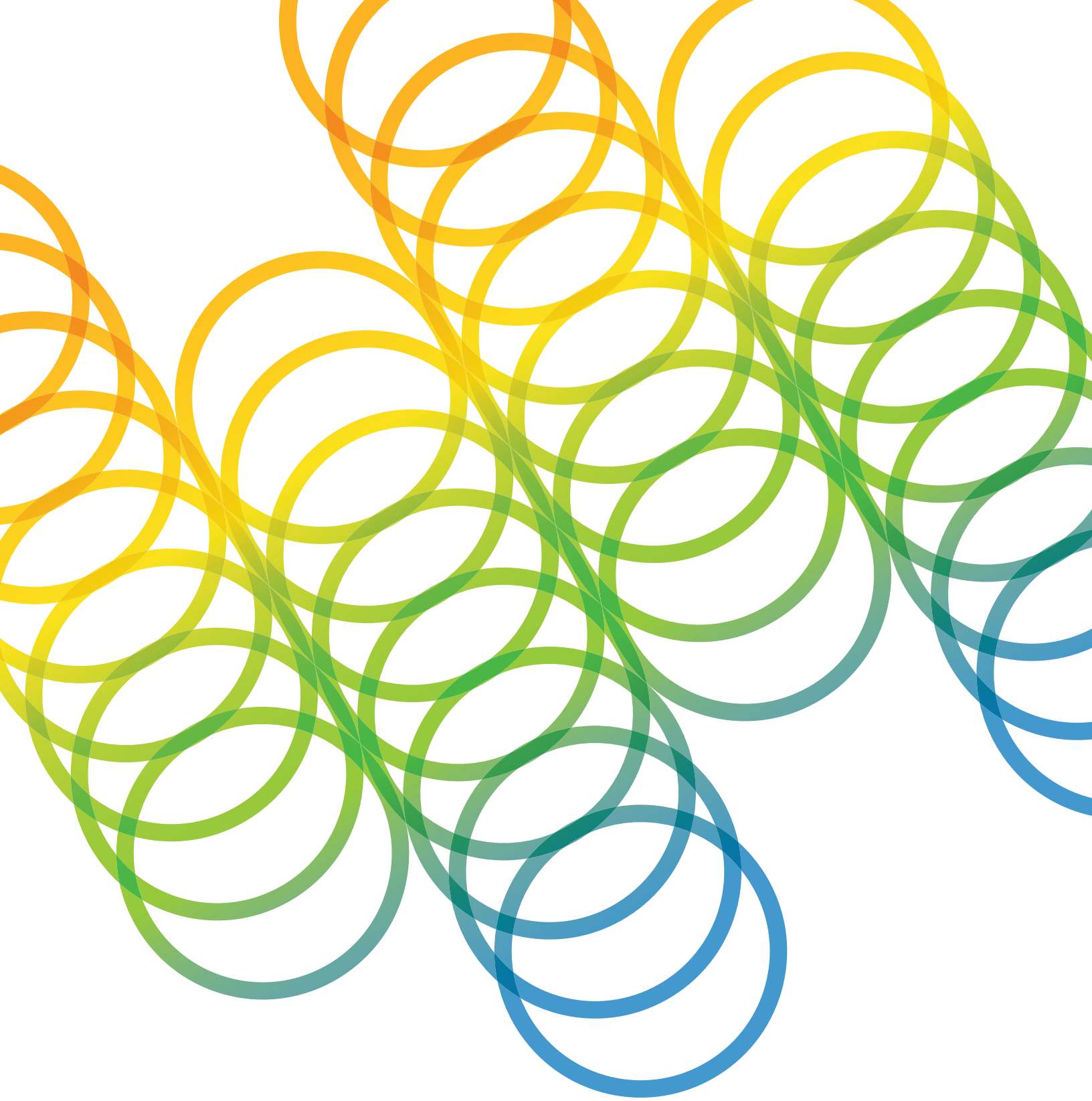


EpiCypher®
Bringing Epigenetics to Life

CUTANA™
ChIC / CUT&RUN Kit Version 5
User Manual Version 5.1

EpiCypher®

CUTANA™

ChIC / CUT&RUN Kit

Kit Version 5
Catalog No. 14-1048: 48 Reactions
Catalog No. 14-1048-24: 24 Reactions

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

Stable for 12 months upon date of receipt.
See p. 7-8 for storage instructions.

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

24 Hour Emergency Phone Number:
US & Canada: 1-800-535-5053 | International: 1-352-323-3500

Table of Contents

See EpiCypher's Tech Support Center at support.epicypher.com for additional FAQs and troubleshooting guidance.

Background and Product Description

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, permeabilized, and treated with a target-specific antibody (Figure 1). pAG-MNase releases target chromatin into solution, where it is easily separated from bead-bound cells. CUT&RUN-enriched DNA is purified and prepared for next-generation sequencing to provide genome-wide profiles of histone post-translational modifications (PTMs) and chromatin proteins (e.g. transcription factors).

The CUTANA™ CUT&RUN Kit (Figure 1) includes additional controls to ensure high-quality results. The SNAP-CUTANA™ K-MetStat Panel of spike-in nucleosomes is added to positive and negative control reactions at the beginning of CUT&RUN to support assay optimization and workflow validation. After pAG-MNase digestion, *E. coli* DNA is added to allow normalization of CUT&RUN sequencing data. Visit support.epicypher.com for additional information about these key controls.

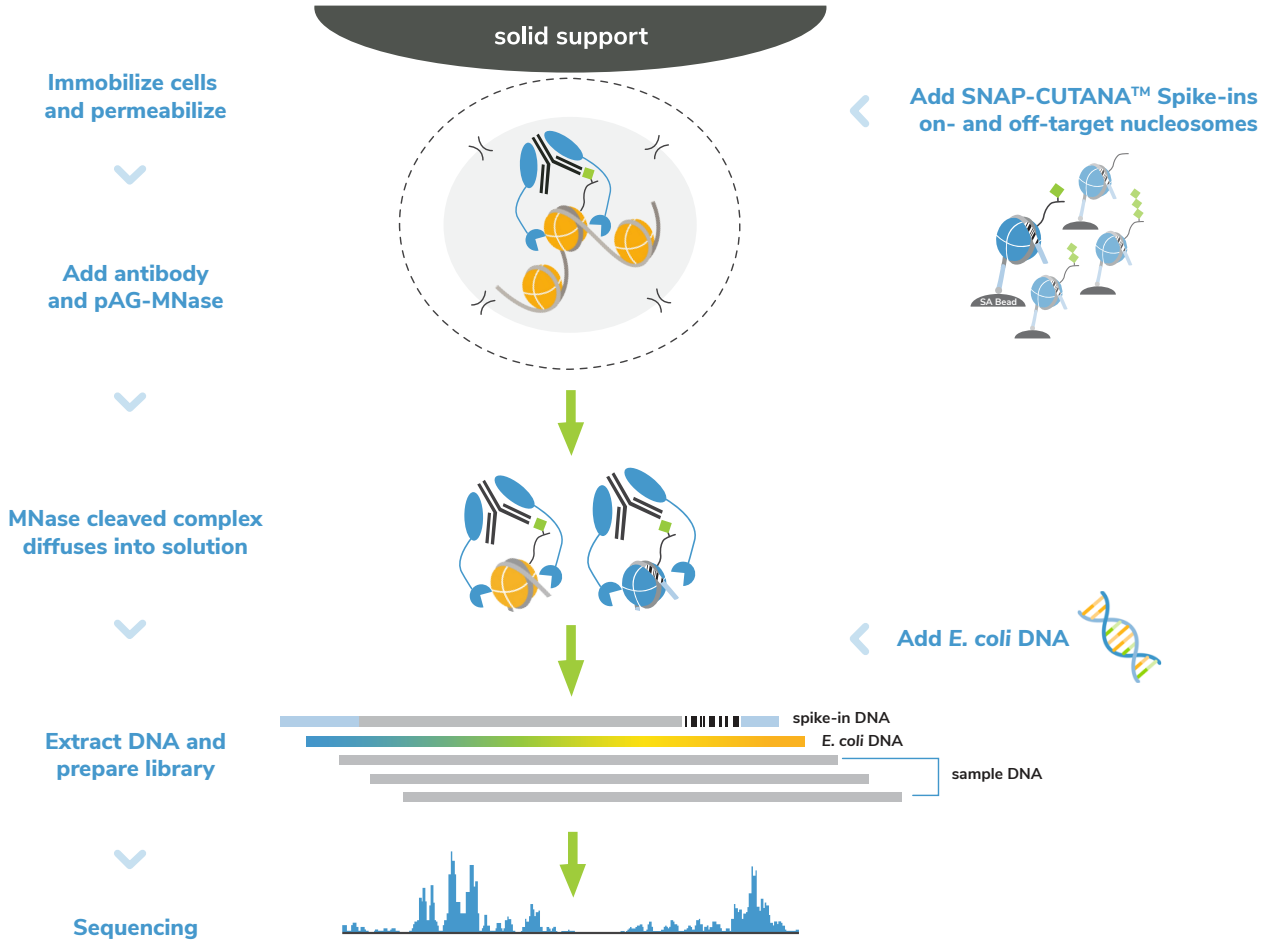


FIGURE 1

Overview of the CUTANA™ CUT&RUN protocol.

Background and Product Description

The CUTANA™ ChIC/CUT&RUN Kit contains materials for 48 reactions (EpiCypher 14-1048) or 24 reactions (EpiCypher 14-1048-24). Features of CUTANA™ CUT&RUN include:

- **A high-throughput workflow.** The protocol is designed for 8-strip tubes and multi-channel pipetting, highlighting the increased throughput made possible by CUT&RUN.
- **Low sequencing depths.** Only 3-8 million unique sequencing reads per reaction are needed to generate high-resolution chromatin profiles (Figure 2).
- **Diverse sample compatibility.** Validated for cells and nuclei, including cryopreserved and cross-linked samples (Figure 3). All sample prep protocols are available at support.epicypher.com.
- **Low cell number requirements.** Although it is recommended to use 500,000 cells per reaction, comparable data can be generated down to 5,000 cells (Figure 4).
- **Comprehensive quality control metrics.** The kit includes multiple control antibodies and spike-ins, as well as detailed success metrics, to guide you along every step of the CUT&RUN workflow. Visit support.epicypher.com for detailed explanations of controls and guidance on assay metrics.
- **On-demand customer support.** Support.epicypher.com contains detailed information about CUT&RUN assay optimization, sample prep, recommended controls, sequencing analysis, and troubleshooting.

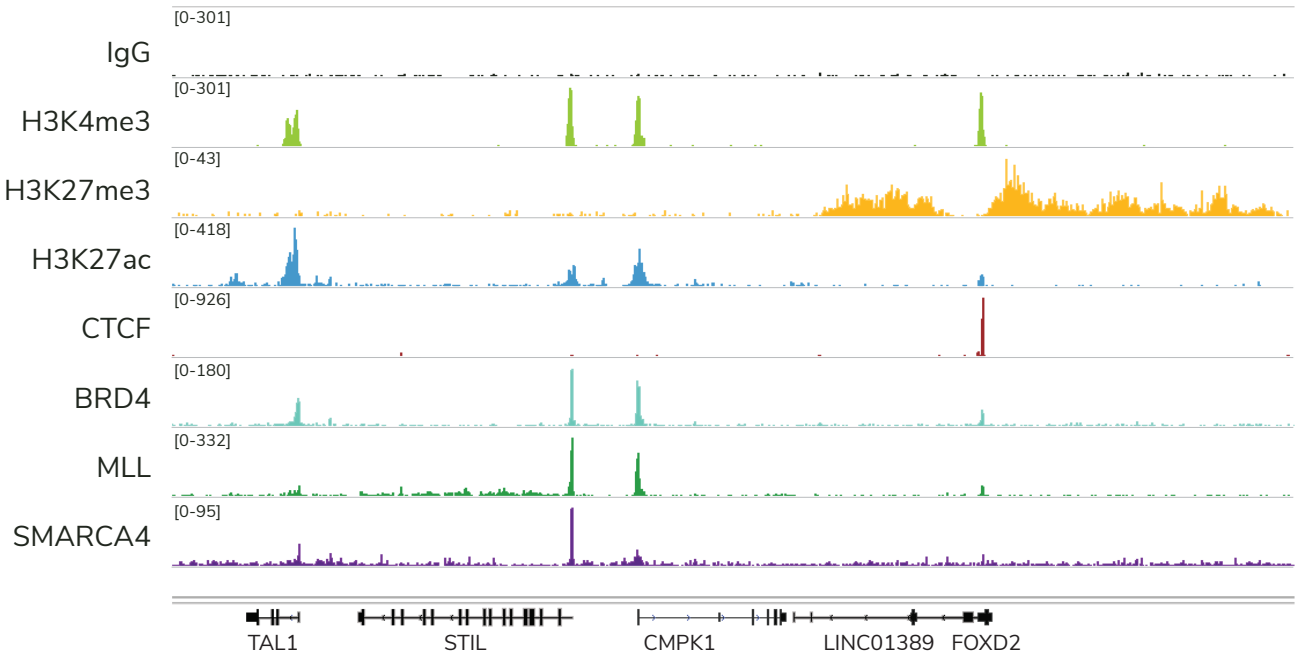


FIGURE 2

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 unique 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.

Background and Product Description

The CUTANA™ CUT&RUN Kit includes multiple controls, which should be used in **each** experiment to validate sample quality and ensure assay success. See support.epicypher.com for details on kit controls, including spike-in analysis, expected results, and their application for troubleshooting.

- **H3K4me3 Positive Control Antibody.** This control provides a representative example of a low-abundance target, with sharp peaks, that is localized at active transcription start sites (TSS).
- **H3K27me3 Positive Control Antibody.** This control offers an example of a high-abundance target with broad peaks, and is enriched at transcriptionally inactive genes.
- **IgG Negative Control Antibody.** Used to assess nonspecific background signal.
- **SNAP-CUTANA™ K-MetStat Panel** (16 DNA-barcoded nucleosomes carrying widely-studied lysine methylation PTMs). The K-MetStat Panel is spiked into each control reaction to directly monitor experimental success and aid troubleshooting.
- **E. coli Spike-in DNA.** Added to all reactions after pAG-MNase cleavage to control for library prep and enable sequencing normalization.

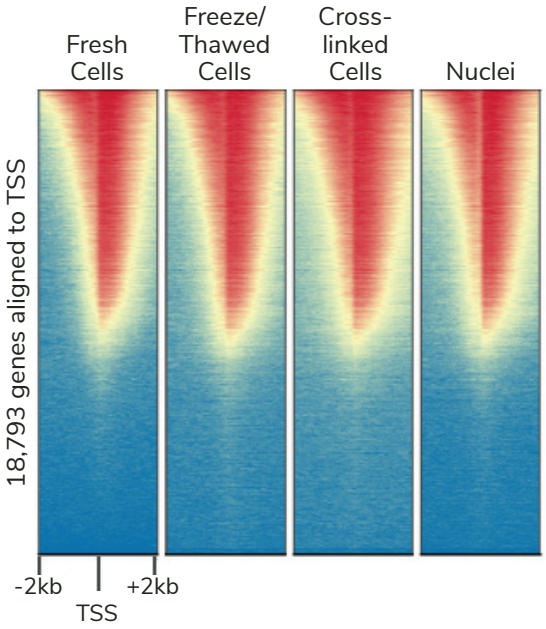


FIGURE 3

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.

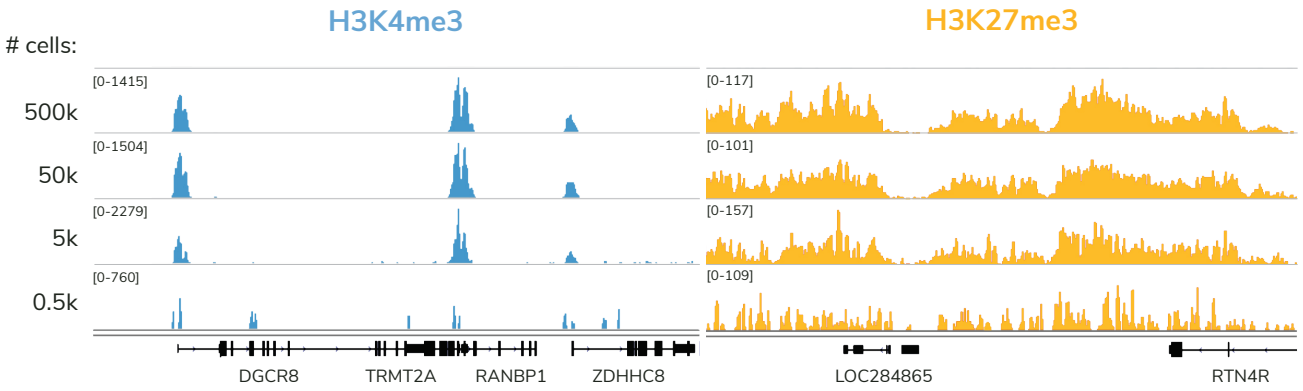


FIGURE 4

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

Included in the Kit

Kit components are stable for 12 months upon date of receipt. Store as outlined below.

Store at room temperature (RT) upon receipt:

Item	Kit 14-1048 (48 reactions)	Kit 14-1048-24 (24 reactions)	Notes before use
8-strip Tubes	10-0009k	10-0009-01	Enables use of multi-channel pipettors.
0.5 M EDTA	21-1006k	21-1006-01	Use to prepare Antibody Buffer.
100 mM Calcium Chloride	21-1007k	21-1007-01	Activates pAG-MNase to cleave DNA.
SPRIselect Reagent Manufactured by Beckman Coulter Inc.	21-1405k	21-1405-01	**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.
0.1X TE Buffer	21-1025k	21-1025-01	Use to elute CUT&RUN DNA.

Store at 4°C upon receipt:

Item	Kit 14-1048 (48 reactions)	Kit 14-1048-24 (24 reactions)	Notes before use
ConA Beads	21-1401k	21-1401-01	**DO NOT FREEZE.** Use to immobilize cells or nuclei for CUT&RUN.
Bead Activation Buffer	21-1001k	21-1001-01	Use to prepare ConA beads.
Pre-Wash Buffer	21-1002k	21-1002-01	Use to prepare CUT&RUN Buffers
Stop Buffer	21-1003k	21-1003-01	Use to terminate pAG-MNase activity.
H3K4me3 Positive Control Antibody	13-0060k	13-0060-02	**SMALL VOLUME: quick spin before use.** Rabbit monoclonal antibody, 0.5 mg/mL stock. Add 1 µL to H3K4me3 control reaction. Sufficient for 8 reactions.
H3K27me3 Positive Control Antibody	13-0055k	13-0055-02	**SMALL VOLUME: quick spin before use.** Rabbit monoclonal antibody, 0.5 mg/mL stock. Add 1 µL to H3K27me3 control reaction. Sufficient for 8 reactions.

Included in the Kit

Kit components are stable for 12 months upon date of receipt. Store as outlined below.

Store at -20°C upon receipt:

Item	Kit 14-1048 (48 reactions)	Kit 14-1048-24 (24 reactions)	Notes before use
5% Digitonin	21-1004k	21-1004-01	Thaw at RT. Use to prepare Cell Permeabilization and Antibody Buffers. Final Digitonin concentration should be optimized for each sample type. Visit support.epicypher.com for a detailed protocol.
1 M Spermidine	21-1005k	21-1005-01	Use to prepare Wash Buffer.
SNAP-CUTANA™ K-MetStat Panel	19-1002k	19-1002-02	**SMALL VOLUME: quick spin before use. Pipette to resuspend Panel — DO NOT VORTEX.** Panel of biotinylated nucleosomes coupled to streptavidin-coated magnetic beads. Pair with IgG, H3K4me3, and H3K27me3 control antibodies. Sufficient for 24 reactions. For more information, visit support.epicypher.com.
Rabbit IgG Negative Control Antibody	13-0042k	13-0042-02	**SMALL VOLUME: quick spin before use.** 0.5 mg/mL stock. Add 1 µL to designated negative control reactions. Sufficient for 8 reactions.
pAG-MNase	15-1016k	15-1016-01	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.
E. coli Spike-in DNA	18-1401k	18-1401-01	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.

Materials Required But Not Supplied

REAGENTS:

- Target-specific antibody. Visit epicypher.com/antibodies or contact techsupport@epicypher.com for recommendations. See support.epicypher.com for guidance on CUT&RUN antibody validation.
- CUTANA™ Protease Inhibitor Tablets (EpiCypher 21-1027, epicypher.com/21-1027)
- 1X PBS
- 0.4% Trypan Blue (e.g. Invitrogen T10282)
- 100% Ethanol (200 proof), any vendor
- Molecular biology grade water, any vendor
- Library prep kit (CUTANA™ CUT&RUN Library Prep Kit, EpiCypher 14-1001 and 14-1002, epicypher.com/14-1002)
- Optional: Additional SNAP-CUTANA™ K-MetStat Panel (EpiCypher 19-1002, epicypher.com/19-1002) for experimental reactions targeting histone lysine methylation PTMs. Download the SNAP-CUTANA Spike-in User Guide to learn more.
- Optional: CUTANA™ Nuclei Extraction Buffer (EpiCypher 21-1026, epicypher.com/21-1026), if isolating nuclei for CUT&RUN.
- Optional: Additional SPRI beads (EpiCypher 14-0052, epicypher.com/14-0052) or DNA spin column kit (e.g. Monarch® Spin PCR & DNA Cleanup Kit, NEB T1130) for chromatin proteins that generate small fragments (<120 bp). See this Tech Support Center article for details.

EQUIPMENT:

- 1.5, 15, and 50 mL tubes
- Low-retention filter pipette tips
- Magnetic separation rack for 1.5 mL tubes (EpiCypher 10-0012, epicypher.com/10-0012) and 8-strip tubes (EpiCypher 10-0008, epicypher.com/10-0008)
- 8-channel multi-pipettor (e.g. VWR 76169-250) and multi-channel reagent reservoirs (e.g. Thermo Fisher Scientific 14-387-072)
- Vortex (e.g. Vortex-Genie® 2, Scientific Industries SI-0236)
- Benchtop centrifuge/mini-centrifuge with an 8-strip tube adapter (e.g. from Fisher Scientific)
- Tube nutator for incubation steps (e.g. VWR 82007-202)
    - A **nutator** rocks tubes gently, without end-over-end rotation. Rotating tubes traps liquid in tube caps, resulting in ConA bead dry out and sample loss - do **NOT** rotate tubes.
- Thermocycler with heated lid (e.g. from BioRad, Applied Biosystems, Eppendorf)
- Qubit™ 4 Fluorometer (or previous version) and 1X dsDNA HS Kit (Invitrogen Q33230)

Outline of Workflow

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

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

Cells are harvested, washed, and bound to activated ConA beads. High-quality sample prep is essential to CUT&RUN workflows. Note that modifications are suggested for certain sample types (frozen cells, nuclei, adherent cells, tissues), which can be found at support.epicypher.com. The protocol includes detailed steps to confirm the quality of samples at initial cell harvest, after wash steps, and following ConA bead binding. Avoid bead dry out and clumping during the assay, which results in sample loss and reduced CUT&RUN yields.

Step 2: Permeabilize cells and 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, following the protocol available at support.epicypher.com.

Selection of an antibody with high target specificity and efficiency is crucial to CUT&RUN 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 and 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 pAG. 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 SPRI bead strategy optimized for mononucleosome-sized fragments. The use of SPRI beads allows the protocol to stay in 8-strip tubes, improving throughput and reliability. Purified DNA is quantified using the Qubit Fluorometer.

Note: If mapping transcription factors or other chromatin proteins that generate small, subnucleosomal fragments (<120 bp), consider purifying CUT&RUN DNA using a higher ratio of SPRI beads or with a spin column kit. See this Tech Support Center article for details.

Step 5: CUT&RUN library prep and next-generation 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 (5-10 million total reads) are needed to generate robust CUT&RUN profiles.

SAMPLE INPUTS FOR CUT&RUN

- Freshly isolated, unfixed (i.e. native) cells are the preferred input for CUT&RUN.
- 500,000 cells per reaction is recommended. Harvest 10% excess cells to account for sample loss and allow for quality control checks.
- See support.epicypher.com if using nuclei, adherent cells, tissues, or cyropreserved samples.
  - Cross-linking may be useful for labile targets, such as histone lysine acetylation PTMs or transiently-interacting chromatin proteins.
  - Isolation of nuclei is recommended for tissue samples, immune cells, and cells prone to lysis and/or clumping. Use the CUTANA™ Nuclei Extraction Buffer (epicypher.com/21-1026).

IMPORTANT NOTE ON SAMPLE QUALITY

- High quality sample prep is essential for CUT&RUN success. Check cell count, viability, and morphology at initial cell harvest and before ConA bead binding. Count washed cells to ensure cells are not being lost during during the centrifugation (spin longer if needed).
- Confirm cell binding to ConA beads — a critical part of the CUT&RUN workflow.
- On **Day 2**, it is important that ConA beads are resuspended during pAG-MNase incubation and digestion. Excessive clumping leads to sample loss and poor yields.

COMPATIBLE TARGETS AND CONSIDERATIONS FOR DNA PURIFICATION

- CUT&RUN is robust for most targets we have tested, including histone PTMs, transcription factors, chromatin reader proteins, modifying enzymes, and ATP-dependent remodeling enzymes.
- For chromatin proteins that generate small fragments (<120 bp), consider purifying CUT&RUN DNA using a higher ratio of SPRI beads (1.8X; additional beads available with EpiCypher 14-0052, epicypher.com/14-0052) or a spin column kit (Monarch® Spin PCR & DNA Cleanup Kit, NEB T1130), which may improve recovery. See this Tech Support Center article for details.

ANTIBODY SELECTION

- Antibodies that work well in ChIP-seq are **NOT** guaranteed success in CUT&RUN.
- Visit epicypher.com/antibodies to shop our entire list of CUT&RUN-validated antibodies or email techsupport@epicypher.com for recommendations.
- For new targets, test antibodies from multiple vendors, as detailed in Figure 5. Select the antibody that gives the best balance of expected target enrichment, low background, and high yields.
- Lysine methylation PTM antibodies can be directly validated in CUT&RUN using the SNAP-CUTANA™ K-MetStat Panel (EpiCypher 19-1002, epicypher.com/19-1002).
- We also offer SNAP-CUTANA Spike-in Controls and validated CUT&RUN antibodies for HA and DYKDDDDK epitope-tagged proteins (see epicypher.com/antibodies).

ASSAY OPTIMIZATION OVERVIEW

- CUT&RUN optimization is reviewed in Figure 5. See support.epicypher.com for guidance.
- CUT&RUN success depends on many factors, including cell type, cell number, target abundance, and antibody quality.
- Using low cell numbers may result in lower signal and increased background, including for control antibodies. In addition, an antibody that performs reliably at 500,000 cells may fail at lower numbers of cells.
- Note that our standard CUT&RUN DNA purification protocol is designed for capture of mononucleosome fragments. For experimental targets that generate small fragments (<120 bp), consider using additional SPRI beads or a spin column kit, which may help improve recovery.
- 5 ng CUT&RUN-enriched DNA is recommended for library prep. For low CUT&RUN yields, use as much DNA as possible for library prep.

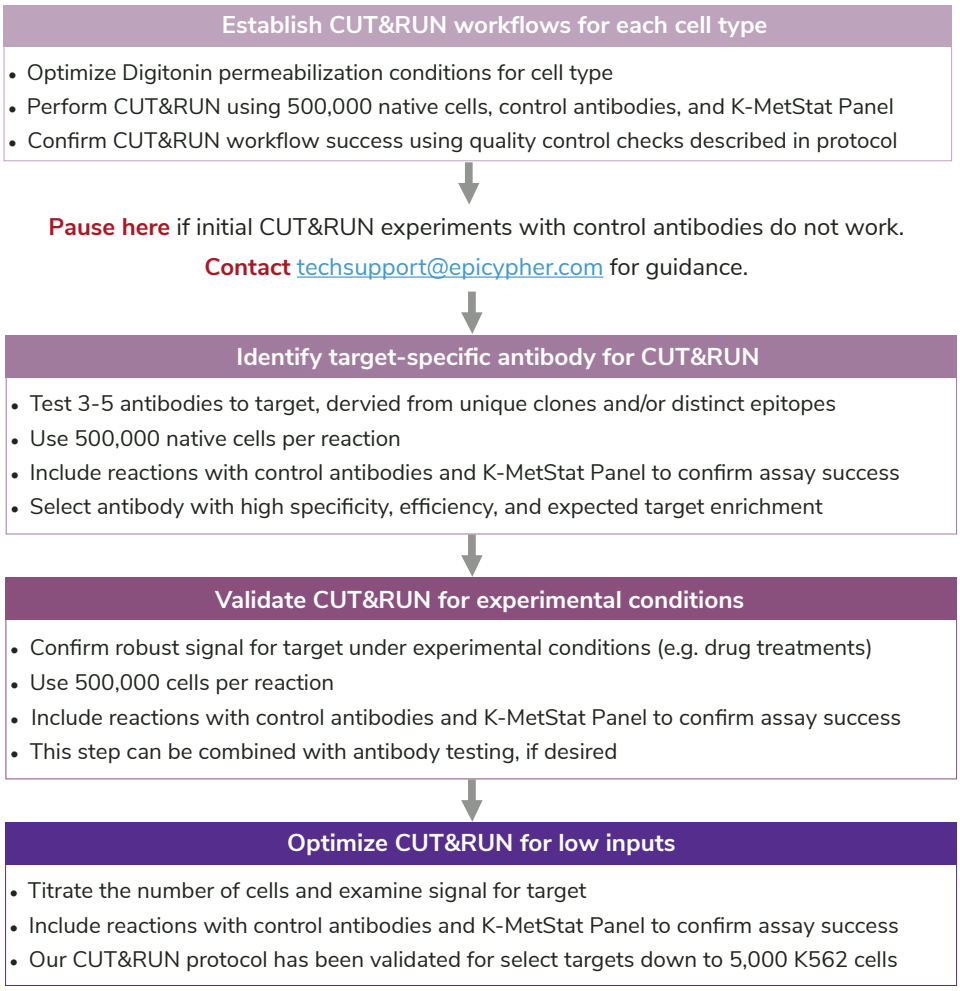


FIGURE 5

Development and optimization guidelines for successful CUT&RUN workflows.

EXPERIMENTAL CONTROLS

- The kit contains multiple quality control checks (Figure 6) to ensure reliable CUT&RUN assays. See support.epicypher.com for guidance regarding assay controls and success metrics.
- The kit also comes with our unique set of control antibodies and accompanying SNAP-CUTANA™ Spike-ins, oulined below. **Control reactions spiked with SNAP-CUTANA™ K-MetStat Panel should be used in every experiment to determine assay success and aid troubleshooting.**
  - H3K4me3 and H3K27me3 Positive Control Antibodies generate representative data for targets with varying abundanace, peak structures, and genomic enrichment, thus providing a general overview of data quality.
  - IgG Negative Control Antibody is used to assess nonspecific background signal.
  - SNAP-CUTANA™ K-MetStat Panel, 16 DNA-barcoded nucleosomes with defined lysine methylation PTMs. The K-MetStat Panel is spiked into each control reaction to validate the CUT&RUN workflow. See this article on the Tech Support Center for information.

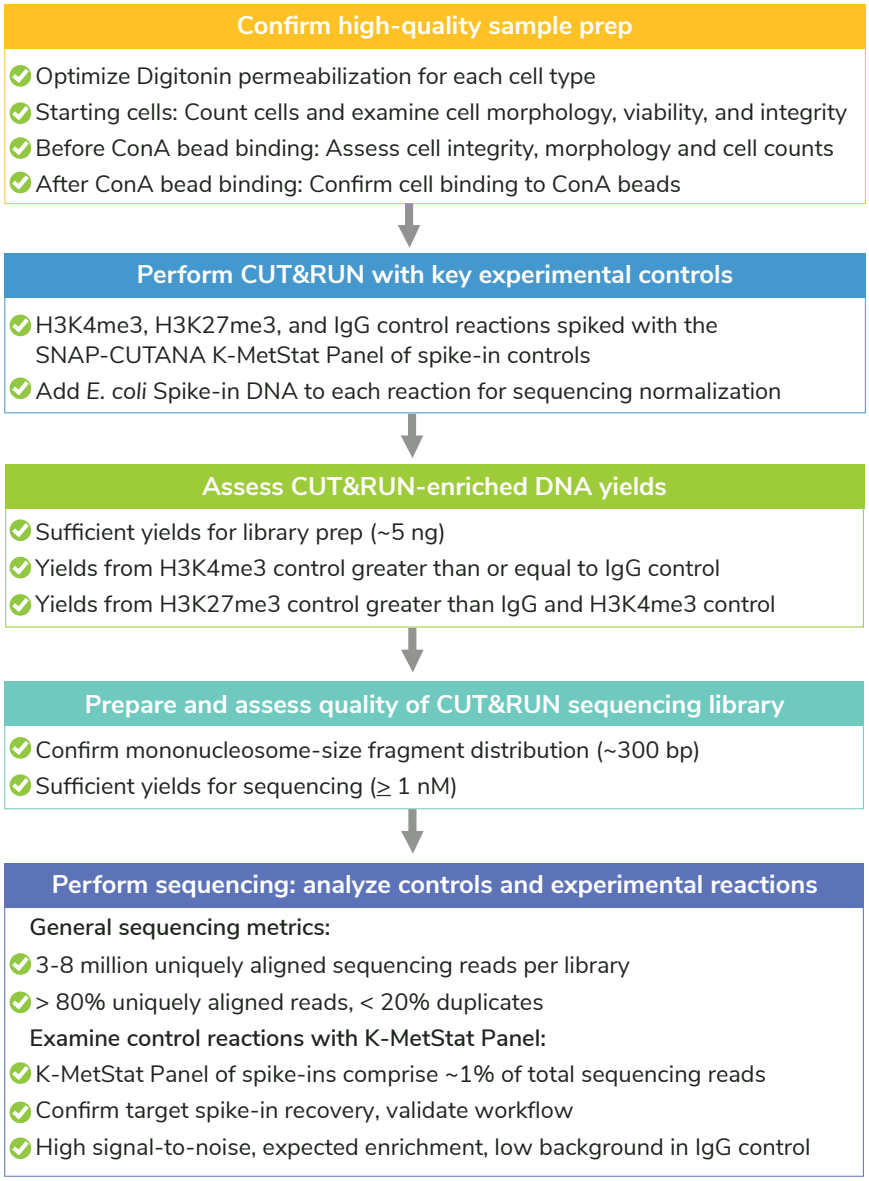


FIGURE 6

The CUTANA™ CUT&RUN Kit contains multiple quality control metrics and checks to ensure successful chromatin profiling.

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

IMPORTANT NOTES ON BUFFER PREP

* Buffers (Figure 7) 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.

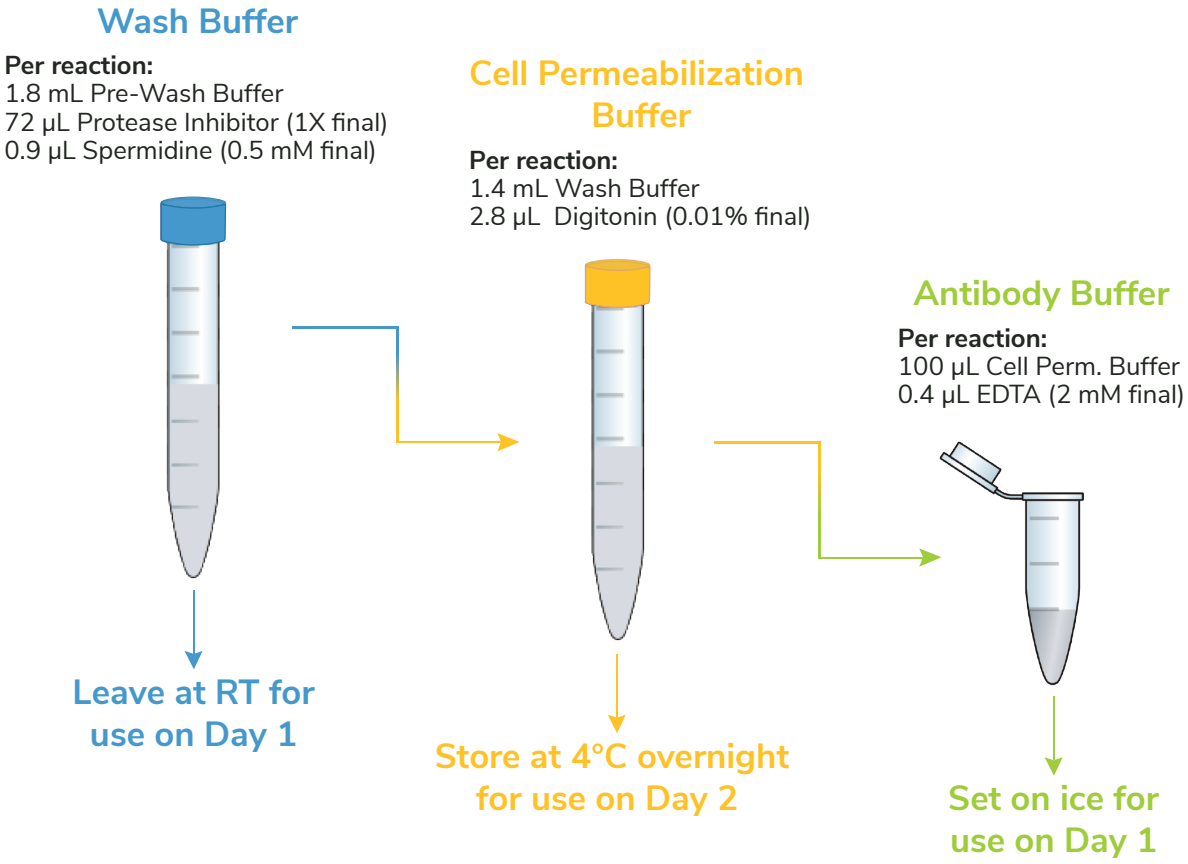


FIGURE 7

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, K-MetStat Panel, H3K4me3, H3K27me3, and IgG Control Antibodies**. Place on ice to thaw or equilibrate.
2. Reconstitute protease inhibitor tablet (EpiCypher 21-1027, epicypher.com/21-1027) as instructed in product data sheet to prepare 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 room temperature (RT).
4. To a new tube labeled **Cell Permeabilization Buffer**, add Wash Buffer as outlined in Table 1. Add 5% Digitonin as optimized for your cell type*. Place **Cell Permeabilization Buffer** on ice.

   * Calculations in Table 1 are for 0.01% Digitonin, which is optimal for most cell types tested at EpiCypher (K562, MCF7, A549, NIH3T3, LNCaP), and is recommended for reactions using nuclei. We recommend optimizing Digitonin concentrations for efficient permeabilization. See support.epicypher.com for instructions.

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

Combine reagents as instructed in the table to prepare CUT&RUN Buffers. Calculations for 8X and 16X reactions are provided. All buffers include 20% extra volume to account for pipetting error - no additional overage is needed.

SECTION II: CONA BEAD ACTIVATION (~30 MIN)

TIPS FOR WORKING WITH 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**. Place on ice.

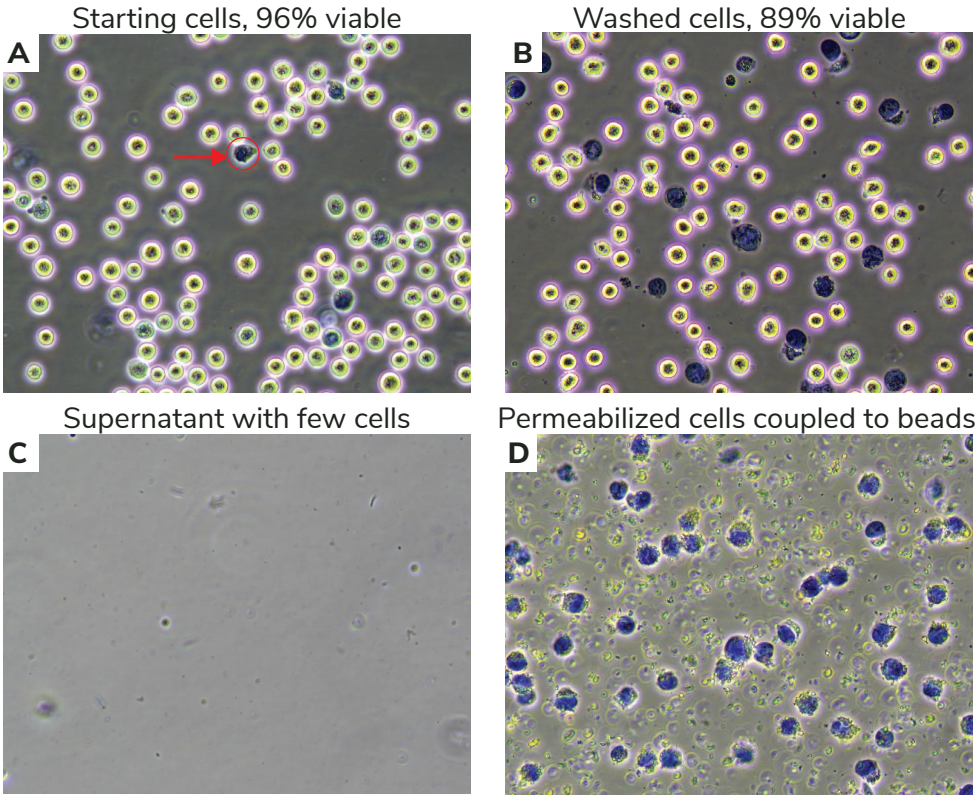
SECTION III: BINDING CELLS TO ACTIVATED BEADS (~30 MIN)

SAMPLE PREP NOTES

* **High-quality sample prep is essential to CUT&RUN.** This protocol includes steps to check the quality of starting cells, washed cells, and cells bound to ConA beads. Perform these steps in every experiment to ensure robust CUT&RUN sequencing data.
* The CUTANA CUT&RUN protocol is designed for native suspension cells. If using nuclei, adherent cells, cross-linked samples, frozen nuclei/cells, or other sample types, visit support.epicypher.com for protocol modifications.

12. Spin cells at 600 x g for 3 min at room temperature (RT). Pipette to remove supernatant, flick tube to loosen pellet, and resuspend cells in 1X PBS.
13. Transfer 10 µL cells to a fresh tube.
14. Add 10 µL 0.4% Trypan Blue to the 10 µL aliquot of cells. Pipette 10 times to mix.
15. Transfer 10 µL of the cell-Trypan Blue mixture to a cell counting slide. Obtain cell counts, determine viability (>80% is ideal), and confirm expected cellular morphology using a brightfield/phase microscope or cell counter. See Figure 8A (p. 18).

16. Harvest 500,000 cells per reaction in a fresh tube. To account for sample loss and allow quality checks, it is recommended to collect 10% excess cells.
17. Spin cells 600 x g, 3 min, RT. Pipette to remove supernatant. If cells are being lost during spins, increase spin time.
18. 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.
19. Repeat the previous step one time.
20. Resuspend cells in 105 µL per reaction RT **Wash Buffer**.
21. To determine total cell counts and evaluate cell integrity, transfer 10 µL cells to a new tube. Perform Trypan Blue staining as in Steps 14-15. Cells should be unclumped and free of cellular debris, with clear borders (see Figure 8B). Total cells counts should be at ~500,000 cells per reaction.
22. To washed cells, add 10 µL per reaction activated ConA beads. Gently vortex to mix and quick spin in a mini-centrifuge to collect slurry (beads should not settle).
23. Incubate bead-cell slurry for 10 min at RT. Cells will adsorb to beads.
24. After the 10 min incubation, place tube on magnet and allow slurry to clear.
25. If bead binding was successful, the supernatant should not contain cells. To confirm, transfer 10 µL supernatant to a new 1.5 mL tube. Perform Trypan Blue staining as in Steps 14-15. See Figure 8C for expected results.
26. Pipette to remove and discard remaining supernatant.
27. Remove tube from magnet and immediately add 55 µL per reaction cold **Antibody Buffer**. Pipette to resuspend. Note that Antibody Buffer contains Digitonin, which permeabilizes cells.
28. To confirm cell permeabilization and binding to ConA beads, transfer 10 µL bead slurry to a new 1.5 mL tube. Perform Trypan Blue staining as in Steps 14-15. Cells should be blue and surrounded by ConA beads, as in Figure 8D.
29. Aliquot 50 µL bead slurry per reaction to **8-strip Tubes**.



Sample	Success Metrics	Troubleshooting Tips
Starting cells Figure 8A	Cells show expected morphology, are unclumped, and >80% viable. Acceptable viability depends on cell type and experimental conditions. A dead cell is circled in red.	Evaluate cell culture conditions; use fresh media, troubleshoot contamination issues. Increase spin time if losing sample. See support.epicypher.com for specific recommendations for your sample type (tissues, adherent cells, etc.)
Washed cells Figure 8B	Total cell counts are ~500,000 cells per reaction. Cells show clear borders, are unclumped, and free of cell debris. Minimal lysis is observed.	Cell viability may be reduced following resuspension in Wash Buffer. Instead, focus on total cell counts. Confirm cells are intact, with expected morphology and minimal lysis.
Cells coupled to ConA beads Figure 8C,D	Supernatant (**C**) contains few cells. The slurry (**D**) contains permeabilized (Trypan Blue positive) cells bound to beads (brown specks).	Ensure that ConA beads were never frozen, cells were not clumped, beads did not become clumped or dried out, and all buffers were correctly prepared.

FIGURE 8

Validation of K562 cell sample prep using Trypan Blue staining.

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

ANTIBODY BINDING NOTES

* Add K-MetStat Panel to control reactions **BEFORE** adding the primary antibody. See support.epicypher.com for additional guidance on using these important controls.
* 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.

30. Quick spin the K-MetStat Panel. Pipette to resuspend - do NOT vortex stock.
31. Add the **K-MetStat Panel** to reactions designated for H3K4me3, H3K27me3, and IgG Control Antibodies. Add 2 µL if using 500,000 cells per reaction.

    For lower cell numbers, prepare a dilution of the K-MetStat Panel in **Antibody Buffer** on the day of the experiment, as outlined below. Add 2 µL diluted K-MetStat Panel to each reaction.

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

32. Gently vortex tubes and quick spin. Add 0.5 µg primary antibody (or manufacturer's recommendation) to each reaction. For control reactions, add 1µL respective **H3K4me3, H3K27me3, or IgG Control Antibody**.

    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.

33. Gently vortex to mix and quick spin. Incubate overnight at 4˚C on a nutator, gently rocking with tube caps elevated (Figure 9). Do **NOT** invert or rotate tubes end-over-end.

FIGURE 9

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)

IMPORTANT NOTES ON CONA BEAD MIXING

* **Resuspension of ConA beads is essential for CUT&RUN success.** Beads often become clumpy after overnight incubation. Mix as instructed in each step to keep beads in solution, ensuring even distribution of pAG-MNase.

34. Gather reagents at 4˚C and -20˚C for Day 2: **Cell Permeabilization Buffer, *E. coli* Spike-in DNA, pAG-MNase, Stop Buffer**. Place on ice to thaw or equilibrate.
35. If using a multi-channel pipettor (recommended), place a multi-channel reagent reservoir on ice. Fill with cold **Cell Permeabilization Buffer**. Throughout the protocol, remove and replace buffers one tube strip at a time to avoid bead dry out.

    Retrieve a compatible 8-strip tube magnetic rack. If using the EpiCypher 8-strip tube magnet, use the high-volume side unless otherwise noted.
36. Remove tubes from 4˚C incubation and quick spin to collect liquid. Note that beads may settle overnight (Figure 10), but this will not impact results.
37. Place tubes on magnet and allow slurry to clear. Pipette to remove supernatant.
38. Keeping tubes on magnet, add 200 µL cold **Cell Permeabilization Buffer** to each reaction. Pipette to remove supernatant. Repeat one time, keeping tubes on magnet.
39. Remove tubes from magnet and immediately add 50 µL cold **Cell Permeabilization Buffer** to each reaction. Gently vortex and/or pipette to mix. If pipetting, avoid losing beads in tips and expel all material back into tubes. Always quick spin after mixing to avoid bead loss.

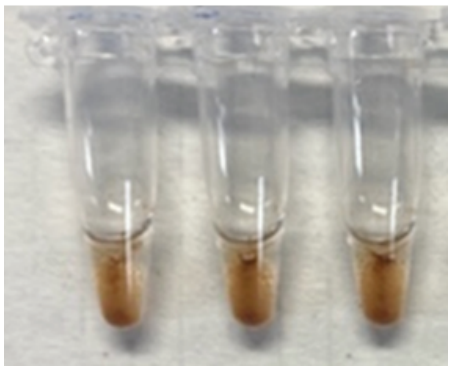


FIGURE 10

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

40. Add 2.5 µL **pAG-MNase** to each reaction. Vortex ~5 sec to thoroughly mix beads and evenly distribute enzyme.
41. Quick spin tubes and incubate reactions for 10 min at RT.
42. Quick spin tubes, place on magnet, and allow slurry to clear. Pipette to remove supernatant.
43. Keeping tubes on magnet, add 200 µL cold **Cell Permeabilization Buffer** directly onto beads. Pipette to remove supernatant. Repeat one time, keeping tubes on magnet.
44. Remove tubes from magnet. Immediately add 50 µL cold **Cell Permeabilization Buffer** to each reaction. Resuspend by pipetting (avoid bead loss, expel all material back into tubes) and/or vortexing, followed by a quick spin.

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 this Tech Support Center article.

45. Place tubes on ice. Add 1 µL **100 mM Calcium Chloride** to each reaction. Gently vortex ~5 sec to evenly resuspend beads and ensure efficient digestion.
46. Quick spin tubes. Incubate on a nutator, capped ends elevated, 2 hours at 4°C.
47. 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.
48. 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.
49. 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 ~5 sec to mix
50. Quick spin tubes and and place in a thermocycler set to 37˚C. Incubate for 10 min.
51. Quick spin tubes to collect liquid and place on a magnet until slurry clears.
52. Transfer supernatants containing CUT&RUN DNA to new **8-strip Tubes**.

SECTION VII: DNA PURIFICATION (~30 MIN)

ALTERNATIVE STRATEGIES FOR PURIFYING SMALL FRAGMENTS (<120 BP)

* Our standard DNA purification method is optimized for mononucleosomal fragments.
* If mapping chromatin proteins that generate small fragments (<120 bp), consider purifying CUT&RUN DNA using a higher ratio of SPRI beads (1.8X; additional beads available with EpiCypher 14-0052, epicypher.com/14-0052) OR with a spin column kit (Monarch® Spin PCR & DNA Cleanup Kit, NEB T1130). See this Tech Support Center article for details.

53. Prepare 85% Ethanol (EtOH) fresh using a 100% EtOH and molecular biology grade water. Make 500 µL per reaction: 425 µL 100% EtOH + 75 µL water. Note that these calculations include extra volume to account for pipetting error.
54. Vortex **SPRIselect** reagent to fully resuspend.

55. Slowly add 118 µL **SPRIselect** reagent (1.4X volume) to each reaction. If using a 1.8X volume (see **Alternative Strategies** box, p. 21), add 151 µL beads. Ensure pipette tip is free of extra droplets before dispensing beads to reactions.
56. Mix well by pipetting and/or vortexing to an even resuspension (critical for bead binding). Quick spin tubes and incubate 5 min at RT.
57. Place tubes on a magnet for 2-5 min at RT, until solution clears. Pipette to remove supernatant without disturbing beads.
58. Keeping tubes on the magnet, add 180 µL **85% EtOH** directly onto beads. Pipette to remove supernatant.
59. Repeat the previous step one time.
60. Remove tubes from magnet. Quick spin to collect liquid, with caps facing in, to avoid dislodging beads. Return to magnet and pipette to remove residual EtOH.
61. Remove tubes from magnet. Air-dry, caps open, 2-3 min at RT. Beads should be damp matte brown (Figure 11). If beads are crackly and/or light brown, they are too dry.

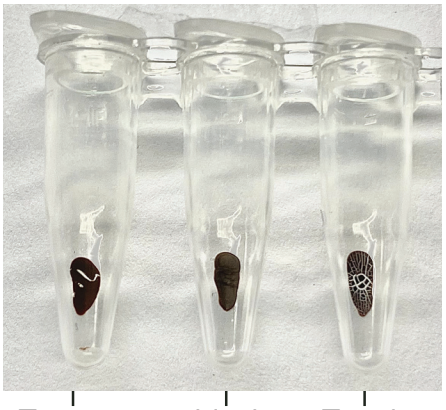


FIGURE 11

Elute DNA before beads dry out.

62. Add 17 µL **0.1X TE Buffer** to each reaction to elute DNA. Pipette and/or vortex to fully resuspend beads and quick spin. Incubate 2 min at RT.
63. For EpiCypher magnet, flip to low-volume side.
64. Place tubes on magnet for 2 min at RT.
65. Transfer 15 µL CUT&RUN-enriched DNA to new **8-strip Tubes**.
66. Quantify 1 µL DNA using the Qubit fluorometer and 1X dsDNA HS Assay Kit.

Safe pause point. Store DNA at -20˚C or continue to library prep.

EXPECTED RESULTS AND FAQS: CUT&RUN YIELDS

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. Aim for ≥5 ng DNA to enable robust library prep. If yields are low, use as much as possible for library prep. See support.epicypher.com for guidance.

Yields from positive and negative controls can also be used to gauge assay success. H3K27me3 should have higher yields compared to IgG. H3K4me3, a low abundance target, should have yields similar to or just slightly greater than IgG.

Can I use Tapestation or Bioanalyzer to examine CUT&RUN DNA yields?

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

CUT&RUN LIBRARY PREP - FAQS

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

Fragment distribution analysis of **purified libraries** on TapeStation or Bioanalyzer. Libraries should show enrichment of mononucleosome-sized DNA fragments (~300 bp, including CUT&RUN DNA + sequencing adapters). See Figure 12.

How should I prepare CUT&RUN sequencing libraries?

Prepare Illumina sequencing libraries using the CUTANA™ CUT&RUN Library Prep Kit (EpiCypher 14-1001 and 14-1002, epicypher.com/14-1002) and ~5 ng CUT&RUN DNA.

If yields are <5 ng, use total amount of recovered DNA for library prep. Our scientists frequently observe low yields for low-abundance targets, including H3K4me and IgG control reactions, and use the full volume of DNA for library prep. See support.epicypher.com for additional guidance regarding low yields.

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 CUTANA™ CUT&RUN Library Prep Kit manual at epicypher.com/protocols.

SEQUENCING CUT&RUN LIBRARIES - FAQS

What is the recommended sequencing depth and read length?

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

What are standard CUT&RUN sequencing metrics?

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

How do I analyze control reactions spiked with the SNAP-CUTANA™ K-MetStat Panel?

See support.epicypher.com for guidance on SNAP- CUTANA Spike-in data analysis.
The K-MetStat Panel should comprise ~1% of total reads. Controls should show expected enrichment and peaks. Experimental replicates should be highly reproducible (Figures 13-14).

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

See support.epicypher.com for instructions. *E. coli* DNA should comprise ~1% of total reads.

See EpiCypher's Tech Support Center at support.epicypher.com for additional FAQs and troubleshooting guidance.

EXAMPLE FRAGMENT DISTRIBUTION TRACE OF CUT&RUN SEQUENCING LIBRARIES

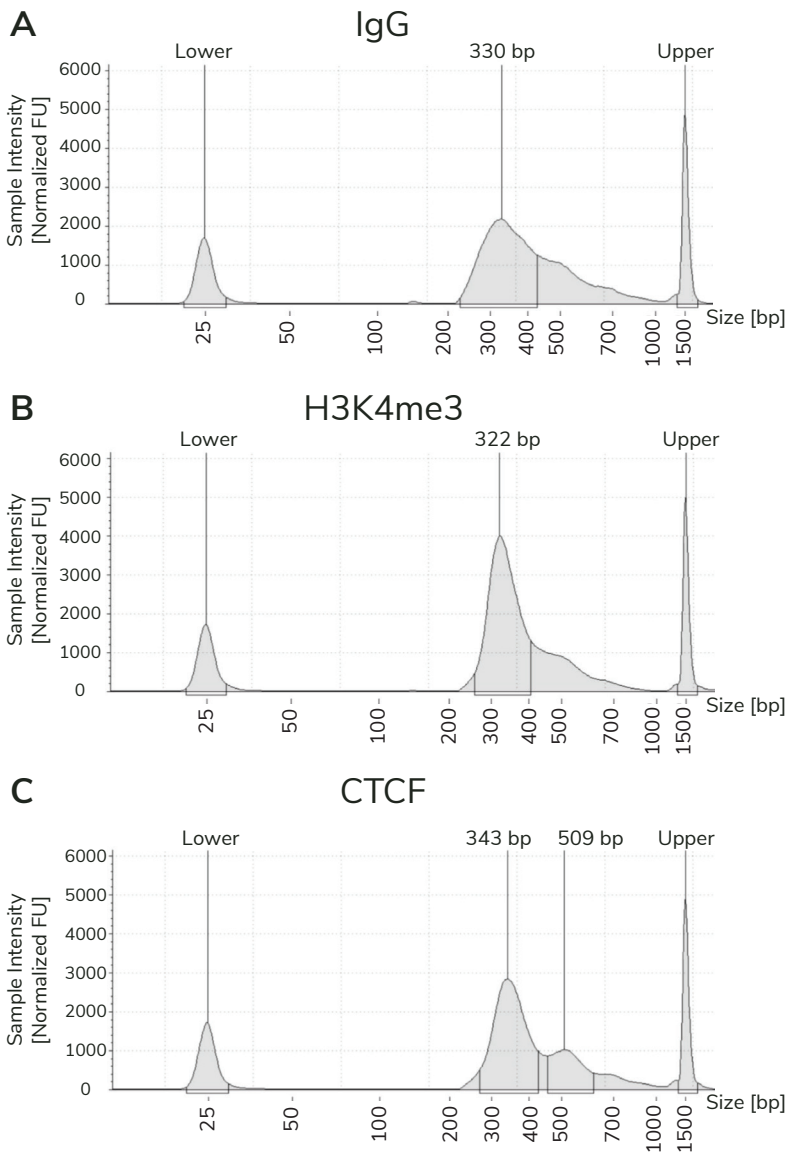


FIGURE 12

Typical TapeStation traces from CUTANA™ CUT&RUN libraries prepared using antibodies targeting IgG, H3K4me3, 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).

See EpiCypher's Tech Support Center at support.epicypher.com for additional FAQs and troubleshooting guidance.

EXAMPLE DATA FROM POSITIVE AND NEGATIVE CONTROL REACTIONS

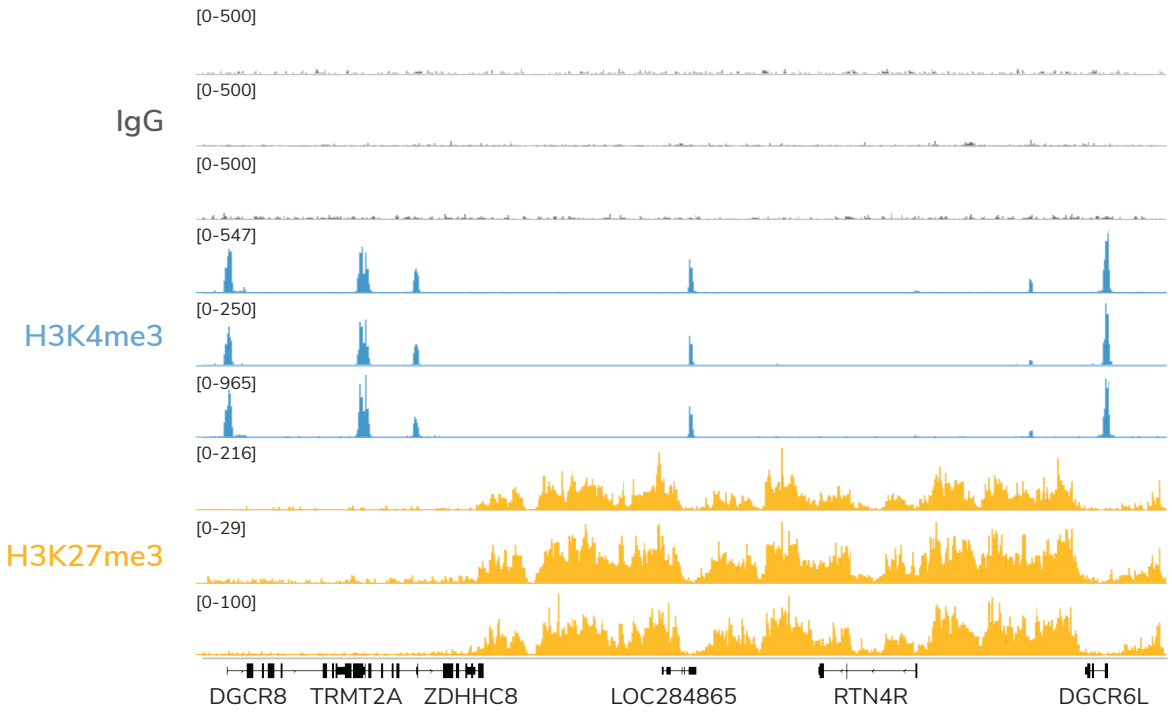
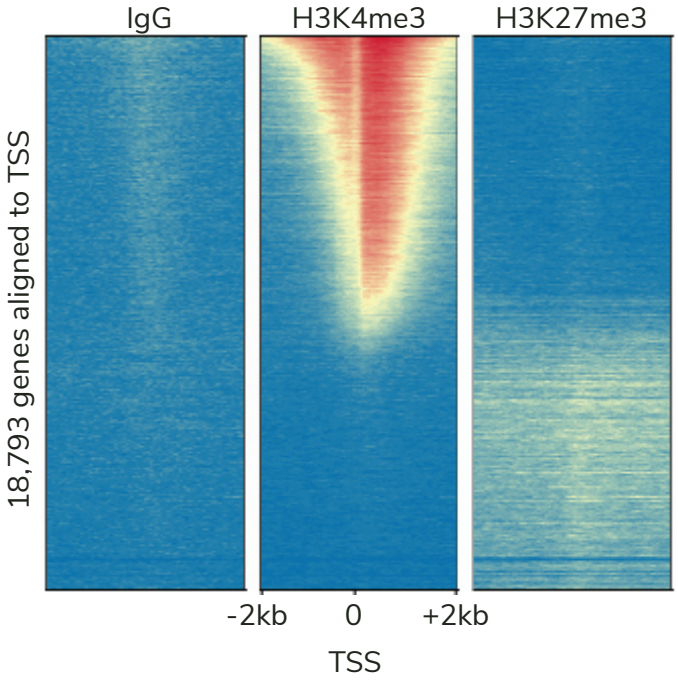


FIGURE 13

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, H3K4me3, and H3K27me3. 3-6 million unique 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 14

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.



References

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).

Revision History

Kit Manual Version #	Date	Notes
5.1	2.17.2025	• Updated recommendations for CUT&RUN DNA purification when mapping targets that enrich small subnucleosomal fragments (<120 bp). See p. 9, 10, 11, 12, 21. • Detailed support available within this Tech Support Center article.