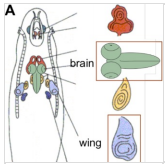

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CUT&Tag with Drosophila tissues

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1 Works for me dx.doi.org/10.17504/protocols.io.bnx5mfq6

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ABSTRACT

This is a modification of the Benchtop CUT&Tag method (dx.doi.org/10.17504/protocols.io.bcuhiwt6) for epigenomic profiling of histone modifications and chromatin proteins in dissected Drosophila tissues. Intact unfixed tissues are permeabilized and incubated under conditions where a factor-specific antibody can infiltrate and bind a chromatin protein *in situ*. This antibody is then decorated with a secondary antibody, and this is used to tether a protein AG-Tn5 fusion protein loaded with sequencing adapters. Upon transposome activation by adding Mg2+ ions, DNA is tagmented around the chromatin binding site. Here, dissected imaginal discs are coated with magnetic beads for handling, and about 5 imaginal discs provides high-quality data for histone modifications or for chromatin factor for which high-quality antibodies are available. The protocol is scaled for 8 samples. Performing CUT&Tag takes 2 days from larvae to sequencing-ready libraries.

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Kami Ahmad, Steven Henikoff 2020. CUT&Tag with Drosophila tissues. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bnx5mfq6

KEYWORDS

chromatin, histone modifications, chromatin proteins

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GUIDELINES

The strategy of this protocol is to coat dissected Drosophila imaginal discs or tissues with ConA magnetic beads, and perform all washes and buffer changes by magnetic capture of the sample. This method uses small numbers of imaginal discs, and uses digitonin to gently permeabilize the unfixed tissues. Bottles where larvae are not too dense provide well-fed crawling larvae that are easiest to dissect and provide the best-quality tissue.

The protocol workflow is:

Day 1: Larvae to primary antibody incubation
Preparing working solutions and conA beads
Dissecting larvae

Binding tissues to beads
Binding primary antibody

Day 2: secondary antibody incubation to Library enrichment
Binding secondary antibody
Tethering transposomes
DNA tagmentation
DNA recovery
Library enrichment

Limitations
The success of CUT&Tag depends on the affinity, specificity, and yield of an antibody for its target under the conditions used for binding. Because antibodies bind to their epitopes in the solid state using CUT&Tag, we expect that antibodies successfully tested for specificity by immunofluorescence (IF) will be likely to work with CUT&Tag, with the caveat that IF generally involves fixation, whereas formaldehyde fixation decreases the efficiency of CUT&Tag for many epitopes.

One of the limitations of working with small amounts of tissues is that the amount of DNA recovered can be very low, such that even after library enrichment DNA is not detectable by sensitive capillary electrophoresis (*eg.* Agilent Tapestation), although may yield good results by NGS sequencing. We recommend using a positive control antibody that targets an abundant epitope so that library DNA can be detected. We have successfully used a rabbit monoclonal antibody raised against the H3K27me3 histone modification, with capillary electrophoresis showing a nucleosomal ladder. For less abundant epitopes such as transcription factors we often do not detect any library by Tapestation, yet libraries can be successfully sequenced. As a negative control, we recommend the use of a non-specific rabbit IgG antibody that will randomly coat the chromatin at low density without sequence bias.

MATERIALS TEXT

MATERIALS

cOmplete™, EDTA-free Protease Inhibitor Cocktail **Sigma Aldrich Catalog #05056489001**

Ethanol 100% **Contributed by users**

Agencourt Ampure XP **Beckman Coulter Catalog #A63880**

Concanavalin-coated magnetic beads **Bangs Laboratories Catalog #BP531**

5% Digitonin **Emd Millipore Catalog #300410**

DMSO

Distilled, deionized or RNAse-free H2O (dH2O e.g., Promega, cat. no. P1197) **Promega Catalog #P1197**

1 M Manganese Chloride (MnCl2) **Sigma Aldrich Catalog #203734**

1 M Calcium Chloride (CaCl2) **Fisher Scientific Catalog #BP510**

1 M Potassium Chloride (KCl) **Sigma Aldrich Catalog #P3911**

1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.5 (HEPES (Na)) **Sigma Aldrich Catalog #H3375**

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1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.9 (HEPES (K)) **Sigma Aldrich Catalog #H3375**

5 M Sodium chloride (NaCl) **Sigma Aldrich Catalog #S5150-1L**

0.5 M Ethylenediaminetetraacetic acid (EDTA) **Research Organics Catalog #3002E**

2 M Spermidine **Sigma Aldrich Catalog #S2501**

BSA ??

10% Sodium dodecyl sulfate (SDS) **Sigma Aldrich Catalog #L4509**

Proteinase K **Thermo Fisher Scientific Catalog #EO0492**

1 M Tris-HCl pH 8.0 **Contributed by users**

Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #5067-4626**

rabbit monoclonal anti-H3K27me3 antibody **Cell Signaling Technology Catalog #9733**

Antibody to an epitope of interest (user-provided)
guinea pig α-rabbit antibody (Antibodies online catalog #ABIN101961)

rabbit anti-mouse IgG **Abcam Catalog #ab46540**

Protein A/G–Tn5 (pAG-Tn5) fusion protein loaded with double-stranded adapters with 19mer Tn5 mosaic ends (Epicypher catalog #15-1117).
NEBNext 2X PCR Master mix (NEB ME541L)
PCR primers: 10 µM stock solutions of i5 and i7 primers with unique barcodes [Buenrostro, J.D. et al. Nature 523:486 (2015)] in 10 mM Tris pH 8. Standard salt-free primers may be used. Do not use Nextera or NEBNext primers.
PCR 8-tube strips (BrandTech #P1200)
PCI
chloroform
phase-lock tubes

EQUIPMENT

- Centrifuge Eppendorf 5810R, refrigerated swinging bucket rotor
- Macsimag magnetic separator (Miltenyi, cat. no. 130-092-168)
- Permagen 0.2 mL PCR 8 Strip Magnetic Separator (SKU:MSRLV08)
- Vortex mixer (e.g., VWR Vortex Genie)
- Micro-centrifuge (e.g., VWR Model V)
- 1.5-mL microcentrifuge tubes (Genesee, cat. no. 22-282)
- Aluminum block with wells for 200 µL tube strips
- PCR machine
- Capillary electrophoresis instrument (e.g. Agilent Tapestation 4200)

SAFETY WARNINGS

Digitonin is toxic and care should be taken especially when weighing out the powder. Use full PPE including a mask. Use gloves while handling any amount of digitonin.

ABSTRACT

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This is a modification of the Benchtop CUT&Tag method (dx.doi.org/10.17504/protocols.io.bcuhiwt6) for epigenomic profiling of histone modifications and chromatin proteins in dissected Drosophila tissues. Intact unfixed tissues are permeabilized and incubated under conditions where a factor-specific antibody can infiltrate and bind a chromatin protein *in situ*. This antibody is then decorated with a secondary antibody, and this is used to tether a protein AG-Tn5 fusion protein loaded with sequencing adapters. Upon transposome activation by adding Mg2+ ions, DNA is tagmented around the chromatin binding site. Here, dissected imaginal discs are coated with magnetic beads for handling, and about 5 imaginal discs provides high-quality data for histone modifications or for chromatin factor for which high-quality antibodies are available. The protocol is scaled for 8 samples. Performing CUT&Tag takes 2 days from larvae to sequencing-ready libraries.

BEFORE STARTING

The following are used as stock solutions that can be prepared and stored in advance:

5% Digitonin/DMSO
Dissolve 50 mg digitonin in 1 mL DMSO. Store at RT for up to 1 week or freeze at -20˚.

Caution: Digitonin is toxic and care should be taken especially when weighing out the powder. Use full PPE including a mask, lab coat and gloves while handling any amount of digitonin. Be aware that DMSO can penetrate through the skin.

Bead Binding Buffer (400 mL)
387 mL H_2O
8 mL 1 M HEPES-KOH pH7.9 final 20 mM
4 mL 1 M KCl final 10 mM
400 µL 1 M $CaCl_2$ final 1 mM
400 µL 1 M $MnCl_2$ final 1 mM

Wash $ Buffer (500 mL)
475 mL water
10 mL 1 M HEPES pH 7.5 final 20 mM
15 mL 5 M NaCl final 150 mM

300Wash $ Buffer
460 mL water
10 mL 1 M HEPES pH 7.5 final 20 mM
30 mL 5 M NaCl final 300 mM

30% BSA

100 mM $MgCl_2$ (100 mL)
90 mL H_2O
10 mL 1 M $MgCl_2$

80% ethanol

Prepare solutions and beads

1 Prepare **Wash+ Buffer** (50 mL):
- Add 50 mL of Wash $ buffer solution to a 50 mL conical tube
- Add 1 large Roche cOmplete EDTA-free tablets
- Add 12.5 µL 2 M Spermidine final 0.5 mM

Keep on ice or store overnight at 4˚C.

2 Prepare **300Wash+ Buffer** (15 mL):
- Add 15 mL of 300Wash $ buffer solution to a 15 mL conical tube
- Add 1 mini Roche cOmplete EDTA-free tablets

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- Add 3.8 µL 2 M Spermidine final 0.5 mM

Store overnight at 4˚C.

3 Prepare Dig-Block-EDTA (**dbe+**) Buffer (1 mL):
- Add 1 mL of Wash+ Buffer to an eppendorf tube
- Add 33 µL 30% BSA final 1%
- Add 4 µL 0.5 M EDTA final 2 mM
- Add 10 µL 5% Digitonin/DMSO final 0.05%

Keep on ice.

4 Prepare **1˚ antibody dilutions** in dbe+ buffer. You'll need 20 µL for each sample, typically at 1:100 dilution.
For example, if you are doing 8 reactions with 1 antibody, aliquot 200 µL dbe+ buffer, add 2 µL 1˚ antibody, and pipette to mix. Antibody dilutions can be stored for 1 week at 4˚.

5 Prepare **ConA bead slurry**:
Vortex bottle of Bio-Mag Plus Concanavalin A-coated beads. Aliquot 200 µL of Bead Binding buffer to an eppendorf tube, add 45 µL of ConA beads, and pipette to mix. Incubate 5' Grab beads with a magnet for 1', remove buffer and resuspend ConA beads in 180 µL of Binding buffer.

Dissect larvae

6 Work with a dissecting microscope with tangential illumination. Collect healthy 3rd instar larvae in a glass dish with PBT. Wash off any yeast and food.

7 Aliquot 200 µL Wash+ buffer into 3 wells of a dissection plate for each genotype.Transfer larvae from PBT to the first well of Wash+ buffer and dissect out imaginal discs. Aim for 4 wing discs or 6 eye discs/sample, so 32 wing discs (or 48 eye discs) for 8 parallel samples. As you dissect, transfer parts to the next wells with Wash+ buffer to minimize contamination with other tissues.

Primary antibody binding

8 Add 20 µL Wash+ buffer to each tube of a 8-tube strip. Transfer imaginal discs with forceps to each tube. Add 20 µL ConA bead suspension to each tube and pipette gently to mix. Incubate 10' RT.

All successive buffer changes are by grabbing beads with the magnet, pipetting off liquid, and replacing with new buffers. Be careful not to withdraw any beads during washes. Beads are not resuspended or agitated at any time in the procedure to minimize sample loss.

9 Place strip on low-volume side of magnet and let bind for 1'. Pipette off the buffer, and replace with 20 µL antibody dilution. Do not resuspend the beads. Remove the strip from the magnet and incubate with no movement 4˚C O/N.

Secondary antibody binding

10 Prepare 2˚ antibody dilution in dbe+ buffer. You'll need 20 µL for each sample at 1:100 dilution, so for 8 reactions with a rabbit 1˚ antibody, aliquot 100 µL dbe+ buffer, add 1 µL 2˚ antibody, and pipette to mix.

11 Place sample strip on low-volume side of magnet and let bind for 1'. Pipette off the 1˚ antibody solution buffer and replace with 20 µL 2˚ antibody dilution. Do not resuspend the beads. Remove the strip from the magnet and incubate with no movement 1 hr at RT.

pAG-Tn5 tethering

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12 *Batches of pAG-Tn5 are calibrated after production. Use the appropriate calibrated dilution for your batch, or recommended dilutions for commercial enzymes.*

Prepare pAG-Tn5 dilution: For 8 samples, Aliquot 200 µL 300Wash+ buffer, add 1 µL pAG-Tn5 and mix by pipetting. Keep on ice or store at 4˚C.

13 Place sample strip on low-volume side of magnet to grab beads, 1'. Remove 2˚ antibody solution and replace with 20 µL pAG-Tn5 dilution. Do not resuspend the beads. Remove the strip from the magnet and incubate with no movement 1 hr at RT.

14 Wash: place strip on low-volume side of magnet to grab beads, 1'. Remove tethering solution and replace with 20 µL 300Wash+ buffer. Do not resuspend the beads. Incubate on the magnet for 2'.

chromatin tagmentation

15 Prepare **300Mg+ tagmentation buffer**: aliquot 180 µL 300Wash+ buffer to an eppendorf and add 20 µL 100 mM $MgCl_2$ and mix.Store at RT. *Final concentration is 10 mM $MgCl_2$.*

16 Place sample strip on low-volume side of magnet to grab beads, 1'. Remove buffer and add 20 µL 300Mg+ tagmentation buffer. Promptly move strip to a preheated PCR machine and incubate 1 hr 37˚.

17 Prepare **300STOP buffer**: aliquot 200 µL 300Wash+ buffer,. Add 3.3 µL 10% SDS and 3 µL 20 mg/mL protease K. Mix by pipetting. Store at RT until tagmentation reactions are complete.

18 At the end of tagmentation, add 20 µL 300STOP buffer to each sample tube and pipette to mix. Incubate samples in a PCR machine 1 hr 58˚C.

DNA recovery

19 Add 60 µL 300Wash+ buffer to each sample.

20 Prepare 8 phase-lock tubes: spin at max, 30". Number the tubes.

21 Work in the hood: aliquot 100 µL PCI into 8 eppendorf tubes. Transfer each sample with beads into each eppendorf, cap, and mix by full-speed vortexing ~2".

22 Transfer PCI/sample mix into a phase-lock tube. Spin at 16,000 x g, 4'.

23 Add 100 µL chloroform to each phase-lock tube and invert 10X to mix (do not vortex). Spin at 16,000 x g, 4'.

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24 Transfer the aqueous layer by pipetting to a fresh 1.5 mL tube containing 250 µL 100% ethanol on ice. Invert tubes 10X to mix. Incubate 1' on ice.

25 Centrifuge 10' at 16,000 x g, 4˚.

26 Carefully pour off ethanol, and replace with 300 µL 80% ethanol. Centrifuge 4' at 16,000 x g, 4˚.

27 Carefully pour off ethanol, and allow samples to air-dry on a paper towel, 15'.

There is no visible pellet.

28 Resuspend DNA in 22 µL 10 mM Tris-HCl, pH 8 and vortex on full to dissolve. Spin briefly to collect.

Library enrichment

29 Aliquot 2 µL of 10 µM Universal or barcoded i5 primer + 2 µL of 10 µM uniquely barcoded i7 primer into each tube of a PCR tube strip, using a different barcode for each sample*. Add 21 µL of sample DNA.

**Indexed primers are described by Buenrostro, J.D. et al. Single-cell chromatin accessibility reveals principles of regulatory variation. Nature 523:486 (2015).*

30 Chill PCR strip on ice. Add 25 µL NEBNext HiFi 2X PCR Master mix. Vortex and spin briefly to mix, and return to ice.

Do not use hot-start enzyme mixes.

31 Start cycling program on thermocycler to get to temperature. When the block gets to 58˚ move the tube strip into the machine.

Heated lid
Cycle 1: 58˚ for 5' (gap filling)
Cycle 2: 72˚ for 5' (gap filling)
Cycle 3: 98˚ for 30"
Cycle 4: 98˚ for 10"
Cycle 5: 60˚ for 10"
Repeat Cycles 4-5 13 times
72˚ for 1' and hold at 8˚

This typically takes 30' to run.

To minimize the contribution of large DNA fragments and excess primers, PCR should be performed for 12-14 cycles, preferably with a 10" 60-63˚ combined annealing/extension step.

*Do not add extra PCR cycles to see a signal by capillary gel electrophoresis (*eg. *Tapestation). Observing no signal for a sparse chromatin protein such as a transcription factor is normal, and the barcoded sample can be concentrated for mixing with the pool of barcoded samples for sequencing. Extra PCR cycles reduce the complexity of the library and may result in an unacceptable level of PCR duplicates.*

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32 Label eppendorfs with library ID and aliquot 65 µL Ampure XP beads to each tube.

This is a 1.3:1 ratio for cleanup.

33 Transfer PCR reactions to eppendorfs with Ampure beads and vortex to mix. Quickly spin the tubes to collect the beads, and incubate 5' RT.

34 Place eppendorfs on magnet and incubate 5' to grab beads.

35 *Beads are somewhat slippery in this step.* Pipette off solution and replace with 700 µL 80% ethanol while still on magnet. Incubate 30".

36 *Beads stick very well in this step.* Aspirate off ethanol and replace with 700 µL 80% ethanol while still on magnet. Incubate 30".

37 Slowly aspirate to remove all traces of ethanol. Do not air-dry the beads but proceed immediately to the next step.

38 Resuspend beads in 25 µL 10 mM Tris-HCl pH 8.

39 Determine the size distribution and concentration of libraries by capillary electrophoresis using an Agilent 4200 TapeStation with D1000 reagents or equivalent.

Sequencing & analysis

40 Mix barcoded libraries to achieve equal representation as desired aiming for a final concentration as recommended by the sequencer manufacturer. After mixing, perform an Ampure bead cleanup if needed to remove any residual PCR primers.

41 Perform paired-end Illumina sequencing on the barcoded libraries following the manufacturer's instructions. PE25 is more than sufficient for mapping to the Drosophila genome.

42 Mapping, spike-in normalization and analysis are described here: dx.doi.org/10.17504/protocols.io.bjk2kkye

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