*).

Preabsorption and antibody addition

16. Take the 20 mg/ml BSA stock solution out of the freezer to defrost.
17. Add 5 μ l of the beta-galactosidase antibody to the control sample, and 360 μ l of the precleared antibody premix to main reaction sample. Incubate at 4 °C overnight on a rotation wheel.
18. For each precipitation, pool 25 μ l of 50% ProtA suspension and wash with 1ml RIPA buffer supplemented with 1 mg/ml BSA. Pellet the beads by centrifugation at 1,000g for 2 minutes, and incubate in 1 ml RIPA buffer supplemented with 1 mg/ml BSA on a Stuart SB2 Fixed Speed rotating wheel at 4 °C overnight.

DAY 2

Purification of immunocomplexes

19. Centrifuge the preblocked ProtA Agarose beads at 1,000g for 2 minutes, discard the supernatant, and resuspend the beads in 100 μ l RIPA buffer per reaction.
20. Precool some RIPA buffer at 4 °C for later.
21. Add 100 μ l of ProtA Agarose bead suspension to each 1 ml chromatin sample, and incubate on a rotating wheel at 4 °C for 3 hours.
22. Pellet the beads by centrifugation at 1,000g for 2 minutes, discard the supernatant, and rinse the beads once with 1 ml cold RIPA buffer.
23. Pellet again at 1,000g for 2 minutes, then wash with 1 ml of each of the following solutions and incubate at 4 °C on a rotating wheel for 10 minutes each time:
 - ◆ 1x with RIPA buffer
 - ◆ 4x with RIPA500
 - ◆ 1x with LiCl buffer
 - ◆ 2x with TE buffer
24. Resuspend the beads in 100 μ l TE buffer supplemented with 50 μ g/ml RNase A, and incubate at 37 °C for 30 minutes. From this point on, also include the 10 μ l input chromatin samples increase the volume to 100 μ l by adding 90 μ l TE buffer, then add RNase and include with the other samples.
25. Add 5 μ l of 10% SDS, 2.5 μ l proteinase K (20 mg/ml), then incubate at 37 °C overnight. *Tip: Perform the incubations in a water bath, as this way the samples are heated more evenly.*

DAY 3

Crosslink reversal and DNA purification

26. Incubate at 65 °C for 6 hours.
27. Pre-spin the phase-lock tubes at 15,000g for 20-30 seconds.
28. Working inside the fume cupboard, add 300 µl Phenol:Chloroform:Isoamylalcohol pH 8.0 (use the lower phase and do not shake the bottle).
29. Apply the sample to the phase-lock tube, shake vigorously for 15 seconds (do not vortex!), centrifuge at 15,000g for 5 minutes.
30. Add 300 µl of chloroform, shake vigorously, centrifuge at 15,000g for 5 minutes.
31. Carefully transfer the aqueous upper phase to new tubes (~100 µl left).
32. Add 1.25 µl of glycogen (20 mg/ml stock), 25 µl of 3M sodium acetate pH5.2, and 550 µl of 100% ethanol.
33. Mix thoroughly, and incubate at -80 °C for at least 30 minutes.
34. Centrifuge at 15,000g for 30 minutes at 4 °C to precipitate DNA.
35. Wash the pellet once with 1 ml of 70% ethanol, centrifuge at 15,000g for 10 minutes at 4 °C.
36. Air-dry the pellet and resuspend in 30 µl miliQ H₂O. Store at -20 °C.

6. Amplification

Overview

Day 1

- Generation of blunt ends (~1h)
- Purification and linker ligation (~1h, then overnight)

Day 2

- PCR 1 (~2.5h)
- PCR 2 (~2.5h)

Final QC steps

- Run gel (~1.5h)
- Purify PCR product (~1h)

Protocol

DAY 1

Generating blunt ends

1. Set up the reaction below in PCR tubes and incubate at 37 °C for 30 minutes. Include a "no DNA" sample containing water as a contamination control.
 - ◆ 7 µl Sample DNA
 - ◆ 5 µl 10x T4 polymerase buffer
 - ◆ 2.5µl 2mM dNTPs
 - ◆ 1 µl T4 DNA polymerase (5 U/µl)
 - ◆ 34.5 µl Water
2. Purify using the Qiagen Qiaquick PCR purification kit.
3. Dry using the speed-vac, then resuspend in 6 µl water.

Ligation

4. In the same tube, set up the following reaction. Add another "no DNA" control at this step. Vortex the buffer to remove of any precipitation.
 - ◆ 6 µl DNA sample from step 3
 - ◆ 2 µl 5x ligation buffer
 - ◆ 1 µl Annealed linkers (1 µM)

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- ◆ 1 µl T4 DNA ligase (5 U/µl)
 - ◆ Water up 10 µl total.
5. Incubate at 16 °C overnight.

DAY 2

LM-PCR

The protocol requires to perform two PCR amplifications to obtain enough DNA for the labeling reactions. The reason for this is to stay in the linear range during the amplification.

IMPORTANT: If you are planning on using Affymetrix arrays please refer to the ChIP for fly genomic DNA using Affymetrix arrays for the following steps!

6. Set up the PCR reaction below in the same tube.
- ◆ 10 µl Ligation from step 5
 - ◆ 10 µl 10x TaqDNA buffer (15 mM Mg²⁺)
 - ◆ 10 µl 2mM dNTPs
 - ◆ 1 µl TaqDNA polymerase (5 U/µl)
 - ◆ 1 µl 100 µM 20-mer oligo
 - ◆ Water up to 68 µl total.
7. Run PCR program:
- ◆ 55 °C 2 min
 - ◆ 72 °C 5 min
 - ◆ 94 °C 5 min
 - ◆ 94 °C 1 min
 - ◆ 55 °C 1 min
 - ◆ 72 °C 1 min
 - ◆ Repeat steps 4-6 for 19 cycles
 - ◆ 72 °C 5 min
 - ◆ 4 °C hold.
8. Take 10 µl of above PCR reaction, and set up exactly the same reaction as above.
- ◆ 10 µl PCR product from step 7
 - ◆ 10 µl 10x TaqDNA buffer (15 mM Mg²⁺)
 - ◆ 10 µl 2mM dNTPs
 - ◆ 1 µl TaqDNA polymerase (5 U/µl)
 - ◆ 1 µl 100 µM 20-mer oligo
 - ◆ Water up to 68 µl total.
9. Run PCR program:
- ◆ 55 °C 2 min
 - ◆ 72 °C 5 min
 - ◆ 94 °C 5 min
 - ◆ 94 °C 1 min
 - ◆ 55 °C 1 min
 - ◆ 72 °C 1 min
 - ◆ Repeat steps 4-6 for 19 cycles
 - ◆ 72 °C 5 min
 - ◆ 4 °C hold.

Quality control

10. Analyse 5 µl of the final PCR product on a 1% agarose gel to see if the sample has been amplified.
11. Purify using the QiaQuick PCR purification kit.
12. Measure concentration using a Nanodrop.

For hybridisation to Nimblegen HD2 arrays 2 µg amplified DNA is required for each sample and control. Continue with Nimblegen processing of ChIP or DamID samples on 2.1M microarrays protocol.

J. Aleksic (05-01-2011)

DamID for fly genomic DNA using Affymetrix arrays

Outline

This protocol is based on the method from Vogel, M.J., Peric-Hupkes, D., Steensel, B. (2007). Nat Protoc 2(6):1467-78. PMID 17545983 ([abstract](#)) with modifications made by [Andrea Brand's lab](#) and [Lisa Meadows](#).

Before you start:

The PCR amplification step varies according to the microarray format that will be used, so first decide whether you are going to use [Affymetrix](#) or [Nimblegen](#) microarrays and then follow the appropriate protocol. You will need to collect samples from the DamID-fusion fly stock and to control for background, also from a control stock (DamID-no fusion). See Vogel *et al.* (2007) for a detailed explanation.

Equipment and reagents

- 1 x Phosphate Buffered Saline (PBS)
- DNeasy Blood and Tissue Kit (Qiagen; Cat No. 69504)
- RNase A (Sigma; Cat. No. R6513)
- 100% Ethanol
- 70% Ethanol in MilliQ H₂O
- 3M Sodium Acetate, pH 5.5 (NaOAc) (Sigma; Cat. No. 241245)
- DpnI 20,000 U/ml (NEB; Cat. No. R0176L)
- DpnII 10,000 U/ml (NEB; Cat. No. R0543S)
- AdRt oligonucleotide: 5'-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGA-3'
- AdRb oligonucleotide: 5'-TCCTCGGCCG-3'
- 5 U/mL T4 DNA ligase (Invitrogen; Cat. No. 799009)
- Advantage cDNA polymerase mix (Clontech; Cat. No. 639105)
- 100mM Deoxynucleotide set (Sigma; Cat. No. DNTP-100A)
- Aminoallyl-UTP-sodium salt (Sigma; Cat. No. A-5660)
- AdR_PCR primer: 5'-GGTCGCGGCCGAGGATC-3'
- QIAquick PCR purification kit, (Qiagen; Cat. No. 28104)
- 1.5ml disposable Polypropylene Pellet Pestle with microtube (Anachem; Cat. No. K-749520-0000)
- Micro 20 centrifuge, Hettich
- Homogeniser
- Hot block (Grant, QBT2)
- Spectrophotometer (Nanadrop, ND-100)
- PCR Machine (Dyad Thermal Cycler)
- Speed vac (Savant)
- Gel electrophoresis equipment

Notes

DpnI and DpnII enzymes should ideally be less than 6 months old.

Do not phosphorylate the 5' or 3' ends of the AdRb and AdRt oligonucleotides to prevent self-ligation of the double-stranded dsAdR adaptor. Order oligonucleotides of standard 'desalted' purity.

50 µM double-stranded (ds) AdR stock is made as follows:

- Add 50 µL AdRt (100 µM) to 50 µL AdRb (100 µM) and mix together by pipetting
- Place tube into a beaker with water that has just boiled
- Allow the beaker to cool to room temperature, so that the adaptors can anneal slowly
- Store aliquots at -20 °C.

Procedure

Embryo collection

1. Collect embryos on yeasted apple- (or grape-) juice agar plates and age accordingly.
2. Wash embryos off plate with water and collect in sieve.
3. Wash thoroughly with water to remove traces of yeast and blot sieve gently on tissue to remove excess water.
4. Using a clean paint brush or small spatula, transfer the embryos to a 1.5 mL microfuge tube containing approx. 400 μ L PBS. Spin briefly to pellet embryos, remove and discard as much PBS as possible and freeze tube on dry ice until you are able to place samples at -80 °C for storage. Samples should be stable at -80 °C for at least several months.

Day 1:

Genomic DNA isolation (Qiagen DNeasy Blood and Tissue Kit):

1. Remove Dam sample tubes from -80 °C freezer.
2. Add 175 μ L 1 x PBS into a 1.5 mL tube to wash and mix the embryos (you can pool embryos from several tubes).
3. Use sterile (wash in 100% ethanol) pestle to homogenise embryos in PBS.
4. Add 20 μ L RNase (12.5 μ g/ μ L, stored in 4 °C fridge) pipette mix and put at room temperature for 2 minutes.
5. Add 20 μ L Proteinase K and 200 μ L Buffer AL (Qiagen DNeasy Blood and Tissue Kit); gently pipette approx. 50 times to mix, pulse spin for 1 second to collect all of the sample and incubate at 70 °C for 10 minutes in a hot block.
6. Add 200 μ L 100% ethanol and mix, apply all solution into the spin column (Qiagen DNeasy Blood and Tissue Kit).
7. Spin 8000 rpm for 1 minute, discard flow-through and collecting tube.
8. Add 500 μ L AW1 solution and spin 8000 rpm for 1 minute, discard flow-through and collecting tube.
9. Add 500 μ L AW2 solution and spin 13000 rpm for 3 minutes, discard flow-through and collecting tube.
10. Add 200 μ L AE buffer and leave at room temperature 1 minute, spin 8000 rpm for 1 minute into a fresh 1.5 mL tube: keep the flow-through (e1 200 μ L).
11. Add 200 μ L AE buffer and leave at room temperature 1 minute, transfer column to another fresh 1.5 mL tube, spin 8000 rpm for 1 minute: keep the flow-through (e2 200 μ L).
12. Run 2 μ L of genomic DNA from e1 and e2 on a 0.7% agarose gel to check quality (should be a single high mass band with no smearing) and measure the concentration of e1 and e2 using a spectrophotometer (*e.g.*, Nanodrop).

A total of 2.5 μ g genomic DNA is required for each sample for the following steps, you can use all of e1 and e2 (approx. 400 μ L) - if necessary. It may also be possible to use as little as 0.5 μ g gDNA starting material in the same volumes, but we use 2.5 μ g.

13. Take a new 1.5 mL microfuge tube, take at least 2.5 μ g genomic DNA from e1 and/or e2, add MilliQ water to a total of 500 μ L.
14. Add 1 mL 100% ethanol and 50 μ L 3M Sodium Acetate, mix and vortex 2 seconds, leave at -20 °C for at least 20 minutes (sample can be stored at -20 °C for at least a year).
15. Spin at 13000 rpm at 4 °C for 30 minutes, carefully remove the supernatant (there will be a very small, almost invisible pellet).
16. Add 500 μ L 70% ethanol and vortex 1 second, spin 13000 rpm at 4 °C for 10 minutes, remove ethanol.
17. Pulse spin, remove last traces of ethanol and leave lid open until genomic DNA pellet is dry.

An alternative to steps 13-17 is to take 2.5 μ g genomic DNA and speed vac until pellet is dry.

For a single replicate comparison of DamID-fusion vs DamID-no fusion control, the following tubes are required:

- #1: 2.5 μ g DamID-fusion gDNA
- #2: 2.5 μ g DamID-fusion gDNA - 'no DpnI' control
- #3: 2.5 μ g DamID-fusion gDNA - 'no T4 ligase' control

ChIP and DamID

- #4: 2.5 µg DamID-no fusion control genomic DNA
- Samples #1-3 should be taken from the same stock preparation of gDNA

DpnI digestion (DpnI cuts methylated GATC sequences):

Master mix:

- 5.0 µL Buffer 4 (10 x, NEB)
- 43.5 µL MilliQ water
- 1.5 µL DpnI (20 U/µL, NEB) (or MilliQ water, for #2 no DpnI control)

18. Add 50 µL DpnI master mix directly to the tube with the dried genomic DNA pellet and gently pipette to mix.
19. Digest at 37 °C overnight in a hot block or a waterbath.

Day 2

20. Take the DpnI digested genomic DNA tube from 37 °C and incubate at 80 °C for 20 minutes to inactivate DpnI.
21. Use a QIAGEN PCR purification kit to purify the DpnI digested product, use 30 µL MilliQ water to elute, put on ice and prepare the ligation buffer.

Ligation (adaptor oligonucleotides are ligated to blunt-ended DpnI fragments):

Master mix:

- 4.0 µL 5X ligation buffer (Invitrogen)
- 0.8 µL ds-AdR (made from AdRt and AdRb, see reagents list)
- 1.0 µL T4 DNA ligase (5 U/µL, Invitrogen) (Replace T4 DNA ligase with MilliQ water for #3 'no ligase' control)

22. Transfer 14.2 µL of purified DpnI digested genomic DNA product to the PCR tube for ligation to the adaptors (store the remaining 15.8 µL of genomic DNA at -20 °C for future use).
23. Mix with 5 µL ligation master mix (total volume is now 20 µL).
24. Using the PCR machine, ligate at 16 °C for 2 hours followed by 65 °C for 10 mins to inactivate the ligase.

Dpn II digestion (this digestion will destroy fragments containing unmethylated GATCs):

Master mix:

- 8 µL DpnII buffer (10x, NEB)
- 1 µL DpnII (10 U/µL, NEB)
- 51 µL MilliQ water

25. Mix 60 µL of the DpnII digestion master mix with 20 µL of ligated DNA product (total 80 µL).
26. Incubate at 37 °C (use incubator or PCR machine) for at least 1 hour (overnight is also fine).

Affymetrix microarray PCR (to amplify ligated products):

Master mix:

- 8.0 µL 10X cDNA PCR reaction buffer (Clontech)
- 1.25 µL AdR-PCR primer (50 µM)
- 1.6 µL dNTP/dUTP mix for Affy (10mM dCTP, 10mM dATP, 10mM dGTP, 8mM dTTP, 2mM dUTP)
- 1.0 µL Advantage DNA polymerase mix (50X, Clontech)
- 48.15 µL MilliQ water

Note: to get sufficient DNA yield for Affymetrix microarrays (7.5 µg) you may need to do more than one PCR in parallel and pool them before Qiagen purification. The number of PCR cycles necessary at step 12 is

ChIP and DamID

typically 14-17, but may have to be adjusted according to your particular sample, so a pilot PCR would be a good idea.

27. Transfer 20 μL from DpnII digested genomic DNA into a fresh PCR tube (store remaining 60 μL at $-20\text{ }^{\circ}\text{C}$ for further amplification).
28. Mix with 60 μL PCR master mix and run PCR (use DamID programme in PCR machine). Replace DpnII digested genomic DNA with MilliQ water as a 'no template' PCR control.

DamID PCR Program:

1. $68\text{ }^{\circ}\text{C}$ 10 min
2. $94\text{ }^{\circ}\text{C}$ 1 min
3. $65\text{ }^{\circ}\text{C}$ 5 min
4. $68\text{ }^{\circ}\text{C}$ 15 min
5. $94\text{ }^{\circ}\text{C}$ 1 min
6. $65\text{ }^{\circ}\text{C}$ 1 min
7. $68\text{ }^{\circ}\text{C}$ 10 min
8. Repeat steps 5-7, 3 times
9. $94\text{ }^{\circ}\text{C}$ 1 min
10. $65\text{ }^{\circ}\text{C}$ 1 min
11. $68\text{ }^{\circ}\text{C}$ 2 min
12. Repeat steps 9-11, *e.g.*, 14-17 times.
13. $4\text{ }^{\circ}\text{C}$, hold

Day 3

29. Check quality of PCR product by running 3 μL on a 1% agarose gel, (should be a smear of $\sim 200\text{-}2000\text{ bp}$).

The DamID-fusion sample (#1) and the DamID-no fusion control sample (#4) should have a smear and the 'no DpnI' (#2) and 'no ligase' (#3) controls should have no PCR product.

30. Use QIAquick PCR purification kit to purify the rest of PCR product, use 20 μL MilliQ water to elute (leave MilliQ water on column for at least 2 minutes before spinning)
31. Measure concentration using a Nanodrop spectrophotometer and store at $-20\text{ }^{\circ}\text{C}$

For Affymetrix arrays you need 7.5 μg purified PCR product. See the Affymetrix microarray hybridisation protocol for how to proceed.

L. Meadows (26-07-2010)

DamID for fly genomic DNA using Nimblegen arrays

Outline

This protocol is based on the method from Vogel, M.J., Peric-Hupkes, D., Steensel, B. (2007). Nat Protoc 2(6):1467-78. PMID 17545983 ([abstract](#)) with modifications made by [Andrea Brand's lab](#) and [Lisa Meadows](#).

Before you start:

The PCR amplification step varies according to the microarray format that will be used, so first decide whether you are going to use [Affymetrix](#) or [Nimblegen](#) microarrays and then follow the appropriate protocol. You will need to collect samples from the DamID-fusion fly stock and to control for background, also from a control stock (DamID-no fusion). See Vogel *et al.* (2007) for a detailed explanation.

Equipment and reagents

- 1 x Phosphate Buffered Saline (PBS)
- DNeasy Blood and Tissue Kit (Qiagen; Cat No. 69504)
- RNase A (Sigma; Cat. No. R6513)
- 100% Ethanol
- 70% Ethanol in MilliQ H₂O
- 3M Sodium Acetate, pH 5.5 (NaOAc) (Sigma; Cat. No. 241245)
- DpnI 20,000 U/ml (NEB; Cat. No. R0176L)
- DpnII 10,000 U/ml (NEB; Cat. No. R0543S)
- AdRt oligonucleotide: 5'-CTAATACGACTCACTATAGGGCAGCGTGGTCGCGGCCGAGGA-3'
- AdRb oligonucleotide: 5'-TCCTCGGCCG-3'
- 5 U/mL T4 DNA ligase (Invitrogen; Cat. No. 799009)
- Advantage cDNA polymerase mix (Clontech; Cat. No. 639105)
- 100mM Deoxynucleotide set (Sigma; Cat. No. DNTP-100A)
- Aminoallyl-UTP-sodium salt (Sigma; Cat. No. A-5660)
- AdR_PCR primer: 5'-GGTCGCGGCCGAGGATC-3'
- QIAquick PCR purification kit, (Qiagen; Cat. No. 28104)
- 1.5ml disposable Polypropylene Pellet Pestle with microtube (Anachem; Cat. No. K-749520-0000)
- Micro 20 centrifuge, Hettich
- Homogeniser
- Hot block (Grant, QBT2)
- Spectrophotometer (Nanadrop, ND-100)
- PCR Machine (Dyad Thermal Cycler)
- Speed vac (Savant)
- Gel electrophoresis equipment

Notes

DpnI and DpnII enzymes should ideally be less than 6 months old.

Do not phosphorylate the 5' or 3' ends of the AdRb and AdRt oligonucleotides to prevent self-ligation of the double-stranded dsAdR adaptor. Order oligonucleotides of standard 'desalted' purity.

50 μ M double-stranded (ds) AdR stock is made as follows:

- Add 50 μ L AdRt (100 μ M) to 50 μ L AdRb (100 μ M) and mix together by pipetting
- Place tube into a beaker with water that has just boiled
- Allow the beaker to cool to room temperature, so that the adaptors can anneal slowly
- Store aliquots at -20 °C.

Procedure

Embryo collection

1. Collect embryos on yeasted apple- (or grape-) juice agar plates and age accordingly.
2. Wash embryos off plate with water and collect in sieve.
3. Wash thoroughly with water to remove traces of yeast and blot sieve gently on tissue to remove excess water.
4. Using a clean paint brush or small spatula, transfer the embryos to a 1.5 mL microfuge tube containing approx. 400 μ L PBS. Spin briefly to pellet embryos, remove and discard as much PBS as possible and freeze tube on dry ice until you are able to place samples at -80 °C for storage. Samples should be stable at -80 °C for at least several months.

Day 1:

Genomic DNA isolation (Qiagen DNeasy Blood and Tissue Kit):

1. Remove Dam sample tubes from -80 °C freezer.
2. Add 175 μ L 1 x PBS into a 1.5 mL tube to wash and mix the embryos (you can pool embryos from several tubes).
3. Use sterile (wash in 100% ethanol) pestle to homogenise embryos in PBS.
4. Add 20 μ L RNase (12.5 μ g/ μ L, stored in 4 °C fridge) pipette mix and put at room temperature for 2 minutes.
5. Add 20 μ L Proteinase K and 200 μ L Buffer AL (Qiagen DNeasy Blood and Tissue Kit); gently pipette approx. 50 times to mix, pulse spin for 1 second to collect all of the sample and incubate at 70 °C for 10 minutes in a hot block.
6. Add 200 μ L 100% ethanol and mix, apply all solution into the spin column (Qiagen DNeasy Blood and Tissue Kit).
7. Spin 8000 rpm for 1 minute, discard flow-through and collecting tube.
8. Add 500 μ L AW1 solution and spin 8000 rpm for 1 minute, discard flow-through and collecting tube.
9. Add 500 μ L AW2 solution and spin 13000 rpm for 3 minutes, discard flow-through and collecting tube.
10. Add 200 μ L AE buffer and leave at room temperature 1 minute, spin 8000 rpm for 1 minute into a fresh 1.5 mL tube: keep the flow-through (e1 200 μ L).
11. Add 200 μ L AE buffer and leave at room temperature 1 minute, transfer column to another fresh 1.5 mL tube, spin 8000 rpm for 1 minute: keep the flow-through (e2 200 μ L).
12. Run 2 μ L of genomic DNA from e1 and e2 on a 0.7% agarose gel to check quality (should be a single high mass band with no smearing) and measure the concentration of e1 and e2 using a spectrophotometer (*e.g.*, Nanodrop).

A total of 2.5 μ g genomic DNA is required for each sample for the following steps, you can use all of e1 and e2 (approx. 400 μ L) - if necessary. It may also be possible to use as little as 0.5 μ g gDNA starting material in the same volumes, but we use 2.5 μ g.

13. Take a new 1.5 mL microfuge tube, take at least 2.5 μ g genomic DNA from e1 and/or e2, add MilliQ water to a total of 500 μ L.
14. Add 1 mL 100% ethanol and 50 μ L 3M Sodium Acetate, mix and vortex 2 seconds, leave at -20 °C for at least 20 minutes (sample can be stored at -20 °C for at least a year).
15. Spin at 13000 rpm at 4 °C for 30 minutes, carefully remove the supernatant (there will be a very small, almost invisible pellet).
16. Add 500 μ L 70% ethanol and vortex 1 second, spin 13000 rpm at 4 °C for 10 minutes, remove ethanol.
17. Pulse spin, remove last traces of ethanol and leave lid open until genomic DNA pellet is dry.

An alternative to steps 13-17 is to take 2.5 μ g genomic DNA and speed vac until pellet is dry.

For a single replicate comparison of DamID-fusion vs DamID-no fusion control, the following tubes are required:

- #1: 2.5 μ g DamID-fusion gDNA
- #2: 2.5 μ g DamID-fusion gDNA - 'no DpnI' control
- #3: 2.5 μ g DamID-fusion gDNA - 'no T4 ligase' control

ChIP and DamID

- #4: 2.5 µg DamID-no fusion control genomic DNA
- Samples #1-3 should be taken from the same stock preparation of gDNA

DpnI digestion (DpnI cuts methylated GATC sequences):

Master mix:

- 5.0 µL Buffer 4 (10 x, NEB)
- 43.5 µL MilliQ water
- 1.5 µL DpnI (20 U/µL, NEB) (or MilliQ water, for #2 no DpnI control)

18. Add 50 µL DpnI master mix directly to the tube with the dried genomic DNA pellet and gently pipette to mix.
19. Digest at 37 °C overnight in a hot block or a waterbath.

Day 2

20. Take the DpnI digested genomic DNA tube from 37 °C and incubate at 80 °C for 20 minutes to inactivate DpnI.
21. Use a QIAGEN PCR purification kit to purify the DpnI digested product, use 30 µL MilliQ water to elute, put on ice and prepare the ligation buffer.

Ligation (adaptor oligonucleotides are ligated to blunt-ended DpnI fragments):

Master mix:

- 4.0 µL 5X ligation buffer (Invitrogen)
- 0.8 µL ds-AdR (made from AdRt and AdRb, see reagents list)
- 1.0 µL T4 DNA ligase (5 U/µL, Invitrogen) (Replace T4 DNA ligase with MilliQ water for #3 'no ligase' control)

22. Transfer 14.2 µL of purified DpnI digested genomic DNA product to the PCR tube for ligation to the adaptors (store the remaining 15.8 µL of genomic DNA at -20 °C for future use).
23. Mix with 5 µL ligation master mix (total volume is now 20 µL).
24. Using the PCR machine, ligate at 16 °C for 2 hours followed by 65 °C for 10 mins to inactivate the ligase.

Dpn II digestion (this digestion will destroy fragments containing unmethylated GATCs):

Master mix:

- 8 µL DpnII buffer (10x, NEB)
- 1 µL DpnII (10 U/µL, NEB)
- 51 µL MilliQ water

25. Mix 60 µL of the DpnII digestion master mix with 20 µL of ligated DNA product (total 80 µL).
26. Incubate at 37 °C (use incubator or PCR machine) for at least 1 hour (overnight is also fine).

Nimblegen microarray PCR (to amplify ligated products):

Master mix:

- 8.0 µL 10X cDNA PCR reaction buffer (Clontech)
- 1.25 µL AdR-PCR primer (50 µM)
- 1.6 µL dNTP mix (Sigma)
- 1.0 µL Advantage DNA polymerase mix (50X, Clontech)
- 48.15 µL MilliQ water

Note: to get sufficient DNA yield for Nimblegen microarrays (2 µg) you may need to do more than one PCR in parallel and pool them before Qiagen purification. The number of PCR cycles necessary at step 12 is typically 14-17, but may have to be adjusted according to your particular sample, so a pilot PCR would be a

good idea.

27. Transfer 20 μ L from DpnII digested genomic DNA into a fresh PCR tube (store remaining 60 μ L at -20 $^{\circ}$ C for further amplification).
28. Mix with 60 μ L PCR master mix and run PCR (use DamID programme in PCR machine). Replace DpnII digested genomic DNA with MilliQ water as a 'no template' PCR control.

DamID PCR Program:

1. 68 $^{\circ}$ C 10 min
2. 94 $^{\circ}$ C 1 min
3. 65 $^{\circ}$ C 5 min
4. 68 $^{\circ}$ C 15 min
5. 94 $^{\circ}$ C 1 min
6. 65 $^{\circ}$ C 1 min
7. 68 $^{\circ}$ C 10 min
8. Repeat steps 5-7, 3 times
9. 94 $^{\circ}$ C 1 min
10. 65 $^{\circ}$ C 1 min
11. 68 $^{\circ}$ C 2 min
12. Repeat steps 9-11, *e.g.*, 14-17 times.
13. 4 $^{\circ}$ C, hold

Day 3

29. Check quality of PCR product by running 3 μ L on a 1% agarose gel, (should be a smear of ~200-2000 bp).

The DamID-fusion sample (#1) and the DamID-no fusion control sample (#4) should have a smear and the 'no DpnI' (#2) and 'no ligase' (#3) controls should have no PCR product.

30. Use QIAquick PCR purification kit to purify the rest of PCR product, use 20 μ L MilliQ water to elute (leave MilliQ water on column for at least 2 minutes before spinning)
31. Measure concentration using a Nanodrop spectrophotometer and store at -20 $^{\circ}$ C

For Nimblegen HD2 arrays you need 2 μ g purified PCR product. See the Nimblegen microarray hybridisation protocol for how to proceed.

L. Meadows (26-07-2010)

Nimblegen Processing of ChIP or DamID DNA on 2.1M Arrays

Outline

Random primers are annealed to denatured DNA templates and extended by Klenow fragment while fluorescent dyes are incorporated. The labelled samples are then subjected to paired competitive hybridisations on Nimblegen 2.1M tiling arrays.

Equipment and reagents

- dATP, dCTP, dTTP and dGTP (Sigma; Cat. No. dNTP-100A)
- Bioprime DNA Labeling System (Invitrogen; Cat. No. 18094-011)
- Cy3 dCTP (GE Healthcare Bio-Sciences AB; Cat. No. PA 53021)
- Cy5 dCTP (GE Healthcare Bio-Sciences AB; Cat. No. PA 55021)
- Isopropanol (BDH; Cat. No. 102246L)
- NimbleGen Hybridization Kit, LS (Roche-Nimblegen; Cat. No. 05583934001)
- NimbleGen Wash Buffer Kit (Roche-Nimblegen; Cat. No. 05584507001)
- NimbleGen Hybridization System Accessory Kit (Roche-Nimblegen; Cat. No. 05327695001)
- NimbleGen Mixer HX1 (10) (Roche-Nimblegen; Cat. No. 05223741001)
- Sigma water (Sigma; Cat. No. W4502)
- Sodium Chloride (Sigma; Cat. No. S-3014)
- Hettich micro 20 centrifuge
- Hettich Rotina 35 microtitre plate centrifuge
- Grant QBT2 hot-block
- Savant Speed Vac
- Dyad thermal cycler (PCR block)
- GenePix 4000B Microarray Scanner
- NimbleGen Hybridization System 4

Procedure

Making the 10 X low-C dNTP mix:

1. Make a large 10 X low-C dNTP mix for the labelling reaction (5 mM A-,G-,T-dNTPs and 3mM C-dNTP)
 - ◆ 25 µl 100mM dNTA
 - ◆ 25 µl 100mM dNTT
 - ◆ 25 µl 100mM dNTG
 - ◆ 15 µl 100mM dNTC
2. Then make up to 500 µl using MilliQ water
3. Aliquot the 10 X low-C dNTP mix and then store at -20 °C

Klenow labelling (Flychip protocol):

The following steps are performed in 200 µl PCR tubes and the PCR block. Perform 2 reactions per sample and control.

1. Take 1 µg DNA and make up to a total volume of 31.2 µl with Sigma water
2. Add 30 µl 2.5x Random Primer Reaction Buffer (supplied in the Bioprime Labelling System Kit)
3. Incubate at 100 °C for 5 minutes
4. Snap freeze on ice
5. Mix together the following to make a master mix:
 - ◆ 7.5 µl 10 X low-C dNTP mix
 - ◆ 4.5 µl Cy3 or Cy5 dCTP
 - ◆ 1.8 µl 40U/µl Klenow (supplied in the Bioprime Labelling System Kit)
6. Add 13.8 µl to each sample and mix by pipetting up and down
7. Incubate at 37 °C for 2 to 3 hours
8. Stop the reaction by adding 7.5 µl Stop Buffer (supplied in the Bioprime Labelling System Kit)
9. Combine the Cy3 and Cy5 pairs

Precipitation purification (as per Nimblegen protocol):

It is important to separate the fluorescently-labelled probe from any unincorporated dye and nucleotides.

10. Combine the 2 same reactions into 1.5 ml microcentrifuge tubes
11. Add 17.25 µl 5M NaCl-solution
12. Add 165 µl Isopropanol
13. Vortex and incubate for 10 minutes at room temp (dark)
14. Centrifuge for 10 minutes at 13,000 rpm
15. Discard the supernatant and wash the DNA pellet with 500 µl 80% ice-cold ethanol
16. Centrifuge for 2 minutes at 13,000 rpm and remove supernatant
17. Dry the DNA pellet for 5 minutes (dark)

Quantify samples (as per Nimblegen protocol):

18. Rehydrate DNA pellet in 50 µl Sigma water and vortex
19. Briefly centrifuge and incubate for 5 minutes (dark), then vortex and centrifuge again
20. Measure concentration on Nanodrop - as Nucleic acid
21. Combine 34 µg Test Sample with 34 µg Reference Sample into a 1.5 ml tube. (Hybridisation can be performed with as little as 24 µg.)
22. Dry contents in SpeedVac, protect from light
23. Store samples at -20 °C until ready for hybridisation (up to 1 month)

Hybridisation to 2.1M arrays (as per Nimblegen protocol):

The Alignment Oligo provided in the Nimblegen Hybridisation Kit are labeled with Cy dyes, which are sensitive to photobleaching and freeze-thawing. After thawing stock tubes for the first time, aliquot 1.2 µl of the Alignment Oligo into PCR tubes and store at -20 °C (protect from light).

24. Set the Hybridisation System to 42 °C, allow at least 3 hours for the temperature to stabilise
25. Resuspend dried sample pair in 12.3 µl of Sigma water and vortex
26. Prepare Hybridisation Master Mix:
 - ◆ 1.2 µl Alignment Oligo
 - ◆ 11.8 µl Hybridisation Component A
 - ◆ 29.5 µl 2X Hybridisation Buffer
27. Add 31.7 µl of Hybridisation Master Mix to each sample pair
28. Vortex and spin
29. Incubate at 95 °C for 5 minutes in hotblock
30. Incubate at 42 °C for at least 5 minutes or until ready to load sample
31. Vortex and spin before loading

Prepare Mixers (as per Nimblegen protocol):

32. Remove HX1 mixer from package
33. Open the Precision Mixer Alignment Tool (PMAT, supplied with the Hybridisation System)
34. Push back the plastic spring with a thumb; place the slide in the base of the PMAT so that the barcode is readable facing outward. Make sure the slide is positioned to the rightmost and closest to you; make sure the slide is lying flat against the PMAT. Gently use the AirDuster to remove any particles from the slide
35. Snap the mixer onto the two alignment pins on the lid of the PMAT, with the end of the mixer pointing towards the hinge and the adhesive gasket facing outward
36. Use forceps to remove the backing from the adhesive gasket of the mixer and close the lid of the PMAT
37. Lift the lid by grasping the long ends of the PMAT while applying pressure with a finger through the window in the lid to free the mixer-slide assembly from alignment pins
38. Remove the mixer-slide from the PMAT and place on clean smooth dark surface
39. Rub the Mixer Brayer (supplied in the Hybridisation System Accessory Kit) over the mixer to adhere the gasket and remove any bubbles, starting in the center and rub outwards. Gasket becomes clear when fully adhered to both surfaces
40. Place the mixer-slide in the slide bay of the Hybridisation System

Load and Hybridise Samples (as per Nimblegen protocol):

41. Using a Gilson P200 slowly load 41 μ l into the fill port until the sample starts to overflow from the vent port (avoid bubbles, keep pipette tip perpendicular, apply gentle pressure of the tip into the port to ensure a tight seal)
42. Dry sample overflow from the ports, e.g. using a cotton swap
43. Adhere a mixer port seal over both ports and press simultaneously to seal
44. Close the bay clamp
45. Turn on the Mixing Panel, set the mix mode to B, and press the mix button to start mixing. Check indicator light is green for each slide position
46. Hybridise for 20 hours at 42 °C

Wash Hybridised Arrays (as per Nimblegen protocol):

It is important to proceed through all the washing and drying steps without interruption. We only process two arrays at a time to minimise the time the arrays are stored until scanned.

47. Prewarm 10X Wash Buffer I at 42 °C for a couple of minutes as it can precipitate before use
48. Prepare 250 ml Wash I and for each array 25 ml of Washes I, II and III according to the following table:

Solution	Wash I (250 ml)	Wash I, II and III (25 ml)
RO-water	225 ml	22.5 ml
10X Wash Buffer I, II or III	25 ml	2.5 ml
1M DTT	25 μ l	2.5 μ l

49. Prewarm 250 ml of Wash I to 42 °C and pour warm solution into shallow dish, so that the Mixer Disassembly Tool (supplied in the Hybridisation System Accessory Kit) is completely covered
50. Insert Mixer Disassembly Tool into dish and load the mixer-slide assembly into the Tool
51. Carefully peel the mixer off the slide (keeping the Mixer Disassembly Tool flat)
52. Remove the slide from the Tool and agitate the slide for 10-15 seconds in the shallow dish
53. Transfer slide into a slide container that contains Wash I (room temperature)
54. Wash for 2 minutes, shaking the container at least 20 times every 10 seconds
55. Blot off excess buffer on tissue and transfer slide into a slide container that contains Wash II (room temperature)
56. Wash for 1 minute, shaking the container at least 20 times every 10 seconds
57. Blot off excess buffer on tissue and transfer slide into a slide container that contains Wash III (room temperature)
58. Wash for 15 seconds, shaking the container at least 20 times every 10 seconds
59. Transfer the slides to a clean microscope slide box with tissue at the base and centrifuge at 1000 rpm for 5 minutes
60. Proceed immediately to scanning

Two-Colour Array Scanning (as per Nimblegen protocol):

Keep arrays in the dark until you are ready to scan them.

61. Launch the GenePix software 10 minutes before scanning to allow lasers to warm
62. Place the slide in the slide carriage so that the array is facing down and the barcode end is closest to you
63. Open the Hardware Settings dialog box and select the following settings for scanning:
 - ◆ 532 PMT Gain = 650
 - ◆ 635 PMT Gain = 750
 - ◆ Power (%) = 100%
 - ◆ Pixel size (μ m) = 5
 - ◆ Lines to average = 1
 - ◆ Focus position (μ m) = 0
64. Press Preview Scan to start a preview scan of the entire slide

ChIP and DamID

65. Then press the Scan Area button:
 - ◆ Move the mouse cursor to the top left of the features on the image
 - ◆ Hold down the mouse cursor and drag a rectangle around the region containing the features
 - ◆ Confirm that all features have been included within the scan area
66. Start a Scan of the array and zoom into a smaller region and adjust the 532 and 635 PMT gains until only few spots remain saturated (saturated spots are displayed white)
67. Zoom into as large a region as possible (exclude areas with high background) and select the Histogram tab:
 - ◆ Make sure that the Wavelength 532 and Wavelength 635 boxes are checked so both wavelength histograms are displayed
 - ◆ Make sure that the Log Axis box is checked
 - ◆ The red and green curves should be superimposed. If the ref curve is above the green, lower the red PMT setting or raise the green PMT setting on the Image tab.
 - ◆ The curve should end above $1e-5$ normalized counts at the 65,000 intensity level (saturation)
 - ◆ The histogram graphs only the region of the image viewable on-screen in the image tab
68. After the PMT settings are properly adjusted, stop the current scan (do not save image)
69. Restart the scan
70. Save the images for both channels (single image .tif files) as: NNNNNN_XXXXXX, where NNNNNN = barcode and XXXXXX = user defined text (sample name)

Proceed to NimbleScan data analysis as outlined in the Nimblegen Arrays User's Guide.

B. Fischer (15-06-2010).

Affymetrix Processing of ChIP or DamID DNA to GeneChip Drosophila Tiling 2.0R Array

Outline

For the following protocol 7.5 µg DNA containing dUTP (see the corresponding DamID or ChIP protocols for Affymetrix microarrays for the sample preparation) are required for the hybridisation to GeneChip Drosophila Tiling 2.0R Array (49 Array Format).

Equipment and reagents

- GeneChip Drosophila Tiling 2.0R Array (Affymetrix; Cat. No. 901021)
- WT Double Stranded DNA Terminal Labeling Kit (Affymetrix; Cat. No. 900812)
- Control Oligo B2, 3 nM (Affymetrix; Cat. No. 900301)
- GeneChip Hybridization Wash and Stain Kit (Affymetrix; Cat. No. 900720)
- Affymetrix GeneChip Hybridization Oven 640
- Affymetrix GeneChip Fluidics Station 450
- Affymetrix GeneChip Scanner 3000 7G
- Hettich micro 20 centrifuge
- Grant QBT2 hot-block
- Dyad thermal cycler (PCR block)
- GenePix 4000B Microarray Scanner
- NimbleGen Hybridization System 4
- Gel electrophoresis equipment

Procedure

Fragmentation using the WT Double Stranded DNA Terminal Labeling Kit:

1. Prepare samples in PCR tubes as follows:
 - ◆ 7.5 µg Double-stranded DNA (purified PCR product)
 - ◆ 4.8 µL 10x cDNA Fragmentation Buffer
 - ◆ 1.5 µL 10U/µl UDG
 - ◆ 2.25 µL 100U/µl APE 1
2. Then make up to 48 µL using Nuclease-free water (supplied in kit)
3. Mix and spin
4. Incubate:
 - ◆ 37 °C for 2 hours
 - ◆ 93 °C for 2 minutes
 - ◆ Cool down to 4 °C
5. Run 2 µL of the reaction on a 1% agarose gel (fragment size should be 100-200 bp, otherwise the fragmentation time needs to be optimised)
6. Transfer 45 µL of the fragmented sample to a new PCR tube

Labelling:

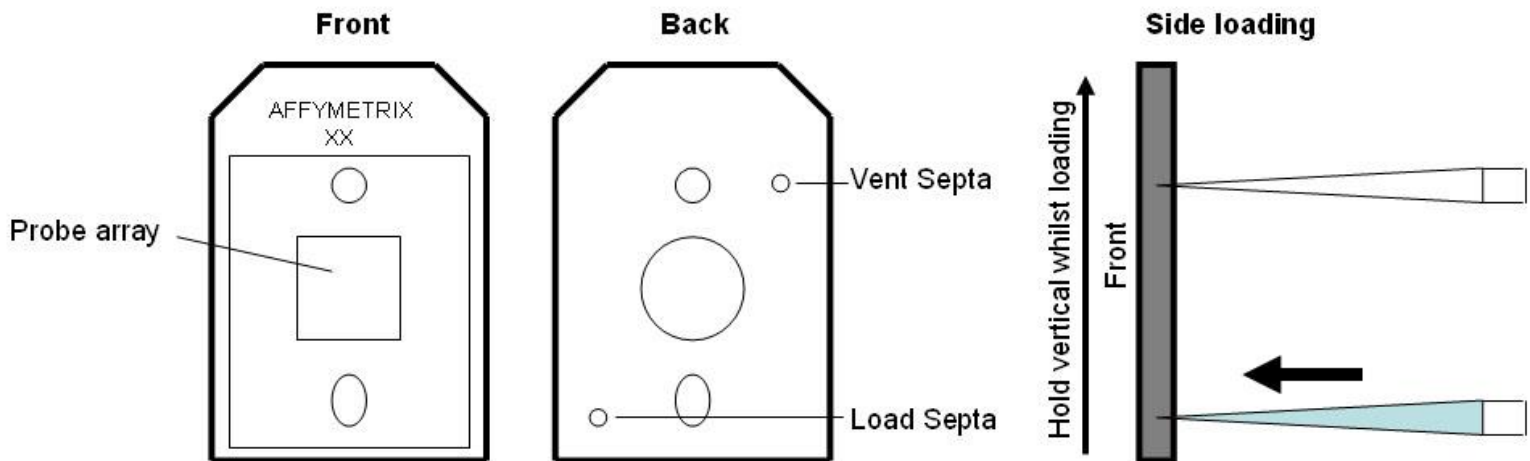
7. Prepare labeling premix:
 - ◆ 12 µL 5x TdT Buffer
 - ◆ 2 µL TdT
 - ◆ 1 µL 5mM DNA Labeling Reagent
8. Add 15 µL of labeling premix to each sample
9. Mix and spin
10. Incubate:
 - ◆ 37 °C for 1 hour
 - ◆ 70 °C for 10 minutes
 - ◆ cool down to 4 °C
11. Switch on the GeneChip Hybridization Oven 640 and set the temperature to 45 °C to allow the temperature to stabilise

Hybridisation using the Hyb Module of the GeneChip Hybridization Wash and Stain Kit:

12. Equilibrate the GeneChip Drosophila Tiling 2.0R Array to room temperature
13. Prepare in 1.5 mL tubes:
 - ◆ 60 μ L Fragmented and Labelled sample
 - ◆ 3.3 μ L Control Oligo B2
 - ◆ 100 μ L 2x Hybridisation Mix
 - ◆ 14 μ L DMSO
14. Make up to 200 μ L with Nuclease-free Water
15. Mix and spin
16. Heat at 99 °C for 5 minutes
17. Incubate to 45 °C for 5 minutes
18. Spin at 13,000 rpm for 1 minute
19. Label each array with the sample name
20. Insert a 250 μ L pipette tip into the vent septa on the back of the array (see Affymetrix array schema below)
21. Inject 200 μ L through the load septa while holding the array vertical

Note: Use a 250 μ L Filter tip for loading the sample. When using 200 μ L Filter tips the sample can get absorbed into the filter when holding the pipette horizontal during the loading process!

Schema of Affymetrix array:



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22. Remove the pipette tips and seal both septa with tough spots
23. Insert the array in the probe array tray and slide it into the GeneChip Hybridization Oven (make sure the carousel is balanced)
24. Position the small hooked end of the tray retainer over the upper rail of the carousel frame
25. Snap the lower end of the tray retainer over the lip of the probe array tray
26. Incubate at 45 °C, 60 rpm for 16 hours

Prime Fluidics Station (takes ~10 minutes):

27. Switch on the Fluidics Station and insert the wash line A into wash solution A and wash line B into wash solution B (light sensitive), make sure there is sufficient deionised water in the DI water bottle
28. Open AGCC Fluidics Control from the Affymetrix Launcher
29. On the screen tick the boxes of the Fluidics Modules to be used (one for each array)
30. Tick the List Maintenance Protocols Only option
31. Select PRIME_450 protocol
32. Click Copy to Selected Modules
33. Change to the Station 1 ID window
34. Click Run for each Module to be used
35. Follow the instructions on the Fluidics Station LCD window as the prime progresses:

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- ◆ When prompted raise the Needle Lever
 - ◆ Place three empty 1.5 mL tubes onto the Sample Holder
 - ◆ Lower the Needle Lever
36. Priming is finished when remove all vials message is displayed in the LCD window
37. Raise the Needle Lever and remove vials

Prepare Staining Reagents using the Stain Module of the GeneChip Hybridization Wash and Stain Kit:

38. For each array prepare three 1.5 mL tubes as follows:
- ◆ Tube 1 - 600 µL Stain Cocktail 1 (light sensitive)
 - ◆ Tube 2 - 600 µL Stain Cocktail 2
 - ◆ Tube 3 - 800 µL Array Holding Buffer
39. Protect tubes from light until use

Prepare Arrays for staining:

40. Remove arrays from the oven after 16 hours
41. Insert a 250 µL pipette tip into the venting septa on the back of the array (see schema above)
42. Remove hybridisation cocktail from the array through the loading septa using a 250 µL pipette tip whilst holding the array vertical (the sample can be stored at -20 °C and re-hybridised in case of a problem with the array)
43. Load 250 µL Wash Buffer A using a 1000 µL pipette tip
44. Remove both pipette tips
45. Arrays which are not stained immediately should be kept at 4 °C in the dark

Create a New Project (skip when adding arrays to an existing project):

46. Open AGCC Portal from the Affymetrix Launcher
47. Go to ADMINISTRATION - Projects - Manage
48. Select the data root in the left panel
49. Add Subfolder name (e.g. your name or project name) and press Create
50. Click on the "new" Subfolder just created on the left panel
51. Add Project name and press Create

Register Samples:

52. Open AGCC Portal from the Affymetrix Launcher
53. Go to SAMPLES - Quick Register
54. Select number of sample files (ARR) to create from drop down list
55. Assign a project and Probe Array Type (DM_tiling2_MR_v01)
56. Add Sample File Name, Array Name and scan the barcode of the array
57. Press Next

Stain Arrays in Fluidics Station (takes ~1.5 hours):

58. In the Fluidics Control window press Refresh button
59. For each Module select Sample File Name from the drop down menu
60. In the Protocol selection tick the All box
61. Select Protocol: FS450_0001 (for Drosophila Tiling 2.0R Array)
62. Click Run for each Module to be used
63. Follow the instructions on the Fluidics Station LCD window as the prime progresses:
- ◆ When fluidics LCD prompts lift the Cartridge Lever
 - ◆ Insert the array and lower the Cartridge Lever
 - ◆ When the fluidics LCD prompts raise the Needle Lever
 - ◆ Place the three Staining tubes onto their respective Sample Holder positions
 - ◆ Lower the Needle Lever
64. After ~1.5 hours when the fluidics LCD prompts:
- ◆ Lift the Cartridge Lever and remove the array
 - ◆ Engage the Cartridge Lever
 - ◆ When the fluidics LCD prompts raise the Needle Lever

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- ◆ Place three empty 1.5 mL tubes onto the Sample Holder
 - ◆ Lower the Needle Lever
65. Run has finished when the LCR displays: Protocol done

Shutdown Fluidics Station (takes ~12 minutes):

66. Remove Wash Solution A and B and place all wash lines into the DI water bottle
67. In the Fluidics Control window select the Shutdown_450 protocol for all used Modules
68. Press Run for each Module
69. Follow the instructions on the Fluidics Station LCD window:
- ◆ Raise the Needle Lever
 - ◆ Place three empty 1.5 mL tubes onto the Sample Holder
 - ◆ Lower the Needle Lever
70. After Shutdown protocol is completed switch off the Fluidics station

Scanning:

71. Inspect the array for air bubbles
72. In case of "larger" air bubbles:
- ◆ Insert a 250 μ L pipette tip into the venting septa on the back of the array (refer to schema above)
 - ◆ Remove solution through the loading septa using a 1000 μ L pipette tip
 - ◆ Load 800 μ L Array Holding Buffer
 - ◆ Remove both pipette tips
73. Seal both septa with tough spots
74. Let the scanner warm up for at least 10 minutes
75. Insert arrays in the carousel starting at position 1
76. Open the AGCC Scan Control from the Affymetrix Launcher
77. Select Edit - Options
- ◆ Clear the enable Manual Mode check box
 - ◆ Tick box the Turn on Laser at Startup
 - ◆ OK
78. Press Start
79. After scan has finished remove arrays
80. Close Scan Control software and switch scanner off

Review the Grid Alignment using the AGCC Viewer:

81. Open the AGCC Viewer from the Affymetrix Launcher
82. Select the array image from the Review Window or select - File - Open File and browse for the *.DAT image
83. Press the Auto Scale button if the features are too bright
84. Navigate to the corners using the shortcut buttons on the left panel
85. Check the grid alignment at the corners and the middle of each array
86. In case the grid alignment has failed or is not optimal follow the help instructions of how to re-align the grids

Raw image files are named *.DAT. The *.CEL files contain the raw intensity values for each probe. Continue with the data normalisation and enrichment detection using any analysis tool of your choice.

B. Fischer (07-09-2010).