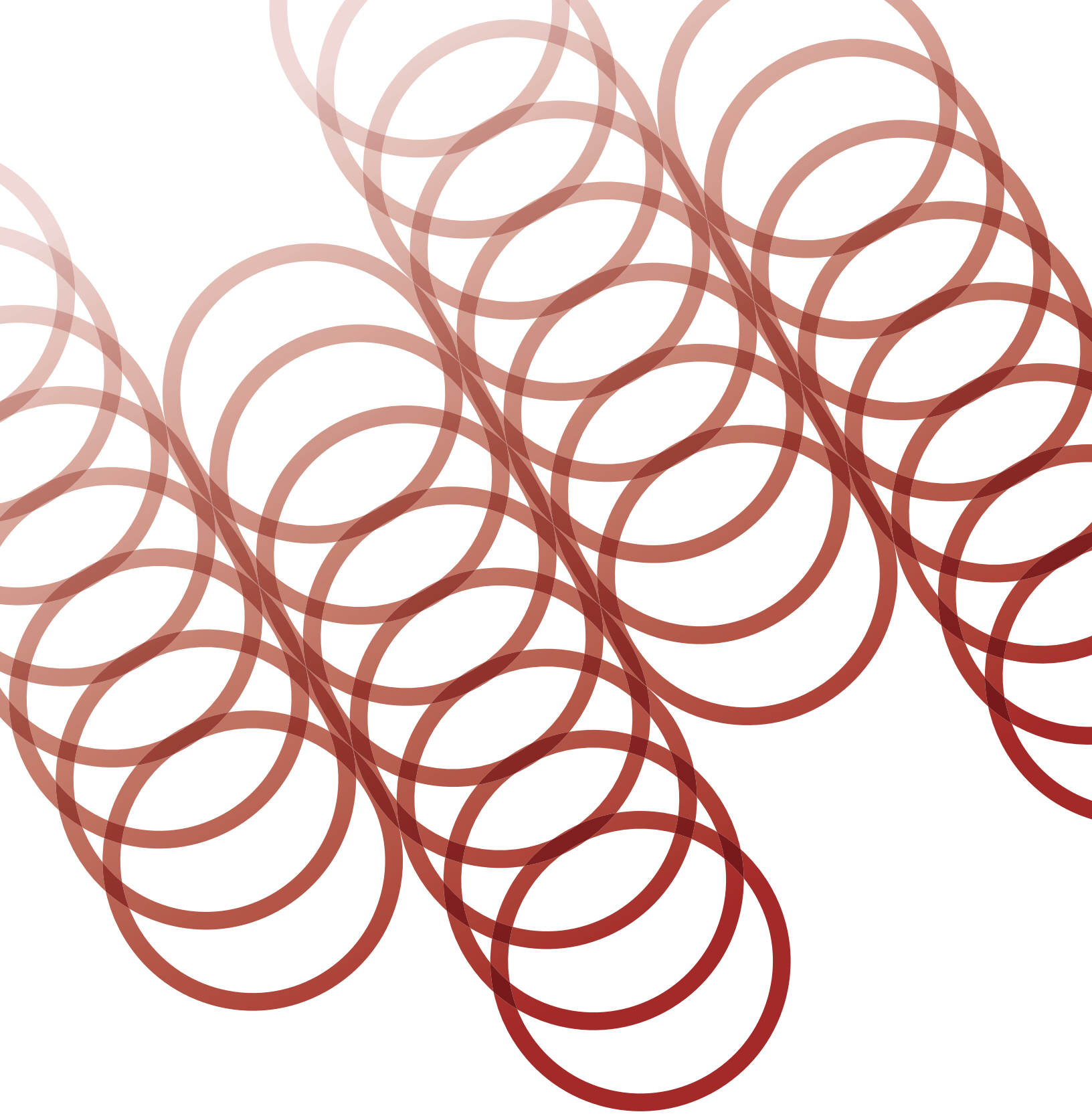


EpiCypher®
Bringing Epigenetics to Life

CUTANA™ meCUT&RUN Kit for DNA Methylation Sequencing

Kit Version 1

User Manual Version 1.0

EpiCypher®

CUTANA™

meCUT&RUN Kit for DNA Methylation Sequencing

Kit Version 1
Catalog No. 14-1060-24

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

Stable for 12 months upon date of receipt.
See p. 6 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

Introduction	**4**
Outline of Workflow	**5**
Included in the Kit	**6**
Materials Required but Not Supplied	**7**
Experimental Design and Controls	**8**
meCUT&RUN Experimental Protocol	**9**
Section I: meCUT&RUN Buffer Prep (~30 min)	9
Section II: ConA Bead Activation (~30 min)	11
Section III: Binding Cells to Activated Beads (~30 min)	11
Section IV: GST-MeCP2 Binding (~30 min + overnight)	14
Section V: Binding of Anti-GST Antibody and pAG-MNase (~1.5 hrs)	14
Section VI: Targeted Chromatin Digestion and Release (~3 hrs)	16
Section VII: DNA Purification (~30 min)	16
Library Prep and Sequencing Option 1: Traditional Library Prep (meCUT&RUN)	**18**
Library Prep and Sequencing Option 2: EM-seq (meCUT&RUN-EM)	**20**
References	**25**

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

Introduction

meCUT&RUN is an innovative DNA methylation profiling technology based on **C**leavage **U**nder **T**argets & **R**elease **U**sing **N**uclease (CUT&RUN)[1-3] and EpiCypher's groundbreaking Reader CUT&RUN technology[4]. meCUT&RUN uses a methyl binding domain to selectively enrich methylated DNA enabling low-cost, ultra-sensitive DNA methylation profiling. Competitive advantages include:

- **Improved 5-methylcytosine coverage.** meCUT&RUN captures 80% of methylated CpGs, outperforming reduced representation bisulfite sequencing (RRBS), arrays, and hybridization panels.
- **Reduced sequencing costs.** Compared to whole-genome sequencing approaches, meCUT&RUN requires >20-fold fewer sequencing reads, offering a budget-friendly alternative.
- **Low cell number requirements.** Although it is recommended to use 500,000 cells per reaction, comparable data can be generated down to ~10,000 cells (Figure 2).
- **Avoids bisulfite conversion, minimizing bias.** By enriching methylated DNA, meCUT&RUN avoids harsh chemical conversion strategies that can reduce yields and introduce unwanted bias.
- **Optional base-pair resolution.** meCUT&RUN can be paired with NEBNext® Enzymatic Methyl Seq (meCUT&RUN-EM) for base-pair resolution CpG methylation analysis (Figure 1).

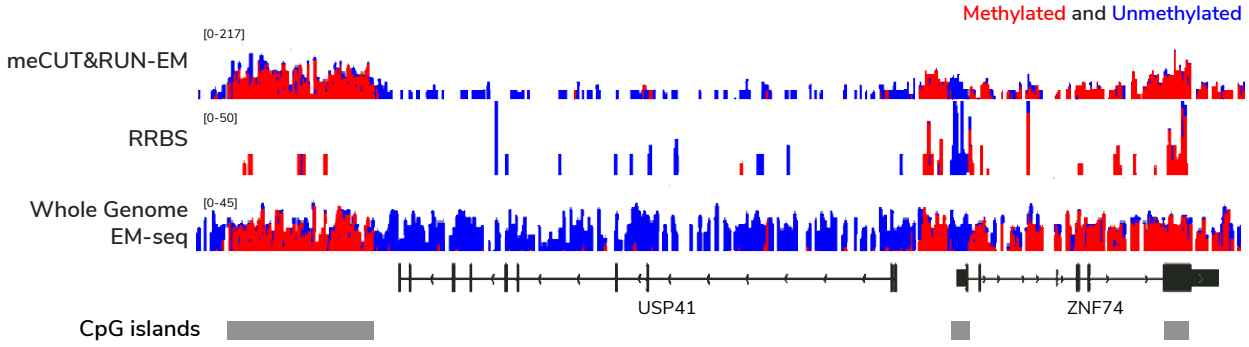


FIGURE 1

CUTANA™ meCUT&RUN generates DNA methylation profiles similar to whole genome EM-seq at greatly reduced sequencing depth, while also providing improved 5-methylcytosine coverage compared to RRBS. meCUT&RUN was paired with EM-seq (meCUT&RUN-EM; 30 M reads) to thoroughly evaluate it's performance against targeted bisulfite-conversion assays (RRBS; data from ENCODE, 57 M reads) and whole-genome, enzymatic-based approaches (EM-seq; 300 M reads) in K562 cells.

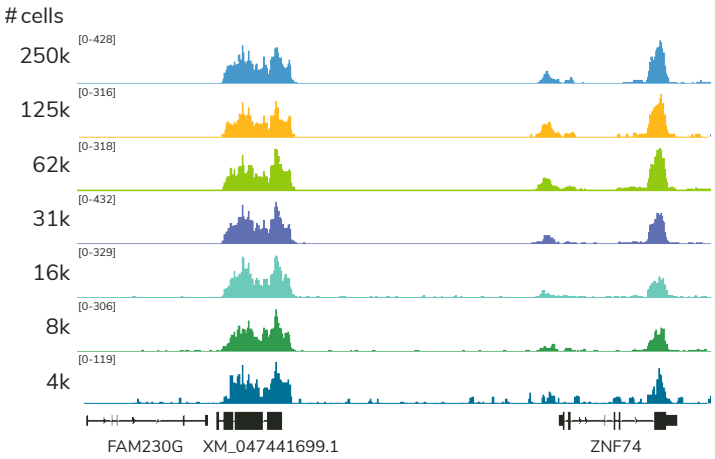


FIGURE 2

Representative genome browser tracks for meCUT&RUN experiments using decreasing amounts of lightly cross-linked K562 cells (0.1 % formaldehyde, 1 min). At 8,000 cells, data are largely indistinguishable from assays using 250,000 cells.

Outline of Workflow

Here, we review the main steps of the CUTANA™ meCUT&RUN workflow (Figure 3).

- Intact cells or nuclei are washed, bound to magnetic ConA Beads, and permeabilized using Digitonin.
- GST-MeCP2 Methyl Binding Domain is added to bind 5-methylcytosine.
- Anti-GST Tag antibody is added, followed by pAG-MNase (protein A/G micrococcal nuclease). 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.
- meCUT&RUN-enriched DNA is purified from supernatant using a SPRI bead strategy.
- Users have two options for library prep (Figure 3). **Option 1 (meCUT&RUN)** uses a traditional library prep strategy for DNA methylation profiles with ~150 bp resolution, and requires 15-20 M total reads. **Option 2 (meCUT&RUN-EM)** uses an EM-seq cytosine conversion and library prep strategy for single-base resolution, and requires 30-50 M total reads.

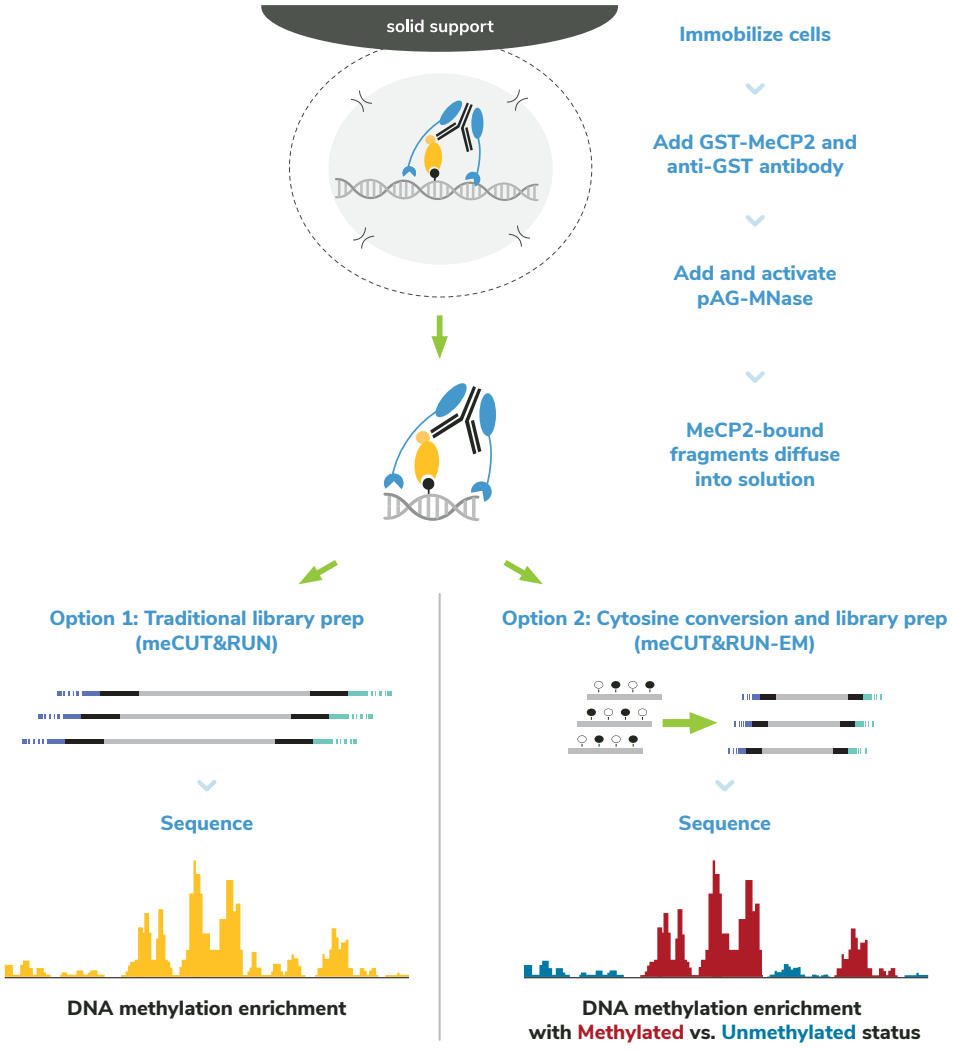


FIGURE 3

Overview of the CUTANA™ meCUT&RUN workflow.

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	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.
5 M NaCl	21-1013-04	Use to prepare Wash 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 meCUT&RUN-enriched DNA.
0.1X TE Buffer	21-1025-01	Use to elute meCUT&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 meCUT&RUN Buffers
Stop Buffer	21-1003-01	Use to terminate pAG-MNase activity.
Rabbit Anti-GST Tag Antibody	13-0073-04	**SMALL VOLUME: quick spin before use.** 0.5 mg/mL stock; sufficient for 24 reactions. Tethers pAG-MNase to MeCP2-bound chromatin. Also use as a negative control (recommended).

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.
GST-MeCP2 Methyl Binding Domain	15-2002-04	**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. Sufficient for 24 reactions. Use to enrich methylated DNA for next-generation sequencing.
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	50 ng lyophilized *E. coli* DNA, an optional spike-in control for meCUT&RUN using traditional library prep. Quick spin and resuspend in 100 µL DNase-free water (0.5 ng/µL). Store at -20˚C.

Materials Required But Not Supplied

REAGENTS:

- CUTANA™ Protease Inhibitor Tablets (EpiCypher 21-1027)
- 1X PBS, any vendor
- 0.4% Trypan Blue (e.g. Invitrogen T10282)
- 100% Ethanol (200 proof), any vendor
- Molecular biology grade water, any vendor
- If isolating nuclei: CUTANA™ Nuclei Extraction Buffer (EpiCypher 21-1026)

LIBRARY PREP REAGENTS:

Option 1: Traditional library prep (meCUT&RUN)

- Library prep kit (CUTANA™ CUT&RUN Library Prep Kit, EpiCypher 14-1001 and 14-1002)

Option 2: Cytosine conversion and library prep (meCUT&RUN-EM)

- 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)

EQUIPMENT:

- 1.5, 15, and 50 mL tubes
- Low-retention filter pipette tips
- Magnetic rack for 1.5 mL tubes (EpiCypher 10-0012) and 8-strip tubes (EpiCypher 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)

SAMPLE INPUTS FOR MECUT&RUN

- Freshly isolated, unfixed (i.e. native) cells are the preferred input for meCUT&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: Establish workflows using 500,000 cells, and then titrate down inputs as desired. The minimum input for meCUT&RUN is 10,000 cells. Note that lower cell inputs may result in reduced signal-to-noise and higher read duplication rates.
- The meCUT&RUN protocol is designed for native suspension cells. If using nuclei, adherent cells, cross-linked samples, frozen nuclei/cells, or other sample types, visit the CUT&RUN Sample Prep section at support.epicypher.com for protocol modifications.

EXPERIMENTAL CONTROLS & SUCCESS METRICS

- The kit contains multiple quality control checks (Figure 4) to ensure reliable meCUT&RUN assays.
- For a negative control, we recommend including a reaction that only recieves the Anti-GST Tag Antibody. This reaction can be used to assess nonspecific background signal in sequencing data.
- The kit also comes with *E. coli* Spike-in DNA, as an optional control for sequencing normalization. This control should only be used when meCUT&RUN is paired with traditional library prep (Option 1, meCUT&RUN). For guidance, visit support.epicypher.com.

FIGURE 4

The CUTANA™ meCUT&RUN Kit contains multiple quality control metrics and checks to ensure successful DNA methylation profiling.

Metric	Option 1: Traditional Library Prep (meCUT&RUN)	Option 2: EM-seq (meCUT&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	✓ Anti-GST Tag Antibody to assess nonspecific background	
Spike-in Control	✓ Optional *E. coli* DNA for sequencing normalization	✓ None
	Assess meCUT&RUN-enriched DNA yields	
Yields	✓ 1-5 ng	✓ 1 ng
Controls	✓ Yields from Anti-GST Tag negative control less than yields from GST-MeCP2 reactions	
	Assess quality of sequencing libraries	
Fragment Size	✓ ~300 bp	✓ ~350 bp
Concentration	✓ >1 nM	✓ ≥ 1 nM
	Sequencing metrics	
Configuration	✓ 2 x 50 bp reads	✓ 2 x 150 bp reads
Total Seq. Depth	✓ 15-20 M reads	✓ 30-50 M reads
Unique Align %	✓ 70-80%	✓ ~60%
Unique Reads	✓ 10-15 M reads	✓ 20-30 M reads

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

IMPORTANT NOTES ON BUFFER PREP

* Buffers (Figure 5) are prepared **FRESH** on Day 1 of every meCUT&RUN experiment.
* Volumes in Table 1 are per meCUT&RUN reaction and include 20% excess to account for pipetting errors. You do **NOT** need to add additional volume.

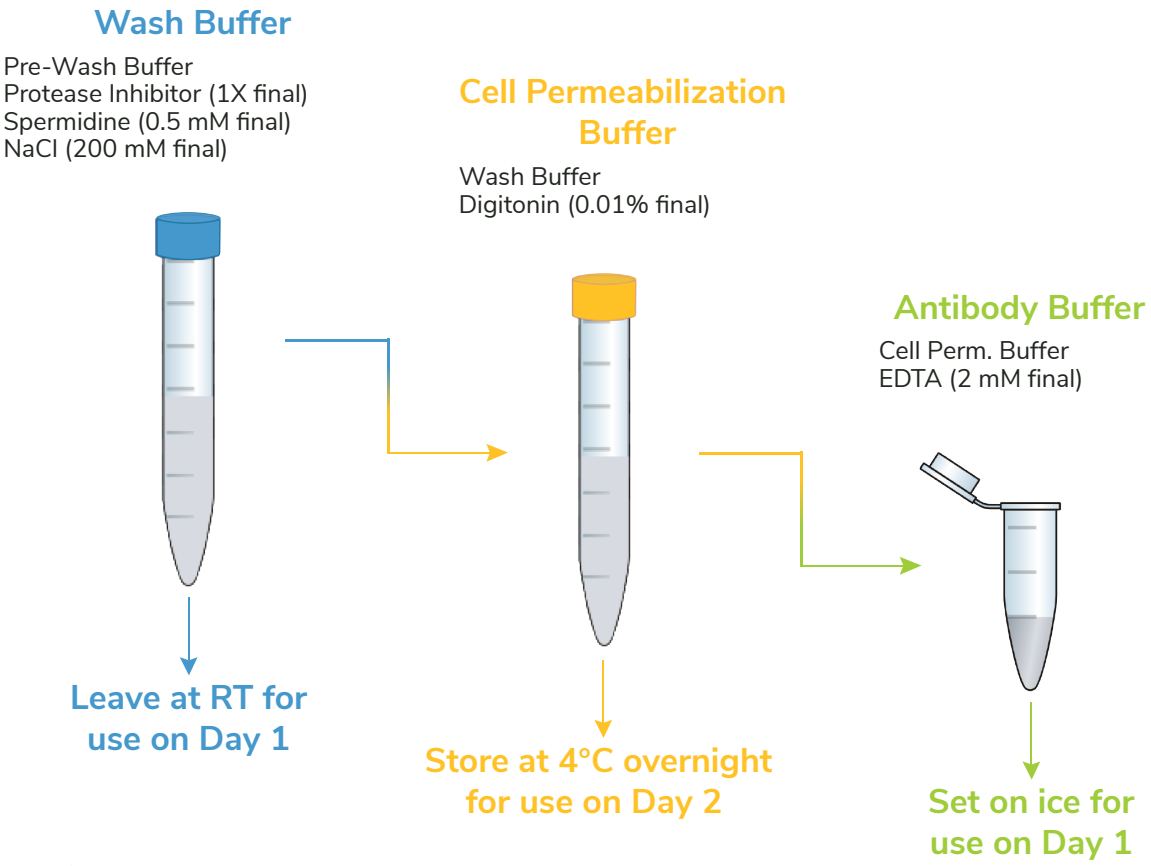


FIGURE 5

Schematic of buffers prepared on Day 1 of the meCUT&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, GST-MeCP2**. Place on ice to thaw or equilibrate.
2. Reconstitute protease inhibitor tablet (EpiCypher 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, 1M Spermidine, and 5M NaCl as outlined in Table 1. meCUT&RUN Wash Buffer contains 200 mM NaCl, a notable increase from standard CUT&RUN buffers—this modification is key to meCUT&RUN success. Store final Wash 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. At the end of Day 1, 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	76 µL	608 µL	1.22 mL
1 M Spermidine	0.5 mM	0.95 µL	7.6 µL	15.2 µL
5 M NaCl*	200 mM	19 µL	152 µL	304 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 meCUT&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.**

* Pre-Wash Buffer contains 150 mM NaCl. Final Wash Buffer, as prepared for meCUT&RUN, contains 200 mM NaCl.

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** (e.g. for 8 reactions, resuspend in 88 µL 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 meCUT&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 meCUT&RUN sequencing data.
* The meCUT&RUN protocol is designed for native suspension cells. If using nuclei, adherent cells, cross-linked samples, frozen nuclei/cells, or other sample types, visit the CUT&RUN Sample Prep section at support.epicypher.com for protocol modifications.

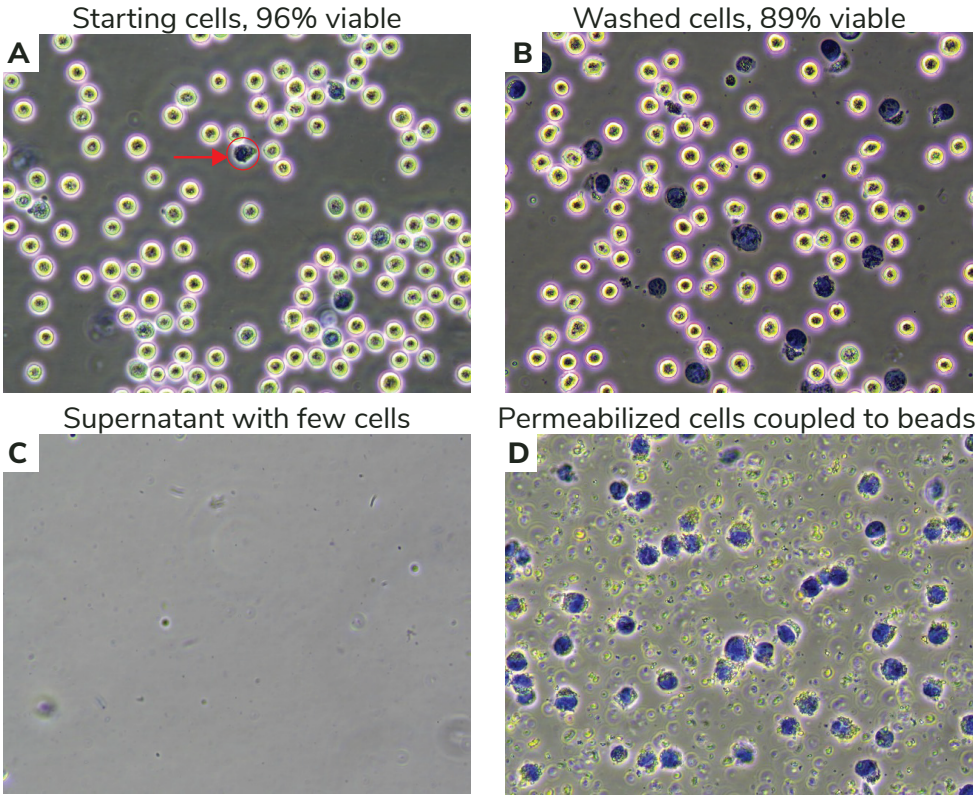
12. Collect starting cells (in tissue culture flask, tube, etc.). Count and confirm cell integrity as follows:

    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. 13).

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 at least 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: GST-MECP2 BINDING (~30 MIN + OVERNIGHT)

GST-MECP2 BINDING NOTES

* GST-MeCP2 is stored in 50% glycerol. **Before each use**, mix GST-MeCP2 thoroughly by pipetting (use a pipettor set to 2.5 µL, pipette up and down 20 times).
* 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. For GST negative control reactions, add 2.5 µL **Antibody Buffer**. For all other reactions, add 2.5 µL **GST-MeCP2**. To ensure accurate pipetting of GST-MeCP2: 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 elevated (Figure 7). Do **NOT** invert or rotate tube 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 ANTI-GST ANTIBODY AND PAG-MNASE (~1.5 HRS)

IMPORTANT NOTES ON CONA BEAD MIXING

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

29. Gather reagents at 4˚C and -20˚C for Day 2: **Cell Permeabilization Buffer, Anti-GST Tag Antibody, 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.

SECTION V: BINDING OF ANTI-GST ANTIBODY AND PAG-MNASE (~1.5 HRS)

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. Remove tubes from magnet and immediately add 50 µL cold **Cell Permeabilization Buffer** to each reaction. Gently pipette to resuspend, vortex tubes to mix, and quick spin to collect liquid. When pipetting, avoid losing beads in tips and expel all material back into tubes. Always quick spin after mixing to avoid bead loss.
34. Add 1 µL **Anti-GST Tag Antibody** to each reaction. Gently vortex to mix and quick spin.

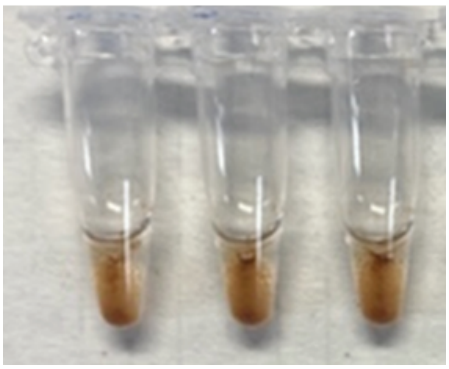


FIGURE 8

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

35. Incubate for 30 min at RT on a nutator, gently rocking with tube caps elevated.
36. At end of incubation, quick spin tubes to collect liquid. Place tubes on magnet and allow slurry to clear. Pipette to remove supernatant.
37. Keeping tubes on magnet, add 200 µL cold **Cell Permeabilization Buffer** to each reaction, directly onto beads. Pipette to remove supernatant.
38. Repeat the previous step 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.
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** to each reaction, directly onto beads. Pipette to remove supernatant.
44. Repeat one time, keeping tubes on magnet.
45. 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)

46. 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.
47. Quick spin tubes. Incubate on a nutator, capped ends elevated, 2 hours at 4°C.
48. Quick spin tubes to collect liquid. Add 33 µL **Stop Buffer** to each reaction and gently vortex ~5 sec to mix.
49. ***Optional*** For meCUT&RUN with Traditional Library Prep (Option 1), add *E. coli* Spike-in DNA:
    A. Before first use, reconstitute lyophilized ***E. coli* Spike-in DNA**: Quick spin tube. Add 100 µL DNase-free water and thoroughly vortex tube on all sides to resuspend *E. coli* DNA.
    B. For 500,000 cells per reaction, add 1 µL ***E. coli* Spike-in DNA**. If using fewer than 500,000 cells per reaction, dilute E. coli Spike-in DNA as outlined in this Tech Support Center article.
    C. Gently vortex tubes ~5 sec to mix and proceed to next step. Store reconstituted *E. coli* DNA at -20˚C.
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 84 µL supernatant to new **8-strip Tubes**. Supernatants contain meCUT&RUN-enriched chromatin.

SECTION VII: DNA PURIFICATION (~30 MIN)

NOTES ON WORKING WITH SPRI BEADS

- When adding SPRIselect reagent to meCUT&RUN supernatants, mix well by pipetting and vortexing to maximize capture of DNA on beads.
- Use a strong magnet (EpiCypher 10-0008) to minimize bead loss and maximize yields.

53. Prepare 85% Ethanol (EtOH) fresh using 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 (beads) to fully resuspend. Slowly add 118 µL beads (1.4X volume) to each reaction. Ensure pipette tip is free of extra droplets before dispensing beads.
55. Mix well by pipetting and/or vortexing to an even resuspension. Quick spin tubes and incubate 5 min at RT.
56. Place tubes on a magnet for 2-5 min at RT, until solution clears. Pipette to remove supernatant without disturbing beads.

SECTION VII: DNA PURIFICATION (~30 MIN)

57. Keeping tubes on the magnet, add 180 µL **85% EtOH** directly onto beads. Pipette to remove supernatant.
58. Repeat the previous step one time.
59. 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.
60. Remove tubes from magnet and air-dry, caps open, for 2-3 min at RT. Beads should appear damp matte brown (Figure 9). If beads are crackly and/or light brown, they are too dry.
61. 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.
62. For EpiCypher magnet, flip to low-volume side.
63. Place tubes on magnet for 2 min at RT.
64. Transfer 15 µL meCUT&RUN-enriched DNA to new **8-strip Tubes**.
65. Quantify 1 µL DNA using the Qubit fluorometer and 1X dsDNA HS Assay Kit.

FIGURE 9

Elute DNA before beads dry out.

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 meCUT&RUN?

There is no typical DNA yield for meCUT&RUN, as yields can vary by cell type, number of cells, and sample prep quality. Aim for ≥1 ng DNA to enable robust library prep. If yields are low, use as much as possible for library prep.

Yields from the Anti-GST Tag negative control reaction should be less than meCUT&RUN reactions with GST-MeCP2, which can also be used to gauge assay success.

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

Do **NOT** assess fragment size distribution of raw meCUT&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.

Library Prep and Sequencing Option 1: Traditional Library Prep (meCUT&RUN)

Option 1 uses a traditional library prep strategy, meaning there is no base-pair conversion and no specialized DNA methylation library prep kit. This workflow provides ~150 bp resolution of DNA methylation enrichment, and data are analyzed using standard CUT&RUN bioinformatics pipelines. Only 15-20 million total reads are required, maximizing cost-savings for genome-wide DNA methylation analysis.

GUIDELINES FOR LIBRARY PREP:

- Prepare Illumina sequencing libraries using 1-5 ng meCUT&RUN DNA and the CUTANA™ CUT&RUN Library Prep Kit (EpiCypher 14-1001/ 14-1002). If yields are below 1 ng, use the total amount of recovered DNA for library prep.
    - For other library prep kits, follow EpiCypher's parameters for indexing PCR. See the CUTANA™ CUT&RUN Library Prep Kit manual at epicypher.com/protocols.
- Examine final prepared sequencing libraries on the Agilent TapeStation® or Bioanalyzer®.
    - Libraries should show enrichment of mononucleosome-sized DNA fragments (~300 bp, including meCUT&RUN DNA + sequencing adapters). See Figure 10.
    - 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).
    - For low-concentration libraries, see support.epicypher.com for considerations. If the experiment cannot be repeated, add as much of the low-concentration library as possible to the sequencing pool. Deeper sequencing may be necessary to fully capture read diversity.

GUIDELINES FOR SEQUENCING

- Paired-end sequencing (2 x 50 bp minimum) is recommended.
- Libraries should be sequenced to a depth of 15-20 million total reads, with a goal of generating 10-15 million uniquely aligned reads.

GUIDELINES FOR DATA ANALYSIS

- meCUT&RUN data can be analyzed using standard CUT&RUN bioinformatic pipelines.
- The majority of reads (70-80%) should align uniquely to the species genome. Sequence duplication levels should be low (< 20% of total sequencing reads).
- The Anti-GST Tag negative control reaction should show low background signal, with no defined peaks.
- If using *E. coli* 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 guidance.

EXAMPLE FRAGMENT DISTRIBUTION TRACE OF MECUT&RUN SEQUENCING LIBRARIES

FAQS: LIBRARIES

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

Libraries should show enrichment of mononucleosome-sized DNA fragments (~300 bp, including meCUT&RUN DNA + sequencing adapters). See Figure 10.

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

Laddering is common in meCUT&RUN assays, and you may see dinucleosome peaks at ~500 bp (Figure 10B).

They do not impact sequencing and we do **NOT** recommend removing them, as it risks loss of the mononucleosome peak (~300 bp).

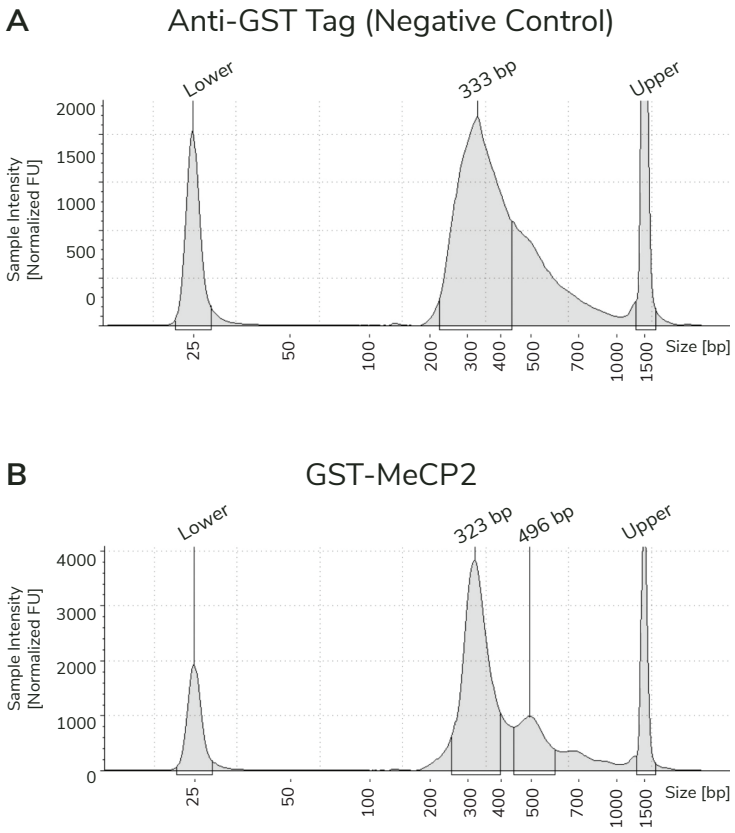


FIGURE 10

Typical TapeStation traces from CUTANA™ meCUT&RUN libraries, generated using K562 cells. All libraries are predominantly enriched for mononucleosome-sized fragments, as indicated by the peak at ~300 bp (~170 bp nucleosomes + sequencing adapters).

Library Prep and Sequencing Option 2: EM-seq (meCUT&RUN-EM)

Option 2 uses NEBNext® Enzymatic Methyl-seq (EM-seq) conversion and library prep to provide base-pair resolution DNA methylation sequencing. Bisulfite conversion may also be used, but has not been thoroughly tested by EpiCypher.

Note that this method requires additional reagents, increased sequencing, and different bioinformatic processing than Option 1.

GUIDELINES FOR LIBRARY PREP:

- Source the following reagents:
  - 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 meCUT&RUN DNA prior to library prep, 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 addition to meCUT&RUN DNA.
- Prepare meCUT&RUN DNA for EM-seq conversion and library prep:
  - Transfer 1 ng meCUT&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.
  - 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.
  - Add 1 µL Fragmented Controls Dilution to 49 µL meCUT&RUN DNA in 8-Strip Tubes. Gently vortex to mix, quick spin, and place tubes on ice.
  - 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.

Library Prep and Sequencing Option 2: EM-seq (meCUT&RUN-EM)

BEGIN THE EM-SEQ WORKFLOW STARTING IN SECTION 1.2.

Section 1.2 – End Prep of Fragmented DNA

End repair of meCUT&RUN DNA prior to enzymatic conversion. The "Sheared DNA" component of the End Prep reaction is the 50 µL meCUT&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 11 for example traces and p. 23 for details on sequencing.

* Libraries should show enrichment of mononucleosome-sized DNA fragments (~350 bp, including meCUT&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 MECUT&RUN-EM LIBRARIES

FAQS: LIBRARIES

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

meCUT&RUN-EM libraries should show enrichment of mononucleosome-sized DNA fragments (~350 bp, including meCUT&RUN DNA + sequencing adapters). See Figure 11.

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

Laddering is more common in meCUT&RUN-EM assays vs. meCUT&RUN with traditional library prep, and you will likely observe dinucleosome peaks at ~500 bp or larger (Figure 11).

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

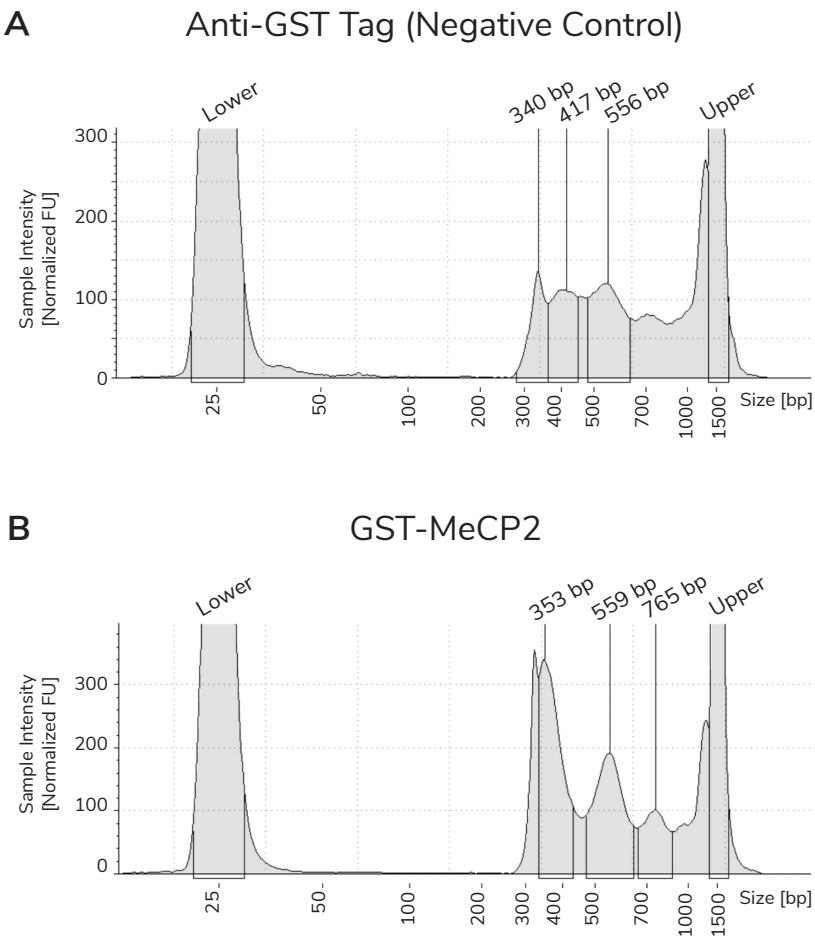


FIGURE 11

Typical TapeStation traces from CUTANA™ meCUT&RUN-EM libraries, generated using K562 cells. All libraries are predominantly enriched for mononucleosome-sized fragments, as indicated by the peak at ~350 bp (~170 bp nucleosomes + sequencing adapters).

Library Prep and Sequencing Option 2: EM-seq (meCUT&RUN-EM)

GUIDELINES FOR SEQUENCING

- 2x150 bp paired-end sequencing is recommended to ensure coverage of 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. This sequencing depth will provide ~80% 5-methylcytosine coverage (vs. whole-genome EM-seq; see Figure 12).

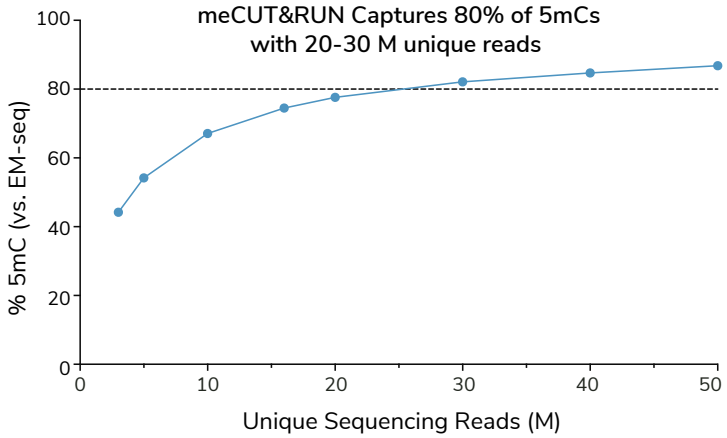


FIGURE 12

CUTANA™ meCUT&RUN-EM recovers 80% of methylated CpGs (5mCs) compared to whole-genome EM-seq in K562 cells, with just 20-30 M unique reads. Here, meCUT&RUN-EM data were downsampled to 3 to 50 M uniquely aligned reads. The number of methylated CpG positions were calculated for each downsampled dataset and normalized to the number of 5mC position detected by EM-seq. EM-seq was sequenced to a total depth of 300 M reads.

GUIDELINES FOR DATA ANALYSIS

- meCUT&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.
  - **Note:** Due to the GST-MeCP2 enrichment step, meCUT&RUN cannot provide quantitative percent DNA 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 Anti-GST Tag negative control reaction should show low background signal.

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 13A).
- Unmethylated Lambda DNA should show ≤1% DNA methylation, which incidates an EM-seq conversion efficiency of ~99% (Figure 13B).

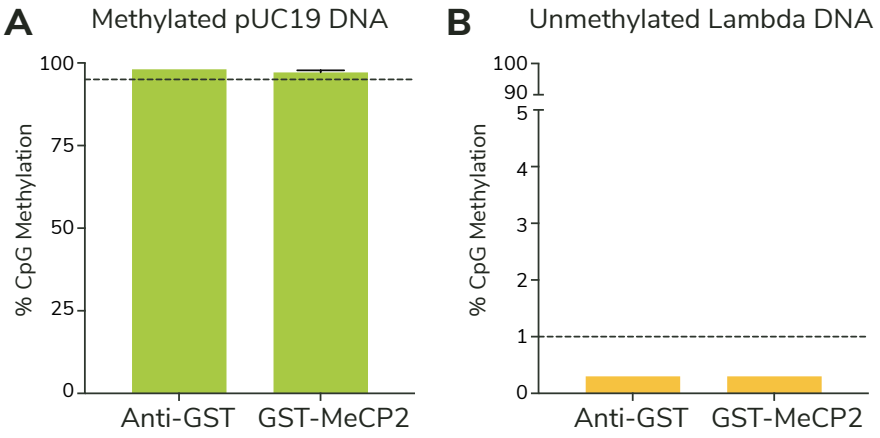


FIGURE 13

meCUT&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.

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. Marunde et al. Nucleosome conformation dictates the histone code. **eLife** 13 (2024).
5. Kruegger & Andrews. Bismark: a flexible aligner and methylation caller for Bisulfite-Seq applications. ***Bioinformatics*** 27, 1571-1572 (2011).
6. Akalin et al. methylKit: a comprehensive R package for the analysis of genome-wide DNA methylation profiles. ***Genome Biol*** 13, R87 (2012).