Analysis of Epigenetic Marks and Mechanisms in Disease: Your Guide to a Successful CUT&RUN Assay.

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ABSTRACT

Like the chromatin immunoprecipitation (ChIP) assay, Cleavage Under Targets & Release Using Nuclease (CUT&RUN) is a powerful and versatile technique used for probing protein-DNA interactions within the natural chromatin context of the cell. The CUT&RUN assay can be combined with downstream qPCR or NG-seq to analyze histone modifications and binding of transcription factors, DNA replication factors, or DNA repair proteins at specific target genes or across the entire genome.

CUT&RUN provides a rapid, robust, and true low cell number assay for detection of protein-DNA interactions in the cell. Unlike the ChIP assay, CUT&RUN is free from formaldehyde cross-linking, chromatin fragmentation, and immunoprecipitation, making it a much faster and more efficient method for enriching protein-DNA interactions and identifying target genes. CUT&RUN can be performed in as little as one to two days, from live cells to purified DNA, and has been shown to work with as few as 500-1000 cells per assay (1,2). Instead of fragmenting all of the cellular chromatin as done in ChIP, CUT&RUN utilizes an antibody-targeted digestion of chromatin, resulting in much lower background signal than seen in the ChIP assay. As a result, CUT&RUN requires only 1/10th the sequencing depth that is required for ChIP-seq assays. Finally, the inclusion of simple spike-in control DNA allows for accurate quantification and normalization of target-protein binding between samples. This provides for effective normalization of signal between samples and between experiments.

I will discuss the basics of the CUT&RUN assay and important factors to consider when setting up your experiment. In addition, I will introduce Cell Signaling Technology's new CUT&RUN Assay Kit and CUT&RUN pAG-MNase enzyme, and present data showing the versatility of this assay for mapping various histone modifications, transcription factor, and transcription cofactor binding across multiple cell types.

FEATURED CST REAGENTS

- CUT&RUN Assay Kit #86652
- CUT&RUN pAG-MNase and Spike-In DNA #40366
- DNA Purification Buffers and Spin Columns (ChIP and CUT&RUN) #14209
- SimpleChIP® Universal qPCR Master Mix #88989
- SimpleChIP® ChIP-seq DNA Library Prep Kit for Illumina®
- SimpleChIP® ChIP-seq Mutiplex Oligos for Illumina® (Dual Index Primers)
- SimpleChIP® ChIP-seq Multiplex Oligos for Illumina® (Single Index Primers)

DESCRIPTION

The CUT&RUN Assay Kit is designed to conveniently provide reagents needed to perform up to 24 digestion reactions from cells and is optimized for 100,000 cells per reaction. The kit has been optimized to work for all types of DNA binding proteins, including histones, transcription factors and cofactors. A complete assay can be performed in as little as one to two days. This kit is compatible with both qPCR and Next Generation sequencing (NG-seq).

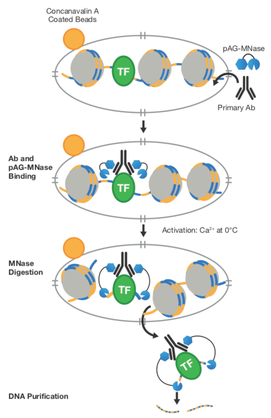
When performing the CUT&RUN assay, there is no need to fix the cells. Instead, live cells are permeabilized with digitonin to allow for subsequent diffusion of primary antibody and Protein A-Protein G-Micrococcal Nuclease (pAG-MNase) fusion enzyme into the cell nuclei. Once inside the cell nuclei, the target-specific antibody seeks out and binds to the chromatin-bound target protein. The target-bound antibody recruits the pAG-MNase enzyme to the chromatin, where upon activation of the enzyme with Ca^{2+} ion, the enzyme gently cleaves and liberates the desired chromatin fragments, which then diffuse away from the genomic chromatin, out of the cell, and into the supernatant. These chromatin fragments are collected from the supernatant and purified using DNA purification spin columns or phenol/chloroform followed by ethanol precipitation. The enriched DNA is identified and quantified using real-time quantitative PCR (qPCR) or NG-seq.

The CUT&RUN Assay Kit also provides important controls to ensure a successful CUT&RUN experiment. The kit contains a positive control Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751 and a negative control Rabbit (DA1E) mAb IgG XP® Isotype Control (CUT&RUN) #66362, both of which can be used for qPCR or NG-seq analysis. PCR primer sets are provided for the human (#7014) and mouse (#7015) RPL30 gene locus to be used in conjunction with the control antibodies.

REFERENCES

1. Skene, J.S. and Henikoff, S. (2017) *eLIFE* 6, e21856.
2. Skene, J.S. et al. (2018) *Nature Protocols* 13, 1006-1019.
3. Meers, M.P. et al. (2019) *eLIFE* 8, e46314.
4. Meers, M.P. et al. (2019) *Molecular Cell* 75, 1-14.

CUT&RUN Method Overview



Antibody and pAG-MNase Binding

1. Cells are immobilized on Concanavalin A Magnetic Beads to allow for subsequent buffer and reagent exchanges.
2. Cell membranes are then permeabilized with digitonin to facilitate the entry of primary antibody and pAG-MNase fusion enzyme into the cell nuclei.
3. The target-specific primary antibody recruits the pAG-MNase to the chromatin through protein-protein interactions between the antibody and the pAG domain of the fusion enzyme.

MNase Digestion:

4. The addition of Ca^{2+} activates the pAG-MNase, which gently cleaves and liberates the desired chromatin fragments, allowing them to diffuse away from the genomic chromatin, out of the cell, and into the supernatant.

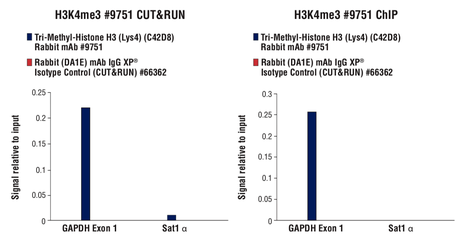
DNA Purification:

5. DNA is purified using DNA purification spin columns or phenol/chloroform extraction followed by ethanol precipitation. The purified, enriched DNA is then identified and quantified using qPCR or NG-seq.

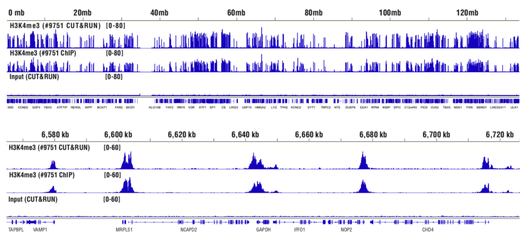
Advantages

- **Low cell number:** Only 100K cells used to generate all CUT&RUN data shown compared to 4M cells used to generate ChIP and ChIP-seq data.
- **Get results quickly:** 1-2 days from cells to DNA.
- **Save on sequencing costs:** Only requires 3-5M reads due to low background.
- **Target versatility:** Generate **qPCR** or **NG-seq** data for histones, transcription factors, and cofactors
- **Reproducibility:** Spike-In DNA control allows for normalization between samples
- **Avoid artifacts:** *in vivo* method using native, NOT cross-linked chromatin

Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751

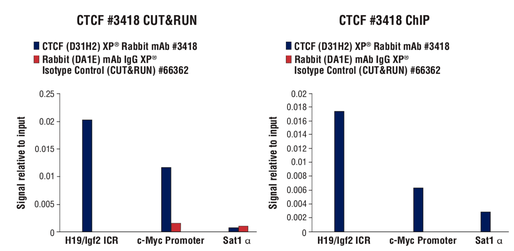


CUT&RUN and ChIP assays were performed with HCT 116 cells using the CUT&RUN Assay Kit #86652 (left panel) or SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads) #9005 (right panel). The enriched DNA was quantified by real-time PCR using SimpleChIP® Human GAPDH Exon 1 Primers #5516 and SimpleChIP® Human α Satellite Repeat Primers #4486. The amount of immunoprecipitated DNA in each sample is represented as signal relative to the total amount of input chromatin, which is equivalent to one.

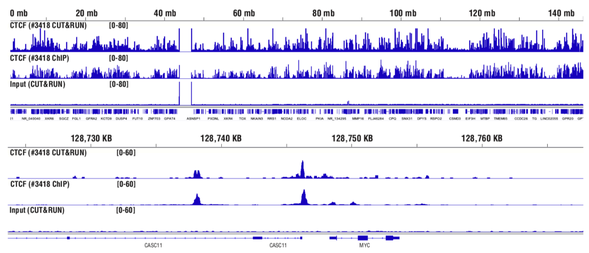


CUT&RUN and ChIP assays were performed with HCT 116 cells using the CUT&RUN Assay Kit #86652 or Simple-ChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads) #9005. DNA Libraries were prepared using SimpleChIP® ChIP-seq DNA Library Prep Kit for Illumina® #56795. The upper panel compares enrichment of H3K4me3 across chromosome 12, while the lower panel compares enrichment at the GAPDH gene, a known target of H3K4me3. The input tracks are from the CUT&RUN input sample.

CTCF (D31H2) XP® Rabbit mAb #3418

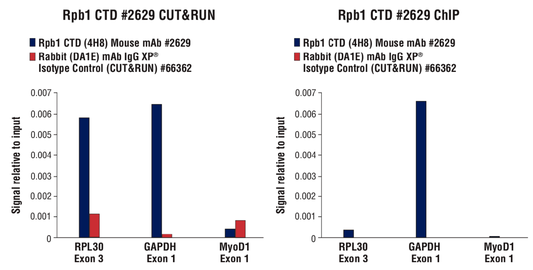


CUT&RUN and ChIP assays were performed with HCT 116 cells using the CUT&RUN Assay Kit #86652 (left panel) or SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads) #9005 (right panel). The enriched DNA was quantified by real-time PCR using human c-Myc promoter primers, SimpleChIP® Human H19/Igf2 ICR Primers #5172, and SimpleChIP® Human α Satellite Repeat Primers #4486. The amount of immunoprecipitated DNA in each sample is represented as signal relative to the total amount of input chromatin, which is equivalent to one.

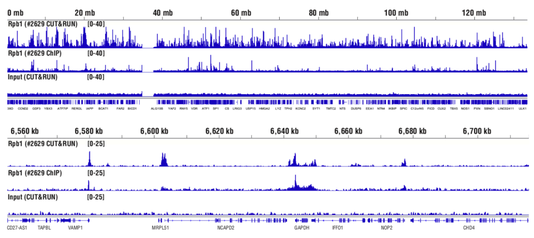


CUT&RUN and ChIP assays were performed with HCT 116 cells using the CUT&RUN Assay Kit #86652 or SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads) #9005. DNA Libraries were prepared using SimpleChIP® ChIP-seq DNA Library Prep Kit for Illumina® #56795. The upper panel compares enrichment of CTCF across chromosome 8, while the lower panel compares enrichment at the MYC gene, a known target of CTCF. The input tracks are from the CUT&RUN input sample.

Rpb1 CTD (4H8) Mouse mAb #2629

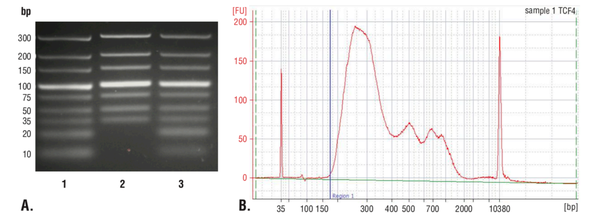


CUT&RUN and ChIP assays were performed with HeLa cells using the CUT&RUN Assay Kit #86652 (left panel) or the SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads) #9005 (right panel). The enriched DNA was quantified by real-time PCR using SimpleChIP® Human RPL30 Exon 3 Primers #7014, SimpleChIP® Human GAPDH Exon 1 Primers #5516, and SimpleChIP® Human MyoD1 Exon1 Primers #4490. The amount of immunoprecipitated DNA in each sample is represented as signal relative to the total amount of input chromatin, which is equivalent to one.



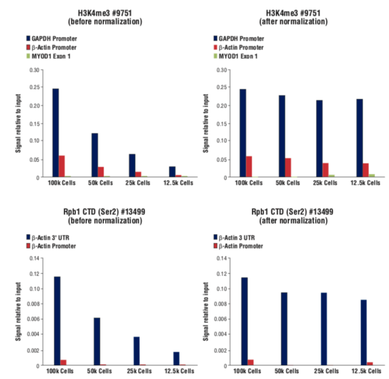
CUT&RUN and ChIP assays were performed with HeLa cells using the CUT&RUN Assay Kit #86652 or the SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads) #9005. DNA Libraries were prepared using SimpleChIP® ChIP-seq DNA Library Prep Kit for Illumina® #56795. The upper panel compares enrichment of Rpb1 across chromosome 12, while the lower panel compares enrichment at the GAPDH gene, a known target of Rbp1. The input tracks are from the CUT&RUN input sample.

CUT&RUN DNA Purification (Spin Columns vs. Phenol Chloroform)

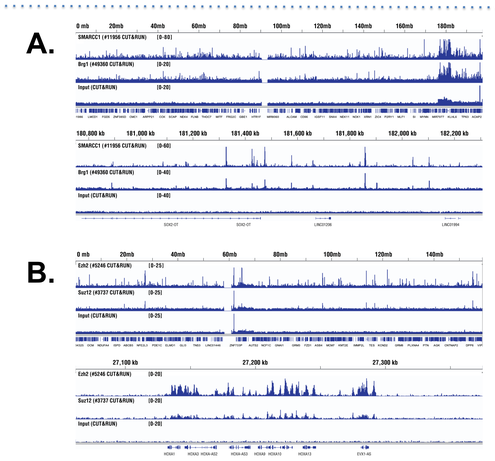


Comparison of DNA purification using spin columns or phenol/chloroform extraction followed by ethanol precipitation. (A) A low range DNA ladder mix (lane 1, unpurified) was purified using either DNA Purification Buffers and Spin Columns (ChIP and CUT&RUN) #14209 (lane 2) or phenol/chloroform extraction followed by ethanol precipitation (lane 3) and separated by electrophoresis on a 4% agarose gel. As shown, phenol/chloroform followed by ethanol precipitation efficiently recovers all DNA fragment sizes, while DNA spin columns recover DNA fragments ≥35 bp. **(B)** DNA was purified using phenol/chloroform extraction followed by ethanol precipitation from a CUT&RUN assay performed using TCF4/TCF7L2 (C48H11) Rabbit mAb #2569. The size of the DNA fragments in the library was analyzed using a Bioanalyzer (Agilent Technologies). The adaptor and barcode sequences added to the library during construction account for 140 bp in fragment length. Therefore, starting 35 bp DNA fragments would be 175 bp in length after library preparation (indicated with blue vertical line in figure). As shown, less than 2% of the total CUT&RUN enriched DNA fragments are less than 175 bp (starting length of 35 bp), suggesting that DNA purification spin columns are sufficient for capture of >98% of the total CUT&RUN DNA fragments.

CUT&RUN Sample Normalization Using Spike-In DNA



Normalization of CUT&RUN signals using spike in DNA for qPCR analysis. CUT&RUN was performed with a decreasing number of HCT 116 using the CUT&RUN Assay Kit #86652. Enriched DNA was quantified by real-time PCR using SimpleChIP® Human GAPDH Exon 1 Primers #5516, SimpleChIP® Human β-Actin Promoter Primers #13653, SimpleChIP® Human β-Actin 3' UTR Primers #13669, and SimpleChIP® Human MyoD1 Exon 1 Primers #4490. The amount of immunoprecipitated DNA in each sample is represented as signal relative to the total amount of input chromatin for 100,000 cells. Non-normalized enrichments are depicted in the left panels. The Sample Normalization Spike-In DNA was added into each reaction proportionally to the starting cell number. Based on the difference of qPCR signals from spike in DNA in each sample, CUT&RUN signals were normalized to the sample containing 100,000 cells. Normalized enrichments are depicted in the right panels.



CUT&RUN assays were performed with NCCIT cells using the CUT&RUN Assay Kit #86652. DNA Libraries were prepared using SimpleChIP® ChIP-seq DNA Library Prep Kit for Illumina® #56795. NG-seq tracks are shown for Ezh2 (D2C9) XP® Rabbit mAb #5246 and SUZ12 (D39F6) XP® Rabbit mAb #3737 **(A)** or SMARCC1/BAF155 (D7F8S) Rabbit mAb #11956 and Brg1 (D1Q7F) Rabbit mAb #49360 **(B)**.

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