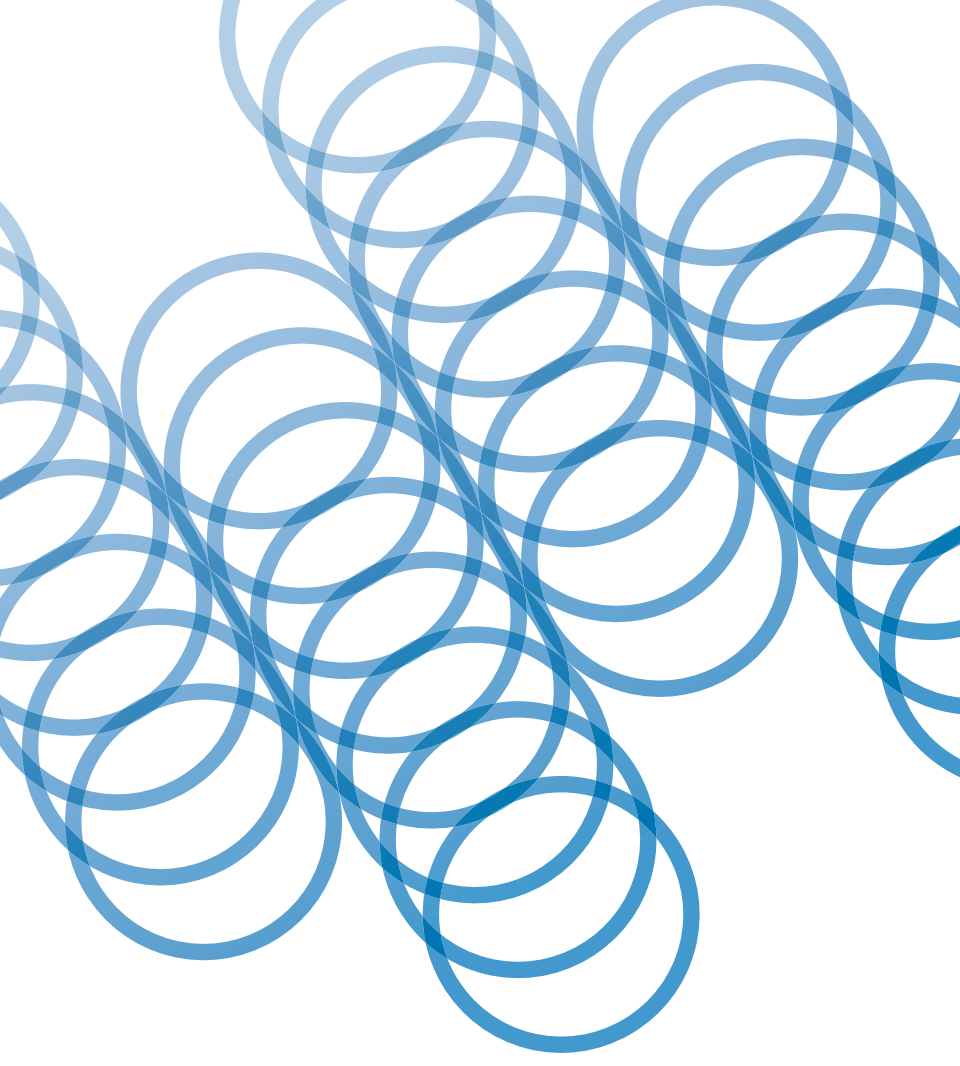

EpiCypher®
Bringing Epigenetics to Life

CUTANA™
CUT&Tag Kit Version 2
User Manual Version 2.0

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EpiCypher®

CUTANA™

CUT&Tag Kit

Catalog No. 14-1102 & 14-1103
48 CUT&Tag Reactions

Upon receipt, store indicated components at 4°C, -20°C and room temperature (RT)
Stable for 6 months upon date of receipt.
See p. 8-9 for storage instructions.

Table of Contents

Cleavage **U**nder **T**argets & **Tag**mentation (CUT&Tag) is a groundbreaking epigenomic mapping strategy that builds on its predecessor immunotethering technologies CUT&RUN and ChIC[1-6]. In CUTANA™ CUT&Tag, nuclei are immobilized to a solid support and antibodies bind their chromatin targets *in situ*. A fusion of proteins A and G with prokaryotic transposase 5 (pAG-Tn5) is used to selectively cleave and tagment antibody-bound chromatin with sequencing adapters (Figure 1). Tagmented fragments are directly PCR amplified using EpiCypher's exclusive single-tube ("Direct-to-PCR") approach, yielding sequence-ready DNA[6,7].

CUT&Tag is best for mapping histone post-translational modifications (PTMs). For chromatin-associated proteins, CUT&RUN assays are strongly recommended.

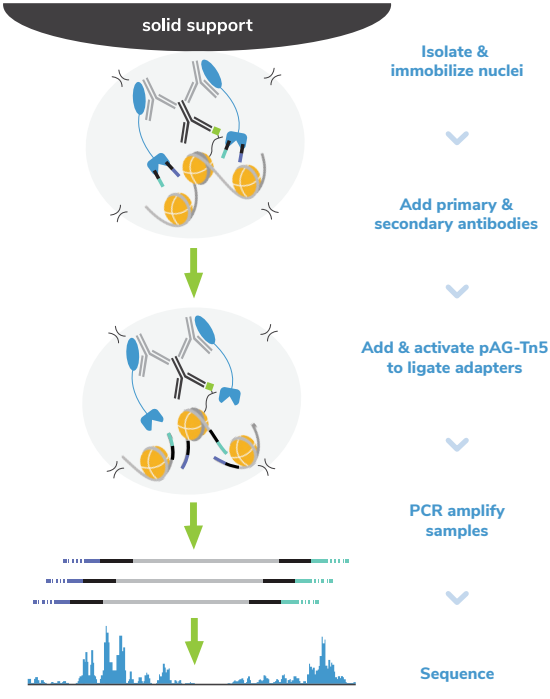


FIGURE 1

Overview of the CUTANA™ CUT&Tag workflow.

Compared to ChIP-seq, the historical go-to chromatin mapping assay, CUT&Tag provides higher quality sequencing data with improved sensitivity. By selectively targeting antibody-labeled chromatin in intact nuclei (without chromatin fragmentation or IP) background is dramatically reduced. Tagmentation eliminates traditional library prep, further streamlining the protocol and mitigating sample loss. These innovations enable high-resolution mapping for histone PTMs using a small number of starting cells and only 5-8 million total reads per reaction (Figure 2).

The CUTANA™ CUT&Tag Kit is ideal for genomic mapping experts that want to increase throughput and scale without sacrificing sensitivity. Our unique single-tube workflow is designed for multi-channel pipetting and can be completed in just two days[6,7]. These features, combined with reduced assay costs and exquisite signal-to-noise, position CUT&Tag to become a leading tool for chromatin research.

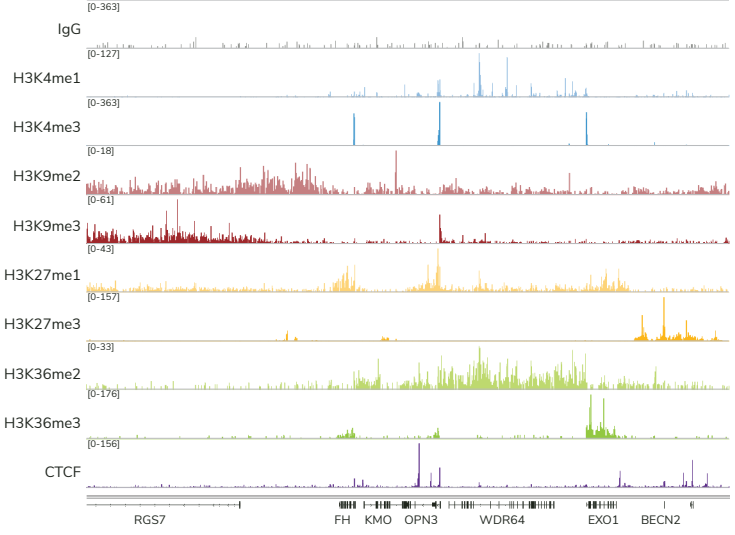


FIGURE 2

CUTANA CUT&Tag generates high-quality profiles for targets in both active and repressed chromatin regions, including active regulatory elements (H3K4me1) and promoters (H3K4me3), constitutive (H3K9me2/me3) and facultative (H3K27me1/me3) heterochromatin, active gene bodies (H3K36me2/me3), and select proteins (CTCF). Rabbit IgG is shown as a negative control. Results were generated using 100,000 K562 nuclei and 5-8 million total reads per reaction.

The kit contains sufficient materials for 48 CUT&Tag reactions, including pAG-Tn5, Concanavalin A (ConA) beads, buffers, and tubes. Key positive, negative, and spike-in controls are provided to optimize workflows, monitor experimental success, and aid troubleshooting.

To facilitate library prep and multiplexed sequencing, the kit also includes indexing primers, a PCR master mix, and DNA purification beads. A combinatorial dual indexing primer strategy[8,9] enables the entire 48-reaction kit to be pooled in a single run. The two versions of this kit (14-1102 & 14-1103) contain distinct primer sets, allowing up to 96 reactions to be multiplexed when kits are used together.

The user-friendly protocol starts with a nuclei isolation step. Although it is recommended to start with 100,000 nuclei per reaction, comparable data can be generated down to 10,000 nuclei for select targets (Figure 3), making CUT&Tag well-suited for rare cell types and low-input applications.

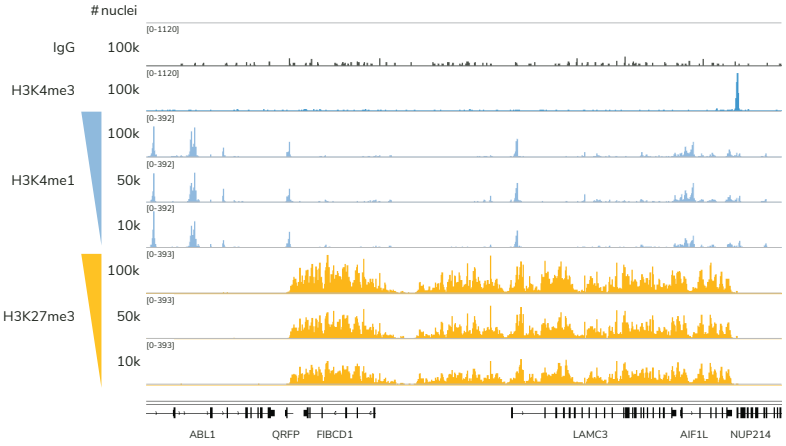


FIGURE 3

CUTANA CUT&Tag enables reliable chromatin profiling from low nuclei numbers. CUT&Tag was used to map H3K4me1 (low abundance target) and H3K27me3 (high abundance target) using decreasing numbers of K562 nuclei. Data quality at 10,000 nuclei is comparable to to standard inputs of 100,000 nuclei. There are some caveats when using low nuclei numbers; see **FAQs** for details. H3K4me3 and IgG are shown as controls.

Included in the Kit

Kit 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
8-strip Tubes	10-0009t	Enables use of multi-channel pipettors.
0.5 M EDTA	21-1014	250X concentration. Use to prepare Antibody Buffer **FRESH** for each experiment.
4.5 M NaCl	21-1013	Use to prepare Wash Buffer 2 **FRESH** for each experiment.
1 M $MgCl_2$	21-1015	Use to prepare Tagmentation Buffer **FRESH** for each experiment.
SDS Release Buffer	21-1017	Use to digest reactions and release tagmented fragments into solution.
SDS Quench Buffer	21-1018	Neutralizes SDS prior to indexing PCR.
SPRIselect Reagent manufactured by Beckman Coulter Inc.	21-1404	**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&Tag libraries.
0.1X TE Buffer	21-1019	Use to elute CUT&Tag libraries.

Store at 4°C upon receipt:

Item	Catalog No.	Notes before use
ConA Beads	21-1401	**DO NOT FREEZE.** Concanavalin A (ConA) beads are used for immobilizing nuclei or cells.
Bead Activation Buffer	21-1022	Use to prepare ConA beads for sample immobilization.
Pre-Nuclear Extract Buffer	21-1021	Use to prepare Nuclear Extraction Buffer **FRESH** for each experiment.
Pre-Wash Buffer	21-1020	Use to prepare Wash Buffer 1 **FRESH** for each experiment. Use to wash reactions after tagmentation.
H3K27me3 Positive Control Antibody	13-0055t	**SMALL VOLUME: quick spin before use.** Rabbit monoclonal antibody, 0.5 mg/mL stock. Add 1µL to positive control reactions. Sufficient for 10 reactions.

Store at -20°C upon receipt:

Item	Catalog No.	Notes before use
5% Digitonin	21-1023	Thaw at RT. Use to prepare Wash Buffer 1 **FRESH** for each experiment.
1 M Spermidine	21-1024	Use to prepare Nuclear Extraction Buffer and Wash Buffer 1 **FRESH** for each experiment.
SNAP-CUTANA™ K-MetStat Panel	19-1002t	**SMALL VOLUME: quick spin before use. Pipette to resuspend - DO NOT VORTEX.** Panel of biotinylated nucleosomes coupled to streptavidin-coated magnetic beads. Pair with IgG and H3K27me3 control antibodies. Sufficient for 20 reactions. See **Appendix 1.4**.
Rabbit IgG Negative Control Antibody	13-0042t	**SMALL VOLUME: quick spin before use.** 0.5 mg/mL stock. Add 1µL to negative control reactions. Sufficient for 10 reactions.
Anti-Rabbit Secondary Antibody	13-0047	**SMALL VOLUME: quick spin before use.** Secondary for positive & negative control reactions and other reactions using a rabbit primary antibody. Sufficient for 50 reactions.
pAG-Tn5	15-1017	20X concentration. Proteins A and G (pAG) bind antibodies of various isotypes and host species including total IgG for rabbit, mouse, goat, donkey, rat, guinea pig, horse, & cow.
Non-Hot Start 2X PCR Master Mix	15-1018	Use for PCR amplification & indexing of CUT&Tag libraries.
i7 Primers	i701-i712	**SMALL VOLUME: quick spin before use.** 14-1102 & 14-1103 contain primers i701-i712.
i5 Primers	Kit 14-1102: i501-i504 Kit 14-1103: i505-i508	**SMALL VOLUME: quick spin before use.** 14-1102 & 14-1103 contain distinct i5 primers. Each kit generates 48 unique pairs of barcodes; combined, they generate up to 96 uniquely barcoded libraries. See **Appendix 3**. ***NOTE:** Download i5 & i7 index sequences at epicypher.com/14-1102 under Documents and Resources

Materials Required but Not Supplied

REAGENTS:

- Antibody to target of interest; see **FAQs 2-4** for guidance on antibody selection
  - * The kit includes anti-rabbit secondary antibody. If using mouse primary antibodies, purchase of anti-mouse secondary antibody is required (EpiCypher 13-0048).
- Protease inhibitor (e.g. cOmplete™, EDTA-free Protease Inhibitor Cocktail, Roche 118735800001)
- 0.4% Trypan blue (e.g. Invitrogen T10282)
- Molecular biology grade water, any vendor
- 100% Ethanol (200 proof), any vendor
- 1X Phosphate buffered saline (1X PBS), any vendor

EQUIPMENT:

- 1.5, 15 and 50 mL tubes
- Low-retention filter pipette tips
- Magnetic separation 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, Benchmark 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 (Invitrogen Q33238 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) & D1000 reagents (5067-5583) or Agilent Bioanalyzer® with High Sensitivity DNA Kit (5067-4626)

The CUTANA™ CUT&Tag Kit uses an exclusive Direct-to-PCR strategy, going from cells to PCR-amplified libraries in one tube. Advantages include:

* Improved throughput - designed for 8-strip tubes, fewer steps vs. standard CUT&Tag
* Low nuclei requirements - streamlined workflow minimizes sample loss, supports low inputs
* High reproducibility - optimized for multichannel pipetting, reduces sample handling variation

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

Step 1: Isolate nuclei and immobilize to Concanavalin A (ConA) beads

Nuclei are prepared from bulk cell populations and immobilized to activated ConA beads. High quality sample prep is essential to CUT&Tag workflows. It is recommended to confirm cell viability, nuclear integrity and binding to ConA beads (**Appendix 1.1**). Avoid ConA bead clumping and dry out during the assay, which results in sample loss and reduced yields.

Step 2: Label chromatin using primary & secondary antibodies

The bead-nuclei mixture is incubated with a target-specific primary antibody overnight, followed by treatment with a species-matched secondary antibody the next day. Selection of a highly specific primary antibody is crucial to CUT&Tag assay success, as off-target binding can significantly impact data quality.

At the conclusion of this step, nuclei are washed with a high-salt buffer (Wash Buffer 2) to remove unbound antibodies and prepare for tagmentation.

Step 3: Perform targeted chromatin tagmentation using pAG-Tn5

pAG-Tn5 is added to each reaction and binds antibody-labeled chromatin via the immunoglobulin binding properties of protein A/G. The addition of secondary antibody in prior steps increases the number of pAG binding sites, amplifying Tn5 localization and on-target signal in sequencing data.

Tn5 is activated by the addition of magnesium (Tagmentation Buffer) to cleave and append sequencing adapters to DNA proximal to antibody-bound chromatin (Figure 4). These steps are performed under high salt to minimize nonspecific Tn5 cleavage (i.e. ATAC-like signal)[5,6]. Rinsing the bead slurry with Pre-Wash Buffer effectively quenches the tagmentation reaction.

SDS Release Buffer is added to digest proteins and release tagmented DNA into solution. Finally, SDS Quench Buffer is added to neutralize SDS and enable PCR.

Step 4: Indexing PCR & library cleanup

The 2X Non-Hot Start PCR Master Mix and selected i5 & i7 indexing primers are added to the entire CUT&Tag reaction mixture. Of note, the kit uses a combinatorial dual indexing primer strategy, meaning that each CUT&Tag library will contain a distinct pair of i5 & i7 barcodes[8,9]. This strategy enables multiplexing of up to 48 libraries using various combinations of the four i5 primers and twelve i7 primers provided with each kit. The two versions of this kit (14-1102 & 14-1103) contain distinct primer sets, allowing up to 96 reactions to be multiplexed when kits are used together. See **Appendix 3** for information.

The kit cycling parameters include two steps prior to PCR amplification (Figure 4). The first step fills in and repairs 3' gaps caused by Tn5 tagmentation. The second step uses ligated adapter DNA as a primer to extend 3' ends.

The subsequent PCR steps are specifically optimized for CUT&Tag fragments. Briefly, i5 primers anneal to i5 adapters on tagmented DNA and i7 primers anneal to i7 adapters (Figure 4). Each primer incorporates a unique barcode (i.e. index) and the required Illumina® P5/P7 flow cell sequences during PCR. Because adapter-ligated chromatin is the only suitable template for PCR using i5 & i7 indexing primers, tagmented DNA is selectively amplified and barcoded – even in the presence of cell debris. This Direct-to-PCR approach enriches target DNA and greatly streamlines library prep[6,7].

Step 5: Analysis of library quality

Purified CUT&Tag libraries are examined using the Qubit™ Fluorometer and the Agilent Bioanalyzer® or TapeStation® to determine library concentration and fragment size distribution. The PCR parameters in this protocol amplify fragments compatible with Illumina paired-end sequencing, with an average fragment size of ~300 bp (including adapter DNA). See **Protocol: Section VIII** and **Appendix 1.2** for discussion of expected results.

Step 6: Illumina® next-generation sequencing

Once libraries are quantified, they are diluted, pooled, and sequenced on an appropriate Illumina sequencing platform; see **Protocol: Section IX** and **Appendix 3** for guidance. For information on expected results from control reactions, see **Appendix 1.3 & 1.4**.

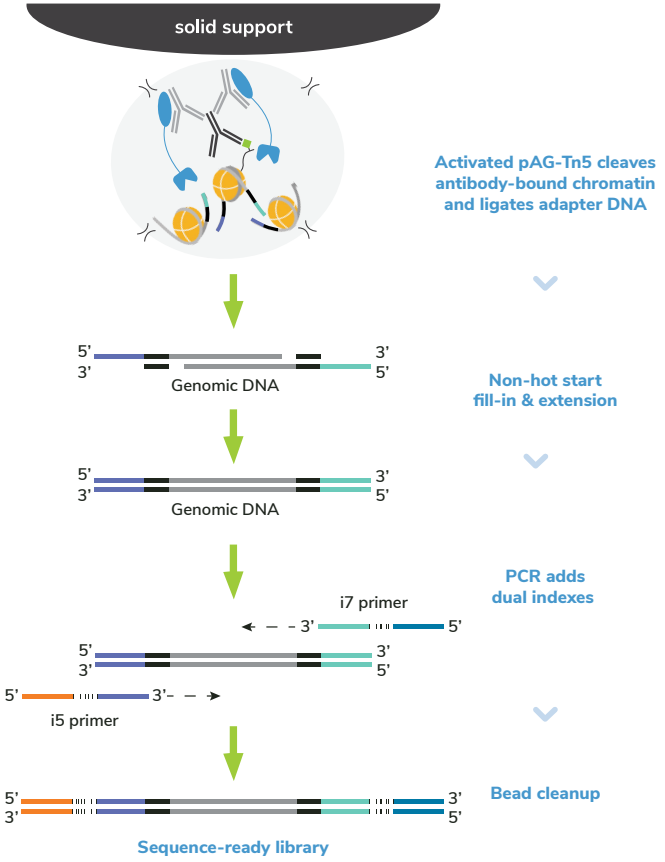


FIGURE 4

Overview of tagmentation, indexing PCR, and bead cleanup sections of the CUTANA CUT&Tag workflow. Activated pAG-Tn5 cleaves and ligates sequencing adapters to DNA proximal to antibody-labeled chromatin. Tagmented DNA is selectively repaired and amplified using i5 & i7 primers that recognize ligated adapter DNA sequences. The i5 & i7 primers add indexes (barcodes) to 5' and 3' ends, respectively, during PCR amplification, generating dual-barcoded libraries for multiplexed sequencing. Final libraries are bead-purified, quantified and analyzed by capillary electrophoresis (e.g. TapeStation), and used for Illumina sequencing.

SAMPLE INPUTS FOR CUT&TAG

- Freshly isolated, unfixed (i.e. native) nuclei are the preferred input for CUT&Tag.
- 100,000 nuclei per reaction is recommended. For nuclei prep, harvest 100,000 cells per reaction plus 10% excess to account for sample loss.
- Using more than 100,000 nuclei does **NOT** improve yield and may inhibit PCR.
- See **Appendix 2** if using whole cells, adherent cells, tissues, cryopreserved samples, or cross-linked nuclei & cells.

COMPATIBLE TARGETS

- Histone PTMs generate the most robust and reliable profiles using CUT&Tag.
- Mapping chromatin-associated proteins with CUT&Tag is **NOT** recommended. For these targets we recommend CUTANA CUT&RUN assays, which generate robust profiles for diverse target classes (epicypher.com/cut-and-run).

EXPERIMENTAL CONTROLS

- This kit includes multiple quality control checks (Figure 5) to ensure reliable CUT&Tag workflows.
- Each kit comes with H3K27me3 positive & IgG negative control antibodies and the SNAP-CUTANA™ K-MetStat Panel of spike-in controls.
- Control reactions spiked with the K-MetStat Panel should be included in **every** experiment to determine assay success and aid troubleshooting (**Appendix 1.4**).

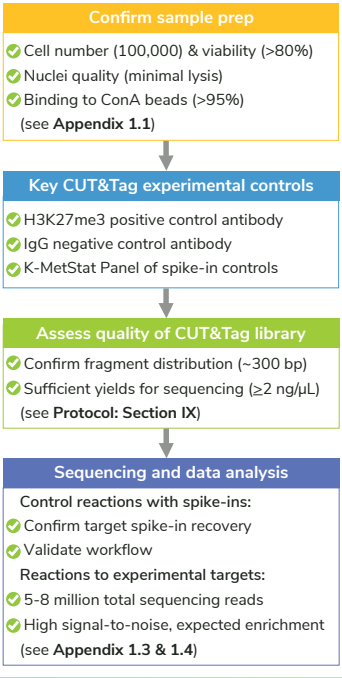


FIGURE 5

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

ANTIBODY SELECTION

- Use a highly specific and efficient antibody that has been validated in CUT&Tag.
- Antibodies that work well in ChIP-seq are **NOT** guaranteed success in CUT&Tag. However, many of our SNAP-Certified™ histone PTM antibodies for CUT&RUN also work for CUT&Tag. Visit epicypher.com/cut-and-tag-antibodies.
- See **FAQs 2-4** for information regarding antibody sourcing and validation.

HOW TO OPTIMIZE CUT&TAG

- Optimization methods are provided in Figure 6. For CUT&Tag troubleshooting, see **Appendix 1.2.** For considerations when using low nuclei numbers, see **FAQ 7**.
- CUT&Tag success depends on many factors, including cell type, nuclei number, target abundance, and antibody quality. CUT&RUN may be preferrable in some cases (epicypher.com/technologies/cutana).

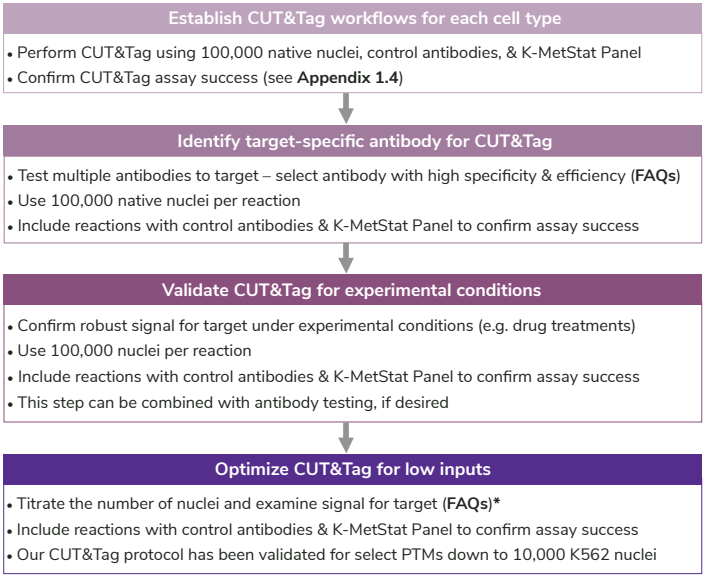


FIGURE 6

Development and optimization guidelines for successful CUT&Tag workflows.

***** Note that an antibody that performs reliably at 100,000 nuclei may fail at lower nuclei inputs.

SECTION I: BUFFER PREP (~30 MIN)

IMPORTANT NOTES ON BUFFER PREP

* These buffers (Figure 7) are prepared **FRESH** on Day 1 of every CUT&Tag experiment.
* If using whole cells, Digitonin conditions **MUST** be optimized for efficient cell permeabilization. See **Appendix 2.1**.
* Volumes in Table 1 are per CUT&Tag reaction and include 20% excess to account for pipetting errors. You do **NOT** need to add additional volume.

1. Gather kit reagents stored at 4˚C and -20˚C needed for Day 1 (**ConA beads, Bead Activation Buffer, Pre-Nuclear Extract Buffer, Pre-Wash Buffer, Digitonin, Spermidine, H3K27me3 and IgG control antibodies, K-MetStat Panel**). Place on ice to thaw or equilibrate.
2. Dissolve 1 protease inhibitor tablet (Roche) in 2 mL water for a **25X Protease Inhibitor** stock. After buffer prep, the remaining 25X stock can be stored for 12 weeks at -20˚C.

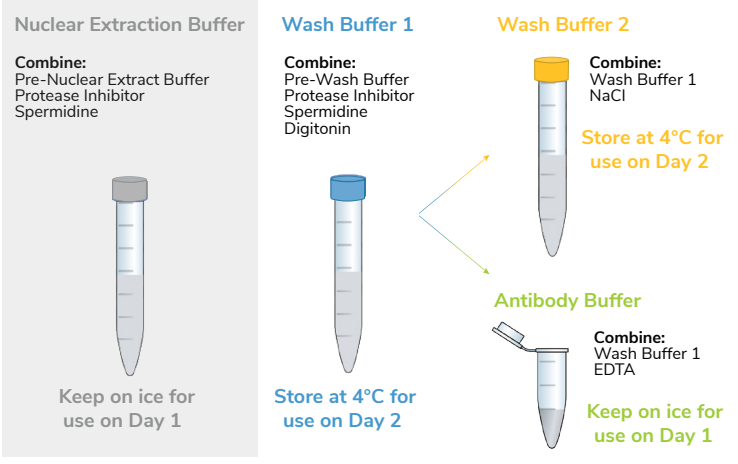


FIGURE 7

Schematic of CUT&Tag buffer preparation. See Table 1 for volume calculations.

3. Prepare **Nuclear Extract Buffer** by combining Pre-Nuclear Extraction Buffer, 25X Protease Inhibitor, and 1M Spermidine as outlined in Table 1. Place on ice.
4. To a tube labelled **Wash Buffer 1**, add Pre-Wash Buffer, 25X Protease Inhibitor, 1M Spermidine, and 5% Digitonin as in Table 1 (see Important notes on buffer prep). As prepared, **Wash Buffer 1** contains 150 mM NaCl. Place on ice.
5. Prepare **Wash Buffer 2** using **Wash Buffer 1** and 4.5 M NaCl as outlined in Table 1. As prepared, **Wash Buffer 2** contains 300 mM NaCl. Store **Wash Buffer 2** at 4˚C for use on Day 2.
6. In a new 1.5 mL tube labeled **Antibody Buffer**, combine **Wash Buffer 1** and 0.5 M EDTA as described in Table 1. Place on ice.
7. Store remaining **Wash Buffer 1** at 4˚C overnight for use on Day 2.

Buffer Sample Scaling Calculations

COMPONENT	[Final]	1X	8X	16X
Nuclear Extraction Buffer - store on ice for use on Day 1				
Pre-Nuclear Extract Buffer	-	235 µL	1.9 mL	3.8 mL
25X Protease Inhibitor	1X	9.8 µL	78.4 µL	157 µL
1 M Spermidine	0.5 mM	0.13 µL	1.0 µL	2.0 µL
Wash Buffer 1 - store at 4°C for use on Day 2				
Pre-Wash Buffer	-	1.3 mL	10.4 mL	20.8 mL
25X Protease Inhibitor	1X	56 µL	448 µL	896 µL
1 M Spermidine	0.5 mM	0.7 µL	5.6 µL	11.2 µL
5% Digitonin	0.01%	2.8 µL	22.4 µL	44.8 µL
Wash Buffer 2 - store at 4°C for use on Day 2				
Wash Buffer 1	-	600 µL	4.8 mL	9.6 mL
4.5 M NaCl	300 mM	20.7 µL	166 µL	331 µL
Antibody Buffer - store on ice for use on Day 1				
Wash Buffer 1	-	60 µL	480 µL	960 µL
0.5 M EDTA	2 mM	0.25 µL	2 µL	4 µL

TABLE 1

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

SECTION II: BEAD ACTIVATION (~30 MIN)

TIPS FOR WORKING WITH MAGNETIC CONA BEADS

* ConA beads should be brown and easily resuspended by gentle pipetting. Do **NOT** use ConA beads that have been frozen and/or appear black, granular, or clumpy (indicates damage).
* 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.

8. Gently resuspend **ConA beads** and transfer 11 µL per reaction to a 1.5 mL tube.
9. Place tube on a magnet, allow slurry to clear. Pipette to remove supernatant.
10. Take tube off 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. Repeat one time for a total of two washes.
11. Take tube off magnet and resuspend in 11 µL per reaction cold **Bead Activation Buffer**. Aliquot 10 µL per reaction of bead slurry into **8-strip tubes**. Keep on ice.

SECTION III: NUCLEI PREP AND BINDING TO BEADS (~30 MIN)

GUIDELINES FOR SUCCESSFUL SAMPLE PREP

* For sample inputs other than native suspension cells (e.g. adherent cells) see **Appendix 2.**
* Confirm cell viability, nuclear integrity, and binding to ConA beads as in **Appendix 1.1**. These steps help ensure low background and good data quality.

12. Counts cells and determine viability (see **Appendix 1.1**). Transfer 100,000 cells per reaction (plus 10% excess) to a 1.5 mL tube.
13. Spin cells 600 x g for 3 min at room temperature (RT). Remove supernatant and resuspend cells in 100 µL per reaction cold **Nuclear Extraction Buffer**.
14. Incubate for 10 min on ice. Spin 600 x g for 3 min at 4°C. Pipette to remove supernatant. The pellet should change from pale yellow (cells) to white (nuclei).
15. Gently resuspend nuclei in 105 µL per reaction cold **Nuclear Extraction Buffer**. Examine nuclei integrity (see **Appendix 1.1**).
16. Add 100 µL nuclei to 10 µL ConA beads in 8-strip tubes. Gently vortex to mix and quick spin in a mini-centrifuge to collect slurry (beads should not settle).

17. Incubate bead-nuclei slurry for 10 min at RT. Nuclei will adsorb to beads.
18. Place tubes on a magnet. Allow slurry to clear and pipette to remove supernatant. Keep 10 µL supernatant to examine bead binding (**Appendix 1.1**).
19. Add 50 µL cold **Antibody Buffer** to each reaction. Remove from magnet and pipette to resuspend. Confirm binding to ConA beads (**Appendix 1.1**).

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

ANTIBODY INCUBATION NOTES

* Add K-MetStat Panel to positive (H3K27me3) and negative (IgG) control reactions **BEFORE** primary antibody. Scale the volume of K-MetStat Panel to the number of nuclei as in Table 2.
* Quick spin antibody stocks to collect liquid and flick to mix. Check pipette tip for accuracy and pipette up and down to fully clear the solution from tip.
* Do **NOT** rotate or invert tubes. Rotation causes ConA beads to stick to tube lids and dry out, reducing yields. Use a nutator for incubations and elevate tube caps as suggested.

20. Quick spin the **K-MetStat Panel** and pipette to resuspend - do **NOT** vortex stock. Add 2 µL **K-MetStat Panel** to reactions designated for H3K27me3 and IgG control antibodies. Gently vortex to mix and quick spin tubes. If using <100,000 nuclei, decrease K-MetStat Panel amount as in Table 2.
21. Add 0.5 µg primary antibody (or manufacturer's recommendation) to each reaction. For positive and negative control reactions, add 1µL **H3K27me3 Positive Control Antibody** & 1µL **IgG Negative Control Antibody**, respectively.
22. Gently vortex to mix. Incubate overnight on a nutator at 4˚C, gently rocking tubes with caps elevated. Do **NOT** invert tubes; see Notes, above.

Number of nuclei	Working stock dilution	Volume added to reaction	Final dilution
100,000	Stock	2 µL	1:25
50,000	1:2	2 µL	1:50
20,000	1:5	2 µL	1:125
10,000	1:10	2 µL	1:250

TABLE 2

Scale the amount of K-MetStat Panel to the number of nuclei. For <100,000 nuclei, prepare a working stock dilution of the K-MetStat Panel in **Antibody Buffer** the day of the experiment.

SECTION V: SECONDARY ANTIBODY BINDING (~1 HR)

IMPORTANT NOTES ON CONA BEAD MIXING

* **Resuspension of ConA beads is essential for assay success.** Mix as instructed in each step.
* Beads often become clumpy after overnight incubation. Vortex frequently to keep in solution. The end of a pipette tip can be cut off to help mix plant nuclei or preserve delicate samples.

GUIDELINES FOR HIGH-THROUGHPUT PROCESSING

* Multi-channel pipettors and reagent reservoirs are recommended for Day 2 of the protocol.
* For 8-strip tubes, remove and replace buffers one strip at a time to avoid ConA bead dry out.

23. Gather reagents from 4˚C and -20˚C needed for Day 2 (**Wash Buffer 1, Wash Buffer 2, Anti-Rabbit Secondary Antibody, pAG-Tn5, Non-Hot Start 2X PCR Master Mix, i5 & i7 Primers**). Place on ice to thaw or equilibrate.
24. Prepare **Tagmentation Buffer** in a new 1.5 mL tube. Per reaction, combine 59.4 µL **Wash Buffer 2** and 0.6 µL **1 M $MgCl_2$** (10 mM final concentration). Place on ice. Recipe includes 20% excess volume to account for pipetting error.
25. Remove 8-strip tubes from 4˚C and quick spin to collect liquid. Place tubes on magnet and allow slurry to clear. Pipette to remove supernatant.
26. Take tubes off magnet. Add 50 µL cold **Wash Buffer 1** to each reaction and resuspend bead slurry by gentle pipetting and/or vortexing. If pipetting, avoid losing beads in tips and expel all material back into tubes. Vortexing can be used to help resuspend beads, and also is recommended for viscous samples. Always quick spin tubes after mixing to avoid bead loss.
27. Add 0.5 µg secondary antibody to each reaction. For H3K27me3, IgG, and rabbit primary antibodies, use 0.5 µL **Anti-Rabbit Secondary Antibody**. Gently vortex tubes ~5 sec to mix.
    * Secondary antibody must match primary antibody host species. Anti-mouse secondary antibody is available (EpiCypher 13-0048).
28. Quick spin tubes and place on nutator, caps elevated, for 30 min at RT.
29. Gently vortex tubes ~5 sec and quick spin. Place tubes on magnet and allow slurry to clear. Pipette to remove supernatant.
30. Keeping tubes on magnet, add 200 µL cold **Wash Buffer 1** to each reaction. Pipette to remove supernatant. Repeat one time for a total of two washes.

31. Take tubes off magnet. Add 50 µL cold **Wash Buffer 2** to each reaction and resuspend by pipetting (avoid bead loss, expel all material back into tubes) and/or vortexing, followed by a quick spin.

SECTION VI: PAG-TN5 BINDING & TARGETED TAGMENTATION (~4 HRS)

32. Add 2.5 µL **pAG-Tn5** to each reaction. Gently vortex tubes ~5 sec to mix.
33. Quick spin tubes and place on a nutator, caps elevated. Incubate 1 hour at RT.
34. Gently vortex tubes ~5 sec and quick spin. Place tubes on magnet and allow slurry to clear. Pipette to remove supernatant.
35. Take tubes off magnet. Add 200 µL cold **Wash Buffer 2** to each reaction and resuspend by pipetting (avoid bead loss, expel all material back into tubes). Quick spin tubes, place on magnet, and allow slurry to clear. Pipette to remove supernatant. Repeat one time for a total of two washes.
36. Take tubes off magnet. Add 50 µL cold **Tagmentation Buffer** to each reaction and resuspend by pipetting (avoid bead loss, expel all material back into tubes).
37. Gently vortex tubes ~5 sec and quick spin. Incubate 1 hour in a thermocycler set to 37˚C (heated lid at 47˚C). During the incubation, transfer 60 µL per reaction **Pre-Wash Buffer** to a new tube and let equilibrate to RT.
38. Gently vortex tubes ~5 sec and quick spin. Place tubes on magnet and allow slurry to clear. Pipette to remove supernatant and take tubes off magnet.
39. Add 50 µL RT **Pre-Wash Buffer** to each reaction. **Do NOT vortex** - you may lose sample. Instead, gently but thoroughly pipette 3-5 times to resuspend the bead slurry. Expel all material back into tubes.
40. Place tubes on magnet, allow slurry to clear. Pipette to remove supernatant.
41. Take tubes off magnet. Add 5 µL RT **SDS Release Buffer** to each reaction, vortex ~10 sec at max speed to mix, and quick spin.
    Do NOT pipette - you may lose sample.
42. Incubate reactions for 1 hour in a thermocycler set to 58˚C (heated lid at 68˚C).
43. Quick spin tubes. Add 15 µL RT **SDS Quench Buffer** to each reaction, carefully pipetting to rinse beads. The slurry may clog tips; avoid loss and expel all material back into the tube. If slurry is too viscous to pipette, move to next step.
44. Vortex ~10 sec at max speed to fully mix and quick spin. Keep tubes at RT.

SECTION VII: INDEXING PCR & LIBRARY CLEANUP (~1 HR)

INDEXING PCR OPTIMIZATION

* Confirm that ConA beads are in solution to maximize PCR efficiency and library yields.
* Use the minimum number of PCR cycles needed to accurately quantify DNA libraries (≥2 ng/µL). EpiCypher typically uses 16 PCR cycles to map PTMs from 100,000 K562 nuclei. Low yields may require additional PCR cycles or deeper sequencing; see **Appendix 1.2**.
* For index sequences, go to epicypher.com/14-1102 and download the Multiplexing Primers spreadsheet under Documents and Resources.

45. Assign a unique pair of **i5 & i7 indexing primers** to each CUT&Tag reaction as instructed in **Appendix 3**. Quick spin the i5 & i7 primers before each use.
46. For indexing PCR, add primers and PCR Master Mix directly to reactions in 8-strip tubes at RT. To each reaction add: 2 µL **i5 primer**, 2 µL **i7 primer**, and 25 µL **Non-Hot Start 2X PCR Master Mix**.

    Do **NOT** remove ConA beads. Do **NOT** put tubes on ice until after primers and PCR Master Mix are added. Change tips between each addition to prevent cross-contamination. Mix well by pipetting, avoiding bubbles. Expel all material back into tubes and quick spin.
47. Place reaction in a thermocycler with a heated lid set to 105˚C. Perform PCR using the parameters below to amplify tagmented DNA from 100-700 bp. We recommend using 16 PCR cycles and optimizing as needed (see **Appendix 1.2**)

Step #	Temperature	Time	Cycles	Notes
1	58˚C	5 min	1	Fill-in step
2	72˚C	5 min	1	Extension
3	98˚C	45 sec	1	DNA melting
4	98˚C	15 sec	14-21	DNA melting
5	60˚C	10 sec		Hybrid annealing/extension
6	72˚C	1 min	1	Final extension
7	4-12˚C	∞	1	Hold temperature

48. After PCR, remove reactions from thermocycler and quick spin to collect liquid.

49. 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.

50. Vortex **SPRIselect** reagent to fully resuspend. Slowly add 65 µL **SPRIselect** reagent (1.3X reaction volume) to each reaction. SPRIselect reagent is added to the **entire** reaction, including ConA beads. Ensure pipette tip is free of extra droplets when dispensing beads to reactions.

51. 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.

52. Place tubes on a magnet for 2-5 min at RT, until solution clears. Pipette to remove supernatant without disturbing beads.

53. Keeping tubes on the magnet, add 180 µL **85% EtOH** directly onto SPRIselect reagent. Pipette to remove supernatant. Repeat one time for a total of two washes.

54. 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.

55. Remove tubes from magnet and air-dry, caps open, for 2-3 min at RT. Beads should appear damp matte brown (Figure 8). If beads are crackly and/or light brown, they are too dry.

FIGURE 8

Elute DNA before beads dry out.

56. Add 17 µL **0.1X TE Buffer** to each reaction to elute CUT&Tag libraries. Pipette and/or vortex to fully resuspend beads and quick spin. Incubate for 2 min at RT.

57. Place tubes on magnet for 2 min at RT.

58. Transfer 15 µL CUT&Tag libraries to new **8-strip tubes**.
Safe pause point. Libraries can be stored at -20˚C.

SECTION VIII: ANALYSIS OF LIBRARY FRAGMENT SIZE (~1 HR)

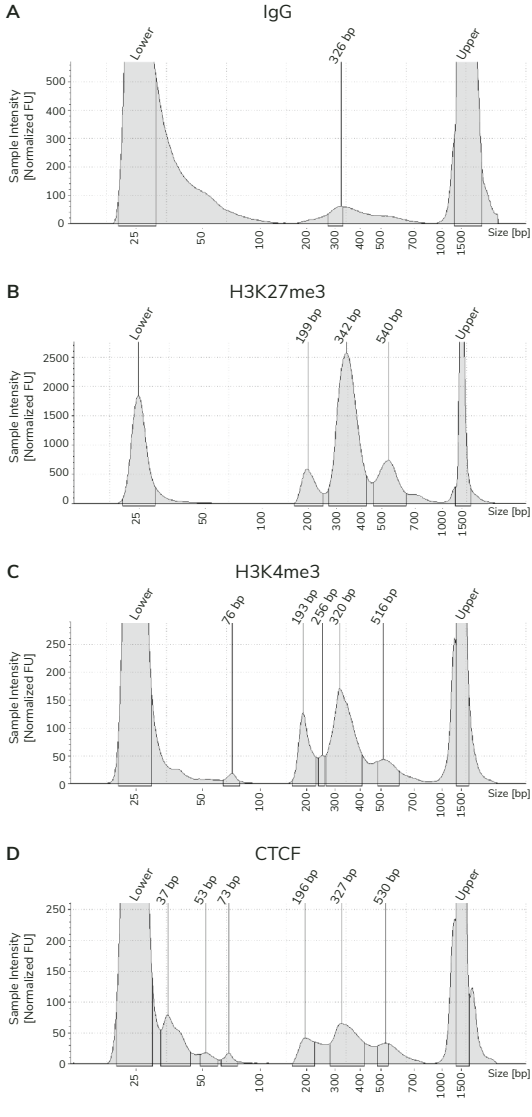
NOTES ON EXPECTED YIELDS AND FRAGMENT SIZE ENRICHMENT

* The **BEST** indicator of CUT&Tag experimental success prior to sequencing is enrichment of mononucleosome-sized fragments (~300 bp = ~170 bp nucleosome + sequencing adapters).
* There is no typical yield for CUT&Tag assays. The minimal DNA amount required for accurate quantification is ≥2 ng/µL or ~30 ng total DNA.
* Because adapters are added by pAG-Tn5 during tagmentation, adapter dimer formation is **NOT** possible. Primer dimers may appear at 25-100 bp, but should have minimal enrichment.
* Traces may show an oligonucleosome ladder with peaks every ~170 bp (see Figure 9). This periodicity does not impact sequencing, and size selection is not necessary.
* See **Appendix 1.2** for troubleshooting low yields and/or fragment distribution results.

59. Use 1 µL to quantify CUT&Tag libraries using the Qubit fluorometer with the 1X dsDNA HS Assay Kit, per the manufacturer's instructions.
60. Use 1 µL library for analysis on the Agilent Bioanayzer (High Sensitivity DNA Kit) or TapeStation (D1000 ScreenTape System), per the manufacturer's instructions.
61. Final traces should show predominant enrichment of mononucleosome-sized fragments, as in Figure 9 (~300 bp: ~170 bp mononucleosomes + sequencing adapters; see Notes above). For additional details and troubleshooting, see **Appendix 1.2**.

FIGURE 9 (opposite page)

Typical TapeStation traces from CUTANA CUT&Tag libraries prepared using antibodies targeting IgG (negative control), H3K27me3 (positive control), H3K4me3 (EpiCypher 13-0041; now replaced by 13-0060), and CTCF (EpiCypher 13-2014). All libraries are predominantly enriched for mononucleosome-sized fragments as indicated by the peak at ~300 bp (~170 bp mononucleosomes + sequencing adapters). The "trident" pattern shown in the H3K27me3 trace is typical for many histone PTMs.


A
IgG
Lower
326 bp
Upper
Sample Intensity [Normalized FU]
Size [bp]
B
H3K27me3
Lower
199 bp
342 bp
540 bp
Upper
C
H3K4me3
Lower
76 bp
193 bp
256 bp
320 bp
516 bp
Upper
D
CTCF
Lower
37 bp
53 bp
73 bp
196 bp
327 bp
530 bp
Upper

SECTION IX: ILLUMINA® SEQUENCING & DATA ANALYSIS

TIPS FOR SEQUENCING CUT&TAG LIBRARIES

* Paired-end sequencing (2 x 50 bp cycles minimum) is recommended for CUT&Tag to enable detection of K-MetStat Panel barcodes.
* Library molarity ≥0.5 nM (200-700 bp region) will allow pooling at standard concentrations for sequencing. For low-concentration libraries see **Appendix 1.2**.
* Sequence to a depth of 5-8 million total reads per library. The end goal to is generate 3-5 million **unique** reads per library. See **Appendix 1.3.**

62. Select appropriate Illumina sequencing platform (see **Appendix 3**) based on the number of CUT&Tag libraries and desired sequencing depth.
63. Dilute and pool libraries using molarity calculations from **Protocol: Section VIII** (200-700 bp region) and load onto the Illumina sequencer. General steps:
    a. Dilute each library to the same concentration using sequencing normalization buffer as recommended by Illumina (support.illumina.com). For NextSeq 2000 and NextSeq 500/550, dilute to ~1-4 nM.
    b. Pool equimolar libraries into one tube.
    c. Dilute pooled libraries to appropriate concentration in the volume required for Illumina platform. Follow guidelines from Illumina (support.illumina.com).
    d. When setting up a multiplexed sequencing run, ensure that each library contains a unique i5 & i7 primer pair and that dual barcodes are correctly assigned. For a full list of index sequences, download the CUTANA CUT&Tag Kit Multiplexing Primers spreadsheet at epicypher.com/14-1102.
64. For H3K27me3 and IgG control reactions spiked with the K-MetStat Panel, align paired-end sequencing reads to PTM-specific DNA barcodes. Use this data to validate your workflow, identify failed reactions, and troubleshoot problematic experiments. See **Appendix 1.4** for guidance and examples.
65. If control reactions generate expected results, proceed to analysis of experimental reactions (Figures 10 & 11). Align paired-end reads to the appropriate reference genome (e.g. using Bowtie 2). See **FAQ 9** for additional information.

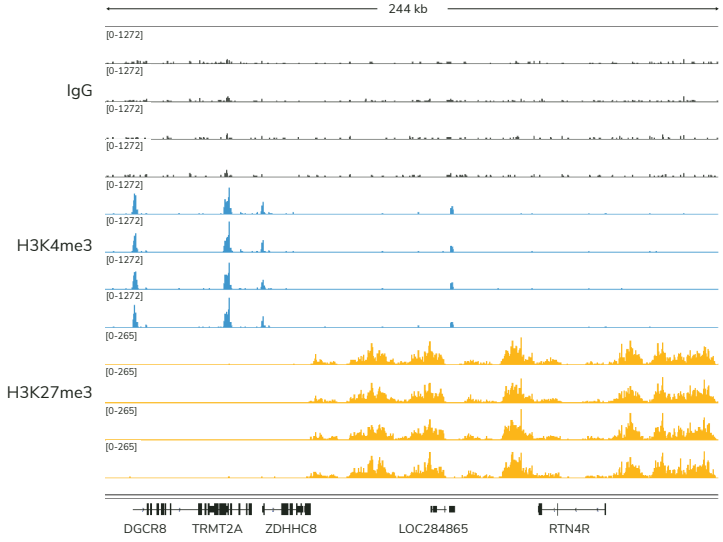
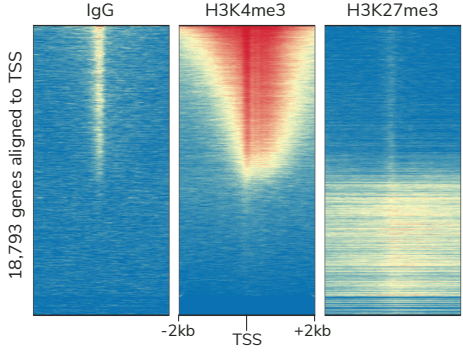


FIGURE 10

Data across four technical replicates in a CUT&Tag experiment demonstrate the reproducibility of the CUTANA™ CUT&Tag Kit. Genome browser tracks are shown for CUT&Tag replicates using 100,000 K562 nuclei with IgG Negative Control antibody, H3K4me3 antibody (EpiCypher 13-0041; now 13-0060), and H3K27me3 Positive Control antibody. H3K4me3 tracks show sharp peaks localized to transcription start sites (TSSs), while H3K27me3 tracks show broad peaks spread over repressed regions. IgG shows typical background enrichment.

FIGURE 11

Expected results from CUTANA CUT&Tag assays using 100,000 K562 nuclei 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).



1.1. QUALITY CONTROL CHECKS FOR SAMPLE PREP

This protocol uses a simple Trypan Blue staining to flag CUT&Tag samples of poor quality or that fail to bind ConA beads. Note that:

* Low cell viability and nuclear lysis increase assay background - **don't skip these steps!**
* Nuclei are resuspended with 5 µL excess buffer to account for pipetting error. It may be helpful to process one extra sample to evaluate nuclei integrity.
* If using whole cells instead of nuclei, also see **Appendix 2.1**.

1. Spin cells 600 x g, 3 min, RT. Remove supernatant, flick tube to loosen cell pellet, and resuspend in 1-2 mL 1X PBS. Transfer 10 µL cells to a 1.5 mL tube.
2. Add 10 µL 0.4% Trypan Blue to 10 µL cells. Pipette 10 times to mix.
3. Transfer 10 µL to a cell counting slide. Obtain cell counts and determine viability using a brightfield/phase microscope or cell counter. See Figure 12, next page.
4. Harvest 100,000 cells per reaction (plus 10% excess) in a new 1.5 mL tube. Spin cells 600 x g, 3 min, RT. Pipette to remove supernatant.
5. Resuspend cells in 100 µL per reaction cold **Nuclear Extraction Buffer**. Incubate for 10 min on ice.
6. Spin 600 x g, 3 min, 4°C. Pipette to remove supernatant. Reuspend nuclei in 105 µL per reaction cold **Nuclear Extraction Buffer**.
7. Transfer 10 µL nuclei to a new tube. Evaluate integrity using Trypan Blue staining as in Steps 2 & 3 (above). See Figure 12 for expected results.
8. Add 100 µL nuclei to 10 µL activated ConA beads (see **Protocol: Section III**).
9. Gently vortex the sample [**sample slurry**] and quick spin. Incubate 10 min at RT.
10. Place tubes on magnet and wait for slurry to clear. Transfer 10 µL supernatant [**unbound fraction**] to a new 1.5 mL tube. Discard remaining supernatant.
11. Add 50 µL cold **Antibody Buffer** to the **sample slurry**. Remove tubes from magnet and pipette to resuspend.
12. Transfer 10 µL **sample slurry** to a new 1.5 mL tube. Place remaining **sample slurry** on ice.
13. To samples that were set aside (**unbound fraction**, **sample slurry**), perform Trypan Blue staining as described in Steps 2 & 3. See Figure 12 for results.
14. Continue to **Protocol: Section IV** (**Primary Antibody Binding**).

Sample	Success Metrics	Troubleshooting Tips
Cells Figure 12A	Cells should be round, white (Trypan excluded), not clumped, and >80% viabile.	Evaluate cell culture conditions; use fresh media, troubleshoot potential contamination issues.
Nuclei Figure 12B	Nuclei should be >95% “dead” (Trypan blue positive) and unclumped.	Monitor cells during nuclear extraction by Trypan Blue staining to optimize incubation time. Increase spin time if losing sample (keep at 600 x g).
Unbound fraction Figure 12C	Little to no material should be present if binding occurred.	Ensure that ConA beads were never frozen (if frozen, beads will appear black, granular/clumpy), cells and nuclei were not clumped, beads did not become clumped or dried out, and all buffers were correctly prepared.
Sample slurry Figure 12D	Successful binding will show permeabilized nuclei/cells surrounded by beads.	

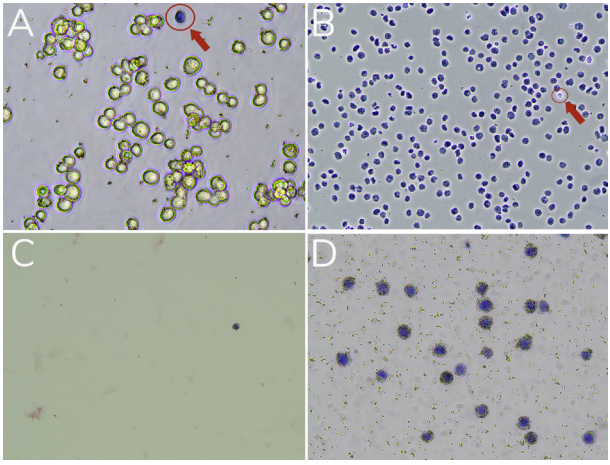


FIGURE 12

Validation of sample prep using Trypan Blue staining. **(A)** Starting cells are mostly viable (bright white and round). A dead cell (blue, Trypan positive) is circled in red. **(B)** Successful nuclei harvest shows Trypan Blue stained nuclei. An intact cell (bright white, Trypan negative) is circled in red. **(C)** Unbound fraction has minimal nuclei. **(D)** Representative sample slurry image showing nuclei (blue) successfully conjugated to activated ConA Beads (brown specks). **Note:** ConA bead-bound cells will also be Trypan positive (blue), due to the presence of Digitonin in buffers.

1.2. EXPECTED CUT&TAG RESULTS & TROUBLESHOOTING

CUT&Tag assay success metrics:

- The **BEST** indicator of CUT&Tag success before sequencing is predominant enrichment of mononucleosome-sized fragments (~300 bp = ~170 bp nucleosome + sequencing adapters) in Bioanalyzer/TapeStation results.
- Do **NOT** use library yields to determine assay success, as yields can vary by cell type, number of nuclei, target abundance, and antibody quality.
- Samples should pass **ALL** quality control checks described in **Appendix 1.1**. If starting cells have poor viability and/or are clumpy, or if nuclei are lysed and sticky, assay background will be higher - reducing the quality of sequencing data.

Typical library yields and requirements for Illumina sequencing:

In general, ≥2 ng/µL or ~30 ng total DNA will allow accurate library quantification and minimize PCR duplicates. Library molarity ≥0.5 nM for the 200-700 bp region will allow pooling at standard concentrations for sequencing.

Shorter and/or larger fragments in Bioanalyzer/TapeStation traces:

Shorter fragments (~175 bp) are partly due to the use of a secondary antibody, which augments pAG-Tn5 binding and tagmentation. Longer fragments may also be observed (i.e. oligonucleosomal ladder). Size selection is **NOT** recommended, since they do not represent assay background and do not impact data quality.

Basic CUT&Tag troubleshooting guidelines:

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

- What is your cell/sample type? Check **Appendix 2** for protocol modifications.
- Have you confirmed sample prep and ConA bead binding? See **Appendix 1.1**.
- Are you using the recommended 100,000 nuclei/cells per reaction? Note that:
  - Using more than 100,000 nuclei per reaction does **NOT** improve yields.
  - Success at low nuclei numbers is dependent on antibody quality and target abundance. See **FAQ 7** for considerations.
- Is indexing PCR optimized? Use the minimum number of cycles for ≥2 ng/µL DNA.
- Have you included reactions with control antibodies & the K-MetStat Panel? These controls are crucial for troubleshooting CUT&Tag (**Appendix 1.4**.)
- Are ConA beads brown? Have reactions been mixed properly using a nutator? Have beads become clumpy or dried out?

TABLE 3 Troubleshooting fragment distribution and library yields

Concern	Causes & troubleshooting approaches
Adapter Dimers	pAG-Tn5 adds sequencing adapters in CUT&Tag. Formation of adapter dimers is **NOT** possible and size selection is not needed.
Most fragments >700 bp	⚠ **Poor sample prep** • See **Basic CUT&Tag troubleshooting guidelines** (opposite page); confirm sample prep quality (**Appendix 1.1**), avoid ConA bead loss ⚠ **Excessive amplification or incorrect PCR cycling parameters** • Check PCR settings and repeat assay using fewer PCR cycles
Most fragments <150 bp (primer dimers)	⚠ **Bad antibody, too many nuclei (may inhibit PCR)** • Repeat experiment using 100,000 native nuclei per reaction and include reactions with control antibodies & K-MetStat Panel • If controls work (**Appendix 1.4**) but experimental targets fail, test additional antibodies; see **FAQs 2-4** for guidance
Low yields, no enrichment in Bioanalyzer or TapeStation results	⚠ **Many potential causes: poor sample prep, low nuclei numbers, ConA bead loss, target requires different processing conditions, bad antibody, low abundance target, poorly optimized PCR** • See **Basic CUT&Tag troubleshooting guidelines** (opposite page); confirm sample prep quality (**Appendix 1.1**), avoid ConA bead loss, and optimize PCR cycling conditions • Repeat experiment using 100,000 native nuclei per reaction and include reactions with control antibodies & K-MetStat Panel **Examine results:** • Controls work but experimental targets fail: Confirm target is correctly localized to chromatin (e.g. stimulation conditions); test additional antibodies and/or cross-linking conditions (**Appendix 2.3**) • If controls **AND** experimental targets show no yield: Try purifying DNA prior to PCR using phenol-chloroform extraction[5] or SPRI beads (e.g. EpiCypher 14-0052) ⚠ **Considerations for low nuclei inputs & low abundance targets:** • Increase the number of PCR cycles to improve yields for sequencing; this strategy may increase read duplicates and requires deeper sequencing to capture library diversity (also see **FAQ 7**)
Sequencing low yield libraries	Use a Speedvac to increase library concentration and add as much of the library as possible to the sequencing pool. Deeper sequencing is recommended to fully capture library diversity

1.3. CUT&TAG SEQUENCING RESULTS & TROUBLESHOOTING

CUT&Tag data sequencing data metrics:

- Libraries should be sequenced to a depth of 5-8 million **total** reads.
- Deeper sequencing is recommended for rare targets and low inputs (Table 4).
- For sufficient coverage, each library should generate 3-5 million **unique** reads (after removing multimapping reads, duplicate reads, reads in blacklisted regions).
- The SNAP-CUTANA K-MetStat Panel should comprise ~1% of unique reads and produce expected results in H3K27me3 and IgG control reactions (**Appendix 1.4**).
- H3K27me3 and IgG controls should show expected enrichment and peak structures. Experimental replicates should be highly reproducible (Figure 10 & 11).

Notes on read duplicates in CUT&Tag sequencing data:

- High read duplicate rates are common in CUT&Tag due to assay sensitivity and extremely low background.
- Based on EpiCypher's CUT&Tag optimization studies and analyses of published datasets[3], duplication rates below 70% do not impact sequencing data quality.
- In general, duplication rates are higher for low abundant PTMs (H3K4me3; 50-70%) vs. abundant PTMs (H3K27me3 positive control; 10-30%).
- Note that excessive PCR amplifcation and/or sequencing (>10 million total reads) may increase read duplicates, but good data are still obtained.
- If duplicate read levels are a problem, see Table 4. We also recommended trying CUT&RUN, which has lower duplication rates.*

Download i5 and i7 index sequences:

See epicypher.com/14-1102, Documents and Resources. The **CUT&Tag Multiplexing Primers** spreadsheet contains index sequences for 14-1102 & 14-1103.

Need help with sequencing analysis?

- For help with CUT&Tag sequencing analysis, including genomic alignment, peak calling, and signal-to-noise calculations, see **FAQ 9**.
- Instructions for K-MetStat spike-in analysis from sequencing data, including examples and expected data, are provided in **Appendix 1.4**.

* CUT&Tag is an expert-level assay. For most users, EpiCypher suggests CUTANA CUT&RUN assays, which has a user-friendly protocol and is robust for most targets and cell types (epicypher.com/cut-and-run).

TABLE 4 Troubleshooting CUT&Tag sequencing results

Concern	Causes & troubleshooting approaches
Signal in IgG negative control and/or Signal in open chromatin for repressive targets	⚠ **Preference of Tn5 for accessible chromatin** • The high-salt wash after pAG-Tn5 binding helps reduce signal at open chromatin, but some background may be present in the IgG control[5,6] and when profiling repressive targets **To minimize background:** • Use freshly harvested, native nuclei • Always include the IgG negative control for comparison • Confirm sample prep quality (**Appendix 1.1**), as cell/nuclear lysis increases background
High read duplication rates	⚠ **Many potential causes: Target abundance, antibody quality, cell type, number of nuclei, number of PCR cycles, library yields, sequencing depth** High read duplicate rates are common in CUT&Tag and generally are not a problem. However, if duplication rates are concerning, optimize as follows: • Assess the number of nuclei, target abundance, PCR conditions, yields, and sequencing depths (see **Appendix 1.2**) • Use 100,000 native nuclei per reaction; include reactions with control antibodies & K-MetStat Panel • Confirm sample prep quality (**Appendix 1.1**), avoid ConA bead loss • Optimize PCR: test 14, 16, 18 cycles; aim for ≥2 ng/µL DNA **Examine results:** • Yields are high: Reduce the number of PCR cycles; use the minimum number of PCR cycles that provides ≥2 ng/µL DNA • Yields are adequate but duplication rates are high despite using recommended input and sequencing depth: Indicates poor antibody and/or mapping a low abundance target ⚠ **Considerations for low nuclei inputs & low abundance targets:** • For low nuclei numbers or low abundance targets, additional PCR and sequencing is needed to capture diversity; high read duplicates may be a necessary trade-off (also see **FAQ 7**) • Duplicates can be assessed and removed using Picard (broadinstitute.github.io/picard)

1.4. SNAP-CUTANA™ K-METSTAT PANEL

To validate experiments and guide troubleshooting, EpiCypher recommends adding the SNAP-CUTANA K-MetStat Panel of nucleosome spike-ins to control reactions in **every** experiment. The kit includes K-MetStat Panel for 10 experiments, or 20 reactions: 10 for the IgG control antibody and 10 for the H3K27me3 control antibody.

What is the K-MetStat Panel?

The K-MetStat Panel comprises designer nucleosomes representing 16 distinct histone lysine methylation states (Figure 13). Each PTM is represented by two unique DNA-barcoded templates (A and B, for an internal technical replicate). Nucleosomes are individually coupled to magnetic beads and pooled into a single panel for convenient one-step addition to CUT&Tag workflows (Figure 14).

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

The K-MetStat Panel is the **only** control that uses purified recombinant nucleosomes, replicating the physiological target of CUT&Tag and providing reliable on- and off-target substrates for control reactions. These spike-ins directly report on antibody specificity, sample quality, pAG-Tn5 activity, and more.

Why should I use the K-MetStat Panel?

It may be unclear from genomic tracks alone that a reaction issue has occurred. The controls in this section are designed to flag failed reactions **AND** indicate the cause. By identifying poor samples or reactions and using the spike-in results to guide troubleshooting, researchers can be confident in their experimental results.

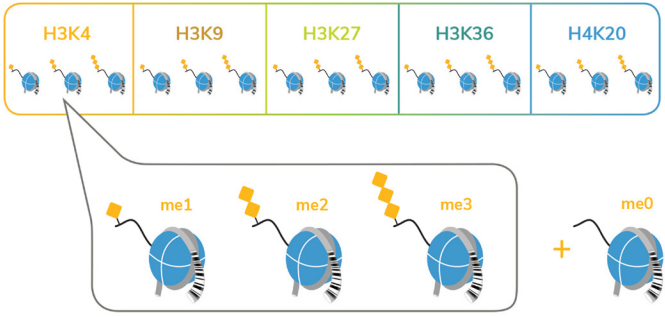


FIGURE 13

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

SPIKE-IN PROCEDURE OUTLINE:

- Add the K-MetStat Panel to designated control reactions immediately prior to the addition of H3K27me3 or IgG control antibody (Figure 14).
- Add antibody, which binds its target in cells **and** in the spike-in panel (Figure 14).
- pAG-Tn5 cleaves and ligates sequencing adapters to antibody-bound chromatin **and** antibody-bound spike-in. Tagmented DNA is prepared for sequencing.
- For each control reaction, determine the number of sequencing reads aligned to each PTM-specific DNA barcode (next page). Barcode read counts provide a useful measurement of PTM recovery and workflow success (Figures 15 & 16).

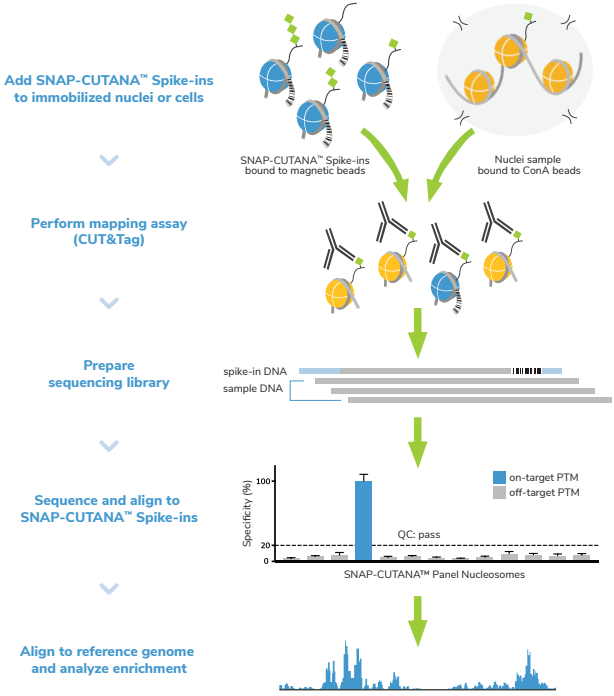


FIGURE 14

Schematic showing addition of SNAP-CUTANA Spike-ins during CUT&Tag workflows. Note that the spike-ins are also compatible with CUT&RUN assays.

K-METSTAT PANEL DATA ANALYSIS:

1. Download R1 & R2 paired-end sequencing files (fastq.gz) for control reactions. Double-click the fastq.gz files to create **fastq files** and save in a **new folder**.
2. At epicypher.com/14-1102, Documents and Resources, download Shell Script (**.sh**) and K-MetStat Panel Analysis (**.xlsx**) files. Save to the **folder** from Step 1.
3. Open the .sh file in TextEdit or any **text editing** program. Do **NOT** open in Word or a PDF program. Scroll past the barcode sequences to find the analysis **script**.
4. The script is a **loop** that **counts the number of reads** aligned to each **PTM-specific DNA barcode** in a reaction. Each PTM in the K-MetStat Panel is represented by two unique barcodes, A & B, for a **total of 32 barcodes**.

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

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

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

   c. In the first loop replace sample1_R1.fastq and sample1_R2.fastq with R1 & R2 fastq file names for **one** control reaction. Repeat for each loop. Press save.
5. In **Terminal**, set the directory to your **folder**: Type **cd** and press space. Drag the folder from your files into Terminal to copy the location. Press return.
6. **Run your script** in Terminal: Type **sh** and press space. Drag your .sh file from your files into Terminal to copy the file location. Press return. Terminal generates barcode read counts from R1 & R2 reads, one loop/reaction at a time.
7. Open the K-MetStat Panel .xlsx file in **Excel**. Fill in reaction names and set the **on-target PTM** in **Column B**. The first reaction is set to IgG (negative control); for other reactions, select a target (i.e. H3K27me3) from the drop-down menu.
8. Copy R1 barcode read counts from the first loop in Terminal. In Excel, paste into the yellow cells for that reaction in **Column C**. Copy & paste the R2 read counts from the same loop to yellow cells in **Column D**. Repeat for each loop/reaction.
9. The Excel file automatically analyzes spike-in data for **each reaction** by:

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

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

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

10. **Column J** auto-populates the **Output Table** (Figure 15). Reactions are separated by **row** and PTM data are sorted into **columns**. A color gradient is used to visualize the recovery of each PTM normalized to on-target PTM, from blue (100%) to orange (less than 20%).

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

EXPECTED RESULTS:

- IgG negative control: No preference among PTMs, low background (Figure 15).
- H3K27me3 positive control: Strong enrichment for H3K27me3 spike-ins, less than 20% off-target PTM recovery, and high signal-to-noise.
- Spike-in barcode reads: Comprise ~1% (0.5-5%) of total sequencing reads.
- If control reactions generate expected spike-in data (Figure 15, Samples 1 & 2), you can be confident in the technical aspects of your workflow.
- More than 20% off-target PTM recovery in H3K27me3 control and/or high background in IgG control indicate experimental problems (Figure 15, Sample 3). See next page for a discussion of troubleshooting using spike-in results.

* **Note:** Although CUT&RUN data are shown, the same principles can be applied for CUT&Tag.

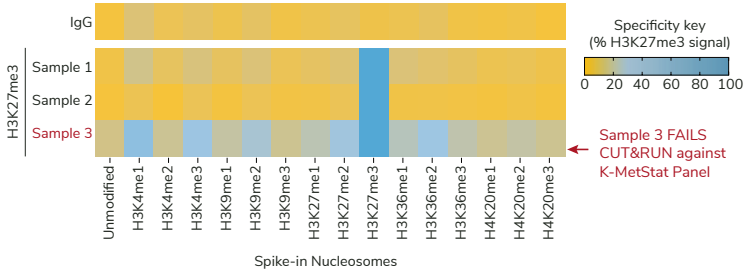


FIGURE 15

K-MetStat Spike-ins validate workflows and flag poor samples in CUTANA CUT&RUN experiments. Spike-in data for H3K27me3 positive control reactions is shown for three independently prepared mouse B cell samples. Samples 1 & 2 show expected results, while Sample 3 was flagged for recovery of off-target PTMs and low signal-to-noise. Representative data from one IgG reaction is shown as a negative control.

TROUBLESHOOTING CUTANA ASSAYS USING THE K-METSTAT PANEL:

Figures 15 & 16 demonstrate the use of control antibodies and K-MetStat Panel for troubleshooting CUT&RUN. The same principles can also be applied for CUT&Tag.

- In Figure 15 we used spike-in data from H3K27me3 & IgG control reactions to validate workflows for three independently prepared mouse B cell samples.
- Samples 1 & 2 showed expected results from control reactions, while Sample 3 displayed low signal-to-noise (S:N) and high off-target PTM recovery (Figure 15).
- Genomic profiles agreed with spike-in data: Samples 1 & 2 generated expected tracks for H3K27me3 & IgG, while Sample 3 profiles had poor S:N (Figure 16).

To troubleshoot Sample 3 reactions, we considered the following:

- All reactions were performed in parallel using the same antibodies and reagents. However, only Sample 3 reactions had problems with background.
- Sample 3 showed poor S:N in both genomic profiles **and** K-MetStat Panel data.
- Sample 3 generated poor profiles across multiple targets (data not shown).

Combined, these results suggested problems with sample prep vs. a complete workflow failure. We subsequently reviewed Sample 3 processing methods, revealing that the number of cells used per reaction was much lower than intended.

For other troubleshooting tactics using the K-MetStat Panel, see Table 5.

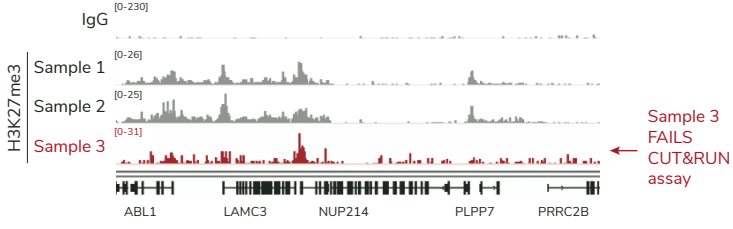


FIGURE 16

CUT&RUN was used to map IgG and H3K27me3 in three independently prepared mouse B cell samples (10,000 cells each; protocol optimization experiment with a multi-lab consortium). A representative 400 kb region is shown. Tracks from sample chromatin show consistent peaks for Samples 1 & 2, while Sample 3 displays low signal-to noise (red).

TABLE 5 Troubleshooting CUT&Tag results using the K-MetStat Panel

Results	Causes & troubleshooting approaches
K-MetStat spike-in data: • High target specificity • High S:N Genomic data: • Poor S:N	pAG-Tn5 cleavage and wash conditions are optimized. Control antibodies are performing as expected. Problems may include: ⚠ **Low numbers of nuclei** • Optimize assay with 100,000 nuclei before decreasing input • If using whole cells, adherent cells, cross-linked samples, or tissues, additional optimization is required (**Appendix 2**) ⚠ **Poor sample prep** • Confirm sample prep and bead binding (**Appendix 1.1**); note that low cell viability and/or nuclear lysis increases background • Avoid ConA bead clumping and dry-out during assay ⚠ **Experimental target requires different processing conditions** • Ensure target is present and localized to chromatin • If using frozen nuclei, try freshly isolated nuclei • Test native vs. lightly cross-linked conditions (**Appendix 2.3**)
K-MetStat spike-in data: • Nonspecific PTM recovery • Poor S:N Genomic data: • Poor S:N	⚠ **Indicates a fundamental failure in the workflow** • Carefully re-read the protocol and important notes • Ensure buffers are prepared fresh on day of use • Ensure ConA beads are in good condition (e.g. never frozen) • Make sure correct parameters are used in indexing PCR ⚠ **Low numbers of nuclei and/or poor sample prep** • Optimize following the guidelines above and **Appendix 1.2**
K-MetStat spike-in data: • Nonspecific PTM recovery • S:N may vary Genomic data: • High S:N	⚠ **Indicates cross-reactive control antibodies** • Examine potential contamination of control reactions with antibodies to other targets • Ensure buffers are prepared fresh on day of use • Change pipette tips after **each** reagent addition to avoid cross-contamination • For concerns about control antibody performance, email us at techsupport@epicypher.com.

* The K-MetStat Panel can be added to any reaction that is mapping a PTM in the panel (e.g. lysine methylation targets). Purchase additional K-MetStat Panel and learn more about SNAP-CUTANA Spike-in applications at epicypher.com/19-1002.

2.1. SAMPLE PREP VARIATIONS

The CUT&Tag kit is compatible with whole cells, adherent cells, tissues, cryopreserved samples, and cross-linked nuclei/cells.

WHOLE CELLS

Digitonin is a nonionic detergent used to permeabilize cell membranes. Because Digitonin is added to CUT&Tag assay buffers, whole cells are compatible with the protocol. However, additional modifications are required when using cells:

- Optimize Digitonin for each cell type to achieve >95% cell permeabilization, with no precipitation or cell lysis. A detailed protocol is in the CUT&RUN Kit manual (epicypher.com/14-1048). The optimal concentration should be used in **ALL** CUT&Tag buffers containing Digitonin.
- When using whole cells in CUT&Tag do **NOT** use Nuclear Extraction Buffer.
- Count cells as in **Appendix 1.1**. Harvest 100,000 cells per reaction (plus 10% excess), and spin 600 x g, 3 min, RT. Remove supernatant, resuspend cells in 105 µL per reaction cold **Wash Buffer 1**, and proceed to ConA bead binding.

ADHERENT CELLS

- Collect adherent cells using a mild Trypsin digestion, which dislodges and disaggregates clumps into monodispersed cells without cell damage. Incubate cells with **0.05% Trypsin at 37°C for the minimal time necessary to dislodge cells**. Add pre-warmed complete media to inactivate Trypsin and then collect cells.
- For nuclei, proceed with **Appendix 1.1**. Trypsin is removed during PBS wash steps.
- If using intact adherent cells, ensure Trypsin has not disrupted cell integrity or ConA bead binding (**Appendix 1.1**, Figure 12). Follow instructions for whole cells above.

TISSUES

Tissues must be processed into a monodispersion of cells, typically by mechanical maceration or douncing. Enzymatic digestion (e.g. collagenase, dispase) can be used for connective tissue and Trypsin may be used for macro-dissected tissues (as above; monitor dissolution to single cells). See literature for additional methods[11,12].

IMMUNE CELLS

Concanavalin A (ConA) is a lectin, which can cause immune cell activation. It is recommended to isolate nuclei for immune cell studies.

2.2 CRYOPRESERVED NUCLEI & CELLS

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

FREEZE/THAWING NUCLEI

1. Isolate nuclei and confirm sample quality as described in **Appendix 1.1.**
2. Aliquot nuclei resuspended in **Nuclear Extraction Buffer**. EpiCypher typically aliquots nuclei for ≥8 reactions, plus 20-30% excess to account for sample loss.
3. Slowly freeze aliquots (-1˚C per minute) in an isopropanol-filled chiller in a -80°C freezer (e.g. "Mr. Frosty"). Nuclei can be shipped on dry ice in this state.
4. When ready to perform CUT&Tag, remove tubes from -80˚C and place on a 37°C block to thaw. Work quickly to avoid nuclear lysis and chromatin fragmentation.
5. Thawed nuclei in Nuclear Extraction Buffer can be directly added to activated ConA beads (**Protocol: Section III**).

FREEZE/THAWING CELLS

1. Make sure Digitonin is optimized for cell types (see previous page).
2. Count cells and confirm viability (**Appendix 1.1**). Spin cells 600 x g, 3 min, RT.
3. Remove supernatant. Resuspend in **cell culture media with 10% DMSO** and aliquot as desired. EpiCypher typically aliquots cells for ≥8 reactions, plus 20-30% excess cells to account for sample loss.
4. Slowly freeze aliquots in an isopropanol-filled chiller in a -80°C freezer.
5. When ready to perform CUT&Tag, remove tubes from -80˚C and quickly place on a 37°C block to thaw. Work quickly to avoid cell lysis.
6. When cells are almost thawed, remove from 37˚C and pipette to fully thaw cells.
7. Spin thawed cells at 600 x g, 3 min, RT. Pipette to remove supernatant.
8. Resuspend cells in 105 µL per reaction RT **Wash Buffer 1**. Take a 10 µL aliquot to count (as in **Appendix 1.1**). Note that viability may be decreased; focus instead on cell integrity, lysis levels, and total cell counts. If significant sample loss has occurred, spin cells again and resuspend in a smaller volume of Wash Buffer.
9. Continue to ConA bead binding (**Protocol: Section III**).

2.3. CROSS-LINKING PROTOCOL

This cross-linking protocol should be considered for:

* Lysine acetylation PTMs (labile targets, impacted by histone deacetylase activity)
* Experiments with tightly controlled variables (e.g. time course drug treatments)
  * Note that for transiently interacting chromatin-associated proteins, it is recommended to use the CUTANA CUT&RUN Kit (EpiCypher 14-1048), which includes methods for light to moderately cross-linked samples.
  * ALWAYS include native samples when testing cross-linking conditions.

Although native conditions are preferred for CUT&Tag, signal for some targets may be improved by light cross-linking. When optimizing cross-linking conditions:

- Start with light cross-linking (0.1% formaldehyde, 1 min), which generally preserves signal without negatively impacting yields and data (Figure 17).
- If light cross-linking is not sufficient, moderate cross-linking (1% formaldehyde, 1 min) can be attempted with the caveat that it may impact DNA yield.
- Avoid heavy cross-linking associated with ChIP protocols (>1% formaldehyde, 1-10 min) which is deleterious to both DNA yield and data quality (Figure 17).

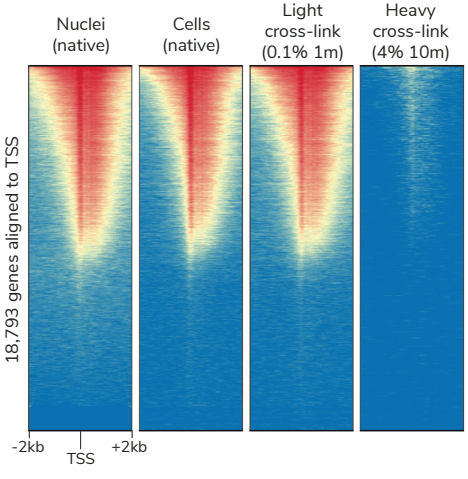


FIGURE 17

H3K4me3 (EpiCypher 13-0041) signal in CUT&Tag is preserved in lightly cross-linked nuclei when compared to native nuclei and cells. However, heavy cross-linking significantly reduces DNA yield. CUT&Tag data from 100,000 K562 nuclei or cells are displayed in a heatmap with each gene row aligned across conditions.

CROSS-LINKING PROTOCOL

Materials Needed	Recipe & Source
37% Formaldehyde	Sigma 252549
Glycine	Sigma 50046

1. For suspension cells: Harvest 100,000 cells per reaction. Due to the increased risk of sample loss when cross-linking cells **and** isolating nuclei, it is recommended to harvest 20-30% excess cells.

   For adherent cells: Cross-link cells while still attached to the plate.

2. Add fresh **37% Formaldehyde** directly to culture to desired final concentration (recommended 0.1-1%). Test a range of formaldehyde concentrations in initial experiments to determine the optimal conditions for target & cell type.
3. Quickly vortex (suspension cells) or swirl plate (adherent cells) to mix.
4. Incubate for 1-10 min at RT (recommended 1 min). Test a range of times in inital experiments to determine optimal conditions.
5. Quench cross-linking by adding **Glycine** to a final concentration of 125 mM. Vortex (suspension cells) or swirl plate (adherent cells) to mix.
6. For adherent cells: See **Appendix 2.1, Adherent Cells** for instructions.

   For suspension cells: Spin at 600 x g for 3 min at RT. Remove supernatant and proceed to next step.

7. **If extracting nuclei**: Begin at **Appendix 1.1**, **Step 1**. Proceed to CUT&Tag.

   If using whole cells: See **Appendix 2.1, Whole Cells** for instructions.

8. No additional protocol modifications are required. Digestion with SDS Release Buffer in **Protocol: Section VI** is sufficient to reverse cross-links.
9. Select the optimal sample prep conditions for the target based on the balance of DNA yield, enrichment, and signal-to-noise in sequencing data.

A detailed CUTANA Cross-Linking Protocol is available at epicypher.com/protocols.

2.4. CUTAC PROTOCOL FOR CHROMATIN ACCESSIBILITY MAPPING

In their 2020 paper, the Henikoff group described CUTAC (Cleavage Under Target Accessible Chromatin), which enables chromatin accessibility profiling using a modified CUT&Tag protocol[7]. Key changes include:

* An H3K4me2 antibody (e.g. EpiCypher 13-0027) is used to label open chromatin.
* A No-Salt Tagmentation Buffer is used to support Tn5 activity in open chromatin.

Figure 18 shows H3K4me2 CUT&Tag profiles generated using the standard tagmentation conditions vs. the modified, no-salt CUTAC tagmentation buffer. When compared to high quality Omni-ATAC-seq profiles[13], data from CUTAC assays display improved signal over background (Figure 18 A&C). Another important feature of CUTAC is the low rate of mitochondrial reads, which enables ~10-fold reduced sequencing depths compared to traditional ATAC-seq[7].

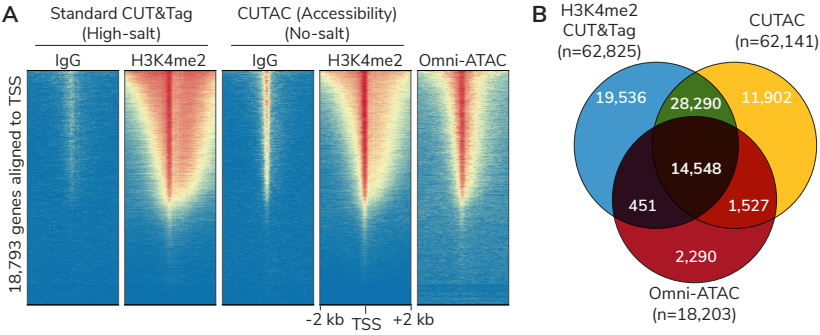


FIGURE 18

Modification of the CUTANA CUT&Tag Kit for chromatin accessibility mapping. Profiles were generated using 100,000 K562 nuclei with EpiCypher H3K4me2 or IgG negative control antibody. Tagmentation was performed under conditions for standard CUT&Tag or CUTAC. Omni-ATAC-seq data from [13] are shown for comparison. **(A)** Heatmaps show signal relative to TSS in each assay. Gene rows are aligned across conditions. **(B)** Venn diagram showing peak overlap between H3K4me2 CUT&Tag, CUTAC, and Omni-ATAC-seq. **(C)** FRiP scores show that CUT&Tag and CUTAC data have higher signal-to-noise vs. Omni-ATAC-seq.

C

Assay	FRiP (%)
H3K4me2 CUT&Tag	63.5%
CUTAC	64.1%
Omni-ATAC	11.3%

FRiP = Fraction of reads in peaks, measure of signal-to-noise

For CUTAC assays, please note the following modifications to the CUTANA CUT&Tag Experimental Protocol:

SECTION IV: PRIMARY ANTIBODY BINDING

- **Use an H3K4me2 primary antibody** (EpiCypher 13-0027). Add 0.5 µg to each reaction. Gently vortex to mix and incubate overnight on a nutator at 4˚C.

SECTION V: SECONDARY ANTIBODY BINDING

- At the start of Day 2, prepare the **No-Salt Tagmentation Buffer** as outlined in the table below. Store on ice.
- Note that 50 µL is needed per CUT&Tag reaction. The recipe provides enough for 10 reactions with 20% overflow to account for pipetting error.

Components	Source	[Final]	10X
1M TAPS, pH 8.5	Boston Bioproducts BB-2375	10 mM	6 µL
1 M $MgCl_2$	Included with CUTANA CUT&Tag Kit	5 mM	3 µL
Molecular biology grade water	Any Vendor	-	591 µL

SECTION V: SECONDARY ANTIBODY BINDING

- Use a species-matched secondary antibody. EpiCypher's H3K4me2 antibody can be used with the **Anti-Rabbit Secondary Antibody** provided in the kit.

SECTION VI: PAG-TN5 BINDING & TAGMENTATION

- Use 50 µL cold **No-Salt Tagmentation Buffer** in place of the standard Tagmentation Buffer. Thoroughly pipette to resuspend.
- At **Step 37**, incubate reactions for **20 minutes** in a thermocycler set to 37˚C (lid to 47˚C).
- Proceed with kit protocol at **Step 38**. No other protocol modifications are required

For guidance on sequencing analysis, refer to [7]. Note that the IgG negative control may show slightly more background (Figure 18A). This is expected, because nonspecific tagmentation in accessible chromatin is increased by low salt.

Appendix 3: Illumina® Sequencing & Primer Selection Guide

ILLUMINA® SEQUENCING PLATFORMS

* The CUTANA CUT&Tag Kit is compatible with Illumina high-throughput sequencing platforms (e.g. NextSeq 1000/2000).
* Paired-end sequencing (2 x 50 bp minimum) is recommended for CUT&Tag.
* Libraries should be sequenced to a depth of 5-8 million total reads. See **Appendix 1.3**.

The table below outlines Illumina next-generation sequencing platforms and kits compatible with the multiplexing capabilities of the CUTANA CUT&Tag Kit. The number of reactions per run assumes ~5 million total reads per CUT&Tag library. Additional user optimization may be required.

* i5 & i7 primer selection is NOT optimized for color balancing on MiSeq systems. We suggest pooling with other labs on a higher throughput sequencing platform.

Platform	Cartridge	Cat#	Expected Read Depth	# CUT&Tag Reactions
MiniSeq	High Output Kit (150 cycles)	FC- 420-1002	25-40 million	~8
HiSeq 3000/4000	SBS Kit (150 cycles) & PE Cluster Kit	FC-410-1002 PE-410-1001	350 million per lane	~70 per lane
NextSeq 500/550	Mid Output v2.5 (150 cycles)	20024904	130 million	~26
	High Output v2.5 (150 cycles)	20024907	400 million	~80
NextSeq 1000/2000	P2 (100 cycles) v3	20046811	400 million	~80
	P2 (200 cycles) v3	20046812		
NovaSeq 6000	SP v1.5 100-cycle	20028401	650 million	~130
NextSeq 2000	P3 100 cycles	20040559	1 billion	~220
	P3 200 cycles	20040560		

INDEXING PRIMER STRATEGY

- Each CUT&Tag library must be assigned a **unique pair of i5 & i7 primers**, included with each kit. Primers are used to add dual barcodes to libraries during PCR.
- Our i5 & i7 primer selection strategy is compatible with high-throughput Illumina sequencing systems (previous page).
- Index sequences are available in an easy-to-copy spreadsheet at epicypher.com/14-1102 under Documents and Resources.

To enable multiplexed sequencing on Illumina systems, this kit uses a combinatorial dual indexing primer strategy, meaning that each CUT&Tag library is prepared with a distinct combination of two 8 bp barcodes, or indexes – one at the 5' end (the i5 index), and the second at the 3' end (the i7 index; see Figure 19).

Dual i5 & i7 indexes, as well as the P5 & P7 sequences required by Illumina flow cells, are added to libraries during indexing PCR (**Protocol: Section VII**). Each kit comes with four i5 primers and twelve i7 primers, which can be used to generate 48 unique pairs of dual barcodes[8,9]. This approach allows up to 48 libraries to be multiplexed in a single sequencing run.

Note that the two versions of this kit contain different i5 primers: 14-1102 contains i501-i504, while EpiCypher 14-1103 comes with i505-i508. Combining the two kits increases the number of unique dual barcode pairs to 96, maximizing flexibility for large-scale projects.

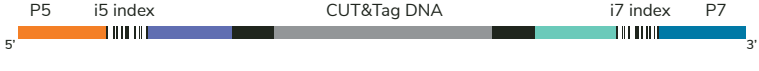


FIGURE 19

CUTANA CUT&Tag sequencing libraries are prepared using a combinatorial dual-indexing primer strategy. Each library is prepared using unique pairs of i5 & i7 primers to add indexes and P5/P7 sequences. See Figure 4, page 47, for a full workflow schematic.

PRIMER SELECTION

Figure 20 illustrates how primers should be organized to facilitate successful i5 & i7 primer pair selection and pipetting. Each i5 primer represents a row in Figure 20, while each i7 primer is in its own column.

Figure 21 provides an example of how i5 & i7 primer pairs can be selected across three different sequencing runs. Although the i5 primers shown are from 14-1102, the same principles apply for 14-1103.

Do **NOT** repeat pairs of i5 & i7 primers in a sequencing run. If an experiment will be combined with others on a single lane or flow cell, ensure that there is no overlap of primer pairs (i.e. identical dual indexes).

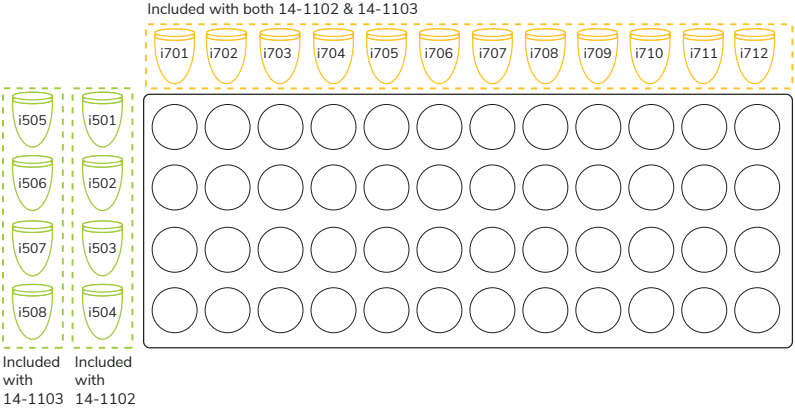


FIGURE 20

i5 & i7 primers are organized to guide primer pair selection and ensure successful multiplexed sequencing. Dashed orange lines indicate i7 primers (i701-i712), which are included with both versions of the CUTANA CUT&Tag Kit (14-1102 & 14-1103). Dashed green lines indicate i5 primers. The two versions of this kit come with distinct i5 primers, with 14-1102 containing i501-i504 and 14-1103 containing i505-i508.

SELECTION OF I5 & I7 PRIMER PAIRS FOR MULTIPLEXING (EXAMPLE)

- For Sequencing Run 1 in Figure 21 (dark blue), begin by pairing the first i5 primer (i501) with the first i7 primer (i701).
- Then work across the row from left to right, pairing the i501 primer with each i7 primer to generate 12 distinct dual barcodes.
- For the six additional libraries in Sequencing Run 1, use the next i5 primer (i502) and pair with i701. Move across the row until the desired number of dual indexes is achieved.
- In subsequent experiments, begin primer selection where the last run stopped. In Figure 21, Sequencing Run 1 ends with i502 & i706 (dark blue). When selecting primers for the next CUT&Tag experiment, begin with i502 & i707 (Sequencing Run 2; light blue).

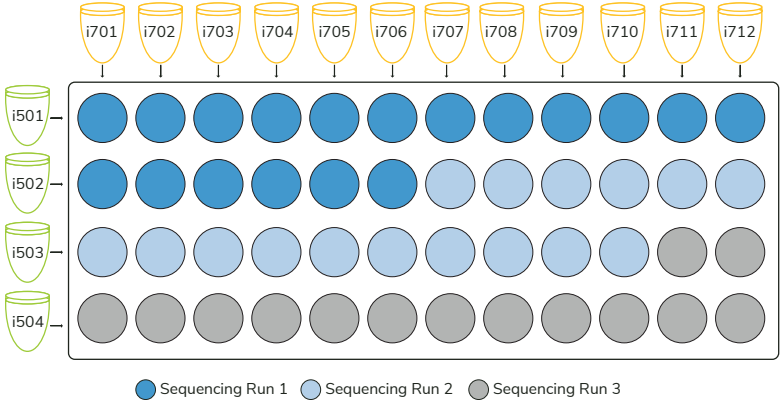


FIGURE 21

Three sequencing runs illustrate appropriate i5 & i7 primer pair selection for dual-indexing of CUT&Tag libraries. In all sequencing runs, each library is generated using a unique pair of i5 & i7 primers. Although primers for 14-1102 are shown, the same rules apply for i5 & i7 primers included with 14-1103.

Appendix 4: Frequently Asked Questions (FAQs)

1. **Can I use CUT&Tag to map chromatin-associated proteins?**
   CUT&Tag is **NOT** recommended for chromatin-associated proteins such as transcription factors or chromatin regulators. The protocol is most robust for histone PTMs and is only validated for select protein targets (e.g. CTCF). For protein targets, the CUTANA CUT&RUN Kit (epicypher.com/14-1048) is recommended, as it generates robust profiles for diverse targets and cell types.

2. **How do I validate an antibody for CUT&Tag?**
   See **Experimental Design & Optimization.** Histone PTM antibodies are susceptible to off-target binding, which can compromise biological interpretations[15]. To select and validate CUT&Tag histone PTM antibodies:

   1. Source 3-5 antibodies (preferably monoclonal) to your PTM. Purchase from various vendors and make sure the antibodies target distinct epitopes.
      - Many of our CUTANA CUT&RUN histone PTM antibodies work for CUT&Tag. Visit epicypher.com/cut-and-run-antibodies to view and email techsupport@epicypher.com to learn about their application in CUT&Tag.
   2. Test candidate antibodies in CUT&Tag using 100,000 native nuclei per reaction; test cross-linked conditions for labile targets (see **Appendix 2.3**). To monitor workflow success, always include reactions with H3K27me3 and IgG control antibodies and the K-MetStat Panel (**Appendix 1.4**).
      - For antibodies to lysine methylation PTMs, add the SNAP-CUTANA K-MetStat Panel (epicypher.com/19-1002) to CUT&Tag reactions for quantitative antibody validation. Learn more: epicypher.com/snap-spike-ins.
   3. Confirm that libraries from positive & negative control reactions show expected K-MetStat Panel data and genomic profiles (**Appendix 1.3 & 1.4**).
   4. Examine sequencing data and compare profiles from target antibodies. Select an antibody based on DNA yield, target enrichment, and signal-to-noise.
      - Antibodies tested using the K-MetStat Panel should show <20% off-target recovery and robust genomic enrichment consistent with target biology.

3. **What should I do if none of my antibodies work?**
   See troubleshooting guidelines in **Appendix 1.2**. Source additional antibodies to test, if desired. If CUT&Tag does not work for your target, consider using CUTANA CUT&RUN assays (epicypher.com/cutana-cut-and-run-kit), which are robust for most targets and can go from cells to sequencing DNA in 3 days.

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

5. **Why are nuclei preferred for CUTANA CUT&Tag assays?**
   Tn5 tagments mitochondrial DNA in the cytoplasm, which consumes significant sequencing bandwidth[14]. Using nuclei in CUT&Tag avoids this problem and allows reduced sequencing depths.

   Intact cells also require optimizing permeabilization and considerations for unique cell types. The use of nuclei bypasses these challenges.

6. **Why is Digitonin added to the buffers?**
   Digitonin prevents ConA beads from forming a thin film on the side of tubes, which causes beads to dry out and reduces yields. The use of Digitonin also makes our assay compatible with whole cells.

7. **My assay requires using 10,000 nuclei (or less). What are my options?**
   Follow the steps outlined in **Experimental Design & Optimization**, Figure 6. Include **ALL** the quality control steps detailed in Figure 5. **Note that using low numbers of nuclei may result in lost signal and increased background.** Briefly:

   1. Establish CUT&Tag workflows for your cell type using 100,000 native nuclei per reaction, H3K27me3 & IgG control antibodies, and the K-MetStat Panel.
   2. Once the assay is validated, identify a high quality antibody for your target using 100,000 nuclei. **Successful CUT&Tag using low nuclei numbers requires a highly specific and efficient antibody.**
   3. After these steps are completed, titrate nuclei and determine the acceptable input range for your cell type and target. Note that an antibody that works well at 100,000 nuclei may perform poorly at lower inputs.
   4. Yields may be low depending on antibody quality and target abundance. The H3K27me3 positive control may also have lower yields, and appear similar to IgG. Additional PCR cycles can be used to increase yields for sequencing.
   5. Deeper sequencing is recommended to fully capture read diversity. Rates of duplicate reads will likely increase, but good quality data can still be obtained (see **Appendix 1.3**).

8. Can I check if my CUT&Tag assay worked before indexing PCR?

The CUTANA CUT&Tag workflow goes from cells to DNA libraries in one tube. As a result, there is no opportunity to examine yields prior to indexing PCR.

However, if you confirm sample quality (**Appendix 1.1**), ensure ConA beads do not dry out, include positive & negative control reactions (**Appendix 1.4**), and use a validated CUT&Tag antibody, you will most likely achieve success.

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

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

- Align raw reads to a reference genome using Bowtie 2. Adapter trimming is not required for the recommended 2 x 50 bp paired-end sequencing. For longer reads (i.e. 2 x 150 bp) adapter sequences can be trimmed using CutAdapt and the local alignment option in Bowtie2.
- The Integrative Genomics Viewer (IGV) and/or deepTools[16] can be used to visualize enrichment (e.g. bigWig files graphed over a genome browser).
- For peak calling, EpiCypher frequently uses MACS2 and SICER, programs for ChIP-seq that work well for CUT&Tag[5,17-19]. SICER can be adjusted for analysis of sharp enrichment peaks (e.g. H3K4me3) vs. broad areas of enrichment (e.g. H3K27me3)[20]. SEACR, a peak caller designed for CUT&RUN data[21], is also frequently cited for CUT&Tag studies[5-7,11,22,23]. Another option is the CUT&RUNTools 2.0 pipeline, which is designed for CUT&RUN and CUT&Tag data, including analysis of single cells[22].
- To determine signal over background, EpiCypher uses bedTools to calculate fractions of reads in peaks (FRiP) and compare FRiP scores from experimental samples vs. controls[24]. Other tools can be applied for differential analysis and heatmap generation (e.g. DESeq2[25], deepTools[16]).

10. Can I use *E. coli* spike-in DNA for CUT&Tag sequencing normalization?

CUTANA pAG-Tn5 is highly purified and depleted of contaminating nucleic acids, so residual *E. coli* DNA cannot be used for sequencing normalization. CUT&Tag data normalization using exogenous *E. coli* spike-in DNA and/or the K-MetStat Panel is under development.

11. Can I use CUTANA pAG-Tn5 for ATAC-seq?

Although CUT&Tag and ATAC-seq use the same Tn5 transposase, EpiCypher's pAG-Tn5 requires anchoring to an antibody to tagment efficiently. **We have not tested pAG-Tn5 in ATAC-seq, and do not support its use for this application.**

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