

## **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)

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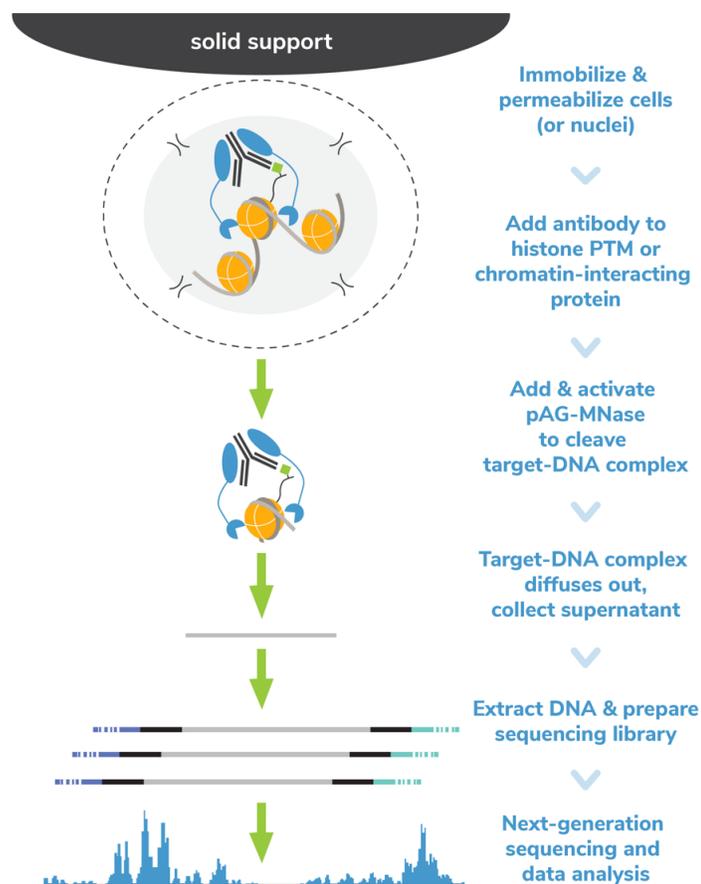
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## 1. Overview

Cleavage Under Targets & Release Using Nuclease (CUT&RUN) is a revolutionary genomic mapping strategy developed by the group of Dr. Steven Henikoff<sup>1</sup>. It builds on Chromatin ImmunoCleavage (ChIC) from Dr. Ulrich Laemmli<sup>2</sup>, whereby a fusion of Proteins A and/or G to Micrococcal Nuclease (pAG-MNase) is recruited to selectively cleave antibody-bound chromatin *in situ*<sup>3</sup>. In CUT&RUN, cells or nuclei are immobilized to a solid support, with pAG-MNase cleaved target 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](https://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:

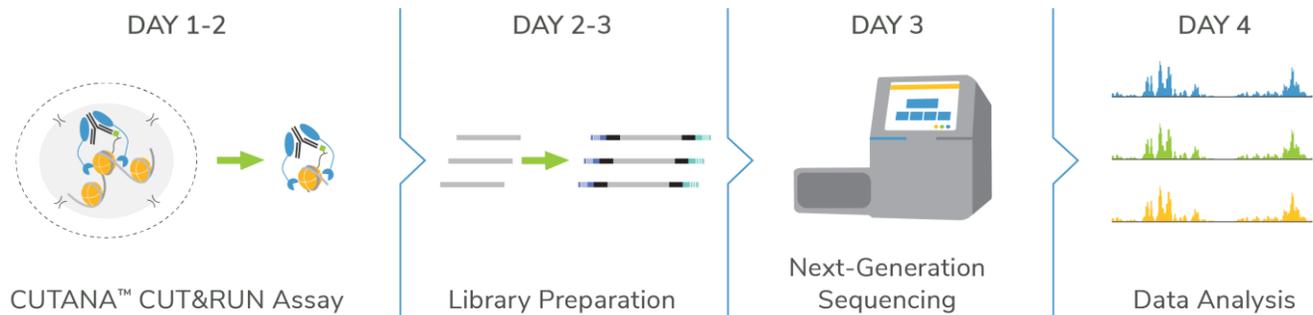
| Platform Comparison      | ChIP-seq                                     | CUTANA™ CUT&RUN  |
|--------------------------|--|--|
| Required cells           | > 1 million                                  | 5,000 – 500,000  |
| Cell Input               | Fragmented chromatin                         | Intact cells or nuclei   |
| Compatible Targets       | Histone PTMs & chromatin-associated proteins | Histone PTMs & chromatin-associated proteins, including difficult ChIP targets |
| 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 ([epicypher.com/cut-and-run-assays](https://epicypher.com/cut-and-run-assays)), 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.
- **ChIC/CUT&RUN Kit** (EpiCypher 14-1018) with all reagents included to go from cells to purified CUT&RUN DNA.
- **CUT&RUN Antibodies** to histone PTMs and chromatin-associated proteins, rigorously validated directly in CUT&RUN. See [epicypher.com/cut-and-run-antibodies](https://epicypher.com/cut-and-run-antibodies) for information.
- **CUT&RUN Spike-in Controls**
  - *E. coli* Spike-in DNA (EpiCypher 18-1401) for data normalization.
  - **SNAP-CUTANA™ Spike-in Controls** are DNA-barcoded semi-synthetic/recombinant nucleosome spike-in panels that control for all aspects of CUT&RUN workflows, including antibody specificity and assay success, guide troubleshooting experiments, and enable quantitative normalization. Now available for histone lysine methylation PTMs (SNAP-CUTANA K-MetStat Panel: [epicypher.com/19-1002](https://epicypher.com/19-1002)).
- **CUT&RUN supporting reagents** selected and validated for optimal performance in the EpiCypher CUT&RUN protocol. See [epicypher.com/cut-and-run-assays](https://epicypher.com/cut-and-run-assays) for more info.
- **CUTANA CUT&Tag** reagents for ultra-low input applications. Go to [epicypher.com/CUT&Tag](https://epicypher.com/CUT&Tag) for more information.
- Inquire for more information or to connect with EpiCypher scientists: [info@epicypher.com](mailto:info@epicypher.com)

### 3. Outline of CUT&RUN Workflow

**Description:** EpiCypher’s in-house optimized protocol for CUTANA™ CUT&RUN assays (**Figure 2**). Before starting, we strongly recommend reading this section, the [Experimental Design & Key Protocol Notes](#) and the [Protocol](#) to carefully plan your experiment and familiarize yourself with the assay.



**Figure 2:** Timeline of CUT&RUN assay using EpiCypher’s CUTANA™ CUT&RUN protocol.

#### Section I: ConA Bead Activation (~30 min)

**Description:** This section “activates” Concanavalin A coated beads (**Con A beads**) to bind and immobilize cell/nuclei samples. If preparing for multiple CUT&RUN reactions, it is recommended to batch process the full volume of beads needed for all reactions in a single 1.5 mL tube. This helps ensure homogeneity across reactions. For these steps, beads can be washed using a 1.5 mL magnetic rack (e.g. EpiCypher 10-0012).

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

**Description:** In this section, cell/nuclei samples are prepared and immobilized to ConA beads in bulk, and then separated into 8-strip PCR tubes for individual CUT&RUN reactions. This protocol section was developed using 500,000 unfixed (*i.e.* native) K562 cells per CUT&RUN reaction and is specifically designed for batch processing of cell samples for multiple CUT&RUN reactions (see [Protocol Notes](#)). Once the cell-coupled beads are transferred to PCR tubes, it is recommended to use multi-channel pipettes and a compatible magnetic rack (EpiCypher 10-0008; **Figure 3**), which helps increase experimental throughput and reproducibility.

Importantly, we have protocol adaptations for many types of inputs (adherent vs. suspension cells, nuclei, cryopreservation, and cross-linking) noted within **Section II** of the protocol and our [FAQs](#). To ensure that cells are immobilized to ConA beads, we have also developed simple quality control checks ([Appendix II](#)) that we strongly recommend using in every experiment.

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

**Description:** After coupling to activated ConA beads, the cell – bead mixture is resuspended in cold **Antibody Buffer**, and a target-specific antibody is added for an overnight incubation. Note

that antibody selection in CUT&RUN is critical to success; see [FAQs](#) for more information. Importantly, **Antibody Buffer** is the first to contain digitonin, which permeabilizes cells and allows the antibody to bind its target *in situ* (histone PTM or chromatin-associated protein). The digitonin concentration required for CUT&RUN assays varies by sample (e.g. cell type, fixation) and must be optimized for every unique input, as described in the [Protocol Notes](#). It is crucial to use the minimum amount of digitonin needed for efficient permeabilization to avoid cell lysis and digitonin precipitation during overnight incubations.

A second feature of this section is the addition of the **SNAP-CUTANA™ K-MetStat Panel** (EpiCypher 19-1002) to CUT&RUN reactions using a methyl-lysine antibody (e.g. H3K4me3) or IgG control. This spike-in panel comprises highly pure, DNA-barcoded semi-synthetic/recombinant nucleosomes carrying defined lysine methylation PTMs, and is useful for in-assay antibody validation, quantitative normalization, and experimental troubleshooting. We outline its appropriate use in **Section III** of the protocol (*i.e.* added immediately before antibody addition; also see **Table 5**). The **SNAP-CUTANA Spike-in User Guide** (available at [epicypher.com/19-1002](http://epicypher.com/19-1002)) includes detailed information about how the spike-ins work and how they can be incorporated as critical controls to master CUT&RUN.

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

Description: At this stage of the protocol, **pAG-MNase** (EpiCypher 15-1016) is added to each reaction and incubated briefly at room temperature to allow binding to antibody-labelled chromatin. This incubation is performed in the **Digitonin Buffer**, which is free of CaCl<sub>2</sub> to avoid premature activation of MNase. ConA bead – cell suspensions often become “clumpy” at this point and are difficult to pipette. Taking care to minimize this clumping is key for quality results. In this case, we recommend using a P200 pipette to gently disperse beads in buffer. Cut-off pipette tips can be used if beads are clogging pipette tips or if cells/nuclei are easily damaged.

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

Description: During this part of the experiment, MNase is activated by addition of **CaCl<sub>2</sub>** to cleave and release antibody-bound chromatin. The fragmented chromatin containing the histone PTM or protein of interest diffuses into the supernatant, where it can be easily separated from bead-coupled cells. The fragmented chromatin is purified, quantified using a Qubit™ fluorometer, and used for next-generation sequencing (NGS) library preparation. Of note, the **CUTANA™ DNA Purification Kit** (EpiCypher 14-0050) is designed to capture CUT&RUN DNA fragments as small as 50 bp, which is adequate for most chromatin targets, including TFs.

We have also included instructions for the addition of exogenous ***E. coli* Spike-in DNA** (EpiCypher 18-1401), which can be added to CUT&RUN reactions following MNase activation as a component of the **Stop Buffer** and used to normalize downstream NGS data. For further instructions on optimizing *E. coli* DNA for CUT&RUN experiments, see [Appendix III](#).

**IMPORTANT:** At this step, many researchers are tempted to assess fragment size distribution of CUT&RUN purified DNA and/or analyze enrichment of known targets by qPCR. We do not recommend either of these analyses, which are derived from ChIP-seq and are not robust indicators of CUT&RUN success (see [FAQs](#) for a detailed explanation). CUT&RUN is distinct from ChIP-seq, and the quality controls applied in one assay cannot be transferred to the other. In fact, raw CUT&RUN DNA yields are often below the sensitivity of Bioanalyzer/TapeStation approaches used to examine fragment distribution. Furthermore, because CUT&RUN is performed *in situ*, there is no chromatin Input, complicating enrichment analysis by qPCR.

The best indicator of CUT&RUN success at this step is that raw DNA yields from positive control reaction(s) are greater than yields from the IgG negative control. For low abundance targets, such as H3K4me3, this difference may be slight, while high abundance targets (e.g. H3K27me3) will display a more pronounced difference. Proceed directly to library preparation.

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

Description: Here, we provide guidance on CUT&RUN sequencing library preparation using the NEBNext® Ultra™ II Library Prep Kit for Illumina®. This includes PCR steps specifically optimized to amplify short CUT&RUN library fragments compatible with Illumina paired-end sequencing (100 bp – 700 bp including universal adapters; see [FAQs](#) for further guidance).

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

Description: Prior to sequencing, CUT&RUN libraries should be examined using the Bioanalyzer, TapeStation, or equivalent approach to assess fragment size, library concentration, and DNA quality. Predominant enrichment of mononucleosome-sized fragments (~300 bp including universal adapters) is the best indicator of CUT&RUN experimental success prior to NGS (e.g. see **Figure 5**). Here we describe the use of Bioanalyzer and the Agilent High Sensitivity DNA Chip for CUT&RUN library analysis along with expected results.

### **Section VIII: Illumina Sequencing**

Description: The final step of the protocol is sequencing your CUT&RUN libraries on an Illumina sequencing system. In this section, we provide guidance on pooling indexed CUT&RUN libraries for multiplexed sequencing. We also offer recommendations for loading pooled libraries onto MiniSeq and NextSeq systems. These suggestions are based on the fact that CUT&RUN only requires 3-8 million reads per sample to generate high signal-to-noise data (vs. 30 million or more with ChIP-seq). This allows researchers to pool >48 samples per sequencing run, if using the Illumina NextSeq, or use a benchtop sequencer (e.g. Illumina MiniSeq) for smaller projects.

For guidelines on NGS analysis see the [FAQ](#) section. For guidance on SNAP-CUTANA K-MetStat Panel analysis, see the SNAP-CUTANA Spike-in User Guide at [epicypher.com/19-1002](http://epicypher.com/19-1002); for *E. coli* spike-in DNA, see [Appendix III](#).

## 4. Experimental Design & Key Protocol Notes

**Description:** This section is considered essential reading for CUTANA™ CUT&RUN assays. For CUT&RUN workflows to be successful you must include proper controls and optimize key steps for your unique cell input (e.g. digitonin permeabilization, number of cells) as detailed in this section. We also offer tips on common problems with the protocol and explain our rationale for using 8-strip PCR tubes in the CUTANA CUT&RUN protocol.

1. **Include controls in every experiment.** We suggest the following controls (at minimum):

- Negative control antibody (e.g. IgG negative control antibody: EpiCypher 13-0042)
- Positive control antibody (e.g. for PTMs, H3K4me3: EpiCypher 13-0041; for chromatin-associated protein targets, BRD4: EpiCypher 13-2003 or CTCF EpiCypher 13-2014)
- SNAP-CUTANA™ K-MetStat Panel of spike-in controls (EpiCypher 19-1002). These spike-ins should be added to reactions designated for H3K4me3 and IgG control antibodies in every experiment, as well as any assays targeting methyl-lysine PTMs.

These controls are especially critical when optimizing CUT&RUN for new experimental conditions (e.g. new cell types, reduced inputs, drug treatments, fixation method), but should also be included as standard controls for continuous monitoring of assay success.

2. Optimize conditions and become familiar with the CUT&RUN workflow using a control cell line (e.g. K562 cells) before attempting different sample types (see [FAQs](#)).

- This protocol was optimized using 500,000 human K562 cells per reaction. 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).

3. We strongly recommend performing the **Quality Control Checks** outlined in [Appendix II](#), which include evaluating the integrity of starting cells/nuclei and binding to Concanavalin A (**ConA**) beads.

4. While the standard protocol is written for native (unfixed) suspension cells, we have included specific sample preparation instructions for working with nuclei ([Appendix I](#)), cryopreserved cells/nuclei ([Appendix I](#)), adherent cells ([CUT&RUN Protocol, Section II](#)), and cross-linked material (see **Cross-linking Protocol** at [epicypher.com/protocols](http://epicypher.com/protocols)).

5. We recommend a 5% digitonin stock solution be prepared in DMSO (as opposed to heated H<sub>2</sub>O), as this improves detergent solubility and protocol reproducibility.

6. To avoid digitonin precipitation and cell lysis, use the minimal concentration of digitonin required for efficient permeabilization in the **Digitonin Buffer** and **Antibody Buffer**. This step should be optimized for every type of cell/nuclei input used in CUT&RUN workflows. A detailed protocol for digitonin optimization is described in the CUTANA™ ChIC/CUT&RUN Kit

manual ([epicypher.com/14-1048](http://epicypher.com/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 (see [Appendix II](#)). Find the minimum digitonin concentration needed to achieve >95% permeabilized cells.

7. This protocol has been adapted to 8-strip PCR tubes (vs. 1.5 mL tubes) for rapid “batch processing” of multiple CUT&RUN reactions from bulk cell samples. The first few steps, *i.e.* cell harvest and conjugation to ConA beads, are performed in 1.5 mL tubes, and then the reactions are split into 8-strip PCR tubes for the remainder of the assay. These steps:
  - Minimize beads sticking to tubes
  - Enable more rapid workflows with multichannel pipettes
  - Provide more consistent sample handling
  - Enable high-throughput sample preparation
8. ConA beads dry out easily, which can result in sample loss. To avoid this problem in the CUT&RUN assay, take caution to prevent ConA beads sticking to the sides/caps of tubes.
  - To avoid ConA beads sticking to tube sides/caps and drying out, it is essential to use a **nutator** rather than a **rotator** (see **Table 3**), since nutators gently agitate by shaking or rocking tubes rather than rotating end-over-end.
  - Take note of steps that indicate when to pipette or vortex to disperse clumps and keep ConA beads in an even suspension.
9. Although protocols with shortened antibody and/or CaCl<sub>2</sub> incubation times have been published<sup>3</sup>, in our hands such changes adversely impact yields and reproducibility.
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 (see [FAQs](#)). Instead, assess DNA yield compared to positive (e.g. H3K4me3, BRD4, CTCF) and negative (IgG) controls, determine fragment size distribution of sequencing-ready libraries (**Figure 5**), and evaluate peak structure and expected genome-wide distribution in sequencing data.

## 5. Buffers, Reagents & Materials Needed

**Table 1: Buffer components**

| Components   | Source                   | Cat #            |
|--|--------------------------|------------------|
| HEPES  | Sigma-Aldrich            | H3375            |
| KCl  | Sigma-Aldrich            | P3911            |
| CaCl <sub>2</sub>  | Sigma-Aldrich            | C1016            |
| MnCl <sub>2</sub>  | Sigma-Aldrich            | 203734           |
| Molecular biology grade H <sub>2</sub> O (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 -20°C)      | Millipore Sigma          | 300410           |
| DMSO   | Sigma-Aldrich            | D8418-100ml      |
| cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail       | Roche                    | 11836170001      |
| Trypan blue  | Thermo Fisher Scientific | T10282           |

\*1M spermidine preparation: Dissolve 1 gram spermidine (MW = 254.63) in 3.93 mL molecular grade H<sub>2</sub>O. 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<sub>2</sub>  
1 mM MnCl<sub>2</sub>  
*Filter sterilize. Store at 4°C for up to 6 months.*

### Pre-Wash Buffer

20 mM HEPES, pH 7.5  
150 mM NaCl  
*Filter sterilize. Store at 4°C 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, 1 tab/10mL)  
*Filter sterilize. Store at 4°C for up to 1 week.*

### Digitonin Buffer

Wash Buffer + 0.01% digitonin\*\*  
*Prepare fresh each day and store at 4°C.*

### Antibody Buffer

Digitonin Buffer\*\*\* + 2 mM EDTA  
*Prepare fresh each day and store at 4°C.*

### Stop Buffer

340 mM NaCl  
20 mM EDTA  
4 mM EGTA  
50 µg/mL RNase A  
50 µg/mL Glycogen  
*Filter sterilize. Store at 4°C for up to 6 months.*

## Buffer Preparation Notes

\* Spermidine is added to compensate for the removal of Mg<sup>2+</sup> from the buffer. Mg<sup>2+</sup> can cause DNA degradation and is typically omitted from CUT&RUN buffers.

\*\* Optimal [digitonin] for each cell type should be determined empirically, as described in **Protocol Notes**. Starting concentration validated for K562, MCF7, and A549 cells is 0.01% digitonin.

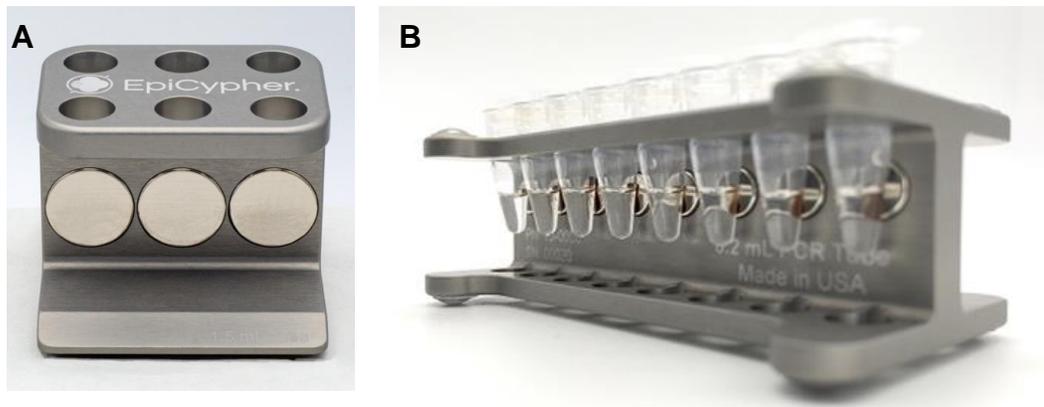
\*\*\* See **Protocol Notes** for guidance on optimizing [digitonin] for a given cell type.

**Table 2: Reagents**

| Item  | Vendor                   | Catalog No.  | Notes   |
|---|--------------------------|--|---|
| Concanavalin A (ConA) Conjugated Paramagnetic Beads                       | EpiCypher                | <a href="#">21-1401</a>                            | ConA is a lectin, which can cause immune cell activation. For technical support re. immune cell studies, see the <a href="#">FAQs</a> section or contact <a href="mailto:info@epicypher.com">info@epicypher.com</a> .   |
| CUTANA™ pAG-MNase   | EpiCypher                | <a href="#">15-1016</a> or <a href="#">15-1116</a> | 50 & 250 reaction pack sizes available. Supplied as 20X stock.  |
| SNAP-CUTANA™ K-MetStat Panel  | EpiCypher                | <a href="#">19-1002</a>                            | <p><b>Spike-in controls for Sample Normalization &amp; Antibody Profiling (SNAP Spike-in Controls):</b><br/>           The K-MetStat panel comprises fifteen semi-synthetic/recombinant nucleosomes carrying unique methyl-lysine modifications (me1/2/3 at H3K4, H3K9, H3K27, H3K36, and H4K20), plus an unmodified control, immobilized to magnetic beads. Each histone octamer is wrapped with two different barcoded DNA templates, providing an internal technical replicate for each histone PTM.</p> <p>Add spike-ins to CUT&amp;RUN reactions targeting one of the PTMs in the panel as well as in CUT&amp;RUN samples designated for H3K4me3 positive and IgG negative control antibodies. For more information about using SNAP-CUTANA Spike-ins, see the User Guide at <a href="http://epicypher.com/19-1002">epicypher.com/19-1002</a>.</p> <p><b>NOTE: Store at -20°C.</b> Lower temperatures can cause freezing and will permanently damage the beads. Pipette-mix (<b>do NOT vortex</b>) before use.</p> |
| Rabbit IgG Negative Control Antibody                                      | EpiCypher                | <a href="#">13-0042</a>                            | See datasheet for application-specific dilutions.   |
| SNAP-ChIP® Certified, CUTANA Compatible H3K4me3 Positive Control Antibody | EpiCypher                | <a href="#">13-0041</a>                            | 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                | <a href="#">13-2014</a>                            | See datasheet for application-specific dilutions.   |
| Antibody to target of interest (histone PTM, TF, or chromatin regulator)  | User-dependent           |  | EpiCypher continues to conduct <a href="#">extensive antibody characterization studies</a> <sup>4</sup> . For antibodies directly validated in CUT&RUN, visit: <a href="http://epicypher.com/cut-and-run-antibodies">epicypher.com/cut-and-run-antibodies</a> . For other targets not found on our site, contact us for recommendations: <a href="mailto:info@epicypher.com">info@epicypher.com</a> .   |
| <i>E. coli</i> Spike-in DNA   | EpiCypher                | <a href="#">18-1401</a>                            | Use as an exogenous spike-in control for experimental normalization. See <a href="#">Appendix III</a> for detailed instructions.  |
| CUTANA™ DNA Purification Kit  | EpiCypher                | <a href="#">14-0050</a>                            | Designed to maximize yield from low [DNA] samples/low cell inputs; is optimized for low elution volume and retention of >50bp fragments. Suitable for both nucleosomal (PTMs) and subnucleosomal (TFs) fragments.   |
| Agencourt AMPure XP Magnetic Beads  | Beckman Coulter          | A63880   | For PCR cleanup and size selection ( <i>i.e.</i> 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  |
|---|--------------------------|-------------------------|--|
| 1.5 mL Magnetic Separation Rack                     | EpiCypher                | <a href="#">10-0012</a> | For bulk or “batch” processing of ConA beads and ConA bead-conjugated cells in <b>Sections I and II</b> of CUT&RUN protocol; see <b>Figure 3A</b> .  |
| 8-strip PCR tube Magnetic Separation Rack           | EpiCypher                | <a href="#">10-0008</a> | For processing of individual CUT&RUN reactions in <b>Section III</b> onward; see <b>Figure 3B</b> . Enables streamlined sample handling for higher experimental throughput and improved reproducibility. |
| 8-strip 0.2 mL PCR tubes                            | EpiCypher                | <a href="#">10-0009</a> | Compatible with the magnetic stand.  |
| Qubit™ 4 Fluorometer                                | Thermo Fisher Scientific | Q33226                  | For DNA quantification.  |
| Agilent 2100 Bioanalyzer                            | Agilent                  | G2939A                  | For analysis of purified CUT&RUN sequencing libraries. May substitute comparable capillary electrophoresis instrument (e.g. Agilent TapeStation).  |
| High Performance Multi-Channel Pipettors, 8-Channel | VWR                      | 76169-252               | For performing CUT&RUN in 8-strip PCR tubes e.g. for aspiration and wash steps. May substitute comparable multi-channel pipettor.  |
| Tube Nutator  | VWR                      | 82007-202               | For bead incubation steps (overnight antibody incubation, pAG-MNase digest reaction). <b>It is critical to use a tube nutator rather than a rotator for these steps.</b>                                 |
| Vortex-Genie  | Scientific Industries    | SI-0236                 | For bead mixing steps.   |



**Figure 3: Magnetic racks for CUT&RUN assays.** (A) For batch processing of ConA beads, use a 1.5 mL magnetic rack (e.g. EpiCypher 10-0012, pictured). (B) For processing samples in 8-strip PCR tubes we recommend using a multi-channel pipette and compatible magnetic rack (e.g. EpiCypher 10-0008, pictured).

## 6. EpiCypher CUTANA™ CUT&RUN Protocol

**Essential Reading:** Before starting, we strongly recommend reading the [Outline of CUT&RUN Workflow](#) and the [Experimental Design & Key Protocol Notes](#) for important information about assay controls and optimization. The [FAQs section](#) also provides guidance on adapting the protocol for unique inputs, targets, and other cell preparation methods.

### 6.1. CUT&RUN Protocol (~5hrs)

---Day 1---

#### Section I: ConA Bead Activation (~30 min)

1. Gently resuspend the **ConA beads** and transfer 11 µL per planned CUT&RUN reaction to a 1.5 mL tube for batch processing.
2. Place the tube on a 1.5 mL magnetic separation rack until slurry clears (30 s – 2 min) and pipette to remove supernatant.
3. Immediately add 100 µL/reaction cold **Bead Activation Buffer**, remove from magnet, and pipette to mix. Place back on magnet until slurry clears and pipette to remove supernatant.
4. Repeat the previous step for total of two washes.
5. Resuspend beads in 11 µL/reaction cold **Bead Activation Buffer**.
6. Keep activated **ConA beads** on ice until needed.

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

7. Harvest 500,000 cells per planned CUT&RUN reaction. If more than one CUT&RUN reaction is planned for the cell sample, cells can be processed together at this step (see **Table 4** for guidance). For suspension cell cultures, spin for 3 min at 600 x g at room temperature (**RT**) in a 1.5 mL tube. Remove supernatant by decanting or pipetting.

#### Notes and alternative cell preparation protocols:

- It is recommended to prepare excess cells (~10% excess if working in batch [preferred] or one extra sample if preparing individual reactions) for **Quality Control (QC) Checks** described in [Appendix II](#). See **Table 4** for guidance.
- 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, frozen nuclei, and frozen cells are compatible with CUT&RUN; for instructions, see [Appendix I](#).
- For samples that require cross-linking (e.g. formaldehyde) to preserve labile marks, such as histone lysine acetylation, see our cross-linking protocol at [epicypher.com/protocols](http://epicypher.com/protocols).
- See [FAQs](#) “[Sample Input Compatibility](#)” section for special considerations when using adherent, cryopreserved and cross-linked cells, immune cells, and tissue.

| Cell Type  | # Reactions | Cells/Reaction | Total Cells* | Vol. Wash Buffer* | Vol. ConA Beads* |
|------------|-------------|----------------|--------------|-------------------|------------------|
| K562 cells | 8           | 500,000        | 4.4 million  | 880 µL            | 88 µL            |
| K562 cells | 1           | 500,000        | 1 million    | 200 µL            | 20 µL            |
|            |             |                |              |                   |                  |
|            |             |                |              |                   |                  |

**Table 4:** Preparation of cells for CUT&RUN reactions with extra volume allotted for key Quality Control Checks (**Appendix II**). \*Total cell number and volumes include a 10% excess for batch processing cells, or one extra sample volume for preparing individual CUT&RUN reactions. Extra rows are provided to customize suggestions for user workflows.

8. Resuspend cells in 100 µL/reaction RT **Wash Buffer**, spin for 3 min at 600 x g at RT, and remove supernatant by decanting or pipetting.
9. Repeat the previous step for total of two washes.
10. Resuspend cells in 100 µL/reaction in RT **Wash Buffer**. Pipette to mix.
11. Proceed with **ConA bead** binding.
  - Note 1: If batch processing cell samples for multiple CUT&RUN reactions (as in **Table 4, row 1**), process bulk cells + ConA beads together in 1.5 mL tubes to ensure homogeneity across reactions. Add 10 µL of activated **ConA beads** per 100 µL washed cells. Gently vortex (setting #7) to mix.
  - Note 2: If working with individual cell samples at this step (e.g. each CUT&RUN reaction uses a distinct cell type or sample input; see **Table 4, row 2**), add 10 µL activated **ConA beads** per 100 µL cells, and transfer individual samples (110 µL) to 8-strip PCR tubes for subsequent steps.
12. **Incubate** cell – bead slurry for **10 min at RT**. Cells will adsorb to the activated ConA beads.

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

13. Place the tube(s) on a magnet until slurry clears (30 s – 2 min). Pipette to remove and discard supernatant.
14. Add 50 µL cold **Antibody Buffer** per reaction quickly, to avoid bead drying. Gently vortex immediately and thoroughly to an even resuspension.  
 If still working in 1.5 mL tubes for batch processing:
  - Take into account 10% excess volume of Antibody Buffer as in **Table 4**. Can save an aliquot of cell – bead slurry for QC checks described in [Appendix II](#).
  - Transfer individual reactions (50 µL) into 8-strip PCR tubes.
  - Continue the remaining steps using an 8-strip PCR tube magnetic rack.
15. For reactions designated for positive control histone PTM (e.g. H3K4me3) and negative control (IgG) antibodies, as well as samples assigned a target in the K-MetStat Panel (me1, me2, and me3 at H3K4, H3K9, H3K27, H3K36 and H4K20): Add 2 µL **SNAP-CUTANA™ K-**

**MetStat Panel** per 500,000 cells. If using less than 500,000 cells, decrease the amount of SNAP-CUTANA Spike-ins linearly by preparing a “working stock” dilution of the panel in **Antibody Buffer**. General starting recommendations are provided in **Table 5**.

Notes:

- Prior to use, mix the K-MetStat Panel by pipetting. Do NOT vortex.
- SNAP-CUTANA Spike-ins must be added before addition of Primary Antibody.
- Aim for spike-ins to comprise ~1% of total sequencing reads; adjust amount added as needed. This bandwidth is typically higher for low abundance targets/negative controls (e.g. IgG = 10-20%) and lower for high abundance targets (e.g. H3K27me3 = 0.1-1%).
- For more information, refer to the SNAP-CUTANA Spike-in User Guide at [epicypher.com/19-1002](http://epicypher.com/19-1002).

| Starting # Cells in CUT&RUN | Working Stock Dilution in Antibody Buffer [use <i>FRESH</i> the day of preparation] | Volume added to reaction | Final dilution in reaction |
|-----------------------------|---|--------------------------|----------------------------|
| <b>500,000</b>              | Stock   | 2 µL                     | 1:25                       |
| <b>250,000</b>              | 1:2   | 2 µL                     | 1:50                       |
| <b>100,000</b>              | 1:5   | 2 µL                     | 1:125                      |
| <b>50,000 or less*</b>      | 1:10  | 2 µL                     | 1:250                      |

**Table 5:** Recommended SNAP-CUTANA Spike-in amounts for varying numbers of starting cells in CUT&RUN. **\*NOTE:** *additional dilutions of the SNAP-CUTANA Panels may be added for lower cell inputs. However, dilution of spike-ins beyond 1:250 increases risk of experimental variation and may require end user optimization.*

16. Add manufacturer’s recommended amount (or 0.5 µg if application is untested) of **Primary Antibody** to each reaction and gently vortex immediately and thoroughly.

- Note: Antibodies stored in glycerol solution may be viscous. Take care to ensure accurate pipetting by aspirating slowly, check tip for accuracy, and pipette up and down ~3x times into CUT&RUN samples to clear remaining glycerol from tip.

17. **Incubate** 8-strip PCR tubes on nutator **overnight at 4°C**.

- Critical step: To keep beads in solution, slightly elevate cap side of 8-strip PCR tubes on nutator to ensure bead solution remains in bottom of conical tube (**Figure 4**). **DO NOT USE** a rotator or turn tubes over end-to-end for this step.



**Figure 4.** ConA 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 8-strip PCR tubes on a magnet until slurry clears (30 s – 2 min) and pipette to remove and discard supernatant.
19. Keeping tubes on the magnet, add 250  $\mu$ L cold **Digitonin Buffer** directly onto beads of each reaction, and then pipette to remove supernatant.
20. Repeat the previous step for total of two washes, keeping tubes + beads on magnet the entire time.
21. After second wash, remove supernatant and discard. Add 50  $\mu$ L cold **Digitonin Buffer** per reaction, and gently vortex the 8-strip PCR tubes to resuspend bead – cell slurry in buffer. Continue to the addition of pAG-MNase.
  - Note: Beads are often clumpy at this point but can easily be dispersed by gentle pipetting with a P200 pipette. A slightly cut-off pipette tip may be used to aid in resuspension and/or preserve delicate cells/nuclei.

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

22. Add 2.5  $\mu$ L **pAG-MNase** (20x stock) to each CUT&RUN reaction, and gently vortex to mix.
  - Critical step: To evenly distribute pAG-MNase across cells/nuclei, ensure beads are thoroughly resuspended by gentle vortexing and/or pipetting using a P200 pipette (cut-off tip optional).
23. **Incubate** reactions for **10 min at RT**.
24. Return 8-strip PCR tubes to magnet, allow slurry to clear (30 s – 2 min). Pipette to remove and discard supernatant.
25. Keeping tubes on the magnet, add 250  $\mu$ L cold **Digitonin Buffer** directly onto beads of each reaction, and then pipette to remove supernatant.
26. Repeat the previous step for total of two washes, keeping tubes + beads on magnet the entire time.
27. After second wash, remove supernatant and discard. Remove 8-strip PCR tubes from the magnet and add 50  $\mu$ L cold **Digitonin Buffer** to each reaction. Gently vortex to mix.
  - Note: If beads are clumpy, gently pipette to mix using a P200 pipette (cut-off tip optional).

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

28. Place 8-strip PCR tubes on ice and add 1  $\mu$ L **100 mM CaCl<sub>2</sub>** to each reaction. Gently vortex to mix.
  - Critical step: For efficient pAG-MNase digestion, ensure the beads are thoroughly resuspended by vortexing and/or pipetting using a P200 pipette (cut-off tip optional).

29. **Incubate** 8-strip PCR tubes on nutator for **2 hours at 4°C**, with tubes slightly elevated as in **Figure 4**. Note that this is the key step wherein MNase tethered to antibody-bound chromatin is activated by calcium to cleave target chromatin.
30. Add 33 µL **Stop Buffer** to each reaction, and gently vortex to mix. This buffer stops MNase enzymatic activity by chelating Ca<sup>2+</sup> ions.
  - Note: If using *E. coli* spike-in DNA for NGS normalization, prepare a **Stop Buffer Master Mix** by adding the optimized amount of ***E. coli* DNA** ([Appendix III](#)) to the Stop Buffer immediately before adding to reactions. Make enough Master Mix for all reactions plus 10% excess volume. Mix thoroughly and add 33 µL **Stop Buffer Master Mix** per reaction.
31. **Incubate** 8-strip PCR tubes for **10 min at 37°C** in a thermocycler. This step releases cleaved chromatin to supernatant and degrades RNA.
32. Perform a quick spin of 8-strip PCR tubes in benchtop microfuge to collect beads/buffer. Place 8-strip PCR tubes on a magnet stand until slurry clears (30 s – 2 min).
33. Transfer supernatant containing CUT&RUN-enriched DNA to new 1.5 mL tubes. The ConA beads can now be discarded.
34. Purify DNA from supernatant using the **CUTANA™ DNA Purification Kit** and provided instructions. This kit captures DNA fragments >50 bp, which is adequate for most targets.
35. Elute DNA in 12 µL **Elution Buffer** and use 1 µL to quantify the CUT&RUN-enriched DNA using the **Qubit™ fluorometer** per manufacturer's instructions.
  - Note 1: Yields are influenced by a variety of factors, including cell type, antibody, and target abundance. The best indicator of experimental success at this stage is that the CUT&RUN DNA yield of the target(s) of interest are greater than IgG negative control (even if slightly so, *i.e.* for low abundance targets such as H3K4me3).
  - Note 2: EpiCypher's typical CUT&RUN DNA yields from 500,000 native K562 cells (pre-PCR) are noted below:
    - IgG → ~ 2-5 ng
    - H3K4me3 (low abundance target) → ~5-10 ng
    - H3K27me3 (high abundance target) → ~20-50 ng
36. **PROCEED DIRECTLY TO LIBRARY PREPARATION**. DO NOT assess fragment size distribution prior to library preparation, as the yields may be below the limit of detection for this analysis and is not indicative of experimental success. See [FAQs](#) for more information.

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

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

37. Use 5-10 ng purified CUT&RUN-enriched DNA to prepare Illumina NGS libraries using the **NEBNext® Ultra™ II Library Prep Kit for Illumina®** per manufacturer's instructions.
  - Note: The NEB Ultra™ II Kit contains hot-start Q5 DNA polymerase.

38. Overview of library prep workflow, according to manufacturer's instructions:
  - a. End repair
  - b. Universal adapter ligation
  - c. DNA cleanup using 1.1x **AMPure beads** : reaction volume (retains fragments >150 bp)
  - d. PCR and primer indexing according to cycling parameters below
39. CUT&RUN-specific PCR cycling parameters. See [FAQs](#) for additional information and suggestions.
  - a. 45 sec at 98°C → activation of hot-start Q5 polymerase
  - b. 15 sec at 98°C → DNA melting
  - c. 10 sec at 60°C → hybrid primer annealing & short extension (<700 bp)
  - d. Repeat Steps b-c for a total of 14 cycles
  - e. 1 min at 72°C → final extension
40. DNA cleanup using 1.1x **AMPure beads** : reaction volume (e.g. 55 µL beads : 50 µL PCR).
41. Elute DNA in 15 µL **0.1x TE buffer**. Use 1 µL to quantify the purified PCR product using the **Qubit fluorometer** 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)

42. For each purified CUT&RUN library, including your IgG negative control, prepare 5 µL at 10 ng/µL for loading on the Agilent Bioanalyzer. 1 µL of each diluted library will be loaded onto the **Agilent High Sensitivity DNA Chip** (Cat# 5067-4626).
  - Note the dilution factor, which will be needed to calculate the library molarity for desired DNA size range (100-1,000 bp) using Bioanalyzer results.
43. Prepare and run the **Agilent High Sensitivity DNA Chip** per the manufacturer's instructions.
44. Typical Bioanalyzer results for CUT&RUN sequencing libraries are shown in **Figure 5**.
  - Note 1: Confirm that positive control histone PTM antibodies (e.g. H3K4me3) enriched for predominantly mononucleosome fragments (~275 bp peak with nucleosomes + sequencing adapters).
  - Note 2: The typical molarity for a 15 µL purified CUT&RUN sequencing library (100-1,000 bp region) is 100-200 nM.

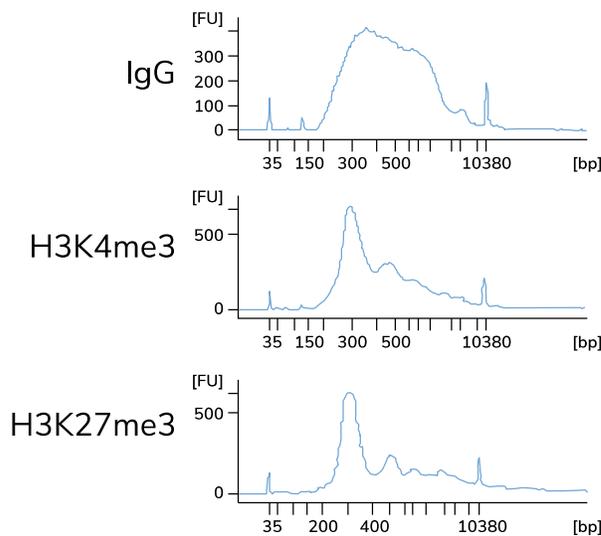
### Section VIII: Illumina Sequencing

45. Based on Bioanalyzer molarity calculations, pool libraries at the desired ratios.
  - Note: Only 3-8 million paired-end (PE) reads are needed for good CUT&RUN coverage. For lower abundance targets (e.g. H3K4me3), 3-5 million reads are adequate. For higher abundance targets (e.g. H3K27me3), aim for 5-8 million reads.
46. To obtain 3-8M 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).

47. To obtain 3-8M 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).



**Figure 5: Typical Bioanalyzer traces for IgG negative control and H3K4me3 and 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 or other 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 for these platforms.

## 7. Frequently Asked Questions (FAQs)

### 7.1 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 and negative controls in every experiment. We have included a series of quality control (QC) checks to analyze sample quality, confirm ConA bead binding, optimize cell permeabilization, assess DNA yields at various stages, and confirm fragment size distribution (see [Appendix II](#) and the **CUTANA™ CUT&RUN Protocol, Sections V - VII**). If the QC checks and positive and 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 (e.g. H3K4me3 for PTMs; CTCF for TFs)
  - iii. Negative control antibody (IgG)
- b. Experimental sample antibodies and cell types
- c. Use spike-in controls whenever possible (e.g. the SNAP-CUTANA™ K-MetStat Panel can be added into the positive and negative control reactions as well as any sample with an antibody to a methyl-lysine histone PTM target).

#### Quality control checks before decision to sequence:

- a. Confirm integrity of cells/nuclei before and after permeabilization ([Appendix II](#)). Samples should not contain clumps and starting counts should be accurate.
- b. Optimize digitonin permeabilization conditions (>95% cells permeabilized).
- c. Confirm sample binding to ConA beads ([Appendix II](#)).
- d. Ideally, the yield of CUT&RUN enriched DNA for the positive control antibody (e.g. H3K4me3) should be greater than negative control antibody (e.g. 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. Yields from PCR amplified sequencing libraries (~750 ng from 5-10 ng library input). See [Section VI](#).
- f. Bioanalyzer or TapeStation traces of sequence-ready libraries for positive control histone PTMs (e.g. H3K4me3) and other PTMs should be enriched for mononucleosome size fragments (~275 bp = ~150 bp nucleosome + 125 bp adapters, see [Section VII](#)).

- **IMPORTANT:** It is not recommended to assess fragment size distribution of CUT&RUN purified DNA (from [Section V](#)) prior to library preparation (see further explanation below). This analysis is not indicative of the success of a CUT&RUN experiment, and the amount of purified CUT&RUN DNA is often below the sensitivity of detection for Bioanalyzer/TapeStation approaches. For these reasons, we recommend assessment of CUT&RUN fragment size distribution after library preparation.
- Examples of library distribution in our protocol (**Figure 5**) are based on CUT&RUN analysis of histone PTMs. However, EpiCypher routinely maps TFs using CUT&RUN, and generally recovers 100-120 bp DNA fragments, similar to PTMs. These data provide reliable motif analysis and expected peak structures.
- Nevertheless, some DNA-bound TFs may have smaller size distributions due to processive MNase cleavage, which can generate sub-nucleosomal (<120 bp) DNA fragments. Our DNA purification kit is designed to recover fragments as small as 50 bp, and PCR conditions can also be adjusted to enrich for smaller library fragments if desired (see below).

## 2. Can I use Bioanalyzer or TapeStation traces to evaluate the success of CUT&RUN prior to library preparation?

We do not advise assessing the fragment size distribution of raw CUT&RUN DNA (from [Section V](#)) before library preparation. This habit is derived from ChIP-seq methods, which requires careful optimization of chromatin shearing to achieve mononucleosome-sized fragments in Input chromatin. Fragment size distribution in Input chromatin and immunoprecipitated (IP'd) DNA is monitored by agarose gel or capillary electrophoresis (e.g. Bioanalyzer) to confirm assay success at multiple steps.

However, CUT&RUN and ChIP-seq are not the same method, and thus do not entail the same quality control steps. ChIP-DNA yields are typically high due to high cell inputs and elevated background, which are inherent to the IP process. CUT&RUN, on the other hand, is a ChIP-less *in situ* technique that bypasses bulk chromatin fragmentation steps. CUT&RUN also has low background and requires ~10-fold fewer cells vs. ChIP-seq. As a result, raw CUT&RUN DNA yields are below the limit of sensitivity for fragment size distribution using the Bioanalyzer or TapeStation.

The best indicator of CUT&RUN success at this step is that raw DNA yields (e.g. from Qubit) from positive control reaction(s) are greater than yields from the IgG negative control. For low abundance targets, such as H3K4me3, this difference may be slight, while high abundance targets (e.g. H3K27me3) will display a more pronounced difference.

### 3. Can I use qPCR to evaluate the success of a CUT&RUN experiment?

We do not recommend qPCR for validation of CUT&RUN enrichment. To understand why, it is important to consider why qPCR is a go-to method for ChIP-seq, and how ChIP-seq approaches differ from CUT&RUN.

ChIP involves antibody-mediated enrichment of targets (histone PTMs or chromatin-associated proteins) from bulk fragmented chromatin, or “Input.” qPCR is traditionally used to verify the enrichment of a known on-target region following a ChIP experiment as a readout for experimental success. To determine enrichment, qPCR for the same region must also be performed on the bulk chromatin Input. Regions not enriched by the ChIP reaction are also included as negative controls for comparison.

Not only are these steps unnecessary for CUT&RUN assays, they are also incredibly challenging. CUT&RUN is an *in situ* assay, meaning it is performed in intact cells. Antibody-bound chromatin is cleaved by pAG-MNase, diffuses outside the cell, and is collected for sequencing. There is no immunoprecipitation step and no bulk chromatin Input for enrichment comparisons. In fact, due to the processive nature of MNase, the CUT&RUN fragments may be 50-100 bp (for some TFs), challenging primer design and qPCR analysis.

Instead, we recommend analysis of post-library prepped, purified CUT&RUN sequencing libraries (using the Agilent Bioanalyzer or TapeStation) to confirm DNA size distribution and molarity (see [Section VII](#)) and proceed to sequencing without qPCR. As you will need only 3-8 million reads per sample, you can multiplex samples, save on sequencing costs, and obtain the genomic data regarding your target. Due to this cost savings, it is much more effective to obtain full sequencing data rather than spending time and money on attempted qPCR optimization.

## 7.2 Spike-in Controls

### 4. 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 at very low levels in EpiCypher’s pAG-MNase preps. However, at a typical sequencing depth of 3-8 million 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](#)).

### 5. Does EpiCypher offer spike-in nucleosome controls for CUT&RUN?

EpiCypher recently launched SNAP Spike-in Controls for CUTANA™ CUT&RUN/CUT&Tag assays, and now offers the SNAP-CUTANA™ K-MetStat Panel for CUT&RUN/CUT&Tag

reactions against histone lysine methylation targets (EpiCypher 19-1002). The panel comprises highly pure, modified semi-synthetic/recombinant nucleosomes wrapped with DNA containing a PTM-specific barcode, allowing detection in next-generation sequencing (NGS). SNAP-CUTANA Spike-ins are the ideal physiological control because they replicate the natural substrate of histone PTM antibodies in CUT&RUN, and thus can provide a direct readout on assay success. Our SNAP-CUTANA Spike-ins offer multiple advantages, including sample normalization and in-assay antibody validation. SNAP-CUTANA Spike-ins also provide essential information on the quality of sample inputs, pAG-MNase activity, DNA purification, and library preparation, making them an essential tool for developing, optimizing, and troubleshooting CUT&RUN workflows. See [epicypher.com/19-1002](https://epicypher.com/19-1002) for product and ordering information and to download the **SNAP-CUTANA Spike-in User Guide**, which contains detailed guidance on their application in CUT&RUN/CUT&Tag assays and how to analyze spike-in data from NGS results.

### 7.3 Sample Input Compatibility

#### 6. What types of cell 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](#). A number of groups have successfully performed CUT&RUN on human and mouse primary tissue<sup>5-8</sup>, FACS isolated cells<sup>9</sup>, and immune cells<sup>10,11</sup>. 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), for the minimal incubation time needed to detach cells as optimized for cell type. Collect cells by centrifugation for ~3 min at 600 x g at RT. Discard supernatant, and then proceed directly to cell counting and CUT&RUN wash steps as outlined in [Section II](#). Trypsin is washed away by subsequent washes that are a standard part of the CUT&RUN protocol. This method detaches and monodisperses cells, resulting in >95% adsorption onto ConA beads.

Tissue samples: While EpiCypher does not have tissue-specific protocols available at this time, the primary requirement is that tissue is processed to a monodispersed cell suspension

(typically by mechanical maceration or douncing). Enzymatic digestion (e.g. collagenase/dispase) can be used for connective tissue and trypsin (as described above; monitoring dissolution to single cells) may be used for macro-dissected tissue.

Working with immune cells: Lectins (e.g. ConA) play a known role in the innate immune system, and immune cell types may be inadvertently stimulated via binding to ConA beads. To circumvent this potential problem in CUT&RUN, EpiCypher recommends using nuclei<sup>9</sup> (see [Appendix I](#)) or a cross-linking strategy<sup>12</sup> (see detailed cross-linking protocol at [epicypher.com/protocols](http://epicypher.com/protocols)).

## 7. Is CUT&RUN compatible with frozen or cross-linked cell preparations?

Yes. General guidelines are noted below; our detailed **CUTANA CUT&RUN Cross-linking Protocol** is provided at [epicypher.com/protocols](http://epicypher.com/protocols), and additional protocols are available upon request (email [info@epicypher.com](mailto:info@epicypher.com)):

Cryopreservation: EpiCypher has confirmed that freeze/thawed cells (e.g. slow freeze in 10% DMSO/media, and then quick thaw at 37°C) 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 CUT&RUN cross-linking conditions and recommended wash buffers<sup>12</sup>. Although yields are significantly lower than from native cells, the resulting data tracks display similar quality (*i.e.* signal-to-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 or 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 light (e.g. 0.1% formaldehyde, 1 min) to moderate (e.g. 1% formaldehyde, 1 min) cross-linking even though total yields are 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 from K562 cells in CUT&RUN. 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-to-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](https://epicypher.com/protocols); similar protocols can be found in the literature<sup>12</sup>.

## 7.4 Antibodies and Targets

### 8. Does EpiCypher's CUT&RUN protocol work on non-PTM targets?

Yes. The current protocol has been used at EpiCypher to generate CUT&RUN data for numerous non-PTM targets, including CTCF, FOXA1/HNF3A, BRD4, and SMARCA4 (BRG1). Antibodies to chromatin-associated protein targets validated for use in CUT&RUN can be found at [epicypher.com/cut-and-run-antibodies](https://epicypher.com/cut-and-run-antibodies); for targets not yet on this list, contact [info@epicypher.com](mailto:info@epicypher.com) for recommendations. No protocol modifications were necessary to generate these data since the columns used to purify the CUT&RUN-enriched chromatin fragments retain >50 bp DNA, allowing for NGS library distributions down to ~175 bp (~50bp DNA + 125 bp sequencing adapters).

For TFs, which may generate sub-nucleosomal size (<120 bp) DNA fragments, modifications to the library protocol have been reported<sup>7,13</sup> to improve the representation of these smaller fragments during library preparation. Briefly, to enrich for CUT&RUN library fragments <120 bp, the authors altered the MNase inactivation step (after end repair) from 65°C for 30 min to 50°C for 1 hr. Also, after the ligation reaction, AMPure bead size-selection ratio was increased from 1.1x volume to 1.75x volume (retains library fragments >100 bp). 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.

### 9. What antibodies does EpiCypher recommend for CUT&RUN? Will reliable ChIP antibodies work for CUT&RUN?

Through our extensive development of CUT&RUN assays to various targets, EpiCypher has found that antibodies that work well in ChIP may not always work in CUT&RUN. To address this unmet need for researchers, EpiCypher has begun screening antibodies for high quality performance in CUT&RUN and its sister technology CUT&Tag (see the growing list of antibodies: [epicypher.com/cut-and-run-antibodies](https://epicypher.com/cut-and-run-antibodies)). Below we outline our criteria for antibodies to chromatin-associated proteins and histone PTMs.

Chromatin-associated protein targets: EpiCypher has recently launched **CUTANA™ CUT&RUN Antibodies to Chromatin-Associated Proteins**. Every lot of CUTANA

Chromatin-Associated Protein Antibody is directly validated in CUT&RUN and/or CUT&Tag assays and must meet our “CUTANA Compatible” criteria for superior performance. To pass testing, the antibody must generate reliable and high signal-to-noise CUT&RUN and/or CUT&Tag data and display genomic distribution profiles consistent with the reported function of the target protein (for example, DNA binding motif analysis for TFs). 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](mailto:techsupport@epicypher.com).

Histone PTM targets: Antibodies to histone PTMs are particularly susceptible to off-target cross-reactivity which can compromise biological interpretations<sup>4</sup>. EpiCypher has conducted extensive studies of histone PTM antibodies in ChIP-seq by using our exclusive SNAP-ChIP® nucleosome spike-in control technology. Although the majority of antibodies exhibit major problems such as poor specificity and/or low efficiency, best-in-class reagents for ChIP-seq do exist, and have been identified using SNAP-ChIP Spike-ins (see ref <sup>4</sup> and [chromatinantibodies.com](http://chromatinantibodies.com) *Maryanski et al., In preparation*).

Ideally, PTM antibodies should be validated using defined on- and-off target nucleosome controls in the final application. EpiCypher has recently launched the **SNAP-CUTANA K-MetStat Panel** and are using these defined spike-ins controls to identify best-in-class histone PTM antibodies for CUT&RUN. To pass testing, the antibody must display <20% cross-reactivity to related PTMs in the K-MetStat Panel and generate consistent genomic enrichment using 500,000 and 50,000 starting cells. Our first **Histone PTM Antibody, SNAP-Certified for CUT&RUN** targeting H3K4me3 is now available (EpiCypher 13-0041; [epicypher.com/13-0041](http://epicypher.com/13-0041)). Antibodies to additional PTM targets are being tested and are expected to launch in 2022.

For more information or for antibody recommendations, please contact [techsupport@epicypher.com](mailto:techsupport@epicypher.com).

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

**Description:** This protocol gives instructions on fresh nuclei isolation, cryopreservation of nuclei, and thawing nuclei – all of which are compatible with CUT&RUN assays. Note that this protocol is designed similarly to the CUTANA CUT&RUN protocol, *i.e.* to batch process cells for multiple CUT&RUN reactions, and was developed using 500,000 K562 cells per CUT&RUN reaction. Adjust volumes and cell numbers for your experiments as needed.

### 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             |             |

\*1M spermidine preparation: Dissolve 1 gram spermidine (MW = 254.63) in 3.93 mL molecular grade H<sub>2</sub>O. Store in single-use aliquots at -20°C for 6 months.

### Nuclear Extraction (NE) Buffer

NE Buffer base:

20 mM HEPES, pH 7.9

10 mM KCl

0.1% Triton X-100

20% Glycerol

*Filter sterilize. Store at 4°C for up to 6 months.*

Add to NE Buffer on day of experiment:

1X cOmplete Mini-Tablet (1 tablet)

0.5 mM Spermidine

*Keep at 4°C throughout experiment. Discard after 1 day.*

### Protocol: Nuclei Harvest, from Beginning of Section II of CUT&RUN Protocol

- Harvest 500,000 cells per planned CUT&RUN reaction by spinning for 3 min at 600 x g at RT in 1.5 mL tube. Pipette or aspirate to discard supernatant.
  - Note 1:** For all steps, scale buffer volumes with number of cells, *e.g.* use 1 mL buffer for 5 million cells. Adjust volumes and cell numbers for your experiment as needed.
  - Note 2:** Prepare ~10% excess cells to confirm cell integrity prior to nuclear isolation and nuclei integrity at the end of the protocol (see **Protocol Notes**, below).
- Resuspend cells in 100 µL/reaction RT **1X PBS**.
  - Note:** Set 10 µL aside for confirmation of cell integrity.
- Spin for 3 min at 600 x g at RT. Decant or pipette to remove and discard supernatant.

4. Resuspend cell pellet in 100  $\mu$ L/reaction cold **NE Buffer**.
5. **Incubate** reactions for **10 min on ice**.
6. Spin for 3 min at 600 x g at **4°C**. Pipette or aspirate to discard supernatant.
  - Note: The pellet should change in appearance from a sticky, pale yellow pellet (cells) to a white, fluffy pellet (nuclei).
7. Resuspend nuclei in 100  $\mu$ L/reaction cold **NE Buffer**.
8. Freeze (see below) or proceed to ConA bead conjugation step ([Section II Step 11](#)). Nuclei in NE Buffer can be directly added to activated ConA beads.
  - Note: Set 10  $\mu$ L aside for confirmation of isolated nuclei integrity

### Protocol Notes (also see Appendix II)

To examine the efficiency of nuclear isolation and ensure intact nuclei:

1. Remove 10  $\mu$ L aliquots from washed cells/final nuclei.
2. Combine 10  $\mu$ L aliquots with 10  $\mu$ L 0.4% Trypan blue dye, mix, and load onto cell counter or hemacytometer slide.
3. Examine under brightfield or phase microscope (**Figure 6**, [Appendix II](#)). Cells should not take up Trypan blue and appear clear/white, whereas nuclei should take up Trypan and appear blue.

### Protocol: Cryopreservation and thawing of nuclei

1. To cryopreserve nuclei, slowly freeze aliquots in an isopropanol-filled chiller in a -80°C freezer.
  - Note: If necessary, nuclei can be shipped on dry ice in this state.
2. To avoid nuclear lysis and chromatin fragmentation, thaw nuclei quickly by placing tubes on 37°C block until thawed.
3. Proceed to ConA bead conjugation step, [Section II Step 11](#). Nuclei in NE Buffer can be directly added to activated ConA beads.

### Protocol: Cryopreservation and thawing of cells

1. To cryopreserve cells, supplement cell culture media with a cryoprotective agent (e.g. 10% DMSO in media) and slowly freeze aliquots in an isopropanol-filled chiller in a -80°C freezer.
2. When ready to perform CUT&RUN, quickly and completely thaw samples at 37°C and then prepare cells as described in [Section II Step 7](#).

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

Description: This Appendix provides detailed instructions on checking the quality of cells/nuclei before and after permeabilization + conjugation to ConA beads. Of note, these Quality Control (QC) checks should also be used to check the integrity of cells before nuclei isolation, as described in [Appendix I](#). All QC checks were developed using batch processed K562 cells and nuclei (*i.e.* for multiple CUT&RUN reactions).

We recommend checking the quality of starting materials (cells or nuclei) prior to every CUT&RUN experiment. For new users or for working with new cell types/sample inputs, we strongly recommend performing the ConA bead conjugation checks. This is a key step in the CUT&RUN protocol, and if cells are not successfully bound and permeabilized, CUT&RUN yields will be dramatically reduced.

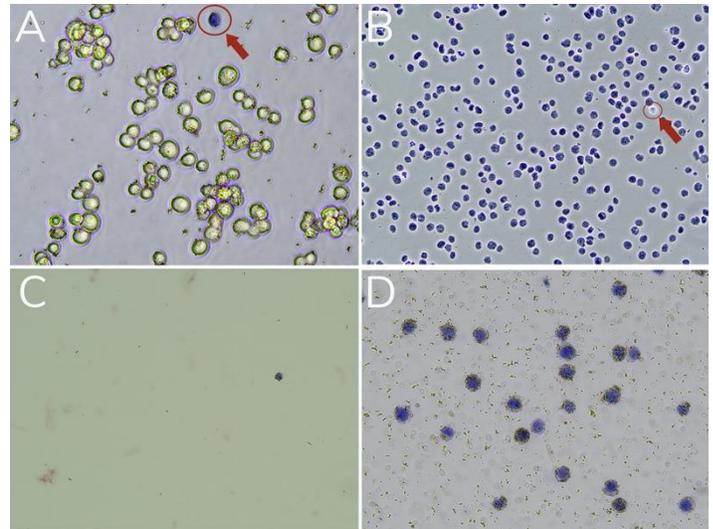
### Reagents, Materials & Equipment Needed

| Item  | Vendor |
|---|--------|
| 0.4% Trypan blue  | Any    |
| Hemocytometer   | Any    |
| Cell counting slides  | Any    |
| Brightfield/phase contrast microscope or automated cell counter | Any    |

### Protocol: Sample Integrity and ConA Bead Conjugation QC Checks, Starting from Beginning of Section II of CUT&RUN Protocol

- In [Section II Step 7](#), process excess cells (or nuclei) to have leftover enough leftover material (*e.g.* prepare ~10% excess volume for batch processing or one extra sample).
- Prior to ConA bead conjugation, take 10  $\mu$ L from washed cells (or prepared nuclei) and evaluate sample integrity as follows:
  - Add 10  $\mu$ L of 0.4% **Trypan blue**.
  - Mix 10 times by pipetting.
  - Transfer 10  $\mu$ L to a counting slide.
  - View under brightfield/phase microscope or cell counter.
  - Confirm sample integrity or troubleshoot as needed (see **Figure 6** and **Table 6**):
    - Cells/nuclei should not be clumped and should show the expected morphology
    - Intact cells will not absorb trypan blue and appear white (**Figure 6A**)
    - Nuclei will take up Trypan and appear blue (**Figure 6B**)
    - Troubleshoot as necessary (**Table 6**)
- Proceed with bead binding (working in batch if possible) by adding 10  $\mu$ L activated **ConA Beads** per 100  $\mu$ L washed cells/nuclei in a 1.5 mL tube. Again, make sure to prepare ~10% extra volume.
- Gently vortex and/or pipette to mix ConA beads with cells/nuclei.

5. **Incubate** bead slurry for **10 min at RT**. Cells/nuclei will adsorb to the activated ConA beads.
6. Place 1.5 mL tube(s) on magnet until slurry clears. Transfer 10  $\mu$ L supernatant into a fresh 1.5 mL tube (**Unbound fraction**) and set aside for bead binding integrity check. Pipette to remove remaining supernatant and discard.
7. Add 50  $\mu$ L/reaction cold **Antibody Buffer** quickly, to avoid bead drying. If working in bulk, scale volume for total number of planned reactions. Gently vortex immediately and thoroughly to an even resuspension.
8. Remove 10  $\mu$ L and transfer to a fresh tube (**Bead-Bound Fraction**). Place remaining sample on ice.
9. Perform Trypan blue staining and bead binding integrity check as described in **Steps 2a-e** (above), comparing **Unbound Fraction** and **Bead-Bound Fraction**.



**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 Fraction** showing nuclei (blue) successfully bound to activated ConA Beads (brown specks).

- Successful binding will show Trypan positive cells/nuclei surrounded by ConA beads with little leftover in the Unbound Fraction; compare **Figures 6C and 6D**. For troubleshooting approaches, see **Table 6**.

9. Continue with the [CUT&RUN Protocol, Section III](#) (Antibody Binding).

| Samples                    | Figure  | Success Metric (Trypan Blue staining)   | Troubleshooting Tips   |
|----------------------------|---------|---|--|
| <b>Cells</b>               | 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.  |
| <b>Nuclei</b>              | Fig. 6B | Nuclei should be >95% Trypan blue positive and unclumped.   | See <a href="#">Appendix I</a> for a detailed nuclei preparation protocol.   |
| <b>Unbound Fraction</b>    | 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. |
| <b>Bead-Bound Fraction</b> | Fig. 6D | Successful ConA bead binding will show Trypan blue positive permeabilized cells/nuclei surrounded by beads.   |  |

**Table 6:** Quality Control testing of cell/nuclei integrity and ConA bead binding steps.

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

**Description:** This Appendix describes the optimization and application of *Escherichia coli* (*E. coli*) Spike-in DNA (EpiCypher 18-1401) for CUT&RUN sequencing normalization, as noted in [Section V](#) of the CUTANA CUT&RUN Protocol. This protocol should be used in combination with **CUTANA pAG-MNase for ChIC/CUT&RUN** (EpiCypher 15-1016), which has very low levels of *E. coli* DNA. These steps were developed using 500,000 native K562 cells.

### Protocol: Optimizing amount of *E. coli* Spike-in DNA for CUT&RUN

1. Add *E. coli* spike-in DNA to CUT&RUN **Stop Buffer** prior to sample addition by preparing a **Stop Buffer Master Mix** (see [Section V](#)). The amount of spike-in added should consider cell/nuclei input, condition (fixed, native, etc.), and target abundance. Thus, optimization may be required. Some general guidelines:
  - For reactions using 500,000 cells add 0.5 – 1 ng *E. coli* spike-in DNA/reaction. This works for most cell types and conditions.
  - The spike-in amount should be scaled linearly from the 500,000 cell input baseline; e.g. using 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 7](#)).

| <i>E. coli</i> Spike-in DNA | Target   | Total Reads | <i>E. coli</i> Reads | % <i>E. coli</i> 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 7.** 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](#)), quench MNase activity with 33  $\mu$ L **Stop Buffer Master Mix** containing *E. coli* spike-in DNA.
3. Complete the CUT&RUN protocol and prepare Illumina sequencing library.

## Protocol: NGS Normalization using *E. coli* Spike-in DNA

Description: To use spike-in *E. coli* DNA for normalization and pairwise comparisons perform the following steps.

4. Align NGS data to the experimental sample reference genome (e.g. human hg19 build) and to the *E. coli* K12, MG1655 reference genome (see Illumina iGenomes Reference Sequences; [support.illumina.com/sequencing/sequencing\\_software/igenome.html](https://support.illumina.com/sequencing/sequencing_software/igenome.html)).
5. For pairwise comparisons, determine the number of *E. coli* spike-in DNA reads and normalize to total sequencing depth (i.e. total number of uniquely mapped reads). Calculate this ratio for each individual NGS dataset.
  - Example: CUT&RUN was performed to map a histone PTM target from treated and untreated cell samples.
  - Treated spike-in: 100,000 *E. coli* reads in 5,000,000 uniquely aligned reads = 2%
  - Untreated spike-in: 30,000 *E. coli* reads in 3,000,000 uniquely aligned reads = 1%
6. Calculate normalization factor<sup>A</sup>, such that after normalization the *E. coli* spike-in signal is equal across all samples. Continuing with the example above:
  - Treated normalization factor = 1/2% spike-in bandwidth = 0.5
  - Untreated normalization factor = 1/1% spike-in bandwidth = 1.0
7. Use single scalar normalization ratio with the `--scaleFactor` option enabled in the `deeptools bamCoverage` tool to generate normalized bigwig files for visualization in IGV (<https://deeptools.readthedocs.io/en/develop/content/tools/bamCoverage.html>). Example:
  - Treatment sample `--scaleFactor = 0.5`
  - Untreated sample `--scaleFactor = 1.0`

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<sup>A,B</sup>.

### Appendix III References

<sup>A</sup> Tay *et al.* (2020) Hdac3 is an epigenetic inhibitor of the cytotoxicity program in CD8 T cells.

<sup>B</sup> Orlando *et al.* (2014) Quantitative ChIP-Seq Normalization Reveals Global Modulation of the Epigenome.