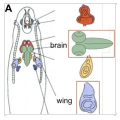

CUT&RUN with Drosophila tissues

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ABSTRACT

We have modified the Cleavage Under Targets and Release Using Nuclease (CUT&RUN) method for epigenomic profiling of histone modifications and chromatin proteins to use dissected Drosophila tissues. In CUT&RUN, cells or tissues are permeabilized and incubated under conditions where a factor-specific antibody can bind at sites of a chromatin protein. This antibody is then used to tether a protein A-micrococcal nuclease fusion protein, which upon activation releases specific protein-DNA complexes into the supernatant for paired-end DNA sequencing, and leaving the vast majority of DNA behind in the cellular pellet. CUT&RUN outperforms the most widely used Chromatin Immunoprecipitation (ChIP) protocols in resolution, signal-to-noise, and depth of sequencing required. Previous versions of CUT&RUN have used isolated nuclei, mammalian cultured cells, or yeast spheroplasts. Here we have modified the CUT&RUN protocol to manipulate imaginal discs and tissues dissected from larvae with magnetic beads without isolation of cells or nuclei. About 10 imaginal discs provides high-quality data for a histone modification or for a chromatin factor. A simple spike-in strategy is used for accurate quantitation of experiment yields and library preparation. From larvae to purified DNA, CUT&RUN requires 1-2 days with minimal handling after dissection.

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

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. Finally, the protocol uses Ampure beads to efficiently recover DNA, avoiding the risk of contamination by phenol or chloroform. Bottles where larvae are not too dense provide well-fed crawling larvae that are easiest to dissect and provide the most tissue.

The protocol workflow is as follows:

Day 1: Larvae to primary antibody incubation
Preparing working solutions and conA beads (Steps 1-4, 15')
Dissecting larvae (Steps 5-6, 15')
Binding tissues to beads (Step 7, 10')
Binding primary antibody (Steps 8-10, O/N)

Day 2: primary antibody incubation to DNA recovery
Binding primary antibody (Steps 11-12, 10')
Optional: binding secondary antibody (Steps 13-16, 1 hr 15')
nuclease tethering (Steps 17-20, 1 hr 20')
DNA cleavage (Steps 21-25, 1 hr 5')
DNA recovery (Steps 26-36, 3 hrs 30')

Preparing Libraries, sequencing, data processing and analysis is performed as perviously described for CUT&RUN protocols (eg. Skene et al (2018). Targeted in situ genome-wide profiling with high efficiency for low cell numbers. Nature Protocols 13:1006-1019. doi: 10.1038/nprot.2018.015.

Limitations

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

One of the limitations of working with small amounts of tissues is that the amount of DNA recovered can be very low, such that analysis even by sensitive capillary electrophoresis or picogreen assays (e.g. Agilent Tapestation and Qubit) are problematic. In addition, high resolution mapping techniques that cleave a minimal footprint are not suitable to PCR-based analysis of known binding loci, as it is not commonly possible to design ~50 bp PCR amplicons. As such, we recommend using a positive control antibody that targets an abundant epitope and therefore the DNA can be readily detected. We have successfully used a rabbit monoclonal antibody raised against the H3K27me3 histone modification, with capillary electrophoresis showing with the amount of cleaved fragments being proportional to the number of starting cells. A nucleosomal ladder is expected by Tapestation or other sensitive electrophoretic analysis method, and the use of a monoclonal antibody avoids potential lot-to-lot variation that can complicate troubleshooting. For less abundant epitopes such as trnascription factors, we often do not detect any cleaved fragments by Tapestation, yet successful libraries can be prepared from these reactions. 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. We do not recommend a no-antibody control, as the lack of tethering increases the possibility that any slight carry-over of pA-MN will result in preferential fragmentation of hyper-accessible DNA. We recommend activating the nuclease for 30 minutes as a starting point that can be tailored based upon epitope abundance and antibody concentration.

In the standard CUT&RUN protocol we recommend allowing the cleaved chromatin complexes to diffuse out of the nuclei, thereby permitting simple isolation of the cut DNA from the supernatant fraction with the undigested genome retained in the intact nuclei. However, it is possible that certain chromatin complexes are too large to diffuse out of the nucleus, or that protein-protein interactions retain the cleaved complex. In such cases, DNA extraction and size-selection of fragments below ~700 bp can be used for profiling.

MATERIALS

NAME	CATALOG #	VENDOR
cOmplete™, EDTA-free Protease Inhibitor Cocktail	05056489001	Sigma Aldrich
Ethanol 100%		
Agencourt Ampure XP	A63880	Beckman Coulter
Concanavalin-coated magnetic beads	BP531	Bangs Laboratories
Negative control antibody to an absent epitope, e.g. guinea pig α-rabbit antibody		
5% Digitonin	300410	Emd Millipore
Protein A–Micrococcal Nuclease (pA-MNase) fusion protein (provided in 50% glycerol by the authors upon request). Store at -20 oC.		
Spike-in DNA (e.g., from Saccharomyces cerevisiae micrococcal nuclease-treated chromatin, provided by authors upon request)		
Distilled, deionized or RNAse-free H2O (dH2O e.g., Promega, cat. no. P1197)	P1197	Promega
1 M Manganese Chloride (MnCl2)	203734	Sigma Aldrich
1 M Calcium Chloride (CaCl2)	BP510	Fisher Scientific
1 M Potassium Chloride (KCl)	P3911	Sigma Aldrich
1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.5 (HEPES (Na))	H3375	Sigma Aldrich
1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.9 (HEPES (K))	H3375	Sigma Aldrich
5 M Sodium chloride (NaCl)	S5150-1L	Sigma Aldrich
0.5 M Ethylenediaminetetraacetic acid (EDTA)	3002E	Research Organics
0.2 M Ethylene glycol-bis(β-aminoethyl ether)-N,N,N,N-tetraacetic acid (EGTA)	E3889	Sigma Aldrich
2 M Spermidine	S2501	Sigma Aldrich
RNase A, DNase and protease-free (10 mg/ml)	EN0531	Thermo Fisher Scientific
10% Sodium dodecyl sulfate (SDS)	L4509	Sigma Aldrich
Proteinase K	EO0492	Thermo Fisher Scientific

NAME	CATALOG #	VENDOR
1 M Tris-HCl pH 8.0		
Agilent High Sensitivity DNA Kit	5067-4626	Agilent Technologies
Qubit dsDNA HS Assay Kit	Q32851	Thermo Fisher Scientific
Drosophila 3rd instar larvae	View	
rabbit anti-mouse IgG	ab46540	Abcam
rabbit monoclonal anti-H3K27me3 antibody	9733	Cell Signaling Technology
Rabbit IgG antibody to a chromatin protein of interest. A mouse IgG antibody can be used with indirect tethering.	View	
yeast spike-in DNA (1 ng/mL). NOTE: this fragmented to ~150 bp by MNase digestion of yeast nuclei and resuspended in water at 1 ng/mL.	none	

MATERIALS TEXT

EQUIPMENT

- Centrifuge Eppendorf 5810R, refridgerated swinging bucket rotor
- Macsimag magnetic separator (Miltenyi, cat. no. 130-092-168)
- Vortex mixer (e.g., VWR Vortex Genie)
- Micro-centrifuge (e.g., VWR Model V)
- 1.5-ml microcentrifuge tubes (Genesee, cat. no. 22-282)
- Tube rotator (Labquake, Thermo Fisher)
- Aluminum block with wells for 1.5-ml microcentrifuge tubes
- Water baths (set to 37°C and 50 °C)
- Capillary electrophoresis instrument (e.g. Agilent Tapestation 4200)
- Qubit Fluorometer (Life Technologies, cat. no. Q33216)

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.

BEFORE STARTING

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

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 (800 mL)

757 mL H_2O
16 mL 1 M HEPES pH7.5 final 20 mM
24 mL 5 M NaCl final 150 mM
2.7 mL 30% BSA final 0.1%

2XSTOP buffer (100 mL)

90 mL H_2O
4 mL 5 M NaCl final 200 mM
4 mL 0.5 M EDTA final 20 mM
2 mL 0.2 mM EGTA final 4 mM

100 mM $CaCl_2$ (100 mL)

90 mL H_2O
10 mL 1 M $CaCl_2$

MXP buffer (50 mL)

10 g PEG8000 final 20%
25 mL 5 M NaCl final 2.5 M
0.5 mL 1 M $MgCl_2$ final 10 mM
bring up to 50 mL with H_2O

HXP buffer

10 g PEG8000 final 20%
25 mL 5 M NaCl 2.5 M
bring up to 50 mL with H_2O

80% ethanol

Prepare solutions and beads

1 Prepare a fresh **5% digitonin** solution as follows:

SAFETY INFORMATION

• CAUTION: Digitonin is toxic and care should be taken especially when weighing out the powder.

Weigh out 50 mg digitonin powder in a 2 ml microcentrifuge tube. Boil some water in a small beaker in a microwave oven, and pipette in and out to warm the 1000 µL pipette tip. Pipette the hot water into the tube with the digitonin powder, close the cap, and quickly vortex on full until the digitonin is completely dissolved. If refrigerated, this stock can be used within a week, but will need reheating as the digitonin slowly precipitates. The effectiveness of digitonin varies between batches, so testing permeability of Trypan blue is recommended to determine effective concentrations. We have obtained excellent results with 0.02-0.1% digitonin.

2 Prepare **Wash+ Buffer** (50 mL):

- Add 50 mL of wash buffer stock solution to a 50 mL conical tube
- Add 1 large Roche complete EDTA-free tablets
- Add 125 µL 200 mM Spermidine final 0.5 mM

Keep on ice or store overnight at 4˚C.

3 Prepare **Dig-Block-EDTA (dbe+) Buffer** (25 mL):

- Add 25 mL of Wash+ Buffer to a 50 mL conical tube
- Add 100 µL 0.5 M EDTA final 2 mM
- Add 250 µL 5% Digitonin final 0.05%

Keep on ice or store overnight at 4˚C.

4 Prepare **ConA beads:**

Gently vortex bottle of Bio-Mag Plus Concanavalin A-coated beads. Transfer 150 µL of ConA bead slurry to an eppendorf. Grab beads with a magnet for 5', remove buffer and replace with 1 mL Binding buffer. Incubate 1'. Wash 2X. Resuspend ConA beads in 150 µL of Binding buffer.

Dissect larvae

5 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.

6 Aliquot 200 µL Wash+ buffer into 2 wells of a clean shallow dissection dish for each sample. Pick up larvae and transfer to the dissection dish, and dissect out 10 wing discs. Aim to take less than 10 minutes, so that discs remain healthy. Use a 200 µL pipette tip to transfer the wing discs to the second well of Wash+ buffer.

Binding tissues to beads

7 Add 15 µL ConA bead suspension to an eppendorf. Use a 200 µL pipette tip to transfer the wing discs to the eppendorf with a minimal volume of Wash+ buffer. Mix by gentle pipetting. 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.

Binding primary antibody

8 Block the tissues: Add 1 mL dbe+ buffer to each eppendorf. Gently flick the tube to mix, and incubate 10' at RT.

9 Prepare antibody dilutions in dbe+ buffer. You'll need 100 µL for each sample, typically at 1:100 dilution.
For example, you are doing 10 reactions with 1 antibody. Aliquot 1 mL dbe+ buffer, add 10 µL 1˚ antibody, and pipette to mix. If you are doing 10 different antibodies, aliquot 100 µL dbe+, add 1 µL 1˚ antibody and mix.

10 Place eppendorfs on magnet, and let bind for 2'. Pipette off the buffer, and replace with 100 µL antibody dilution. Incubate eppendorfs at a 60˚ angle on an orbital shaker, orbiting slowly, 4˚C O/N.

11 Very briefly spin tubes to collect liquid if necessary. Place tubes on magnet to grab beads, 2'. Remove buffer, and replace with 500 µL dbe+ buffer to wash the beads. Resuspend sample by pipetting and incubate 2' RT.

12 Repeat Wash. Very briefly spin tubes to collect liquid if necessary. Place tubes on magnet to grab beads, 2'. Remove buffer, and replace with 500 µL dbe+ buffer to wash the beads. Resuspend sample by pipetting and incubate 2' RT.

Optional: Binding secondary antibody

13 *This section uses a secondary antibody to adapt mouse IgG primary antibody for protein-A binding. If not needed, skip to Step 17.*

Prepare secondary rabbit anti-mouse IgG antibody dilution in dbe+ buffer. You'll need 100 µL for each sample at 1:100 dilution. For 10 reactions with a mouse primary antibody, aliquot 1 mL dbe+ buffer, add 10 µL 1˚ antibody, and pipette to mix.

14 Place sample eppendorfs on magnet, and let bind for 2'. Pipette off the buffer, and replace with 100 µL secondary antibody dilution. Incubate eppendorfs at a 60˚ angle on an orbital shaker, orbiting slowly, 1 hr at RT.

15 Very briefly spin tubes to collect liquid if necessary. Place tubes on magnet to grab beads, 2'. Remove buffer, and replace with 500 µL dbe+ buffer to wash the beads. Resuspend sample by pipetting and incubate 2' RT.

16 Repeat Wash. Very briefly spin tubes to collect liquid if necessary. Place tubes on magnet to grab beads, 2'. Remove buffer, and replace with 500 µL dbe+ buffer to wash the beads. Resuspend sample by pipetting and incubate 2' RT.

nuclease tethering

17 *Dilution of different batches of pAMN are calibrated. This step uses Batch #6, which is used at a 1:400 dilution. Use the appropriate calibrated dilution of other batches.*

Prepare pAMN dilution: Aliquot 1 mL dbe+ buffer, add 2.5 µl pAMN#6 and mix by pipetting. This is enough for 10 samples.

18 Place sample tubes on magnet to grab beads, 2'. Remove buffer, and replace with 100 µL pAMN dilution. Resuspend sample by pipetting and incubate orbiting slowly 1 hr, RT.

19 Wash: very briefly spin tubes to collect liquid if necessary. Place samples on magnet to grab beads, 2'. Remove buffer, and replace with 500 µL Wash+ buffer. Resuspend samples by pipetting and incubate 2'.

20 Repeat wash: very briefly spin tubes to collect liquid if necessary. Place samples on magnet to grab beads, 2'. Remove buffer, and replace with 500 µL Wash+ buffer. Resuspend samples by pipetting and incubate 2'.

DNA cleavage

21 Prepare Wash+C buffer: Aliquot 1.5 mL Wash+ buffer, add 30 µL 100 mM $CaCl_2$ and mix. Place buffer on ice and chill.

Final concentration is 2 mM $CaCl_2$.

22 Place sample tubes on magnet to grab beads, 2'. Remove buffer, and place samples on ice to chill tubes. Promptly resuspend sample with 150 µl Wash+C buffer and start timer. Incubate 30' on ice. Occasionally flick the tube to resuspend the beads.

23 Prepare 2XSTOPyR buffer: aliquot 1600 µL 2XSTOP buffer. Add 3.2 µL 1 ng/mL yeast spike-in DNA and 10 µL RNaseA. Chill on ice until cleavage reaction is complete.

24 Prepare Pellet buffer: Aliquot 800 µL Wash+ buffer, add 800 µL 2XSTOP buffer. Add 3.2 µL of 1 ng/mL yeast spike-in DNA. Add 17 µL 10% SDS and 22 µL of 20mg/mL proteinase K. Store at RT.

25 At the end of 30' incubation, add 150 µL 2XSTOPyR buffer to each sample tube and pipette to mix. Incubate samples in a 37˚C water bath, 30'.

DNA recovery

26 *This section includes DNA recovery from both supernatant and pellet fractions. In initial experiments with a new antibody, we compare the abundance of cleaved DNA in supernatant and pellet fractions to confirm that most of the cleaved DNA is soluble. in most cases this is the case, and then library construction and sequencing is performed only with supernatant DNA.*

The following steps describe DNA recovery from both supernatant and pellet fractions.

27 Chromatin fractionation: Grab beads on magnet, 2'. Transfer the supernatant containing cleaved chromatin particles to a new tube, marked 'sample#S'. Resuspend pellet in 150 µL Pellet buffer, add a 'P' to the tube label.

The 'P' tube is a transient tube.

28 Add 2 µL 10% SDS and 2.5 µL of 20 mg /mL Proteinase K to each 'S' sample and mix by brief vortexing. Incubate 'S' and 'P' samples in a 50˚C water bath, 2 hr.

29 To 'S' tubes: Use wide-bore green tips to add 40 µL AmpureXP bead slurry and 560 µL MXP buffer to each tube, mix thoroughly by 10X pipetting. Incubate 15' RT.

This is a non-size-selective recovery of DNA fragments; the PEG:sample ratio is 2V. Total volume is ~900 µL.

30 To 'P' tubes: grab ConA beads with magnet, 2'. Transfer supernatant to a new tube. **Beads are very slippery in this buffer.** Use wide-bore green tips to add 40 µL AmpureXP bead slurry and 65 µL HXP buffer to each tube, mix thoroughly by 10X pipetting. Incubate 15' RT.

This is a size-selective recovery of DNA; the PEG:sample ratio is 0.7V. Total volume is ~255 µL. The fragments you are looking for will be in the supernatant!

31 To 'P' samples: grab Ampure beads with magnet, 5'. **Beads are very slippery in this buffer.** Transfer supernatant to a new tube, tag-labeled as 'sample#PX'. Add 40 µL AmpureXP bead slurry and 155 µL MXP buffer to each tube, mix thoroughly by 10X pipetting. Incubate 15' RT.

This is a non-size-selective recovery of DNA; the PEG:sample ratio is 2V. Total volume is ~450 µL.

32 24) To 'S' and 'PX' tubes: grab Ampure beads with magnet, 5'. **Beads are very slippery in this buffer.** Pipette off buffer, making sure not to remove beads at the end. Keep tubes on the magnet, and add 1 mL 80% ethanol to each tube. Incubate 30".

33 **Beads stick very well in this step.** Aspirate off ethanol and replace with 1 mL 80% ethanol. Incubate 30".

34 Slowly aspirate to remove all traces of ethanol. Leave tubes on magnet and allow beads to air-dry, 15'.

35 Resuspend beads in 40 µL 10 mM Tris pH8.

36 Determine the size distribution of DNA in samples by Agilent 4200 TapeStation analysis.

The positive control (anti-H3K27me3) should show a nucleosomal ladder in the 'S' sample, and a weak high-molecular weight DNA band in the 'PX' sample.
The negative control (non-specific rabbit IgG) should show no DNA in the 'S' sample, and a weak high-molecular weight DNA band in the 'PX' sample.
Most antibodies (eg to transcription factors) will show no nucleosomal ladder, but we find that they often have sufficient material for library construction and produce excellent genomic profiles.

*See **Guidelines** for more discussion.*

37 Quantify library yield using dsDNA-specific assay, such as Qubit.