EpiCypher® CUTANA™ CUT&RUN Protocol v3.0

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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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 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 for regular protocol updates.

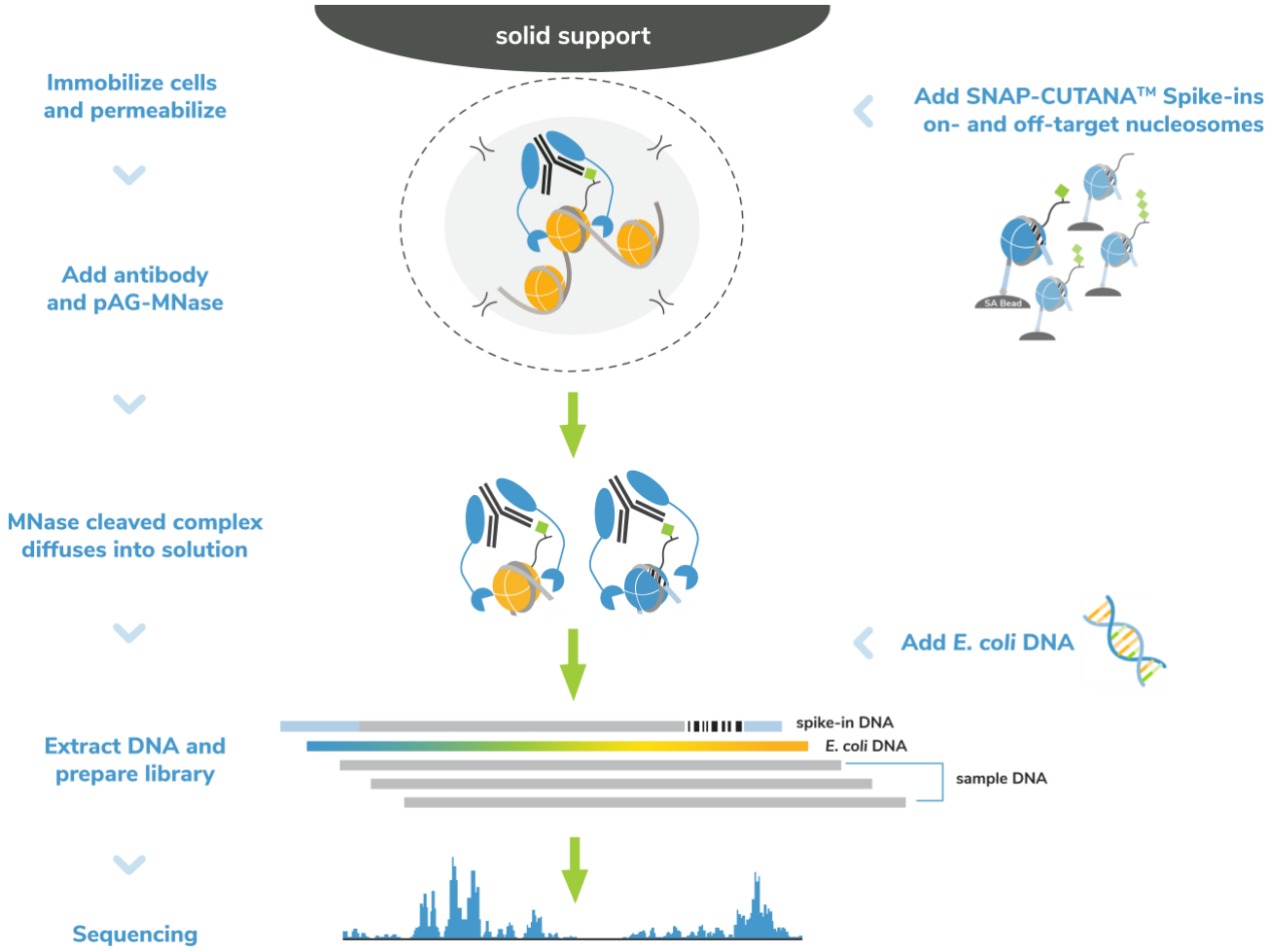

Figure 1. Overview of the CUTANA™ CUT&RUN protocol.

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), including:

- **pAG-MNase** (EpiCypher 15-1016 & 15-1116), the essential reagent 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 14-1048) with all reagents included to go from cells to purified CUT&RUN DNA.
- **CUT&RUN Library Prep Kit** (EpiCypher 14-1001 & 14-1002) for robust, user-friendly CUT&RUN library preparation. Includes DNA purification beads and indexing primers.
- **CUT&RUN Antibodies** to histone PTMs and chromatin-associated proteins, rigorously validated directly in CUT&RUN. See epicypher.com/cut-and-run-antibodies for information.
- **CUT&RUN Spike-in Controls**
  - ***E. coli* Spike-in DNA** (EpiCypher 18-1401) for sequencing 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. Now available for histone lysine methylation PTMs (SNAP-CUTANA K-MetStat Panel: EpiCypher 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 for more info.
- **CUTANA Cloud** (cloud.epicypher.com) online platform for streamlined CUT&RUN data analysis. See epicypher.com/cutana-cloud for more information.
- **CUTANA CUT&Tag** reagents for ultra-low input applications. Go to epicypher.com/CUT&Tag for more information.
- Inquire for more information or to connect with EpiCypher scientists: 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 recommend reading this section, the Experimental Design & Key Protocol Notes and the Protocol to carefully plan your experiment as well as familiarize yourself with the assay.

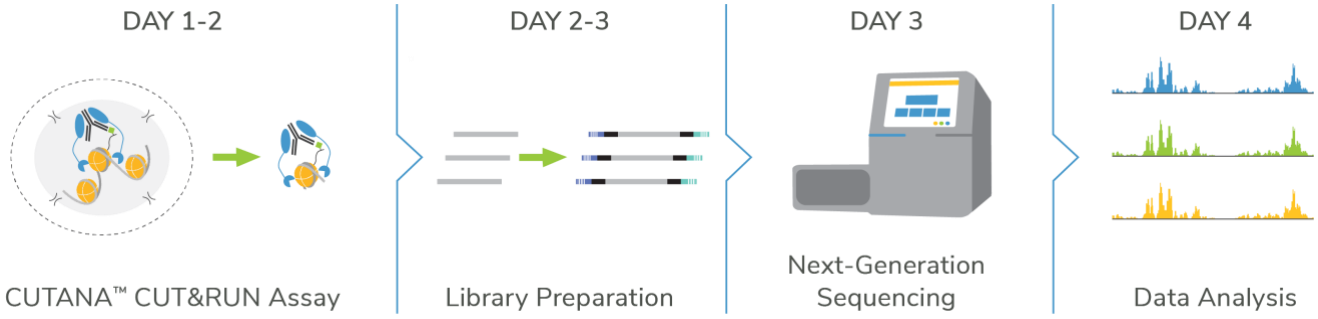

Figure 2: Timeline of CUT&RUN assay using EpiCypher's CUTANA™ CUT&RUN protocol.

Section I: ConA Bead Activation (~30 min)
Description: This section "activates" Concanavalin A coated beads (**ConA 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.

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. To reduce bead clumping and improve sample handling, we recommend adding the Wash Buffer Enhancer Set (EpiCypher 14-1803) to **Wash Buffer**.

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 in our Tech Support Center. To ensure that cells are immobilized to ConA beads, we have also developed simple quality control checks that we strongly recommend using in every experiment.

Section III: Binding of Antibodies (~30 min + overnight)
Description: Next, the ConA bead – cell mixture is resuspended in cold **Antibody Buffer**, and a target-specific antibody is added for overnight incubation. Note that antibody selection in

CUT&RUN is critical to success, and antibodies that work well in ChIP-seq are **NOT** guaranteed success in CUT&RUN; see our Tech Support Center for more antibody selection guidelines. Importantly, **Antibody Buffer** is the first buffer to contain digitonin, which permeabilizes cells and allows the antibody to bind its target *in situ* (PTM or chromatin-associated protein). The digitonin concentration required for CUT&RUN varies by sample (*e.g.* cell type, fixation) and must be optimized for every unique input, as described in 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.* positive control H3K4me3) or IgG negative 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. The K-MetStat Panel is added immediately before antibody addition, as outlined in **Section III** of the protocol. The **SNAP-CUTANA Spike-in User Guide** (epicypher.com/protocols) includes detailed information about how the spike-ins work and their incorporation 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 & 15-1116) 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 $CaCl_2$ 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 **$CaCl_2$** 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.

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 Master Mix** and used to normalize downstream NGS data. For further instructions on optimizing *E. coli* DNA for CUT&RUN experiments, see this Tech Support Center section.

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 this Tech Support Center article). 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 Agilent TapeStation® or Bioanalyzer® approaches used to examine fragment distribution. Further, 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: We recommend using the **CUTANA™ Library Prep Kit** (EpiCypher 14-1001 or 14-1002) to prepare CUT&RUN sequencing libraries. The CUTANA Library Prep Kit is specifically optimized for CUT&RUN, eliminating the guesswork surrounding adaptation of multi-purpose or ChIP library kits. The protocol is also robust for the low inputs generated by CUT&RUN, providing high quality libraries using 10 to 0.5 ng DNA.

Each kit includes everything you need for library prep, including DNA purification beads and indexing primers. A combinatorial dual indexing primer strategy enables the entire 48-reaction kit to be multiplexed in a single NGS run. The two versions of the kit (14-1001 & 14-1002) contain distinct primer sets, combined allowing up to 96 CUT&RUN libraries to be multiplexed.

Section VII: Agilent TapeStation® Analysis (~1 hr)

Description: Before sequencing, CUT&RUN libraries are examined using the TapeStation or Bioanalyzer to assess fragment size, library concentration and DNA quality. Predominant enrichment of ~300 bp-sized fragments (~170 bp excised DNA plus adapters) is the best indicator of CUT&RUN experimental success prior to NGS. Here we describe the use of the Agilent TapeStation for CUT&RUN library analysis and 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. These suggestions are based on the fact that CUT&RUN only requires 3-8 million uniquely aligned 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 this Tech Support Center section. For guidance on SNAP-CUTANA K-MetStat Panel analysis, see the SNAP-CUTANA Spike-in User Guide at epicypher.com/19-1002; for *E. coli* spike-in DNA, see Tech Support Center. If expertise and timelines are limited, we recommend using CUTANA™ Cloud (cloud.epicypher.com), an online data analysis platform for streamlined CUT&RUN and CUT&Tag bioinformatics with built-in pipelines for spike-in analyses.

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 13-0060; 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 this Tech Support Center section).
   - 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 13-0060), H3K27me3 (EpiCypher 13-0055), and BRD4 (EpiCypher 13-2003).
3. We strongly recommend performing the **Quality Control Checks**, which include evaluating the integrity of starting cells/nuclei and binding to Concanavalin A (**ConA**) beads (See **Section II, Step 8** and **Figure 4**).
4. While the standard protocol is written for native (unfixed) suspension cells, we have sample preparation instructions for working with nuclei, cryopreserved cells/nuclei, adherent cells,

and cross-linked material (see Appendix I and Tech Support Center for more protocols on sample prep).

5. 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 (*e.g,* EpiCypher 21-1004).
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 this Tech Support Center article. 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 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

   Note: The **CUTANA CUT&RUN Library Prep Kit** has also been designed for use with 8-strip PCR Tubes and multi-channel pipettors, further streamlining CUT&RUN workflows from cells to sequencing-ready libraries.
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.
   - Add **CUTANA™ Wash Buffer Enhancers** (EpiCypher 14-1803) to Wash Buffer to reduce bead clumping.
9. Although protocols with shortened antibody and/or $CaCl_2$ incubation times have been published[3], 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 this Tech Support Center article). 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 6**), and evaluate peak structure and expected genome-wide distribution in sequencing data.

5. Buffers, Reagents & Materials Needed

Table 1: Buffer components

Components	Source	Cat #
1X PBS	Any vendor	
$CaCl_2$	Sigma-Aldrich	C1016
CUTANA™ 5% Digitonin	EpiCypher	21-1004
CUTANA™ Bead Activation Buffer	EpiCypher	21-1001
CUTANA™ Protease Inhibitor Tablets	EpiCypher	21-1027
CUTANA™ Stop Buffer	EpiCypher	21-1003
CUTANA™ Wash Buffer Enhancer Set	EpiCypher	14-1803
DMSO	Sigma-Aldrich	D8418-100ml
EDTA (prepare 0.5 M stock at pH 8.0)	Sigma-Aldrich	E5134
HEPES	Sigma-Aldrich	H3375
KCl	Sigma-Aldrich	P3911
Molecular biology grade H_2O (RNase, DNase free)	VWR	VWRV02-0201-0500
NaCl	Sigma-Aldrich	S5150-1L
Spermidine trihydrochloride*	Sigma-Aldrich	S2501
Trypan blue	Thermo Fisher Scientific	T10282

*1M spermidine preparation: Dissolve 1 gram spermidine (MW = 254.63) in 3.93 mL molecular grade H_2O. Store in single-use aliquots at -20°C for 6 months.

Buffer recipes

Bead Activation Buffer

CUTANA™ Bead Activation Buffer (EpiCypher 21-1001) is ready to use with no additives.
See Appendix II for recipe that can be used in place of EpiCypher 21-1001.

Pre-Wash Buffer

20 mM HEPES, pH 7.5
150 mM NaCl
Filter sterilize. Store at 4ºC for up to 6 months.

Wash Buffer

Pre-Wash Buffer (recipe above)
0.5 mM Spermidine*
1X CUTANA™ Protease Inhibitor (1 tab / 420 µL small or 1 tab / 2 mL large)
1X CUTANA™ Wash Buffer Enhancer Set**
Filter sterilize. Store at 4ºC for up to 1 week.

Digitonin Buffer

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

Antibody Buffer

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

Stop Buffer

CUTANA™ Stop Buffer (EpiCypher 21-1003) is ready to use with no additives.
See Appendix II for recipe that can be used in place of EpiCypher 21-1003.

0.1X TE

1 mM Tris-HCl pH 8.0
0.1 mM EDTA, pH 8.0 (NaOH)

Buffer Preparation Notes

* Spermidine is added to compensate for the removal of Mg^{2+} from the buffer. Mg^{2+} can cause DNA degradation and is typically omitted from CUT&RUN buffers.
** CUTANA Wash Buffer Enhancer reduces bead clumping and improves sample handling.
*** 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
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/products/antibodies. For other targets not found on our site, contact us for recommendations: info@epicypher.com.
CUTANA Compatible CTCF Positive Control Antibody	EpiCypher	13-2014	See datasheet for application-specific dilutions.
CUTANA™ Concanavalin A (ConA) Conjugated Paramagnetic Beads	EpiCypher	21-1401 or 21-1411	ConA is a lectin, which can cause immune cell activation. For technical support re. immune cell studies, see this Tech Support Center article or contact info@epicypher.com.
CUTANA™ CUT&RUN Library Prep Kit	EpiCypher	14-1001 or 14-1002	Library Prep Kit specifically optimized for CUT&RUN applications. Each kit contains sufficient materials to prep 48 CUT&RUN libraries. A combinatorial dual indexing primer strategy is used to generate 48 distinct barcode pairs for multiplexed NGS. The two kit versions (14-1001 and 14-1002) contain unique indexing primer sets, and can be combined to multiplex up to 96 libraries in a single sequencing run.
CUTANA™ DNA Purification Beads	EpiCypher	21-1407	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.
CUTANA™ pAG-MNase	EpiCypher	15-1016 or 15-1116	50 & 250 reaction pack sizes available. Supplied as 20X stock.
E. coli Spike-in DNA	EpiCypher	18-1401	Use as an exogenous spike-in control for experimental normalization. See Tech Support Center for detailed instructions.
Qubit™ 1x dsDNA HS Assay Kit	Thermo Fisher Scientific	Q33230	For DNA quantification.
Rabbit IgG Negative Control Antibody	EpiCypher	13-0042	Use 0.5 µg per reaction.
SNAP-ChIP® Certified H3K27me3 Positive Control Antibody	EpiCypher	13-0055	Use 0.5 µg per reaction.
SNAP-ChIP® Certified, CUTANA Compatible H3K4me3 Positive Control Antibody	EpiCypher	13-0060	Use 0.5 µg per reaction.
SNAP-CUTANA™ K-MetStat Panel	EpiCypher	19-1002	**Spike-in controls for Sample Normalization & Antibody Profiling (SNAP Spike-in Controls)**: 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. Add spike-ins to CUT&RUN reactions targeting one of the PTMs in the panel as well as in CUT&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 epicypher.com/19-1002. **NOTE: Store at -20˚C.** Lower temperatures can cause freezing and will permanently damage the beads. Pipette-mix (**do NOT vortex**) before use.

Table 3: Equipment

Item	Vendor	Catalog No.	Notes
1.5 mL Magnetic Separation Rack	EpiCypher	10-0012	For bulk or "batch" processing of ConA beads and ConA bead-conjugated cells in **Sections I and II** of CUT&RUN protocol; see **Figure 3A.**
8-strip 0.2 mL PCR tubes	EpiCypher	10-0009	Compatible with the magnetic stand.
8-strip PCR tube Magnetic Separation Rack	EpiCypher	10-0008	For processing of individual CUT&RUN reactions in **Section III** onward; see **Figure 3B**. Enables streamlined sample handling for higher experimental throughput and improved reproducibility.
Brightfield/phase contrast microscope or automated cell counter	Various		For counting cells and confirming sample integrity.
Cell counting slides	Various, needs to be compatible with cell counter		For counting cells and confirming sample integrity.
Hemacytometer	Various		For counting cells and confirming sample integrity.
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.
Qubit™ 4 Fluorometer	Thermo Fisher Scientific	Q33228	For DNA quantification. Older versions of the Qubit 4 are also compatible with this protocol.
TapeStation® 4200 or 4150	Agilent	G2991BA or G2992AA	For analysis of purified CUT&RUN sequencing libraries. May substitute comparable capillary electrophoresis instrument (*e.g.* Agilent Bioanalyzer® & High Sensitivity DNA Kit).
D1000 ScreenTape & D1000 Reagents	Agilent	5067-5582 & 5067-5583	For analysis of purified CUT&RUN sequencing libraries. May substitute comparable capillary electrophoresis instrument (*e.g.* Agilent Bioanalyzer® & High Sensitivity DNA Kit).
Tube Nutator	VWR	82007-202	For bead incubation steps (overnight antibody incubation, pAG-MNase digest reaction). **It is critical to use a tube nutator rather than a rotator for these steps**.
Vortex-Genie	Scientific Industries	SI-0236	For bead mixing steps.

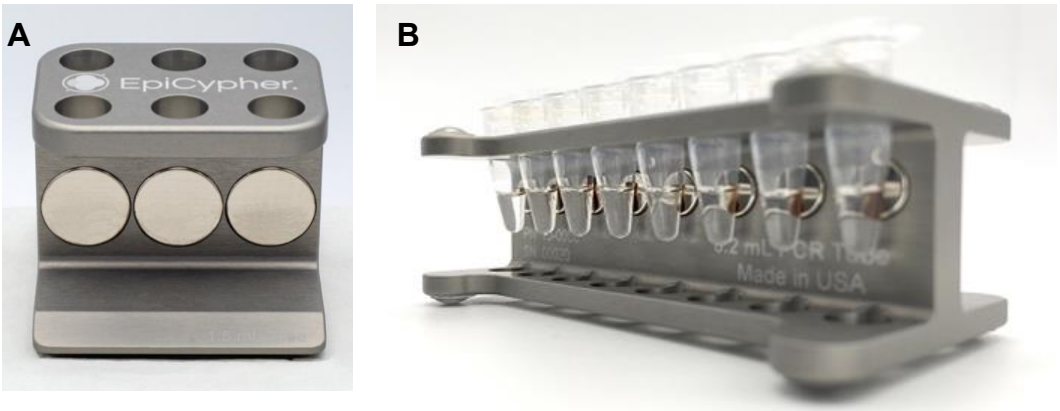

Figure 3: Magnetic racks for CUT&RUN assays. (A) For batch processing of ConA beads, use a 1.5 mL magnetic rack (*e.g.* EpiCypher 10-0012, pictured). **(B)** For processing samples in 8-strip PCR tubes we recommend using a multi-channel pipette and compatible magnetic rack (*e.g.* EpiCypher 10-0008, 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 and our Tech Support Center also provide guidance on adapting the protocol for unique inputs, targets, and other cell preparation methods.

6.1. CUT&RUN Protocol (~5 hrs)

---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 **CUTANA™ 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 **CUTANA™ Bead Activation Buffer**.
6. Keep activated **ConA beads** on ice until needed.

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

7. For suspension cell cultures, spin cells at 600 x g for 3 min at room temperature (RT). Pipette to remove supernatant, flick tube to loosen pellet, and resuspend cells in 1 mL 1X PBS.

   Note:
   0.01% Digitonin (included in **Digitonin Buffer** and **Antibody Buffer**) is optimal for most cell types tested at EpiCypher (K562, MCF7, A549, NIH3T3, LNCaP), and is recommended for reactions using nuclei. We recommend optimizing Digitonin concentrations for efficient permeabilization. See **Experimental Design & Key Protocol Notes** for suggestions and protocols.

   Notes and alternative cell preparation protocols:
   - 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 this Tech Support Center article).
   - 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 this Tech Support Center article for our cross-linking protocol.

- See FAQs "Sample Input Compatibility" section for special considerations when using adherent, cryopreserved and cross-linked cells, immune cells, and tissue.

8. Transfer 10 µL cells to a fresh tube and mix with 10 µL 0.4% Trypan Blue. Transfer 10 µL of the cell-Trypan Blue mixture to a cell counting slide. Obtain cell counts, determine viability (>80% is ideal), and confirm expected cellular morphology using a brightfield/phase microscope or cell counter. See **Figure 4A**.
9. 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). 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 as in Step 8. Perform these steps in every experiment to ensure robust CUT&RUN sequencing data. Spin cells 600 x g, 3 min, RT. Pipette to remove supernatant. If cells are being lost during spins, increase spin time.

Cell Type	# Reactions	Cells/Reaction	Total Cells*	Vol. Wash Buffer*	Vol. ConA Beads*
K562 cells	8	500,000	4.4 million	924 µL	88 µL
K562 cells	1	500,000	1 million	210 µL	20 µL

Table 4: Preparation of cells for CUT&RUN reactions with extra volume allotted for key Quality Control Checks. ***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 batch processing for user workflows.***

10. 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.
11. Repeat the previous step for total of two washes.
12. Resuspend cells in 105 µL/reaction in RT **Wash Buffer.** Pipette to mix. Perform cell count and evaluate cell integrity as described in step 8. Cells should be unclumped and free of cellular debris, with clear borders (see **Figure 4B**). Total cell counts should be at 500,000 cells per reaction.
13. 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 and quick spin (beads should not settle).
    - 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.

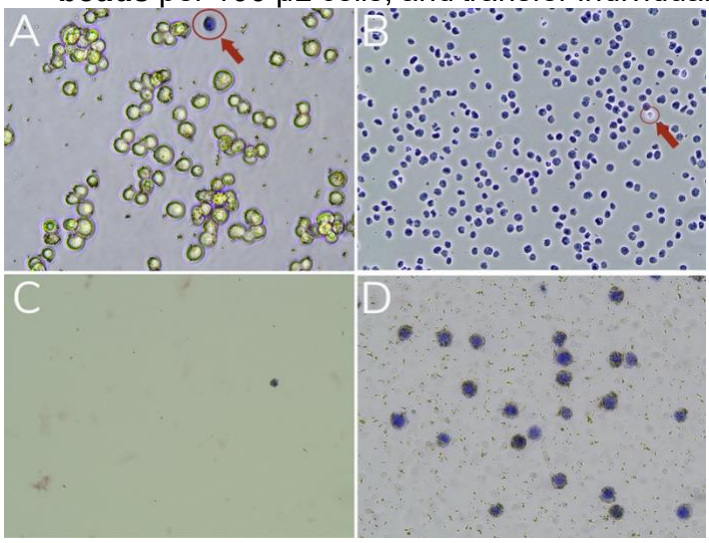

Figure 4: **Representative images of cell and nuclei samples for ConA Bead binding.** Samples were stained with Trypan blue and visualized under brightfield microscope. **(A) Cells** before bead binding. A dead cell (blue; Trypan positive) is circled in red, whereas the remaining live cells are bright 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).

14. **Incubate** cell – bead slurry for **10 min at RT**. Cells will adsorb to the activated ConA beads. Pace tube(s) on magnet and allow slurry to clear (30 s – 2 min). Transfer 10 µL supernatant to a new 1.5 mL tube for QC checks as described in step 8. If bead binding was successful, the supernatant should not contain cells. See **Figure 4C** for expected results.

Samples	Figure	Success Metric (Trypan Blue staining)	Troubleshooting Tips
Cells	Fig. 4A	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. 4B	Nuclei should be >95% Trypan blue positive and unclumped.	See **Appendix I** for a detailed nuclei preparation protocol.
Unbound Fraction	Fig. 4C	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. 4D	Successful ConA bead binding will show Trypan blue positive permeabilized cells/nuclei surrounded by beads.	

Table 5: Quality Control testing of cell/nuclei integrity and ConA bead binding steps.

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

15. Pipette to remove and discard remaining supernatant.
16. 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 (55 µL per reaction). Transfer 10 µL cell – bead slurry to a new 1.5 mL tube to confirm cell permeabilization and binding to ConA beads as described in Step 8. Cells should be blue and surrounded by ConA beads, as in **Figure 4D**.
- Transfer individual reactions (50 µL) into 8-strip PCR tubes**.**
- Continue the remaining steps using an 8-strip PCR tube magnetic rack.

17. For reactions designated for positive control histone PTM (*e.g.* H3K4me3, H3K27me3) 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 6**.
    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.

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

Table 6: 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.***

18. 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. For control reactions using EpiCypher's antibodies, add 1 µL respectively of H3K4me3, H3K27me3, or IgG Control Antibody.
    - 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.
19. **Incubate** 8-strip PCR tubes on nutator **overnight at 4ºC**.
    - 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 5**). DO NOT USE a rotator or turn tubes over end-to-end for this step. Rotation causes ConA beads to stick to tube sides and dry out, reducing yields.

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

Figure 5. 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)

20. Place the 8-strip PCR tubes on a magnet until slurry clears (30 s – 2 min) and pipette to remove and discard supernatant.
21. Keeping tubes on the magnet, add 200 µL cold **Digitonin Buffer** directly onto beads of each reaction, and then pipette to remove supernatant.
22. Repeat the previous step once for total of two washes. Keep tubes + beads on magnet entire time.
23. After second wash, remove supernatant and discard. Add 50 µL 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. Always quick spin after mixing to avoid bead loss.

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

24. Add 2.5 µL **pAG-MNase** (20x stock) to each CUT&RUN reaction, and gently vortex to mix.
    - Critical step: To evenly distribute pAG-MNase, ensure beads are thoroughly resuspended by gentle vortexing and/or pipetting using a P200 pipette (cut-off tip optional).
25. **Incubate** reactions for **10 min at RT.**
26. Quick spin and return 8-strip PCR tubes to magnet, allow slurry to clear (30 s – 2 min). Pipette to remove and discard supernatant.
27. Keeping tubes on the magnet, add 200 µL cold **Digitonin Buffer** directly onto beads of each reaction, and then pipette to remove supernatant.
28. Repeat the previous step for total of two washes, keeping tubes + beads on magnet the entire time.
29. After second wash, remove supernatant and discard. Remove 8-strip PCR tubes from the magnet and add 50 µL 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 (~3.5 hrs)

30. Place 8-strip PCR tubes on ice and add 1 µL **100 mM $CaCl_2$** 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).
31. **Incubate** 8-strip PCR tubes on nutator for **2 hours at 4ºC**, with tubes slightly elevated as in **Figure 5**. Note that this is the key step wherein MNase tethered to antibody-bound chromatin is activated by calcium to cleave target chromatin.
32. Add 33 µL **CUTANA™ Stop Buffer** to each reaction, and gently vortex to mix. This buffer stops MNase enzymatic activity by chelating Ca^{2+} 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** (See Tech Support Center) to the **CUTANA™ 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.
33. **Incubate** 8-strip PCR tubes for **10 min at 37ºC** in a thermocycler. This step releases cleaved chromatin to supernatant and degrades RNA.
34. 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).
35. Transfer supernatant containing CUT&RUN-enriched DNA to new 8-strip PCR tubes. The ConA beads can now be discarded.
36. Purify DNA from supernatant by adding a ratio of **1.8X CUTANA™ DNA Purification Beads** to reaction volume. This ratio captures smaller DNA fragments (<120 bp) generated by some transcription factors.
37. Mix well by pipetting and/or vortexing to an even resuspension (critical for bead binding). Quick spin tubes and incubate 5 min at RT.
38. Place tubes on a magnet for 2-5 min at RT, until solution clears. Pipette to remove supernatant without disturbing beads.
39. Keeping tubes on the magnet, add 180 µL freshly prepared 85% EtOH directly onto beads. Pipette to remove supernatant.
40. Repeat the previous step one time.
41. Remove tubes from magnet. Quick spin to collect liquid, with caps facing in, to avoid dislodging beads. Return to magnet and pipette to remove residual EtOH.
42. Remove tubes from magnet. Air-dry, caps open, 2-3 min at RT. Beads should be damp matte brown. If beads are crackly and/or light brown, they are too dry.
43. Add 17 µL **0.1X TE** to each reaction to elute DNA. Pipette and/or vortex to fully resuspend beads and quick spin. Incubate 2 min at RT.
44. Quick spin tubes and place on magnet for 2 min at RT.

45. Transfer 15 µL CUT&RUN-enriched DNA to new tubes and use 1 µL to quantify the CUT&RUN-enriched DNA using the **Qubit™ fluorometer** per manufacturer's instructions.
    - Note: 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). See this Tech Support Center article on DNA yields for CUT&RUN.
    -
46. **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 this Tech Support Center article for more information.

6.2. Library prep (~4 hrs), Agilent TapeStation® Analysis (~1 hr), & Illumina® Sequencing

Section VI: Library Preparation (~4 hrs)

47. Use ~5 ng purified CUT&RUN-enriched DNA to prepare Illumina NGS libraries using the **CUTANA CUT&RUN Library Prep Kit**.
    - Note 1: The CUTANA Library Prep Kit contains sufficient materials for the preparation of 48 CUT&RUN sequencing libraries. A combinatorial dual indexing primer strategy enables the entire 48-reaction kit to be multiplexed in a single run, if desired.
    - Note 2: ~5 ng input DNA for library prep is recommended for most CUT&RUN targets, but robust and reliable sequencing data have been obtained from less input. We strongly recommend proceeding to library prep regardless of yield, as even very low raw yields can produce adequate libraries for sequencing. See this Tech Support Center article for guidance.
48. Use 1 µL to quantify the purified PCR product using the **Qubit™ fluorometer** as per the manufacturer's instructions.
    - Note: Typical library yield using the CUTANA Library Prep Kit and 5 ng input DNA is ~300-500 ng.

Section VII: Agilent TapeStation® System (~1 hr)

49. For each purified CUT&RUN library, including your IgG negative control, prepare 5 µL at 10 ng/µL for loading on the Agilent TapeStation. The Agilent Bioanalyzer and High Sensitivity DNA Kit can also be used for this step.
    - Record the dilution factor, which will be needed to calculate the library molarity for desired DNA size range (200-700 bp) using TapeStation results.
50. Load samples onto the TapeStation and analyze using the **Agilent D1000 ScreenTape &**

Reagents, as per the manufacturer's instructions.

51. Example TapeStation results for final CUT&RUN libraries are shown in **Figure 6**.
    - Note 1: Confirm that positive control histone PTM antibodies (*e.g.* H3K4me3) predominantly enriched mononucleosome fragments (~300 bp peak with nucleosomes + sequencing adapters). Experimental targets (*e.g.* CTCF) should enrich for fragments of similar size. Adapter dimers, if present, would be observed as a sharp peak at ~125 bp.
    - Note 2: Typical concentration for a CUT&RUN library (200-700 bp region) is 100-200 nM.

Section VIII: Illumina® Sequencing

52. Select appropriate Illumina® sequencing platform (see **Appendix I**) based on the number of CUT&RUN libraries and desired sequencing depth.

    Notes:
    - Paired-end sequencing (2 x 50 cycles minimum) is recommended for CUT&RUN. This ensures accurate alignment to the K-MetStat Panel and provides information on DNA fragment sizes, enabling bioinformatic filtering and DNA footprinting (*e.g.* for TFs).
    - 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.
    - Make sure that each library in a sequencing run has a unique pair of barcodes. Libraries with the same pair of indexes must be sequenced in separate lanes/flow cells.

53. Based on TapeStation molarity calculations (200-700 bp region), pool libraries at the desired ratios.

    General steps:
    - Dilute each library to the same nM concentration, depending on final yields. For NextSeq 2000 and NextSeq 500/550, dilute to 1-4 nM.
    - Pool equimolar libraries into one tube.
    - Dilute library pool to appropriate concentration and in the volume required for Illumina platform. Follow guidelines from specific Illumina kit to load onto sequencer (support.illumina.com) or see examples below.
    - When setting up the NGS run, make sure dual i5 & i7 barcodes are correctly assigned for each library. For a full list of NGS indexes in an easy-to-copy format, see the CUTANA™ Library Prep Multiplexing Primers Excel spreadsheet at epicypher.com/protocols.

Example 1: For 8 libraries, we typically load 0.8 pM (500 µL) into a cartridge for a MiniSeq High Output Reagent Kit, 150-cycles (FC-420-1002). Flow cells using the MiniSeq High Output Kit yield ~25-40 million pass-filter clusters, or ~25-40 million PE reads.

Example 2: For >48 libraries, we typically load 0.8 pM (1,500 µL) into a cartridge for a NextSeq 500/550 High Output Kit v2.5, 150-cycles (20024907). Flow cells using the NextSeq High Output Kit v2.5 yield ~400 million pass-filter clusters, or ~400 million PE reads.

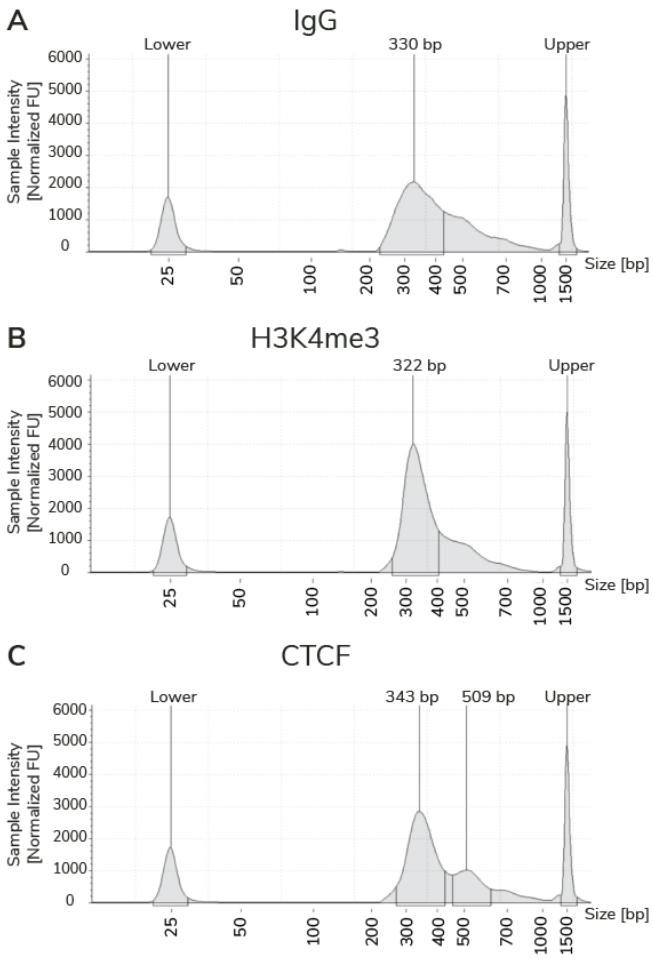

Figure 6: **Typical Agilent TapeStation® traces from CUTANA™ CUT&RUN libraries**. Quality TapeStation (or Bioanalyzer) traces are the best indicator of success prior to sequencing. CUT&RUN was performed in K562 cells using antibodies targeting IgG (EpiCypher 13-0042), H3K4me3 (EpiCypher 13-0041, ; now use 13-0060), and CTCF (EpiCypher 13-2014). Libraries were prepared using the CUTANA Library Prep Kit and ~5 ng CUT&RUN enriched DNA. All CUT&RUN libraries, including IgG negative control reactions, predominantly enriched ~300 bp DNA (reflecting ~170 bp excised DNA + sequencing adapters). Adapter dimers, if present, would be observed as a peak at ~125 bp.

54. We recommend using EpiCypher's CUTANA™ Cloud (cloud.epicypher.com), an online data analysis platform for streamlined CUT&RUN and CUT&Tag bioinformatics with built-in pipelines for SNAP-CUTANA Spike-in analyses. Visit epicypher.com/cutana-cloud to learn more, and see our Tech Support Center for additional FAQs and troubleshooting guidance.

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?**

   Fragment distribution analysis of purified libraries on TapeStation or Bioanalyzer. Libraries should show enrichment of mononucleosome-sized DNA fragments (~300 bp, including CUT&RUN DNA + sequencing adapters).

   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. 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.).

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

   Do **NOT** assess fragment size distribution of raw CUT&RUN DNA before library prep. Yields are too low for detection on Bioanalyzer/TapeStation and **will not provide useful information at this step**. Wait until after library prep.

3. **Is there a technical resource guide for CUT&RUN library prep?**
   For questions about the **CUTANA™ CUT&RUN Library Prep Kit** (EpiCypher 14-1001 & 14-1002), please visit the Tech Support Center. It contains detailed information about our multiplexing strategy, ideal input amounts, and how to avoid adapter dimer contamination.

4. **What is the recommended sequencing depth and read length?**
   Paired-end sequencing (2 x 50 bp minimum) is recommended for CUT&RUN. Libraries should be sequenced to a depth of 3-8 million uniquely aligned reads (5-10 million total reads).

5. **What are standard CUT&RUN sequencing metrics?**
   The majority of reads (>80%) should align uniquely to the species genome. Sequence duplication levels should be low (<20% of total sequencing reads).

7.2 Spike-in Controls

6. **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 uniquely aligned 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 (See Tech Support Center articles on optimizing *E.coli* Spike-in DNA and normalizing CUT&RUN data using *E.coli* Spike-in DNA).

7. **Does EpiCypher offer spike-in nucleosome controls for CUT&RUN?**
   EpiCypher 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 for ordering information and to download the **SNAP-CUTANA Spike-in User Guide**, which contains detailed instructions on their application in CUT&RUN/CUT&Tag assays and how to analyze spike-in data from NGS results.

7.3 Sample Input Compatibility

8. **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[5-8], FACS isolated cells[9], and immune cells[10,11]. See the Tech Support Center section for sample prep recommendations.

9. **Is CUT&RUN compatible with frozen or cross-linked cell preparations?**
   Yes. General guidelines and protocols working with frozen or cross-linked cells can be found in the Tech Support Center section.

7.4 Antibodies and Targets

10. **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 SP1, JUN/c-Jun, 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; for targets not yet on this list, contact info@epicypher.com for recommendations.

11. 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. see our Tech Support Center for more guidelines for antibody selection.

12. Does EpiCypher offer bioinformatic assistance for CUT&RUN data analysis?

Yes! EpiCypher now supports streamlined bioinformatic analysis of CUT&RUN and CUT&Tag data with CUTANA™ Cloud (cloud.epicypher.com), a fast, secure, user-friendly cloud computing platform to help researchers rapidly unlock insights from their data. CUTANA Cloud automates genomic alignment, SNAP-CUTANA Spike-in analysis, and compilation of all necessary QC statistics to determine experimental success. Visit epicypher.com/cutana-cloud to learn more, and see Tech Support Center for additional FAQs and troubleshooting guidance.

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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.
CUTANA™ Protease Inhibitor Tablets	EpiCypher	21-1027
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_2O. Store in single-use aliquots at -20°C for 6 months.

Nuclear Extraction (NE) Buffer
This recipe can be used in place of CUTANA™ Nuclei Extraction Buffer (EpiCypher 21-1026)

NE Buffer base:
20 mM HEPES, pH 7.9
10 mM KCl
0.1% Triton X-100
20% Glycerol
Filter sterilize. Store at 4ºC for up to 6 months.

Add to NE Buffer on day of experiment:
1X CUTANA™ Protease Inhibitor
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

1. 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).
2. Resuspend cells in 100 µL/reaction RT **1X PBS**.
   - Note: Set 10 µL aside for confirmation of cell integrity.

3. Spin for 3 min at 600 x g at RT. Decant or pipette to remove and discard supernatant.
4. Resuspend cell pellet in 100 µL/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 105 µL/reaction cold **Wash Buffer**.
8. Freeze (see below) or proceed to ConA bead conjugation step (**Section II Step 13**). Nuclei in NE Buffer can be directly added to activated ConA beads.
    - Note: Set 10 µL aside for confirmation of isolated nuclei integrity

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, mix, and load onto cell counter or hemacytometer slide.
3. Examine under brightfield or phase microscope (**Figure 4**). 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 13**. 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: Alternative buffer recipes

Bead Activation Buffer

This recipe can be used in place of CUTANA™ Bead Activation Buffer (EpiCypher 21-1001)

20 mM HEPES, pH 7.9
10 mM KCl
1 mM $CaCl_2$
1 mM $MnCl_2$
Filter sterilize. Store at 4ºC for up to 6 months.

Stop Buffer

This recipe can be used in place of CUTANA™ Stop Buffer (EpiCypher 21-1003)

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

Components	Source	Cat #
$CaCl_2$	Sigma-Aldrich	C1016
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
Glycogen	Sigma-Aldrich (Roche)	10930193001
HEPES	Sigma-Aldrich	H3375
KCl	Sigma-Aldrich	P3911
$MnCl_2$	Sigma-Aldrich	203734
Molecular biology grade H_2O (RNase, DNase free)	VWR	VWRV02-0201-0500
NaCl	Sigma-Aldrich	S5150-1L
RNase A	Thermo Fisher Scientific	EN0531

Table 7: Alternative buffer components