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CUT&RUN and CUT&Tag Handbook

Method Overview, Protocols and Reagents

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CUT&RUN (Cleavage Under Targets and Release Using Nuclease) and CUT&Tag (Cleavage Under Targets and Tagmentation) offer a novel approach to pursue epigenetics. Both methods are designed to map genome wide transcription factor binding sites, chromatin-associated complexes, and histone variants and post-translational modifications.

They are performed in situ on immobilized, intact cells without crosslinking. DNA fragmentation is achieved using either micrococcal nuclease (MNase) for CUT&RUN or a hyperactive transposase (Tn5) for CUT&Tag fused to Protein A and/or Protein G. The fusion protein is directed through binding of the Protein A/G moiety to the Fc region of an antibody bound to the target of interest. The DNA under the target is subsequently cleaved and released and the fusion protein-antibody-chromatin complex is free to diffuse out of the cell. DNA cleavage products are extracted and then processed by next generation sequencing (NGS).

This handbook gives a short overview of both methods and their respective advantages. Subsequently detailed step-by-step protocols are provided based on Skene PJ and Henikoff S (2017) Nature Protocols and Meers MP et al. (2019) eLIFE for CUT&RUN and Kaya-Okur HS et al. (2019) Nature Communications and Kaya-Okur HS et Kaya-Okur HS et al. (2020) Nature Protocols for CUT&Tag.

Content

1. CUT&RUN

Advantages of CUT&RUN

ChIP (Chromatin Immunoprecipitation) has been the primary technique to map epigenetic markers for the last decades. More recently, ChIP followed by NGS (ChIP-seq) allows localization of epigenetic markers and protein binding sites on a genomic scale and has become a mainstay application to study gene regulation. However, in spite of the evolution of the readout, the basic method to enrich the DNA of interest has remained unchanged – including its drawbacks.

CUT&RUN introduces some major modifications to eliminate shortcomings inherent to ChIP-seq. Samples are not fixed, as it is the case for ChIP-seq, which can lead to epitope masking. Chromatin is fragmented in a targeted manner by a directed nuclease cleavage from intact cells reversibly permeabilized with the mild, nonionic detergent digitonin. The nuclear envelope remains intact since digitonin replaces cholesterol, which is only present in the plasma membrane. In contrast, chromatin for ChIP is prepared by sonication or enzymatic treatment of whole cells leading to a substantial background due to genomic DNA even after immunoprecipitation DNA enrichment. As a consequence of this superior selectivity for chromatin containing the desired epitope, CUT&RUN has considerably lower background and better signal-to-noise ratio than ChIP-seq. This leads to a higher sensitivity and renders genomic features visible that are undetectable using ChIP-seq. In addition, less sequencing depth is required. Transcription factor binding sites can be mapped at bp resolution with 10^6 reads. For abundant antigens such as H3K27me3, it is even possible to start with as few as 100 cells. Single-cell profiling using combinatorial indexing genomic analysis using CUT&RUN is possible since intact cells are being used.

In contrast to other methods for the genome-wide mapping of chromatin accessibility improving upon ChIP-seq – e.g. DNase1 footprinting, MNase-seq, or ATAC-seq – CUT&RUN maps specific antigens or chromatin structure markers. Other tethering approaches like DNA adenine methyltransferase identification (DamID) and Chromatin Endogenous Cleavage (ChEC) also allow specific chromatin fragmentation depending on the protein of interest. Expression of recombinant fusion protein does, however, limit their scalability and they are not suitable to address specific histone modifications.

Chromatin Immunocleavage (ChIC) relies on a Protein A-MNase fusion protein that is tethered to an antibody against the protein of interest to direct DNA cleavage. However, ChIC read-out is based on a Southern blot. Combination of ChIC on native cells or isolated nuclei immobilized on magnetic beads and high-throughput NGS gave rise to CUT&RUN.

CUT&RUN advantages:

- Performed in situ on non-fixed cells; no chromatin fragmentation necessary.
- Low background and high sensitivity require low sequencing depth.
- Depending on the antigen, only low cell numbers are needed – as few as 100 cells.
- Simple, fast, amenable to automation.
- Accurate quantitation using heterologous spike-in DNA or carry-over *E. coli* DNA from the pA/G-MNase purification.

Direct or indirect CUT&RUN

An antibody specific for the protein of interest is crucial to direct the pA/G-MNase mediated nucleic acid cleavage to the intended site. The Protein A/G portion tethers the fusion protein to the Fc region of the antibody bound to its antigen. This allows the pA/G-MNase nuclease portion to cleave the nucleic acid under the targeted protein and to release the nucleic acid.

Depending on the host species and isotype of the antibody and the Protein A and/or Protein G MNase fusion protein, a secondary antibody may be necessary for pA/G-MNase binding. For example, if the pA-MNase is used in conjunction with a primary mouse IgG1 or goat IgG antibody, it is recommended to use a rabbit secondary antibody. Protein A binds well to rabbit or guinea pig IgG antibodies but only poorly to mouse IgG1 or goat IgG. No additional secondary antibody is needed when using pA/G-MNase.

The CUT&RUN Positive Control and CUT&RUN Negative Control are important to assess cleavage and chromatin release without the need to sequence the released DNA fragments. Do not use a no-antibody negative control: untethered pA/G-MNase will non-specifically bind and cleave any accessible DNA, thus increasing background signal.

Chromatin cleavage at high Ca^{2+}/low salt concentration

In the original CUT&RUN protocol chromatin is cleaved by MNase at a low concentration of divalent cations (2 mM Ca^{2+}) and a high salt concentration (150 mM). Cleavage products are released in the presence of Ca^{2+} and the MNase is free to cut accessible DNA irrespective of the antigen of interest it is tethered to via the Protein A or Protein G moiety and the antigen-specific antibody. MNase off-site DNA cleavage can cause undesired background.

A more recent improvement of the CUT&RUN protocol is intended to reduce background due to DNA overdigestion by free pA/G-MNase-antibody-chromatin complexes. The protocol takes advantage of the fact that nucleosomes aggregate in the presence of high concentrations of divalent cations (10 mM Ca^{2+}) and at low salt concentrations to reduce premature release of the pA/G-MNase-antibody-chromatin cleavage products. Subsequently to the digestion of the samples in high Ca^{2+}/low salt conditions, cleavage products are released in a high salt buffer containing a chelator to prevent further DNA cleavage.

As mentioned above, premature release of cleavage product particles during the digestion step can cause MNase off-site cleavage and thus increased background signal. This is particularly relevant when cleaving chromatin under abundant targets for longer digestions times. Longer retention of the cleavage product particles within the nucleus may improve CUT&RUN with lower cell numbers.

Advantages of chromatin cleavage at high Ca^{2+} and low salt concentration:

- Prevent premature release of the pA/G-MNase-antibody-chromatin complex after cleavage.
- Minimize unspecific off-site cleavage due to free MNase in the presence of divalent cations.
- Reduce variability of the cleavage products and background depending on the incubation time.

Accurate quantitation with heterologous spike-in DNA or carry-over *E.coli* DNA

The original CUT&RUN protocol includes heterologous spike-in DNA to quantify binding event. Heterologous spike-in DNA in the Stop Buffer allows the comparison of DNA yields between different samples. The total number of spike-in DNA sequencing reads serve as a normalization factor and are inversely proportional to the total number of sample DNA sequencing reads. Spike-in DNA should be fragmented down to an average length of approximately 200 bp. The amount of spike-in DNA can be adjusted based on the number of cells collected for each sample: use 100 pg/mL for 10^4-10^6 cells and 2 pg/mL for 10^2-10^4 cells.

However, in the improved CUT&RUN protocol the authors show that accurate quantitation is possible using heterologous spike-in DNA or carry-over *E. coli* DNA from the pA/G-MNase purification. As it is introduced earlier at step 46, it is digested by the MNase and released at the same time as the sample chromatin DNA. Consequently, no heterologous spike-in DNA needs to be added to the Stop Buffer.

Reagents Required

CUT&RUN Positive Control (ABIN6923144)
CUT&RUN Negative Control (ABIN101961)
CUT&RUN anti-DYKDDDDK Antibody R (ABIN6923143)
CUT&RUN Concanavalin A Beads (ABIN6952467)
CUTANA™ pAG-MNase for ChIC/CUT&RUN Assays (ABIN6950951)
2.5 M Manganese Chloride ($MnCl_2$) (ABIN2958419)
1 M Calcium Chloride ($CaCl_2$)
1 M Potassium Chloride (KCl) (ABIN412574)
1 M HEPES pH 7.5 (NaOH)
5 M NaCl (ABIN412560)
0.5 M EDTA (ABIN925554)
0.2 M EGTA
2 M Spermidine
EZBlock™ Protease Inhibitor Cocktail V, EDTA-Free (ABIN4991987)
5% Digitonin (ABIN1304051)
10% BSA (ABIN412576)
20 mg/mL Glycogen
Trypan Blue (ABIN413910)
RNase A (DNase and protease free)
10% Sodium Dodecyl Sulfate (SDS) (ABIN925555)
10 mg/mL Proteinase K (ABIN920948)
Phenol-Chloroform-Isoamyl Alcohol (PCI)
Chloroform:Isoamyl Alcohol 24:1
3 M Sodium Acetate (NaOAc) pH 5.2 (ABIN925556)
5 M Ammonium Acetate (NH_4OAc) (ABIN925566)
1 mM Tris-HCl pH 8.0

antibodies-online™ CUT&RUN Sets

Get the antibodies-online™ CUT&RUN set tailored to your needs and save money compared to purchasing the components separately.

	Positive & Negative Control	Rabbit anti-Mouse Secondary	Rabbit anti-DYKDDDDK Primary	Mouse anti-DYKDDDDK Primary	ConA Beads
CUT&RUN Pro ABIN6923135	✓	✓		✓	✓
CUT&RUN Pro Direct ABIN6923135	✓		✓		✓
CUT&RUN Pro Sec ABIN6923135	✓	✓			✓
CUT&RUN Pro ABIN6923135	✓				✓
CUT&RUN Core Complete ABIN6923135	✓	✓		✓	
CUT&RUN Core Direct ABIN6923135	✓		✓		
CUT&RUN Core Sec ABIN6923135	✓	✓			
CUT&RUN Core ABIN6923135	✓				

The CUTANA™ pAG-MNase (ABIN6950951) is also available in our shop online.

Reagent Preparation

(for 12 samples)

» Wash Buffer (165 mL)

Component	Volume	Final concentration
ddH_2O	156.7 mL	-
1 M HEPES pH 7.5	3.3 mL	20 mM
5 M NaCl	4.95 mL	150 mM
2 M Spermidine	41.25 µL	0.5 mM

- Store Wash Buffer without protease inhibitors for up to one week at 4 °C.
- Add protease inhibitors fresh before use, e.g.:

Protease Inhibitor Cocktail 100x	1.65 mL	1x

» Binding Buffer (45 mL)

Component	Volume	Final concentration
ddH_2O	43.6 mL	-
1 M HEPES pH 7.5	900 µL	20 mM
1 M KCl	450 µL	10 mM
1 M $CaCl_2$	45 µL	1 mM
2.5 M $MnCl_2$	16 µL	1 mM

- Store Binding Buffer for up to six months at 4 °C.

» Digitonin Wash Buffer (82.5mL)

Component	Volume	Final concentration
5% Digitonin	825 µL	0.05%
Wash Buffer	82 mL	-

- Store Digitonin Wash Buffer for up to one day at 4 °C.
- Recommended Digitonin concentration ranges from 0.025% to 0.1%.
- The effectiveness of Digitonin varies between batches. Test cell permeability using Trypan Blue to determine the optimal concentration to use.

» Antibody Buffer (1.5 mL)

Component	Volume	Final concentration
0.5 M EDTA	4 µL	2 mM
10% BSA	10 µL	0.1%
Digitonin Wash Buffer	1.5 mL	-

- Store Antibody Buffer for up to one day at 4 °C until use.

» Low Salt Rinse Buffer (27 mL)

Component	Volume	Final concentration
ddH_2O	26.2 mL	-
1 M HEPES pH 7.5	540 µL	20 mM
2 M Spermidine	6.75 µL	0.5 mM
5% Digitonin	270 µL	0.05%

- Store Low Salt Rinse Buffer for up to one week at 4 °C until use.

» Low Salt Incubation Buffer (3 mL)

Component	Volume	Final concentration
ddH_2O	3 mL	-
1 M HEPES pH 7.5	10.5 µL	3.5 mM
1 M $CaCl_2$	30 µL	10 mM
5% Digitonin	30 µL	0.05%

- Store Low Salt Incubation Buffer for up to one week at 4 °C until use.

» Low Salt Stop Solution (3 mL)

Component	Volume	Final concentration
ddH_2O	2.6 mL	-
5 M NaCl	102 µL	170 mM
0.2 M EGTA	300 µL	20 mM

- Store Low Salt Stop Buffer at 4 °C until use.
- Add fresh before use

5% Digitonin	30 µL	0.05%
RNase A (10 mg/mL)	15 µL	50 µg/mL
Glycogen (20 mg/mL)	7.5 µL	25 µg/mL

Optional:

heterologous spike-in DNA	-	100 pg/mL

Protocol

Please read the entire protocol carefully!

General remarks

- The original CUT&RUN protocol recommends sample sizes of 100 to 1,000 mammalian cells for abundant antigens such as H3K27me3 or CTCF. This protocol adapted from the improved CUT&RUN protocol is suitable for up to 500,000 cells.

  This protocol is intended to give a general outline of the CUT&RUN workflow. It has to be adjusted according to the:

  » Cell type.
  Your specific cell type might necessitate different treatments prior to the CUT&RUN procedure, e.g. disintegration of tissue, generation of spheroblasts.

  » MNase digestion time points during the optimization.
  Different samples, approaches, and digestion time points are uniformly referred to in the protocol as “samples”.

- To minimize DNA breakage during sample preparation, avoid cavitation through vigorous resuspension and vigorous vortexing.

- Keep cells at room temperature during all steps prior to the addition of antibody to minimize stress on the cells and DNA breakage.

- All steps from the incubation with the primary antibodies forward should be carried out at 4 °C.

Additional remarks or particular protocol steps

- **Step 1**
  Prepare single cell suspension from your sample material according to your established protocol.

- **Step 6:**
  Cells can be used directly harvested from fresh cultures or cryopreserved with 10% DMSO as cryoprotectant. Avoid flash freezing, as this can cause undesired DNA breakage, thus increasing background.

- **Step 23:**
  EDTA in the Antibody Buffer chelates excess divalent cations in the Binding Buffer used to activate Concanavalin A. This prevents carry-over of Ca^{2+} and premature pA/G-MNase DNA cleavage and endogenous DNase activity.

- **Step 30:**
  The quick spin minimizes carry-over of antibody and pA/G-MNase that could result in overall background cleavage during the digestion step.

- **Step 33/43:**
  Washing out EDTA before pA/G-MNase addition prevents removal of divalent cations necessary for MNase nuclease acid cleavage.

- **Step 46:**
  It is important that no divalent cations are present during the pA/G-MNase binding to prevent premature DNA cleavage.
  MNase binds DNA in the Digitonin Wash Buffer that does not contain divalent cations. It only cleaves DNA upon addition of Ca^{2+} in step 58. Thus, the digestion is a zero-order reaction that is less temperature sensitive than the diffusion of the pA/G-MNase-antibody-chromatin complex out of the cells in step 63. Minimizing diffusion of the digestions products helps to keep unspecific cleavage of non-antibody-bound sites low. Binding of MNase to its abundant nucleic acid substrate in the absence of Ca^{2+} helps to overcome effects of the MNase's sequence preference.

- **Step 58:**
  The high Ca^{2+} concentration in the Low Salt Incubation Buffer will compact chromatin. Compacted chromatin does not diffuse out of the nucleus and the liquid will contain very little pA/G-MNase-bound particles. To be safe, keep the Low Salt Incubation Buffer at -20 °C for troubleshooting in case of a low DNA yield.

- **Step 59:**
  Digestion times typically vary between 5 min and 30 min. In case you observe excessive background signal use shorter incubation times.

- **Step 62:**
  EGTA in the Low Salt Stop Solution remaining Ca^{2+} and allows the digested chromatin fragments to freely diffuse out of the cells.
  Heterologous spike-in DNA with an average fragment length of approximately 200 bp in the Stop Buffer can serve as a reference to allow normalization of DNA yields from different samples. Adjust the amount of spike-in DNA according to the number of cells collected for each sample: use 100 pg/mL for 10^4-10^6 cells and 2 pg/mL for 10^2-10^4 cells. Alternatively, *E. coli* carry-over

DNA from the purification of the pA/G-MNase fusion protein has been shown to be a viable calibration standard replacing spike-in DNA in the Stop Buffer. As it is introduced earlier at step 43, it is digested by the MNase and released at the same time as the sample chromatin DNA.

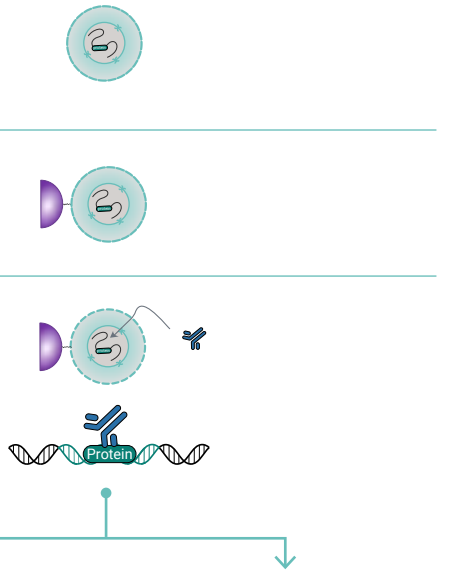
- **Step 65:**
  Keep the Concanavalin A bead-bound cells at -20 °C for troubleshooting in case of a low DNA yield.

Overview CUT&RUN

Steps 1-7
Cell harvest

Steps 8-20
Cell immobilization

Steps 21-33
Cell permeabilization and primary antibody binding

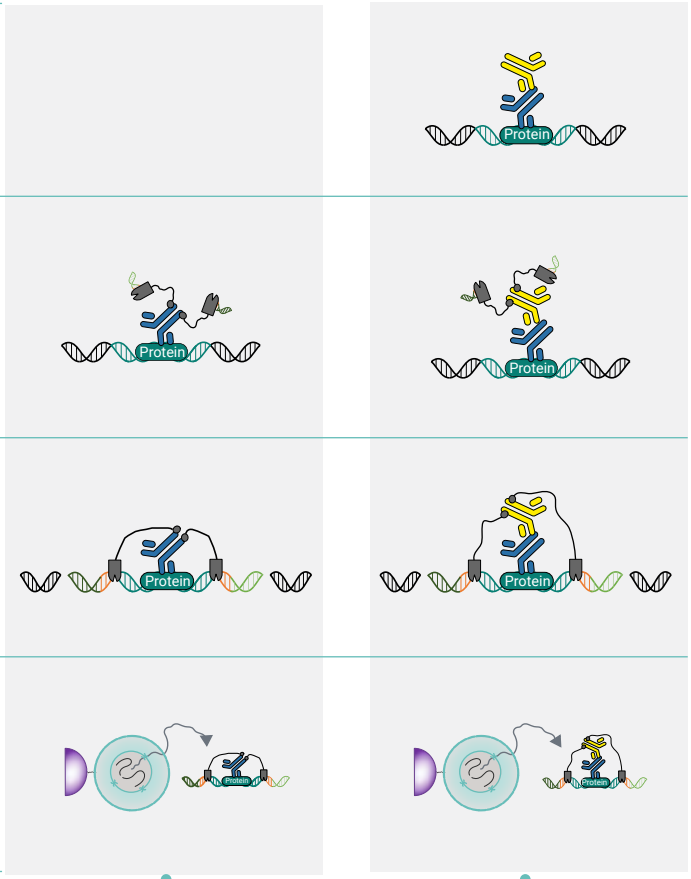


Steps 34-43
Secondary antibody binding (optional)

Steps 44-51
pA/G-MNase binding

Steps 52-62
MNase digestion

Steps 63-65
Chromatin release



Steps 66-85
DNA extraction

Step-by-step protocol

I. Cell harvest – at room temperature

1. Harvest 10,000 to 500,000 cells for each sample at room temperature. Keep cells for each sample in separate tubes. [1]
2. Centrifuge cell solution 3 min at 600 x g at room temperature.
3. Remove the liquid carefully.
4. Gently resuspend cells in **1 mL Wash Buffer** by pipetting and transfer cell solution to a 1.5 mL microcentrifuge tube.
5. Centrifuge cell solution 3 min at 600 x g at room temperature and discard the supernatant.
6. Repeat steps 4-5 thrice for a total of four washes. [6]
7. Resuspend cell pellet for each sample in **1 mL Wash Buffer** by gently pipetting.

II. Concanavalin A beads preparation

8. Prepare one 1.5 mL microcentrifuge tube for each sample.
9. Gently resuspend the **CUT&RUN Concanavalin A Beads.**
10. Pipette **10 µL CUT&RUN Concanavalin A Beads** slurry for each sample into the 1.5 mL microcentrifuge tubes.
11. Place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.
12. Remove the microcentrifuge tube from the magnet stand.
13. Pipette **1 mL Binding Buffer** into each tube and resuspend **CUT&RUN Concanavalin A Beads** by gentle pipetting.
14. Spin down the liquid from the lid with a quick pulse in a table-top centrifuge (max. 100 x g).
15. Place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.
16. Remove the microcentrifuge tube from the magnet stand.
17. Repeat steps 13-16 twice for a total of three washes.
18. Gently resuspend the **CUT&RUN Concanavalin A Beads** in a volume of **Binding Buffer** corresponding to the original volume of bead slurry, i.e. **10 µL per sample**.

III. Cell immobilization – binding to Concanavalin A beads

19. Carefully vortex the cell suspension from step 7 and add 10 µL of the **CUT&RUN Concanavalin A Beads** in **Binding Buffer** from step 18 to each sample.
20. Close tubes tightly and rotate for 5-10 min at room temperature.

IV. Cell permeabilization and primary antibody binding

21. Place the microcentrifuge tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.
22. Remove the microcentrifuge tubes from the magnet stand.
23. Place each tube at a low angle on the vortex mixer set to a low speed (approx. 1,100 rpm) and add **100 µL Antibody Buffer** containing digitonin. [23]

24. Gently vortex the microcentrifuge tubes until the beads are resuspended.
25. For the positive control, add **5 µL CUT&Tag rabbit anti-H3K4me3 IgG Positive Control** corresponding to a 1:20 dilution to the corresponding tube.
26. For the negative control, add **5 µL CUT&RUN guinea pig anti-rabbit IgG Negative Control** corresponding to a 1:20 dilution to the corresponding tube.
27. In case you are using one of the **CUT&RUN anti-DYKDDDDK antibodies**, add **5 µL** corresponding to a 1:20 dilution to the corresponding tube.
28. For the remaining samples, add **1 µL primary rabbit antibody** against your protein of interest corresponding to a 1:100 dilution (or a volume corresponding to the manufacturer's recommended dilution for immunofluorescence).
29. Rotate the microcentrifuge tubes for 2 h at room temperature or 4 h to overnight at 4 °C.
30. Spin down the liquid and place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully. 30
31. Remove the microcentrifuge tubes from the magnet stand.
32. Resuspend with **1 mL Digitonin Wash Buffer** and mix by inversion. If clumping occurs, gently remove the clumps with a 1 mL pipette tip.
33. Repeat steps 30-32 once for a total of two washes. 33

V. Anti-rabbit secondary antibody binding (optional)

Binding of the CUT&RUN guinea pig anti-rabbit IgG Negative Control antibody increases the number of Fc fragments for pAG-MNase binding in the vicinity of the protein of interest's binding site, thus leading to an amplification of the CUT&RUN signal. This optional step is relevant when working with less abundant proteins. It is not necessary for the positive and negative controls.

If no secondary antibody is used proceed directly to step 44.

34. Place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.
35. Remove the microcentrifuge tubes from the magnet stand.
36. Vortex the sample at low speed (approx. 1,100 rpm) and add **100 µL Digitonin Wash Buffer** per sample along the side of the tube.
37. Tap to remove the remaining beads from the tube side.
38. Add **5 µL CUT&RUN guinea pig anti-rabbit IgG Negative Control antibody** corresponding to a 1:20 dilution to the positive control and your samples.
39. Rotate the microcentrifuge tubes for 1 h at 4 °C.
40. Spin down the liquid and place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.
41. Remove the microcentrifuge tubes from the magnet stand.
42. Resuspend with **1 mL Digitonin Wash Buffer** and mix by inversion. If clumping occurs, gently remove the clumps with a 1 mL pipette tip.
43. Repeat steps 40-42 once for a total of two washes. 43

VI. pA/G-MNase binding

44. Place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.
45. Remove the microcentrifuge tubes from the magnet stand.

46. Vortex the sample at low speed (approx. 1,100 rpm) and add **50 µL Digitonin Wash Buffer** per sample along the side of the tube. Add **2.5 µl CUTANA™ pAG-MNase for ChIC/CUT&RUN Assays ABIN6950951/ABIN6950952.** 46
    Alternatively:
    Vortex the sample at low speed (approx. 1,100 rpm) and add **150 µl Digitonin Wash Buffer** containing 700 ng/mL of your own **pA/G-MNase** preparation per sample along the side of the tube.
47. Rotate the microcentrifuge tubes for 1 h at 4 °C.
48. Spin down the liquid and place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.
49. Remove the microcentrifuge tubes from the magnet stand.
50. Resuspend with **1 mL Digitonin Wash Buffer** and mix by inversion. If clumping occurs, gently remove the clumps with a 1 mL pipette tip.
51. Repeat steps 48-50 once for a total of two washes.

VII. MNase digestion and release of pA/G-MNase-antibody-chromatin complexes

52. Spin down the liquid from the lid with a quick pulse in a table-top centrifuge (max. 100 x g).
53. Place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.
54. Resuspend with **1 mL Low Salt Rinse Buffer** and mix by inversion. If clumping occurs, gently remove the clumps with a 1 mL pipette tip.
55. Spin down the liquid from the lid with a quick pulse in a table-top centrifuge (max. 100 x g)
56. Place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.
57. Repeat steps 54-56 once for a total of two washes.
58. Place each tube at a low angle on the vortex mixer set to a low speed (approx. 1,100 rpm) and add **200 µL ice cold Low Salt Incubation Buffer** per sample along the side of the tube. 58
59. Incubate tubes at 0 °C for the desired time (default is 30 min).
60. Place the tubes on a cold magnet stand until the fluid is clear. Remove the liquid carefully.
61. Remove the microcentrifuge tubes from the magnet stand.
62. Resuspend with **200 µL Low Salt Stop Solution** and mix by gentle vortexing. 62
63. Incubate tubes at 37 °C for 30 min.
64. Place the tubes on a magnet stand until the fluid is clear.
65. Transfer the supernatant containing the pA/G-MNase-bound digested chromatin fragments to fresh 1.5 mL microcentrifuge tubes. 65

VIII. DNA extraction

66. Add **2 µL 10% SDS** to a final concentration of 0.1% and **5 µL Proteinase K (10 mg/mL)** to a final concentration of 2.5 mg/mL to each supernatant from step 62.
67. Gently vortex tubes at a low speed of approx. 1,100 rpm.
68. Incubate tubes at 50 °C for 1 h or at 37 °C overnight.

69. Add **200 µL PCI** to tube.
70. Vortex tubes thoroughly at high speed until the liquid appears milky.
71. Centrifuge tubes in a table-top centrifuge at 16,000 x g at 4 °C for 5 min.
72. Carefully transfer the upper aqueous phase to a fresh 1.5 mL microcentrifuge tube containing **200 µL Chloroform:Isoamyl Alcohol 24:1**.
73. Vortex tubes thoroughly at high speed until the liquid appears milky.
74. Centrifuge tubes in a tabletop centrifuge at 16,000 x g at 4 °C for 5 min.
75. Carefully transfer the upper aqueous phase to a fresh 1.5 mL microcentrifuge tube containing **2 µL glycogen** (diluted 1:10 to 2 mg/mL from the 20 mg/mL stock solution).
76. Add **20 µL 3 M NaOAc pH 5.2** or **100 µL 5 M NH_4OAc**.
77. Add **500 µL 100% ethanol**.
78. Place tubes for 10 min in a dry ice/ethanol mix or overnight at -20 °C.
79. Centrifuge tubes in a table-top centrifuge at 16,000 x g at 4 °C for 5 min.
80. Remove the liquid carefully with a pipette.
81. Add **1mL 70% ethanol**.
82. Centrifuge tubes in a table-top centrifuge at 16,000 x g at 4 °C for 1 min.
83. Remove the liquid carefully with a pipette.
84. Air-dry the pellet or dry the pellet in a SpeedVac.
85. Dissolve the pellet in **30 µL 1 mM Tris-HCl, 0.1 mM EDTA**.

IX. Sample quality control

Size distribution and concentration of the CUT&RUN products can be assessed at this point, e.g. using a Qubit or Nanodrop fluorometer or a Bioanalyzer or Tapestation. It is possible that the concentration of the recovered DNA is below the instrument's detection limit. It is also to be expected that the extracted DNA includes some large DNA fragments that will mask the signal of the CUT&RUN products. In this case, it may be useful to PCR-amplify the DNA and check the library on a Bioanalyzer or Tapestation.

X. Sequencing library preparation

Prepare the CUT&RUN products sequencing libraries according to your established work-flow. Because of the very low background with CUT&RUN, typically 5 million paired-end reads suffice for epitopes with a multitude of genomic binding sites, e.g. transcription factors or nucleosome modifications.

XI. Peak calling

The sparse background signal in CUT&RUN samples compared to ChIP-seq samples represents a challenge for peak callers that employ statistical models relying on a high sequencing depth and high recall to identify true positives and avoid false positives. In contrast, peak calling for CUT&RUN data sets requires high specificity for true signal peaks.

To this end, the Henikoff lab developed the Sparse Enrichment Analysis for CUT&RUN (SEACR) peak caller that can be easily accessed using its web server at https://seacr.fredhutch.org/.
Alternatively, the Orkin and Yuan labs have streamlined processing of CUT&RUN data using their CUT&RUN Tools pipeline https://bitbucket.org/qzhudfci/cutruntools/.

Frequently Asked Question

Why is the DNA yield so low?

CUT&RUN is performed using low cell numbers and the background signal is considerable lower than e.g. for ChIP. Due to these two factors, the amount of recovered DNA is often times too low to be reliably measured based on a fluorometric assay or by capillary electrophoresis. PCR amplification of small CUT&RUN products, i.e. less than 50 bp, can be problematic and is therefore not any option.
To assess the success of the CUT&RUN method each of the antibodies-online CUT&RUN product sets includes the CUT&RUN Positive Control antibody ABIN6923144 against the abundant H3K27me3 histone modification. DNA fragments prepared using this antibody can be measured by sensitive electrophoresis on a Bioanalyzer or Tapestation or fluorometrically on a Qubit or Nanodrop fluorometer. When using the CUT&RUN Positive Control ABIN6923144 (or any other antibody specific for nucleosomal markers) a ladder corresponding to multiples of the 147 bp long nucleosomes should be visible by capillary electrophoresis.

How can I validate that my primary antibody is working for CUT&RUN?

For a CUT&RUN experiment the validation data could include e.g. a Tapestation or Bionalyzer plot showing the size distribution and qPCR data showing target enrichment.
As mentioned above, the DNA yield of an CUT&RUN experiment appears typically very low compared e.g. to ChIP-seq because of the lower initial sample size and the substantially lower DNA background. In particular for less abundant target protein the concentration is often times too low to be reliably measured using a fluorometric assay or by capillary electrophoresis. PCR amplification of small CUT&RUN products, i.e. less than 50 bp, can be problematic and is therefore not an option.
Once a sequencing library has been generated and sequenced map sequencing reads and verify the accumulation of reads at known binding sites.

Why do I need a negative control antibody? Why not just use a no-antibody control?

MNase is an endo- and exonuclease that will unspecifically bind and cleave unprotected DNA in hyper-accessible DNA, e.g. in regions surrounding regulatory elements. Free MNase will preferentially cut DNA within these hyper-accessible regions, thus potentially causing false positives and increased background signal in general.

To avoid this undesired effect of untethered MNase, the chromatin is randomly coated with the CUT&RUN Negative Control (ABIN101961) prior to the addition of pA/G-MNase is added to the samples. pA/G-MNase is then tethered via its Protein A or Protein G portion to the antibody's Fc fragment and background DNA fragmentation is dictated by the random antibody binding as opposed to the nuclease digestion of hyper-accessible DNA regions.

Can I replace the antibody negative control using a knock-out (or knock-down) of my protein?

Both controls are useful but address different aspects of the experiment and are therefore not interchangeable.

The CUT&RUN Negative Control (ABIN101961) antibody is used to establish a reference background for peak calling. This is necessary because of the sparse background signal in CUT&RUN samples compared to ChIP-seq samples. The ko (or kd) control on the other hand gives an impression of unspecific binding of the antibody specific for the protein of interest to other proteins. It is useful to avoid identification of false positive signals.

Do I need to use a secondary antibody? Other CUT&RUN protocols do not use a secondary.

Depending on the host species and isotype of the antibody and the Protein A and/or Protein G MNase fusion protein, a secondary antibody may be necessary for pA/G-MNase binding.

Protein A has good high affinity to all rabbit IgG antibodies but low affinity to rat, goat and sheep IgG isotype antibodies and certain mouse IgG antibody subclasses, in particular IgG1. Protein G on the other hand binds well to the Fc region of mouse, goat, sheep, and most rat IgG. Its affinity to rabbit IgG however is lower than that of Protein A.

When using pAG-MNase introduced with the improved CUT&RUN protocol it is therefore generally not necessary to use a secondary antibody. Use of the pA-MNase of the original protocol however might require the use of a secondary antibody raised in rabbit to assure efficient binding of the fusion protein to the antibody.

Should I include heterologous spike-in DNA for quantitation?

Our protocol is largely based on the improved CUT&RUN protocol[3]. Here, the authors show that accurate quantitation is possible using heterologous spike-in DNA or carry-over *E. coli* DNA from the pA/G-MNase purification. Therefore, the addition of heterologous spike-in DNA is not necessary.

Is it possible to fix the cells prior to immobilization?

It is possible to fix your samples, e.g. to avoid dissociation of larger protein complex from the DNA during the course for the experiment. You can either follow your established cross-linking procedure or the mild cross-linking conditions described in PMID 31164146 using formaldehyde at a lower concentration of 0.1%. Cross-linking at 1% formaldehyde can actually reduce signal, possibly due to epitope masking. In these cases, a lower concentration of cross-linker is preferable.

Are there protocols to make this work for tissue, where I cannot necessarily isolate single cells?

The Henikoff lab that published the original CUT&RUN method in 2017 has since developed several variations. One of these modification is AutoCUT&RUN which outlines a workflow to automate CUT&RUN for high-throughput characterization of single cells but also patient samples and xenografts.

In short, the tissue is processed manually or enzymatically into single, intact cell that are subsequently bound to ConA beads. The CUT&RUN protocol that serve as basis for the protocol outlined in this handbook can be adapted accordingly.

Is it possible to use the CUT&RUN product sets with plant tissue samples?

The CUT&RUN method can be applied to plant tissue samples. An essential step in addition to those lined out in the protocol is the generation of spheroblasts so that it becomes possible to permeabilize the plasma membrane for the application of the antibodies and the MNase fusion protein. Alternatively, use isolated nuclei as sample material.

The CUT&RUN Positive Control H3K27me3 antibody (red dot) and the CUT&RUN Negative Control guinea pig anti-rabbit IgG antibody as well as the ConA beads are suitable for use with plant samples. The antibodies that are included in some of the sets, such as the anti-DYKDDDDK antibodies or the secondary antibody, can also be used with plant samples. Whether they are needed or not depends on your experiment.

Instead of the proteinase K digestion can I denature the proteins in the CUT&RUN product complexes by heat?

We recommend against this option: the DNA of interest is at his point present in a complex consisting of the DNA, the antigen, the corresponding antibody, and the pA/G-MNase. Boiling this complex will likely precipitate the DNA together with denatured protein. This will also primarily affect the short CUT&RUN products and not the larger DNA molecules, leading to a decreased signal to noise ratio in your library and potentially also reducing the library's complexity. This effect is further exacerbated because of the lower melting temperature of these short molecules compared to the longer contaminating DNA molecules.

What is preferable for DNA extractions prior to library preparation: extraction using phenol-chloroform or affinity purification using a column?

A potential issue when using SPRI beads for the DNA fragment clean-up is the carry-over of active Proteinase K, which can interfere with the downstream PCR amplification. Therefore, a phenol-chloroform extraction is preferable to assure complete denaturation of Proteinase K.

Is it possible to do single-end instead of paired-end sequencing of the CUT&RUN libraries?

Single-end sequencing instead of paired-end sequencing is possible. However, it has drawbacks compared to paired-end sequencing: (i) For abundant targets like histone marks or transcription factors a large number of binding is expected. Paired-end sequencing facilitates unambiguous mapping to the correct genomic position. This additional information reduces the necessary sequencing depth. (ii) MNase will digest the target DNA until the section covered by the protein of interest. Paired-end sequencing will reveal this footprint while the information is lost in single-end sequencing.

2. CUT&Tag

Advantages of CUT&Tag

ChIP (Chromatin Immunoprecipitation) has been the primary technique to map epigenetic markers for the last decades. More recently, ChIP followed by NGS (ChIP-seq) allows localization of epigenetic markers and protein binding sites on a genomic scale and has become a mainstay application to study gene regulation. However, in spite of the evolution of the readout the basic method to enrich the DNA of interest has remained unchanged – including its drawbacks.

In 2017, CUT&RUN introduced some major modifications in order to eliminate some of the ChIP-seq shortcomings. Samples are immobilized on magnetic Concanavalin A beads as a solid support. However, they are not fixed, as it is the case for ChIP-seq, which can lead to epitope masking. Chromatin is fragmented in a targeted manner by a directed nuclease cleavage from intact cells reversibly permeabilized with the mild, nonionic detergent digitonin. The nuclear envelope remains intact since digitonin replaces cholesterol, which is only present in the plasma membrane. In contrast, chromatin for ChIP is prepared by sonication or enzymatic treatment of whole cells leading to a substantial background due to genomic DNA even after immunoprecipitation DNA enrichment. As a consequence of this selectivity for chromatin containing the desired epitope CUT&RUN has considerably lower background and a better signal-to-noise ratio than ChIP-seq. This leads to a higher sensitivity and renders genomic features visible that are undetectable using ChIP-seq. In addition, less sequencing depth is required. Transcription factor binding sites can be mapped at bp resolution with 10^6 reads. For abundant antigens such as H3K27me3 it is even possible to start with as few as 100 cells. Single-cell profiling using combinatorial indexing genomic analysis[4] using CUT&RUN is possible since intact cells are being used.

Chromatin Immunocleavage (ChIC) does also rely on a Protein A-MNase fusion protein that is tethered to an antibody against the protein of interest to direct DNA cleavage. However, ChIC read-out is based on a Southern blot. Combination of ChIC on native cells or isolated nuclei immobilized on magnetic beads and high-throughput NGS gave rise to CUT&RUN.

One of the drawbacks of CUT&RUN is carried over from ChIP-seq: the prepared DNA fragments need end-polishing and sequencing adapter ligation prior to the preparation of a sequencing library. A combination of the CUT&RUN protocol and tagmentation by a hyperactive Tn5 transposase[6] resulted in the CUT&Tag method. Cells are immobilized using magnetic Concanavalin A beads and reversibly permeabilized using digitonin. Instead of the directed nuclease cleavage however, DNA is fragmented by a pA/G-Tn5 loaded with sequencing adapter duplexes. Sequencing adapters are attached to the DNA fragments directly during tagmentation. No further DNA end processing is necessary and the fragments can be used for sequencing library preparation. In addition, CUT&Tag is inherently less sensitive to endogenous DNA damage than CUT&RUN because the transposition takes place on intact double stranded DNA molecules.

CUT&Tag advantages:

- Performed In situ on non-fixed cells; no chromatin fragmentation necessary.
- Low background and high sensitivity require low sequencing depth.
- No end-polishing and sequencing adapter ligation steps necessary.
- Possible with low cell numbers down to 100 cells depending on the antigen.
- Simple, fast, amenable to automation.
- Accurate quantitation using carry-over *E. coli* DNA from the pAG-Tn5 purification.

In contrast to other methods for the genome-wide mapping of chromatin accessibility improving upon ChIP-seq – e.g. DNase1 footprinting, MNase-seq, or ATAC-seq – CUT&RUN and CUT&Tag map specific antigens or chromatin structure markers. Other tethering approaches like DNA adenine methyltransferase identification (DamID) and Chromatin Endogenous Cleavage (ChEC) also allow specific chromatin fragmentation depending on the protein of interest. Expression of recombinant fusion proteins does however limit their scalability and they are not suitable to address specific histone modifications.

Reagents Required

Positive Control Antibody H3K27me3 (ABIN6923144)
Positive Control Antibody H3K4m3 (ABIN3023254)
Secondary Antibody (ABIN101961)
CUT&RUN Concanavalin A Beads (ABIN6952467)
Protein A and/or protein G Tn5 fusion protein (pA/G-Tn5) preloaded (coming shortly)
Protein A and/or protein G Tn5 fusion protein (pA/G-Tn5) unloaded (coming shortly)
Distilled, deionized or RNase-free H_2O
2.5 M Manganese Chloride ($MnCl_2$) (ABIN2958419)
1 M Calcium Chloride ($CaCl_2$)
1 M Potassium Chloride (KCl) (ABIN412574)
1 $MgCl_2$ (ABIN412564)
1 M HEPES pH 7.5 HEPES (NaOH)
5 M NaCl (ABIN412560)
0.5 M EDTA (ABIN925554)
2 M Spermidine
EZBlock Protease Inhibitor Cocktail V (EDTA-free) (ABIN4991987)
5% Digitonin (ABIN1304051)
Trypan Blue (ABIN413910)
10% Sodium dodecyl sulfate (SDS) (ABIN925555)
10 mg/mL Proteinase K (ABIN920948)
Phenol-chloroform-isoamyl alcohol (PCI)
Phase-lock tubes (optional)
Chloroform:Isoamyl Alcohol 24:1
100% Ethanol
80% Ethanol
10 mM Tris-HCl pH 8.0
TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA)
10 mg/mL RNase A (DNase and protease free)

CUT&Tag Reagents

(for 12 samples)

» Binding Buffer (5 mL)

Component	Volume	Final concentration
ddH_2O	4.85 mL	-
1 M HEPES pH 7.5	100 µL	20 mM
1 M KCl	50 µL	10 mM
1 M $CaCl_2$	5 µL	1 mM
2.5 M $MnCl_2$	2 µl	1 mM

- Store Binding Buffer for up to six months at 4 °C.

» Wash buffer (70 mL)

Component	Volume	Final concentration
ddH_2O	66 mL	-
1 M HEPES pH 7.5	1.4 mL	20 mM
5 M NaCl	2.1 mL	150 mM

- Add protease inhibitor fresh before use

2 M Spermidine	17.5 µL	0.5 mM
Protease Inhibitor (EDTA-free) 100x	700 µL	1x

- Once Spermidine and Protease Inhibitor have been added, store the Wash Buffer at 4°C and use up within two days or store at -20°C.

» Digitonin Wash Buffer (45 mL)

Component	Volume	Final concentration
5% Digitonin	225 µL	0.025%
Wash Buffer	45 mL	-

- Store Digitonin Wash Buffer for up to one day at 4 °C.
- Recommended Digitonin concentration ranges from 0.025% to 0.1%.
- The effectiveness of Digitonin varies between batches, so testing cell permeability using Trypan Blue is recommended to determine the optimal concentration to use.

» Antibody Buffer (1.5 mL)

Component	Volume	Final concentration
0.5 M EDTA	6 µL	2 mM
10% BSA	15 µL	0.1%
Digitonin Wash Buffer	1.5 mL	-

- Store Antibody Buffer for up to one day at 4 °C until use.

» Dig-300 Buffer (48 mL)

Component	Volume	Final concentration
ddH_2O	154 mL	-
1 M HEPES pH 7.5	960 µL	20 mM
5 M NaCl	2.88 ml	300 mM
2 M Spermidine	12 µL	0.5 mM

- Store Dig-300 Buffer without protease inhibitors and Digitonin for up to one week at 4 °C.
- Add protease inhibitor and Digitonin fresh before use, e.g.

Protease Inhibitor Cocktail (EDTA-free) 100x	480 µL	1x
5% Digitonin	96 µL	0.01%

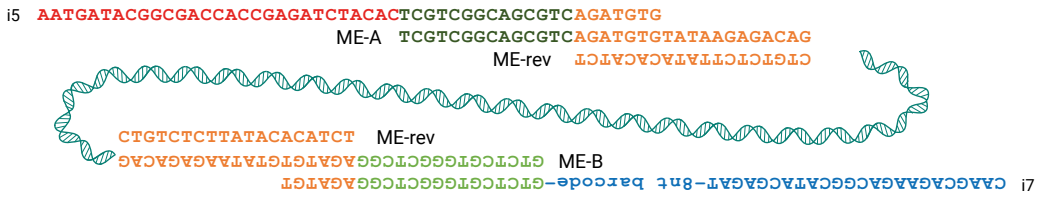
» Tagmentation Buffer (4.2 mL)

Component	Volume	Final concentration
Dig-300 Buffer	4.2 mL	-
1 M $MgCl_2$	42 µl	10 mM

- Prepare Tagmentation Buffer fresh before use.

» Oligonucleotides (for Illumina)[5]

Oliognucleotide	Nucleotide sequence	Concentration
Mosaic end - adpater A (ME-A)	TCGTCGGCAGCGTCAGATGTGTA TAAGAGACAG	100 µM
Mosaid end - adapter B (ME-B)	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	100 µM
Mosaic end – reverse (ME-rev)	Phos-CTGTCTCTTATACACATCT	100 µM
Universal i5 primer	AATGATACGGCGACCACCGAGATCT ACACTCGTCGGCAGCGTCAGATGTG	10 µM
Uniquely barcoded i7 primer	CAAGCAGAAGACGGCATACGAGAT -8nt barcode- GTCTCGTGGGCTCGGAGATGT	10 µM



CUT&Tag adapter and sequencing primers based on Picelli et al[6].

Protocol

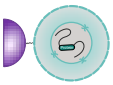
General remarks

- This protocol adapted from the original CUT&Tag protocol[1] is suitable for sample sizes of 100,000 intact mammalian cells for abundant antigens such as H3K27me3.
  It is intended to give a general outline of the CUT&RUN workflow and must be adjusted according to factors such as
  1. Cell type.
     Your specific cell type might necessitate different treatments prior to the CUT&RUN procedure, e.g. tissue homogenization, generation of spheroblasts.
  2. Number of samples.
     Different samples, experimental conditions approaches, or antibody incubation time points are uniformly referred to in the protocol as “samples”.
- To minimize DNA breakage during sample preparation, avoid cavitation through vigorous pipetting and vortexing. Gently pipette sample or vortex the microcentrifuge tubes at low speeds.
- Keep cells at room temperature during all steps prior to the addition of antibody to minimize stress on the cells and DNA breakage.
- All steps from the incubation with the primary antibodies until the end of the protocol should be carried out at 4°C.

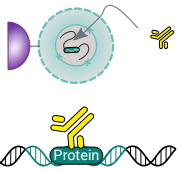
Overview CUT&Tag

Steps 7-15
Cell harvest

Steps 16-26
Cell immobilization



Steps 27-38
Cell permeabilization and primary antibody binding



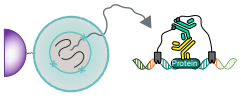
Steps 39-46
Secondary antibody

Steps 47-55
pA/G-Tn5 binding

Steps 56-61
Tagmentation

and

Chromatin release



Steps 62-76
DNA prep

steps 77-91
PCR amplification

Step-by-step protocol

I. pA/G-Tn5 adapter complex assembly

This section is optional and is only relevant when using unloaded pA/G-Tn5

1. Prepare one 0.5 mL PCR tube for each of the ME-A/ME-rev and ME-B/ME-rev oligonucleotide duplexes.
2. Combine 10 µL 100 µM ME-A or ME-B oligonucleotide with 10 µM ME-rev oligonucleotide in the respective tubes.
3. Place tubes in a heating block at 95 °C for 5 min.
4. Keep tubes in the heating block and remove the heating block from the dry block incubator. Let the heating block cool down on the bench top to RT.
5. Mix 8 µl of each of the preannealed ME-A/ME-rev and ME-B/ME-rev oligonucleotide duplexes at 100 µM **with 100 µL of 5.5 µM Protein A and/or Protein G-Tn5 fusion protein (pA/G-Tn5) unloaded**.
6. Rotate the mixture for 1 h at RT and then store at -20 °C.

II.Cell harvest

7. Harvest a cell number corresponding to up to 100,000 mammalian cells for the positive control, negative control, and each sample plus one at room temperature; e.g. 1.3x10⁶ cells for 10 samples and the two controls. [7]
8. Centrifuge cell solution 3 min at 600 x g at room temperature.
9. Remove the liquid carefully.
10. Resuspend cells in a volume of **Wash Buffer** corresponding to the volume of the cell solution or at most 10 mL by pipetting.
11. Centrifuge cell solution 3 min at 600 x g at room temperature.
12. Remove the liquid carefully.
13. Resuspend cells in **1.2 mL Wash Buffer** by pipetting and transfer cell solution to a 1.5 mL microcentrifuge tube.
14. Centrifuge cell solution 3 min at 600 x g at room temperature and discard the supernatant.
15. Resuspend cell pellet in **100 µL Wash Buffer** for each sample plus one by gently pipetting; e.g. 1.3 mL for 10 samples and the two controls.

III.Concanavalin A beads preparation

16. Gently resuspend the **CUT&RUN Concanavalin A Beads**. Pipette a volume of **CUT&RUN Concanavalin A Beads** slurry corresponding to **10 µL** for the positive control, negative control, and each sample plus one into a 1.5 mL microcentrifuge tube containing **1.2 mL Binding Buffer**; e.g. **130 µL CUT&RUN Concanavalin A Beads** slurry for 10 samples and the two controls.
17. Place the tube on a magnet stand until the fluid is clear.
18. Remove the liquid carefully and remove the microcentrifuge tubes from the magnetic stand.
19. Resuspend **CUT&RUN Concanavalin A Beads** in **1 mL Binding Buffer** by gentle pipetting.
20. Spin down the liquid from the lid with a quick pulse in a table-top centrifuge (max 100 x g).

21. Place the tubes on a magnet stand until the fluid is clear.
22. Remove the liquid carefully and remove the microcentrifuge tubes from the magnetic stand.
23. Repeat steps 19-22 once for a total of two washes.
24. Gently resuspend the **CUT&RUN Concanavalin A Beads** in a volume of **Binding Buffer** corresponding to the original volume of bead slurry, i.e. **10 µL per sample and control**; e.g. **130 µL CUT&RUN Binding Buffer** for 10 samples and the two controls.

IV. Cell immobilization – binding to Concanavalin A beads

25. Carefully vortex the cell suspension from step 15 and add the **CUT&RUN Concanavalin A Beads** in **Binding Buffer** from step 24.
26. Close tube tightly and rotate for 5-10 min at room temperature.

V. Cell permeabilization and primary antibody binding

27. Prepare one 1.5 mL microcentrifuge tube for each sample and the two controls.
28. Place the microcentrifuge tube from step 26 on a magnetic stand until the fluid is clear.
29. Carefully remove the liquid from the cells immobilized on the **CUT&RUN Concanavalin A Beads.**
30. Remove the microcentrifuge tubes from the magnetic stand.
31. Gently resuspend the beads in a volume of ice cold **Antibody Buffer** containing digitonin corresponding to 100 µL per sample and control; e.g. 1.3 mL **Antibody Buffer** for 10 samples and the two controls.
32. Pipette 100 µL aliquots of the **CUT&RUN Concanavalin A Beads in Antibody Buffer** into the 1.5 mL microcentrifuge tubes prepared in step 27. 32
33. For the positive control, add **5 µL CUT&Tag rabbit anti-H3K4me3 IgG Positive Control** (turquois dot) corresponding to a 1:20 dilution to the corresponding tube.
34. For the negative control, do not add anything else to the corresponding tube. 34
35. For the remaining samples, add **1 µL primary rabbit antibody** against your protein of interest corresponding to a 1:100 dilution. 35
36. Rotate the microcentrifuge tubes for 2 h at room temperature or overnight at 4 °C.
37. Quickly spin down the liquid and place the tubes on a magnet stand until the fluid is clear.
38. Remove the liquid carefully and remove the microcentrifuge tubes from the magnetic stand.

VI. Secondary antibody binding

39. Add **100 µL Digitonin Wash Buffer** per tube along the side of the microcentrifuge tube and vortex at low speed (approximately 1,100 rpm).
40. Tap to remove the remaining beads from the tube side.
41. Add **5 µL CUT&Tag Secondary Antibody** corresponding to a 1:20 dilution. 41

42. Rotate the microcentrifuge tubes for 1 h at room temperature.
43. Spin down the liquid and place the tubes on a magnet stand until the fluid is clear.
44. Remove the liquid carefully and remove the microcentrifuge tubes from the magnetic stand.
45. Resuspend with **1 mL Digitonin Wash Buffer** and mix by inversion. If clumping occurs, gently remove the clumps with a 1 ml pipette tip.
46. Repeat steps 44-46 twice for a total of three washes. 46

VII. pA/G-Tn5 adapter complex binding

47. Dilute the **pA/G-Tn5 adapter complex** from step 6 1:250 in a volume of **Dig-300 Buffer** corresponding to 100 µL per sample; e.g. 5.2 µL **pA/G-Tn5 adapter complex** in 1.3 mL for 10 samples and the two controls.
48. Place the tubes from step 46 on a magnet stand until the fluid is clear.
49. Remove the liquid carefully and remove the microcentrifuge tubes from the magnetic stand.
50. Place each tube at a low angle on the vortex mixer set to a low speed (approximately 1,100 rpm) and add 100 µL **pAG-Tn5 adapter complex in Dig-300 Buffer** from step 47 along the side of the tube. 50
51. Rotate the microcentrifuge tubes for 1 h at room temperature.
52. Spin down the liquid and place the tubes on a magnet stand until the fluid is clear.
53. Remove the liquid carefully and remove the microcentrifuge tubes from the magnetic stand.
54. Resuspend with **1 ml Dig-300 Buffer** and mix by inversion. If clumping occurs, gently remove the clumps with a 1 ml pipette tip.
55. Repeat steps 52-54 twice for a total of three washes.

VIII. Tagmentation

56. Spin down the liquid from the lid with a quick pulse in a table-top centrifuge (max 100 x g).
57. Place the tubes on a magnet stand until the fluid is clear.
58. Remove the liquid carefully and remove the microcentrifuge tubes from the magnetic stand.
59. Place each tube at a low angle on the vortex mixer set to a low speed (approximately 1,100 rpm) and add **300 µL Tagmentation Buffer** along the side of the tube.
60. Spin down the liquid from the lid with a quick pulse in a table-top centrifuge (max 100 x g)
61. Rotate the microcentrifuge tubes for 1 h at 37 °C.

IX. DNA extraction

62. Add **10 µL 0.5 M EDTA** to a final concentration of 16 mM, 3 µL 10% SDS to a final concentration of 0.1%, and **7.5 µL Proteinase K (10 mg/mL)** to a final concentration of 0.25 mg/mL to each reaction.
63. Vortex tubes thoroughly at a high speed.
64. Incubate tubes at 50 °C for 1 h or at 37 °C ON.

65. Without separating the liquid supernatant and the beads add **300 µL PCI** to each tube.
66. Vortex tubes thoroughly at high speed until the liquid appears milky.
67. Optional: Transfer liquid to a 1.5 mL phase-lock tube. [67]
68. Add 300 µL chloroform and mix by inversion.
69. Centrifuge tubes in a tabletop centrifuge at 16,000 x g at room temperature for 3 min.
70. Using a pipette, transfer the aqueous layer to a new tube containing 750 µL 100% ethanol.
71. Transfer tubes to a cold tabletop centrifuge and centrifuge at 16,000 x g at 4 °C for 15 min.
72. Carefully pour off the liquid and remove the remaining liquid with a pipette.
73. Add **1 mL 100% ethanol**.
74. Carefully pour off the liquid, remove the remaining liquid with a pipette, and air dry the tubes.
75. Dissolve the pellet in **23 µL TE containing RNase A diluted 1:400 to 25 ng/mL**.
76. Incubate tubes at 37 °C for 10 min.

X. PCR amplification and Clean-Up

77. Transfer 21 µl into a 0.5 mL PCR tube.
78. Add 2 µL Universal i5 Primer at 10 µM and 2 µL i7 Primer at 10 µM with a unique barcode for each sample.
79. Add 25 µL 2x PCR master mix of a non-hot start, high fidelity polymerase (e.g. NEBNext Ultra II Q5 Master Mix, Roche KAPA Library Amplification Kit). [79]
80. Mix tubes thoroughly by vortexing.
81. Spin down the liquid from the lid with a quick pulse (max 100 x g).
82. PCR program: [82]

step 1	58 °C	5 min
step 2	72 °C	30 sec
step 3	98 °C	30 sec
step 4	98 °C	10 sec
step 5	60 °C	10 sec
step 6	goto step 4	14 times
step 7	72 °C	1min
step 8	4 °C	hold

83. Transfer the PCR reactions to 1.5 mL microcentrifuge tubes.
84. Add 1.3x volumes (65 µL for a 50 µL PCR mix) SPRI bead slurry and mix by pipetting. [84]
85. Place the tubes on a magnet stand until the fluid is clear.
86. Remove the liquid carefully with a pipette and keep the microcentrifuge tubes on the magnetic stand.
87. Add **200 µL 80% ethanol**.

88. Remove the liquid carefully with a pipette and remove the microcentrifuge tubes from the magnetic stand.
89. Immediately add 25 µL 10 mM Tris-HCl pH 8.0 and mix by pipetting. Elute DNA for at RT for 5 min.
90. Place the tubes on a magnet stand until the fluid is clear.
91. Transfer liquid to fresh 1.5 mL microcentrifuge tubes.

XI. Sample quality control

Size distribution and concentration of the CUT&Tag products can be assessed at this point, e.g. using a Qubit or Nanodrop fluorometer or a Bioanalyzer or Tapestation. It is possible that the concentration of the recovered DNA is below the instrument's detection limit. It is also to be expected that the extracted DNA includes some large DNA fragments that will mask the signal of the CUT&Tag products. In this case, it may be useful to PCR-amplify the DNA and check the library on a Bioanalyzer or Tapestation.

XII. Sequencing library preparation

Prepare the CUT&Tag products sequencing libraries according to your established workflow. Because of the very low background with CUT&Tag, typically 5 million paired-end reads suffice for antigens with a multitude of genomic binding sites, e.g. transcription factors or nucleosome modifications.

XIII. Peak calling

The sparse background signal in CUT&Tag samples compared to ChIP-seq samples represents a challenge for peak callers that employ statistical models relying on a high sequencing depth and high recall to identify true positives and avoid false positives. In contrast, peak calling for CUT&RUN data sets requires high specificity for true signal peaks.

To this end, the Henikoff lab developed the Sparse Enrichments analysis for CUT&RUN (SEACR) peak caller that can be easily accessed using their web server at https://seacr.fredhutch.org/ . Alternatively, the Orkin and Yuan labs have streamlined processing of CUT&RUN data using their CUT&RUNTools pipeline https://bitbucket.org/qzhudfci/cutruntools/ .

Additional remarks for particular protocol steps

Please read through these remarks carefully prior to executing the protocol.

- **Step 7**
  Prepare single cell suspension from your sample material according to established protocol.
  Cells can be used directly harvested from fresh cultures or cryopreserved with 10% DMSO as cryoprotectant. Avoid flash freezing, as this can cause undesired DNA breakage, thus increasing background.

- **Step 32:**
  EDTA in the Antibody Buffer chelates excess divalent cations in the Binding Buffer used to activate Concanavalin A. This prevents carry-over of Ca^{2+} and Mn^{2+} which could trigger premature tagmentation by the pAG-Tn5 adapter complex and endogenous DNase activity.

- **Step 34:**
  For the negative control, do not include a primary antibody at this stage. The **CUT&Tag Secondary Antibody** alone, added later on in Step 41, serves as a negative control.

- **Step 35:**
  This protocol foresees the use of rabbit primary antibodies, which are bound in section VI step 42 by the **CUT&Tag guinea pig anti-rabbit IgG antibody** to increase the number of protein A and protein G binding sites. In case you use primary antibodies from a different species select a different secondary antibody accordingly.
  Your primary antibody should be diluted 1:100 or according to the manufacturer's recommendation for immunofluorescence.

- **Step 41:**
  Binding of the **CUT&Tag guinea pig anti-rabbit IgG antibody** to the rabbit primary antibodies used in step 35 increases the number of protein A and protein G binding sites available to the pAG-Tn5 fusion proteins in step 50. In case you used a non-rabbit primary antibody in step 36 select a corresponding secondary antibody the respective samples.

- **Step 46:**
  Washing out EDTA before pAG-Tn5 adapter complex addition prevents removal of divalent cations necessary for tagmentation.

- **Step 50:**
  Increasing the NaCl concentration to 300 mM prevents Tn5 binding to accessible chromatin site. To avoid clumping and cell lysis in the presence of digitonin the digitonin concentration is reduced to 0.01% in the Dig-300 Buffer.
  E. coli carry-over DNA from the purification of the pAG-Tn5 fusion protein has been shown to be a viable calibration standard, rendering additional heterologous spike-in DNA unnecessary. The carry-over DNA is released by the transposase at the same time as the sample chromatin DNA upon tagmention. It can therefore be used as standard across different experiments assuming constant amounts of pAG-Tn5.

- **Step 67:**
  Use of phase-lock tubes is optional. Alternatively, use 1.5 mL microcentrifuge tubes without the phase-lock gel and use chloroform:isoamyl alcohol 24:1 instead of chloroform in step 64.

- **Step 79:**
  Avoid using a hot-start polymerase for the PCR amplification of the library. The active Tn5 is essential for gap-filling during the first two steps of the PCR program. In a hot-start PCR program the Tn5 will be inactivated before the fill-in reaction can take place, which would result in a reduced PCR yield.

- **Step 82:**
  The PCR conditions are optimized for the amplification of the short CUT&Tag DNA fragments. The short extension step 5 favors PCR products of 100 bp-700 bp. The limited number of PCR cycles is intended to minimize the contribution of larger DNA fragments to the sequencing library. Please keep these factors in mind in case the PCR program has to be adjusted depending on the utilized polymerase.
  The program does not contain a dedicated annealing step. Assuming a ramp rate of 3 °C/sec, primer annealing takes place during the cool-down from 98 °C to 60 °C. In case a rapid cycler is used, the ramp rate has to be adjusted accordingly or a dedicated annealing step should be added to the cycling program.

- **Step 84:**
  1.3x volumes of SPRI beads relative to the PCR mix selects for DNA fragment sizes >100 bp. In case you are concerned about the presence of smaller fragments, e.g. the i5 and i7 primers from step 79, you can reduced the amount of SPRI to 1.1x volumes of the PCR mix.

Frequently Asked Questions

How do I choose between CUT&RUN and CUT&Tag?

In CUT&Tag, sequencing primers are being attached to the cleaved DNA and no additional annealing step is necessary. It works well for nucleosomal and tightly bound proteins. The method is also less likely to produce background signal due to DNA damage.
CUT&RUN on the other hand is preferable for transcription factors (complexes) or proteins less tightly bound to DNA. It also has a better resolution than CUT&Tag.

Is it possible to use CUT&Tag with plant tissue samples?

The CUT&Tag method can be applied to plant tissue samples similar to CUT&RUN (see e.g. https://www.ncbi.nlm.nih.gov/pubmed/30719569). An essential step in addition to those lined out in the protocol is the generation of spheroblasts so that it becomes possible to permeabilize the plasma membrane for the application of the antibodies and the MNase fusion protein. Alternatively, use isolated nuclei as sample material.

The positive control H3K27me3 and H3K4me3 antibodies and the negative control guinea pig anti-rabbit IgG antibody as well as the ConA beads are suitable for use with plant samples. The antibodies that are included in some of the kits, such as the anti-DYKDDDDK antibodies or the secondary antibodies, can also be used with plant samples. Whether they are needed or not depends on your experiment.

Can I denature the proteins in the CUT&Tag product complex instead of a proteinase K treatment?

We recommend against this option: the DNA of interest is at his point present in a complex consisting of the DNA, the protein of interest, the corresponding antibody, and the pA/G-Tn5. Boiling this complex will likely precipitate the DNA together with denatured protein. This will also primarily affect the short CUT&Tag products and not larger DNA molecules, leading to a decreased signal to noise ratio in your library and potentially also reducing the library's complexity. This effect is further exacerbated because of the lower melting temperature of these short molecules compared to the longer contaminating DNA molecules.

Can I use AMPure CP beads to purify tagmentation products instead of phenol-chloroform extraction?

In the original publication describing the CUT&Tag method the authors mention the use of AMPure XP beads for the purification of the DNA subsequently to tagmentation and Proteinase K digest. A potential issue is the carry-over of active Proteinase K, which can interfere with the downstream PCR amplification. Therefore, the authors recommend now the phenol-chloroform extraction to assure complete denaturation of Proteinase K.

Why is the DNA yield so low?

CUT&Tag is performed using low cell numbers and the background signal is considerable lower than e.g. for ChIP. Due to these two factors the amount of recovered DNA is often times too low to be reliably measured based on a fluorometric assay or by capillary electrophoresis. PCR amplification of small CUT&RUN products, i.e. less than 50 bp, can be problematic and is therefore not any option.

In order to assess the success of the CUT&Tag method each of the antibodies-online CUT&RUN product sets includes the Positive Control antibodies ABIN6923144 and against the abundant H3K27me3 histone modification. DNA fragments prepared using this antibody can be measured by sensitive electrophoresis on a Bioanalyzer or Tapestation or fluorometrically on a Qubit or Nanodrop fluorometer. When using the CUT&RUN Positive Control ABIN101961 (or any other antibody specific for nucleosomal markers) a ladder corresponding to multiples of the 147 bp long nucleosomes should be visible by capillary electrophoresis.

3. Supplementary Material

Recommended Antibodies for CUT&RUN and CUT&Tag

We are constantly working on extending our list of antibodies that are suitable for CUT&RUN and CUT&Tag. These comprise antibodies against a wide range of histone modifications, pluripotency markers and transcription factors, and enzymes involved in chromatin plasticity.

Click here to see all available CUT&RUN and CUT&Tag antibodies online

Recommended CUT&RUN Antibodies

Product ID	Antibody	Application
ABIN101961	Guinea Pig anti-Rabbit IgG negative control antibody	CUT&RUN
ABIN101785	Rabbit anti-Mouse IgG secondary antibody	CUT&RUN
ABIN6923143	Rabbit anti-DYKDDDDK antibody	CUT&RUN
ABIN6923142	Mouse anti-DYKDDDDK antibody	CUT&RUN
ABIN6939594	Histone H1 antibody	CUT&RUN
ABIN6579169	Histone H2B antibody	CUT&RUN
ABIN6971798	Histone H2B antibody	CUT&RUN
ABIN3023248	Histone 3 (H3K4me) antibody	CUT&RUN
ABIN3023251	Histone 3 (H3K4me) antibody	CUT&RUN
ABIN2668472	Histone 3 (H3K4me3) antibody	CUT&RUN
ABIN3023254	Histone 3 (H3K4me3) antibody	CUT&RUN
ABIN2668475	Histone 3 (H3K27ac) antibody	CUT&RUN
ABIN6952337	Histone 3 (H3K27ac) antibody	CUT&RUN
ABIN6952339	Histone 3 (H3K27me3) antibody	CUT&RUN
ABIN2668403	Histone 3 (H3K36me3) antibody	CUT&RUN
ABIN3434046	Histone 3 (H3K36me3) antibody	CUT&RUN
ABIN2830942	Histone 3 (H3) (acLys56) antibody	CUT&RUN
ABIN6971847	Histone H3.X/Y (AA 9-20) antibody	CUT&RUN
ABIN2668232	BRD3 antibody (C-Term)	CUT&RUN
ABIN2668282	CTCF antibody (N-Term)	CUT&RUN
ABIN2668651	Nanog Homeobox antibody (N-Term)	CUT&RUN
ABIN6972403	Nanog Homeobox antibody (N-Term)	CUT&RUN
ABIN2855042	beta Catenin antibody (N-Term)	CUT&RUN

Recommended CUT&RUN Antibodies (continued)

Product ID	Antibody	Application
ABIN2855074	SOX2 (Center) antibody	CUT&RUN
ABIN2668643	SOX2 (C-Term) antibody	CUT&RUN
ABIN6972778	SOX2 (C-Term) antibody	CUT&RUN
ABIN4283788	BCL9L antibody	CUT&RUN
ABIN560046	BCL9 (AA 1036-1135) antibody	CUT&RUN
ABIN563109	TBX3 (AA 311-410) antibody	CUT&RUN
ABIN6265491	TBX3 (AA 311-410) antibody	CUT&RUN
ABIN2856044	mtRNA Polymerase antibody (Center)	CUT&RUN
ABIN6655366	Pol II (pSer2) antibody	CUT&RUN
ABIN6655367	Pol II (pSer5) antibody	CUT&RUN
ABIN2668768	HDAC1 (AA 1-5) antibody	CUT&RUN
ABIN6971704	HDAC1 (AA 1-5) antibody	CUT&RUN
ABIN6971708	HDAC2 (AA 473-488) antibody	CUT&RUN

Recommended CUT&Tag Antibodies

Product ID	Antibody	Application
ABIN101961	Guinea Pig anti-Rabbit IgG negative control antibody	CUT&Tag
ABIN6971963	anti-Histone 3 (H3) (2meLys4) antibody	CUT&Tag
ABIN6971918	Histone 3 (H3) (3meLys27) antibody	CUT&Tag
ABIN6972014	Histone 3 (H3) (acLys9) antibody	CUT&Tag
ABIN6972040	Histone H1 antibody	CUT&Tag
ABIN6971956	Histone 3 (H3) (meLys4) antibody	CUT&Tag
ABIN6971888	Histone 3 (H3) (acLys27) antibody	CUT&Tag
ABIN6972043	Histone 3 (H3) (3meLys9) antibody	CUT&Tag
ABIN6971554	CTCF antibody (CCCTC-Binding Factor (Zinc Finger Protein))	CUT&Tag
ABIN6971895	Histone 3 (H3) (acLys27) antibody	CUT&Tag
ABIN6971556	CTCF antibody (CCCTC-Binding Factor (Zinc Finger Protein))	CUT&Tag
ABIN101785	Histone 3 (H3K27ac) antibody	CUT&Tag
ABIN101785	Rabbit anti-Mouse IgG (Heavy & Light Chain) Antibody	CUT&Tag

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