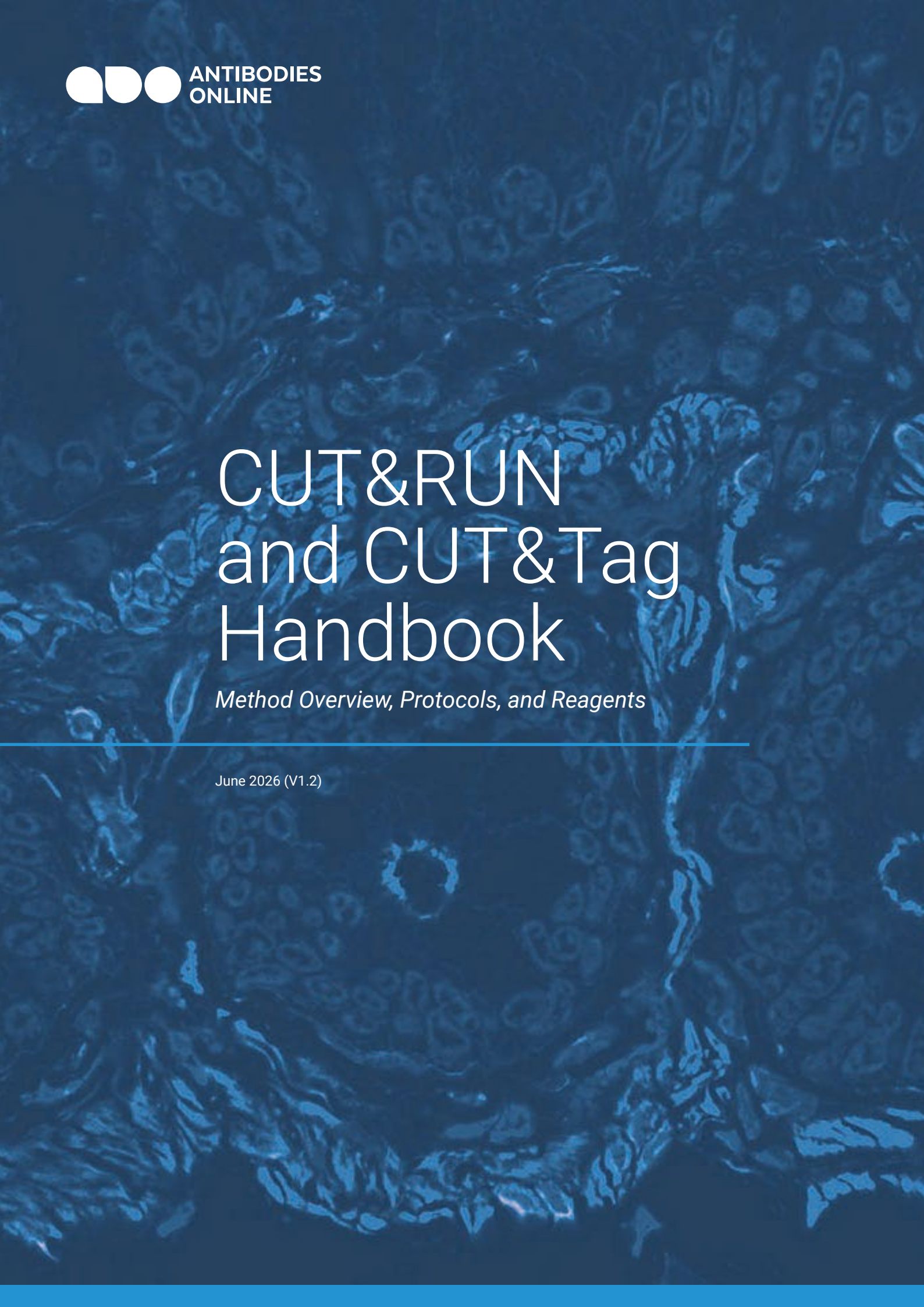

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# CUT&RUN and CUT&Tag Handbook

*Method Overview, Protocols, and Reagents*

June 2026 (V1.2)

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# Introduction

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# Background

CUT&RUN (Cleavage Under Targets and Release Using Nuclease) and CUT&Tag (Cleavage Under Targets and Tagmentation) are two novel methods offering a targeted approach to epigenetics. Both methods are designed to map genome wide transcription factor binding sites, chromatin associated complexes, 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 procedures are provided for:

- CUT&RUN – based on *Skene PJ and Henikoff S (2017) Nature Protocols*
- CUT&RUN Low Salt and Low Volume Urea – based on *Meers MP et al. (2019) eLIFE, Zambanini G et al. (2022) bioRxiv*
- CUT&Tag – based on *Kaya-Okur HS et al. (2019) Nature Communications and Kaya-Okur HS et Kaya-Okur HS et al. (2020) Nature Protocols*

## Overview of Selected Epigenetics Techniques

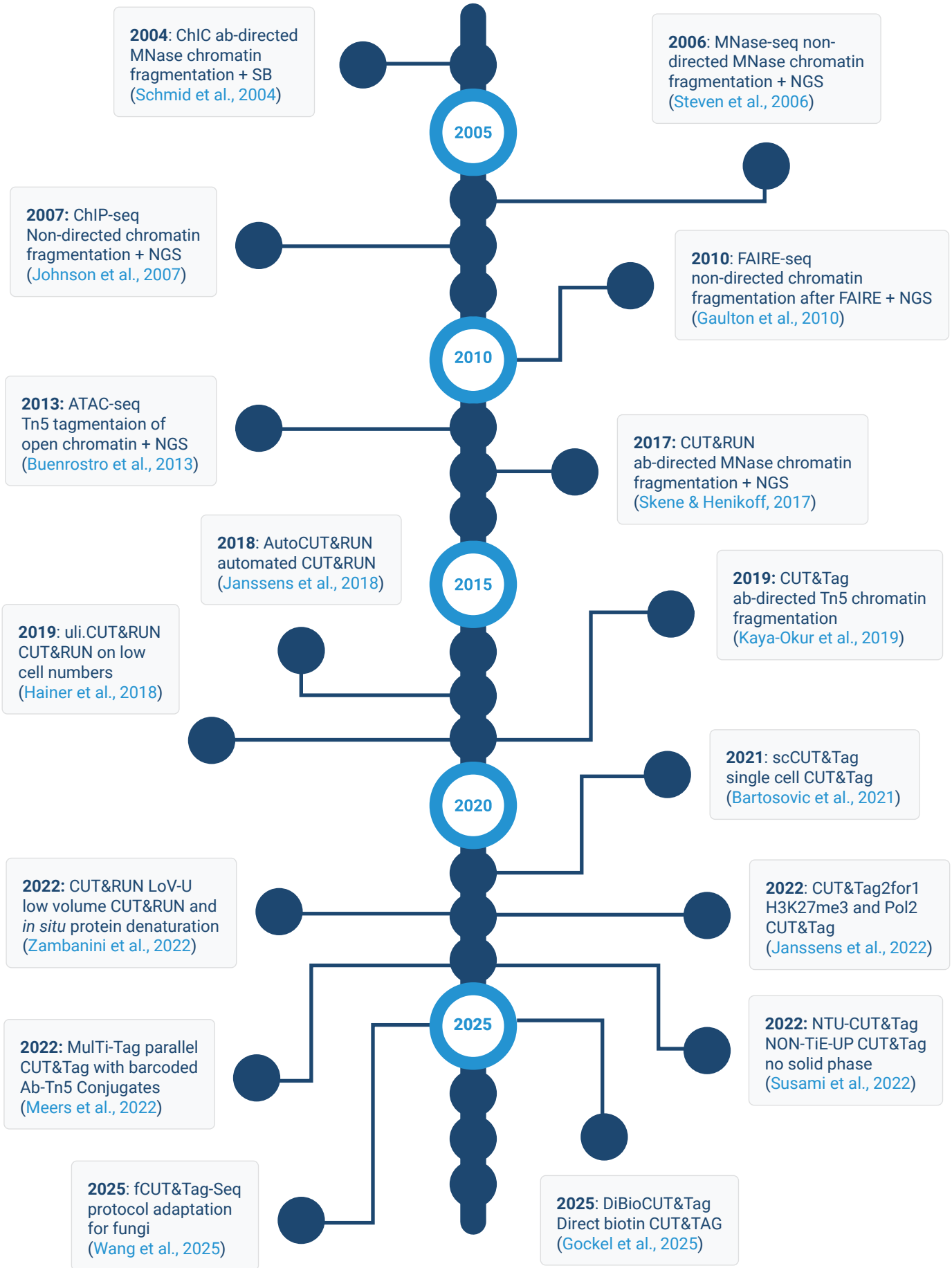
Various methods have been developed to characterize genome-wide interaction sites of chromatin proteins and their influence on chromatin organization and gene expression.

Chromatin Immunocleavage (ChIC) was described in 2004 as a method to generate localized DNA breaks using an antibody guided MNase. It reduced the background signal that plagues Chromatin Immunoprecipitation (ChIP) and improved its resolution. The method's shortcoming is the low throughput readout via Southern blot, which limited its usefulness for mapping genome-wide protein-DNA-interactions. High-throughput sequencing downstream of chromatin fragmentation by different means has been used in DNase-seq, MNase-seq, FAIRE-seq, and most recently, ATAC-seq to assess genome-wide regions of accessible DNA. However, these techniques are not directed towards a particular target of interest.

Chromatin fragmentation with subsequent target enrichment via ChIP combined with high-throughput sequencing gave rise to ChIP-seq. This had been the *de facto* standard for mapping genome-wide protein DNA interactions in spite of several drawbacks, such as fixation that leads to epitope masking and whole cell lysis during DNA fragmentation causing considerable background signal and leading to substantial requirements for the starting material and sequencing depth.

CUT&RUN and CUT&Tag address the issues inherent to the established methods. Many variations of both techniques have already been described for specific uses, illustrating their adaptability.

# Timeline of Selected Epigenetics Techniques



# CUT&RUN

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# About the Method

CUT&RUN (Cleavage Under Targets and Release Using Nuclease) offers a novel approach to pursue epigenetics. The method is designed to map genome wide transcription factor binding sites, chromatin-associated complexes, and histone variants and post-translational modifications.

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

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 106 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.

### Advantages of CUT&RUN

- 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 pAG-MNase 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 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.

## Direct or Indirect CUT&RUN

An antibody specific for the protein of interest is crucial to direct the pAG-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 pAG-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 pAG-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 pAG-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 as untethered pAG-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 Ca2+ and the MNase is free to cut accessible DNA irrespective of its tethered antigen of interest (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 pAG-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 pAG-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 pAG-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.

**For:** $10^4$-$10^6$ cells: use 100 pg/mL
$10^2$-$10^4$ cells: use 2 pg/mL

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 pAG-MNase purification. 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.

# Protocol: Low Salt

! Please read the entire protocol carefully!

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:

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

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

**Additional Notes:**
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.

# CUT&RUN: Low Salt Workflow

**Step 1-7**
Cell harvest

**Steps 8-19**
Cell immobilization

**Steps 20-37**
Cell permeabilization and primary antibody binding

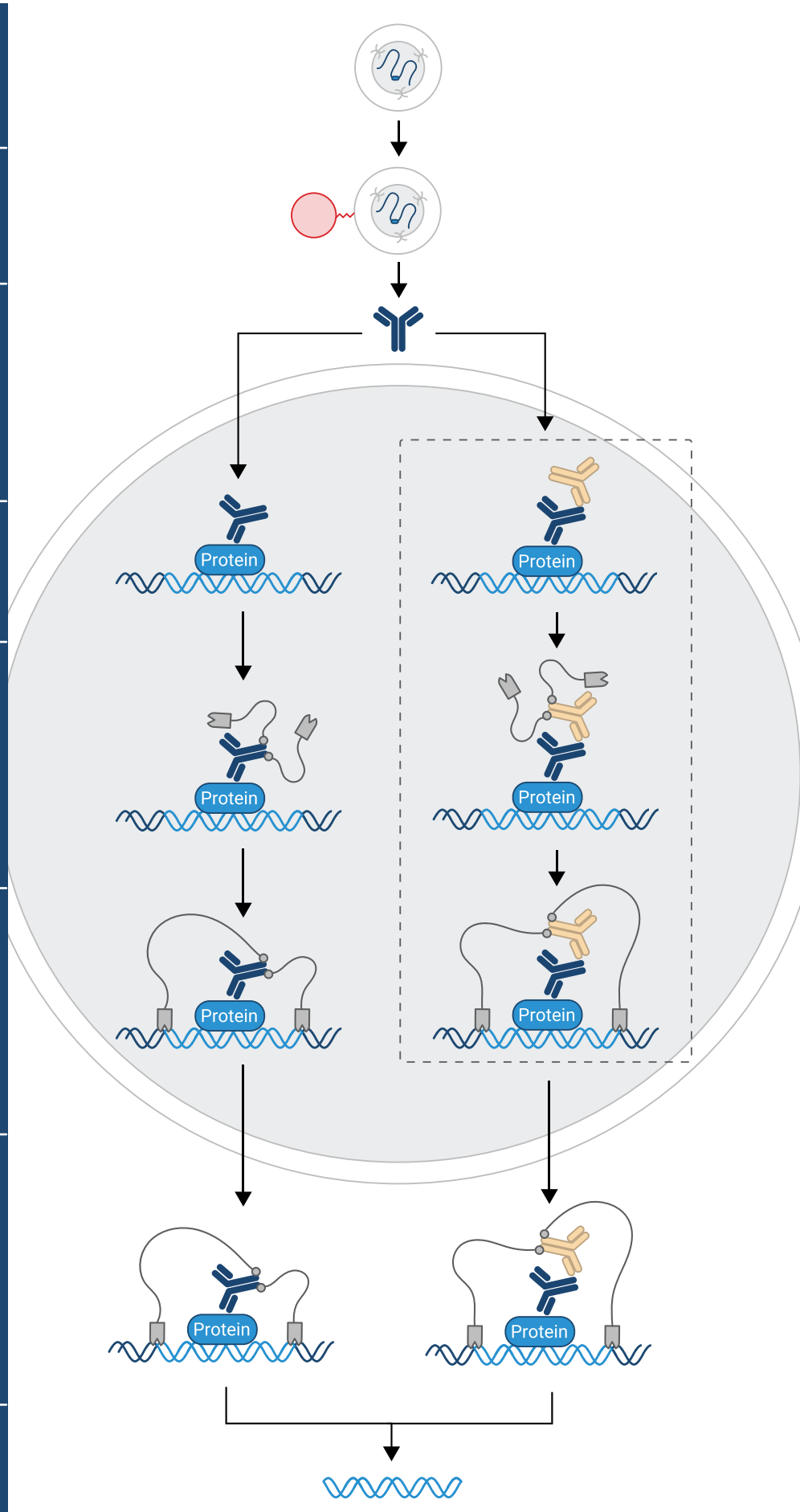
**Steps 38-47**
Secondary antibody binding (optional)

**Steps 48-55**
pAG-MNase binding

**Steps 56-66**
MNase digestion

**Steps 67-69**
Chromatin release

**Steps 70-89**
DNA Extraction



# Reagents Required

| Product | Item No. (if applicable) |
|---|---|
| CUT&RUN Positive Control H3K27me3 | ABIN6923144 |
| CUT&RUN Positive Control Antibody H3K4me3 | ABIN3023254 |
| CUT&RUN Negative Control | ABIN101961 |
| CUT&RUN anti-DYKDDDDK Antibody | ABIN6923143 |
| CUT&RUN Concanavalin A Beads | ABIN6952467 |
| CUTANA™ pAG-MNase for ChIC/CUT&RUN Assays | ABIN6950951 |
| 2.5 M Manganese Chloride ($MnCl_2$) | |
| 1 M Calcium Chloride ($CaCl_2$) | |
| 1 M Potassium Chloride (KCl) | |
| 1 M HEPES pH 7.5 (NaOH) | |
| 5 M NaCl | |
| 0.5 M EDTA | ABIN925554 |
| 0.2 M EGTA | |
| 2 M Spermidine | |
| Protease Inhibitor Cocktail, EDTA-Free | |
| 1.1% Digitonin | ABIN3220553 |
| 10% BSA | |
| 20 mg/mL Glycogen | |
| Trypan Blue | |
| RNase A (DNase and protease free) | |
| 10% Sodium Dodecyl Sulfate (SDS) | ABIN925555 |
| 10 mg/mL Proteinase K | |
| 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 | |

# Reagent Preparation (for 12 samples)

## Binding Buffer (45 mL)

**Note:** Store Binding Buffer for up to six months at 4 °C.

| 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 |

## Wash Buffer (165 mL)

**Note:** Store Wash Buffer without protease inhibitors for up to one week at 4 °C. Add protease inhibitor fresh before use.

| 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 |
| Protease Inhibitor (EDTA-free) 100x | 1.65 mL | 1x |

## Digitonin Wash Buffer (45 mL)

**Note:** 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 permeabilityusing Trypan Blue is recommended to determine the optimal concentration to use.

| Component | Volume | Final Concentration |
|---|---|---|
| 1.1% Digitonin | 3.75 mL | 0.05% |
| Wash Buffer | 78.75 mL | - |

## Antibody Buffer (1.5 mL)

**Note:** Store Antibody Buffer for up to one day at 4°C until use.

| Component | Volume | Final Concentration |
|---|---|---|
| 0.5 M EDTA | 6 µL | 2 mM |
| 10% BSA | 15 µL | 0.10% |
| Digitonin Wash Buffer | 1.5 mL | - |

## EDTA Wash Buffer (3 mL)

| Component | Volume | Final Concentration |
|---|---|---|
| Digitonin Wash Buffer | 3 mL | - |
| 0.5 M EDTA | 12 µL | 2 mM |

## Low Salt Rinse Buffer (27 mL)

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

| Component | Volume | Final Concentration |
|---|---|---|
| $ddH_2O$ | 25.3 mL | - |
| 1 M HEPES pH 7.5 | 540 µL | 20 mM |
| 2 M Spermidine | 6.75 µL | 0.5 mM |
| 1.1% Digitonin | 1,125 µL | 0.05% |

## Low Salt Incubation Buffer (3 mL)

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

| Component | Volume | Final Concentration |
|---|---|---|
| $ddH_2O$ | 2.8 mL | - |
| 1 M HEPES pH 7.5 | 10.5 µL | 3.5 mM |
| 1 M $CaCl_2$ | 30 µL | 10 mM |
| 1.1% Digitonin | 125 µL | 0.05% |

## Low Salt Stop Solution (3 mL)

**Note:** Store Low Salt Stop Solution at 4°C until use. Add Digitonin, RNase A, and Glycogen fresh before use.

| Component | Volume | Final Concentration |
|---|---|---|
| $ddH_2O$ | 2.5 mL | - |
| 5 M NaCl | 102 µL | 170 mM |
| 0.2 M EGTA | 300 µL | 20 mM |
| 1.1% Digitonin | 125 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 |

# Procedure

## 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.

   **Note:** Prepare single cell suspension from your sample material according to your established protocol.

2. Centrifuge cell solution 5 minutes 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 minutes at 600 x g at room temperature and discard the supernatant.
6. Repeat steps 4-5 thrice for a total of four washes.

   **Note:** 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.

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 a new 1.5 mL microcentrifuge tubes; for 12 samples pipette 120 µL bead slurry.
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. Place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.
15. Remove the microcentrifuge tube from the magnet stand.
16. Repeat steps 13-15 twice for a total of three washes.
17. 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; 120 µL for 12 samples.

## III. Cell Immobilization – Binding to Concanavalin A Beads

18. 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.
19. Close tubes tightly and rotate for 5-10 minutes at room temperature.

## IV. Cell Permeabilization and Primary Antibody Binding

20. Place the microcentrifuge tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.
21. Remove the microcentrifuge tubes from the magnet stand.
22. Resuspend the beads in 100 µL Wash Buffer and incubate for 5 minutes at room temperature.
23. Place the tubes on the magnet stand until the fluid is clear. Remove the liquid carefully.
24. Remove the microcentrifuge tubes from the magnet stand.
25. Resuspend each sample in 200 µL EDTA Wash Buffer and incubate for 5 minutes at room temperature.

    **Note:** The presence of EDTA in the EDTA Wash Buffer and Antibody Buffer assures that no bivalent cations are carried over from the Binding Buffer which might cause premature MNase digestion during later steps.

26. Place the microcentrifuge tubes on the magnet stand until the fluid is clear. Remove the liquid carefully.
27. 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.

    **Note:** The presence of EDTA in the EDTA Wash Buffer and Antibody Buffer assures that no bivalent cations are carried over from the Binding Buffer which might cause premature MNase digestion during later steps.

28. Gently vortex the microcentrifuge tubes until the beads are resuspended.
29. For the positive control, add 5 µL CUT&RUN rabbit anti-H3K4me3 IgG Positive Control corresponding to a 1:20 dilution to

the corresponding tube.

30. For the negative control, add 1 µL CUT&RUN guinea pig anti-rabbit IgG Negative Control corresponding to a 1:100 dilution to the corresponding tube.
31. When using one of the CUT&RUN rabbit anti-DYKDDDDK antibodies, add 5 µL corresponding to a 1:20 dilution to the corresponding tube.
32. 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).
33. Rotate the microcentrifuge tubes for 2 hours at room temperature or 4 hours to overnight at 4 °C.
34. Spin down the liquid and place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.

    **Note:** The quick spin minimizes carry-over of antibody and pAG-MNase that could result in overall background cleavage during the digestion step.

35. Remove the microcentrifuge tubes from the magnet stand.
36. Resuspend with 1 mL Digitonin Wash Buffer and mix by inversion. If clumping occurs, gently remove the clumps with a 1 mL pipette tip.
37. Repeat steps 34-36 once for a total of two washes.

    **Note:** Washing out EDTA before pAG-MNase addition prevents removal of divalent cations necessary for MNase nuclease acid cleavage.

## V. (Optional) Anti-rabbit Secondary Antibody Binding

Binding of the CUT&RUN guinea pig anti-rabbit IgG Negative Control antibody in addition to rabbit primary 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 48.

38. Place the relevant tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.
39. Remove the microcentrifuge tubes from the magnet stand.
40. 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.
41. Tap to remove the remaining beads from the tube side.
42. 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.
43. Nutate the microcentrifuge tubes for 1 hours at 4 °C.
44. Spin down the liquid and 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. Resuspend with 1 mL Digitonin Wash Buffer and mix by inversion. If clumping occurs, gently remove the clumps with a 1 mL pipette tip.
47. Repeat steps 44-46 once for a total of two washes.

    **Note:** Washing out EDTA before pAG-MNase addition prevents removal of divalent cations necessary for MNase nuclease acid cleavage.

## VI. pAG-MNase Binding

48. 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. 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.

    **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 pAG-MNase preparation per sample along the side of the tube.

    **Note:** It is important that no divalent cations are present during the pAG-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 pAG-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.

51. Nutate the microcentrifuge tubes for 1 hours at 4 °C.
52. Spin down the liquid and place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.
53. Remove the microcentrifuge tubes from the magnet stand.
54. Resuspend with 1 mL Digitonin Wash Buffer and mix by inversion. If clumping occurs, gently remove the clumps with a 1 mL pipette tip.
55. Repeat steps 52-54 once for a total of two washes.

## VII. MNase Digestion and Release of pAG-MNase-antibody-chromatin Complexes

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. Remove the liquid carefully.
58. 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.
59. Spin down the liquid from the lid with a quick pulse in a table-top centrifuge (max. 100 x g)
60. Place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.
61. Repeat steps 58-60 once for a total of two washes.
62. 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.

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

63. Incubate tubes at 0 °C for the desired time (default is 30 minutes).
64. Place the tubes on a cold magnet stand until the fluid is clear. Remove the liquid carefully.
65. Remove the microcentrifuge tubes from the magnet stand.
66. Resuspend with 200 µL Low Salt Stop Solution and mix by gentle vortexing.
67. Incubate tubes at 37 °C for 30 minutes.

    **Note:** Digestion times typically vary between 5 minutes and 30 minutes. In case you observe excessive background signal use shorter incubation times.

68. Place the tubes on a magnet stand until the fluid is clear.
69. Transfer the supernatant containing the pAG-MNase-bound digested chromatin fragments to fresh 1.5 mL microcentrifuge tubes.

    **Note:** 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 pAG-MNase fusion protein has been shown to be a viable calibration standard replacing spike-in DNA in the Stop Buffer. It is digested by the MNase and released at the same time as the sample chromatin DNA. Keep the Concanavalin A bead-bound cells at -20 °C for troubleshooting in case of a low DNA yield.

## VIII. DNA Extraction

70. Add 2µL 10% SDS to a final concentration of 0.1% and 5 µL Proteinase K (10mg/mL) to a final concentration of 0.25 mg/mL to each supernatant from step 69.
71. Gently vortex tubes at a low speed of approx. 1,100 rpm.
72. Incubate tubes at 50 °C for 1 hours or at 37 °C overnight.
73. Add 200 µL PCI to tube.
74. Vortex tubes thoroughly at high speed until the liquid appears milky.
75. Centrifuge tubes in a table-top centrifuge at 16,000 x g at 4 °C for 5 minutes.
76. Carefully transfer the upper aqueous phase to a fresh 1.5 mL microcentrifuge tube containing 200 µL Chloroform:Isoamyl Alcohol 24:1.
77. Vortex tubes thoroughly at high speed until the liquid appears milky.
78. Centrifuge tubes in a tabletop centrifuge at 16,000 x g at 4 °C for 5 minutes.
79. 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).

80. Add 20 µL 3 M NaOAc pH 5.2 or 100 µL 5 M NH4OAc.
81. Add 500 µL 100% ethanol.
82. Place tubes for 10 minutes in a dry ice/ethanol mix or overnight at -20 °C.
83. Centrifuge tubes in a table-top centrifuge at 16,000 x g at 4 °C for 5 minutes.
84. Remove the liquid carefully with a pipette.
85. Add 1mL 70% ethanol.
86. Centrifuge tubes in a table-top centrifuge at 16,000 x g at 4 °C for 1 minutes.
87. Remove the liquid carefully with a pipette.
88. Air-dry the pellet or dry the pellet in a SpeedVac.
89. 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 workflow. Because of the very low background with CUT&RUN, typically 5 million paired-end reads is sufficient 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. Alternatively, the Orkin and Yuan labs have streamlined processing of CUT&RUN data using their CUT&RUN Tools pipeline.

# Protocol: Low Volume Urea

The standard CUT&RUN protocol is primarily intended for profiling of histone proteins and transcription factors that bind to chromatin. Reliable detection of rare transcription factors, transient interactions, or proteins in complexes that are not directly associated with DNA have proven difficult using CUT&RUN on uncrosslinked samples. Preserving protein-protein and DNA-protein interactions using crosslinkers on the other hand can introduce artifacts.

CUT&RUN Low Volume and Urea (CUT&RUN Lov-U) is a modification of the original procedure aimed at these difficult to characterize interactions in situ while avoiding crosslinking. Initial nuclear extraction minimizes sequestration of the primary antibody and pAG-MNase by non-not chromatin-bound targets in the cytosol. Low volumes throughout the protocol facilitate parallel processing of samples which benefits scalability and reproducibility. Lastly, urea is used as chaotropic reagent to denature samples in situ and release CUT&RUN products, followed by DNA clean-up on beads.

## Advantages of CUT&RUN LoV-U

- *In situ* profiling of rare transcription factors and non-direct DNA binding cofactors which are part of larger transcription regulation complexes.
- Nuclear extraction and in situ protein degradation using urea improve recovery of DNA fragments after MNase digest.
- Low volumes facilitate simultaneous processing of a higher number of samples, thus reducing costs and increase throughput and reproducibility.

# CUT&RUN: Low Volume Urea Workflow

**Step 1-8**
Cell harvest and nuclear extraction

**Steps 9-19**
Nuclei immobilization

**Steps 20-31**
Primary antibody binding

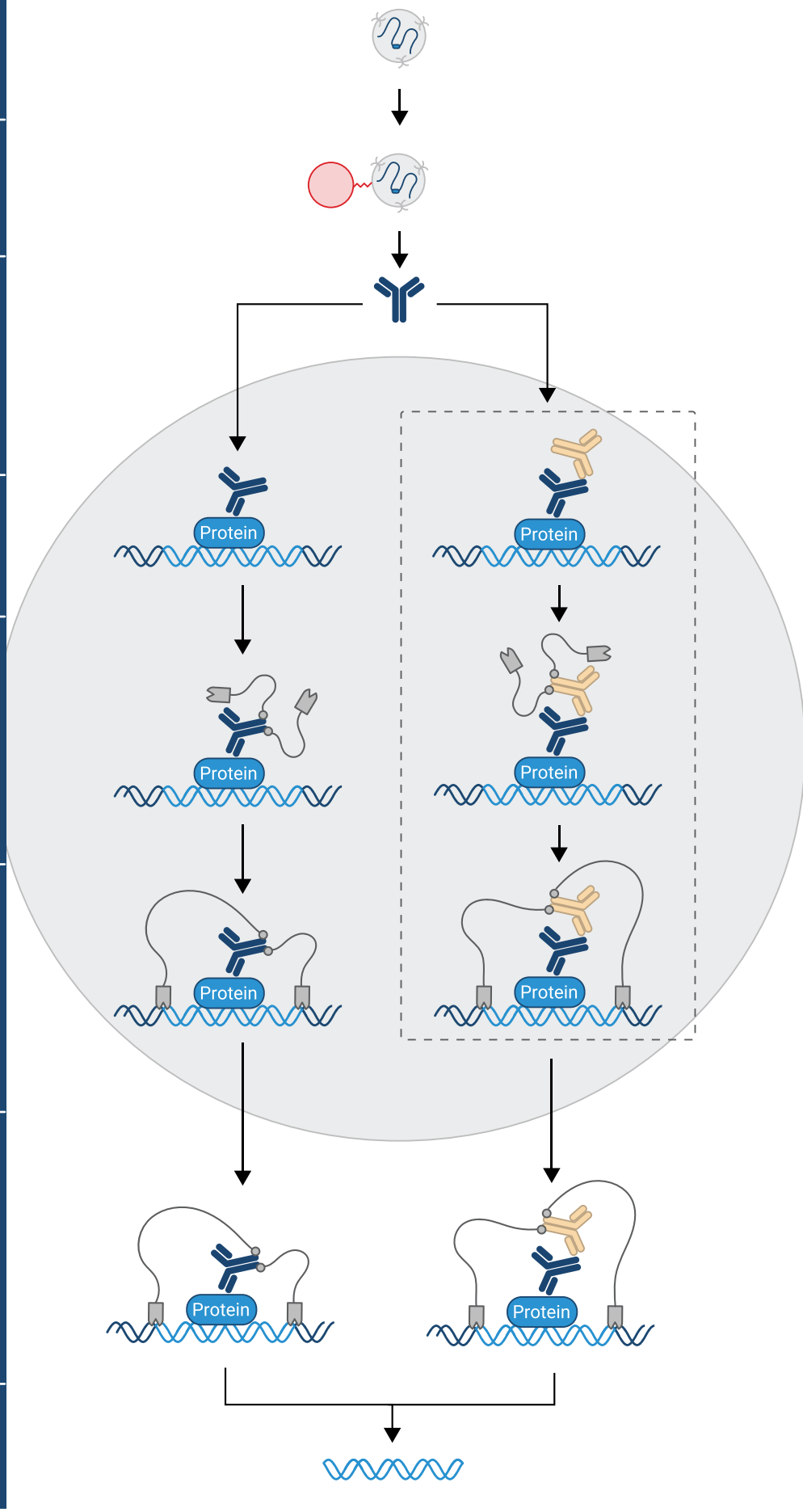
**Steps 32-39**
Secondary antibody binding (optional)

**Steps 40-57**
pAG-MNase binding

**Steps 58-62**
MNase digestion

**Steps 63-64**
Chromatin release

**Steps 65-82**
DNA Extraction



# Reagents Required

| Product | Item No. (if applicable) |
|---|---|
| Distilled, deionized or RNase-free $H_2O$ | |
| CUT&RUN Positive Control | ABIN6923144 |
| CUT&RUN Negative Control | ABIN101961 |
| CUT&RUN rabbit anti-DYKDDDDK Antibody | ABIN6923143 |
| CUT&RUN Concanavalin A Beads | ABIN6952467 |
| CUTANA™ pAG-MNase for ChIC/CUT&RUN Assays | ABIN6950951 |
| 2.5 M Manganese Chloride ($MnCl_2$) | |
| 1 M HEPES pH 8.2 (NaOH) | |
| 1 M HEPES pH 7.5 (NaOH) | |
| 1 M Potassium Chloride (KCl) | |
| 1 M Calcium Chloride ($CaCl_2$) | |
| 2.5 M $MnCl_2$ | |
| IGEPAL | |
| 5 M NaCl | |
| 2 M Spermidine | |
| Protease Inhibitor Cocktail, EDTA-Free | |
| 8.8 M Urea | |
| 0.5 M EDTA | ABIN925554 |
| 0.2 M EGTA | |
| Mag-Bind® TotalPure NGS beads (Omega Bio-Tek, M1378-01) | |
| 10 mM Tris-HCl pH 8.2 | |
| EtOH 80% | |

# Reagents Preparation (for 12 samples)

## Nuclear Extraction Buffer (75 mL)

**Note:** Store Wash Buffer without protease inhibitors for up to one week at 4 °C.

| Component | Volume | Final Concentration |
|---|---|---|
| $ddH_2O$ | 42.25 mL | - |
| 1 M HEPES-KOH pH 8.2 | 1.25 mL | 20 mM |
| 1 M KCl | 750 µL | 10 mM |
| 100% IGEPAL | 37.5 µL | 0.05% |
| Glycerol | 37.5 µL | 20% |
| 2 M Spermidine | 18.75 µL | 0.5 mM |
| Protease Inhibitor (EDTA-free) 100x | 750 µL | 1x |

**Note:** Add protease inhibitors fresh before use.

## Binding Buffer (40 mL)

**Note:** Store Binding Buffer for up to six months at 4 °C.

| Component | Volume | Final Concentration |
|---|---|---|
| $ddH_2O$ | 38.8 ml | - |
| 1 M HEPES pH 7.5 | 800 µl | 20 mM |
| 1 M KCl | 400 µl | 10 mM |
| 1 M $CaCl_2$ | 40 µl | 1 mM |
| 2.5 M $MnCl_2$ | 16 µl | 1 mM |

## Wash Buffer (50 mL)

**Note:** Store Wash Buffer without protease inhibitors for up to one week at 4 °C.

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

**Note:** Add protease inhibitors fresh before use.

## EDTA Wash Buffer (3 mL)

| Component | Volume | Final Concentration |
|---|---|---|
| Wash Buffer | 3 mL | - |
| 0.5 M EDTA | 12 µL | 2 mM |

## 1x Urea STOP Buffer (50 mL)

**Note:** Store Wash Buffer without protease inhibitors for up to one week at 4 °C.

| Component | Volume | Final Concentration |
|---|---|---|
| 8.8 M Urea | 961 µL | 8.5 M |
| 5 M NaCl | 20 µL | 100 mM |
| 0.5 M EDTA | 4 µL | 2 mM |
| 0.2 M EGTA | 10 µL | 2 mM |
| 100% IGEPAL | 5 µL | 0.50% |

# Procedure

## I. Cell Harvest and Nuclear Extraction – at Room Temperature

1. Harvest 50,000-500,000 cells for each sample at room temperature.

   **Note:** The protocol is suitable for single adherent cells, cells in suspension, or cells prepared from tissue. In latter case, prepare a single cells suspension from your sample material according to your established protocol.

2. Centrifuge cell solution for each sample in separate 2 mL microcentrifuge tubes for 5 minutes at 600 x g at room temperature.
3. Remove the liquid carefully.
4. Gently resuspend cells in 2 mL of Nuclear Extraction Buffer.
5. Pellet the nuclei 5 minutes at 800 x g at room temperature and discard the supernatant.
6. Repeat steps 4-5 twice for a total of three washes.
7. Resuspend the nuclei from each sample in 20 µL Nuclear Extraction Buffer.
8. Transfer the nuclei solution for each sample to a fresh 1.5 mL microcentrifuge tube and store on ice.

## II. Concanavalin A Beads Preparation

9. Gently resuspend the CUT&RUN Concanavalin A Beads.
10. Pipette 20 µL CUT&RUN Concanavalin A Beads slurry for each sample into a new 1.5 mL microcentrifuge tube; for 12 samples pipette 240 µL bead slurry.
11. Place the tube on a magnet stand until the fluid is clear. Remove the liquid carefully.
12. Remove the microcentrifuge tube from the magnetic stand.
13. Pipette 1 mL Binding Buffer into the tube and resuspend CUT&RUN Concanavalin A Beads by gentle pipetting.
14. Place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.
15. Remove the microcentrifuge tube from the magnetic stand.
16. Repeat steps 13-16 twice for a total of three washes.
17. Gently resuspend the CUT&RUN Concanavalin A Beads in a volume of Binding Buffer corresponding to the original volume of bead slurry, i.e. 20 µL per sample; 240 µL for 12 samples.

## III. Nuclei Immobilization – Binding to Concanavalin A beads

18. Carefully vortex the nuclei suspension from step 8 and add 20 µL CUT&RUN Concanavalin A Beads in Binding Buffer from step 18 to each sample.
19. Close tube tightly and incubate for 15 minutes at 4 °C.

## IV. Primary Antibody Binding

20. Place the microcentrifuge tube for each sample on the magnet stand until the liquid is clear. Remove the liquid carefully.
21. Remove the microcentrifuge tubes from the magnetic stand.
22. Resuspend the beads in 100 µL Wash buffer and incubate for 5 minutes at room temperature.
23. Place the tubes on the magnet stand until the fluid is clear. Remove the liquid carefully.
24. Resuspend each sample in 200 µL EDTA Wash buffer and incubate for 5 minutes at room temperature.

    **Note:** A wash step with EDTA Wash Buffer assures that no bivalent cations are carried over from the binding buffer which might premature MNase digestion during later steps.

25. Place the microcentrifuge tubes on the magnet stand until the fluid is clear. Remove the liquid carefully.
26. Resuspend each sample in 200 µl Wash Buffer.
27. For the positive control, add 10 µL CUT&RUN rabbit anti-H3K27me3 IgG Positive Control corresponding to a 1:20 dilution (final antibody concentration 0.02 µg/µL).
28. For the negative control, add 2 µL CUT&RUN CUT&RUN guinea pig anti-rabbit IgG Negative Control corresponding to a 1:100 dilution.
29. When using the CUT&RUN rabbit anti-DYKDDDDK antibody, add 10 µL corresponding to a 1:20 dilution to the corresponding tube.
30. For the remaining samples, add 2 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.

    **Note:** If no recommendation is available for the antibody's use in CUT&RUN select a dilution corresponding to the manufacturer's recommended dilution for immunofluorescence or a similar *in situ* method.

31. Nutate the microcentrifuge tubes for 1 hours at room temperature or overnight at 4 °C.

## V. (Optional) Anti-rabbit Secondary Antibody Binding

**Note:** Binding of the CUT&RUN guinea pig anti-rabbit IgG Negative Control antibody in addition to rabbit primary 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 40.

32. Place the relevant tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.
33. Resuspend each sample with 200 µL Wash Buffer and mix carefully using a pipette.
34. Place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.
35. Remove the microcentrifuge tubes from the magnetic stand.
36. Repeat steps 33-35 four times for a total of five washes.
37. Resuspend each sample with 200 µL Wash Buffer.
38. Add 2 µL CUT&RUN CUT&RUN guinea pig anti-rabbit IgG Negative Control antibody ABIN101961 corresponding to a 1:100 dilution.
39. Nutate the microcentrifuge tubes for 1 hours at 4 °C.

## VI. pAG-MNase Binding

40. Place the tubes from steps 31 and step 39 on a magnet stand until the fluid is clear. Remove the liquid carefully.
41. Remove the microcentrifuge tubes from the magnetic stand.
42. Resuspend each sample with 200 µL Wash Buffer and mix carefully using a pipette.
43. Transfer the beads solution to 200 µL PCR tubes in strips.

    **Note:** Working with 200 µL offers to possibility to use a multi-pipette to speed up processing of multiple samples. It is also possible to continue with microcentrifuge tubes. In this case, reduce the number of wash steps and increase the volume per wash step to reach the same buffer volume per wash (e.g. twice 500 µL instead of five times 200 µL for a total volume of 1 mL).

44. Place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.
45. Remove the tubes from the magnetic stand.
46. Repeat steps 42-45 four times for a total of five washes.
47. Resuspend the samples in 100 µL Wash Buffer per sample and keep on ice.
48. Prepare a 1.5 mL microcentrifuge tube containing 100 µL Wash Buffer and 0.12 µg CUTANA™ pAG-MNase for ChIC/CUT&RUN Assays for each sample.
49. Add 100 µL Wash Buffer/pAG-MNase mix to each sample from step 47.
50. Nutate the samples for 30 minutes at 4 °C.
51. Place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.
52. Remove the tubes from the magnetic stand.
53. Resuspend each sample with 200 µL Wash Buffer and mix carefully using a pipette.
54. Place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.
55. Remove the tubes from the magnetic stand.
56. Repeat steps 53-55 four times for a total of five washes.
57. Resuspend in 100 µL of Wash Buffer.

    **Note:** For targets with a small footprint resuspend the samples in 50 µL Wash Buffer each.

## VII. MNase Digestion and Release of pAG-MNase-antibody-chromatin Complexes

**Note:** Samples must be kept at 4 °C during MNase digestion and fragment release.

58. Place PCR tubes on ice for 5 minutes and allow to chill.
59. Add 2 µL 100 mM CaCl2 per sample corresponding to a 1:50 dilution and mix carefully using a pipette.
60. Incubate on ice for exactly 30 minutes.
61. Place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.

    **Note:** For targets with a small footprint collect the supernatant and add EDTA and EGTA to 2 mM final concentration

each to stop the reaction.
62. Resuspend each sample in 50 µl 1x Urea STOP Buffer.
63. Nutate the samples for 1 hours at 4°C.
64. Transfer the supernatant containing the pAG-MNase-bound digested chromatin fragments to fresh 200 µL tubes. Proceed immediately to DNA clean-up to avoid DNA denaturation by the highly concentrated urea.

## VIII. DNA Clean-up

65. Warm up Mag-Bind® TotalPure NGS beads to room temperature.
66. Add 2x volume of beads to each sample, i.e. 100 µL Mag-Bind® TotalPure NGS beads to each 50 µL sample from step 64. Mix well using a pipette or vortex.
67. Incubate the beads and the samples for 15 minutes at room temperature.
68. During the incubation step prepare 200 µL fresh EtOH 80% per sample.
69. Place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.
70. Without removing the tubes from the magnet stand, add 200 µl freshly prepared 80% EtOH to the sample without disturbing the bead; do NOT resuspend the beads or remove the tubes from the magnet stand to avoid sample loss.
71. Incubate the beads and the samples for 30 sec at room temperature.
72. Remove the EtOH carefully and dry beads for 5 minutes at room temperature.
73. For each sample resuspend the beads in 25 µL 10 mM Tris-HCl pH 8.2.
74. Incubate the samples for 5 minutes at room temperature.
75. Add 2x volume of beads to each sample, i.e. 50 µL Mag-Bind® TotalPure NGS beads to each 25 µL sample. Mix well using a pipette or vortex.
76. Without removing the tubes from the magnet stand, add 200 µL freshly prepared 80% EtOH to the sample without disturbing the bead.
77. Incubate the beads and the samples for 30 sec at room temperature.
78. Remove the EtOH carefully and repeat steps 76-77 once for a total of two washes.
79. Remove the EtOH carefully and dry beads for 5 minutes at room temperature.
80. For each sample resuspend the beads in 20 µL 10 mM Tris-HCl pH 8.2.
81. Incubate the samples for 5 minutes at room temperature.
82. Place the tubes on a magnet stand and when the liquid is clear transfer 20 µL of each sample to a new 1.5 mL microcentrifuge tube.

## IX. Sample Quality Control (Optional)

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.

## X. Sequencing Library Preparation

The preparation of sequencing libraries from CUT&RUN DNA fragments comprises end repair and A-tailing, ligation of sequencing adapters, a post-ligation clean-up, PCR amplification of the library, and a post-PCR clean-up step. Commercial kits such as the KAPA HyperPrep Kit (Roche, KR0961) or the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (NEB, E7645) are convenient all-in-one solutions suitable for CUT&RUN. Alternatively, libraries can be prepared with separate components using PCR and clean-up conditions optimized for the expected product sizes; e.g. approximately 250 bp for nucleosomal DNA plus sequencing adapters. Quantify sequencing library concentration and size distribution using a Qubit or Nanodrop fluorometer or a Bioanalyzer or Tapestation.

## XI. Sequencing and Data Analysis

Because of the very low background with CUT&RUN, a low coverage with approximately 5 to 10 million short (i.e. 25-36 bp) paired-end reads per sample suffice even for difficult CUT&RUN targets. Single-end sequencing should be avoided because information regarding the protein of interest's footprint and position is lost.
Trim sequencing reads to remove adapters, artifacts and repeat sequences. Align reads to the relevant genome e.g. using Bowtie 2. 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. Alternatively, the Orkin and Yuan labs have streamlined processing of CUT&RUN data using their CUT&RUN Tools pipeline.

# CUT&Tag

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Protocol

# About the Method

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 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 pAG-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.

## Advantages of CUT&Tag

- 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

# Protocol

This protocol adapted from the original CUT&Tag protocol 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”.

**Additional Notes:**
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.

# CUT&Tag Workflow

**Steps 7-15**
Cell harvest

**Steps 16-26**
Cell immobilization

**Steps 27-38**
Cell permeabilization and primary antibody binding

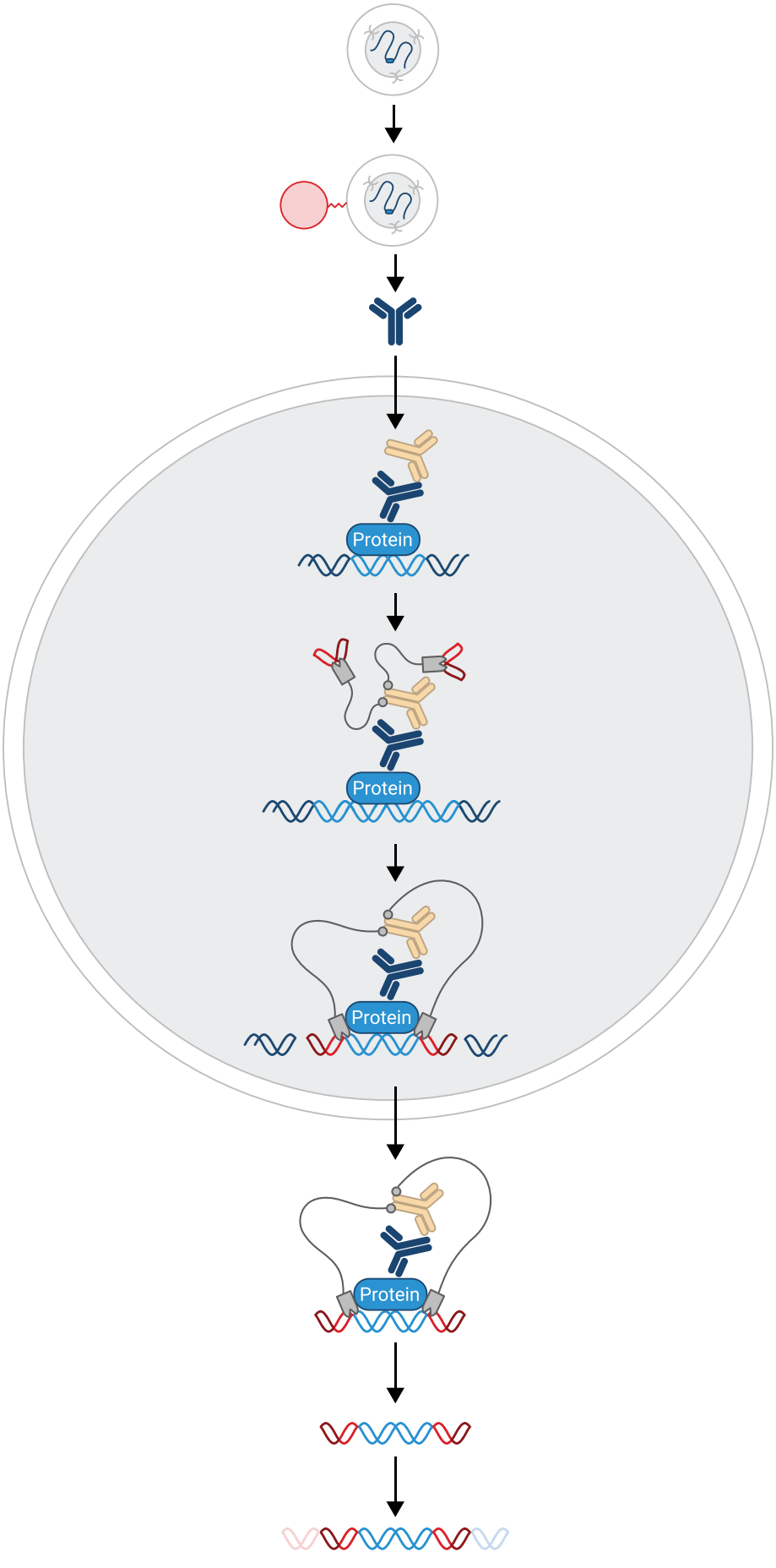
**Steps 39-46**
Secondary antibody binding

**Steps 47-55**
pAG-Tn5 binding

**Steps 56-61**
Tagmentation and chromatin release

**Steps 62-76**
DNA extraction

**Steps 77-91**
PCR amplification



# Reagents Required

| Product | Item No. (if applicable) |
|---|---|
| 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 (pAG-Tn5) preloaded | |
| Protein A and/or protein G Tn5 fusion protein (pAG-Tn5) unloaded | |
| Distilled, deionized or RNase-free $H_2O$ | |
| 2.5 M Manganese Chloride ($MnCl_2$) | |
| 1 M Calcium Chloride ($CaCl_2$) | |
| 1 M Potassium Chloride (KCl) | |
| 1 $MgCl_2$ | ABIN925573 |
| 1 M HEPES pH 7.5 HEPES (NaOH) | |
| 5 M NaCl | |
| 0.5 M EDTA | ABIN925554 |
| 2 M Spermidine | |
| Protease Inhibitor Cocktail, EDTA-free | |
| 1.1% Digitonin | ABIN3220553 |
| Trypan Blue | |
| 10% Sodium dodecyl sulfate (SDS) | ABIN925555 |
| 10 mg/mL Proteinase K | |
| Phenol-chloroform-isoamyl alcohol (PCI) | |
| Chloroform:Isoamyl Alcohol 24:1 | |
| 100% Ethanol | |
| 80% Ethanol | |
| 10 mM Tris-HCl pH 8.0 | ABIN5706418 |
| TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA) | |
| 10 mg/mL RNase A (DNase and protease free) | |

# Reagent Preparation (for 12 samples)

## Binding Buffer (5 mL)

**Note:** Store Binding Buffer for up to six months at 4°C.

| Component | Volume | Final Concentration |
|---|---|---|
| ddH2O | 4.85 mL | - |
| 1 M HEPES pH 7.5 | 100 µL | 20 mM |
| 1 M KCl | 50 µL | 10 mM |
| 1 M CaCl2 | 5 µL | 1 mM |
| 2.5 M MnCl2 | 2 µl | 1 mM |

## Wash Buffer (70 mL)

**Note:** Add protease inhibitor fresh before use.

| Component | Volume | Final Concentration |
|---|---|---|
| ddH2O | 66 mL | - |
| 1 M HEPES pH 7.5 | 1.4 mL | 20 mM |
| 5 M NaCl | 2.1 mL | 150 mM |
| 2 M Spermidine | 17.5 µL | 0.5 mM |
| Protease Inhibitor (EDTA-free) 100x | 700 µL | 1x |

**Note:** 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)

**Note:** 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 permeabilityusing Trypan Blue is recommended to determine the optimal concentration to use.

| Component | Volume | Final Concentration |
|---|---|---|
| 1.1% Digitonin | 1 mL | 0.03% |
| Wash Buffer | 44 mL | - |

## Antibody Buffer (1.5 mL)

**Note:** Store Antibody Buffer for up to one day at 4°C until use.

| Component | Volume | Final Concentration |
|---|---|---|
| 0.5 M EDTA | 6 µL | 2 mM |
| 10% BSA | 15 µL | 0.10% |
| Digitonin Wash Buffer | 1.5 mL | - |

## Dig-300 Buffer (48 mL)

**Note:** 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

| Component | Volume | Final Concentration |
|---|---|---|
| ddH2O | 43.4 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 |
| Protease Inhibitor Cocktail | 480 µL | 1x |
| (EDTA-free) 100x | | |
| 1.1% Digitonin | 400 µL | 0.01% |

## Tagmentation Buffer (4.2 mL)

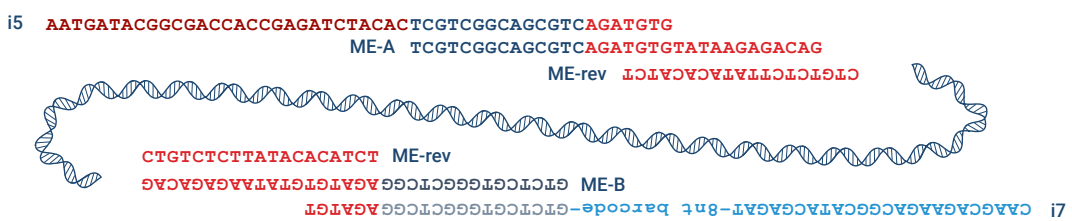
**Note:** Store Dig-300 Buffer without protease inhibitors and Digitonin for up to one week at 4°C. Add protease inhibitor and Digitonin fresh before use

| Component | Volume | Final Concentration |
|---|---|---|
| Dig-300 Buffer | 4.2 mL | - |
| 1 M MgCl2 | 42 µl | 10 mM |

## Oligonucleotides (for Illumina)[5]

**Note:** 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

| Component | 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].

# Procedure

## I. (Optional) pAG-Tn5 adapter complex assembly

**Note:** This section is optional and is only relevant when using unloaded pAG-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 minutes.
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 (pAG-Tn5) unloaded.
6. Rotate the mixture for 1 hours 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. $1.3x10^6$ cells for 10 samples and the two controls.

   **Note:** Prepare single cell suspension from your sample material according to established protocol. Cells can be used directly harvested from fresh cultures or cryopreserved with10% DMSO as cryoprotectant. Avoid flash freezing, as this can cause undesired DNA breakage, thus increasing background.

8. Centrifuge cell solution 3 minutes 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 minutes 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 minutes 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 minutes 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.

    **Note:** EDTA in the Antibody Buffer chelates excess divalent cations in the Binding Buffer used to activate Concanavalin A. This prevents carry-over of Ca2+ and Mn2+ which could trigger premature tagmentation by the pAG-Tn5 adapter complex and endogenous DNase activity.

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.

    **Note:** 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.

35. For the remaining samples, add 1 µL primary rabbit antibody against your protein of interest corresponding to a 1:100 dilution.

    **Note:** 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.

36. Nutate the microcentrifuge tubes for 2 hours 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.

    **Note:** 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.

42. Rotate the microcentrifuge tubes for 1 hour 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.

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

## VII. pAG-Tn5 Adapter Complex Binding

47. Dilute the pAG-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 pAG-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.

    **Note:** 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.

51. Rotate the microcentrifuge tubes for 1 hour 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 hours 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 hours 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.

    **Note:** 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.

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 minutes.
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 minutes.
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 minutes.

## 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).

    **Note:** 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.

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: 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.

**Note:** 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.

| Step | Temperature | Time |
|---|---|---|
| 1 | 58°C | 5 minutes |
| 2 | 72°C | 30 seconds |
| 3 | 98°C | 30 seconds |
| 4 | 98 °C | 10 seconds |
| 5 | 60°C | 10 seconds |
| 6 | Go to step 4 | 14 times |
| 7 | 72°C | 1 minute |
| 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.

    **Note:** 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.

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 minutes.
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/ .

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# Frequently Asked Questions

## 1. How do I choose between CUT&RUN and CUT&Tag?

Advantages of CUT&RUN and CUT&Tag compared to ChIP-seq are a better signal-to-noise ratio, higher sensitivity, a wider dynamic range, a lower requirement of sequencing reads and cell number.

CUT&Tag has the advantage that the sequencing primers are being attached to the cleaved DNA fragments and requires fewer library preparation steps than CUT&RUN. No additional annealing is necessary. The method works particularly well for nucleosomal and tightly bound proteins. It has also been streamlined by the Henikoff lab into a protocol where the entire process takes place in one tube and high throughput variations amenable for automation are available.

CUT&RUN on the other hand is preferable for transcription factors and other less tightly bound DNA binding proteins that are sensitive to the higher salt concentration in CUT&Tag necessary to prevent off-target tagmentation of accessible chromatin by Tn5. In addition, the spatial resolution of the MNase digestion is higher than that of the tagmentation, enabling a clearer footprint of the protein of interest.

## 2. Why is the DNA yield so low?

CUT&RUN and CUT&Tag are performed using low cell numbers and the background signal is considerably lower than e.g. for ChIP. This can make reliable measurements of the DNA concentration using a fluorometric assay or by capillary electrophoresis challenging.

To assess the success of the CUT&RUN and CUT&Tag methods it is recommended to include a reaction using an antibody against and abundant histone modification such as H3K27me (ABIN6923144) or H3K4me3 (ABIN2668472) as a positive control. DNA fragments prepared using such an antibody can be measured by capillary electrophoresis on a Bioanalyzer or Tapestation or fluorometrically on a Qubit or Nanodrop fluorometer.

## 3. How do I choose a primary antibody for CUT&RUN or CUT&Tag?

Antibodies that are recommended for ChIP-seq do not necessarily work in CUT&RUN in CUT&Tag. In contrast to ChIP-seq, the antigen is generally in its native state without additional fixation. Unless an antibody has already been tested for CUT&RUN/Tag, a recommendation for a method in which the antigen is expected to be in a native state is helpful, e.g. Immunofluorescence. Unless indicated otherwise, the recommended dilution for immunofluorescence is also a good starting point for the antibody's concentration in CUT&RUN/Tag.

## 4. 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 pAG-MNase is added to the samples. pAG-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.

## 5. 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.

## 6. 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 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.

For CUT&Tag a secondary antibody is recommended to increase the local concentration of Fc fragment binding site in the vicinity of the intended transposition site around the antigen of interest. This step is necessary to increase the specific signal.

## 7. Should I include heterologous spike-in DNA for quantitation?

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

## 8. 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 by Oh et al., 2019 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.

## 9. Is it possible to use CUT&RUN and CUT&Tag with plant tissue samples?

CUT&RUN and CUT&Tag can be applied to plant tissue samples (e.g. Zheng & Gehring, 2019). 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 rabbit anti-H3K27me3 positive control antibody (ABIN6923144) and the CUT&RUN guinea pig anti-rabbit IgG negative control antibody (ABIN6923140) as well as the ConA beads (ABIN6952467) are suitable for use with plant samples.

## 10. 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 pAG-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.

## 11. 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.

## 12. One of my antibodies is mouse. Has your pAG-MNase good affinity for mouse antibodies, or do you advise to use a rabbit anti-mouse secondary antibody?

The pAG-MNase will work well with your murine antibody. The addition of protein G to the MNase is primarily to accomodate the use of mouse IgG1 monoclonal antibodies that bind poorly to protein A. The other IgG isotypes bind well to either protein A or protein G.c

## 13. 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.

# Recommended Antibodies for CUT&RUN

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

## Controls & Secondaries for CUT&RUN

| Product | Host | Item No. |
|---|---|---|
| Anti-Rabbit IgG negative control Antibody | Guinea Pig | ABIN101961 |
| Positive control H3K27me3 Antibody | Rabbit | ABIN6923144 |
| Anti-Mouse IgG secondary Antibody | Rabbit | ABIN101785 |

## Histone Modification

| Product | Host | Item No. |
|---|---|---|
| H2A Histone Family, Member Z (H2AFZ) (C-Term) Antibody | Rabbit | ABIN4889649 |
| Histone 3 (H3) (3meLys36) Antibody | Rabbit | ABIN6971937 |
| Histone 3 (H3) (3meLys4) Antibody | Rabbit | ABIN6971977 |
| Histone 3 (H3) (acLys56) Antibody | Rabbit | ABIN2830942 |
| Histone 3 (H3) (H3K27ac) Antibody | Rabbit | ABIN2668475 |
| Histone 3 (H3) (H3K36me3) Antibody | Mouse | ABIN2668403 |
| Histone 3 (H3) (H3K36me3) Antibody | Rabbit | ABIN3434046 |
| Histone 3 (H3) (H3K4me) Antibody | Rabbit | ABIN3023251 |
| Histone 3 (H3) (H3K4me2) Antibody | Mouse | ABIN2668392 |
| Histone 3 (H3) (H3K4me3) Antibody | Rabbit | ABIN3023254 |
| Histone 3 (H3) (H3K4me3) Antibody | Rabbit | ABIN2668472 |
| Histone 3 (H3) (H3K4me3) Antibody | Rabbit | ABIN2668474 |
| Histone 3 (H3) (H3K9ac) Antibody | Rabbit | ABIN2668415 |
| Histone H1 Antibody | Mouse | ABIN6939594 |
| Histone H2B Antibody | Rabbit | ABIN6579169 |
| Histone H3.X/Y Antibody | Rat | ABIN2668484 |

## NA Metabolism Nucleic Acid

| Product | Host | Item No. |
|---|---|---|
| Polymerase (RNA) Mitochondrial (POLRMT) Antibody | Rabbit | ABIN2856044 |

## Chromatin Remodeler

| Product | Host | Item No. |
|---|---|---|
| Bromodomain Containing 3 (BRD3) (C-Term) Antibody | Rabbit | ABIN2668232 |
| Bromodomain Containing 3 (BRD3) (C-Term) Antibody | Rabbit | ABIN6971459 |
| Histone Deacetylase 1 (HDAC1) (AA 1-5) Antibody | Rabbit | ABIN2668768 |
| Histone Deacetylase 2 (HDAC2) (AA 473-488) Antibody | Mouse | ABIN7540288 |
| Histone Deacetylase 2 (HDAC2) (AA 473-488) Antibody | Mouse | ABIN7540291 |

## Transcription Regulator

| Product | Host | Item No. |
|---|---|---|
| Catenin (Cadherin-Associated Protein), beta 1, 88kDa (CTNNB1) (N-Term) Antibody | Rabbit | ABIN2855042 |
| CCCTC-Binding Factor (Zinc Finger Protein) (CTCF) Antibody | Rabbit | ABIN6731034 |
| Distal-Less Homeobox 5 (DLX5) (Center) Antibody | Rabbit | ABIN5619487 |
| Jun D Proto-Oncogene (JUND) (N-Term) Antibody | Rabbit | ABIN2668613 |
| Jun D Proto-Oncogene (JUND) (N-Term) Antibody | Rabbit | ABIN6972242 |
| Nanog Homeobox (NANOG) (N-Term) Antibody | Rabbit | ABIN2668651 |
| Nanog Homeobox (NANOG) (N-Term) Antibody | Rabbit | ABIN6972403 |
| SMAD Family Member 4 (SMAD4) (N-Term) Antibody | Rabbit | ABIN2668839 |
| SMAD Family Member 4 (SMAD4) (N-Term) Antibody | Rabbit | ABIN6972727 |
| SRY (Sex Determining Region Y)-Box 2 (SOX2) (C-Term) Antibody | Rabbit | ABIN2668643 |
| SRY (Sex Determining Region Y)-Box 2 (SOX2) (C-Term) Antibody | Rabbit | ABIN6972778 |
| SRY (Sex Determining Region Y)-Box 2 (SOX2) Antibody | Rabbit | ABIN2855074 |
| Transcription Factor 7 (T-Cell Specific, HMG-Box) (TCF7) (N-Term) Antibody | Rabbit | ABIN5620945 |
| Transcription Factor 7-Like 1 (T-Cell Specific, HMG-Box) (TCF7L1) (N-Term) Antibody | Rabbit | ABIN2668896 |
| Transcription Factor 7-Like 1 (T-Cell Specific, HMG-Box) (TCF7L1) (N-Term) Antibody | Rabbit | ABIN6972849 |
| YY1 Transcription Factor (YY1) Antibody | Rabbit | ABIN5563972 |

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

| Product | Host | Item No. |
|---|---|---|
| Guinea Pig anti-Rabbit IgG negative control antibody | Rabbit | ABIN101961 |
| Anti-Mouse IgG secondary Antibody | Rabbit | ABIN101785 |
| Histone 3 (H3K4me2) Antibody | Mouse | ABIN2668392 |
| Histone 3 (H3K9ac) Antibody | Rabbit | ABIN6972014 |
| Histone 3 (H3K9ac) Antibody | Rabbit | ABIN2668415 |
| Histone 3 (H3K9me3) Antibody | Rabbit | ABIN6972043 |
| Histone 3 (H3K27me3) Antibody | Rabbit | ABIN6971918 |
| Histone 3 (H3K27ac) Antibody | Rabbit | ABIN2668475 |
| Histone 3 (H3K27ac) Antibody | Rabbit | ABIN2667903 |
| Histone 3 (H3K27ac) Antibody | Rabbit | ABIN6952337 |
| Histone 3 (H3k36me3) Antibody | Mouse | ABIN2668403 |
| STAT 1 (pSer727) Antibody | Rabbit | ABIN2668935 |
| STAT 1 (C-Term) Antibody | Rabbit | ABIN7299460 |

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