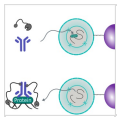

Nov 07, 2019

Bench top CUT&Tag with antibodies-online™ CUT&RUN Sets V.2

Forked from Bench top CUT&Tag

antibodies online GmbH[1]

[1]antibodies-online

1 Works for me dx.doi.org/10.17504/protocols.io.85hhy36

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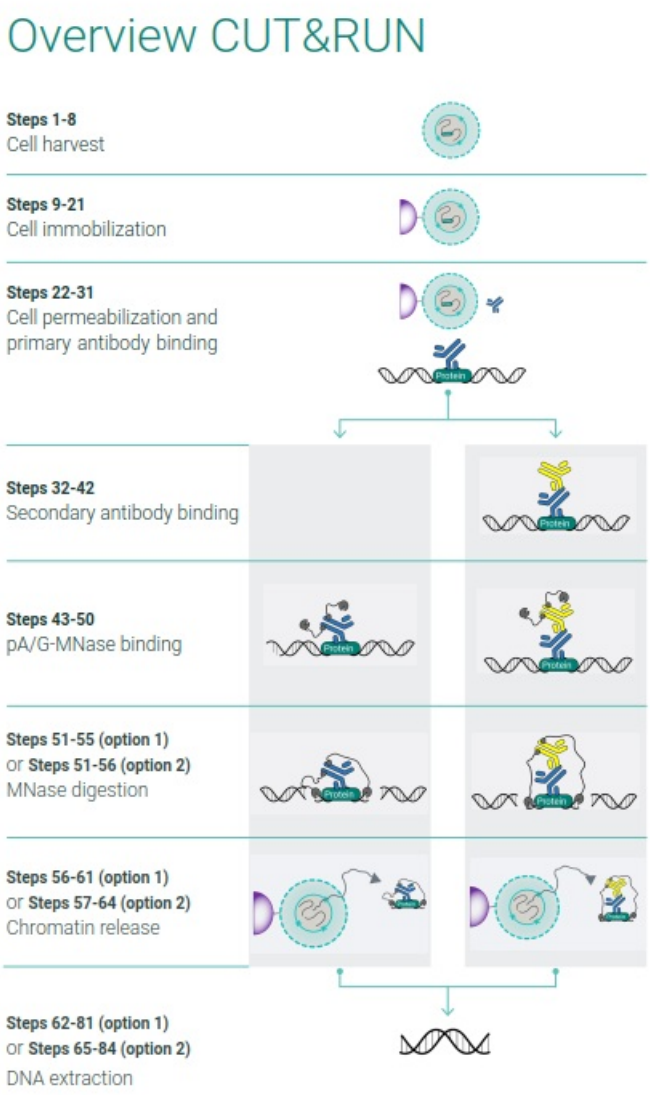
ABSTRACT

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.



In CUT&Tag 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 in one day.

EXTERNAL LINK

https://campaigns.antibodies-online.com/cutandrun-sets/?utm_source=protocolsio&utm_medium=protocol&utm_content=cutrun

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

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

GUIDELINES

Frequently Asked Questions

Why is the DNA yield so low?

CUT&RUN is performed using low cell numbers and the background signal is considerable lower than e.g. for ChIP. Due to these two factors the amount of recovered DNA is often times too low to be reliably measured based on a fluorometric assay or by capillary electrophoresis. PCR amplification of small CUT&RUN products, i.e. less than 50 bp, can be problematic and is therefore not any option. In order to assess the success of the CUT&RUN method each of the antibodies-online CUT&RUN product sets includes the CUT&RUN Positive Control antibodyABIN6923144•against the abundant H3K27me3 histone modification. DNA fragments prepared using this antibody can be measured by sensitive electrophoresis on a Bioanalyzer or Tapestation or fluorometrically on a Qubit or Nanodrop fluorometer. When using the CUT&RUN Positive Control antibodyABIN6923144•(or any other antibody specific for nucleosomal markers) a ladder corresponding to multiples of the 147 bp long nucleosomes should be visible by capillary electrophoresis.

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.

In order to avoid this undesired effect of untethered MNase, the chromatin is randomly coated with the CUT&RUN Negative Control (ABIN6923140•) prior to the addition of pA/G-MNase is added to the samples. pA/G-MNase is then tethered via its Protein A or Protein G portion to the antbodies' Fc fragment and background DNA fragmentation is dictated by the random antibody binding as opposed to the nuclease digestion of hyper-accessible DNA regions.

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

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

In short, the tissue is processed manually or enyzmatically into single, intact cell that are subsequently bound to ConA beads. The improved (and standard) CUT&RUN protocol that serve as basis for the protocol outlined in our CUT&RUN handbook can be adapted accordingly. Presently there is no work published documenting the use of intact tissue.

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

The CUT&RUN method can be applied without a problem to plant tissue samples (see e.g.PMID30719569). One 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.
The positive control H3K27me3 antibody and the negative control guinea pig anti-rabbit IgG antibody as well as the ConA beads are suitable for use with plant samples. The antibodies that are included in some of the sets, such as the anti-DYKDDDDK antibodies or the secondary antibodies, can also be used with plant samples. Whether they are needed or not depends on your experiment.

Can CUT&RUN be adapted for RIP-seq?

It should be possible to adapt the CUT&RUN protocol for use with RNA as an alternative to RIP-seq. RNA in the cytoplasm will attract the degradation machinery if it is lacking the 5' cap and the 3' poly-A tail. Thus, it is advisable to use isolated nuclei as sample material. Consequently, no digitonin in the different buffers is needed since the nuclear envelope does not contain cholesterol. Isolated nuclei may be immobilized via glycoproteins on the nuclear envelope to the ConA beads like for a CUT&RUN experiment. An antibody against the protein of interest is added and subsequently the pA/G-MNase is tethered to the antibody, thus bringing the MNase into proximity of the RNA of interest. Similar to RIP-seq, isolated RNA is then translated into cDNA and can be sequenced and mapped.

Is it possible to use a cell free extract of a bacterial cell instead of immobilizing and permeabilizing the cell?

It should indeed be possible to use a bacterial extract as starting material instead of immobilized eukaryotic cells. There are a couple of things that should be considered:
One of the factors that makes CUT&RUN so much better than ChIP-seq in a eukaryotic system is that it is done in situ. The intact cells (or isolated nuclei) retain the large, undigested chromatin. Consequently, much fewer off-sites are present in the prepared DNA which is one of the reasons why CUT&RUN data sets have so much less background signal when compared to ChIP-seq data. This enrichment step will be missing when using a cell lysate as starting material. The CUT&RUN result should still be superior to the CHIP-seq result for the same starting material. However, it will be more "noisy" than CUT&RUN sequencing data from intact eukaryotic cells/nuclei.
The buffers described in the CUT&RUN protocol are designed to maintain the cells in the samples intact. The binding buffer contains some divalent cations that are necessary for the binding of the cells to the Concanavalin on the beads that are used for the immobilization. Subsequently, this buffer is changed for an antibody buffer that contains EDTA to chelate the divalent cations and prevent DNA cleavage prior to pA/G-MNase antibody binding. Since you are working with cell lysates as opposed to intact cells there is no need to include digitonin in any of the buffers. You don't need the binding buffer used to immobilize cells on the ConA beads. In order to control premature DNA cleavage by the MNase I would include some EDTA and/or EGTA in the lysis buffer.
Probably, you will have to titrate the amount of pA/G-MNase and try different digestion times to find the best balance between efficient cleavage of the intended sites and too much off-site cleavage.
The original CUT&RUN protocol foresees the use of heterologous spike-in DNA, e.g. from *E. coli*. In its latest iteration it is argued that there is no need to add this DNA since the pA/G-MNase preparation contains sufficient levels of *E. coli* DNA to serve as a standard for quantitation. In a prokaryotic system, spike-in DNA from a eukaryotic organism (e.g. *S. cerevisiae*) is recommended. Also keep in in mind that the *E. coli* DNA carried over with the pA/G-MNase preparation may contain sequences that show homology to the bindings sites of your protein of interest.
Our product sets are optimized for use with eukaryotic cells. The positive control in all sets is a recombinant rabbit anti-H3K27me3 antibody. This control is not suitable for use with prokaryotic samples.

MATERIALS TEXT

Reagents required

CUT&RUN Pro Complete Set (antibodies-online, cat no. ABIN6923135) with following components:

- Positive control Recombinant Rabbit anti-H3K27me3 Antibody (ABIN6923144)
- Negative control Polyclonal Guinea Pig anti-Rabbit IgG Antibody (ABIN101961)
- Concanavalin A Beads (ABIN6923139)
- Mouse anti-DYKDDDDK Tag Antibody (ABIN6923141)
- Secondary Rabbit anti-Mouse IgG (H&L) Antibody (ABIN6923141)

- Antibody to an epitope of interest
- Distilled, deionized or RNAse-free H2O
- 1 M Manganese Chloride (MnCl2)
- 1 M Calcium Chloride (CaCl2)
- 1 M Potassium Chloride
- 1 M HEPES pH 7.5 HEPES (NaOH)
- 5 M NaCl
- 0.5 M EDTA (ABIN925554)
- 0.2 M EGTA
- 2 M Spermidine
- EZBlock Protease Inhibitor Cocktail II (ABIN1995262)
- 5% Digitonin (ABIN1304051)
- 20 mg/ml Glycogen
- Trypan Blue (ABIN413910)
- RNase A (DNase and protease free)
- 10% Sodium dodecyl sulfate (SDS)
- Proteinase K (ABIN920948)
- Phenol-chloroform-isoamyl alcohol (PCI)
- 7.5 M Ammonium Acetate (NH4OAc)
- 100% Ethanol
- 1 mM Tris-HCl pH 8.0

Materials not provided with the package

Protein A/G-tethered micrococcal nuclease (pA/G-MNase)
Spike-in DNA (e.g. S. cerevisiae, D. melanogaster, E. coli), 200 bp mean size

SAFETY WARNINGS

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.

BEFORE STARTING

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 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, 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 16 samples)

1

» Wash buffer (100 mL)

Component	Volume	Final concentration
ddH2O	94 mL	-
1 M HEPES pH 7.5	2 mL	20 mM
5 M NaCl	3 mL	150 mM
2 M Spermidine	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 mL EZBlockTM Protease Inhibitor Cocktail II 100x

» Binding Buffer (40 mL)

Component	Volume	Final concentration
ddH2O	39 mL	-
1 M HEPES pH 7.5	800 µL	20 mM
1 M KCl	400 µL	10 mM
1 M CaCl2	40 µL	1 mM
1 M MnCl2	40 µL	1 mM

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

» Digitonin Wash Buffer (70 mL)

Component	Volume	Final concentration
5% Digitonin	350-1400 µL	0.025% - 0.1%
Wash Buffer	69 mL	-

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

» Antibody Buffer (2 mL)

Component	Volume	Final concentration
0.5 M EDTA	8 µL	2 mM
Digitonin Wash Buffer	2 mL	-

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

» 100 mM CaCl2 (2 mL)

Component	Volume	Final concentration
1M CaCl2	200 µL	100 mM
ddH2O	1,800 µL	-

• Store 100 mM CaCl2 at 4 °C until use.

Attention! The Buffer composition differs with options chosen

Option 1 – Standard CUT&RUN

• is suitable for most target proteins that are not too abundant.
• is typically the best starting point when using untested antibodies.

Option 2 – High Ca2+/low salt chromatin cleavage

• prevents premature release of the pA/G-MNase-antibody-chromatin complex after cleavage.
• minimizes unspecific off-site cleavage due to free MNase in the presence of divalent cations.
• reduces variability of the cleavage products and background depending on the incubation time.
• is preferable when working with low cell numbers and abundant antigens.

step case

Option 1 – Standard CUT&RUN

Chromatin is cleaved by MNase at a low concentration of divalent cations (2 mM Ca2+) 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 the antigen of interest that it is tethered to via the Protein A or Protein G residue and the antigen-specific antibody. This can cause undesired background.

The enzymatic MNase nucleic acid cleavage is less temperature sensitive than the subsequent diffusion of the pA/G-MNase-antibody-chromatin cleavage products. Therefore, chromatin digestion is carried out at 0 °C to reduce DNA overdigestion by free pA/G-MNase-antibody-chromatin complexes.

» 2x Stop Buffer (5 mL)

Component	Volume	Final concentration
ddH2O	4.3 mL	-
5 M NaCl	340 µL	340 mM
0.5 M EDTA	200 µL	20 mM
0.2 M EGTA	100 µL	4 mM

• Store 100 mM CaCl2 at 4 °C until use.
• Add fresh before use

Component	Volume	Final concentration
5% Digitonin	50 µL	0.05%
RNase A (10 mg/mL)	50 µL	100 µg/mL
Glycogen (20 mg/mL)	12.5 µL	50 µg/mL
heterologous spike-in DNA	-	100 pg/mL

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 .

4 Remove the liquid carefully.

5 Gently resuspend cells in 1 ml **Wash Buffer** by pipetting and transfer cell solution to a 1.5 ml microcentrifuge tube.

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

7 Repeat steps 5-6 thrice for a total of four washes. go to step #5

8 Resuspend cell pellet for each sample in 1 ml **Wash Buffer** by gently pipetting.

II. Concanavalin A beads preparation

9 Prepare one 1.5 ml microcentrifuge tube for each sample.

10 Gently resuspend the **CUT&RUN Concanavalin A Beads**.

11 Pipette 10 µl **CUT&RUN Concanavalin A Beads** slurry for each sample into the 1.5 ml microcentrifuge tubes.

12 Place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.

13 Remove the microcentrifuge tube from the magnet stand.

14 Pipette 1 ml **Binding Buffer** into each tube and resuspend **CUT&RUN Concanavalin A Beads** by gentle pipetting.

15 Spin down the liquid from the lid with a quick pulse in a table-top centrifuge (max **100 x g**).

16 Place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.

17 Remove the microcentrifuge tube from the magnet stand.

18 Repeat steps 14-17 **go to step #14** twice for a total of three washes.

19 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

20 Carefully vortex the cell suspension from step **go to step #8** and add **10 µl** of the **CUT&RUN Concanavalin A Beads** in **Binding Buffer** prepared in **section II** to each sample.

21 Close tubes tightly and rotate for **00:05:00** to **00:10:00** at **Room temperature** .

IV. Cell permeabilization and primary antibody binding

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

23 Remove the microcentrifuge tubes from the magnet stand.

24 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**.

25 Gently vortex the microcentrifuge tubes until the beads are resuspended.

26 Add **1 µl** **primary antibody** - against your antigen of interest, the **CUT&RUN Positive Control** , and the **CUT&RUN Negative Control** - corresponding to a 1:100 dilution (or a volume corresponding to the manufacturer's recom- mended dilution for immunofluorescence).

27 Rotate the microcentrifuge tubes for **00:05:00** to **00:10:00** at **Room temperature** or **02:00:00** to **Overnight** at **4 °C** .

28 Spin down the liquid and place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.

29 Remove the microcentrifuge tubes from the magnet stand.

30 Resuspend with **1 ml** **Digitonin Wash Buffer** and mix by inversion. If clumping occurs, gently remove the clumps with a **1 ml** pipette tip.

31 Repeat steps 28-31 **go to step #28** once for a total of two washes.

V. Secondary antibody binding (optional)

32 ⚠

The polyclonal **CUT&RUN Secondary** 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.

If no secondary antibody is used proceed directly to section VI. pA/G-MNase binding (Step 43).

33 Place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.

34 Remove the microcentrifuge tubes from the magnet stand.

35 Vortex the sample at low speed (approximately **1100 rpm**) and add **100 µl** **Digitonin Wash Buffer** per sample along the side of the tube.

36 Tap to remove the remaining beads from the tube side.

37 Add **1 µl** **CUT&RUN Secondary** corresponding to a 1:100 dilution (or a volume corresponding to the manufacturer's recommended dilution for immunofluorescence).

38 Rotate the microcentrifuge tubes for **01:00:00** at **4 °C** .

39 Spin down the liquid and place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.

40 Remove the microcentrifuge tubes from the magnet stand.

41 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.

42 Repeat steps 35-37 **go to step #35** once for a total of two washes.

VI. Protein A-MNase or Protein AG-MNase Binding

43 Place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.

44 Remove the microcentrifuge tubes from the magnetic stand.

45 Place each tube at a low angle on the vortex mixer set to a low speed (approximately **1100 rpm**) and add **150 µl** **Digitonin Wash Buffer** containing **Protein A-MNase** or **Protein AG-MNase fusion protein** (pA/G-MNase) at **100 ng/mL** per sample of the along the side of the tube.

46 Rotate the microcentrifuge tubes for **01:00:00** at **4 °C** .

47 Spin down the liquid and place the tubes on a magnet stand until the fluid is clear. Remove the liquid carefully.

48 Remove the microcentrifuge tubes from the magnetic stand.

49 Resuspend with **1 ml** **Digitonin Wash Buffer** and mix by inversion. If clumping occurs, gently remove the clumps with a 1 ml pipette tip.

50 Repeat steps 47-49 **go to step #47** once for a total of two washes.

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

51 ⚠

Spin down the liquid from the lid with a quick pulse in a table-top centrifuge (max **100 x g**).

The following steps are according to **: Option 1**

go here to switch to Option 2 – High Ca2+/low salt chromatin cleavage

52 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 Place each tube at a low angle on the vortex mixer set to a low speed (approximately **1100 rpm**) and add **100 µl** **Digitonin Wash Buffer** per sample along the side of the tube.

55 Chill the tubes down to **0 °C** .

56 Add **2 µl** **100 Milimolar (mM)** **$CaCl_2$** per sample to a final concentration of **2 Milimolar (mM)** **$CaCl_2$** while gently vortexing at a low speed of approximately **1100 rpm** .

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

58 Add **100 µl** **2x Stop Buffer** per sample.

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

60 Place the tubes on a magnet stand until the fluid is clear.

61 Transfer the supernatant containing the pA/G-MNase-bound digested chromatin fragments to fresh **1.5 ml** microcentrifuge tubes.

VIII. DNA extraction

62 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 61.

63 Gently vortex tubes at a low speed of approximately **1100 rpm** .

64 Incubate tubes at **50 °C** for **01:00:00** .

65 Add **200 µl** **PCI** to tube.

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

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

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

69 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).

70 Add **100 µl** **7.5 Molarity (M)** NH4OAc and **500 µl** **100% ethanol**.

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

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

73 Remove the liquid carefully with a pipette.

74 Add **1 ml** **100% ethanol**.

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

76 Remove the liquid carefully with a pipette.

77 Air-dry the pellet or dry the pellet in a SpeedVac

.

78 Dissolve the pellet in **30 µl** **1 Milimolar (mM)** **Tris-HCl**, **0.1 Milimolar (mM)** **EDTA**.

IX. Sample quality control

79 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

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

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

XI. Peak calling

81 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/.

I. Cell harvest – at room temperature

step case

Option 2 – High Ca2+/low salt chromatin cleavage

It is intended to reduce background due to DNA overdigestion by free pA/G-MNase-antibody-chromatin complexes.
The protocol takes advantage of the fact that nucleosomes aggregate in the presence of high concentrations of divalent cations (10 mM Ca2+) and at low salt concentrations to reduce release of the pA/G-MNase-antibody-chromatin cleavage products. Subsequently to the digestion of the samples are in high Ca2+/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 digestion times causes increased background. Longer retention of the cleavage product particles within the nucleus could also improve CUT&RUN with lower cell numbers.

» 2x Low Salt Rinse Buffer (35 mL)

Component	Volume	Final concentration
ddH2O	34 mL	-
1 M HEPES pH 7.5	700 µL	3,5 mM
2 M Spermidine	8,75 µL	10 mM
5% Digitonin	350 µL	0.05 %

• Store 100 mM CaCl2 at 4 °C until use.
• Add fresh before use

» Low Salt Incubation Buffer (4 mL)

Component	Volume	Final concentration
ddH2O	3900 µL	-
1 M HEPES pH 7.5	14 µL	3.5 mM
1 M CaCl2	40 µL	10 mM
5% Digitonin	40 µL	0.05%

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

» Low Salt Stop Buffer (4 mL)

Component	Volume	Final concentration
ddH2O	4.3 mL	-
5 M NaCl	340 µL	340 mM
0.5 M EDTA	200 µL	20 mM
0.2 M EGTA	100 µL	4 mM

• Store 100 mM $CaCl^2$ at 4 °C until use.
• Add fresh before use

Component	Volume	Final concentration
5% Digitonin	40 µL	0.05%
RNase A (10 mg/mL)	20 µL	50 µg/mL
Glycogen (20 mg/mL)	5 µL	25 µg/mL
heterologous spike-in DNA	-	100 pg/mL

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** .