ChIC (Chromatin Immuno-Cleavage) Kits

ChIC (Chromatin Immuno-Cleavage) Kit (pA-MN, Catalog No. CHR100)
ChIC (Chromatin Immuno-Cleavage) Kit (pG-MN, Catalog No. CHR101)
12 reactions

ChIC (Chromatin Immuno-Cleavage) Kit (pA-MN/pG-MN, Catalog No. CHR102)
24 reactions

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Not for use in diagnostic procedures

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Introduction

DNA in eukaryotic cells is associated with a plethora of structural, enzymatic, and regulatory proteins which interact with regional DNA sequences and affect genomic functions via packaging as chromatin. These dynamic interactions influence the expression and cellular utilization of each gene locus, whether coding or non-coding. Additionally, such proteins and non-coding RNAs influence genomic DNA's ability to replicate and repair itself in a cell cycle dependent manner. Thus, it is crucial to elucidate these DNA-protein interactions to decipher the nuclear mechanisms underlying a wide variety of biological processes such as development, differentiation, and cellular response to environmental changes.

Over the last three decades, chromatin immunoprecipitation (ChIP) has been used to interrogate association of proteins with genomic DNA sequences. In the process of ChIP, cells are cross-linked with formaldehyde, chromatin is fragmented and solubilized, and immunoprecipitation is applied to solubilized chromatin. Although the readout methods for ChIP have evolved over years from endpoint PCR to high-throughput sequencing, the fundamentals of ChIP have remained unchanged. Issues remain with high background that limits sensitivity, high amounts of cellular input, and artifacts resulting from cross-linking and solubilization.

From the Laemmli lab, Schmid et al. previously reported an alternative strategy to detect binding sites of transcription factors in the genome by targeting micrococcal nuclease (MNase) conjugated with protein A (pA-MN) through a specific antibody, termed chromatin immune-cleavage (ChIC)[1]. Furthermore, Skene et al. in the Henikoff lab combined ChIC with sequencing to detect genome-wide transcription factor binding sites and histone modifications on native chromatin and termed Cleavage under targets and release using nuclease (CUT&RUN)[2].

In the ChIC/CUT&RUN methods, only the targeted fragments are released into solution and the majority of DNA is left behind, leading to an exceptionally low background level. As a result, ChIC/CUT&RUN provides high-quality results using only 100 cells for a histone modification analysis and 1,000 cells for a transcription factor binding site analysis. In contrast to ChIP, ChIC/CUT&RUN is free of solubility issues and DNA accessibility artifacts from cross-linking solubilization. ChIC/CUT&RUN outperforms ChIP in resolution, signal-to-noise ratio, and depth of sequencing required.

Recently, the ChIC/CUT&RUN technique was further evolved to be applied to low cell numbers or single-cell assays, entitled Ultra-low input CUT&RUN (uliCUT&RUN)[3] and single-cell ChIC (scChIC-seq)[4].

The MilliporeSigma ChIC (Chromatin Immuno-Cleavage) kits allow researchers to perform ChIC/CUT&RUN experiments using antibodies directed against proteins in nuclei. The kits supply all key reagents and buffers to prepare ChIC/CUT&RUN samples before the DNA sequencing library preparation step. There are three kit configurations namely with protein A-micrococcal nuclease (pA-MN, Cat. No. CHR100), protein G-micrococcal nuclease (pG-MN, Cat. No. CHR101) and pA-MN/pG-MN fusion protein blend (Cat. No. CHR102). pA-MN has higher affinity to most antibodies including rabbit IgG, whereas pG-MN is more suitable for antibodies that have low affinity to protein A for example mouse IgG1. pA-MN/pG-MN blend allows for either choice. MilliporeSigma also supply DNA

extraction kit for ChIC (12 rxns, Cat. No. CHR104), and 2% digitonin solution (Cat. No. CHR103) as accessory products for ChIC/CUT&RUN assays.

Overview of the ChIC/CUT&RUN Method

To perform ChIC/CUT&RUN, cells are harvested and bound to concanavalin A–coated magnetic beads. Cell membranes are permeabilized with digitonin to allow the specific antibody to find its target. Following antibody incubation, beads are briefly washed and incubated with pA-MN. Cells are chilled to 0 °C, and digestion begins with addition of incubation buffer with Ca^{2+}. Reactions are stopped by chelation and the DNA fragments released into solution by cleavage are extracted from the supernatant.

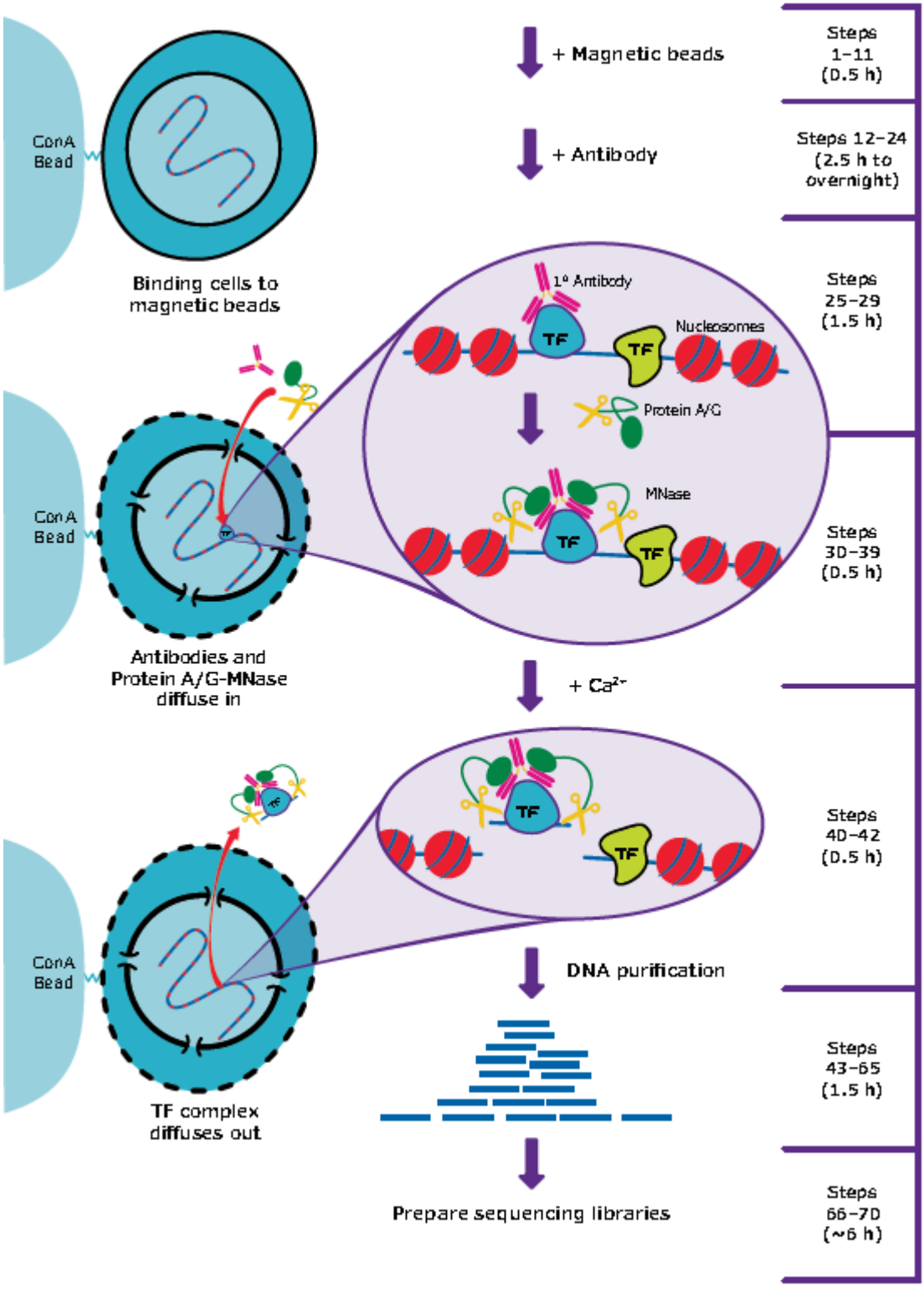

Figure 1. The ChIC/CUT&RUN Process

Materials Provided (Kit Configurations)

The ChIC kit provides sufficient reagents for 12 (CHR100 and CHR101) or 24 (CHR102) ChIC/CUT&RUN reactions. Please refer to the table below for details on kit components.

CHR100-1, CHR101-1, CHR102-1 (ChIC Component box 1, Store at 2°C to 8°C)		
Component	**Part #**	**Quantity**
Concanavalin A-coated magnetic beads	CS226334	120 µL (CHR100, CHR101)
	CS226313	240 µL (CHR102)
Binding Buffer	CS226311	28.5 mL
Wash Buffer	CS226309	200 mL
Spermidine	CS226308	1g
0.5M EDTA	CS203175	0.5 mL
Rinse Buffer	CS226306	24 mL
Incubation buffer	CS226352	4.8 mL
Stop Solution	CS226346	4.8 mL

** DNA Wash Buffer requires the addition of 100% Ethanol, see the Reagent Preparation section of the protocol for additional details

CHR100-2, CHR101-2, CHR102-2 (ChIC Component box 2, Store at -20°C)			
Component		**Part #**	**Quantity**
2% Digitonin **Contains DMSO		CS226345	1mL x 5 vials
RNase A (10 mg/mL)		20-297	100 µL
Spike-in DNA (10 ng/mL)		CS226342	0.1 ng (100 pg)
Precipitate Enhancer (5 mg/mL)		CS203208	100 µL
Proteinase K Solution, 600mAU/mL, 0.2mL (20 mg/mL)		CS207286	200 µL
Protease Inhibitor Cocktail III, Animal Free **Contains DMSO		535140-1ML	1.0 mL
Rabbit IgG Purified (1mg/mL)		PP64B	125 µg
Anti-H3K27me3 rabbit monoclonal antibody		CS226331	10 µL
Micrococcal nuclease fusion protein	Protein A–micrococcal nuclease (pA-MN) fusion protein (300 µg/mL, CHR100 only)	CS226337	2.8 µg
	Protein G–micrococcal nuclease (pG-MN) fusion protein (300 µg/mL, CHR101 only)	CS226336	2.8 µg
	Protein A-micrococcal nuclease and protein G-micrococcal nuclease fusion proteins blend (pA-MN/pG-MN) (300 µg/mL, CHR102 only)	CS226335	2.8 µg

** 2% Digitonin and Protease Inhibitor Cocktail III contains DMSO. Handle those solutions with caution, as DMSO is known to facilitate the entry of organic molecules into tissues. Avoid contact with skin.

Materials Required but Not Supplied

Reagents

- Cells stimulated or treated as needed for the experimental system
- Antibodies of interest
- Secondary antibody (Optional) rabbit α-mouse (MilliporeSigma Cat # 06-371)
- Nuclease Free Water (MilliporeSigma Cat # W4502)

Reagents for DNA extraction by columns

- DNA extraction kit for ChIC (12 rxns, MilliporeSigma, Cat. No. CHR104), or similar DNA purification columns

Reagents for DNA extraction by phenol chloroform

- Phase Lock Gel® – Heavy (Quantabio, Cat. # 2302830)
- Chloroform (EMD Millipore, Cat. #3150)
- 100% Ethanol (molecular biology grade)
- Phenol: Chloroform: Isoamyl alcohol, 25:24:1, TE Buffered Saturated pH 8.0 (EMD Millipore Cat. # 6805)
- 10% SDS solution
- 1 mM Tris-HCl, pH 8.0 and 0.1mM EDTA buffer

Reagents for DNA evaluation

- Qubit® dsDNA HS kit (Life Tech, Cat. # Q3851)
- Agilent High Sensitivity DNA Kit (Agilent, Cat. # 5067-4626)

Reagents for Seq Library Construction

- DNA sequence library preparation kits and barcoded adapters

Equipment

- Magnetic Separation Rack
  - PureProteome™ Magnetic Stand, (EMD Millipore Cat. # LSKMAGS08)
- Vortex mixer
- Centrifuge for cell culture
- Microcentrifuge
- Thermomixer® (60°C capable)
- Variable temperature water bath or incubator
- Rotating microtube mixer or nutator
- Timer
- Variable volume (5-1000 µL) pipettes
- Nuclease-free filter pipette tips
- Bioanalyzer®
- Qubit Fluorometer
- Nuclease-free Microcentrifuge tubes, 1.5 mL, 2.0 mL
- Conical tubes (15 mL and 50 mL)

Warnings and Precautions

- Wear hand and eye protection and follow good laboratory practices when preparing and handling reagents and samples.
- 2% Digitonin and Protease Inhibitor Cocktail III contains DMSO. Handle those solutions with caution as DMSO is known to facilitate the entry of organic molecules into tissues. Avoid contact with skin.

Storage and Stability

Store ChIC component box 1 at 2°C-8ºC and ChIC component box 2 at -20°C; performance guaranteed for 6 months from date of receipt when reagents are stored properly.

Important Notes Before Starting

Please read through the entire protocol and carefully plan your work before starting. The ChIC/CUT&RUN method requires multiple steps and can be done under a day or over multiple days. There are several stopping points to allow the method to be carried out over multiple days. The approximate time required for each step and potential stopping points are provided in the tables below.

Binding Cells to Beads, Permeabilization, and Bind Antibodies

Protocol Step	Time Required	Stopping Points and Protocol Notes
Binding cells to beads	~0.5 hours	Cells can be cryopreserved in 10% DMSO using a Mr. Frosty isopropyl alcohol chamber before binding
Permeabilize cells and bind primary antibodies	~2.5 hours to overnight	Antibody incubation can be done overnight at 4 °C.
Bind secondary antibody (Optional)	~15 minutes to 1.5 hours	Although protein A, protein G and protein A/G fused MNase are used in some cases a secondary antibody may still be required.

Bind MNase Fusion Proteins, Digestion, and Release

Protocol Step	Time Required	Stopping Points and Protocol Notes
Bind MNase fusion proteins	~1.5 hours	Continue to Chromatin Digestion and Release step
Chromatin Digestion and Release	~1 hour	Continue to DNA Extraction step

DNA Extraction, Sample Evaluation, and Library Preparation

Protocol Step	Time Required	Stopping Points and Protocol Notes
DNA Extraction	~1.5 hours	DNA can be Store at -20°C for up to 2 months
DNA sample Evaluation	~1.5 hours	Because of low amount and too small size, DNA may not to be detected by gel analysis or even by Bioanalyzer.
Library Preparation	~6 hours	Time required for Library Preparations are depending on the kits and systems

Cells

Since introduction of ChIC/CUT&RUN assay by Henikoff lab, the assay has been adopted by many research groups.[5-7] In addition to K562 cells, other cells, including: HUDEP-2 cells, primary stem/progenitor cells, and primary human T cells have successfully been used in this assay. The following ChIC/CUT&RUN protocol may be executed with up to 500,000 mammalian cells in 100 µL per sample however, optimum cellular numbers may still need to be determined empirically.

Digitonin

This protocol uses cells permeabilized by the non-ionic detergent digitonin, which has been successfully used in other in situ methods, including ATAC-seq. Digitonin partitions into membranes and extracts cholesterol, therefore Membranes that lack cholesterol are minimally impacted by digitonin. Nuclear envelopes are relatively devoid of cholesterol compared to plasma membranes and as such, treatment of cells with digitonin represents a robust method for permeabilizing cells without compromising nuclear integrity.

This kit provides 5 bottles (each bottle contains 1 mL) of 2% High Purity Digitonin solution in DMSO. The effectiveness of digitonin varies between cell types, so testing permeability of Trypan blue is recommended to determine the concentration (0.02-0.1%) to be used for a particular cell type Additional 2% Digitonin is available as a separate product. (MilliporeSigma, Cat. No. CHR103)

To test for digitonin effectiveness, mix 10 µl of Trypan blue with 10 µl of cells ~10 min after addition of varying concentrations of digitonin (0–0.1% (wt/vol)), apply ~20 µl to a hemocytometer and observe within 10 min. All or nearly all the cells should be blue for successful permeabilization. For example: Using K562 cells, ~1/4 of the cells were permeable by 0.0125% (wt/vol) digitonin and all cells were permeable by 0.025% (wt/vol) digitonin.

Antibodies

As is the case with ChIP, the success of ChIC/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 situ with ChIC/CUT&RUN, it would be expected that antibodies successfully tested for specificity by immunofluorescence (IF) would likely work with ChIC/CUT&RUN, with the exception that IF generally involves formaldehyde fixation.

Positive and negative controls

The amount of DNA recovered is sometimes very low therefore, the DNA cannot be detected with analysis even by sensitive capillary electrophoresis or picogreen assays (Agilent Bioanalyzer or Qubit). This is especially the case if the assay was performed with low cell numbers or low abundant targets, including many transcription factors. 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.

To evaluate success of the procedure before sequence library prep, it is recommended to include positive control antibody (anti-H3K27me3, part No. CS226331) and a negative control antibody (rabbit IgG purified, part No. PP64B) with 200,000 to 500,000 cells. If the number of cells of interest

are limited, you may run the positive control antibody (H3K27me3) on a different cell culture sample (e.g. K563 cells) in parallel. Since the epitope for anti-H3K27me3 is abundant, a nucleosomal DNA ladder can be observed by Bioanalyzer analysis. (Appendix A) If a nucleosome ladder is observed, you may assume the reactions run in parallel were at least executed correctly.

Do not include a no-antibody control, as the lack of tethering may allow any unbound pA-MN to digest any accessible DNA, resulting in a background of DNA-accessible sites.

Spike-in DNA (Optional)

Adding heterologous spike-in DNA to the Stop Solution can be useful for comparison of DNA yields between samples. The total number of mapped spike-in reads can then be used as a normalization factor, where the number of spike-in reads is inversely proportional to the DNA yield from the sample.

This kit includes 100 pg of Spike-in DNA from Yeast, which was fragmented to ~200 bp mean size. The amount of spike-in DNA should be changed based on the starting number of the cells. When starting with low cell numbers (i.e. 100 - 10,000 cells), 2 pg/mL(final) in the Stop Solution is recommended. For samples with high cell numbers (i.e. 10,000 -500,000 cells), 100 pg/mL (final) in the STOP Buffer is recommended to obtain enough number of reads.

Recently, The Henikoff lab introduced the method to use E. coli DNA that is carried-over from the production of the fusion protein to serve as a spike-in for sample calibrations.[8] If you choose this method, no additional heterologous spike-in needs to be included in the Stop Solution.

DNA extraction

MilliporeSigma supply 12 (Cat. No. CHR104) DNA purification column kit as separate product. The column purification was recommended for large protein-DNA complexes such as nucleosomes. For quantitative recovery of <80-bp fragments, the alternative extraction method is recommended.

Too large chromatin complex

In the standard ChIC/CUT&RUN protocol the cleaved chromatin complexes can diffuse out of the cells, thereby simple isolation of the cut DNA from the supernatant fraction can be processed with the undigested genome retained in the intact cells.

However, it is possible that a chromatin complex is too large to diffuse out or that protein-protein interactions retain the cleaved complex. In such cases, total DNA may be extracted after the digestion. By doing a very simple negative size selection using Solid Phase Reversible Immobilization beads (e.g. Agencourt AMPure XP beads) large genomic DNA can be removed prior to preparing sequencing libraries.

DNA sequencing library preparation

The DNA sequencing library for Illumina sequencing can be prepared with several different kits including the Hyper prep kits (KAPA Biosystem), True-Seq kits (Illumina), and NEBNext Ultra I DNA Library Kit (E7645). Successful protocols were developed by Henikoff's lab (dx.doi.org/10.17504/protocols.io.zcpf2vn) and Nan Liu in Stuart Orkin's lab (dx.doi.org/10.17504/protocols.io.wvgfe3w).

Detailed Protocol

If you are new to the ChIC/CUT&RUN method, please read and understand this entire protocol before starting. This version of the protocol contains important details and helpful tips to facilitate successful results. For advanced users, a summary protocol is presented starting on page 21.

- The same protocol can be used for up to 500,000 mammalian cells per sample

I. Binding cells to beads

- Multiple samples can be processed as bulk and divided at step 14. For example, 10 samples (total up to 5,000,000 mammalian cells) can be bulk processed in a 1.5 mL microcentrifuge tube.
- All steps prior to the addition of antibody are performed at room temperature to minimize stress on the cells. it is crucial that DNA breakage is minimized throughout the protocol, therefore avoid cavitation and vigorous vortexing.
- To evaluate the success of the procedure without requiring library preparation, include in parallel Anti-H3K27me3 rabbit monoclonal antibody (part # CS226331) as a positive-control antibody, and Rabbit IgG Purified (part # PP64B) as a negative-control antibody. Do not include a no-antibody control, as the lack of tethering may allow any unbound pA-MN to digest accessible DNA, resulting in a background of DNA-accessible sites.

1. Allow following parts to equilibrate to room temperature for 30 minutes.
    - Concanavalin A-coated magnetic beads (part # CS226333 or CS226314)
    - Binding buffer (part # CS226311)
    - Spermidine (part # CS226308) or 2M Spermidine aliquots
    - Protease Inhibitor Cocktail III, Animal Free (part # 535140-1ML)
    - Wash buffer (part # CS226309)
2. Activate Concanavalin A-coated magnetic beads.
    - 10 µL Concanavalin A-coated magnetic beads will be used for each final sample.
        a. Completely disperse and re-suspend Concanavalin A-coated magnetic beads (part # CS226333 or CS226314) by pipetting or end-over-end rotation. No clumps of beads should be visible.
        b. Transfer (10 µL x Sample number) of Concanavalin A-coated magnetic bead slurry to a 1.5 ml microcentrifuge tube.

	Each	X N
Concanavalin A-coated magnetic bead	10 µL	µL

c. Place the tube on the magnetic separator (e.g. Millipore Cat. # LSKMAGS08) for 30s to 2 minutes. Remove the liquid.

d. Add (100 µL x Sample number) Binding buffer (Part# CS226311) to the tube and mix the beads by gently pipetting to completely resuspend beads. Centrifuge the tube briefly to remove liquid from the cap and side of the tube and place it on the magnetic separator (e.g. Millipore Cat. # LSKMAGS08) for 30s to 2 minutes.

	Each	X N
Binding buffer	100 µL	µL

e. Remove the supernatant making sure not to aspirate any magnetic beads. Remove the tubes from the magnet.

f. Repeat step c and d for an additional wash.

g. Resuspend the beads in a volume of Binding buffer equal to the volume of beginning bead slurry (10 µL x Sample number).

	Each	X N
Binding buffer	10 µL	µL

3. Prepare Complete wash buffer.

a. Dissolve entire (1g) Spermidine (part # CS226308) in Nuclease-free water. (2M Spermidine)

- 1 g of Spermidine is around 1.1 mL of liquid at room temperature. Mix it with 2.3 mL of Nuclease free water and adjust the total volume to 3.4 mL.
- Store the solution in small aliquots at -20°C (up to one month). Avoid multiple freeze and thaw cycles.

b. Add 1.8 µL of 2M Spermidine and 36.5 µL of Protease Inhibitor Cocktail III, Animal Free (part # 535140-1ML) to 7.3 mL of Wash buffer (part # CS226309) for each sample.

Complete wash buffer	Each	X N
2M Spermidine	1.8 µL	µL
Protease Inhibitor Cocktail III	36.5 µL	µL
Wash buffer	7.3 mL	mL

4. Harvest fresh culture(s) at room temperature and count cells. (up to 500,000 mammalian cells per sample)
    - If necessary, cells can be cryopreserved in 10% DMSO using a Mr. Frosty isopropyl alcohol chamber. Flash freezing is not recommended since it can cause background DNA breakage that may impact final data quality.
5. Centrifuge at 600 x g for 3 min at room temperature and aspirate supernatant.
6. Resuspend the cell pellet in 1.0 mL room temperature complete wash buffer by gently pipetting and transfer the cell suspension to a 1.5 mL microcentrifuge tube.
7. Centrifuge the tube at 600 x g for 3 min at room temperature and aspirate supernatant.
8. Wash the cells twice with Complete wash buffer
    a. Resuspend the cell pellet in 1.0 mL room temperature Complete wash buffer by gently pipetting.
    b. Centrifuge 3 min 600 x g at room temperature and aspirate supernatant.
    c. Repeat steps a and b one more time.
        - Thorough washing removes free sugars and other molecules that can compete for binding to the Concanavalin A coated-beads, ensuring efficient binding and recovery of the cells of interest.
9. Resuspend the cell pellet in (100 μL x Sample number) of room temperature Complete wash buffer by gently pipetting.

	Each	X N
Complete wash buffer	100 μL	μL

10. While gently vortexing the cells at room temperature, add the entire activated Concanavalin A-coated magnetic bead slurry prepared at step 2.
11. Rotate the microcentrifuge tube for 5-10 min at room temperature and continue to Permeabilize cells and bind primary antibodies section.

II. Permeabilize cells and bind primary antibodies

12. Prepare Dig-wash buffer
    - The effectiveness of digitonin varies between cell types, therefore testing permeability of Trypan blue empirically is recommended to determine the concentration (0.02-0.1%) to be used for a cell type. Please see the Digitonin section at page 7 of this manual.
    - This kit supplies 5 bottles (each bottle contains 1 mL) of 2% Digitonin. Additional 2% Digitonin is available as a separate product. (Cat. No. CHR103)

a. Thaw required number of bottles of 2% Digitonin at room temperature or with 37˚C heat block.

b. For each sample Mix 42-210 µL 2% digitonin with 4.2 mL of complete wash buffer prepared in step 3 for a final concentration of digitonin between 0.02% and 0.1% (wt/vol).

Dig-wash buffer	Each	X N
2% Digitonin	42- 210 µL	µL
Complete wash buffer	4.2 mL	mL

- **Store this buffer on ice or at 4 °C** for up to 1 day and mix well before use.

13. Prepare Antibody buffer

    Add 0.4 µL 0.5 M EDTA (part # CS203175) with 100 µL of Dig-wash buffer for each sample and place on ice.

Antibody buffer	Each	X N
0.5M EDTA	0.4 µL	µL
Dig-wash buffer	100 µL	µL

14. Mix well the microcentrifuge tube from Step 11 of Section I by vigorous inversion to ensure the bead-bound cells are in a homogenous suspension and divide into 100µL aliquots in 1.5-mL microcentrifuge tubes, one for each final sample.

15. Place the microcentrifuge tubes on the magnetic separator for 30 s to 2 minutes. Remove the supernatant making sure not to aspirate any magnetic bead-bound cells. Remove the microcentrifuge tubes from the magnet.

16. Place each tube at a low angle on the vortex mixer set to low (~1100 rpm) and dispense 100 µL of the Antibody buffer prepared at step 13 along the side while gently vortexing to allow the solution to dislodge most or all bead-bound cells. Tap to dislodge the remaining bead-bound cells.

    - Permeabilizing the cells with digitonin and chelating divalent cations with EDTA serves to halt metabolic processes and prevent endogenous DNase activity. This helps to preserve the native chromatin state and reduce background noise in the final CUT&RUN libraries. Therefore, it is recommended to work quickly to get cells into Antibody Buffer.

17. Add the primary antibody to a final concentration of 1:100 or to the manufacturer's recommended concentration for immunofluorescence to each tube and mix well.

    - To evaluate success of the procedure without requiring sequencing, include in parallel a positive control antibody and a negative control antibody as described in the beginning of Section I.

18. Place the tubes on a nutator or a tube rotator at room temperature for ~2 hours or at 4 °C overnight.

    - When using more than 200K cells per 10 µL beads, cells may clump or stick to the side of the tube during incubation. First, attempt to resuspend stuck bead-bound cells by inverting the tube. If bead-bound cells remain attached to the side of the tube, pipette gently to push bead-bound cells back into solution.

III. Bind secondary antibody (Optional: If you don't use secondary antibodies, skip this section and proceed to section IV Bind MNase fusion protein.

- The binding efficiency of Protein A and Protein G to the primary antibody depends on host species and IgG isotype. For example, Protein A binds well to rabbit and guinea pig IgG but poorly to mouse IgG1 and goat IgG. Therefore, use of a secondary antibody, such as rabbit α-mouse (MilliporeSigma Cat# 06-371, not included in the kits) is recommended for these latter antibodies. Although pG-MN or pA-MN/pG-MN blend increases antibody compatibility relative to pA-Mn, in some cases a secondary antibody may still be necessary.

19. Remove the tubes from the nutator or tube rotator. Centrifuge the tubes briefly to remove liquid from the cap and side of the tubes and place them on the magnetic separator for 30s to 2 minutes.

    - After mixing with a nutator or a tube rotator, brief centrifuge (no more than 100 x g) before placing a tube on the magnet stand is recommended. It will minimize carry-over of antibody and pA-MN that could result in overall background cleavages during the digestion step.

20. Remove the supernatant making sure not to aspirate any magnetic bead-bound cells. Remove the tubes from the magnet.

21. Wash the bead-bound cells once with Dig-wash buffer.

    a. Add 1 mL Dig-wash buffer to the tube and mix the bead-bound cells by gently pipetting to completely resuspend bead-bound cells. Centrifuge the tube briefly to remove liquid from the cap and side of the tube and place it on the magnetic separator for 30 s to 2 minutes.

    b. Remove the supernatant making sure not to aspirate any magnetic bead-bound cells. Remove the tubes from the magnet.

22. Place each tube at a low angle on the vortex mixer set to low (~1100 rpm) and dispense 100 µL of the Dig-wash buffer per sample along the side while gently vortexing to allow the solution to dislodge most or all of the bead-bound cells. Tap to dislodge the remaining bead-bound cells.

23. Add secondary antibody to each tube to a final concentration of 1:100 or to the manufacturer's recommended concentration for immunofluorescence.

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

IV. Bind MNase fusion protein

25. Remove the tubes from the nutator or tube rotator. Centrifuge the tubes briefly to remove liquid from the cap and side of the tubes and place them on the magnetic separator for 30s to 2 minutes.
26. Remove the supernatant making sure not to aspirate any magnetic bead-bound cells. Remove the tubes from the magnet.
27. Wash the bead-bound cells once with Dig-wash buffer.
    a. Add 1 mL Dig-wash buffer to the tube and mix the bead-bound cells by gently pipetting to completely resuspend bead-bound cells. Centrifuge the tube briefly to remove liquid from the cap and side of the tube and place it on the magnetic separator for 30 s to 2 minutes.
    b. Remove the supernatant making sure not to aspirate any magnetic bead-bound cells. Remove the tubes from the magnet.
28. Place each tube at a low angle on the vortex mixer set to low (~1100 rpm) and dispense 100 µL of the pA-MN (part # CS226337), pG-MN (part # CS226336) or pA-MN/pG-MN blend (part # CS226335) at 700 ng/mL (e.g., 1:430 of a 300 µg/mL glycerol stock) in Dig-wash buffer, per sample, along the side while gently vortexing to allow the solution to dislodge most or all of the bead-bound cells. Tap to dislodge the remaining bead-bound cells.
29. Place the tubes on the nutator or tube rotator at 4 °C for ~1 hr.

V. Chromatin Digestion and Release

- The modified High Ca^{2+} / Low Salt ChIC/CUT&RUN digestion protocol prevents premature release of the nuclease-bound complex, thereby preventing premature diffusion of the MNase complex which has the potential to cause nonspecific cleavage and increase the background signal. This is ideal for targets that are prevalent at regions of active chromatin.

30. Thaw a bottle of 2% Digitonin (part # CS226345) at room temperature or with 37˚C heat block.
31. Prepare complete rinse buffer.

    Add 25 µL of 2% Digitonin (part # CS226345) and 0.25 µL of 2M Spermidine, prepared at step 3, to 1 mL of Rinse buffer (part # CS226306) for each sample and place on ice.

Complete rinse buffer	Each	X N
2% Digitonin	25 µL	µL
2M Spermidine	0.25 µL	µL
Rinse buffer	1 mL	mL

32. Prepare complete incubation buffer.

    Add 5 µL of 2% Digitonin (part # CS226345) to 200 µL of Incubation buffer (part # CS226352) for each sample and place on ice.

Complete incubation buffer	Each	X N
2% Digitonin	5 µL	µL
Incubation buffer	200 µL	µL

33. Prepare complete Stop Solution.

    a. (Optional) Dilute Spike-in DNA from Yeast (10 ng/mL, part # CS226342) to 0.1- 10 ng/mL

    b. Add 5 µL of 2% Digitonin (part # CS CS226345), 0.5 µL of RNaseA (Part # 20-297), 1 µL of Precipitation Enhancer (Part # CS203208) and (optional 2 µL of Spike-in DNA from Yeast (0.1-10 ng/ mL)) to 200 µL of Stop solution (part # CS226346) for each sample and place on ice.

    - The amount of spike-in DNA should be changed based on the starting number of the cells. When starting with low cell numbers (i.e. 100 - 10,000 cells), 2 pg/mL(final) in the Stop Solution is recommended. For samples with high cell numbers (i.e. 10,000 -500,000 cells), 100 pg/mL (final) in the Stop Solution is recommended to obtain enough number of reads.
    - Recently, The Henikoff lab introduced the method to use E. coli DNA that is carried-over from the production of the fusion proteins to serve as a spike-in for sample calibrations.[8] If you choose this method, no additional heterologous spike-in needs to be included in the Stop Solution

Complete Stop Solution	Each	X N
2% Digitonin	5 µL	µL
RNase A	0.5 µL	µL
Precipitation Enhancer	1 µL	µL
Optional: Spike-in DNA from Yeast (diluted to 0.1-10 ng/mL)	2 µL	µL
Stop Solution	200 µL	µL

34. Remove the tubes from step 29 from the nutator or tube rotator. Centrifuge the tubes briefly to remove liquid from the cap and side of the tubes and place them on the magnetic separator for 30s to 2 minutes.

35. Remove the supernatant making sure not to aspirate any magnetic bead-bound cells. Remove the tubes from the magnet.

36. Wash the bead-bound cells twice with Dig-wash buffer.

    a. Add 1 mL Dig-wash buffer to the tube and mix the bead-bound cells by gently pipetting to completely resuspend bead-bound cells. Centrifuge the tube briefly to remove liquid from the cap and side of the tube and place it on the magnetic separator for 30 s to 2 minutes.

    b. Remove the supernatant making sure not to aspirate any magnetic bead-bound cells. Remove the tubes from the magnet.

    c. Repeat step a and b one more time.

37. Wash the bead-bound cells once with complete rinse Buffer.

    a. Add 1 mL complete rinse buffer to the tube and mix the bead-bound cells by gently pipetting to completely resuspend bead-bound cells. Centrifuge the tube briefly to remove liquid from the cap and side of the tube and place it on the magnetic separator for 30 s to 2 minutes.

    b. Remove the supernatant making sure not to aspirate any magnetic bead-bound cells. Remove the tubes from the magnet and put them on ice.

38. Place each tube at a low angle on the vortex mixer set to low (~1100 rpm) and add 200 µL of ice-cold complete incubation buffer along the side while gently vortexing to allow the solution to dislodge most or all of the bead-bound cells. Tap to dislodge the remaining bead-bound cells.

39. Incubate at 0 °C (on ice with some ice cold water) for the desired digestion time (default is 5 to 30 min).

    - MNase binds DNA but only cleaves when Ca^{2+} is present, resulting in a zero-order reaction that seems to be less temperature-dependent than the subsequent diffusion of released pA-MN-bound particles, which can digest accessible regions of the genome. Cleavage and release of particles in most cell population can be obtained at 0 °C while minimizing background cleavages attributable to diffusion. Digestion at ambient temperature or higher, results in unacceptable background cleavage levels.
    - The targeted cleavage occurs within seconds of adding Ca^{2+} ions, and by virtue of being a sterically regulated tethered reaction and the cleavage pattern was constant over time. However, longer digestion times releases more material with no apparent change in the signal-to-noise ratio. Therefore, digesting for 5 minutes as a starting point is recommended and that can be extended to 30 minutes.

40. Place each tube on a (cold) magnet separator and allow to clear for ≥10 s and transfer the supernatant to a new 1.5 mL centrifuge tube and put it aside on ice or discard the supernatant. Add 200 µL of complete Stop Solution to the **ConA bead-bound cells** and mix by gentle vortexing.

- The high concentration of Ca^{2+} in the Incubation Buffer will compact chromatin and prevent diffusion out of the nucleus during digestion. Thus, following the digestion, the Incubation Buffer will contain very little MNase fusion protein digested chromatin.[8] However, to be safe, the Incubation Buffer can be saved for potential future analysis. The addition of 200 µL complete Stop Solution will then chelate away remaining Ca^{2+} and allow the digested chromatin fragments to freely diffuse out of the cells.

41. Incubate 30 min at 37 °C to release ChIC/CUT&RUN fragments from the insoluble nuclear chromatin.
42. Place each tube on a magnet separator and allow to clear for 30 s to 2 minutes. Transfer the supernatant containing digested chromatin to a new 1.5 mL centrifuge tube.
    - When performing ChIC/CUT&RUN using untested antibodies, following removal of the Stop Solution w/ Chromatin, it may be prudent to spin down and freeze the ConA bead-bound cells containing the insoluble nuclear chromatin that is left behind after MNase digestion. These cells may be useful for troubleshooting in cases where the user suspects chromatin solubility issues may be limiting yields. By preparing a sequencing library from the total DNA extracted from the "left-over" ConA bead bound material, as described in Skene and Henikoff (eLife, 2017)[2], and comparing the sequencing data, the user can determine if the majority of the cleaved DNA was in fact being retained in the nuclear fraction.

VI. DNA Extraction by using optionally supplied DNA extraction kits (Spin columns)

The DNA extraction by using a spin column is recommended for recovery of large protein–DNA complexes such as nucleosomes. If you are processing recovery of small protein-DNA complexes such as transcription factors, the alternative DNA extraction (phenol chloroform extraction, section VII) is recommended.

43. Add 400 µL of DNA Binding Buffer to each sample from step 42. Mix briefly ensuring that the sample and binding buffer are completely mixed.
44. For each sample, place a DNA Purification Column into a 2 mL Collection Tube and load the entire sample into the spin column.
45. Centrifuge the column at 11,000 x g for 1 minute to bind the DNA to the column membrane. Discard the flow through.
46. Add 700 µL of DNA Wash Buffer to each spin column and centrifuge at 11,000 x g for 1 minute and discard the flow through.
    - Make sure that ethanol has been added to the wash buffer. Adding wash buffer that does not contain ethanol will result in the loss of your sample.
47. Repeat the wash step as described in step 47.
48. Centrifuge at 11,000 x g for 5 minute to remove residual Wash Buffer.
    - Failure to remove the wash buffer will result in residual ethanol in your final eluted material. Residual ethanol can result in a lower recovery of your DNA.
49. Place the spin column in a new 1.5 mL microcentrifuge tube. Add 30-50 µL of DNA Elution Buffer to the center of the membrane and incubate on the bench top for 1 minute. Spin at full speed for 1 minute.

50. DNA samples are ready for sequencing library creation or store DNA at -20°C for up to 2 months

VII. Alternative DNA extraction method (preferred for quantitative recovery of ≤80-bp fragments)

51. Add 2 µL of 10% SDS and 2.5 µL of Proteinase K (Cat. # CS207286) to each tube from Step 42.
52. Incubate at 50 °C for 1 hour with mixing.
53. Centrifuge the tubes briefly and place them at room temperature.
    - Immediately before use pre-spin Phase-Lock gel tubes (ex. 5 PRIME, Cat. # 2302810) at 12,000 - 16,000 x g for 20 to 30 seconds.
54. Transfer the samples to a phase-lock gel tube.
55. Add 200 µL of Phenol:Chloroform:Isoamyl Alcohol (25:24:1, pH 8.0) per sample.
56. Vortex the tube vigorously and centrifuge at 16,000 x g for 5 minutes at room temperature.
57. Add 200 µL of Chloroform per sample.
58. Vortex the tube vigorously and centrifuge at 16,000 x g for 5 minutes at room temperature.
59. Remove the upper aqueous phase (~200 µL) and place it in a new 1.5 mL microcentrifuge tube.
60. Add 0.8 µL of Precipitate Enhancer (part # CS203208) followed by 500 µL of 100% ethanol. Mix well.
61. Chill the tubes on ice for 10 minutes or store them at -20 °C overnight.
62. Centrifuge the tubes at 16,000 x g for 10 minutes at 4 °C and remove the supernatant being careful not to disturb the pellet.
63. Wash the pellet once by adding 1 mL of 100% ethanol. Centrifuge at 16,100 x g for 5 minutes at 4°C. Carefully discard the supernatant and allow pellets to air dry.
64. Resuspend DNA pellet in 30 to 50 µL of 1 mM Tris-HCl, pH 8.0 and 0.1mM EDTA.
65. DNA samples are ready for sequencing library creation or store DNA at -20°C for up to 2 months

VIII. DNA sample Evaluation

66. Quantify 1-2 µL, for example using fluorescence detection with a Qubit instrument.
67. Evaluate the presence of cleaved fragments and the size distribution by capillary electrophoresis with fluorescence detection, for example using a Bioanalyzer instrument. (Figure 4, Appendix A)
    - Some long undigested DNA will leak through, and this is what will dominate the Qubit fluorescence for ChIC/CUT&RUN of typical transcription factors. For these samples, the targeted DNA recovered is too low in concentration and too small in size to be detected

by gel analysis or even by Bioanalyzer. In such cases the DNA cannot be evaluated by Bioanalyzer until DNA sequence library is produced.

IX. Library preparation and sequencing

- The DNA sequencing library for Illumina sequencing can be prepared with several different kits including the Hyper prep kits (KAPA Biosystem), True-Seq kits (Illumina), and NEBNext Ultra I DNA Library Kit (E7645). Please refer the protocols developed by Henikoff''s lab (dx.doi.org/10.17504/protocols.io.zcpf2vn) and Nan Liu in Stuart Orkin's lab (dx.doi.org/10.17504/protocols.io.wvgfe3w).

68. 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 ChIC/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.

    - To minimize the contribution of large DNA fragments, the number of PCR cycles should be at least 12–14, preferably with a combined annealing/extension time of **10 seconds** at 60 °C. Good results have been obtained with the Hyper Prep Kit (KAPA Biosystems).

Step	Temperature	Time	Cycles
Initial Denaturation	98 °C	45 seconds	1
Denaturation	98 °C	15 seconds	12-14
Anneal/Extension	60 °C	10 seconds	
Final Extension	72 °C	1 minute	1
Hold	4-10°C	Hold	

    - After PCR is complete, be extra careful to avoid contamination of any pre-PCR reagents with post-PCR products, which have P5 and P7 primer binding sites at either end. Even a minute amount of contamination in a solution or from pipette tip can dominate the final library. Ideally, post-PCR manipulations should be done at a workbench that is never used for pre-PCR steps.

69. Quantify the library yield using a dsDNA-specific assay, such as the Qubit dsDNA HS Assay Kit, following the manufacturer's instructions**.**

70. Determine the size distribution of libraries by Bioanalyzer, following the manufacturer's instructions.

71. Perform paired-end Illumina sequencing on the barcoded libraries using an Illumina massively parallel DNA sequencer, following the manufacturer's instructions.

- 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, 24 barcoded samples per lane can be run on a two-lane flow cell and perform paired-end 25 × 25-bp sequencing. Single-end sequencing is not recommended for ChIC/CUT&RUN, as it sacrifices resolution and discrimination between TFs and neighboring nucleosomes.

X. Data Processing and Analysis

- Align paired-end reads using Bowtie2 version 2.2.5 with options: --local --very-sensitive-local --no-unal --no-mixed --no-discordant --phred33 -I 10 -X 700. For mapping spike-in fragments, use the --no-overlap --no-dovetail options to avoid cross-mapping of the experimental genome to that of the spike-in DNA.
- Separation of sequenced fragments into ≤120 bp and ≥150 bp size classes provides mapping of the local vicinity of a DNA-binding protein, but this can vary depending on the steric access to the DNA by the tethered MNase.
- Scripts are available from https://github.com/Henikoff/Cut-and-Run for spike-in calibration and for peak-calling.

Summary Protocol for Experienced Users

If this is your first time using this kit please follow the detailed protocol above for best results. Once you are comfortable with all the steps of the protocol this summarized version can be used.

- The same protocol can be used for up to 500,000 mammalian cells per sample

I. Binding cells to beads

1. Allow following parts to **equilibrate to room temperature** for 30 minutes.
   - Concanavalin A-coated magnetic beads (part # CS226333 or CS226314)
   - Binding buffer (part # CS226311)
   - Spermidine (part # CS226308) or 2M Spermidine aliquots
   - Protease Inhibitor Cocktail III, Animal Free (part # 535140-1ML)
   - Wash buffer (part # CS226309)
2. **Activate Concanavalin A-coated magnetic beads**.
   a. Completely disperse and re-suspend Concanavalin A-coated magnetic beads (part # CS226333 or CS226314)
   b. Transfer (10 µL x Sample number) of Concanavalin A-coated magnetic bead slurry to a 1.5 ml microcentrifuge tube. Place the tube on the magnetic separator (e.g. Millipore Cat. # LSKMAGS08) for 30s to 2 minutes. Remove the liquid.
   c. Add (100 µL x Sample number) Binding buffer (Part# CS226311) to the tube and mix the beads by gently pipetting. Centrifuge the tube briefly and place it on the magnetic separator for 30 s to 2 minutes.
   d. Remove the supernatant. Remove the tubes from the magnet.
   e. Repeat step c and d for an additional wash.
   f. Resuspend the beads in a volume of Binding buffer equal to the volume of beginning bead slurry (10 µL x Sample number).
3. **Prepare Complete wash buffer**.
   a. Dissolve entire (1g) Spermidine (part # CS226308) in Nuclease-free water and adjust the total volume to 3.4 mL (2M Spermidine). Store the solution in small aliquots at -20°C.
   b. Add 1.8 µL of 2M Spermidine and 36.5 µL of Protease Inhibitor Cocktail III, Animal Free (part # 535140-1ML) to 7.3 mL of Wash buffer (part # CS226309) for each sample.

Complete wash buffer	Each	X N
2M Spermidine	1.8 µL	µL
Protease Inhibitor Cocktail III	36.5 µL	µL
Wash buffer	7.3 mL	mL

4. **Harvest fresh culture(s)** at room temperature and count cells. (up to 500,000 mammalian cells per sample)
5. **Centrifuge** at 600 x g for 3 min at room temperature and **aspirate supernatant**.
6. **Resuspend the cell pellet** in 1.0 mL room temperature complete wash buffer and **transfer the cell suspension** to a 1.5 mL microcentrifuge tube.
7. **Centrifuge the tube** at 600 x g for 3 min at room temperature and **aspirate supernatant**.
8. **Wash the cells twice** with Complete wash buffer
   d. Resuspend the cell pellet in 1.0 mL room temperature Complete wash buffer by gently pipetting.
   e. Centrifuge 3 min 600 x g at room temperature and aspirate supernatant.
   f. Repeat steps a and b one more time.
9. **Resuspend the cell pellet** in (100 µL x Sample number) of room temperature Complete wash buffer by gently pipetting.

10. While gently vortexing the cells at room temperature, **add the entire activated Concanavalin A-coated magnetic bead slurry** prepared at step 2.
11. **Rotate the microcentrifuge tube** for 5-10 min at room temperature and continue to Permeabilize cells and bind primary antibodies section.

II. Permeabilize cells and bind primary antibodies

12. **Prepare Dig-wash buffer**
    a. Thaw required number of bottles of 2% Digitonin at room temperature or with 37˚C heat block.
    b. For each sample mix 42-210 µL 2% digitonin with 4.2 mL of complete wash buffer prepared in step 3 for a final concentration of digitonin between 0.02% and 0.1% (wt/vol).

Dig-wash buffer	Each	X N
2% Digitonin	42- 210 µL	µL
Complete wash buffer	4.2 mL	mL

- **Store this buffer on ice or at 4 °C** for up to 1 day and mix well before use.

13. **Prepare Antibody buffer**
    Add 0.4 µL 0.5 M EDTA (part # CS203175) with 100 µL of Dig-wash buffer per each sample and place on ice.
14. **Mix well the microcentrifuge tube** from Step 11 of Section I and **divide into 100µL aliquots** in 1.5-mL microcentrifuge tubes, one for each final sample.
15. **Place the microcentrifuge tubes on the magnetic separator** for 30 s to 2 minutes. **Remove the supernatant. Remove the microcentrifuge tubes from the magnet**.
16. Place each tube at a low angle on the vortex mixer set to low (~1100 rpm) and **dispense 100 µL of the Antibody buffer** prepared at step 13 along the side while gently vortexing to allow the solution to dislodge most or all bead-bound cells. Tap to dislodge the remaining bead-bound cells.
17. **Add the primary antibody** to a final concentration of 1:100 or to the manufacturer's recommended concentration for immunofluorescence to each tube and **mix well**.
18. **Place the tubes on a nutator or a tube rotator** at room temperature for ~2 hours or at 4 °C overnight.

III. Bind secondary antibody (Optional: If you don't use secondary antibodies, skip this section and proceed to section IV Bind MNase fusion protein.

19. **Remove the tubes from th nutator**. **Centrifuge the tubes briefly and place them on the magnetic separator** for 30s to 2 minutes.
20. **Remove the supernatant**. **Remove the tubes from the magnet**.
21. **Wash the bead-bound cells once with Dig-wash buffer**.
    a. Add 1 mL Dig-wash buffer to the tube and mix the bead-bound cells by gently pipetting. Centrifuge the tube briefly and place it on the magnetic separator for 30 s to 2 minutes.
    b. Remove the supernatant. Remove the tubes from the magnet.
22. Place each tube at a low angle on the vortex mixer set to low (~1100 rpm) and **squirt 100 µL of the Dig-wash buffer** per sample along the side while gently vortexing to allow the solution to dislodge most or all of the bead-bound cells. Tap to dislodge the remaining bead-bound cells.
23. **Add secondary antibody** to each tube to a final concentration of 1:100 or to the manufacturer's recommended concentration for immunofluorescence.
24. **Place the tubes on the nutator or tube rotator** at 4 °C for ~1 hr.

IV. Bind MNase fusion proteins

25. **Remove the tubes from the nutator. Centrifuge the tubes briefly and place them on the magnetic separator for 30s to 2 minutes.**
26. **Remove the supernatant. Remove the tubes from the magnet.**
27. **Wash the bead-bound cells once with Dig-wash buffer.**
    a. Add 1 mL Dig-wash buffer to the tube and mix the bead-bound cells by gently pipetting. Centrifuge the tube briefly and place it on the magnetic separator for 30 s to 2 minutes.
    b. Remove the supernatant. Remove the tubes from the magnet.
28. Place each tube at a low angle on the vortex mixer set to low (~1100 rpm) and **dispense 100 µL of the MNase fusion protein at 700 ng/mL in Dig-wash buffer** per sample along the side while gently vortexing to allow the solution to dislodge most or all of the bead-bound cells. Tap to dislodge the remaining bead-bound cells.
29. **Place the tubes on the nutator or tube rotator** at 4 °C for ~1 hour.

V. Chromatin Digestion and Release

30. **Thaw a bottle of 2% Digitonin** (part # CS226345) at room temperature or with 37˚C heat block.
31. **Prepare complete rinse buffer.**
    Add 25 µL of 2% Digitonin (part # CS226345) and 0.25 µL of 2M Spermidine prepared at step 3 to 1 mL of Rinse buffer (part # CS226306) per each sample and place on ice.
32. **Prepare complete incubation buffer.**
    Add 5 µL of 2% Digitonin (part # # CS226345) to 200 µL of Incubation buffer (part # CS226352) per each sample and place on ice.
33. **Prepare complete Stop Solution.**
    a. (Optional) Dilute Spike-in DNA from Yeast (10 ng/mL, part # CS226342) to 0.1- 10 ng/mL
    b. Add 5 µL of 2% Digitonin (part # CS CS226345), 0.5 µL of RNaseA (Part # 20-297), 1 µL of Precipitation Enhancer (Part # CS203208) and (optional 2 µL of Spike-in DNA from Yeast (0.1-10 ng/ mL)) to 200 µL of Stop solution (part # CS226346) for each sample and place on ice.

Complete Stop Solution	Each	X N
2% Digitonin	5 µL	µL
RNase A	0.5 µL	µL
Precipitation Enhancer	1 µL	µL
Optional: Spike-in DNA from Yeast (diluted to 0.1-10 ng/mL)	2 µL	µL
Stop Solution	200 µL	µL

34. **Remove the tubes from step 29 from the nutator. Centrifuge the tubes briefly and place them on the magnetic separator** for 30s to 2 minutes.
35. **Remove the supernatant. Remove the tubes from the magnet.**
36. **Wash the bead-bound cells twice with Dig-wash buffer.**
    a. Add 1 mL Dig-wash buffer to the tube and mix the bead-bound cells by gently. Centrifuge the tube briefly and place it on the magnetic separator for 30 s to 2 minutes.
    b. Remove the supernatant. Remove the tubes from the magnet.
    c. Repeat step a and b one more time.
37. **Wash the bead-bound cells once with complete rinse Buffer.**
    a. Add 1 mL complete rinse buffer to the tube and mix the bead-bound cells by gently pipetting. Centrifuge the tube briefly and place it on the magnetic separator for 30 s to 2 minutes.
    b. Remove the supernatant. Remove the tubes from the magnet and put them on ice.
38. Place each tube at a low angle on the vortex mixer set to low (~1100 rpm) and **add 200 µL of ice-cold complete incubation buffer** along the side while gently vortexing to allow the solution to dislodge most or all of the bead-bound cells. Tap to dislodge the remaining bead-bound cells.
39. **Incubate at 0 °C** for the desired digestion time (default is 5 to 30 min).

40. **Place each tube on a (cold) magnet separator** and allow to clear for ≥10 s and **transfer the supernatant** to a new 1.5 mL centrifuge tube and store them at -20C. **Add 200 µL of complete Stop Solution to the cells and mix** by gentle vortexing.
41. **Incubate 30 min at 37 °C** to release ChIC/CUT&RUN fragments from the insoluble nuclear chromatin.
42. **Place each tube on a magnet separator** and allow to clear for 30 s to 2 minutes. **Transfer the supernatant containing digested chromatin** to a new 1.5 mL centrifuge tube.

VI. DNA Extraction by using optionally supplied DNA extraction kits (Spin columns)

43. **Add 400 µL of DNA Binding Buffer** to each sample from step 42 and **mix**.
44. For each sample, **place a DNA Purification Column** into a 2 mL Collection and **load the entire sample** into the spin column.
45. **Centrifuge the column** at 11,000 x g for 1 minute to bind the DNA to the column membrane. Discard the flow through.
46. **Add 700 µL of DNA Wash Buffer** to each spin column and **centrifuge** at 11,000 x g for 1 minute and **discard the flow through**.
47. **Repeat the wash step** as described in step 47.
48. **Centrifuge** at 11,000 x g for 5 minute to remove residual Wash Buffer.
49. **Place the spin column** in a new 1.5 mL microcentrifuge tube. **Add 30 to 50 µL of DNA Elution Buffer** to the center of the membrane and incubate on the bench top for 1 minute. Spin at full speed for 1 minute.
50. **DNA samples are ready** for sequencing library creation or **store at -20°C** for up to 2 months

VII. Alternative DNA extraction method (preferred for quantitative recovery of ≤80-bp fragments)

51. **Add 2 µL of 10% SDS and 2.5 µL of Proteinase K** (Cat. # CS207286) to each tube from Step 42.
52. **Incubate at 50 °C for 1 hour with mixing.**
53. **Centrifuge the tubes** briefly and place them at room temperature.
54. **Transfer the samples** to a phase-lock gel tube.
55. **Add 200 µL of Phenol:Chloroform:Isoamyl Alcohol (25:24:1, pH 8.0)** per sample.
56. **Vortex the tube vigorously** and **centrifuge** at 16,000 x g for 5 minutes at room temperature.
57. **Add 200 µL of Chloroform** per sample.
58. **Vortex the tube vigorously** and **centrifuge** at 16,000 x g for 5 minutes at room temperature.
59. **Remove the upper aqueous phase** (~200 µL) and **place it in a new 1.5 mL microcentrifuge tube.**
60. **Add 0.8 µL of Precipitate Enhancer** (part # CS203208) followed by **500 µL of 100% ethanol. Mix well.**
61. **Chill the tubes on ice** for 10 minutes or store them at -20 °C overnight.
62. **Centrifuge the tubes** at 16,000 x g for 10 minutes at 4 °C and **remove the supernatant**.
63. **Wash the pellet once** by adding 1 mL of 100% ethanol. **Centrifuge** at 16,100 x g for 5 minutes at 4°C. Carefully **discard the supernatant** and allow pellets to **air dry**.
64. **Resuspend DNA pellet** in 30 to 50 µL of 1 mM Tris-HCl, pH 8.0 and 0.1mM EDTA.
65. **DNA samples are ready** for preparation of DNA sequencing libraries. **Store at -20°C** for up to 2 months

VIII. DNA sample Evaluation

66. **Quantify** 1-2 µL, for example using fluorescence detection with a Qubit instrument.
67. **Evaluate the presence of cleaved fragments and the size distribution** by capillary electrophoresis with fluorescence detection, for example using a Bioanalyzer instrument.

Example of ChIC/CUT&RUN results

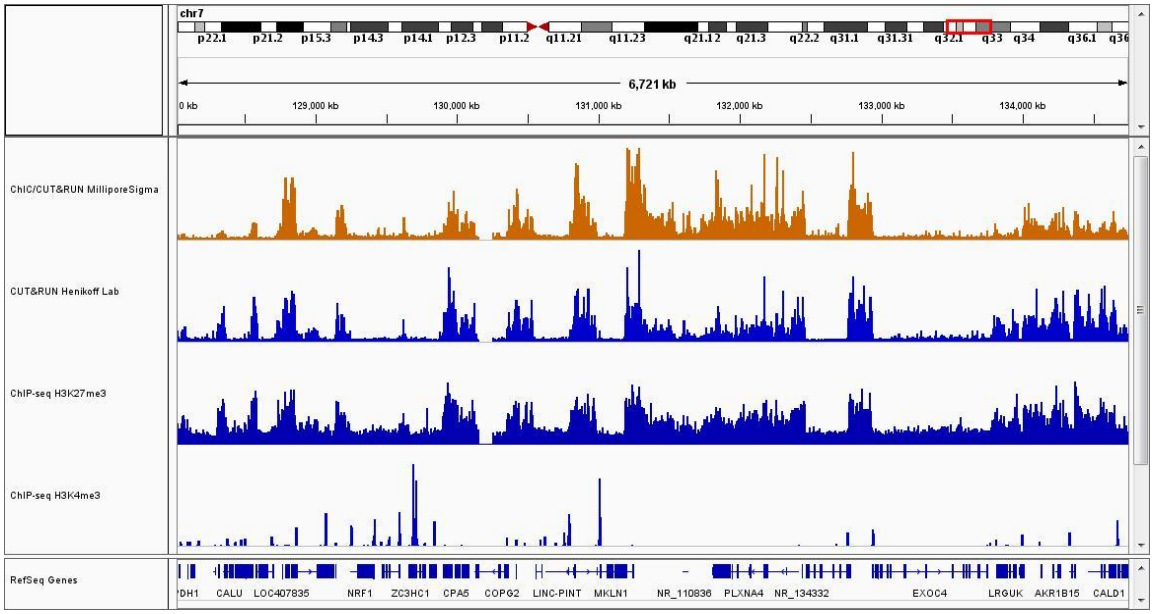

Figure 2: Successful Sequence results of ChIC/CUT&RUN assay, H3K27me3

ChIC/CUT&RUN assay was performed with anti-H3K27me3 antibody (Part No. CS226331) and 200,000 of K562 cells (1st track). The results were compared with published CUT&RUN assay results from Henikoff lab (GSE104550) with anti- H3K27me3 antibody (MilliporeSigma Cat. No. 07-449) (2nd tack) and ENCODE ChIP-seq data (GSM733658) with anti- H3K27me3 antibody (MilliporeSigma Cat. No. 07-449) (3rd track). ENCODE ChIP-seq results (GSM733680) with Anti-H3K4me3 (MilliporeSigma Cat. No. 07-473) is also shown as a negative control (4th track).

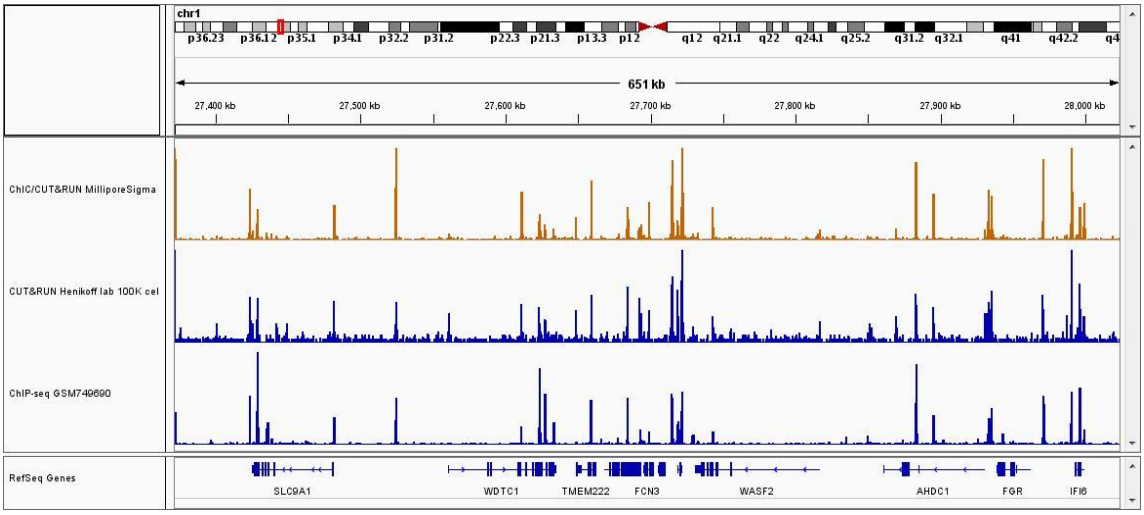

Figure 3. Successful Sequence results of ChIC/CUT&RUN assay, CTCF

ChIC/CUT&RUN assay was performed with anti-CTCF antibody (MilliporeSigma Cat. No. 07-729) and 200,000 of K562 cells (1st track). The results were compared with published CUT&RUN assay results from Henikoff lab (GSE104550) (2nd tack) and ENCODE ChIP-seq data (GSM749690) with the same antibody (3rd track).

Appendix: Bioanalyzer analysis of purified ChIC/CUT&RUN DNA with positive control antibody (anti-H3K27me3, part No. CS226331)

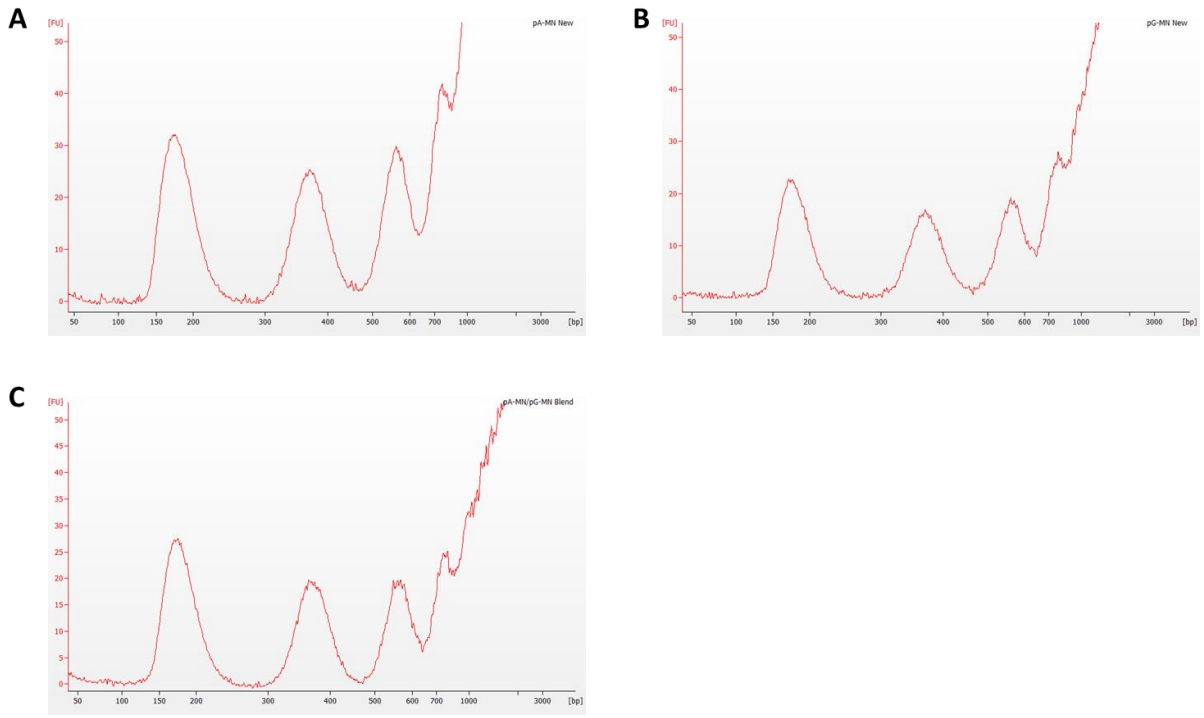

Figure 4. Bioanalyzer analysis of purified ChIC/CUT&RUN DNA

ChIC/CUT&RUN assays were performed with anti-H3K27me3 antibody (Part No. CS226331) and 200,000 of K562 cells. A. with pA-MN kit (Cat. No. CHR100), B. with pG-MN kit (Cat. No. CHR102), C. pA-MN/pG-MN kit (Cat. No. CHR103)

ChIC Optimization and Troubleshooting

Step	Potential Problems	Experimental Suggestions
Binding antibodies and MNase fusion proteins	Beads aggregate and cannot be separated, stick to the tube, or come out from the solution	• Cells might be lysed by the digitonin. Reduce the digitonin concentration at step 12. • There might be excessive movement of solution within the tube during incubation. Proceed the antibody incubation on a Nutator or a stationary tube rack. Use low-binding tubes.
DNA sample evaluation	No DNA <200 bp is detected by Bioanalyzer analysis or Qubit measurement	• This is typical for low cell number (<10,000 cells) or low abundant epitopes (like transcription factors). • It may also indicate a failure of antibody binding. Test the antibody binding by immunofluorescence. Use different antibody.
	A nucleosome ladder is detected by Bioanalyzer analysis	• This is typical for abundant nucleosomal epitopes. • It may also indicate the release of MNase fusion protein during digestion step. Run a negative-control rabbit IgG sample.
	Small DNA or a nucleosome ladder is seen in the negative control by Bioanalyzer analysis	Divalent cations have not been removed by the EDTA in the antibody solution, or the negative-control antibody failed to bind. Try to use different negative control antibody (not provided in the kit). Reduce the digestion time. Add another wash step with dig-wash buffer before digestion.
Library Preparation	A prominent peak at ~130 bp is detected by Bioanalyzer analysis in a large number of samples	This peak corresponds to self-ligated adapters when relatively little digested chromatin was released. (Low cell numbers or low abundant epitopes) • Remove the adapter by another SPRI Beads purification. • Reduce the ratio of SPRI beads to sample. • Increase MNase Digestion time at step 39.
	A small peak at ~130 bp is detected by Bioanalyzer analysis in some samples	This indicate self-ligated adapter remains in the library. Remove the adapter by another SPRI Beads purification.
	No DNA <300bp is detected by Bioanalyzer analysis after library preparation	Sub-nucleosomal particles (often protected by transcription factors) are being denatured during end repair and ligation or being removed during library cleanup. • Ensure the dA-Tailing temperature is <58˚C. • Increase the concentration of adaptors. • Increase the ratio of SPRI beads to sample.

Step	Potential Problems	Experimental Suggestions
Library Preparation	Larger DNA 400 bp < is detected by Bioanalyzer analysis	Annealing/Extension time is too long. Check the PCR cycles for the amplification. The Annealing/Extension time should be as short as 10 seconds to minimize the contribution of large DNA fragments.
Data analysis	Data quality from a sample of interest has high background or is indistinguishable from the IgG control	Sub-nucleosomal particles (often protected by transcription factors) are being denatured. It causes the signal from the target to be lost. • Ensure the dA-Tailing temperature is <58˚C. • Work quickly to get cells into antibody buffer to halt endogenous DNase activity. Excessive DNA damage and fragmentation may be overwhelming the signal from MNase digestion. • Avoid mechanically shearing the DNA. Diffusion of MNase during the digestion step may cause excessive off-target digestion. • Increase antibody concentration. • Test antibody binding by Immunofluorescence. The reaction might have failed because of poor antibody binding. • Use different antibody.
	Mapped spike-in reads are too few	The amount of Spike-in DNA was insufficient. The amount of spike-in DNA should be changed based on the starting number of the cells. When starting with low cell numbers (i.e. 100 - 10,000 cells), add 2 pg/mL. and for samples with high cell numbers (i.e. 10,000 -500,000 cells), add ~100 pg/mL. to the Stop solution.

Related Products

Product	Description	Catalog Number
2% Digitonin Solution	Additional 2% digitonin solution	CHR103
DNA extraction kit for ChIC (Chromatin Immuno-Cleavage) (12 rxns)	12 DNA extraction columns kit	CHR104
Magna ChIP™ HiSens Chromatin Immunoprecipitation Kit	24 Chromatin immunoprecipitation reactions. Includes reagents, buffers and beads for ChIP as well as reagents and buffers for chromatin preparation and isolation	17-10460
EZ-Magna ChIP™ HiSens Chromatin Immunoprecipitation Kit	24 Chromatin immunoprecipitation reactions plus positive and negative control antibodies and validated qPCR primer set. Includes reagents, buffers and beads for ChIP as well as reagents and buffers for chromatin preparation and isolation	17-10461
ChIPAb+™ Validated Antibody Primer Set	Proven for RIP or ChIP and lot tested for performance. See the complete selection at www.millipore.com/antibodies	Multiple
RIPAb+™ Validated Antibody Primer Set	Proven for RIP or ChIP and lot tested for performance. See the complete selection at www.millipore.com/antibodies	Multiple
Magna Nuclear RIP™ (Cross-Linked)	Complete set of reagents for performing 24 cross-linked nuclear RIP assays	17-10520
EZ-Magna Nuclear RIP™ (Cross-Linked)	Complete set of reagents for performing 24 cross-linked nuclear RIP assays, plus positive and negative control primers and positive control antibody	17-10521
Magna Nuclear RIP (Native)	Complete set of reagents for performing 24 native nuclear RIP assays	17-10522
EZ-Magna Nuclear RIP (Native)	Complete set of reagents for performing 24 native nuclear RIP assays, plus positive and negative control primers and positive control antibody	17-10523
Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit	Complete set of reagents for performing 12 standard RIP assays	17-700
EZ-Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit	Complete set of reagents for performing 12 standard RIP assays, plus positive control antibody and control primers	17-701
Magna ChIRP™ RNA Interactome kits	Complete set of reagents for performing 12 standard ChIRP assays	17-10494
Magna ChIRP™ RNA Interactome kits	Complete set of reagents for performing 12 standard ChIRP assays plus positive control probe sets and control primers	17-10495

References

1. Schmid, M., Durussel, T. & Laemmli, U. K. Mol. Cell 16, 147–157 (2004).
2. Skene, P. J. & Henikoff, S. eLife 6, e21856 (2017).
3. Ku, W. L. et al. Nat. Methods 16, 323-325 (2019)
4. Hainer, S. J. et al. Cell 177, 1319–1329 (2019)
5. Liu, N. et al. Cell. 2018;173(2):430-42.
6. Roth, T.L. et al. Nature. 2018;559(7714):405-9.
7. de Bock, C. E. et al. Cancer discovery. 2018;8(5):616-31.
8. Michael, P. M. et al. eLife 2019;8:e46314

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