EpiCypher®

Bringing Epigenetics to Life

CUTANA™
ChIC / CUT&RUN Kit Version 4
User Manual Version 4.0

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CUTANA™

ChIC / CUT&RUN Kit

Kit Version 4

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.

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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).

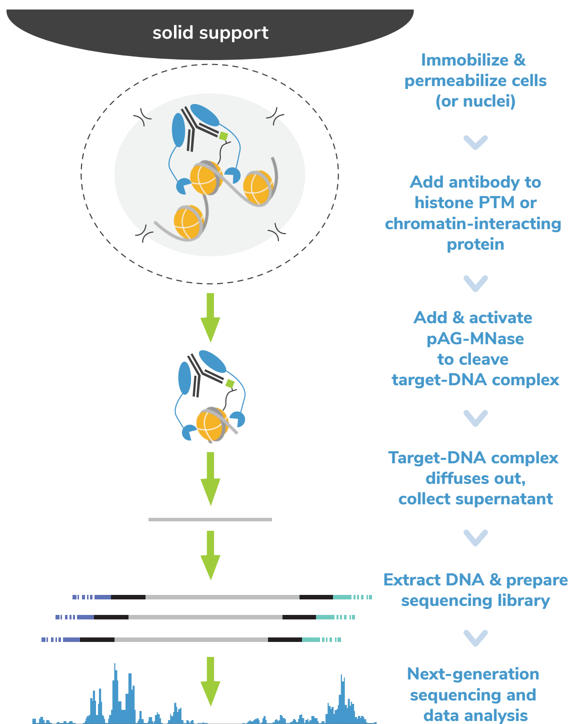


FIGURE 1

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.

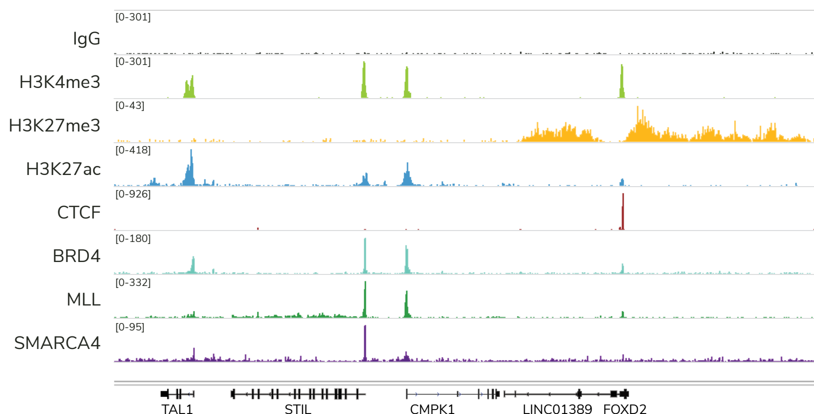


FIGURE 2

Representative genome browser tracks show CUTANA CUT&RUN results using 500,000 K562 cells. Clear peaks with expected distribution profiles 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 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.

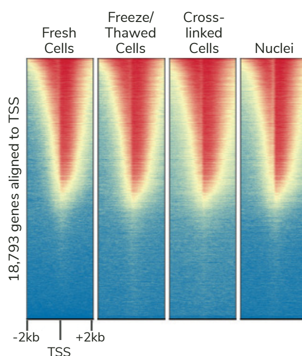


FIGURE 3

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.

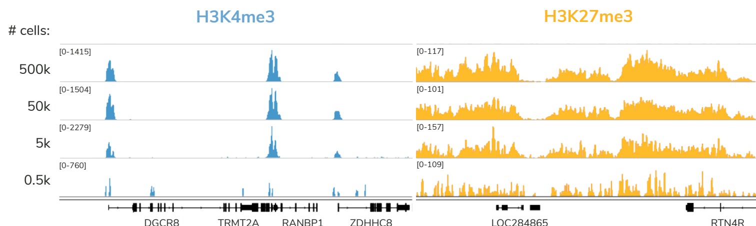


FIGURE 4

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

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

Store at room temperature (RT) upon receipt:

Item	Catalog No.	Notes before use
8-strip Tubes	10-0009a	Enables use of multi-channel pipettors.
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.
SPRIselect Reagent Manufactured by Beckman Coulter Inc.	21-1405	**DO NOT FREEZE.** Reagent is slightly viscous. Thoroughly mix prior to use and pipette carefully to ensure correct volume is transferred. Use to purify CUT&RUN-enriched DNA from supernatant.
0.1X TE Buffer	21-1025	Use to elute CUT&RUN-enriched 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. Before first use, quick spin and reconstitute in 200 µL DNase-free water (0.5 ng/µL). Add to reactions for sequencing normalization. **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	21-1004k	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 - DO NOT VORTEX.** Panel of biotinylated nucleosomes coupled to streptavidin-coated magnetic beads. Pair with IgG and H3K4me3 control antibodies. Sufficient for 20 reactions. See **Appendix 1.3**.
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, and cow.

Materials Required but Not Supplied

REAGENTS:

- DMSO and PBS (for optimizing Digitonin permeabilization; **Appendix 1.1**)
- Antibody to target of interest; see **FAQs 5-7** 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 this kit is sufficient 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)
- 100% Ethanol (200 proof), any vendor
- Molecular biology grade water, any vendor
- Library prep kit (i.e. CUTANA CUT&RUN Library Prep Kit, EpiCypher 14-1001 & 14-1002)

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)
- 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** 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 reduced 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. The CUTANA™ CUT&RUN kit is validated for robust profiling of histone PTMs, transcription factors, remodeling enzymes, chromatin writers and readers, and epitope-tagged proteins.

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 CUT&RUN-enriched DNA

Bead-bound cells are removed using a magnet. CUT&RUN-enriched DNA is purified from supernatant using a bead-based strategy optimized for mononucleosome-sized fragments. DNA is quantified using the Qubit Fluorometer.

Step 5: CUT&RUN library prep and Illumina® sequencing

CUT&RUN DNA is prepared for Illumina® sequencing using the CUTANA™ CUT&RUN Library Prep Kit (or equivalent). Libraries are sequenced on an Illumina sequencing platform. Only 3-8 million uniquely aligned reads are needed to generate roubust CUT&RUN profiles.

Experimental Protocol: Day 1

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

IMPORTANT NOTES ON BUFFER PREP

* Buffers (Figure 5) are prepared **FRESH** on Day 1 of every CUT&RUN experiment.
* 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.

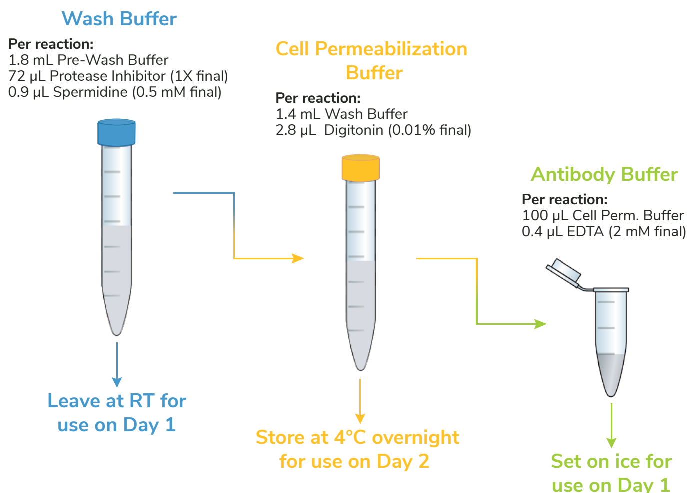


FIGURE 5

Schematic of buffers prepared on Day 1 of the CUT&RUN protocol. **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 as outlined in Table 1. Add 5% Digitonin as optimzed for your cell type.

   0.01% Digitonin is optimal for permeabilizing K562, MCF7, and A549 cells (as in Table 1), and is recommended for reactions using nuclei. For other cell types, Digitonin conditions MUST be optimized for efficient cell permeabilization. See **Appendix 1.1** for instructions.

   Place **Cell Permeabilization Buffer** 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 20% extra volume to account for pipetting error.

SECTION II: CONA BEAD ACTIVATION (~30 MIN)

QUALITY CONTROL CHECKS FOR 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.

SAMPLE PREP FAQS

How important is sample prep for CUT&RUN?

High quality sample prep is essential to CUT&RUN success, and is the main variable we see when troubleshooting customer experiments.

How do I check my sample?

Count and examine cells using a Trypan Blue staining protocol (**Appendix 1.2**). Check cellular morphology, integrity, and viability at **three steps**:

* **Initial cell harvest:** Cells have high viability and expected morphology. Acceptable viability is dependent on cell type and experimental conditions.
* **Before ConA bead binding:** Cells in CUT&RUN Wash Buffer have expected morphology and minimal lysis and/or sample loss (**Appendix 1.2**).
* **After binding to ConA beads:** The supernatant contain very few cells and the sample shows that all cells are bound to ConA beads (**Appendix 1.2**).

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

MODIFICATIONS FOR SAMPLE TYPES

* Freshly isolated, native cells are the preferred input for CUT&RUN.
* See **Appendix 3** for adherent cells, tissues, immune cells, nuclei, cross-linked cells, or cryopreserved samples.

GUIDELINES FOR HIGH-THROUGHPUT PROCESSING

* Multi-channel pipetting is recommended to improve reliability and experimental throughput.

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. If using a multi-channel pipettor (recommended), place a multi-channel reagent reservoir on ice. Fill with cold **Antibody Buffer**.

    Retrieve compatible 8-strip tube magnetic rack. If using the EpiCypher 8-strip tube magnet, use the high-volume side unless otherwise noted.
20. After the 10 min incubation, place tubes on a magnet and allow slurry to clear. If bead binding was successful, the supernatant should not contain cells. To confirm, save 10 µL supernatant for Trypan Blue staining (**Appendix 1.2**).
21. Pipette to remove and discard the remaining supernatant. Remove tubes from magnet and immediately add 50 µL cold **Antibody Buffer** to each reaction.
22. Pipette to resuspend. Confirm ConA bead binding (**Appendix 1.2)**.

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

QUALITY CONTROL CHECKS FOR ANTIBODY BINDING

* Add K-MetStat Panel to control reactions **BEFORE** adding the primary antibody.
* 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 in Figure 6.

23. 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 and IgG negative control antibodies. Gently vortex to mix and quick spin tubes. If using fewer than 500,000 cells, decrease K-MetStat Panel as in Table 2.

Number of cells	Panel dilution	Volume per reaction
500,000	Use stock	2 µL
250,000	1:2	2 µL
100,000	1:5	2 µL
50,000 or fewer	1:10	2 µL

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.

24. Add 0.5 µg primary antibody (or manufacturer's recommendation) to each reaction. For positive and negative control reactions, add 1µL **H3K4me3 Positive Control Antibody** and 1µL **IgG Negative Control Antibody**, respectively. For antibodies stored in viscous glycerol solutions, ensure accurate pipetting: aspirate slowly, check tip for accuracy, and pipette up and down to clear the solution from tip.

25. Gently vortex reactions to mix. Incubate overnight on a nutator at 4˚C with tube caps elevated (Figure 6). Do **NOT** rotate - see Antibody Binding QC Checks, above.

FIGURE 6

8-strip tubes should be placed on a nutator at a 45 degree angle with caps elevated.

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

NOTES ON CONA BEAD-CELL CLUMPING

* It is essential that ConA beads remain in solution during pAG-MNase binding and digestion. Excessive bead clumping leads to sample loss, reduced yields, and poor data quality.

26. If using a multi-channel pipettor (recommended), place a multi-channel reagent reservoir on ice. Fill with cold **Cell Permeabilization Buffer**.
27. Remove tubes from 4˚C incubation and quick spin to collect liquid. Note that beads may settle overnight (Figure 7), but this will not impact results.
28. Place tubes on magnet and allow slurry to clear. Pipette to remove supernatant. If using a multi-channel pipettor, remove and replace buffers one tube strip at a time to avoid ConA bead dry out.
29. Keeping tubes on magnet, add 200 μL cold **Cell Permeabilization Buffer** to each reaction. Pipette to remove supernatant.
30. Repeat the previous step one time (keep tubes on magnet).
31. Remove tubes from magnet and immediately add 50 μL cold **Cell Permeabilization Buffer** to each reaction. Gently vortex to mix. Beads may become clumpy at this stage of the protocol, but can be dispersed by gentle pipetting. The end of a pipette tip can be cut off to help mix delicate cells.
32. Add 2.5 μL **pAG-MNase** to each reaction. Gently vortex and/or pipette to thoroughly mix beads and evenly distribute enzyme.
33. Incubate reactions for 10 min at RT.
34. Quick spin tubes, place on magnet, and allow slurry to clear. Pipette to remove supernatant.
35. Keeping tubes on magnet, add 200 μL cold **Cell Permeabilization Buffer** directly onto beads. Pipette to remove supernatant.
36. Repeat one time.
37. Remove tubes from magnet. Immediately add 50 μL cold **Cell Permeabilization Buffer** to each reaction. Gently vortex to mix and disperse clumps by pipetting.

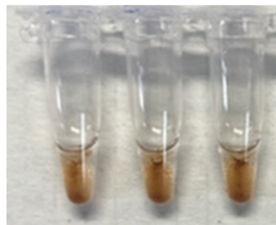


FIGURE 7

Settling of ConA beads after overnight incubation at 4˚C.

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

GUIDELINES FOR *E. COLI* SPIKE-IN DNA

* Reconstitute the lyophilized ***E. coli* Spike-in DNA** prior to first use: Quick spin tube before opening to collect *E. coli* DNA in bottom of tube. Add 200 μL DNase-free water and thoroughly vortex tube on all sides to resuspend *E. coli* DNA. Store at -20˚C.
* This protocol is optimized for 500,000 cells per reaction. If using fewer than 500,000 cells per reaction, dilute ***E. coli* Spike-in DNA** as outlined in **Appendix 2**.

38. 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.
39. Incubate tubes on nutator (capped ends elevated; Figure 6) for 2 hours at 4°C.
40. Retrieve ***E. coli* Spike-in DNA**. Reconstitute DNA prior to first use (Guidelines, above) or thaw previously resuspended DNA on ice and quick spin before use.
41. Prepare a **Stop Master Mix** in a 1.5 mL tube. Per reaction, combine 33 μL **Stop Buffer** and 1 μL ***E. coli* Spike-in DNA** (0.5 ng). Gently vortex to mix.
42. At the end of the 2 hour incubation, quick spin 8-strip tubes to collect liquid. Add 34 μL **Stop Master Mix** to each reaction and gently vortex to mix.
43. Place reactions in a thermocycler set to 37˚C. Incubate for 10 min.
44. Quick spin tubes to collect liquid, and place on a magnet until slurry clears.
45. Transfer supernatants containing CUT&RUN DNA to new **8-strip tubes**.

SECTION VII: DNA PURIFICATION (~30 MIN)

46. Prepare 85% Ethanol (EtOH) **FRESH** using a 100% EtOH stock and molecular biology grade water. Make 500 μL per reaction: 425 μL 100% EtOH + 75 μL water. These calculations include extra volume to account for pipetting error.
47. Vortex **SPRIselect** reagent (beads) thoroughly to resuspend.
48. Slowly add 119 μL **SPRIselect** reagent (1.4X volume) to each reaction. Ensure pipette tip is free of extra droplets before dispensing beads to reactions.
49. Gently vortex tubes to mix and quick spin to collect liquid in tube bottom. Incubate 5 min at RT.
50. Place tubes on magnet for 2-5 min at RT. Pipette to remove supernatant, being careful not to disturb beads with pipette tip.

51. Keeping tubes on magnet, add 180 µL **85% EtOH** directly onto beads. Pipette to remove supernatant.

52. Repeat the previous step one time.

53. Remove tubes from magnet and quick spin, caps facing in. Beads should stay in place on side of tube. Return to magnet and pipette to remove residual EtOH.

54. Remove tubes from magnet, leaving caps open. Air-dry beads for 2-3 min at RT, or until liquid has evaporated but beads still appear damp matte brown. If beads become crackly, they are too dry (Figure 8).

55. Add 17 µL **0.1X TE Buffer** to each reaction to elute DNA.

56. Gently vortex tubes to resuspend beads and quick spin. Incubate 2 min at RT.

57. For EpiCypher magnet, flip to low-volume side. Place tubes on magnet for 2 min, RT.

58. Transfer 15 µL CUT&RUN-enriched DNA to new **8-strip tubes**.

FIGURE 8

Elute DNA before beads dry out.

58. Quantify 1 µL DNA using the Qubit fluorometer and 1X dsDNA HS Assay Kit.

59. **Safe pause point. Continue to library prep or store DNA at -20˚C.**

CUT&RUN EXPECTED RESULTS & FAQS

What yields can I expect from CUT&RUN?

There is no typical DNA yield for CUT&RUN, as yields vary by cell type, number of cells, target abundance, and antibody quality. EpiCypher suggests to:

* Check that yields from the H3K4me3 control are similar to or slightly greater than IgG. Similar and/or low yields do **NOT** imply assay failure; see **FAQ 2**.
* Aim for ≥5 ng DNA to enable robust library prep. For low yields, see **FAQ 3**.

Can I use Bioanalzyer or TapeStation traces to examine my CUT&RUN DNA?

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

CUT&RUN LIBRARY PREP - FAQS

What is the single BEST method to confirm CUT&RUN success?

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

How should I prepare CUT&RUN sequencing libraries?

- Prepare Illumina sequencing libraries using the CUTANA™ CUT&RUN Library Prep Kit (EpiCypher 14-1001 & 14-1002) and ~5 ng CUT&RUN DNA.
- For low-abundance targets or if yields <5 ng, use total amount of recovered DNA. Note that IgG and H3K4me3 control antibodies often generate low yields (**FAQ 2**).
- If using other library prep kits, follow EpiCypher's parameters for indexing PCR and library amplification, which are specifically optimized for CUT&RUN. For guidance, see the Library Prep Kit manual at epicypher.com/protocols.

SEQUENCING CUT&RUN LIBRARIES - FAQS

What is the recommended sequencing depth and read length?

- Libraries should be sequenced to a depth of 3-8 million reads.
- Paired-end sequencing (2 x 50 bp minimum) is recommended for CUT&RUN.

What are standard CUT&RUN sequencing metrics?

- Majority of reads (>80%) should align uniquely to the species genome.
- Sequence duplication levels should be low (<20% of total sequencing reads).

How do I analyze the H3K4me3 positive and and IgG negative control reactions spiked with the SNAP-CUTANA™ K-MetStat Panel?

- See **Appendix 1.3**. The K-MetStat Panel should comprise ~1% of total reads.
- H3K4me3 and IgG controls should show expected enrichment and peaks. Experimental replicates should be highly reproducible (Figures 10 & 11).

How do I use *E. coli* Spike-in DNA for sequencing normalization?

- See **Appendix 2**. *E. coli* DNA should comprise ~1% of total reads.

EXAMPLE FRAGMENT DISTRIBUTION ANALYSIS OF CUT&RUN SEQUENCING LIBRARIES

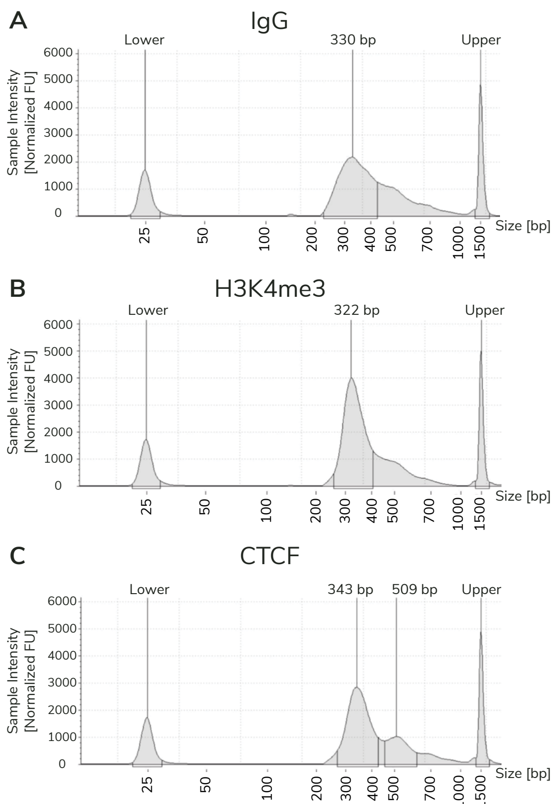


FIGURE 9

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).

EXAMPLE DATA FROM POSITIVE AND NEGATIVE CONTROL REACTIONS

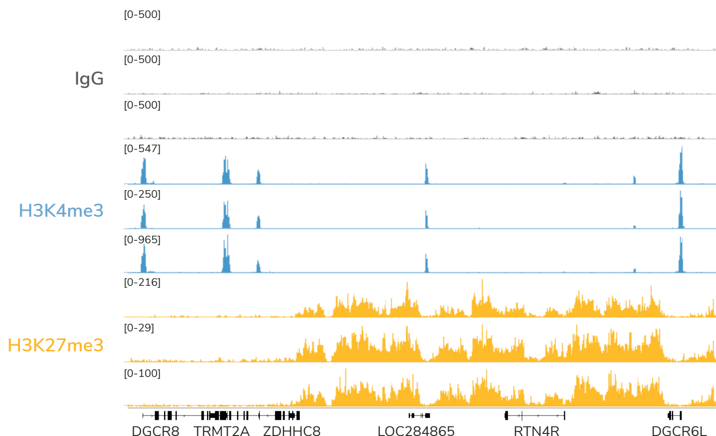
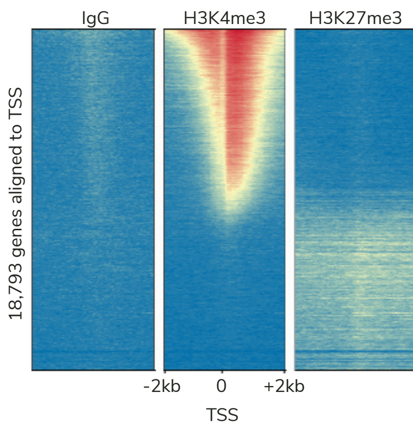


FIGURE 10

Data generated by three 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 11

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.



EpiCypher's end-to-end CUT&RUN workflow includes multiple quality control checks for reliable CUT&RUN assays. A summary of quality control checks is provided in Figure 12.

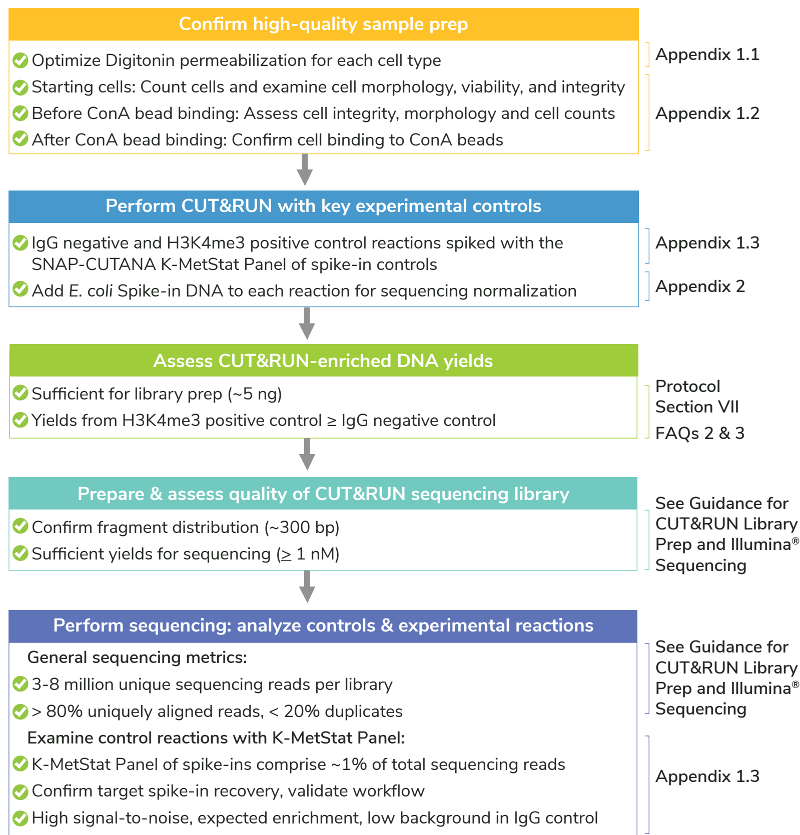


FIGURE 12

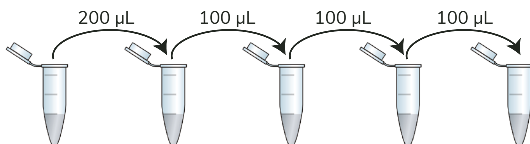
The CUTANA CUT&RUN Kit contains multiple quality control metrics and checks to ensure successful chromatin profiling. Quality control metrics are listed for each section of the CUT&RUN workflow, along with the reference material located in this manual. For more information on library prep and sequencing, see the CUTANA CUT&RUN Library Prep Manual at epicypher.com/protocols.

1.1 OPTIMIZATION OF CELL PERMEABILIZATION

CUT&RUN uses Digitonin to permeabilize cells, which is crucial to assay success. Insufficient Digitonin prevents antibody and pAG-MNase from entering cells, 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, permeabilization is not required. Add Digitonin to a final concentration of 0.01% to prevent 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).
    b. Add the appropriate volume of **Wash Buffer** to each tube.
    c. Add 10 μL **5% Digitonin** to the first tube, labelled 0.05%. Vortex to mix.
    d. Prepare the other four Cell Permeabilization Buffers by serial dilution (see table). In a sixth 1.5 mL tube, prepare 0.05% DMSO in **Wash Buffer** as a control. 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 hemocytometer/cell counter slide.
7. Count live (intact, Trypan negative) vs. dead (permeabilized, Trypan positive) cells. Select minimum Digitonin concentration that permeabilizes >95% of cells.

In Figure 13, 0.01% Digitonin is the minimum concentration necessary to permeabilize >95% of total K562 cultured cells (red arrow).

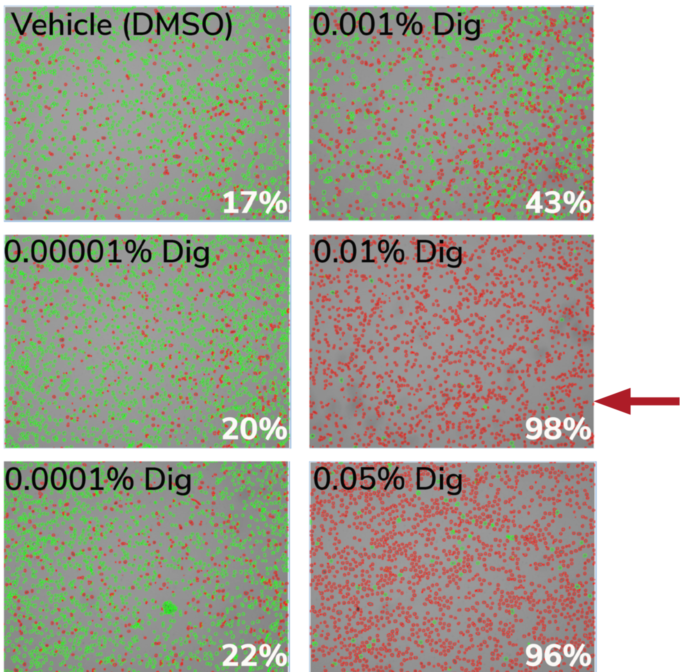


FIGURE 13

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 assess quality of cells in CUT&RUN Wash Buffer (i.e. morphology, integrity) AND to confirm cell binding to ConA beads. Note that:

* Poor cell integrity and/or cell can lysis increase assay background - **don't skip these steps!**
* Cells are resuspended with 5 µL excess Wash 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.**

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 integrity of washed cells 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 hemocytometer/cell counter slide.
   c. View under brightfield/phase microscope or cell counter.
   d. Confirm sample integrity or troubleshoot as needed (see Figure 14A).
3. Add 100 µL washed cells to 10 µL of activated ConA beads in 8-strip tubes. Gently vortex to resuspend [bead fraction]. Quick spin to collect beads.
4. Incubate bead fraction 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 bead fraction. Remove tubes from magnet and pipette to resuspend.
7. Move 10 µL bead fraction to a 1.5 mL tube. Place remaining bead fraction on ice.
   - Alternative: Take 1-5 µL bead fraction. Dilute with 1X PBS to 10 µL volume.
8. To 10 µL samples set aside (unbound fraction, bead fraction), perform Trypan Blue staining as in Step 2. Successful ConA bead binding is indicated when:
   - The unbound fraction contains few cells (or nuclei; Figure 14C).
   - The bead fraction cotains permeabilized, Trypan Blue positive cells (or nuclei) surrounded by beads (Figure 14D).
9. Continue with the **Protocol: Section IV (Antibody Binding).**

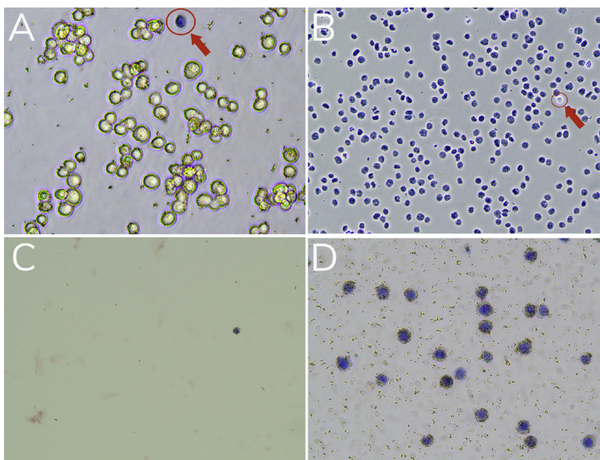


FIGURE 14

Validation of sample prep using Trypan Blue staining. **(A)** Washed K562 cells show normal morphology/integrity and minimal lysis. 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 bead fraction image showing nuclei (blue) bound to ConA beads (brown specks). **Note:** ConA bead-bound cells will also be Trypan positive (blue), due to the presence of Digitonin in Antibody Buffer.

<table>
<tr><th>Sample</th><th colspan="2">Success Metrics / Tips</th></tr>
<tr><td>Cells
Figure 14A</td><td colspan="2">Cells should show normal morphology/integrity, minimal cell lysis, and be unclumped. Cells in Wash Buffer may appear less viable compared to initial cell harvest. Instead, focus on total cell counts, confirming ~500,000 cells per reaction.
To troubleshoot cell loss: Increase spin time (keep at 600 x g). Leave ~50 μL liquid on cell pellet when removing supernatant to minimize cell loss, and gently pipette to resuspend.</td></tr>
<tr><td>Nuclei
Figure 14B</td><td colspan="2">>95% nuclei are Trypan Blue positive and unclumped. See the EpiCypher Nuclei Extraction Protocol at epicypher.com/protocols.</td></tr>
<tr><td>Unbound Fraction
Figure 14C</td><td>Little to no material is present.</td><td rowspan="2">To troubleshoot: 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.</td></tr>
<tr><td>Bead Fraction
Figure 14D</td><td>Permeabilized cells/nuclei are surrounded by beads.</td></tr>
</table>

1.3 SNAP-CUTANA™ K-METSTAT PANEL

* The K-MetStat Panel is a fast, easy, and reliable control that confirms CUT&RUN success.
* Download the SNAP-CUTANA Spike-in User Guide (epicypher.com/protocols) for more info.

The K-MetStat Panel is a defined spike-in control that is added to IgG negative and H3K4me3 positive control reactions (Figure 16). This spike-in control replicates chromatin structure (i.e. nucleosomes; Figure 15), the natural target of CUT&RUN, thus allowing you to validate multiple steps of the workflow. Benefits include:

- **Fast**: The K-MetStat Panel is added to control reactions in one step (Figure 16).
- **Easy**: No protocol modifications are necessary. Examine spike-in results in your final sequencing data using our provided analysis tools (p. 30-31).
- **Reliable**: K-MetStat data directly report on CUT&RUN success. Use to examine sample quality, MNase activity, and troubleshoot challenging workflows (p. 32).

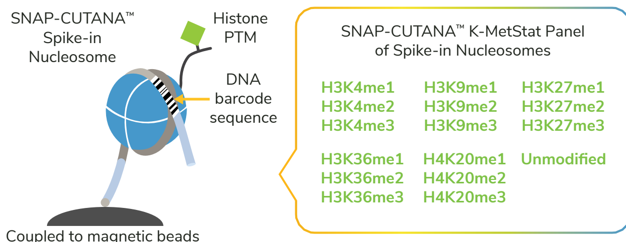
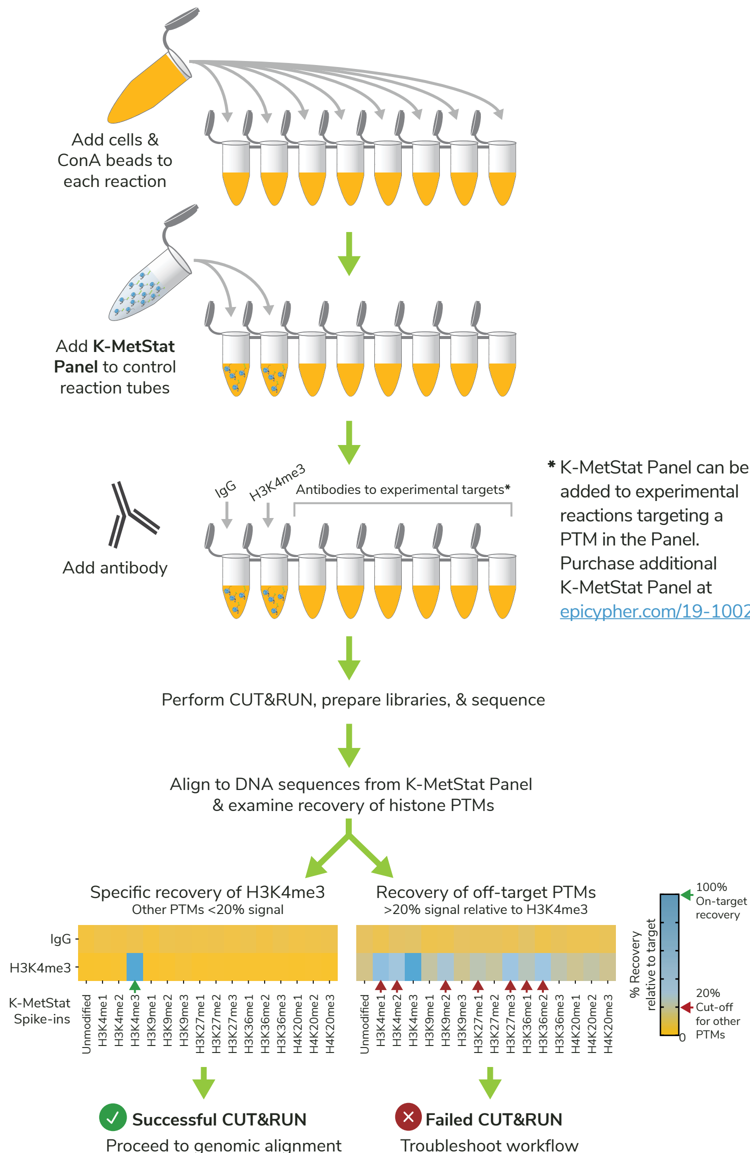


FIGURE 15

The K-MetStat Panel is a pool of spike-in nucleosomes representing 15 histone lysine methylation PTMs plus an unmodified control. The nucleosomes are pre-immobilized on beads, allowing them to be processed alongside bead-coupled cells. All antibody-bound targets (both sample chromatin and spike-in nucleosomes) are cleaved by pAG-MNase and released into solution. The PTM-specific DNA barcode sequences enable detection of K-MetStat Panel nucleosomes in sequencing data.

FIGURE 16 (pg. 29)

The K-MetStat Panel is used with control antibodies to determine CUT&RUN success. Two different outcomes are shown. Left heatmap: Workflow success is indicated by specific recovery of H3K4me3 in the positive control (dark green arrow) and low background from IgG. Right heatmap: Failed CUT&RUN workflows show off-target PTM recovery (red arrows) and elevated background.


Add cells &
ConA beads to
each reaction
Add **K-MetStat Panel** to control
reaction tubes
Add antibody
IgG
H3K4me3
Antibodies to experimental targets*
* K-MetStat Panel can be added to experimental reactions targeting a PTM in the Panel. Purchase additional K-MetStat Panel at epicypher.com/19-1002.
Perform CUT&RUN, prepare libraries, & sequence
Align to DNA sequences from K-MetStat Panel
& examine recovery of histone PTMs
Specific recovery of H3K4me3
Other PTMs <20% signal
Recovery of off-target PTMs
>20% signal relative to H3K4me3
IgG
H3K4me3
K-MetStat
Spike-ins
Unmodified
H3K4me1
H3K4me2
H3K4me3
H3K9me1
H3K9me2
H3K9me3
H3K27me1
H3K27me2
H3K27me3
H3K36me1
H3K36me2
H3K36me3
H4K20me1
H4K20me2
H4K20me3
% Recovery
relative to target
100%
On-target
recovery
20%
Cut-off
for other
PTMs
0
Successful CUT&RUN
Proceed to genomic alignment
Failed CUT&RUN
Troubleshoot workflow

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 sequence** 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 ##`. Under the last "done," paste one copy per control reaction.

   b. In the first loop replace `sample1_R1.fastq` and `sample1_R2.fastq` with R1 & R2 fastq file names for **one** 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 17). 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 17).
- H3K4me3 positive control: Strong enrichment for H3K4me3 spike-ins, less than 20% off-target PTM recovery, and high signal-to-noise.
- Number of spike-in reads: ~1% (0.5-5%) of total sequencing reads (**FAQ 12**).
- If control reactions generate expected spike-in data (Figure 17, 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 17, Sample 3). See next page for a discussion of troubleshooting using spike-in results.

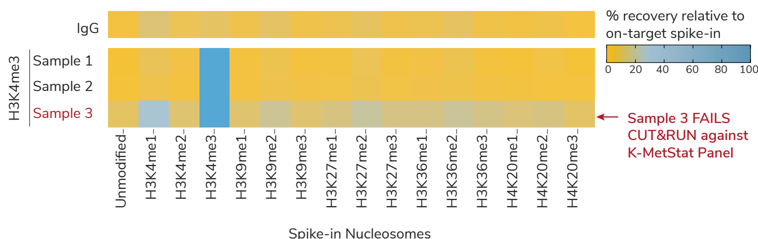


FIGURE 17

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.

TABLE 3 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 3.1**)
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, include all quality control checks • 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 per the guidelines in the first section of this Table
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.

Aim for *E. coli* Spike-in DNA to comprise ~1% (0.5-5%) of total sequencing reads (**FAQ 12**). 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 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 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 the normalization factor (see Tay et al.[5]) such that after normalization the *E. coli* spike-in signal is equal across paired 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].

Appendix 3: Protocol Variations

3.1 SAMPLE PREP VARIATIONS

NUCLEI

- Nuclei may be preferred for some sample types, such as immune cells and tissues.
- EpiCypher offers a Nuclei Extraction Buffer Bundle (EpiCypher 21-1026), which includes the necessary reagents for isolating nuclei for CUT&RUN.
- Our validated nuclei isolation protocol is available now at epicypher.com/protocols.

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.

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].

CROSS-LINKING

- Light cross-linking may improve signal for labile targets (e.g. lysine acetylation PTMs) or help capture signal for time points or other variables.
- Do **NOT** use heavy cross-linking conditions used in ChIP (>1% formaldehyde, 1-10 min) which significantly reduce CUT&RUN DNA yield and data quality.
- EpiCypher's CUT&RUN cross-linking protocol provides the optimal balance of target stabilization and good assay yields. Download at epicypher.com/protocols.

IMMUNE CELLS

Concanavalin A (ConA) is a lectin, which can cause immune cell activation. It is recommended to use nuclei[11] or a cross-linking strategy[12] for immune cell studies.

3.2 CRYOPRESERVED CELLS & NUCLEI

Freeze/thaw samples under conditions that minimize lysis, which can elevate assay background. Avoid vortexing thawed samples prior to ConA bead binding.

FREEZE/THAWING CELLS

1. Make sure Digitonin is optimized for cell types (**Appendix 1.1**).
2. Count cells and confirm viability, integrity, and morphology (**Appendix 1.2**). Spin cells 600 x g, 3 min, RT.
3. Remove supernatant. Resuspend in **cell culture media with 10% DMSO** and aliquot as desired. EpiCypher typically aliquots 5 million cells for 8 reactions, which allows for ~20% sample loss during freeze/thaw.
4. Slowly freeze aliquots (-1˚C per minute) in an isopropanol-filled chiller in a -80°C freezer (e.g. "Mr. Frosty").
5. When ready to perform CUT&RUN, remove tubes from -80˚C and quickly place on a 37°C block to thaw. Work quickly to avoid cell lysis.
6. When cells are almost thawed, remove from 37˚C and pipette to fully thaw cells.
7. Spin cells at 600 x g, 3 min, RT. Pipette to remove supernatant.
8. Resuspend cells in 105 μL per reaction RT **Wash Buffer**. Take a 10 μL aliquot to count (as in **Appendix 1.2**). Note that viability may be decreased; focus instead on cell integrity, lysis levels, and total cell counts. If significant sample loss has occurred, spin cells again and resuspend in a smaller volume of Wash Buffer.
9. Continue to ConA bead binding (**Protocol: Section III**).

FREEZE/THAWING NUCLEI

1. Isolate nuclei (epicypher.com/protocols) and resuspend in **Nuclei Extraction Buffer** (EpiCypher 21-1026). Confirm sample quality as in **Appendix 1.2.**
2. Aliquot nuclei as desired. EpiCypher typically aliquots for ≥8 reactions, plus 20-30% excess 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 place on a 37°C block to thaw. Work quickly to avoid lysis and chromatin fragmentation.
5. Thawed nuclei in Nuclei Extraction Buffer can be directly added to activated ConA beads (**Protocol: Section III**).

Appendix 4: Frequently Asked Questions (FAQs)

1. I ran my CUT&RUN DNA on the TapeStation/Bioanalyzer and don't see anything! What went wrong?

Do **NOT** run your raw CUT&RUN DNA on the TapeStation or Bioanalyzer. 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, CUT&RUN DNA yields are often below the limit of sensitivity for these approaches, meaning you won't see any enrichment.

We only recommend analysis by TapeStation/Bioanalyzer AFTER library prep.

2. My yields from the H3K4me3 positive control are low (i.e. below 5 ng), about the same as the IgG negative control. Did my experiment fail?

Similar yields from H3K4me3 and IgG controls do **NOT** imply CUT&RUN failure. H3K4me3 is a low abundance target, resulting in lower yields that are often similar to IgG. In these cases, use the total amount of CUT&RUN-enriched DNA for library prep. In EpiCypher's experience, good sequencing data with high signal-to-noise are still obtained.

If desired, a high abundance target (e.g. H3K27me3, EpiCypher 13-0055) can be used as an additional positive control, as yields will be much higher than IgG.

3. What should I do if my yields for experimental targets are low (i.e. below 5 ng)?

Low CUT&RUN DNA yields are common for low abundance targets (e.g. H3K4me3), but also depend on the number/quality of starting cells, cell type, and antibody performance. If you are consistently generating low yields for experimental targets:

- See **Basic Troubleshooting Guidelines** on the next page. Pay careful attention to the quality and count of your cells during sample prep and avoid ConA bead dry out, which causes sample loss.
- Include H3K4me3 and IgG control reactions. If these controls work but experimental targets fail, confirm that your target is correctly localized to chromatin (e.g. stimulation conditions) and test additional antibodies and/or cross-linking conditions.
- If the experiment cannot be repeated, use the total amount of CUT&RUN enriched DNA for library prep. See the CUTANA CUT&RUN Library Prep Kit Manual for guidance (epicypher.com/protocols).

Basic CUT&RUN Troubleshooting Guidelines

If your CUT&RUN experiment isn't working, review the following questions:

* Are ConA beads brown and stored at 4˚C? **NEVER** freeze ConA beads.
* What is your cell/sample type? Check **Appendix 3** for protocol modifications.
* Are Digitonin permeabilization conditions optimized? See **Appendix 1.1**.
* Are you using the recommended 500,000 cells per reaction? Success from low cell numbers depends on antibody quality and target abundance. See **FAQ 9**.
* Have you confirmed the integrity, morphology, and count of starting cells (i.e. at initial cell harvest) **and** reconfirm before ConA bead binding (i.e. cells in Wash Buffer)? You may be losing cells during low-speed wash steps or cells may be lysing in Wash Buffer, all of which can impact yield. See **Protocol Section II-III** for details and **Appendix 1.2** for examples.
* Have you confirmed ConA bead binding? See **Appendix 1.2**.
* Have you included reactions with the control antibodies and K-MetStat Panel? These controls are crucial for troubleshooting CUT&RUN (**Appendix 1.3**).
* Are you using a CUT&RUN-validated antibody? See **FAQs 5-7**.
* Have reactions been mixed properly using a nutator?
* Have ConA beads become clumpy or dried out during the protocol?

4. Where can I get help with library prep and/or sequencing?

The CUTANA CUT&RUN Library Prep Kit Manual has extensive guidance for optimizing CUT&RUN library prep and sequencing (epicypher.com/protocols). The CUTANA CUT&RUN Library Prep Kit can be purchased at epicypher.com/14-1001.

5. 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.

6. 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. 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), 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 transcription factors).

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. We also validate antibody efficiency, allowing users to be confident when using reduced cell numbers. 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.

7. How do I validate an antibody for CUT&RUN?

- We recommend using an EpiCypher CUT&RUN antibody, if possible, or contact techsupport@epicypher.com for antibody recommendations.
- If an EpiCypher CUT&RUN antibody is not available for your target, follow the steps outlined in Figure 18. 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 cells. Select a specific antibody based on DNA yield, enrichment, and signal-to-noise in sequencing data.
- For methyl-lysine PTM antibodies, use the K-MetStat Panel (EpiCypher 19-1002) for direct validation in CUT&RUN. Aim for <20% antibody cross-reactivity and consistent genomic enrichment with 500,000 cells. To help ensure high antibody efficiency, validation at 50,000 is also recommended.

8. How do I optimize CUT&RUN for new cell types?

Optimization guidelines for new cell types are outlined in Figure 18.

9. How do I optimize CUT&RUN for low cell numbers?

See Figure 18 for optimization guidelines. Note that using low cell numbers may result in lower signal and increased background, including for control antibodies.

Lower yields may be partly due to antibody quality and target abundance, as well as reduced cell numbers. **For these reasons, an antibody that works well with 500,000 cells may fail at lower inputs**.

Library prep can be optimized for low yields (see the CUTANA Library Prep Manual at epicypher.com/protocols). Deeper sequencing is often recommended.

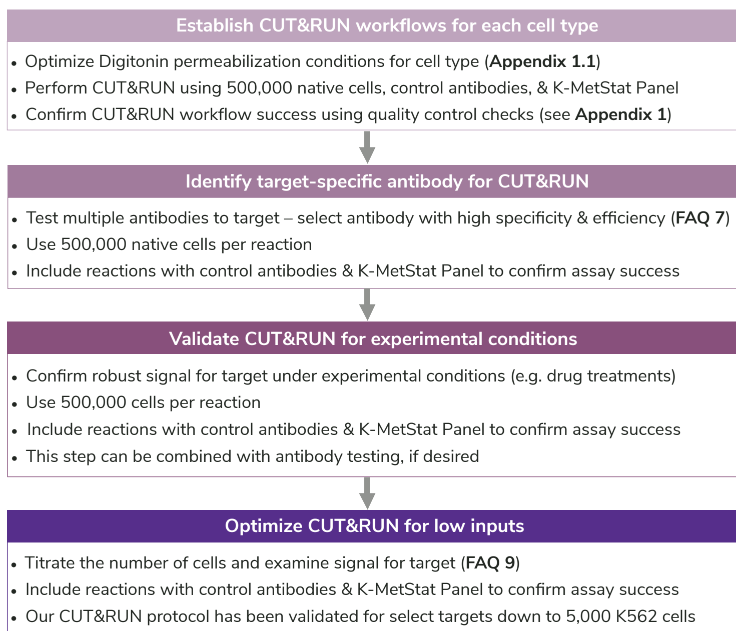


FIGURE 18

Development and optimization guidelines for successful CUT&RUN workflows.

10. Do I need to adjust the protocol to map transcription factors or other chromatin-associated proteins?

No. While a few exceptions exist (e.g. RNA Pol II), the standard CUTANA CUT&RUN protocol is sufficient for the vast majority of protein targets tested by EpiCypher, including transcription factor motif analysis.

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

CUT&RUN analysis methods are similar to those used for ChIP-seq, with key differences. Briefly, align raw reads to a reference genome using Bowtie2[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]. 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]).

12. How many reads do I need for *E. coli* Spike-in DNA and the K-MetStat Panel?

In general, ~1% of total sequencing reads should be assigned to spike-in controls. This sequencing bandwidth provides many thousands of spike-in reads, which is adequate to examine SNAP-CUTANA Spike-in recovery and/or to use *E. coli* reads for normalization. It also prevents spike-in reads from swamping sequencing data, ensuring that sufficient reads (3-8 million) are aligned to the species reference genome for biological analysis.

Note that the spike-in sequencing bandwidth may be higher or lower depending on target abundance, sequencing depth, and other factors. For instance, the IgG negative control often has 10-20% of reads assigned to the K-MetStat Panel, while a high abundance target (e.g. H3K27me3) may have 0.1-1%. Outside of this range, consider adjusting the spike-in dilution to be optimal for future experiments.

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