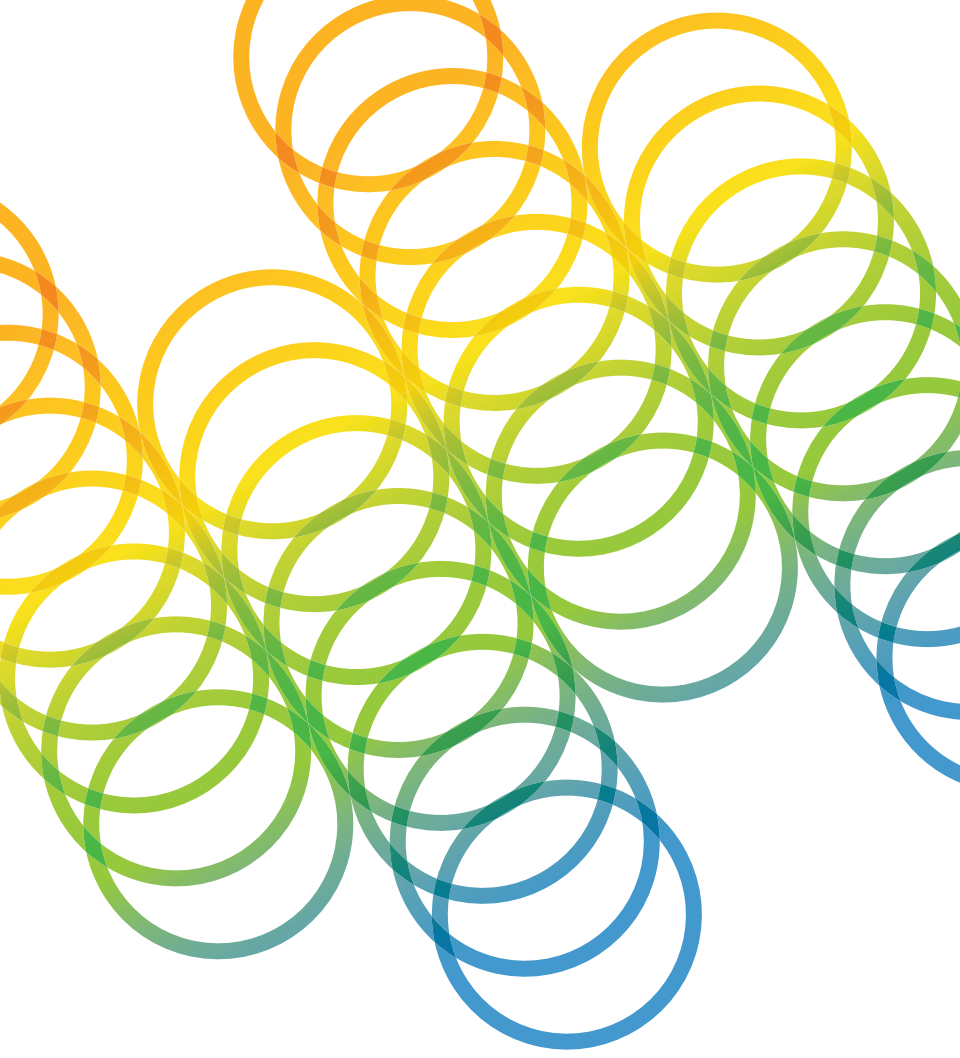

EpiCypher®
Bringing Epigenetics to Life

CUTANA™
ChIC / CUT&RUN Kit Version 3
User Manual Version 3.5

EpiCypher, Inc.
PO Box 14453
Durham, NC 27709

www.epicypher.com
Ph: 1-855-374-2461 F: 1-855-420-6111
Email: info@epicypher.com
Tech Support: techsupport@epicypher.com

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CUTANA™

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

Cleavage **U**nder **T**argets & **R**elease **U**sing **N**uclease (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], 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 to provide high-quality, genome-wide profiles of histone post-translational modifications (PTMs) and chromatin-associated proteins (e.g. transcription factors; Figure 1).

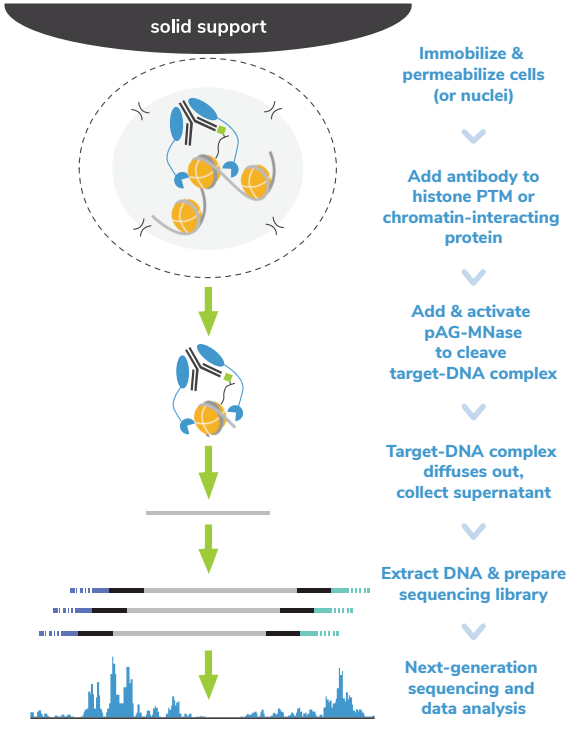


FIGURE 1

Overview of the CUTANA™ CUT&RUN protocol.

Historically, ChIP-seq was 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.

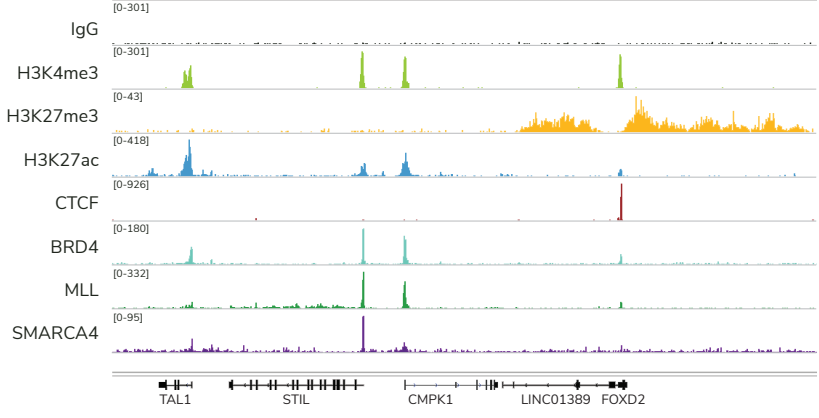


FIGURE 2

Representative genome browser tracks show CUTANA CUT&RUN results using 500,000 K562 cells. Clear peaks with 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 is shown as a negative control.

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 sequencing 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 down to 5,000 cells (Figure 4). The inclusion of rigorous controls as well as compatibility with diverse targets, sample types, and cell numbers make the kit ideal for a variety of research applications.

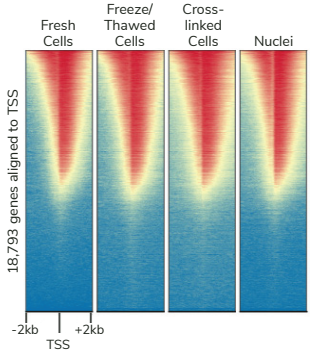


FIGURE 3

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

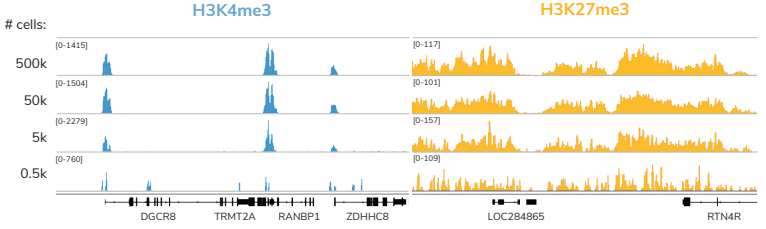


FIGURE 4

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

Store at room temperature (RT) upon receipt:

Item	Catalog No.	Notes before use
8-strip Tubes	10-0009k	Enables use of multi-channel pipettors.
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 ≥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. Because ConA can activate immune cells, we recommend using nuclei for immune cell studies (**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 µL DNase-free water (0.5 ng/µL). **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 shoud 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 - DO NOT VORTEX.** Panel of biotinylated nucleosomes coupled to streptavidin-coated magnetic beads. Pair with IgG and H3K24me3 control antibodies. Sufficient for 20 reactions. See **Appendix 1.6**.
H3K4me3 Positive Control Antibody	13-0041k	**SMALL VOLUME: quick spin before use.** 0.5 mg/mL rabbit mixed monoclonal antibody. 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 1µL to negative control reactions. Sufficient 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, goat, donkey, rat, guinea pig, horse, & cow.

* Kit components are stable for 6 months upon date of receipt. Store components as outlined in this section.

Materials Required but Not Supplied

REAGENTS:

- Antibody to target of interest; see **FAQs 1–4** for guidance on antibody selection
- Additional SNAP-CUTANA K-MetStat Panel (EpiCypher 19-1002) for experimental reactions targeting histone lysine methylation PTMs
  - * The K-MetStat Panel in the kit is meant for positive and negative control reactions only.
- 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 the kits are used together.

EQUIPMENT:

- 1.5, 15 and 50 mL tubes
- Low-retention filter pipette tips
- 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 CUTANA™ CUT&RUN assay:

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

Cells are harvested, washed, and bound to activated ConA beads. Modifications are required for certain sample types (**Appendix 3**). Quality control checks in **Appendix 1.2** are used to confirm cell integrity and ConA bead binding. 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 1–4** for guidance on antibody sourcing and validation.

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. The 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.

CUT&RUN DNA is prepared for Illumina® sequencing using the CUTANA™ 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 **Protocol: Section IX** for examples).

Step 5: Illumina® sequencing and data analysis

Libraries are diluted, pooled, and sequenced on an Illumina sequencing platform. Only 3-8 million uniquely aligned sequencing reads are needed to generate roubust CUT&RUN profiles (see **Protocol: Section X** for examples). For troubleshooting assay yields, library prep, and sequencing, see **Appendices 1.3–1.5**.

SAMPLE INPUTS FOR CUT&RUN

- Freshly isolated, unfixed (i.e. native) cells are the preferred input for CUT&RUN.
- Harvest 500,000 cells per reaction plus 10% excess to account for sample loss.
- See **Appendix 3** if using adherent cells, tissues, immune cells, nuclei, cross-linked cells, or cryopreserved samples.

COMPATIBLE TARGETS

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

- Histone PTMs
- Transcription factors
- Chromatin remodeling enzymes
- Chromatin writers & readers
- Epitope-tagged proteins

EXPERIMENTAL CONTROLS

- The kit includes multiple quality control checks (Figure 5) for reliable CUT&RUN workflows.
- Each kit comes with H3K4me3 positive & 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 (**Appendix 1.6**).
- The provided ***E. coli* Spike-in DNA** can be added to any reaction for sequencing normalization (**Appendix 2**).

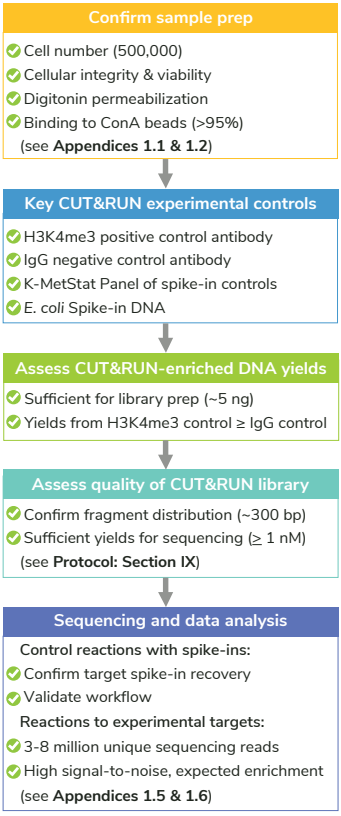


FIGURE 5

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

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.
- See **FAQs 1-4** for information regarding antibody sourcing and validation.

HOW TO OPTIMIZE CUT&RUN

- Optimization methods are provided in Figure 6. For CUT&RUN troubleshooting, see **Appendices 1.3–1.6**.
- CUT&RUN success depends on many factors, including cell type, cell number, target abundance, and antibody quality. For considerations when using low cell numbers, see **FAQ 5**.

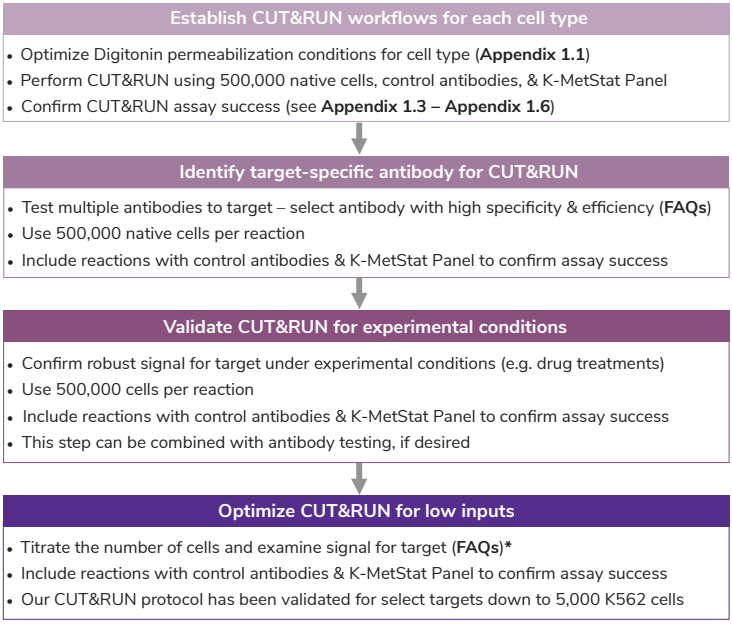


FIGURE 6

Development and optimization guidelines for successful CUT&RUN workflows.

* Note that an antibody that performs reliably at 500,000 cells may fail at lower inputs.

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

IMPORTANT NOTES ON BUFFER PREP

* These buffers (Figure 7) are prepared **FRESH** on Day 1 of every CUT&RUN experiment.
* 0.01% Digitonin is optimal for permeabilizing K562, MCF7, and A549 cells (Table 1). For other cell types, Digitonin conditions **MUST** be optimized for efficient cell permeabilization. See **Appendix 1.1** for instructions.
* Volumes in Table 1 are per CUT&RUN reaction and include 20% excess to account for pipetting errors. You do **NOT** need to add additional volume.

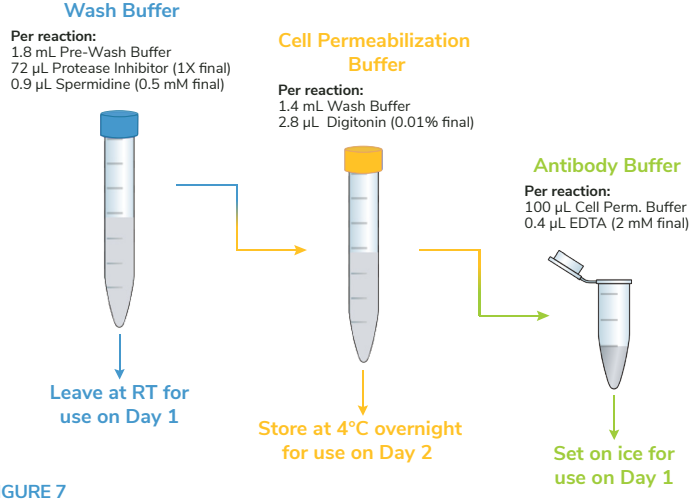


FIGURE 7

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

1. Gather kit reagents stored at 4˚C and -20˚C needed for Day 1: **ConA beads, Bead Activation Buffer, Pre-Wash Buffer, Digitonin, Spermidine, H3K4me3 and IgG control antibodies, K-MetStat Panel**. Place on ice to thaw/equilibrate.
2. Dissolve 1 protease inhibitor tablet (Roche) in 2 mL water for a **25X Protease Inhibitor** stock. After buffer prep, the remaining 25X stock can be stored for 12 weeks at -20˚C.

3. Prepare **Wash Buffer** by combining Pre-Wash Buffer, 25X Protease Inhibitor, and 1M Spermidine as outlined in Table 1. Store final buffer at RT.
4. To a new tube labeled **Cell Permeabilization Buffer**, add Wash Buffer and 5% Digitonin as in Table 1 (see Important Notes on Buffer Prep). Place on ice.
5. In a new 1.5 mL tube labeled **Antibody Buffer**, combine **Cell Permeabilization Buffer** and 0.5 M EDTA as described in Table 1. Place final buffer on ice.
6. Store remaining **Cell Permeabilization Buffer** at 4˚C for use on Day 2.

Buffer Sample Scaling Calculations:

COMPONENT	[FINAL]	1 RXN	8 RXN	16 RXN
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 µL	576 µL	1.15 mL
1 M Spermidine	0.5 mM	0.9 µL	7.2 µL	14.4 µL
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 µL	22.4 µL	44.8 µL
Antibody Buffer - store on ice for use on Day 1				
Cell Permeabilization Buffer	-	100 µL	800 µL	1.6 mL
0.5 M EDTA	2 mM	0.4 µL	3.2 µL	6.4 µL

TABLE 1

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

INTENTIONALLY BLANK FOR USER NOTES:

SECTION II: CONA BEAD ACTIVATION (~30 MIN)

TIPS FOR WORKING WITH MAGNETIC CONA BEADS

* Do **NOT** use ConA beads that have been frozen and/or appear black, granular, or clumpy.
* Do **NOT** let ConA beads dry out. Avoid disturbing beads with pipette while on magnet.
* Activated ConA beads should be kept on ice and used within four hours of activation.

7. Gently resuspend **ConA beads** and transfer 11 µL per reaction to a 1.5 mL tube.
8. Place tube on a magnet, allow slurry to clear. Pipette to remove supernatant.
9. Remove tube from magnet. Immediately add 100 µL per reaction cold **Bead Activation Buffer** and pipette to resuspend. Return tube to magnet, allow slurry to clear, and pipette to remove supernatant.
10. Repeat the previous step one time.
11. Resuspend beads in 11 µL per reaction cold **Bead Activation Buffer**.
12. Aliquot 10 µL per reaction of bead slurry 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) see **Appendix 3.**
* Assess the integrity of starting cells and washed cells and confirm binding to ConA beads (**Appendix 1.2**). High-quality sample prep is critical for successful CUT&RUN.

13. Count starting cells and confirm integrity. Harvest 500,000 cells per reaction (plus 10% excess) and spin at 600 x g for 3 min at RT. Remove supernatant.
14. Resuspend cells in 100 µL per reaction RT **Wash Buffer** by gentle yet thorough pipetting. Spin at 600 x g, 3 min, RT. Pipette to remove supernatant.
15. Repeat the previous step one time.
16. Resuspend cells in 105 µL per reaction RT **Wash Buffer**. Count and examine integrity of prepared cells (**Appendix 1.2**).
17. Add 100 µL cells to 10 µL ConA beads in 8-strip tubes. Gently vortex to mix and quick spin in a mini-centrifuge to collect slurry (beads should not settle).
18. Incubate bead-cell slurry for 10 min at RT. Cells will adsorb to beads.

19. Place tubes on a magnet, allow slurry to clear, pipette to remove supernatant. Save 10 µL supernatant to confirm cells are bound to beads (**Appendix 1.2**). Discard the remaining supernatant and **move quickly** to the next step.

20. Remove tubes from magnet. Immediately add 50 µL cold **Antibody Buffer** to each reaction and pipette to resuspend. Confirm ConA bead binding (**Appendix 1.2**).

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

ANTIBODY INCUBATION NOTES

* Add K-MetStat Panel to control reactions **BEFORE** adding the primary antibody. Scale the amount of K-MetStat Panel to the number of cells as in Table 2.
* For antibodies stored in viscous glycerol solutions, take care to ensure accurate pipetting: aspirate slowly, check tip for accuracy, and pipette up and down to clear the solution from tip.
* Do **NOT** rotate or invert tubes. Rotation causes ConA beads to stick to tube sides and dry out, reducing yields. Use a nutator for incubations and elevate tube caps as suggested.

21. Quick spin the **K-MetStat Panel** and pipette to resuspend - do **NOT** vortex. Add 2 µL **K-MetStat Panel** to reactions designated for H3K4me3 positive & IgG negative control antibodies. Gently vortex to mix and quick spin tubes. If using less than 500,000 cells, decrease K-MetStat Panel amount as in Table 2.

22. Add 0.5 µg primary antibody (or manufacturer's recommendation) to each reaction. For positive & negative control reactions, add 1µL **H3K4me3 Positive Control Antibody** & 1µL **IgG Negative Control Antibody**, respectively.

23. Gently vortex to mix. Incubate overnight on a nutator at 4˚C with tube caps elevated. Do **NOT** rotate - see Antibody Incubation Notes, above.

Number of cells	K-MetStat Panel dilution	Volume added to reaction	Final dilution
500,000	Use 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

Scale the amount of K-MetStat Panel to the number of cells. For <500,000 cells, prepare a working stock dilution of the K-MetStat Panel in **Antibody Buffer** the day of the experiment.

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

NOTES ON CONA BEAD-CELL CLUMPING

* Beads often become clumpy/sticky after overnight incubation at 4˚C but can be dispersed by gentle pipetting. The end of a pipette tip can be cut off to help mix or preserve delicate cells.
* It is essential that ConA beads remain in solution during pAG-MNase binding and digestion. Excessive bead clumping leads to sample loss, reduces yields, and negatively impacts quality.

GUIDELINES FOR HIGH-THROUGHPUT PROCESSING

* Multi-channel pipetting is recommended to improve reliability and experimental throughput. To easily dispense buffers, use multi-channel reagent reservoirs (keep on ice).
* For 8-strip tubes, remove and replace buffers one strip at a time to avoid ConA bead dry out.

24. Remove tubes from 4˚C incubation and quick spin to collect liquid. Note that beads may settle overnight - this is normal and will not impact results.
25. Place tubes on magnet and allow slurry to clear. Pipette to remove supernatant.
26. Keeping tubes on magnet, add 200 µL cold **Cell Permeabilization Buffer** to each reaction. Pipette to remove supernatant.
27. Repeat the previous step one time (keep tubes on magnet).
28. Remove tubes from magnet and immediately add 50 µL cold **Cell Permeabilization Buffer** to each reaction. Gently vortex and/or pipette with a P200 to mix.
29. Add 2.5 µL **pAG-MNase** to each reaction. Gently vortex and/or pipette to thoroughly mix beads and evenly distribute enzyme.
30. Incubate reactions for 10 min at RT.
31. Quick spin tubes, place on magnet, and allow slurry to clear. Pipette to remove supernatant.
32. Keeping tubes on magnet, add 200 µL cold **Cell Permeabilization Buffer** directly onto beads. Pipette to remove supernatant.
33. Repeat one time.
34. Remove tubes from magnet and immediately add 50 µL cold **Cell Permeabilization Buffer** to each reaction. Gently vortex to mix and disperse clumps by pipetting.

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

GUIDELINES FOR USING *E. COLI* SPIKE-IN DNA

* Prior to first use, quick spin lyophilized *E. coli* DNA and reconstitute in 200 µL DNase-free water. Thoroughly vortex the tube on all sides to resuspend DNA. Store at -20˚C.
* This protocol is optimized for 500,000 cells per reaction. If using less than 500,000 cells per reaction, dilute ***E. coli* Spike-in DNA** as outlined in **Appendix 2**.
* 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**.

35. 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.
36. Incubate tubes on nutator (capped ends elevated) for 2 hours at 4ºC.
37. Prepare a **Stop Master Mix** in a new 1.5 mL tube (see Guidelines for *E. coli* Spike-in DNA, above). Per reaction, add 33 µL **Stop Buffer** and 1 µL ***E. coli* Spike-in DNA** (0.5 ng). Gently pipette/vortex to mix.
38. At the end of the 2 hour incubation, quick spin tubes to collect liquid. Add 33 µL **Stop Master Mix** to each reaction and gently vortex to mix.
39. Place reactions in a thermocycler set to 37˚C. Incubate for 10 min.
40. Quick spin tubes to collect liquid, and place on a magnet until slurry clears.
41. Transfer supernatants containing CUT&RUN-enriched DNA to 1.5 mL tubes.

SECTION VII: DNA PURIFICATION (~30 MIN)

NOTES BEFORE STARTING DNA PURIFICATION

* Prior to first use, add 6.9 mL isopropanol to **DNA Binding Buffer**.
* Prior to first use, add 20 mL ≥95% ethanol to **Wash Buffer**.
* The **DNA Cleanup Columns** retain fragments >50 bp. For enrichment of smaller fragments for footprinting analysis, see **FAQ 8**.
* 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.

42. Add 420 µL **DNA Binding Buffer** to each reaction. Mix well by vortexing.
43. 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.
44. Spin at 16,000 x g, 30 sec, RT. Discard flow-through. Place column back into collection tube.
45. Add 200 µL **DNA Wash Buffer** to each column. Spin at 16,000 x g, 30 sec, RT. Discard flow-through. Place column back into collection tube.
46. Repeat previous step one time.
47. Spin at 16,000 x g, 30 sec, RT to completely dry the column.
48. 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.
49. Add 12 µL **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 µL; 12 µL 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.
50. Let sit 5 minutes, then spin at 16,000 x g, 1 min, RT.
51. Vortex eluted material to mix. Use 1 µL to quantify the CUT&RUN DNA using the Qubit fluorometer with the 1X dsDNA HS Assay Kit per the manufacturer's instructions.

Safe pause point. DNA can be stored at -20˚C for future processing.

CONFIRMING CUT&RUN SUCCESS PRIOR TO LIBRARY PREP

* DNA yields from experimental reactions should **NOT** be used as a definitive metric of success. There is no typical yield; instead, aim for ≥5 ng CUT&RUN enriched DNA, which will enable robust library prep. See **Table 3 (Appendix 1.3)** for troubleshooting low yields.
* In general, 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.
* For some cell types/targets, low CUT&RUN DNA yields are unavoidable. To optimize library prep for low DNA inputs, see **Table 4 (Appendix 1.3)**.
* Electrophoretic analysis (e.g. TapeStation) and/or qPCR of CUT&RUN DNA prior to library prep is **NOT** recommended (see **FAQs 6 & 7**).
* 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: LIBRARY PREP (~4 HRS)

52. 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 FRAGMENT SIZE ENRICHMENT

* The **BEST** indicator of CUT&RUN experimental success prior to sequencing is enrichment of mononucleosome-sized fragments (~300 bp = ~170 bp nucleosome + sequencing adapters).
* Final CUT&RUN library concentration (200-700 bp region) is usually 100-200 nM. Libraries ≥1 nM allow pooling at standard concentrations for sequencing, but good data are obtained down to 0.5 nM. If library concentrations are <0.5 nM, see **Appendix 1.4**.
* 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.
* Adapter dimer contamination appears as a peak at ~125 bp, and is caused by low input DNA, inefficent adapter ligation, and/or using excess beads during library purification. See **Appendix 1.4** and the CUTANA CUT&RUN Library Prep Kit Manual for details.
* See **Appendix 1.4** for troubleshooting low library yields and/or fragment distribution results.

53. Use 1 µL purified CUT&RUN library for quantification. Use the Qubit fluorometer with the 1X dsDNA HS Assay Kit per the manufacturer's instructions.
54. For each library, prepare 5 µL at 10 ng/µL 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).
55. Load and analyze 1 µL diluted sequencing library using the High Sensitivity DNA Kit (Bioanalyzer) or the D1000 ScreenTape System & Reagents (TapeStation) as per the manufacturer's instructions.
56. The final traces should show predominant enrichment of mononucleosome-sized fragments, such as those yielded by H3K4me3 and CTCF antibodies in Figure 8 (~300 bp: ~170 bp + 125 bp sequencing adapters). Adapter dimers, if present, are observed as a peak at ~125 bp; see **Appendix 1.4**.

Safe pause point. Libraries can be stored at -20˚C for future processing.

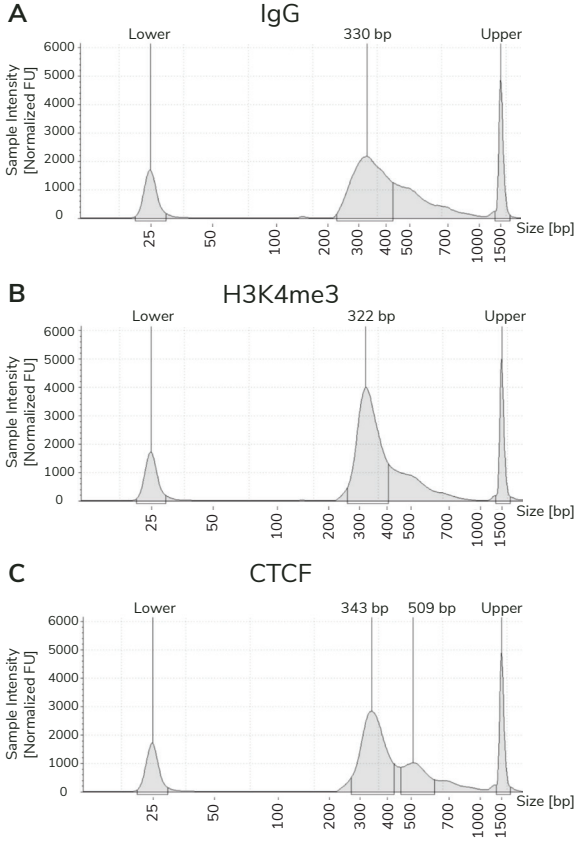


FIGURE 8

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 & DATA ANALYSIS

TIPS FOR SEQUENCING CUT&RUN LIBRARIES

* Only 3-8 million uniquely aligned reads are needed for adequate CUT&RUN coverage.
* Paired-end sequencing (2 x 50 bp cycles minimum) is recommended for CUT&RUN to enable detection of K-MetStat Panel barcodes.
* See **Appendix 1.5** for considerations when sequencing low-concentration libraries.
* See **FAQ 9** for basic information on CUT&RUN sequencing analysis.

57. Select appropriate Illumina sequencing platform based on the number of CUT&RUN libraries and desired sequencing depth.
58. 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.
59. 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.
60. If control reactions generate expected results, proceed to analysis of experimental reactions (Figures 9 & 10). Align paired-ends reads to the appropriate reference genome (e.g. using Bowtie 2). See **FAQ 9** for more information.
61. For sequencing normalization using ***E. coli* Spike-in DNA**, see **Appendix 2**.

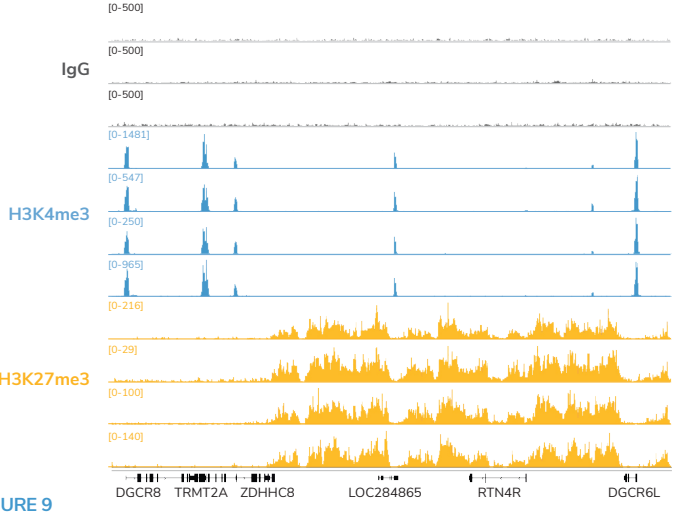
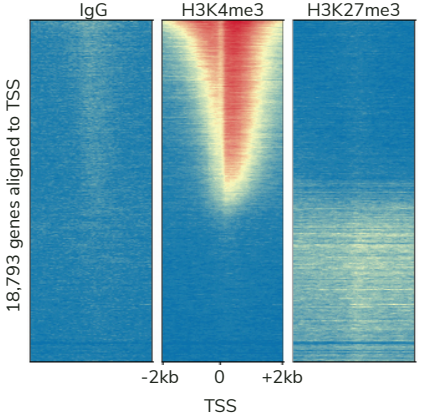


FIGURE 9

Data generated by four independent users demonstrate the reproducibility of the CUTANA CUT&RUN Kit. CUT&RUN was performed using 500,000 K562 cells and antibodies to IgG (negative control), H3K4me3 (positive control), and H3K27me3. 3-6 million reads were generated per library. H3K4me3 tracks show sharp peaks localized to transcription start sites (TSSs), while H3K27me3 tracks show broad peaks over repressed regions. IgG shows typical low background.

FIGURE 10

Expected results from CUTANA CUT&RUN assays using 500,000 K562 cells with antibodies to IgG, H3K4me3, and H3K27me3. 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). These data 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.

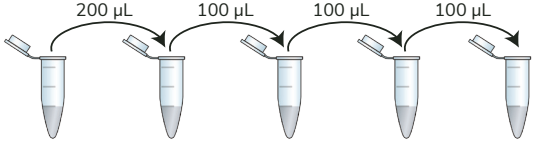


1.1 OPTIMIZATION OF CELL PERMEABILIZATION

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 prevent the beads from forming a film on the side of tubes.

1. Prepare a series of Cell Permeabilization Buffers using **5% Digitonin** and **Wash Buffer** (see **Protocol: Section I**). Prepare **FRESH** on the day of use.
   a. Label five fresh 1.5 mL tubes with percent Digitonin (see table, below). In a sixth 1.5 mL tube, prepare 0.05% DMSO in **Wash Buffer** as a control.
   b. Add the appropriate volume of **Wash Buffer** to each tube.
   b. Add 10 µL **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.
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) x 6.2 (include 20% excess volume for pipetting errors).
3. Spin 600 x g, 3 min, RT. Remove supernatant. Resuspend in 620 µL RT 1X PBS.
4. Aliquot 100 µL 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 µL	800 µL	900 µL	900 µL	900 µL
5% Digitonin	10 µL	-	-	-	-

5. Spin cells at 600 x g, 3 min, RT. Remove supernatant. Resuspend each cell pellet in 100 µL assigned buffer and incubate 10 minutes at RT.

6. For each of the six tubes: Mix 10 µL cells + 10 µL 0.4% Trypan blue. Load 10 µL 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 11, 0.01% Digitonin is the minimum concentration necessary to permeabilize >95% of total K562 cultured cells (red arrow).

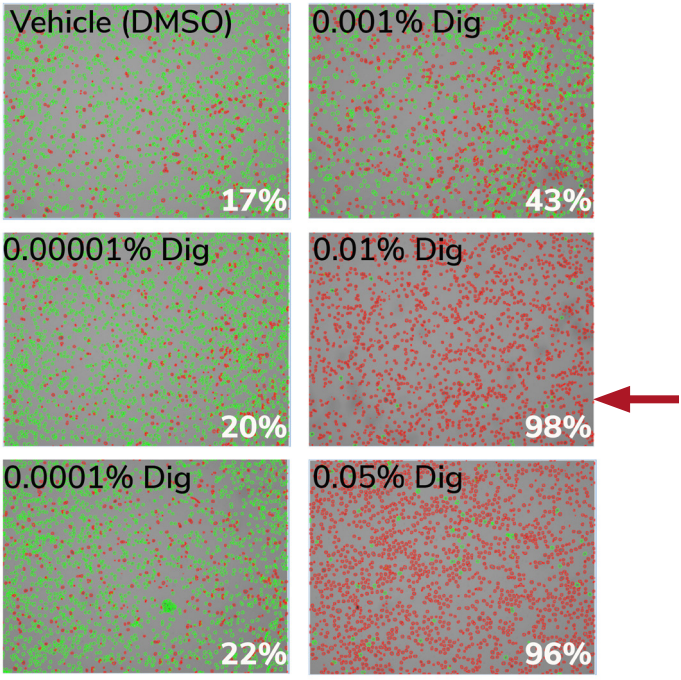


FIGURE 11

The minimum amount of Digitonin ("Dig") needed to permeabilize K562 cells was determined by serial dilution of 5% Digitonin in Wash 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 uses a simple Trypan Blue staining to flag CUT&RUN samples of poor quality or that fail to bind ConA beads. Note that:

* Low cell viability and nuclear lysis increase assay background - **don't skip these steps!**
* Cells are resuspended with 5 µL excess buffer per reaction to account for pipetting error. It may be helpful to process one extra sample to evaluate cell integrity.
* If using nuclei instead of whole cells, also see **Appendix 3.2**.

1. Starting at **Step 16** in **Protocol: Section III**, resuspend cells in 105 µL per reaction RT **Wash Buffer** (i.e. for 8 reactions, resuspend in 840 µL). Transfer 10 µL washed cells to a new 1.5 mL tube.
2. Evaluate cell integrity immediately prior to ConA bead binding as follows:
    a. Add 10 µL of **0.4% Trypan Blue** to 10 µL washed cells. Pipette to mix.
    b. Transfer 10 µL to a counting slide.
    c. View under brightfield/phase microscope or cell counter.
    d. Confirm sample integrity or troubleshoot as needed (see Figure 12A).
3. Add 100 µL washed cells to 10 µL 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 cells to beads.
5. Place tubes on magnet and allow slurry to clear. Transfer 10 µL supernatant [**unbound fraction**] to a 1.5 mL tube and discard remaining supernatant.
6. Immediately add 50 µL cold **Antibody Buffer** to the **sample slurry**. Remove tubes from magnet and pipette to resuspend.
7. Move 10 µL **sample slurry** to a 1.5 mL tube. Place remaining **sample slurry** on ice.
    • Alternative: Take 1-5 µL **sample slurry**. Dilute with 1X PBS to 10 µL volume.
8. To 10 µL samples set aside (**unbound fraction**, **sample slurry**), perform Trypan Blue staining as in Step 2. Successful ConA bead binding is indicated when:
    • The **unbound fraction** contains few cells (Figure 12C).
    • The **sample slurry** cotains permeabilized, Trypan Blue positive cells surrounded by beads (Figure 12D).
9. Continue with the **Protocol: Section IV (Antibody Binding).**

Sample	Success Metrics	Troubleshooting Tips
Cells Figure 12A	Cells are bright white (Trypan excluded) and show minimal clumping, expected morphology, and high viability. Fresh K562 cells should be >90% viable.	Acceptable viability is dependent on cell type and experimental conditions. In general, aim for >60% viable cells with normal cell morphology. If cell integrity is a problem, evaluate culture technique and use fresh media.
Nuclei Figure 12B	>95% nuclei are Trypan Blue positive or "dead" and unclumped.	Use Trypan Blue staining to monitor and optimize nuclear extraction. Increase spin time if losing sample.
Unbound Fraction Figure 12C	Little to no material is present.	Ensure ConA beads were never frozen, cells/nuclei were not clumped, beads did not become clumped or dried out, and all buffers were correctly prepared.
Sample Slurry Figure 12D	Permeabilized cells/nuclei are surrounded by beads.	

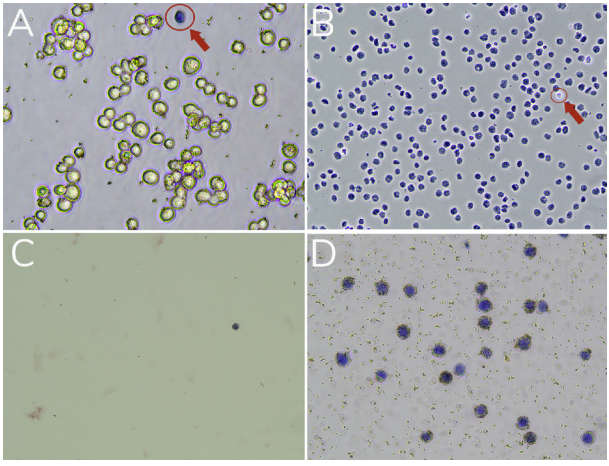


FIGURE 12

Validation of sample prep using Trypan Blue staining. **(A)** Washed K562 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. **(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 Antibody Buffer.

1.3 CUT&RUN ASSAY: EXPECTED RESULTS & TROUBLESHOOTING

CUT&RUN success metrics (pre-library prep):

- Samples should pass **ALL** quality control checks described in **Appendix 1.1**. If starting cells have poor viability and/or are clumpy, assay background will be higher - reducing the quality of sequencing data.
- In general, yields from the H3K4me3 positive control should be slightly greater than IgG. However, similar yields do **NOT** imply assay failure. If a positive control with higher yields is needed, we recommend H3K27me3 (EpiCpher 13-0055.)

Typical CUT&RUN yields and suggestions for library prep:

- **There is no typical DNA yield for CUT&RUN**, as yields can vary by cell type, number of cells, target abundance, and antibody quality.
- Aim for ≥5 ng CUT&RUN-enriched DNA, which will enable robust library prep.

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

- Low CUT&RUN DNA yields are common for low abundance targets, but also depend on the number of starting cells, cell type, and antibody performance
- See Basic troubleshooting guidelines below and Table 3 to optimize workflows.
- If the experiment cannot be repeated, use the total amount of CUT&RUN enriched DNA for library prep. See Table 4 for suggested modifications for library prep.

Basic CUT&RUN troubleshooting guidelines:

Follow the steps outlined in **Experimental Design & Optimization** Figure 6 and include **ALL** quality control steps in Figure 5. Review the following questions:

- What is your cell/sample type? Check **Appendix 3** for protocol modifications.
- Are Digitonin permeabilization conditions optimized for your cell type? See **Appendix 1.1**.
- Have you confirmed sample prep and ConA bead binding? See **Appendix 1.2**.
- Are you using the recommended 500,000 cells per reaction? Success from low cell numbers depends on antibody quality and target abundance. See **FAQ 5**.
- Have you included reactions with control antibodies & the K-MetStat Panel? These controls are crucial for troubleshooting CUT&RUN (**Appendix 1.6**).
- Are ConA beads brown and stored at 4˚C? ConA beads should **NEVER** be frozen.
- Have reactions been mixed properly using a nutator? Have ConA beads become clumpy or dried out during the protocol?

TABLE 3 Troubleshooting low CUT&RUN yields (pre-library prep)

Potential causes and troubleshooting approaches
⚠ **Poor sample prep, low cell numbers, ConA bead damage or loss, bad antibody, target is low abundance and/or requires different conditions**
• See **Basic CUT&RUN troubleshooting guidelines** (opposite page); optimize Digitonin permeabilization (**Appendix 1.1**) and confirm sample prep quality (**Appendix 1.2**)
• Repeat experiment using 500,000 native cells per reaction and include reactions with control antibodies & K-MetStat Panel
• Use a nutator for overnight incubation to avoid ConA bead dry out and sample loss
Examine raw CUT&RUN DNA yields:
• If controls work but experimental targets fail: Confirm target is correctly localized to chromatin (e.g. stimulation conditions); test additional antibodies and/or cross-linking conditions (**Appendix 3.3**)
• If controls **AND** experimental targets show no yield: Evaluate cell culture and CUT&RUN technique for possible contamination issues

TABLE 4 Using low DNA inputs for CUT&RUN library prep & sequencing

Considerations and tips for best results
⚠ **For some targets and cell types, low CUT&RUN yields are unavoidable**
⚠ **Although useful sequencing data can be obtained, the resulting libraries often have low concentrations with elevated adapter dimers, reduced read diversity, and low signal over background, all of which impact data quality**
Use the CUTANA CUT&RUN Library Prep Kit (EpiCypher 14-1001 & 14-1002), which is specifically optimized for CUT&RUN workflows and includes guidelines for library prep from low CUT&RUN yields (see manual at epicypher.com/protocols).
General tips:
• Remove adapter dimers, which take up valuable sequencing bandwidth
• Increase number of cycles for indexing PCR (**Protocol: Section IV**) to improve library yields for Bioanalyzer/TapeStation analysis and sequencing
• Deeper sequencing is recommended to capture read diversity
• Read duplicates may be increased, but can be filtered out with Picard (broadinstitute.github.io/picard)

1.4 LIBRARY PREP: EXPECTED RESULTS & TROUBLESHOOTING

CUT&RUN library prep success metrics:

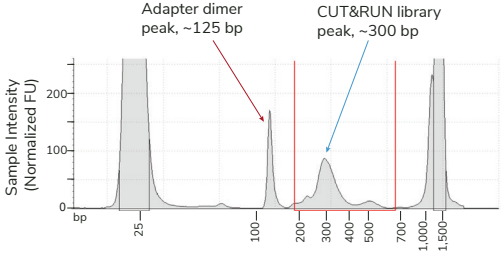
- The **BEST** indicator of CUT&RUN success before sequencing is predominant enrichment of mononucleosome-sized fragments (~300 bp = ~170 bp nucleosome + sequencing adapters) in Bioanalyzer/TapeStation results.
- Do **NOT** use library yields to determine assay success, as yields can vary by cell type, number of cells, target abundance, and antibody quality.
- A library concentration of ≥1 nM will enable pooling for multiplexed sequencing.
- For library concentrations below 0.5 nM, we recommend further optimization per the guidelines in **Tables 3 & 4**. If experiments cannot be repeated, see **Table 6**.

TABLE 5 Troubleshooting fragment distribution and library yields

Concern	Causes / Troubleshooting approaches
Low library yields, no enrichment in Bioanalyzer or TapeStation results	⚠ Low CUT&RUN yields, low inputs for library prep • See previous page for guidance ⚠ Library prep technique • Use the EpiCypher CUTANA CUT&RUN Library Prep Kit (EpiCypher 14-1001 & 14-1002), specifically developed for CUT&RUN
Adapter dimers	⚠ Self-ligation of sequencing adapters, preferentially amplified due to their small size (Figure 13) • Keep adapter ligation reagents on ice during ligation setup • Remove adapter dimers comprising >5% of a library; see the CUT&RUN Library Prep Kit Manual

FIGURE 13

Example TapeStation trace from 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 SEQUENCING DATA: EXPECTED RESULTS & TROUBLESHOOTING

CUT&RUN data sequencing data metrics:

- Libraries should be sequenced to a depth of 3-8 million reads. Majority of reads (>80%) should align uniquely to the species genome.
- Sequence duplication levels should be low (<20% of total sequence reads).
- The SNAP-CUTANA K-MetStat Panel should comprise ~1% of unique reads and produce expected results in H3K4me3 and IgG control reactions (**Appendix 1.6**).
- H3K4me3 and IgG controls should show expected enrichment and peak structures. Experimental replicates should be highly reproducible (Figures 9 & 10).
- *E. coli* Spike-in DNA should comprise ~1% of total unique reads (**Appendix 2**).

Need help with sequencing analysis?

- For help with CUT&RUN sequencing analysis, including genomic alignment, peak calling, and signal-to-noise calculations, see **FAQ 9**.

TABLE 6 Troubleshooting CUT&RUN sequencing and results

Concern	Causes / Troubleshooting approaches
Sequencing a low-concentration DNA library	⚠ If it is not possible to repeat library prep: • Use a Speedvac to increase the library concentration • Add as much of the library as possible to the sequencing pool • Deeper sequencing is recommended
Background in open chromatin	⚠ Indicates over-digestion by MNase • Repeat assay with fresh buffers • Make sure MNase digestion is performed on ice
Experimental target shows high background and/or is indistinguishable from IgG negative control	⚠ Over-digestion by MNase, DNA damage, antibody failure Use 500,000 cells per reaction; include reactions with control antibodies & the K-MetStat Panel. General tips: • Process cells quickly and resuspend in cold Antibody Buffer • Test multiple antibodies to experimental target • Ensure MNase digestion is incubated on ice for 2 hours • Keep adapter ligation reagents on ice during ligation setup

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, or 20 reactions: 10 for the IgG control antibody and 10 for the H3K4me3 control antibody.

What is the K-MetStat Panel?

The K-MetStat Panel contains designer nucleosomes representing 16 distinct histone lysine methylation states (Figure 14). Each PTM is represented by two unique DNA-barcoded templates (A and B, for an internal technical replicate). Nucleosomes are individually coupled to magnetic beads and pooled into a single panel for convenient one-step addition to CUT&RUN workflows (Figure 15).

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

The K-MetStat Panel is the **only** control that uses 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 H3K4me3 positive control antibody specificity, sample quality, pAG-MNase activity, and more.

Why should I use the K-MetStat Panel?

It may be unclear from genomic tracks alone if a reaction issue has occurred. The controls 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.

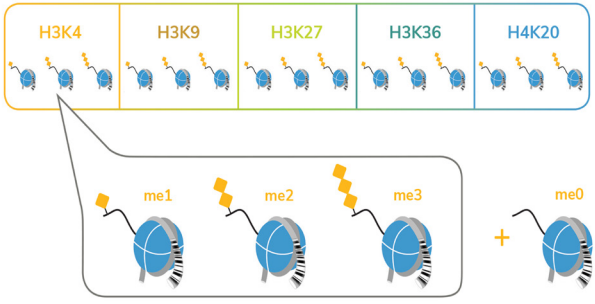


FIGURE 14

The SNAP-CUTANA K-MetStat Panel comprises 16 recombinant nucleosomes. DNA barcodes denote unique histone lysine methylation PTMs and are used to determine spike-in recovery from sequencing data.

SPIKE-IN PROCEDURE OUTLINE

- Add the SNAP-CUTANA K-MetStat Panel to designated control reactions immediately prior to the addition of H3K4me3 or IgG control antibody (Figure 15).
- Add antibody, which binds its target in cells **and** in the spike-in panel (Figure 15).
- pAG-MNase cleaves antibody-bound chromatin **and** antibody-bound spike-in. Cleaved DNA is purified and prepared for sequencing.
- For each control reaction, determine the number of sequencing reads aligned to each PTM-specific DNA barcode (next page). Barcode read counts provide a useful measurement of PTM recovery and workflow success (Figures 15 & 16).

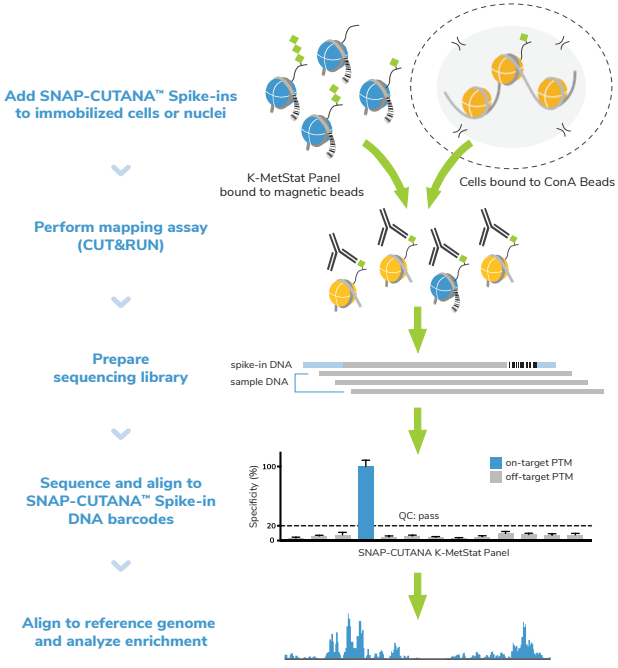


FIGURE 15

Schematic showing addition of SNAP-CUTANA Spike-ins during CUT&RUN workflows. Note that a larger pack size of the K-MetStat Panel is available for purchase, if you want to add it to experimental reactions targeting histone lysine methylation PTMs.

K-METSTAT PANEL DATA ANALYSIS:

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. At epicypher.com/14-1048, Documents and Resources, download Shell Script (**.sh**) and K-MetStat Panel Analysis (**.xlsx**) files. Save to the **folder** from Step 1.
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 **counts the number of reads** aligned to each **PTM-specific DNA barcode** in a reaction. Each PTM in the K-MetStat Panel is represented by two unique barcodes, A & B, for a **total of 32 barcodes**.

   For the script, you need to create **one loop per control reaction**. To customize:

   a. Copy lines **between** # template loop begin ## and # template loop end ##.

   b. Paste the loop under the last **done**. Paste one copy per control reaction.

   c. In the first loop replace sample1_R1.fastq and sample1_R2.fastq with R1 & R2 fastq file names for **one** control reaction. Repeat for each loop. Press save.
5. In **Terminal**, set the directory to your **folder**: Type **cd** and press space. Drag the folder from your files into Terminal to copy the location. Press return.
6. **Run your script** in Terminal: Type **sh** and press space. Drag your .sh file from your files into Terminal to copy the file location. Press return. Terminal generates barcode read counts from R1 & R2 reads, one loop/reaction at a time.
7. Open the K-MetStat Panel .xlsx file in **Excel**. Fill in reaction names and set the **on-target PTM** in **Column B**. The first reaction is set to IgG (negative control); for other reactions, select a target (i.e. H3K4me3) from the drop-down menu.
8. Copy R1 barcode read counts from the first loop in Terminal. In Excel, paste into the yellow cells for that reaction in **Column C**. Copy & paste the R2 read counts from the same loop to yellow cells in **Column D**. Repeat for each loop/reaction.
9. The Excel file automatically analyzes spike-in data for **each reaction** by:

   a. Calculating total read counts for each DNA barcode (R1 + R2) in **Column E**.

   b. Calculating total barcode read counts for each PTM (A + B) in **Column F**.

   c. Expressing total read counts for each PTM as a percentage of on-target PTM read counts (**Columns G & J**), providing a readout of on- vs. off-target PTM recovery and antibody specificity.

10. **Column J** auto-populates the **Output Table** (Figure 16). Reactions are separated by **row** and PTM data are sorted into **columns**. A color gradient is used to visualize the recovery of each PTM normalized to on-target PTM, from blue (100%) to orange (less than 20%).

11. For each reaction, calculate the percent of unique sequencing reads that have been assigned to spike-ins. In Excel, type the **total number of unique reads** in the yellow cell **Uniq align reads** (in Column B). The **% total barcode reads** is calculated in the cell immediately below and is added to the Output Table.

EXPECTED RESULTS:

- IgG negative control: No preference among PTMs, low background (Figure 16).
- H3K4me3 positive control: Strong enrichment for H3K4me3 spike-ins, less than 20% off-target PTM recovery, and high signal-to-noise.
- Spike-in barcode reads: Comprise ~1% (0.5-5%) of total sequencing reads.
- If control reactions generate expected spike-in data (Figure 16, Samples 1 and 2), you can be confident in the technical aspects of your workflow.
- More than 20% off-target PTM recovery in H3K4me3 control and/or high background in IgG control indicate experimental problems (Figure 16, Sample 3). See next page for a discussion of troubleshooting using spike-in results.

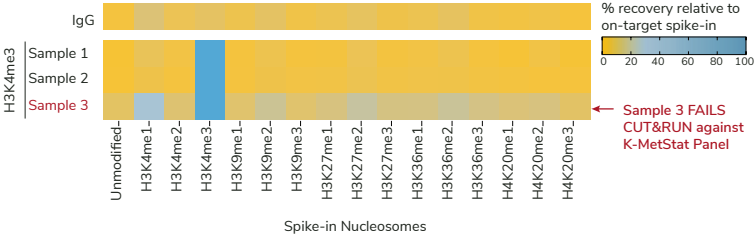


FIGURE 16

K-MetStat Spike-ins validate workflows and flag poor samples in CUTANA CUT&RUN experiments. Spike-in data for H3K4me3 positive control reactions is shown for three independently prepared mouse B cell samples (10,000 cells each; protocol optimization experiment with a multi-lab consortium). Samples 1 & 2 show expected results, while Sample 3 was flagged for recovery of off-target PTMs and low 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 16 & 17 demonstrate the use of K-MetStat Spike-in data for troubleshooting.

- In Figure 16 we used spike-in data from H3K4me3 & IgG control reactions to validate workflows for three independently prepared mouse B cell samples.
- Samples 1 & 2 showed expected results from control reactions, while Sample 3 displayed low signal-to-noise (S:N) and high off-target PTM recovery (Figure 16).
- Genomic profiles agreed with spike-ins: Samples 1 & 2 generated expected tracks for H3K4me3 & H3K27me3, while Sample 3 profiles had poor S:N (Figure 17).

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 had 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.

Combined, these results suggested problems with sample prep vs. a complete workflow failure. We subsequently reviewed Sample 3 processing methods, revealing that the number of cells used per reaction was much lower than intended. For other troubleshooting tactics using the K-MetStat Panel, see Table 7.

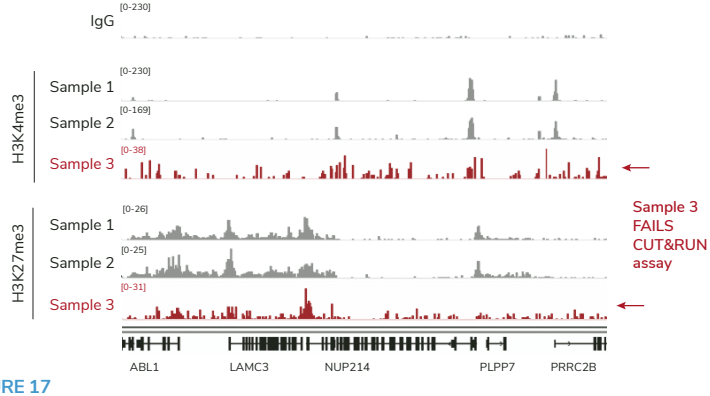


FIGURE 17

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).

TABLE 7 Troubleshooting CUT&RUN results using the K-MetStat Panel

Results	Causes & troubleshooting approaches
K-MetStat spike-in data: • High target specificity • High S:N Genomic data: • Poor S:N	pAG-MNase cleavage and wash conditions are optimized. Control antibodies are performing as expected. Problems may include: ⚠ **Low numbers of cells** • Optimize assay with 500,000 cells before decreasing input • If using nuclei, adherent cells, cross-linked cells, tissues, or cryopreserved samples, see **Appendix 3** for modifications ⚠ **Poor sample prep** • Optimize Digitonin permeabilization of cells (**Appendix 1.1**) • Confirm sample integrity and bead binding (**Appendix 1.2**) • Avoid ConA bead clumping and dry out during assay ⚠ **Experimental target requires different processing conditions** • Ensure target is present and localized to chromatin • If using frozen cells, try freshly isolated cells • Test native vs. lightly cross-linked conditions (**Appendix 3.3**)
K-MetStat spike-in data: • Nonspecific PTM recovery • Poor S:N Genomic data: • Poor S:N	⚠ **Indicates a fundamental failure in the workflow** • Carefully re-read the protocol and important notes • Ensure buffers are prepared fresh on day of use • Ensure ConA beads are in good condition (e.g. never frozen) • Make sure correct parameters are used in indexing PCR; consider using the CUTANA CUT&RUN Library Prep Kit ⚠ **Low numbers of cells and/or poor sample prep** • Optimize following the guidelines above and **Appendix 1.3**
K-MetStat spike-in data: • Nonspecific PTM recovery • S:N may vary Genomic data: • High S:N	⚠ **Indicates cross-reactive control antibodies** • Examine potential contamination of control reactions with antibodies to other targets • Ensure buffers are prepared fresh on day of use • Change pipette tips after **each** reagent addition to avoid cross-contamination • For concerns about control antibody performance, email us at techsupport@epicypher.com.

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, mouse).

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 reads.

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

   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 sequencing 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. Incubate with **0.05% Trypsin at 37°C for the minimal time necessary to dislodge cells**.
- Add pre-warmed complete media to inactivate Trypsin and then collect cells. Trypsin will be removed during subsequent wash steps.
- Check cell integrity and ConA bead binding (**Appendix 1.2**). Proceed with assay.

FREEZE/THAWING CELLS

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

1. Count cells and confirm integrity (**Appendix 1.2**). Spin cells 600 x g, 3 min, RT.
2. Remove supernatant, resuspend in **media with 10% DMSO**, and aliquot cells. EpiCypher aliquots for ≥8 reactions plus ≥20% excess cells to account for 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 x g, 3 min, RT. Remove supernatant.
6. Resuspend cells in 105 µL per reaction cold **Wash Buffer** and count (**Appendix 1.2**). If significant sample loss has occured, spin cells and resuspend in a smaller volume of Wash Buffer. Continue to ConA bead binding (**Protocol: Section III**).

TISSUES

Tissues must be processed into a monodispersion of cells, typically by mechanical maceration or 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 methods[7-10].

IMMUNE CELLS

Concanavalin A (ConA) is a lectin, which can cause immune cell activation. To avoid this potential problem in CUT&RUN, use nuclei[11] or a cross-linking strategy[12].

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
Protease Inhibitor	Roche 11873580001
1 M Spermidine	CUTANA CUT&RUN Kit EpiCypher 14-1048
Phosphate Buffered Saline (PBS)	Any vendor
0.4% Trypan Blue	Invitrogen T10282
Brightfield or phase microscope + hemacytometer slides	Any vendor

PROTOCOL

1. Prepare **Nuclear Extraction Buffer** as 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 **Protease Inhibitor** (1X final concentration) to the **Nuclear Extraction Buffer**. Place final buffer on ice.
3. Counts cells and confirm starting cell integrity by Trypan Blue staining (see Figure 18 & **Appendix 1.2**). Harvest 500,000 cells per reaction plus 10-20% excess to account for sample loss.
4. Spin at 600 x g, 3 min, RT. Remove supernatant and resuspend cells in 100 µL per reaction cold **Nuclear Extraction Buffer**.
5. Incubate on ice for 10 min.

6. Spin 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).
7. Gently resuspend nuclei in 105 µL per reaction cold **Nuclear Extraction Buffer** (i.e. for 8 reactions, resuspend in 840 µL).
8. Take a 10 µL aliquot to examine nuclear integrity by Trypan Blue staining. See **Appendix 1.2** for staining instructions and Figure 18 for expected results.
9. To cryopreserve nuclei, slowly freeze in isopropanol-filled chiller in -80°C freezer.
10. 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.
11. Thawed nuclei in Nuclear Extraction Buffer can be directly added to activated ConA beads (**Protocol: Section III**).

* 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.

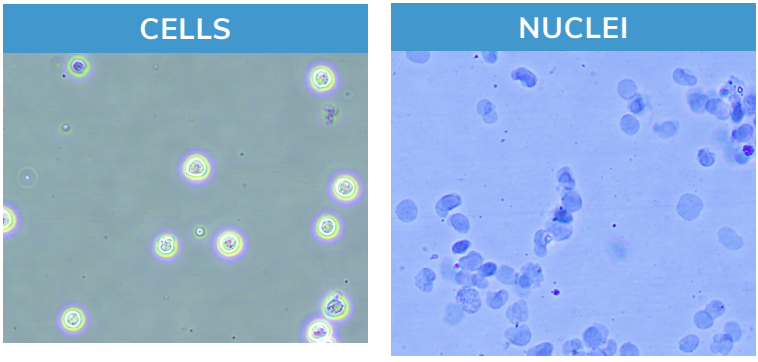


FIGURE 18

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 PROTOCOL

This cross-linking protocol should be considered for:

* Lysine acetylation PTMs (labile targets, impacted by histone deacetylase activity)
* Experiments with tightly controlled variables (e.g. time course drug treatments)
* Transiently interacting chromatin-associated proteins (e.g. acetyl-lysine reader proteins, chromatin remodeling enzymes)
* **ALWAYS** include native samples when testing cross-linking 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 19).
- 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 19).

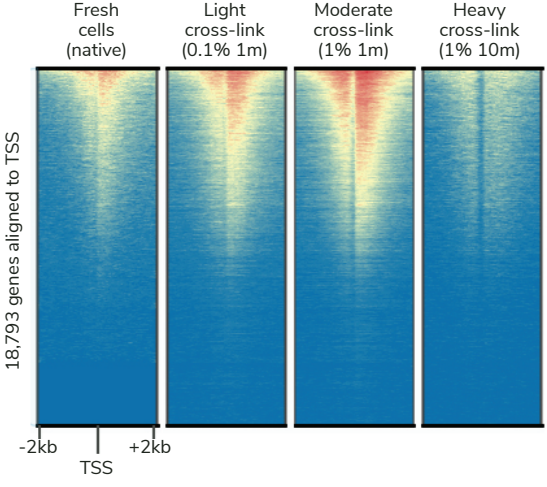


FIGURE 19

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

CROSS-LINKING PROTOCOL (CUT&RUN DAY 1)

- Prepare **Wash**, **Cell Permeabilization**, **& Antibody buffers** using a **Pre-Wash Buffer** supplemented with Triton X-100 and SDS (see Table, above).
- Cross-linking is performed at the beginning of **Protocol: Section III** as follows:

a. From suspension tissue culture, transfer 500,000 cells per reaction to a 15 or 50 mL tube. For adherent cells, cross-link cells while still attached to plate.

b. Add fresh **37% Formaldehyde** directly to culture for a final concentration of 0.1-1%. Test a range of concentrations to optimize for target & cell type.

c. Quickly vortex (suspension cells) or swirl plate (adherent cells) to mix.

d. Incubate for 1-10 min at RT (recommended 1 min). Test a range of times to determine optimal fixation conditions.

e. Quench cross-linking by adding **Glycine** to a final concentration of 125 mM. Vortex (suspension cells) or swirl (adherent cells) to mix.

f. Suspension cells: Spin at 600 x g, 3 min, RT. Proceed to **Section III: Step 14**. For adherent cells: See **Appendix 3.1, Adherent Cells** for instructions.

CROSS-LINKING PROTOCOL (CUT&RUN DAY 2)

- Following collection of supernatants containing CUT&RUN-enriched DNA (**Protocol: Section VI**), it is crucial to reverse cross-links. To each supernatant:

a. Add 0.8 µL **10% SDS** and 1 µL of 20 µg/µL **Proteinase K**. Vortex to mix.

b. Place supernatant in a thermocycler set to 55˚C. Incubate overnight.

c. The next day, quick spin tubes and resume CUT&RUN at **Protocol: Section VII**.

Appendix 4: Frequently Asked Questions (FAQs)

1. **What antibodies does EpiCypher offer for CUT&RUN?**
   EpiCypher is actively screening antibodies for high-quality performance in CUT&RUN. Visit epicypher.com/cut-and-run-antibodies for the most up-to-date list, or contact techsupport@epicypher.com for antibody recommendations. Below we outline the lot-specific testing criteria for EpiCypher CUT&RUN antibodies across various target classes.

   - **Chromatin-associated protein targets:** We offer CUT&RUN antibodies to transcription factors (e.g. CTCF), chromatin reader proteins (e.g. BRD4), chromatin modifying enzymes (e.g. MLL1), and remodelers (e.g. SMARCA2 & SMARCA4). Each antibody displays high signal-to-noise in CUT&RUN and generates genomic distribution profiles consistent with the reported function of the target protein (for example, DNA binding motif analysis for TFs).
   - **Histone PTM targets:** Histone PTM antibodies are particularly susceptible to off-target binding, which can compromise biological interpretations[4]. To address these problems, EpiCypher developed the SNAP-CUTANA™ K-MetStat Panel (EpiCypher 19-1002), and is using these defined nucleosome spike-in controls to identify best-in-class histone lysine methylation PTM antibodies for CUT&RUN. This strategy is the **only** method that directly confirms antibody specificity in CUT&RUN against physiological on- and off-target substrates. Each of our **SNAP-Certified™ Antibodies** show:
     - High specificity: <20% recovery of off-target PTMs in the K-MetStat Panel
     - High target efficiency: Robust profiling at 500,000 and 50,000 starting cells

2. **How do I validate an antibody for CUT&RUN?**
   If an EpiCypher CUT&RUN antibody is not available for your target, we recommend the following steps:

   - Contact techsupport@epicypher.com for antibody recommendations.
   - **Lysine methylation PTMs:** Use the 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 are unique clones or 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 sequencing data.

3. **Can I use my ChIP-validated antibody for CUT&RUN?**
   EpiCypher has found that antibodies validated for ChIP are **NOT** guaranteed to be successful in CUT&RUN. This is likely due to differences in sample prep and wash steps, because ChIP requires heavy cross-linking, stringent washes, and bead-coupled antibodies to attempt to improve signal over background. In contrast, CUT&RUN uses native chromatin, mild washes, and antibodies in solution, reflecting its increased sensitivity compared to ChIP.

4. **What should I do if none of my antibodies work?**
   See **Appendices 1.3 – 1.6** and follow troubleshooting guidelines in Table 3.

5. **My assay requires low numbers of cells. What are my options?**
   See **Experimental Design & Optimization** for basic optimization steps. Follow guidelines in Figure 6 and include **ALL** the quality control steps detailed in Figure 5. **Note that using low cell numbers may result in lower signal and increased background.** Futhermore, CUT&RUN yields may be low depending on antibody quality and target abundance. Note that:
   - An antibody that works with 500,000 cells may fail at lower inputs.
   - The H3K4me3 positive control may have lower yields, similar to IgG.
   - Library prep can be optimized for low yields; see Table 4 & **Appendix 1.3**.
   - Deeper sequencing is often recommended; see Table 4 (**Appendix 1.3**) & Table 6 (**Appendix 1.5**).

6. **Can I use Bioanalyzer or TapeStation traces to evaluate the success of CUT&RUN prior to library prep?**
   Do **NOT** assess fragment size distribution of CUT&RUN DNA before library prep. CUT&RUN uses intact cells and bypasses bulk chromatin fragmentation steps, resulting in high signal-to-noise and low cell input requirements vs ChIP. As a result, raw CUT&RUN DNA yields are often below the limit of sensitivity for fragment size distribution using the TapeStation or Bioanalyzer.

7. **Can I use qPCR to evaluate the success of a CUT&RUN experiment?**
   Do **NOT** use qPCR to validate CUT&RUN assays. qPCR is used in ChIP to verify the enrichment of a known on-target region compared to the bulk chromatin input. However, CUT&RUN is performed in intact cells. There is no immunoprecipitation step and no bulk chromatin input for comparison.

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

CUT&RUN generates nucleosomal (~170 bp) & sub-nucleosomal (<120 bp) DNA fragments. The DNA Cleanup Columns in this kit capture >50 bp fragments, which our scientists found to be sufficient for all targets tested.

However, modifications to library prep that enrich sub-nucleosomal CUT&RUN fragments have been reported[9]. Briefly, 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 or tested by EpiCypher.

9. How do I analyze CUT&RUN sequencing data?

CUT&RUN analysis methods are similar to those used for ChIP-seq datasets, with key differences. Briefly:

- Align raw reads to a reference genome using Bowtie 2[15]. The Integrative Genomics Viewer (IGV) and/or deepTools[16] can be used to visualize enrichment (e.g. bigWig files graphed over a genome browser).
- For peak calling, EpiCypher frequently uses MACS2[18] and SICER[19], programs for ChIP-seq that work well for CUT&RUN[20]. 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 SEACR[22], 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 cells[23]. 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. controls[24]. Other tools can be applied for differential analysis and heatmap generation (e.g. DESeq2[25], deepTools[17]).

1. Skene & Henikoff. An efficient targeted nuclease strategy for high-resolution mapping of DNA binding sites. **eLife** 6 (2017).
2. Schmid et al. ChIC and ChEC; genomic mapping of chromatin proteins. ***Mol Cell*** 16, 147-157 (2004).
3. Skene et al. Targeted *in situ* genome-wide profiling with high efficiency for low cell numbers. **Nat *Prot*** 13, 1006-1019 (2018).
4. Shah et al. Examining the roles of H3K4 methylation states with systematically characterized antibodies. ***Mol Cell*** 72, 162-177 (2018).
5. Tay et al. Hdac3 is an epigenetic inhibitor of the cytotoxicity program in CD8 T cells. ***J Exp Med*** 217 (2020).
6. Orlando et al. Quantitative ChIP-Seq normalization reveals global modulation of the epigenome. ***Cell* Rep** 9, 1163-1170 (2014).
7. de Bock et al. HOXA9 cooperates with activated JAK/STAT signaling to drive leukemia development. ***Cancer Discov*** 8, 616-631 (2018).
8. Janssens et al. Automated *in situ* chromatin profiling efficiently resolves cell types and gene regulatory programs. ***Epigenetics Chromatin*** 11 (2018).
9. Liu et al. Direct promoter repression by BCL11A controls the fetal to adult hemoglobin switch. ***Cell*** 173, 430-442 (2018).
10. Uyehara & McKay. Direct and widespread role for the nuclear receptor EcR in mediating the response to ecdysone in *Drosophila*. ***Proc Natl Acad Sci USA*** 116, 9893-9902 (2019).
11. Hainer et al. Profiling of pluripotency factors in single cells and early embryos. ***Cell*** 177, 1319-1329.e11 (2019).
12. Zheng & Gehring. Low-input chromatin profiling in Arabidopsis endosperm using CUT&RUN. ***Plant Reprod*** 32, 63-75 (2019).
13. Mathsyaraja et al. Max deletion destabilizes MYC protein and abrogates Emicro-Myc lymphomagenesis. ***Genes Dev*** 33, 1252-1264 (2019).
14. Roth et al. Reprogramming human T cell function and specificity with non-viral genome targeting. **Nature** 559, 405-409 (2018).
15. Langmead & Salzberg. Fast gapped-read alignment with Bowtie 2. ***Nat Methods*** 9, 357-359 (2012).

16. Robinson et al. Integrative Genomics Viewer. ***Nat Biotechnol*** 29, 24–26 (2011).

17. Ramírez et al. deepTools2: a next generation web server for deep-sequencing data analysis. ***Nucleic Acids Res*** 8, 44 (2016).

18. Liu T. Use model-based Analysis of ChIP-Seq (MACS) to analyze short reads generated by sequencing protein-DNA interactions in embryonic stem cells. ***Methods Mol Biol*** 1150, 81-95 (2014).

19. Zang C et al. A clustering approach for identification of enriched domains from histone modification ChIP-Seq data. ***Bioinformatics*** 25, 1952-1958 (2009).

20. Evans et al. Ybx1 fine-tunes PRC2 activities to control embryonic brain development. ***Nat Commun*** 11, 4060 (2020).

21. Laczik M et al. Iterative Fragmentation Improves the Detection of ChIP-seq Peaks for Inactive Histone Marks. ***Bioinform Biol Insights*** 10, 209-224 (2016).

22. Meers et al. Peak calling by Sparse Enrichment Analysis for CUT&RUN chromatin profiling. ***Epigenetics Chromatin*** 12, 42 (2019).

23. Yu F et al. CUT&RUNTools 2.0: A pipeline for single-cell and bulk-level CUT&RUN and CUT&Tag data analysis. ***Bioinformatics*** 38, 252-254 (2021).

24. Schep AN et al. chromVAR: inferring transcription-factor-associated accessibility from single-cell epigenomic data. ***Nat Methods*** 14, 975-978 (2017).

25. Love MI et al. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. ***Genome Biol*** 15, 550 (2014).

24 Hour Emergency Phone Number:

US & Canada: 1-800-535-5053

International: 1-352-323-3500

EpiCypher, Inc.
6 Davis Dr. Ste 755
Durham, NC 27709 USA

www.epicypher.com
Ph: 1-855-374-2461 F: 1-855-420-6111
Email: info@epicypher.com Tech Support: techsupport@epicypher.com