# EpiCypher® CUTANA™ CUT&RUN Protocol

For histone PTMs, transcription factors (TFs), and chromatin regulators

## 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) from Dr. Ulrich Laemmli [2], whereby a fusion of Protein A to Micrococcal Nuclease (pA-MNase) is recruited to selectively cleave antibody-bound chromatin *in situ* [3]. In CUT&RUN, cells or nuclei are immobilized to a solid support, with pAG-MNase cleaved DNA fragments isolated from solution. The workflow is compatible with next-generation sequencing (NGS) to provide high resolution genome-wide profiles of histone post-translational modifications (PTMs) and chromatin-associated proteins (*e.g.* TFs and chromatin remodelers; **Figure 1**).

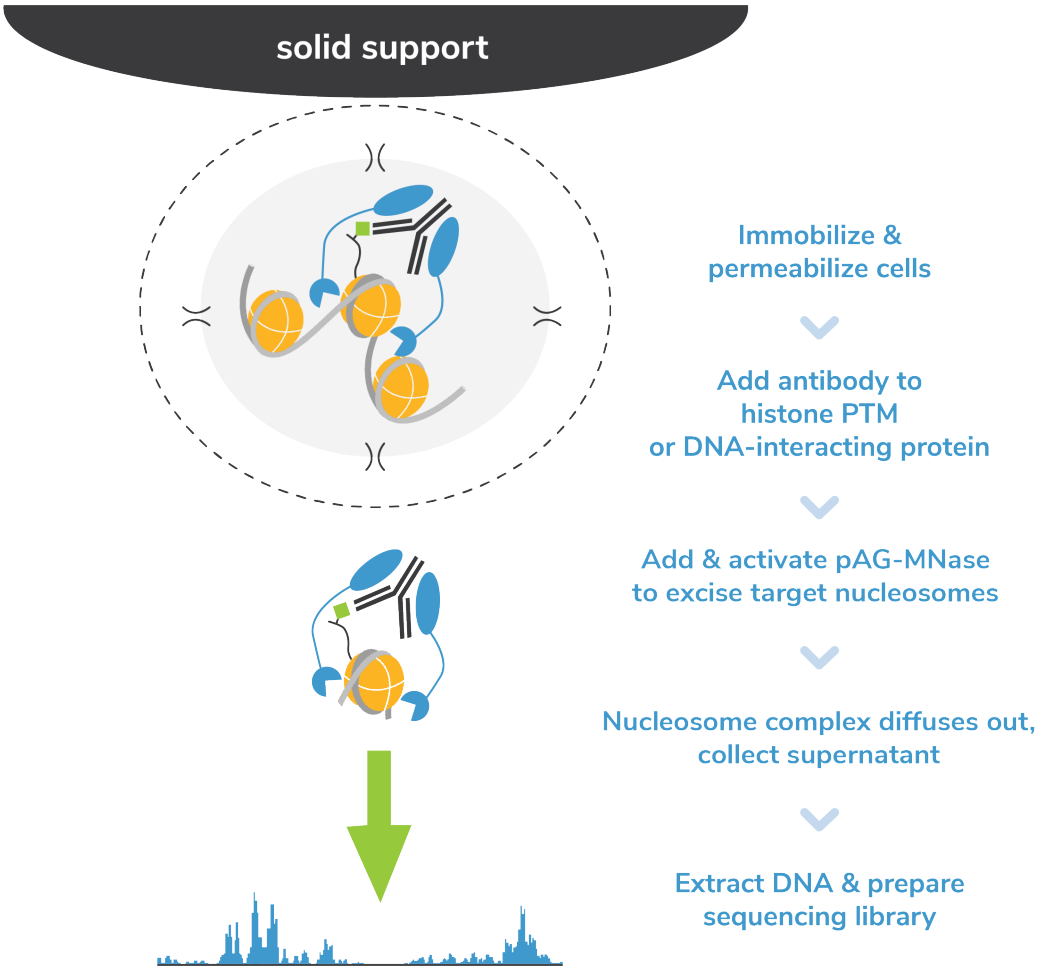



**Figure 1**. Overview of the CUTANA™ CUT&RUN protocol.

## 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 < 2 days
- Empowers benchtop sequencers (*e.g.* Illumina® MiniSeq or MiSeq)
- Dramatically increases experimental throughput (transforming 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) is directly compatible with a broad range of antibody isotypes
- 50 and 250 reaction pack sizes, enabling greater experimental throughput
- Check www.epicypher.com for regular updates of optimized protocols
- Additional CUTANA™ products coming soon:
    - CUT&RUN Compatible Antibodies
    - CUT&RUN Kits
    - CUT&RUN spike-in controls
    - pAG-Tn5 (for CUT&Tag applications)
    - *Inquire for more information or to connect to EpiCypher scientists*: info@epicypher.com

## 3. Protocol Notes

**This protocol has been validated for genomic profiling of:**

- Histone PTMs (*e.g.* lysine methylation, lysine acetylation, lysine ubiquitylation, etc.)
- Transcription factors (*e.g.* CTCF)
- Chromatin remodelers (*e.g.* SMARCA4-SWI/SNF, ISWI, INO80, CHD)
- Chromatin writers & readers (*e.g.* MLL, BRD4)

1. Always include control conditions (*e.g.* positive control cells & antibody; see FAQ section). This is especially critical when evaluating CUT&RUN using previously untested targets and/or antibodies.

2. Protocol optimized using 0.5 million human K562 cells per sample. We have also validated variations to the protocol for working with nuclei, adherent cells, cryopreserved cells/nuclei, and crosslinked material (available upon request).

3. Protocol adapted from 1.5 ml tubes to 300 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.

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

5. Although protocols with shortened antibody and/or $CaCl_2$ incubation times have been published [3], in our hands such changes adversely impact yield and reproducibility.

6. We recommend a 5% digitonin stock solution be prepared in DMSO (as opposed to heated $H_2O$) because this dramatically increases detergent solubility and standardizes cell permeabilization / protocol reproducibility.

7. To avoid digitonin precipitation and cell lysis, use the minimal concentration of digitonin in **Digitonin Buffer** / **Antibody Buffer,** which are required for efficient cell permeabilization. This should be empirically determined for different samples before proceeding with the full CUT&RUN experiment.

## 4. Buffers, Reagents & Materials Needed

### Buffer recipes

**Bead Activation Buffer**

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*

**Wash Buffer**

20 mM HEPES, pH 7.5
150 mM NaCl
0.5 mM Spermidine
1x Roche Complete Protease Inhibitor-mini (CPI-mini), EDTA-free (Roche catalog # 11836170001), 1tab/10ml
*Filter sterilize*
*Store @ 4ºC for up to 1 week*

**Digitonin Buffer**

Wash Buffer + 0.01% Digitonin *
* Optimal [Digitonin] for each cell type should be empirically determined. Starting concentration validated for K562, MCF7, and A549 cells = 0.01% digitonin.
*Prepare fresh each day and store @ 4ºC*

**Antibody Buffer**

Digitonin Buffer* + 2 mM EDTA
* See note above for empirically determining optimal digitonin concentration for a given cell type
*Prepare fresh each day and store @ 4ºC*

**Stop Buffer**

340 mM NaCl
20 mM EDTA
4 mM EGTA
50 µg/ml RNase A
50 µg/ml Glycogen
*Filter sterilize*
*Store @ 4ºC for up to 6 months*

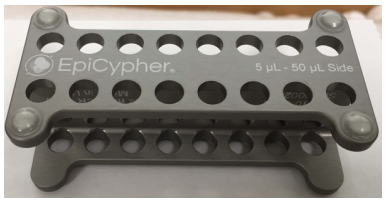
## Reagents

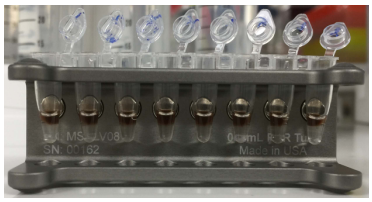
| Item | Vendor | Catalog No. | Notes |
|---|---|---|---|
| Concanavalin A (ConA) Magnetic Beads | EpiCypher | 21-1401 | ConA is a lectin, which can cause immune activation. For technical support re. immune cell studies, contact info@epicypher.com |
| pAG-MNase | EpiCypher | 15-1016 | Supplied as 20X stock |
| Rabbit IgG Negative Control Antibody | EpiCypher | 13-0042 | Dilute 1:10 in Antibody buffer, store @ 4ºC for 1 month. |
| H3K4me3 Positive Control Antibody: SNAP-ChIP Certified, CUTANA CUT&RUN Compatible | EpiCypher | 13-0041 | 0.5 µg/µl → Use at 1:100 dilution in CUT&RUN. |
| Antibody to histone PTM, TF, or chromatin regulator | User-dependent | | EpiCypher continues to conduct extensive antibody characterization (most particularly for those against histone PTMs [4]). Contact us for recommendations: info@epicypher.com |
| NEB Monarch PCR & DNA Cleanup Kit | New England Biolabs | T1030S | Chosen for high yields of low [DNA] samples, low elution volume, and retention of >50bp fragments (thus suitable for nucleosomal (PTMs) and subnucleosomal (TFs) fragments) |
| 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 |
| NEBNext Ultra II DNA Library Prep Kit for Illumina | New England Biolabs | E7645S | For DNA sequencing applications |

## Equipment

| Item | Vendor | Catalog No. | Notes |
|---|---|---|---|
| 8-strip PCR tube magnetic stand | EpiCypher | 10-0008 | For all bead separation steps |
| 8-strip 0.2 mL PCR tubes | EpiCypher | 10-0009 | Compatible with the 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-MNase digest reaction) |
| Vortex-Genie | Scientific Industries | SI-0236 | For bead mixing steps. |

EpiCypher's 8-strip PCR tube magnetic stand (#10-0008):
*Enables streamlined sample handling for higher experimental throughput and improved reproducibility.*



# 5.1. EpiCypher CUTANA: CUT&RUN Protocol (~5hrs)

**Day 1**

## Section I: ConA Bead Activation (~30 min)

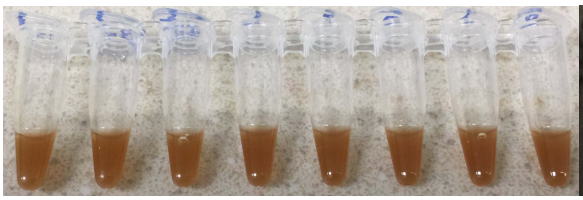
1. Gently resuspend the **ConA beads (Concanavalin A)** and transfer 11 µl/sample to 1.5 ml tube for batch processing.
2. Place the tube on a magnet until slurry clears and pipet to remove supernatant (supe).
3. Add 100 µl/sample cold **Bead Activation Buffer** and pipet to mix. Place the tube on a magnet 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.

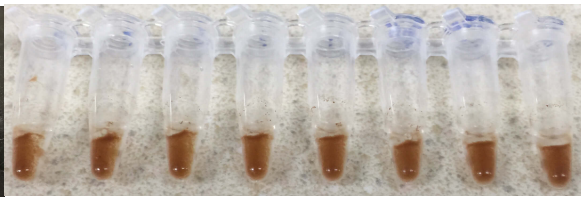
## Section II: Binding Cells to Activated Beads (~30 min)

7. Harvest 0.5 million cells/sample by spinning for 3 min at 600g at room temperature (RT) in 1.5ml tube, and decant supe.
8. Resuspend cells in 100 µl/sample RT **Wash Buffer**; spin for 3 min at 600g at RT; decant supe.
9. Repeat previous step for total of two washes.
10. Resuspend cells in 100 µl/sample in RT **Wash Buffer** and aliquot 100µl washed cells to each 8-strip tube containing 10 µl of activated bead. Gently vortex (setting #7) to mix.
11. **Incubate** cell : bead slurry for **10 min at RT**. *Cells will adsorb to the activated ConA beads.*

## Section III: Binding of Antibodies (~30 min + overnight)

12. Place the tube on a magnet until slurry clears and pipet to remove supe.
13. Add 50 µl cold **Antibody Buffer** to each sample and gently vortex.
14. Add 0.5 µl **Antibody** (1:100 dilution) to each sample and gently vortex.
15. **Incubate** 8-strip tube (caps slightly elevated) on nutator **overnight at 4ºC**.
    a. *Critical step: To keep beads in solution, slightly elevate cap side of 8-strip on nutator.*

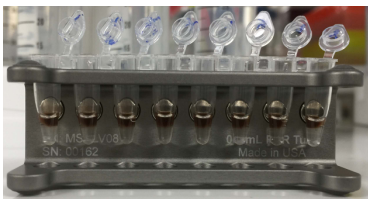




Bead slurry before o/n incubation @ 4C | nutator o/n | Bead slurry after o/n incubation @ 4C

**Day 2**

16. Place the tube on a magnet until slurry clears and pipet to remove supe.



17. While beads are on magnet, add 250 µl cold **Digitonin Buffer** directly onto beads of each sample, and then pipet to remove supe.
18. Repeat previous step for total of two washes.
19. Add 50 µl cold **Digitonin Buffer** to each 8-strip tube, and gently vortex. Beads are often clumpy at this point, but can easily be dispersed with gentle pipetting with a P200 pipet.

## Section IV: Binding of pAG-MNase (~30 min)

20. Add 2.5 µl **CUTANA™ pAG-MNase** (20x stock) to each sample, and gently vortex.
    - *Critical step: to evenly distribute pAG-MNase across cells/nuclei, ensure beads are thoroughly resuspended by gentle vortexing and/or pipetting with a P200 pipet.*
21. **Incubate** samples for **10 min at RT**, and then return 8-strip tube to magnet.
22. While beads are on magnet, add 250 µl cold **Digitonin Buffer** directly to each sample, and then pipet to remove supe.
23. Repeat previous step for total of two washes.
24. Add 50 µl cold **Digitonin Buffer** to each sample, and gently vortex. Beads are often clumpy at this point, but can easily be dispersed with gentle pipetting with a P200 pipet.

## Section V: Targeted Chromatin Digestion and Release (~3 hrs)

25. Place 8-strip on ice, add 1 µl **100 mM $CaCl_2$** to samples, and gently vortex.
    - *Critical step: For efficient pAG-MNase digestion, ensure beads are thoroughly resuspended by vortexing and/or pipetting with a P200 pipet.*
26. **Incubate** 8-strip tube on nutator for **2 hours at 4ºC**. Key step wherein MNase tethered to chromatin is activated by CaCl2 to cleave chromatin.
27. Add 33 µl **Stop Buffer** to each sample, and gently vortex to mix. Stops MNase activity by chelating $Ca^{++}$ ions.
28. **Incubate** 8-strip tube for **10 min at 37ºC** in thermocycler. *Releases chromatin to supernatant and degrades RNA.*
29. Place 8-strip tube on a magnet stand until slurry clears and transfer supe to 1.5 ml tube.
30. Purify DNA using the **NEB Monarch DNA Cleanup Kit** as per manufacturer's instructions.
31. Elute DNA in 10 µl **Elution Buffer** and use 1 µl to quantify the CUT&RUN-enriched DNA using the **Qubit fluorometer** as per manufacturer's instructions.

Typical CUT&RUN DNA total yields from 500k native K562 cells, pre-PCR:

- *Note: Total yields are influenced by a number of considerations, including antibody specificity and efficiency, and epitope abundance in each cell type.*

IgG → ~ 3ng
H3K27me3 → ~200ng
H3K4me3 → ~50ng
CTCF → ~10ng
BRD4 → ~20ng
SMARCA4/BRG1 → ~15ng

## 5.2. Library prep (4 hrs), Bioanalyzer (1 hr), & Illumina sequencing

### Section VI: NEB Ultra II Library Preparation (~4 hrs)

32. Using 5-10ng purified CUT&RUN-enriched DNA, prepare Illumina library using the **NEB Ultra II Library Prep Kit** per manufacturer's instructions (~4hrs).
    - *Note: NEB Ultra II kit contains hot-start Q5 DNA polymerase.*
33. Overview of workflow, according to manufacturer's instructions.
    a. End repair
    b. Universal adapter ligation
    c. DNA cleanup using 1.1x Ampure beads to sample volume (retains fragments >150bp)
    d. PCR and primer indexing according to cycling parameters below
34. CUT&RUN-specific PCR cycling parameters:
    a. 45 sec @ 98ºC → activation of hot-start Q5 polymerase
    b. 15 sec @ 98ºC → DNA melting
    c. 10 sec @ 60ºC → hybrid primer annealing & short extension (<700 bp)
    d. Repeat "step b-c" for a total of 14x
    e. 1 min @ 72ºC → final extension
    - *Critical step: PCR cycling parameter are designed to enrich for DNA fragment within the sequence-able range (100bp-700bp). Thus, contaminating higher molecular weight DNA fragments are not enriched by PCR.*

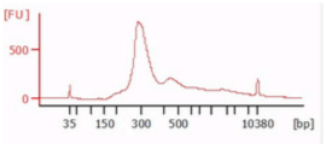
35. DNA cleanup using 1.1x Ampure beads to sample volume (eg: 55 µl Ampure to 50 µl PCR).
36. Elute DNA in 15 µl **0.1x TE buffer** and use 1 µl to quantify the purified PCR product using the **Qubit fluorometer** as per manufacturer's instructions.
    - *Typical yield purified PCR DNA library, ~500-750ng (30-50ng/µl in 15µl)*

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

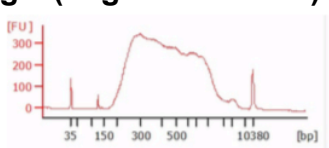
37. For each sample, prepare 5µl at 10ng/µl for loading on the Agilent Bioanalyzer.
    - *Note: record dilution factor such that original sample molarity can be calculated from Bioanalyzer nM for desired DNA size range (100-1,000bp).*
38. For each purified PCR DNA library for Illumina sequencing, load 1ul of 10ng/µl sample on **Agilent High Sensitivity DNA Chip** (Cat# 5067-4626) as per manufacturer's instructions.
    - *Typical molarity for 15 µl purified PCR DNA library (100-1,000bp region) = 100-200 nM*

Typical Bioanalyzer traces (5ng PCR amplified libraries loaded on Bioanalyzer)

**H3K4me3**




**IgG (negative control)**



## Section VIII: Illumina sequencing

39. Based on Bioanalyzer molarity calculations, pool libraries at the desired ratios.
    - Only 3-5 million paired-end (PE) reads are needed for good CUT&RUN coverage.
40. To obtain 3-5M PE reads for 8 samples, we typically load 0.8 pM (500 µl) into a cartridge for a MiniSeq High Output Reagent Kit, 150-cycles (catalog # FC-420-1002).
    - Flow cells using the MiniSeq High Output Kit typically yield 25-40M pass-filter clusters (i.e. 25-40M PE reads).
41. To obtain 3-5M PE reads for >48 samples, we typically load 0.8 pM (1,500 µl) into a cartridge for a NextSeq 500/550 High Output Kit v2.5, 150-cycles (catalog # 20024907).
    - Flow cells using the NextSeq High Output Kit v 2.5 typically yield 400M pass-filter clusters (i.e. 400M PE reads).

## 6. Buffer components

| Components | Source | Cat # |
|---|---|---|
| **HEPES** | Sigma-Aldrich | H3375 |
| **KCl** | Sigma-Aldrich | P3911 |
| **CaCl2** | Sigma-Aldrich | C1016 |
| **MnCl2** | Sigma-Aldrich | 203734 |
| **Molecular biology grade $H_2O$ (RNase, DNase free)** | VWR | 020201--500 |
| **NaCl** | Sigma-Aldrich | S5150-1L |
| **EDTA** | Sigma-Aldrich | E5134 |
| **EGTA** | Sigma-Aldrich | E3889 |
| **RNase A** | Thermo Fisher Scientific | EN0531 |
| **Glycogen** | Sigma-Aldrich (Roche) | Roche 10930193001 |
| **Pure Spermidine (6.4M)** | Sigma-Aldrich | S0266 |
| **Digitonin** | Millipore Sigma | 300410 |
| **DMSO** | Sigma | D8418-100ml |
| **CaCl2** | Sigma-Aldrich | C1016 |
| **Trypan Blue** | Thermo Fisher Scientific | T10282 |

## 7. Frequently Asked Questions (FAQs)

1. What is the best way to know if a CUT&RUN experiment worked prior to sequencing?
   Results from challenging cell inputs / targets may be ambiguous, so EpiCypher recommends including positive / negative controls in every experiment. If the QC checks for the positive control perform as expected, 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, digitonin permeabilization, antibody concentration / alternate vendors, etc.).

   Recommended experimental design:
   a. Always include control antibodies and control cells
      i. Start with 500k K562 cells
      ii. Positive control antibody (H3K4me3 or H3K27me3)
      iii. Negative control antibody (IgG or no antibody)
   b. Experimental sample antibodies and cell types

   Quality control checks before decision to sequence:
   a. Yield of CUT&RUN enriched DNA (~200 ng for positive control H3K27me3 antibody, ~3ng for negative control IgG antibody). See **Section V.**
   b. Yield of library prep and PCR (~750ng from 5-10ng library input). See **Section VI.**
   c. Bioanalyzer/Tapestation traces should be enriched with mono-nucleosomal size fragments (~275bp = ~150bp nucleosome+ 125bp adapters, see **Section VII**)

DNA spike-in controls

2. Can residual *E. coli* in the pAG-MNase prep be used for sample input normalization? What spike-in DNA control does EpiCypher recommend?
   Carry-over *E. coli* DNA is present in EpiCypher's pAG-MNase preps. However, at a typical sequencing depth of 3-5M reads, too few *E. coli* DNA fragments (~hundreds) are recovered for reliably computing sample normalization. Thus, we recommend considering other normalization strategies, such as exogenous DNA spike-in (*E. coli* / yeast / fly) or recombinant CUTANA nucleosomes (in development).

3. Does EpiCypher offer spike-in DNA controls for CUT&RUN?
   EpiCypher plans to offer multiple DNA spike-in options for CUT&RUN, in addition to exogenous *E. coli* DNA spike-in. In particular, CUTANA spike-in nucleosomes (i.e. fully-defined semi-synthetic nucleosomes) for CUT&RUN 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 activity; and **4.** experimental success).

Cell input compatibility

4. What types of cell inputs are compatible with CUT&RUN?
   EpiCypher has confirmed that CUT&RUN is compatible with whole cells and nuclei derived from mammalian suspension and adherent cancer lines.

   EpiCypher has not yet directly tested, but a number of groups have successfully performed CUT&RUN on human and mouse primary tissue [5-8], FACS sorted [9] and immune cells [10,11].

   Note that lectins (*e.g.* ConA-beads) play a role in the innate immune system and so immune cells types may be inadvertently stimulated via binding to ConA-beads. To circumvent this potential problem in CUT&RUN, EpiCypher recommends using nuclei [9] or a crosslinking strategy [12].

5. Is CUT&RUN compatible with frozen or crosslinked cells?
   Yes. EpiCypher has confirmed that freeze/thawed cells (*e.g.* slow freeze in 10% DMSO/media, and then quick thaw at 37ºC) generate data of indistinguishable quality to fresh material. EpiCypher has also tested previously reported crosslinking conditions and recommended wash buffers [12], and found that while yields are significantly lower than native cells, the resulting data tracks display similar quality. We continue to explore crosslinking protocols and believe these will preserve labile PTMs, cell stimulation, or facilitate time-course studies.

Antibodies and targets

6. Does EpiCypher's CUT&RUN protocol work on non-PTM targets?
   Yes. The current protocol has been used at EpiCypher to generate numerous non-PTM CUT&RUN data, including CTCF, BRD4, and SMARCA4 (BRG1). No protocol modifications were necessary to generate these data since the columns we use to purify the CUT&RUN-enriched chromatin fragments retain >50bp DNA.

   However, for TFs in particular, which generate sub-nucleosomal size (<120bp) DNA fragments, modifications to the library protocol have been reported [7,13] to improve the representation of these smaller fragments during library preparation. Briefly, to enrich for sub-120bp fragments, the authors reduced the inactivation temperature after end repair from 65ºC for 30m to 50ºC for 1hr. Also, after the ligation reaction, Ampure bead size-selection ratio was increased from 1.1x volume to 1.75x volume. For TF CUT&RUN fragment sizes, both nucleosomal (~150bp) and sub-nucleosomal (<120bp) reflect TF chromatin occupancy locations, however, the sub-nucleosomal fragments provide higher resolution mapping for their locations.

7. What PTM antibodies do EpiCypher recommend for CUT&RUN?
   We currently recommend SNAP-ChIP Certified histone PTM antibodies, since these display high specificity and efficiency in a ChIP experiment. However, we remain cautious about transferring ChIP capability directly to CUT&RUN, and our ongoing studies include the creation of *in situ* nucleosome standards to interrogate antibodies with the highest possible resolution. For non-PTM targets, we recommend testing a few different antibodies to each target and proceed with the reagent that generates the highest quality data.

## 8. References

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