**The following protocol is from:**

[**http://blocks.fhcrc.org/steveh/papers/CUT&RUN\_protocol.htm**](http://blocks.fhcrc.org/steveh/papers/CUT&RUN_protocol.htm) **This page is no longer available.**

**This webpage is the original ~~and most updated~~ protocol for CUT&RUN from Steve Henikoff lab (Skene, *Elife*, 2017). They will no longer update it.**

**(They have released a new protocol here:**

[**https://www.nature.com/articles/nprot.2018.015**](https://www.nature.com/articles/nprot.2018.015)**) The new protocol is easier to use and can be applied to very limited number of cells. However the following is the old protocol from Skene, *Elife*, 2017.**

**My comments are in blue. Please tell me if you have further improvement to the protocol. The permanent link to this page are** [**here**](https://docs.google.com/document/d/1Rfr-qXMJwGJHsxetkO_JwIPylSjMdtDK8LeLIcT_mXQ/edit?usp=sharing) **or** [**here**](http://nanliulab.com/cutrun-protocol-2017/)**, please check occasionally for updates.**

**Libary preparation protocol is** [**here**](http://dx.doi.org/10.17504/protocols.io.wvgfe3w)**.**

**See here for exciting development and evolution of CUT&RUN, CUT&Tag technology!**

[**https://www.encodeproject.org/documents/f843b460-826b-431f-a1f0-54710f096316/@@download/attachment/ENCODE-2019\_Henikoff.pdf**](https://www.encodeproject.org/documents/f843b460-826b-431f-a1f0-54710f096316/@@download/attachment/ENCODE-2019_Henikoff.pdf)

**CUT&RUN protocol (March 20, 2017)1**

**Buffers2**

**Nuclear extraction (NE 50 ml)**

\_ 1 ml 1M HEPES-KOH pH 7.9 (20 mM)

\_ 500 µL 1M KCl (10 mM)

\_ 12.5 µL 2M Spermidine (0.5 mM)

\_ 500 µL 10% Triton X-100 (0.1%)

\_ 12.5 ml 80% Glycerol (20%)

\_ water to 50 ml

\_ 1 large Roche complete EDTA-free tablet

**Phosphate buffered saline (PBS)**

**Binding (20 ml)**

\_ 400 µL 1M HEPES-KOH pH 7.9 (20 mM)

\_ 200 µL 1M KCl (10 mM)

\_ 20 µL 1M CaCl2 (1 mM)

\_ 20 µL 1M MnCl2 (1 mM)

\_ water to 20 ml

**Wash (100 ml)**

\_ 2 ml 1 M HEPES pH 7.5 (20 mM)

\_ 3 ml 5 M NaCl (150 mM)

\_ 25 µL Spermidine (0.5 mM)

\_ 333 µL 30% BSA (0.1%)

\_ water

\_ 2 large Roche complete EDTA-free tablets

**Blocking3 (5 ml)**

\_ 5 ml Wash buffer

\_ 20 µL 0.5 M EDTA (2 mM)

**2XRSTOP+ (5 ml)**

\_ 200 µl 5M NaCl (200 mM)

\_ 200 µL 0.5M EDTA (20 mM)

\_ 100 µL 0.2M EGTA (4 mM)

\_ 4.46 ml water

\_ 25 µL RNase A 10 mg/ml (50 µg/ml)

\_ 10 µL glycogen 20 mg/ml (40 µg/ml) Add RNase and glycogen freshly.

\_ 5 µL heterologous DNA @10 ng/ml**4** (10 pg/ml) I don’t use spike in DNA.

**Procedure5**

**1) Lysis**

\_ Harvest fresh culture(s)**6,7** and count cells. The following is for ~~6 million~~ 2 million mammalian cells (~~10~~ 1 time-point samples)

I now use 2 million cells for each experiment, and do only one time point. The numbers below are for 2 million cells. I’ve succeeded with as low as 100k cells using this protocol.

\_ Spin down at 600g 3’ 4oC swing-bucket rotor and decant.

\_ Resuspend in 1 ml cold PBS by gently pipetting and transfer to a 1.7 ml Eppendorf tube.

\_ Spin down at 600g 3’ 4oC swing-bucket rotor and decant.

\_ Resuspend in ~~1~~ 0.4 ml NE buffer by gentle pipetting. Cells may be aliquoted and frozen at this point**8**

\_ Spin down at 600g 3’ 4oC swing-bucket rotor and decant.

\_ Resuspend in ~~600~~ 200 µL NE buffer per ~~6~~ 2 million mammalian cells.

**2) Bind to magnetic beads9**

\_ Gently resuspend Bio-Mag Plus Concanavalin A coated beads (Polysciences, Inc. #86057).

\_ Withdraw ~~200~~ 50 µL bead slurry (for ~~10 time points~~ 2 million cells at Step 7), and transfer to 800 µL Binding buffer in a 1.7 ml Eppendorf tube.

\_ Place on a magnet stand and wash twice in 1 ml Binding buffer**10,11**.

\_ Resuspend in ~~300~~ 100 µL Binding buffer.

\_ While *gently* vortexing the nuclei, slowly add the bead slurry.

\_ Rotate 5-10 min at room temperature.

**3) Block**

\_ Place on the magnet stand, allow to clear (~20 s –>2 min) and pull off the liquid.

\_ Add 1 ml Blocking buffer and mix either with gentle pipetting or invert ~10x.

\_ Incubate 5’.

**4)** **Bind primary antibody**

\_ Place on the magnet stand and pull off the liquid.

\_ Add 1 ml Wash buffer, invert ~10x.

\_ Place on the magnet stand and pull off the liquid.

\_ Resuspend in ~~250~~ 100 µL Wash buffer with gentle pipetting or invert ~10x.

Here I will transfer the beads to 0.5 mL tubes. Rinse the tubes with wash buffer. These will decrease beads sticking and drying on the wall.

\_ While *gently* vortexing add ~~250~~ 100 µL primary antibody in Wash buffer (typically 1:100).

\_ Incubate on rotator ≥2 hr at 4oC.

\_ Quick spin and wash twice in ~~1~~ 0.4 ml Wash buffer**12**.

**5) Bind secondary antibody (as required)13** (if you use mouse primary antibody, you need to do this step, otherwise go to step 6).

\_ Pull off the liquid, and resuspend in 250 µL Wash buffer.

\_ While *gently* vortexing add 250 µL secondary antibody in Wash buffer (typically 1:100).

\_ Incubate ≥1 hr on rotator at 4oC.

\_ Quick spin and wash twice in 1 ml Wash buffer.

\_ Resuspend in 500 µL Wash buffer.

**6) Bind pA-MNase**

\_ Pull off the liquid and resuspend each sample in ~~250~~ 100 µL Wash buffer.

\_ While *gently* vortexing add ~~250~~ 100 µL pA-MN in Wash buffer for a final pA-MN concentration of 1:1000 for Batch #5 or 1:400 for Batch #6**14**.

\_ Incubate ≥1 hr on rotator at 4oC.

\_ Quick spin and wash twice in ~~1~~ 0.4 ml Wash buffer.

**7) Digestion**

\_ Pull off the liquid and resuspend in 150 µL Wash buffer per 2 million cells ~~time-point sample (total 0.6 – 1.5 ml)~~. Use tubes that fit the metal blocks to keep constant temperature.

\_ Equilibrate to 0oC in ice water or in metal blocks fitted for Eppendorf tubes in ice water.

\_ Remove a tube from 0oC, add 3 µL 100 mM CaCl2 per 150 µL while vortexing, then return to 0oC. Do not remove the tube from metal block after adding Ca2+.

\_ Stop ~~each time point at the designated time (5 s to 30 min)~~ with 150 µL 2XSTOP+.

I do 30 min digestion as default.

**8) Fractionation**

\_ Incubate 20’ 37 oC to RNase and release CUT&RUN fragments from the insoluble nuclear chromatin.

\_ Spin 5’ 16,000 x g 4oC, and pull off supernatants to fresh tubes.

\_ ~~Optional: For DNA quantitation, add 300 µL 1XSTOP (100 mM NaCl, 10 mM EDTA, 4 mM EGTA).~~

**9) Extractions**

\_ To each sample add 3 µL 10% SDS (to 0.1%), and 2.5 µL Proteinase K (20 mg/ml).

\_ Mix by inversion and incubate 10 min 70oC.

I worried about 70oC incubation, since this temperature will melt short DNA fragments. I use 50oC overnight and it works fine.

\_ Add 300 µL buffered phenol-chloroform-isoamyl solution (25:24:1) and vortex.

\_ Transfer to a phase-lock tube, and spin 5 min full speed.

\_ ~~Add 300 µL chloroform and invert ~10x to mix.~~

\_ Remove aqueous to a fresh tube containing 2 µL of 20 mg/ml glycogen.

\_ Add 750 µL 100% ethanol and mix by vortexing or tube inversion.

\_ Chill on ice, spin 10 min full speed 4oC.

\_ Pour off the liquid and drain on a paper towel.

\_ Wash the pellet (hardly visible) in 1 ml 100% ethanol, spin briefly full speed.

\_ Carefully pour off the liquid and drain on a paper towel. Air dry.

\_ When the pellet is dry, dissolve in 25-50 µL 0.1xTE8 (1x = 10 mM Tris, 1 mM EDTA pH8).

**10) Prepare DNA sequencing libraries15**

\_ Optional: Quantify (*e.g.*, 1 µL in Qubit), and/or resolve (*e.g.*, 2 µL in Tapestation)**16**.

\_ Follow the protocol described in PMID:23139805 (without fragmentation or size selection)**17** to make libraries for *paired-end* Illumina sequencing**18**.

If you are using illumina adapters, you can use Henikoff’s protocol (please see their note 17).

If you are using NEB adapters and kits (I use ultra II), please see the protocol [here](http://dx.doi.org/10.17504/protocols.io.wvgfe3w). There are critical changes to the protocol to make library with CUT&RUN DNA.

One reason for those changes is NEB adapters are much shorter than illumina adapters. You need to adjust the amount of AMPure beads to avoid losing your DNA.

\_ Our scripts available from <https://github.com/peteskene> are customized for processing and analyzing CUT&RUN data**19**.

My collaborator Qian Zhu has developed an easy to use pipeline for CUT&RUN data processing [here](https://bitbucket.org/qzhudfci/cutruntools/src). Some details can be found in the method section in our 2018 [paper](https://www.sciencedirect.com/science/article/pii/S0092867418302964).

**Options**

**For yeast nuclei**

\_ Prepare nuclei according to Orsi et al. *Current Protocols* PMID:25827087.

\_ Divide into aliquots of ~500 million cells, flash freeze and store at -80oC.

\_ Thaw aliquots on ice.

\_ Begin at Step 2 using 300 µL beads per aliquot (for 10 samples).

**Total DNA extraction option (substitute for Steps 8 and 9) Didn’t do this.**

\_ Incubate 20 min 37 oC.

\_ To each sample add 3 µL 10% SDS (to 0.1%), and 2.5 µL Proteinase K (20 mg/ml) and vortex.

\_ Incubate 10 min 70oC.

\_ Add 300 µL buffered phenol-chloroform-isoamyl solution (25:24:1) and vortex.

\_ Transfer to a phase-lock tube, and spin 5 min full speed

\_ Add 300 µL chloroform and invert ~10x to mix.

\_ Remove aqueous to a fresh tube containing 2 µL of 2 mg/ml glycogen.

\_ Add \_ volume (150 µL) Beckmann Agencourt AMPure XP beads, pipetting 10x up/down or while gently vortexing**20**

\_ .

\_ Let sit 10 min, and place on a magnet stand to clear.

\_ Transfer the liquid to a fresh tube (to remove the remaining beads).

\_ Add 1 ml ethanol, and mix by vortexing or tube inversion.

\_ Chill on ice, spin 10 min full speed 4oC.

\_ Pour off the liquid and drain on a paper towel.

\_ Wash the pellet (hardly visible) in 1 ml 100% ethanol, spin briefly on full.

\_ Carefully pour off the liquid and drain on a paper towel.

\_ When the pellet is dry, dissolve in 25-50 µL 0.1xTE8.

**For CUT&RUN with salt fractionation (substitute for Step 7) Didn’t do this.**

\_ Modify Step 7 (Digestion) by reducing the volume of the bead slurry to 1/3rd (600 µL -> 200 µL Wash buffer for 50 µL aliquots).

\_ Add 1 µL 100 mM CaCl2 to start the reaction.

\_ To stop the reaction, add 50 µL 2XSTOP+N (2XSTOP with twice the desired salt concentration, *e.g.*, 4M for a 2M salt extraction, without RNase).

\_ Add 200 µL RNase (100 µg/ml) in water, then continue with Step 8.

**Notes and tips**

1. This protocol updates Appendices 2 and 3 of PJ Skene & S Henikoff (2017) *eLife* <https://elifesciences.org/content/6/e21856>. We continue to refine the protocol and will keep this link updated as needed. Before beginning a CUT&RUN experiment, please reload this page for the most recent version.

2. Buffers should be held on ice. Except as noted, all steps may be performed at room temperature.

3. After the cells bind to the beads, an EDTA-containing blocking solution removes excess divalent cation used to activate the ConA, because carry-over of Ca++ from the beads can prematurely initiate strand cleavage after addition of pA-MN. Spermidine in the wash buffer is intended to compensate for removal of Mg++, which might otherwise affect chromatin properties.

4. Heterologous spike-in DNA should be fragmented down to ~200 bp mean size, for example an MNase-treated sample of mononucleosome-sized fragments. As we use the total number of mapped reads as a normalization factor only, very little spike-in DNA is needed. For example, addition of 1.5 pg results in 1,000-10,000 mapped spike-in reads for 1-10 million mapped experimental reads (in inverse proportion). An aliquot of yeast (or Drosophila) DNA from MNase-treated nucleosomes that can be used as a heterologous spike-in control will be included with the pA-MN aliquot that we are sending out upon request.

5. Because it is crucial that DNA breakage is minimized throughout the protocol, we recommend that freezing of cells, cavitation during resuspension and vigorous vortexing be avoided during Steps 1 to 8.

6. CUT&RUN can be performed on formaldehyde cross-linked cells using the Total DNA extraction option (*e.g.* Figure 5D of our *eLife* 2017 paper). Use a detergent cocktail to permeabilize the cells to allow antibody and pA-MN to gain access (1% Triton X-100 and 0.05% SDS in the wash and blocking buffers), However, cross-linking slows digestion, so 30 min 0oC digestion in Ca++ is recommended.

7. Our NE buffer lysis procedure has been tested on human and Drosophila cultured cells. But because nuclei isolation procedures vary based on organism, cell type and tradition, starting at Step 2 with native (fresh or frozen) nuclei as we do with yeast is a suitable alternative, as long as care is taken to avoid DNA damage by breakage or apoptosis.

8. For CUT&RUN without magnetic beads, perform wash steps by centrifugation for 3’ at 600g in a swing-bucket rotor and careful decanting as described in Appendix 3 of our *eLife* 2017 paper.

9. Maintaining DNA integrity in CUT&RUN is crucial, and we have found that the flash-freezing mammalian cells in glycerol can result in increased background cleavages relative to using fresh cells, similar to to [what has been observed for ATAC-seq](http://www.nature.com/articles/srep25474). We recommend cryopreservation in 10% DMSO using a [Mr. Frosty isopropyl alcohol chamber](https://www.thermofisher.com/order/catalog/product/5100-0001), which minimizes background degradation.

10. Before placing a tube on the magnet stand, a very quick spin on a mini-centrifuge (e.g. no more than 100 x g for one second) will remove liquid adhering to the cap and sides without crushing the nuclei.

11. A sturdy magnet stand is highly recommended for clean separations. For example, the MACSiMAG Separator (Miltenyi Biotec) requires ~10-30 seconds to clear and allows clean removal of the liquid from the bottom of 1.7 ml Eppendorf tubes.

12. For each wash step: After pulling off the liquid, remove the tube from the magnet stand, add Wash buffer, invert the tube(s) (or gently pipette up/down) until beads release from the tube surface (some clumping is normal), quick spin (≤ 100 x g), and replace on the magnet stand to clear.

13. The binding efficiency of Protein A to the primary antibody depends on host species and IgG isotype (for example see here <https://www.thermofisher.com/us/en/home/references/molecular-probes-the-handbook/tables/binding-profiles-of-protein-a-and-protein-g.html>). A secondary antibody can be added as an adapter, for example, rabbit anti-mouse after a mouse primary antibody.

14. We will FedEx aliquots of purified pA-MNase protein at 140 µg/ml (Batch #6) in 50% glycerol upon request. We have not observed any loss of activity after several months at –20oC, or after more than a week at room temperature. We find that a 1:400 final concentration of pA-MN Batch #6 for 200 µL of bead slurry in a 500 µL volume is enough to saturate IgG, even for abundant epitopes such those on histones.

15. Paired-end 25x25 bp sequencing is sufficient for large mammalian genomes. Separation of sequenced fragments into ≤120 bp and ≥150 bp size classes provides mapping of the local vicinity of a DNA-binding protein, but this can vary depending on the steric access to the DNA by the tethered MNase. Single-end sequencing is not recommended for CUT&RUN, as it sacrifices resolution and discrimination between transcription factors and neighboring nucleosomes.

16. The soluble DNA recovered is too low in amount and too small to be detected by gel analysis, and for typical transcription factors the amounts are also too low to be detected by Tapestation (or equivalent) analysis, and in general it is necessary to make a library to quantify, either by Tapestation analysis or Illumina sequencing. However, using a histone epitope, it is possible to detect and quantify CUT&RUN DNA by Tapestation analysis. [An example is shown for H3K27me3 from K562 cells using this protocol.](http://blocks.fhcrc.org/steveh/papers/CUT&RUN_H3K27me3_compare.pdf) By using this as a quantitative positive control, the likely success of an experiment can be determined without the need to construct and sequence libraries.

17. See Christine Codomo’s [step-by-step protocol](http://blocks.fhcrc.org/steveh/papers/Codomo_Solexa_library_prep_protocol.docx) for preparing Illumina libraries from available reagents. We follow this protocol for preparing all of our CUT&RUN libraries.

18. We have also obtained good results with the KAPA hyper-prep kit (<https://www.kapabiosystems.com/product-applications/products/next-generation-sequencing-2/dna-library-preparation/kapa-hyper-prep-kits/>). Rapid PCR cycles favor exponential amplification of the desired CUT&RUN fragments over linear amplification of intervening fragments that are too long for polymerase to completely transit. Therefore, the number of PCR cycles should be at least 12 cycles, preferably with a 10 second 60oC combined annealing/extension step. This is especially important when using the Total DNA protocol, because AMPure bead size selection only partially removes the undesirable large fragments.

19. We align paired-end reads using Bowtie2 version 2.2.5 with options: --local --very-sensitive-local --no-unal --no-mixed --no-discordant --phred33 -I 10 -X 700. For mapping spike-in fragments, we also use the --no-overlap --no-dovetail options to avoid cross-mapping of the experimental genome to that of the spike-in.

20. For the AMPure bead size selection, you are separating short fragments from very large high MW DNA. As such, this mixture is very “gloopy” and easiest to pipette with cut-off tips.

21. We and others have found that CUT&RUN fails for profiling RNA Polymerase II. We recommend that you use our X-ChIP protocol instead: https://elifesciences.org/articles/09225.