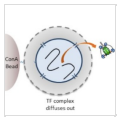


Mar 06, 2019

Working

CUT&RUN: Targeted in situ genome-wide profiling with high efficiency for low cell numbers

Version 2

Steven Henikoff[1]

[1]Howard Hughes Medical Institute, Basic Sciences Division, Fred Hutchinson Cancer Research Center, 1100 Fairview Ave N, Seattle, Washington, USA 98109

dx.doi.org/10.17504/protocols.io.n6wdhfe

Human Cell Atlas Method Development Community

Steven Henikoff
Fred Hutchinson Cancer Research Center

ABSTRACT

We previously described a novel alternative to Chromatin Immunoprecipitation, Cleavage Under Targets & Release Using Nuclease (CUT&RUN), in which unfixed permeabilized cells are incubated with antibody, followed by binding of a Protein A-Micrococcal Nuclease (pA/MNase) fusion protein (1). Upon activation of tethered MNase, the bound complex is excised and released into the supernatant for DNA extraction and sequencing. Here we introduce four enhancements to CUT&RUN: 1) a hybrid Protein A-Protein G-MNase construct that expands antibody compatibility and simplifies purification; 2) a modified digestion protocol that prevents premature release of the nuclease-bound complex; 3) a calibration strategy based on carry-over of *E. coli* DNA introduced with the fusion protein; and 4) a novel peak-calling strategy customized for the low-background profiles obtained using CUT&RUN. These new features, coupled with the previously described low-cost, high efficiency, high reproducibility and high-throughput capability of CUT&RUN make it the method of choice for routine epigenomic profiling.

EXTERNAL LINK

https://www.biorxiv.org/content/10.1101/569129v1

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

A streamlined protocol and analysis pipeline for CUT&RUN chromatin profiling: Michael P. Meers, Terri D Bryson and Steven Henikoff, *bioRxiv* doi: https://doi.org/10.1101/569129.

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

GUIDELINES

The protocol workflow is as follows:

Day 1, Cells to DNA

Binding cells to beads (Steps 1-9, 30 min)
Bind (primary) antibodies (Steps 10-15, 15 min–overnight, with longer incubations providing higher yields)
Bind secondary antibody (as required) (Steps 16-21, 15 min-1.5 hr)
Bind Protein A-MNase fusion protein (Steps 22-30, 15 min–1.5 hr)
Targeted digestion (Steps 31-36, 45 min)
Target chromatin release (Steps 37-38, 20 min)
Option A: Fast DNA extraction by spin column (Steps 39-46, 20 min_
Option B: Alternate DNA extraction (preferred for quantitative recovery of ≤80 bp fragments) (Steps 47-58, 1.5 hr)

Days 2-4

Library preparation and sequencing (Steps 59-64, 2-4 days)

Day 5

Data processing and analysis (Steps 65-66, variable timing)

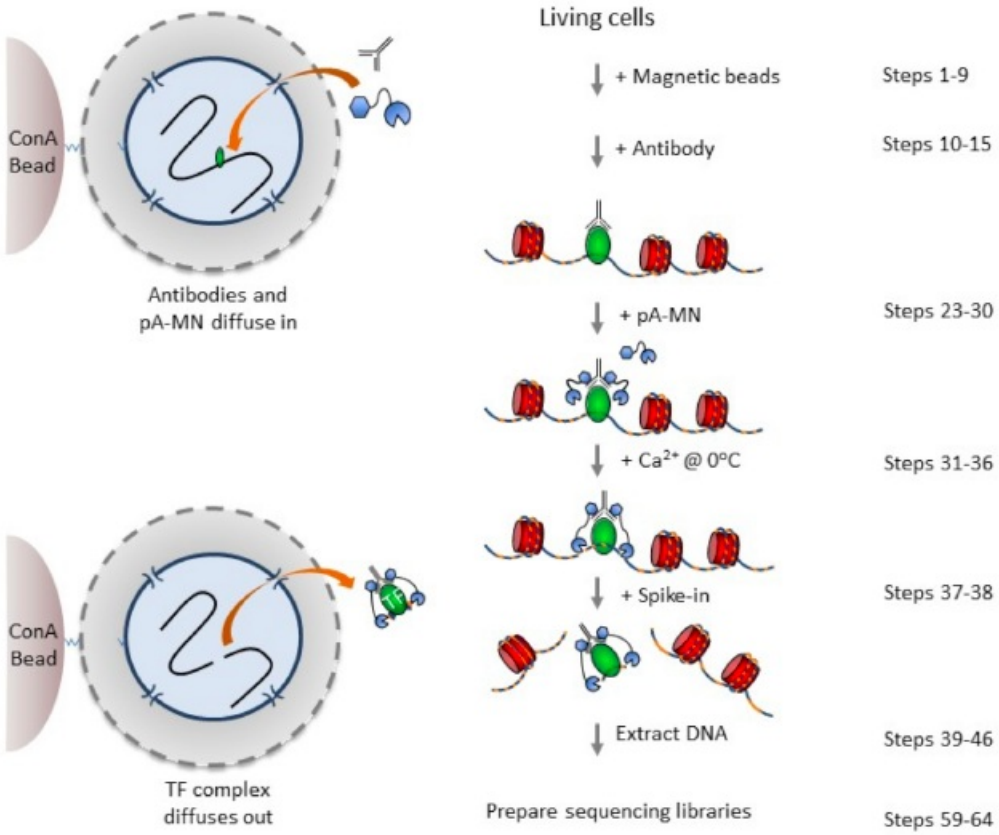


Figure 1: CUT&RUN requires less than a day from cells to DNA. A schematic overview of the CUT&RUN protocol. Cells are harvested and bound to concanavalin A-coated magnetic beads. Cell membranes are permeabilized with digitonin to allow the specific antibody to find it's target. After incubation with antibody, beads are briefly washed, and then incubated with pA-MN. Cells are chilled to 0 °C, and digestion begins with addition of Ca^{2+}. Reactions are stopped by chelation including spike-in DNA and the DNA fragments released into solution by cleavage are extracted from the supernatant.

EQUIPMENT

- Centrifuge Eppendorf 5810, swinging bucket
- Centrifuge Eppendorf 5424, fixed angle rotor
- Centrifuge Eppendorf 5415R, refrigerated fixed angle rotor
- Macsimag magnetic separator (Miltenyi, cat. no. 130-092-168), which allows clean withdrawal of the liquid from the bottom of 1.7 and 2 ml microfuge tubes.
- Vortex mixer (e.g., VWR Vortex Genie)
- Micro-centrifuge (e.g., VWR Model V)
- 1.5-ml microcentrifuge tubes (Genesee, cat. no. 22-282)
- 2-ml microcentrifuge tubes (Axygen, cat. no. MCT-200-C)
- Tube rotator (Labquake, Thermo Fisher)
- Heater block with wells for 1.5-ml microcentrifuge tubes
- Water baths (set to 37°C and 70 °C)
- MaXtract phase-lock microcentrifuge tubes (Qiagen, cat. no. 139046)

- Capillary electrophoresis instrument (e.g. Agilent Tapestation 4200)
- Qubit Fluorometer (Life Technologies, cat. no. Q33216)

INTRODUCTION

Experimental Design

The CUT&RUN method for the in situ targeted cleavage and release of chromatin complexes is straightforward and can be completed in under a day using standard lab equipment. Here we provide a detailed protocol and also provide various options that might be used to tailor the protocol to specific situations. One of the strengths of CUT&RUN is that the entire reaction is performed in situ, whereby the antibody and pA-MN are free to diffuse into the nucleus. The original protocol used nuclei prepared by a combination of hypotonic lysis and treatment of cells with Triton X-100. This has been successful with a number of cell lines, but we have recently adapted the protocol to use cells permeabilized by the non-ionic detergent digitonin, which has been successfully used in other in situ methods, including ChEC-seq[23] and ATAC-seq[24]. Digitonin partitions into membranes and extracts cholesterol. Membranes that lack cholesterol are minimally impacted by digitonin[25,26]. Nuclear envelopes are relatively devoid of cholesterol compared to plasma membranes. As such, treatment of cells with digitonin represents a robust method for permeabilizing cells without compromising nuclear integrity[26]. The protocol described here uses digitonin, but it is possible that individual experimental situations call for generating intact nuclei by other means, and such nuclei can be prepared by a suitable method, bound to concanavalin A-coated beads as per our previously published work and then enter the protocol below at step 10[20].

One of the limitations of a protocol that has inherently low background and is amenable to low cell numbers 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 H3K27me3, 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 sensitiveelectrophoretic analysis method (**Fig. 2**), and the use of a monoclonal antibody avoids potential lot-to-lot variation that can complicate troubleshooting. For less abundant epitopes such as CTCF, it is harder to detect the cleaved fragments by even sensitive electrophoretic analysis (**Supplementary Figure 1**). Once the expected digested DNA pattern is observed for the positive control by capillary electrophoresis such as H3K27me3, it is not necessary to sequence this sample. As a negative control, we recommend the use of a non-specific rabbit IgG antibody that will randomly coat the chromatin at low efficiency without sequence bias. We do not recommend a no-antibody control, as the lack of tethering increases the possibility that slight carry-over of pA-MN will result in preferential fragmentation of hyper-accessible DNA.

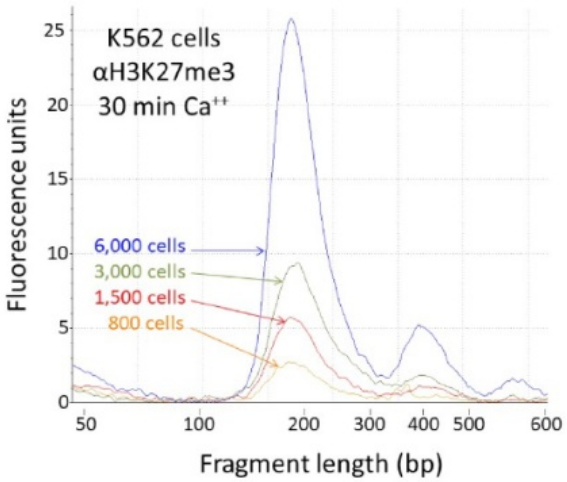
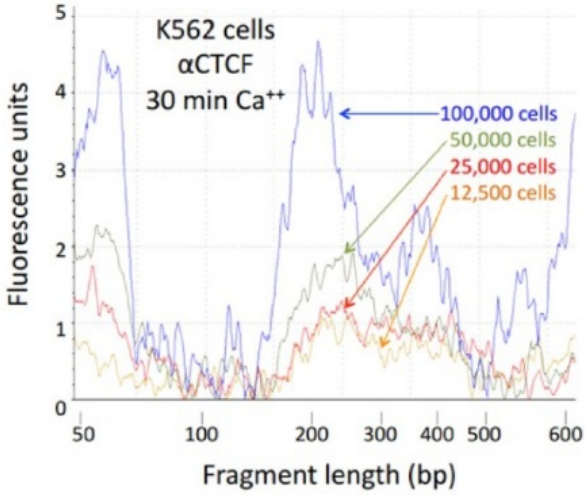
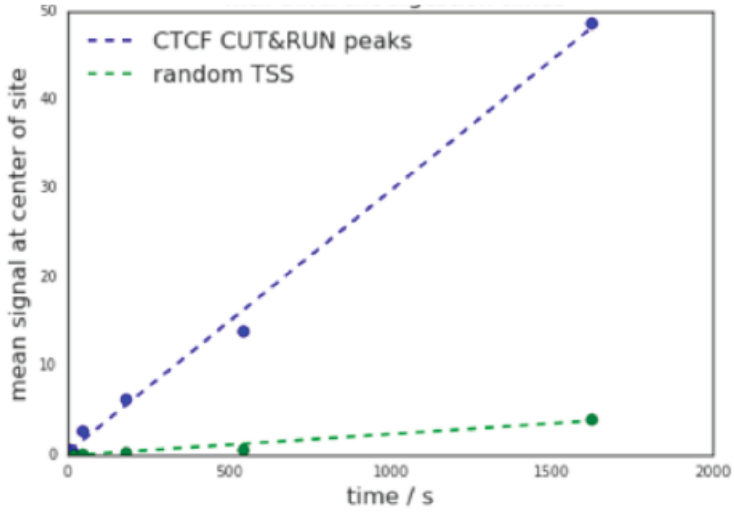


Figure 2: Tapestation analysis of an abundant histone epitope (H3K27me3) as a same-day positive control. The remainder of these samples were used to make libraries for sequencing, with results shown in Figure 3.



Supplementary Figure 1: Tapestation analysis of CUT&RUN cleaved fragments using an anti-CTCF antibody. The remainder of these samples were used to make libraries for sequencing, with results shown in Figure 4.



Supplementary Figure 2: Yield increases with digestion time with little change in signal-to-noise. By scaling to spike-in DNA, quantitative measurement of the amount of cleaved DNA fragments is possible. The average signal over ~20,000 CTCF CUT&RUN binding sites is compared to an equal number of non-overlapping transcriptional start sites (TSS) as a negative control regions. Spike-in scaled signal was summed over the -50 to +50 bp region relative to the center of the site or TSS.

In our previously published study, we showed that targeted cleavage occurred within seconds of adding Ca2+ ions, and by virtue of being a sterically regulated tethered reaction, the cleavage pattern was constant over time. However, longer digestion times release more material with no apparent change in the signal-to-nois ratio (**Supplementary Figure 2**). We therefore recommend digesting for 30 minutes as a starting point that can be tailored based upon epitope abundance and antibody concentration.

Limitations

As is the case with ChIP, the success of CUT&RUN depends in large part on the affinity of the 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 would expect that antibodies successfully tested for specificity by immunofluorescence (IF) would be likely to work with CUT&RUN, with the caveat that IF generally involves fixation, whereas formaldehyde fixation decreases the efficiency of CUT&RUN.

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 a chromatin complex is too large to diffuse out or that protein-protein interactions retain the cleaved complex. In such cases, total DNA may be extracted after the digestion. By doing a very simple size selection using ½ volume of paramagnetic carboxylated beads (e.g. Agencourt AMPure XP beads) fragments below ~700 bp will be selected for. We previously showed that this strategy was successful for the ~1 MDa yeast RSC complex[20].

TROUBLESHOOTING

Table 2: Troubleshooting table.

Steps	Problem	Possible reasons	Solutions
13	Beads clump and cannot be disaggregated	Cells lyse	Reduce the digitonin concentration
59	No DNA is detected by Qubit fluorimetry	This is typical for low cell numbers (<10,000 cells) but otherwise may indicate an antibody failure.	Replace antibody. Antibody binding may be tested by immunofluorescence.
60	No DNA <200 bp is detected by Tapestation analysis	This is typical for most DNA-binding proteins, but otherwise may indicate failure of antibody binding or digestion.	Run a positive control sample for an abundant epitope, *e.g.* H3K27me3.
60	A nucleosome ladder is detected by Tapestation analysis	This is typical for abundant nucleosomal epitopes, but otherwise may indicate release of pA-MNase during digestion.	Run negative control sample using an IgG, *e.g.* guinea pig α-rabbit.
60	Small DNA or a ladder is seen in the negative control by Tapestation analysis	Divalent cations have not been removed by the EDTA in the antibody solution, or the negative control antibody failed to bind, allowing the pA-MNase to behave as a "time-bomb" when Ca^{++} is added.	Replace antibody. Reduce pA-MN concentration. Reduce digestion time. Add a third wash step before digestion.

MATERIALS

NAME	CATALOG #	VENDOR
Cell suspension. We have used human K562 cells, Drosophila S2 cells and dissected Drosophila tissues such as brains and imaginal disks, and spheroplasted yeast.		
Concanavalin-coated magnetic beads	BP531	Bangs Laboratories
Antibody to an epitope of interest. For example, rabbit α-CTCF polyclonal antibody (Millipore 07-729) for mapping 1D and 3D interactions by CUT&RUN		
Positive control antibody to an abundant epitope, e.g. α-H3K27me3 rabbit monoclonal antibody (Cell Signaling Technology, cat. no. 9733)		
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

NAME	CATALOG #	VENDOR
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
Roche Complete Protease Inhibitor EDTA-Free tablets	5056489001	Sigma Aldrich
2 mg/ml Glycogen (1:10 dilution)	10930193001	Sigma Aldrich
RNase A, DNase and protease-free (10 mg/ml)	EN0531	Thermo Fisher Scientific
Gel and PCR Clean-up kit	740609.250	Macherey and Nagel
Agencourt AMPure XP magnetic beads	A63880	Beckman Coulter
10% Sodium dodecyl sulfate (SDS)	L4509	Sigma Aldrich
Proteinase K	EO0492	Thermo Fisher Scientific
Phenol-chloroform-isoamyl alcohol 25:24:1 (PCI)	15593049	Invitrogen - Thermo Fisher
Chloroform	366919-1L	Sigma
1 M Tris-HCl pH 8.0		
Ethanol	2716	Decon Labs
Qubit dsDNA HS kit	Q32851	Life Technologies

SAFETY WARNINGS

- Digitonin is toxic and care should be taken especially when weighing out the powder. A digitonin stock may be prepared by dissolving in dimethylsulfoxide (DMSO), but be aware that DMSO can absorb through the skin.

BEFORE STARTING

REAGENT SETUP

5% Digitonin To reconstitute enough digitonin for an experiment, weigh out the powder in a 2 ml microcentrifuge tube, boil 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 to make 5% (w/v), 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 the concentration to use for a cell type. We have obtained excellent results for K562 cells with 0.02-0.1% digitonin.

- **CAUTION**: Digitonin is toxic and care should be taken especially when weighing out the powder. A digitonin stock may be prepared by dissolving in dimethylsulfoxide (DMSO), but be aware that DMSO can absorb through the skin.

Binding buffer Mix 400 µL 1M HEPES-KOH pH 7.9, 200 µL 1M KCl, 20 µL 1M $CaCl_2$ and 20 µL 1M $MnCl_2$, and bring the final volume to 20 ml with dH_2O. Store the buffer at 4 °C for 6 months.

Concanavalin A-coated beads Gently resuspend and withdraw enough of the slurry such that there will be 10 µL for each final sample and/or digestion time point. Transfer into 1.5 ml Binding buffer in a 2 ml tube. Place the tube on a magnet stand to clear (30 s to 2 min). Withdraw the liquid, and remove from the magnet stand. Add 1.5 ml Binding buffer, mix by inversion or gentle pipetting, remove liquid from the cap and side with a quick pulse on a microcentrifuge. Resuspend in a volume of Binding buffer equal to the volume of bead slurry (10 µL per final sample).

Wash buffer Mix 1 ml 1 M HEPES pH 7.5, 1.5 ml 5 M NaCl, 12.5 µL 2 M Spermidine, bring the final volume to 50 ml with dH_2O, and add 1 Roche Complete Protease Inhibitor EDTA-Free tablet. Store the buffer at 4 °C for up to 1 week.

Dig-wash buffer Mix 160-800 µL 5% Digitonin with 40 ml Wash buffer. The effectiveness of digitonin varies between batches, so testing permeability of Trypan blue is recommended to determine the concentration to use. Count blue cells manually, not using a Vi-Cell counter. We have obtained excellent results for K562 cells with 0.02-0.1% digitonin. Store the buffer at 4 °C for up to 1 day, and heat briefly to redissolve if a precipitate forms.

Low-salt rinse buffer Mix 200 µL 1 M HEPES pH 7.5, 100 µL 5% Digitonin, bring to a final volume of 4 ml. Store the buffer at 4 °C for up to 1 week.

Incubation buffer Mix 14 µL 1 M HEPES pH 7.5, 40 µL 1 M $CaCl_2$, 40 µL 5% Digitonin, bring to a final volume of 4 ml. Store the buffer at 4 °C for up to 1 week.

Antibody buffer Mix 8 µL 0.5 M EDTA with 2 ml Dig-wash buffer and place on ice. Divide into aliquots for each antibody and add antibody solution or serum to a final concentration of 1:100 for to the manufacturer's recommended concentration for immunofluorescence.

STOP solution To 3.44 ml dH_2O add 136 µl 5M NaCl, 400 µL 0.2M EGTA, 40 µL 5% Digitonin, 10 µL RNase A, 10 µL 20 mg/ml glycogen and 2 pg/ml heterologous spike-in DNA. Store the buffer at 4 °C for up to 1 week.

- **CRITICAL STEP**: Heterologous spike-in DNA for calibration should be fragmented down to ~200 bp mean size, for example, an MNase-treated sample of mononucleosome-sized fragments. As we use the total number of mapped reads as a normalization factor only, very little spike-in DNA is needed. For example, addition of 1.5 pg results in 1,000- 10,000 mapped spike-in reads for 1-10 million mapped experimental reads (in inverse proportion).

Binding cells to beads

1 Harvest fresh culture(s) at room temperature and count cells. The same protocol can be used for up to 500,000 mammalian cells per sample and/or digestion time point.

CRITICAL STEP: All steps prior to the addition of antibody are performed at room temperature to minimize stress on the cells. Because it is crucial that DNA breakage is minimized throughout the protocol, we recommend that cavitation during resuspension and vigorous vortexing be avoided.

PAUSE POINT: If necessary, cells can be cryopreserved in 10% DMSO using a Mr. Frosty isopropyl alcohol chamber. We do not recommend flash freezing, as this can cause background DNA breakage that may impact final data quality.

This section is approximately 30 minutes.

2 Centrifuge 3 min 600 x g at room temperature and withdraw liquid.

00:03:00 **Centrifugation**

3 Resuspend in 1.5 ml room temperature Wash buffer by gently pipetting and transfer if necessary to a 2 ml tube. (wash 1/2)

1.5 ml **Wash buffer**

4 Centrifuge 3 min 600 x g at room temperature and withdraw liquid. (wash 1/2)

00:03:00 **Centrifugation**

5 Again, resuspend in 1.5 ml room temperature Wash buffer by gently pipetting. Centrifuge 3 min 600 x g at room temperature and withdraw liquid. (wash 2/2)

1.5 ml **Wash buffer**

00:03:00

6 Resuspend in 1 ml room temperature Wash buffer by gently pipetting.

1 ml **Wash buffer**

7 While gently vortexing the cells at room temperature, add the bead slurry.

8 Rotate 5-10 min at room temperature.

00:10:00 **Rotation**

9 Divide into aliquots in 1.5-ml tubes, one for each antibody to be used.

CRITICAL STEP: To evaluate success of the procedure without requiring library preparation, include in parallel a positive control antibody (e.g. α-H3K27me3) and a negative control antibody (e.g. α-rabbit). Do not include a no-antibody control, as the lack of tethering may allow any unbound pA-MN to act as a "time-bomb" and digest accessible DNA, resulting in a background of DNA-accessible sites.

Bind (primary) antibodies

10 Place on the magnet stand to clear and pull off the liquid.

CRITICAL STEP: Although low-retention pipette tips are preferred for accurate solution transfers, use only conventional (not low-binding) microcentrifuge tubes to avoid loss of beads while decanting.

11 Place each tube at a low angle on the vortex mixer set to low (~1100 rpm) and squirt 100 µL of the Antibody buffer (per sample and/or digestion time point) along the side while gently vortexing to allow the solution to dislodge most or all of the beads. Tap to dislodge the remaining beads.

CRITICAL STEP: The presence of EDTA during antibody treatment removes excess divalent cation used to activate the ConA, because carry-over of Ca++ from the beads can prematurely initiate strand cleavage after addition of pA-MN. Chelation of divalent cations when cells are permeabilized also serves to quickly halt metabolic processes and prevent endogenous DNAse activity. Washing out the EDTA before pA-MN addition avoids inactivating the enzyme. Spermidine in the wash buffer is intended to compensate for removal of Mg++, which might otherwise affect chromatin properties.

12 Place on a nutator or tube rotator at room temperature for ~2 hr or at 4 ^{O}C overnight. When using on the order of 1 million cells per 20 µL beads it is normal for beads to clump or stick to the side during the incubation.

02:00:00 Nutator or tube rotator at 4°C

PAUSE POINT Antibody incubation may proceed overnight at 4 °C.

13 Remove liquid from the cap and side with a quick pulse on a micro-centrifuge.

CRITICAL STEP: After mixing, but before placing a tube on the magnet stand, a very quick spin on a micro-centrifuge (no more than 100 x g) will minimize carry-over of antibody and pA-MN that could result in overall background cleavages during the digestion step.

14 Place on the magnet stand to clear (~30 s) and pull off all of the liquid.

00:00:30 Magnet stand

15 Add 1 ml Dig-wash buffer, mix by inversion, or by gentle pipetting using a 1 ml tip if clumps persist, and remove liquid from the cap and side with a quick pulse on a micro-centrifuge.

1 ml Dig-wash buffer

Bind secondary antibody (as required)

16 Place on the magnet stand to clear and pull off all of the liquid.

CRITICAL STEP: The binding efficiency of Protein A to the primary antibody depends on host species and IgG isotype. For example, Protein A binds well to rabbit and guinea pig IgG but poorly to mouse and goat IgG, and so for these latter antibodies a secondary antibody, such as rabbit α-mouse is recommended.

This section is 15 min to 1.5 hours

17 Place each tube at a low angle on the vortex mixer set to low (~1100 rpm) and squirt 100 µL of the Secondary antibody solution (per sample and/or digestion time point to a final concentration of 1:100 or to the manufacturer's recommended concentration for immunofluorescence) along the side while gently vortexing to allow the solution to dislodge most or all of the beads. Tap to dislodge the remaining beads.

100 µl Secondary antibody solution (per sample and/or digestion time point)

18 Place on the nutator or tube rotator at 4 °C for ~1 hr.

01:00:00 Nutator or tube rotator at 4 °C

19 Remove liquid from the cap and side with a quick pulse on a micro-centrifuge.

20 Place on the magnet stand to clear and pull off all of the liquid.

21 Add 1 ml Dig-Wash buffer, mix by inversion, or by gentle pipetting if clumps persist, and remove liquid from the cap and side with a quick pulse on a micro-centrifuge.

1 ml Dig-Wash buffer

Bind Protein A-MNase or Protein AG-MNase fusion protein

22 Place on the magnet stand to clear and pull off all of the liquid.

23 Place each tube at a low angle on the vortex mixer set to low (~1100 rpm). Squirt 100 µL of the Protein A-MNase or Protein AG-MNase fusion protein at 700 ng/ml (e.g., 1:200 of a 140 µg/ml glycerol stock) in Dig-wash buffer (per sample and/or digestion time point) along the side while gently vortexing to allow the solution to dislodge most or all of the beads. Tap to dislodge the remaining beads.

100 µl Protein A-MNase (700 ng/ml) in Dig-wash buffer

24 Place on the nutator or tube rotator at 4 °C for ~1 hr.

01:00:00 Nutator or tube rotator at 4 °C

25 Remove liquid from the cap and side with a quick pulse on a micro-centrifuge.

26 Place on the magnet stand to clear and pull off all of the liquid.

27 Add 1 ml Dig-wash buffer, mix by inversion, or by gentle pipetting if clumps persist, and remove liquid from the cap and side with a quick pulse on a micro-centrifuge.

1 ml Dig-wash buffer

28 Repeat Dig-wash steps 28-29.

Repeat Dig-wash steps

Low-salt wash

29 Place on the magnet stand to clear and pull off all of the liquid.
Add 1 ml Low-salt wash buffer, mix by inversion, or by gentle pipetting if clumps persist, and remove liquid from cap and side with a quick pulse on a micro-centrifuge.

1 ml Low-salt wash buffer

Targeted digestion

30 Place on the magnet stand to clear and pull off all of the liquid. Insert tubes into the 1.5 ml wells of a heater block sitting in wet ice to chill down to 0 °C.

0 °C

31 Place each tube at a low angle on the vortex mixer set to low (~1100 rpm) and add 200 µL of the Incubation Buffer (per sample and/or digestion time point) along the side while gently vortexing to allow the solution to dislodge most or all of the beads. Tap to dislodge the remaining beads.

200 µl Incubation buffer

0 °C

32 Incubate at 0 °C for the desired digestion time (default is 5 min).

00:05:00 Incubation

CRITICAL STEP: MNase binds DNA but only cleaves when Ca++ is present, so that digestion is a zero-order reaction that seems to be less temperature-dependent than the subsequent diffusion of released pA-MNase-bound particles that can digest accessible regions of the genome. Cleavage and release of particles in most of the cell population can be obtained at

0 oC while minimizing background cleavages attributable to diffusion. We have found that digestion at ambient temperature or higher results in unacceptable background cleavage levels.

0 °C Incubation

33 Place on (cold) magnet stand and allow to clear for ≥10 s. Add 200 µL STOP buffer and mix by gentle vortexing.

200 µl STOP

CRITICAL STEP: Heterologous spike-in DNA may be present in the STOP buffer to calibrate DNA amounts, for example to compare treatments or digestion time points. Alternatively, mapping to E. coli carry-over DNA from the pA/MNase and pAG/MNase that gets fragmented during digestion suffices for calibration.

Target chromatin release

34 Incubate 30 min 37 °C to release CUT&RUN fragments from the insoluble nuclear chromatin.

00:30:00 Incubation

37 °C Incubation

35 Remove liquid from the cap and side with a quick pulse on a micro-centrifuge. Place on magnet stand.

Option A: Fast DNA extraction by spin column

36 Place a spin column into a collection tube and add 400 µL Buffer NT1 (from NucleoSpin kit or equivalent).

400 µl Buffer NT1

If you are performing Option B: Alternate DNA extraction (preferred for quantitative recovery of ≤80 bp fragments), please directly proceed to Step 47.

This section is approximately 20 minutes

37 Decant the supernatant cleanly from the pellet and transfer to the NT1 in the spin column pipetting gently up and down to mix.

38 Centrifuge 30 s at 11,000 x g. Discard flow-through.

00:00:30 Centrifugation

39 Add 700 µL Buffer NT3. Centrifuge 30 s at 11,000 x g. Discard flow-through.

700 µl Buffer NT3

00:00:30 Centrifugation

40 Add 700 µL Buffer NT3. Centrifuge 30 s at 11,000 x g. Discard flow-through and replace in rotor.

700 µl Buffer NT3

00:00:30 Centrifugation

41 Centrifuge for 1 min at 11,000 x g. Let dry 5 min.

00:01:00 **Centrifugation**

00:05:00 **Drying**

42 Place in a fresh tube and add 20-40 µL Buffer NE to membrane.

40 µl **Buffer NE**

43 After 1 min, centrifuge for 1 min at 11,000 x g.

00:01:00 **Wait**

00:01:00 **Centrifugation**

Option B: Alternate DNA extraction (preferred for quantitative recovery of ≤80 bp fragments)

44 Decant the supernatant cleanly from the pellet and transfer to a fresh 1.5-ml microcentrifuge tube.

If you are performing Option A: Fast DNA extraction by spin column, please directly proceed to Step 59.

This section is approximately 1.5 hours

45 To each sample add 2 µL 10% SDS (to 0.1%), and 2.5 µL Proteinase K (20 mg/ml). Mix by inversion and incubate 1 hr 50 °C.

2 µl **10% SDS (to 0.1%)/sample**

2.5 µl **Proteinase K (20 mg/ml)/sample**

50 °C **Incubation**

01:00:00 **Incubation**

46 Add 200 µL PCI and mix by full-speed vortexing ~2 s.

200 µl **PCI**

00:00:02 **Vortexing**

47 Transfer to a phase-lock tube (e.g., Qiagen MaXtract), and centrifuge 5 min room temperature at 16,000 x g.

00:05:00 **Centrifugation**

48 Add 200 µL chloroform and invert ~10x to mix.

200 µl **Chloroform**

49 Remove liquid by pipetting to a fresh tube containing 2 µL 2 mg/ml glycogen.

2 µl **2 mg/ml glycogen**

50 Add 500 µL 100% ethanol and mix by vortexing or tube inversion.

500 µl **100% ethanol**

51 Chill on ice and centrifuge 10 min at 4 °C 16,000 x g.

00:10:00 **Centrifugation**

4 °C **Centrifugation**

52 Pour off the liquid and drain on a paper towel.

53 Rinse the pellet in 1 ml 100% ethanol and centrifuge 1 min at 4 °C 16,000 x g.

1 ml **100% ethanol**

00:01:00 **Centrifugation**

4 °C **Centrifugation**

54 Carefully pour off the liquid and drain on a paper towel. Air dry.

55 When the pellet is dry, dissolve in 25-50 µL 1 mM Tris-HCl pH8 0.1 mM EDTA.

50 µl **1 mM Tris-HCl pH8 0.1 mM EDTA**

Library preparation and sequencing

56 Optional: Quantify 1-2 µL, for example using fluorescence detection with a Qubit instrument.

This section is 2-4 days.

57 Optional: Evaluate the presence of cleaved fragments and the size distribution by capillary electrophoresis with fluorescence detection, for example using a Tapestation instrument.

CRITICAL STEP: Some long undigested DNA will leak through, and this is what will dominate the Qubit fluorescence for CUT&RUN of typical transcription factors. For these, the targeted DNA recovered is too low in amount and too small in size to be detected by gel analysis or even by Tapestation. In such cases it may be necessary to make a PCR-amplified library to quantify by Tapestation or Bioanalyzer analysis.

58 Prepare barcoded libraries for Illumina sequencing with Tru-Seq adapters using a single-tube protocol, following the manufacturer's instructions, except that the high-temperature dA-tailing step should be 58
o
C 1:15 hr. Rapid PCR cycles favor exponential amplification of the desired CUT&RUN fragments over linear amplification of large DNA fragments that are too long for polymerase to complete.

CRITICAL STEP: To minimize the contribution of large DNA fragments, PCR cycles should be at least 12-14 cycles, preferably with a 10 s 60°C combined annealing/extension step. Good results have been obtained with the Hyper-prep kit (KAPA Biosystems).

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

60 Determine the size distribution of libraries by Agilent 4200 TapeStation analysis.

61 Perform paired-end Illumina sequencing on the barcoded libraries following the manufacturer's instructions.

CRITICAL STEP: Because of the very low background with CUT&RUN, typically 5 million paired-end reads suffices for transcription factors or nucleosome modifications, even for the human genome. For maximum economy, we mix up to 24

barcoded samples per lane on a 2-lane flow cell, and perform paired-end 25x25 bp sequencing. Single-end sequencing is not recommended for CUT&RUN, as it sacrifices resolution and discrimination between transcription factors and neighboring nucleosomes.

Data processing and analysis

62 We align paired-end reads using Bowtie2 version 2.2.5 with options: --local --very-sensitive- local --no-unal --no-mixed --no-discordant --phred33 -I 10 -X 700. For mapping spike-in fragments, we also use the --no-overlap --no-dovetail options to avoid cross-mapping of the experimental genome to that of the spike-in DNA.

CRITICAL STEP: Separation of sequenced fragments into ≤120 bp and ≥150 bp size classes provides mapping of the local vicinity of a DNA-binding protein, but this can vary depending on the steric access to the DNA by the tethered MNase. Single-end sequencing is not recommended for CUT&RUN, as it sacrifices resolution and discrimination between transcription factors and neighboring nucleosomes.

63 Scripts are available from https://github.com/Henikoff/Cut-and-Run for spike-in calibration and for peak-calling.

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