

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'-CTAATACGACTCACTATAGGGCAGCGTGGTCCGCGGCCGAGGA-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 $^{\circ}$ 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 $^{\circ}$ C for storage. Samples should be stable at -80 $^{\circ}$ C for at least several months.

Day 1:

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

1. Remove Dam sample tubes from -80 $^{\circ}$ 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 $^{\circ}$ 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 $^{\circ}$ 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
- #4: 2.5 μ g 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 μ L Buffer 4 (10 x, NEB)
- 43.5 μ L MilliQ water
- 1.5 μ L DpnI (20 U/ μ L, NEB) (or MilliQ water, for #2 no DpnI control)

18. Add 50 μ L 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 μ L 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 μ L 5X ligation buffer (Invitrogen)
- 0.8 μ L ds-AdR (made from AdRt and AdRb, see reagents list)
- 1.0 μ L T4 DNA ligase (5 U/ μ L, Invitrogen) (Replace T4 DNA ligase with MilliQ water for #3 'no ligase' control)

22. Transfer 14.2 μ L of purified DpnI digested genomic DNA product to the PCR tube for ligation to the adaptors (store the remaining 15.8 μ L of genomic DNA at -20 °C for future use).
23. Mix with 5 μ L ligation master mix (total volume is now 20 μ L).
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 µL from DpnII digested genomic DNA into a fresh PCR tube (store remaining 60 µL at -20 °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 °C 10 min
2. 94 °C 1 min
3. 65 °C 5 min
4. 68 °C 15 min
5. 94 °C 1 min
6. 65 °C 1 min
7. 68 °C 10 min
8. Repeat steps 5-7, 3 times
9. 94 °C 1 min
10. 65 °C 1 min
11. 68 °C 2 min
12. Repeat steps 9-11, *e.g.*, 14-17 times.
13. 4 °C, hold

Day 3

29. Check quality of PCR product by running 3 µL 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 $-20\text{ }^{\circ}\text{C}$

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)