CUTANA™ CUT&RUN Protocol for Histone PTMs

1. Overview

Cleavage Under Targets & Release Using Nuclease (CUT&RUN) is a revolutionary genomic mapping strategy developed by the group of Dr. Steven Henikoff [1]. It builds on Chromatin ImmunoCleavage (ChIC), whereby a fusion of Protein A to Micrococcal Nuclease (pA-MNase) selectively cleaves antibody-bound chromatin *in situ* [2]. In CUT&RUN, cells or nuclei are immobilized to a solid support, with pA-MNase cleaved DNA fragments isolated from solution. The workflow is compatible with next-generation sequencing (NGS) to generate high resolution genome-wide profiles of histone post-translational modifications (PTMs) and chromatin-associated proteins (**Figure 1**).

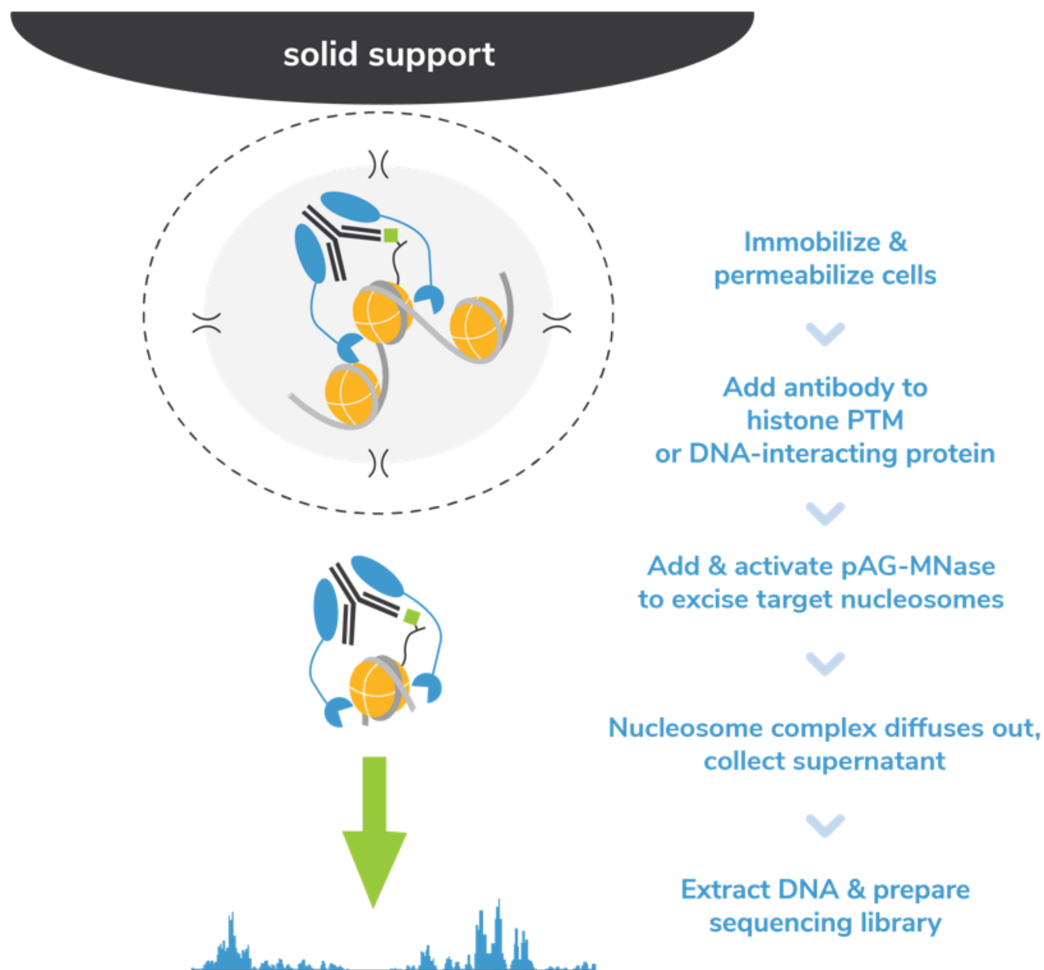

Figure 1. Overview of the CUTANA™ EpiCypher CUT&RUN protocol.

2. CUTANA™ Products & Services: Advantages

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

- Requires substantially fewer cells
- Compatible with low sequencing depths
- Markedly improved [signal : noise]
- Rapid workflow: cells → data in < 1 week
- Empowers benchtop sequencers (*e.g.* Illumina® MiniSeq or MiSeq)
- Dramatically increased experimental throughput (changing the way scientists approach genomic mapping studies)

EpiCypher now offers CUTANA™ pAG-MNase, the essential reagent for ChIC/CUT&RUN workflows:

- First-in-class commercial product for ChIC/CUT&RUN assays
- Optimized fusion of Proteins A and G with Micrococcal Nuclease (pAG-MNase) enables direct compatibility with a broad range of antibody isotypes
- 50 and 250 reaction pack sizes, enabling greater experimental throughput
- Check back at www.epicypher.com for regular updates of optimized protocols
- Additional CUTANA™ products coming soon:
    - CUT&RUN Kits
    - CUT&RUN spike-in controls
    - pAG-Tn5 (for CUT&Tag applications)
    - CUT&Tag Kits
    - CUT&Tag spike-in controls
    - *Inquire for more information or to connect to EpiCypher scientists*: info@epicypher.com

3. Specific Protocol Notes

1. Protocol is optimized using 0.5 million human K-562 cells per sample.

2. Although protocols with shortened antibody and/or $CaCl_2$ incubation times have been published, in our hands such changes adversely impact reproducibility.

3. Take caution throughout to avoid ConA beads sticking to the sides / caps of tubes, as they dry out easily and can result in sample loss.

4. We recommend a 5% digitonin stock solution be prepared in DMSO (as opposed to dissolution in heated H_2O).

5. To avoid digitonin precipitation use the minimal digitonin concentration in the **Digitonin Buffer** and **Antibody Buffer** required for efficient cell permeabilization (see Protocol Part III, Step 6). This should be empirically determined for different cell types before proceeding with the full CUT&RUN experiment.

4. Reagents & Materials Needed

Buffer Stocks

Item	Vendor	Catalog No.	Notes
HEPES	Amresco	7365	
KCl	EMD Millipore	PX1405	
$CaCl_2$	Amresco	1B1110	
$MnCl_2$	Sigma-Aldrich	M1787	
NaCl	Amresco	0241	
Spermidine	Sigma-Aldrich	S2501	Prepare 1 M stock in ddH_2O and make single use aliquots stored at -20˚C. The stock is good for 1 month.
Protease Inhibitor	Sigma-Aldrich	11873580001	
Digitonin	EMD Millipore	300410	Prepare as 5% (wt/vol) stock in DMSO at room temperature (RT). The stock is good for 3 months when stored at -20˚C.
EDTA	Amresco	M101	
EGTA	Amresco	0732	
Trypan Blue	Thermo Fisher Scientific	T10282	
RNase A	Thermo Fisher Scientific	5056489001	
Glycogen	Sigma-Aldrich	10901393001	

Reagents

Item	Vendor	Catalog No.	Notes
Concanavalin A (ConA) Magnetic Beads	Bangs Laboratories	BP531	ConA is a lectin, which can cause immune activation. For technical support re. immune cell studies, contact info@epicypher.com
pAG-MNase	EpiCypher	15-1016	Supplied as 20X stock
Histone PTM Antibody	User-dependent		EpiCypher continues to conduct extensive studies of histone PTM antibodies [3]. Contact us for recommendations: info@epicypher.com
MinElute PCR Purification Kit	Qiagen	28004	
Qubit 1x dsDNA HS Assay Kit	Thermo Fisher Scientific	Q33230	
Library Preparation Kit	User-dependent		

Equipment

Item	Vendor	Catalog No.	Notes
Magnetic Stand	Thermo Fisher Scientific	12321D	
Qubit 4 Fluorometer	Thermo Fisher Scientific	Q33226	
Agilent 2100 Bioanalyzer	Agilent	G2939A	Or other capillary electrophoresis instruments (*e.g.* Agilent TapeStation)
ThermoMixer	Eppendorf	5384000020	
Tube Rotator	Grant-Bio	PTR-35	
Tube Nutator	Labnet	S0500	

5. CUT&RUN Protocol for Histone PTMs

Part I: ConA Bead Activation *(estimated time = 10 - 20 minutes)*	
1. Gently resuspend the ConA magnetic beads and withdraw calculated total volume of the bead suspension (based on 10 µL per sample). 2. Mix into 1.5 mL of **Binding Buffer** in a 2 mL tube[a]. 3. Place the tube on a magnet stand and remove the liquid. 4. Add 1.5 mL of **Binding Buffer** and mix by gentle pipetting. 5. Place the tube on a magnet stand and remove the liquid. 6. Resuspend the beads in (N + 1) x 10 µL **Binding Buffer** (where N = number of samples)[b].	a. After each wash, remove the liquid from the cap by a pulse spin on a microcentrifuge. b. The activated beads may be held on ice for a day before use.

Part II: Binding Cells to Activated Beads[c] *(estimated time = 0.5 – 1 hours)*	
1. Count and harvest calculated number of fresh cells by centrifuging for 3 min at 600 *x g*[d]. 2. Decant supernatant and resuspend cells in 1.5 mL room temperature (RT) **Wash Buffer** and transfer to a 2 mL screw-cap tube. 3. Centrifuge for 3 min at 600 *x g*. 4. Repeat steps 2 & 3 for two total washes. 5. Resuspend cells in 1 mL in RT **Wash Buffer** by gently pipetting. 6. While gently vortexing the cells, add activated bead suspension from Part I. 7. Place the cell bead mixture on a tube rotator and rotate at RT for 10 min.	c. Keep buffers and steps in this section at RT to avoid cell stress. d. **Tested at EpiCypher**: 0.5 million human K-562 cells per sample.

Part III: Binding of Antibodies *(estimated time = 10 – 30 min + overnight)*	
1. Quick spin, then place the tube on a magnet stand and remove the liquid. 2. Place the tube at a low angle on the vortex mixer, squirt (N + 1) x 50 µL **Antibody Buffer** along the side while gentle vortexing[e]. 3. Gently tap to dislodge all beads. 4. Aliquot 50 µL bead suspension to N x Eppendorf tubes and add 0.5 - 1 µL antibody to each tube. 5. Place the tubes on the tube nutator and nutate **overnight at 4˚C**[f]. 6. *(Optional) Test cell permeabilization efficiency by digitonin*[g]*:* *After nutating the cell bead suspension in* ***Antibody Buffer*** *for 10 min at RT, mix 10 µL with 10 µL Trypan blue dye. Apply 10 µL to a glass slide and observe under a microscope. >95% cells should be stained blue if successfully permeabilized.*	e. **Tested at EpiCypher:** 0.01% digitonin and 1:100 dilution of antibodies (~ 0.5-1 mg/mL stocks) for 0.5 million K-562 cells. f. **CRITICAL STEP:** In contrast to shorter incubation times, overnight antibody incubation increases protocol reproducibility. g. Empirically determine the minimal [digitonin] in **Digitonin buffer** and **Antibody buffer** for efficient cell permeabilization.

Step	Note
7. Quick spin, then place the tube on a magnet stand and remove the liquid. 8. Add 1 mL of **Digitonin Buffer**, thoroughly mix with the beads by inversion[h]. 9. Quick spin, then place the tube on a magnet stand and remove the liquid. 10. Repeat steps 8 & 9 for a total of two washes.	**h.** Avoid pipetting as much as possible to avoid bead loss on pipet tips.

Part IV: Binding of pAG-MNase *(estimated time = 30 min)*

Step	Note
1. Place the tube at a low angle on the vortex mixer, squirt 50 µL of **Digitonin Buffer** along the side while gentle vortexing. 2. Gently tap to dislodge all beads[i]. 3. Add 2.5 µL of EpiCypher CUTANA™ pAG-MNase (20X stock). 4. Place the tubes on a tube nutator and nutate for 10 min at room temperature. 5. Quick spin, then place the tube on a magnet stand and remove the liquid. 6. Add 1 mL of **Digitonin Buffer**, thoroughly mix with the beads by inversion[i]. 7. Quick spin, then place the tube on a magnet stand and remove the liquid. 8. Repeat steps 6 & 7 for a total of two washes.	**i.** Avoid pipetting as much as possible to avoid bead loss on pipet tips.

Part V: Targeted Chromatin Digestion and Release *(estimated time = 3 - 4 hours)*

Step	Note
1. Place the tube at a low angle on the vortex mixer, squirt 150 µL of **Digitonin Buffer** along the side while gentle vortexing. 2. Pre-chill the tubes and 100 mM $CaCl_2$ on ice. 3. Add 3 µL of 100 mM $CaCl_2$ to each tube with gentle vortexing and immediately replace the tube on ice. 4. Move the tubes to a tube nutator and **nutate for 2 hours at 4˚C**[j,k].	**CRITICAL STEPS:** **j.** In contrast to shorter incubation times, 2 hour digestion increases protocol reproducibility. **k.** It is critical to keep the tubes at 0-4˚C to minimize background chromatin cleavage by the pAG-MNase.
5. Add 100 µL of **STOP Buffer** and mix by gentle vortexing. 6. Incubate the tubes at 37˚C for 10 min on a ThermoMixer at 500 rpm to release fragments into solution. 7. Centrifuge at 4˚C for 5 min at 16,000 *x g.* 8. Place the tube on a magnet stand and transfer the liquid to a 2 mL tube.	
9. Extract the DNA with the Qiagen MinElute PCR Purification Kit by following the manufacturer's instructions[l]. 10. Quantify the CUT&RUN DNA by Qubit analysis, following the manufacturer's instructions[m,n].	**l.** Use 10 µL EB buffer for the final elution. **m.** 2 - 5 ng total DNA is desired for the library preparation.

	n. Tested at EpiCypher: For H3K27me3 CUT&RUN, ~ 30-50 ng final DNA yield is typically expected; For H3K4me3 CUT&RUN, ~ 4-10 ng final DNA yield is typically expected.

Part VI: Sequencing Library Preparation (estimated time = 1 – 2 days)

1. Prep indexed DNA libraries following the manufacturer's instructions[o]. 2. For PCR amplification of CUT&RUN fragments, perform the following procedures to enrich for mononucleosome sized fragments: a. 98˚C 45 s, b. 98˚C 15 s, c. 60˚C 10 s, d. Repeat b-c for 13x, e. 72˚C 1 min f. 8˚C Hold 3. Quantify the DNA library by Qubit analysis, following the manufacturer's instructions. 4. Assess the DNA library size distribution by Agilent 2100 BioAnalyzer analysis, following the manufacturer's instructions[p].	**o. Tested at EpiCypher:** Both Illumina TruSeq ChIP Sample Prep Kit and NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® work well for CUT&RUN library preparation. **p.** Typical BioAnalyzer traces for H3K27me3 and H3K4me3 after library preparation can be found in the EpiCypher CUTANA™ pAG-MNase Technical Data Sheet.

6. Buffer Recipes

Binding Buffer

20 mM HEPES, pH 7.9
10 mM KCl
1 mM $CaCl_2$
1 mM $MnCl_2$
Filter sterilize
Store @ 4˚C for up to 6 months

Wash Buffer

20 mM HEPES, pH 7.5
150 mM NaCl
0.5 mM Spermidine
1X Protease Inhibitor
Filter sterilize
Store @ 4˚C for up to 1 week

Digitonin Buffer

Wash Buffer + x% Digitonin*
*[Digitonin] needs to be empirically determined, see Protocol Part III, Step 6
Store @ 4˚C for up to 1 day

Antibody Buffer

Digitonin Buffer + 2 mM EDTA
Store @ 4˚C for up to 1 hour upon antibody addition

STOP Buffer

340 mM NaCl
20 mM EDTA
4 mM EGTA
0.02% Digitonin
50 µg/mL RNase A
50 µg/mL Glycogen
Filter sterilize
Store @ 4˚C for up to 1 week

7. References

[1] Skene PJ, Henikoff JG & Henikoff S (2018) Targeted in situ genome-wide profiling with high efficiency for low cell numbers. *Nature Protocols* **13:**1006-1019 (doi:10.1038 /nprot.2018.015).

[2] Schmid M, Durussel T & Laemmli UK (2004) ChIC and ChEC; genomic mapping of chromatin proteins. *Molecular Cell* **16:**147-157 (doi:10.1016/j.molcel.2004.09.007).

[3] Shah RN, Gryybowski AT, Cornett EM, Johnstone AL, Dickson BM, Boone BA, Cheek MA, Cowles MW, Maryanski D, Meiners MJ, Tiedemann RL, Vaughan RM, Sun ZW, Rothbart SB, Keogh M-C & Ruthenburg AJ (2018) Examining the roles of H3K4 methylation states with systematically characterized antibodies. *Molecular Cell* **72:**162-177 (doi: 10.1016/j.molcel.2018.08.015).