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

Optimized for Histone Post-Translational Modifications (PTMs)

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. The tagmentation reaction appends sequence adapters to the antibody-bound DNA, which can be directly PCR amplified *in situ* to yield sequence-ready DNA[2]. High resolution genome-wide profiles of histone PTM enrichment can then be generated using as few as 3-5 million sequencing reads.

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

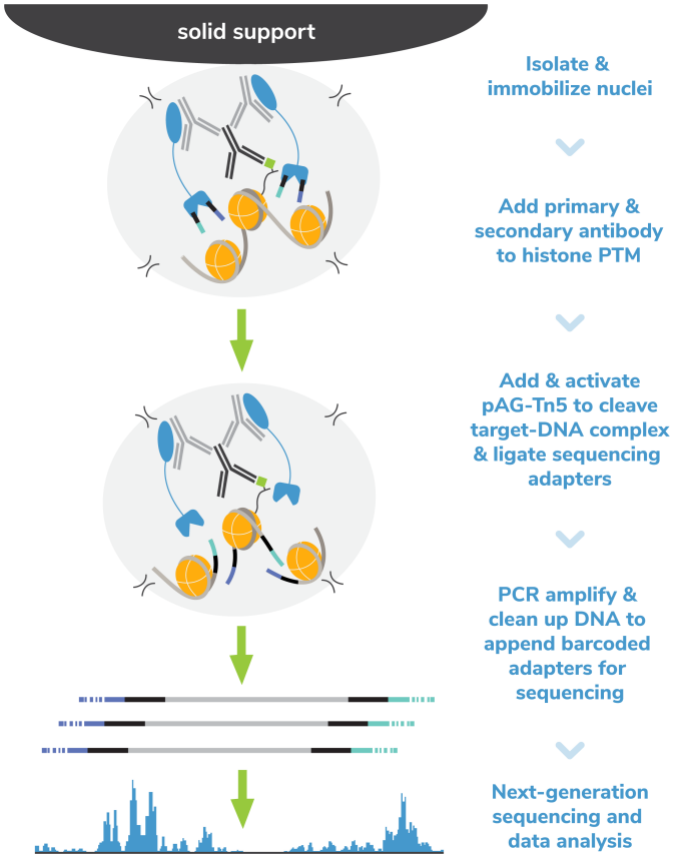

Figure 1. Overview of the CUTANA Direct-to-PCR CUT&Tag protocol.

2. CUTANA Products & Services: Advantages

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

- Optimized for ultra-low cell inputs (<5,000 cells; for >5,000 cells, try CUT&RUN)
- More cost effective: less antibody and required sequencing depth
- Markedly improved [signal : noise]
- Rapid workflow: cells → sequence-ready libraries in < 2 days
- Empowers benchtop sequencers (*e.g.* Illumina® MiniSeq or MiSeq)
- Dramatically increases experimental throughput (transforming the way scientists approach experimental design for genomic mapping studies)

EpiCypher now offers CUTANA pAG-Tn5, the essential reagent for CUT&Tag workflows:

- Optimized fusion of Proteins A and G with hyperactive transposase 5 (pAG-Tn5) is directly compatible with a broad range of antibody isotypes (*e.g.* mouse, rabbit).
- 50 and 250 reaction pack sizes, enabling greater experimental throughput
- Check epicypher.com/resources/protocols for regular protocol updates
- Additional CUTANA products:
    - CUT&RUN Kits
    - Spike-in controls for normalization:
        - *E. coli* Spike-in DNA: currently optimized for CUT&RUN. Inquire for recommendations related to CUT&Tag.
        - CUT&Tag spike-in controls coming soon, including defined recombinant nucleosome spike-ins
    - Platform validated CUTANA Compatible Antibodies: rigorous, lot-specific testing to verify robust performance in CUTANA assays
    - *Inquire for more information or to connect to EpiCypher scientists*: info@epicypher.com

3. Protocol Notes

1. This version of the Direct-to-PCR CUT&Tag protocol is validated for histone PTMs. For genomic mapping of chromatin-associated proteins (*e.g.* transcription factors, epigenetic writers and reader proteins), CUT&RUN is recommended.

2. Always include control conditions (*e.g.* positive control anti-H3K4me3 or anti-H3K27me3 and negative control IgG; see Reagents & FAQ sections). This is especially critical when evaluating CUT&Tag to previously untested targets and/or antibodies.

3. Unlike CUT&RUN where cells are immobilized to ConA beads, it is recommended to use bead-immobilized nuclei for CUT&Tag. This circumvents undesirable tagmentation of mitochondrial DNA. However, cells are compatible with this protocol since digitonin remains in the buffers for cellular input flexibility and to control bead behavior. For more information on cells, see FAQ section.

4. Start by optimizing the protocol using 100,000 native (i.e. unfixed) cells/nuclei per sample. Once conditions are optimized for the target and cell type of interest, scale down to desired final number of cells. For experimental applications that do not require less than 5,000 cells/nuclei, CUT&RUN is recommended (CUT&RUN protocol optimized for 5,000 – 500,000 cells for a variety of targets).

5. Protocol optimized in 200 µL 8-strip PCR tubes to: **a)** minimize beads sticking to tubes; **b)** enable more rapid workflow with multichannel pipets; **c)** provide more consistent sample handling; and **d)** enable high-throughput sample preparation.

6. Take caution throughout to avoid ConA beads sticking to the tube sides and caps: the beads dry out easily, which can result in sample loss.

7. We recommend a 5% digitonin stock solution in DMSO (as opposed to heated H_2O); this improves solubility and standardizes cell permeabilization / protocol reproducibility.

8. CUT&Tag PCR yields tend to vary by target and by 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 to accurately quantify (*i.e.* by Qubit, Bioanalyzer, and/or Tapestation), typically between 14-21 cycles. If a sample is overamplified, PCR duplicates can be removed using Picard.

9. The single best indicator of CUT&Tag success prior to sequencing is enrichment of ~300 bp fragments post-PCR, assessed by a Bioanalyzer or Tapestation.

10. **IMPORTANT**: Since CUT&Tag has lower background and is compatible with low cell inputs, **it is not recommended to assess fragment size distribution by agarose gel or capillary electrophoresis prior to library preparation**. Such analysis is not indicative of the success of a CUT&Tag experiment, and further the amount of DNA recovered is often below the sensitivity of detection for each approach. Instead, assess DNA yield compared to positive (*e.g.* H3K4me3, H3K27me3) and negative (IgG) controls, determine fragment size distribution of sequence-ready libraries (**Figure 5**), and evaluate peak structure and expected genome-wide distribution in NGS data.

4. Buffers, Reagents & Materials Needed

Buffer recipes (*note: see order information for buffer components, Section 6*)

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

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

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

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

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

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

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

Tagmentation Buffer (50 µL/sample)
Wash300 Buffer + 10 mM $MgCl_2$
Store @ 4ºC for up to 1 week

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

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

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

**Digitonin 5% stock solution should be prepared in DMSO and aliquots stored at -20ºC for six months. Note that digitonin is not necessary for nuclei permeabilization in the CUT&Tag workflow (purified nuclei are inherently permeable to antibody and pAG-Tn5). Rather, it helps to prevent the nuclei-conjugated beads from forming a thin film on tubes. Digitonin also makes the protocol compatible with cells if used, although not recommended.*

Reagents

Item	Vendor	Catalog No.	Notes
Concanavalin A (ConA) Conjugated Paramagnetic Beads	EpiCypher	21-1401	ConA is a lectin, which can cause immune activation. For technical support re. immune cell studies, contact info@epicypher.com.
CUTANA pAG-Tn5	EpiCypher	15-1017 and 15-1117	50 & 250 reaction pack sizes available. Supplied as 20X stock.
Rabbit IgG Negative Control Antibody	EpiCypher	13-0042	Use 0.5 µg in CUT&Tag
SNAP-ChIP® Certified 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[4]). Contact us for recommendations: info@epicypher.com
Anti-Mouse Secondary Antibody	EpiCypher	13-0048	Required for CUT&Tag. Use with primary antibodies made in mouse.
Anti-Rabbit Secondary Antibody	EpiCypher	13-0047	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

Universal i5 primer: prepare at 10 µM in Molecular Biology grade H_2O (RNase, DNase free)
5' AATGATACGGCGACCACCGAGATCTACACTAGATCGCTCGTCGGCAGCGTCAGATGTGTAT 3'

Uniquely barcoded i7 primers: prepare at 10 µM in Molecular Biology grade H_2O (RNase, DNase free)

Name	Sequence (5' → 3')	Barcode	Illumina rev. compl. read
i7_1	CAAGCAGAAGACGGCATACGAGATTCGCCTTAGTCTCGTGGGCTCGGAGATGTG	TCGCCTTA	TAAGGCGA
i7_2	CAAGCAGAAGACGGCATACGAGATCTAGTACGGTCTCGTGGGCTCGGAGATGTG	CTAGTACG	CGTACTAG
i7_3	CAAGCAGAAGACGGCATACGAGATTTCTGCCTGTCTCGTGGGCTCGGAGATGTG	TTCTGCCT	AGGCAGAA
i7_4	CAAGCAGAAGACGGCATACGAGATGCTCAGGAGTCTCGTGGGCTCGGAGATGTG	GCTCAGGA	TCCTGAGC
i7_5	CAAGCAGAAGACGGCATACGAGATAGGAGTCCGTCTCGTGGGCTCGGAGATGTG	AGGAGTCC	GGACTCCT
i7_6	CAAGCAGAAGACGGCATACGAGATCATGCCTAGTCTCGTGGGCTCGGAGATGTG	CATGCCTA	TAGGCATG
i7_7	CAAGCAGAAGACGGCATACGAGATGTAGAGAGGTCTCGTGGGCTCGGAGATGTG	GTAGAGAG	CTCTCTAC
i7_8	CAAGCAGAAGACGGCATACGAGATCCTCTCTGGTCTCGTGGGCTCGGAGATGTG	CCTCTCTG	CAGAGAGG
i7_9	CAAGCAGAAGACGGCATACGAGATAGCGTAGCGTCTCGTGGGCTCGGAGATGTG	AGCGTAGC	GCTACGCT
i7_10	CAAGCAGAAGACGGCATACGAGATCAGCCTCGGTCTCGTGGGCTCGGAGATGTG	CAGCCTCG	CGAGGCTG
i7_11	CAAGCAGAAGACGGCATACGAGATTGCCTCTTGTCTCGTGGGCTCGGAGATGTG	TGCCTCTT	AAGAGGCA
i7_12	CAAGCAGAAGACGGCATACGAGATTCCTCTACGTCTCGTGGGCTCGGAGATGTG	TCCTCTAC	GTAGAGGA

Equipment

Item	Vendor	Catalog No.	Notes
1.5 mL Magnetic Separation Rack	EpiCypher	10-0012	For batch processing ConA beads
8-strip PCR tube Magnetic Separation Rack	EpiCypher	10-0008	For bead separation steps after batch processing ConA beads; enables multi-channel sample processing
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	Or comparable capillary electrophoresis instrument (*e.g.* Agilent TapeStation)
High Performance Multi-Channel Pipettors, 8-Channel	VWR	76169-252	For aspiration and wash steps to accelerate workflow
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

5. EpiCypher CUTANA Direct-to-PCR CUT&Tag Protocol (~5hrs)

---Day 1---

Section I: ConA Bead Activation (~30 min)

1. Gently resuspend the **ConA beads (Concanavalin A)** and transfer 11 µL/sample to 1.5 mL tube for batch processing.
2. Place the tube on a 1.5 mL magnetic stand until slurry clears and pipet to remove supernatant (supe).
3. Remove from magnet and add 100 µL/sample cold **Bead Activation Buffer** and pipet to mix. Place the tube on a magnet until slurry clears and pipet to remove supe.
4. Repeat previous step for total of two washes.
5. Resuspend beads in 11 µL/sample cold **Bead Activation Buffer**.
   Split activated ConA beads into separate tubes for different cell types and/or antibodies.
6. Aliquot 10 µL/sample of activated bead slurry into 8-strip tube. Keep beads on ice until needed.

Figure 2. For ConA bead activation steps, it is recommended to batch process the full volume of beads needed for all experimental samples in a single 1.5 mL tube (11 µL beads per sample). For these steps, beads can be washed using a 1.5 mL magnetic rack (*e.g.* EpiCypher 10-0012, pictured), ensuring homogeneity across samples.

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

7. Harvest 100,000 cells/sample by centrifugation for 3 min at 600 x g at room temperature (RT) in 1.5 mL tube, and decant supe.
8. Resuspend cells in 100 µL/sample cold **NE Buffer**; and **incubate for 10 min on ice**.
9. Spin for 3 min at 600 x g at RT; aspirate supe.
10. Resuspend nuclei pellet in 100 µL/sample cold **NE Buffer**.
11. Aliquot 100 µL nuclei to each 8-strip tube containing 10 µL of activated beads. Gently vortex (setting #7) to mix.
12. **Incubate** nuclei : bead slurry for **10 min at RT**. *Nuclei will adsorb to the activated ConA beads.*

Figure 3. For processing individual samples, it is recommended to use multi-channel pipetting using 8-strip PCR tubes and compatible magnetic rack (*e.g.* EpiCypher 10-0008, pictured), increasing experimental throughput and reproducibility.

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

13. Place the tube on a magnet until slurry clears and pipet to remove supe.
14. Add 50 µL cold **Antibody150 Buffer** to each sample, remove from magnet, and thoroughly pipet to resuspend.
15. Add 0.5 µg **Primary Antibody** to each sample and gently vortex.

16. **Incubate** 8-strip tube (caps slightly elevated) on nutator **overnight at 4ºC** (**Figure 4**).

Bead slurry before o/n incubation @ 4ºC | nutator o/n | Bead slurry after o/n incubation @ 4ºC

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.

---Day 2---

17. Place the tube on a magnet until slurry clears and pipet to remove supe.
18. Remove from magnet, add 50 µL cold **Digitonin150 Buffer** to each 8-strip tube, and thoroughly pipet to resuspend.
19. Add 0.5 µg **Secondary Antibody** (matched to primary antibody animal source), and gently vortex.
20. **Incubate** 8-strip tube on nutator for **30 min at RT**.
21. Place the tube on a magnet until slurry clears and pipet to remove supe.
22. While beads are on magnet, add 200 µL cold **Digitonin150 Buffer** directly onto beads of each sample, and then pipet to remove supe.
23. Repeat previous step for total of two washes.
24. Remove from magnet, add 50 µL cold **Digitonin300 Buffer** to each 8-strip tube, and thoroughly pipet to resuspend. Beads are often clumpy at this point, but can easily be dispersed with gentle pipetting with a P200 pipet.
    - *Critical step: 300 mM NaCl is critical in the Digitonin300 buffer to minimize non-specific binding of pAG-Tn5 to accessible DNA.*

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

25. Add 2.5 µL **CUTANA pAG-Tn5** (20x stock) to each sample, and gently vortex.
    - *Critical step: to evenly distribute pAG-Tn5 across nuclei, ensure beads are thoroughly resuspended by gentle vortexing and/or pipetting with a P200 pipet.*
26. **Incubate** samples on nutator for **1 hr at RT**, return 8-strip tube to magnet, and pipet to remove supe.
27. Add 200 µL cold **Digitonin300 Buffer** directly to each sample, thoroughly resuspend by pipetting, return to magnet, and then pipet to remove supe.
28. Repeat previous step for total of two washes.

Section V: Targeted Chromatin Tagmentation (~3 hrs)

29. Remove from magnet, add 50 µL cold **Tagmentation Buffer** to each sample, and

thoroughly pipet to resuspend. Beads are often clumpy at this point, but can easily be dispersed by gentle pipetting with a P200 pipet.

30. **Incubate** 8-strip tube for **1 hr at 37ºC** in thermocycler. *Initiates tagmentation reaction.*
31. Place the tube on a magnet until slurry clears and pipet to remove supe.
32. Remove from magnet and resuspend beads in 50 µL RT **TAPS Buffer** by pipetting, return to magnet, and pipet to remove supe.
33. Remove from magnet, add 5 µL RT **SDS Release Buffer** (containing 0.1% SDS) to each sample, and vortex on max speed for 7 seconds. Quick spin to collect. *Quenches tagmentation reaction. Sample may be chunky and/or sticky, but this is normal since nuclei are being partially lysed.*
    - *Critical step: Do not pipet to mix after adding SDS Release Buffer because it will likely lead to sample loss as the bead slurry is viscous and sticky.*
34. **Incubate** 8-strip tube for **1 hr at 58ºC** in thermocycler.
    - *Critical step: Required to release tagmented chromatin fragments into solution, for both fixed and unfixed nuclei.*
35. Add 15 µL RT **SDS Quench Buffer** (containing 0.67% Triton-X) to each sample, and vortex on max speed. *Neutralizes SDS, which potently inhibits PCR.*

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

36. For each sample, add 2 µL each of universal i5 and barcoded i7 primers (10 µM stocks).
37. Add 25 µL non-hot start **CUTANA High Fidelity 2x PCR Master Mix** to each sample and mix.
38. CUT&Tag-specific PCR cycling parameters:
    a. 5 min @ 58ºC → Fill-in step
    b. 5 min @ 72ºC → Extension step
    c. 45 sec @ 98ºC → DNA melting
    d. 15 sec @ 98ºC → DNA melting
    e. 10 sec @ 60ºC → hybrid primer annealing & short extension (<700 bp)
    f. Repeat "step d-e" for a total of 14-21 cycles, depending on antibody target and cellular input amount. Use minimal number of cycles necessary to accurately quantify library.
    g. 1 min @ 72ºC → final extension
    h. hold @ 4ºC
    - *Critical step: PCR cycling parameter is designed to amplify DNA fragments within the sequence-able range (100 bp-700 bp). Thus, contaminating higher molecular weight DNA fragments are not enriched by these PCR parameters.*
39. DNA cleanup using **1.3x** AMPure beads to sample volume (*e.g.* 65 µL AMPure to 50 µL PCR; recovers > ~75 bp DNA fragments), as per manufacturer's recommendations.
40. Elute DNA in 15 µL **0.1x TE buffer** and use 1 µL to quantify the purified PCR product using the **Qubit™ fluorometer** as per manufacturer's recommendations.

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

41. For each purified PCR DNA library for Illumina sequencing, load 1 µL of library sample on **Agilent High Sensitivity DNA Chip** (Cat# 5067-4626) as per manufacturer's recommendations.
42. Upon Bioanalyzer (or equivalent approach) confirmation that mononucleosome fragments were predominantly enriched (~300 bp with sequence adapters**, Figure 5**), proceed to Illumina sequencing as per manufacturer's recommendations.
    - *Note: Only 3-5 million paired-end reads are needed for good coverage in CUT&Tag.*

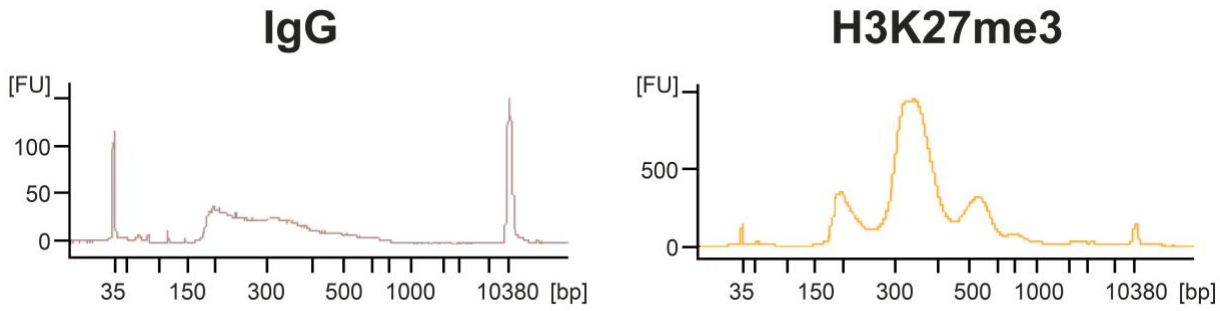

Figure 5. Typical Bioanalyzer traces for IgG negative control and H3K27me3 positive control CUT&Tag sequencing libraries (1 µL PCR amplified / AMPure purified libraries loaded on Bioanalyzer). The positive control H3K27me3 library is predominantly enriched for mononucleosomes as indicated by the peak at ~300 bp (~150 bp mononucleosomes + 150 bp sequence adapters). Quality Bioanalyzer (or Tapestation / equivalent approach) traces are the best indicator of success prior to sequencing. However, this analysis should not be performed prior to NGS library preparation, since the amount of CUT&Tag DNA is likely to be below the limit of detection prior to library amplification.

6. 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 (Pure, 6.4M)	Sigma-Aldrich	S0266
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

7. Frequently Asked Questions (FAQs)

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 / negative controls (H3K4me3 or H3K27me3 / IgG) in every experiment. If QC checks for controls perform as expected (below), then proceeding to sequencing with all samples is recommended. If sequencing results for challenging cell inputs / targets are not satisfactory, further optimization may be necessary (*e.g.* cell type and / or number, antibody concentration, alternate antibodies, etc.).

   First, including control antibodies (IgG and H3K4me3 or H3K27me3) in the experimental design is critical for assessing the success of a CUT&Tag experiment. In CUT&Tag, libraries are quality-controlled at two stages prior to sequencing: 1. PCR yields, and 2. Bioanalyzer DNA fragment distribution. PCR yields are typically lower than CUT&RUN, but the positive control antibody PCR yields are greater than IgG, particularly for high abundance PTMs (*e.g.* H3K27me3; H3K4me3 yields are often comparable or very slightly higher than IgG, since this is a lower abundance PTM). The expected PCR-amplified fragment distribution is predominantly mononucleosomes (~300 bp = 150 insert + 150 adapters). In short, prior to sequencing, enrichment of mononucleosome size fragments is the best indicator of success in CUT&Tag 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 NGS libraries. The entire workflow from isolated nuclei to sequence-ready libraries can be performed in a single tube, enabling increased experimental throughput and supporting automation. By circumventing chromatin purification steps, sample loss is minimized, which increases sensitivity for low cell inputs.

DNA spike-in controls

3. Can residual *E. coli* in the pAG-Tn5 prep be used for sample input normalization? What spike-in DNA control does EpiCypher recommend?
   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: 1. ensures lot-to-lot consistency; 2. maintains high specific activity of pAG-Tn5; and 3. prevents contaminating *E. coli* DNA from dominating signal in ultra-low cell input experiments. Nevertheless, EpiCypher is currently working on two spike-in solutions for CUT&Tag: exogenous DNA and fully defined semi-synthetic nucleosomes.

4. Does EpiCypher offer spike-in DNA controls for CUT&Tag?
   EpiCypher plans to offer multiple spike-in controls for CUT&RUN and CUT&Tag, in addition to exogenous *E. coli* DNA spike-in. In particular, CUTANA spike-in nucleosomes (*i.e.* fully-

defined semi-synthetic nucleosomes) compatible with each approach are in active development and offer multiple advantages, including enabling sample input normalization and multiple control points (1. antibody specificity; 2. antibody efficiency; 3. pAG-MNase /pAG-Tn5 activity; and 4. experimental success).

Cell input compatibility

5. 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.

6. What types of cell inputs are compatible with CUT&Tag?
   While we do not recommend using whole cells (for the reasons listed in #5), these 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). We routinely use native nuclei derived from K562 cells in our CUT&Tag studies. If cells must be used, optimize the digitonin concentration to ensure cells are effectively permeabilized (*e.g.* perform a digitonin titration and monitor cell permeability using trypan blue staining).

7. Is CUT&Tag compatible with frozen or crosslinked cells?
   CUT&Tag is compatible with frozen and/or lightly crosslinked cells (*i.e.* 0.1% formaldehyde for 1 min). These protocol variations are currently under internal development and will be publicized when fully tested.

Antibodies and targets

8. 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 TFs may be incompatible with CUT&Tag. Therefore, for non-PTM targets, CUT&RUN is recommended as a robust and extensively validated approach. See more information about CUT&RUN here.

9. What PTM antibodies does EpiCypher recommend for CUT&Tag?
   We recommend starting with SNAP-ChIP® certified PTM antibodies since they exhibit high specificity and efficiency in ChIP when tested against designer nucleosome panels (representing sets of related on- and off-target PTMs). In particular, we strongly recommend starting with positive control antibodies that have been verified to exhibit robust performance in CUT&Tag (EpiCypher H3K4me3 antibody, Catalog No. 13-0041). We will update our CUTANA Antibodies product page as we continue to identify those best-in-class antibodies for CUT&RUN and CUT&Tag applications: https://www.epicypher.com/antibodies/cutana-

cut-run-compatible-antibodies/. Contact us directly for potential antibody recommendations to additional targets (info@epicypher.com).

Miscellaneous

10. 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 (purified nuclei are inherently permeable to 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 if used, although not recommended.

8. References

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