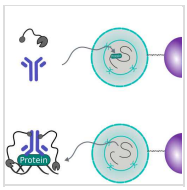

6

May 19, 2022

# Bench top CUT&RUN with antibodies-online™ CUT&RUN Sets V.6

Bench top CUT&Tag

Antibodies Online Gmbh[1]

[1]antibodies-online

1

dx.doi.org/10.17504/protocols.io.kqdg3557pv25/v6

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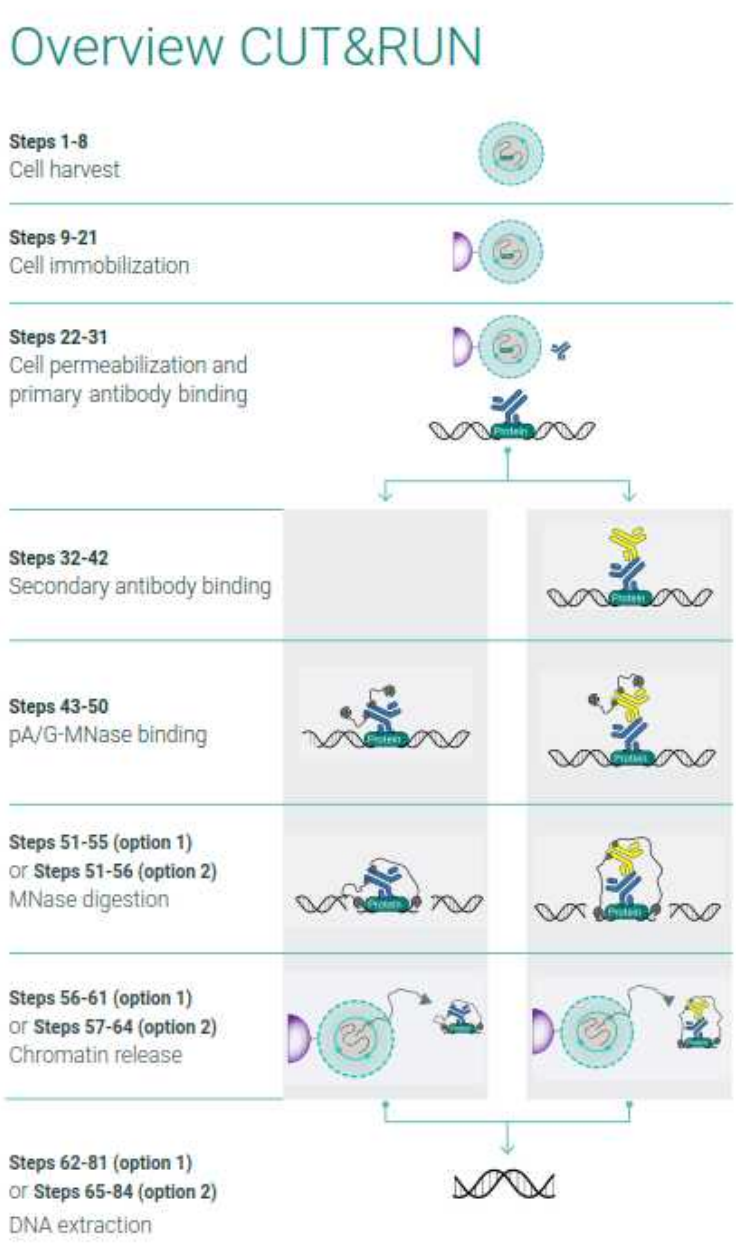
Antibodies Online Gmbh
antibodies online GmbH

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.
Here we provide an updated CUT&RUN protocol that incorporates two variants, one is optimized to further reduce background noise which especially helps when working with low cell numbers and abundant antigens.



**Citation:** Antibodies Online Gmbh Bench top CUT&RUN with antibodies-onlineÃ¢ÂÂ¢ CUT&RUN Sets https://dx.doi.org/10.17504/protocols.io.kqdg3557pv25/v6




In CUT&RUN is performed in situ on immobilized, intact cells without crosslinking. DNA fragmentation is achieved using micrococcal nuclease that is fused to Protein A and/or Protein G (pA/G-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 pA/G-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).

All steps from live cells to sequencing-ready libraries can be performed in a single tube on the benchtop or a microwell in a high-throughput pipeline, and the entire procedure can be performed



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in one day.

DOI

dx.doi.org/10.17504/protocols.io.kqdg3557pv25/v6

https://www.antibodies-online.com/resources/17/5366/cutrun/?utm_content=cutrun&utm_medium=protocol&utm_source=protocolsio

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https://dx.doi.org/10.17504/protocols.io.kqdg3557pv25/v6
Antibodies Online Gmbh

protocol

The protocol is based on Skene; Henikoff JG; Henikoff S (2018): "Targeted in situ genome-wide profiling with high efficiency for low cell numbers"

This version features updates on: -protocol steps -FAQ -recommended reagents

Bench top CUT&Tag, Steven Henikoff

CUT&Tag, CUT&RUN, bench top protocol, epigenetics

protocol ,

Aug 24, 2020

May 19, 2022

40949

## Frequently Asked Questions

1. What are the advantages and disadvantages of CUT&RUN and CUT&Tag compared to ChIP-seq? How do I choose between both methods.
2. Why is the DNA yield so low?
3. How do I choose a primary antibody for CUT&RUN or CUT&Tag?
4. Why do I need a negative control antibody for CUT&RUN? Why not just use a no-antibody control?
5. Can I replace the antibody negative control for CUT&RUN using a knock-out (or knock-down) of my protein?
6. Do I need to use a secondary antibody for CUT&RUN and CUT&Tag?
7. 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?
8. Should I include heterologous spike-in DNA for quantitation?
9. Is it possible to fix the cells prior to immobilization?

10. Is it possible to use the antibodies-online CUT&RUN product sets with plant tissue samples?
11. Is it possible to adapt CUT&RUN to RNA binding proteins?
12. Instead of the proteinase K digestion can I denature the proteins in the CUT&RUN product complexes by heat?
13. What is preferable for DNA extractions prior to library preparation: extraction using phenol-chloroform or affinity purification using a column?
14. Is it possible to do single-end instead of paired-end sequencing of the CUT&RUN libraries?

What are the advantages and disadvantages of CUT&RUN and CUT&Tag compared to ChIP-seq? How do I choose between both methods.
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.

### 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 h4K27me ( ABIN6923144) or h4K4me3 ( 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.

### 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.

### Why do I need a negative control antibody for CUT&RUN? Why not just use a no-antibody control?

The MNase used for CUT&RUN 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 increase the background signal in general.
To avoid this undesired effect of untethered MNase, chromatin is randomly coated with the CUT&RUN guinea pig anti-rabbit IgG negative control antibody ( ABIN6923140) prior to the addition of pAG-MNase ( ABIN6950951) to the samples. pAG-MNase is then tethered via its Protein A or



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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 for CUT&RUN 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 guinea pig anti-rabbit IgG negative control antibody ( ABIN6923140) 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 directed against 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 for CUT&RUN and CUT&Tag?

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.

### 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.

### 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.

### 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 mild cross-linking conditions 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.

### Is it possible to use the antibodies-online 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 rabbit anti-h4K27me3 positive control antibody ( ABIN6923144) and the CUT&RUN guinea pig anti-rabbit IgG negative control antibody ( ABIN6923140) as well as the ConA beads ( ABIN6923139orABIN6952467) are suitable for use with plant samples.

### Is it possible to adapt CUT&RUN to RNA binding proteins?



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The MNase used for CUT&RUN also accepts RNA as substrate so it might be possible to adapt the CUT&RUN protocol for use on RNA binding proteins.
RNA in the cytoplasm will attract the degradation machinery if it is lacking the 5' cap and the 3' poly-A tail. Therefore, I would suggest to use work with isolated nuclei. This has the additional benefit, that you can omit the digitonin in the buffers, which is used to replace the cholesterol and permeabilize the cell membrane. The isolated nuclei may then be immobilized using magnetic ConA beads like for a CUT&RUN experiment. An antibody against the protein of interest is added and subsequently the pAG-MNase is tethered to the antibody, thus bringing the MNase into proximity of the RNA of interest. Isolated RNA can then be reverse transcribed into cDNA to produce your sequencing library.
In order to dispose of any contaminating DNA include a DNase treatment in the protocol subsequently to the RNA-prep and before the library prep. Normalization based on the *E. coli* DNA carried over with the MNase or a spike-in DNA is not an option in this case. Instead, consider using the total read numbers to normalize across samples or include a reference RNA of a known concentration.

### 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.

### 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 sites 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.

MATERIALS

antibodies-online™ CUT&RUN Pro Complete Set **antibodies-online Catalog #ABIN6923135**

antibodies-online™ CUT&RUN Negative Control **antibodies-online Catalog #ABIN6923140** Step 26

antibodies-online™ CUT&RUN Positive Control **antibodies-online Catalog #ABIN6923144** Step 25

antibodies-online™ CUT&RUN Concanavalin A Beads **antibodies-online Catalog #ABIN6923139** Step 9



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antibodies-online™ CUT&RUN Secondary **antibodies-online Catalog #ABIN6923141** Step 38

0.5 M EDTA pH 8.0 **antibodies-online Catalog # ABIN925554**

CUT&RUN Pro Sec Set **antibodies-online Catalog #ABIN6923137**

Spike-in Chromatin **antibodies-online Catalog #ABIN4889666**

CUTANA™ pAG-MNase for ChIC/CUT&RUN Assays **antibodies-online Catalog #ABIN6950951** Step 46

10% Sodium Dodecyl Sulfate (SDS) **antibodies-online Catalog #ABIN925555**

3 M Sodium Acetate (NaOAc) pH 5.2 **antibodies-online Catalog # ABIN925556**

5 M Ammonium Acetate (NH4OAc) **antibodies-online Catalog #ABIN925566**

**Reagents required**

CUT&RUN Pro Sec Set (antibodies-online, cat no.ABIN6923137) with following components:

- Positive control Recombinant Rabbit anti-H3K27me3 Antibody (ABIN6923144)
- Negative control Polyclonal Guinea Pig anti-Rabbit IgG Antibody (ABIN6923140)
- Concanavalin A Beads (ABIN6952467)
- Secondary Rabbit anti-Mouse IgG (H&L) Antibody (ABIN6923141)

- Antibody to an epitope of interest
- Distilled, deionized or RNAse-free H2O
- 2.5 M Manganese Chloride (MnCl2)
- 1 M Calcium Chloride ($CaCl_2$)
- 1 M Potassium Chloride (KCl)
- 1 M HEPES pH 7.5 HEPES (NaOH)
- 5 M NaCl (ABIN412560)
- 0.5 M EDTA (ABIN925554)
- 0.2 M EGTA
- 2 M Spermidine
- Protease Inhibitor Cocktail , EDTA-Free
- 1.2% Digitonin (ABIN4878637)
- 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



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- 3 M Sodium Acetate (NaOAc) pH 5.2 (ABIN925556)
- 5 M Ammonium Acetate (NH4OAc) (ABIN925566)
- 70 % Ethanol
- 100% Ethanol
- 1 mM Tris-HCl pH 8.0

**Materials not provided with the package**
CUTANA™ pAG-MNase for ChIC/CUT&RUN Assays ABIN6950951

Spike-in DNA 200 bp mean size (e.g, D. melanogaster, ABIN4889666)

## Storage

Short term storage of the antibodies for up to two weeks at 4 °C. For long term storage for up to one year at -20 °C, prepare 20 µL aliquots of the antibodies to avoid repeated free-ze-thaw-cycles.
CUT&RUN Concanavalin A Beads must be stored at 4C. Do not freeze CUT&RUN Concanavalin A Beads at -20 °C.

## General remarks

• The original CUT&RUN protocol in Skene et al. (2018)(Skene, PJ; Henikoff JG; Henikoff, 2018) recommends sample sizes of 100 to 1000 mammalian cells for abundant antigens such as H3K27me3 or CTCF. This protocol adapted from Meers et al. (2019)(Meers, Bryson, et al., 2019) is suitable for up to 500.000 cells.

This protocol is intended to give a general outline of the CUT&RUN protocol. 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 on should be carried out at 4°C.

## Antibody selection

An antibody specific for your 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



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acid under the targeted protein and to release the nucleic acid.

Validated CUT&RUN antibodies - available at antibodies-online

Depending on the host species and isotype of the antibody and the Protein A and/or Protein G MNase fusion protein, it can be necessary to include a secondary antibody for pA/G-MNase binding (Skene & Henikoff, 2017). If the pA-MNase is used in conjunction e.g. with a primary mouse IgG1 or goat IgG antibody it is recommended to use a rabbit secondary antibody (Section V). 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 (Meers, Bryson, et al., 2019).

The positive Control H3K27me3 antibody (ABIN6923144) and Negative Control Guinea Pig anti-Rabbit IgG antibody (ABIN6923140) 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 unspecifically bind and cleave any accessible DNA, thus increasing background signal.

## REAGENT SETUP (for 12 samples)

1

### » Wash buffer (165 mL)

| A | B | C |
|---|---|---|
| Component | Volume | Final concentration |
| ddH2O | 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.:
1.65 mL Protease Inhibitor (EDTA-free)

### » Binding Buffer (45 mL)



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| A | B | C |
|---|---|---|
| Component | Volume | Final concentration |
| ddH2O | 43.6 mL | - |
| 1 M HEPES pH 7.5 | 900 µL | 20 mM |
| 1 M KCl | 450 µL | 10 mM |
| 1 M CaCl2 | 45 µL | 1 mM |
| 2.5 M MnCl2 | 16 µL | 1 mM |

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

### » Digitonin Wash Buffer (82.5 mL)

| A | B | C |
|---|---|---|
| Component | Volume | Final concentration |
| 1.2 % Digitonin | 3354 µL | 0.05 % |
| Wash Buffer | 79 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)

| A | B | C |
|---|---|---|
| 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.

### » Low Salt Rinse Buffer (27 mL)



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| A | B | C |
|---|---|---|
| Component | Volume | Final concentration |
| ddH2O | 25.3 mL | - |
| 1 M HEPES pH 7.5 | 540 µL | 20 mM |
| 2 M Spermidine | 6.75 µL | 0.5 mM |
| 1.2% Digitonin | 1125 µL | 0.05 % |

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

### » Low Salt Incubation Buffer (3 mL)

| A | B | C |
|---|---|---|
| Component | Volume | Final concentration |
| ddH2O | 2.8 mL | - |
| 1 M HEPES pH 7.5 | 10.5 µL | 3.5 mM |
| 1 M CaCl2 | 30 µL | 10 mM |
| 1.2% Digitonin | 125 µL | 0.05% |

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

### » Low Salt Stop Buffer (3 mL)

| A | B | C |
|---|---|---|
| Component | Volume | Final concentration |
| ddH2O | 2.8 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: | | |
| 1.2 % 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 |



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## I.Cell Harvest – at room temperature

2 Harvest 10,000 to 500,000 cells for each sample at **Room temperature** . Keep cells for each sample in separate tubes.

3 Centrifuge cell solution **00:03:00** at **600 x g** at **Room temperature** . 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 **00:03:00** at **600 x g** at **Room temperature** and discard the supernatant.

6 Repeat steps 4-5 thrice for a total of four washes.

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 antibodies-online™ CUT&RUN Concanavalin A Beads **antibodies-online Catalog #ABIN6923139**

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.



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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 and add **10 µL** of the **CUT&RUN Concanavalin A Beads** in **Binding Buffer** prepared in **section II** to each sample.

20 Close tubes tightly and rotate for **00:05:00** to **00:10:00** 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.



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23 Place each tube at a low angle on the vortex mixer set to a low speed (approximately **1100 rpm** ) and add **100 µL** **Antibody Buffer** containing **digitonin**.

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.

antibodies-online™ CUT&RUN Positive Control **antibodies-online Catalog #ABIN6923144**

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.

antibodies-online™ CUT&RUN Negative Control **antibodies-online Catalog #ABIN6923140**

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 **00:05:00** to **00:10:00** at **Room temperature** or **02:00:00** 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.

31 Remove the microcentrifuge tubes from the magnet stand.



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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 **go to step #30** once for a total of two washes.

**If no secondary antibody is used proceed directly to section VI. pA/G-MNase binding (Step 41).**

## V. Secondary antibody binding (optional)

34

The antibodies-online™ CUT&RUN Secondary **antibodies-online Catalog #ABIN6923141** is raised in rabbit against mouse IgG (H&L). In case a **primary antibody** from a different host species or isotype is used a different **secondary** must be selected accordingly.

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 (approximately **1100 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 **1 µL** antibodies-online™ CUT&RUN Secondary **antibodies-online Catalog #ABIN6923141** corresponding to a 1:100 dilution (or a volume corresponding to the manufacturer's recommended dilution



**Citation:** Antibodies Online Gmbh Bench top CUT&RUN with antibodies-onlineÃ¢ÂÂ¢ CUT&RUN Sets https://dx.doi.org/10.17504/protocols.io.kqdg3557pv25/v6

for immunofluorescence).

39 Rotate the microcentrifuge tubes for **01:00:00** 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 Buffe**r and mix by inversion. If clumping occurs, gently remove the clumps with a 1 mL pipette tip.

43 Repeat steps40-42 **go to step #40** once for a total of two washes.

## VI. Protein A-MNase or Protein AG-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 magnetic stand.

46 Vortex the sample at low speed (approximately **1100 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 **antibodies-online Catalog #ABIN6950951**

**Alternatively:**

Vortex the sample at low speed (approximately **1100 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 **01:00:00** at **4 °C** .



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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 magnetic 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 **go to step #48** once for a total of two washes.

## VII. MNase digestion and release of pA/G bound chromatin fragments

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 **go to step #54** 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. **1100 rpm** ) and add **200 µL** **ice cold Low Salt Incubation Buffer** per sample along the side of the tube.



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59 Incubate tubes at **0 °C** for the desired time (default is **00:30:00** ).

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.

63 Incubate tubes at **37 °C** for **00:30:00** .

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.

## 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 approximately **1100 rpm** .

68 Incubate tubes at **50 °C** for **01:00:00** or at **37 °C** **Overnight**

69 Add **200 µL** **PCI** to tube.



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70 Vortex tubes thoroughly at high speed until the liquid appears milky.

71 Optional: Transfer liquid to a phase-lock tube.

72 Centrifuge tubes in a table-top centrifuge at **16000 x g** at **4 °C** for **00:05:00** .

73 Carefully transfer the upper aqueous phase to a fresh **1.5 mL** microcentrifuge tube containing **200 µL** **Chloroform:Isoamyl Alcohol 24:1**.

74 Vortex tubes thoroughly at high speed until the liquid appears milky.

75 Centrifuge tubes in a table-top centrifuge at **16000 x g** at **4 °C** for **00:05:00** .

76 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).

77 Add **20 µL** **3 Molarity (M)** **NaOAc** or **100 µL** **5 Molarity (M)** **$NH_4OAc$**

78 Add **500 µL** **100% ethanol**.

79 Place tubes for **00:10:00** in a **dry ice/Ethanol mix** or **Overnight** at **-20 °C** .

80 Centrifuge tubes in a table-top centrifuge at **16000 x g** at **4 °C** for **00:05:00** .

81 Remove the liquid carefully with a pipette.



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82 Add **1 mL** **70% ethanol**.

83 Centrifuge tubes in a table-top centrifuge at **16000 x g** at **4 °C** for **00:01:00** .

84 Remove the liquid carefully with a pipette.

85 Air-dry the pellet or dry the pellet in a SpeedVac.

86 Dissolve the pellet in **30 µL** **1 millimolar (mM)** **Tris-HCl**, **0.1 millimolar (mM)** **EDTA**.

## IX. Sample quality control

87 Size distribution and concentration of the CUT&RUN products can be assessed at this point, e.g. using a

Qubit
Flurometer
**Invitrogen** **Q33228**

or

NanoDrop™ 3300 Fluorospectrometer
Fluorospectrometer
**NanoDrop™ 3300** **nd-3300**



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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

88 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 suffice for epitopes with a multitude of genomic binding sites, e.g. transcription factors or nucleosome modifications.

## XI. Peak calling

89 The sparse background signal in CUT&RUN samples compared to ChIP-seq samples represents 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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