EpiCypher® CUTANA™ Direct-to-PCR CUT&Tag Protocol
Optimized for Histone Post-Translational Modifications (PTMs)

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1. Overview

Cleavage Under Targets & Tagmentation (CUT&Tag) is a breakthrough genomic mapping strategy that builds on its predecessor immunotethering technology, CUT&RUN[1]. In CUT&Tag, nuclei (or cells) are immobilized to a solid support. A fusion of proteins A and G with prokaryotic transposase 5 (pAG-Tn5) is then used to selectively cleave DNA and insert sequencing adapters at antibody-bound chromatin (**Figure 1**). In the CUTANA™ CUT&Tag Protocol, these tagmented fragments are PCR amplified *in situ* to yield sequence-ready DNA[1]. Only 5-8 million total sequencing reads are needed for robust profiles.

CUTANA™ CUT&Tag can be used for projects that require histone post-translational modification (PTM) mapping using ultra-low cell numbers (100,000 down to 10,000 cells). Our protocol is optimized for 8-strip tubes and multichannel pipetting to allow high-throughput processing and improved sample handling. The exclusive direct-to-PCR strategy allows the entire reaction to be performed in a single tube, resulting in a rapid workflow with exquisite sensitivity. However, as with any protocol, there are some considerations for optimal results:

- This Do-It-Yourself (DIY) CUT&Tag Protocol may be challenging for researchers new to epigenomics. For more guidance, we recommend our CUTANA™ CUT&Tag Kit (epicypher.com/14-1102), which comes with everything you need to go from cells to sequence-ready libraries.
- CUTANA™ CUT&Tag should not be used to map chromatin-associated proteins, such as transcription factors; for these cases, we recommend CUTANA™ CUT&RUN assays (epicypher.com/cut-and-run).
- For a more detailed comparison of CUT&Tag and CUT&RUN, read our blog: epicypher.com/resources/blog/chipseq-vs-cutrun-vs-cuttag-which-should-you-use/.

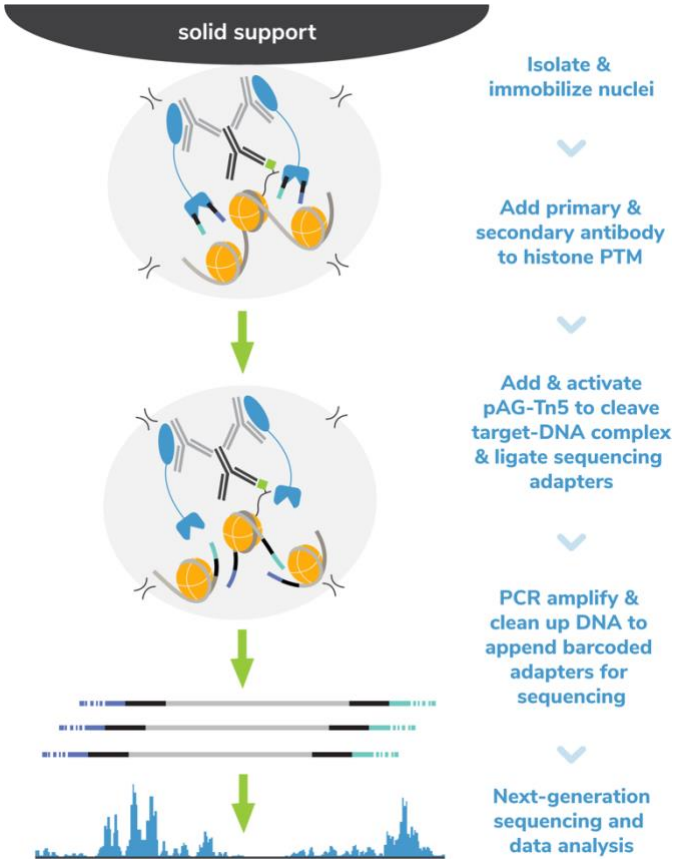

Figure 1. Overview of the CUTANA™ Direct-to-PCR CUT&Tag protocol.

2. Experimental Design and Key Protocol Notes

This section is considered essential reading for CUTANA™ Direct-to-PCR CUT&Tag assays.

Inputs:

- Freshly isolated, native (i.e. unfixed) nuclei are the preferred input for CUT&Tag.
- The recommended input is 100,000 nuclei per reaction. Harvest 100,000 cells per reaction plus 10% excess to account for sample loss during nuclei prep.
  - Using more than 100,000 nuclei does NOT improve yield and may inhibit PCR.
- To confirm high quality sample prep, see the Trypan Blue staining protocol in **Appendix I**. These quality control checks are crucial to experimental success. Samples are assessed at three points: at initial cell harvest, after nuclei isolation, and after nuclei are bound to ConA beads.
- If using whole cells, adherent cells, tissues, cryopreserved samples, or cross-linked cells, see the CUTANA™ CUT&Tag Kit Manual (epicypher.com/14-1102).

Targets:

- This CUT&Tag protocol is only validated for histone PTMs. If mapping chromatin-associated proteins, such as transcription factors, CUT&RUN is recommended (epicypher.com/cut-and-run).

Antibodies:

- Use a highly specific and efficient antibody that has been validated in CUT&Tag.
- Antibodies that work well in ChIP-seq are NOT guaranteed to work in CUT&Tag.
- See optimization guidelines on the following page for help with screening antibodies, or epicypher.com/resources/blog/how-to-cut-and-tag.
- For further guidance and recommendations, reach out to techsupport@epicypher.com.

Control Reactions:

- Include reactions with the following control antibodies in each experiment:
  - Negative control antibody: IgG, EpiCypher 13-0042.
  - Positive control antibody, low abundance target: H3K4me3, EpiCypher 13-0060.
  - Positive control antibody, high abundance target: H3K27me3, EpiCypher 13-0055.
  - Spike each control reaction with the SNAP-CUTANA™ K-MetStat Panel (EpiCypher 19-1002) as instructed to validate your CUT&Tag workflow, guide troubleshooting, and monitor experimental success. See the SNAP-CUTANA™ Spike-in User Guide (epicypher.com/19-1002) for details.
- For experimental reactions targeting histone lysine methylation PTMs, add the SNAP-CUTANA™ K-MetStat Panel (EpiCypher 19-1002) for antibody validation. See the SNAP-CUTANA™ Spike-in User Guide (under Documents & Resources at epicypher.com/19-1002) for more information.

Optimize for new antibodies, new cell types, and/or low inputs:

- First, validate CUT&Tag in your lab using control antibodies (H3K4me3, H3K27me3, IgG) and 100,000 native nuclei per reaction. For new users, we recommend using nuclei from a cell line.
- Once the workflow is validated, test antibodies to your experimental target. Source 3-5 antibodies from various vendors – see if an EpiCypher antibody is available at epicypher.com/antibodies.
- Test antibodies in parallel, and select the antibody that gives the best balance of yields, high signal-to-noise ratios, and expected target enrichment. Include control reactions as outlined above.
- After selecting an antibody, optimize the CUT&Tag workflow for your specific cell type, including processing conditions, cell stimulation, or drug treatment. Include control reactions as outlined above.
- Once conditions are optimized for the target and cell type, scale down to desired number of nuclei. This protocol has been validated for select PTMs down to 10,000 nuclei.
- Note that PTM antibodies that perform well at 100,000 nuclei may fail at lower input. See the CUTANA™ CUT&Tag Kit Manual (epicypher.com/14-1102) for further guidance.

How to know if experiment worked before sequencing:

- Consider total DNA yields, fragment size distribution, and library concentration, using the suggested metrics outlined below. Combined, these quality control checks support overall assay success.
- **Total DNA yields following indexing PCR:** There is no typical yield for CUT&Tag assays. Instead:
  - Aim for ≥ 2ng/µL or ~30 ng total DNA, which will allow for accurate quantification.
  - Compare yields from positive and negative controls. In general, H3K27me3 yields should be > IgG. Yields for H3K4me3 are often similar to or just slightly higher than those for IgG.
- **Library fragment size distribution:** The single best indicator of success prior to sequencing is enrichment of ~300 bp fragments in sequencing libraries, assessed by Bioanalyzer or TapeStation.
  - Traces may show an oligonucleosome ladder with peaks every ~170 bp. This periodicity does not impact sequencing and size selection is not necessary.
  - Primer dimers may appear at ~25-100 bp, and are more common when profiling from low inputs. If primer dimers comprise >5% of a library, remove them using the CUTANA™ Quick Cleanup DNA Purification Kit (EpiCypher 14-0052, epicypher.com/14-0052).
- **Library concentration (200-700 bp region):** Aim for library molarity ≥0.5 nM, which will allow pooling at standard concentrations for Illumina® sequencing.
- For further guidance, see the CUTANA CUT&Tag Kit Manual (epicypher.com/14-1102).

Sequencing:

- Sequence libraries to a depth of 5-8 million total reads. The goal is to generate 3-5 million unique reads per library.
- Paired-end sequencing (2 x 50 bp) is recommended. Longer read lengths can be used, but will require adapter trimming prior to bioinformatic analysis.
- For sequencing data analysis, refer to the CUTANA CUT&Tag Kit Manual (epicypher.com/14-1102).

3. Buffers, Reagents and Materials Needed

Buffer Components and Reagent Preparation

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
CUTANA™ Protease Inhibitor Tablets	EpiCypher	21-1027
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
Optional Nuclei Extraction Buffer*	EpiCypher	21-1026

Reagent Prep

1M spermidine: Dissolve 1 gram spermidine (MW = 254.63) in 3.93 mL molecular biology grade H_2O. Filter sterilize using a 0.22 µM filter. *Store in single-use aliquots at -20°C for 6 months.*

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

5% digitonin stock solution: Prepare in DMSO for optimal detergent solubility and protocol reproducibility (compared to preparation in heated H_2O). *Aliquots can be stored at -20ºC for 6 months.*

> Digitonin is added to buffers to help prevent ConA bead from precipitation/clumping and forming a thin film on tubes. It is not required for nuclei permeabilization.

10mM TAPS: 10 mM TAPS, pH 8.5. *Store at RT for up to 6 months.*

25X Protease Inhibitor: Dissolve one CUTANA™ Protease Inhibitor Tablet in 420 µL (15-1027-S) or 2 mL (15-1027-L) molecular biology grade H_2O. *Aliquots can be stored at -20ºC for 6 months.*

* Nuclei Extraction Buffer is available for purchase at epicypher.com/21-1026. The recipe is also provided on the next page.

Buffer Recipes

Stock Buffers – Can be prepared in advance

Pre-Nuclei Extraction (NE) Buffer
(**Note:** Available for purchase as part of EpiCypher 21-2026; epicypher.com/21-2026)
20 mM HEPES–KOH, pH 7.9
10 mM KCl
0.1% Triton X-100
20% Glycerol
Stable at 4°C for up to 6 months.

Bead Activation Buffer
20 mM HEPES, pH 7.9
10 mM KCl
1 mM $CaCl_2$
1 mM $MnCl_2$
Filter sterilize, 0.22 µM filter. Store at 4°C for up to 6 months.

Pre-Wash150 Buffer
20 mM HEPES, pH 7.5
150 mM NaCl
Filter sterilize, 0.22 µM filter. Store at 4°C for up to 6 months.

Pre-Wash300 Buffer
20 mM HEPES, pH 7.5
300 mM NaCl
Filter sterilize, 0.22 µM filter. Store at 4°C for up to 6 months.

SDS Release Buffer
10 mM TAPS, pH 8.5
0.1% SDS
Store at RT for up to 6 months.

SDS Quench Buffer
0.67% Triton-X 100 in molecular biology grade H_2O
Store at RT for up to 6 months.

Buffers prepared FRESH for each CUT&Tag experiment

Nuclei Extraction (NE) Buffer (250 µL/reaction)
(**Note:** Pre-NE Buffer + spermidine available for purchase; epicypher.com/21-2026)
Pre-NE Buffer
0.5 mM Spermidine
1X CUTANA™ Protease Inhibitor
After spermidine and CPI are added, store at 4ºC for up to 1 week.

Digitonin150 Buffer (600 µL/reaction)
Pre-Wash150 Buffer
0.5 mM Spermidine*
1X CUTANA™ Protease Inhibitor
0.01% Digitonin
Prepare fresh for each experiment and store at 4ºC.

Antibody150 Buffer (60 µL/reaction)
Digitonin150 Buffer
2 mM EDTA
Prepare fresh for each experiment and keep on ice until use.

Digitonin300 Buffer (500 µL/reaction)
Pre-Wash300 Buffer
0.5 mM Spermidine
1X CUTANA™ Protease Inhibitor
0.01% Digitonin
Prepare fresh for each experiment and store at 4ºC.

Tagmentation Buffer (60 µL/reaction)
Digitonin300 Buffer
10 mM $MgCl_2$
Prepare on Day 2 of each experiment and keep on ice.

85% EtOH (400 µL/reaction)
85% ethanol
Prepare fresh for each experiment.

0.1X TE (20 µL/reaction)
1 mM Tris-HCl pH 8.0
0.1 mM EDTA, pH 8.0 (NaOH)

Assay Reagents

Item	Vendor, Cat. No.	Notes
Concanavalin A (ConA) Paramagnetic Beads	EpiCypher 21-1401	To immobilize nuclei/cells. ConA can cause immune cell activation; use nuclei for immune cell studies.
CUTANA™ pAG-Tn5	EpiCypher 15-1017/15-1117	20X stock. 50 & 250 reaction pack sizes available.
SNAP-CUTANA™ K-MetStat Panel	EpiCypher 19-1002	Spike-in control for reactions mapping histone lysine methylation PTMs. Use with control antibodies to IgG, H3K4me3, and H3K27me3. Can also be added to reactions targeting any other PTM in the panel. **Store at -20˚C.** Lower temperatures can cause freezing and will permanently damage the beads. Pipette-mix the stock (**do NOT vortex**) before dispensing to reactions. The Panel is a pool of DNA-barcoded nucleosomes carrying histone methyl-lysine PTMs (me1, me2, or me3 on H3K4, H3K9, H3K27, H3K36, and H4K20). Nucleosomes are bound to magnetic streptavidin beads for easy one-step addition to CUT&Tag. DNA barcodes enable detection of on- and off-target PTM recovery in sequencing data. See the User Guide at epicypher.com/19-1002.
Rabbit IgG Negative Control Antibody	EpiCypher 13-0042	Add 0.5 µg to negative control reactions in CUT&Tag.
H3K4me3 and H3K27me3 SNAP-Certified™ Antibodies	EpiCypher 13-0060 EpiCypher 13-0055	Add 0.5 µg to designated positive control reactions in CUT&Tag. We recommend including separate H3K4me3 and H3K27me3 positive control reactions in each experiment.
Antibodies to target of interest	User-dependent	Start with 0.5 µg and titrate as needed. We continue to conduct extensive antibody screening[2]. For recommendations, contact techsupport@epicypher.com.
Anti-Mouse Secondary Antibody	EpiCypher 13-0048	Use with primary antibodies made in mouse.
Anti-Rabbit Secondary Antibody	EpiCypher 13-0047	Use with primary antibodies made in rabbit.
CUTANA™ Non-Hot Start 2X PCR Master Mix	EpiCypher 15-1018	Must be the non-hot start version of Q5 for CUT&Tag.
Universal i5 Primer	IDT	Primer sequence provided below.
Uniquely Barcoded i7 Primers	IDT	Primer sequences provided below.
CUTANA™ DNA Purification Beads	EpiCypher 21-1407	For cleanup of PCR-amplified sequencing libraries.
Qubit™ 1x dsDNA HS Assay Kit	Thermo Fisher Scientific Q33230	For quantification of purified CUT&Tag sequencing libraries.
Agilent TapeStation® or Bioanalyzer® kits	TapeStation: D1000 reagents Agilent 5067-5582 & 5583. Bioanalyzer: High Sensitivity DNA Kit Agilent 5067-4626.	For fragment size distribution analysis of purified CUT&Tag sequencing libraries.

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

Name	Sequence (5’ → 3’)	Oligo Barcode	Expected 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.

Equipment

Item	Vendor, Catalog No.	Notes
1.5, 15, 50 mL tubes and centrifuge	Various	For making buffers, harvesting cells, etc.
8-strip 0.2 mL tubes	EpiCypher 10-0009	The CUT&Tag Protocol is optimized for 8-strip tubes for streamlined sample handling, higher throughput, and improved reproducibility.
1.5 mL Magnetic Separation Rack	EpiCypher 10-0012	For bulk processing of ConA beads in **Section I** of CUT&Tag protocol.
8-strip PCR tube Magnetic Separation Rack	EpiCypher 10-0008	For processing CUT&Tag in 8-strip tubes.
High Performance Multi-Channel Pipettors, 8-Channel	Various, e.g. VWR 76169-250	For performing CUT&Tag in 8-strip tubes.
Multi-channel reagent reservoirs	Various, e.g. Thermo Fisher Scientific 14-387-072	For dispensing buffers when using multi-channel pipettors.
Vortex	Various, e.g. Vortex Genie, Scientific Industries SI-0236	For bead mixing steps.
Benchtop mini-centrifuge with 8-strip tube adapter	Various, e.g. from Fisher Scientific, Benchmark Scientific	For collecting liquid from sides of tubes/caps after vortexing.
Tube Nutator	Various, e.g. VWR 82007-202	For bead incubation steps, to keep beads in solution/mixing.
Thermocycler with a heated lid	Various: e.g. from Biorad, Applied Biosystems, Eppendorf.	For incubation steps and indexing PCR.
Qubit™ 4 Fluorometer	Thermo Fisher Scientific Q33226	For quantification of CUT&Tag sequencing libraries.
Agilent 2100 Bioanalyzer® or TapeStation®	Agilent	For fragment distribution analysis of purified CUT&Tag sequencing libraries. For platform details see: agilent.com/en/product/automated-electrophoresis

4. EpiCypher CUTANA™ Direct-to-PCR CUT&Tag Protocol

---Day 1---

Section I: ConA Bead Activation (~30 min)

Preparation of ConA beads for CUT&Tag. ***Note*** *that ConA bead stocks should be brown, and beads should be easily resuspended by gentle pipetting. Do NOT use ConA beads that have been frozen or appear black, granular, or clumpy, as this indicates bead damage. During the protocol, do NOT scrape beads with pipette tip to dislodge from tube wall, as this will damage the coated beads.*

1. Fully resuspend ConA beads by gently pipetting. Transfer 11 µL per reaction to a 1.5 mL tube.
2. Place tube on a 1.5 mL magnetic separation rack and allow slurry to clear. Pipette to remove supernatant, without disturbing beads with pipette tip. Move quickly to avoid ConA bead dry-out.
3. Remove tube from magnet and immediately add 100 µL per reaction cold Bead Activation Buffer. Pipette to resuspend. Return tube to magnet, allow slurry to clear, and pipette to remove supernatant.
4. Repeat the previous step one time, for a total of two washes. Move quickly to avoid ConA bead dry-out.
5. Resuspend beads in 11 µL per reaction cold Bead Activation Buffer.
6. Aliquot 10 µL per reaction activated ConA beads into 8-strip tubes. Place on ice.

Section II: Nuclei Prep and Binding to ConA Beads (~30 min)

Good sample prep is critical for CUT&Tag. In this section, you will harvest cells, extract nuclei, and couple nuclei to ConA beads. ***Note*** *that this protocol is designed for fresh, native suspension cells – for other sample types, please refer to the CUTANA CUT&Tag Kit Manual (epicypher.com/19-1001).*

7. Count cells, confirm expected morphology, and determine cell viability (as in **Appendix I**). Ideally, cells should be at least 80% viable, unclumped and with minimal cell lysis or debris. However, ideal viability may vary by sample type, treatments, or processing conditions. The goal is to harvest cells with good integrity and minimal lysis, which will help minimize background in sequencing data.
8. Transfer 100,000 cells per reaction (plus 10% excess) to a 1.5 mL tube.
9. Spin cells 600 x g for 3 min at room temperature (RT). Remove supernatant and resuspend cells in 100 µL per reaction cold Nuclei Extraction Buffer.
10. Incubate for 10 min on ice.
11. Spin 600 x g for 3 min at 4ºC. Pipette to remove supernatant. The pellet should change from pale yellow (cells) to a white, fluffy pellet (nuclei).
12. Gently resuspend nuclei in 105 µL per reaction cold Nuclei Extraction Buffer.
13. Take a 10 µL aliquot to examine nuclei integrity using Trypan Blue staining (as in **Appendix I**). Nuclei should be >95% Trypan Blue positive, unclumped, and show minimal lysis.
14. Transfer 100 µL nuclei per reaction to 10 µL ConA beads in 8-strip tubes. Gently vortex to mix (setting #7) and quick spin in a mini-centrifuge to collect slurry (beads should not settle).
15. Incubate bead-nuclei slurry for 10 min at RT. Nuclei will adsorb to activated ConA beads.

16. Place tubes on an 8-strip tube magnetic rack and allow slurry to clear. Transfer 10 µL supernatant to a 1.5 mL tube and place on ice; use to confirm bead binding in Step 19, below.
17. Pipette to remove and discard remaining supernatant in 8-strip tubes. Quickly add 50 µL cold Antibody150 Buffer to each reaction to avoid ConA bead dry-out.

    A multi-channel pipettor can be used for adding and removing buffers in 8-strip tubes. Use multi-channel reagent reservoirs on ice to dispense buffers. Always remove and replace buffers one strip at a time to avoid ConA bead dry-out. Dried out ConA beads precipitate in solution, become clumpy, and result in sample loss – all of which impact downstream data.
18. Remove tubes from magnet and pipette to resuspend. Transfer 10 µL of the slurry to a 1.5 mL tube.
19. Use the 10 µL aliquots of supernatant (Step 16) and slurry (Step 18) and confirm nuclei binding to ConA beads (as in **Appendix I**). It is critical to confirm the quality of your sample prep prior to starting CUT&Tag.

Optional: Add SNAP-CUTANA K-MetStat Panel before primary antibody.

*The K-MetStat Panel can be added to reactions for me1, me2, or me3 on H3K4, H3K9, H327, H3K36, or H4K20. Do **NOT** add to reactions targeting proteins or PTMs that are not in the K-MetStat Panel.*

The panel must be added prior to addition of antibody. We recommend adding the K-MetStat Panel to reactions designated for positive (H3K4me3, H3K27me3) and negative (IgG) control antibodies.

20. Quick spin the K-MetStat Panel stock tube and pipette to resuspend - do NOT vortex stock tube.
21. Add the K-MetStat Panel to designated reactions. The amount of K-MetStat Panel to add is scaled to the number of nuclei per reaction, and can be found in **Table 1**. If using the recommended 100,000 nuclei per reaction, add 2 µL K-MetStat Panel stock to the designated reactions. If using less than 100,000 nuclei, dilute the panel as instructed in **Table 1**.

Number of nuclei	Panel dilution	Volume added
100,000	Stock	2 µL
50,000	1:2	2 µL
20,000	1:5	2 µL
10,000	1:10	2 µL

Table 1: Scale the amount of SNAP-CUTANA Panel to the number of nuclei. For less than 100,000 nuclei, prepare a dilution of the Panel stock in Antibody150 Buffer the day of the experiment. Discard the dilution after use.

22. Gently vortex reactions to mix and quick spin 8-strip tubes to collect liquid in tube bottom.

Section III: Primary Antibody Binding (~30 min and overnight)

Nuclei are incubated with a target-specific primary antibody overnight, gently rocking on a nutator.

23. Retrieve primary antibodies. Gently flick to mix and quick spin to collect liquid.
24. Add 0.5 µg primary antibody (or manufacturer's recommendation for CUT&Tag assays) to each reaction. **Note** that antibodies stored in glycerol may be viscous: pipette carefully. Aspirate slowly, check tip for accuracy, and pipette up and down 3 times into reaction to fully clear glycerol from tip.
25. Gently vortex tubes to mix and quick spin. Incubate overnight on a gently rocking nutator at 4˚C. Slightly elevate cap side of 8-strip tubes to ensure bead solution remains in bottom of tube (**Figure 2**). Do **NOT** rotate tubes end-over-end, as beads will get stuck in the caps of tubes and dry out, reducing yields.

Figure 2. Ensure tube caps are elevated during incubation steps on tube nutator to keep beads in solution.

---Day 2---

Section IV: Secondary Antibody Binding (~1 hr)

Nuclei are incubated with a species-specific secondary antibody to help amplify pAG-Tn5 binding and tagmentation efficiency. ***Note*** *that resuspension of ConA beads is essential for CUT&Tag success. Users often note that ConA beads become clumpy or sticky after the overnight incubation. We provide specific instructions on bead mixing in this protocol.*

26. Prepare a Secondary Antibody Master Mix in a fresh 1.5 mL tube: per reaction, combine 50 µL cold Digitonin150 Buffer and 0.5 µg Secondary Antibody. **Note** that the secondary antibody must be matched to primary antibody host species (e.g. mouse, rabbit). Mix by pipetting, quick spin to collect liquid, and place on ice.
27. Retrieve 8-strip tubes from 4˚C incubation on the nutator. You may see some bead settling in the bottom of the tubes, which is normal (**Figure 3**). Quick spin to collect liquid. Place tubes on magnet, allow slurry to clear, and pipette to remove supernatant.
28. Remove tubes from magnet. Add 50 µL cold Secondary Antibody Master Mix to each reaction, working quickly to avoid bead dry-out. **General bead slurry mixing instructions**: Resuspend slurry by gently pipetting and/or vortexing. If pipetting, avoid losing beads in tips and expel all material back into the tube. The end of a pipette tip can be cut off to help preserve delicate samples or to help mix plant nuclei. Vortexing can be used help resuspend beads and is recommended for viscous samples. Always quick spin tubes after mixing to avoid sample loss.

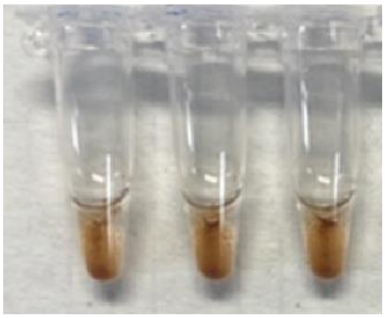

Figure 3. ConA beads settle to bottom of tubes after overnight incubation at 4˚C.

29. Quick spin tubes and place on nutator, caps elevated, for 30 min at RT.
30. Gently vortex tubes ~5 sec and quick spin. Place on magnet, allow slurry to clear, and pipette to remove supernatant.

31. Keeping tubes on magnet, add 200 µL cold Digitonin150 Buffer to each reaction. Pipette to remove supernatant.
32. Repeat the previous step one time, for a total of two washes.
33. Remove tubes from magnet. Add 50 µL cold Digitonin300 Buffer to each reaction and resuspend by pipetting (avoid bead loss, expel all material back into the tubes) and/or vortexing, followed by a quick spin.

Section V: pAG-Tn5 and Targeted Tagmentation (~4 hr)

Reactions are incubated with pAG-Tn5, which binds at antibody-labelled chromatin regions. The addition of magnesium in Tagmentation Buffer activates Tn5 to insert adapters and cleave chromatin. ***Note*** *that resuspension of the ConA bead slurry during these steps is critical to ensure even pAG-Tn5 distribution and efficient tagmentation. Excessive bead clumping will reduce yields.*

34. Quick spin the CUTANA pAG-Tn5 stock tube and flick to mix. Add 2.5 µL pAG-Tn5 to each reaction. Gently vortex to evenly distribute pAG-Tn5 across nuclei and quick spin.
35. Place tubes on a gently rocking nutator with caps elevated and incubate for 1 hour at RT.
36. Gently vortex and quick spin tubes. Place tubes on magnet, allow slurry to clear, and pipette to remove supernatant.
37. Remove tubes from magnet. Add 200 µL cold Digitonin300 Wash Buffer to each reaction and thoroughly pipette to resuspend and disperse bead clumps. Avoid bead loss and expel all material back into tubes.
38. Quick spin tubes, place on magnet, and allow slurry to clear. Pipette to remove supernatant.
39. Repeat steps 37 & 38 one time, for a total of two washes.
40. Remove tubes from magnet. Add 50 µL cold Tagmentation Buffer to each reaction and thoroughly pipette to resuspend and disperse bead clumps. Avoid bead loss and expel all material back into tubes.
41. Gently vortex and quick spin tubes. Incubate reactions for 1 hour in a thermocycler set to 37˚C (heated lid at 47˚C). **Note** that this is the key step wherein Tn5 is activated by magnesium to tagment antibody-bound chromatin.

    During this incubation, transfer 60 µL per reaction Pre-Wash150 Buffer to a new tube and equilibrate to RT.
42. Remove sample tubes from thermocycler, gently vortex and quick spin tubes. Place tubes on magnet, allow slurry to clear, and pipette to remove supernatant.
43. Remove tubes from magnet. Add 50 µL RT Pre-Wash150 Buffer to each reaction. Do **NOT** vortex – you may lose sample! Instead, gently but thoroughly pipette 3-5 times to resuspend the bead slurry. Expel all material back into tubes.
44. Place tubes on magnet, allow slurry to clear. Pipette to remove supernatant.
45. Remove tubes from magnet. Add 5 µL SDS Release Buffer to each reaction to quench tagmentation. Vortex ~10 sec at max speed to mix (do **NOT** pipette) and quick spin tubes.

    Note that addition of SDS partially lyses nuclei, causing the bead slurry to become viscous/sticky. Pipetting may result in sample loss.

46. Incubate reactions for 1 hour in a thermocycler set to 58˚C (heated lid at 68˚C). This step is required to release tagmented chromatin fragments into solution.
47. Quick spin tubes. Add 15 µL SDS Quench Buffer to each reaction to neutralize SDS and enable PCR. Carefully pipette to rinse beads if possible. If the slurry is too viscous to pipette, move to the next step. The slurry may clog tips; avoid loss and expel all material back into the tubes.
48. Vortex ~10 sec at max speed to fully mix and quick spin tubes to collect liquid. Keep tubes at RT.

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

Indexing PCR selectively amplifies tagmented fragments and adds i5 and i7 barcodes for Illumina sequencing. This dual indexing strategy applies a universal i5 primer and a reaction-specific i7 primer (see p. 9). ***Note*** *that ConA beads and nuclei are NOT removed prior to PCR. Indexing PCR is performed on the entire reaction slurry.*

49. Thaw i5 and i7 primers (10 µM stocks) and the CUTANA Non-Hot Start 2X PCR Master Mix and equilibrate on ice. Assign a unique i7 indexing primer to each reaction (p. 9).
50. Ensure primers and 2X PCR master mix tubes are fully mixed by vortexing and/or flicking tubes. Quick spin and return to ice.
51. To each CUT&Tag reaction add: 2 µL universal i5 primer, 2 µL assigned i7 primer, 25 µL Non-Hot Start 2X PCR Master Mix. Do **NOT** remove ConA beads from tubes--they are included in PCR. Keep tubes at RT during PCR setup. Change tips between each reagent addition to avoid cross-contamination.
52. Mix PCR reactions by pipetting. Avoid bubbles and make sure ConA beads are in solution to maximize PCR efficiency. Expel all material back into tubes, flick tubes for a final mix, quick spin, and proceed to PCR.
53. Place reactions in a thermocycler with a heated lid set to 105˚C. Amplify tagmented DNA directly from reaction slurry using PCR cycling parameters outlined below. We recommend using 16 PCR cycles and optimizing as needed (see the CUTANA CUT&Tag Kit Manual for guidance, epicypher.com/14-1102).

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

54. Purify CUT&Tag sequencing libraries using a ratio of **1.3X CUTANA™ DNA Purification Beads** to reaction volume (e.g. add 65 µL CUTANA™ DNA Purification Beads to 50 µL PCR reaction volume). This ratio recovers fragments >75 bp.
55. Mix well by pipetting and/or vortexing to an even resuspension (critical for bead binding). Quick spin tubes and incubate 5 min at RT.
56. Place tubes on a magnet for 2-5 min at RT, until solution clears. Pipette to remove supernatant without disturbing beads.
57. Keeping tubes on the magnet, add 180 µL freshly prepared 85% EtOH directly onto beads. Pipette to remove supernatant.
58. Repeat the previous step one time.
59. Remove tubes from magnet. Quick spin to collect liquid, with caps facing in, to avoid dislodging beads. Return to magnet and pipette to remove residual EtOH.
60. Remove tubes from magnet. Air-dry, caps open, 2-3 min at RT. Beads should be damp matte brown. If beads are crackly and/or light brown, they are too dry.
61. Add 17 µL **0.1X TE** to each reaction to elute DNA. Pipette and/or vortex to fully resuspend beads and quick spin. Incubate 2 min at RT.
62. Quick spin tubes and place on magnet for 2 min at RT.
63. Transfer 15 µL CUT&Tag libraries to new tubes and use 1 µL per reaction to quantify the purified PCR product using the Qubit™ fluorometer and 1X dsDNA HS Assay Kit, per the manufacturer's instructions.

    Note that there is **NO** typical yield for CUT&Tag assays. Instead we recommend aiming for the minimal amount of DNA that allows accurate quantification: at least 2 ng/µL or ~30 ng total DNA.

    Low yields may require additional PCR cycles and/or deeper sequencing. See the CUTANA CUT&Tag Kit Manual (epicypher.com/14-1102) for additional guidance.
64. **Safe pause point.** Store DNA at -20˚C or continue to fragment distribution analysis.

Section VII: Analysis of Library Fragment Size (~1 hr)

*Purified CUT&Tag libraries should show predominant enrichment of mononucleosome-sized fragments, at ~300 bp (**Figure 4**). **Note** that traces may show an oligonucleosome ladder with peaks every ~170 bp. This periodicity does not impact sequencing and size selection is NOT necessary.*

65. Use 1 µL library for analysis on the Agilent Bioanayzer (High Sensitivity DNA Kit) or TapeStation (D1000 ScreenTape System) per the manufacturer's instructions.
66. Final traces should show predominant enrichment of mononucleosome-sized fragments, as in **Figure 4** on the following page (~300 bp: ~170 bp DNA + sequencing adapters). This is the **BEST** indicator of CUT&Tag Experimental success prior to sequencing.

    Safe pause point. Store DNA at -20˚C or continue to sequencing.

Section VIII: Illumina® Next-Generation Sequencing

67. Dilute libraries and pool for sequencing per manufacturer's recommendations. General steps:

- Dilute each library to the same concentration. For NextSeq 2000 and 500/550, dilute to ~1-4 nM.
- Pool equimolar libraries into one tube.
- Dilute library pool as required for Illumina platform (support.illumina.com).
- Check that each library contains a unique i7 primer and that barcodes are correctly assigned. If dual barcoding is desired, consider using the CUTANA CUT&Tag Kit (epicypher.com/14-1102).
- For low-yield libraries: Add as much of the library as possible to the sequencing pool. Deeper sequencing is recommended to capture full library diversity.

68. Sequence to a depth of 5-8 million total reads per library (2x50 bp minimum). The end goal is to generate 3-5 million unique reads per library. See the CUTANA CUT&Tag Kit Manual for additional guidance (epicypher.com/14-1102).

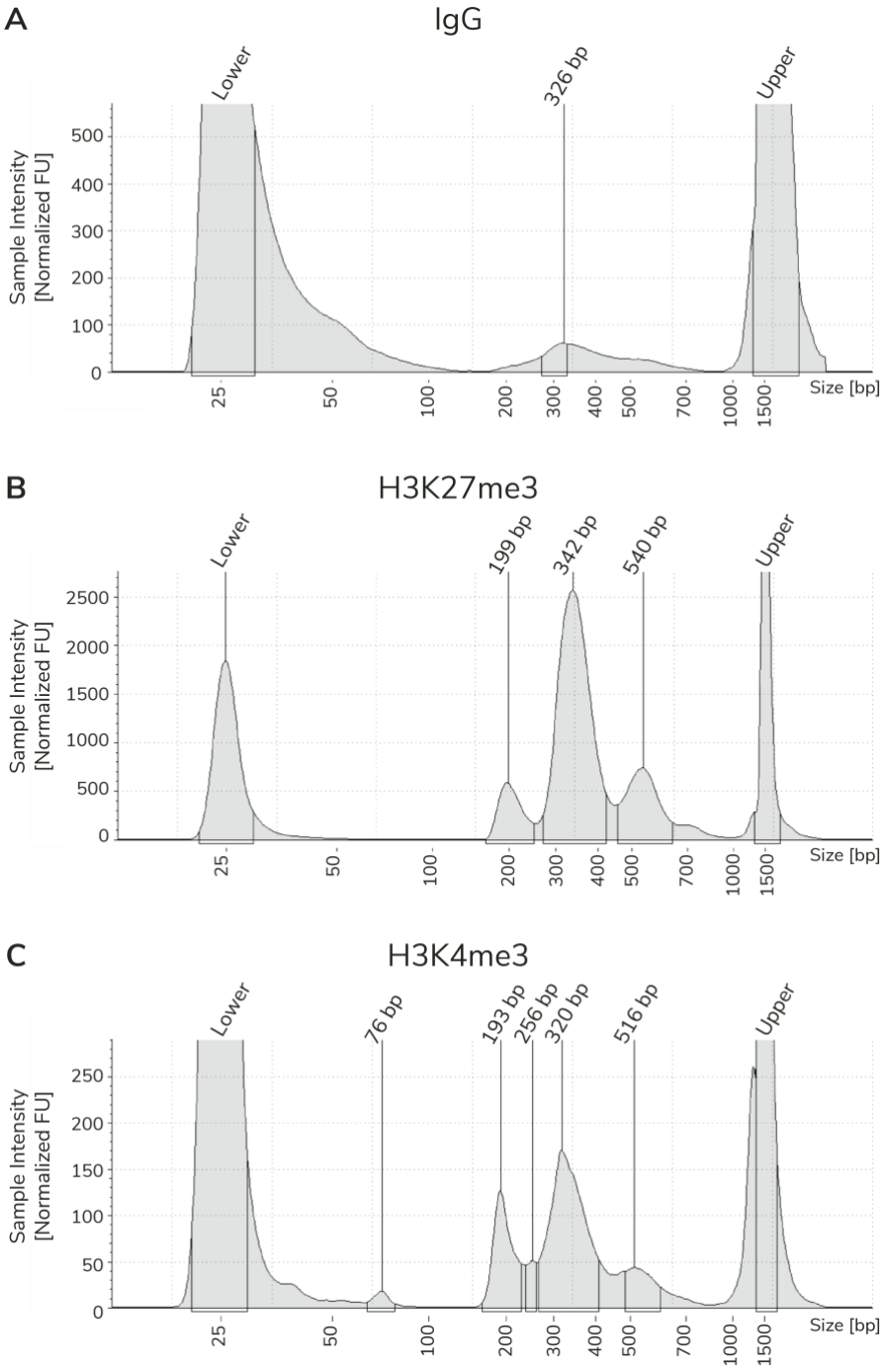

Figure 4
Typical TapeStation traces from CUTANA CUT&Tag libraries prepared using antibodies targeting IgG (**A**, negative control; EpiCypher 13-0042), H3K27me3 (**B**, positive control; EpiCypher 13-0055), and H3K4me3 (**C**, EpiCypher 13-0041; now use 13-0060). All libraries are predominantly enriched for mononucleosome-sized fragments as indicated by the peak at ~300 bp (~170 bp mononucleosomes + sequencing adapters). The "trident" pattern shown in the H3K27me3 trace is typical for many PTMs.

5. Appendix I: Quality Control Checks for Sample Prep

Description: This Appendix provides detailed instructions on checking the quality of starting cells, isolated nuclei, and nuclei conjugated 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 native K562 cells and nuclei.

We recommend these quality control checks for every CUT&Tag experiment. 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
1X PBS	Any
0.4% Trypan blue	Any
Hemacytometer	Any
Cell counting slides	Any
Brightfield/phase contrast microscope or automated cell counter	Any

Protocol: Harvesting cells and nuclei for CUT&Tag with built-in quality control checks

Starting from Section II of the CUTANA CUT&Tag Protocol, Step 7.

1. Spin cells 600 x g, 3 min, at room temperature (RT). Remove supernatant, flick tube to loosen cell pellet, and resuspend in 1-2 mL 1X PBS. Transfer 10 µL to a 1.5 mL tube.
2. Add 10 µL 0.4% Trypan Blue to 10 µL cells. Pipette 10 times to mix.
3. Transfer 10 µL to a cell counting slide. Obtain cell counts and determine viability using a brightfield/phase microscope or cell counter. See **Figure 5** for expected results.
4. Harvest 100,000 cells per reaction (plus 10% excess) in a new 1.5 mL tube. Spin cells 600 x g, 3 min, RT. Pipette to remove supernatant.
5. Resuspend cells in 100 µL per reaction cold Nuclei Extraction Buffer. Incubate for 10 min on ice.
6. Spin 600 x g, 3 min, 4˚C. Pipette to remove supernatant. Resuspend nuclei in 105 µL per reaction cold Nuclei Extraction Buffer.
7. Transfer 10 µL nuclei to a new 1.5 mL tube. Evaluate integrity using Trypan Blue staining as in steps 2 and 3 above. See **Figure 5** for expected results.
8. Add 100 µL nuclei to 10 µL activated ConA beads in 8-strip PCR tubes [**sample slurry**].
   Note that bead binding can also be performed in 1.5 mL tubes for batch processing of large numbers of cells. In this case, simply aliquot to 8-strip tubes before adding primary antibodies.
9. Gently vortex to mix and quick spin. Incubate sample slurry for 10 min at RT.
10. Place tubes on magnet and allow slurry to clear. Transfer 10 µL supernatant [**unbound fraction**] into a fresh 1.5 mL tube and place on ice. Remove and discard remaining supernatant.

11. Remove tubes from magnet and add 50 µL cold Antibody150 Buffer to each reaction, moving quickly to avoid bead dry-out. Pipette to resuspend the sample slurry.
12. Transfer 10 µL **sample slurry** to a fresh 1.5 mL tube. Place 8-strip tubes on ice.
13. To 10 µL aliquots that were set aside (**unbound fraction**, **sample slurry**), perform Trypan Blue staining as described in steps 2 & 3 above. See **Figure 5** for expected results.
14. Continue with the CUT&Tag Protocol, Section III (Antibody Binding).

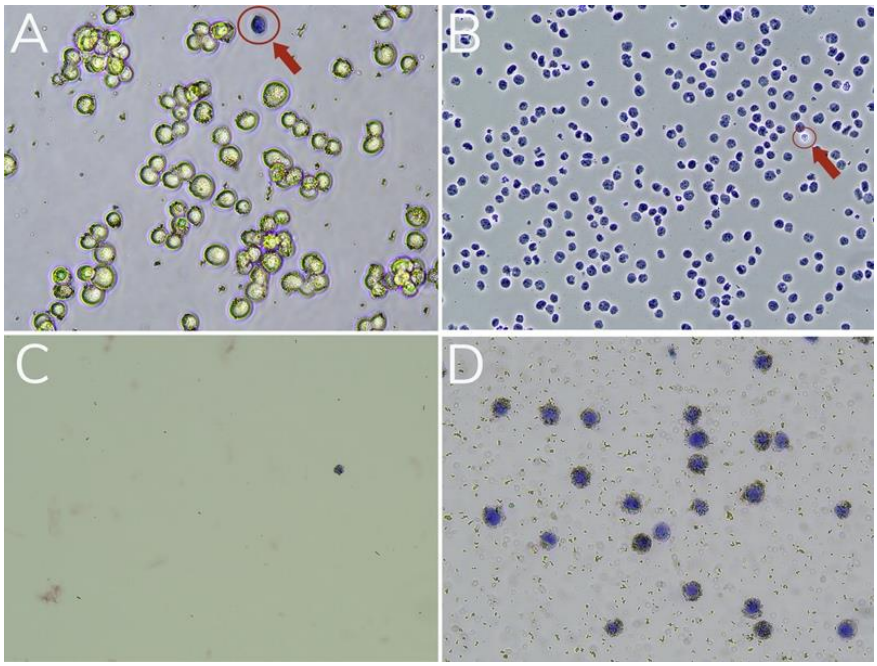

Figure 5: Representative images of cell and nuclei samples for ConA bead binding. Samples were stained with Trypan Blue and visualized under brightfield microscope. **(A) Cells** before bead binding. A dead cell (blue; Trypan positive) is circled in red, whereas the remaining live cells are bright white 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 **sample slurry** showing nuclei (blue) successfully bound to activated ConA beads (brown specks).

Sample	Figure	Success Metric (Trypan Blue staining)	Troubleshooting Tips
Cells	**Fig. 5A**	Cells should be bright white (Trypan Blue excluded), round, unclumped, and ideally show >80% viability. Excess dead cells increase background in CUT&Tag.	Optimize cell culture conditions; use fresh media, evaluate potential contamination issues.
Nuclei	**Fig. 5B**	Nuclei should be >95% Trypan Blue positive and unclumped.	Monitor cells during nuclei extraction by Trypan Blue staining to optimize incubation time. Increase spin time if you are losing nuclei during spins (keep at 600 x g).
Unbound Fraction	**Fig. 5C**	Little to no material should be present if binding is successful.	Ensure that ConA beads were never frozen (if frozen, beads will be black/grainy), cells/nuclei were not clumped, beads did not become clumped or dried out, and all buffers were correctly prepared.
Sample Slurry	**Fig. 5D**	Successful ConA bead binding will show Trypan Blue positive nuclei surrounded by beads.	

6. References

1. 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).
2. 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).