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.

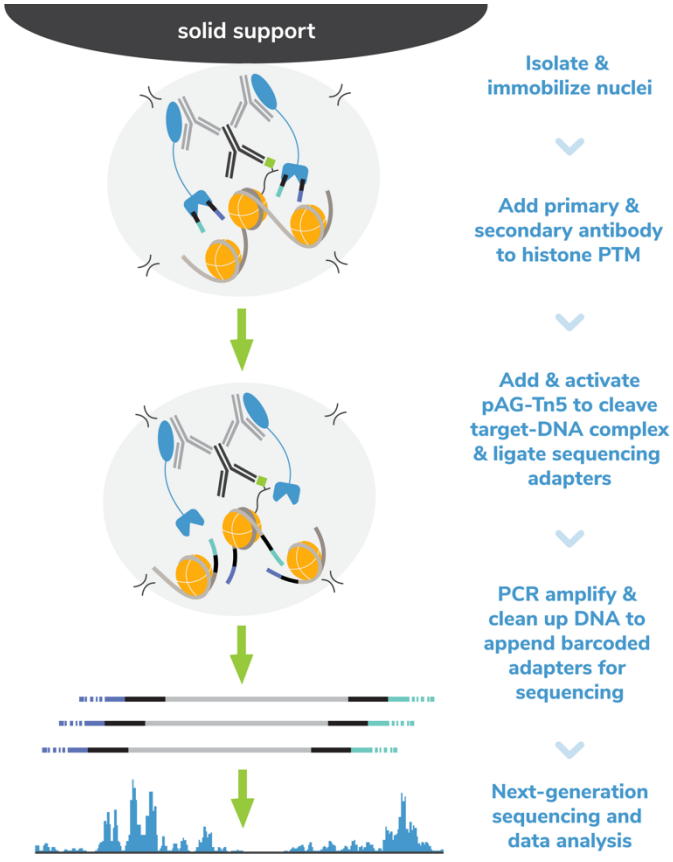

Figure 1. Overview of the CUTANA™ Direct-to-PCR CUT&Tag protocol.

2. CUTANA™ Products & Services: Advantages

CUT&RUN and CUT&Tag have revolutionized epigenomics studies. Compared to ChIP-seq, the current leading chromatin profiling approach, CUT&RUN/CUT&Tag offer multiple 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

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 mapping approach. EpiCypher now offers a suite of products to support CUT&RUN and CUT&Tag workflows under our CUTANA™ assay platform (epicypher.com/technologies/cutana), including:

- **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 DNA-barcoded semi-synthetic/recombinant nucleosome spike-in panels that control for all aspects of CUT&Tag workflows, including antibody specificity and assay success. Available for histone methyl-lysine PTMs (K-MetStat Panel: epicypher.com/19-1002), with additional panels in development.
- **CUT&Tag supporting reagents**, selected and validated for optimal performance in the EpiCypher CUT&Tag protocol (epicypher.com/technologies/cutana/cut-and-tag).
- ***Now Available*: CUT&Tag Core Reagents Bundle** (EpiCypher 14-1101) provides all the essential components required for CUT&Tag assays at a significant cost savings. Included are pAG-Tn5, ConA magnetic beads, control antibodies, anti-rabbit secondary antibody, the SNAP-CUTANA K-MetStat Panel, and PCR master mix for library amplification. For a detailed overview of this bundle and ordering information, visit epicypher.com/14-1101.
- **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**). We strongly recommend reading this section, the Experimental Design & Key Protocol Notes section, and the Protocol to plan your experiment and familiarize yourself with the assay.

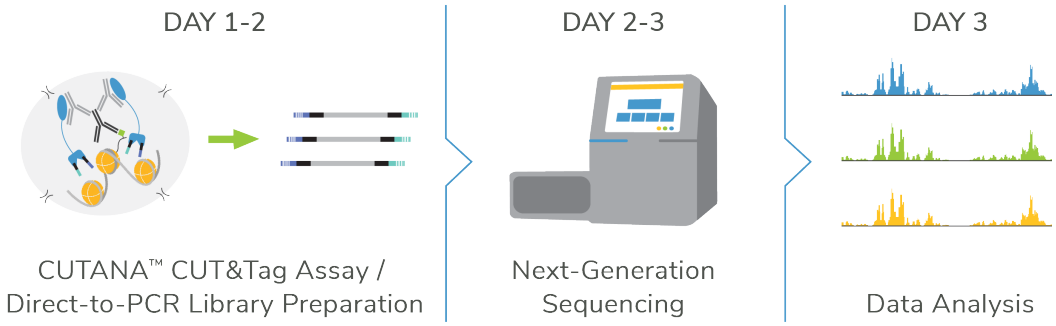

Figure 2: Timeline of CUT&Tag assay using EpiCypher's CUTANA™ Direct-to-PCR CUT&Tag protocol.

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, 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 **Section III** of the Protocol (*i.e.* added immediately before antibody addition; also see **Table 5**). The **SNAP-CUTANA Spike-in User Guide** (available at epicypher.com/19-1002) includes detailed information about how the spike-ins work 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 reactions 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 loss of sample/reaction volume 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 XP 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® or TapeStation®. In EpiCypher's experience, the minimal amount of DNA needed for accurate library quantification is >2 ng/µl 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 library to generate high signal-to-noise data (vs. 30 million or more with ChIP-seq). This allows researchers to pool >48 reactions 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 K-MetStat Panel analysis from sequencing datasets, see the **SNAP-CUTANA Spike-in User Guide**, available at epicypher.com/protocols.

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. If mapping chromatin-associated proteins (*e.g.* TFs, epigenetic enzymes), CUT&RUN is preferred.
2. We strongly recommend using bead-immobilized nuclei for CUT&Tag assays, which avoids undesirable tagmentation and sequencing of mitochondrial DNA. 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. See Appendix II for key Quality Control Checks, include evaluating the integrity of starting cells/nuclei and bead binding.
   - 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). These spike-ins should be added to reactions designated for 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, scale down to desired input.
   - 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 (optimized for 5,000 – 500,000 cells; see epicypher.com/CUT&RUN).

5. This 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 handling
   - Allow high-throughput reaction 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 library 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, 1 tab/10 mL)
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/reaction)
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, 1 tab/10 mL)
Filter sterilize. Store at 4ºC for up to 1 week.

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

Antibody150 Buffer (50 µL/reaction)
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, 1 tab/10 mL)
Filter sterilize. Store at 4ºC for up to 1 week.

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

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

TAPS Buffer (50 µL/reaction)
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/reaction)
10 mM TAPS, pH 8.5
0.1% SDS
Store at RT for up to 6 months.

SDS Quench Buffer (15 µL/reaction)
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

Note: **A CUTANA™CUT&Tag Core Reagent Bundle** is now available (epicypher.com/14-1101). This reagent bundle includes pAG-Tn5, ConA beads, control antibodies, anti-rabbit secondary antibody, the SNAP-CUTANA K-MetStat Panel (20 reactions), and High Fidelity 2X PCR Master Mix for library amplification.

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 the User Guide at epicypher.com/19-1002. **NOTE: Store at -20˚C.** Lower temperatures can cause freezing and will permanently damage the beads. Pipette-mix (**do NOT vortex**) before use.
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.

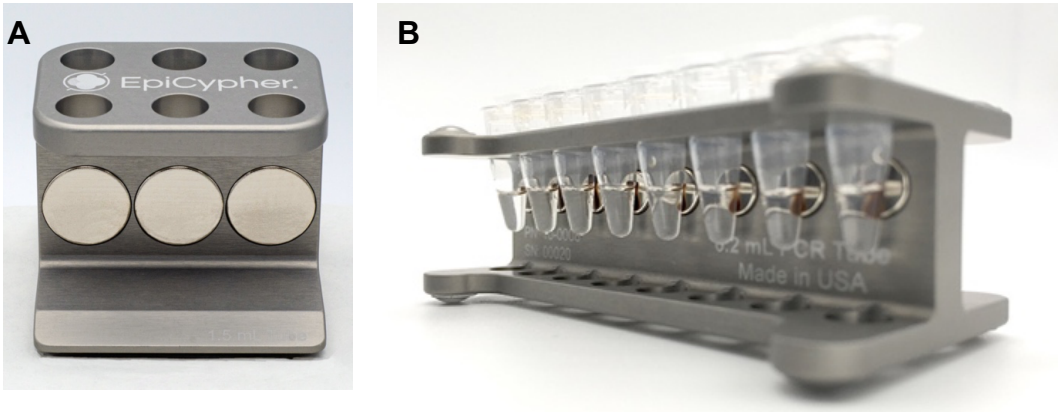

Figure 3: Magnetic racks for CUT&Tag assays. (A) For batch processing of ConA beads, use a 1.5 mL magnetic rack (*e.g.* EpiCypher 10-0012, pictured). **(B)** For processing reactions in 8-strip PCR tubes we recommend using a multi-channel pipette and compatible magnetic rack (*e.g.* EpiCypher 10-0008, 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 FAQs 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 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 for positive (H3K4me3) and negative (IgG) control antibodies, as well as reactions 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**.
    Notes:
    - Prior to use, mix the K-MetStat Panel by pipetting. Do NOT vortex.
    - SNAP-CUTANA Spike-ins must be added before addition of Primary Antibody.
    - Aim for spike-ins to comprise ~1% of total sequencing reads; adjust amount added as needed. This bandwidth is typically higher for low abundance targets/negative controls (*e.g.* IgG = 10-20%) and lower for high abundance targets (*e.g.* H3K27me3 = 0.1-1%).
    - Refer to the SNAP-CUTANA Spike-in User Guide (epicypher.com/19-1002) for more info.

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 30-31** 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 46**).
    g. 1 min at 72ºC → Final extension
    h. Hold at 4ºC
45. DNA cleanup using **1.3x AMPure beads** : reaction 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**.
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.

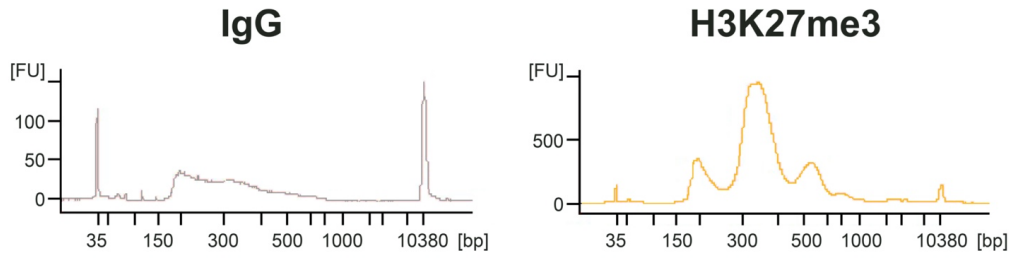

Figure 5. Typical Bioanalyzer® traces for IgG negative control and H3K27me3 positive control CUTANA™ CUT&Tag sequencing libraries (1 µL PCR amplified/AMPure purified libraries loaded on Bioanalyzer). The positive control H3K27me3 library is predominantly enriched for mononucleosomes as indicated by the peak at ~300 bp (~170 bp mononucleosomes + ~125 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 reactions 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 reactions with an antibody to a methyl-lysine histone PTM target).

   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. Circumventing chromatin purification steps minimizes sample loss and 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 library, you can multiplex reactions, 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 (EpiCypher 19-1002). 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 NGS 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 epicypher.com/19-1002 for product and ordering information and to download the **SNAP-CUTANA Spike-in User Guide**, which contains detailed guidance on their application in CUT&Tag assays and how to analyze spike-in data from NGS results.

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.com/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 and CUT&Tag 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™ Cross-linking Protocol** for cells/nuclei is compatible with CUT&RUN and 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*).

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. EpiCypher has recently launched the **SNAP-CUTANA K-MetStat Panel** and are using these defined spike-ins controls to identify best-in-class histone PTM antibodies for CUT&RUN and CUT&Tag. EpiCypher's H3K4me3 antibody (EpiCypher 13-0041) has been verified to exhibit robust and specific performance in CUT&Tag, and is SNAP-certified for CUT&RUN assays. Additional targets are being tested. 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

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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.

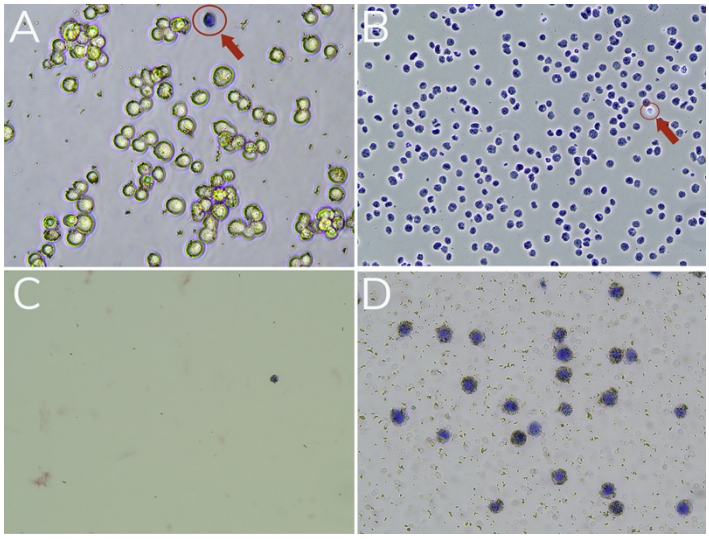

Figure 6: Representative images of cell and nuclei samples for ConA Bead binding. Samples were stained with Trypan blue and visualized under brightfield microscope. **(A) Cells** before bead binding. A dead cell (blue; Trypan positive) is circled in red, whereas the remaining live cells are bright white 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.