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

Peter J. Skene,Steven Henikoff

Abstract

Cleavage Under Targets and Release Using Nuclease (CUT&RUN) is an epigenomic profiling strategy in which antibody-targeted controlled cleavage by micrococcal nuclease releases specific protein-DNA complexes into the supernatant for paired-end DNA sequencing. As only the targeted fragments enter into solution, and the vast majority of DNA is left behind, CUT&RUN has exceptionally low background levels. CUT&RUN outperforms the most widely used Chromatin Immunoprecipitation (ChIP) protocols in resolution, signal-to-noise, and depth of sequencing required. In contrast to ChIP, CUT&RUN is free of solubility and DNA accessibility artifacts and can be used to profile insoluble chromatin and to detect long-range 3D contacts without cross-linking. Here we present an improved CUT&RUN protocol that does not require isolation of nuclei and provides high-quality data starting with only 100 cells for a histone modification and 1000 cells for a transcription factor. From cells to purified DNA CUT&RUN requires less than a day at the lab bench.

In summary, CUT&RUN has several advantages over ChIP-seq: (1) The method is performed in situ in non-crosslinked cells and does not require chromatin fragmentation or solubilization; (2) The intrinsically low background allows low sequence depth and identification of low signal genomic features invisible to ChIP; (3) The simple procedure can be completed within a day and is suitable for robotic automation; (4) The method can be used with low cell numbers compared to existing methodologies; (5) A simple spike-in strategy can be used for accurate quantitation of protein-DNA interactions. As such, CUT&RUN represents an attractive replacement for ChIPseq, which is one of the most popular methods in biological research.

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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 16-22, 15 min-1.5 hr)
- Bind Protein A-MNase fusion protein (Steps 23-30, 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)

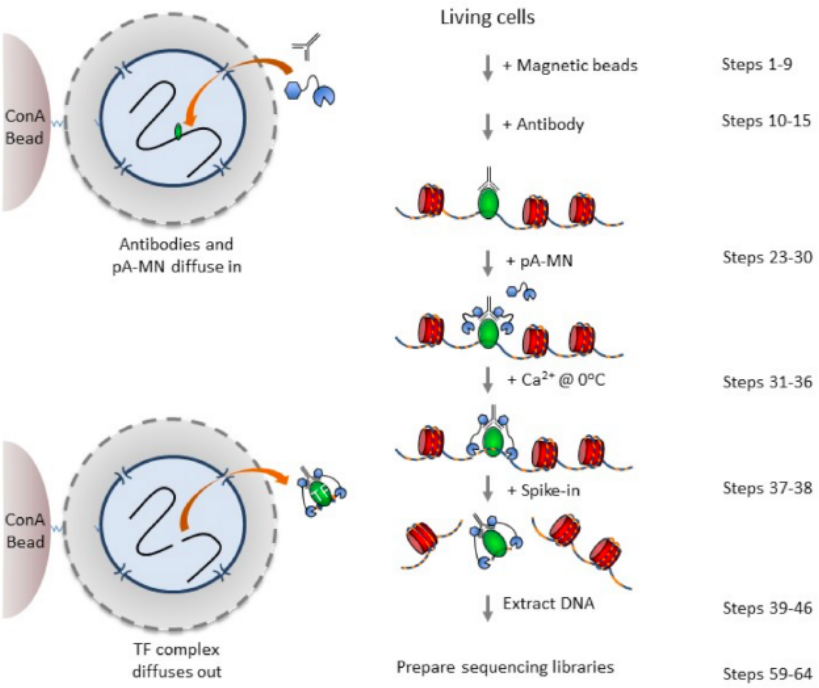


Figure 1: CUT&RUN requires less than a day from cells to DNA. A schematic overview of the CUT&RUN protocol. Cells are harvested and bound to concanavalin A-coated magnetic beads. Cell membranes are permeabilized with digitonin to allow the specific antibody to find it's target. After incubation with 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.

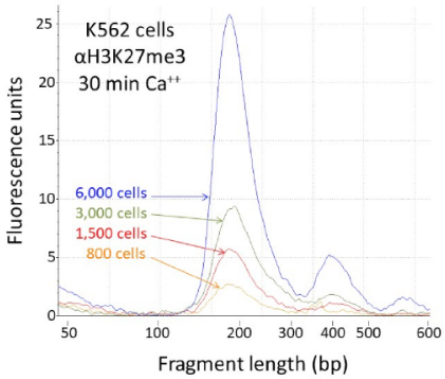
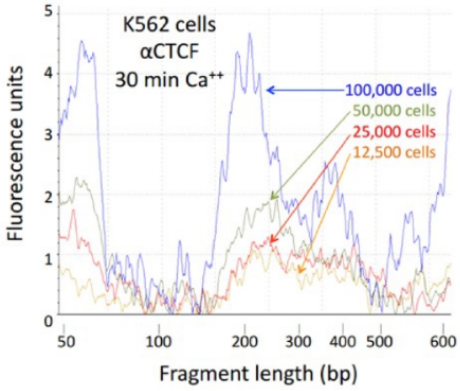
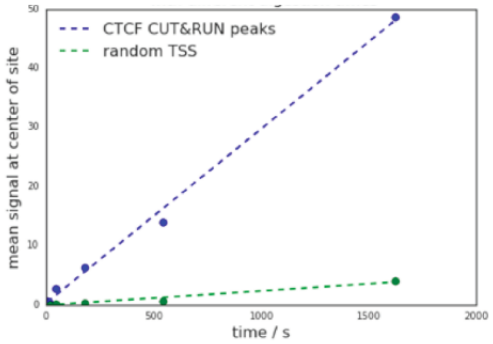


Figure 2: Tapestation analysis of an abundant histone epitope (H3K27me3) as a same-day positive control. The remainder of these samples were used to make libraries for sequencing, with results shown in Figure 3.



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.

AUTHOR CONTRIBUTIONS

P.S. and S.H developed the protocol, performed the experiments, analyzed the data and wrote the manuscript.

ACKNOWLEDGEMENTS

We thank Paul Talbert for helpful comments on the manuscript, Christine Codomo for preparing Illumina sequencing libraries and Jorja Henikoff for bioinformatics.

For additional information, comparison with other methods, and references please see the full manuscript.

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.

- **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 withdH_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. 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.

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.

2XSTOP To 4.2 ml dH_2O add 340 µl 5M NaCl, 200 µL 0.5M EDTA, 100 µL 0.2M EGTA, 20 µL 5% Digitonin, 25 µL RNase A, 125 µL 2 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).

Materials

✓ Cell suspension. We have used human K562 cells, Drosophila S2 cells and dissected Drosophila tissues such as brains and imaginal disks, and spheroplasted yeast. by Contributed by users

Concanavalin-coated magnetic beads BP531 by 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 by Contributed by users

✓ Positive control antibody to an abundant epitope, e.g. α-H3K27me3 rabbit monoclonal antibody (Cell Signaling Technology, cat. no. 9733) by Contributed by users

✓ Negative control antibody to an absent epitope, e.g. guinea pig α-rabbit antibody by Contributed

by users

5% Digitonin 300410 by Emd Millipore

✓ Protein A–Micrococcal Nuclease (pA-MNase) fusion protein (provided in 50% glycerol by the authors upon request). Store at -20 oC. by Contributed by users

✓ Spike-in DNA (e.g., from Saccharomyces cerevisiae micrococcal nuclease-treated chromatin, provided by authors upon request) by Contributed by users

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

1 M Manganese Chloride (MnCl2) 203734 by Sigma Aldrich

1 M Calcium Chloride (CaCl2) BP510 by Fisher Scientific

1 M Potassium Chloride (KCl) P3911 by Sigma Aldrich

1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.5 (HEPES (Na)) H3375 by Sigma Aldrich

1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.9 (HEPES (K)) H3375 by Sigma Aldrich

5 M Sodium chloride (NaCl) S5150-1L by Sigma Aldrich

0.5 M Ethylenediaminetetraacetic acid (EDTA) 3002E by Research Organics

0.2 M Ethylene glycol-bis(β-aminoethyl ether)-N,N,N,N-tetraacetic acid (EGTA) E3889 by Sigma Aldrich

2 M Spermidine S2501 by Sigma Aldrich

Roche Complete Protease Inhibitor EDTA-Free tablets 5056489001 by Sigma Aldrich

2 mg/ml Glycogen (1:10 dilution) 10930193001 by Sigma Aldrich

RNase A, DNase and protease-free (10 mg/ml) EN0531 by Thermo Fisher Scientific

Gel and PCR Clean-up kit 740609.250 by Macherey and Nagel

Agencourt AMPure XP magnetic beads A63880 by Beckman Coulter

10% Sodium dodecyl sulfate (SDS) L4509 by Sigma Aldrich

Proteinase K EO0492 by Thermo Fisher Scientific

Phenol-chloroform-isoamyl alcohol 25:24:1 (PCI) 15593049 by Invitrogen - Thermo Fisher

Chloroform 366919-1L by Sigma

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

Ethanol 2716 by Decon Labs

Qubit dsDNA HS kit Q32851 by Life Technologies

Protocol

Binding cells to beads

Step 1.

Harvest fresh culture(s) at room temperature and count cells. The same protocol can be used for 100 to 250,000 mammalian cells per sample and/or digestion time point.

NOTES

Steven Henikoff 05 Jan 2018

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.

Steven Henikoff 05 Jan 2018

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.

Steven Henikoff 08 Jan 2018

This section is approximately 30 minutes.

Binding cells to beads

Step 2.

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

Binding cells to beads

Step 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)

AMOUNT

1.5 ml Additional info: Wash buffer

Binding cells to beads

Step 4.

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

Binding cells to beads

Step 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)

AMOUNT

1.5 ml Additional info: Wash buffer

Binding cells to beads

Step 6.

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

AMOUNT

1 ml Additional info: Wash buffer

Binding cells to beads

Step 7.

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

Binding cells to beads

Step 8.

Rotate 5-10 min at room temperature.

Binding cells to beads

Step 9.

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

NOTES

Steven Henikoff 05 Jan 2018

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

Step 10.

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

NOTES

Steven Henikoff 05 Jan 2018

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.

Steven Henikoff 08 Jan 2018

This section can be 15 min–overnight, with longer incubations providing higher yields.

Bind (primary) antibodies

Step 11.

Place each tube at a low angle on the vortex mixer set to low (1100 rpm) and squirt 50 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.

NOTES
Steven Henikoff 05 Jan 2018

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.

Bind (primary) antibodies
Step 12.

Place on the tube rotator at 4°C for 2 hr (or at room temperature for 5-10 min)

NOTES
Steven Henikoff 05 Jan 2018

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

Bind (primary) antibodies
Step 13.

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

NOTES
Steven Henikoff 05 Jan 2018

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.

Bind (primary) antibodies
Step 14.

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

Bind (primary) antibodies
Step 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.

AMOUNT

1 ml Additional info: Dig-wash buffer

Bind secondary antibody (as required)

Step 16.

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

NOTES

Steven Henikoff 05 Jan 2018

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.

Steven Henikoff 08 Jan 2018

This section is 15 min to 1.5 hours

Bind secondary antibody (as required)

Step 17.

Place each tube at a low angle on the vortex mixer set to low (1100 rpm) and squirt 50 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.

AMOUNT

50 µl Additional info: Dig-wash buffer (per sample and/or digestion time point)

Bind secondary antibody (as required)

Step 18.

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

Bind secondary antibody (as required)

Step 19.

Place on the tube rotator at 4 °C for 1 hr (or at room temperature for 5-10 min).

Bind secondary antibody (as required)

Step 20.

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

Bind secondary antibody (as required)

Step 21.

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

Bind secondary antibody (as required)

Step 22.

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.

AMOUNT

1 ml Additional info: Dig-Wash buffer

Bind Protein A-MNase fusion protein

Step 23.

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

NOTES

Steven Henikoff 08 Jan 2018

This section is 15 min - 1.5 hours

Bind Protein A-MNase fusion protein

Step 24.

Place each tube at a low angle on the vortex mixer set to low (1100 rpm) and squirt 50 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.

AMOUNT

50 µl Additional info: Dig-wash buffer (per sample and/or digestion time point)

Bind Protein A-MNase fusion protein

Step 25.

Mix in the pA-MNase to a final concentration of 700 ng/ml (e.g. 2.5 µL/50 µL of a 1:10 dilution of the 140 µg/ml glycerol stock provided upon request).

Bind Protein A-MNase fusion protein

Step 26.

Place on the tube rotator at 4 °C for 1 hr (or at room temperature for 5-10 min).

Bind Protein A-MNase fusion protein

Step 27.

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

Bind Protein A-MNase fusion protein

Step 28.

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

Bind Protein A-MNase fusion protein

Step 29.

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.

AMOUNT

1 ml Additional info: Dig-wash buffer

Bind Protein A-MNase fusion protein

Step 30.

Repeat Dig-wash steps 28-29.

GOTO

Repeat Dig-wash steps -> go to step #28

Targeted digestion

Step 31.

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

NOTES

Steven Henikoff 08 Jan 2018

This section is approximately 45 minutes

Targeted digestion

Step 32.

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.

AMOUNT
100 µl Additional info: Dig-wash buffer (per sample and/or digestion time point)

Targeted digestion

Step 33.

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

TEMPERATURE
0 °C Additional info:

Targeted digestion

Step 34.

Remove each tube from the block, mix in 2 µL 100 mM $CaCl_2$ (diluted 1:10 from a 1 M stock) with gentle vortexing and immediately replace the tube in the 0 °C block.

AMOUNT
2 µl Additional info: 100 mM CaCl2

TEMPERATURE
0 °C Additional info:

Targeted digestion

Step 35.

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

TEMPERATURE
0 °C Additional info: Incubation

NOTES
Steven Henikoff 05 Jan 2018

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.

ANNOTATIONS
Jay Sarthy 16 Jan 2018

Should be 10% SDS

Targeted digestion

Step 36.

Add 100 µL 2XSTOP and mix by gentle vortexing. When there are multiple time points, remove 100 µL to 100 µL 2XSTOP and mix by gentle vortexing.

AMOUNT
100 µl Additional info: 2XSTOP

NOTES
Steven Henikoff 05 Jan 2018

CRITICAL STEP: Heterologous spike-in DNA should be present in the 2XSTOP to calibrate DNA amounts, for example to compare treatments or digestion time points. This is especially important for CUT&RUN, as there is too little background cleavage for normalization of samples.

Target chromatin release

Step 37.

Incubate 10 min 37 °C to release CUT&RUN fragments from the insoluble nuclear chromatin.

TEMPERATURE
37 °C Additional info: Incubation

Target chromatin release

Step 38.

Centrifuge 5 min at 4 °C and 16,000 x g and place on magnet stand.

TEMPERATURE
4 °C Additional info: Centrifugation

Option A: Fast DNA extraction by spin column

Step 39.

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

AMOUNT
400 µl Additional info: Buffer NT1

NOTES
Steven Henikoff 05 Jan 2018

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

Steven Henikoff 08 Jan 2018

This section is approximately 20 minutes

Option A: Fast DNA extraction by spin column

Step 40.

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

Option A: Fast DNA extraction by spin column

Step 41.

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

Option A: Fast DNA extraction by spin column

Step 42.

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

AMOUNT

700 µl Additional info: Buffer NT3

Option A: Fast DNA extraction by spin column

Step 43.

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

AMOUNT

700 µl Additional info: Buffer NT3

Option A: Fast DNA extraction by spin column

Step 44.

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

Option A: Fast DNA extraction by spin column

Step 45.

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

AMOUNT

40 µl Additional info: Buffer NE

Option A: Fast DNA extraction by spin column

Step 46.

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

Option B: Alternate DNA extraction (preferred for quantitative recovery of ≤80 bp fragments)
Step 47.

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

NOTES
Steven Henikoff 05 Jan 2018

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

Steven Henikoff 08 Jan 2018

This section is approximately 1.5 hours

Option B: Alternate DNA extraction (preferred for quantitative recovery of ≤80 bp fragments)
Step 48.

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 10 min 70 °C.

AMOUNT
2 µl Additional info: 10% SDS (to 0.1%)/sample
AMOUNT
2.5 µl Additional info: Proteinase K (20 mg/ml)/sample
TEMPERATURE
70 °C Additional info: Incubation

Option B: Alternate DNA extraction (preferred for quantitative recovery of ≤80 bp fragments)
Step 49.

Add 300 µL PCI and mix by full-speed vortexing 2 s.

AMOUNT
300 µl Additional info: PCI

Option B: Alternate DNA extraction (preferred for quantitative recovery of ≤80 bp fragments)
Step 50.

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

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

Step 51.

Add 300 µL chloroform and invert 10x to mix.

AMOUNT

300 µl Additional info: Chloroform

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

Step 52.

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

AMOUNT

2 µl Additional info: 2 mg/ml glycogen

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

Step 53.

Add 750 µL 100% ethanol and mix by vortexing or tube inversion.

AMOUNT

750 µl Additional info: 100% ethanol

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

Step 54.

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

TEMPERATURE

4 °C Additional info: Centrifugation

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

Step 55.

Pour off the liquid and drain on a paper towel.

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

Step 56.

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

AMOUNT
1 ml Additional info: 100% ethanol
TEMPERATURE
4 °C Additional info: Centrifugation

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

Step 57.

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

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

Step 58.

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

AMOUNT
50 µl Additional info: 1 mM Tris-HCl pH8 0.1 mM EDTA

Library preparation and sequencing

Step 59.

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

NOTES
Steven Henikoff 08 Jan 2018

This section is 2-4 days.

Library preparation and sequencing

Step 60.

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

NOTES
Steven Henikoff 05 Jan 2018

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.

Library preparation and sequencing

Step 61.

Prepare barcoded libraries for Illumina sequencing with Tru-Seq adapters using a single-tube protocol,

following the manufacturer's instructions. 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.

NOTES
Steven Henikoff 05 Jan 2018

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).

Library preparation and sequencing

Step 62.

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

Library preparation and sequencing

Step 63.

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

Library preparation and sequencing

Step 64.

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

NOTES
Steven Henikoff 05 Jan 2018

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

Step 65.

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.

NOTES

Steven Henikoff 05 Jan 2018

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.

Data processing and analysis

Step 66.

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

NOTES

Steven Henikoff 16 Jan 2018

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

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.