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CUT&RUN Chromatin Profiling of Human Kidney Tissue

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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.

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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 TEXT

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 @ 4ºC 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 @ 4ºC for up to 1 week
1x Roche Complete Protease Inhibitor-mini (CPI-mini), EDTA-free (Roche catalog # 11836170001), 1tab/10ml, added fresh before use

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5% Digitonin
Dissolve 5 mg in 100 ul DMSO.Store @ 4ºC.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 @ 4ºC

Antibody Buffer
Wash Buffer + 0.01% Digitonin + 2 mM EDTA
Prepare fresh each day and store @ 4ºC

Stop Buffer
340 mM NaCl
20 mM EDTA
4 mM EGTA
Filter sterilize. Store @ 4ºC 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
$CaCl_2$
$MnCl_2$
Molecular biology grade water (RNase, DNase free)
NaCl

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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
QubitTM 4 Fluorometer
Agilent Bioanalyzer
Nutator
Vortex
Water Bath
Centrifuge
Hemocytometer

BEFORE STARTING

1. Prepare **50 mL** Bead Activation Buffer, **10 mL** 100 mM $CaCl_2$, 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/mm^3 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 °C** for Day 2.

2 Make the Antibody Buffer (**100 µL** /sample) by adding **2.0 µL** of 5% digitonin and **4.0 µL** 0.5 M EDTA to **1.0 mL** of Wash Buffer + PIs and put **On ice** . Leave the other **4 mL** at **Room temperature** .

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sample= 1 CUT&RUN antibody reaction

1 mL antibody buffer is enough for 10 samples

3 Activate the Concanavalin A beads by transferring **11 µL** of beads/sample to a 1.5 ml low adhesion tube and place on magnet. Discard sup. Wash the beads 3x with **100 µL** cold Bead Activation Buffer/sample.

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

4 Resuspend the tube of beads in **11 µL** cold Bead activation buffer/sample. Aliquot **10 µL** 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 µL** **Room temperature** Wash Buffer + PIs/ **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 µL** Wash Buffer + PIs/ **10 mg** tissue and pipet up and down 10 times with a 200 µl pipet.

8 Spin again and discard sup.

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9 Add **500 µL** Wash Buffer + PIs and pipet up and down 10 times with a 200 µl pipet.

10 Wet a 30 µm filter with **500 µL** Wash Buffer + PIs and discard buffer from tube. Add tissue homogenate to the wet filter, and rinse the filter with an additional **500 µL** Wash Buffer + PIs.

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 µL** cold Antibody Buffer to each tube and vortex gently. Add 1.0-2.0 µl antibody (negative control IgG or specific antibody) to each tube and vortex gently. Nutate overnight at **4 °C** with caps elevated.

Binding of pAG-MNase 30m

14 In the morning, prepare **2.5 mL** Digitonin Buffer/sample by adding **5 µL** 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 µL** of cold Digitonin Buffer over the beads and then remove the sup.

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16 Repeat static washes 2 more times for a total of 3 washes.

17 Resuspend each tube of beads with **50 µL** cold Digitonin Buffer and chill **00:02:00** on ice. 2m

18 Add **2.5 µL** 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 µL** 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 µL** 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** . 2h

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 Epicypher E.coli spike-in DNA (optional, 150 pg/150,000 cells) into **300 µL** Stop Buffer.

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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. 10m

28 Spin briefly to collect sup, place the tubes on the magnet, let clear, and transfer sups into a new tube.

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.

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

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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.

2m 13s

31.5 CUT&RUN-specific PCR cycling parameters:

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.

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

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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.

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

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 -I 10 -X 700 -p 10 -x genome -1 R1.trim.fastq.gz -2 R2.trim.fastq.gz -S sample.sam

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

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

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 Epicypher Cut&Run protocol).

a. 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%).
b. 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

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

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

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