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CUT&RUN Chromatin Profiling of Human Kidney Tissue

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We use this protocol and it's working

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Abstract

Histone post-translational modifications are key epigenetic features that define gene regulatory elements in the genome. Identifying changes in the epigenome has the potential to uncover novel disease signatures. Chromatin immunoprecipitation with sequencing (ChIP-seq) is a commonly used method to define the chromatin landscape. A limitation of this method is that the amount of starting material ($\geq 10^6$ cells) does not lend itself to interrogation of kidney biopsies. Cleavage Under Targets and Release Using Nuclease (CUT&RUN) is a recently described method that provides genome-wide mapping of chromatin profiles for histone binding. In this method, cells are immobilized on beads, permeabilized with detergent (digitonin) and incubated with an antibody that recognizes specific histone post-translational modification in nucleosomes, DNA binding proteins (e.g., transcription factor) or other factors that associate with chromatin. After antibody binding, a fusion protein of Micrococcal nuclease and protein A/G (pAG-MNase) is added. The protein A/G moiety binds the antibody and the nuclease cuts accessible chromatin adjacent to the nucleosomes. The released DNA fragments, representing the genomic regions enriched for antibody binding, are column purified and used to construct a library for sequencing. Sequence is aligned to the human genome and peaks that represent statistically significant binding (e.g., histone H3 lysine 27 trimethylation, H3K27me3) are called using the Model-based Analysis for ChIP-seq Method (Macs2) that we have adapted for CUT& RUN.



Guidelines

The full CUT&RUN protocol is from Epicypher (<https://www.epicypher.com/content/documents/protocols/cutana-cut&run-protocol.pdf>). Please reference this protocol for full details and required positive and negative controls for experiments.

We outline a brief protocol with modifications for liquid nitrogen frozen human kidney tissue using 1.5 mL tubes and as little as 50,000 cells/CUT&RUN antibody reaction. Depending on the abundance of the protein target and specificity of the antibody, it is possible to use less than 50,000 cells; however, we recommend using 50,000-500,000 cells/reaction.



Materials

Buffer recipes

Bead Activation Buffer (pH with KOH) pH HEPES to 7.9 before adding salts

20 mM HEPES, pH 7.9, 10 mM KCl, 1 mM CaCl₂, 1 mM MnCl₂

Filter sterilize Store @ 4°C for up to 6 months

Wash Buffer (pH with KOH)

20 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mM Spermidine

Filter sterilize

Store @ 4°C for up to 1 week

1x Roche Complete Protease Inhibitor-mini (CPI-mini), EDTA-free (Roche catalog # 11836170001), 1tab/10ml, added fresh before use

5% Digitonin

Dissolve 5 mg in 100 ul DMSO. Store @ 4°C. Good for one 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 @ 4°C

Antibody Buffer

Wash Buffer + 0.01% Digitonin + 2 mM EDTA

Prepare fresh each day and store @ 4°C

Stop Buffer

340 mM NaCl

20 mM EDTA

4 mM EGTA

Filter sterilize. Store @ 4°C for up to 6 months

Before using, add:

50 µg/ml RNase A

50 µg/ml Glycogen

*ADD 150 pg/150,000 cells of Epicypher's E. coli spike-in (optional)

Materials

IgG antibody, Cell Signaling, Cat# 2729



H3K27ac antibody, Cell Signaling, Cat# 8173
H3K27me3 antibody, Cell Signaling, Cat# 9733
H3K4me1 antibody, Cell Signaling, Cat# 5326
CUTANA Concanavalin A magnetic beads, Epicypher, Cat# 21-1401
CUTANA pAG-MNase, Epicypher, Cat# 15-1016
CUTANA E. coli Spike-in DNA, Epicypher, Cat# 18-1401
CUTANA DNA purification kit, Epicypher, Cat# 14-0050
NEBNext® UltraTM II Library Prep Kit for Illumina®, NEB, Cat# E7645
NEBNext® Multiplex Oligos for Illumina® (96 Unique Dual Index Primer Pairs), NEB, Cat# E6440S
Digitonin, Millipore Sigma, Cat# 300410
RNase, Sigma Millipore, Cat# R4642
Glycogen, Roche, Cat# 10901393001
Spermidine, Sigma Millipore, Cat# S2501
cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail, Roche, Cat# 11836170001
HEPES
KCl
CaCl₂
MnCl₂
Molecular biology grade water (RNase, DNase free)
NaCl
EDTA (0.5M stock, pH 8.0)
EGTA (0.5M stock, pH 8.0)
DMSO

Fisherbrand RNase-Free Disposable Pellet Pestles, Fischer Scientific, Cat# 12-141-364
CellTrics 30 µm Filter, Sysmex, Cat# 04-004-2323
GeneMate 1.7 mL Low-Adhesion tubes, VWR, Cat# 490003-230
1.5 mL Magnetic Separation Rack
Qubit™ 4 Fluorometer
Agilent Bioanalyzer
Nutator
Vortex
Water Bath
Centrifuge
Hemocytometer



Before start

1. Prepare  50 mL Bead Activation Buffer,  10 mL 100 mM CaCl₂, and  10 mL Stop Buffer without RNase, glycogen, and spike-in DNA. Filter sterilize all and store at  4 °C . These are good for 6 months.
2. The day before the experiment, prepare  3 mL Wash Buffer/sample (sample= 1 CUT&RUN antibody reaction) and filter sterilize. Store at  4 °C . This buffer is good for one week. Prepare 5% digitonin by dissolving 5 mg in  100 µL DMSO. Store at  4 °C . This buffer is good for one week.

*For human kidney cells we recover ~100,000 cells/mm³ of liquid nitrogen frozen tissue.

Antibody Buffer Prep and ConA Bead Activation

30m

- 1 The day of the experiment, add protease inhibitors (PIs) to 1X concentration in 5 mL of Wash Buffer and leave at Room temperature . Store the remaining Wash Buffer at $4\text{ }^{\circ}\text{C}$ for Day 2.
- 2 Make the Antibody Buffer ($100\text{ }\mu\text{L}$ /sample) by adding $2.0\text{ }\mu\text{L}$ of 5% digitonin and $4.0\text{ }\mu\text{L}$ 0.5 M EDTA to 1.0 mL of Wash Buffer + PIs and put On ice . Leave the other 4 mL at Room temperature .

Note

sample= 1 CUT&RUN antibody reaction

1 mL antibody buffer is enough for 10 samples

- 3 Activate the Concanavalin A beads by transferring $11\text{ }\mu\text{L}$ of beads/sample to a 1.5 ml low adhesion tube and place on magnet. Discard sup. Wash the beads 3x with $100\text{ }\mu\text{L}$ cold Bead Activation Buffer/sample.

Note

Low adhesion tubes need to be used throughout the CUT&RUN protocol to limit DNA loss.

- 4 Resuspend the tube of beads in $11\text{ }\mu\text{L}$ cold Bead activation buffer/sample. Aliquot $10\text{ }\mu\text{L}$ beads/sample into new 1.5 mL low adhesion tubes and keep On ice until needed.



Tissue Prep and Binding Cells to Activated Beads

45m

- 5 Thaw tissue On ice and measure size and weight. Transfer tissue to 1.5 mL low adhesion tube and add 100 μ L Room temperature Wash Buffer + Pls/
 10 mg tissue and dounce 10 times with a hand held pellet pestle.
- 6 Spin 00:03:00 at 600 x g at Room temperature and discard sup. 3m
- 7 Add 100 μ L Wash Buffer + Pls/ 10 mg tissue and pipet up and down 10 times with a 200 μ l pipet.
- 8 Spin again and discard sup.
- 9 Add 500 μ L Wash Buffer + Pls and pipet up and down 10 times with a 200 μ l pipet.
- 10 Wet a 30 μ m filter with 500 μ L Wash Buffer + Pls and discard buffer from tube. Add tissue homogenate to the wet filter, and rinse the filter with an additional 500 μ L Wash Buffer + Pls.

Antibody Binding

16h 30m

- 11 Count cells using a hemocytometer and aliquot enough cells (50,000-500,000) per antibody reaction into each of the prepared tubes with activated ConA beads.
- 12 Gently vortex and let sit at Room temperature for 00:10:00 . 10m
- 13 Place tubes on magnet and remove sup. Add 100 μ L cold Antibody Buffer to each tube and vortex gently. Add 1.0-2.0 μ l antibody (negative control IgG or specific antibody) to each tube and vortex gently. Nutate overnight at 4 $^{\circ}$ C with caps elevated.



Binding of pAG-MNase

30m

- 14 In the morning, prepare  2.5 mL Digitonin Buffer/sample by adding  5 μ L of 5% digitonin in DMSO to  2.5 mL cold Wash Buffer. Add protease inhibitors to 1X. Keep Digitonin Buffer with PIs on ice.
- 15 Place tubes on the magnet, let beads clear from the reaction and remove the sup. Leaving the tubes on the magnet, gently pipet  300 μ L of cold Digitonin Buffer over the beads and then remove the sup.
- 16 Repeat static washes 2 more times for a total of 3 washes.
- 17 Resuspend each tube of beads with  50 μ L cold Digitonin Buffer and chill  00:02:00 on ice. 2m
- 18 Add  2.5 μ L CUTANA pAG-MNase (20X stock) to each tube and pipet to mix.
- 19 Incubate the tubes at  Room temperature for  00:10:00 . 10m
- 20 Place tubes on the magnet and immediately add  300 μ L cold Digitonin Buffer on top of the beads and pAG-MNase.
- 21 Remove sup and do 2 more static washes with the tubes on the magnet.
- 22 Resuspend beads in  50 μ L cold Digitonin Buffer and chill  00:02:00  On ice . 2m



Chromatin Digestion and Release

3h

- 23 Add  1 μL 100 mM CaCl_2 to each tube and gently vortex briefly.
- 24 Immediately place back on ice. Nutate  02:00:00 at  4 °C .
- 25 In the meantime, prepare  300 μL Reaction Stop Buffer by mixing  0.75 μL of 20 mg/mL RNase,  0.75 μL of 20 mg/mL glycogen, and Epicypther E.coli spike-in DNA (optional, 150 pg/150,000 cells) into  300 μL Stop Buffer.

Note

300 μL Reaction Stop Buffer is enough for 8 samples. We add RNase, glycogen, and spike-in DNA fresh for each experiment.

- 26 Add  33 μL Stop Buffer to each reaction and vortex gently.
- 27 Incubate  00:10:00 at  37 °C in a water bath to release the chromatin and degrade the RNA.
- 28 Spin briefly to collect sup, place the tubes on the magnet, let clear, and transfer sups into a new tube.

10m

Purify DNA

20m

- 29 Clean up reactions using CUTANA DNA Purification kit according to their instructions.
- 30 Elute in  20 μL and quantify DNA recovery using a Qubit fluorometer per manufacturer's instructions. Use 1-2 μL to measure concentration.

**Note**

If using less than 500,000 cells per antibody reaction, and depending on the antibody (IgG), DNA recovery may not be detectable with a Qubit. If this is the case, proceed to library prep using all of the DNA recovery.

Library Prep

4h

- 31 Using up to 5 ng purified CUT&RUN enriched DNA, prepare Illumina library using the NEBNext Ultra II Library Kit for Illumina per manufacture's instructions with the noted modifications:
- 31.1 End repair modification: 00:30:00 at 20 °C , 00:30:00 at 50 °C , to avoid melting smaller fragments when antibodies used are for transcription factors. 1h
- 31.2 Universal adapter ligation modification: dilute adapter 1:25 if starting with > 2.5 ng; dilute adapter 1:50 for 1.25-2.5 ng; dilute adapter 1:100 if starting with < 1.25 ng.
- 31.3 DNA cleanup using 1.1x AMPure XP beads to sample volume for histone modification antibodies, or 1.35x beads for transcription factor antibodies.
- 31.4 PCR and primer indexing according to cycling parameters below.
- 31.5 CUT&RUN-specific PCR cycling parameters: 2m 13s
- a. 00:00:45 @ 98 °C - activation of hot-start Q5 polymerase
 - b. 00:00:15 @ 98 °C - DNA melting
 - c. 00:00:13 @ 60 °C - hybrid primer annealing and short extension (<700bp)
 - d. Repeat "step b-c" for a total of 15-18 cycles, depending on the amount of input DNA. If the amount of input was undetectable, use 18 cycles.
 - e. 00:01:00 @ 72 °C - final extension
- 31.6 DNA cleanup using 1.1x AMPure beads to sample volume (e.g. 55 µL beads, 50 µL PCR reaction).
- 31.7 Elute DNA in 30 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 of



a PCR DNA library: ~500-750 ng (15-25 ng/μl in 30 μl).

Agilent Bioanalyzer System

1h

- 32 For each sample, prepare  5 μL at 1-5 ng/μl for loading on the Agilent Bioanalyzer.

Note

Record dilution factor such that the original sample molarity can be calculated from Bioanalyzer nM for desired DNA size range (150-700 bp).

- 33 For each purified PCR DNA library for Illumina sequencing, load  1 μL of 5 ng/μl sample on Agilent High Sensitivity DNA Chip (Cat# 5067-4626) as per manufacturer's instructions.

Note

Typical molarity for 15 μl purified PCR DNA library (150-700 bp region) = 100-200 nM.

- 34 Confirm that positive control antibodies enriched for predominantly mononucleosome fragments (~275 bp peak with nucleosomes + sequence adapters).

Sequencing

- 35 For Illumina libraries 0.8 pm is loaded and sequenced at 10-50 million paired end reads on a NovaSeq 6000 platform.

Bionformatic Analysis

- 36 Trim fastq files to remove adapters using Cutadapt.

Command Line Example: cutadapt -j 10 -m 10 -a AGATCGGAAGAG -A AGATCGGAAGAG -o R1.trim.fastq.gz -p R2.trim.fastq.gz R1.fastq.gz R2.fastq.gz

**Note**

By default, empty reads are kept. Set-minimum-length 10 to remove short/empty reads after trimming. Illumina Universal Adapters can be removed by using sequence: AGATCGGAAGAG. Run fastQC on fastq files to confirm adapter presence in raw fastq files and removal after trimming.

- 37 Align trimmed fastq files to genome using Bowtie2 end to end mode.

Command Line Example: bowtie2 --end-to-end --very-sensitive --no-mixed --no-discordant --phred33 -l 10 -X 700 -p 10 -x genome -1 R1.trim.fastq.gz -2 R2.trim.fastq.gz -S sample.sam

Note

Set minimum and maximum fragment length as 10 and 700, respectively.

- 38 Use SAMtools view and sort commands to extract aligned reads from the resulting sam file and convert to a sorted bam file. Index the sorted bam file using SAMtools index.

Command Line Example:

- samtools view -bS -F 0x04 sample.sam > aligned.bam
- samtools sort aligned.bam -o aligned.sort.bam
- samtools index aligned.sort.bam

Note

The SAMtools view and sort commands can be piped to reduce writing output of unnecessary temporary files.

- 39 Calculate sample normalization factor based on % of aligned E. coli reads (as described in Epicypther Cut&Run protocol).

- Calculate percent of aligned E. coli reads in all uniquely aligned reads (ex: 100,000 E. coli reads in 5,000,000 uniquely aligned reads = 2%).
- Calculate normalization factor to make E. coli spike in signal equal across all samples (ex: 1/2% = 0.5).



- 40 Generate scaled, binned bigWig file using Deeptools bamCoverage command with --scaleFactor parameter.

Command Line Example: `bamCoverage -b aligned.sort.bam --scaleFactor 0.5 --binSize 50 --outFileFormat bigwig -o sample.bw`

Note

Blacklisted regions can be removed from the output bigwig file by including the -blackListFileName argument and providing a bed file of regions to exclude.

- 41 Call peaks using Macs2. Use broad peak calling option for histone modifications.

Command Line Example: `macs2 callpeak -t aligned.sort.bam -c IgG.sort.bam -f BAM -g mm -n sample.name --broad --max-gap 1000 --min-length 160 --keep-dup all --fe-cutoff 2.7`

Note

--fe-cutoff parameter may be modified depending on sample quality and desired stringency level in peak calling.