# EpiCypher® CUTANA™ CUT&RUN Protocol

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

**This protocol has been validated for genomic profiling of:**

- Histone PTMs (*e.g.* lysine methylation, acetylation and ubiquitylation)
- Transcription factors (*e.g.* CTCF, FOXA1)
- Chromatin remodelers (*e.g.* ATPase subunits of SWI/SNF, ISWI, INO80, CHD)
- Chromatin writers & readers (*e.g.* MLL1, BRD4)
- Nuclear hormone receptor signaling factors (*e.g.* Estrogen Receptor)
- Epitope-tagged proteins (*e.g.* HA, FLAG tags)

## Table of Contents

1. Overview ..... 2
2. CUTANA Products & Services: Advantages ..... 3
3. Protocol Notes ..... 4
4. Buffers, Reagents & Materials Needed ..... 5
5. EpiCypher CUTANA CUT&RUN Protocol ..... 8
6. Frequently Asked Questions (FAQs) ..... 14
7. References ..... 18
Appendix I: Nuclei Isolation & Cryopreservation Protocol for CUT&RUN ..... 19
Appendix II: Sample Integrity & Bead Conjugation Quality Control Checks ..... 21
Appendix III: Use of *E. coli* Spike-in DNA for Experimental Normalization ..... 23

## 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 Proteins A and/or G to Micrococcal Nuclease (pAG-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**).

The following protocol describes detailed recommendations for performing CUT&RUN, based on extensively optimized workflows developed by EpiCypher scientists. Check back at epicypher.com/protocols for regular protocol updates.

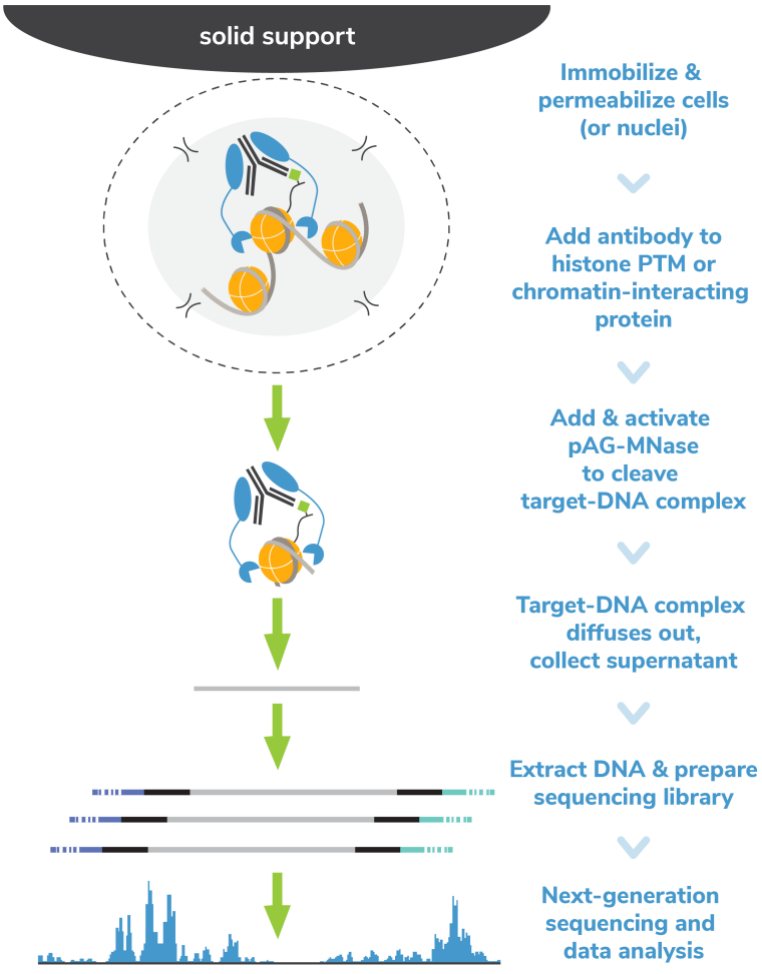


**Figure 1**. Overview of the CUTANA CUT&RUN protocol.

## 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:

| Platform Comparison | ChIP-seq | CUTANA CUT&RUN |
|---|---|---|
| **Required cells** | > 1 million | 5,000- 500,000 |
| **Cell Input** | Fragmented chromatin | Intact cells or nuclei |
| **Sequencing Depth** (Reads) | > 30 million | 3-8 million |
| **Experimental throughput** | Low | High |
| **Signal : Noise** | Low | High |
| **Assay Automation** | Difficult | Yes |

EpiCypher now offers a suite of products to support CUT&RUN workflows under our CUTANA™ assay platform, including:

- **pAG-MNase** (EpiCypher 15-1016), the essential reagent and first-in-class commercial product for ChIC/CUT&RUN assays. The EpiCypher enzyme contains an optimized fusion of Proteins A and G with Micrococcal Nuclease (pAG-MNase) to enable compatibility with a broad range of antibody isotypes.
- **CUT&RUN supporting reagents** selected and validated for optimal performance in the EpiCypher CUT&RUN protocol. See epicypher.com/cut-and-run-assays for more information.
- **CUT&RUN Compatible Antibodies** rigorously lot-to-lot validated directly in CUT&RUN. See epicypher.com/cut-and-run-compatible-antibodies for more information.
- **CUT&RUN Spike-in Controls**
  - *E. coli* Spike-in DNA (EpiCypher 18-1401) for data normalization.
  - [*coming soon*] Recombinant nucleosome spike-in controls based on our proprietary SNAP-ChIP® technology (epicypher.com/snap-chip).
- **ChIC/CUT&RUN Kit** (EpiCypher 14-1018) with all reagents included to go from cells to purified CUT&RUN DNA.
- **CUTANA CUT&Tag** supporting reagents for ultra-low input applications. See epicypher.com/CUT&Tag for more information.
- *Inquire for more information or to connect with EpiCypher scientists:* info@epicypher.com

## 3. Protocol Notes

1. Always include control conditions: IgG negative control antibody (EpiCypher 13-0042) and a positive control antibody (*e.g.* H3K4me3 (EpiCypher 13-0041) is recommended for histone PTMs, while BRD4 (EpiCypher 13-2003) or CTCF (EpiCypher 13-2014) can be used for chromatin-associated proteins). This is especially critical when doing CUT&RUN for new targets and/or antibodies.

2. Optimize conditions and become familiar with the workflow using a control cell line (*e.g.* K562) before attempting different sample types (see **FAQs**).

3. 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 (EpiCypher 13-0041), H3K27me3 (Thermo Fisher MA5-11198), and BRD4 (EpiCypher 13-2003).

4. While the standard protocol is written for native suspension cells, specific sample preparation recommendations are described for working with nuclei (**Appendix I**), adherent cells (**CUT&RUN Protocol, Section II**), cryopreserved cells/nuclei (**Appendix I**), and cross-linked material (see full cross-linking protocol at epicypher.com/protocols).

5. Protocol has been adapted to 8-strip PCR tubes (vs. 1.5 mL tubes) to: **a)** minimize beads sticking to tubes; **b)** enable more rapid workflow with multichannel pipettes; **c)** provide more consistent sample handling; and **d)** enable high-throughput sample preparation.

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

7. 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.

8. We recommend a 5% digitonin stock solution be prepared in DMSO (as opposed to heated $H_2O$), as this improves detergent solubility and protocol reproducibility.

9. To avoid digitonin precipitation and cell lysis, use the minimal concentration of digitonin in **Digitonin Buffer** / **Antibody Buffer** required for efficient permeabilization. A detailed protocol for digitonin optimization is described in the CUTANA™ ChIC/CUT&RUN Kit manual (EpiCypher 14-1048). In general, perform a digitonin titration (*e.g.* 3-fold dilutions from 0.1% down to 0.001%) and monitor cell lysis using Trypan blue staining. Find the minimum [digitonin] needed to achieve >95% permeabilized cells.

10. **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, CTCF) 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

## Table 1: 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 |
| Spermidine trihydrochloride* | Sigma-Aldrich | S2501 |
| 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 |

***Spermidine preparation**

*For 1M, dissolve 254.63 mg spermidine in 1 mL molecular grade $H_2O$*
*Store in single use aliquots at -20°C for 6 months*

## 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*

## Table 2: 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 H3K27me3 Positive Control Antibody | Thermo Fisher | MA5-11198 | Use 0.5 µg per reaction |
| CUTANA Compatible CTCF Positive Control Antibody | EpiCypher | 13-2014 | 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: epicypher.com/cut-and-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 III** 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 |

## Table 3: 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. EpiCypher CUTANA CUT&RUN Protocol

## 5.1. 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 pipette to remove supernatant (sup).
3. Immediately add 100 µL/sample cold **Bead Activation Buffer** and pipette to mix. Place the tube on a magnet until slurry clears and pipette to remove sup.
4. Repeat previous step for total of two washes.
5. Resuspend beads in 11 µL/sample cold **Bead Activation Buffer**.
6. Keep activated ConA 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 to ensure homogeneity across samples. For these steps, beads can be washed using a 1.5 mL magnetic rack (*e.g.* EpiCypher 10-0012, pictured).

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

7. Harvest 0.5 million cells/sample by spinning suspension culture for 3 min at 600 x g at room temperature (RT) in a 1.5 mL tube. Remove sup by decanting or pipetting.

   *Notes and alternative protocols:*

   - *It is recommended to prepare excess cells (~10% excess if working in batch or one extra sample) for quality control checks (**Appendix II**).*
   - *To harvest adherent cells, we recommend a very mild trypsin treatment: 0.05% trypsin, incubated at 37°C, for the **minimal time** necessary to detach cells (see **FAQs**).*
   - *Freshly isolated nuclei and frozen nuclei are compatible with CUT&RUN; for instructions, see **Appendix I**.*
   - *For samples that require cross-linking to preserve labile marks (e.g. formaldehyde), see our **Cross-linking Protocol**.*
   - *See **FAQs "Sample Input Compatibility"** section for special considerations when using adherent, cryopreserved, cross-linked, immune cells, and tissue.*
8. Resuspend cells in 100 µL/sample RT **Wash Buffer**; spin for 3 min at 600 x g at RT; remove sup.
9. Repeat previous step for total of two washes.
10. Resuspend cells in 100 µL/sample in RT **Wash Buffer.** Pipette to mix.
11. Proceed with ConA bead binding, working in batch using 1.5 mL tubes if possible, to ensure homogeneity across samples. Mix 100 µL washed cells/sample with 10 µL of activated ConA beads/sample. Gently vortex (setting #7) to mix.

- *Note: If it is not possible to work in batch (e.g. each sample uses a distinct cell type or sample input), transfer individual samples (100 µL cells + 10 µL beads) to 8-strip tubes for subsequent steps (**Figure 3**).*

12. **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 multi-channel pipette 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)

13. Place the tube(s) on a magnet until slurry clears. Pipette to remove sup and discard.
14. Add 50 µL cold **Antibody Buffer** per sample quickly, to avoid bead drying. *Gently vortex immediately and thoroughly to an even resuspension.*
15. If working in batch, transfer 50 µL per experimental sample into individual 8-strip PCR tubes**.** Continue the remaining steps using an 8-strip tube magnetic rack (**Figure 3**).
16. Add **Antibody** to each sample and gently vortex immediately and thoroughly.
    - *Note: See manufacturer's recommendations for the optimal amount of antibody to use in CUT&RUN. If unknown, 0.5 µg antibody is the recommended starting point.*
17. **Incubate** 8-strip tube (caps slightly elevated at a 30º to 45º angle) on nutator **overnight at 4ºC**.
    - *Critical step: To keep beads in solution, slightly elevate cap side of 8-strip on nutator to ensure bead solution remains in bottom of conical tube (**Figure 4**).*

Bead slurry before O/N incubation at 4°C | Nutator O/N | Bead slurry after O/N incubation at 4°C

**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 to keep beads in solution.

---Day 2---

## Section III (*continued*)

18. Place the tube on a magnet until slurry clears and pipette to remove sup.
19. While beads are on magnet, add 250 µL cold **Digitonin Buffer** directly onto beads of each sample, and then pipette to remove sup.
20. Repeat previous step for total of two washes, keeping beads on magnet.
21. Remove sup and 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 by gently pipetting with a P200 pipette. A slightly cut-off pipette tip may be used to aid in resuspension and/or preserve delicate cells or nuclei.*

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

22. 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 pipette (cut-off pipette tip optional).*
23. **Incubate** samples for **10 min at RT**, and then return 8-strip tube to magnet. Remove sup.
24. While beads are on magnet, add 250 µL cold **Digitonin Buffer** directly to each sample, and then pipette to remove sup.
25. Repeat previous step for total of two washes, keeping beads on magnet.
26. Remove sup and remove beads from magnet. 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 pipette (cut-off pipette tip optional).*

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

27. 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 pipette (cut-off pipette tip optional).*
28. **Incubate** 8-strip tube on nutator for **2 hours at 4ºC**, with tubes slightly elevated as in **Figure 4**. *This is the key step wherein MNase tethered to chromatin is activated by $CaCl_2$ to cleave chromatin.*
29. Add 33 µL **Stop Buffer** to each sample, and gently vortex to mix. *Stops MNase activity by chelating $Ca^{2+}$ ions.*
    - *Note: If using E. coli Spike-in DNA for experimental normalization, first prepare a Stop Buffer Master Mix by adding the optimized amount of spike-in DNA (**Appendix III**) directly to the Stop Buffer before sample addition. Mix thoroughly and distribute 33 µL Stop Buffer Master Mix per sample.*
30. **Incubate** 8-strip tube for **10 min at 37ºC** in thermocycler. *Releases chromatin to supernatant and degrades RNA.*
31. Quick spin tubes in benchtop microfuge. Place 8-strip tube on a magnet stand until slurry clears and transfer sup to 1.5 mL tube.

32. Purify DNA using the **CUTANA DNA Purification Kit** as per the provided instructions.
33. 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. Typical CUT&RUN DNA total yields from 500k native K562 cells, pre-PCR are noted below:

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

    - *Note: Total yields are influenced by a number of factors, including the antibody used, target abundance, and cell type. In general, a good indicator of experimental success is that the DNA yield of the target(s) of interest are above that of IgG control.*

34. 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 experimental success.

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

### Section VI: Library Preparation (~4 hrs)

35. 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.*
36. 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
37. 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 parameters are designed to enrich for DNA fragments within the sequence-able range (100 bp-700 bp). Thus, contaminating higher molecular weight DNA fragments are not enriched by PCR.*
38. DNA cleanup using 1.1x AMPure beads to sample volume (*e.g.* 55 µL beads, 50 µL PCR).
39. 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.
    - *Note: 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)

40. 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).*
41. 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.
    - *Note: Typical molarity for 15 µL purified PCR DNA library (100-1,000 bp region) = 100-200 nM*
42. 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.*

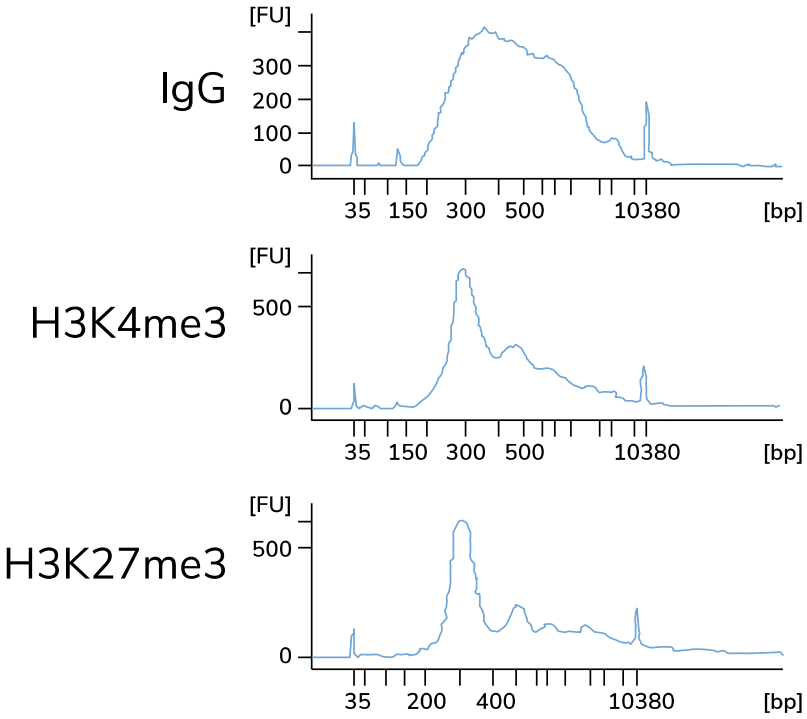


**Figure 5**: Typical Bioanalyzer traces for IgG negative control and H3K4me3 / H3K27me3 positive control CUT&RUN sequencing libraries (5 ng PCR amplified libraries loaded on Bioanalyzer). H3K4me3 and H3K27me3 libraries are predominantly enriched for mononucleosomes as indicated by the peak at 275 bp (~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

43. Based on Bioanalyzer molarity calculations, pool libraries at the desired ratios.
    - *Note: Only 3-5 million paired-end (PE) reads are needed for good CUT&RUN coverage.*
44. 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).
    - *Note: Flow cells using the MiniSeq High Output Kit typically yield 25-40 million pass-filter clusters (i.e. 25-40 million PE reads).*
45. 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).
    - *Note: Flow cells using the NextSeq High Output Kit v 2.5 typically yield 400 million pass-filter clusters (i.e. 400 million PE reads).*

# 6. Frequently Asked Questions (FAQs)

## 6.1 General

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. We have included a series of quality control (QC) checks to analyze sample quality, permeabilization, confirm ConA bead binding, assess DNA yields at various stages, and confirm fragment size distribution (see **Appendix II** and **CUT&RUN Protocol Sections V - VII**). If the QC checks and positive / negative controls 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 500,000 K562 cells
      ii. Positive control antibody (H3K4me3 for PTMs; CTCF for transcription factors)
      iii. Negative control antibody (IgG or no antibody)
   b. Experimental sample antibodies and cell types

   Quality control checks before decision to sequence:
   a. Integrity of starting cells / nuclei is confirmed (**Appendix II**). Samples should not contain clumps and starting counts should be accurate.
   b. Confirm sample binding to ConA beads (**Appendix II**).
   c. Optimize digitonin permeabilization conditions (>95% cells permeabilized).
   d. Ideally, the yield of CUT&RUN enriched DNA for positive control antibody should be greater than negative control IgG. See **Section V.** Note that at low cell inputs, this difference may not be observed, but good quality sequencing data can still be obtained.
   e. Yield of library prep and PCR (~750 ng from 5-10 ng library input). See **Section VI.**
   f. 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**.

## 6.2 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 (**Appendix III**) 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 (**Appendix III**). 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, including confirmation of antibody specificity, target-specific pAG-MNase cleavage, and overall experimental success.

## 6.3 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. Protocols to isolate nuclei for CUT&RUN are provided in **Appendix I**. 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: Adherent cells present a special challenge for CUT&RUN, as the process must be strong enough to detach cells from culture plates and disaggregate cell clumps, yet gentle enough to preserve cell and nuclear membranes for binding to ConA beads. A major concern is that strong or prolonged enzymatic treatment (*e.g.* trypsin) can degrade glycoproteins, thereby impairing cell adsorption to the ConA beads.

EpiCypher has compared different methods of collecting adherent cells for CUT&RUN, including cell scraping, trypsin, and accutase. We analyzed the impact of each method on the cell adsorption rate onto ConA beads, using MCF-7 cells. **Based on these optimization experiments, we recommend a very mild trypsin treatment (0.05% trypsin at 37°C, minimal incubation time as optimized for cell type).** This method detaches and monodisperses cells, resulting in >95% adsorption onto beads. The cells are collected and pelleted by centrifugation for ~3 min at 600 x g, RT. Proceed directly to the CUT&RUN wash steps. Trypsin is then washed away by subsequent washes that are a standard part of the CUT&RUN protocol.

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] (see **Appendix I**) or a cross-linking strategy[12] (see detailed cross-linking protocol at epicypher.com/protocols).

5. Is CUT&RUN compatible with frozen or cross-linked cells?

Yes. General guidelines are noted below; our detailed **CUTANA CUT&RUN Cross-linking Protocol** is provided at epicypher.com/protocols, and additional 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. We use similar methods for freeze / thawing isolated nuclei for CUT&RUN (see **Appendix I**).

Cross-linking: It is recommended to first try native samples in CUT&RUN, since this works well for most targets. Of note, EpiCypher has tested previously reported cross-linking conditions and recommended wash buffers[12]. Although yields are significantly lower than from native cells, the resulting data tracks display similar quality (*i.e.* signal : noise). Furthermore, for labile targets or highly transient chromatin binding proteins, CUT&RUN signal may be improved by light cross-linking.

When using native CUT&RUN, histone deacetylase activity may contribute to incomplete / low resolution genomic profiles for certain targets. For example, EpiCypher has observed that certain acetyl PTMs such as H3K27ac and H3K18ac may show enhanced signal after mild (*e.g.* 0.1% formaldehyde, 1 min) to moderate (*e.g.* 1% formaldehyde, 1 min) cross-linking even though total yield is reduced. However, heavy cross-linking such as that typically used for ChIP-seq (*e.g.* 1% formaldehyde, 10 min) has been shown to damage histone acetylation signal in K562 cells. Therefore, optimal cross-linking 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 cross-linking for high quality data (*e.g.* H3K9ac, BRD4). Therefore, cross-linking should only be used as a last resort to improve signal : noise, since (1) cross-linking reduces yield; (2) cross-linking can decrease specificity and increase artifacts; and (3) key protocol adaptations are required when applying CUT&RUN to cross-linked samples (supplementing Wash, Digitonin, and antibody buffers with Triton X-100 and SDS detergents). EpiCypher continues to optimize cross-linking protocols and determine cases where this approach may improve signal. Our current **CUTANA CUT&RUN Cross-linking Protocol** is available at epicypher.com/protocols; similar protocols can be found in the literature[12].

## 6.4 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, FOXA1/HNF3A, 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 transcription factors (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 MNase 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 antibodies does EpiCypher recommend for CUT&RUN?

**Chromatin-associated protein targets**: Antibodies that work well in ChIP may not always work in CUT&RUN. To address this unmet need for researchers, EpiCypher has developed "CUTANA Compatible Antibodies" for chromatin associated protein targets (see the expanding list of targets at: epicypher.com/cut-and-run-compatible-antibodies). Every lot of a CUTANA Compatible Antibody is validated directly in CUT&RUN and/or CUT&Tag. The antibody is determined to yield peaks that show a genomic distribution pattern consistent with reported function(s) of the target protein (for example, DNA binding motif analysis for transcription factors). Our scientists are continuously testing antibodies to novel targets. For targets of interest not on this list, contact us for antibody recommendations: techsupport@epicypher.com.

**Histone PTM targets**: Antibodies to histone PTMs are particularly susceptible to off-target cross reactivity which can compromise biological interpretations[4]. EpiCypher has conducted extensive studies of histone PTM antibodies in ChIP-seq by using our exclusive SNAP-ChIP® nucleosome spike-in control technology. While the majority of antibodies are exhibit major problems such as poor specificity and/or low efficiency, this approach has enabled the identification of best-in-class reagents for ChIP-seq (see ref [4] and chromatinantibodies.com *Maryanski et al., In preparation*). EpiCypher is currently developing SNAP Spike-in controls for CUT&RUN that are currently being used for antibody validation (*coming soon*). For more information or for antibody recommendations, please contact techsupport@epicypher.com.

## 7. 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).
2 Schmid, M., Durussel, T. & Laemmli, U. K. ChIC and ChEC; genomic mapping of chromatin proteins. *Molecular cell* **16**, 147-157, doi:10.1016/j.molcel.2004.09.007 (2004).
3 Skene, P. J., Henikoff, J. G. & Henikoff, S. Targeted in situ genome-wide profiling with high efficiency for low cell numbers. *Nature protocols* **13**, 1006-1019, doi:10.1038/nprot.2018.015 (2018).
4 Shah, R. N. *et al.* Examining the Roles of H3K4 Methylation States with Systematically Characterized Antibodies. *Molecular cell* **72**, 162-177.e167, doi:10.1016/j.molcel.2018.08.015 (2018).
5 de Bock, C. E. *et al.* HOXA9 Cooperates with Activated JAK/STAT Signaling to Drive Leukemia Development. *Cancer Discov* **8**, 616-631, doi:10.1158/2159-8290.CD-17-0583 (2018).
6 Janssens, D. H. *et al.* Automated in situ chromatin profiling efficiently resolves cell types and gene regulatory programs. *Epigenetics & chromatin* **11**, 74, doi:10.1186/s13072-018-0243-8 (2018).
7 Liu, N. *et al.* Direct Promoter Repression by BCL11A Controls the Fetal to Adult Hemoglobin Switch. *Cell* **173**, 430-442 e417, doi:10.1016/j.cell.2018.03.016 (2018).
8 Uyehara, C. M. & McKay, D. J. Direct and widespread role for the nuclear receptor EcR in mediating the response to ecdysone in Drosophila. *Proceedings of the National Academy of Sciences of the United States of America* **116**, 9893-9902, doi:10.1073/pnas.1900343116 (2019).
9 Hainer, S. J., Boskovic, A., McCannell, K. N., Rando, O. J. & Fazzio, T. G. Profiling of Pluripotency Factors in Single Cells and Early Embryos. *Cell* **177**, 1319-1329.e1311, doi:10.1016/j.cell.2019.03.014 (2019).
10 Mathsyaraja, H. *et al.* Max deletion destabilizes MYC protein and abrogates Emicro-Myc lymphomagenesis. *Genes & development*, doi:10.1101/gad.325878.119 (2019).
11 Roth, T. L. *et al.* Reprogramming human T cell function and specificity with non-viral genome targeting. *Nature* **559**, 405-409, doi:10.1038/s41586-018-0326-5 (2018).
12 Zheng, X. Y. & Gehring, M. Low-input chromatin profiling in Arabidopsis endosperm using CUT&RUN. *Plant reproduction* **32**, 63-75, doi:10.1007/s00497-018-00358-1 (2019).
13 Zhu, Q., Liu, N., Orkin, S. H. & Yuan, G. C. CUT&RUNTools: a flexible pipeline for CUT&RUN processing and footprint analysis. *Genome Biol* **20**, 192, doi:10.1186/s13059-019-1802-4 (2019).

# Appendix I: Nuclei Isolation & Cryopreservation Protocol for CUT&RUN

## Reagents & Buffers Needed

| Item | Vendor | Catalog No. |
|---|---|---|
| cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail Tablets | Roche | 11836170001 |
| Spermidine* | Sigma Millipore | S2501 |
| 0.4% Trypan blue | Any | |
| 1X Phosphate Buffered Saline (PBS) | Any | |

*Spermidine preparation
*For 1M, dissolve 254.63 mg spermidine in 1 mL molecular grade $H_2O$*
*Store in single use aliquots at -20°C for 6 months*

## Buffer Recipes

**Nuclear Extraction (NE) Buffer**
20 mM HEPES, pH 7.9
10 mM KCl
0.1% Triton X-100
20% Glycerol
1 mM $MnCl_2$
*Filter sterilize*
*Store at 4°C for up to 6 months*
*On day of experiment, add **fresh**:*
1X cOmplete Mini-Tablet (1 tablet)
0.5 mM Spermidine
** Keep at 4°C & discard after 1 day*

## Protocol Notes

To examine the efficiency of nuclear isolation and ensure intact nuclei:
1. Remove 10 µL aliquots from washed cells final nuclei.
2. Combine 10 µL aliquots with 10 µL 0.4% Trypan blue dye, and load onto cell counter or hemacytometer slide.
3. Examine under brightfield or phase microscope (**Figure 6**). Cells should not take up Trypan blue and appear clear/white, whereas nuclei should take up Trypan and appear blue.

## Nuclei Harvest Protocol – From Beginning of **Section II** of CUT&RUN Protocol

1. Harvest 0.5 million cells/sample by spinning for 3 min at 600 x g at RT in 1.5 mL tube. Aspirate sup.
2. Resuspend cells in 100 µL/sample RT **1x PBS**. Spin for 3 min at 600 x g at RT. Pipette or decant sup to remove.
   - *Note: For all steps, scale buffer volumes with number of cells, e.g. use 1 mL buffer for 5 million cells. Prepare excess cells to have leftover volume for quality control checks.*
   - *Note: Set 10 µL aside for confirmation of cell integrity prior to nuclear isolation (see Protocol Notes above).*
3. Resuspend cells in 100 µL/sample cold **NE Buffer**. sup.
4. **Incubate** samples for **10 min on ice**.
5. Spin for 3 min at 600 x g at **4°C**, and aspirate sup.
   - *Critical Step: The pellet should change in appearance from a sticky, pale yellow pellet (cells) to a white, fluffy pellet (nuclei).*
6. Resuspend nuclei in 100 µL/sample in cold **NE Buffer**. Freeze (below) or proceed to ConA bead conjugation step (**CUT&RUN Protocol, Step 11**).
   - *Note: Set 10 µL aside for confirmation of isolated nuclei integrity (see Protocol Notes above).*

## Cryopreservation Protocol

7. To cryopreserve nuclei, slowly freeze samples in an isopropanol-filled chiller in -80°C freezer.
   - *Note: If necessary, nuclei can be shipped on dry ice in this state.*

## Protocol for Thawing Frozen Nuclei

8. To avoid nuclear lysis and chromatin fragmentation, thaw nuclei quickly by placing samples on 37°C block until samples are thawed.
9. Proceed to ConA bead conjugation step (**CUT&RUN Protocol, Step 11**).

# Appendix II: Sample Integrity & Bead Conjugation Quality Control Checks

## Materials & Equipment Needed

| Item | Vendor | Catalog No. |
|---|---|---|
| 0.4% Trypan blue | Any | |
| Hemacytometer | Any | |
| Cell counting slides | Any | |
| Brightfield/phase contrast microscope or automated cell counter | Any | |

## Protocol Notes

1. **Always** check the quality of starting cells/nuclei prior to proceeding with CUT&RUN.
2. It is recommended to check sample binding to ConA beads when starting CUT&RUN for the first time and / or working with new cell types / sample inputs.
3. Account for excess volume during the protocol to have leftover material for the quality control checks (*e.g.* prepare ~10% excess volume or one extra sample).

## Protocol

1. Prior to starting with ConA bead conjugation (**CUT&RUN Protocol, Step 11**), take 10 µL from washed cells (or prepared nuclei) and evaluate sample integrity as follows:
   a. Add 10 µL of 0.4% Trypan blue.
   b. Mix 10x times by pipetting.
   c. Transfer 10 µL to a counting slide.
   d. View under brightfield/phase microscope or cell counter. *Intact cells will not absorb trypan blue and appear white (**Figure 6A**), while nuclei will take up Trypan and appear blue (**Figure 6B**).*
      - *Critical step: Cells/nuclei should not be clumped and should show the expected morphology.*
   e. Confirm sample integrity or troubleshoot as needed (**Table 4**).
2. Proceed with bead binding (working in batch if possible) by adding 100 µL washed cells or nuclei/sample to 10 µL activated ConA Beads/sample.

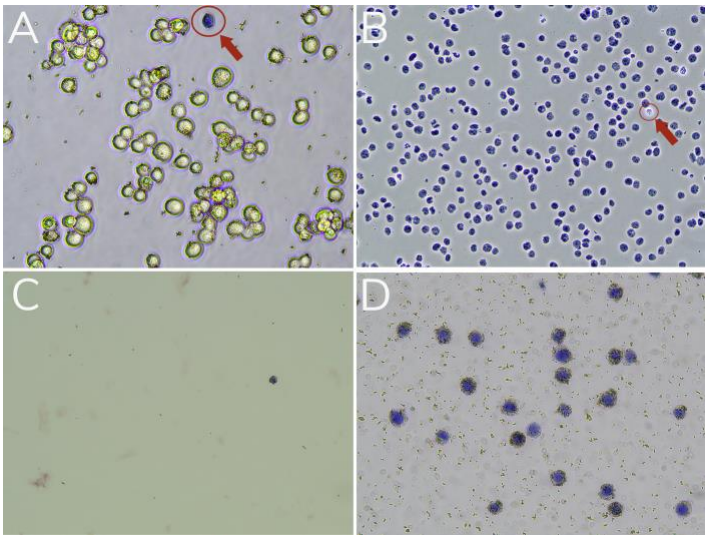


**Figure 6**: Representative images of cell and nuclei samples for ConA Bead binding. Samples were stained with Trypan blue and visualized under brightfield microscope. **(A) Cells** before bead binding. A dead cell (blue; Trypan positive) is circled in red, whereas the remaining live cells are bright white and round. **(B) Nuclei** before bead binding. An intact cell (Trypan negative) is circled in red, whereas isolated nuclei are Trypan stained (*see **Appendix I** for preparation of nuclei*). **(C) Unbound Fraction** shows little to no material leftover after ConA Bead binding. **(D)** Representative **Bead-Bound Faction** showing nuclei (blue) successfully bound to activated ConA Beads (brown specks).

3. Gently vortex and/or pipette mix cell/nuclei : bead mixture to mix.
4. Incubate cell : bead slurry for 10 min at RT. *Cells will adsorb to the activated ConA beads.*
5. Place tube(s) on magnet until slurry clears. Transfer 10 µL sup into a fresh 1.5 mL tube (**Unbound fraction**); set aside for bead binding integrity check. Pipette to remove remaining sup and discard.
6. Add 50 µL per sample cold Antibody Buffer quickly, to avoid bead drying. *Gently vortex immediately and thoroughly to an even resuspension.*
7. Remove 10 µL and transfer to a fresh tube (**Bead-Bound Fraction**). Place remaining sample on ice.
10. Perform Trypan blue staining and bead binding integrity check as described in **Steps 1a-e** (above), comparing **Unbound Fraction** and **Bead-Bound Fraction**.
    - *Critical step: Successful binding will show Trypan positive cells/nuclei surrounded by ConA beads; compare **Figure 6C and 6D**. For troubleshooting approach, see **Table 4**.*
8. Continue on with the **CUT&RUN Protocol, Section III** (antibody binding).

| Samples | Figure | Success Metric (Trypan Blue staining) | Troubleshooting Tips |
|---|---|---|---|
| **Cells** | Fig. 6A | Cells should be bright (Trypan blue excluded), round, unclumped, and ideally show >90% viability. Over 80% minimum viability is recommended, as excess dead cells increase background in CUT&RUN. | Optimize cell culture conditions; use fresh media, evaluate potential contamination issues. |
| **Nuclei** | Fig. 6B | Nuclei should be >95% Trypan blue positive and unclumped. | See **Appendix I** for a detailed nuclei preparation protocol. |
| **Unbound Fraction** | Fig. 6C | Little to no material should be present if binding to beads occurred. | Ensure that ConA Beads were never frozen, cells/nuclei were not clumped, beads did not become clumped or dried out, and all buffers were correctly prepared. |
| **Bead-Bound Fraction** | Fig. 6D | Successful ConA bead binding will show Trypan blue positive permeabilized cells/nuclei surrounded by beads. | |

**Table 4:** Quality control testing of cell / nuclei integrity and ConA bead binding steps.

# Appendix III: Use of *E. coli* Spike-in DNA for Experimental Normalization

## Protocol 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.

### E. coli Spike-in DNA Protocol

1. Add 0.5-1 ng* *E. coli* Spike-in DNA to CUT&RUN **Stop Buffer** prior to sample addition (33 µL Stop Buffer per sample; see protocol).
   - **Note: 0.5-1 ng E. coli Spike-in DNA per sample is recommended for most conditions using 500,000 cells. However, scale the spike-in amount linearly with increasing or decreasing starting cell numbers (e.g. 0.05 – 0.1 ng for 50,000 cells). The major goal is to aim for the spike-in to comprise ~1% (0.2-5%) of total sequencing reads (**Table 5**). Note that this may vary based on a variety of experimental factors (target abundance, antibody), so the amount of spike-in DNA should be optimized.*

| *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 5**. 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 13-0041), a high abundance target (H3K27me3, EpiCypher 13-0030) and IgG negative control (EpiCypher 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 (see **Section V, Step 31**), 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 E. coli reads in 5,000,000 uniquely aligned reads = 2%*
   *b. Untreated spike-in = 30,000 E. coli 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 III 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)