

EpiCypher®

Bringing Epigenetics to Life

CUTANA™

ChIC / CUT&RUN Kit Version 3

User Manual Version 3.3

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CUTANATM

ChIC / CUT&RUN Kit

Kit Version 3

Catalog No. 14-1048

48 ChIC / CUT&RUN Reactions

**Upon receipt, store indicated components
at 4°C, -20°C and room temperature (RT)**

Stable for 6 months upon date of receipt.

See p. 8-9 for storage instructions.

Table of Contents

Background & Product Description	5
Included in the Kit	8
Materials Required but Not Supplied	10
Outline of Workflow	11
Experimental Design & Optimization	12
Experimental Protocol: Day 1	14
Section I: CUT&RUN Buffer Prep (~30 min)	14
Section II: Bead Activation (~30 min)	15
Section III: Binding Cells to Activated Beads (~30 min)	16
Section IV: Antibody Binding (~30 min + overnight)	17
Experimental Protocol: Day 2	18
Section V: Binding of pAG-MNase (~40 min)	18
Section VI: Targeted Chromatin Digestion and Release (~3 hrs)	18
Section VII: DNA Purification (~30 min)	19
Experimental Protocol: Library Prep and NGS	21
Section VIII: NGS Library Prep (~4 hrs)	21
Section IX: Analysis of Library Fragment Size (~1 hr)	21
Section X: Illumina® Sequencing	23
Section XI: Data Analysis	23
Appendix 1: Quality Control Checks & Troubleshooting	24
1.1. Optimization of Cell Permeabilization	24
1.2. Quality Control Checks for Sample Prep	26
1.3. Quality Control Checks After CUT&RUN	28
1.4. Quality Control Checks for Sequencing Libraries	29
1.5. Quality Control Checks After Sequencing	30
1.6. SNAP-CUTANA™ K-MetStat Panel	32
Appendix 2: Experimental Normalization Using E. coli Spike-in DNA	38
Appendix 3: Protocol Variations	39
Appendix 4: Frequently Asked Questions (FAQs)	44
References	50

Cleavage Under Targets & Release Using Nuclease (CUT&RUN) is a revolutionary genomic mapping strategy developed by the group of Dr. Steven Henikoff¹. It builds on Chromatin ImmunoCleavage (ChIC) from Dr. Ulrich Laemmli², wherein a fusion of Protein A to Micrococcal Nuclease (pA-MNase) is recruited to selectively cleave antibody-bound chromatin *in situ*³. In CUT&RUN, cells or nuclei are immobilized to a solid support, with pAG-MNase cleaved DNA fragments isolated from solution. The workflow is compatible with next-generation sequencing (NGS) to provide high quality genome-wide profiles of histone post-translational modifications (PTMs) and chromatin-associated proteins (e.g. transcription factors; [Figure 1](#)).

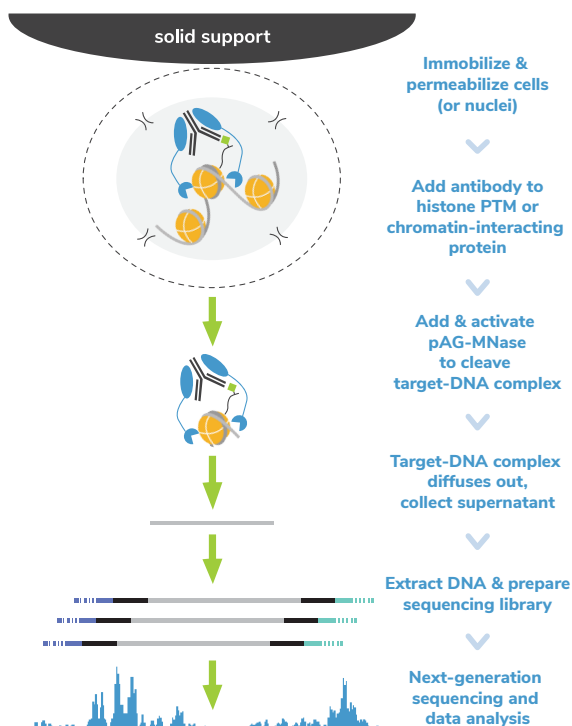


FIGURE 1

Overview of the CUTANA™ CUT&RUN protocol.

Historically, ChIP-seq is the leading approach for genome-wide mapping of histone PTMs and chromatin-associated proteins. In this approach, bulk chromatin is fragmented by sonication or enzymatic digestion. Target-specific fragments are then immunoprecipitated. Despite extensive optimization and stringent wash conditions, ChIP-seq requires large numbers of cells (typically $10^5 - 10^6$ cells) and deep sequencing of both input chromatin and immunoprecipitated material (typically >30 million reads each) to resolve signal from background.

ChIC and CUT&RUN have revolutionized the study of chromatin regulation by enabling targeted release of genomic fragments into solution. With this innovation, background is dramatically reduced, allowing high-resolution genomic mapping for histone PTMs and chromatin-associated proteins using a small number of cells and only 3-8 million sequencing reads per reaction (Figure 2). The streamlined workflow and cost savings make ChIC/CUT&RUN amenable to greater experimental throughput, allowing deeper and more rapid investigations to uncover epigenetic biology.

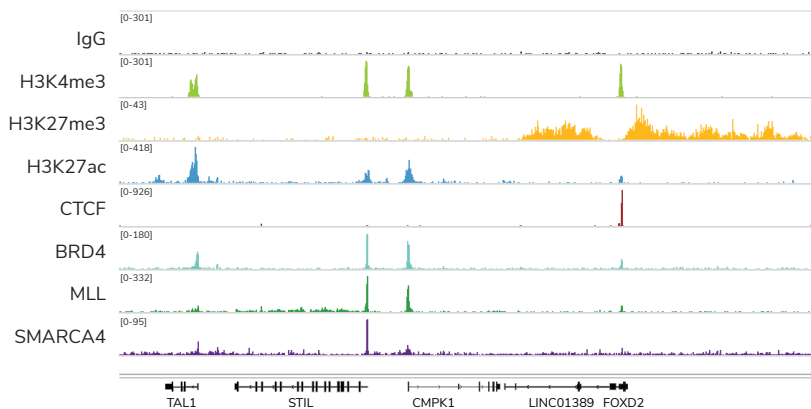


FIGURE 2

Representative genome browser tracks show CUTANAT[™] CUT&RUN results using 500,000 K562 cells. Clear peaks with the expected distribution profile are observed using 3-8 million sequencing reads per reaction for a variety of epigenetic targets, including histone PTMs (H3K4me3, H3K27me3, H3K27ac), transcription factors (CTCF), epigenetic reader proteins (BRD4), writer enzymes (MLL1), and chromatin remodelers (SMARCA4). Rabbit IgG antibody shown as a negative control (top track).

The CUTANA™ ChIC/CUT&RUN Kit contains materials for 48 reactions and is designed for multi-channel pipetting to realize the increased throughput advantage of CUT&RUN. The kit includes positive (H3K4me3) and negative (Rabbit IgG) control antibodies, and an aliquot of the SNAP-CUTANA™ K-MetStat Panel (16 DNA-barcoded designer nucleosomes carrying widely-studied lysine methylation PTMs). The K-MetStat Panel is spiked into control reactions to directly monitor experimental success and aid troubleshooting. Additionally, sheared *E. coli* DNA is added to all reactions after pAG-MNase cleavage to control for library prep and enable NGS normalization. The kit is compatible with cells and nuclei, including cryopreserved and cross-linked samples (Figure 3). Although it is recommended to start with 500,000 cells, comparable data can be generated using as few as 5,000 cells (Figure 4). The inclusion of rigorous controls as well as compatibility with diverse target types, sample inputs, and cell numbers make the kit ideal for a variety of research applications.

FIGURE 3

Heatmaps show CUT&RUN signal (red) and background (blue) of H3K4me3-enriched regions flanking annotated transcription start sites (TSS, +/- 2 kb). Gene rows are aligned across conditions, showing that genome-wide enrichment is preserved across sample types.

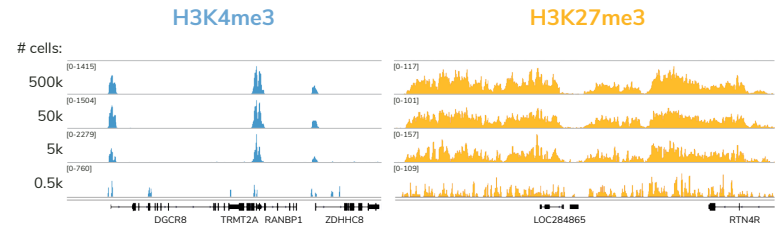
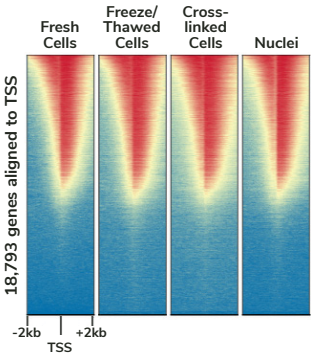


FIGURE 4

Representative genome browser tracks for H3K4me3 (low abundance target) and H3K27me3 (high abundance target) CUT&RUN experiments using decreasing amounts of K562 cells. At 5,000 cells, data quality is largely indistinguishable from standard conditions (500,000 cells).

Included in the Kit

Kit components are stable for 6 months upon date of receipt. Store as outlined below.

Store at room temperature (RT) upon receipt:

Item	Catalog No.	Notes before use
8-strip Tubes	10-0009k	Enables use of multi-channel pipettors and magnetic racks (e.g. EpiCypher 10-0008).
DNA Cleanup Columns	10-0010	Use with the DNA Collection Tubes.
DNA Collection Tubes	10-0011	Use with the DNA Cleanup Columns.
0.5 M EDTA	21-1006	250X concentration. Use to prepare Antibody Buffer FRESH for each experiment.
100 mM Calcium Chloride	21-1007	Activates chromatin-tethered pAG-MNase to cleave DNA.
DNA Binding Buffer	21-1008	Before first use, add 6.9 mL isopropanol. WARNING: Contains toxic ingredients (see Safety Data Sheet).
DNA Wash Buffer	21-1009	Before first use, add 20 mL of $\geq 95\%$ ethanol.
DNA Elution Buffer	21-1010	Use to elute final CUT&RUN DNA.

Store at 4°C upon receipt:

Item	Catalog No.	Notes before use
ConA Beads	21-1401	DO NOT FREEZE. Concanavalin A (ConA) beads are used for immobilizing cells or nuclei. For immune cell studies it is recommended to use nuclei, since ConA can cause immune cell activation (see Appendix 3.1).
E. coli Spike-in DNA	18-1401	100 ng lyophilized E. coli DNA. Added to reactions for sequencing normalization. Before first use, quick spin and reconstitute in 200 μ L DNase-free water (0.5 ng/ μ L). NOTE: After reconstitution, store at -20°C .
Bead Activation Buffer	21-1001	Use to prepare ConA beads prior to sample immobilization.
Pre-Wash Buffer	21-1002	Use to prepare Wash, Cell Permeabilization, and Antibody Buffers FRESH for each experiment.
Stop Buffer	21-1003	3X concentration. Use to terminate pAG-MNase cleavage activity.

Store at -20°C upon receipt:

Item	Catalog No.	Notes before use
5% Digitonin	21-1004	Thaw at RT. Use to prepare Cell Permeabilization and Antibody Buffers FRESH for each experiment. Final Digitonin concentration should be optimized for each sample type; see Appendix 1.1 .
1 M Spermidine	21-1005	2,000X concentration. Use to prepare Wash Buffer FRESH for each experiment.
SNAP-CUTANA™ K-MetStat Panel	19-1002k	SMALL VOLUME: quick spin before use. Pipette to resuspend beads; DO NOT VORTEX. Panel of biotinylated designer nucleosomes coupled to streptavidin-coated magnetic beads. Use as a spike-in control with H3K4me3 and IgG control antibodies. Sufficient volume for 20 reactions. See Appendix 1.6 for additional background info.
H3K4me3 Positive Control Antibody	13-0041k	SMALL VOLUME: quick spin before use. Rabbit mixed monoclonal antibody, 0.5 mg/mL stock. Add 1 µL to positive control reactions. Sufficient volume for 10 reactions.
Rabbit IgG Negative Control Antibody	13-0042k	SMALL VOLUME: quick spin before use. 0.5 mg/mL stock. Add 1 µL to negative control reactions. Sufficient volume for 10 reactions.
pAG-MNase	15-1016	20X concentration. Proteins A and G (pAG) bind antibodies of various isotypes and host species, including total IgG for rabbit, mouse, human, goat, donkey, rat, guinea pig, horse, and cow.

Materials Required but Not Supplied

REAGENTS:

- Antibody to target of interest (user-dependent). See **FAQs** for more information.
 - * EpiCypher continues to conduct extensive investigations of antibody performance⁴. Visit epicypher.com/cut-and-run-antibodies to browse our validated CUT&RUN antibodies.
- Protease inhibitor (e.g. cOmplete™, EDTA-free Protease Inhibitor Cocktail, Roche 11873580001)
- 0.4% Trypan blue (e.g. Invitrogen T10282)
- Isopropanol
- Molecular biology grade water, any vendor
- CUTANA™ CUT&RUN Library Prep Kit, 48 reactions (EpiCypher 14-1001 & 14-1002)
 - * The two versions of this kit contain distinct primer sets, allowing up to 96 CUT&RUN libraries to be multiplexed when kits are used together.

EQUIPMENT:

- 1.5, 15 and 50 mL tubes
- Magnetic separation rack for 1.5 mL tubes (e.g. EpiCypher 10-0012) and 8-strip Tubes (e.g. EpiCypher 10-0008)
- Qubit™ 4 Fluorometer (Invitrogen Q33238) and 1X dsDNA HS Kit (Q33230)
- Capillary electrophoresis machine and required reagents (e.g. Agilent TapeStation® and D1000 ScreenTape 5067-5582 & D100 Reagents 5067-5583, or Agilent Bioanalyzer® and High Sensitivity DNA Analysis Kit 5067-4626)
- 8-channel multi-channel pipettor (e.g. VWR 76169-250) and multi-channel reagent reservoirs (e.g. Thermo Fisher Scientific 14-387-072)
- Vortex (e.g. Vortex-Genie®, Scientific Industries SI-0236)
- Thermocycler (e.g. from BioRad, Applied Biosystems, Eppendorf)
- Tube nutator for incubation steps (e.g. VWR 82007-202)
 - * It is critical to use a **nutator** rather than a rotator to keep liquid in tube conical bottom and avoid bead drying.

Here, we review the main steps of the CUTANATM CUT&RUN assay:

Step 1: Isolate cells & immobilize to concanavalin A (ConA) beads

High quality sample prep is essential to CUT&RUN workflows. Here, cells are washed and bound to activated ConA Beads. Quality control checks in **Appendix 1.2** are used to confirm cell viability and binding to ConA Beads. Avoid bead dry-out and clumping during the assay, which results in sample loss and reduces yields.

Step 2: Permeabilize cells & incubate with target-specific antibody

The bead-cell mixture is resuspended in cold Antibody Buffer and a target-specific antibody is added for overnight incubation at 4°C. Permeabilization is required for antibody binding *in situ*, and is achieved by addition of Digitonin to assay buffers. Digitonin concentrations must be optimized for each cell type (**Appendix 1.1**).

Selection of an antibody with high target specificity and efficiency is also crucial to CUT&RUN assay success. See **Experimental Design & Optimization** and **FAQs** for guidance on antibody testing and target compatibility.

Step 3: Perform targeted chromatin digestion & release

The next day, reactions are washed several times to remove unbound antibody. pAG-MNase is added and binds antibody-labeled chromatin via the immunoglobulin binding properties of Protein A/G. Addition of calcium activates MNase, which cleaves DNA proximal to where the antibody is bound.

Clipped fragments diffuse into the supernatant, while bulk chromatin remains in bead-immobilized cells. A Stop Buffer containing EDTA is added to chelate free calcium and halt MNase activity.

Step 4: Purify DNA & prepare sequencing libraries

Bead-bound cells are removed using a magnet, and CUT&RUN-enriched DNA is purified from the supernatant using a column-based clean up kit optimized for small fragments. Total DNA yields are quantified using the Qubit Fluorometer (**Appendix 1.3**).

CUT&RUN DNA is prepared for Illumina® sequencing using the CUTANATM CUT&RUN Library Prep Kit (or equivalent). Purified libraries are examined using the Bioanalyzer or TapeStation to determine library concentration and fragment size distribution (see **Experimental Protocol, Section IX** and **Appendix 1.4**).

Step 5: Illumina® Next-Generation Sequencing (NGS)

Libraries are diluted, pooled, and sequenced on an Illumina sequencing platform. Only 3-8 million uniquely aligned sequencing reads are needed to generate robust CUT&RUN profiles. See **Appendix 1.5** for expected results.

The CUTANA™ CUT&RUN kit is validated for robust profiling of:

- Histone PTMs (e.g. lysine methylation, acetylation, and ubiquitylation)
- Transcription factors (e.g. CTCF, FOXA1, GATA3)
- Chromatin remodelers (e.g. ATPase subunits of SWI/SNF, ISWI, INO80, CHD)
- Chromatin writers & readers (e.g. MLL1, BRD4)
- Epitope-tagged proteins (e.g. HA, FLAG tags)

SAMPLE INPUTS FOR CUT&RUN

- Freshly isolated, unfixed (i.e. native) cells are the preferred input for CUT&RUN.
- 500,000 cells per reaction is recommended.
- **Appendix 3** contains methods and recommendations for:
 - Harvesting adherent cells & tissues
 - Nuclei isolation, quality analysis, and freeze-thaw methods
 - Cross-linking cells
 - Freeze-thawing cells

ANTIBODY SELECTION

- Use a highly specific and efficient antibody that has been validated in CUT&RUN.
- Antibodies that work well in ChIP-seq are **NOT** guaranteed success in CUT&RUN.
- EpiCypher offers a collection of CUTANA™ CUT&RUN validated antibodies to various histone PTM and protein targets (epicypher.com/cut-and-run-antibodies).
- See **FAQs** for additional guidance regarding antibody sourcing and validation.

EXPERIMENTAL CONTROLS

- **Quality Control Checks** are noted throughout the protocol to support assay optimization and enable continuous performance monitoring ([Figure 5](#)).
- Each kit comes with H3K4me3 positive and IgG negative control antibodies and the SNAP-CUTANA™ K-MetStat Panel of spike-in controls. Control reactions spiked with the K-MetStat Panel should be included in **every** experiment to determine assay success and aid troubleshooting. See **Appendix 1.6** for details.
- The provided **E. coli Spike-in DNA** can be added to any CUT&RUN reaction for sequencing normalization (**Appendix 2**).

HOW TO OPTIMIZE CUT&RUN ASSAYS

1. **Before** starting CUT&RUN experiments, optimize Digitonin permeabilization conditions for each cell type. See **Appendix 1.1** for instructions.
2. Validate the CUT&RUN protocol for desired cell type. Use 500,000 native cells per reaction and control antibodies with the **K-MetStat Panel** of spike-ins.
3. Optimize CUT&RUN for experimental conditions (e.g. cross-linking) and target(s) using 500,000 cells per reaction. Antibody should generate robust, target-specific profiles.
4. (Optional) Scale down to desired number of cells per reaction. **CUTANA CUT&RUN has been validated on as few as 5,000 K562 cells.**

* Compatibility with low inputs depends on antibody quality, target abundance, and other factors. Additional optimization may be required; see **Appendix 1.3 & 1.4** and **FAQs**.

Confirm sample prep	
✓ Cell permeabilization	✓ Cell viability & number
✓ Binding to ConA beads	
Key CUT&RUN experimental controls	
✓ Positive control antibody (H3K4me3)	
✓ Negative control antibody (IgG)	
✓ SNAP-CUTANA™ K-MetStat Panel of spike-ins	
✓ E. coli spike-in DNA	
Examine CUT&RUN enriched DNA	
✓ Determine DNA concentration	
✓ Generally, yields from positive control \geq negative control	
Assess quality of NGS library	
✓ Confirm fragment distribution	✓ Examine yields
Sequencing and data analysis	
Control reactions with spike-ins:	
✓ Show expected profiles & validate workflows	
Experimental targets:	
✓ 3-8 million reads	
✓ Use reads from E.coli spike-in DNA to normalize data	
✓ High signal-to-noise & expected peaks (e.g. promoters)	

FIGURE 5

The CUTANA™ CUT&RUN Kit comes with multiple controls to help ensure success.

Experimental Protocol: Day 1

SECTION I: CUT&RUN BUFFER PREP (~30 MIN)

Important notes on buffer prep

- * These buffers need to be prepared **FRESH** on Day 1 of the experiment (Figure 6).
- * Volumes are per CUT&RUN reaction and include overfill to account for pipetting errors. See Table 1 to scale buffer recipes for multiple reactions.
- * 0.01% Digitonin is optimal for permeabilizing K562, MCF7, and A549 cells (Table 1). For other cell types, optimize conditions as described in Appendix 1.1.

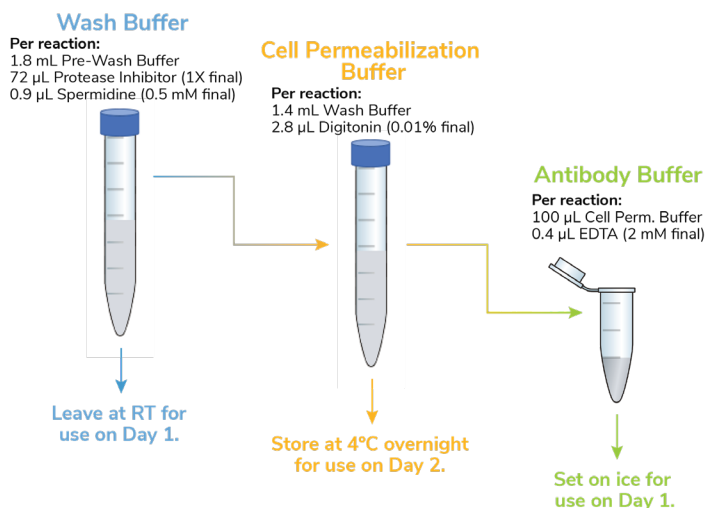


FIGURE 6

Schematic of buffers to be prepared fresh the day of use. RT, room temperature.

1. Gather kit reagents needed for Day 1: **0.5 M EDTA, ConA Beads, Bead Activation Buffer, Pre-Wash Buffer, 5% Digitonin, 1 M Spermidine, H3K4me3 and IgG Control Antibodies, K-MetStat Panel**. Place on ice to thaw/equilibrate.
2. Add 1.8 mL Pre-Wash Buffer per reaction to a conical tube labeled **Wash Buffer**.
3. Dissolve 1 protease inhibitor tablet (Roche) in 2 mL water (25X stock). Add 72 μ L per reaction to the **Wash Buffer**. Store remaining stock 12 weeks at -20°C.
4. Dilute **1 M Spermidine 1:2,000** in **Wash Buffer**. Store final buffer at RT.

5. In a new tube labeled **Cell Permeabilization Buffer**, transfer 1.4 mL **Wash Buffer** per reaction. Add **5% Digitonin** as optimized for your cell type ([notes](#), pg 14).
6. Transfer 100 μ L **Cell Permeabilization Buffer** per reaction to a new tube labeled **Antibody Buffer**. Add **0.5M EDTA** (1:250 dilution). Store final buffer on ice.
7. Store remaining **Cell Permeabilization Buffer** at 4°C for use on Day 2.

Buffer Sample Scaling Calculations:

COMPONENT	[FINAL]	1X	8X	16X
Wash Buffer - store at room temperature (RT) for use on Day 1				
Pre-Wash Buffer	-	1.8 mL	14.4 mL	28.8 mL
25X Protease Inhibitor	1X	72 μ L	576 μ L	1.15 mL
1 M Spermidine	0.5 mM	0.9 μ L	7.2 μ L	14.4 μ L
Cell Permeabilization Buffer - store at 4°C for use on Day 2				
Wash Buffer	-	1.4 mL	11.2 mL	22.4 mL
5% Digitonin	0.01%	2.8 μ L	22.4 μ L	44.8 μ L
Antibody Buffer - store on ice for use on Day 1				
Cell Permeabilization Buffer	-	100 μ L	800 μ L	1.6 mL
0.5 M EDTA	2 mM	0.4 μ L	3.2 μ L	6.4 μ L

TABLE 1

Buffer recipes for CUT&RUN. Includes extra volume to account for pipetting error.

SECTION II: BEAD ACTIVATION (~30 MIN)

Tips for working with magnetic ConA Beads

- * ConA Beads should be brown. Do **NOT** use black ConA Beads (indicates damage).
 - * Do **NOT** let ConA Beads dry out - this will lead to loss of sample and reduced yields.
 - * Avoid disturbing ConA Beads with pipette tips when on magnet.
8. Gently resuspend **ConA Beads** and transfer 11 μ L per reaction to a 1.5 mL tube.
 9. Place tube on a magnet and wait for slurry to clear. Pipette to remove supernatant.
 10. Immediately add 100 μ L per reaction cold **Bead Activation Buffer**. Remove tube from magnet and gently pipette to mix. Return tube to magnet and wait for slurry to clear. Pipette to remove supernatant.

SECTION II: BEAD ACTIVATION (~30 MIN) *continued*

11. Repeat the previous step one time.
12. Immediately add 11 μL per reaction cold **Bead Activation Buffer**. Remove tube from magnet, and gently pipette to mix.
13. Aliquot 10 μL activated beads per reaction into **8-strip Tubes**. Place on ice.

SECTION III: BINDING CELLS TO ACTIVATED BEADS (~30 MIN)

Guidelines for successful sample prep

- * For sample inputs other than native suspension cells (e.g. adherent cells, frozen or cross-linked cells, tissues, nuclei) see **Appendix 3**.
 - * Confirm cell viability/integrity and binding to ConA Beads as detailed in **Appendix 1.2**. EpiCypher often prepares one extra reaction for quality control checks.
 - * Once cells are added, ConA Beads are prone to clumping and may form a film on the side of the tube. Frequently (but gently) vortex or pipette to an even suspension.
14. Harvest 500,000 cells per reaction in a 1.5 mL tube. Spin cells at $600 \times g$ for 3 min at room temperature (**RT**). Pipette to remove supernatant.
 15. Resuspend cells in 100 μL per reaction **RT Wash Buffer** by gentle yet thorough pipetting. Spin at $600 \times g$, 3 min, **RT**. Pipette to remove supernatant.
 16. Repeat the previous step one time.
 17. Resuspend cells in 105 μL per reaction **RT Wash Buffer** by pipetting. Assess viability and integrity of prepared cells as in **Appendix 1.2**.
 18. Aliquot 100 μL cells into 8-strip tubes containing 10 μL activated ConA Beads. Gently vortex to mix. Perform a quick spin in a benchtop mini-centrifuge to collect beads from sides of tubes (beads should not settle).
 19. Incubate bead-cell slurry for 10 min at **RT**. Cells will adsorb to beads.
 20. Place tubes on a compatible magnet and wait for slurry to clear. Take a 10 μL aliquot of supernatant to evaluate bead binding as in **Appendix 1.2**.
 21. Pipette to remove remaining supernatant. Immediately add 50 μL cold **Antibody Buffer** to each reaction (avoid drying out ConA Beads).
 22. Remove tubes from magnet and gently vortex to an even resuspension. Confirm binding to ConA Beads as in **Appendix 1.2**.

SECTION IV: ANTIBODY BINDING (~30 MIN + OVERNIGHT)

23. Add the SNAP-CUTANA™ K-MetStat Panel to CUT&RUN reactions designated for the H3K4me3 Positive and IgG Negative Control Antibodies:
- Quick spin the **K-MetStat Panel**. Gently mix by pipetting (do **NOT** vortex).
 - Add 2 µL of the **K-MetStat Panel** per 500,000 cells. See [Table 2](#).
 - Gently vortex CUT&RUN reactions to mix.

Number of cells	Working stock dilution	Volume added to reaction	Final dilution
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 2

For fewer than 500,000 cells, decrease the amount of K-MetStat Panel linearly by preparing a working stock dilution of the panel in **Antibody Buffer**. The working stock should be prepared **FRESH** the day of the experiment and discarded after use. Starting recommendations are provided, but optimization may be required. See the SNAP-CUTANA™ Spike-in User Guide for information (epicypher.com/19-1002 under Documents and Resources).

24. Add primary antibody to each reaction as recommended by the manufacturer. If no guidance is provided, use 0.5 µg per reaction. **H3K4me3 Positive and IgG Negative Control Antibodies** are provided at 0.5 mg/mL; add 1 µL to designated reactions. Gently vortex all reactions to mix.
- * Antibodies stored in glycerol may be viscous. Flick stock tube to mix. When adding to reactions check tip for accuracy and pipette up and down to clear solution from tip.
25. Incubate reactions overnight on a nutator at 4°C. Prop tubes at a slight angle to elevate caps, keeping ConA Beads in the bottom of the tube.
- * **DO NOT ROTATE TUBES END OVER END** during incubations. Rotation causes ConA Beads to stick to the sides of tubes and dry out, reducing yield.
26. Store the **Cell Permeabilization Buffer** at 4°C overnight for use on Day 2.

SECTION V: BINDING OF PAG-MNASE (~40 MIN)

Guidelines for high-throughput processing

- * Multi-channel pipetting is recommended through the rest of the experiment. This helps avoid bead dry out, improves yield, and increases experimental throughput.
 - * To easily dispense assay buffers, use multi-channel reagent reservoirs (keep on ice).
 - * If processing >8 reactions (multiple 8-strip tubes), remove & replace buffer for a single strip before processing the next to avoid drying out beads during wash steps.
27. Remove tubes from 4°C incubation and quick spin to collect liquid. Note that beads may settle overnight and become clumpy at this stage of the protocol.
 28. Place tubes on magnet until slurry clears. Pipette to remove supernatant.
 29. Keeping tubes on magnet, add 200 µL cold **Cell Permeabilization Buffer** directly onto beads. Pipette to remove supernatant. Repeat one time.
 30. Add 50 µL cold **Cell Permeabilization Buffer** to each reaction. Remove tubes from magnet and gently vortex and/or pipette with a P200 to mix. Slightly cut-off pipette tips may be used to disperse clumps and/or preserve delicate cells.
 31. Add 2.5 µL **pAG-MNase** to each reaction. Gently vortex and/or pipette to thoroughly mix beads and evenly distribute enzyme.
 32. Incubate reactions for 10 min at RT.
 33. Quick spin to collect liquid. Place tubes on magnet until slurry clears and pipette to remove supernatant.
 34. Keeping tubes on magnet, add 200 µL cold **Cell Permeabilization Buffer** directly onto beads. Pipette to remove supernatant. Repeat one time.
 35. Add 50 µL cold **Cell Permeabilization Buffer** to each reaction and remove tubes from magnet. Gently vortex to mix and disperse clumps by pipetting.

SECTION VI: TARGETED CHROMATIN DIGESTION AND RELEASE (~3 HRS)

36. Place tubes on ice. Add 1 µL **100 mM Calcium Chloride** to each reaction. Gently vortex and/or pipette to evenly resuspend beads and ensure efficient digestion.
37. Incubate tubes on nutator (capped ends elevated) for 2 hours at 4°C.
 - * Set a thermocycler to 37°C for Step 40.

38. Prepare a **Stop Master Mix** by adding **E. coli Spike-in DNA** to **Stop Buffer**:
- Prior to first use, quick spin lyophilized DNA and reconstitute in 200 μ L DNase-free water. Thoroughly vortex to fully resuspend. Store at -20°C .
 - For the master mix, add 1 μ L (0.5 ng) **E. coli Spike-in DNA** to 33 μ L **Stop Buffer** per CUT&RUN reaction. Pipette or gently vortex to mix.
 - * Aim for **E. coli Spike-in DNA** to comprise 0.5 – 5% (ideally ~1%) total sequencing reads. This may require further optimization; see **Appendix 2**.
39. At the end of the 2 hour incubation, quick spin tubes to collect liquid. Add 33 μ L **Stop Master Mix** to each reaction and gently vortex to mix.
40. Place reactions in a thermocycler set to 37°C . Incubate for 10 min.
41. Quick spin tubes to collect liquid, and place on a magnet until slurry clears.
42. Transfer supernatants containing CUT&RUN enriched DNA to 1.5 mL tubes.

SECTION VII: DNA PURIFICATION (~30 MIN)

Notes before starting

- * Prior to first use, add 6.9 mL isopropanol to **DNA Binding Buffer**.
 - * Prior to first use, add 20 mL $\geq 95\%$ ethanol to **Wash Buffer**.
 - * The **DNA Cleanup Columns** retain fragments >50 bp. For enrichment of smaller fragments for footprinting analysis, see **FAQs**.
 - * A vacuum manifold can be used in place of centrifugation. For each step, add the indicated buffer, turn the vacuum on, and allow the solution to pass through the column before turning the vacuum off.
43. Add 420 μ L **DNA Binding Buffer** to each reaction. Mix well by vortexing.
44. For every CUT&RUN reaction, place a **DNA Cleanup Column** into a **DNA Collection Tube**. Load each reaction onto a column and label the top.
45. Spin at $16,000 \times g$, 30 sec, RT. Discard flow-through. Place column back into collection tube.
46. Add 200 μ L **DNA Wash Buffer** to each column. Spin at $16,000 \times g$, 30 sec, RT. Discard flow-through. Place column back into collection tube.
47. Repeat previous step one time.

SECTION VII: DNA PURIFICATION (~30 MIN) *continued*

48. Spin at 16,000 x g, 30 sec, RT to completely dry the column.
49. Carefully remove the column from the collection tube, ensuring it does not contact the flow-through. Transfer column to a clean pre-labeled 1.5 mL tube.
50. Add 12 μ L **DNA Elution Buffer**, taking care to ensure the buffer is added to the center of the column rather than the wall. Tap the column & microfuge collection tube on the benchtop to ensure all droplets are absorbed onto the resin.
 - * DNA can be eluted in 6–20 μ L; 12 μ L is recommended. Larger elution volumes, longer incubation times, and/or multiple rounds of elution may improve DNA yield. However, DNA concentration will be reduced with larger elution volume.
51. Let sit 5 minutes, then spin at 16,000 x g, 1 min, RT.
52. Vortex eluted material to mix. Use 1 μ L to quantify the CUT&RUN DNA using the Qubit fluorometer with the 1X dsDNA HS Assay Kit per the manufacturer's instructions. See notes below and **Appendix 1.3**.
53. CUT&RUN DNA can be stored at -20°C for future processing.

CONFIRMING CUT&RUN SUCCESS PRIOR TO LIBRARY PREP

- The best indicator of success before library prep is that yields from the H3K4me3 positive control are slightly greater than the IgG negative control. However, if yields are similar, this does not imply assay failure; see **Appendix 1.3**.
- DNA yields from experimental reactions should **NOT** be used as a definitive metric of success (**Appendix 1.3**). There is no typical yield; instead, aim for ≥ 5 ng CUT&RUN enriched DNA, which will enable robust library prep.
- **Electrophoretic analysis (e.g. TapeStation) and/or qPCR of CUT&RUN DNA prior to library prep is NOT recommended.** These strategies are not robust indicators of CUT&RUN success (see **FAQs**). We suggest comparing yields from positive and negative controls and proceeding directly to library prep.
- **Do NOT shear or fragment DNA before library prep.** Our PCR conditions for library prep specifically amplify DNA fragments from 200 to 700 bp, which eliminates large fragments.

SECTION VIII: NGS LIBRARY PREP (~4 HRS)

54. Prepare Illumina sequencing libraries using ~5 ng purified CUT&RUN DNA and the CUTANA™ CUT&RUN Library Prep Kit (EpiCypher 14-1001 & 14-1002). See **FAQs** for considerations when using low DNA inputs for library prep.

If using a different kit for library prep, follow EpiCypher's recommended PCR parameters for indexing PCR and library amplification. These conditions are specifically optimized for small CUT&RUN fragments (200-700 bp):

Step #	Temperature	Time	Cycles	Notes
1	98°C	45 sec	1	Hot start activation of DNA Polymerase
2	98°C	15 sec	14	DNA melting
3	60°C	10 sec		Hybrid annealing/extension
4	72°C	60 sec	1	Final extension
5	4°C	∞		Hold temperature

SECTION IX: ANALYSIS OF LIBRARY FRAGMENT SIZE (~1 HR)

Notes on expected yields and control reactions:

- * There is no typical yield for purified CUT&RUN sequencing libraries. Instead, aim for library concentrations ≥ 1 nM, which will allow pooling at standard concentrations for multiplexed sequencing. See **Appendix 1.4** and **FAQs**.
 - * Fragment distributions for positive (e.g. H3K4me3) and negative (e.g. IgG) control reactions can be used to assess yields and validate library prep workflows. Note that library yields for H3K4me3 and IgG may be similar, but this does not indicate assay failure. See **Appendix 1.4** for additional details.
55. Use 1 μ L to quantify CUT&RUN libraries using the Qubit fluorometer with the 1X dsDNA HS Assay Kit as per the manufacturer's instructions.
56. For each library, prepare 5 μ L at 10 ng/ μ L for loading onto the Bioanalyzer or TapeStation system. Record the dilution factor, which is needed to calculate library molarity from the results (reported as DNA concentrations in nM for the desired 200 - 700 bp region).

SECTION IX: ANALYSIS OF LIBRARY FRAGMENT SIZE (~1 HR) *continued*

57. Load and analyze 1 μ L diluted sequencing library using the High Sensitivity DNA Kit (Bioanalyzer) or the D1000 ScreenTape System & Reagents (TapeStation) as per the manufacturer's instructions.
58. The final traces should show predominant enrichment of mononucleosome-sized fragments, such as those yielded by H3K4me3 and CTCF antibodies in [Figure 7](#) (~300 bp: ~170 bp + 125 bp sequencing adapters). Adapter dimers, if present, would be observed as a peak at ~125 bp; see [Appendix 1.4](#).
59. CUT&RUN libraries can be stored at -20°C for future processing.

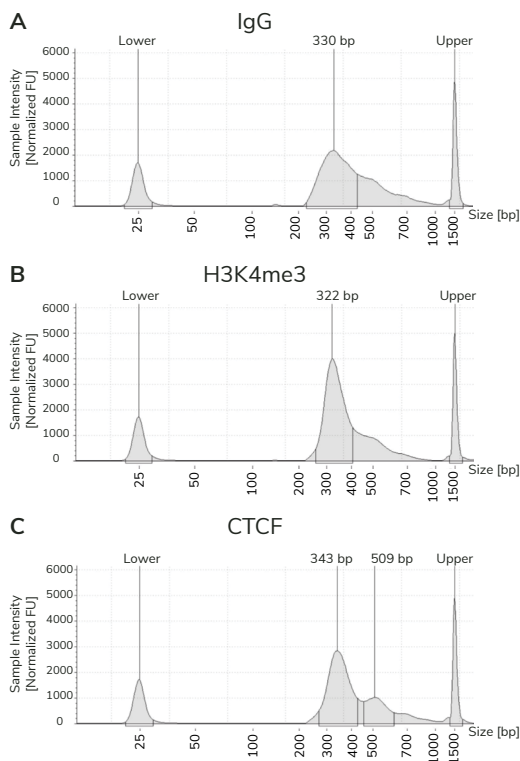


FIGURE 7

Typical TapeStation traces from CUTANA™ CUT&RUN libraries prepared using antibodies targeting IgG (EpiCypher 13-0042), H3K4me3 (EpiCypher 13-0041), and CTCF (EpiCypher 13-2014). All libraries are predominantly enriched for mononucleosome-sized fragments, as indicated by the peak at ~300 bp (~170 bp nucleosomes + sequencing adapters).

SECTION X: ILLUMINA® SEQUENCING

Tips for sequencing CUT&RUN libraries:

- * Only 3-8 million uniquely aligned reads are needed for adequate CUT&RUN coverage. For low abundance targets (e.g. H3K4me3), aim for 3-5 million reads. For high abundance targets (e.g. H3K27me3), aim for 5-8 million. See **Appendix 1.5** for examples.
- * Paired-end sequencing (2 x 50 bp cycles minimum) is recommended to detect the K-MetStat Panel, and will allow target footprinting analysis.

60. Select appropriate Illumina® sequencing platform based on the number of CUT&RUN libraries and desired sequencing depth.
61. Pool libraries at desired ratios using the molarity calculations from **Section IX** (200-700 bp region) and load onto Illumina sequencer. General steps:
 - a. Confirm that each library in a multiplexed sequencing run has a unique pair of i5 & i7 indexes. Libraries with the same pair of indexes must be sequenced in separate lanes/flow cells.
 - b. Dilute each library to the same nM concentration, depending on final yields. For NextSeq 2000 and NextSeq 500/550, dilute to 1-4 nM.
 - c. Pool equimolar libraries into one tube.
 - d. Dilute pooled libraries to appropriate concentration and in the volume required for Illumina platform. Follow guidelines from specific Illumina kit to load onto sequencer (support.illumina.com).
 - e. When setting up the sequencing run, make sure dual i5 & i7 indexes are correctly assigned for each library.

SECTION XI: DATA ANALYSIS

62. For H3K4me3 and IgG control reactions spiked with the **K-MetStat Panel**, align paired-end sequencing reads to the PTM-specific DNA barcodes. Use this data to validate your workflow, identify failed reactions, and troubleshoot problematic experiments. See **Appendix 1.6** for guidance and examples.
63. If control reactions generate expected spike-in results, proceed to analysis of experimental reactions. Align paired-ends reads to the appropriate reference genome (e.g. using Bowtie 2). See **FAQs** for more information.
64. For NGS normalization using the **E. coli Spike-in DNA**, see **Appendix 2**.

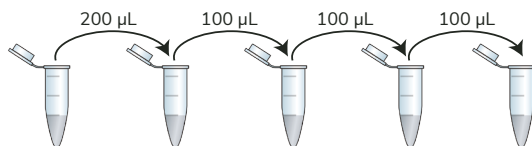
Appendix 1: Quality Control Checks & Troubleshooting

1.1. OPTIMIZATION OF CELL PERMEABILIZATION

CUT&RUN uses Digitonin to permeabilize cells, and represents a crucial step in the protocol. Insufficient Digitonin prevents antibody and pAG-MNase binding, while excess amounts may result in cell lysis. EpiCypher recommends using the minimal amount of Digitonin required to permeabilize >95% of cells. Optimize Digitonin concentrations for each cell type used in CUT&RUN as outlined below.

If using nuclei, Digitonin optimization is not required. Use 0.01% Digitonin in CUT&RUN buffers to reduce bead clumping.

1. Prepare a series of Cell Permeabilization Buffers using **5% Digitonin** and **Wash Buffer** (see **Experimental Protocol, Section I**). Prepare **FRESH** on the day of use.
 - a. Label five fresh 1.5 mL tubes with percent Digitonin (see table, below). Add the appropriate volume of **Wash Buffer** to each tube.
 - b. Add 10 μ L **5% Digitonin** to the first tube, labelled 0.05%. Vortex to mix.
 - c. Prepare the other four Cell Permeabilization Buffers by serial dilution (see table). Vortex each buffer to mix and place on ice.
 - d. In a sixth 1.5 mL tube, prepare 0.05% DMSO in **Wash Buffer** as a control.
2. Harvest cultured cells in a 1.5 mL tube. To determine the number of cells needed for Digitonin optimization, multiply the number of cells used per CUT&RUN reaction (e.g. 500,000) \times 6.2 (include 20% excess volume for pipetting errors).
3. Spin 600 \times g, 3 min, RT. Remove supernatant. Resuspend in 620 μ L RT 1X PBS.
4. Aliquot 100 μ L cells to six new 1.5 mL tubes. Assign each buffer to one tube.



Final % Digitonin	0.05%	0.01%	0.001%	0.0001%	0.00001%
Wash Buffer	990 μ L	800 μ L	900 μ L	900 μ L	900 μ L
5% Digitonin	10 μ L	-	-	-	-

5. Spin 600 x g, 3 min, RT. Remove supernatant. Resuspend in 100 μ L assigned buffer and incubate cells for 10 minutes at RT.
 6. For each of the six tubes: Mix 10 μ L cells + 10 μ L 0.4% Trypan blue. Load 10 μ L onto a hemacytometer slide.
 7. Count live (intact, Trypan negative) vs. dead (permeabilized, Trypan positive) cells. Select minimum Digitonin concentration that permeabilizes >95% of cells.
- In [Figure 8](#), 0.01% Digitonin is the minimum concentration necessary to permeabilize >95% of total K562 cultured cells (red arrow).

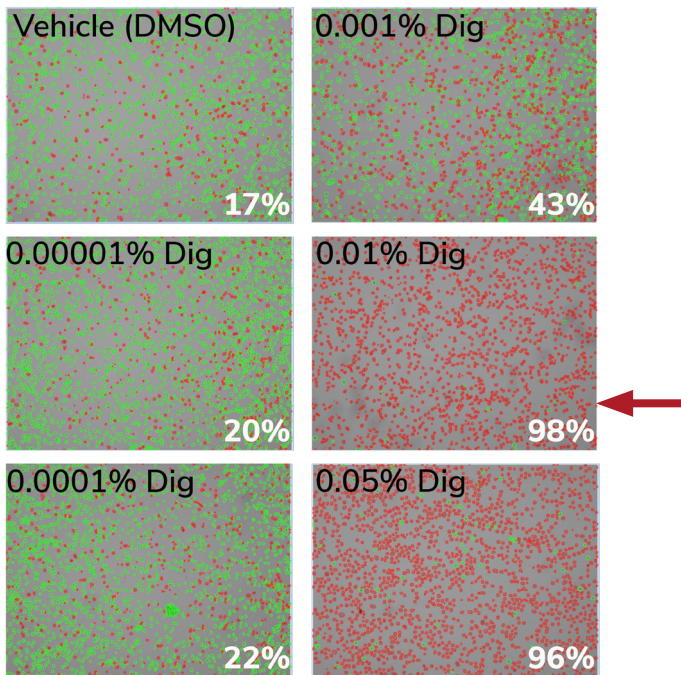


FIGURE 8

The minimum amount of Digitonin ("Dig") needed to permeabilize K562 cells was determined by varying the amount of 5% Digitonin added to the final Cell Permeabilization Buffer. Trypan blue staining was used to evaluate permeability with an automated cell counter (live/dead cell viability analysis). Green cells (Trypan negative) are intact, whereas permeabilized/dead cells (Trypan positive) are red. Values (bottom right of each panel) indicate percent of dead/permeabilized cells.

1.2. QUALITY CONTROL CHECKS FOR SAMPLE PREP

This protocol flags samples of poor quality or that fail to bind ConA beads. Perform these checks for every experiment to confirm sample quality prior to CUT&RUN.

- * Cells are resuspended with 5 μ L excess **Wash Buffer** to account for pipetting error. It may be helpful to process one extra sample to evaluate sample integrity.
- * If using nuclei instead of whole cells, also see **Appendix 3.2**.

1. Starting at **Step 17** in **Experimental Protocol**, resuspend cells in 105 μ L per reaction RT **Wash Buffer**.
2. Take 10 μ L from washed cells and evaluate sample integrity as follows:
 - a. Add 10 μ L of **0.4% Trypan blue**.
 - b. Mix 10 times by pipetting.
 - c. Transfer 10 μ L to a counting slide.
 - d. View under brightfield/phase microscope or cell counter.
 - e. Confirm sample integrity or troubleshoot as needed (See [Figure 9](#)).
3. Add 100 μ L washed cells to 10 μ L of activated ConA Beads in 8-strip tubes. Gently vortex to resuspend [**sample slurry**]. Quick spin to collect beads.
4. Incubate **sample slurry** for 10 min at RT to adsorb nuclei to beads.
5. Place tubes on magnet and wait for slurry to clear. Transfer 10 μ L supernatant [**unbound fraction**] to a 1.5 mL tube and set aside. Discard remaining supernatant.
6. Immediately add 50 μ L cold **Antibody Buffer** to the **sample slurry**. Remove tubes from magnet and gently vortex to an even resuspension. Quick spin to collect beads from tube lid.
7. Move 10 μ L **sample slurry** to a 1.5 mL tube. Place remaining **sample slurry** on ice.
 - * Alternative: Take 1-5 μ L **sample slurry**. Dilute with 1X PBS to 10 μ L volume.
8. To 10 μ L samples that were set aside (**unbound fraction**, **sample slurry**), perform Trypan Blue staining as described in Step 2. See [Figure 9](#) for results.
9. Continue with the **Experimental Protocol, Section IV: Antibody Binding**.

Sample	Success Metrics (Trypan Blue Stained)	Troubleshooting Tips
Cells Figure 9A	Cells should be bright white (Trypan excluded), round, and show >95% viability.	Evaluate cell culture conditions; use fresh media, determine contamination issues.
Nuclei Figure 9B	Nuclei should be >95% “dead” (Trypan blue positive) and unclumped. See Appendix 3.2 .	Monitor cells during nuclear extraction by Trypan Blue staining to optimize incubation time. If losing sample during spin, increase to >3 min (keep at 600-800 x g).
Unbound Fraction Figure 9C	Little to no material should be present if binding occurred.	Ensure that ConA Beads were never frozen (if frozen, beads will appear black), cells/nuclei were not clumped, beads did not become clumped or dried out, and all buffers were correctly prepared.
Sample Slurry Figure 9D	Successful conjugation will show permeabilized cells/ nuclei surrounded by beads.	

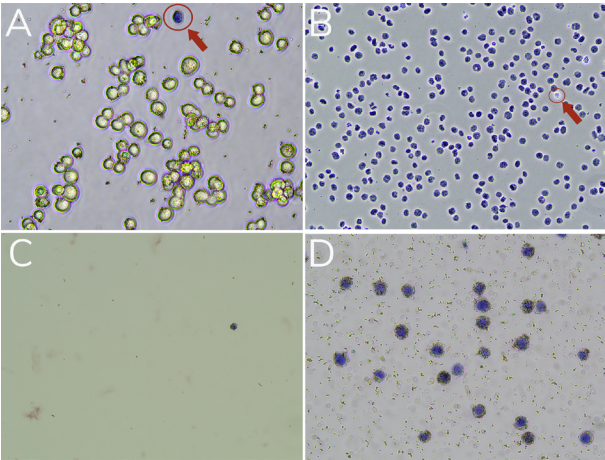


FIGURE 9

Validation of sample prep using Trypan Blue staining. **(A)** Starting cells are mostly viable (bright white and round). A dead cell (blue, Trypan positive) is circled in red. **(B)** Successful nuclei harvest shows Trypan Blue stained nuclei. An intact cell (bright white, Trypan negative) is circled in red. See **Appendix 3.2** **(C)** **Unbound fraction** has minimal nuclei. **(D)** Representative **sample slurry** image showing nuclei (blue) successfully conjugated to activated ConA Beads (brown specks). **Note:** ConA Bead-bound cells will also be Trypan positive (blue), due to the presence of Digitonin in Buffers.

1.3. QUALITY CONTROL CHECKS AFTER CUT&RUN

- Confirm integrity of starting cells, cell permeabilization, and ConA Bead binding.
- The **best indicator of assay success before library prep** is that yields from H3K4me3 positive control are slightly greater than IgG negative control. Similar yields from positive and negative controls do **NOT** indicate assay failure.
- **There is no typical yield for CUT&RUN**, since results vary by cell type and number, target abundance, antibody specificity, and so forth. Instead, aim for ≥ 5 ng CUT&RUN enriched DNA to enable robust library prep.
- See the Table below for troubleshooting CUT&RUN assays with low yields. See **FAQs** for considerations when using less than 5 ng for CUT&RUN library prep.

Troubleshooting CUT&RUN DNA yields (pre-library prep)

Unexpected Low Yields - Causes & Troubleshooting Approaches

- Possible causes: poor sample quality, inefficient permeabilization, ConA bead damage or dry-out and loss, bad antibody and/or low abundance target, contaminated buffers, etc.
- Repeat experiment with fresh buffers. Follow the kit protocol and include reactions with H3K4me3 and IgG control antibodies spiked with the K-MetStat Panel. Confirm experimental details and/or optimize as follows:
 1. To reduce ConA Bead aggregation and dry-out, store beads at 4°C (do **NOT** freeze*). Gently vortex stock to evenly resuspend; double check that beads are brown in color. Activate beads in bulk before aliquoting to 8-strip tubes.
 - * Freezing ConA Beads causes irreversible damage. The beads will appear black and clump/aggregate despite vigorous mixing. Do **NOT** use damaged ConA Beads for CUT&RUN.
 2. Use 500,000 cells per reaction. Confirm cell viability and ConA Bead binding as detailed in **Appendix 1.2**.
 3. Use the lowest concentration of Digitonin that permeabilizes >95% cells (**Appendix 1.1**).
 4. Use a **nutator** for the overnight incubation to avoid bead dry-out and sample loss.
 5. Examine raw DNA yields. If controls work (**Appendix 1.3**) but experimental targets fail:
 - Test additional antibodies as outlined in **Experimental Design & Optimization** and **FAQs**.
 - Consider target abundance. For low-abundance targets, low yields may be unavoidable. Library prep can be modified to increase yields for sequencing; see **FAQs** and the CUTANATM CUT&RUN Library Prep Kit Manual (epicypher.com/protocols) for guidance.

1.4. QUALITY CONTROL CHECKS FOR SEQUENCING LIBRARIES

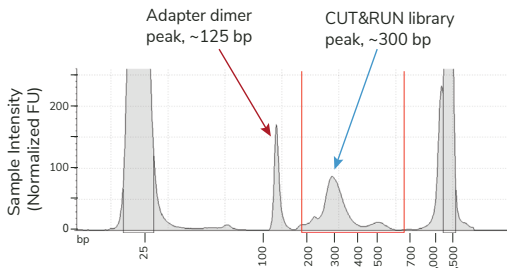
- In general, H3K4me3 library yields should be slightly higher than IgG. However, similar yields from H3K4me3 positive and IgG negative controls does not indicate experiment failure, and good sequencing data can still be obtained.
- Library yields for an abundant target (i.e. H3K27me3) should be higher than IgG.
- Examine fragment size distribution of libraries. Predominant enrichment of mononucleosome-sized fragments (~300 bp = ~170 bp nucleosome + sequencing adapters) is the **best indicator of CUT&RUN success prior to NGS**.
- Aim for a final library concentration ≥ 1 nM to enable pooling for multiplexed sequencing. If library concentrations fall below 0.5 nM, see **FAQs** for guidance.

Troubleshooting fragment distribution of CUT&RUN libraries

Concern	Causes / Troubleshooting approaches
No peaks or most peaks >300 bp	<p>Indicates problems with CUT&RUN assay and/or library prep; can also result from low target abundance and/or low DNA input for library prep (FAQs).</p> <ul style="list-style-type: none"> • See table on previous page for guidance on sample prep and assay controls. Ensure control reactions pass quality control checks prior to library prep. • Ensure adapter ligation reagents/reactions are kept on ice during ligation setup. • Use EpiCypher's CUT&RUN PCR parameters to ensure optimal enrichment.
Adapter dimers	<p>Adapter dimers result from self-ligation of sequencing adapters and are preferentially amplified due to their small size (Figure 10).</p> <ul style="list-style-type: none"> • Ensure adapter ligation reagents/reactions are kept on ice during ligation setup. • Remove adapter dimers that comprise >5% of a library (200-700 bp region). See CUTANA CUT&RUN Library Prep Kit Manual (epicypher.com/protocols).

FIGURE 10

Example TapeStation trace from CUTANA™ CUT&RUN H3K27me3 library containing an adapter dimer peak (~125 bp peak; red arrow) and expected library peak (~300 bp, blue arrow). Orange lines denote the 200-700 bp range, used to determine library concentration.



1.5. QUALITY CONTROL CHECKS AFTER SEQUENCING

- Ensure appropriate read depth (3-8 million reads per reaction) was achieved.
- Majority of reads (>80%) should align uniquely to the species genome.
- Sequence duplication levels should be low (<10% of total sequence reads), but can be filtered out if needed.
- *E. coli* Spike-in DNA should comprise ~1% of total unique reads (**Appendix 2**).
- The SNAP-CUTANA™ K-MetStat Panel should comprise ~1% (0.5-5%) of total unique sequence reads and produce expected results in H3K4me3 positive control and IgG negative control reactions (see **Appendix 1.6**).
- H3K4me3 and IgG controls should show expected genomic distributions and peak structures. Results for experimental replicates should be highly reproducible. Representative examples for human K562 cells are shown in [Figures 11](#) and [12](#).

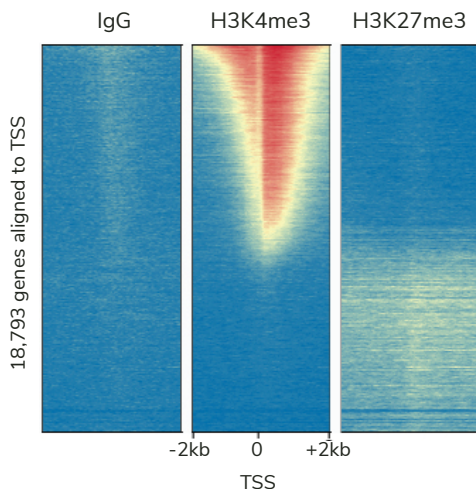


FIGURE 11

Representative CUT&RUN data for K562 cells using Rabbit IgG Negative Control Antibody, H3K4me3 Positive Control Antibody, and H3K27me3 antibody. Data are presented as a heatmap of signal intensity aligned to the TSS of 18,793 genes (+/- 2kb). Genes are aligned across conditions and ranked by H3K4me3 intensity from top (high signal, red) to bottom (low signal, yellow). The heatmaps show that H3K4me3, a mark of active gene transcription, is enriched proximal to the TSS and is anti-correlated with H3K27me3, a mark of transcriptional repression. IgG shows low nonspecific background signal.

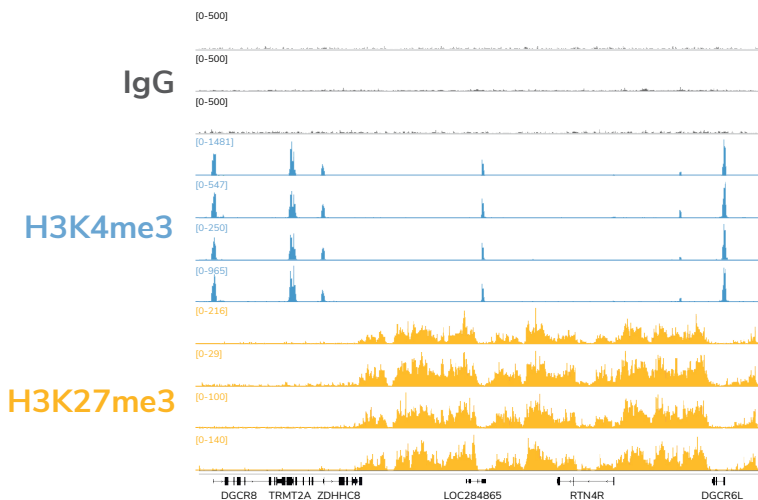


FIGURE 12

Data generated by four independent users demonstrate the reproducibility of the CUTANATM CUT&RUN Kit. CUT&RUN was performed using 500,000 K562 cells with Rabbit IgG Negative Control Antibody, H3K4me3 Positive Control Antibody, and H3K27me3 antibody. Total read counts were ~3 million for IgG and H3K4me3 and ~6 million for H3K27me3. H3K4me3 tracks show sharp peaks localized to TSSs, while H3K27me3 tracks show broad peaks over repressed regions. IgG shows low background when scaled to H3K4me3.

Troubleshooting CUT&RUN sequencing results

Concern	Causes / Troubleshooting approaches
Background in open chromatin	Indicates over-digestion by MNase. Repeat assay with fresh buffers, and make sure MNase digestion is performed on ice (temperature is critical).
Experimental target shows high background and/or is indistinguishable from IgG negative control	<p>Possible causes: over-digestion by MNase, DNA damage, antibody failure. Repeat assay with fresh buffers and 500,000 cells per reaction. Include reactions with control antibodies spiked with the K-MetStat Panel. Ensure:</p> <ul style="list-style-type: none"> • Cells are quickly processed and resuspended in cold Antibody Buffer. • Antibody incubation (varying dilutions) is performed overnight at 4°C. • MNase digestion is incubated on ice for 2 hours. • Adapter ligation is kept on ice until all reagents are added. <p>If problems persist, test additional antibodies; see FAQs for guidance.</p>

1.6. SNAP-CUTANA™ K-METSTAT PANEL

To validate experiments and guide troubleshooting, EpiCypher recommends adding the SNAP-CUTANA™ K-MetStat Panel of nucleosome spike-ins to control reactions in **every** experiment. The kit includes K-MetStat Panel for 10 experiments (i.e. 20 reactions; 10 per control antibody).

What are spike-in controls?

Spike-ins are synthetic or exogenous DNA sequences that are added directly to genomics reactions to gauge assay performance.

What is the K-MetStat Panel?

The K-MetStat Panel is a pool of 16 unique nucleosomes, each containing a distinct histone lysine methylation PTM (or unmodified; see [Figure 13](#)) and a PTM-specific DNA barcode. Spike-ins are already coupled to magnetic beads, which allows the panel to be processed alongside cell samples bound to ConA Beads.

How is the K-MetStat Panel different from other spike-ins?

The K-MetStat Panel is the **only** control that uses purified recombinant nucleosomes, replicating the physiological target of CUT&RUN and providing reliable on- and off-target substrates for control reactions. These spike-ins directly report on antibody specificity, pAG-MNase activity, sample quality, and more.

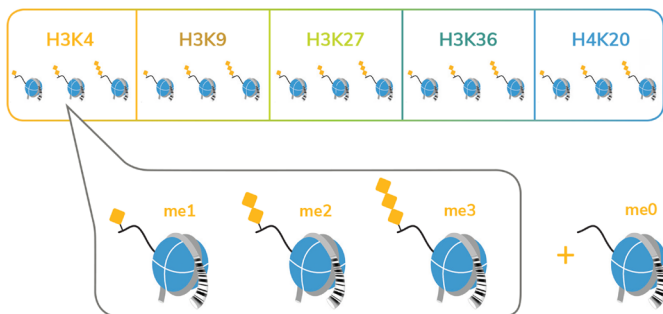


FIGURE 13

Schematic of SNAP-CUTANA™ K-MetStat Panel, comprising 16 DNA-barcoded recombinant nucleosomes. DNA barcodes designate unique histone lysine methylation states in NGS.

SPIKE-IN PROCEDURE OUTLINE

- Add the K-MetStat Panel to designated control reactions immediately prior to the addition of H3K4me3 or IgG Control Antibody (Figure 14).
- Add antibody, which binds its target in cells **and** in the spike-in panel (Figure 14).
- Add and activate pAG-MNase to cleave antibody-bound chromatin. DNA fragments are released from immobilized cells **and** spike-ins into the supernatant.
- Purify DNA from the supernatant and prepare for sequencing.
- For each control reaction, determine the number of sequencing reads assigned to each PTM-specific DNA barcode (next page). Barcode read counts provide a useful measurement of PTM recovery and workflow success (Figures 15 and 16).

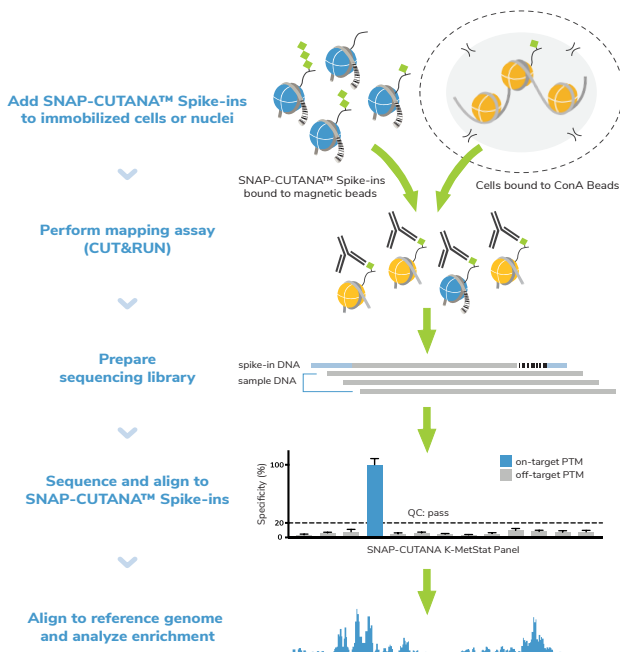


FIGURE 14

Schematic showing addition of SNAP-CUTANATM spike-ins during CUT&RUN workflows. Note that the spike-ins are also compatible with CUT&Tag assays.

DATA ANALYSIS (PERFORMED ON A MAC):

1. Download R1 & R2 paired-end sequencing files (fastq.gz) for control reactions. Double-click the fastq.gz files to create fastq files and save in a new folder.
2. Download the shell script (.sh) file from the K-MetStat Panel product web page (epicypher.com/19-1002, under Documents and Resources).
3. Open the .sh file in TextEdit or any **text editing** program. Do **NOT** open in Word or a PDF program. Scroll past the barcode sequences to find the analysis script.
4. The script is a loop that reports the number of R1 & R2 reads assigned to each PTM-specific barcode. You need **one loop per control reaction**. To customize:
 - a. Copy lines **between** # template loop begin ## and # template loop end ##.
 - b. Paste the loop **immediately under** the last "done." Repeat until you have a copy of the loop for each control reaction.
 - c. In the first loop, replace sample1_R1.fastq and sample1_R1.fastq with R1 & R2 fastq file names for **one** control reaction. Repeat for other loops.
 - d. Save your .sh file in the **same folder** containing your fastq files.
5. Open Terminal. Set the directory to your folder: Type "cd" and press space once. Drag the **folder** from Finder into Terminal to copy the location path. Press return.
6. Run the script in Terminal: Type "sh" and press space once. Drag your **.sh file** from Finder into Terminal to copy the location path. Press return.
7. Terminal generates the barcode read counts from **both** R1 & R2 sequencing reads, one loop (i.e. one reaction) at a time.
8. To visualize the data, download the Excel file from the K-MetStat Panel web page (epicypher.com/19-1002, under Documents and Resources).

Navigate to Sheet 1, Column B. The first two reactions are pre-set for IgG (cell B4) and H3K4me3 (cell B46) as the On-Target PTM.

* Additional analysis templates are provided in the Excel workbook; use as needed.
9. In Terminal, copy R1 barcode read counts from one loop. In Excel, find the matching reaction and paste into yellow R1 cells in Column C. Copy & paste R2 read counts to yellow R2 cells in Column D. Repeat for each loop/reaction.
10. **Per reaction**, the sheet calculates the number of barcode reads assigned to each PTM in the K-MetStat Panel. Data are normalized to barcode read counts for the On-Target PTM, providing a readout of PTM recovery (see Column G).

11. PTM recovery data populate the Output Table (Figure 15). Reactions are separated by **row** and PTM data are sorted into **columns**. Data are expressed as a gradient from high (blue) to low (orange); On-Target PTM is set to 100%.
12. Finally, calculate the percentage of total sequencing reads assigned to spike-in barcodes. In the Excel file, type the number of uniquely mapped genomic reads in the yellow cell denoted "Uniq align reads," which generates the "% total barcode reads" in the cell immediately below. Repeat for each reaction.

EXPECTED RESULTS:

- IgG negative control: No preference among PTMs and low background (Figure 15).
- H3K4me3 positive control: High signal-to-noise (**S:N**) and strong enrichment for H3K4me3 spike-ins (<20% off-target recovery; typically <5% for native cells).
- Spike-in barcode reads: Comprise ~1% (0.5-5%) of total sequencing reads.
- If control reactions generate expected spike-in data (Figure 15, Samples 1 and 2), you can be confident in the technical aspects of your workflow.
- Off-target PTM recovery in H3K4me3 control (>20%; Figure 15, Sample 3) and/or high background in IgG control indicate significant experimental problems. See next page for a discussion of troubleshooting using spike-in results.

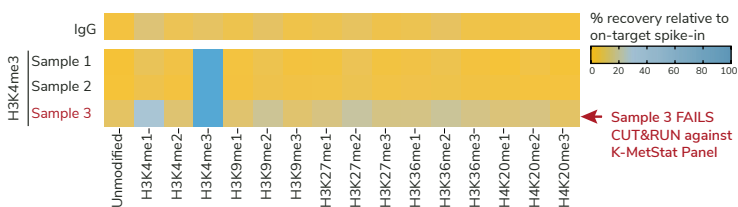


FIGURE 15

K-MetStat Spike-ins validate workflows and flag poor samples in CUTANA™ CUT&RUN workflows. Spike-in data for H3K4me3 positive control reactions is shown for three independently prepared mouse B cell samples. Samples 1 and 2 show expected results, while Sample 3 was flagged for recovery of off-target PTM spike-ins and low S:N (signal-to-noise). Representative data from one IgG reaction is shown as a negative control.

TROUBLESHOOTING CUT&RUN ASSAYS USING THE K-METSTAT PANEL:

Figures 15 & 16 demonstrate the use of K-MetStat Spike-in data for troubleshooting.

- Figure 15 (previous page) uses spike-in data to validate workflows for Samples 1 and 2, while Sample 3 displays low S:N and high recovery of off-target PTMs.
- Next, compare the quality of genomic profiles to spike-in results for each sample. Figure 16 shows tracks for IgG, H3K4me3, and H3K27me3 for all three samples.
- Genomic profiles agree with spike-in data: Samples 1 and 2 show expected tracks with high S:N, while Sample 3 profiles exhibit poor S:N (red arrows, Figure 16).

To troubleshoot Sample 3 reactions, we considered the following:

- All reactions were performed in parallel using the same antibodies and reagents. However, only Sample 3 reactions displayed problems with background.
- Sample 3 showed poor S:N in both genomic profiles **and** K-MetStat Panel data.
- Sample 3 generated poor profiles across multiple targets (H3K4me3, H3K27me3).

Combined, these results suggest problems with sample prep vs. a workflow failure. In this case, the number of cells used for the assay was much lower than intended.

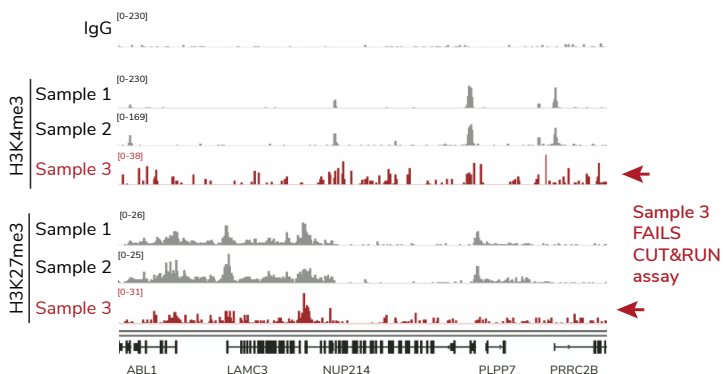


FIGURE 16

CUT&RUN was used to map IgG (negative control), H3K4me3 (positive control) and H3K27me3 in three independently prepared mouse B cell samples (10,000 cells each; protocol optimization experiment with a multi-lab consortium). A representative 400 kb region is shown. Samples 1 and 2 show consistent peaks, while Sample 3 displays low S:N (red).

These results illustrate the utility of including control reactions with the **K-MetStat Panel in ALL experiments**. It may be unclear from genomic tracks alone that a reaction issue has occurred. The controls outlined in this section are designed to flag failed reactions **AND** indicate the cause. By identifying poor samples or reactions and using the spike-in results to guide troubleshooting, researchers can be confident in their experimental results.

Troubleshooting experiments using H3K4me3 and IgG control antibodies paired with the SNAP-CUTANA™ K-MetStat Panel

Control reactions	Interpretations & troubleshooting approaches
Spike-in data: <ul style="list-style-type: none"> • High target specificity • High S:N H3K4me3 genomic data: <ul style="list-style-type: none"> • Poor S:N 	<p>pAG-MNase conditions, library prep, and control antibodies are performing as expected. Other problems are indicated:</p> <ul style="list-style-type: none"> ⚠ Low numbers of cells <ul style="list-style-type: none"> • Optimize assay with 500,000 cells before decreasing input • Consider using control cells (K562 cells) to optimize workflow • If using nuclei, adherent cells, cross-linked samples, or tissues, additional optimization is required (Appendix 3) ⚠ Poor sample prep <ul style="list-style-type: none"> • Optimize Digitonin permeabilization of cells (Appendix 1.1) • Confirm cell viability and binding to beads (Appendix 1.2); note that low cell viability increases background signal • Avoid ConA Bead clumping and/or dry-out during assay ⚠ Experimental target may require different processing conditions <ul style="list-style-type: none"> • Ensure target is present and localized to chromatin • If using frozen nuclei, try freshly isolated nuclei • Test native vs. lightly cross-linked conditions (Appendix 3.3)
Spike-in data: <ul style="list-style-type: none"> • Recover off-target PTMs • Poor S:N H3K4me3 genomic data: <ul style="list-style-type: none"> • Poor S:N 	<ul style="list-style-type: none"> ⚠ Indicates a fundamental failure in the workflow. <ul style="list-style-type: none"> • Carefully re-read the protocol and important notes • Ensure buffers are prepared fresh on day of use and Digitonin is added to buffers as indicated in the protocol • Ensure ConA Beads are in good condition (e.g. never frozen) • Make sure correct PCR parameters are used in Indexing PCR; consider using CUTANA™ CUT&RUN Library Prep Kit ⚠ Low numbers of cells and/or poor sample prep <ul style="list-style-type: none"> • Optimize following the guidelines above
Spike-in & Genomic data show expected results	✔ None needed - Happy data analysis!

* SNAP-CUTANA™ Spike-ins can also be added to any experimental reaction targeting a PTM included in the panel (e.g. lysine methylation targets). To purchase additional K-MetStat Panel and learn more about spike-in applications, visit epicypher.com/19-1002.

Appendix 2: Experimental Normalization Using *E. coli* Spike-in DNA

Aim for *E. coli* Spike-in DNA to comprise ~1% (0.5-5%) of total sequencing reads. In the protocol, 0.5 ng is recommended for 500,000 cells. Generally, this can be decreased linearly with decreasing cell number (e.g. 0.1 ng per 100,000 cells). The amount may need to be adjusted to achieve read counts in the optimal range due to variables such as target abundance, antibody efficiency, etc.

To normalize sequencing results using *E. coli* Spike-in DNA:

1. Align sequencing reads to the *E. coli* K12, MG1655 reference genome:

https://support.illumina.com/sequencing/sequencing_software/igenome.html

Filter out reads that do **NOT** align uniquely.

Note that this alignment is separate from the experimental reference genome (e.g. human hg19 build).

2. For pairwise comparisons, quantify *E. coli* Spike-in DNA reads for each CUT&RUN reaction and and normalize to the total number of uniquely aligned NGS reads.

Example: CUT&RUN was used to map H3K4me3 in treated and untreated cell samples.

Treatment spike-in = 100,000 Ec reads in 5,000,000 total reads = 2%

Untreated spike-in = 30,000 Ec reads in 3,000,000 total reads = 1%

3. Calculate normalization factor (see Tay et al.⁵) such that after normalization the *E. coli* spike-in signal is set to be equal across all reactions.

Example from above, comparing H3K4me3 in treated vs. untreated cells:

Treatment normalization factor = $1 / 2\%$ spike-in bandwidth = 0.5

Untreated normalization factor = $1 / 1\%$ spike-in bandwidth = 1.0

4. Use single scalar normalization ratio with the `--scaleFactor` option enabled in `deeptools bamCoverage` tool to generate normalized bigwig files for visualization in IGV (<https://deeptools.readthedocs.io/en/develop/content/tools/bamCoverage.html>). Continuing with the **Example** from above:

Treatment sample `--scaleFactor` = 0.5

Untreated sample `--scaleFactor` = 1.0

- * The effect of normalization on a dataset is inversely proportional to the *E. coli* Spike-in bandwidth. In other words, reactions with the highest bandwidth will receive the largest reduction in signal after normalization. For further information on experimental NGS normalization using exogenous spike-in controls, see ⁵ and ⁶.

3.1. SAMPLE PREP VARIATIONS

Freshly isolated cells are the preferred input for CUT&RUN. The kit is also compatible with adherent cells, cryopreserved samples, tissues, and cross-linked cells or nuclei.

ADHERENT CELLS

Collect adherent cells using a mild Trypsin digestion, which dislodges and disaggregates clumps into monodispersed cells without cell damage.

1. Incubate cells with **0.05% Trypsin at 37°C for the minimal time necessary to dislodge cells.**
2. Add pre-warmed complete media to inactivate Trypsin and then collect cells (**Experimental Protocol, Step 14**). Trypsin will be removed in wash steps.
3. Ensure Trypsin has not disrupted cell integrity or ConA Bead binding (as in **Appendix 1.2**). Proceed with CUT&RUN experiment.

CRYOPRESERVATION

Use conditions that minimize lysis, which can contribute to elevated background.

1. Count cells and confirm viability (**Appendix 1.2**). Spin cells 600-800 x g, 3 min, RT.
2. Resuspend in **media with 10% DMSO** and aliquot. EpiCypher typically aliquots cells for ≥ 8 reactions, plus 20-30% excess cells to account for sample loss.
3. Slowly freeze aliquots in an isopropanol-filled chiller in a -80°C freezer.
4. When ready to perform CUT&RUN, remove tubes from -80°C and quickly place on 37°C block to thaw. Work quickly to avoid cell lysis.
5. Spin thawed cells at 600-800 x g, 3 min, RT. Remove supernatant.
6. Resuspend cells in 105 μ L per reaction cold **Wash Buffer** (based on cell count before freezing). Take a 10 μ L aliquot to count (as in **Appendix 1.2**).

TISSUES

Tissues must be processed into a mono-dispersion of cells, typically by mechanical maceration/douncing. Enzymatic digestion (e.g. collagenase, dispase) can be used for connective tissue and Trypsin may be used for macro-dissected tissues (as above; monitor dissolution to single cells). See literature for additional methods⁷⁻¹⁰.

IMMUNE CELLS

Concanavalin A (ConA) is a lectin, which can cause immune cell activation. To avoid this potential problem in CUT&RUN, use nuclei¹¹ or a cross-linking strategy¹².

3.2. NUCLEI PREP AND CRYOPRESERVATION

Materials Needed	Recipe / Source
Nuclear Extraction Buffer	20 mM HEPES, pH 7.9, 10 mM KCl, 0.1% Triton X-100, 20% glycerol, 1mM MnCl ₂
Protease Inhibitor	Roche 11873580001
1 M Spermidine	EpiCypher CUTANA™ CUT&RUN Kit 14-1048
Phosphate Buffered Saline (PBS)	Any vendor
0.4% Trypan blue	Invitrogen T10282
Brightfield or phase microscope + hemacytometer slides	Any vendor

1. Prepare **Nuclear Extraction Buffer** with final concentration of ingredients outlined in the Table above. Filter sterilize; can be stored at 4°C for up to 6 months.
2. On the day of the experiment, add 1:2,000 dilution of **1 M Spermidine** and **1X Protease Inhibitor** to the **Nuclear Extraction Buffer**. Place final buffer on ice.
3. Harvest 500,000 cells per CUT&RUN reaction, plus 10-20% extra cells for Trypan blue staining. Centrifuge at 600 x g, 3 min, RT.
4. Remove and discard supernatant. Resuspend cells in 100 µL per reaction **1X PBS**.
5. Set aside 10 µL cells for Trypan blue staining (intact cell control). Centrifuge at 600 x g, 3 min, RT. Remove and discard supernatant.
6. Resuspend cells in 100 µL per reaction cold **Nuclear Extraction Buffer**. Incubate on ice for 10 min.
7. Centrifuge 600 x g, 3 min, 4°C. Remove and discard supernatant.
The pellet should change in appearance from sticky, pale yellow (cells) to white and fluffy (nuclei).

8. Resuspend nuclei in 100 μ L per reaction cold **Nuclear Extraction Buffer**. Set aside 10 μ L nuclei for Trypan blue staining.
 9. Add 10 μ L **0.4% Trypan blue** to the intact cell control (**Step 4**) and the isolated nuclei (previous step). Mix 10 times by pipetting.
 10. Load onto hemacytometer slide and examine under brightfield/phase microscope to determine whether nuclei have been efficiently isolated (**Figure 17**).
 11. To cryopreserve nuclei, slowly freeze in isopropanol-filled chiller in -80°C freezer.
 12. When ready to use samples for CUT&RUN, thaw nuclei quickly by placing on 37°C block. Move quickly to avoid nuclear lysis and chromatin fragmentation.
 13. Proceed to CUT&RUN ConA Bead conjugation step (**Experimental Protocol, Step 18**). Nuclei in **Nuclear Extraction Buffer** can be added directly to activated ConA beads (no need to resuspend in Wash Buffer).
- * If CUT&RUN yields are low or if there are concerns with data quality, it is recommended to start with ~50% excess cells to account for loss of nuclei during isolation and/or freeze-thaw process.

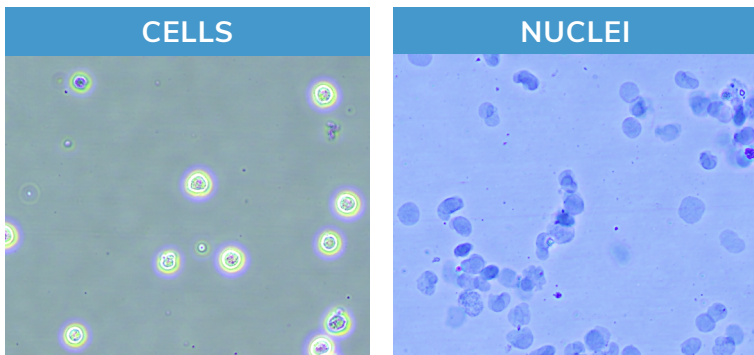


FIGURE 17

Morphology characteristic of intact K562 cells (left) compared to isolated nuclei (right) when visualized under brightfield microscope after Trypan blue staining. Isolated nuclei will stain blue, while cells will be bright white and round. For accurate nuclei counts, record "dead" cell numbers on an automated cell counter or manually count blue stained nuclei.

3.3. CROSS-LINKING

This cross-linking protocol should be considered for:

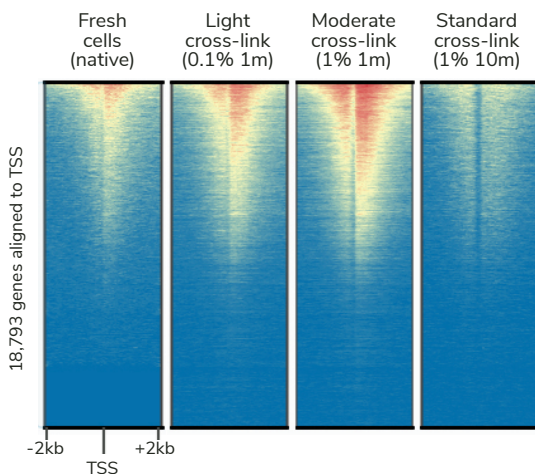
- Lysine acetylation PTMs (labile targets, impacted by histone deacetylase activity)
- Transiently interacting chromatin-associated proteins (e.g. acetyl-lysine reader proteins, chromatin remodeling enzymes)
- Experiments with tightly controlled variables (e.g. time course drug treatments)
- **Always test antibodies under cross-linked AND native conditions**

Although native conditions are preferred for CUT&RUN, signal for some targets may be improved by cross-linking. When optimizing cross-linking conditions:

- Start with light cross-linking (0.1% formaldehyde, 1 min), which generally preserves signal without negatively impacting data ([Figure 18](#)).
- If light cross-linking is not sufficient, moderate cross-linking (1% formaldehyde, 1 min) can be attempted with the caveat that it may impact DNA yield.
- Avoid heavy cross-linking conditions used for ChIP (>1% formaldehyde, 1-10 min) which is deleterious to both DNA yield and data quality ([Figure 18](#)).

FIGURE 18

H3K27ac CUT&RUN signal is improved by light to moderate fixation (0.1 – 1% formaldehyde for 1 min) compared to native cells. However, heavy (1%, 10 min) cross-linking significantly reduces CUT&RUN DNA yield. CUT&RUN data from 500,000 K562 cells are displayed in a heatmap with each gene row aligned across the conditions.



Materials Needed	Recipe / Source
Pre-Wash Buffer supplemented with detergent	Add 1% Triton X-100 + 0.05% SDS to Pre-Wash Buffer (EpiCypher CUTANA™ CUT&RUN Kit 14-1048)
37% Formaldehyde	Sigma 252549
Glycine	Sigma 50046
10% SDS	Any vendor
20 µg/µL Proteinase K	Ambion AM2546

1. From suspension tissue culture, transfer 500,000 cells per reaction into a 15 or 1.5 mL tube. For adherent cells, cross-link cells while still attached to plate.
2. Add fresh **37% Formaldehyde** directly to culture to desired final concentration (recommended 0.1-1%). Test a range of formaldehyde concentrations in initial experiments to determine optimal conditions for target and cell type of interest.
3. Quickly vortex (suspension cells) or swirl plate (adherent cells) to mix. Incubate for 1-10 min at RT (recommended 1 min). Test a range of times in initial experiments to determine optimal conditions.
4. Quench cross-linking by adding **Glycine** to a final concentration of 125 mM. Vortex (suspension cells) or swirl (adherent cells) to mix.
5. Collect cells (see **Appendix 3.1**, for adherent cells). Begin CUT&RUN starting at **Experimental Protocol, Step 14**.

Complete CUT&RUN protocol with the following modifications:

- Prepare **Pre-Wash Buffer** supplemented with Triton X-100 and SDS (see table above). Use to prepare CUT&RUN Buffers (**Experimental Protocol, Section I**).
- After isolation of supernatants containing CUT&RUN enriched DNA (last step of **Experimental Protocol, Section VI**), it is critical to reverse cross-links.
 - a. To each supernatant add 0.8 µL **10% SDS** and 1 µL of 20 µg/µL **Proteinase K**. Mix by vortexing.
 - b. Place tubes in a thermocycler set to 55°C. Incubate overnight.
 - c. Next day, quick spin reactions in benchtop microfuge. Resume **Experimental Protocol** at **Section VII: DNA Purification**.

Appendix 4: Frequently Asked Questions

TOPICS: Input types, Target Compatibility, Assay Optimization

Related sections: Experimental Design & Optimization, Appendix 3

What types of cell inputs are compatible with CUT&RUN?

Protocol modifications for diverse sample types are outlined in **Appendix 3**. A number of groups have also successfully performed CUT&RUN on human and mouse primary tissue⁷⁻¹⁰, FACS sorted cells¹¹ and immune cells^{13,14}.

What are the considerations for using immune cells?

See **Appendix 3.1**.

Is CUT&RUN compatible with frozen and/or cross-linked cells?

Yes. See **Appendix 3** for recommendations.

Does CUT&RUN work on non-PTM targets, such as transcription factors (TFs)?

Yes. The current protocol has been used at EpiCypher to profile diverse targets, including TFs, chromatin reader proteins, and ATP-dependent chromatin remodeling enzymes (see **Experimental Design & Optimization**).

How should I optimize CUT&RUN for low cell numbers and/or new targets?

See **Experimental Design & Optimization** for basic optimization steps.

Additional steps are recommended, especially for assays using <500,000 cells:

- **Identify the best antibody for your target.** It is important to test multiple antibodies for your target (next pages). Note that an antibody that performs reliably at 500,000 cells may fail at lower inputs. This could be due to poor antibody binding efficiency and/or specificity, problems that are worsened with low cell numbers. A poor antibody also challenges analysis of low-abundance targets, e.g. TFs, some PTMs (H3K4me3).
- **Confirm sample prep and binding to ConA beads.** Low quality sample prep is a common culprit in failed or low-yield CUT&RUN assays. Follow instructions in **Appendix 1.1** to ensure optimal digitonin conditions for permeabilization, and see **Appendix 1.2** for protocols to validate cell viability and binding to ConA Beads. In addition, make sure that ConA Beads do not dry-out or clump during the protocol.
- **Optimize indexing PCR conditions & sequencing depth.** DNA yields and overall signal may be reduced with low cell numbers. The number of PCR cycles can be increased to generate adequate yields for library quantification (>2 ng/ μ L). In this case, increased sequencing depth is recommended to capture full library diversity. See questions below on assay yields and using low DNA inputs for library prep.

TOPICS: Antibodies, K-MetStat Panel

Related sections: Experimental Design & Optimization, Experimental Protocol Section IV, Appendixes 1.3-1.6

What antibodies are recommended for CUT&RUN? Can I use ChIP antibodies?

Through our extensive development of CUT&RUN assays to various targets, EpiCypher has found that antibodies validated for ChIP or orthogonal approaches (e.g. immunoblot) are **NOT** guaranteed to be successful in CUT&RUN. To address this unmet need for researchers, EpiCypher has begun screening antibodies for high quality performance in CUT&RUN (see the expanding list of available targets at: epicypher.com/cut-and-run-antibodies). Below we outline our criteria for antibodies to chromatin-associated proteins and histone PTMs.

Chromatin-associated protein targets: We offer CUT&RUN antibodies to diverse chromatin-associated proteins, including transcription factors (e.g. CTCF), chromatin reader proteins (e.g. BRD4), chromatin modifying enzymes (e.g. MLL1) and remodelers (e.g. SMARCA2 & SMARCA4). To pass testing, the antibody must display high signal-to-noise in CUT&RUN and generate genomic distribution profiles consistent with the reported function of the target protein (for example, DNA binding motif analysis for TFs). Every lot of antibody is validated directly in CUT&RUN mapping assays to ensure reliable results.

Histone PTM targets: Histone PTM antibodies are particularly susceptible to off-target binding, which can compromise biological interpretations⁴. EpiCypher developed SNAP Spike-in technology to directly quantify PTM antibody performance in epigenomics assays (**Appendix 1.6**). Although the majority of antibodies exhibit poor specificity and/or low efficiency, best-in-class reagents do exist, and have been identified using SNAP Spike-ins⁴.

EpiCypher has recently launched the SNAP-CUTANA™ K-MetStat Panel (EpiCypher 19-1002) and are using these defined nucleosome spike-in controls to identify best-in-class histone lysine methylation PTM antibodies for CUT&RUN. The collection of **SNAP-Certified™ Antibodies** meet each of the following criteria:

- High specificity: <20% cross-reactivity to related PTMs in the K-MetStat Panel.
- High target efficiency: Consistent genomic enrichment in CUT&RUN at 500,000 and 50,000 starting cells.

Our scientists are continuously testing antibodies to novel targets. Visit epicypher.com/cut-and-run-antibodies for the most up-to-date list, or contact techsupport@epicypher.com for antibody recommendations.

TOPICS: Antibodies, K-MetStat Panel

Related sections: Experimental Design & Optimization, Experimental Protocol Section IV, Appendixes 1.3-1.6

How should I validate my antibody for CUT&RUN?

If an EpiCypher CUT&RUN antibody is not available for your target, we recommend the following steps:

- **Lysine methylation PTMs:** Use the SNAP-CUTANA™ K-MetStat Panel (EpiCypher 19-1002) to validate methyl-lysine PTM antibodies in CUT&RUN. Aim for <20% antibody cross-reactivity and consistent genomic enrichment with 500,000 cells.
- **Other PTMs or protein targets:** Source 3-5 antibodies from reputable vendors that target different epitopes. Test side-by-side in CUT&RUN assays using 500,000 native cells (**Experimental Design & Optimization**). Select a specific antibody based on DNA yield, enrichment, and signal-to-noise in NGS data.

None of the antibodies to my target are working in CUT&RUN. What should I do?

See **Experimental Design & Optimization** and **Appendixes 1.3-1.6**. If the workflow was validated with control antibodies and K-MetStat Spike-ins, try these steps:

- Confirm that your experimental target is present and localized to chromatin (e.g. correct cell type, stimulation conditions).
- Test additional antibodies to your target.
- Try cross-linked vs. native cell samples (**Appendix 3.3**).
- If the target has low abundance and/or generates low yields, adjustments to library prep and sequencing may be required. See **FAQs: CUT&RUN DNA Yields**.

Where can I find more information on the SNAP-CUTANA™ K-MetStat Panel?

Visit epicypher.com/19-1002 under Documents and Resources:

- **The SNAP-CUTANA™ Spike-in User Guide**, which contains background, detailed examples, and step-by-step instructions for analysis of spike-in data.
- **A shell script** for paired-end sequencing alignment to the K-MetStat Panel.
- **An Excel template** to streamline K-MetStat data processing and analysis.

SNAP-CUTANA Spike-ins are useful for antibody validation and workflow standardization/monitoring. A 50-reaction pack size is available, allowing users to add the K-MetStat Panel to experimental reactions targeting methyl-lysine PTMs.

TOPICS: Analysis of CUT&RUN DNA Before Library Prep

Related sections: Experimental Protocol Section VII, Appendix 1.3

Can I use Bioanalyzer or TapeStation traces to evaluate the success of CUT&RUN prior to library prep?

It is **not** recommended to assess fragment size distribution of raw CUT&RUN DNA before library prep. This habit is derived from ChIP-seq methods, which require careful optimization of chromatin shearing to achieve mononucleosome-sized fragments in input chromatin. Fragment size distribution in input chromatin and immunoprecipitated DNA is monitored using capillary electrophoresis (e.g. TapeStation or Bioanalyzer) to confirm assay success.

However, CUT&RUN and ChIP-seq are not the same method, and thus do not entail the same quality control steps. DNA yields from ChIP are typically high due to high cell inputs and elevated background, which are inherent to immunoprecipitation. In contrast, CUT&RUN 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 often below the limit of sensitivity for fragment size distribution using the TapeStation or Bioanalyzer.

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

qPCR is **not** recommended for validation of CUT&RUN target enrichment. qPCR is used in ChIP protocols to verify the enrichment of a known on-target region compared to the bulk chromatin input. However, this analysis is both unnecessary and challenging for CUT&RUN assays. CUT&RUN is an *in situ* assay, meaning it is performed in intact cells. There is no immunoprecipitation step and no bulk chromatin input for enrichment comparisons. Due to the processive nature of MNase, CUT&RUN fragments can be 50-100 bp (e.g. for some TFs), further challenging qPCR analysis.

Instead, we recommend analysis of purified CUT&RUN sequencing libraries to confirm DNA size distribution and molarity (see **Experimental Protocol, Section IX**) and proceed to NGS without qPCR. As you will need only 3-8 million reads per reaction, you can multiplex, save on sequencing costs, and obtain the genomic data regarding your target.

TOPICS: CUT&RUN DNA Yields, Considerations for Library Prep, Modifications for Footprinting/Small Fragments

Related sections: Experimental Protocol Section VII, Appendix 1.3 & 1.4

What should I do if my raw CUT&RUN DNA yields are below 5 ng?

If the CUT&RUN experiment yields <5 ng DNA, it is recommended to use the total amount of CUT&RUN enriched DNA for library prep. Low CUT&RUN DNA yields are common for low abundance targets (e.g. H3K4me3), but also depend on the number of starting cells, cell type, and antibody binding specificity and efficiency.

What caveats and modifications should be considered when using low DNA inputs for CUT&RUN library prep?

Although useful sequencing data can still be obtained, these libraries often have low yields with elevated adapter dimers, reduced read diversity, and low signal over background, all of which impact data quality. To optimize library prep and sequencing for low CUT&RUN yields, see the CUTANA™ CUT&RUN Library Prep Kit Manual (EpiCypher 14-1001 & 14-1002; epicypher.com/protocols). General tips:

- **Remove adapter dimers.** Takes up valuable sequencing bandwidth.
- **Increase number of cycles for indexing PCR.** Improves library yields for fragment distribution analysis and enable pooling at standard concentrations for NGS.
- **Increase sequencing depth.** Additional PCR cycles may increase read duplication rates, necessitating greater sequencing depths to capture read diversity.
- **Remove duplicate reads** in fastq.gz files with Picard (broadinstitute.github.io/picard).

Are there modifications to enrich for small fragments (i.e. footprinting analysis)?

CUT&RUN can generate both nucleosomal (~170 bp) and sub-nucleosomal (<120 bp) sized DNA fragments. The DNA Cleanup Columns in this kit capture >50 bp fragments, which EpiCypher scientists find to be sufficient for all targets tested.

However, modifications to enrich sub-nucleosomal fragments during CUT&RUN library prep have been reported⁹. Briefly, to enrich for sub-120 bp fragments, the authors altered the inactivation step after end repair from 65°C for 30 minutes to 50°C for 1 hr. Also, after the ligation reaction, the AMPure® bead size-selection ratio was increased from 1.1X to 1.75X volume (retains library fragments >100 bp).

Note that technical support regarding these protocol changes is limited, as the library prep modifications have **NOT** been optimized by EpiCypher.

TOPICS: Low Library Yields and Sequencing, Bioinformatic Analysis
Related sections: Experimental Protocol Sections IX-XI, Appendix 1.4

What should I do if my library yields are low?

If library yields are below the threshold required for Illumina sequencing (i.e. less than 0.5 nM), it is recommended to repeat library prep using more CUT&RUN enriched DNA input. If desired, CUT&RUN reactions from technical replicates may be combined for library prep.

Alternatively, the number of PCR cycles used during indexing PCR (**Section IV**) can be increased, which will improve yields for fragment distribution analysis and enable sample pooling at standard concentrations for NGS. Note that increasing the number of PCR cycles may lead to higher read duplication rates and require deeper sequencing to capture read diversity.

If it is not possible to repeat library prep, use a Speedvac to increase the library concentration and add as much of the library as possible to the sequencing pool. Deeper sequencing is recommended in these cases to ensure sufficient read depth for the the low-concentration library.

What pipeline is recommended for CUT&RUN sequencing analysis and peak calling?

CUT&RUN data analysis methods described in the literature are similar to those used for ChIP-seq datasets, with key differences. Briefly, raw sequence reads can be aligned to a reference genome using Bowtie 2¹⁵. The Integrative Genomics Viewer (IGV)¹⁶ and/or deepTools¹⁷ can be used for data visualization (e.g. bigWig files graphed over a genome browser to visualize enrichment).

There are special considerations for peak calling since CUT&RUN has very low background compared to ChIP-seq and an input sample is not sequenced. EpiCypher frequently uses MACS2¹⁸ and SICER¹⁹, peak calling programs for ChIP-seq that work well for CUT&RUN²⁰. SICER can be adjusted for analysis of sharp enrichment peaks (e.g. H3K4me3) vs. broad areas of enrichment (e.g. H3K27me3)²¹. Other options include SEACR²², a peak caller designed for CUT&RUN data, and the CUT&RUNTools 2.0 pipeline, which is designed for CUT&RUN and CUT&Tag data, including analysis of single cells²³. It is recommended to test several programs and select the one that faithfully represents the target of interest. To determine signal over background, EpiCypher uses bedTools to calculate fractions of reads in peaks (FRiP) and compare FRiP scores from experimental samples vs. controls²⁴. Other tools can be applied for differential analysis and heatmap generation (e.g. DESeq2²⁵, deepTools¹⁷).

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