EpiCypher® CUTANA™ Cross-linking Protocol

For analysis of labile histone PTMs and transiently-interacting chromatin regulators in CUT&RUN and CUT&Tag assays

Compatible with CUTANA™ CUT&RUN & CUT&Tag Protocols: epicypher.com/protocols

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1. Overview

Cleavage Under Targets & Release Using Nuclease (CUT&RUN) and Cleavage Under Targets & Tagmentation (CUT&Tag) are revolutionary genomic mapping strategies that generate high resolution chromatin profiles with very low background, using a fraction of the cells and sequencing depth required by ChIP-seq[1-3]. In both assays, permeabilized cells or nuclei* are immobilized to a solid support and antibodies bind their chromatin targets *in situ*. In CUT&RUN, a fusion of Protein A/G and Micrococcal Nuclease (pAG-MNase) is used to cleave antibody-labelled DNA fragments, which are released into solution and collected for next-generation sequencing (NGS). In CUT&Tag, pAG is fused with prokaryotic transposase 5 (pAG-Tn5) to cleave and tagment antibody-bound chromatin with sequencing adapters. Tagmented fragments are then selectively amplified by PCR for NGS. Compared to CUT&RUN, CUT&Tag is uniquely optimized for profiling histone post-translational modifications (PTMs) in cutting-edge research applications, including ultra-low cell inputs. However, for the majority of applications and targets, CUT&RUN assays are recommended as the go-to genomic mapping approach.

While the majority of targets work well under native conditions, there are situations (*e.g.* labile histone PTMs, transient chromatin binding proteins) where CUT&RUN and CUT&Tag signal is improved by light to moderate cross-linking. As an example, deacetylase activity towards histone lysine acetylations can lead to incomplete or low resolution genomic profiles in native CUT&RUN/CUT&Tag. In the case of H3K27ac CUT&RUN (**Figure 1**), light to moderate cross-linking may improve signal:noise (although total yields may be reduced; see FAQs). However, heavy cross-linking (1% formaldehyde for 10 min; standard for ChIP-seq) dramatically reduces DNA yield and H3K27ac signal (**Figure 1**). Therefore, optimal cross-linking conditions should be empirically determined in the model system of interest.

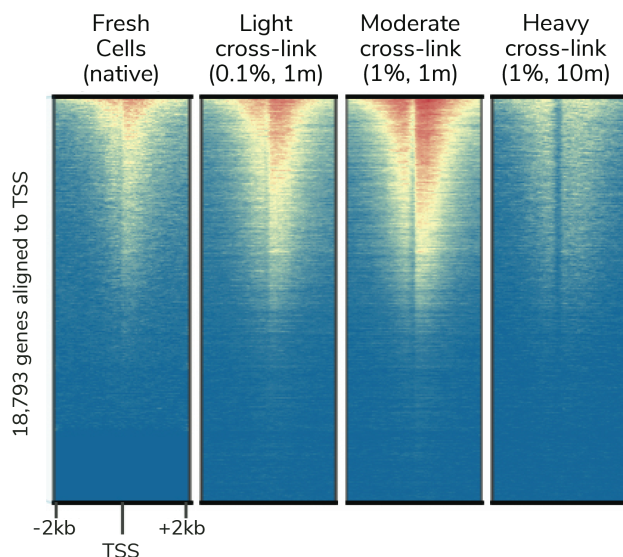

Figure 1: H3K27ac CUT&RUN signal is improved by light to moderate fixation (0.1 – 1% formaldehyde for 1 min) compared to native conditions. However, cross-linking negatively impacts DNA yield even under moderate conditions. Eventually, heavy cross-linking such as that used in standard ChIP procedures (1%, 10 min) has a profound impact on DNA yield and also degrades signal quality. CUT&RUN data using H3K27ac antibody (EpiCypher 13-0045) with 500,000 K562 cells are displayed in a heatmap. Signal -/+ 2 kb of annotated transcription start sites (TSS) of ~18k genes is colored by intensity and ranked from high (top) to low (bottom) with each gene row aligned across conditions relative to native cells.

*NOTE: Nuclei are preferred for CUT&Tag; see the CUTANA CUT&Tag Protocol at epicypher.com/protocols.

2. Protocol Notes

This cross-linking protocol should be considered for:

- Labile histone PTMs (*e.g.* Histone lysine acetylation [Kac] marks that are dynamically regulated by lysine deacetylases [KDACs])
- Chromatin binding complexes that recognize histone acetylation (*e.g.* Bromodomain-containing proteins and complexes)
- Transiently interacting chromatin-associated proteins (*e.g.* transcription factors, chromatin remodelers)
- Experiments where variables should be tightly controlled (*e.g.* time course drug treatments)

NOTE: Not all members of the above classes require cross-linking. Native and cross-linked conditions should be compared in early optimization experiments. See FAQs for details.

1. **For the majority of targets, CUT&RUN performed under native conditions is the preferred workflow.** We recommend testing antibodies using native CUT&RUN prior to using cross-linking conditions or CUT&Tag workflows, based on the following observations:
   a. Most targets (*e.g.* lysine methylation, many transcription factors, most chromatin remodelers, and even some acetylation marks and readers such as H3K9ac and BRD4) work well under native conditions using the CUTANA CUT&RUN protocol.
   b. CUT&Tag is only recommended for histone PTMs and/or applications requiring low cell inputs. However, native conditions are also preferred for CUT&Tag assays.
2. **For CUT&RUN (or CUT&Tag) to new targets, we recommend the following:**
   a. Source antibodies to desired target.
      i. Check EpiCypher's expanding list of CUT&RUN antibodies at epicypher.com/cut-and-run-antibodies. The accompanying technical datasheet will provide optimal conditions.
      ii. If an antibody is not offered, contact us at info@epicypher.com for recommendations.
      iii. Source 3-5 antibodies to the target, preferentially from multiple reputable vendors and to different epitopes. Note that antibody success in ChIP or claims of "ChIP-grade" status do **not** guarantee success in CUT&RUN.
   b. Test all antibodies under native and cross-linked conditions.

      IMPORTANT: **Light cross-linking (0.1% formaldehyde, 1 min) is recommended to start**, as this is generally sufficient to preserve labile PTMs without negatively impacting DNA yield. If light cross-linking is not sufficient, moderate cross-linking (1% formaldehyde, 1 min) can be attempted with the caveat that it may impact DNA yield (potentially

problematic for low abundance targets and low cell inputs). Heavy cross-linking (1% formaldehyde, 10 min) is deleterious to both DNA yield and data quality (**Figure 1**) and should be avoided in CUT&RUN.

c. Select the optimal sample prep conditions for the target based on the balance of DNA yield, enrichment, and signal-to-noise in the sequence data.

3. **Note that for CUT&RUN, cross-linking is accompanied by changes to the wash conditions in the CUTANA™ CUT&RUN protocol** (see Protocol, Section 4). The addition of Triton and SDS may improve nuclear pore permability after cross-linking to increase diffusion of target complexes into the supernatant[4].

3. Buffers, Reagents & Equipment Needed

Table 1: CUT&RUN Buffer Components

Components	Source	Catalog No.
HEPES (prepare 1 M stock at pH 7.9 using KOH)	Sigma-Aldrich	H3375
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	S2501
Spermidine trihydrochloride*	Sigma-Aldrich	S0266
Digitonin (store aliquots of 5% stock in DMSO at -20ºC)	Millipore Sigma	300410
DMSO	Sigma-Aldrich	D8418-100ml
cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail	Roche	11836170001
Triton X-100	Sigma-Aldrich	T8787

*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.

Cross-linking buffer recipes (for use in CUT&RUN; see Protocol for details)
Note: No buffer modifications are required if performing CUT&Tag.

Pre-XL Wash Buffer
- 20 mM HEPES-KOH, pH 7.9
- 150 mM NaCl
- 1% Triton X-100
- 0.05% SDS
- *Filter sterilize*
- *Store @ 4ºC for up to 6 months*

XL Wash Buffer
- Pre-XL Wash Buffer
- 1x cOmplete™, Mini, EDTA-free Protease Inhibitor (CPI-mini, 1 tablet/10 mL)
- 0.5 mM Spermidine
- *Stable for 2 days @ 4ºC*

XL Digitonin Buffer
- XL Wash Buffer
- 0.01% Digitonin
- *Stable for 2 days @ 4ºC*

XL Antibody Buffer
- XL Digitonin Buffer
- 2 mM EDTA
- *Stable for 2 days @ 4ºC*

Table 2: Cross-linking Reagents

Item	Vendor	Catalog No.	Notes
37% Formaldehyde	Sigma	252549	Handle using appropriate safety precautions (see Safety Data Sheet)
2.5 M Glycine	Sigma	50046	For 2.5M, dissolved 9.4 g Glycine in 50 mL molecular grade water. Filter sterilize and store at room temperature.
10% SDS	Sigma	71736	
20 µg/µL Proteinase K	Ambion	AM2546	Only required for CUT&RUN

Table 3: Equipment

Item	Vendor	Catalog No.	Notes
Vortex-Genie	Scientific Industries	SI-0236	For mixing steps
Thermocycler	Any	Any	For incubation steps

4. EpiCypher CUTANA™ Cross-linking Protocol (30 min + Overnight)

Note: EpiCypher suggests using 500,000 cells per CUT&RUN reaction and 100,000 cells per CUT&Tag reaction (epicypher.com/protocols).

IMPORTANT: If using nuclei (preferred for CUT&Tag), note that cross-linking is performed prior to nuclei harvest.

1. From suspension tissue culture, transfer cells into a 15 mL or 1.5 mL tube.

   For adherent cells, cross-link cells *in situ* (*i.e.* while cells are still attached to plate).

   Note: Although it is common to wash cells with PBS prior to fixation, we prefer to fix in media so that the protocol is more widely applicable. In addition, washing with PBS can significantly diminish the effect of transient cell perterbations (*e.g*. cell stimulation, drug treatment).
2. Add X µL **37% formaldehyde** directly to culture to desired final concentration.

   Some general guidelines on formaldehyde concentrations:
   - Light cross-linking (0.1% formaldehyde for 1 min) preserves most labile PTMs without negatively impacting DNA yield, and is recommended for new targets/antibodies.
   - If light cross-linking is not sufficient, moderate cross-linking (1% formaldehyde, 1 min) can be attempted with the caveat that it may impact DNA yield (potentially problematic for low abundance targets and low cell inputs).
   - Heavy cross-linking (1% formaldehyde, 10 min) is deleterious to both DNA yield and data quality (**Figure 1**) and should be avoided in CUT&RUN and CUT&Tag.
3. Quickly vortex suspension cells to mix. For adherent cells, swirl plate/flask to mix.
4. Incubate for desired time at room temperature (RT). It is recommended to start with a 1 min incubation, and increase time if needed.
5. Quench fixation by adding **2.5 M Glycine** to a final concentration of 125 mM (1:20 dilution).
6. Vortex suspension cells to mix.

   For adherent cells, swirl plate to mix. Scrape cells from the plate, transfer to 15 mL tube, and centrifuge for 3 min at 600 x g, RT.
7. [*Optional*] If it is desired to isolate cross-linked nuclei – recommended for CUT&Tag experiments – follow the nuclei isolation protocol exactly as described in the respective CUTANA™ CUT&RUN or CUT&Tag protocol (epicypher.com/protocols). Our CUTANA workflows are compatible with lightly cross-linked cells (0.1% formaldehyde, 1 min).

If continuing to CUT&RUN, proceed to Step 8. If using nuclei for CUT&Tag, go to Step 9.

8. For CUT&RUN: Cross-linked cells or nuclei enter the CUTANA™ CUT&RUN Protocol at Section II: Binding Cells to Activated Beads. Note the following protocol modifications:
   - Replace Wash, Digitonin, and Antibody buffers in the native protocol with **XL Wash Buffer**, **XL Digitonin Buffer** and **XL Antibody Buffer** (see Section 3, above).

     NOTE: Detergents are included in cross-linking buffers to help cell/nuclei permeability, which supports comparable yields vs. native CUT&RUN assays.
   - After the 37ºC incubation step in **Section V: Targeted Chromatin Digestion and Release**, reverse cross-links as follows:
     i. Place reactions on magnet.
     ii. Transfer 89 µL supernatant, containing fragmented CUT&RUN DNA, to a new tube.
     iii. Add 0.8 µL **10% SDS** and 1 µL of 20 µg/µL **Proteinase K** to each supernatant.
     iv. Incubate overnight at 55ºC using a thermocycler.

     IMPORTANT: Only add SDS and Proteinase K to the isolated supernatant containing CUT&RUN enriched DNA. If SDS and Proteinase K are added to the entire sample:bead slurry, then total chromatin will be extracted. When this is done, the yields are high for all samples and do not reflect experimental success.
   - Carry on with the CUT&RUN Protocol as normal by placing the 8-strip tube on a magnet until slurry clears and transfer supernatant to 1.5 mL tube. Purify DNA using the CUTANA™ DNA Purification Kit (EpiCypher 14-0050).

9. For CUT&Tag: Cross-linked nuclei enter the CUTANA™ CUT&Tag Protocol at Section II: Nuclei Preparation and Binding Nuclei to Activated Beads. No additional modifications to the protocol are required. Note the following:
   - Digestion with SDS Release Buffer at 58˚C following tagmentation (Section V: Targeted Chromatin Tagmentation) is sufficient to reverse cross-links.
   - Do not add Proteinase K for the Direct-to-PCR CUT&Tag protocol. Proteinase K will inhibit PCR amplification.

5. Frequently Asked Questions (FAQs)

1. **Do lower yields from a cross-linked sample vs. a native sample indicate a problem?**
DNA yields are often diminished relative to the native protocol, and are inversely correlated with the amount of cross-linking (**Table 1**). For example, moderate (1% formaldehyde, 1 min) and heavy cross-linking (*i.e.* standard ChIP conditions: 1% formaldehyde, 10 min) can have a deleterious effect on DNA yield and eventually on data quality. While this can be particularly problematic under certain conditions (*e.g.* low adundance targets and small cell numbers), moderate cross-linking can be tolerated if the resulting data tracks are of good quality. Importantly, we have found that light cross-linking (0.1% formaldehyde, 1 min) can help to preserve some labile PTMs with little to no adverse impact on CUT&RUN DNA yields. For this reason, we suggest first testing light cross-linking conditions (0.1% formaldehyde, 1 min) vs. native cells to see if CUT&RUN or CUT&Tag signal is improved.

Cross-linking Condition	DNA Yields	Data Quality / Notes
Light: 0.1% Formaldehyde, 1 min	Unchanged vs. native cells	May improve signal vs. native conditions by preserving or stabilizing labile targets.
Moderate: 1% Formaldehyde, 1 min	~10x reduced vs. native cells	Very dependent on experimental conditions (target, antibody, cell type & number): data quality may be improved vs. light cross-linking, but could also be negatively impacted. Use as a second option when light cross-linking does not work.
Heavy: 1% Formaldehyde, 10 min	>10x reduced vs. native cells	Very poor data quality, do not use in CUT&RUN or CUT&Tag.

Table 4: Summary of cross-linking conditions tested by EpiCypher in CUT&RUN and CUT&Tag.

2. **When should cross-linking be considered?**
If labile PTMs are suspected as a source of poor data quality, we recommend testing cross-linked cell/nuclei samples. In these cases, we have found that light cross-linking most often improves data quality without sacrificing yields, and recommend this strategy as a starting point. Of note, EpiCypher continues to explore cross-linking protocols. Our early work suggests cross-linking can preserve labile PTMs and cell stimulation states, thus facilitating CUT&RUN/ CUT&Tag for diverse targets and highly controlled studies (*e.g.* time-course, cell stimulation, or drug treatment).

3. **Which targets require cross-linking?**
In our experience, the vast majority of targets work well under native conditions and using our robust CUTANA™ CUT&RUN protocol. To avoid potential artifacts and reduced yield, cross-linking is a secondary option when native conditions do not produce the expected enrichment pattern.

We have performed extensive antibody testing for potentially labile targets:

- **Lysine acetylation (Kac):** In our hands some specific KAc signals are equally robust in native vs. cross-linked conditions (*e.g.* H3K9ac, EpiCypher 13-0033) while others benefit from (*e.g.* H3K27ac, EpiCypher 13-0045) or absolutely require (*e.g.* H3K18ac, Thermo Fisher Scientific PA5-85523) cross-linking.
- **Bromodomains (BRDs):** BRDS are a class of chromatin reader proteins that selectively bind histone lysine acetylation PTMs, and may also benefit from light cross-linking. However, many BRDs show robust enrichment under native conditions (*e.g.* BRD4 antibody, EpiCypher 13-2003).
- **Chromatin remodelers:** These enzymes transiently interact with chromatin and have been challenging to profile by ChIP-seq. We have found that some antibodies to chromatin remodelers work well under native conditions (*e.g.* BRG1/SMARCA4, EpiCypher 13-2002; SNF2H/SMARCA5, EpiCypher 13-2007), while others benefit from light cross-linking (*e.g.* BRM/SMARCA2, EpiCypher 13-2006; SNF2L/SMARCA1, EpiCypher 13-2005).

When investigating a new target, always compare native and light to moderate cross-linking conditions in optimization studies before proceeding to valuable samples. For a full list of CUT&RUN antibodies available from EpiCypher, visit epicypher.com/cut-and-run-antibodies or contact us (info@epicypher.com) for recommendations.

4. **Instead of cross-linking, can I use inhibitors to stabilize labile PTMs (*e.g.* for lysine acetylation)?**

   EpiCypher uses this approach for profiling lysine acetylation marks in native ChIP, by adding histone deacetylase inhibitors (*e.g.* sodium butyrate, trichostatin A) to buffers prior to nuclear lysis[5]. A similar approach is currently being evaluated for CUT&RUN and CUT&Tag. One potential benefit of this approach is that the CUT&RUN buffers are not modified (see Protocol), which aids sample parallelization. Limitations of this approach and specific recommendations for when it may be beneficial are still being explored.

6. References

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