Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE63536R2
Full Title:	Single-cell factor localization on chromatin using ultra-low input Cleavage Under Targets and Release Using Nuclease
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Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Biology
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
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TITLE:

Single-Cell Factor Localization on Chromatin Using Ultra-Low Input Cleavage Under Targets and Release Using Nuclease

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SUMMARY:

CUT&RUN and its variants can be used to determine protein occupancy on chromatin. This protocol describes how to determine protein localization on chromatin using single-cell uliCUT&RUN.

ABSTRACT:

Determining the binding locations of a protein on chromatin is essential for understanding its function and potential regulatory targets. Chromatin Immunoprecipitation (ChIP) has been the gold standard for determining protein localization for over 30 years and is defined by the use of an antibody to pull out the protein of interest from sonicated or enzymatically digested chromatin. More recently, antibody tethering techniques have become popular for assessing protein localization on chromatin due to their increased sensitivity. Cleavage Under Targets & Release Under Nuclease (CUT&RUN) is the genome-wide derivative of Chromatin Immunocleavage (ChIC) and utilizes recombinant Protein A tethered to micrococcal nuclease (pA-MNase) to identify the IgG constant region of the antibody targeting a protein of interest, therefore enabling site-specific cleavage of the DNA flanking the protein of interest. CUT&RUN can be used to profile histone modifications, transcription factors, and other chromatin-binding proteins such as nucleosome remodeling factors. Importantly, CUT&RUN can be used to assess the localization of either euchromatic- or heterochromatic-associated proteins and histone modifications. For these reasons, CUT&RUN is a powerful method for determining the binding profiles of a wide range of proteins. Recently, CUT&RUN has been optimized for transcription factor profiling in low populations of cells and single cells and the optimized protocol has been termed ultra-low input CUT&RUN (uliCUT&RUN). Here, a detailed protocol is presented for single-cell factor profiling using uliCUT&RUN in a manual 96-well format.

INTRODUCTION:

Many nuclear proteins function by interacting with chromatin to promote or prevent DNA-templated activities. To determine the function of these chromatin-interacting proteins, it is

important to identify the genomic locations at which these proteins are bound. Since its development in 1985, Chromatin Immunoprecipitation (ChIP) has been the gold standard for identifying where a protein binds to chromatin[1,2]. The traditional ChIP technique has the following basic workflow: cells are harvested and crosslinked (usually with formaldehyde), chromatin is sheared (usually with harsh sonication methods, necessitating crosslinking), the protein of interest is immunoprecipitated using an antibody that targets the protein (or tagged protein) followed by a secondary antibody (coupled to agarose or magnetic beads), crosslinking is reversed, protein and RNA are digested to purify DNA, and this ChIP enriched-DNA is used as the template for analysis (using radiolabeled probes[1,2], qPCR[3], microarrays[5,6], or sequencing[4]). With the advent of microarrays and massively parallel deep sequencing, ChIP-chip[5,6] and ChIP-seq[4] have more recently been developed and allow for genome-wide identification of protein localization on chromatin. Crosslinking ChIP has been a powerful and reliable technique since its advent with major advances in resolution by ChIP-exo[7] and ChIP-nexus[8]. In parallel to the development of ChIP-seq, native (non-crosslinking) protocols for ChIP (N-ChIP) have been established, which utilize nuclease digestion (often using a micrococcal nuclease or MNase) to fragment the chromatin, as opposed to sonication performed in traditional crosslinking ChIP techniques[9]. However, one major drawback to both crosslinking ChIP and N-ChIP technologies has been the requirement for high cell numbers due to low DNA yield following the experimental manipulation. Therefore, in more recent years, many efforts have been toward optimizing ChIP technologies for low cell input. These efforts have resulted in the development of many powerful ChIP-based technologies that vary in their applicability and input requirements[10–18]. However, single-cell ChIP-seq based technologies have been lacking, especially for non-histone proteins.

In 2004, an alternative technology was developed to determine protein occupancy on chromatin termed Chromatin Endogenous Cleavage (ChEC) and Chromatin Immunocleavage (ChIC)[19]. These single-locus techniques utilize a fusion of MNase to either the protein of interest (ChEC) or to protein A (ChIC) for direct cutting of DNA adjacent to the protein of interest. In more recent years, both ChEC and ChIC have been optimized for genome-wide protein profiling on chromatin (ChEC-seq and CUT&RUN, respectively)[20,21]. While ChEC-seq is a powerful technique for determining factor localization, it requires developing MNase-fusion proteins for each target, whereas ChIC and its genome-wide variation, CUT&RUN, rely on an antibody directed toward the protein of interest (as with ChIP) and recombinant Protein A-MNase, where the Protein A can recognize the IgG constant region of the antibody. As an alternative, a fusion Protein A/Protein G-MNase (pA/G-MNase) has been developed that can recognize a broader range of antibody constant regions[22]. CUT&RUN has rapidly become a popular alternative to ChIP-seq for determining protein localization on chromatin genome-wide.

Ultra-low input CUT&RUN (uliCUT&RUN), a variation of CUT&RUN that enables the use of low and single-cell inputs, was described in 2019[23]. Here, the methodology for a manual 96-well format single-cell application is described. It is important to note that since the development of uliCUT&RUN, two alternatives for histone profiling, CUT&Tag and iACT-seq have been developed, providing robust and highly parallel profiling of histone proteins[24,25]. Furthermore, scCUT&Tag has been optimized for profiling multiple factors in a single cell (multiCUT&Tag) and for application to non-histone proteins[26]. Together, CUT&RUN provides an attractive alternative to

low input ChIP-seq where uliCUT&RUN can be performed in any molecular biology lab that has access to a cell sorter and standard equipment.

PROTOCOL:

Ethics statement: All studies were approved by the Institutional Biosafety Office of Research Protections at the University of Pittsburgh.

1. Prepare magnetic beads

NOTE: Perform prior to cell sorting and hold on ice until use.

1.1. Pipette 30 µL of ConA-conjugated paramagnetic microspheres bead slurry mix per reaction to a fresh 1.5 mL microfuge tube and add 850 µL of Binding Buffer, pipetting gently to mix.

NOTE: ConA-conjugated paramagnetic microspheres are lectin-coated magnetic beads that permit lipid membrane binding.

1.2. Place the tube on a magnetic rack and allow the beads to magnetize for 1–2 min. Once the supernatant has cleared, remove, and discard the supernatant without disturbing the beads.

1.3. Remove the tube from the magnetic rack and wash the beads by resuspending in 1 mL of Binding Buffer.

1.4. Repeat steps 1.2 and 1.3.

1.5. Magnetize the beads for 2 min and remove the supernatant to discard.

1.6. Remove the tube from the magnetic rack and resuspend the beads in 30 µL of Binding Buffer per reaction.

1.7. Hold the washed bead mix on ice until cells are sorted.

2. Harvest cells

NOTE: This step is written for adhered cells and optimized for murine E14 embryonic stem cells. Culturing and harvesting the cells depend on the cell type.

2.1. Remove the cells from the 37 °C incubator and examine them under a microscope to assure quality.

2.2. Aspirate the media from the cell plate and rinse with 5 mL of 1x PBS.

2.3. Aspirate PBS from the plate and harvest the cells (using traditional cell harvesting methods which will differ by cell type). Obtain single-cell suspension by gently pipetting up and down with a serological pipette against the culture dish, if necessary.

2.4. Transfer the cell suspension to a 15 mL conical tube and spin down at 200 x *g* for 5 min.

2.5. Aspirate off the media to discard and wash the cell pellet with 5 mL of PBS + 1% FBS.

2.6. Spin down the cells at 200 x *g* for 5 min, discard the supernatant, and resuspend the cell pellet in 5 mL of PBS + 1% FBS.

2.7. Count the cells and transfer 1 mL of 1 x 10^6 cells into a fresh 1.5 mL microfuge tube.

2.8. Add 5 µL of 7-Amino-Actinomycin D (7-AAD), invert the well to mix, and then apply the sample to the cell sorter to sort live single cells into individual wells of a 96-well plate.

NOTE: 7-AAD dye is excluded from live cells, and therefore can be used in live-cell sorting.

3. Cell sorting and lysis

3.1. Prepare a cell sorter-compatible 96-well plate with 100 µL of Nuclear Extraction (NE) Buffer in each well prior to cell sorting.

3.2. Sort the cells into 96-well plates following the manufacturer's instructions.

3.3. Quickly spin the plate (600 x *g* for 30 s) to assure cells are in the buffer within the wells.

NOTE: It is worth testing in a preliminary experiment whether the cells being used are reliably brought to the bottom of the wells.

3.4. Hold the samples on ice for 15 min.

3.5. Spin down the samples at 600 x *g* for 5 min at 4 °C and carefully pipette to remove the supernatant (leaving behind 5 µL).

3.6. Resuspend each sample in 55 µL of NE buffer and add 30 µL of the prewashed ConA-conjugated paramagnetic microspheres (from step 1.7; in Binding Buffer) to each reaction.

3.7. Incubate at room temperature for 10 min.

4. Pre-block samples to prevent early digestion by MNase

4.1. Place the plate on a 96-well magnetic rack, allow the beads to bind for a minimum of 5 min, and then remove and discard the supernatant.

4.2. Add 100 µL of Blocking Buffer to the nucleus-bound beads and mix with gentle pipetting.

4.3. Incubate for 5 min at room temperature.

5. Addition of primary antibody

5.1. Place the plate on a 96-well magnetic rack, allow the supernatant to clear for a minimum of 5 min, and then remove and discard the supernatant without disturbing the beads.

5.2. Remove the plate from the magnetic rack and resuspend the beads in 100 µL of Wash Buffer per reaction with gentle pipetting.

5.3. Place the plate back on the 96-well magnetic rack, allow the supernatant to clear, and then remove and discard the supernatant.

5.4. Resuspend the beads in 25 µL of Wash Buffer per reaction with gentle pipetting.

5.5. Make a primary antibody master mix: 25 µL of Wash Buffer + 0.5 µL of antibody per reaction.

5.6. While gently vortexing the nuclei-bound beads, add 25 µL of the primary antibody master mix to each sample being treated with an antibody targeting the protein of interest (typically 1:100 final dilution). Add 25 µL of Wash Buffer with no antibody, if performing a control.

5.7. Incubate for 1 h at room temperature.

5.8. Place the samples on a 96-well magnetic rack, allow the supernatant to clear for a minimum of 5 min, and then remove and discard the supernatant without disturbing the beads.

5.9. Remove the plate from the magnetic rack and wash the beads with 100 µL of Wash Buffer, resuspending by pipetting.

6. Addition of pA-MNase or pA/G-MNase

NOTE: Protein A has a high affinity for IgG molecules from certain species such as rabbits but is not suitable for IgGs from other species such as mice or rats. Alternatively, Protein A/G-MNase can be used. This hybrid binds rabbit, mouse, and rat IgGs, avoiding the need for secondary antibodies when mouse or rat primary antibodies are used.

6.1. Place the plate back on a 96-well magnetic rack, allow the supernatant to clear for a minimum of 5 min, and then remove and discard the supernatant without disturbing the beads.

6.2. Remove the plate from the magnetic rack and resuspend each sample in 25 µL of Wash Buffer.

6.3. Make a pA-MNase master mix (25 µL of Wash Buffer + optimized amount of pA-MNase per reaction).

6.4. While gently vortexing, add 25 µL of the pA-MNase master mix to each sample, including the control samples.

NOTE: The concentration of pA-MNase varies upon preparation, if homemade, and should be therefore tested prior to use upon each independent purification. For pA/G-MNase, 2.5 µL of the 20x stock should be used.

6.5. Incubate the samples for 30 min at room temperature.

6.6. Place the plate on a 96-well magnetic rack, allow the supernatant to clear for a minimum of 5 min, and then remove and discard the supernatant without disturbing the beads.

6.7. Remove the plate from the magnetic rack and wash the beads with 100 µL of Wash Buffer, resuspending by gentle pipetting.

7. Directed DNA digestion

7.1. Place the plate on a 96-well magnetic rack, allow the supernatant to clear for a minimum of 5 min, and then remove and discard the supernatant.

7.2. Remove the samples from the magnetic rack and resuspend the beads in 50 µL of Wash Buffer by gentle pipetting.

7.3. Equilibrate the samples to 0 °C in an ice/water mixture for 5 min.

7.4. Remove the samples from the 0 °C ice/water bath and add 1 µL of 100 mM $CaCl_2$ using a multichannel pipette. Mix well (3–5 times) with gentle pipetting using a larger volume multichannel pipette, and then return the samples to 0 °C.

NOTE: Mixing well here is essential. $CaCl_2$ is added to activate MNase digestion of DNA flanking the protein of interest.

7.5. Start a 10 min timer as soon as the plate is back in the ice/water bath.

7.6. Stop the reaction by pipetting 50 µL of 2XRSTOP+ Buffer into each well, in the same order as the $CaCl_2$ was added.

NOTE: Make 2XRSTOP+ Buffer before the 10 min digestion is over to prevent over digestion.

8. Sample fractionation

8.1. Incubate the samples for 20 min at 37 ˚C.

8.2. Spin the plate at 16,000 x *g* for 5 min at 4 °C.

8.3. Place the plate on a 96-well magnetic rack, allow the supernatant to clear for a minimum of 5 min, and transfer supernatants to a fresh 96-well plate. Discard the beads.

9. DNA extraction

9.1. Add 1 µL of 10% Sodium Dodecyl Sulfate (SDS) and 0.83 µL of 20 mg/mL Proteinase K to each sample.

CAUTION: SDS powder is harmful if inhaled. Users should use in well-ventilated spaces wearing goggles, gloves, and an N95-grade respirator, handling with care.

9.2. Mix the samples by gentle pipetting.

9.3. Incubate the samples for 10 min at 70 °C.

9.4. Return the plate to room temperature and add 46.6 µL of 5 M NaCl and 90 µL of 50% PEG 4000. Mix by gentle pipetting.

9.5. Add 33 µL of polystyrene–magnetite beads to each sample and incubate for 10 min at room temperature.

NOTE: Be sure to bring polystyrene–magnetite beads to room temperature (~30 min) and mix well before using.

9.6. Place the plate on a magnetic rack and allow the supernatant to clear for ~5 min, and then carefully discard the supernatant without disturbing the beads.

9.7. Rinse 2x with 150 µL of 80% ethanol without disturbing the beads.

CAUTION: Ethanol is highly flammable and causes skin, eye, and lung irritation. Perform this step with appropriate laboratory clothing and in a vented hood.

9.8. Spin the plate briefly at 1000 x *g* for 30 s. Place the plate back on a 96-well magnetic rack and remove all residual EtOH without disturbing the beads.

9.9. Air-dry the samples for ~2–5 min.

NOTE: Do not dry the beads for longer than 5 min. If diligent about removing the EtOH, 2–3 min of drying is sufficient.

9.10. Resuspend the beads with 37.5 µL of 10 mM Tris-HCl (pH 8) and incubate for 5 min at room temperature.

9.11. Place the plate on a magnetic rack, and allow the beads to bind for 5 min.

9.12. Transfer 36.5 µL of the supernatant to a fresh thermocycler compatible 96-well plate. Discard the beads.

NOTE: The experiment can be stopped here by storing samples at -20 °C or can continue with the library build (steps 10–15).

10. End repair, phosphorylation, adenylation

NOTE: The reagents are sourced as referenced in the **Table of Materials**. The below protocol follows a similar method to the commercial kit such as NEBNext Ultra DNA II kit.

10.1. Dilute 5 U/µL of T4 DNA polymerase 1:20 in 1x T4 DNA ligase buffer.

10.2. Prepare an end-repair/3'A master mix: 2 µL of 10x T4 DNA ligase buffer, 2.5 µL of 10 mM dNTPs, 1.25 µL of 10 mM ATP, 3.13 µL of 40% PEG 4000, 0.63 µL of 10 U/µL T4 PNK, 0.5 µL of diluted T4 DNA polymerase, 0.5 µL of 5U/µL Taq DNA polymerase, with a total volume of 13.5 µL per reaction.

NOTE: Be sure to bring 40% PEG 4000 to room temperature before pipetting.

10.3. Add 13.5 µL of end-repair/3'A master mix to 36.5 µL of DNA.

10.4. Mix the reaction by quick vortex, and then a quick spin (500 x *g* for 10 s).

10.5. Incubate using the following reaction conditions in a pre-cooled thermocycler with a heated lid for temperatures >20 °C. Use the reaction conditions: 12 °C for 15 min, 37 °C for 15 min, 72 °C for 20 min, hold at 4 °C.

11. Adapter ligation

NOTE: Keep the samples on ice while setting up the following reaction. Allow ligase buffer to come to room temperature before pipetting. Dilute the Adaptor (see **Table of Materials**) in a solution of 10 mM Tris-HCl containing 10 mM NaCl (pH 7.5). Due to the low yield, do not pre-quantify the CUT&RUN-enriched DNA. Rather, generate 25-fold dilutions of the adaptor, using a final working adaptor concentration of 0.6 µM.

11.1. Make a ligation master mix: 55 µL of ligase buffer (2x), 5 µL of T4 DNA ligase, and 5 µL of diluted Adaptor, with a total volume of 65 µL per reaction.

11.2. Add 65 µL of the master ligation mix to 50 µL of DNA from step 10.5.

11.3. Mix by quick vortexing, followed by quick spinning (500 x *g* for 10 s).

11.4. Incubate at 20 °C in a thermocycler (without a heated lid) for 15 min.

NOTE: Proceed immediately to the following step.

12. USER digestion

12.1. Add 3 µL of USER enzyme to each sample, vortex, and spin (500 x *g* for 10 s).

12.2. Incubate in thermal cycler at 37 °C for 15 min (heated lid set to 50 °C).

13. Polystyrene–magnetite bead clean-up following ligation reaction

NOTE: Allow polystyrene–magnetite beads to equilibrate at room temperature (~30 min). Vortex to homogenize the bead solution before using. Perform the following steps at room temperature.

13.1. Add 39 µL (0.33x) of polystyrene–magnetite bead solution to each well containing adaptor-ligated DNA.

13.2. Mix thoroughly by pipetting, and then incubate the samples at room temperature for 15 min to allow DNA to bind to the beads.

13.3. Place the samples on a 96-well magnetic rack and incubate for 5 min until the supernatant is clear.

13.4. Keep the plate on the magnetic rack and carefully remove and discard the supernatant without disturbing the beads.

13.5. Rinse the beads with 200 µL of 80% EtOH without disturbing the beads.

13.6. Incubate for 30 s on a 96-well magnetic rack to allow the solution to clear.

13.7. Remove and discard the supernatant without disturbing the beads.

13.8. Repeat steps 13.5–13.7 for a total of two washes.

13.9. Spin the plate briefly at 500 x *g* for 10 s, place the plate back on a 96-well magnetic rack, and remove residual EtOH without disturbing the beads.

13.10. Keep the plate on the magnetic rack and air-dry the samples for 2 min.

NOTE: Do not over-dry the beads.

13.11. Remove the plate from the magnetic rack and resuspend the beads in 28.5 µL of 10 mM Tris-HCl (pH 8).

CAUTION: Hydrochloric acid is very corrosive. Users should handle it with care in a chemical fume hood wearing goggles, gloves, and a lab coat.

13.12. Thoroughly resuspend beads by pipetting, taking care to not produce bubbles.

13.13. Incubate for 5 min at room temperature.

13.14. Place the plate on a 96-well magnetic rack and allow the solution to clear for 5 min.

13.15. Transfer 27.5 µL of supernatant to a new PCR plate and discard the beads.

14. Library enrichment

NOTE: Primers are diluted with the same solution as the adaptor. For this library build, use a final working primer concentration of 0.6 µM.

14.1. Add 5 µL of diluted indexed primer (see **Table of Materials**) to each sample.

NOTE: Each sample needs a different index to be identified during sequencing.

14.2. Prepare a PCR master mix: 10 µL of 5x high fidelity PCR buffer, 1.5 µL of 10 mM dNTPs, 5 µL of diluted Universal primer, 1 µL of 1 U hot start high fidelity polymerase, with a total master mix volume of 17.5 µL per sample.

14.3. Add 17.5 µL pf PCR mix to 32.5 µL of purified adaptor-ligated DNA (5 µL of indexed primer is included in this volume).

14.4. Mix the solution by pipetting.

14.5. Incubate in a thermocycler using the following reaction conditions with a maximum ramp rate of 3 °C/s: 98 °C for 45 s, 98 °C for 45 s, 60 °C for 10 s, repeat the second and third steps 21 times, 72 °C for 1 min, hold at 4 °C.

NOTE: The samples can be kept at 4 °C for short-term storage or -20 °C for long-term storage.

15. Polystyrene–magnetite bead clean-up

15.1. Add 60 µL (1.2x) of polystyrene–magnetite beads to each sample.

15.2. Resuspend the beads by pipetting and incubate for 15 min at room temperature.

15.3. Place the plate on a magnetic rack for 5 min until the solution is clear.

15.4. Discard the supernatant and rinse the beads with 200 µL of 80% EtOH without disturbing the beads.

15.5. Repeat the wash step for a total of two 80% EtOH washes.

15.6. Spin the plate at 500 x *g* for 10 s, place the plate on a 96-well magnetic rack, and allow the beads to bind for 5 min.

15.7. Pipette to remove excess EtOH without disturbing the beads and allow the beads to air-dry for 2 min.

NOTE: Do not over-dry the beads.

15.8. Resuspend the beads in 21 µL of nuclease-free water and incubate for 5 min at room temperature.

15.9. Place the plate on a magnetic rack and allow the solution to clear for 5 min.

15.10. Transfer 20 µL of the supernatant to a new plate.

NOTE: The experiment can be stopped here by storing the sample at -20 °C.

15.11. Quantify library concentrations with a fluorometer (see **Table of Materials**), using a 1x HS reagent.

15.12. If the concentration allows, run 30 ng of sample on 1.5% agarose gel with a low molecular weight ladder to visualize. Alternatively, visualize on a Fragment Analyzer or related instrument.

15.13. Sequence libraries on an Illumina platform to obtain ~50,000–100,000 uniquely mapped reads.

REPRESENTATIVE RESULTS:

Here, a detailed protocol is presented for single-cell protein profiling on chromatin using a 96-well manual format uliCUT&RUN. While results will vary based on the protein being profiled (due to protein abundance and antibody quality), cell type, and other contributing factors, anticipated results for this technique are discussed here. Cell quality (cell appearance and percent of viable cells) and single-cell sorting should be assessed prior to or at the time of live-cell sorting into the NE buffer. An example of ES cell colonies and cell sorting is shown in **Figure 2A,B**. Specifically, low-quality cells should not be used, and if the quality is an issue, care should be taken to follow

guidelines for the specific cell type. In addition, accurate cell sorting by the cell sorting instrument should be assessed in advance of experimentation. For example, test cells could be sorted and stained using Hoechst 33342 stain and counted to assure either 0 or 1 cell is found in each well. If single cells are not found, sorting conditions must be optimized. After library preparation, samples can be assessed on either an agarose gel (if the concentration permits for loading of 30 ng or more, as there is a lower limit to DNA visualization on an agarose gel) or a Fragment Analyzer (or similar device such as a Tapestation or Bioanalyzer) prior to sequencing and example results are shown in **Figure 2C,D**. Specifically, the expected size distribution is from ~150 to ~500 bp. In higher cell amounts, CUT&RUN performed on large proteins (such as histones) will have a right-sided size distribution, where the majority of DNA will be seen ~270 bp; however, this shoulder is typically not observed in single-cell experiments.

After sequencing, the quality of the sequencing reads should be assessed using FASTQC. The percent of uniquely mapping reads should be determined. Typically, 0.5%–10% uniquely mapping reads are observed in single-cell experiments. These mapping percentages are similar to other DNA-based single-cell techniques[30]. Next, the size distribution of the reads after mapping should be determined to assure the profile is similar to pre-sequencing (with the adaptor sequence no longer contributing to the read sizes).

After data quality has been assessed, protein occupancy can be visualized using various methods: single locus genome browser images can be visualized using UCSC genome browser or IGV (**Figure 3A**) and genome-wide occupancy patterns over specific genomic coordinates can be visualized using metaplots (**Figure 3B, bottom**), heatmaps (**Figure 3B, top**), or 1D heatmaps (**Figure 3C**). For more information on data analysis, refer to the study by Patty and Hainer[27]. Single-cell data from a diploid cell will result in up to four reads contributing to each locus (four reads if the cell was in mitosis), but more often one or two reads. Therefore, the data is binary, and a high background can be more easily mistaken for occupancy, relative to high cell experiments. Therefore, it is recommended to perform a parallel CUT&RUN experiment on a high cell number (5,000 to 100,000 cells), if possible, to acquire all the possible binding locations for the protein of interest. Then, single-cell data can be compared to possible binding locations. In the examples shown in **Figure 3**, single-cell CTCF uliCUT&RUN results are compared to high cell CTCF uliCUT&RUN (**Figure 3A**) or ChIP-seq (**Figure 3B,C**). As demonstrated previously, it is found that CTCF, SOX2, and NANOG single-cell uliCUT&RUN peaks largely represented stronger peaks from high cell ChIP-seq datasets[23].

FIGURE AND TABLE LEGENDS:

Figure 1: **Schematic of the uliCUT&RUN protocol**. Cells are harvested and sorted into a 96-well plate containing NE buffer. Individual nuclei are then bound to ConA-conjugated paramagnetic microspheres, and sequentially an antibody (targeting the protein of interest) and pA-MNase or pA/G-MNase are added. Protein-adjacent DNA is cleaved *via* MNase, and DNA is then purified for use in library preparation. This figure was created with Biorender.com.

Figure 2: **Example results from cell sorting and quality control of uliCUT&RUN libraries.** (**A**) Image of high-quality murine embryonic stem (ES) cells. Scale bar: 200 μm. (**B**) Output from

single-cell sorting after adding 7AAD to harvested ES cells and sorting on a FACS instrument. (**C**) Ethidium bromide-stained agarose gel depicting completed uliCUT&RUN libraries. Lane 1 is a low molecular weight ladder and lanes 2–21 are examples of successful individual single-cell uliCUT&RUN libraries prior to sequencing. (**D**) Ethidium bromide-stained agarose gel depicting sub-optimal and optimal completed uliCUT&RUN libraries. Lane 1 is a low molecular weight ladder, lane 2 is a sub-optimal library due to inefficient MNase digestion, and lane 3 is a successful library with appropriate digestion. (**E**) Fragment analyzer distribution of one single cell uliCUT&RUN library prior to sequencing.

Figure 3: **Example of expected results for single ES cell uliCUT&RUN data.** (**A**) Browser track of high cell number (5,000 cells) CTCF or negative control (No Antibody, No Ab) uliCUT&RUN (top two tracks) and single-cell CTCF uliCUT&RUN. The image is reproduced, with permission, from Patty and Hainer[27]. (**B**) Heatmaps (top) and metaplots (bottom) of single-cell CTCF or negative control (No Ab) uliCUT&RUN over previously published CTCF ChIP-seq sites (GSE11724). The image is reproduced, with permission, from Hainer et al.[23]. (**C**) 1D heatmaps of single-cell CTCF or negative control (No Ab) uliCUT&RUN over previously published CTCF ChIP-seq sites (GSE11724). The image is reproduced, with permission, from Hainer et al.[23].

Table 1: **Composition of various buffers used in this protocol.** Volume of stock solution required is listed with final concentration written in parentheses.

DISCUSSION:

CUT&RUN is an effective protocol to determine protein localization on chromatin. It has many advantages relative to other protocols, including: 1) high signal-to-noise ratio, 2) rapid protocol, and 3) low sequencing read coverage required thus leading to cost savings. The use of Protein A- or Protein A/G-MNase enables CUT&RUN to be applied with any available antibody; therefore, it has the potential to quickly and easily profile many proteins. However, adaptation to single-cell for any protein profiling on chromatin has been difficult, especially when compared to single-cell transcriptomics (i.e., scRNA-seq), due to the low copy number of DNA relative to RNA (two to four copies of DNA versus possible thousands of RNA copies).

In the protocol detailed above, several critical steps should be considered. First, the appropriate sorting of cells is dependent on the cell (or tissue) type, and care should be taken for accurate sorting. It is recommended to test how effective single-cell sorting is with the instrument in advance of any experiment. Second, effective antibody choice is essential. Before proceeding to a single-cell application, it is recommended to test the antibody in a high cell number experiment (as well as other standard antibody tests, such as confirming specificity using western blot after knockdown, titration of the antibody in CUT&RUN experiments, etc.). Third, use a negative control, such as IgG or no primary antibody, because an appropriate comparison to the experimental samples is essential for the interpretation of the data quality and biological results. When comparing single-cell experimental results to a high cell number dataset, the negative control single-cell experiments should have less read coverage over those binding sites identified in the high cell experiment and rather have a random distribution of reads across the genome (with a bias for open regions of chromatin). Fourth, care should be taken when adding and

activating the Protein A- or Protein A/G-MNase so as not to over digest the chromatin: do not overheat the samples with your hands, maintain the samples in an ice/water-bath temperature (0 °C), and chelate the reaction at the appropriate time. Fifth, care should be taken throughout the uliCUT&RUN experiment and library preparation, due to low material. For example, extended incubation on the magnetic rack during bead binding to assure the supernatant has cleared and taking care not to disturb the beads when removing the supernatant are essential for sufficient yield. Sixth, depending on the questions being addressed, the number of single-cell experiments being performed is an important consideration. Some of the single-cell experiments will fail (as with all low-input experiments), and, therefore, the number of positive experimental results required for appropriate interpretation should be considered in advance of beginning the experiment. The number of cells to include in the experiment is dependent on the amount of experimental data required by the investigator and the quality of the antibody. Finally, note that equivalent results could be achieved with reduced volumes of many aspects of this protocol. Steps, where the volume was reduced by 50%, include the volume of NE buffer, wash buffer, primary antibody mixture (including the amount of primary antibody), pA-MNase mixture (including the amount of pA-MNase), and all steps in the library preparation.

Based on the complicated nature of factor profiling on chromatin, there are many potential sources of issues and places where troubleshooting may become necessary. While there may be many steps where issues can arise, three major issues have been observed here: 1) low DNA yield for input into library build; 2) high background signal in experimental samples, and 3) low yield after library build. If there is insufficient DNA for library preparation and sequencing (point 1), note the following troubleshooting advice: a) there may have been incomplete membrane lysis and therefore the lysis time with NE buffer can be increased; b) there may have been inefficient binding of the nucleus to the ConA-conjugated paramagnetic microspheres and this could be remedied through appropriate mixing upon addition of these beads; c) there may have been too little antibody added, and therefore it is recommended to perform a titration of antibody to identify the most effective amount; d) incubation times with either the primary antibody or the Protein A- or Protein A/G-MNase are either too short (i.e., not enough time to permit binding) or too long (these are native, uncrosslinked samples), and could be optimized; e) the interaction of the target protein with chromatin could be too transient to capture in native conditions and therefore crosslinking CUT&RUN could be performed[28]. While single-cell datasets will yield high background, there may be excessively high background where the signal is hard to interpret from the background (point 2). For this issue, note the following advice: a) the blocking step with EDTA to prevent pre-emptive MNase digestion could be increased or optimized; b) if there is excessive cutting, it could be due to having too much Protein A- or Protein A/G-MNase, and therefore a titration of appropriate amounts can be performed; c) over digestion by MNase could result in the high background, and therefore the appropriate mixing of calcium chloride upon addition and optimization for MNase digestion time should be assessed. Finally, efficient uliCUT&RUN-enriched DNA may have been recovered, but a low amount of library may be recovered (point 3). For this issue, the following are recommended: a) appropriate handling and use of polystyrene–magnetite beads to assure the correct purification and no DNA loss; specifically, it is recommended to have 15 min incubations and a minimum of 5 min to magnetize beads to prevent loss; b) under-amplification of the library at the PCR stage would result in low yield and

therefore the appropriate cycles should be determined using qPCR (as previously established for ATAC-seq libraries[29]).

As with all technologies, there are limitations to uliCUT&RUN that should be considered before initiating any experimentation. First, these experiments are designed and optimized for native conditions, and therefore if a protein is only transiently interacting with chromatin, a crosslinking approach may be necessary to ensure recovery of the interaction. Second, as with all antibody-based techniques, the quality of the antibody is important. Care should be taken to assure the quality and consistency of the antibody in advance of undertaking any experiments. Third, MNase background cutting can occur and, while there is a consistently lower background signal in CUT&RUN relative to other experiments, the background signal can be high in single-cell experiments and therefore appropriate controls and analyses should be performed. While the binary nature of single-cell profiling data limits the visualization, more advanced computational genomic technologies, such as dimensional reduction and others can be performed (as previously described[27]). Finally, while single-cell profiling has been expanded here to 96-well format, this is low throughput relative to other single-cell technologies that utilize 10xGenomics or other formats.

Tethering-based profiling technologies such as ChEC-seq[20], CUT&Tag[24], CUT&RUN[21], and uliCUT&RUN[23], can determine factor localization on chromatin with a faster experimental timeline, lower background, and lower cost than traditional profiling technologies, such as ChIP-seq. Therefore, these are very exciting technologies for application to precious samples such as patient samples or early developmental samples. Furthermore, application to single cells can provide complementary studies performed using other single-cell experiments such as scRNA-seq and scATAC-seq[30]. As described using these more broadly used single-cell technologies, novel insights can be gained relative to bulk cell experiments. Single-cell protein profiling on chromatin is anticipated to become more regularly used as the technologies continue to improve and permit more parallelization.

ACKNOWLEDGEMENTS:
We thank members of the Hainer Lab for reading and comments on an earlier version of this manuscript. This project used the NextSeq500 available at the University of Pittsburgh Health Sciences Sequencing Core at UPMC Children's Hospital of Pittsburgh for sequencing with special thanks to its director, William MacDonald. This research was supported in part by the University of Pittsburgh Center for Research Computing through the computer resources provided. This work was supported by the National Institutes of Health Grant Number R35GM133732 (to S.J.H.).

DISCLOSURES:
The authors declare no competing interests related to this project.

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Figure 1

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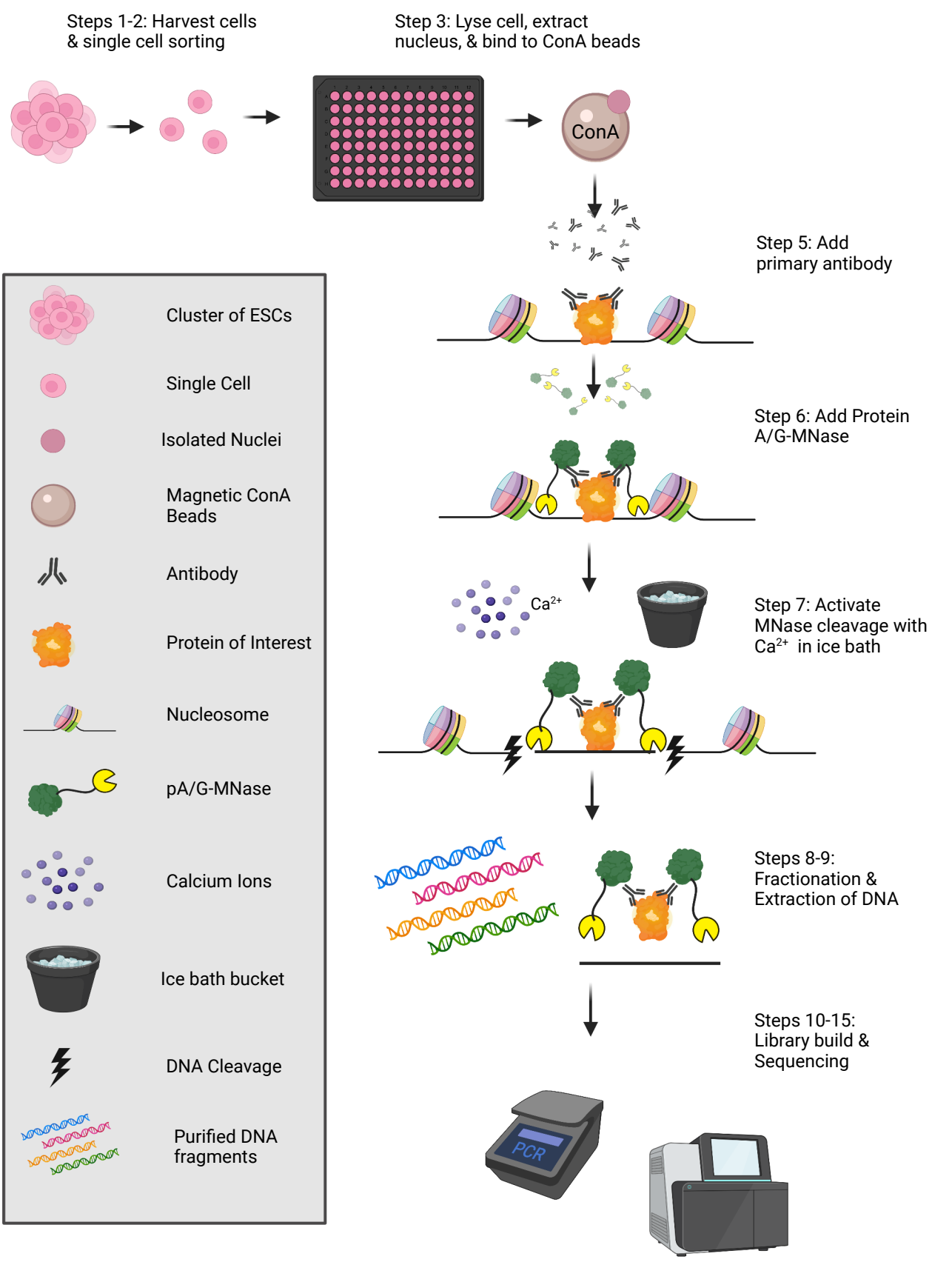

Figure 2

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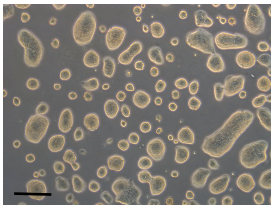
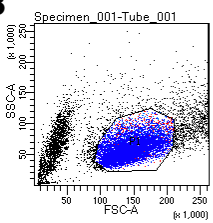
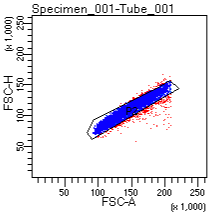
A

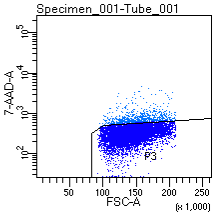


Cell Number	1.2x10^6 cells/mL
% Cell Viability	98%

B







Tube: Tube_001

Population	#Events	%Parent	%Total
All Events	13,566	####	100.0
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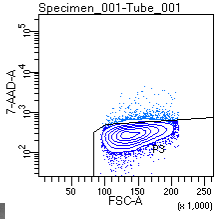
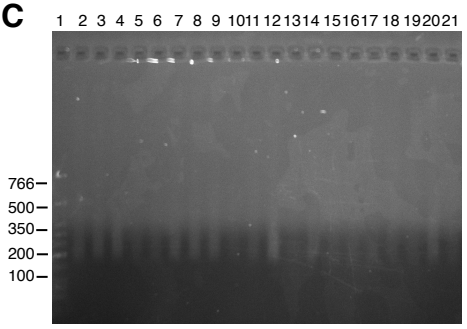
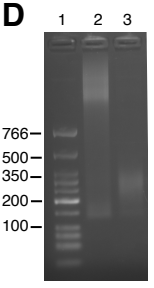
P2	7,557	94.0	55.7
P3	6,848	90.6	50.5



C



D



E

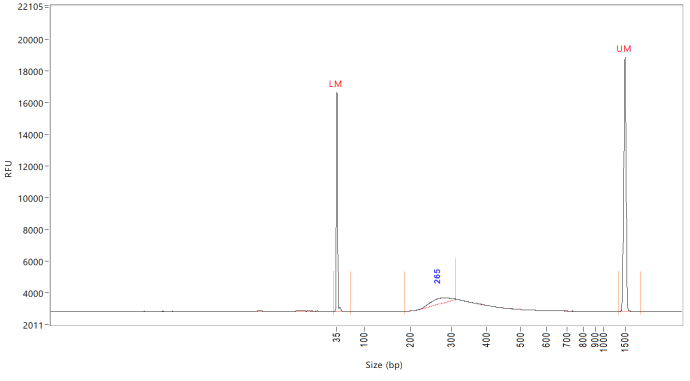


Figure 3

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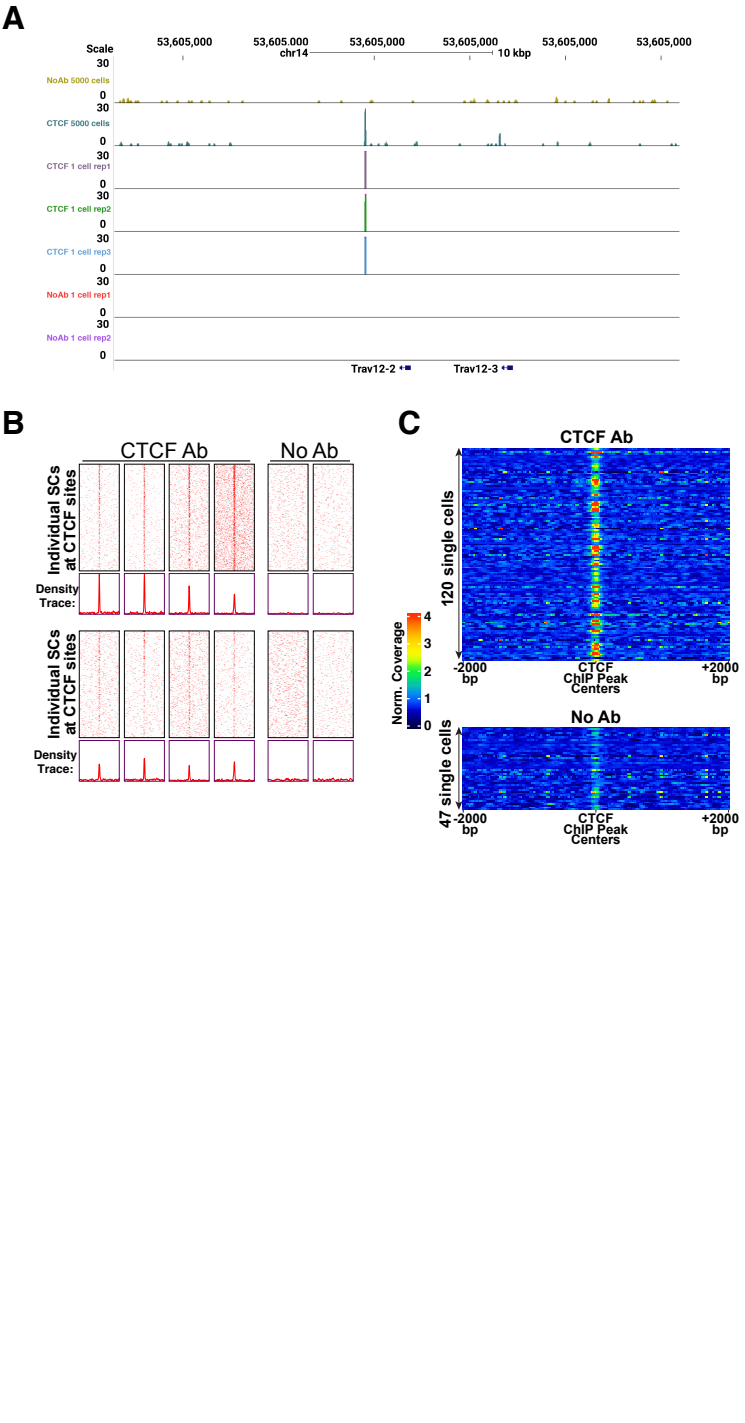

Table

Click here to access/download;Table;Solution Composition Table_SH.xlsx

Volume of buffers needed is dependent on number of s
All buffers can be stored at 4 °C for up to one year, unle

Nuclear Extraction Buffer (NE; 50 mL):

Binding Buffer (20 mL):

Wash Buffer (100 mL):

Blocking Buffer (5 mL):

2XRSTOP+ Buffer (15 mL):

PBS + 1% FBS (5 mL):

amples.
ss otherwise noted.

1 mL of 1M HEPES-KOH pH 7.9 (20 mM)
500 µL of 1 M KCl (10 mM)
12.5 µL of 2 M Spermidine (0.5 mM)
500 µL of 10% Triton X-100 (0.1%)
12.5 mL of 80% Glycerol (20%)
Volume up to 50 mL with autoclaved double distilled water
Store at 4 °C up to 1 year
Add protease inhibitors fresh upon each use of buffer
Caution: KOH and Triton X-100 are both hazardous. Use in a chemical fume hood wearing gloves, googles

400 µL of 1 M HEPES-KOH pH 7.9 (20 mM)
200 µL of 1 M KCl (10 mM)
20 µL of 1 M $CaCl_2$ (1 mM)
20 µL of 1 M $MnCl_2$ (1 mM)
Volume up to 20 mL with autoclaved double distilled water
Store at 4 °C up to 1 year

2 mL of 1 M HEPES-KOH pH 7.5 (20 mM)
3 mL of 5 M NaCl (150 mM)
25 µL of 2 M Spermidine (0.5 mM)
333 µL of 30% BSA (0.1%)
Volume up to 100 mL with autoclaved double distilled water
Store at 4 °C up to 1 year
Add protease inhibitors fresh upon each use of buffer

Made on the same day experiment is being performed
5 mL of pre-made Wash Buffer with protease inhibitors included
20 µL of 0.5M EDTA (2 mM)

600 µL of 5 M NaCl (200 mM)
600 µL of 0.5 M EDTA (20 mM)
300 µL of 0.2 M EGTA (4 mM)
Volume up to 15 mL with autoclaved double distilled water
Store at 4 °C up to 1 year
Add fresh per 1 mL of STOP buffer:
5 µL of 10 mg/mL RNase A (50 µg/mL)
2 µL of 20 mg/mL Glycogen (40 µg/mL)
1 µL of 10 ng/mL heterologous spike-in DNA (10 pg/mL)

5 mL of PBS
1% FBS
Mix well and filter sterilize
Store at 4 °C up to 1 month

and a lab coat for safety.

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Table of Materials
JoVE_Materialsv4.xlsx

Click here to access/download;Rebuttal Letter;Reviewer Responses.docx

We thank the Reviewers for their comments. Our responses are in Red.

Reviewer #1:

Major Concerns:

The authors should specify the improvement of this protocol to the one they published on Benjamin J. Patty & Sarah J. Hainer et al. Nature protocols and Sarah J. Hainer, Thomas G. Fazzio et la. Currentprotocols. Otherwise, I don't see the point to publish the same thing again and again.

We appreciate the Reviewers comment. When invited to write this article, we noted to the editors that we had previously published those above-mentioned articles. The editors still wanted us to contribute and noted that JOVE finds novelty in the accompanying video that is generated. Therefore, we obliged and put together this manuscript. Of note, we have updated our protocol to include 96-well format for single cell application, which is uniquely discussed in this protocol.

Reviewer #2:

Manuscript Summary:

Lardo and Hainer., "Single cell factor localization on chromatin using uliCUT&RUN"

In this manuscript, Lardo and Hainer detail their protocol for single cell CUT&RUN assays in a plate (96well) format. CUT&RUN and other similar methods have become the predominant method for characterizing protein-DNA interactions in the context of the nucleus and methods that can provide single cell resolution are increasingly important. Critically, single cell experiments typically require high numbers of cells so optimized methods for high-throughput work are valuable.

The methods paper clearly describes most of the steps in the protocols used by the laboratory. However, the rationale and requirements for many critical steps could use additional detail or discussion, particularly to highlight potential pitfalls. Including critical lessons learned as part of establishing these methods would facilitate other researchers in make modifications, such as automation, that would further take advantage of the laboratory's work. A subset of these are highlighted below but the authors should review their manuscript for additional locations where specific reagent choices were critical for the success of their method.

Thank you for these suggestions. We have gone through all your suggestions and added discussion of these items as well as a few other aspects that, upon re-reading, we identified as requiring more discussion.

Major Concerns:

The selection of the cell sorter (and it's set up) is likely valuable. Many of the details of the protocol cited here likely directly tied to the instrumentation and its limitations. These would include the number of cells to start with (2.7), target volume of lysis buffer (3.1), etc. Is 100uL used because that is the lowest volume that would successfully give high fractions of cells loaded into the well? What is the fraction of wells with cells you expect?

Thank you for this discussion. The instrument and cell input go hand in hand. We have used FACS Aria II instruments (included in our Materials Table), and the recommended input is 1 million cells (in 1mL), which is why that is described here. We recovered 80-95% of wells with single cells, and the remainder with 0 cells. We have now recommended that readers test the sorting efficiency in advance (Lines 157-158; 380-381; 454-456). We have always

initially lysed the cells in 100µL; partly to make sure it is a high volume and we can capture the cell, and also out of habit. This is not a volume we have altered in our protocol to this point.

Along the same lines, the choice of plate you are using is likely critical as well - presumably favoring low bind v-bottom plates? More explicit inclusion of details where an uninformed user could scuttle their experiment would be beneficial throughout the manuscript.
Thank you for this recommendation, we have included the plates in the materials table.

With the cell spin down (3.5) how much loss is expected? Are there cell types tested where the nucleus is not reliably brought to the bottom of the well.
With this low-speed spin, we do not observe lysis of murine ES cells. We have performed CUT&RUN on a few other cell types, and in bulk cells, we do not observe lysis; however we have not performed single cell CUT&RUN in other cell types.

In all cases, the volumes of reagents used are enormous given the very tiny amount of sample. Why were such large volumes chosen? Have the authors explored the impact of lowering these volumes? Many reactions would be expected to work better in a lower volume situation, but what is the impact on the MNase reaction? Is that less well controlled? A further discussion on these points would be helpful.
Thank you for pointing this out. We have been testing reductions in some of the reagents recently in our lab and have obtained robust results for some but not all reagents. We are including here what we are 100% confident in working; but have now added a discussion about how some of these volumes could be decreased based on your suggestion (Lines 481-485).

For the adaptor ligation/library construction, how different is this method from kitted protocols such as NEB UltraII where miniaturization of the reactions has been demonstrated with robust results?
The library construction method we use follows a similar protocol to the Illumina kit, but by sourcing the reagents separately we can save money. However, we have also tested using the kit for library preparation and it works as well.

In analysis, if only 0.5%-10% of the reads are alignable, what are the other 90+% of the reads? Are they all just the spike in? If so, to get 100k aligned reads, you are needing 1m-20m reads per cell?
Yes, as is common in all single cell DNA-based datasets (including scATAC-seq and scDNase-seq), there are small % mapped reads in these datasets (we have noted this on Line 394). The majority of the reads are spike in and random bacterial reads. In our original 2019 paper (Hainer et al Cell 2019) where we originally described this method, we compare CUT&RUN data to scATAC-seq data and actually find a higher percent mapping. And yes, you are correct that given the low mapping, the amount of raw sequencing reads required is high.

The negative control in all ChIP experiments is always challenging to interpret since so few molecules would be expected to be released. What results are you observing in failed experiments where the negative control is proving valuable as a control for the experiment? What peaks or data points are you able to exclude?

In single cell experiments, the negative control is also hard to interpret. However, if one has high cell CUT&RUN (or ChIP-seq) data to compare the single cell datasets to (as we suggest in this article), the negative control would have less reads contributing to the locations that high cell datasets have identified as a binding site for the protein of interest, but rather have a random distribution of reads across the majority of the genome (with a bias for open regions of chromatin). In single cell experiments, the negative control can be used as an “input” type, where reads could be subtracted. However, given the sparse nature of single cell datasets, we do not recommend this, but rather use the negative control as a comparison of reads over the locations of interest (as defined by high cell datasets). (Lines 460-466)

The authors indicate that, because of the expected failure rate, additional samples should be done (line 460). How do you decide on the number of cells you want to process to compensate for an expected failure rate?
This is complicated, as it is largely dependent on the factor being profiled. For robust antibodies, the number of cells is likely lower than for a weak antibody. In addition, it is possibly cell type dependent. (Lines 476-480)

The authors indicate a number of possible failure modes but were these observed or simply hypothetical? For example, incomplete lysis of a single cell would seem to be quite unlikely.
These are observed failures in CUT&RUN experiments we have performed, although this includes both high cell and single cell experiments. We wanted to be thorough in what we included, and therefore included everything we have experienced.

Minor Concerns:
Typo in line 110: presumably this should be pg/uL?
It is actually pg/mL, however, thank you for identifying that we did not detail carefully enough the volume added here (15 µL).

For 96 well work, the choice of the magnetic rack can be a critical step and should be included.
Thank you for this suggestion! We have included the magnetic rack we prefer to use in the Materials Table.

The baseline for the trace in figure 2D is clearly set up incorrectly.
Thank you for this comment. This is an example of a library pre-sequencing, and therefore adapters are still present, so the size distribution is ~200-400bp. This trace is reflective of a typical distribution before adapters are removed for the analysis.

Reviewer #3:
Manuscript Summary:
It is a well-written manuscript although the method has been described and published on Cell (2019) and Nature Protocols (2021). A few questions/ comments are listed as minor concerns.

Minor Concerns:
Line 117: Please briefly describe Bio-Mag ConA beads.

Thank you for this comment; we have included a “NOTE” describing these beads (Line 119-120)

Line 145: Please briefly describe the purpose of adding 7-AAD.
Thank you for this comment; we have included a “NOTE” describing the purpose of 7-AAD (Line 149-150).

Line 216: Please briefly describe the purpose of adding CaCl2.
Thank you for this comment; we have included a “NOTE” regarding the purpose of $CaCl_2$ (Line 225-226).

Figure 2C: The DNA visualization on agarose gel is not clear enough.
Thank you for this comment; the gel is hard to see. We have provided a higher quality image for the reads to visualize more easily (Figure 2C)

Figure 3: The figure has been published in Reference 23. Authors may consider using another set of uliCUT&RUN data as example, either on different cell or using different antibody to convince readers this protocol's reliability and wide application.
Thank you for the comment; JOVE allows previously published work to be included and since we do not want to “scoop” our own work and publish work for a primary dataset, we have chosen to use previously published datasets.

Line197: add the example of optimal MNase digestion vs. sub-optimal MNase digestion will be beneficial for the readers.
Thank you for the suggestion, we have included this example as Figure 2D.

Line233: It should be 20 mg/mL instead of 20 mg/mm
Thank you for catching this typo. It has been corrected.

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Are you the author of this Elsevier article?	Yes
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Title of new article	Single cell factor localization on chromatin using uliCUT&RUN
Lead author	Sarah Hainer
Title of targeted journal	JOVE
Publisher	MyJove Corp.
Expected publication date	Mar 2022
Portions	Figure 4A, Figure 4B
Requestor Location	Sarah Hainer 4249 Fifth Ave 527 Langley Hall PITTSBURGH, PA 15260 United States Attn: University of Pittsburgh
Publisher Tax ID	98-0397604

Total 0.00 USD

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