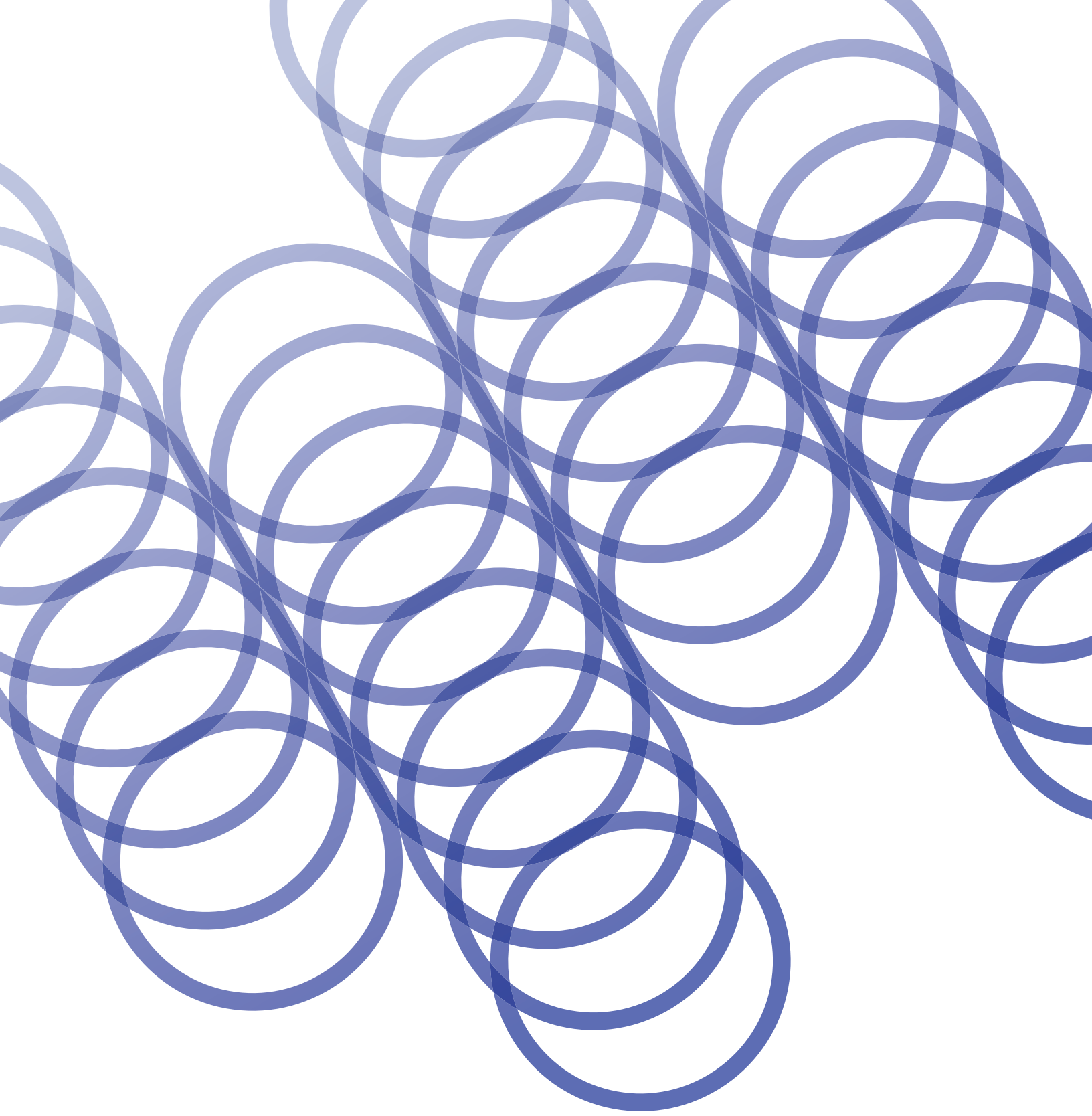

EpiCypher®
Bringing Epigenetics to Life

CUTANA™
Multiomic CUT&RUN Workflow
User Manual Version 1.0

EpiCypher®

CUTANA™

Multiomic CUT&RUN Workflow

User Manual Version 1.0

Catalog No. 14-1802 & 14-1048-24

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

See p. 6 for storage instructions.

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

Introduction

CUTANA™ Multiomic CUT&RUN is a next-generation epigenomic mapping strategy that enables direct, simultaneous analysis of DNA methylation and chromatin proteins in a single workflow. This assay leverages EpiCypher's CUTANA™ Cleavage Under Targets & Release Using Nuclease (CUT&RUN) technology, which uses a Protein A/G-micrococcal nuclease fusion (pAG-MNase) to selectively enrich antibody-bound chromatin from intact cells. CUT&RUN fragments are then processed using either an Enzymatic Methyl-seq (EM-seq) or bisulfite conversion kit, enabling base-pair resolution of DNA methylation at chromatin targets. **EM-seq is the preferred approach for Multiomic CUT&RUN (CUT&RUN-EM)** because it is less harsh than bisulfite conversion, preserves DNA integrity, and is optimized for low-input samples—making it ideal for CUT&RUN applications.

The sensitivity and specificity of CUT&RUN-EM results in a highly efficient workflow compatible with histone post-translational modifications (PTMs), transcription factors, and other proteins, revealing distinct patterns of crosstalk with DNA methylation (Figures 1, 2). The results will enable unprecedented new discoveries at the intersection of chromatin proteins and DNA methylation.

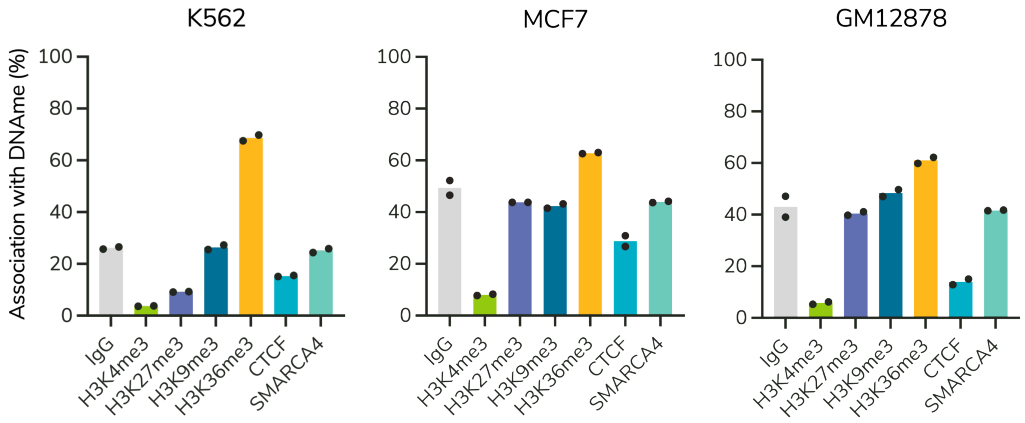
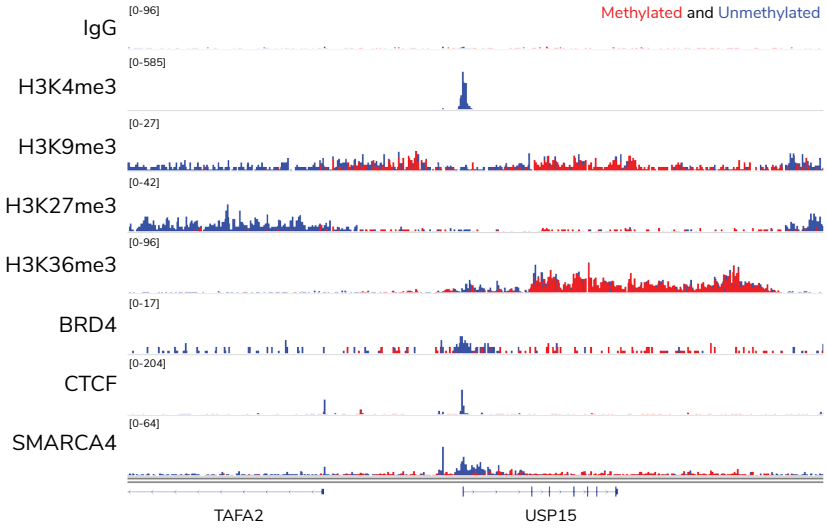


FIGURE 1

CUT&RUN-EM reveals levels of CpG methylation associated with distinct chromatin proteins, across various cells lines. Black dots represent replicates.

FIGURE 2

Representative CUT&RUN-EM genome browser tracks show differential DNAme across of variety of chromatin proteins, including histone PTMs (H3K4me3, H3K9me3, H3K27me3, H3K36me3), epigenetic reader proteins (BRD4), transcription factors (CTCF), and chromatin remodelers (SMARCA4). Red and blue tracks show methylated and unmethylated cytosines, respectively. Rabbit IgG is shown as a negative control.



Outline of Workflow

Here, we review the main steps of the CUTANA™ Multiomic CUT&RUN workflow (Figure 3):

PART 1: PERFORM CUT&RUN TO ENRICH TARGET CHROMATIN

- Intact cells or nuclei are washed, bound to magnetic ConA Beads, and permeabilized using Digitonin.
- A target-specific antibody is added for overnight incubation.
- The next day, pAG-MNase is added and binds antibody-labeled chromatin via immunoglobulin binding properties of pAG. Addition of calcium activates MNase, which cleaves proximal DNA.
- Clipped fragments diffuse into the supernatant. The Stop Buffer containing EDTA is added to chelate free calcium and halt MNase activity. Bead-bound cells are removed using a magnet.
- CUT&RUN-enriched DNA is purified from supernatant using a SPRI bead strategy.

PARTS 2 & 3: PERFORM CYTOSINE CONVERSION, LIBRARY PREP, AND SEQUENCE

- EM-seq (or bisulfite conversion) is used to annotate methylated and unmethylated CpGs and prep libraries for Illumina sequencing. With CUT&RUN-EM, just 30-50 million total reads are needed to generate robust multiomic profiles.

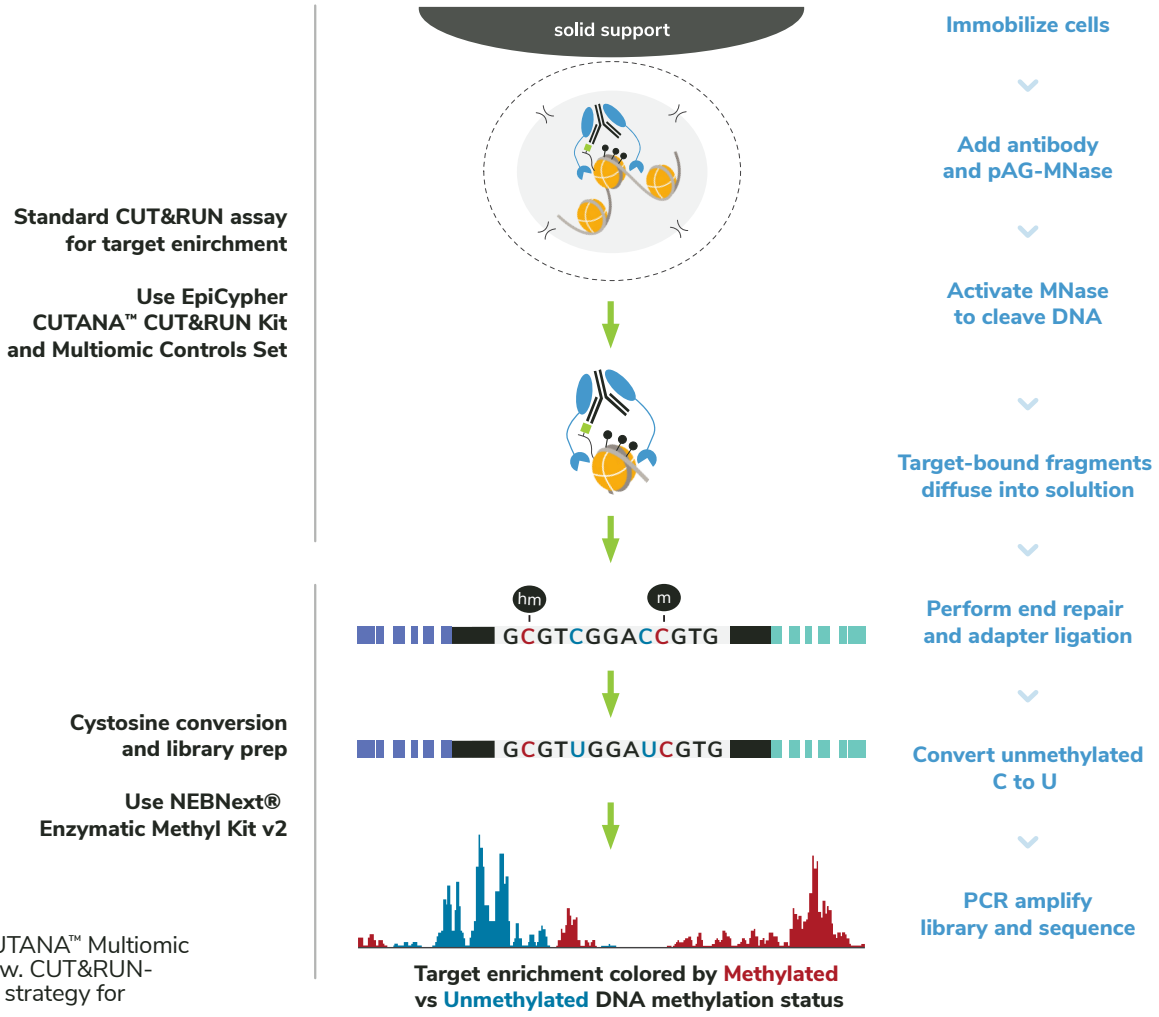


FIGURE 3

Overview of the CUTANA™ Multiomic CUT&RUN workflow. CUT&RUN-EM is the preferred strategy for multiomic analysis.

Required Reagents and Materials

CUTANA™ CUT&RUN KIT (14-1048-24)

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

Store at room temperature (RT) upon receipt:

Item	Catalog No.	Notes before use
8-strip Tubes	10-0009-01	Enables use of multi-channel pipettors.
0.5 M EDTA	21-1006-01	Use to prepare Antibody Buffer.
100 mM Calcium Chloride	21-1007-01	Activates pAG-MNase to cleave DNA.
SPRIselect Reagent Manufactured by Beckman Coulter Inc.	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	21-1025-01	Use to elute CUT&RUN DNA.

Store at 4°C upon receipt:

Item	Catalog No.	Notes before use
ConA Beads	21-1401-01	**DO NOT FREEZE.** Use to immobilize cells or nuclei.
Bead Activation Buffer	21-1001-01	Use to prepare ConA beads.
Pre-Wash Buffer	21-1002-01	Use to prepare CUT&RUN Buffers
Stop Buffer	21-1003-01	Use to terminate pAG-MNase activity.
H3K4me3 Positive Control Antibody	13-0060-02	**SMALL VOLUME: quick spin before use.** Rabbit monoclonal antibody, 0.5 mg/mL stock. Sufficient for 8 reactions (1 µL/reaction).
H3K27me3 Positive Control Antibody	13-0058-02	**SMALL VOLUME: quick spin before use.** Rabbit monoclonal antibody, 0.5 mg/mL stock. Sufficient for 8 reactions (1 µL/reaction).

Store at -20°C upon receipt:

Item	Catalog No.	Notes before use
5% Digitonin	21-1004-01	Thaw at RT. Use to prepare Cell Perm. and Antibody Buffers.
1 M Spermidine	21-1005-01	Use to prepare Wash Buffer.
SNAP-CUTANA™ K-MetStat Panel	19-1002-02	**Included with Kit, but NOT compatible with Multiomic CUT&RUN workflows.**
Rabbit IgG Negative Control Antibody	13-0042-02	**SMALL VOLUME: quick spin before use.** 0.5 mg/mL stock. Add 1 µL to negative control reactions. Sufficient for 8 reactions.
pAG-MNase	15-1016-01	**GLYCEROL STOCK: Quick spin each use; flick tube to mix. Aspirate slowly; pipette up and down several times to clear solution from tip.** 20X concentration. pAG binds IgG antibodies from various species including rabbit, mouse, goat, donkey, rat.
E. coli Spike-in DNA	18-1401-01	**Included with Kit, but NOT compatible with Multiomic CUT&RUN workflows.**

Required Reagents and Materials

CUTANA™ MULTIOMIC CUT&RUN CONTROLS SET (14-1802)

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

Store at room temperature (RT) upon receipt:

Item	Catalog No.	Notes before use
0.1X TE Buffer	21-1025-05	Use to dilute Methylated pUC19 and Unmethylated Lambda Fragmented Control DNA

Store at 4°C upon receipt:

Item	Catalog No.	Notes before use
H3K36me3 Positive Control Antibody	13-0058-03	**SMALL VOLUME: quick spin before use.** Rabbit monoclonal antibody, 0.5 mg/mL stock. Sufficient for 8 reactions (1 µL/reaction). Use as a positive control; H3K36me3 should show strong association with methylated DNA.

Store at -20°C upon receipt:

Item	Catalog No.	Notes before use
Methylated pUC19 Fragmented Control DNA	18-8001-05	Methylated DNA positive control. Dilute and add to reactions prior to EM-seq as instructed. 20 µL provided.
Unmethylated Lambda Fragmented Control DNA	18-8002-05	Spike-in control to monitor cytosine conversion efficiency. Dilute and add to reactions prior to EM-seq as instructed. 20 µL provided.

ADDITIONAL REQUIRED REAGENTS:

- DMSO and PBS (for optimizing Digitonin permeabilization; find details at support.epicypher.com)
- Antibody to target of interest; visit support.epicypher.com for guidance on antibody selection
- CUTANA™ Protease Inhibitor Tablets (EpiCypher 21-1027)
- 0.4% Trypan Blue (e.g. Invitrogen T10282)
- 100% Ethanol (200 proof), any vendor
- Molecular biology grade water, any vendor
- DNA methylation conversion kit and library prep kit. **We optimized this workflow using EM-seq, and provide specific recommendations for CUT&RUN-EM in this manual.** Materials include:
  - NEBNext® Enzymatic Methyl-seq v2 Kit (E8015)
  - NEBNext® LV Unique Dual Index Primers (e.g. E3390, E3392)

Required Reagents and Materials

EQUIPMENT:

- 1.5, 15, and 50 mL tubes and low-retention filter pipette tips
- Magnetic separation rack for 1.5 mL tubes and 8-strip tubes (EpiCypher 10-0012 and 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)
- Capillary electrophoresis machine and required reagents, e.g. Agilent TapeStation® with D1000 ScreenTape (5067-5582) and D1000 reagents (5067-5583) or Agilent Bioanalyzer® with High Sensitivity DNA Kit (5067-4626)

OPTIONAL REAGENTS:

- CUTANA™ Nuclei Extraction Buffer (EpiCypher 21-1026) if isolating nuclei for CUT&RUN.
- Additional SPRI beads (EpiCypher 14-0052) if mapping protein targets that generate fragments < 120 bp. See our Tech Support Center for up-to-date information.

SAMPLE INPUTS FOR MULTIOMIC CUT&RUN

- Freshly isolated, unfixed (i.e. native) cells are the preferred input for Multiomic CUT&RUN. Optimize Digitonin concentrations for each cell type as outlined at support.epicypher.com.
- 500,000 cells per reaction is recommended. Harvest at least 10% excess cells to account for sample loss and allow for quality control checks.
- To optimize for low cell numbers and/or new cell types:
  - Establish workflows using 500,000 cells and recommended controls (Figure 4).
  - Validate workflows for experimental targets using 500,000 cells per reaction. For new targets, test multiple antibodies from different vendors and/or targeting distinct epitopes.
  - Titrate down cell numbers as desired. The minimum recommended input for Multiomic CUT&RUN is 5,000 cells. Success at low cell numbers depends on cell type, antibody quality, and target abundance. As such, 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.
- Visit support.epicypher.com if using nuclei, adherent cells, tissues, cryopreserved samples, or cross-linked cells and nuclei.
  - 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 21-1026).

COMPATIBLE TARGETS AND CONSIDERATIONS FOR DNA PURIFICATION

- Multiomic CUT&RUN (CUT&RUN-EM) is robust for most targets we have tested, including histone PTMs, transcription factors, chromatin reader proteins, and modifying enzymes.
- If mapping proteins that generate small fragments (< 120 bp), consider purifying CUT&RUN DNA using a higher ratio of SPRI beads to improve yields. See this Tech Support Center article.

ANTIBODY SELECTION

- Antibodies that work well in ChIP-seq are **NOT** guaranteed success in Multiomic CUT&RUN.
- Antibodies validated for standard CUT&RUN workflows also work well in Multiomic 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. Use 500,000 cells per reaction and include recommended control reactions (Figure 4). Select the antibody that gives the best balance of expected target enrichment, low background, and high yields.
  - Note that an antibody that performs reliably at 500,000 cells may fail at lower numbers of cells.

Experimental Controls and Success Metrics

The workflow contains multiple quality control checks and success metrics (Figure 4). **Include the following controls in every experiment to help ensure Multiomic CUT&RUN success**:

- **H3K4me3 and H3K36me3 Positive Control Antibodies** represent targets with low and high association with DNA methylation, respectively, providing a general overview of data quality.
- **IgG Negative Control Antibody** is included to assess nonspecific background signal.
- **Methylated pUC19 Spike-in DNA** is added to each reaction prior to cytosine conversion, and serves as a positive control for detection of methylated CpGs.
- **Unmethylated Lambda Spike-in DNA** is also added to each reaction prior to cytosine conversion, and is used to monitor conversion efficiency.

FIGURE 4

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

Metric	Multiomic CUT&RUN with EM-seq (CUT&RUN-EM)		
Confirm high-quality sample prep			
Cell Quality	✓ Starting cells >70% viable with expected morphology		
Permeabilization and Binding to ConA Beads	✓ >95% of cells are permeabilized, bound to ConA beads ✓ Total cell counts are ~500,000 per reaction ✓ Cells are intact, unclumped, and show minimal lysis		
Include key experimental controls			
Negative Control	✓ IgG antibody to assess nonspecific background		
Positive Controls	✓ H3K4me3 and H3K36me3 antibodies (targets showing low and high association with DNA methylation)		
Assess CUT&RUN-enriched DNA yields			
Yields	✓ 1 ng		
Controls	✓ Yields from H3K36me3 greater than IgG Yields from H3K4me3 equal to or greater than IgG		
Assess quality of sequencing libraries			
Fragment Size	✓ ~350 bp		
Concentration	✓ >1 nM		
Examine next-generation sequencing data			
Sequencing metrics		**Results from Controls**	
Configuration	✓ 2 x 150 bp reads	IgG	✓ Low signal, no specific peaks
Total Seq. Depth	✓ 30-50 M reads		
Unique Align %	✓ ~60%	H3K4me3, H3K36me3	✓ Low vs. high overlap with 5mC
Unique Reads	✓ 20-30 M reads		

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.

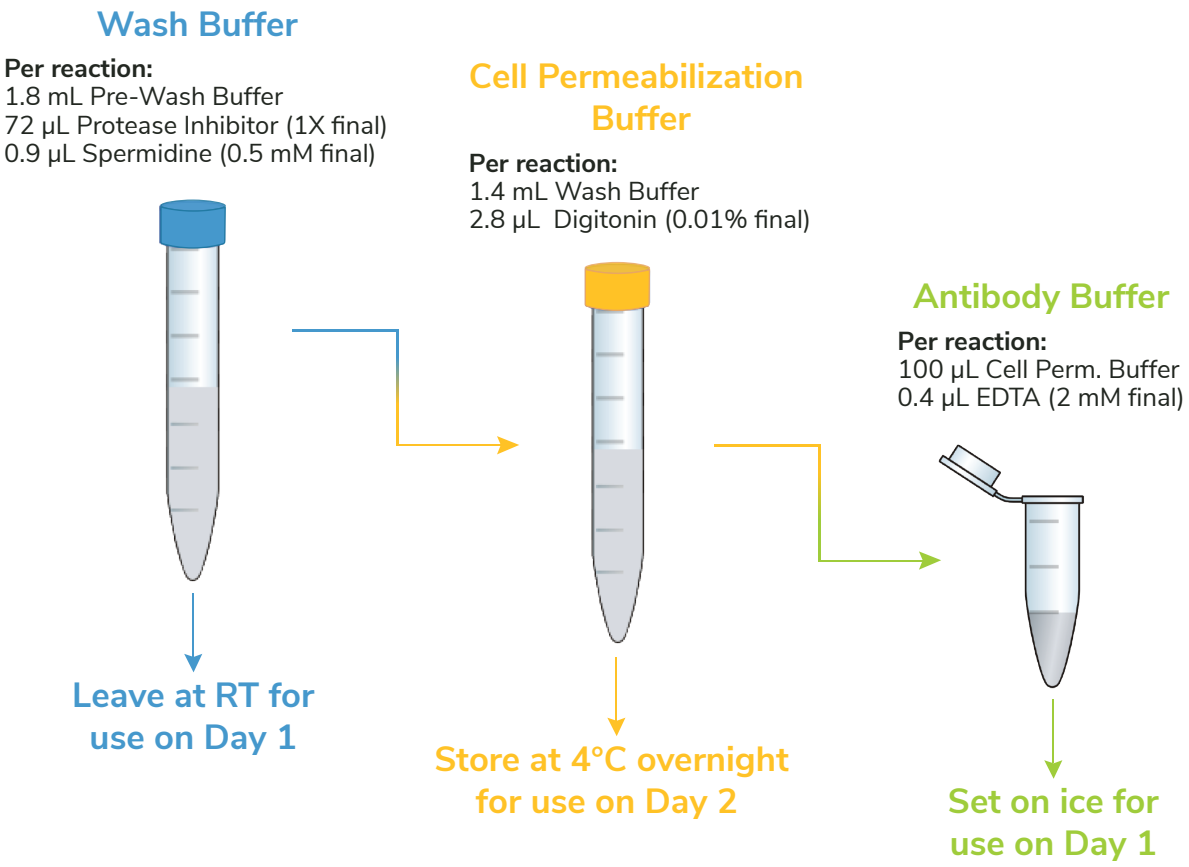


FIGURE 5

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, H3K36me3, and IgG Control Antibodies, Target-Specific Primary Antibody**. Place on ice to thaw or equilibrate.
2. Reconstitute protease inhibitor tablet (EpiCypher 21-1027) as instructed in the 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 used for processing 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** (for 8 reactions, resuspend in 88 µL). Place on ice.

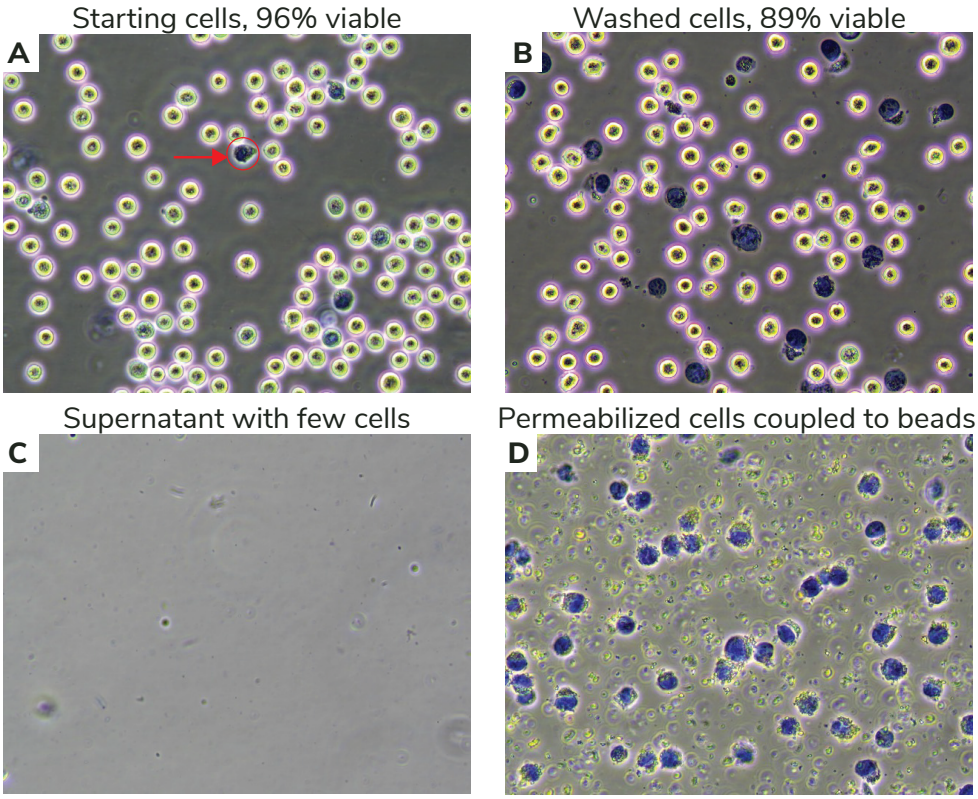
SECTION III: BINDING CELLS TO ACTIVATED BEADS (~30 MIN)

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 Multiomic 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. Collect starting cells (in tissue culture flask, tube, etc.). Count and confirm cell integrity as follows:
    A. Transfer 10 µL cells to a fresh tube.
    B. Add 10 µL 0.4% Trypan Blue. Pipette 3 times to mix.
    C. 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 6A (p. 15).

13. 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 (e.g. for 8 reactions, harvest ~4.4 million cells).
14. Spin cells 600 x g, 3 min, RT. Pipette to remove supernatant. If cells are being lost during spins, increase spin time.
15. To the cell pellet, add 100 µL per reaction RT **Wash Buffer** (e.g. for 8 reactions, add 800 µL Wash Buffer). Resuspend cells by gentle yet thorough pipetting. Spin at 600 x g, 3 min, RT. Pipette to remove supernatant.
16. Repeat the previous step one time.
17. Resuspend cells in 105 µL per reaction RT **Wash Buffer**.
18. To determine total cell counts and evaluate cell integrity, transfer 10 µL cells to a new tube. Perform Trypan Blue staining as in Step 12A-C. Cells should be unclumped and free of cellular debris, with clear borders (see Figure 6B). Total cells counts should be at ~500,000 cells per reaction.
19. 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.
20. Incubate bead-cell slurry for 10 min at RT. Cells will adsorb to beads.
21. After the 10 min incubation, place tube on magnet and allow slurry to clear.
22. 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 12A-C. See Figure 6C for expected results.
23. Pipette to remove and discard remaining supernatant.
24. 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.
25. 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 12A-C. Cells should be blue and surrounded by ConA beads, as in Figure 6D.
26. Aliquot 50 µL bead slurry per reaction to **8-strip Tubes**. Gently vortex tubes and quick spin.



Sample	Success Metrics	Troubleshooting Tips
Starting cells Figure 6A	Cells show expected morphology, are unclumped, and >70% 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 6B	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 6C,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 6

Validation of K562 cell sample prep using Trypan Blue staining.

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

ANTIBODY BINDING NOTES

* 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.

27. Add 0.5 µg (or manufacturer's recommendation) target-specific primary antibody to each reaction. For control reactions, add 1µL **H3K4me3, H3K36me3, or IgG Control Antibody**.

    For any experimental 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.

28. Gently vortex to mix and quick spin. Incubate overnight at 4˚C on a nutator, gently rocking with tube caps elevate (Figure 7). Do **NOT** invert or rotate tubes end-over-end.

End of Day 1

FIGURE 7

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 Multiomic 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.

29. Gather reagents at 4˚C and -20˚C for Day 2: **Cell Permeabilization Buffer, pAG-MNase, Stop Buffer**. Place on ice to thaw or equilibrate.
30. 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 and work quickly 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.
31. Remove tubes from 4˚C incubation and quick spin to collect liquid. Note that beads may settle overnight (Figure 8), but this will not impact results.
32. Place tubes on magnet and allow slurry to clear. Pipette to remove supernatant.
33. 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.
34. 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.

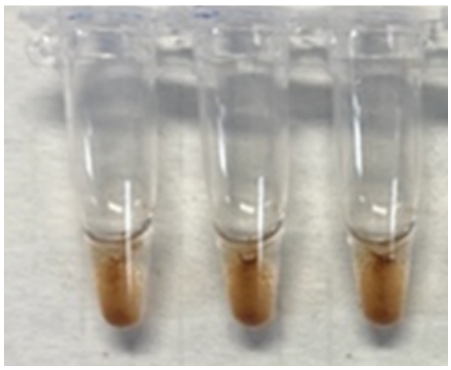


FIGURE 8

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

35. Add 2.5 µL **pAG-MNase** to each reaction. Vortex ~5 sec to thoroughly mix beads and evenly distribute enzyme.
36. Quick spin tubes and incubate reactions for 10 min at RT.
37. Quick spin tubes, place on magnet, and allow slurry to clear. Pipette to remove supernatant.
38. 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.
39. 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)

40. 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.
41. Quick spin tubes. Incubate on a nutator, capped ends elevated, 2 hours at 4°C.
42. Quick spin tubes to collect liquid. Add 33 µL **Stop Buffer** to each reaction and gently vortex ~5 sec to mix.
43. Quick spin tubes and and place 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 84 µL supernatants containing CUT&RUN-enriched chromatin to new **8-strip Tubes**.

SECTION VII: DNA PURIFICATION (~30 MIN)

ALTERNATIVE STRATEGY 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). See this Tech Support Center article for details.

46. 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.
47. Vortex **SPRIselect** reagent to fully resuspend.
48. Slowly add 118 µL **SPRIselect** reagent (1.4X volume) to each reaction. If using a 1.8X volume (see **Alternative Strategies** box), add 151 µL beads. Ensure pipette tip is free of extra droplets before dispensing beads to reactions.
49. Mix well by pipetting and/or vortexing to an even resuspension (critical for **SPRIselect** reagent binding). Quick spin tubes and incubate 5 min at RT.
50. Place tubes on a magnet for 2-5 min at RT, until solution clears. Pipette to remove supernatant without disturbing beads.
51. Keeping tubes on the magnet, add 180 µL **85% EtOH** directly onto SPRIselect reagent (beads). Pipette to remove supernatant.
52. Repeat the previous step one time.

53. 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.
54. Remove tubes from magnet. Air-dry, caps open, 2-3 min at RT. Beads should be damp matte brown (Figure 9). If beads are crackly and/or light brown, they are too dry.
55. 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.
56. For EpiCypher magnet, flip to low-volume side.
57. Place tubes on magnet for 2 min at RT.
58. Transfer 15 µL CUT&RUN-enriched DNA to new **8-strip Tubes**.

    Safe pause point. Store DNA at -20˚C or continue to Section VIII: Preparing DNA for EM-seq Conversion (p. 20).

FIGURE 9

Elute DNA before beads dry out.

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 ≥1 ng DNA to enable robust EM-seq and library prep. If yields are low, use as much as possible for library prep.

Yields from positive and negative controls can also be used to gauge assay success. H3K36me3 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 cytosine conversion and 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.

SECTION VIII: PREPARING CUT&RUN DNA FOR EM-SEQ CONVERSION

THE FOLLOWING REAGENTS ARE NEEDED:

- NEBNext® Enzymatic Methyl-seq v2 Kit (E8015)
- NEBNext® LV Unique Dual Index Primers (e.g. E3390, E3392)
- CUTANA™ Fragmented Controls for DNA Methylation Sequencing (EpiCypher 14-1804). These controls are pre-fragmented, so they can be directly spiked into CUT&RUN DNA, as outlined below. If using intact pUC19 and Lambda plasmid DNA provided in the NEBNext® Enzymatic Methyl-seq v2 Kit, the plasmids **must** be sonicated prior to use.

59. Quantify 1 µL CUT&RUN DNA using the Qubit Fluorometer and 1X dsDNA HS Assay Kit.
60. Transfer 1 ng CUT&RUN DNA to a new 8-Strip Tube and adjust final volume to 49 µL with **0.1X TE Buffer**. If meCUT&RUN yields are < 1 ng, use the total amount of recovered DNA. Place on ice.
61. Prepare a **fresh** dilution of CUTANA™ Fragmented Controls for DNA Methylation Sequencing. Combine 1 µL **Methylated pUC19 Control DNA** and 1 µL **Unmethylated Lambda Control DNA** with 98 µL 0.1X TE Buffer. Mix well.

    NOTE: Additional modifications may be required to achieve desired sequencing depth for controls. The minimum required reads for accurate conversion estimates are 500 paired-end Methylated pUC19 reads and 5,000 paired-end Unmethylated Lambda reads.
62. Add 1 µL **Fragmented Controls Dilution** to 49 µL CUT&RUN DNA in 8-Strip Tubes. Gently vortex to mix, quick spin, and place tubes on ice.
63. Proceed to EM-seq conversion and library prep using the NEBNext® Enzymatic Methyl-seq v2 Kit, starting at Section 1.2. Follow the guidelines on the next page.

SECTION IX: EM-SEQ AND LIBRARY PREP GUIDANCE

Begin the EM-seq workflow starting in Section 1.2.

Section 1.2 – End Prep of Fragmented DNA

End repair of CUT&RUN DNA prior to enzymatic conversion. The "Sheared DNA" component of the End Prep reaction is the 50 µL CUT&RUN DNA (with Fragmented Controls Dilution) prepared on the previous page.

Section 1.3 – Ligation of EM-seq Adaptor

Ligation of adapter for EM-seq and library prep. No protocol adaptation required.

Section 1.4 – Clean-Up of Adaptor Ligated DNA

Purification of adapter-ligated DNA. Follow Elution Option B for clean-up of ≤ 10 ng DNA input.

Section 1.5 – Protection of 5-Methylcytosines and 5-Hydroxymethylcytosines

Oxidation of 5-mC and 5-hmC protects methylated DNA from enzymatic conversion. Follow instructions for ≤ 10 ng DNA input. Note that the Fe(II) Solution required for TET2 activity is extremely unstable, and if this stock solution has oxidized, the entire reaction will fail. Treat the Fe(II) Solution with caution: thaw on ice, do not expose to air longer than is necessary, and return to freezer immediately after preparing the dilution. Note kit expiration date, and source new EM-seq reagents upon expiration.

Section 1.6 – Clean-Up of Protected DNA

Purification of oxidized DNA. No protocol adaptation required.

Section 1.7 – Denaturation of DNA

DNA must be denatured prior to APOBEC addition. Follow Option A for Formamide-based DNA denaturation protocol.

Section 1.8 – Deamination of Cytosines

Unmethylated cytosines are enzymatically converted to uracil. No protocol adaptation required.

Section 1.9 – PCR Amplification

Enzymatically-converted DNA is amplified and indexed for next-generation sequencing. During PCR, thymine is substituted for uracil at converted bases. Perform 11 cycles as instructed for 1 ng DNA input. If using < 1 ng DNA input, perform 12-14 cycles.

Section 1.10 – Clean-Up of Amplified Libraries

Sequencing libraries are purified using a bead-based strategy. No modification needed.

Section 1.11 – Library Quantification and Sequencing

Perform fragment distribution analysis of purified libraries on TapeStation or Bioanalyzer. See Figure 10 for example traces and p. 23 for details on sequencing.

* Libraries should show enrichment of mononucleosome-sized DNA fragments (~300 bp, including CUT&RUN DNA + sequencing adapters).
* Aim for library molarity (200-700 bp band) to be ≥ 1 nM, which will allow pooling of sequencing libraries at normal concentrations (1-4 nM).

EXAMPLE FRAGMENT DISTRIBUTION TRACE OF CUT&RUN-EM LIBRARIES

FAQS: LIBRARIES

What should I expect the Bioanalyzer/Tapestation traces to show?

Libraries should show enrichment of mononucleosome-sized DNA fragments (~350 bp, including CUT&RUN DNA + sequencing adapters). See Figure 10.

Why do I see multiple peaks in my Bioanalyzer/ TapeStation traces?

Laddering is common in CUT&RUN-EM compared to traditional CUT&RUN, and you will likely observe dinucleosome peaks at ~500 bp or larger (Figure 10).

They do not impact sequencing and we do **NOT** recommend removing them, as it risks loss of the mononucleosome peak (~350 bp).

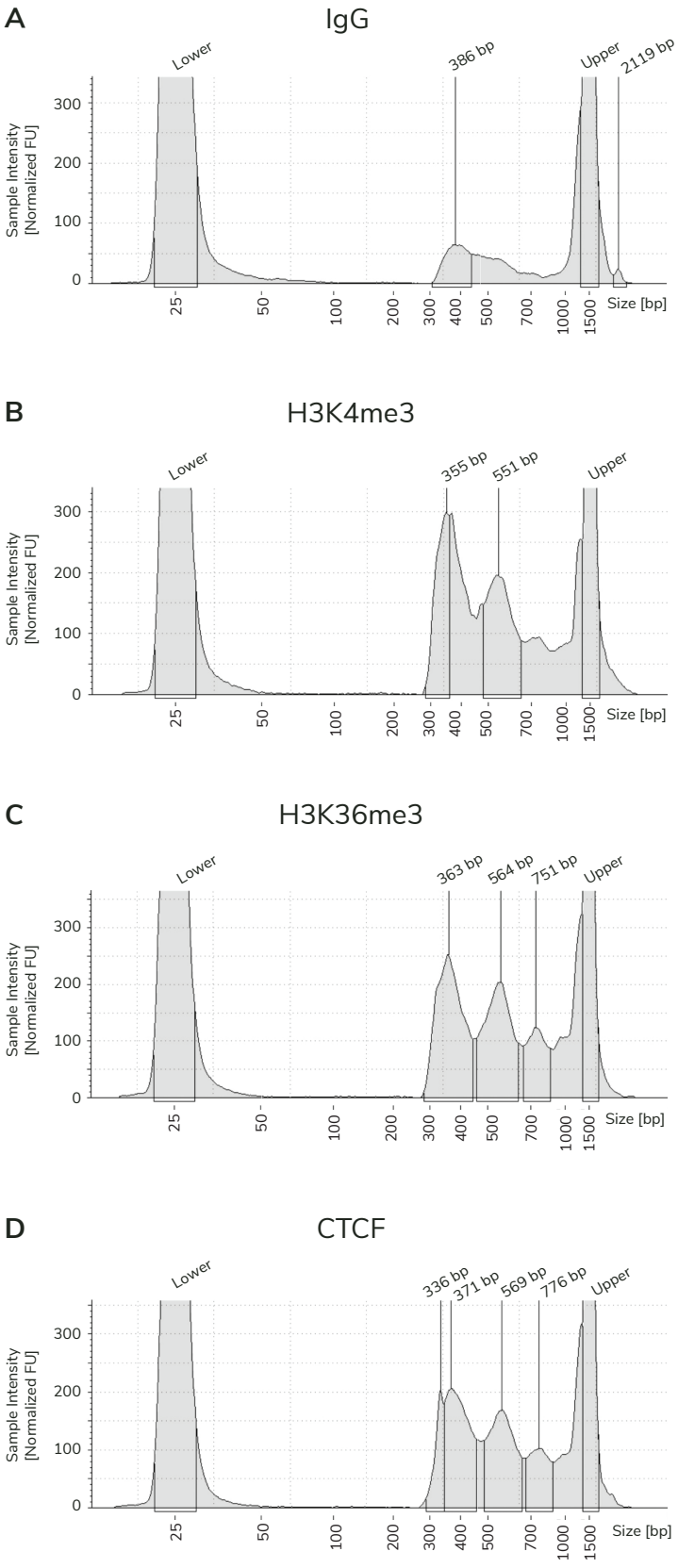


FIGURE 10

Typical TapeStation traces from CUTANA™ CUT&RUN-EM libraries, generated using K562 cells and antibodies to IgG, H3K4me3, H3K36me3, and CTCF (EpiCypher 13-2014). All libraries are predominantly enriched for mononucleosome-sized fragments, as indicated by the peak at ~350 bp (~170 bp nucleosomes + sequencing adapters).

GUIDELINES FOR SEQUENCING

- 2x150 bp paired-end sequencing is recommended to ensure coverage of entire DNA fragments.
- Libraries should be sequenced to a depth of 30-50 million total reads, with a goal of generating 20-30 million uniquely aligned reads.
- For low-concentration libraries, add as much as possible to the sequencing library pool. Deeper sequencing may be necessary to fully capture read diversity.

GUIDELINES FOR DATA ANALYSIS

- CUT&RUN-EM data can be analyzed using standard bioinformatic tools developed for bisulfite sequencing and EM-seq.
  - Map sequencing reads and call methylation status using Bismark[5].
  - Upload Bismark coverage files for further analysis via methylKit[6], an R package specifically designed for analysis of genome-wide DNA methylation sequencing data.
  - methylKit can be used to generate descriptive statistics, visualize data, and perform differential methylation analysis.
- The majority of reads (> 60%) should align uniquely to the species genome. Sequence duplication levels should be relatively low (< 30% of total sequencing reads).
- The IgG negative control reaction should show low background and no specific peaks.
- The H3K4me3 positive control denotes active promoters. Peaks should show low levels of DNA methylation.
- The H3K36me3 positive control is enriched in active gene bodies and intergenic regions, and should display high association with DNA methylation.
- Representative results are shown in Figure 11. Whole genome EM-seq is shown for comparison.

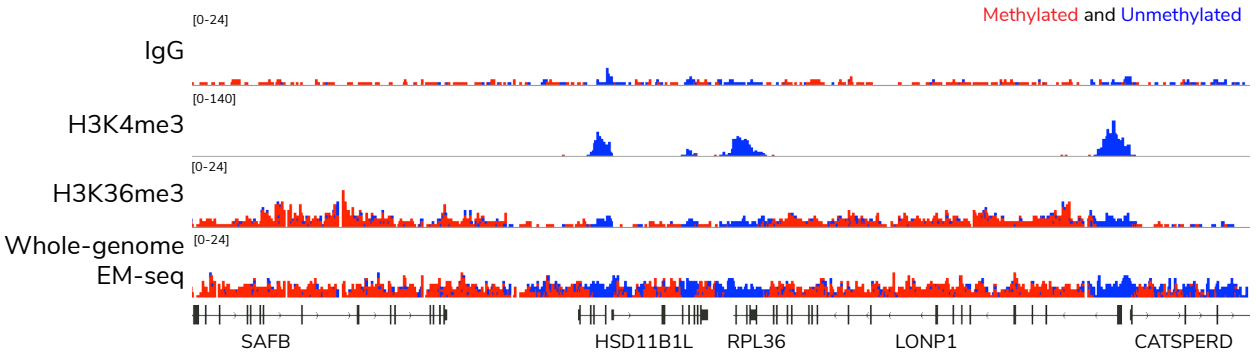


FIGURE 11

CUT&RUN-EM was performed in K562 cells using IgG, H3K4me3, and H3K36me3 control antibodies. 500,000 cells were used per reaction, and libraries were sequenced to a depth of ~30 M total reads. Whole-genome EM-seq data was generated by EpiCypher (300 M total reads) and shown to illustrate how different targets associate with DNA methylation, compared to genome-wide DNA methylation patterns.

ANALYSIS OF FRAGMENTED PUC19 AND LAMBDA CONTROL SPIKE-IN DNA

Part 1: Reference genome preparation

Retrieve pUC19 and Lambda sequences and prepare them for alignment in Bismark. Genome prep only needs to be performed once; in future experiments, reuse the pUC19 and Lambda files.

- Download pUC19 and Lambda sequences: github.com/nebiolabs/EM-seq/blob/master/methylation_controls.fa
- Save files to directory folder. Files must be in a FastA format with `.fa` or `.fasta` extension.
- Index the pUC19 and Lambda sequences using the bismark_genome_preparation command:
  `bismark_genome_preparation [options*] <path_to_directory_folder>`

Part 2: Alignment

Align meCUT&RUN-EM sequencing data to indexed Lamba and pUC19 genomes.
Note: The directory folder must also contain the sequencing files to be analyzed.

- Align paired-end reads to indexed Lambda and pUC19 genomes using the bismark command:
  `bismark [options*] <path_to_directory_folder> -1 <read_1> -2 <read_2>`
- Two output files are generated: `test_dataset.fastq_bismark.bam` (all alignments plus methylation call strings) and `test_dataset.fastq_bismark_SE_report.txt` (summary).

`[options*]` See www.bioinformatics.babraham.ac.uk/projects/bismark/ for analysis options.

Expected Results

- Aim for a minimum of 500 reads assigned to Methylated pUC19 DNA and 5,000 reads assigned to Unmethylated Lambda DNA.
- Methylated pUC19 DNA is protected from EM-seq conversion, and should show >95% methylation (Figure 12A).
- Unmethylated Lambda DNA should show ≤1% DNA methylation, which incidates an EM-seq conversion efficiency of ~99% (Figure 12B).

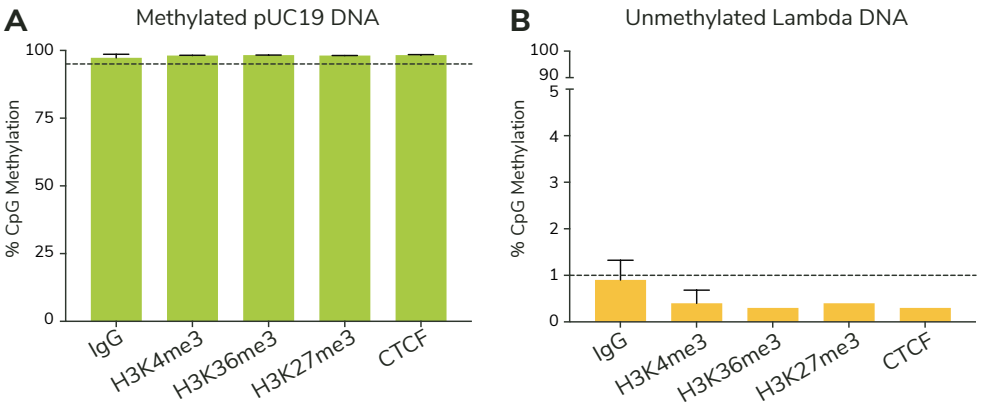


FIGURE 12

CUT&RUN-EM was performed in K562 cells with Methylated pUC19 and Unmethylated Lambda Control DNA. (**A**) Methylated pUC19 DNA shows >95% methylated CpGs, as expected. (**B**) Unmethylated Lambda DNA shows <1% DNA methylation.

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5. Akalin et al. methylKit: a comprehensive R package for the analysis of genome-wide DNA methylation profiles. ***Genome Biol*** 13, R87 (2012).