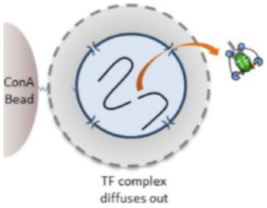
Apr 27, 2024 Version 1

CUT&RUN abbreviated protocol V.1



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

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Derek Janssens[1], Steven Henikoff[2], CG -[3]

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

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-

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We use this protocol and it's working

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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. Upon activation of tethered MNase, the bound complex is excised and released into the supernatant for DNA extraction and sequencing. In the manuscript attached to this version of the protocol, Meers et al. introduce four enhancements to CUT&RUN: 1) a hybrid Protein A-Protein G-MNase construct that expands antibody compatibility; 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. Here we provide an updated CUT&RUN protocol that incorporates these enhancements, and provides three different options for the CUT&RUN MNase digestion reaction that are helpful to improve data quality or to increase throughput. 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.

Guidelines

The protocol workflow is as follows:

Day 1, Cells to DNA
Binding cells to beads (Steps 1-8, 30 min)
Permeabilize cells and bind primary antibodies (Steps 9-13, 2.5 hr–overnight, longer incubations provide higher yields)
Bind secondary antibody as required (Steps 14-20, 15 min-1.5 hr)
Bind Protein A-MNase or Protein A/G-MNase fusion protein (Steps 21-26, 1.5 hr)
Chromatin Digestion and Release Option 1: Standard CUT&RUN (Steps 27-37, 1.5 hr)
Chromatin Digestion and Release Option 2: High Ca^{2+}/ Low Salt (Steps 38-47, 1 hr)
Chromatin Digestion and Release Option 3: Direct Ligation (Steps 48-56, 1.5 hr)
Phenol Chloroform Extraction (only required for Chromatin Digest Options 1 and 2) (Steps 57-67, 1.5 hr)

Days 2-4, Library preparation and sequencing
Sample Analysis Pre-Library Prep (optional) (Steps 68-69, 1 hr)
End Repair and Adapter Ligation (Steps 70-78, 3 hr–overnight)
PCR Enrichment of CUT&RUN Libraries (Steps 79-109, 2-3 hr)
CUT&RUN Library Analysis and Sequencing (Steps 110-112, variable timing)

Day 5
Data processing and analysis (Steps 113-114, variable timing)

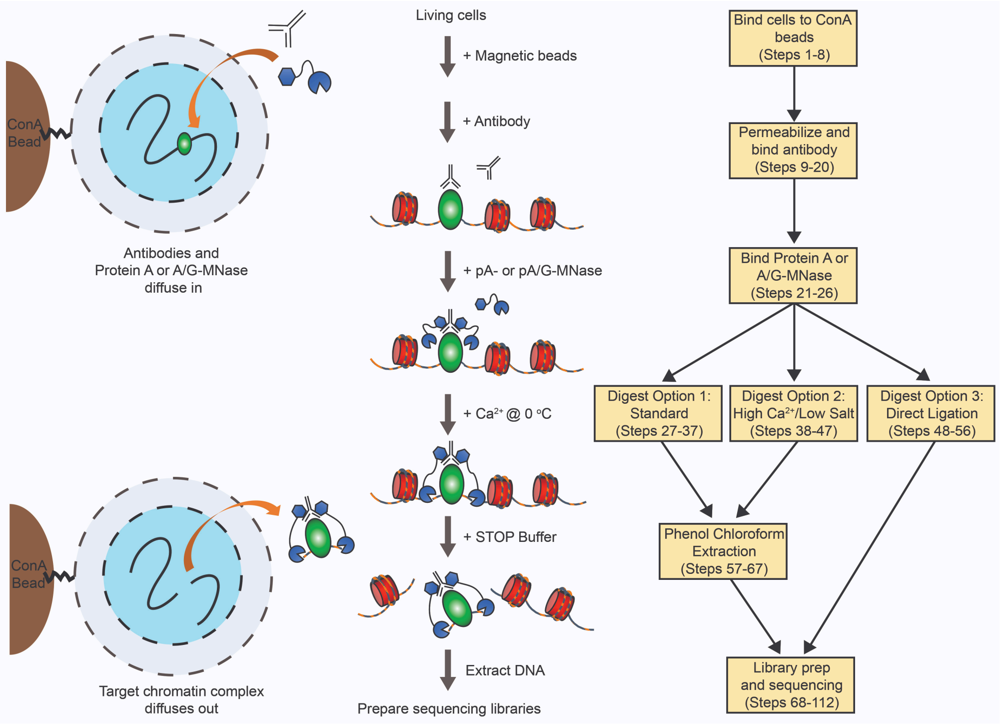


Figure 1: CUT&RUN workflow. A schematic overview of the CUT&RUN protocol. Cells are harvested and bound to concanavalin A-coated magnetic beads. Cell membranes are permeabilized with digitonin to allow the specific antibody to find it's targets. Afer incubation with antibody, beads are briefly washed, and then incubated with pA- or pA/G-MNase. The user then selects one of three different options for the MNase digestion reaction to best fit the needs of their experiment. The first option for digestion is the same as the previously released CUT&RUN protocol, and is refered to as standard CUT&RUN. The second option includes high Ca^{2+} to compact chromatin and hold it in place during digestion, limiting the amount of MNase that is able to freely diffuse, and reducing the background for targets that are enriched at active chromatin (e.g. H3K27ac). The third option is for direct ligation of Illumina-compatible adapters to the cleaved chromatin, avoiding the DNA puritification steps that are required for options 1 and 2. Regardless of the digestion option that is selected, cells are first chilled to 0 °C, and digestion begins upon addition of Ca^{2+}. Reactions are stopped by chelating away the calcium and the DNA fragments released into solution by cleavage are used to prepare CUT&RUN sequencing libraries.

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 CUT&RUN protocol that now includes various optional modifications to the MNase digestion reaction that can be used to improve data quality or increase throughput in specific situations. One of the strengths of CUT&RUN is that the entire reaction is performed in situ, whereby the antibody and pA- or pA/G-MNase 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 and ATAC-seq. Digitonin partitions into membranes and extracts cholesterol. Membranes that lack cholesterol are minimally impacted by digitonin. 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. 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 the protocol provided in Skene and Henikoff (*eLife,* 2017), and then enter the protocol below at step 9.

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 sensitive electrophoretic 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, including many transcription factors, 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-MNase will result in preferential fragmentation of hyper-accessible DNA.

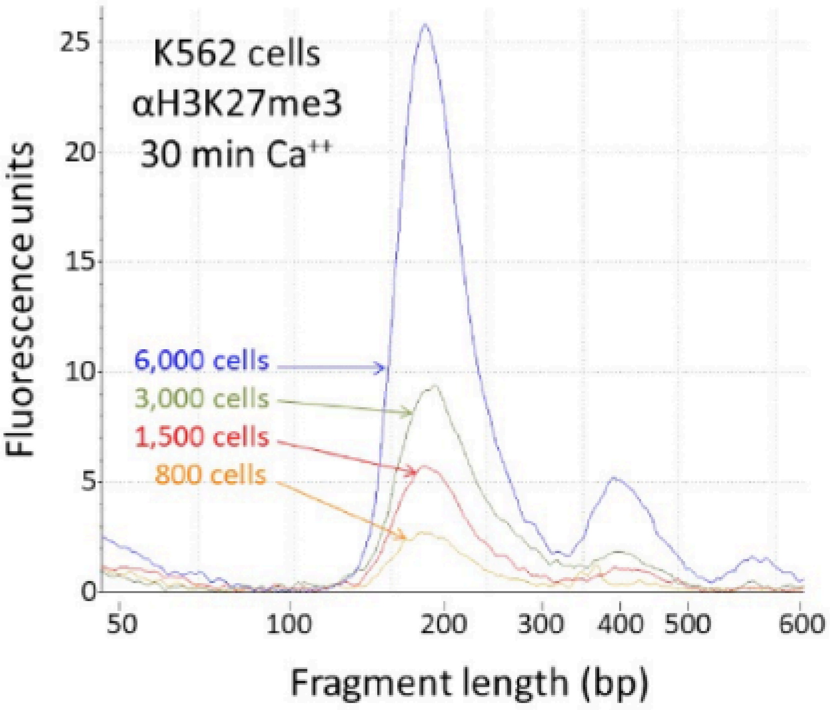
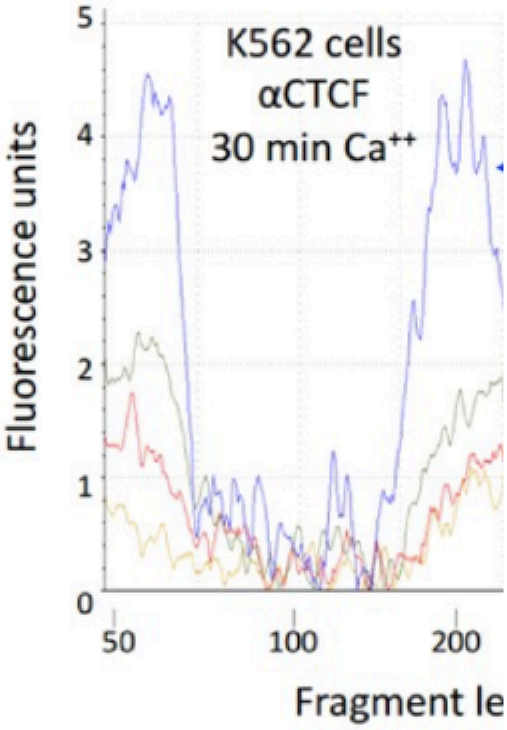
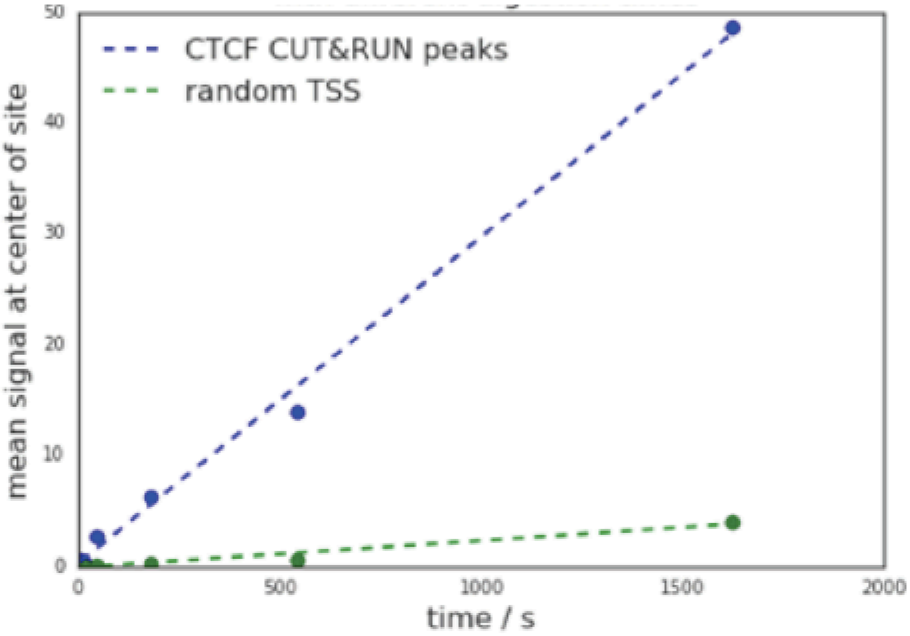


Figure 2: Tapestation analysis of an abundant histone epitope (H3K27me3) as a same-day positive control. The remainder of these samples were used to make libraries for sequencing, with results shown in Figure 3.



Supplementary Figure 1: T
CUT&RUN cleaved fragme
antibody. The remainder
used to make libraries
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.

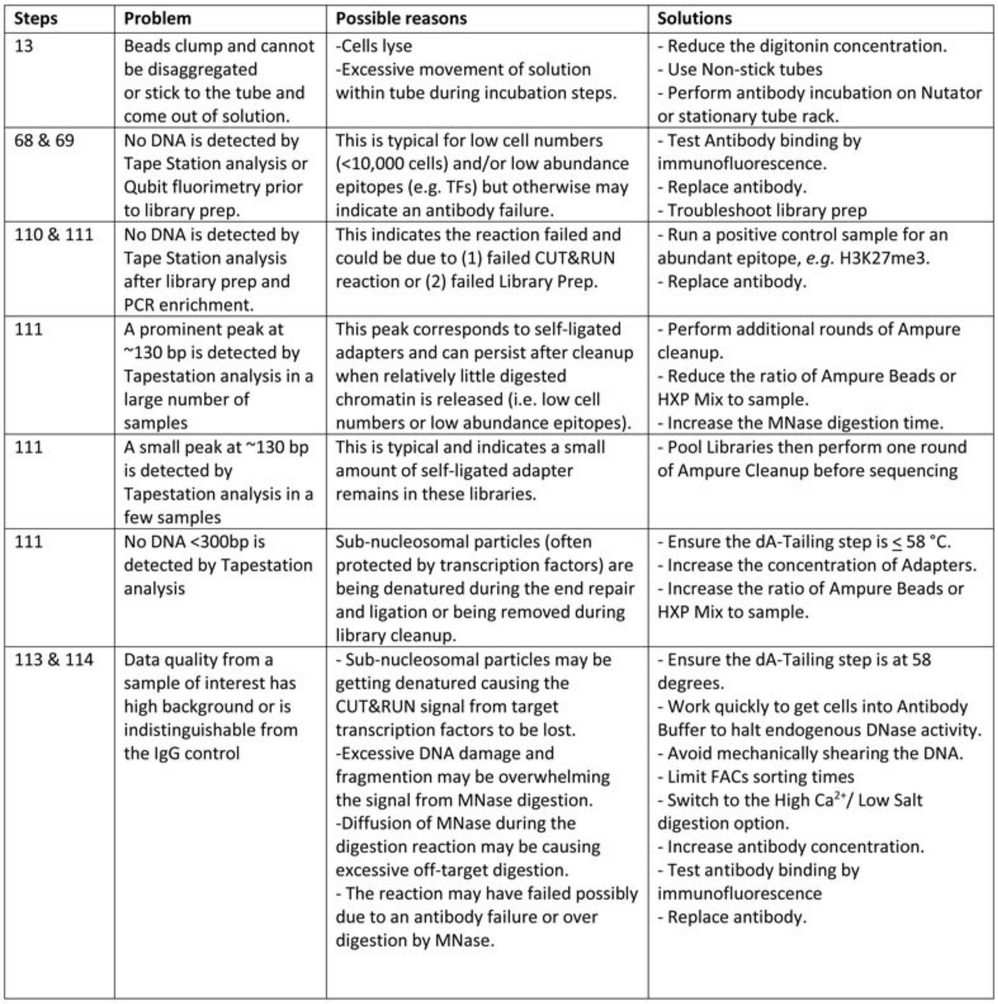
In our previously published study, we showed that targeted cleavage occurred within seconds of adding Ca^{2+} ions, and by virtue of being a sterically regulated tethered reaction, the cleavage pattern was constant over time. However, longer digestion times release more material with no apparent change in the signal-to-noise 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 negative size selection using paramagnetic carboxylated beads (e.g. Agencourt AMPure XP beads) large genomic DNA can be removed prior to preparing CUT&RUN sequencing libraries. In Skene and Henikoff (*eLife,* 2017) we showed that this strategy was successful for the ~1 MDa yeast RSC complex.

TROUBLESHOOTING



Steps	Problem	Possible reasons	Solutions
13	Beads clump and cannot be disaggregated or stick to the tube and come out of solution.	-Cells lyse -Excessive movement of solution within tube during incubation steps.	- Reduce the digitonin concentration. - Use Non-stick tubes - Perform antibody incubation on Nutator or stationary tube rack.
68 & 69	No DNA is detected by Tape Station analysis or Qubit fluorimetry prior to library prep.	This is typical for low cell numbers (<10,000 cells) and/or low abundance epitopes (e.g. TFs) but otherwise may indicate an antibody failure.	- Test Antibody binding by immunofluorescence. - Replace antibody. - Troubleshoot library prep
110 & 111	No DNA is detected by Tape Station analysis after library prep and PCR enrichment.	This indicates the reaction failed and could be due to (1) failed CUT&RUN reaction or (2) failed Library Prep.	- Run a positive control sample for an abundant epitope, *e.g.* H3K27me3. - Replace antibody.
111	A prominent peak at ~130 bp is detected by Tapestation analysis in a large number of samples	This peak corresponds to self-ligated adapters and can persist after cleanup when relatively little digested chromatin is released (i.e. low cell numbers or low abundance epitopes).	- Perform additional rounds of Ampure cleanup. - Reduce the ratio of Ampure Beads or HXP Mix to sample. - Increase the MNase digestion time.
111	A small peak at ~130 bp is detected by Tapestation analysis in a few samples	This is typical and indicates a small amount of self-ligated adapter remains in these libraries.	- Pool Libraries then perform one round of Ampure Cleanup before sequencing
111	No DNA <300bp is detected by Tapestation analysis	Sub-nucleosomal particles (often protected by transcription factors) are being denatured during the end repair and ligation or being removed during library cleanup.	- Ensure the dA-Tailing step is ≤ 58 °C. - Increase the concentration of Adapters. - Increase the ratio of Ampure Beads or HXP Mix to sample.
113 & 114	Data quality from a sample of interest has high background or is indistinguishable from the IgG control	- Sub-nucleosomal particles may be getting denatured causing the CUT&RUN signal from target transcription factors to be lost. -Excessive DNA damage and fragmention may be overwhelming the signal from MNase digestion. -Diffusion of MNase during the digestion reaction may be causing excessive off-target digestion. - The reaction may have failed possibly due to an antibody failure or over digestion by MNase.	- Ensure the dA-Tailing step is at 58 degrees. - Work quickly to get cells into Antibody Buffer to halt endogenous DNase activity. - Avoid mechanically shearing the DNA. - Limit FACs sorting times - Switch to the High Ca^{2+}/ Low Salt digestion option. - Increase antibody concentration. - Test antibody binding by immunofluorescence - Replace antibody.

Materials

MATERIALS

- 10 mM Adenosine 5-Triphosphate (ATP) **New England Biolabs Catalog** #P0756S
- 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 **Bangs Laboratories Catalog** #BP531
- 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 **Merck Millipore (EMD Millipore) Catalog** #300410
- 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) **Promega Catalog** #P1197
- 1 M Manganese Chloride (MnCl2) **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #203734
- 1 M Calcium Chloride (CaCl2) **Fisher Scientific Catalog** #BP510
- 1 M Potassium Chloride (KCl) **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #P3911
- 1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.5 (HEPES (Na)) **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #H3375
- 5 M Sodium chloride (NaCl) **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #S5150-1L
- 0.5 M Ethylenediaminetetraacetic acid (EDTA) **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #3002E
- 0.2 M Ethylene glycol-bis(β-aminoethyl ether)-N,N,N,N-tetraacetic acid (EGTA) **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #E3889
- Roche Complete Protease Inhibitor EDTA-Free tablets **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #5056489001
- RNase A, DNase and protease-free (10 mg/ml) **Thermo Fisher Scientific Catalog** #EN0531
- Agencourt AMPure XP magnetic beads **Beckman Coulter Catalog** #A63880
- 10% Sodium dodecyl sulfate (SDS) **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #L4509
- Proteinase K **Thermo Fisher Scientific Catalog** #EO0492
- Phenol-chloroform-isoamyl alcohol 25:24:1 (PCI) **Invitrogen - Thermo Fisher Catalog** #15593049
- Chloroform **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #366919-1L
- 1 M Tris-HCl pH 8.0

Ethanol **Decon Labs Catalog** #2716

Qubit dsDNA HS kit **Life Technologies Catalog** #Q32851

10mM dNTPs **Kapa Biosystems Catalog** #KK1017

T4 Polynucleotide Kinase - 500 units **New England Biolabs Catalog** #M0201S

T4 DNA polymerse **Invitrogen - Thermo Fisher Catalog** #18005025

Taq DNA polymerase **Thermo Scientific Catalog** #EP0401

2X Rapid ligase buffer **Catalog** #B101L

Enzymatics DNA ligase **Catalog** #L6030-HC-L

5X KAPA buffer **Kapa Biosystems Catalog** #KK2502

KAPA HS HIFI polymerase **Kapa Biosystems Catalog** #KK2502

10X NEB T4 DNA ligase buffer **New England Biolabs**

40% PEG 4000 **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #81242

40% PEG 8000 **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #202452

Protein A/G–Micrococcal Nuclease (pA/G-MNase) fusion protein (plasmid for protein prep available from Addgene ID:123461). Store in 50% glycerol at -20 oC. **addgene Catalog** #123461

Protein A–Micrococcal Nuclease (pA-MNase) fusion protein (plasmid for protein prep available from Addgene ID: 86973). Store in 50% glycerol at -20 oC.

20 mg/ml Glycogen **Merck MilliporeSigma (Sigma-Aldrich) Catalog** #10930193001

5g Spermidine (mix up to 17.2 mL in water to make 2M stock and store @ -20 oC) **Merck MilliporeSigma (Sigma-Aldrich) Catalog** ##S0266-5G

In this protocol we provide an optional library prep strategy for Illumina sequencing that uses TruSeq-Y Adapters with a free 3'T overhang. Alternatively, many users have also had success with the NEBNext Ultra I DNA Library Kit (E7645) following a protocl developed by Nan Liu in Stuart Orkin's lab (**dx.doi.org/10.17504/protocols.io.wvgfe3w**). To follow the library prep protocol described here the following oligos can be ordered from any company that provides custom oligo synthesis (e.g. IDT or Sigma-Aldrich):

TruSeq Universal Adapter (PAGE purification):
AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T
TruSeq Indexed Adapters (PAGE purification):
P-GATCGGAAGAGCACACGTCTGAACTCCAGTCAC(**INDEX**)ATCTCGTATGCCGTCTTCTGCTT*G

Adapter master stocks should be prepared by annealing the TruSeq Universal adapter to each of the TruSeq Indexed Adapters individually by mixing them at a concentration of 25 µM, and then heating them to 100 ^{0}C and allowing them to slowly cool either at RT on a bench top or in a thermocycler (1 degree per minute).

P5 primer (HPLC purification):

AATGATACGGCGACCACCGA∗G
P7 primer (HPLC purification):
CAAGCAGAAGACGGCATACGA∗G

(* = phosphorothioate bond; P = phosphate group; INDEX = 6 nucleotide barcode)

Troubleshooting

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.

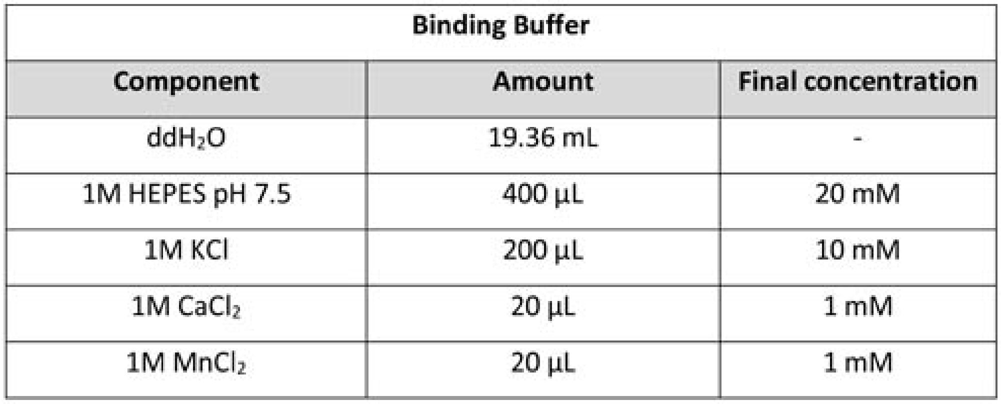
Before start

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.

- **NOTE**: The 5% digitonin stock may also be prepared by dissolving in dimethylsulfoxide (DMSO), and can then be stored at -20 °C for up to 6 months. Be aware that DMSO can absorb through the skin.
- **CAUTION**: Digitonin is toxic and care should be taken especially when weighing out the powder.

Binding buffer: Mix 20 mL of Binding Buffer in a 50 mL conical tube. Store the buffer at 4 °C for up to 6 months.

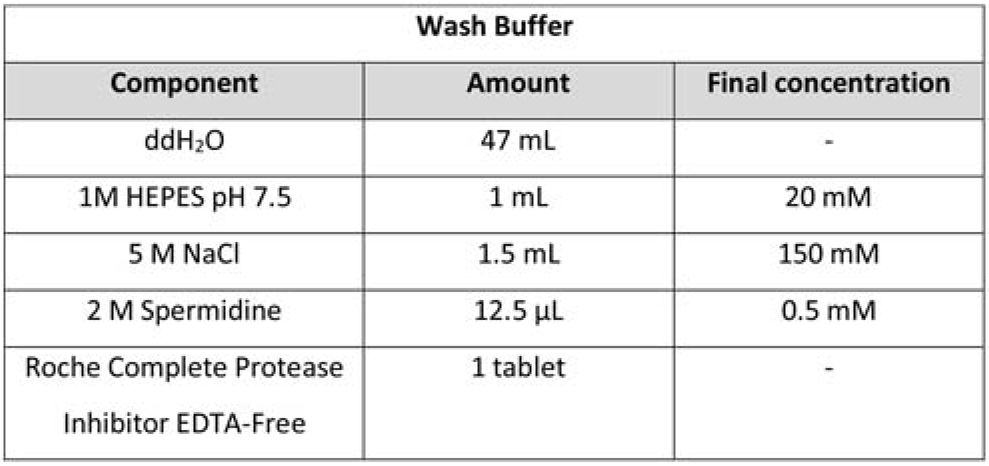


Binding Buffer		
Component	**Amount**	**Final concentration**
ddH_2O	19.36 mL	-
1M HEPES pH 7.5	400 µL	20 mM
1M KCl	200 µL	10 mM
1M $CaCl_2$	20 µL	1 mM
1M $MnCl_2$	20 µL	1 mM

Activate Concanavalin A-coated beads in Binding Buffer: 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 50 mL of Wash Buffer. This buffer can be stored at 4 °C for up to 1 week, however, Roche Complete Protease Inhibitor tablet should be added fresh on the day of use.

- **NOTE:** A concentration of salt that is in the physiological range avoids stress when washing the cells and mixing with beads. Spermidine in the wash buffer is intended to compensate for removal of Mg^{2+} during incubation in the Antibody Buffer, which might otherwise affect chromatin properties.



Wash Buffer		
Component	**Amount**	**Final concentration**
ddH$_2$O	47 mL	-
1M HEPES pH 7.5	1 mL	20 mM
5 M NaCl	1.5 mL	150 mM
2 M Spermidine	12.5 µL	0.5 mM
Roche Complete Protease Inhibitor EDTA-Free	1 tablet	-

Dig-wash buffer: Mix 150-600 µL 5% (wt/vol) digitonin with 30 mL Wash Buffer for a final concentration of digitonin between 0.025% and 0.1% (wt/vol). Store this buffer on ice or at 4 °C for up to 1 day, and vortex before use.

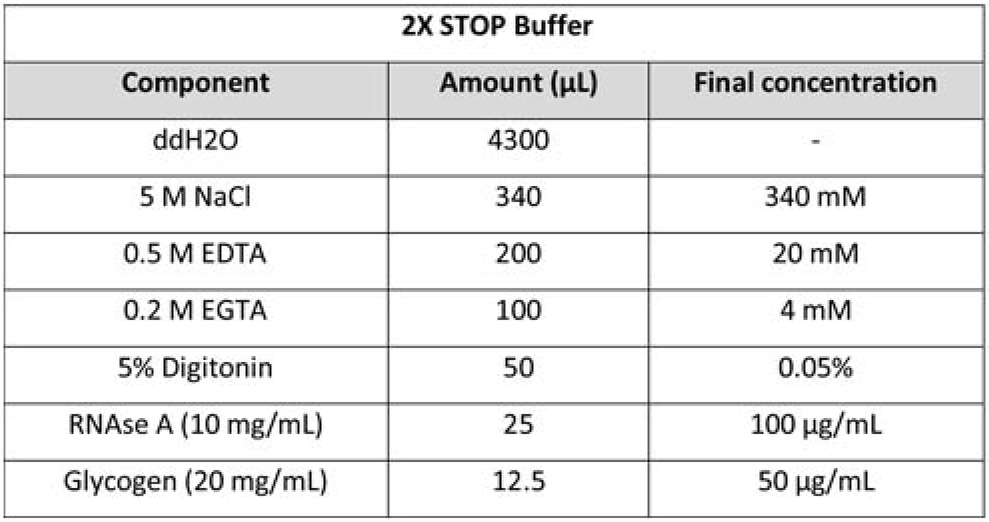
- **NOTE:** The effectiveness of digitonin varies between batches, so testing for full permeability of Trypan blue is recommended to determine the concentration to use for a cell type. We have obtained excellent results for H1 and K562 cells with 0.05% digitonin (300 µL 5% (wt/vol) digitonin in 30 mL Wash Buffer). For simplicity, we use this same buffer for all steps starting from the incubation in primary antibody until the chromatin digestion.

Antibody buffer: Mix 8 µL 0.5 M EDTA with 2 mL Dig-wash buffer and place on ice.

- **NOTE:** The presence of EDTA during antibody treatment removes excess divalent cations used to activate the Concanavalin A-coated beads, as well as endogenous cations from the cells of interest. This serves to halt metabolic processes, stop endogenous DNAse activity, and prevent carry-over of Ca^{2+} from the Binding Buffer that might prematurely initiate strand cleavage after addition of pA-MNase. Washing out the EDTA before pA-MNase addition avoids inactivating the enzyme.

Protocol Option 1: Standard CUT&RUN specific reagents

2X STOP Buffer: Mix 5 mL of 2X STOP Buffer. Store the buffer at 4 °C for up to 1 week.

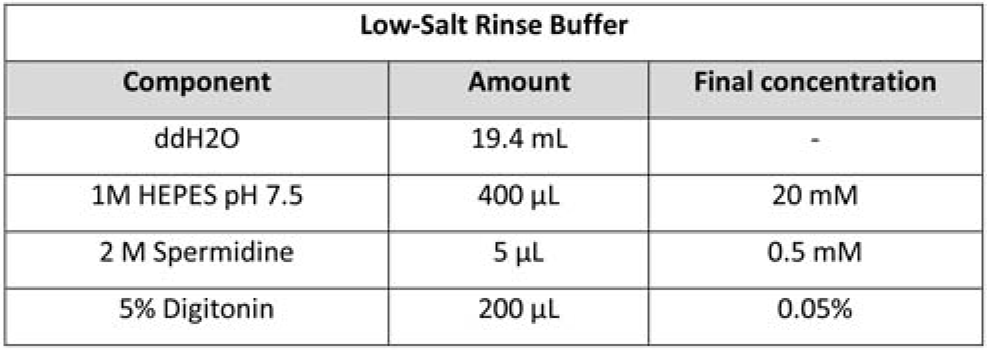


2X STOP Buffer		
Component	**Amount (µL)**	**Final concentration**
ddH2O	4300	-
5 M NaCl	340	340 mM
0.5 M EDTA	200	20 mM
0.2 M EGTA	100	4 mM
5% Digitonin	50	0.05%
RNAse A (10 mg/mL)	25	100 µg/mL
Glycogen (20 mg/mL)	12.5	50 µg/mL

- **CRITICAL STEP**: Adding heterologous spike-in DNA to the STOP Buffer can be useful for comparison of DNA yields between samples. The total number of mapped spike-in reads can then be used as a normalization factor, where the amount of spike-in reads is inversly proportional to the DNA yeild from the sample. The spike-in DNA should be fragmented down to ~200 bp mean size, for example, an MNase-treated sample of mononucleosome-sized fragments. When starting with low cell numbers (i.e. 100 - 10,000 cells) very little spike-in DNA is required, we recommend a final concentration of 2 pg/mL in the STOP buffer. For samples with high cell numbers (i.e. 10,000 -1 million cells) more spike-in DNA is required to obtain sufficient reads and we recommend 100 pg/mL in the STOP Buffer. Alternatively, *E. coli* DNA that is carried-over from the production of the fusion protein can also serve as a spike-in for sample calibrations, in which case no additional heterologous spike-in needs to be included in the STOP Buffer.

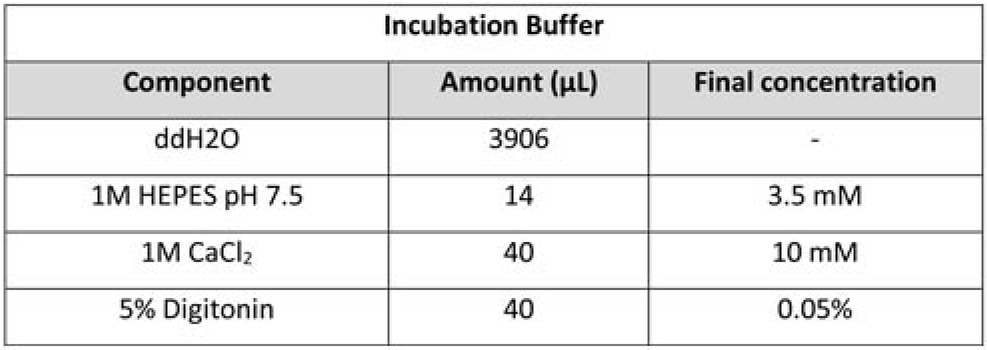
Protocol Option 2: High Ca^{2+} / Low Salt specific reagents

Low-Salt Rinse Buffer: Mix 20 mL of Low-Salt Rinse Buffer. Store the buffer at 4 °C for up to 1 week.



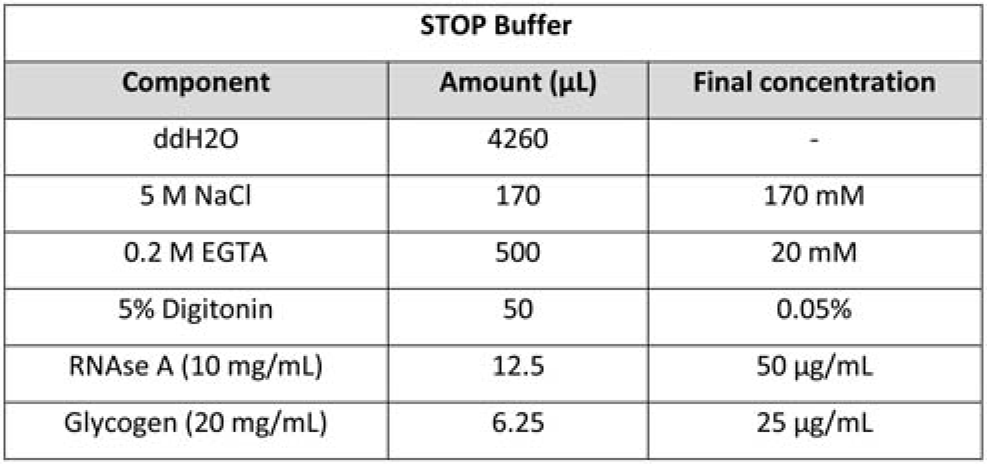
Low-Salt Rinse Buffer		
Component	**Amount**	**Final concentration**
ddH2O	19.4 mL	-
1M HEPES pH 7.5	400 µL	20 mM
2 M Spermidine	5 µL	0.5 mM
5% Digitonin	200 µL	0.05%

Incubation Buffer: Mix 4 mL of Incubation Buffer. Store the buffer at 4 °C for up to 1 week. Briefly chill on ice before use.



Incubation Buffer		
Component	**Amount (µL)**	**Final concentration**
ddH2O	3906	-
1M HEPES pH 7.5	14	3.5 mM
1M $CaCl_2$	40	10 mM
5% Digitonin	40	0.05%

STOP Buffer: Mix 5 mL of STOP Buffer. Store the buffer at 4 °C for up to 1 week.

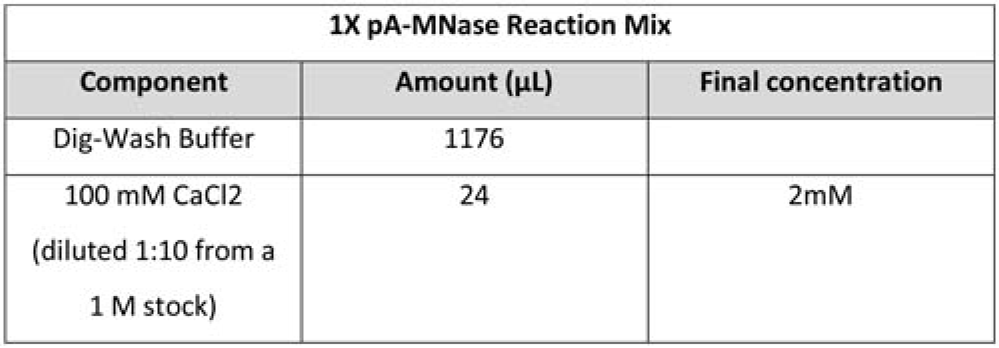


STOP Buffer		
Component	**Amount (µL)**	**Final concentration**
ddH2O	4260	-
5 M NaCl	170	170 mM
0.2 M EGTA	500	20 mM
5% Digitonin	50	0.05%
RNAse A (10 mg/mL)	12.5	50 µg/mL
Glycogen (20 mg/mL)	6.25	25 µg/mL

CRITICAL STEP: Adding heterologous spike-in DNA to the STOP Buffer can be useful for comparison of DNA yields between samples. For recommended concentrations see 2X STOP Buffer in Protocol Option 1 specific reagents. Alternatively, *E. coli* DNA that is carried-over from the production of the fusion protein can also serve as a Spike-In for sample calibrations, in which case no additional heterologous spike-in needs to be included in the STOP Buffer.

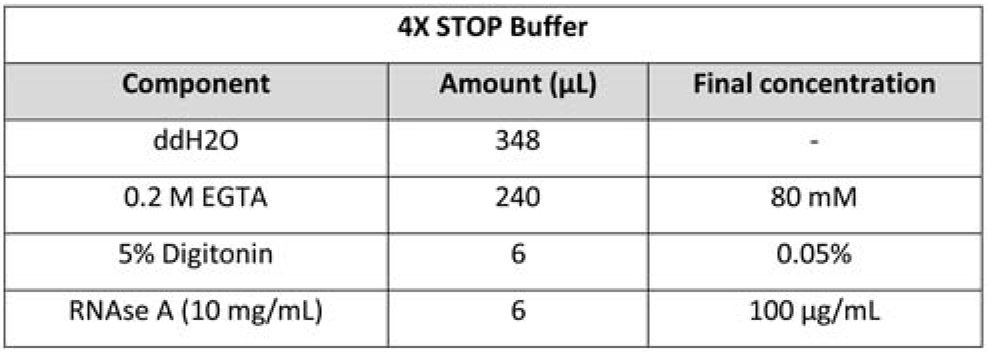
Protocol Option 3: Direct Ligation specific reagents

1X pA-MNase Reaction Mix: Prepare 1.2 mL of 1X pA-MNase Reaction Mix. Store the buffer at 4 °C for up to 1 week.



1X pA-MNase Reaction Mix		
Component	**Amount (µL)**	**Final concentration**
Dig-Wash Buffer	1176	
100 mM CaCl2 (diluted 1:10 from a 1 M stock)	24	2mM

4X STOP Buffer: Mix 600 µL of 4X STOP Buffer. Store the buffer at 4 °C for up to 1 week.



4X STOP Buffer		
Component	**Amount (µL)**	**Final concentration**
ddH2O	348	-
0.2 M EGTA	240	80 mM
5% Digitonin	6	0.05%
RNAse A (10 mg/mL)	6	100 µg/mL

CRITICAL STEP: Adding heterologous spike-in DNA to the STOP Buffer can be useful for comparison of DNA yields between samples. For recommended concentrations see 2X STOP Buffer in Protocol Option 1 specific reagents. Alternatively, *E. coli* DNA that is carried-over from the production of the fusion protein can also serve as a Spike-In for sample calibrations, in which case no additional heterologous spike-in needs to be included in the STOP Buffer.

Binding cells to beads (~30 min)

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.

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.

1.5 mL Wash buffer

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

00:03:00 Centrifugation

5 Repeat steps 3 and 4 two more times.

Repeat Dig-wash steps

Note

CRITICAL STEP: Thorough washing removes free sugars and other molecules that can compete for binding to the Concanavalin A coated-beads, ensuring efficient binding and recovery of the cells of interest.

6 Resuspend in 1 mL room temperature Wash Buffer by gently pipetting.

7 While gently vortexing the cells at room temperature, add the ConA-coated magnetic bead slurry.

8 Rotate 5-10 min at room temperature.

00:10:00 Rotation

Permeabilize cells and bind primary antibodies (2.5 hours - overnight)

9. Mix well by vigorous inversion to ensure the bead-bound cells are in a homogenous suspension and divide into aliquots in 1.5-mL tubes, one for each antibody to be used.

   Note

   NOTE: Some users have experienced issues with ConA beads sticking to the sides of the tube and coming out of solution during antibody incubation steps. In this case, 0.6-mL Lo-bind microcentrifuge tubes can be used, and subsequent Dig-wash volumes reduced to 300 µL. However, when bound to low cell numbers (e.g. <100K) the ConA beads will be extremely slippery on the sides of lo-bind tubes, and so great care must be taken while removing solutions on the magnet during wash steps etc. to avoid losing the sample.

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

11. Place each tube at a low angle on the vortex mixer set to low (~1100 rpm) and squirt 50-150 µL of the Antibody buffer per sample along the side while gently vortexing to allow the solution to dislodge most or all of the beads. Tap to dislodge the remaining beads

    150 µL Antibody buffer

12. Mix in the primary antibody to a final concentration of 1:100 or to the manufacturer's recommended concentration for immunofluorescence.

13. Place on a nutator or tube rotator at room temperature for ~2 hr or at 4 °C overnight.

    02:00:00 Nutator or tube rotator at RT

Bind Protein A-MNase or Protein A/G-MNase fusion protein (1.5 hours)

14. Remove liquid from the cap and side with a quick pulse on a micro-centrifuge. Place on the magnet stand (~30 sec) to clear 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 if clumps persist.

    1 mL Dig-Wash buffer

16. Repeat Dig-wash steps 21-22.

    Repeat Dig-wash steps

17. Remove liquid from the cap and side with a quick pulse on a micro-centrifuge. Place on the magnet stand to clear (~30 s) and pull off all of the liquid.

00:00:30 Magnet stand

18 Place each tube at a low angle on the vortex mixer set to low (~1100 rpm). Squirt 150 µL of the Protein A-MNase or Protein A/G-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.

150 µL Protein A-MNase or Protein A/G-MNase (700 ng/mL) in Dig-wash buffer

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

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

Chromatin Digestion and Release Option 1: Standard CUT&RUN (1.5 hours)

20 Remove liquid from the cap and side with a quick pulse on a micro-centrifuge. Place on the magnet stand (~30 s) to clear and pull off all of the liquid.

00:00:30 Magnet stand

21 Add 1 mL Dig-wash buffer, mix by inversion, or by gentle pipetting if clumps persist.

1 mL Dig-wash buffer

22 Repeat Dig-wash steps 27-28.

Repeat Dig-wash steps

23 Remove liquid from the cap and side with a quick pulse on a micro-centrifuge. Place on the magnet stand to clear (~30 s) and pull off all of the liquid.

00:00:30 Magnet stand

24 Place each tube at a low angle on the vortex mixer set to low (~1100 rpm) and add 100 µL of the 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 Dig-wash buffer (per sample or digestion time point)

25 Insert tubes into the 1.5 mL wells of a heater block sitting in wet ice to chill down to 0 °C.

0 °C

26 Remove each tube from the block, mix in 2 µL 100 mM CaCl2 (per sample and/or digestion time point), diluted 1:10 from a 1 M stock, with gentle vortexing and immediately replace the tube in the 0 °C block

2 µL 100 mM CaCl2 (per sample or digestion time point) 0 °C

27 Incubate at 0 °C for the desired digestion time (default is 30 min).

00:30:00 Incubation

0 °C Incubation

28 Add 100 µL 2X STOP Buffer and mix by gentle vortexing. When there are multiple digestion time points, remove 100 µL and add to a new tube containing 100 µL 2X STOP Buffer and mix by gentle vortexing.

100 µL 2XSTOP

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

00:30:00 Incubation

37 °C Incubation

30 Place on the magnet stand to clear. Cleanly transfer the supernatant containing digested chromatin to a fresh 1.5-mL microcentrifuge tube and proceed with Phenol Chloroform Extraction (Step 57).

200 µL STOP w/ Chromatin

Phenol Chloroform Extraction (~1.5 hours)

31 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

32 Add an equal volume of Phenol Chloroform to the sample (e.g. to 200 µL sample add 200 µL Phenol Chloroform). Mix by full-speed vortexing ~2 s.

200 µL PCI

00:00:02 Vortexing

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

00:05:00 Centrifugation

34 Add an equivalent volume of chloroform to the initial sample volume (e.g. for a 200 µL starting sample volume add 200 µL chloroform). Invert ~10X to mix and then centrifuge 5 min, room temperature @ 16,000 x g.

200 µL Chloroform

00:05:00 Centrifugation

35 Remove the top liquid phase by pipetting to a fresh tube containing 2 µL 2 mg/mL glycogen (diluted 1:10 from 20 mg/mL glycogen stock).

2 µL 2 mg/ml glycogen

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

500 µL 100% ethanol

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

00:10:00 Centrifugation

4 °C Centrifugation

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

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

1 mL 100% ethanol

00:01:00 Centrifugation

4 °C Centrifugation

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

00:05:00 Air Dry

41 When the pellet is dry, dissolve in 30-50 µL 1 mM Tris-HCl pH 8 0.1 mM EDTA, then transfer to a new 0.6 mL Lo-Bind microcentrifuge tube.

30 µL 1 mM Tris-HCl pH 8 0.1 mM EDTA

CUT&RUN Library Analysis and Sequencing

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

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

44 Pool samples at equimolar concentrations and perform paired-end Illumina sequencing on the barcoded libraries following the manufacturer’s instructions.

Data Processing and Analysis

45 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.

Note

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.

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