EpiCypher® CUTANA™ Direct-to-PCR CUT&Tag Protocol

Optimized for Histone Post-Translational Modifications (PTMs)

This protocol has been validated for genomic profiling of:

- Histone PTMs (*e.g.* lysine methylation, acetylation)
- Low cell inputs: Starting with 100,000 cells - down to as low as 1,000 cells
- For profiling chromatin-associated proteins (*e.g.* transcription factors, epigenetic enzymes, etc.) or for applications that do not require ultra-low cell inputs, see EpiCypher CUTANA™ CUT&RUN assays.

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1. Overview

Cleavage Under Targets & Tagmentation (CUT&Tag) is an emerging genomic mapping strategy that builds on the revolutionary advances enabled by its predecessor immunotethering technology CUT&RUN[1]. In CUT&Tag, nuclei (recommended) or cells are immobilized to a solid support. A fusion of proteins A and G with prokaryotic transposase 5 (pAG-Tn5) is used to selectively cleave and tagment antibody-bound chromatin (**Figure 1**). The tagmentation reaction appends sequencing adapters to the antibody-bound DNA, which can be directly PCR amplified *in situ* to yield sequence-ready DNA[2]. High-resolution profiles of histone PTM enrichment can then be generated using as few as 3-8 million sequencing reads.

CUT&RUN, the sister technology of CUT&Tag, enables the use of low cell inputs (500,000 down to 5,000 cells) for mapping genome occupancy of histone PTMs and chromatin-associated proteins (*e.g.* transcription factors). While CUT&RUN is recommended for most applications, CUT&Tag can be used for specific projects that require histone PTM mapping using ultra-low inputs (100,000 down to 1,000 cells). The entire CUT&Tag workflow can be conducted in a single tube using multichannel pipettes, enabling high experimental throughput.

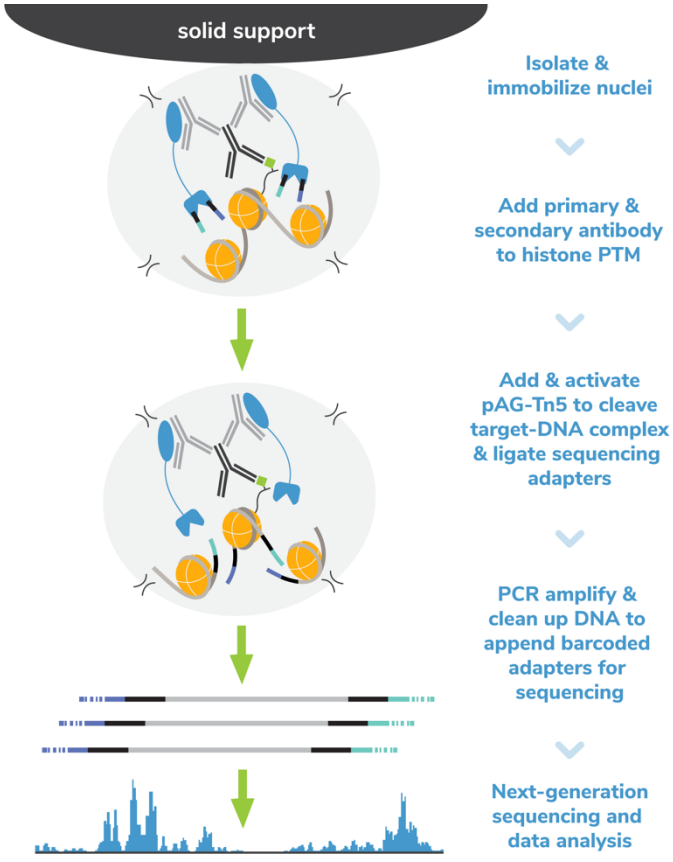

Figure 1. Overview of the CUTANA™ Direct-to-PCR CUT&Tag protocol.

2. CUTANA™ Products & Services: Advantages

CUT&RUN and CUT&Tag have revolutionized the study of chromatin regulation. Compared to ChIP-seq, the historically leading approach for genome-wide mapping of histone PTMs and chromatin-associated proteins, CUT&RUN/CUT&Tag offer the following advantages:

Platform Comparison	ChIP-seq	CUTANA™ CUT&RUN	CUTANA™ CUT&Tag
Required cells	> 1 million	5,000 – 500,000	1,000 – 100,000
Cell Input	Fragmented chromatin	Intact cells or nuclei	Nuclei (preferred) or intact cells
Compatible Targets	Histone PTMs & chromatin-associated proteins	Histone PTMs & chromatin-associated proteins, including difficult ChIP targets	Histone PTMs only
Sequencing Depth (Reads)	> 30 million	3-8 million	3-8 million
Experimental throughput	Low	High	High
Signal : Noise	Low	High	High
Assay Automation	Difficult	Yes	Yes

Compared to CUT&RUN, CUT&Tag is uniquely optimized for histone PTM profiling in cutting-edge research applications, including ultra-low cell inputs. However, for the vast majority of applications and targets, CUT&RUN assays are recommended as the go-to genomic mapping approach. EpiCypher now offers a suite of products to support CUT&RUN and CUT&Tag workflows under our CUTANA™ assay platform, including:

- **CUTANA pAG-Tn5** (EpiCypher 15-1017), the essential reagent for CUT&Tag workflows. EpiCypher pAG-Tn5 contains an optimized fusion of Proteins A and G with hyperactive transposase 5 (pAG-Tn5) for compatibility with a broad range of antibody isotypes (*e.g.* mouse, rabbit). Available in two pack sizes (50 and 250 reactions).
- **SNAP-CUTANA™ Spike-in Controls** are proprietary DNA-barcoded recombinant nucleosome spike-in panels that control for all aspects of CUT&Tag workflows, including antibody specificity and technical variability, guide troubleshooting experiments, and enable quantitative normalization (see **Appendix III**). Now available for histone lysine methylation PTMs (SNAP-CUTANA K-MetStat Panel, EpiCypher 19-1002).
- **CUT&Tag supporting reagents**, selected and validated for optimal performance in the EpiCypher CUT&Tag protocol. See epicypher.com/cut-and-run-assays for more info.
- **CUT&RUN applications:** For more information, see our CUT&RUN Kit (EpiCypher 14-1018) which includes all reagents needed to go from cells to purified CUT&RUN DNA.

3. Outline of CUT&Tag Workflow

Description: EpiCypher's in-house optimized protocol for CUTANA™ CUT&Tag assays (**Figure 2**). Before starting, we strongly recommend reading this section, the **Experimental Design & Key Protocol Notes** section, and the **Protocol** to carefully plan your experiment and familiarize yourself with the assay.

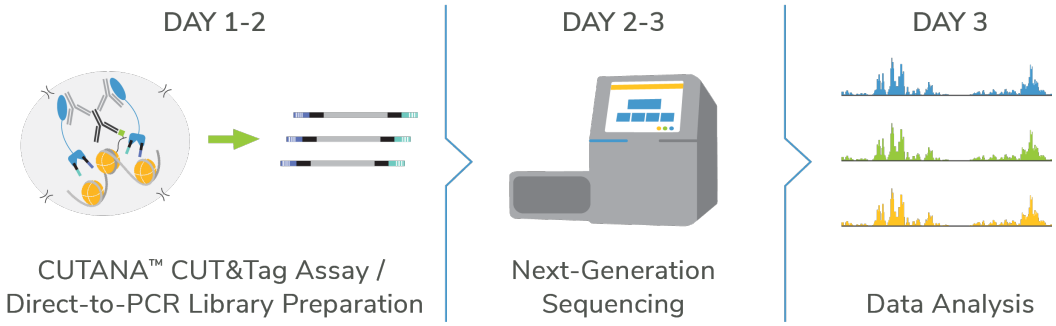

Figure 2: Timeline of CUT&Tag assay using EpiCypher's CUTANA™ Direct-to-PCR CUT&Tag protocol.

Section I: ConA Bead Activation (~30 min)

Description: During this section Concanavalin A coated magnetic beads (**Con A beads**) are "activated" to bind and immobilize nuclei (or cell) samples. If preparing for multiple CUT&Tag reactions, it is recommended to batch process the full volume of beads needed for all reactions in a single 1.5 mL tube. This helps ensure homogeneity across reactions. For these steps, beads can be washed using a 1.5 mL magnetic rack (*e.g.* EpiCypher 10-0012). At the conclusion of this section, activated ConA beads are aliquoted into 8-strip PCR tubes for CUT&Tag reactions.

Section II: Nuclei Preparation and Binding to Activated Beads (~30 min)

Description: Nuclei are prepared from bulk cell populations and immobilized to activated ConA beads. Importantly, this section was developed using 100,000 unfixed (*i.e.* native) K562 cell nuclei per CUT&Tag reaction, and is specifically designed for batch processing of cell samples for multiple CUT&Tag reactions (see **Protocol Notes**). Isolated nuclei are aliquoted to 8-strip PCR tubes containing activated ConA beads. Following bead binding, it is recommended to use multi-channel pipettes and a compatible magnetic rack (EpiCypher 10-0008; **Figure 3**), which helps increase experimental throughput and reproducibility.

As discussed in the **Experimental Design & Key Protocol Notes** and **FAQs** sections, EpiCypher recommends using nuclei vs. intact cells for CUT&Tag, to avoid mitochondrial DNA contamination in sequencing data. In addition, the use of cells requires optimizing digitonin for permeabilization, and comes with other considerations for unique cell types (*e.g.* adherent, immune cells etc. see **FAQs**). The use of nuclei bypasses many of these challenges, allowing for a streamlined workflow. However, for scientists needing to use cells, we do offer guidelines in the **FAQs** for a variety of inputs, including cells, tissues, and cross-linked samples. We also provide a protocol for preparing and using cryopreserved nuclei in CUT&Tag (**Appendix I**).

Importantly, EpiCypher also strongly recommends performing the Quality Control Checks outlined in **Appendix II**, which include confirmation of (1) starting cell sample viability, (2) integrity of isolated nuclei, and (3) success of ConA bead + nuclei conjugation. These are important controls for every experiment because they report the quality of CUT&Tag inputs (*i.e.* bead-coupled nuclei), directly impacting assay yields. For new users or for researchers attempting novel cell types or processing conditions, these QC checks should be considered essential parts of the protocol.

Section III: Binding of Primary and Secondary Antibodies (~30 min + overnight + 1 hr)

Description: After coupling to activated ConA beads, the nuclei – bead mixture is resuspended in cold **Antibody150 Buffer**, and a target-specific histone PTM antibody is added for overnight incubation. Note that antibody selection in CUT&Tag is critical to success; see **FAQs** for more information. After overnight incubation of the primary antibody, a secondary antibody targeted to the species isotype of the primary antibody (*e.g.* anti-rabbit, anti-mouse) is added to aid in more efficient tagmentation at the antibody-bound loci.

Antibody150 Buffer is the first buffer in the protocol that contains digitonin (see **Buffer Recipes**), which permeabilizes cells (if used as opposed to nuclei, though not recommended) and allows the antibody to bind its histone PTM target *in situ*. When cells are used as sample input, digitonin concentration must be optimized for every unique input (*e.g.* cell type, fixation) as described in the **FAQs**. It is crucial to use the minimum amount of digitonin needed for efficient permeabilization to avoid cell lysis and digitonin precipitation during overnight incubations. While nuclei are inherently permeable and do not require digitonin permeabilization for antibody and pAG-Tn5 binding, the inclusion of digitonin minimizes bead clumps and precipitation, thereby improving data quality.

A second feature of this section is the addition of the **SNAP-CUTANA™ K-MetStat Panel** (EpiCypher 19-1002) to CUT&Tag reactions that use a methyl-lysine antibody (*e.g.* H3K4me3) or IgG control. This spike-in panel comprises highly pure, DNA-barcoded semi-synthetic/recombinant nucleosomes carrying defined lysine methylation PTMs, and is useful for in-assay antibody validation, quantitative normalization, and experimental troubleshooting. We outline its appropriate use in the Protocol **Section III** (just before antibody addition; also see **Table 5**). **Appendix III** includes detailed information about SNAP-CUTANA Spike-ins and how they can be incorporated as critical controls to master CUT&Tag.

As a final step before the addition of pAG-Tn5 in the next section, the reactions are washed under high stringency conditions using the **Digitonin300 Buffer**. The inclusion of high salt (300 mM NaCl) is essential to minimize non-specific binding of pAG-Tn5 to accessible DNA. The requirement for high salt washing is also the reason why CUT&Tag is generally not

recommended for profiling chromatin-associated proteins, as these transient binding interactions can be destabilized by the stringent wash conditions.

Section IV: Binding of pAG-Tn5 (~1 hr)

Description: At this stage of the protocol, **pAG-Tn5** pre-loaded with Illumina sequence adapters (EpiCypher 15-1017) is added to each reaction and incubated briefly at room temperature to allow binding to antibody-labelled chromatin. This incubation is performed in the **Digitonin300 Buffer**, which is free of $MgCl_2$ to avoid premature activation of Tn5. ConA bead – nuclei suspensions often become "clumpy" at this point and are difficult to pipette. In this case, we recommend using a P200 pipette to gently disperse beads in buffer. Cut-off pipette tips can also be used if beads are clogging pipette tips or if nuclei or cells are easily damaged.

Section V: Targeted Chromatin Tagmentation (~3 hrs)

Description: During this part of the experiment, Tn5 is activated by addition of $MgCl_2$ to simultaneously cleave proximal antibody-bound DNA and append library sequencing adapters ("tagmentation"). Unlike in CUT&RUN where MNase-cleaved fragments diffuse into solution, pAG-Tn5 remains bound to target chromatin, retaining tagmented fragments in the nucleus. The samples are washed using **TAPS Buffer** and **SDS Release Buffer** is added to quench the tagmentation reaction. As the Release Buffer contains SDS, nuclei can become lysed at this step, causing the solution to become viscous or sticky. It is important not to pipette the reactions at this stage, which may cause sample loss. Instead, vortex to mix and quick-spin to collect the reaction at the bottom of the tube. The reactions can then be heated to release tagmented chromatin fragments into solution. Finally, **SDS Quench Buffer** is added to neutralize SDS, which potently inhibits PCR.

Section VI: Non-hot Start PCR and Library Cleanup (~1 hr)

Description: Here, we complete library amplification for CUT&Tag reactions *in situ* using the ligated sequencing adapters as PCR templates. This strategy minimizes sample loss and streamlines the workflow, resulting in greater assay sensitivity and throughput. Library amplification is performed directly on the entire reaction mixture by adding i5 and i7 sequencing primers as well as non-hot start **CUTANA™ High Fidelity 2x PCR Master Mix**. Note that these PCR steps are designed to amplify CUT&Tag DNA fragments compatible with Illumina paired-end sequencing (100 bp – 700 bp). Thus, contaminating high-molecular weight DNA fragments are not enriched by these PCR parameters. After PCR, final sequence library DNA is purified using **AMPure beads** and quantified using the **Qubit™ fluorometer**.

There is no "typical yield" for a CUT&Tag assay, as the reaction varies widely by cell type, target abundance, number of cells, fixation or treatment conditions, and so forth. It is far more useful to consider the minimal post-PCR DNA yields necessary to accurately quantify sequencing libraries using the Qubit fluorometer and Bioanalyzer/TapeStation. In EpiCypher's experience, the minimal amount of DNA needed for accurate library quantification is >2ng/ul in 15 µL (or 30

ng total DNA). EpiCypher includes positive (H3K4me3 and/or H3K27me3) and negative (IgG) control antibodies in all CUT&Tag experiments (see **FAQs** and **Experimental Design**), and compares yields as part of our quality control analysis. However, there is no set ratio for differential recovery and it can vary by cell type, target, and input amount. In general, yields for an abundant target (*i.e.* H3K27me3) should always be higher than IgG.

IMPORTANT: Following tagmentation, many researchers are tempted to purify CUT&Tag DNA to assess fragment size distribution and/or analyze enrichment of known targets by qPCR before PCR amplification. We do not recommend either of these analyses, which are derived from ChIP-seq and are not robust indicators of CUT&Tag success (see **FAQs** for a detailed explanation). Purification of bulk chromatin at this stage is not useful, because the isolated DNA contains both tagmented and non-tagmented DNA, *i.e.* there is no method to selectively purify tagmented DNA from nuclei. PCR is required to determine assay success, even immediately following tagmentation. Our method provides the same information about assay success while bypassing an unnecessary DNA purification step, saving time, resources, and mitigating sample loss.

Section VII: Agilent 2100 Bioanalyzer System (~1 hr)

Description: Prior to sequencing, CUT&Tag libraries should be examined using the Agilent Bioanalyzer, TapeStation, or equivalent approach to assess fragment size, library concentration, and DNA quality. Predominant enrichment of mononucleosome-sized fragments (~300 bp, including universal adapters) is the best indicator of CUT&Tag experimental success prior to NGS (*e.g.* see **Figure 5**; also see **FAQs** for expanded discussion on quality control checks for CUT&Tag). Here we describe the use of Bioanalyzer and Agilent High Sensitivity DNA Chip for CUT&Tag library analysis along with expected results.

Following confirmation of CUT&Tag library fragment distribution and quality, proceed to Illumina next-generation sequencing (NGS) and analysis*. **Importantly,** CUT&Tag only requires 3-8 million reads per sample to generate high signal-to-noise data (vs. 30 million or more with ChIP-seq). This allows researchers to pool >48 samples per sequencing run, if using the Illumina NextSeq, or use a benchtop sequencer (*e.g.* Illumina MiniSeq) for smaller projects.

*For guidance on SNAP-CUTANA Spike-in analysis from NGS datasets, see **Appendix III**.

4. Experimental Design & Key Protocol Notes

Description: This section is considered essential reading for CUTANA™ Direct-to-PCR CUT&Tag assays. For CUT&Tag workflows to be successful you must include proper controls and optimize key steps for your unique cell input (*e.g.* number of cells, antibody) as detailed in this section. We also offer tips on common problems with the protocol and explain our rationale for using 8-strip PCR tubes in the CUTANA CUT&Tag protocol.

1. This version of the Direct-to-PCR CUT&Tag protocol is validated for histone PTMs. For genomic mapping of chromatin-associated proteins (*e.g.* transcription factors, epigenetic writers and reader proteins, etc.), CUT&RUN is recommended.
2. We strongly recommend using bead-immobilized nuclei for CUT&Tag assays. This strategy circumvents undesirable tagmentation and sequencing of mitochondrial DNA, which can contaminate results and may require deeper sequencing per reaction. However, cells are compatible with our CUT&Tag protocol, since digitonin remains in the buffers for input flexibility and to control bead behavior.
    - For more information on sample inputs, see **FAQs** section. See **Appendix II** for key Quality Control Checks, including evaluating integrity of starting cells, isolated nuclei and binding to Concanavalin A (ConA) beads.
    - When using cells in CUT&Tag rather than nuclei, it is imperative to optimize digitonin concentration for efficient permeabilization. See **FAQs** for more information.
3. Include controls in every experiment. We suggest the following controls (at minimum):
    - Negative control antibody (*e.g.* IgG antibody: EpiCypher 13-0042).
    - Positive control antibody (*e.g.* H3K4me3: EpiCypher 13-0041).
    - SNAP-CUTANA™ Spike-in Controls (EpiCypher 19-1002; see **Appendix III**). They should be included with H3K4me3 and IgG control antibodies in every experiment, as well as any reactions targeting histone methyl-lysine PTMs.

    These controls are especially critical when optimizing CUT&Tag for new experimental conditions (*e.g.* new cell types, reduced inputs, drug treatments, fixation method), but should also be included as standard controls for continuous monitoring of assay success.
4. To optimize for reduced cell inputs (less than 100,000 per reaction):
    - Start by optimizing the protocol using 100,000 native (*i.e.* unfixed) nuclei per reaction and control antibodies (*e.g.* H3K4me3 and IgG as noted above).
    - Then optimize for any cell treatments (*e.g.* fixation, etc.) and for your target of interest. Continue to include reactions using control antibodies to monitor assay success.

- Once conditions are optimized for the target and cell type of interest, scale down to desired number of cells.
- Without any further modifications, **this protocol has been validated on as few as 1,000 cells** using antibodies to H3K4me3 and H3K27me3.
- For experimental applications that do not require ultra-low cell inputs, CUT&RUN is recommended (CUTANA™ CUT&RUN assays optimized for 5,000 – 500,000 cells; see epicypher.com/CUT&RUN).

5. Protocol has been adapted to 8-strip PCR tubes (vs. 1.5 mL tubes) for rapid "batch processing" of multiple CUT&Tag reactions from bulk cell samples. The first few steps, *i.e.* nuclei harvest and ConA bead activation, are performed in 1.5 mL tubes, and then the reactions are split into 8-strip PCR tubes for the remainder of the assay. These steps:
   - Minimize beads sticking to tubes
   - Enable more rapid workflow with multi-channel pipettes
   - Provide more consistent sample handling
   - Allow high-throughput sample preparation
6. ConA beads dry out easily, which can result in sample loss. To avoid this problem in CUT&Tag, take caution to prevent ConA beads sticking to the sides or caps of tubes.
   - To avoid ConA beads sticking to tube sides/caps and drying out, it is essential to use a **nutator** rather than a **rotator** (see **Table 4**), since nutators gently agitate by shaking or rocking tubes rather than rotating end-over-end.
   - Take note of steps that indicate when to pipette or vortex to disperse clumps and keep ConA beads in an even suspension.
7. We recommend a 5% digitonin stock solution in DMSO (as opposed to heated H_2O), as this improves detergent solubility and protocol reproducibility.
8. Direct-to-PCR CUT&Tag yields tend to vary by target and cellular input amount. Rather than suggesting an arbitrary number of PCR cycles, we recommend determining the minimum number of PCR cycles that generate enough material for accurate quantification (*i.e.* by Qubit, Bioanalyzer, and/or Tapestation), typically between 14-21 cycles. If a sample is overamplified, PCR duplicates can be removed using Picard (https://broadinstitute.github.io/picard/). Aim to meet or exceed the minimal DNA amount necessary for accurate CUT&Tag sequencing library quantification (>2ng/ul in 15 µL or 30 ng total DNA).
9. **IMPORTANT**: **It is not recommended to purify CUT&Tag DNA prior to PCR.** It is tempting to purify DNA after tagmentation to check size distribution by Bioanalyzer or TapeStation, as in ChIP-seq. However, purifying bulk chromatin at this stage is not useful,

and will still require PCR to enrich tagmented DNA and determine assay success (see **FAQs** for expanded discussion). Our method bypasses this DNA purification step, streamlining the workflow and reducing sample loss for improved sensitivity. **The single best indicator of CUT&Tag success prior to sequencing is enrichment of ~300 bp fragments post-PCR**, as assessed by the Agilent Bioanalyzer or TapeStation (**Figure 5**). It is also useful to assess DNA yields compared to positive (*e.g.* H3K4me3) and negative (IgG) controls. In general, yields for an abundant target (*i.e.* H3K27me3) should always be higher than IgG.

5. Buffers, Reagnts & Materials Needed

Table 1: Buffer components

Components	Source	Cat #
HEPES	Sigma-Aldrich	H3375
KCl	Sigma-Aldrich	P3911
$CaCl_2$	Sigma-Aldrich	C1016
$MnCl_2$	Sigma-Aldrich	203734
Molecular biology grade H_2O (RNase, DNase free)	VWR	VWRV02-0201-0500
NaCl	Sigma-Aldrich	S5150-1L
EDTA (prepare 0.5 M stock at pH 8.0)	Sigma-Aldrich	E5134
Spermidine trihydrochloride*	Sigma-Aldrich	S2501
Digitonin	Millipore Sigma	300410
DMSO	Sigma	D8418-100ml
Trypan Blue	Thermo Fisher Scientific	T10282
cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail	Roche	11836170001
Triton X-100	Sigma-Aldrich	X100
Glycerol	Millipore Sigma	G5516
1 M TAPS, pH 8.5	Boston Bioproducts	BB-2375
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	L4509

*1M spermidine preparation: Dissolve 1 gram spermidine (MW = 254.63) in 3.93 mL molecular grade H_2O. Store in single-use aliquots at -20°C for 6 months.

Buffer recipes

Nuclear Extraction (NE) Buffer (200 µL/reaction)
20 mM HEPES–KOH, pH 7.9
10 mM KCl
0.1% Triton X-100
20% Glycerol
0.5 mM Spermidine*
1x Roche cOmplete™, Mini, EDTA-free Protease Inhibitor (CPI-mini, 1tab/10mL)
After spermidine and CPI are added, store at 4ºC for up to 1 week.
NE buffer without spermidine and CPI is stable at 4ºC for up to 6 months.

Bead Activation Buffer (211 µL/sample)
20 mM HEPES, pH 7.9
10 mM KCl
1 mM $CaCl_2$
1 mM $MnCl_2$
Filter sterilize. Store at 4ºC for up to 6 months.

Wash150 Buffer (use to prepare Digitonin150 Buffer)
20 mM HEPES, pH 7.5
150 mM NaCl
0.5 mM Spermidine*
1x Roche cOmplete™, Mini, EDTA-free Protease Inhibitor (CPI-mini, 1tab/10mL)
Filter sterilize. Store at 4ºC for up to 1 week.

Digitonin150 Buffer (450 µL/sample)
Wash150 Buffer + 0.01% Digitonin**
Prepare fresh each day and store at 4ºC.

Antibody150 Buffer (50 µL/sample)
Digitonin150 Buffer** + 2 mM EDTA
Prepare fresh each day and store at 4ºC.

Wash300 Buffer (use to prepare Digitonin300 and Tagmentation Buffers)
20 mM HEPES, pH 7.5
300 mM NaCl
0.5 mM Spermidine*
1x Roche cOmplete™, Mini, EDTA-free Protease Inhibitor (CPI-mini, 1tab/10mL)
Filter sterilize. Store at 4ºC for up to 1 week.

Digitonin300 Buffer (450 µL/sample)
Wash300 Buffer + 0.01% Digitonin**
Prepare fresh each day and store at 4ºC.

Tagmentation Buffer (50 µL/sample)
Digitonin300 Buffer** + 10 mM $MgCl_2$
Store at 4ºC for up to 1 week.

TAPS Buffer (50 µL/sample)
10 mM TAPS, pH 8.5
0.2 mM EDTA
Store at Room Temperature (RT) for up to 6 months.

SDS Release Buffer (5 µL/sample)
10 mM TAPS, pH 8.5
0.1% SDS
Store at RT for up to 6 months.

SDS Quench Buffer (15 µL/sample)
0.67% Triton-X 100 in Molecular grade H_2O
Store at RT for up to 6 months.

Buffer Preparation Notes

*Spermidine is added to compensate for the removal of Mg^{2+} from the buffer. Mg^{2+} can cause DNA degradation and is typically omitted from CUT&Tag and CUT&RUN buffers.

**Digitonin 5% stock solution should be prepared in DMSO. Aliquots can be stored at -20ºC for 6 months. Note that digitonin is not necessary for nuclei permeabilization in the CUT&Tag workflow, as purified nuclei are inherently permeable to antibody and pAG-Tn5. Rather, digitonin helps prevent the nuclei-conjugated beads from precipitating/clumping and forming a thin film on tubes. Digitonin also makes the protocol compatible with cells, although the use of cells is not recommended.

Table 2: Reagents

Item	Vendor	Catalog No.	Notes
Concanavalin A (ConA) Conjugated Paramagnetic Beads	EpiCypher	21-1401	ConA is a lectin, which can cause immune activation. For technical support re. immune cell studies, contact info@epicypher.com.
CUTANA™ pAG-Tn5	EpiCypher	15-1017 and 15-1117	50 & 250 reaction pack sizes available. Supplied as 20X stock.
SNAP-CUTANA™ K-MetStat Panel	EpiCypher	19-1002	**Spike-in controls for Sample Normalization & Antibody Profiling (SNAP Spike-in Controls)**: The K-MetStat panel comprises fifteen semi-synthetic/recombinant nucleosomes carrying unique methyl-lysine modifications (me1/2/3 at H3K4, H3K9, H3K27, H3K36, and H4K20), plus an unmodified control, immobilized to magnetic beads. Each histone octamer is wrapped with two different barcoded DNA templates, providing an internal technical replicate for each histone PTM. Add spike-ins to CUT&Tag reactions targeting one of the PTMs in the panel as well as in CUT&Tag reactions designated for H3K4me3 positive and IgG negative control antibodies. For more information about using SNAP-CUTANA Spike-ins, see **Appendix III**.
Rabbit IgG Negative Control Antibody	EpiCypher	13-0042	Use 0.5 µg in CUT&Tag.
SNAP-ChIP® Certified, CUTANA Compatible H3K4me3 Positive Control Antibody	EpiCypher	13-0041	Use 0.5 µg in CUT&Tag.
Antibody to histone PTM	User-dependent		EpiCypher continues to conduct extensive antibody characterization (most particularly for those against histone PTMs[2]). Contact us for recommendations: info@epicypher.com.
Anti-Mouse Secondary Antibody	EpiCypher	13-0048	A secondary antibody is required for CUT&Tag. Use with primary antibodies made in mouse.
Anti-Rabbit Secondary Antibody	EpiCypher	13-0047	A secondary antibody is required for CUT&Tag. Use with primary antibodies made in rabbit.
Agencourt AMPure XP magnetic beads	Beckman Coulter	A63880	For PCR cleanup and size selection (*i.e.* adapter dimer and primer removal)
Qubit™ 1x dsDNA HS Assay Kit	Thermo Fisher Scientific	Q33230	For DNA quantification
CUTANA High-Fidelity 2X PCR Master Mix™	EpiCypher	15-1018	Must be the non-hot start version of Q5 for CUT&Tag
Universal i5 Primer	IDT	NA	Primer sequence provided below.
Uniquely Barcoded i7 Primers	IDT	NA	Primer sequences provided below.

Library Preparation Primers

Universal i5 primer

5' AATGATACGGCGACCACCGAGATCTACACTAGATCGCTCGTCGGCAGCGTCAGATGTGTAT 3'
Prepare at 10 µM in Molecular Biology grade H_2O (RNase, DNase free)

Uniquely barcoded i7 primers

Table 3: i7 Barcoded primer sequences

Name	Sequence (5' → 3')	Oligo Barcode	Expected NGS Read*
i7_1	CAAGCAGAAGACGGCATACGAGAT**TCGCCTTA**GTCTCGTGGGCTCGGAGATGTG	TCGCCTTA	TAAGGCGA
i7_2	CAAGCAGAAGACGGCATACGAGAT**CTAGTACG**GTCTCGTGGGCTCGGAGATGTG	CTAGTACG	CGTACTAG
i7_3	CAAGCAGAAGACGGCATACGAGAT**TTCTGCCT**GTCTCGTGGGCTCGGAGATGTG	TTCTGCCT	AGGCAGAA
i7_4	CAAGCAGAAGACGGCATACGAGAT**GCTCAGGA**GTCTCGTGGGCTCGGAGATGTG	GCTCAGGA	TCCTGAGC
i7_5	CAAGCAGAAGACGGCATACGAGAT**AGGAGTCC**GTCTCGTGGGCTCGGAGATGTG	AGGAGTCC	GGACTCCT
i7_6	CAAGCAGAAGACGGCATACGAGAT**CATGCCTA**GTCTCGTGGGCTCGGAGATGTG	CATGCCTA	TAGGCATG
i7_7	CAAGCAGAAGACGGCATACGAGAT**GTAGAGAG**GTCTCGTGGGCTCGGAGATGTG	GTAGAGAG	CTCTCTAC
i7_8	CAAGCAGAAGACGGCATACGAGAT**CCTCTCTG**GTCTCGTGGGCTCGGAGATGTG	CCTCTCTG	CAGAGAGG
i7_9	CAAGCAGAAGACGGCATACGAGAT**AGCGTAGC**GTCTCGTGGGCTCGGAGATGTG	AGCGTAGC	GCTACGCT
i7_10	CAAGCAGAAGACGGCATACGAGAT**CAGCCTCG**GTCTCGTGGGCTCGGAGATGTG	CAGCCTCG	CGAGGCTG
i7_11	CAAGCAGAAGACGGCATACGAGAT**TGCCTCTT**GTCTCGTGGGCTCGGAGATGTG	TGCCTCTT	AAGAGGCA
i7_12	CAAGCAGAAGACGGCATACGAGAT**TCCTCTAC**GTCTCGTGGGCTCGGAGATGTG	TCCTCTAC	GTAGAGGA

Prepare at 10 µM in Molecular Biology grade H_2O (RNase, DNase free)

*Note that expected barcode reads are reverse complement of 5' → 3' sequence.

Table 4: Equipment

Item	Vendor	Catalog No.	Notes
1.5 mL Magnetic Separation Rack	EpiCypher	10-0012	For bulk or "batch" processing of ConA beads in **Section I** of CUT&Tag protocol; see **Figure 3A.**
8-strip PCR tube Magnetic Separation Rack	EpiCypher	10-0008	For processing of individual CUT&Tag reactions in **Section III** onward; see **Figure 3B**. Enables streamlined sample handling for higher experimental throughput and improved reproducibility.
8-strip 0.2 mL PCR tubes	EpiCypher	10-0009	Compatible with 8-strip magnetic stand.
Qubit™ 4 Fluorometer	Thermo Fisher Scientific	Q33226	For DNA quantification.
Agilent 2100 Bioanalyzer	Agilent	G2939A	For analysis of purified CUT&Tag sequencing libraries. May substitute comparable capillary electrophoresis instrument (*e.g.* Agilent TapeStation).
High Performance Multi-Channel Pipettors, 8-Channel	VWR	76169-252	For performing CUT&Tag in 8-strip PCR tubes *e.g.* for aspiration and wash steps. May substitute comparable multi-channel pipettor.
Tube Nutator	VWR	82007-202	For bead incubation steps (overnight antibody incubation, pAG-Tn5 digest reaction).
Vortex-Genie	Scientific Industries	SI-0236	For bead mixing steps.

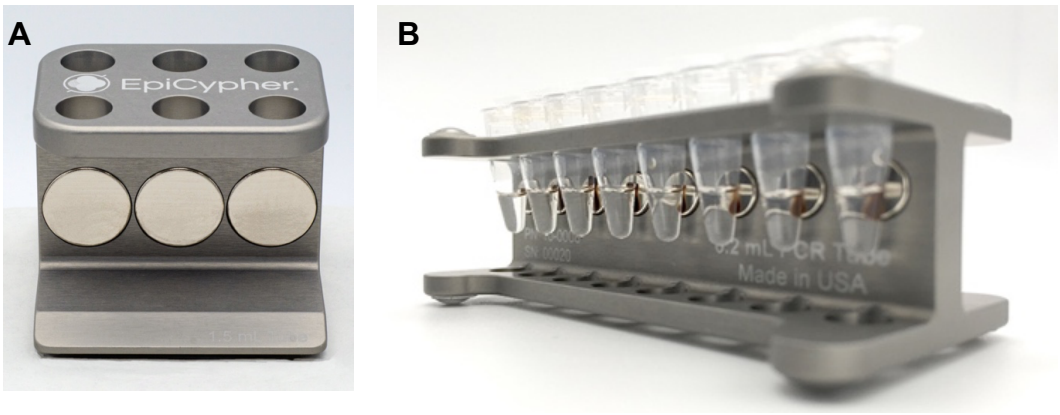

Figure 3: Magnetic racks for CUT&Tag assays. (A) For batch processing of ConA beads, use a 1.5 mL magnetic rack (*e.g.* EpiCypher 10-0012, pictured). **(B)** For processing samples in 8-strip PCR tubes we recommend using a multi-channel pipette and compatible magnetic rack (*e.g.* EpiCypher 10-0008, pictured).

6. EpiCypher CUTANA Direct-to-PCR CUT&Tag Protocol

Essential Reading: Before starting, we strongly recommend reading the **Outline of CUT&Tag Workflow** and the **Experimental Design & Key Protocol Notes** for important information about assay controls and optimization. The **FAQ section** also provides guidance on adapting the protocol for unique inputs, targets, and other cell preparation methods.

6.1. CUT&Tag Protocol (~5hrs)

---Day 1---

Section I: ConA Bead Activation (~30 min)

1. Gently resuspend the **ConA beads** and transfer 11 µL per planned CUT&Tag reaction to a 1.5 mL tube for batch processing.
1. Place the tube on a 1.5 mL magnetic separation rack until slurry clears and pipette to remove supernatant.
2. Immediately add 100 µL/reaction cold **Bead Activation Buffer**, remove from magnet, and pipette to mix. Place back on magnet until slurry clears and pipette to remove supernatant.
3. Repeat the previous step for total of two washes.
4. Resuspend beads in 11 µL/reaction cold **Bead Activation Buffer**.
5. Aliquot 10 µL of activated **ConA beads** into 8-strip PCR tubes for individual CUT&Tag reactions (*i.e.* 10 µL activated beads per reaction). Keep on ice until needed.

Section II: Nuclei Preparation and Binding Nuclei to Activated Beads (~30 min)

6. Harvest 100,000 cells per planned CUT&Tag reaction. Spin cells for 3 min at 600 x g at room temperature (**RT**) in a 1.5 mL tube. Pipette or aspirate to discard supernatant.

   Notes and alternative cell preparation protocols:
   - We strongly recommend using CUT&Tag with nuclei, as opposed to cells (see the **Workflow Outline**, **Experimental Design & Key Protocol Notes** and **FAQs**).
   - It is recommended to prepare ~10% excess cells to confirm (1) cell integrity prior to nuclei isolation, (2) nuclei integrity at the end of harvest, and (3) nuclei binding to ConA beads. These Quality Control (**QC**) Checks are described in **Appendix II**.
   - If using preparing or using frozen nuclei/cells, see **Appendix I**.
   - See **FAQs "Sample Input Compatibility"** section for special considerations when using adherent, cryopreserved and cross-linked cells, immune cells, and tissue.

7. Resuspend cells in 100 µL/reaction **RT 1x PBS**. Scale volumes based on cell number as needed (*e.g.* 1 mL buffer for 5 million cells).
   - Note: For QC Checks, set 10 µL aside to confirm cell integrity (**Appendix II**).

8. Spin for 3 min at 600 x g at RT. Decant or pipette to remove and discard supernatant.
9. Resuspend cell pellet in 100 µL/reaction cold **NE Buffer** and **incubate for 10 min on ice**.
10. Spin for 3 min at 600 x g at **4°C.** Pipette or aspirate to discard supernatant.
    - Note: The pellet should change in appearance from a sticky, pale yellow pellet (cells) to a white, fluffy pellet (nuclei).
11. Resuspend nuclei in 100 µL/reaction cold **NE Buffer**.
    - Note: For QC Checks set 10 µL aside to confirm isolated nuclei integrity (**Appendix II**).
12. Aliquot 100 µL nuclei into 8-strip PCR tubes containing 10 µL of activated beads. Gently vortex (setting #7) to mix.
13. **Incubate** nuclei – bead slurry for **10 min at RT**. Nuclei will adsorb to the activated ConA beads.
    - Note 1: If performing QC Checks see **Appendix II** for guidance.
    - Note 2: ConA beads remain in reaction tube throughout protocol, including PCR and Post-PCR DNA Cleanup steps.

Section III: Binding of Primary and Secondary Antibodies (~30 min + overnight + 1 hr)

14. Place the tubes on a 8-strip PCR tube magnet until slurry clears (30 s – 2 min). Pipette to remove and discard supernatant.
15. Add 50 µL cold **Antibody150 Buffer** per reaction quickly, to avoid bead drying. Remove from magnet, and thoroughly pipette to resuspend.
16. For reactions designated to positive (H3K4me3) and negative (IgG) control antibodies, as well as samples assigned a target in the K-MetStat Spike-in Panel (me1, me2, and me3 at H3K4, H3K9, H3K27, H3K36 and H4K20): Add 2 µL **SNAP-CUTANA™ K-MetStat Panel** per 100,000 cells. If using less than 100,000 nuclei, decrease the amount of SNAP-CUTANA spike-in linearly by preparing a "working stock" dilution of the panel in **Antibody150 Buffer**. General starting recommendations are provided in **Table 5**.
    - Note 1: SNAP-CUTANA Spike-ins must be added before addition of Primary Antibody.
    - Note 2: Aim for spike-ins to comprise ~1% of total sequencing reads adjust amount added as needed. It is acceptable for this bandwidth to be higher for low abundance targets and negative controls (*e.g.* IgG = 10-20%) and lower for high abundance targets (*e.g.* H3K27me3 = 0.1-1%).
    - See **Appendix III** for more information about SNAP-CUTANA Spike-ins.

Starting # Nuclei In CUT&Tag	Working Stock in Antibody150 Buffer [*use FRESH the day of preparation*]	Volume added to reaction	Final dilution in reaction
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 or less	1:10	2 µL	1:250

Table 5: Recommended SNAP-CUTANA™ Spike-in amounts for varying numbers of starting cells in CUT&Tag. ***NOTE: additional dilutions of the SNAP-CUTANA Panels may be added for lower inputs. However, dilution of spike-ins beyond 1:250 increases risk of experimental variation and may require end user optimization.***

17. Add manufacturer's recommended amount (or 0.5 µg if application is untested) of **Primary Antibody** to each reaction and gently vortex immediately and thoroughly.
    - Note: Antibodies stored in glycerol solution may be viscous. Take care to ensure accurate pipetting by aspirating slowly, check tip for accuracy, and pipette up and down ~3x times into CUT&Tag reactions to clear remaining glycerol from tip.
18. **Incubate** 8-strip PCR tubes on nutator **overnight at 4ºC**.
    - Critical step: To keep beads in solution, slightly elevate cap side of 8-strip PCR tubes on nutator to ensure bead solution remains in bottom of conical tube (**Figure 4**). DO NOT USE a rotator or turn tubes over end-to-end for this step.

Bead slurry before O/N incubation at 4°C | Nutator O/N | Bead slurry after O/N incubation at 4°C

Figure 4. Beads should appear homogenous and not clumpy throughout the procedure. Vortex or pipette as needed to disperse clumps. Ensure tube caps are elevated during incubation steps on tube nutator to keep beads in solution.

---Day 2---

Section III (continued)

19. Prior to continuing the CUT&Tag experiment on Day 2, prepare master mix for addition of Secondary Antibody by diluting **Secondary Antibody** in cold **Digitonin150 Buffer** in a 1.5 mL tube **(Table 6)**. The secondary antibody must be matched to primary antibody host species (*e.g.* mouse, rabbit). Mix by pipetting, and keep on ice until needed.

Secondary Antibody Master Mix		
# Reactions	1X	8X
Cold Digitonin150 Buffer	50 µL	400 µL
Secondary Antibody	0.5 µg	4 µg
Final Volume added/Reaction	50 µL	50 µL

Table 6: Prepare Secondary Antibody Master Mix.

20. Place the 8-strip PCR tubes from overnight incubation on a magnet until slurry clears (30 s – 2 min) and pipette to remove and discard supernatant.
21. Remove tubes from magnet and add 50 µL cold **Secondary Antibody Master Mix** to each reaction. Thoroughly pipette or gently vortex to resuspend.
    - Note: Mix the Secondary Antibody Master Mix before adding to CUT&Tag reactions.

22. **Incubate** 8-strip PCR tubes on nutator for **30 min at RT**. Keep caps elevated (**Figure 4**).
23. Place the 8-strip PCR tubes on a magnet until slurry clears (30 s – 2 min) and pipette to remove and discard supernatant.
24. Keeping tubes on the magnet, add 200 µL cold **Digitonin150 Buffer** directly onto beads of each reaction, and then pipette to remove supernatant.
25. Repeat the previous step for total of two washes, keeping tubes + beads on magnet the entire time.
26. After second wash, remove supernatant and discard. Add 50 µL cold **Digitonin300 Buffer** per reaction, and thoroughly pipette to resuspend. Continue to the addition of pAG-Tn5.
    - Note: Beads are often clumpy at this point but can easily be dispersed by gentle pipetting with a P200 pipette. A slightly cut-off pipette tip may be used to aid in resuspension and/or preserve delicate nuclei.
    - Critical step: 300 mM NaCl is essential in the Digitonin300 Buffer to minimize non-specific binding of pAG-Tn5 to accessible DNA.

Section IV: Binding of pAG-Tn5 (~1 hr)

27. Add 2.5 µL **CUTANA pAG-Tn5** (20x stock) to each reaction, and gently vortex.
    - Critical step: To evenly distribute pAG-Tn5 across nuclei, ensure beads are thoroughly resuspended by gentle vortexing and/or pipetting using a P200 pipette.
    - Note: pAG-Tn5 can be prepared in Digitonin300 Buffer as a Master Mix, by combining 2.5 µL pAG-Tn5 + 50 µL Digitonin300 Buffer per reaction. Add 50 µL of this master mix to each reaction (*i.e.* combine with previous step).
28. **Incubate** reactions on nutator for **1 hr at RT**, caps elevated (**Figure 4**).
29. Return 8-strip PCR tubes to magnet, allow slurry to clear (30 s – 2 min). Pipette to remove and discard supernatant.
30. Remove tubes from magnet. Add 200 µL cold **Digitonin300 Buffer** to each reaction, and thoroughly pipette to resuspend.
31. Return 8-strip PCR tubes to magnet, allow slurry to clear (30 s – 2 min). Pipette to remove and discard supernatant.
32. Repeat **Steps 31-32** for total of two washes. Pipette to remove and discard supernatant.

Section V: Targeted Chromatin Tagmentation (~3 hrs)

33. Remove tubes from magnet and add 50 µL cold **Tagmentation Buffer** to each reaction. Thoroughly pipette to resuspend.
    - Note: Beads are often clumpy at this point, but can easily be dispersed by gentle pipetting with a P200 pipette (cut-off pipette tip optional).
34. **Incubate** 8-strip PCR tubes for **1 hr at 37ºC** in a thermocycler. Note that this is the key step wherein Tn5 tethered to antibody-bound chromatin is activated by magnesium to tagment target chromatin.

35. Return 8-strip PCR tubes to magnet, allow slurry to clear (30 s – 2 min). Pipette to remove and discard supernatant.
36. Remove tubes from magnet and resuspend beads in 50 µL RT **TAPS Buffer** by pipetting.
37. Return tubes to magnet, allow slurry to clear (30 s – 2 min). Pipette to remove and discard supernatant.
38. Remove tubes from magnet and add 5 µL RT **SDS Release Buffer** (containing 0.1% SDS) to each reaction to quench tagmentation. Vortex tubes on max speed for 7 s to mix.
    - Critical Step: Do not pipette to mix! Addition of SDS partially lyses nuclei, causing the bead slurry to become viscous/sticky. Pipetting may result in sample loss.
39. Perform a quick spin of 8-strip PCR tubes in benchtop microfuge to collect beads/buffer.
40. **Incubate** 8-strip PCR tube(s) for **1 hr at 58ºC** in a thermocycler.
    - Critical step: Required to release tagmented chromatin fragments into solution, for both fixed and unfixed nuclei.
41. Add 15 µL RT **SDS Quench Buffer** (containing 0.67% Triton-X) to each reaction, and vortex briefly on max speed.
    - Note: Neutralizes SDS, which potently inhibits PCR.

Section VI: Non-hot Start PCR and Library Cleanup (~1 hr)

42. Add 2 µL universal i5 and 2 µL barcoded i7 primer (10 µM stocks) to each reaction.
43. Add 25 µL non-hot start **CUTANA High Fidelity 2x PCR Master Mix** to each reaction and vortex thoroughly.
44. Amplify DNA directly from cell pellet using CUT&Tag-specific PCR cycling parameters:
    a. 5 min at 58ºC → Fill-in step
    b. 5 min at 72ºC → Extension step
    c. 45 sec at 98ºC → DNA melting
    d. 15 sec at 98ºC → DNA melting
    e. 10 sec at 60ºC → Hybrid primer annealing & short extension (<700 bp)
    f. Repeat Steps d-e for a total of 14-21 cycles. Number of cycles depends on the target abundance and cellular input. Use the minimal number of cycles needed to accurately quantify the sequencing library (*i.e.* >2 ng/µL in a 15 µL elution; see **Step 47**).
    g. 1 min at 72ºC → Final extension
    h. Hold at 4ºC
45. DNA cleanup using **1.3x AMPure beads** : sample volume (*e.g.* 65 µL AMPure : 50 µL PCR) to recover > ~75 bp DNA fragments. Follow manufacturer's recommendations.
46. Elute DNA in 15 µL **0.1x TE buffer** and use 1 µL to quantify the purified PCR product using the **Qubit™ fluorometer** per manufacturer's instructions.
    - Note 1: There is no "typical yield" for a CUT&Tag assay, as discussed in the **Workflow Outline**, **Experimental Design & Key Protocol Notes** and **FAQs** sections. Instead, we recommend aiming to meet or exceed the minimal DNA amount necessary for accurate

library quantification (>2ng/ul in 15 µL or 30 ng total DNA).

- Note 2: It is useful to compare yields between reactions using positive (H3K4me3 and/or H3K27me3) and negative (IgG) control antibodies, which should be included in all experiments. In general, yields for an abundant target (*i.e.* H3K27me3) should always be higher than IgG.

Section VII: Agilent 2100 Bioanalyzer System (~1 hr)

47. For each purified CUT&Tag sequencing library, including your IgG negative control, load 1 µL of library on the **Agilent High Sensitivity DNA Chip** (Cat# 5067-4626).
48. Prepare and run the **Agilent High Sensitivity DNA Chip** per the manufacturer's instructions.
49. Typical Bioanalyzer results for CUT&Tag sequencing libraries are shown in **Figure 5**. Confirm that antibodies enriched for predominantly mononucleosome fragments (~300 bp peak with nucleosomes + sequencing adapters).
50. Proceed to Illumina sequencing as per manufacturer's recommendations.
    - Note: Only 3-8 million paired-end reads are needed for good coverage in CUT&Tag. For lower abundance targets (*e.g.* H3K4me3), 3-5 million reads are adequate. For higher abundance targets (*e.g.* H3K27me3), aim for 5-8 million reads.

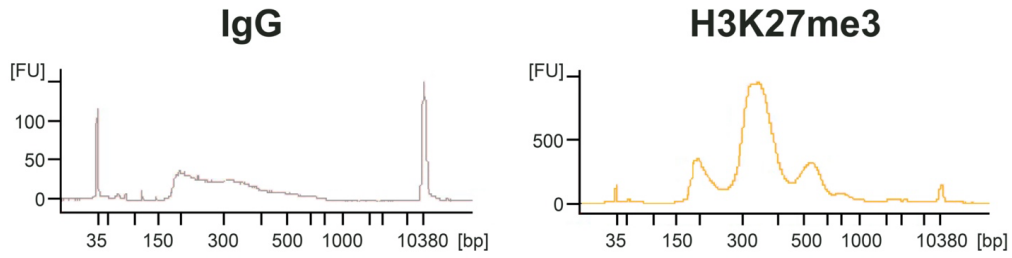

Figure 5. Typical Bioanalyzer traces for IgG negative control and H3K27me3 positive control CUT&Tag sequencing libraries (1 µL PCR amplified/AMPure purified libraries loaded on Bioanalyzer). The positive control H3K27me3 library is predominantly enriched for mononucleosomes as indicated by the peak at ~300 bp (~150 bp mononucleosomes + 150 bp sequence adapters). Quality Bioanalyzer (or Tapestation/equivalent approach) traces of purified CUT&Tag libraries are the best indicator of success prior to sequencing.

7. Frequently Asked Questions (FAQs)

7.1 General

1. **What is the best way to know if a CUT&Tag experiment worked prior to sequencing?**
   Results from challenging cell inputs/targets may be ambiguous, so EpiCypher recommends including positive and negative controls in every experiment. We have included a series of quality control (QC) checks to analyze sample quality, permeabilization, confirm ConA bead binding, assess DNA yields post-PCR, and confirm fragment size distribution (see **Appendix II** and **CUT&Tag Protocol, Sections VI - VII**). If the QC checks and positive/negative controls perform as expected, then proceeding to sequencing with all samples is recommended. If sequencing results for challenging cell inputs/targets are not satisfactory, further optimization may be necessary (*e.g.* cell type and/or number, antibody concentration/alternate vendors).

   Recommended experimental design (see **Experimental Design & Key Protocol Notes**):
   a. Always include reactions with control antibodies and control cells
      i. Start with 100,000 native K562 cells
      ii. Positive control antibody (*e.g.* H3K27me3 for abundant targets, H3K4me3 for low abundance targets)
      iii. Negative control antibody (IgG)
   b. Experimental cell types and antibodies
   c. Use spike-in controls whenever possible (*e.g.* the SNAP-CUTANA™ K-MetStat Panel can be added into the positive/negative control reactions as well as any sample with an antibody to a methyl-lysine histone PTM target; see **Appendix III**).

   Quality control checks before decision to sequence:
   a. Integrity of starting cells and nuclei are confirmed (**Appendix II**). Samples should not contain clumps and starting counts should be accurate.
   b. Confirm sample binding to ConA beads (**Appendix II**).
   c. Aiming to meet or exceed the minimal DNA amount necessary for accurate library quantification (>2ng/ul in 15 µL or 30 ng total DNA). PCR yields from reactions with positive control antibody should be greater than those from the IgG negative control, particularly for high abundance PTMs (*e.g.* H3K27me3). At low cell inputs, this difference may not be observed, but good quality sequencing data can still be obtained.
      If using H3K4me3 as the positive control, note that H3K4me3 PCR yields are often comparable or only slightly higher than IgG, since this is a lower abundance PTM. However H3K4me3 is still an extremely helpful control, since in sequencing results H3K4me3 peaks are sharp with high signal-to-noise and are specifically localized at gene transcription start sites, in contrast to the broader, ill-defined peaks characteristic of H3K27me3.

d. Bioanalyzer/Tapestation traces of PCR-amplified CUT&Tag libraries should be enriched with mononucleosome size fragments (~300 bp = nucleosome + sequencing adapters, see **Figure 5**). **Enrichment of mononucleosome size fragments in CUT&Tag DNA libraries is the best indicator of assay prior to sequencing.**

2. **Why are chromatin purification steps prior to PCR omitted from this protocol?**
Direct-to-PCR CUT&Tag is a parsimonious workflow that offers many advantages over the chromatin purification option. In the direct approach, tagmented DNA is directly PCR amplified *in situ* to generate next-generation sequencing (NGS) libraries. The entire workflow from isolated nuclei to sequence-ready libraries can be performed in a single tube, enabling increased experimental throughput and supporting automation. By circumventing chromatin purification steps, sample loss is minimized, which increases sensitivity for low cell inputs.

3. **Can I use Bioanalyzer or TapeStation traces to evaluate the success of CUT&Tag prior to library preparation?**
We do not advise assessing the fragment size distribution of raw CUT&Tag DNA before library preparation. In our approach, DNA is only purified at one step – after PCR for library preparation – and there is no opportunity to examine raw CUT&Tag DNA fragment size distribution (*e.g.* Bioanlayzer) or target enrichment (*e.g.* qPCR), standard controls in ChIP-seq. This feature underscores an important point: CUT&Tag and ChIP-seq are not the same method, and thus do not entail the same quality control steps.

ChIP-seq involves antibody-mediated enrichment of targets from bulk fragmented chromatin, or "Input." Fragment size distribution in Input chromatin is carefully optimized and monitored by agarose gel or capillary electrophoresis (*e.g.* Bioanalyzer) to confirm assay success at multiple steps. At the conclusion of a ChIP assay, immunoprecipitated (IP'd) DNA is easily isolated and purified for quantification, size distribution analysis, and comparison to Input.

In contrast, CUT&Tag is a ChIP-less *in situ* technique, in which antibody-bound chromatin is tagmented and retained in nuclei with bulk chromatin. There is no method for selectively "pulling down" tagmented DNA; PCR is required to examine tagmented DNA. Our strategy saves time and resources by bypassing post-tagmentation DNA purification and directly amplifying target DNA fragments from nuclei. Thus, the best metric for CUT&Tag assay success is enrichment of mononucleosome size fragments in sequence-ready libraries.

4. **Can I use qPCR of to evaluate the success of a CUT&Tag experiment?**
We do not recommend qPCR for validation of CUT&Tag enrichment. qPCR is traditionally used to verify the enrichment of a known on-target region following a ChIP experiment as a readout for experimental success prior to library preparation. To determine enrichment, qPCR for the same region must also be performed on the bulk chromatin Input used for the

ChIP reaction. Regions not enriched by the ChIP reaction are also included as negative controls for comparison.

Not only are these steps unnecessary for CUT&Tag assays, they are also technically unfeasible. Our CUT&Tag protocol is a one-tube, direct-to-PCR method, in which antibody-bound chromatin is tagmented by pAG-Tn5 *in situ*. Tagmented fragments are selectively PCR amplified from the pelleted nuclei, generating sequencing-ready CUT&Tag libraries. There is no IP step, and thus no bulk chromatin Input for enrichment comparisons. In addition, there is no equivalent for ChIP-DNA; the protocol goes immediately from tagmentation to PCR, and the only assay output is the sequencing library.

Instead, we recommend analysis of post-PCR CUT&Tag sequencing libraries (using the Qubit and Agilent Bioanalyzer/TapeStation) to confirm DNA size distribution and concentration (see **Section VI-II**), and proceed to sequencing without qPCR. As you will need only 3-8 million reads per sample, you can multiplex samples, save on sequencing costs, and obtain the genomic data regarding your target.

7.2 Spike-in Controls

5. **Can residual *E. coli* in the pAG-Tn5 prep be used for sample input normalization?**
   The EpiCypher pAG-Tn5 preparation is highly purified and depleted of contaminating nucleic acids, so residual *E. coli* DNA cannot be used for sample input normalization. The primary advantages of our optimized purification strategy are:
   - Ensures lot-to-lot consistency
   - Maintains high specific activity of pAG-Tn5
   - Prevents contaminating *E. coli* DNA from dominating signal in ultra-low cell input experiments

   EpiCypher is currently working on multiple exogenous spike-in control solutions for CUT&Tag (see below).

6. **What spike-in controls does EpiCypher recommend for CUT&Tag?**
   EpiCypher recently launched SNAP-CUTANA™ Spike-in Controls for CUT&Tag assays, and now offers the SNAP-CUTANA™ K-MetStat Panel for CUT&Tag reactions against histone lysine methylation (see **Appendix III**). The panel comprises highly pure, modified semi-synthetic/recombinant nucleosomes wrapped with DNA containing a PTM-specific barcode, allowing detection in NGS assays. SNAP-CUTANA Spike-ins are the ideal physiological control because they replicate the natural substrate of histone PTM antibodies in CUT&Tag. In addition, because these spike-ins contain a panel of on- and off-target epitopes and are carried throughout the workflow alongside sample chromatin, they can be used:

- As a direct readout of assay success (including pAG-Tn5 activity)
- To determine antibody specificity in the context of your assay
- To monitor technical variation/assay stability across experiments
- For quantitative sample normalization, enabling reliable and accurate cross-sample comparisons

SNAP-CUTANA spike-ins also provide essential information on the quality of sample inputs and DNA purification, making them an essential tool for developing, optimizing, and troubleshooting CUT&Tag assays. As noted above, the first-in-class commercial SNAP-CUTANA product is the K-MetStat panel for lysine methylation targets (see **Appendix III**). However, additional targets are in development, including lysine acetylation, ubiquitylation, and even chromatin-associated protein targets. EpiCypher is also developing exogenous *E. coli* DNA spike-in controls for CUT&Tag data normalization (compatible with any target of interest). Sign up for emails to stay up to date!

7.3 Sample Input Compatibility

7. **Why are nuclei used in the default protocol?**
   Tn5 is known to efficiently tagment mitochondrial DNA in the cytoplasm, which consumes sequencing bandwidth. Using nuclei in CUT&Tag circumvents this problem, allowing reduced sequencing depths that are enriched for target cellular chromatin.

   While we do not recommend using whole cells, cells may be used in CUT&Tag with the caveat that a percentage of reads will be lost to mitochondrial DNA (degree of acceptability to be experimentally determined).

8. **If I use whole cells, are there additional steps required?**
   If whole cells must be used, optimize the digitonin concentration to ensure cells are effectively permeabilized (*e.g.* perform a digitonin titration for every cell type and monitor cell permeability using trypan blue staining). A detailed protocol for digitonin optimization is described in the CUTANA™ ChIC/CUT&RUN Kit manual (EpiCypher 14-1048). In brief, perform a digitonin titration in **Antibody150 Buffer** (*e.g.* 3-fold dilutions from 0.1% down to 0.001%) and monitor cell lysis using Trypan blue staining (see **Appendix II**). Find the minimum digitonin concentration needed to achieve >95% permeabilized cells to avoid precipitation and cell lysis. Use this optimal concentration in all buffers containing digitonin (**Digitonin150**, **Antibody150, Digitonin300, and Tagmentation Buffers**).

9. **What types of cell inputs (whole cells and nuclei) can be used in CUT&Tag?**
   The EpiCypher Direct-to-PCR CUT&Tag protocol was developed using native (unfixed) nuclei from K562 cells, a suspension human cell line. Recommendations for working with

specific cell types other than suspension cells are outlined below. Many of the notes detailed here are also relevant when harvesting nuclei from these cell types.

Adherent cells: Adherent cells present a special challenge for CUT&Tag, as the process must be strong enough to detach cells from culture plates and disaggregate cell clumps, yet gentle enough to preserve cell membranes for binding to ConA beads. A major concern is that strong or prolonged enzymatic treatment (*e.g.* trypsin) can degrade glycoproteins, thereby impairing cell adsorption to the ConA beads.

EpiCypher has compared different methods of collecting adherent cells for CUT&Tag, including cell scraping, trypsin, and accutase. We analyzed the impact of each method on the cell adsorption rate onto ConA beads using MCF-7 cells. Based on these optimization experiments, we recommend a very mild trypsin treatment (0.05% trypsin at 37°C), for the minimal incubation time as optimized for cell type. Collect cells and pellet by centrifugation for ~3 min at 600 x g at RT. Discard supernatant, and then proceed directly to cell counting and CUT&Tag wash steps as outlined in **Section II**. Trypsin is washed away by subsequent washes that are a standard part of the CUT&Tag protocol. This method detaches and monodisperses cells, resulting in >95% adsorption onto ConA beads (see Quality Control Checks outlined in **Appendix II**).

Tissue samples: While EpiCypher does not have tissue-specific protocols available at this time, the primary requirement is that tissue is processed to a monodispersed cell suspension (typically by mechanical maceration or douncing). Enyzmatic digestion (*e.g.* collagenase/dispase) can be used for connective tissue and trypsin (as described above; monitoring dissolution to single cells) may be used for macro-dissected tissue.

Working with immune cells: Note that lectins (*e.g.* ConA) play a role in the innate immune system and so immune cell types may be inadvertently stimulated via binding to ConA beads. To circumvent this potential problem in CUT&Tag, EpiCypher recommends using nuclei or a cross-linking strategy (see a detailed cross-linking protocol at epicypher.com/protocols).

10. Is CUT&Tag compatible with frozen or cross-linked nuclei and cells?

Yes. General guidelines are noted below. Our detailed **CUTANA CUT&RUN Cross-linking Protocol** is recommended when fixing nuclei or cells for CUT&Tag and is provided at epicypher.com/protocols; additional protocols are available upon request (email info@epicypher.com). Note that when using cross-linked or frozen materials in CUT&Tag assays, EpiCypher still recommends the use of nuclei over cells, for the reasons stated above.

Cryopreservation: EpiCypher has confirmed that freeze/thawed cells and nuclei generate data of indistinguishable quality to fresh material. Our cryopreservation method, outlined in

Appendix I, is optimized to preserve native physiological interactions, minimize lysis, and reduce background signal in CUT&Tag.

Cross-linking: It is recommended to first try native samples in CUT&Tag, since this works well for most targets. Of note, EpiCypher has tested previously reported cross-linking conditions and recommended wash buffers. Although yields are lower than from native cells, the resulting data tracks display similar quality (*i.e.* signal-to-noise). Furthermore, for labile targets or highly transient chromatin binding proteins, CUT&Tag signal may be improved by light cross-linking (*i.e.* not standard ChIP conditions; see below).

When using native CUT&Tag, histone deacetylase activity may contribute to incomplete or low-resolution genomic profiles for certain targets. For example, EpiCypher has observed that certain acetyl PTMs such as H3K27ac and H3K18ac may show enhanced signal after mild (*e.g.* 0.1% formaldehyde, 1 min) to moderate (*e.g.* 1% formaldehyde, 1 min) cross-linking even though total yields are reduced. However, heavy cross-linking such as that typically used for ChIP-seq (*e.g.* 1% formaldehyde, 10 min) has been shown to damage histone acetylation signal from K562 cells in CUT&Tag and CUT&RUN. Therefore, optimal cross-linking conditions for profiling histone acetylation PTMs should be empirically determined in the model system of interest.

Importantly, not all acetyl-PTMs require cross-linking for high quality data (*e.g.* H3K9ac). Therefore, cross-linking should only be used as a last resort to improve signal-to-noise, since (1) cross-linking reduces yield; (2) cross-linking can decrease specificity and increase artifacts; and (3) key protocol adaptations are required when applying CUT&Tag to cross-linked samples (supplementing Wash, Digitonin, and Antibody Buffers with Triton X-100 and SDS detergents). EpiCypher continues to optimize cross-linking protocols and determine cases where this approach may improve signal. Our current **CUTANA™ CUT&RUN Cross-linking Protocol** for cells/nuclei is compatible with CUT&Tag, and is available at epicypher.com/protocols; similar protocols can be found in the literature[3].

7.4 Antibodies and Targets

11. Does EpiCypher's CUT&Tag protocol work on non-PTM targets?

Kaya-Okur et al. report several non-PTM targets in CUT&Tag (CTCF, SOX2, NPAT, and Pol II)[3]. However, because CUT&Tag is performed at twice the salt concentration of CUT&RUN and tagmentation is performed at 37ºC, many transcription factors (TFs) may be incompatible with CUT&Tag. Therefore, for non-PTM targets, CUT&RUN is recommended as a more robust and extensively validated approach. See more information about CUT&RUN at epicypher.com/CUT&RUN.

12. What PTM antibodies does EpiCypher recommend for CUT&Tag?

It is critical to evaluate antibody performance against a defined control directly in the application of interest. Antibodies to histone PTMs are particularly susceptible to off-target cross-reactivity which can compromise biological interpretations[3]. EpiCypher has conducted extensive studies of histone PTM antibodies in ChIP-seq by using our exclusive SNAP-ChIP® nucleosome spike-in control technology. Although the majority of antibodies exhibit major problems such as poor specificity and/or low efficiency, best-in-class reagents for ChIP-seq do exist, and have been identified using SNAP-ChIP® Spike-ins (see ref [3] and chromatinantibodies.com *Maryanski et al., In preparation*).

As noted above, EpiCypher has recently launched the first SNAP Spike-in Controls for CUTANA™ CUT&RUN and CUT&Tag assays (*i.e.* the SNAP-CUTANA™ K-MetStat Panel). Through our extensive development of CUT&Tag/CUT&RUN assays to various histone PTMs and chromatin-associated proteins, EpiCypher has found that robust antibody performance in ChIP does **not** guarantee success in CUT&Tag/CUT&RUN. To address this unmet need for researchers, EpiCypher has begun screening antibodies for high quality performance in CUT&Tag and its sister technology CUT&RUN. EpiCypher's H3K4me3 antibody (EpiCypher 13-0041) has been verified to exhibit robust and specific performance in CUT&Tag. We are currently using our novel SNAP-CUTANA Spike-ins for histone PTM antibody validation in CUT&Tag and CUT&RUN, and plan to launch the first set of validated histone lysine methylation antibodies in the coming months. For more information or for antibody recommendations, please contact techsupport@epicypher.com.

7.5 Miscellaneous

13. Why is digitonin still used in the protocol if nuclei do not require digitonin for permeabilization?

Digitonin is not necessary for nuclei permeabilization in the CUT&Tag workflow, since purified nuclei are inherently permeable to both the antibody and pAG-Tn5. However, without digitonin, the bead-nuclei mixture tends to form a thin film on the side of the tubes, resulting in reduced yields. Digitonin also makes the protocol compatible with cells, although we strongly recommend using nuclei (see above).

8. References

1. Skene, P. J., Henikoff, J. G. & Henikoff, S. Targeted in situ genome-wide profiling with high efficiency for low cell numbers. *Nature protocols* **13**, 1006-1019, doi:10.1038/nprot.2018.015 (2018).
2. Kaya-Okur, HS, Wu, SJ, Codomo, CA, Pledger, ES, Bryson, TD, Henikoff, JG, Ahmad, K, Henikoff, S. CUT&Tag for Efficient Epigenomic Profiling of Small Samples and Single Cells. *Nature Communications.* **10**(1):1930. doi: 10.1038/s41467-019-09982-5 (2019).
3. Shah, R. N. *et al.* Examining the Roles of H3K4 Methylation States with Systematically Characterized Antibodies. *Molecular cell* **72**, 162-177.e167, doi:10.1016/j.molcel.2018.08.015 (2018).

Appendix I: Cryopreservation and Thawing Protocols for CUT&Tag

Description: This protocol gives detailed instructions on cryopreservation and thawing of nuclei and cells for CUT&Tag assays. Note that this protocol is designed similarly to the CUTANA™ CUT&Tag protocol, *i.e.* to batch process cells for multiple CUT&Tag reactions, and was developed using 100,000 K562 cells per reaction. Adjust volumes and cell numbers for your experiments as needed. Start with at least 10% excess cells, to ensure sufficient cells and nuclei for quality controls checks (**Appendix II**).

Protocol: Cryopreservation of Nuclei, from Section II, Step 12 of the CUTANA CUT&Tag protocol

1. Harvest nuclei per the **CUTANA CUT&Tag Protocol, Section II, Steps 7-12.**
2. Confirm integrity and number of starting cells, the efficiency of nuclear isolation, and ensure that final isolated nuclei are intact (as in **Appendix II, Figure 6**).
   a. Remove 10 µL aliquots from washed cells and nuclei, noted in **Section II** of the protocol.
   b. Combine each 10 µL aliquot with 10 µL 0.4% **Trypan blue dye**, and load onto a cell counter or hemacytometer slide.
   c. Examine under brightfield or phase microscope (**Figure 6, Appendix II**). Cells should not take up Trypan blue and appear clear/white, with >90% viability. Nuclei should take up Trypan and appear blue, with >95% Trypan Blue positive and unclumped.
3. Following confirmation of nuclear integrity, aliquot nuclei as desired.
4. Cryopreserve nuclei by slowly freezing aliquots in an isopropanol-filled chiller in a -80°C freezer.
   - Note: If necessary, nuclei can be shipped on dry ice in this state.

Protocol: Thawing Frozen Nuclei

1. To avoid nuclear lysis and chromatin fragmentation, thaw nuclei quickly by placing tubes on 37°C block until thawed.
2. Proceed to ConA bead conjugation step, **Section II Step 13**. Nuclei in NE Buffer can be directly added to activated ConA beads.

Protocol: Cryopreservation and Thawing of Cells

1. To cryopreserve cells, supplement cell culture media with a cryoprotective agent (*e.g.* 10% DMSO in media) and slowly freeze aliquots in an isopropanol-filled chiller in a -80°C freezer.
2. When ready to perform CUT&Tag, quickly and completely thaw samples at 37°C.
3. Wash cells 2X with **Wash150 Buffer**, and confirm cell quality (**Appendix II**).
4. Resuspend in **Wash150 Buffer** and proceed with ConA bead binding (**Section II Step 13**).

Appendix II: Sample Integrity & Bead Conjugation Quality Control Checks

Description: This Appendix provides detailed instructions on checking the quality of starting cells and isolated nuclei (as in **Appendix I**), as well as the success of nuclei conjugation to ConA beads. If using cells, these steps can also be used to validate cell permeabilization and ConA bead binding. All QC checks were developed using batch processed K562 cells and nuclei (*i.e.* for multiple CUT&Tag/CUT&RUN reactions).

We recommend checking the quality of starting materials (both cells and nuclei) prior to every CUT&Tag experiment. For new users or for working with new cell types/sample inputs, we strongly recommend performing the ConA bead conjugation checks. This is a key step in the CUT&Tag protocol, and if cells/nuclei are of poor quality or not successfully bound to ConA beads, CUT&Tag yields will be dramatically reduced.

Reagents, Materials & Equipment Needed

Item	Vendor
0.4% Trypan blue	Any
Hemacytometer	Any
Cell counting slides	Any
Brightfield/phase contrast microscope or automated cell counter	Any

Protocol: Sample Integrity and ConA Bead Conjugation QC Checks, Starting fom Section II, Step 12 of CUT&Tag Protocol

1. In **Section II, Steps 7-12,** process excess cells to have leftover material (*e.g.* prepare ~10% excess volume for batch processing or one extra sample).
2. Prior to starting with ConA bead conjugation (**Section II, Step 13**), take 10 µL from washed cells and final prepared nuclei and evaluate sample integrity as follows:
    - Add 10 µL of 0.4% **Trypan blue** to each sample.
    - Mix 10 times by pipetting.
    - Transfer 10 µL to a counting slide.
    - View under brightfield/phase microscope or cell counter.
    - Confirm sample integrity or troubleshoot as needed (see **Figure 6** and **Table 7**):
        - Cells and nuclei should not be clumped and should show the expected morphology
        - Intact cells will not absorb trypan blue and appear white (**Figure 6A**)
        - Nuclei will take up Trypan and appear blue (**Figure 6B**)
        - Troubleshoot as necessary (**Table 7**)
3. Proceed with bead binding by 100 µL nuclei to 10 µL activated **ConA Beads** in 8-strip PCR tubes. Again, make sure to prepare ~10% extra volume.
4. Gently vortex and/or pipette to mix ConA beads with nuclei.

5. **Incubate** nuclei – bead slurry for **10 min at Room Temperature (RT)**. Cells will adsorb to the activated ConA beads.
6. Place 8-strip PCR tubes on magnet until slurry clears. Transfer 10 µL supernatant into a fresh 1.5 mL tube (**Unbound fraction**); set aside for bead binding integrity check. Pipette to remove remaining supernatant and discard.
7. Add 50 µL/reaction cold **Antibody150 Buffer** quickly, to avoid bead drying. If working in bulk, scale volume for total number of planned reactions. Gently vortex immediately and thoroughly to an even resuspension.
   - Note: Antibody150 Buffer contains Digitonin, which will permeabilize cells.
8. Remove 10 µL and transfer to a fresh tube (**Bead-Bound Fraction**). Place remaining sample on ice.

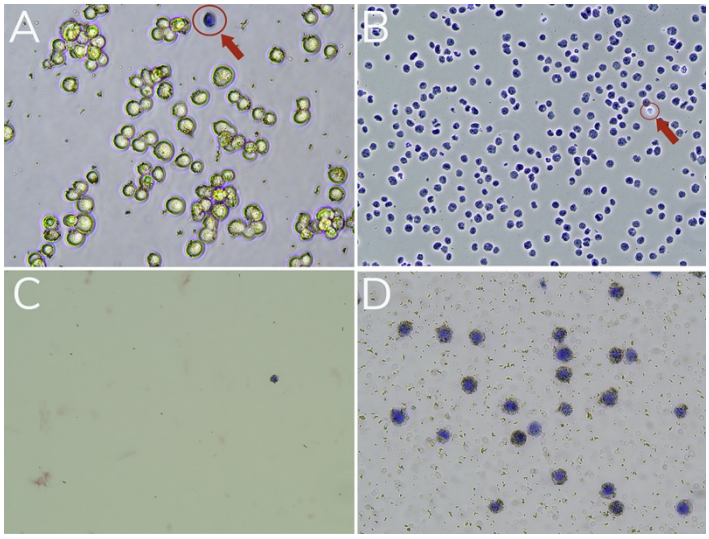

Figure 6: Representative images of cell and nuclei samples for ConA Bead binding. Samples were stained with Trypan blue and visualized under brightfield microscope. **(A) Cells** before bead binding. A dead cell (blue; Trypan positive) is circled in red, whereas the remaining live cells are bright white and round. **(B) Nuclei** before bead binding. An intact cell (Trypan negative) is circled in red, whereas isolated nuclei are Trypan stained. **(C) Unbound Fraction** shows little to no material leftover after ConA Bead binding. **(D)** Representative **Bead-Bound Faction** showing nuclei (blue) successfully bound to activated ConA Beads (brown specks).

5. Perform Trypan blue staining and bead binding integrity check as described in **Steps 2a-e** (above), comparing **Unbound Fraction** and **Bead-Bound Fraction**.
   - Successful binding will show Trypan positive nuclei (or cells) surrounded by ConA beads; compare **Figures 6C and 6D**. For troubleshooting approaches, see **Table 7**.
9. Continue with the **CUT&Tag Protocol, Section III** (Antibody Binding).

Samples	Figure	Success Metric (Trypan Blue staining)	Troubleshooting Tips
Cells	Fig. 6A	Cells should be bright (Trypan blue excluded), round, unclumped, and ideally show >90% viability. Over 80% minimum viability is recommended, as excess dead cells increase background in CUT&Tag.	Optimize cell culture conditions; use fresh media, evaluate potential contamination issues.
Nuclei	Fig. 6B	Nuclei should be >95% Trypan blue positive and unclumped.	See main Protocol and **Appendix I** for detailed nuclei preparation protocol.
Unbound Fraction	Fig. 6C	Little to no material should be present if binding to beads occurred.	Ensure that ConA Beads were never frozen, cells/nuclei were not clumped, beads did not become clumped or dried out, and all buffers were correctly prepared.
Bead-Bound Fraction	Fig. 6D	Successful ConA bead binding will show Trypan blue positive permeabilized cells/nuclei surrounded by beads.	

Table 7: Quality Control testing of cell/nuclei integrity and ConA bead binding steps.

Appendix III: SNAP-CUTANA™ Spike-in Controls

Description: This Appendix describes EpiCypher's quantitative nucleosome spike-in technology, or SNAP (Sample Normalization and Antibody Profiling) Spike-in Controls for CUTANA™ CUT&RUN/CUT&Tag assays. In **Section I**, we provide an overview of SNAP-CUTANA™ Spike-ins and how they should be incorporated into CUT&RUN/Tag workflows. **Section II** contains more detailed descriptions and examples of SNAP-CUTANA Spike-in applications, including antibody specificity profiling, flagging failed reactions, and next-generation sequencing (NGS) normalization. In **Section III**, we offer detailed instructions on extracting SNAP-CUTANA Spike-in data from CUT&RUN/CUT&Tag datasets using the shell script and Excel template provided on the EpiCypher product page. **Table 7** contains the DNA barcodes associated with each nucleosome in the K-MetStat Spike-in Panel (EpiCypher 19-1002).

Section I: An Introduction to SNAP-CUTANA™ Spike-in Controls

What are SNAP-CUTANA Spike-ins?

SNAP-CUTANA Spike-in Controls are panels of semi-synthetic/recombinant nucleosomes carrying defined histone post-translational modifications (PTMs), which can be distinguished in NGS by unique PTM-specific DNA barcodes. Panels are grouped by PTM class, such as that related to histone lysine methylation (K-MetStat Panel, **Figure 7**) and contain widely studied and disease-relevant modifications. This strategy enables a direct readout of assay success, *in situ* antibody validation against closely related PTMs (which have high risk for cross-reactivity) and provides quantitative tools for valuable epigenetic targets.

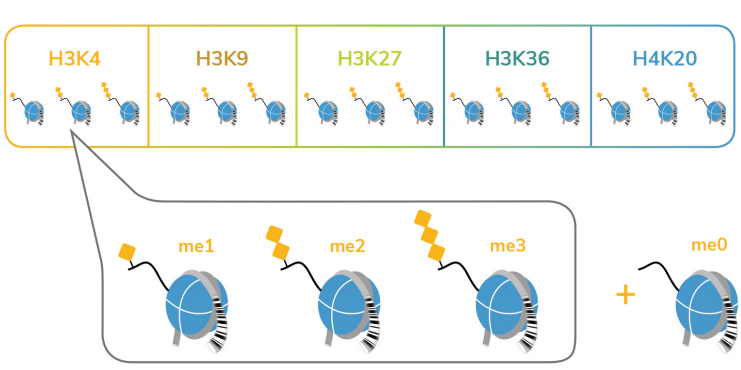

Figure 7: Histone methyl-lysine PTMs in the SNAP-CUTANA™ K-MetStat Panel.

How are the spike-ins detected? What is the "barcode"?

As in **Figure 8**, each nucleosome is wrapped with a DNA template containing the Widom 601 nucleosome positioning sequence (Lowary & Widom, *J. Mol. Biol.* 1998),

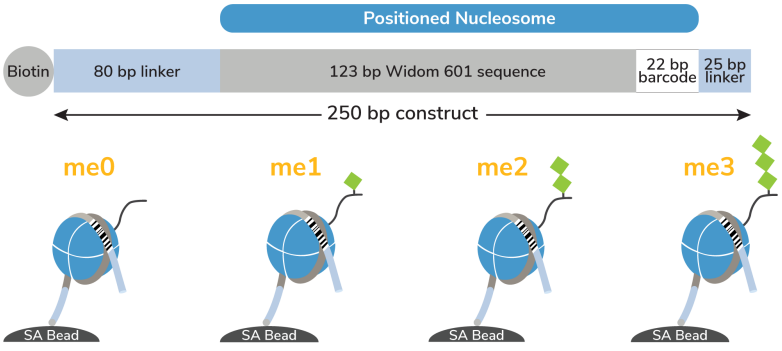

Figure 8: Schematic showing the DNA barcoding and magnetic bead immobilization used in SNAP-CUTANA™ Spike-in Controls.

and a 22 bp barcode unique to each PTM in the panel. This nucleosome assembly DNA is flanked by linker DNA, providing an appropriate substrate for pAG-MNase cleavage or pAG-Tn5 tagmentation in CUT&RUN or CUT&Tag, respectively. The 5' linker DNA also contains a biotin tag (**Figure 8**), allowing spike-ins to be immobilized to streptavidin (SA) magnetic beads for streamlined incorporation in CUT&RUN/CUT&Tag workflows alongside cells (or nuclei) immobilized onto ConA magnetic beads.

One important feature of SNAP-CUTANA Spike-in Panels is that each histone PTM is present in "duplicate," allowing scientists to monitor technical variation of spike-in recovery. This means that for a given panel we prepare each modified nucleosome using two unique DNA-barcoded templates. As an example, the **SNAP-CUTANA K-MetStat Spike-in panel** (EpiCypher 19-1002) contains 16 PTM-defined states (15 methyl-lysines: me1, me2, me3 at H3K4, H3K9, H3K27, H3K36 and H4K20, and an unmodified control). However, each modified histone octamer was wrapped with two different barcoded DNAs, so the final panel contains 32 distinct DNA-barcoded nucleosomes to be monitored by NGS (**Table 7**).

PTM	Barcode A (Nuc Replicate 1)	Barcode B (Nuc) Replicate 2
Unmodified	TTCGCGCGTAACGACGTACCGT	CGCGATACGACCGCGTTACGCG
H3K4me1	CGACGTTAACGCGTTTCGTACG	CGCGACTATCGCGCGTAACGCG
H3K4me2	CCGTACGTCGTGTCGAACGACG	CGATACGCGTTGGTACGCGTAA
H3K4me3	TAGTTCGCGACACCGTTCGTCG	TCGACGCGTAAACGGTACGTCG
H3K9me1	TTATCGCGTCGCGACGGACGTA	CGATCGTACGATAGCGTACCGA
H3K9me2	CGCATATCGCGTCGTACGACCG	ACGTTCGACCGCGGTCGTACGA
H3K9me3	ACGATTCGACGATCGTCGACGA	CGATAGTCGCGTCGCACGATCG
H3K27me1	CGCCGATTACGTGTCGCGCGTA	ATCGTACCGCGCGTATCGGTCG
H3K27me2	CGTTCGAACGTTCGTCGACGAT	TCGCGATTACGATGTCGCGCGA
H3K27me3	ACGCGAATCGTCGACGCGTATA	CGCGATATCACTCGACGCGATA
H3K36me1	CGCGAAATTCGTATACGCGTCG	CGCGATCGGTATCGGTACGCGC
H3K36me2	GTGATATCGCGTTAACGTCGCG	TATCGCGCGAAACGACCGTTCG
H3K36me3	CCGCGCGTAATGCGCGACGTTA	CCGCGATACGACTCGTTCGTCG
H4K20me1	GTCGCGAACTATCGTCGATTCG	CCGCGCGTATAGTCCGAGCGTA
H4K20me2	CGATACGCCGATCGATCGTCGG	CCGCGCGATAAGACGCGTAACG
H4K20me3	CGATTCGACGGTCGCGACCGTA	TTTCGACGCGTCGATTCGGCGA

Table 7: SNAP-CUTANA K-MetStat Spike-in DNA barcode sequences.

Importantly, the DNA barcodes do not overlap with known sequences in the human, mouse, fly, or yeast genome and can therefore be distinguished from sample chromatin. We provide instructions on DNA barcode detection in this Appendix (see **Section III**).

How are they "spiked" into assays?

SNAP-CUTANA nucleosomes are supplied pre-immobilized to magnetic SA beads via 5' biotinylated linker DNA (**Figure 8**), allowing them to be processed alongside ConA bead-conjugated cells in CUT&RUN and CUT&Tag reactions. SNAP-CUTANA Spike-in controls are added to CUT&RUN and CUT&Tag reactions just prior to antibody addition, in one simple pipetting step (**Figure 9**). The antibody will bind its target epitope in sample cells and the spike-in panel. Addition and activation of pAG-MNase (in CUT&RUN) or pAG-Tn5 (in CUT&Tag) will cleave or tagment the associated spike-in nucleosome, respectively. In both cases, the targeted standard will be processed with sample chromatin through library preparation, PCR amplification, and NGS (**Figure 9**).

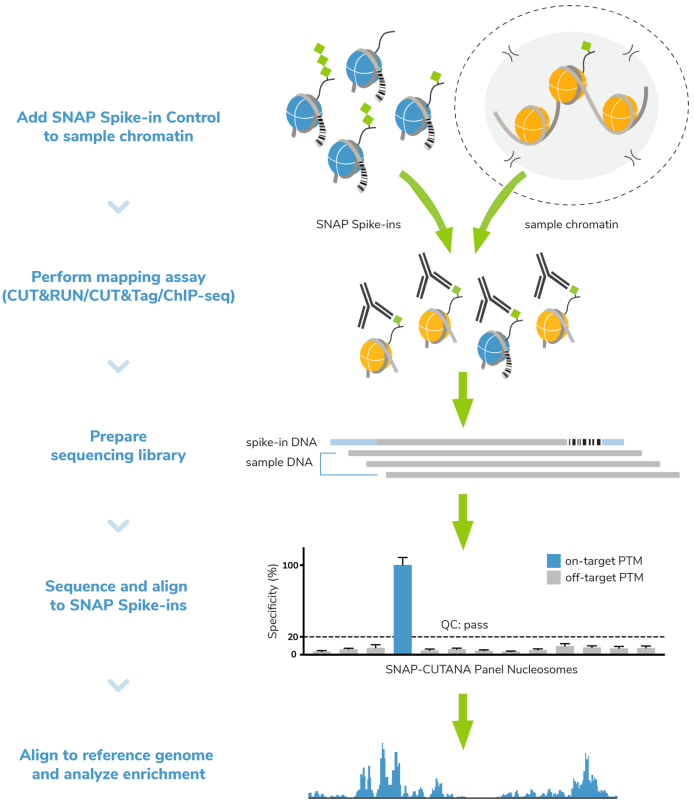

Figure 9: Schematic showing addition of SNAP-CUTANA™ Spike-in Controls during CUT&RUN workflow.

How can the SNAP-CUTANA Spike-in data be used? Why should I add these into my CUT&RUN or CUT&Tag workflow?

SNAP-CUTANA Spike-ins are the only fully defined, quantitative chromatin mapping assay control that represents the nucleosome target of histone-PTM antibodies. Thus, relative recovery of SNAP-CUTANA Spike-in Controls serve as a straightforward proxy for assay success. Because the spike-ins contain a panel of related PTM states (**Figure 7**), assessing the recovery of each nucleosome can also be used as a direct readout of antibody specificity. This is crucial, as many histone PTM antibodies display cross-reactivity, lot variation, and/or assay-specific performance (*e.g.* ChIP-seq vs. CUT&RUN/Tag). Finally, because the spike-in nucleosomes are carried through the protocol alongside sample chromatin, SNAP-CUTANA Spike-ins can be used to normalize NGS data and enable reliable cross-sample comparisons. See **Figure 10** for a general outline of how SNAP-CUTANA Spike-ins can be used to examine the success of CUT&RUN and CUT&Tag workflows; for more detail on each of these steps, see **Section II**.

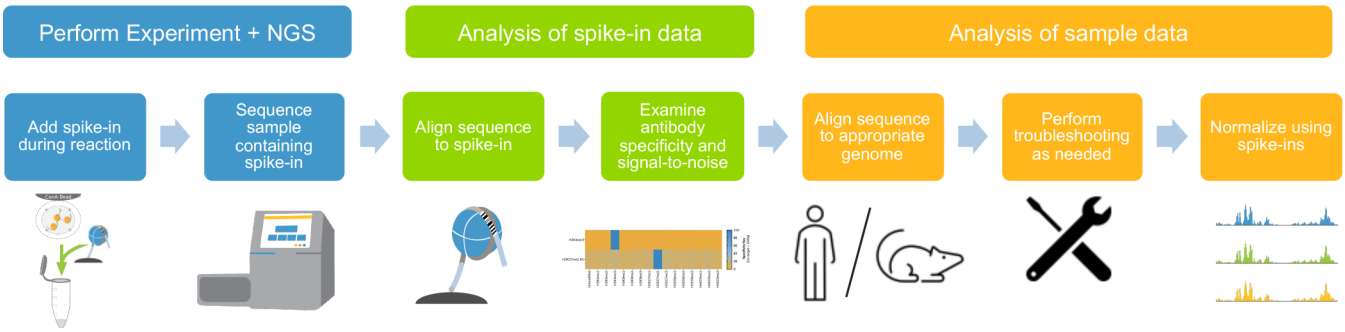

Figure 10: **SNAP-CUTANA™ Spike-ins offer a quantitative control for CUT&RUN/CUT&Tag assay workflows.** These defined nucleosome spike-ins can be used to determine the success of your assay, including on-target antibody specificity, as well as assay normalization and experimental comparisons.

Section II: How to use SNAP-CUTANA™ Spike-ins for CUT&RUN and CUT&Tag Workflows

1. **Combine SNAP-CUTANA Spike-ins with positive and negative control antibodies for CUT&RUN/CUT&Tag assay optimization.** When first starting CUT&RUN/CUT&Tag, or when trying these approaches under new experimental conditions (new cell types, changing cell number inputs, fixed vs. native preps, etc.) we strongly recommend using the SNAP-CUTANA K-MetStat spike-ins with positive (*e.g.* H3K4me3, EpiCypher 13-0041) and negative (*e.g.* IgG, EpiCypher 13-0042) control antibodies to validate your CUT&RUN workflow. These antibodies are lot-validated for superior performance and reliability in CUT&RUN/Tag. In fact, EpiCypher includes these exact positive and negative control antibodies and SNAP-CUTANA K-MetStat Spike-ins in every experiment, and have found them to be essential tools for initial workflow optimization and daily performance monitoring.

   As an example, we used CUT&RUN to map H3K27me3 in K562 cells, with IgG and H3K4me3 antibodies as controls, and SNAP-CUTANA K-MetStat Spike-ins added to each reaction (**Figure 11**). The first confirmation of optimized experimental conditions was relative recovery of each spike-in DNA barcode in NGS data (see **Section III** for detailed analysis instructions). Here the IgG control showed no target preference (as expected), while the H3K4me3 control and H3K27me3 antibodies each enriched their target of interest with very low background (**Figure 11A**). This provides confidence that multiple technical aspects of the CUT&RUN experiment (*i.e.* antibodies and pAG-MNase cleavage conditions) were successful, supporting further analysis of the sample. NGS data for the sample chromatin showed the expected PTM enrichment patterns (*e.g.* H3K4me3 showed tight peaks at transcription start sites [TSS] with minimal IgG background; **Figure 11B-C**). These robust controls allowed us to have high confidence in the accuracy of our H3K27me3 sequencing results.

Note: If the spike-in analysis reveals off-target cleavage or low signal-to-noise this indicates problems with the assay workflow. Results from the spike-ins can guide troubleshooting experiments, as outlined in **Table 8**, below.

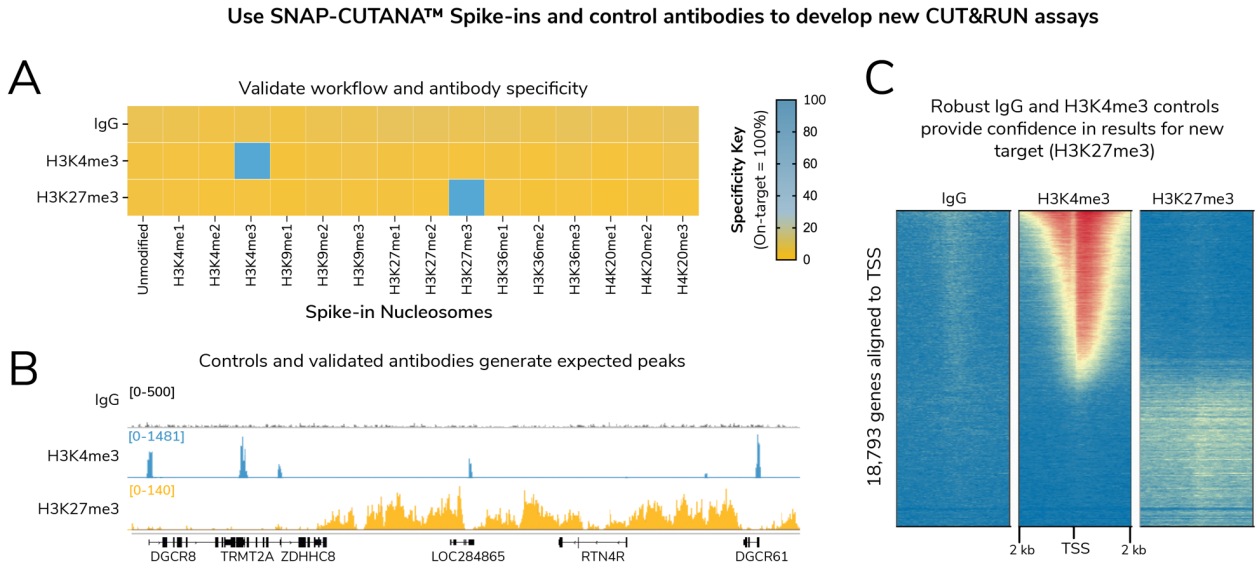

Figure 11: SNAP-CUTANA™ Spike-in Controls and control antibodies were used to validate CUT&RUN workflows for K562 cells. (A) PTM antibodies recovered on-target spike-in, while IgG antibody displayed no preference. Heatmap data normalized to DNA barcodes from on-target PTM; IgG normalized to total reads. (**B,C**) CUT&RUN generated expected H3K4me3 and H3K27me3 maps profiles from K562 sample chromatin. (**B**) RPKM normalized peaks on representative regions. (**C**) Heatmaps display signal intensity +/- 2 kb from transcription start site (TSS). Genes in each heatmap are ordered by signal intensity from H3K4me3 profiles.

2. **Use SNAP-CUTANA Spike-ins to find fit-for-purpose antibodies to lysine methylation targets.** Test your favorite antibody or inquire at info@epicypher.com for recommendations (**Figure 12**). EpiCypher performs extensive (and ongoing) antibody testing in epigenomic applications (to date: >1,000 commercial reagents) and has determined that the vast majority (>70%!!) of histone PTM antibodies are non-specific and/or display poor enrichment[1]. From these studies we have determined that:

- Antibody capability on modified histone peptide arrays does not transfer to epigenomic assays that consider full nucleosome structure (*e.g.* CUT&RUN, CUT&Tag, or ChIP-seq).
- Validation of an antibody in one chromatin mapping assay does not guarantee success in another (*e.g.* ChIP validation does not transfer to CUT&RUN or CUT&Tag, see **FAQs**).
- Specific PTM recognition under one set of conditions does not guarantee specific PTM recognition in all conditions. Antibodies must always be validated for user-specific conditions with spike-in controls.

Thus, application-specific (*i.e. in situ*) testing with PTM-defined, target-representative controls is essential to success. As minimum criteria, EpiCypher recommends selecting

antibodies that show <20% cross-reactivity to **each** off-target PTM in the K-MetStat panel. Below, we address some common questions when using nucleosome spike-ins for antibody specificity testing:

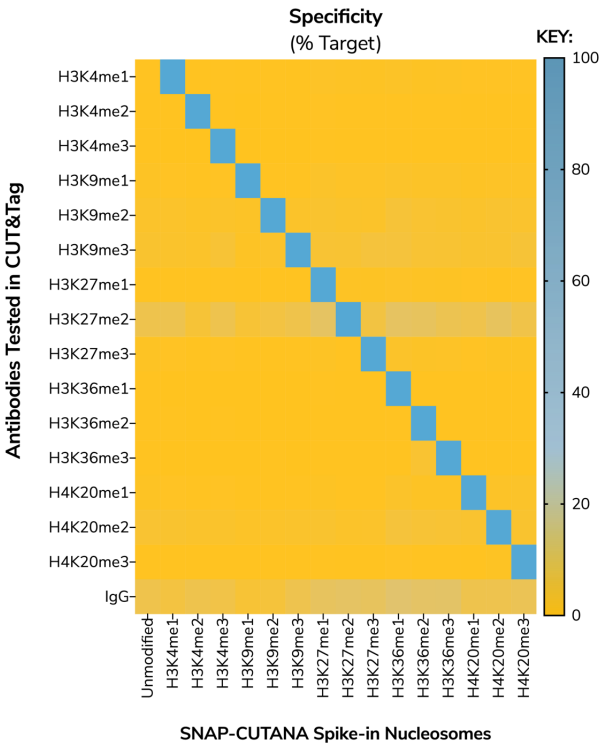

Figure 12: **EpiCypher's best-to-date CUT&RUN antibodies to lysine methylation PTMs.** Each row displays SNAP-CUTANA™ K-MetStat Spike-in data for a PTM antibody validated in CUT&RUN. Heatmap data for each antibody are normalized to DNA barcodes from the on-target PTM.

Question: *What does antibody cross-reactivity look like?*

EpiCypher recommends a cutoff of <20% binding to each off-target nucleosome in the K-MetStat panel. This is determined as described in **Section III** of this Appendix, using SNAP-CUTANA Spike-in data with **on-target** PTM barcode recovery set to 100%. Cross-reactivity <20% supports the accuracy of biological findings and interpretations[1]. As an example of how antibody cross-reactivity can affect NGS data, we show specificity profiles for one H3K4me3 and three H3K27me2 antibodies (CUT&RUN with SNAP-CUTANA K-MetStat panel in K562 cells: **Figure 13**). Read counts for each DNA-barcoded nucleosome were normalized to the stated PTM target and used to generate the heatmaps in **Figure 13A**. Note the anti-H3K4me3 and anti-H3K27me2 showing high specificity for their respective PTM target, relative to two H3K27me2 antibodies that displayed substantial cross-reactivity to H3K4me3 spike-in nucleosome. This cross-reactivity identified by SNAP-CUTANA Spike-ins was reflected by CUT&RUN sequencing from the cell sample (**Figure 13B**, discussed in detail below). Without the SNAP-CUTANA Spike-in Controls to flag antibody cross-reactivity, such data could misinform biological conclusions.

Question: *Is antibody cross-reactivity from spike-ins an accurate representation of antibody cross-reactivity in my sample chromatin?*

Figure 13B shows heatmaps of CUT&RUN data from K562 cells aligned to gene transcription start sites (TSS, +/- 2 kb) using antibodies characterized with SNAP-CUTANA K-MetStat Spike-in Controls (**Figure 13A**). Note the "anti-correlated" enrichment pattern from highly specific H3K4me3 and H3K27me2 antibodies (**Figure 13B**). The red boxes highlight a contaminating, TSS-enriched (*i.e.* H3K4me3-like) signal using the H3K27me2 antibodies that cross-react with H3K4me3. These results demonstrate that SNAP-CUTANA Spike-ins correctly predicted antibody specificity and performance in CUT&RUN cell sample data, in agreement with our previous studies using SNAP Spike-ins for ChIP-seq[1]. Thus, including

SNAP-CUTANA Spike-ins allows researchers to directly assess the accuracy of NGS data, providing a powerful tool for advanced epigenomics research.

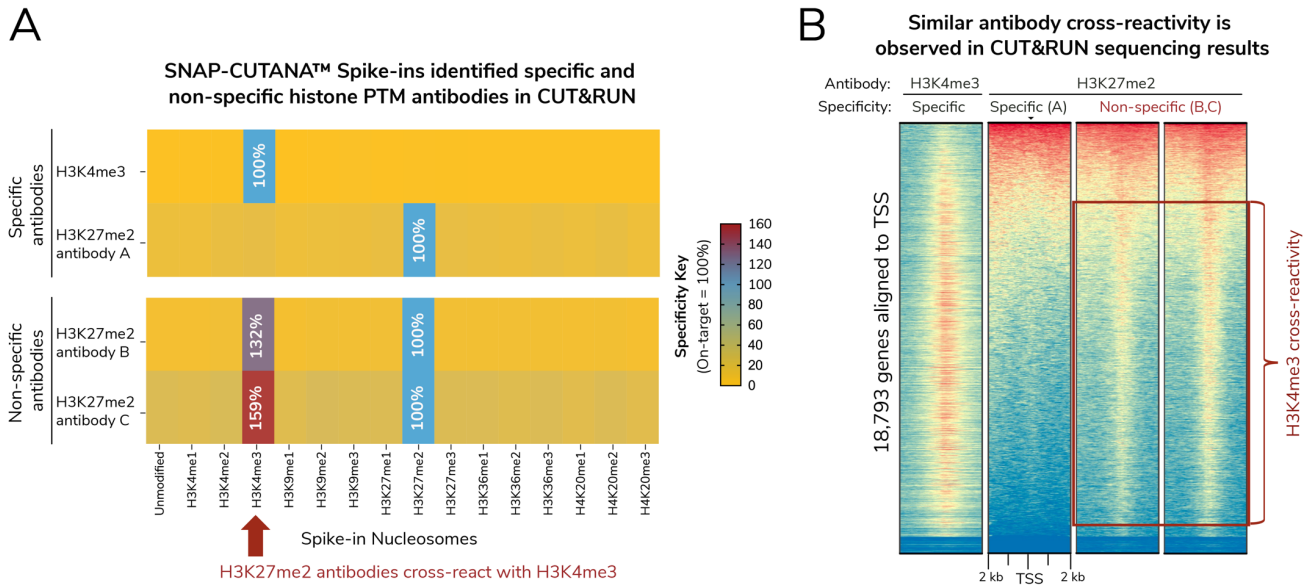

Figure 13: **Antibody cross-reactivity identified by SNAP-CUTANA Spike-ins (A) is reflected in CUT&RUN sequencing results (B).** (**A**) SNAP-CUTANA K-MetStat spike-ins were used to test H3K4me3 and H3K27me2 antibodies in CUT&RUN workflows. Data are shown normalized to on-target PTM. (**B**) CUT&RUN data from K562 cells was generated using the antibodies characterized in (**A**). Heatmaps display signal intensity +/- 2 kb from TSS. Gene rows in each heatmap are linked and ordered by signal intensity sorted by H3K4me3 specific antibody profile.

3. **Use SNAP-CUTANA Spike-ins to monitor experimental success in every possible reaction.** While EpiCypher CUTANA protocols are extensively optimized and robust, CUT&RUN/CUT&Tag reactions can occasionally go awry. Including a SNAP-CUTANA Spike-in Control Panel in every reaction identifies problematic experiments and guides troubleshooting. For now, we **minimally** suggest adding SNAP-CUTANA K-MetStat Spike-ins to control reactions containing the positive (*e.g.* H3K4me3) and negative (IgG) control antibodies. We strongly recommend including these controls in every experiment.

   However, the K-MetStat panel has obvious application to every covered lysine methylation target (me0-1-2-3 at H3K4, H3K9, H3K27, H3K36 and H4K20). Similarly comprehensive panels or targeted reagents are in development for histone lysine acylation (K-AcylStat), ubiquitylation (K-UbStat), arginine methylation (R-MetStat), histone oncomutations, histone variants, and even chromatin associated proteins (inquire for specific progress on each). Here we will discuss how SNAP-CUTANA Spike-ins can be used to identify aberrant CUT&RUN/Tag reactions. See **Table 8** for specific troubleshooting approaches.

Question: *What do successful assays look like using SNAP-CUTANA Spike-ins?*

In a successful assay to a given histone PTM, spike-ins reveal specific recovery of the target PTM with minimal background and/or cross-reactivity, and genomic enrichment patterns appear as expected. This is shown in **Figure 11** (explained above), and in **Figure 14** (below). In **Figure 14**, we used SNAP-CUTANA K-MetStat Spike-ins in CUT&RUN to map H3K4me3 and H3K27me3 from four independently prepared mouse primary B cell samples (10K cells each; protocol optimization pilot experiments with a multi-lab consortium). **Figure 14A** shows NGS results from the spike-in nucleosomes for each CUT&RUN reaction using the same antibodies. Samples 1-3 displayed expected antibody performance, with low cross-reactivity to off-target nucleosomes in the panel. These results were reflected in the sample sequencing data (**Figure 14B**), as Samples 1-3 generated expected peak structures and NGS results were consistent across samples. In contrast, Sample 4 had low signal-to-noise (**S:N**) in both the SNAP-CUTANA Spike-in data and the sample data (see next question).

Question: *What do failed assays look like using SNAP-CUTANA Spike-ins?*

In a failed experiment, an antibody with previously characterized specificity exhibits unusual behavior, such as cross-reactivity to an off-target PTM and/or high background. To demonstrate this in an experimental context, reference Sample 4 in **Figure 14**. In this experiment we used well-characterized H3K4me3 and H3K27me3 antibodies and added the

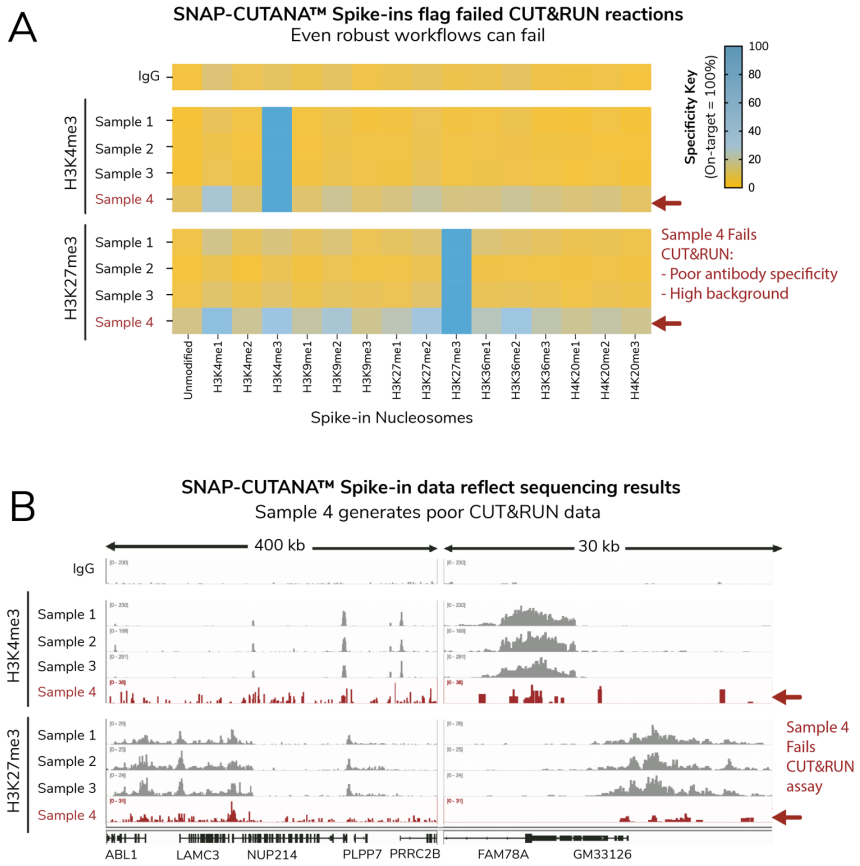

Figure 14: SNAP-CUTANA™ K-MetStat Spike-in Controls identify failed CUT&RUN reactions. CUT&RUN was used to map H3K4me3 and H3K27me3 in mouse B cells (four independently processed samples). **(A)** SNAP-CUTANA Spike-ins were included in each reaction. Heatmaps show antibody binding relative to on-target PTM; Sample 4 displays increased cross-reactivity (red arrows). (**B**) RPKM-normalized tracks from sample chromatin shows consistent peaks for Samples 1-3, while Sample 4 displayed low S:N and excessive background (red arrows).

K-MetStat Spike-in Panel to each reaction. Samples 1-3 show high antibody specificity. In contrast, Sample 4 displayed uncharacteristic high recovery of multiple off-target PTMs in the panel for both H3K4me3 and H3K27me3 reactions (**Figure 14A**, red arrows), suggesting high background and low S:N in these CUT&RUN reactions. When analyzing sample NGS data, we observed similarly low S:N for Sample 4, for both H3K4me3 and H3K27me3 (**Figure 14B**, red arrows).

Of note, all eight CUT&RUN reactions were performed in parallel using the same antibodies, but only Sample 4 displayed problems with S:N. In addition, both H3K4me3 and H3K27me3 reactions using Sample 4 cells displayed cross-reactivity against SNAP-CUTANA Spike-ins. Combined, these results suggest overall problems with the sample material *vs.* a simple failed reaction, and we focused our troubleshooting approaches on this aspect (in this case the consortium lab had delivered less than the expected 10,000 cells).

Importantly, these results illustrate the utility of including SNAP Spike-in Controls in all experiments. It may be unclear from the genome tracks alone that a reaction issue had occurred. **SNAP-CUTANA Spike-ins can flag failed reactions and indicate the cause**. By flagging failed samples and using the SNAP Spike-in results to guide troubleshooting, researchers can be confident in their experimental results.

Question: ***Why are SNAP-CUTANA Spike-ins better than other methods to identify failed reactions?***
Although outliers can be identified using other methods (*e.g.* separation in Principal Component Analysis [PCA], poor Pearson correlation), SNAP-CUTANA Spike-in Controls provide an added layer of experimental context. PCA/Pearson are only a proxy for assay stability and cannot determine which part of the experiment is at fault (*e.g.* antibody specificity, cell preparation, workflow). In contrast, SNAP-CUTANA Spike-in Controls provide a direct and quantitative readout of experimental success, guiding troubleshooting or providing confidence for researchers to proceed with data analysis and interpretation.

Question: ***How can I use SNAP-CUTANA Spike-ins to guide troubleshooting?***
When the spike-in controls work as expected, users can trust that their antibody was specific, and most CUT&RUN/CUT&Tag experimental conditions are optimal. If the spike-in results **and/or** genomic enrichment patterns are not as expected, the specific form of the data can provide valuable insights for diagnosing and troubleshooting the problem (**Table 8**).

Spike-in results	Genomic results	Result confirms	Troubleshooting approach
Specific, high S:N	Poor S:N	✓ pAG-MNase cleavage confirmed ✓ Optimal wash conditions ⚠ Cell integrity may be in question	➢ Confirm cell viability ➢ Try higher cell numbers ➢ Ensure target is present & localized to chromatin ➢ Use native or lightly cross-linked cells ➢ Optimize permeabilization conditions
Recover off-target PTMs	Good S:N, but unexpected peaks	⚠ Antibody cross-reactivity	➢ Test additional antibodies
Poor S:N	Poor S:N	⚠ Fundamental workflow failure	➢ Restart with recommended control cells & antibodies ➢ Assess bead clumping ➢ Prepare fresh buffers ➢ Try using our kit (EpiCypher 14-1048)
Specific, high S:N	High S:N, expected peaks	➢ Happy data analysis!	❌ None needed!

Table 8: Interpreting SNAP-CUTANA™ Spike-in and NGS results to guide troubleshooting. S:N, signal-to-noise.

4. **Use SNAP-CUTANA Spike-ins to normalize data and quantitatively compare samples.** In addition to profiling antibody specificity, guiding assay development, and monitoring assay success, SNAP-CUTANA Spike-ins can also be used to normalize NGS data for reliable, quantitative cross-sample comparisons. EpiCypher is currently investigating multiple normalization methodologies and will share recommendations when available. Below, we describe an emerging application of this exciting technology.

   Question: ***How does normalization to spike-ins compare with other normalization methods?***
   To demonstrate the application of SNAP-CUTANA Spike-ins for NGS normalization and drug response studies, we show a direct comparison of CUT&Tag data normalization using the K-MetStat Spike-ins vs. standard RPKM methods (**Figure 15**). Briefly, CAL27 squamous cell carcinoma cells were treated with either vehicle (0hr) or 3 µM of the EZH2 methyltransferase inhibitor tazemetostat (24, 48hr). CUT&Tag was performed with an antibody to H3K27me3 (CST #9733) using the EpiCypher CUTANA™ Direct-to-PCR CUT&Tag Protocol (epicypher.com/protocols). The SNAP-CUTANA K-MetStat Panel was added to each sample prior to antibody addition. Sequencing data were RPKM normalized (top panel) or normalized to the K-MetStat Spike-in Controls (similar to reported methods[2]; bottom panel).

   For spike-in normalization, a scale factor was calculated for each sample by dividing the percent of total reads aligned to human genome by the percent of total reads aligned to the spike-in barcodes (Scale Factor = % Human Reads / % Spike-in Reads) and applying this

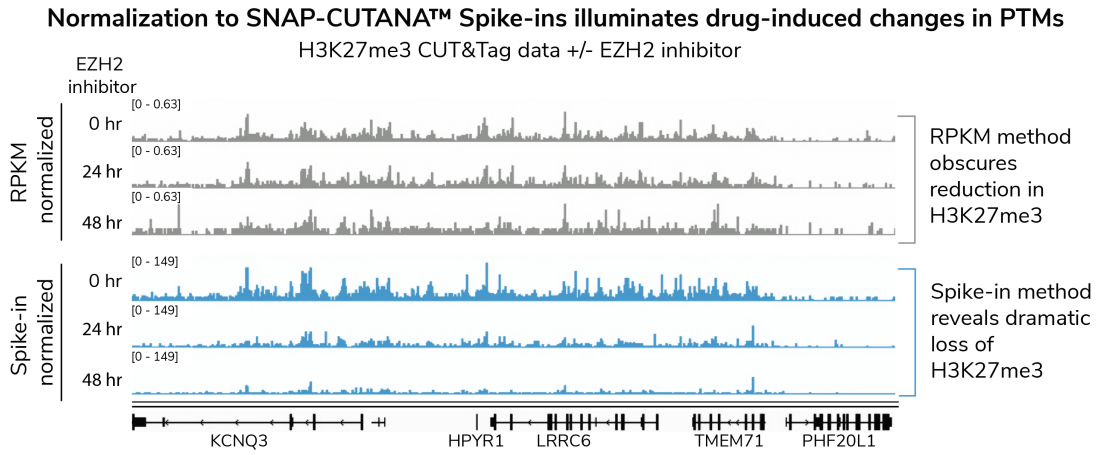

Figure 15: CUT&Tag data normalization using SNAP-CUTANA™ K-MetStat Spike-in Controls illuminates drug-induced differences in histone PTM enrichment. Cancer cells treated with the EZH2 inhibitor tazemetostat for the indicated times show a modest difference in H3K27me3 enrichment compared to vehicle (0hr) when using RPKM normalization. However, spike-in normalization reveals a pronounced and time-dependent reduction in H3K27me3 enrichment following treatment. *Special thanks to Drs. Yinglu Li, Xiao Chen and Chao Lu (Columbia University) for performing the experiment and sharing the data.*

factor to adjust the total sequencing reads of each respective sample. A very modest difference in H3K27me3 enrichment after EZH2 inhibition is observed in RPKM normalized samples. In contrast, spike-in normalization reveals a pronounced and time-dependent reduction in H3K27me3 enrichment following tazemetostat treatment, consistent with immunoblot analysis of total H3K27me3 levels (not shown). Thus, normalization using SNAP-CUTANA Spike-ins can illuminate drug-induced differences in histone PTM enrichment that are not discernable by other approaches.

Q: *What resources are there for developing normalization strategies using SNAP-CUTANA Spike-ins?*

Several methods have been reported for experimental normalization using exogenous spike-ins; these approaches can be adopted by calculating a single scalar normalization ratio using total read counts from SNAP-CUTANA Spike-ins:

SNAP-ChIP:

Tay *et al.* Hdac3 is an epigenetic inhibitor of the cytotoxicity program in CD8 T cells. *J. Exp. Med.* 217, e20191453 (2020). (PMID: 32374402)

Lam *et al.* Cell-type-specific genomics reveals histone modification dynamics in mammalian meiosis. *Nat. Commun.* 10, 3821 (2019). (PMID: 31444359)

ChIP-Rx:

Orlando *et al.* Quantitative ChIP-Seq Normalization Reveals Global Modulation of the Epigenome. *Cell Rep.* 9, 1163-1170 (2014). (PMID: 25437568)

5. **Use SNAP-CUTANA Spike-ins to push the boundary.** At EpiCypher, we use SNAP-CUTANA Spike-ins in all CUT&RUN and CUT&Tag reactions to monitor assay stability, define antibody specificity, and much more. We envision SNAP-CUTANA Spike-ins as an essential tool in broadening the clinical applications of CUT&RUN and CUT&Tag assays, particularly for low-input assays and the use of valuable patient samples. As these new technologies develop, quantitative controls are crucial to standardize workflows across lab environments and/or different experiments.

   Use SNAP-CUTANA Spike-in Controls to address key problems and tackle advanced applications in the field:

   - Optimize CUT&RUN/CUT&Tag assays for reduced cell numbers or rare sample types
   - Validate your workflow for new cell types/preparation methods
   - Standardize CUT&RUN/CUT&Tag assays for quantitative clinical experiments
   - Identify and avoid widely cited antibodies that are not fit for CUT&RUN/CUT&Tag assays

Section III: How to Analyze Sequencing Data Using SNAP-CUTANA™ Spike-in Controls

1. **Align paired-end sequencing reads to the SNAP-CUTANA Spike-in Controls and determine the number of reads assigned to each barcoded nucleosome in the panel.** Each spike-in nucleosome contains a unique, PTM-specific barcode (**Figure 8**) that will be detected by NGS. Importantly, each modified nucleosome is assembled using two distinct DNA barcoded templates, providing a technical replicate within each reaction. Thus, for the K-MetStat Spike-in Panel, which contains 16 PTM states, there are 32 DNA barcodes.

   These barcode sequences are not contained in mouse, human, fly, or yeast genomes, and thus require an independent alignment procedure. In addition, depending on the directionality of adaptor ligation during library prep, the barcode reads may be contained with R1 reads (adaptor P5) or R2 reads (adaptor P7) from paired-end sequencing. As a result, both R1 and R2 fastq files should be aligned to DNA barcodes.

   These steps are easily accomplished using the shell script available on the respective SNAP-CUTANA panel product page. A shell script is a .sh extension file that can be opened with any basic text editor program (*e.g.* TextEdit on a Mac or any text editing app). It should not be opened or saved as a PDF/Word Doc.

   The instructions for aligning sequencing reads to the spike-in DNA barcodes are contained in the shell script, and expanded upon here:

1. In Finder (on a Mac), duplicate the template shell script provided by EpiCypher to save an experiment-specific copy. It should be saved as a .sh file.
2. Download your R1 and R2 fastq.gz files. Unzip (extract) all fastq.gz to fastq on Mac or Linux by double-clicking on the .gz files. Place these fastq files and your shell script in a specific folder on your computer.
3. Open your experiment-specific shell script file in a text editor program.
4. Within the provided shell script, below the instructions, there is a "template loop" for barcode alignment. Copy&Paste the lines between "# template loop begin ##" and "# template loop end ##" (from the first "echo" to the last "done") in the shell script as many times as needed: you will need 1 template loop per paired-end R1 & R2 data set.
   Example: For three sequencing reactions, copy the template loop three times.
5. Next, replace the template file names in the loops (Sample1_R1.fastq and Sample1_R2.fastq) with your R1 and R2 file names. Each loop should contain matched R1 and R2 file names for a given sequencing reaction. Add the R1 fastq. file name in the first section of the loop, and the R2 fastq. file name in the second section of the loop.
   Example: I replace the dummy file names with file names for my H3K4me3 experiment. File names "H3K4me3_Rep1_R1.fastq" and "H3K4me3_Rep1_R2.fastq" go in the first loop, "H3K4me3_Rep2_R1.fastq" and "H3K4me3_Rep2_R2.fastq" go in the second loop, and so on.
6. If using a Linux or Mac operating system, use Terminal to run your script, per the instructions in the shell file. In Terminal, be sure to change the directory of Terminal to the location of your fastq. files.
   Example: My files are saved to my Desktop, in a folder called "NGS Analysis." On a Mac, I would open Terminal and type "cd" followed by a space, and then drag the folder onto the Terminal window to copy the file path. Press Return.
   Alternatively, you can also type in the path to your folder containing the fastq files (*eg*: cd path_to_fastq).
7. To execute your shell script in the Terminal application, type: "sh" followed by a space, and then drag the .sh file into Terminal to copy the file path. Press return to run the script. Again, you can also type this in manually by typing "sh filepath_to_shell.sh". Press return to run the script.
8. Terminal will generate read counts one "loop" (*i.e.* paired R1 & R2 file set) at a time, for all 32 barcodes. For each loop, the script will first generate read counts from R1 files, and then from the R2 files. The barcodes will be in the order listed under "# Barcode identities" in the shell script instructions, and datasets will also be annotated based on the filenames.
9. Now you can move forward with analysis in Excel.

2. **Use the provided Excel file on the SNAP-CUTANA Product Page to generate a heatmap of the spike-in reads.** When the barcode counts generated in Terminal are copied and pasted into the appropriate cells of the Excel file, a heatmap of the results (*e.g.* similar to that shown in **Figure 11A**) will automatically be generated. This heatmap will provide information

on the success of control reactions and antibody specificity. Instructions on using the Excel template:

a. The template consists of two sheets: the "K-MetStat" sheet, where you paste in your R1 and R2 data, and the "Output Table" which generates the full antibody specificity heatmap.
b. We provide space to copy in SNAP-CUTANA read count data for an IgG negative control, an H3K4me3 positive control, and 6 additional reactions (scroll down). Copy and paste to create additional sample analyses as needed.
c. Select your target(s) in the Excel sheet from the drop-down menu in Column B. Note that the Target is pre-set for the IgG and H3K4me3 analyses.
d. For each reaction, copy the R1 and R2 barcode read counts generated from running the script and paste into the appropriate highlighted yellow cells in Excel. Terminal generates the R1 and R2 read counts in the order we have provided on the Excel Template.
e. Once your Target is selected and your R1 and R2 reads are pasted in, the template will auto-generate a heatmap analysis normalized to the Target PTM (*i.e.* on-target will be set as 100% binding). Note that for IgG, binding is automatically normalized to the sum total of barcode reads.
f. Assess antibody binding specificity, starting with the control reactions. Antibody specificity data for all 8 samples are summarized in one large heatmap, found in the "Output Table" sheet in the Excel Template. Some guidance on using the controls:
   - IgG should not display specific enrichment for any SNAP-CUTANA Spike-in (all boxes yellow/orange).
   - H3K4me3 should display specific recovery of the H3K4me3 spike-in (blue) and <20% binding to all other PTMs (yellow).
   - Confirmation of these controls is considered a general readout of workflow success: *i.e.* cells were prepared properly, your pAG-MNase or pAG-Tn5 enzyme behaved as expected, library preparation and sequencing proceeded normally, etc.
g. Once these controls have been confirmed, proceed to analysis of other reactions/antibodies. Each antibody should display 100% binding to target (blue), and <20% binding to off-target PTMs.
   - **EpiCypher considers an antibody with <20% binding to all off-target PTMs specific and suitable for downstream data analysis.**
h. Finally, we recommend calculating the percentage of total reads attributed to SNAP-CUTANA Spike-ins for each reaction. To determine the percent of total reads assigned to the spike-ins, fill in the "Uniq align reads" with the full number of uniquely aligned reads from the NGS reaction. The total number of barcode reads will be auto-filled from R1 and R2 counts for each reaction. Percentages (% total barcode reads) will auto-populate.
   This is also referred to as the spike-in "bandwidth" and should be ~1% for reliable analysis of antibody specificity. However, the range may be higher or lower depending on target abundance and antibody used. For example:

- For H3K4me3 (low abundance target in cells), panel barcode reads are typically 1-10% of total sequencing reads.
- For H3K27me3 (high abundance target in cells), panel barcode reads are typically 0.1-1% of total sequencing reads.
- For IgG negative control antibody (no target present in sample), panel barcode reads are typically 10-20% of total sequencing reads.

Outside of this range, consider adjusting the spike-in dilution to be optimal for future experiments. The main goal is that thousands of sequencing reads are aligned to the spike-ins for adequate sampling of the panel and reliable use in antibody specificity assessment and data normalization.

Appendix III References

[1] Shah, R. N. *et al.* (2018) Examining the Roles of H3K4 Methylation States with Systematically Characterized Antibodies.

[2] Orlando *et al.* (2014) Quantitative ChIP-Seq Normalization Reveals Global Modulation of the Epigenome.