EpiCypher® CUTANA™ CUT&RUN Protocol

For histone PTMs, transcription factors (TFs), and chromatin regulators

1. Overview

Cleavage Under Targets & Release Using Nuclease (CUT&RUN) is a revolutionary genomic mapping strategy developed by the group of Dr. Steven Henikoff[1]. It builds on Chromatin ImmunoCleavage (ChIC) from Dr. Ulrich Laemmli[2], whereby a fusion of Protein A to Micrococcal Nuclease (pA-MNase) is recruited to selectively cleave antibody-bound chromatin *in situ*[3]. In CUT&RUN, cells or nuclei are immobilized to a solid support, with pAG-MNase cleaved DNA fragments isolated from solution. The workflow is compatible with next-generation sequencing (NGS) to provide high resolution genome-wide profiles of histone post-translational modifications (PTMs) and chromatin-associated proteins (*e.g.* TFs and chromatin remodelers; **Figure 1**).

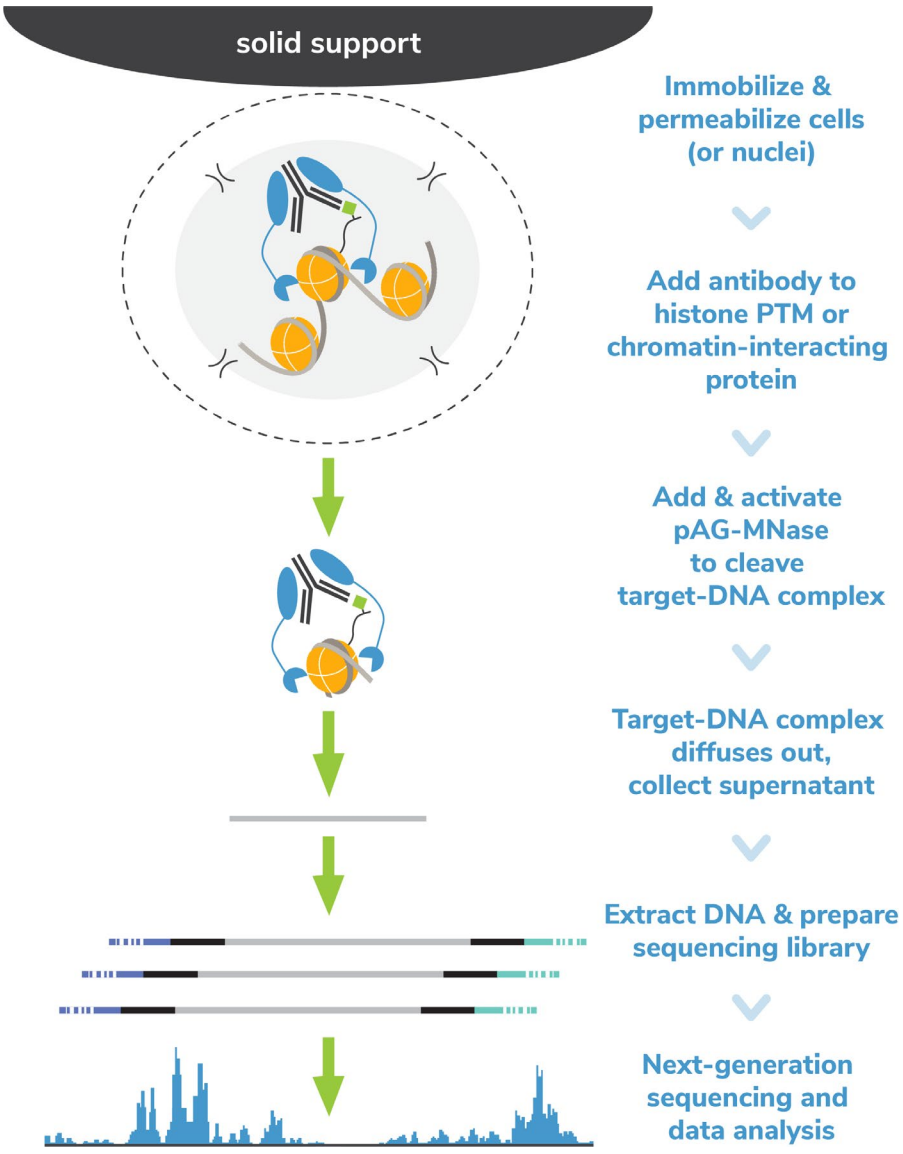

Figure 1. Overview of the CUTANA CUT&RUN protocol.

2. CUTANA Products & Services: Advantages

ChIC and CUT&RUN have revolutionized the study of chromatin regulation. Compared to ChIP-seq (the current leading approach for genome-wide mapping of histone PTMs and chromatin-associated proteins), CUT&RUN offers the following advantages:

- Requires substantially fewer cells
- More cost effective: less antibody and required sequencing depth
- Markedly improved [signal : noise]
- Streamlined workflow: cells → data in < 1 week
- Empowers benchtop sequencers (*e.g.* Illumina® MiniSeq or MiSeq)
- Dramatically increases experimental throughput (transforming the way scientists approach experimental design for genomic mapping studies)

EpiCypher now offers CUTANA pAG-MNase, the essential reagent for ChIC/CUT&RUN workflows:

- First-in-class commercial product for ChIC/CUT&RUN assays
- Optimized fusion of Proteins A and G with Micrococcal Nuclease (pAG-MNase) is directly compatible with a broad range of antibody isotypes
- 50 and 250 reaction pack sizes, enabling greater experimental throughput
- Check EpiCypher.com/resources/protocols for regular protocol updates
- Additional CUTANA products:
    - CUT&RUN Compatible Antibodies
    - CUT&RUN spike-in controls
    - CUT&RUN Kit
    - pAG-Tn5 (for CUT&Tag applications)
    - *Inquire for more information or to connect to EpiCypher scientists*: info@epicypher.com

3. Protocol Notes

This protocol has been validated for genomic profiling of:

- Histone PTMs (*e.g.* lysine methylation, acetylation and ubiquitylation)
- Transcription factors (*e.g.* CTCF)
- Chromatin remodelers (*e.g.* SMARCA4-SWI/SNF, ISWI, INO80, CHD)
- Chromatin writers & readers (*e.g.* MLL, BRD4)

1. Always include control conditions (*e.g.* positive control cells & antibody; see FAQs). This is especially critical when doing CUT&RUN for new targets and/or antibodies.

2. Protocol optimized using 500,000 human K562 cells per sample. However, without any further modifications, **this protocol has been validated on as few as 5,000 cells** with antibodies against H3K4me3, H3K27me3, and BRD4. We have also validated variations to the protocol for working with nuclei, adherent cells, cryopreserved cells/nuclei, and crosslinked material (see FAQs, protocols also available upon request).

3. Protocol adapted from 1.5 mL tubes to 300 µL 8-strip PCR tubes to: **a)** minimize beads sticking to tubes; **b)** enable more rapid workflow with multichannel pipets; **c)** provide more consistent sample handling; and **d)** enable high-throughput sample preparation.

4. Take caution throughout to avoid ConA beads sticking to the sides / caps of tubes: they dry out easily, which can result in sample loss.

5. Although protocols with shortened antibody and/or $CaCl_2$ incubation times have been published[3], in our hands such changes adversely impact yield and reproducibility.

6. We recommend a 5% digitonin stock solution be prepared in DMSO (as opposed to heated H_2O) because this dramatically increases detergent solubility and standardizes cell permeabilization / protocol reproducibility.

7. To avoid digitonin precipitation and cell lysis, use the minimal concentration of digitonin in **Digitonin Buffer** / **Antibody Buffer,** which are required for efficient permeabilization. This should be empirically determined by titrating digitonin (*e.g.* 3-fold dilutions from 0.1% down to 0.001%) and monitoring cell lysis using trypan blue staining (goal is >95% permeabilized cells) for different samples before proceeding with the full experiment.

8. **IMPORTANT**: Since CUT&RUN has lower background and is compatible with fewer cells compared to ChIP-seq, **it is not recommended to assess fragment size distribution using agarose gel or capillary electrophoresis prior to library preparation**. Such analysis is not indicative of the success of a CUT&RUN experiment, and further the amount of DNA recovered is often below the sensitivity of detection for these approaches. Instead, assess DNA yield compared to positive (*e.g.* H3K4me3, H3K27me3) and negative (IgG) controls, determine fragment size distribution of sequence-ready libraries (**Figure 5**), and evaluate peak structure and expected genome-wide distribution in NGS data.

4. Buffers, Reagents & Materials Needed

Buffer recipes

Bead Activation Buffer
20 mM HEPES, pH 7.9
10 mM KCl
1 mM $CaCl_2$
1 mM $MnCl_2$
Filter sterilize
Store @ 4ºC for up to 6 months

Pre-Wash Buffer
20 mM HEPES, pH 7.5
150 mM NaCl
Filter sterilize
Store @ 4ºC for up to 6 months

Wash Buffer
Pre-Wash Buffer (recipe above)
0.5 mM Spermidine
1x Roche cOmplete™, Mini, EDTA-free Protease Inhibitor (CPI-mini, 1tab/10mL)
Filter sterilize
Store @ 4ºC for up to 1 week

Digitonin Buffer
Wash Buffer + 0.01% Digitonin*
**Optimal [Digitonin] for each cell type should be empirically determined. Starting concentration validated for K562, MCF7, and A549 cells = 0.01% digitonin.*
Prepare fresh each day and store @ 4ºC

Antibody Buffer
Digitonin Buffer** + 2 mM EDTA
***See note above for empirically determining optimal digitonin concentration for a given cell type*
Prepare fresh each day and store @ 4ºC

Stop Buffer
340 mM NaCl
20 mM EDTA
4 mM EGTA
50 µg/mL RNase A
50 µg/mL Glycogen
Filter sterilize
Store @ 4ºC for up to 6 months

Reagents

Item	Vendor	Catalog No.	Notes
Concanavalin A (ConA) Conjugated Paramagnetic Beads	EpiCypher	21-1401	ConA is a lectin, which can cause immune activation. For technical support re. immune cell studies, contact info@epicypher.com.
pAG-MNase	EpiCypher	15-1016 or 15-1116	50 & 250 reaction pack sizes available. Supplied as 20X stock.
Rabbit IgG Negative Control Antibody	EpiCypher	13-0042	See datasheet for application-specific dilutions
SNAP-ChIP® Certified, CUTANA Compatible H3K4me3 Positive Control Antibody	EpiCypher	13-0041	See datasheet for application-specific dilutions
SNAP-ChIP® Certified, CUTANA Compatible H3K27me3 Positive Control Antibody	EpiCypher	13-0030	See datasheet for application-specific dilutions
Antibody to target of interest (histone PTM, TF, or chromatin regulator)	User-dependent		EpiCypher continues to conduct extensive antibody characterization studies[4]. For antibodies directly validated in CUT&RUN, visit: https://www.epicypher.com/antibodies/cutana-cut-run-compatible-antibodies/. For other targets not found on our webpage, contact us for recommendations: info@epicypher.com.
E. coli Spike-in DNA	EpiCypher	18-1401	Use as an exogenous spike-in control for experimental normalization. See **Appendix 1** for detailed instructions.
CUTANA DNA Purification Kit	EpiCypher	14-0050	Designed to maximize yield from low [DNA] samples, low elution volume, and retention of >50bp fragments; thus suitable for nucleosomal (PTMs) and subnucleosomal (TFs) fragments
Agencourt AMPure XP Magnetic Beads	Beckman Coulter	A63880	For PCR cleanup and size selection (*i.e.* adapter dimer and primer removal)
Qubit™ 1x dsDNA HS Assay Kit	Thermo Fisher Scientific	Q33230	For DNA quantification
NEBNext® Ultra™ II DNA Library Prep Kit for Illumina®	New England Biolabs	E7645S	For DNA sequencing applications

Equipment

Item	Vendor	Catalog No.	Notes
6 rack 1.5 mL Magnetic Separation Rack	EpiCypher	10-0012	For batch processing ConA beads.
8-strip PCR tube Magnetic Separation Rack	EpiCypher	10-0008	Enables streamlined sample handling for higher experimental throughput and improved reproducibility.
8-strip 0.2 mL PCR tubes	EpiCypher	10-0009	Compatible with the magnetic stand.
Qubit™ 4 Fluorometer	Thermo Fisher Scientific	Q33226	For DNA quantification.
Agilent 2100 Bioanalyzer	Agilent	G2939A	Or comparable capillary electrophoresis instrument (*e.g.* Agilent TapeStation).
High Performance Multi-Channel Pipettors, 8-Channel	VWR	76169-252	Or comparable multi-channel pipettor, for aspiration and wash steps to accelerate workflow.
Tube Nutator	VWR	82007-202	For bead incubation steps (overnight antibody incubation, pAG-MNase digest reaction).
Vortex-Genie	Scientific Industries	SI-0236	For bead mixing steps.

5.1. EpiCypher CUTANA CUT&RUN Protocol (~5hrs)

---Day 1---

Section I: ConA Bead Activation (~30 min)

1. Gently resuspend the **ConA beads (Concanavalin A)** and transfer 11 µL/sample to 1.5 mL tube for batch processing.
2. Place the tube on a 1.5 mL magnetic separation rack (**Figure 2**) until slurry clears and pipet to remove supernatant (supe).
3. Add 100 µL/sample cold **Bead Activation Buffer** and pipet to mix. Place the tube on a magnet until slurry clears and pipet to remove supe.
4. Repeat previous step for total of two washes.
5. Resuspend beads in 11 µL/sample cold **Bead Activation Buffer**.
6. Aliquot 10 µL/sample of activated bead slurry into 8-strip tube. Keep beads on ice until needed.

Figure 2: For ConA bead activation steps, it is recommended to batch process the full volume of beads needed for all experimental samples in a single 1.5 mL tube (11 µL beads per sample). For these steps, beads can be washed using a 1.5 mL magnetic rack (*e.g.* EpiCypher 10-0012, pictured), ensuring homogeneity across samples.

Section II: Binding Cells to Activated Beads (~30 min)

7. Harvest 0.5 million cells/sample by spinning for 3 min at 600 x g at room temperature (RT) in 1.5 mL tube. Decant supe.
   - *Note: See FAQs "Sample Input Compatibility" section for special considerations for adherent, cryopreserved, crosslinked, and immune cells as well as tissue.*
8. Resuspend cells in 100 µL/sample RT **Wash Buffer**; spin for 3 min at 600 x g at RT; decant supe.
9. Repeat previous step for total of two washes.
10. Resuspend cells in 100 µL/sample in RT **Wash Buffer** and aliquot 100 µL washed cells to each 8-strip tube containing 10 µL of activated bead. Gently vortex (setting #7) to mix.
11. **Incubate** cell : bead slurry for **10 min at RT**. *Cells will adsorb to the activated ConA beads.*

Figure 3: For processing individual samples, it is recommended to use multi-channel pipetting using 8-strip PCR tubes and compatible magnetic rack (*e.g.* EpiCypher 10-0008, pictured), increasing experimental throughput and reproducibility.

Section III: Binding of Antibodies (~30 min + overnight)

12. Place the tube strip on a magnet (**Figure 3**) until slurry clears and pipet to remove supe.
13. Add 50 µL cold **Antibody Buffer** to each sample. Gently vortex.
14. Add 0.5 µL **Antibody** (1:100 dilution) to each sample and gently vortex.

15. **Incubate** 8-strip tube (caps slightly elevated) on nutator **overnight at 4ºC**.
    - *Critical step: To keep beads in solution, slightly elevate cap side of 8-strip on nutator (**Figure 4**).*

Bead slurry before o/n incubation @ 4C | nutator o/n | Bead slurry after o/n incubation @ 4C

Figure 4. Beads should appear homogenous and not clumpy throughout the procedure. Vortex or pipette as needed to disperse clumps. Ensure tube caps are elevated during incubation steps on tube nutator.

---Day 2---

16. Place the tube on a magnet until slurry clears and pipet to remove supe.
17. While beads are on magnet, add 250 µL cold **Digitonin Buffer** directly onto beads of each sample, and then pipet to remove supe.
18. Repeat previous step for total of two washes.
19. Add 50 µL cold **Digitonin Buffer** to each 8-strip tube, and gently vortex. Beads are often clumpy at this point, but can easily be dispersed with gentle pipetting with a P200 pipet.

Section IV: Binding of pAG-MNase (~30 min)

20. Add 2.5 µL **CUTANA pAG-MNase** (20x stock) to each sample, and gently vortex.
    - *Critical step: to evenly distribute pAG-MNase across cells/nuclei, ensure beads are thoroughly resuspended by gentle vortexing and/or pipetting with a P200 pipet.*
21. **Incubate** samples for **10 min at RT**, and then return 8-strip tube to magnet. Remove supe.
22. While beads are on magnet, add 250 µL cold **Digitonin Buffer** directly to each sample, and then pipet to remove supe.
23. Repeat previous step for total of two washes.
24. Add 50 µL cold **Digitonin Buffer** to each sample, and gently vortex. Beads are often clumpy at this point, but can easily be dispersed with gentle pipetting with a P200 pipet.

Section V: Targeted Chromatin Digestion and Release (~3 hrs)

25. Place 8-strip tubes on ice, add 1 µL **100 mM $CaCl_2$** to samples, and gently vortex.
    - *Critical step: For efficient pAG-MNase digestion, ensure beads are thoroughly resuspended by vortexing and/or pipetting with a P200 pipet.*
26. **Incubate** 8-strip tube on nutator for **2 hours at 4ºC**. Key step wherein MNase tethered to chromatin is activated by $CaCl_2$ to cleave chromatin.
27. Add 33 µL **Stop Buffer** to each sample, and gently vortex to mix. Stops MNase activity by chelating Ca^{++} ions.
    - *Note: If using E. coli Spike-in DNA for experimental normalization, add spike-in DNA*

(0.5 – 1 ng or optimized amount) directly to Stop Buffer prior to mixing with samples. Aim for Spike-in DNA to comprise ~1% of sequencing reads. See Appendix 1 for full instructions.

28. **Incubate** 8-strip tube for **10 min at 37ºC** in thermocycler. *Releases chromatin to supernatant and degrades RNA.*
29. Place 8-strip tube on a magnet stand until slurry clears and transfer supe to 1.5 mL tube.
30. Purify DNA using the **CUTANA DNA Purification Kit** as per the provided instructions.
31. Elute DNA in 12 µL **Elution Buffer** and use 1 µL to quantify the CUT&RUN-enriched DNA using the **Qubit™ fluorometer** as per manufacturer's instructions.
32. PROCEED STRAIGHT TO LIBRARY PREPARATION. DO NOT assess fragment size distribution prior to library preparation, as the yield may be below the limit of detection for this analysis and is not indicative of the experimental success.

Typical CUT&RUN DNA total yields from 500k native K562 cells, pre-PCR:

- *Note: Total yields are influenced by a number of considerations, including antibody specificity and efficiency, and epitope abundance in each cell type.*

IgG → ~ 3 ng
H3K27me3 → ~200 ng
H3K4me3 → ~50 ng
CTCF → ~10 ng
BRD4 → ~20 ng
SMARCA4/BRG1 → ~15 ng

5.2. Library prep (4 hrs), Bioanalyzer (1 hr), & Illumina sequencing

Section VI: Library Preparation (~4 hrs)

33. Using 5-10 ng purified CUT&RUN-enriched DNA, prepare Illumina library using the **NEBNext® Ultra™ II Library Prep Kit for Illumina®** per manufacturer's instructions (~4 hrs).
    - *Note: NEB Ultra™ II kit contains hot-start Q5 DNA polymerase.*
34. Overview of workflow, according to manufacturer's instructions.
    a. End repair
    b. Universal adapter ligation
    c. DNA cleanup using 1.1x AMPure beads to sample volume (retains fragments >150 bp)
    d. PCR and primer indexing according to cycling parameters below
35. CUT&RUN-specific PCR cycling parameters:
    a. 45 sec @ 98ºC → activation of hot-start Q5 polymerase
    b. 15 sec @ 98ºC → DNA melting
    c. 10 sec @ 60ºC → hybrid primer annealing & short extension (<700 bp)
    d. Repeat "step b-c" for a total of 14x
    e. 1 min @ 72ºC → final extension
    - *Critical step: PCR cycling parameter are designed to enrich for DNA fragment within the sequence-able range (100 bp-700 bp). Thus, contaminating higher molecular weight DNA fragments are not enriched by PCR.*
36. DNA cleanup using 1.1x AMPure beads to sample volume (*e.g.* 55 µL beads, 50 µL PCR).
37. Elute DNA in 15 µL **0.1x TE buffer** and use 1 µL to quantify the purified PCR product using the **Qubit™ fluorometer** as per manufacturer's instructions.
    - *Typical yield of purified PCR DNA library: ~500-750 ng (30-50 ng/µL in 15 µL)*

Section VII: Agilent 2100 Bioanalyzer System (~1 hr)

38. For each sample, prepare 5 µL at 10 ng/µL for loading on the Agilent Bioanalyzer.
    - *Note: record dilution factor such that original sample molarity can be calculated from Bioanalyzer nM for desired DNA size range (100-1,000 bp).*
39. For each purified PCR DNA library for Illumina sequencing, load 1 µL of 10 ng/µL sample on **Agilent High Sensitivity DNA Chip** (Cat# 5067-4626) as per manufacturer's instructions.
    - *Typical molarity for 15 µL purified PCR DNA library (100-1,000 bp region) = 100-200 nM*
40. Typical Bioanalyzer results for CUT&RUN sequence libraries are shown (**Figure 5**). Confirm that positive control antibodies enriched for predominantly mononucleosome fragments (~275 bp peak with nucleosomes + sequence adapters).
    - *Note: Bioanalyzer (or Tapestation/equivalent approach) of PCR amplified sequence libraries is the best indicator of CUT&RUN experimental success prior to NGS.*

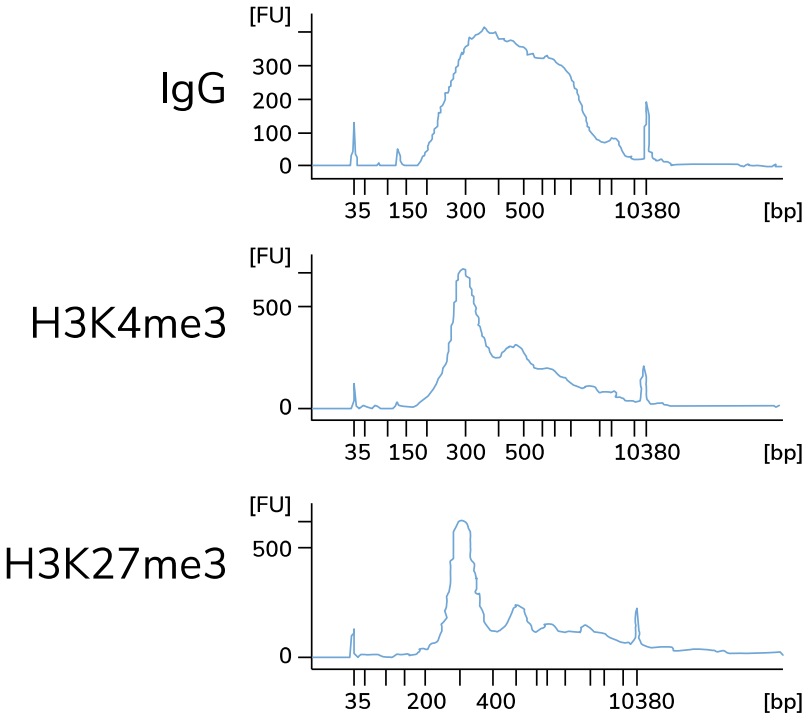

Figure 5. Typical Bioanalyzer traces for IgG negative control and H3K4me3 / H3K27me3 positive control CUT&RUN sequencing libraries (5 ng PCR amplified libraries loaded on Bioanalyzer). H3K4me3 and H3K27me3 libraries are predominantly enriched for mononucleosomes as indicated by the peak at 275 bp (~150 bp mononucleosomes + 125 bp sequence adapters). Quality Bioanalyzer (or Tapestation / equivalent approach) traces are the best indicator of success prior to sequencing. However, this analysis should not be performed prior to NGS library preparation, since the amount of CUT&RUN DNA is likely to be below the limit of detection prior to library amplification.

Section VIII: Illumina sequencing

41. Based on Bioanalyzer molarity calculations, pool libraries at the desired ratios.
    - Only 3-5 million paired-end (PE) reads are needed for good CUT&RUN coverage.
42. To obtain 3-5M PE reads for 8 samples, we typically load 0.8 pM (500 µL) into a cartridge for a MiniSeq High Output Reagent Kit, 150-cycles (catalog # FC-420-1002).
    - Flow cells using the MiniSeq High Output Kit typically yield 25-40M pass-filter clusters (*i.e.* 25-40M PE reads).
43. To obtain 3-5M PE reads for >48 samples, we typically load 0.8 pM (1,500 µL) into a cartridge for a NextSeq 500/550 High Output Kit v2.5, 150-cycles (catalog # 20024907).
    - Flow cells using the NextSeq High Output Kit v 2.5 typically yield 400M pass-filter clusters (*i.e.* 400M PE reads).

6. Buffer components

Components	Source	Cat #
HEPES	Sigma-Aldrich	H3375
KCl	Sigma-Aldrich	P3911
$CaCl_2$	Sigma-Aldrich	C1016
$MnCl_2$	Sigma-Aldrich	203734
Molecular biology grade H_2O (RNase, DNase free)	VWR	VWRV02-0201-0500
NaCl	Sigma-Aldrich	S5150-1L
EDTA (prepare 0.5 M stock at pH 8.0)	Sigma-Aldrich	E5134
EGTA (prepare 0.5 M stock at pH 8.0)	Sigma-Aldrich	E3889
RNase A	Thermo Fisher Scientific	EN0531
Glycogen	Sigma-Aldrich (Roche)	10930193001
Pure Spermidine (6.4M)	Sigma-Aldrich	S0266
Digitonin (store aliquots of 5% stock in DMSO at -20ºC)	Millipore Sigma	300410
DMSO	Sigma-Aldrich	D8418-100ml
cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail	Roche	11836170001
Trypan Blue	Thermo Fisher Scientific	T10282

7. Frequently Asked Questions (FAQs)

1. What is the best way to know if a CUT&RUN experiment worked prior to sequencing?
   Results from challenging cell inputs / targets may be ambiguous, so EpiCypher recommends including positive / negative controls in every experiment. If the QC checks for the positive control perform as expected, then proceeding to sequencing with all samples is recommended. If sequencing results for challenging cell inputs / targets are not satisfactory, further optimization may be necessary (*e.g.* cell type and / or number, digitonin permeabilization, antibody concentration / alternate vendors, etc.).

   Recommended experimental design:
   a. Always include control antibodies and control cells
      i. Start with 500k K562 cells
      ii. Positive control antibody (H3K4me3 or H3K27me3)
      iii. Negative control antibody (IgG or no antibody)
   b. Experimental sample antibodies and cell types

   Quality control checks before decision to sequence:
   a. Yield of CUT&RUN enriched DNA (~200 ng for positive control H3K27me3 antibody, ~3 ng for negative control IgG antibody). See **Section V.**
   b. Yield of library prep and PCR (~750 ng from 5-10 ng library input). See **Section VI.**
   c. Bioanalyzer/Tapestation traces of sequence-ready libraries* should be enriched with mononucleosome size fragments (~275 bp = ~150 bp nucleosome+ 125 bp adapters, see **Section VII**)

      ***IMPORTANT**: It is **not** recommended to assess fragment size distribution using agarose gel or capillary electrophoresis **prior to library preparation**. This analysis is not indicative of the success of a CUT&RUN experiment, and further the amount of DNA recovered may be below the sensitivity of detection for these approaches. **Always assess CUT&RUN fragment size distribution after library preparation**.

DNA spike-in controls

2. Can residual *E. coli* in the pAG-MNase prep be used for sample input normalization? What spike-in DNA control does EpiCypher recommend?
   Carry-over *E. coli* DNA is present in EpiCypher's pAG-MNase preps. However, at a typical sequencing depth of 3-5M reads, too few *E. coli* DNA fragments (~hundreds) are recovered for reliably computing sample normalization. Thus, EpiCypher now offers *E. coli* Spike-in DNA to provide sufficient read depth for experimental normalization (EpiCypher Catalog No. 18-1401; **Appendix 1**) and is actively developing recombinant nucleosome spike-in controls.

3. Does EpiCypher offer spike-in DNA controls for CUT&RUN?
   EpiCypher now offers *E. coli* Spike-in DNA for experimental normalization (EpiCypher Catalog No. 18-1401; **Appendix 1**). However, CUTANA Spike-in nucleosomes (*i.e.* fully-defined semi-synthetic nucleosomes) for CUT&RUN are in active development and offer multiple advantages, including enabling sample input normalization and multiple control points (**1.** antibody specificity; **2.** antibody efficiency; **3.** pAG-MNase activity; and **4.** experimental success).

Sample input compatibility

4. What types of sample inputs are compatible with CUT&RUN?
   EpiCypher has confirmed that CUT&RUN is compatible with whole cells and nuclei derived from mammalian suspension and adherent cell lines. EpiCypher has not yet directly tested, but a number of groups have successfully performed CUT&RUN on human and mouse primary tissue[5-8], FACS sorted[9] and immune cells[10,11]. Below are general recommendations for specific sample types:

   Adherent cells: Enzymatic treatment (*e.g.* trypsin) will degrade glycoproteins, thereby impairing cell adsorption to the ConA beads. Therefore, **do not use trypsin to prepare adherent cells for CUT&RUN**. Instead, scrape adherent cells to dislodge them from the dish, then pellet by centrifugation for ~3 min at 600 x g, RT. Proceed directly to the CUT&RUN wash steps.

   Tissue samples: While EpiCypher does not have tissue-specific protocols available at this time, the primary requirement is that tissue is processed to a mono-dispersion of cells (typically by mechanical maceration or douncing).

   Working with immune cells: Note that lectins (*e.g.* ConA-beads) play a role in the innate immune system and so immune cells types may be inadvertently stimulated via binding to ConA-beads. To circumvent this potential problem in CUT&RUN, EpiCypher recommends using nuclei[9] or a crosslinking strategy[12].

5. Is CUT&RUN compatible with frozen or crosslinked cells?
   Yes. General guidelines are noted below and detailed protocols are available upon request (email info@epicypher.com):

   Cryopreservation: EpiCypher has confirmed that freeze/thawed cells (*e.g.* slow freeze in 10% DMSO/media, and then quick thaw at 37ºC) generate data of indistinguishable quality to fresh material. This cryopreservation method is optimized to preserve native physiological interactions, minimize cell lysis, and reduce background signal in CUT&RUN.

   Crosslinking: It is recommended to first try native samples in CUT&RUN, since this works well for most targets. However, EpiCypher has also tested previously reported crosslinking conditions and recommended wash buffers[12], and found that while yields are significantly lower than native cells, the resulting data tracks display similar quality. Therefore, in some cases CUT&RUN signal for labile targets or highly transient chromatin binding proteins may be improved by light crosslinking.

   In some cases, histone deacetylase activity may contribute to incomplete/low resolution genomic profiles in native CUT&RUN. For example, EpiCypher has observed that certain acetyl PTMs such as H3K27ac and H3K18ac may show enhanced signal after mild (*e.g.* 0.1% paraformaldehyde, 1 min) to moderate (*e.g.* 1% paraformaldehyde, 1 min) crosslinking even though total yield is reduced. However, heavy crosslinking such as that typically used for ChIP-seq (*e.g.* 1% paraformaldehyde, 10 min) has been observed to damage histone

acetylation signal in K562 cells. Therefore, optimal crosslinking conditions for profiling histone acetylation and histone acetylation binding proteins (*e.g.* some bromodomain-containing proteins) should be empirically determined in the model system of interest.

Importantly, not all acetyl-PTM and readers require crosslinking for high quality data (*e.g.* H3K9ac, BRD4). Therefore, crosslinking should only be used as a last resort to improve signal-to-noise, since (1) crosslinking reduces yield; (2) crosslinking can decrease specificity and increase artifacts; and (3) key protocol adaptations are required when adapting CUT&RUN to crosslinked samples (supplementing Wash, Digitonin, and antibody buffers with Triton X-100 and SDS detergents). EpiCypher continues to optimize crosslinking protocols and determine cases where this approach may improve signal. CUT&RUN crosslinking protocols can be found in the literature[12], and are also available from EpiCypher upon request (email info@epicypher.com).

Antibodies and targets

6. Does EpiCypher's CUT&RUN protocol work on non-PTM targets?
   Yes. The current protocol has been used at EpiCypher to generate numerous non-PTM CUT&RUN data, including CTCF, BRD4, and SMARCA4 (BRG1). No protocol modifications were necessary to generate these data since the columns we use to purify the CUT&RUN-enriched chromatin fragments retain >50bp DNA.

   However, for TFs in particular, which generate sub-nucleosomal size (<120 bp) DNA fragments, modifications to the library protocol have been reported[7,13] to improve the representation of these smaller fragments during library preparation. Briefly, to enrich for sub-120 bp fragments, the authors reduced the inactivation temperature after end repair from 65ºC for 30m to 50ºC for 1hr. Also, after the ligation reaction, AMPure bead size-selection ratio was increased from 1.1x volume to 1.75x volume. For TF CUT&RUN fragment sizes, both nucleosomal (~150 bp) and sub-nucleosomal (<120 bp) reflect TF chromatin occupancy locations, however, the sub-nucleosomal fragments provide higher resolution mapping for their locations.

7. What PTM antibodies do EpiCypher recommend for CUT&RUN?
   EpiCypher now offers CUTANA Compatible Antibodies, which are rigorously tested for specificity using EpiCypher's SNAP-ChIP recombinant nucleosome spike-in control panels[4] and are verified to yield robust results in CUT&RUN: https://www.epicypher.com/antibodies/cutana-cut-run-compatible-antibodies/.

   Since we have not yet developed recombinant nucleosome spike-in control panels for CUT&RUN, we remain cautious about transferring SNAP-ChIP specificity directly to CUT&RUN. Our ongoing studies include the creation of *in situ* nucleosome standards to interrogate antibodies with the highest possible resolution. For non-PTM targets, we recommend testing a few different antibodies to each target and proceed with the reagent that generates the highest quality data.

8. References

1 Skene, P. J. & Henikoff, S. An efficient targeted nuclease strategy for high-resolution mapping of DNA binding sites. *eLife* **6**, doi:10.7554/eLife.21856 (2017).
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Appendix 1: Use of *E. coli* Spike-in DNA for experimental normalization

Notes

- Use in combination with **CUTANA pAG-MNase for ChIC/CUT&RUN** (EpiCypher 15-1016), which has very low levels of *Escherichia coli* (*E. coli*) DNA.

Protocol

1. Add 0.5-1 ng* *E. coli* Spike-in DNA directly to CUT&RUN Stop Buffer (33 µL Stop Buffer per sample; see protocol).
    - *Note: 0.5-1 ng E. coli Spike-in DNA per sample is recommended for most samples. However, based on target abundance and antibody efficiency, the amount of spike-in DNA may need to be adjusted. Aim for spike-in to comprise ~1% (0.2-5%) of total sequencing reads (**Table 1**).*

E. coli Spike-in DNA	Target	Total Reads	*E. coli* Reads	% *E. coli* Reads
0.5 ng	IgG	3,644,233	155,549	4.27%
	H3K4me3	3,121,112	42,210	1.35%
	H3K27me3	5,254,299	8,511	0.16%
1.0 ng	IgG	2,569,291	241,645	9.41%
	H3K4me3	3,127,912	147,565	4.72%
	H3K27me3	9,650,258	22,419	0.23%

Table 1. EpiCypher *E. coli* Spike-in DNA (0.5 and 1.0 ng) was added to CUT&RUN samples using 500,000 K562 cells enriched for a low abundance target (H3K4me3, EpiCypher Catalog No. 13-0041), a high abundance target (H3K27me3, EpiCypher Catalog No. 13-0030) and IgG negative control (EpiCypher Catalog No. 13-0042). Total numbers of paired-end sequencing reads, reads aligned to *E. Coli*, and percentage of total sequencing reads aligned to *E. coli* spike-in DNA are shown. Green boxes highlight the spike-in amounts recommended for each target.

2. At the appropriate step in the CUTANA CUT&RUN protocol, quench pAG-MNase reaction with 33 µL Stop Buffer + *E. coli* Spike-in DNA.
3. Complete protocol and prepare Illumina sequencing library.
4. After sequencing, in addition to the experimental sample reference genome (*e.g.* human hg19 build) align reads to the *E. coli* K12, MG1655 reference genome:
    a. https://support.illumina.com/sequencing/sequencing_software/igenome.html
5. For pairwise comparisons, quantify *E. coli* Spike-in DNA reads for treatment and untreated samples and normalize to sequencing depth.
    Example:
    a. *Treatment spike-in = 100,000 Ec reads in 5,000,000 uniquely aligned reads = 2%*
    b. *Untreated spike-in = 30,000 Ec reads in 3,000,000 uniquely aligned reads = 1%*
6. Calculate normalization factor (see reference A, below), such that after normalization the *E. coli* spike-in signal is set to be equal across all samples:
    Example:
    a. *Treatment normalization factor = 1 / 2% spike-in bandwidth = 0.5*
    b. *Untreated normalization factor = 1/ 1% spike-in bandwidth = 1.0*

7. Use single scalar normalization ratio with the --scaleFactor option enabled in deeptools bamCoverage tool to generate a normalized bigwig files for visualization in IGV (https://deeptools.readthedocs.io/en/develop/content/tools/bamCoverage.html).
   Example:
   a. *Treatment sample --scaleFactor = 0.5*
   b. *Untreated sample --scaleFactor = 1.0*

Note: The effect of normalization on a sample is inversely proportional to the E. coli spike-in bandwidth. In other words, samples with the highest bandwidth will receive the largest reduction in signal after normalization. For further information on experimental normalization using exogenous spike-in controls, see references below [A,B].

Appendix 1 references

[A] Tay et al. Hdac3 is an epigenetic inhibitor of the cytotoxicity program in CD8 T cells. (2020)
"Reads for the entire barcoded spike-in histone K-acetyl panel were quantified before and after ChIP. We evaluated the reads from all barcoded spike-in acetylation marks to calculate the enrichment of ChIPs from both Hdac3-KO and Hdac3-WT samples, and used the ratio of the barcode signals to normalize the sequencing Bedgraph files. Normalization was done by taking the union set of all peaks from the two H3K27ac ChIP samples (Hdac3-KO and Hdac3-WT), calculating the read depth- normalized ratio of reads at each peak location, and then applying the normalization factor derived from the spike-in panel barcodes to the sequencing results. Finally, the normalized Bed- graph files were converted to Bigwiggle format for viewing in the Integrative Genomics Viewer platform and downstream analysis."

[B] Orlando et al. Quantitative ChIP-Seq Normalization Reveals Global Modulation of the Epigenome. (2014)