Targeted *in situ* genome-wide profiling with high efficiency for low cell numbers

Peter J Skene[1–3], Jorja G Henikoff[1] & Steven Henikoff[1,2]

[1]Basic Sciences Division, Fred Hutchinson Cancer Research Center, Seattle, Washington, USA. [2]Howard Hughes Medical Institute, Seattle, Washington, USA. [3]Present address: NanoString Technologies, Seattle, Washington, USA. Correspondence should be addressed to S.H. (steveh@fhcrc.org).

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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 ratio and depth of sequencing required. In contrast to ChIP, CUT&RUN is free of solubility and DNA accessibility artifacts and has been 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 when starting with only 100 cells for a histone modification and 1,000 cells for a transcription factor. From cells to purified DNA, CUT&RUN requires less than a day at the laboratory bench and requires no specialized skills.

INTRODUCTION

Development of the protocol

All the cells in a multicellular organism have the same genomic sequence, but different gene-expression patterns underpin tissue specification. Differences in gene expression arise from the binding of transcription factors (TFs) and their recruitment of chromatin-associated complexes that modify and mobilize nucleosomes[1,2]. As a result, genome-wide mapping of TFs, chromatin-associated complexes and chromatin states, including histone variants and post-translational modifications (PTMs), has become a major focus of research. For more than 30 years, ChIP has been the predominant method of mapping protein–DNA interactions. With ChIP, cells are cross-linked with formaldehyde, then the entire cellular content is solubilized to fragment the chromatin fiber and an antibody is added to isolate the chromatin fragments of interest[3]. Although the readout strategies for ChIP have evolved over 30 years from gel electrophoresis[3] to massively parallel sequencing[4,5], the fundamentals of ChIP have remained largely unchanged. Although improvements in ChIP-seq allow base-pair-resolution mapping of TFs[6–8], issues remain with high background that limits sensitivity, requirements for large numbers of cells, and artifacts resulting from cross-linking and solubilization[9–13]. Without an alternative method that is based on different principles than ChIP, it has been difficult to distinguish true positives from misleading false-positive artifacts.

Alternative strategies have been used for the genome-wide mapping of protein–DNA interactions that can address some of these limitations of ChIP. For example, several methods, including DNase1 footprinting[14], FAIRE-seq[15], Sono-seq[16], MNase-seq[17,18] and ATAC-seq[19], are being used to map TF binding genome-wide using a sequencing readout. However, as these approaches are not targeted to specific proteins, they are not specific to any one TF. Furthermore, they cannot be used to map specific chromatin states such as those demarcated by histone PTMs, which may be used to clinically differentiate healthy and disease states[20].

In contrast to methods that solubilize chromatin such as ChIP and chromatin accessibility mapping, methods that tether DNA modification enzymes or nucleases to map TFs or chromatin proteins are performed on intact cells or nuclei. In the case of DamID, a fusion of the protein of interest and an enzyme that methylates the surrounding DNA is genetically engineered[21], and in the case of chromatin endogenous cleavage (ChEC), the protein's footprint is targeted for cleavage[22]. Enzyme tethering can be performed *in vivo* (DamID) or *in situ* (ChEC) without the need to cross-link, fragment and solubilize chromatin, thus avoiding potential artifacts caused by these procedures. However, these methods require a transgenic approach, limiting scalability for large infrastructural consortiums such as ENCODE and transferability to a clinical setting. In addition, these methods cannot map histone PTMs. These limitations were partially overcome by the chromatin immunocleavage (ChIC) method, whereby crude nuclei from cross-linked cells were first treated with a TF-specific antibody and then a fusion protein composed of protein A and micrococcal nuclease (pA-MN), which can be activated by calcium ions[22]. However, ChIC was developed using a Southern blot readout, and so its applicability to genome-wide profiling remained unclear for more than a decade.

We recently reported a major development of the enzyme-tethering strategy that we termed CUT&RUN (**Fig. 1**)[23]. Our protocol took unfixed nuclei and attached them to a solid support using concanavalin A–coated magnetic beads to allow simple handling. Following *in situ* binding of the antibody and pA-MN specifically to the target protein, seconds after exposure to calcium at 0 °C, cleavage occurred on either side of the TF. As non-cross-linked nuclei were used, cleaved fragments released with two cuts were free to diffuse out of the nuclei. After removing the intact nuclei by centrifugation, the supernatant containing released chromatin fragments could be used to extract DNA directly for sequencing. We found that performing the Ca^{2+}-dependent digestion reaction at 0 °C was essential to limiting the diffusion of the cleaved chromatin complexes, which would otherwise cleave and release accessible DNA. Overall, we showed that CUT&RUN has a much higher signal-to-noise ratio than cross-linking ChIP-seq, thereby

allowing identification of previously unknown genomic features. CUT&RUN achieved base-pair resolution of mammalian TFs with only 10 million sequenced reads.

The need for quantitative mapping of protein–DNA interactions has become increasingly apparent[24]. However, due to the complexity of ChIP, which requires genome-wide solubilization of chromatin and immunoprecipitation, an involved quantitation strategy is required whereby a fixed number of cells from a different species that has antibody cross-reactivity is spiked in[25]. The requirement for conserved epitopes limits its general applicability. By contrast, due to the inherent simplicity of CUT&RUN, a straightforward spike-in strategy with heterologous DNA sufficed to accurately quantify binding events.

In our original description of CUT&RUN, we applied the method to TFs (budding yeast Abf1 and Reb1, and human CCTC-binding factor (CTCF), Myc and Max), histones (yeast H2A and CenH3) and chromatin regulators (yeast Mot1 and Sth1). We also showed that the method can be applied to as few as 600,000 mammalian cells. Our subsequent work has shown that this report was overly conservative by ~3 orders of magnitude. In the protocol described here, we show that high-quality data can be obtained using only ~100 cells for an abundant histone modification and ~1,000 cells for a TF. These findings demonstrate that CUT&RUN is applicable to many basic and translational applications for which only low cell numbers are available, such as studies of development and analysis of clinical samples.

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

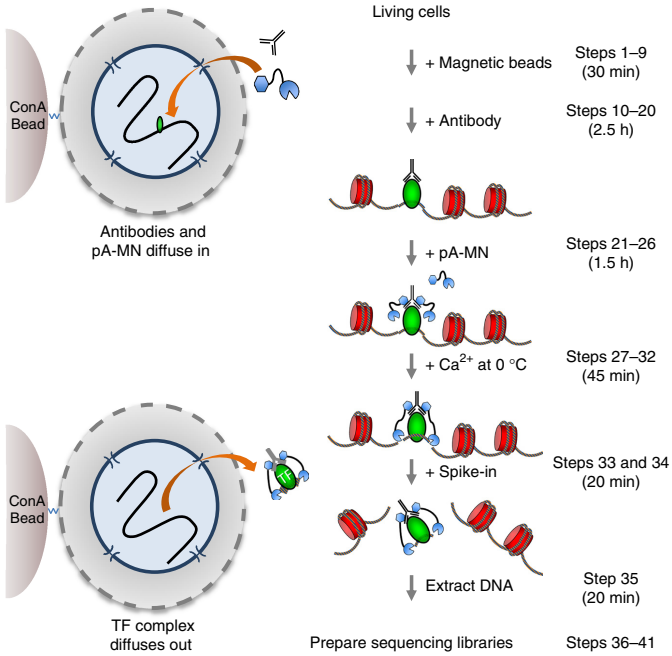

Figure 1 | A schematic overview of the CUT&RUN protocol. CUT&RUN requires less than a day from cells to DNA. Cells are harvested and bound to concanavalin A–coated magnetic beads. Cell membranes are permeabilized with digitonin (indicated by holes in the membrane) to allow the specific antibody to find its target. After incubation with the 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. ConA, concanavalin A–coated.

Applications of the method

CUT&RUN has the potential to replace all ChIP-based applications. For a typical research project in which ChIP-seq is currently used, transitioning to CUT&RUN is simple, as it can be done entirely on the benchtop using standard equipment that is already present in most molecular biology laboratories. Furthermore, as CUT&RUN is performed *in situ* in permeabilized cells that can readily be attached to a solid support such as magnetic beads, coated plates or glass slides, this method will readily transfer to robotics, allowing high throughput from cell to sequencing library. Adapting CUT&RUN to robotics should be more straightforward than is the case for ChIP-seq, as CUT&RUN does not require equipment such as sonicators or high-speed spin steps to remove insoluble material, which are difficult to automate.

Standard cross-linking ChIP protocols are not suitable for the low cell numbers that are often obtained after fluorescence-activated cell sorting or dissection, or in clinical settings. In light of this limitation of ChIP, ATAC-seq has been used for samples as small as 5,000 cells[26]. But ATAC-seq is limited to nonspecific identification of TFs that are in accessible regions of chromatin and is unable to distinguish chromatin states demarcated by histone PTMs. Problems of epitope masking in cross-linking ChIP, leading to low efficiency, can be mitigated by using a native ChIP strategy, which was shown to provide high-quality data with a few thousand cells for abundant nucleosome epitopes, but was not applied to TFs[27]. Here, we show that CUT&RUN is suitable for application to 100 cells for profiling H3K27me3, a histone modification involved in developmental silencing, or to 1,000 cells for CTCF, a sequence-specific DNA-binding protein. Therefore, CUT&RUN makes possible targeted genome-wide maps of protein–DNA interactions for rare cell types.

Further development of the protocol could include a replacement for sequential ChIP to map co-occupancy of subunits within a protein complex. Sequential ChIP-seq[28] has typically been challenging, and because of the very low yield after the second immunoprecipitation step, it is generally suitable only for abundant chromatin complexes. However, by first performing CUT&RUN, the cleaved chromatin complexes that are liberated into the supernatant at high efficiency could be immunoprecipitated with a second antibody. This application should allow compositional analysis and mapping of chromatin complexes genome wide.

We previously showed that by virtue of CUT&RUN being an *in situ* cleavage approach and the inherent flexibility of the chromatin fiber, it is possible to probe the local chromatin structure, including adjacent nucleosomes and 3D contacts[23]. We found that CTCF CUT&RUN identified 93% of high-scoring CTCF ChIA-PET contact sites with base-pair resolution. By introducing a native ChIP

TABLE 1 | Comparison of CUT&RUN with ChIP-seq protocols.

Method	CUT&RUN	X-ChIP-seq	ChIP-exo	N-ChIP-seq
Number of cells required	~100	~5 million	~50 million	~5,000
Resolution	<5 bp	~300 bp	<5 bp	<5 bp
Number of reads required	~3 million	~20 million	~100 million	~40 million
Profiles insoluble complexes	Yes	Yes	Yes	No
Signal-to-noise ratio	High	Low	Low	Medium
Fragmentation bias	No	Yes	Probably	Yes
Speed (cells to DNA)	1 d	3 d	1 week	2 d
Spike-in for quantitation	Simple	Possible	Complicated	Possible

protocol to identify sites directly bound by CUT&RUN, we were able to distinguish bona fide CTCF sites from sequences that are not bound by CTCF but rather are in contact[23]. Hi-C, ChIA-PET and Hi-ChIP, which are popular technologies for genome-wide mapping of 3D nuclear organization, rely on formaldehyde cross-linking to stabilize protein–protein interactions[29–31]. As such, these techniques have no formal distance constraint for mapping a positive genomic interaction, as very large nuclear structures could be cross-linked. By contrast, TSA-seq[32] and genome architecture mapping[33] have distance constraints and therefore measure cytological distance, either by the limited diffusion of a reactive species or the cryosectioning of cells. Similarly, in CUT&RUN, the reach of protein A-MNase provides an intrinsic limit to how far cleavage can occur from an epitope, and therefore how close two interacting DNA loci must be in order to be cleaved by tethering to one of them. By combining CUT&RUN with a proximity-based ligation method, it will be possible to generate factor-specific high-resolution maps of nuclear architecture.

Other novel applications can be envisioned. Any epitope for which an antibody is available can potentially be subjected to profiling using CUT&RUN, and CUT&RUN *in situ* mapping of long non-coding RNAs (lncRNAs) would seem to be an attractive alternative to DRIP-seq[34]. In addition, the ability of CUT&RUN to profile insoluble chromatin[23] suggests that combining CUT&RUN with salt fractionation will allow for an epigenomic map to be based on chromatin solubility, which has traditionally been used to define classic 'active' chromatin[35–37]. In this way, each DNA-binding protein or chromatin feature being profiled can be enriched with information about its solubility, a key physical property. Although salt fractionation can be performed with MNase-based ChIP-seq[37], high salt concentrations can disrupt the complex and cause loss of the epitope before antibody binding, whereas with CUT&RUN, salt fractionation is performed only after the antibody is bound and the fragments are cleaved.

Comparison with other methods

Table 1 lists metrics for CUT&RUN and three ChIP-seq methods, X-ChIP-seq[5], ChIP-exo[6] and N-ChIP-seq[8]. Compared with these ChIP-seq methods, CUT&RUN requires fewer cells and fewer reads, has a higher signal-to-noise ratio, has no fragmentation bias, is faster and is amenable to spike-in for quantitation.

An important advance in ChIP-based technologies has been the leveraging of next-generation sequencing to generate base-pair-resolution genome-wide maps of protein–DNA interactions[38]. In contrast to standard cross-linking ChIP, in which sonication is used to fragment the chromatin to a minimum of ~200-bp fragments, exonuclease treatment in ChIP-exo or MNase digestion in high-resolution X-ChIP-seq or native ChIP approaches allows limit or near-limit digestion[6–8,23,39]. However, this improvement in resolution in cross-linking strategies has often come at the price of increases in sequence depth requirements and the number of cells required. For example, in ChIP-exo, any sonicated fragments that contain more than just the target protein, such as an adjacent nucleosome, will form a block to the exo-nuclease in generating minimal TF footprints and as such will contribute to an apparent localized background, requiring increased cell numbers and sequencing depths to call high-resolution peak pairs. Native ChIP often does not suffer from these associated problems but has limited general applicability due to the requirement to generate soluble chromatin extracts in the absence of harsh detergents and therefore is best suited to stably bound proteins and may require optimization on a case-by-case basis. It has previously been shown that sonication, such as that used for cross-linking ChIP methods, is nonrandom and therefore is subject to a fragmentation bias[7,40]. As CUT&RUN is performed on intact cells or nuclei without fragmentation, it can be used to probe all genomic compartments. Technologies that use MNase for genome-wide digestion can suffer from A/T bias of the enzyme[41] and will preferentially digest open chromatin. By contrast, CUT&RUN involves a sterically regulated cleavage reaction, and we have shown that it does not suffer from any detectable A/T or DNA accessibility bias[23].

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, and 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 could be 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 a half volume of paramagnetic carboxylated beads (e.g., Agencourt AMPure XP beads), fragments smaller than ~700 bp will be selected for. We previously showed that this strategy was successful for the ~1-MDa yeast RSC complex, which was not released from the nuclei[23].

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 various options that might be used to tailor the protocol to specific situations. In brief, living cells are immobilized on magnetic beads (Steps 1–9), permeabilized and incubated with a primary antibody (Steps 10–15) and optionally with a secondary antibody for antibodies such as mouse IgGs that are not strongly bound by protein A (Steps 16–20), washing the beads after each step. Cells are next incubated with protein A-MNase (Steps 21–26), washed, and then the MNase is activated by addition of Ca^{2+} (Steps 27–32). The DNA–protein complex is then allowed to diffuse out of the cells (Steps 33 and 34), the supernatant is collected, and the DNA is extracted by either a spin column (Step 35A) or an organic extraction (Step 35B) procedure. Library preparation and paired-end sequencing are performed using standard protocols (Steps 36–41).

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 multiple cell lines, but we have recently adapted the protocol to use cells permeabilized by the nonionic detergent digitonin (Step 11), which has been successfully used in other *in situ* methods, including ChEC-seq[42] and ATAC-seq[26]. Digitonin permeates membranes and extracts cholesterol. Membranes that lack sterols are minimally impacted by digitonin[43,44]. Nuclear envelopes are relatively devoid of cholesterol, as compared with plasma membranes. As such, treatment of cells with digitonin represents a robust method for permeabilizing cells without compromising nuclear integrity[44]. The protocol described here uses digitonin, but it is possible that individual experimental situations call for the generation of intact nuclei by other means, and such nuclei can be prepared by a suitable method, bound to concanavalin A–coated beads per our previously published work[23] and then used in the protocol below starting at Step 10.

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) is problematic. In addition, high-resolution mapping techniques that cleave a minimal footprint are not suitable for 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 allows the DNA to be readily detected. We have successfully used a rabbit monoclonal antibody raised against H3K27me3, with capillary electrophoresis showing that the amount of cleaved fragments is proportional to the number of starting cells. A nucleosomal ladder is expected by TapeStation or another 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 such as CTCF, it is harder to detect the cleaved fragments by even sensitive electrophoretic analysis (**Supplementary Fig. 1**). Once the expected digested DNA pattern is observed for the positive control by capillary electrophoresis

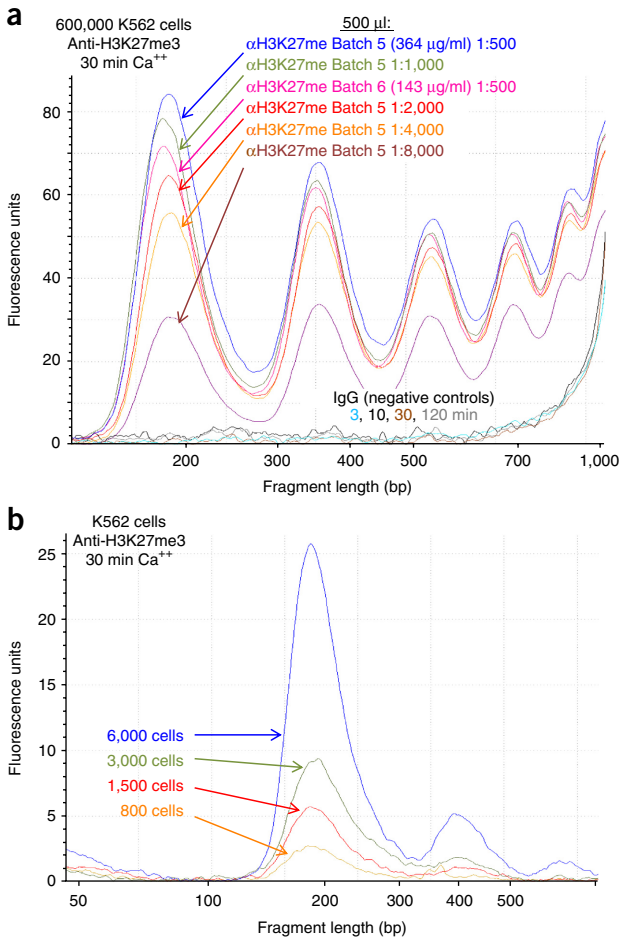

Figure 2 | TapeStation analysis of an abundant histone epitope (H3K27me3). (**a**) Dilutions of pA-MN for two batches, showing the approach to saturation for pA-MN for 600,000 cells in a 500-µl volume. Eight percent of each sample was loaded. (**b**) TapeStation analysis as a same-day positive control for a low-cell-number experiment. Ten percent of each sample was used for this analysis, and the remainder was used to make libraries for sequencing, with results shown in **Figure 3**.

such as H3K27me3, it is not necessary to sequence the sample. As a negative control, we recommend the use of a nonspecific 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 carryover of pA-MN will result in preferential fragmentation of hyperaccessible DNA.

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 Fig. 2**). We therefore recommend digesting for 30 min as a starting point that can be tailored based upon epitope abundance (shorter times for more abundant epitopes) and antibody concentration.

Spin-column extraction (Step 35A) is simple and fast, providing good recovery of fragments in the range of nucleosomes while reducing the concentration of very large fragments that can interfere with library preparation (**Supplementary Fig. 3**). Therefore, this DNA extraction option is preferred for most applications of CUT&RUN. However, for CUT&RUN of TFs at low cell numbers, organic extraction (Step 35B) is preferred for better recovery of small fragments.

MATERIALS

REAGENTS

- Cell suspension. We have used human K562 (ATCC, cat. no. CCL-243) and other mammalian cell lines, *Drosophila* S2 cells (Drosophila Genomics Resource Center, stock no. 181) and dissected *Drosophila* tissues such as brains and imaginal disks, and spheroplasted yeast (W1588-4C *Saccharomyces cerevisiae*, T. Tsukiyama, Fred Hutchinson Cancer Research Center).
  ! CAUTION The cell lines used in your research should be regularly checked to ensure they are authentic and are not infected with mycoplasma.
- Concanavalin A–coated magnetic beads (Bangs Laboratories, cat. no. BP531)
- Antibody to an epitope of interest, for example, rabbit anti-CTCF polyclonal antibody (Millipore, cat. no. 07-729), for mapping 1D and 3D interactions by CUT&RUN
- Positive-control antibody to an abundant epitope, e.g., anti-H3K27me3 rabbit monoclonal antibody (Cell Signaling Technology, cat. no. 9733)
- Negative-control antibody to an absent epitope, e.g., guinea pig anti-rabbit antibody (Antibodies-Online, cat. no. ABIN101961)
- Digitonin (EMD Millipore, cat. no. 300410)
- Trypan blue solution (0.4% (wt/vol); Fisher, cat. no. 15250061)
- Protein A–micrococcal nuclease (pA-MN) fusion protein (provided in 50% (vol/vol) glycerol by the authors upon request). Store at −20 °C for up to 1 year.
- Spike-in DNA for calibration (e.g., from *Saccharomyces cerevisiae* micrococcal nuclease-treated chromatin, provided by the authors upon request). This reagent can be stored for at least 1 year at 4 °C.
- Distilled, deionized or RNase-free H_2O (dH_2O; e.g., Promega, cat. no. P1197)
- 1 M Manganese chloride ($MnCl_2$; Sigma-Aldrich, cat. no. 203734)
- 1 M Calcium chloride ($CaCl_2$; Fisher, cat. no. BP510)
- 1 M Potassium chloride (KCl; Sigma-Aldrich, cat. no. P3911)
- 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES; Sigma-Aldrich, cat. no. H3375)
- 5 M Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S5150-1L)
- 0.5 M EDTA (Research Organics, cat. no. 3002E)
- 0.2 M Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA; Sigma-Aldrich, cat. no. E3889)
- 2 M Spermidine (Sigma-Aldrich, cat. no. S2501)
- Roche Complete Protease Inhibitor (EDTA-free) tablets (Sigma-Aldrich, cat. no. 5056489001)
- Glycogen (20 mg/ml; Sigma-Aldrich, cat. no. 10930193001)
- RNase A, DNase- and protease-free (10 mg/ml; Thermo Fisher Scientific, cat. no. EN0531)
- NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, cat. no. 740609.250)
- Agencourt AMPure XP magnetic beads (Beckman Coulter, cat. no. A63880)
- SDS (Sigma-Aldrich, cat. no. L4509)
- Proteinase K (Thermo Fisher Scientific, cat. no. EO0492)
- Phenol–chloroform–isoamyl alcohol 25:24:1 (PCI; Invitrogen, cat. no. 15593049) **! CAUTION** Phenol and chloroform are toxic, so they should be handled in a hood while wearing disposable gloves.
- Chloroform (Sigma, cat. no. 366919-1L)
- 1 M Tris-HCl (pH 8.0; Fisher, cat no. BP1521)
- Ethanol (Decon Labs, cat. no. 2716)
- Qubit dsDNA HS Assay Kit (Life Technologies, cat. no. Q32851)

EQUIPMENT

- Centrifuge with swinging bucket (Eppendorf, model no. 5810)
- Centrifuge with fixed-angle rotor (Eppendorf, model no. 5424)
- Centrifuge, refrigerated, with fixed-angle rotor (Eppendorf, model no. 5415R)
- 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 microcentrifuge tubes
- Vortex mixer (e.g., Vortex Genie; VWR)
- Microcentrifuge (e.g., VWR, model no. 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 and 70 °C)
- MaXtract phase-lock microcentrifuge tubes (Qiagen, cat. no. 139046)
- Capillary electrophoresis instrument (e.g., Agilent, model no. TapeStation 4200)
- Qubit Fluorometer (Life Technologies, cat. no. Q33216)
- Massively parallel DNA sequencer (e.g., Illumina, model no. HiSeq 2500)
- **Software**
- Bowtie 2 v2.2.5 (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml)
- Picard v2.15 (https://broadinstitute.github.io/picard/index.html)
- Peak-calling software (callpeaks2.pl:https://github.com/Henikoff/Cut-and-Run)
- gnuplot v3.13.0 (http://gnuplot.info/)
- Java Treeview v1.1.6r2 (http://jtreeview.sourceforge.net/)

REAGENT SETUP

5% (wt/vol) Digitonin solution 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 1,000-µl pipette tip. Pipette the hot water into the tube with the digitonin powder to make a 5% (wt/vol) digitonin solution, close the cap and quickly vortex at top speed until the digitonin is completely dissolved. If saved and refrigerated at 4 °C, this stock can be used within a week, but will need reheating to ~100 °C to redissolve the digitonin precipitate that accumulates. **! CAUTION** Digitonin is toxic and a face mask should be worn when weighing out the powder. A digitonin stock may be prepared by dissolving the powder in DMSO, but be aware that DMSO can be absorbed through the skin.

1 M HEPES-KOH Dissolve 23.8 mg of HEPES powder in 50 ml of water, add 10 M potassium hydroxide dropwise with stirring until pH 7.9 is reached and bring the volume to 100 ml with water. Sterilize by filtration and store at room temperature (RT; ~22 °C). Sterile stock solutions are stable for at least 1 year.

1 M HEPES (pH 7.5) Follow the procedure for 1 M HEPES-KOH, but bring the pH to 7.5 with 10 M NaOH.

20 mg/ml Glycogen Dissolve 200 mg of glycogen in 10 ml of water and sterilize by filtration or autoclaving. Store indefinitely as 1-ml aliquots at −20 °C.

10% (wt/vol) SDS Dissolve 10 g of SDS powder in water, and bring the volume to 10 ml (10% wt/vol). Autoclave or filter-sterilize. Stock solutions are stable indefinitely when stored at RT.

Binding buffer Mix 400 µl of 1 M HEPES-KOH at pH 7.9, 200 µl of 1 M KCl, 20 µl of 1 M $CaCl_2$ and 20 µl of 1 M $MnCl_2$, 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 the beads and withdraw enough of the bead suspension such that there will be 10 µl for each final sample and/or digestion time point. Mix into 1.5 ml of binding buffer in a 2-ml tube. Place the tube on a magnet stand until it is clear (30 s–2 min). Withdraw the liquid and remove the tube from the magnet stand. Add 1.5 ml of binding buffer, mix by inversion or gentle pipetting, remove the liquid from the cap and sides of the tube with a quick pulse on a microcentrifuge (<100*g*, 22 °C, 1 s). Place the tube on a magnet stand until it is clear (30 s–2 min). Aspirate and discard the liquid, and resuspend the beads in a volume of binding buffer equal to the initial volume of bead suspension (10 µl per final sample). Activated beads may be held on ice for a day before use.

Wash buffer For simplicity, we use a single buffer throughout the protocol, from binding to beads through digestion. A concentration of salt that is in the physiological range avoids stress when washing the cells and mixing with beads. Mix 1 ml of 1 M HEPES at pH 7.5, 1.5 ml of 5 M NaCl and 12.5 µl of 2 M Spermidine, bring the final volume to 50 ml with dH_2O and add one Roche Complete Protease Inhibitor (EDTA-free) tablet. Store the buffer at 4 °C for up to 1 week.

Digitonin buffer Mix 160–800 µl of 5% (wt/vol) Digitonin with 40 ml of wash buffer. Store the buffer at 4 °C for up to 1 d. ▲ **CRITICAL** 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 K562 cells with 0.02–0.1% (wt/vol) digitonin. For one lot we tested, we observed ~1/4 of the cells to be permeable by 0.0125% (wt/vol) digitonin and all of the cells to be permeable by 0.025% (wt/vol) digitonin. To test for digitonin effectiveness, mix 5 µl of Trypan blue with 5 µl of cells ~10 min after addition of varying concentrations of digitonin (0–0.1% (wt/vol)), apply ~5 µl to a glass slide, cover and observe within 10 min. All or nearly all the cells should be clear for no digitonin and blue for successful permeabilization.

Antibody buffer Mix 8 µl of 0.5 M EDTA with 2 ml of digitonin buffer and place on ice. Divide into aliquots for each antibody and add antibody solution or serum to a final concentration of 1:100 or to the manufacturer's recommended concentration for IF. Hold on ice and use within 1 h.

2× Stop buffer To 4.2 ml of dH_2O, add 340 µl of 5 M NaCl, 200 µl of 0.5 M EDTA, 100 µl of 0.2 M EGTA, 20 µl of 5% (wt/vol) digitonin, 25 µl of RNase A, 125 µl of 2 mg/ml glycogen and 2 pg/ml heterologous spike-in DNA. Store the buffer at 4 °C for up to 1 week.

▲ **CRITICAL** Heterologous spike-in DNA is highly recommended for calibration, for example, to compare time points in a digestion series[45]. Spike-in DNA should be fragmented down to an ~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).

PROCEDURE

Binding of cells to beads ● TIMING 30 min

▲ **CRITICAL** All steps before the addition of antibody (Step 11) are performed in succession at RT to minimize stress on the cells. Because it is critical that DNA breakage be minimized throughout the protocol, we recommend that cavitation during resuspension and vigorous vortexing be avoided.

1| Harvest fresh cell culture(s) at RT in 15- to 50-ml conical centrifuge tubes or 2-ml tubes and count the cells. The same protocol can be used for 100–250,000 mammalian cells per sample and/or digestion time point.

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

2| Centrifuge for 3 min at 600*g* at RT and withdraw the liquid.

3| Resuspend in 1.5 ml of wash buffer at RT by gently pipetting and, if necessary, transfer to a 2-ml tube.

4| Centrifuge for 3 min at 600*g* at RT and withdraw the liquid.

5| Repeat Steps 3 and 4.

6| Resuspend in 1 ml of wash buffer at RT by gently pipetting.

7| While gently vortexing the cells at RT, add the bead suspension.

8| Rotate for 5–10 min at RT.

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

▲ **CRITICAL STEP** To evaluate the success of the procedure without requiring library preparation, include in parallel a positive-control antibody (e.g., anti-H3K27me3) and a negative-control antibody (e.g., anti-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.

Permeabilization of cells and binding of (primary) antibodies ● TIMING 15 min to overnight, with longer incubations providing higher yields

10| Place the tubes on the magnet stand until the solution turns clear, then remove and discard 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 (which should be set to a low (~1,100 r.p.m.) speed) 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.
▲ **CRITICAL STEP** The presence of EDTA during antibody treatment removes excess divalent cations used to activate the concanavalin A, because carryover of Ca^{2+} 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 inactivation of the enzyme. Spermidine in the wash buffer is intended to compensate for the removal of Mg^{2+}, which might otherwise affect chromatin properties.

12| Place the tubes on the tube rotator at 4 °C for ~2 h, or at RT for 5–10 min.
■ **PAUSE POINT** Antibody incubation can proceed overnight at 4 °C.

13| Remove the liquid from the caps and the sides of the tubes with a quick pulse on a microcentrifuge (<100*g*, 22 °C, 1 s).
▲ **CRITICAL STEP** After mixing, but before placing a tube on the magnet stand, a very quick spin on a microcentrifuge (<100*g*, 22 °C, 1 s) will minimize carryover of antibody and pA-MN that could result in overall background cleavages during the digestion step.
? TROUBLESHOOTING

14| Place the tube on the magnet stand until the solution turns clear (~30 s) and remove all the liquid.

15| Add 1 ml of digitonin buffer, mix by inversion, or by gentle pipetting using a 1-ml tip if clumps persist, and remove the liquid from the cap and the sides of the tube with a quick pulse on a microcentrifuge (<100*g*, 22 °C, 1 s).

(Optional) Binding of secondary antibody ● TIMING 15 min–1.5 h
▲ **CRITICAL** The binding efficiency of protein A to the primary antibody depends on the 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 anti-mouse is recommended.

16| Place the tube on the magnet stand until the solution turns clear and remove all the liquid.

17| Place each tube at a low angle on the vortex mixer (which should be set to a low (~1,100 r.p.m.) speed) and squirt 50 µl of the digitonin buffer (per sample and/or digestion time point) along the sides of the tube while gently vortexing to allow the solution to dislodge most or all of the beads. Tap to dislodge the remaining beads.

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

19| Place on the tube rotator at 4 °C for ~1 h, or at RT for 5–10 min.
■ **PAUSE POINT** Antibody incubation can proceed overnight at 4 °C.

20| Repeat Steps 13–15.

Binding of protein A–MNase fusion protein ● TIMING 15 min–1.5 h
21| Place the tube on the magnet stand until the solution turns clear and remove all the liquid.

22| Place each tube at a low angle on the vortex mixer (which should be set to a low (~1,100 r.p.m.) speed) and squirt 50 µl of digitonin buffer (per sample and/or digestion time point) along the sides of the tube while gently vortexing to allow the solution to dislodge most or all of the beads. Tap to dislodge the remaining beads.

23| Mix in the pA-MN 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).
▲ **CRITICAL STEP** CUT&RUN is relatively insensitive to the concentration of pA-MN, as is evident from the titration test of two different batches (**Fig. 2a**), in which increasing the concentration of pA-MN above ~100 ng/ml resulted in little additional release of H3K27me3-bound nucleosomes from 600,000 human cells after 30 min of digestion in a 500-µl volume.

24| Place on the tube rotator at 4 °C for ~1 h, or at RT for 5–10 min.

25| Remove the liquid from the cap and sides of the tube with a quick pulse (<100*g*, ~22 °C, ~1 s) on a microcentrifuge.

26| Repeat Steps 14 and 15 twice.

Targeted digestion ● TIMING 45 min
27| Place the tube on the magnet stand until the solution turns clear and remove all the liquid.

28| Place each tube at a low angle on the vortex mixer (which should be set to a low (~1,100 r.p.m.) speed) and add 150 µl of digitonin buffer (per sample and/or digestion time point) along the sides of the tube while gently vortexing to allow the solution to dislodge most or all of the beads. Tap to dislodge the remaining beads.

29| Insert the tubes into 1.5-ml wells of a heater block sitting in wet ice to chill down to 0 °C (~5 min).

30| Remove each tube from the block, mix in 3 µl of 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.

31| Incubate at 0 °C for the desired digestion time (default is 30 min).
▲ **CRITICAL STEP** MNase binds DNA but only cleaves when Ca^{2+} is present, so that digestion is a zero-order reaction that seems to be less temperature-dependent than the subsequent diffusion of released pA-MN-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 °C while minimizing background cleavages attributable to diffusion. We have found that digestion at ambient temperature or higher results in unacceptable background cleavage levels.

32| Add 100 µl of 2× stop buffer and mix by gentle vortexing.
▲ **CRITICAL STEP** Heterologous spike-in DNA should be present in the 2× stop buffer 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 ● TIMING 20 min
33| Incubate the tube for 10 min at 37 °C to release CUT&RUN fragments from the insoluble nuclear chromatin.

34| Centrifuge for 5 min at 4 °C at 16,000*g* and place the tube on a magnet stand.

DNA extraction
35| Extract the DNA. Follow option A to quickly extract the DNA by using a spin column (recommended for recovery of large protein–DNA complexes such as nucleosomes) or option B for the alternative DNA extraction method (recommended for TFs).
(A) Fast DNA extraction by spin column ● TIMING 20 min
(i) Place a spin column into a collection tube and add 400 µl of buffer NT1 (from the NucleoSpin kit or equivalent) to the spin column.
(ii) Decant the supernatant from Step 34 cleanly from the pellet to the spin column containing buffer NT1 and mix by pipetting gently up and down.
(iii) Centrifuge the spin column for 30 s at 11,000*g* at RT. Discard the flow-through.
(iv) Add 700 µl of buffer NT3. Centrifuge for 30 s at 11,000*g* at RT. Discard the flow-through.
(v) Add 700 µl of buffer NT3. Centrifuge for 30 s at 11,000*g* at RT. Discard the flow-through and replace the spin column in the rotor.
(vi) Centrifuge for 1 min at 11,000*g* at RT. Remove the tube from the rotor and let it dry for 5 min at RT.
(vii) Place the spin column in a fresh tube and add 20 µl of buffer NE to the membrane to elute the DNA.

(viii) After 1 min, centrifuge for 1 min at 11,000*g* at RT.
(ix) Add 20 µl of buffer NE to the membrane to elute any remaining DNA.
(x) After 1 min, centrifuge for 1 min at 11,000*g* at RT.

(B) Alternative DNA extraction method (preferred for quantitative recovery of ≤80-bp fragments) ● **TIMING 1.5 h**

(i) Decant the supernatant from Step 34 cleanly from the pellet into a fresh 1.5-ml microcentrifuge tube.
(ii) To each sample, add 2 µl of 10% (wt/vol) SDS (to a final concentration of 0.1%) and 1.5 µl of proteinase K (20 mg/ml). Mix by inversion and incubate for 10 min at 70 °C.
(iii) Add 200 µl of PCI and mix by full-speed vortexing for ~2 s.
! CAUTION Phenol and chloroform are toxic, so they should be handled in a hood while wearing disposable gloves.
(iv) Transfer the solution to a phase-lock tube, and centrifuge for 5 min at RT at 16,000*g*.
(v) Add 200 µl of chloroform, invert ~10× to mix, and centrifuge for 5 min at RT at 16,000*g*.
(vi) Remove the liquid by pipetting into a fresh 1.5-ml Eppendorf tube containing 2 µl of 2 mg/ml glycogen (1:9 of the 20 mg/ml stock solution).
(vii) Add 500 µl of 100% ethanol and mix by vortexing or tube inversion.
(viii) Chill on ice for 5–10 min and centrifuge for 10 min at 4 °C at 16,000*g*.
(ix) Pour off the liquid and drain on a paper towel.
(x) Rinse the pellet by adding 1 ml of 100% ethanol, invert ~10× to mix, and centrifuge for 1 min at 4 °C at 16,000*g*.
(xi) Carefully pour off the liquid and drain on a paper towel. Air-dry for at least 5 min.
(xii) When the pellet is dry, dissolve it in 40 µl of 1 mM Tris-HCl at pH 8 and 0.1 mM EDTA.

Library preparation and sequencing ● **TIMING 2–4 d**

36| (Optional) Quantify 1–2 µl of the solution from Step 35A(x) or 35B(xii), for example, by using fluorescence detection with a Qubit instrument following the manufacturer's instructions.

37| (Optional) Evaluate the presence of cleaved fragments and the size distribution by capillary electrophoresis with fluorescence detection, for example, by using a TapeStation 4200 instrument with D1000 high-sensitivity reagents, following the manufacturer's instructions.
▲ **CRITICAL STEP** Some long undigested DNA will always leak through, and this is what will dominate the Qubit fluorescence for CUT&RUN of typical TFs. 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.

38| Prepare barcoded libraries for Illumina sequencing using a single-tube protocol, following the manufacturer's instructions. Rapid PCR cycles, as in the table below, favor exponential amplification of the desired CUT&RUN fragments over linear amplification of large DNA fragments that are too long for polymerase to replicate in a single cycle, resulting in failure to exponentially amplify these background fragments.
▲ **CRITICAL STEP** To minimize the contribution of large DNA fragments, the number of PCR cycles should be at least 12–14, preferably with a 10-s 60 °C combined annealing/extension step. Good results have been obtained with the Hyper Prep Kit (KAPA Biosystems).

Cycle number	Denature	Anneal	Extend	Final
1	98 °C, 45 s			
2–15	98 °C, 15 s	60 °C, 10 s		
16			72 °C, 1 min	
17				8 °C, hold

39| Quantify the library yield using a dsDNA-specific assay, such as the Qubit dsDNA HS Assay Kit, following the manufacturer's instructions.
? TROUBLESHOOTING

40| Determine the size distribution of libraries by Agilent 4200 TapeStation analysis, following the manufacturer's instructions.
? TROUBLESHOOTING

41| Perform paired-end Illumina sequencing on the barcoded libraries using an Illumina HiSeq 2500 or another massively parallel DNA sequencer, following the manufacturer's instructions.
▲ **CRITICAL STEP** Because of the very low background with CUT&RUN, typically 5 million paired-end reads per sample suffice for TFs or nucleosome modifications, even for the human genome. For maximum economy, we mix up to 24 barcoded samples per lane on a two-lane flow cell, and perform paired-end 25 × 25-bp sequencing. Single-end sequencing is not recommended for CUT&RUN, as it sacrifices resolution and discrimination between TFs and neighboring nucleosomes.

Data processing and analysis ● TIMING >1 d
42| Align paired-end reads using Bowtie2 v2.2.5 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 and map to a repeat-masked version of the spike-in genome to avoid cross-mapping of the experimental genome to that of the spike-in DNA.
▲ **CRITICAL STEP** Separation of sequenced fragments into ≤120- 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.

43| Analyze the data. A Unix-compatible script for processing CUT&RUN spike-in data is provided as an example (**Supplementary Methods**). We used the Picard v2.15 'MarkDuplicates' command to mark presumed PCR duplicates for removal from low-cell-number data. Peak-calling software is available from https://github.com/Henikoff/Cut-and-Run. Scatterplots were produced using gnuplot v3.13.0, and the correlation plot and heat maps were displayed using Java Treeview v1.1.6r2. Profiles shown in **Figures 3** and **4** were displayed using IGV version 2.3.32 (ref. 46).

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
13	Beads clump and cannot be disaggregated	Cells lyse	Reduce the digitonin concentration
39	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 the antibody. Antibody binding can be tested by immunofluorescence
40	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
	A nucleosome ladder is detected by Tapestation analysis	This is typical for abundant nucleosomal epitopes, but otherwise may indicate the release of pA-MN during digestion	Run a negative-control sample using an IgG, e.g., guinea pig anti-rabbit
	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-MN to behave as a 'time bomb' when Ca^{2+} is added	Replace the antibody. Reduce the pA-MN concentration. Reduce the digestion time. Add a third wash step before digestion

● TIMING

Day 1, cells to DNA

Steps 1–9, binding of cells to beads: 30 min

Steps 10–15, binding of (primary) antibody: 15 min to overnight

Steps 16–20, (optional) binding of secondary antibody: 15 min–1.5 h

Steps 21–26, binding of protein A–MNase fusion protein: 15 min–1.5 h

Steps 27–32, targeted digestion: 45 min

Steps 33 and 34, target chromatin release: 20 min

Step 35A, fast DNA extraction by spin column: 20 min

Step 35B, alternative DNA extraction method: 1.5 h

Days 2–4, library preparation and sequencing

Steps 36–41, library preparation and sequencing: 2–4 d

Day 5 and beyond, data processing and analysis

Steps 42 and 43, ≥1 d

ANTICIPATED RESULTS

We previously demonstrated that CUT&RUN can provide high-quality data with as few as 600,000 cells, but we suspected that the method could be used with much lower cell numbers. Therefore, we applied the current protocol to decreasing numbers of cells for two typical epitopes, an abundant histone modification (H3K27me3) and a general DNA-binding protein (CTCF). K562 cells were harvested and diluted such that between 100 and 6,000 cells were profiled for H3K27me3 and between 1,000 and 100,000 cells for CTCF. To verify cell numbers, DNA from pellets was quantified by Qubit fluorescence (**Supplementary Fig. 4**). DNA from selected supernatant fractions was resolved by TapeStation analysis (**Fig. 2b**) and subjected to Illumina PE25 × 25 sequencing.

Typical ChIP-seq experiments use high starting cell numbers that result in a large number of unique sonicated fragments that are immunoprecipitated. By contrast, as CUT&RUN has a relatively low background, the number of unique fragments is less than typical sequence depths. Therefore, high sequencing depths from low-cell-number experiments could result in

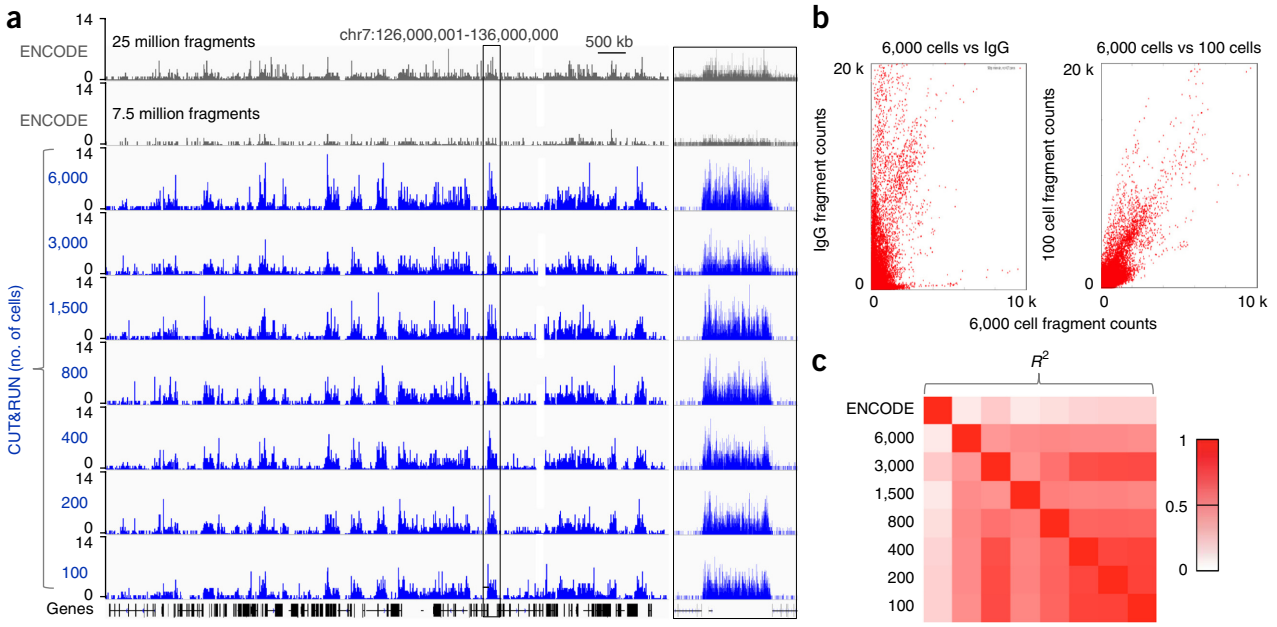

Figure 3 | CUT&RUN of H3K27me3 requires only 100 cells to profile the human polycomb chromatin landscape. (**a**) Varying numbers of K562 cells were used as the starting material for profiling H3K27me3 by CUT&RUN. Following paired-end 25 × 25-bp Illumina sequencing and removal of duplicates, 7.5 million fragments were randomly selected and used to generate bedgraph files representing raw counts, as indicated on the *y* axis. A 10-Mb region is shown. ENCODE X-ChIP-seq data (GSM733658) are shown for the full profile (top track, 25 million fragments (before removal of duplicates)) and for the randomly selected subset. The boxed 300-kb region is expanded on the right. (**b**) Scatterplot comparison of selected data sets. After fragment selection and removal of duplicates, data sets were binned in 50-bp intervals genome-wide. Relative to a negative-control data set, a strong increase in correlation to the 6,000-cell data set is seen for the 100-cell data set. (**c**) High H3K27me3 data quality down to 100 cells. To obtain an unbiased comparison between data sets without applying an arbitrary threshold, all 50-bp bins with zero in one or more data sets were removed, and pairwise R^2 values were calculated (N = 3,263,028) as a summary description of the rich information that is revealed in the scatterplots. Data sets are available at the NCBI GEO repository under accession no. GSE104550.

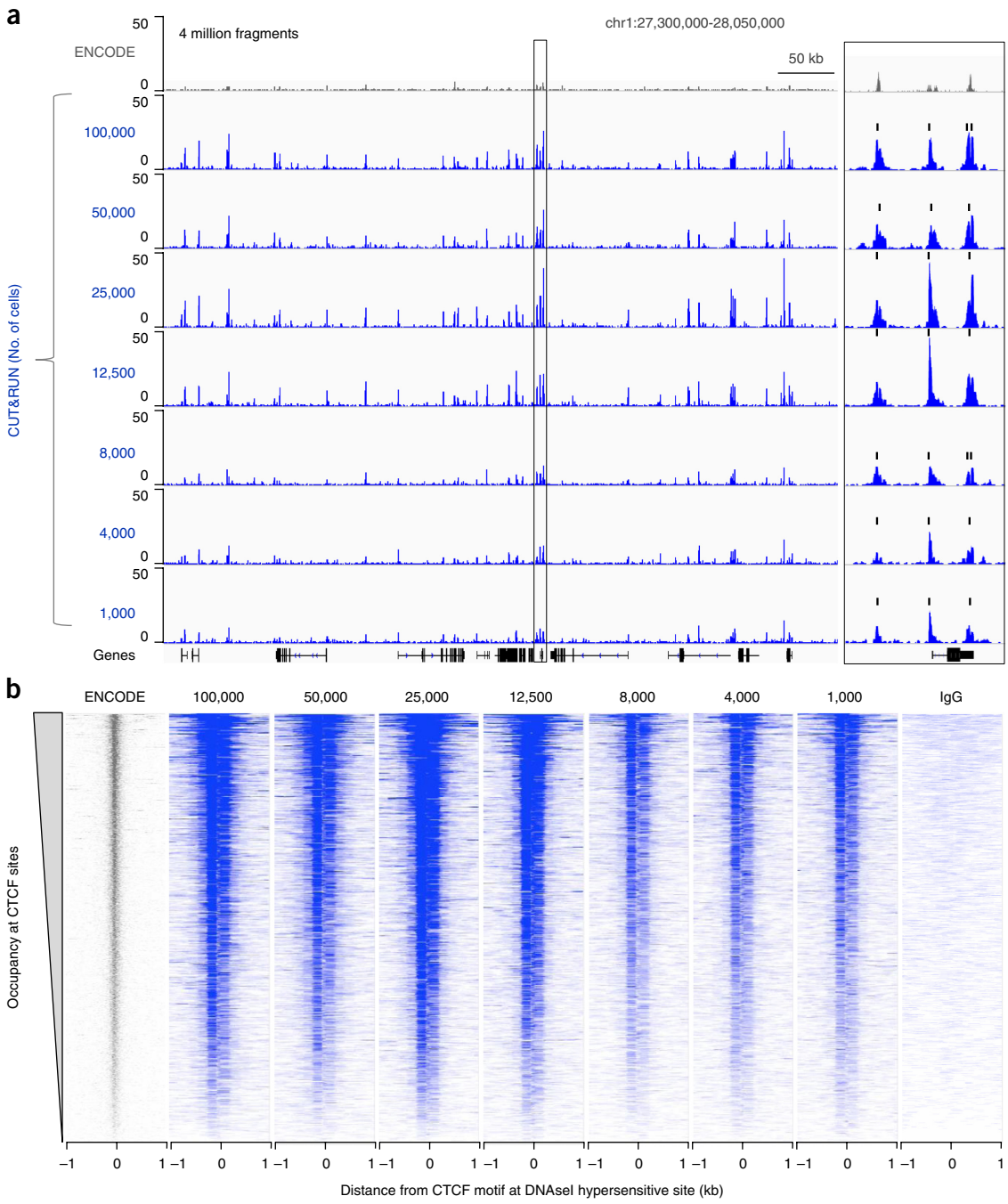

Figure 4 | CUT&RUN requires only 1,000 cells and 4 million fragments to delineate human CTCF peaks. (**a**) CUT&RUN was used to map CTCF binding sites in varying numbers of K562 cells. Following paired-end sequencing, 4 million nonduplicated fragments were randomly selected and used to generate bedgraphs representing raw counts, as indicated on the *y* axis. For comparison, ENCODE X-ChIP-seq data (GSE30263) were similarly analyzed. The boxed 12-kb region is expanded on the right, where tick marks indicate peak calls (**Supplementary Fig. 5**) using a threshold method described in ref. 47. (**b**) To compare CTCF peak quality between the CUT&RUN data sets, we aligned normalized counts from each data set over the 1,039 CTCF sites ±1 kb on chromosome 1 identified as having both a CTCF motif and a DNAseI hypersensitive site[23]. CTCF ENCODE ChIP-seq, CTCF CUT&RUN (with cell numbers as indicated) and IgG background control sites were ordered by decreasing occupancy over the 2-kb region shown. Heat maps were displayed using Java Treeview with contrast = 10 (http://jtreeview.sourceforge.net/). The dominant double-track pattern results from cleavage and release of large fragments protected by CTCF-adjacent nucleosomes, as previously described[23]. Data sets are available at the NCBI GEO repository under accession no. GSE104550.

redundant sequencing of PCR duplicates. Presumed PCR and optical duplicates were removed, and mapped fragments were randomly sampled without replacement, resulting in 7.5 million unique fragments per sample, displayed as normalized counts from stacked fragments (**Fig. 3a**). For comparison, this region from the full ENCODE data set for H3K27me3 in K562 cells (GSM733658) and a sample of 7.5 million unique fragments were similarly displayed. It is evident that very little if any loss

of data quality occurred with reduction in cell number down to 100 cells, confirmed by correlation analysis (**Fig. 3b**). By contrast, the ENCODE profile sampled at the same depth shows a blurry profile, owing to the high background inherent to ChIP.

CUT&RUN using an anti-CTCF antibody (1:100, Millipore, cat. no. 07-729) yielded profiles with little loss of data quality down to samples of 1,000 cells (**Fig. 4a**). Consistent data quality was confirmed by peak calling as described[47] (**Supplementary Fig. 5**) and by heatmap analysis of peak densities (**Fig. 4b**). The double-track profile results from excision of neighboring nucleosomes. Importantly, an IgG negative control shows that there is no perceptible background digestion flanking the CTCF sites. The consistent detection of peaks for all CUT&RUN samples, regardless of cell number, and failure to detect these peaks in ENCODE ChIP-seq data is largely attributable to the much higher signal-to-noise ratio of CUT&RUN (**Supplementary Fig. 5**).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS P.J.S. and S.H. developed the protocol and performed the experiments. P.J.S., J.G.H. and S.H. analyzed the data. S.H. wrote the manuscript with input from P.J.S. and J.G.H.

COMPETING INTERESTS
The authors declare no competing interests.

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