SOP62v1_TGD_CUT&RUN and Library prep

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FEB 11, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.yxmvm2z39g3p/v1

Protocol Citation: Yunkyeong Lee 2023. SOP62v1_TGD_CUT&RUN and Library prep. **protocols.io** https://dx.doi.org/10.17504/protocols.io.yxmvm2z39g3p/v1

Protocol status: Working
We use this protocol and it's working

Created: Feb 09, 2023

Last Modified: Feb 11, 2023

PROTOCOL integer ID:
76760

ABSTRACT

Cleavage Under Targets & Release Using Nuclease (CUT&RUN) is a revolutionary genomic mapping strategy developed by the group of Dr. Steven Henikoff.

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 quality genome-wide profiles of histone post-translational modifications (PTMs) and chromatin-associated proteins (e.g. TFs and chromatin remodelers)

Historically, ChIP-seq is the leading approach for genome-wide mapping of histone PTMs and chromatin-associated proteins. ChIP-seq requires large numbers of cells (typically 10^5 - 10^6 cells) and deep sequencing of both input chromatin and immunoprecipitated material (typically > 30 million read each) to resolve signal from background.

With this innovation, background is dramatically reduces, allowing high resolution genomic mapping for histone PTMs and chromatin-associated proteins using a small number of cells and only 3-8 million sequencing reads per sample.

Although it is recommended to start with 500,000 cells, comparable data can be generated using as few as 5,000 cells.

Overview of the CUTANA CUT&RUN protocol

1. Immobilize & permeabilize cells (or nuclei)

2. Add antibody to histone PTM or chromatin-interacting protein

3. Add & activate pAG-MNase to cleave target-DNA complex

4. Target-DNA complex diffuses out, collect supernatant

5. Extract DNA & prepare sequencing library 6. Next-generation sequencing and data analysis

from CUTANA CUT&RUN kit manual

GUIDELINES

Thaw all reagents stored at -20 and keep on ice during experiment.
And you should check the note in protocol.

For selection of I5 & i7 primer pairs for multiplexing, please see the Library prep protocol p. 22-25

MATERIALS

Epicypher CUTANA ChIC/CUT&RUN Kit User manual version 2.1 Kit version 2.0
Catalog No. 14-1048, 48 ChIC/CUT&RUN Samples

BOX 1 (at RT under the PCR machine): DNA Binding Buffer (25 mL), DNA Wash Buffer (5 mL), EDTA 100 mM (500 uL), $CaCl_2$(500 uL), DNA Elution Buffer (1.2 mL), DNA Cleanup Columns (50 Columns), DNA Collection Tubes (50 Tubes), 8-Strip Tubes (56 Tubes)

BOX 2 (at 4°C under the PCR machine): SA Beads (40 uL) (X), ConA Beads (550 uL) -> (Jan 2023 Re-ordering), Stop Buffer (1.5 mL), Bead Activation Buffer (12 mL), Pre-Wash Buffer (105 mL)

BOX 3 (at-20 °C in Franklin(Nicole's)): IgG Negative Control (10 uL), H3K4me3 Positive Control (10 uL), Spermidine (100 uL), pAG-MNase (48 runs), Spike-in DNA (100 ng), 5% Digitonin (100 uL) + EpiCypher 50 uL - 0.2 mL

+ Magnetic Rack, Invivogen 1.5 mL tube Magnetic Rack (in drawer near the PCR machine)

Epicypher CUTANA CUT&RUN Library Prep Kit User manual version 1.0 Kit version 1
Catalog No. 14-1001 & 14-1002, 48 CUT&RUN Library Prep Reactions

BOX 1 (at RT under the PCR machine, in CUT&RUN kit box): 8-Strip Tubes, SPRIselect reagent manufactured by Beckman Coulter, Inc. (DO NOT FREEZE), 0.1X TE Buffer

BOX 2 (at -20 °C between 2 benches in Tissue Culture room, Thaw and store on ice during experiment. Thoroughly mix before using): End Prep Enzyme, End Prep Buffer, Adapter for Illumination, Ligation Mix, Ligation Enhancer, U-Excision Enzyme, High Fidelity 2X PCR Master Mix, i5 and i7 Primers (Primer set 1(14-1001))

+ Molecular biology grade water, 100% Ethanol (200 proof), Multi-channel pipettes (P200), Multi-channel reagent reservoir, Thermocycler with heated lid, Bioanalyzer

SAFETY WARNINGS

Wear your own lab coat and gloves.

BEFORE START INSTRUCTIONS

You should check if there are all the materials in the kit, especially ConA beads.

1 CUT&RUN

1. Buffer preparation: Make CUT&RUN buffers fresh the day of use.

1) Add 1.8 mL Pre-Wash Buffer per sample to a 50 mL conical tube labeled "Wash Buffer".
2) Dissolve 1 protease inhibitor tablet (Roche) in 2 mL water (25X stock). Add 72 uL per sample to the Wash Buffer. Store remaining 25X stock for 12 weeks at -20°C.
3) Dilute 1M Spermidine 1:2,000 in the Wash Buffer. Store final buffer at RT.
4) Transfer 1.4 mL of Wash Buffer per sample into a new 50 mL conical tube labeled "Cell Perm Buffer". Add 5% Digitonin (1:500 dilution).
5) Transfer 100 uL per sample of Cell Permeabilization Buffer into a new 50 mL tube labeled "Antibody Buffer". Add 0.5 M EDTA (1:250 dilution). Store final buffer on ice.
6) Store the remaining Cell Perm Buffer at 4°C overnight (for Day 2 use).

A. **Wash buffer**: Leave at RT for use on Day 1 Per sample: 1.8 mL Pre-Wash Buffer, 72 uL Protease inhibitor (1X final), 0.9 uL Spermidine (0.5 mM final)

B. **Cell Permeabilization Buffer**: Store at 4°C overnight for use on Day 2 Per sample: 1.4 mL Wash Buffer, 2.8 uL Digitonin (0.01% final)
C. **Antibody Buffer**: Set on ice for use on Day 1 Per sample: 100 ul Cell Perm. Buffer, 0.4 uL EDTA (2 mM final)

2 2. Bead activation

1) Gently resuspend the ConA Beads by pipetting. Transfer 11 uL/sample to a 1.5 mL tube for batch processing. 6 samples: Total 66 ul
2) Place the tube on a magnet until slurry clears and pipette to remove sup.
3) To avoid drying the beads, immediately add 100 uL/sample cold Bead Activation Buffer. Pipette gently to mix.
4) Place the tube on a magnet until slurry clears and pipette to remove sup. Repeat previous step for total of two washes.
5) Resuspend beads in 11 uL/sample cold Bead Activation Buffer.
6) Aliquot 10 uL/sample of activated bead slurry into separate 8-strip tubes. Keep on ice until needed.
7) Harvest 0.5 million cells/sample in 1.5 mL tube. Centrifuge at 600 x g, 3 min at RT. Decant or pipette culture media sup.

a) 0.5 M: FLAG, b) 1.0 M: FLAG, c) 0.5 M: V5, d) 1.0 M: V5, e) 0.5 M: IgG negative control, f) 0.5 M: H3K4me3 positive control
8) Resuspend cells in 100 uL/sample RT Wash Buffer. Pipette to thoroughly resuspend.Centrifuge at 600 x g, 3 min at RT. Decant or pipette sup.
9) Repeat previous step for total of two washes.
10) Resuspend cells in 105 uL/sample in RTWash Buffer and thoroughly pipette to mix. Aliquot 100 uL washed cells to each 8-strip tube containing 10 uL of activated beads. Gently vortex and/or pipette until evenly resuspended.
11) Incubate cell-bead slurry on benchtop for 10 min at RT to adsorb cells to beads.

3 3. Antibody binding

12) If using a multi-channel pipette (recommended) place a multi-channel reagent reservoir on ice.
13) Place the 8-strip tubes on an 8-strip magnet (high volume setting) until slurry clears. Pipette to remove sup, taking care to avoid disturbing beads with tip.
14) Immediately add 50 uL cold Antibody Buffer to each sample and gently vortex and/or pipette mix to prevent beads from drying.
15) Add 2 uL CUTANA H3K4 MetStat Spike-in Control dNucs to the cell samples designated for the positive (H3K4me3) and negative (IgG) control antibodies.
16) Add 0.5 ug antibody to each sample and gently vortex.
*** Control antibodies included in this kit are provided at 0.5 mg/mL, therefore add 1.0 uL per sample.
FLAG antibody: 1 mg/mL -> 0.5 uL V5 antibody: 1 mg/mL -> 0.5 uL IgG, H3K4me3 antibodies: 1.0 u
17) Incubate 8-strip tubes on nutator (capped ends elevated) overnight at 4°C
18) Store the Cell Permeabilization Buffer at 4°C overnight for use on Day 2.

4 4. Antibody binding, continued

19) If using a multi-channel pipette (recommended) place a multi-channel reagent reservoir on ice. Fill with Cell Permeabilization Buffer.
20) Place the 8-strip tubes on magnet until slurry clears. Pipette to remove sup.
21) While beads are on magnet, add 200 uL cold Cell permeabilization Buffer directly onto beads. Pipette to remove sup.
22) Repeat previous step for total of two washes, without removing 8-strip tubes from the magnet. 23) Add 50 uL cold Cell Permeabilization Buffer to each sample. Gently vortex and/or disperse clumps by thorough pipetting.
*** Permeabilization: the puncturing of the cell membrane

5 5. Binding of pAG-MNase: pAG-MNase enzyme is a fusion of protein A and protein G to micrococcal nuclease.

24) Add 2.5 uL pAG-MNase (20X stock) to each sample. Gently vortex/pipette mix.
25) Incubate samples for 10 min at RT. Return 8-strip tube to magnet. Remove sup.
26) While beads are on magnet, add 200 uL cold Cell permeabilization Buffer directly onto beads.

Pipette to remove sup.
27) Repeat previous step for total of two washes, without removing 8-strip tubes from the magnet. 28) Remove 8-strip tubes from the magnet. Add 50 uL cold Cell permeabilization Buffer to each sample. Gently vortex and/or disperse clumps by thorough pipetting.

6 6. Targeted chromatin digestion and release

29) Place 8-strip tubes on ice. Add 1 uL 100 mM Calcium Chloride to each sample and gently vortex. Ensure efficient digestion by making sure beads are thoroughly resuspended. Gently pipette with a P200 if needed.
30) Incubate 8-strip tubes on nutator for 2 hours at 4°C.
31) Add 33 uL Stop Buffer to each sample. Gently vortex to mix.
32) Prior to first use, reconstitute E.coli Spike-in DNA in 200 uL DNase free water.
33) Add 1 uL (0.5 ng) Spike-in DNA to each sample. Gently vortex to mix.
34) Incubate 8-strip tubes for 10 min at 37°C in a thermocycler. (Setting: Incubate mode)
35) Quick spin in benchtop microfuge.
36) Place 8-strip tubes on a magnet stand until slurry clears. Transfer sups containing CUT&RUN enriched DNA to 1.5 mL tubes and discard ConA Beads

7 7. DNA purification

37) Add 420 uL DNA Binding Buffer to each sample. Mix well by vortexing.
38) For every sample, place a DNA Cleanup Column into a DNA Collection Tube. Load each sample onto a column and label the top.
39) Centrifuge at 16,000 x g, 30 sec, RT. Discard the flow-through. Place the column back into the collection tube.
40) Prior to first use, add 20 mL > 95% ethanol to DNA Wash Buffer.

8 8. DNA purification, continued

41) Add 200 uL DNA Wash Buffer to each sample column.
42) Centrifuge at 16,000 x g, 30 sec, RT. Discard the flow-through. Place the column back into the collection tube.
43) Repeat for a total of two washes.
44) Discard the flow-through. Centrifuge one additional time at 16,000 x g, 30 sec to completely dry the column (Empty spin).
45) Carefully remove the column from the collection tube, ensuring it does not come into contact with the flow-through. Transfer column to a clean pre-labeled 1.5 mL microfuge tube.
46) Elute DNA by adding 12 uL DNA Elution Buffer, taking care to ensure the buffer is added to the center of the column rather than the wall. Tap the column + microfuge collection tube on the benchtop to ensure all droplets are absorbed onto the resin.
*** 12 uL is recommended, however DNA can be eluted in 6-20 uL volumes depending on anticipated yield and desired final concentration. Larger elution volumes, longer incubation times, and/or multiple rounds of elution may improve DNA yield. However, sample concentration will be reduced with larger total elution volume.

47) Let sit 5 minutes, then centrifuge at 16,000 x g, 1 min, RT.
48) Vortex eluted material and use 1 uL to quantify the CUT&RUN-enriched DNA using the Qubit fluorometer as per the manufacturer's instructions. See Quality Control Checks section for typical DNA yields.
49) CUT&RUN DNA can be stored at -20°C for future processing.

9 9. Measurement of DNA concentration

50) Measure the DNA concentration using Nanodrop
*** Blank: DNA Elution Buffer 1ul

10 Library Prep

1. End repair

1) Transfer 5 ng of CUT&RUN enriched DNA to a new 8-strip PCR tube and adjust final volume to 25 uL with 0.1X TE Buffer.
2) Prepare an End Repair Master Mix by combining the following in a fresh 1.5 mL tube on ice. Prepare sufficient master mix for N reactions using the recipe below (includes 20% excess volume to account for pipetting error):
4.2 uL End Prep Buffer x N reactions = uL
1.8 uL End Prep Enzyme x N reactions = uL
3) Gently vortex End Repair Master Mix, quick spin, and return to ice.
4) Add 5 uL End Repair Master Mix to 25 uL CUT&RUN enriched DNA. Pipette up and down 5 times to clear tips, gently vortex to mix, and quick spin.
5) Place reactions in a thermocycler and run the following program with heated lid set to ≥ 75°C
Step 1. 20°C , 20 min, 1 cycle: Reaction temperature
Step 2. 65°C , 30 min, 1 cycle: Enzyme activation
Step 3. 4°C , infinity: Hold temperature
6) Proceed immediately to Section II.

11 2. Adaptor ligation and U-excision

7) Place 8-strip tubes directly on ice or in a pre-chilled aluminum block on ice.
8) Add 1.25 µL of 1.5 µM Adapter for Illumina® to each reaction on ice.
9) Prepare a Ligation Master Mix by combining the following in a fresh 1.5 mL tube on ice. Prepare sufficient master mix for N reactions using the recipe below (includes 10% excess volume to account for pipetting error):
16.5 µL Ligation Mix x N reactions = _____ µL
0.55 µL Ligation Enhancer x N reactions = _____ µL
10) Gently vortex Ligation Master Mix, quick spin, and return to ice.
11) Add 15.5 µL Ligation Master Mix to each reaction on ice. Thoroughly vortex to mix, quick spin, and return to ice. If processing multiple tube strips, dispense master mix to one 8-tube strip, vortex and quick spin, before continuing to the next strip.

12) Incubate tubes in a thermocycler without a heated lid for 15 minutes at 20°C
13) Remove tubes from thermocycler to a room temperature (RT) rack. Add 1 µL of U-Excision Enzymeto each reaction. Pipette up and down 3 times to clear tip. Gently vortex and briefly spin. Final volume of each reaction is now 47.75 µL.
14) Place tubes in a thermocycler with a heated lid. Set lid temperature to ≥ 47°C, block temperature to 37°C, and incubate reactions for 15 minutes.
15) Remove tubes from thermocycler and quick spin.

12 **3. DNA Cleanup**

*NOTE:For steps denoted with a red flag use of multi-channel pipettors is recommended.
16) Prepare 85% Ethanol (EtOH) FRESH on the day of the experiment using a 100% EtOH stock and molecular biology grade water. Make 900 µL 85% EtOH per reaction (e.g. for one reaction, combine 765 µL 100% ETOH + 135 µL water).
17) Vortex SPRIselect reagent (beads) thoroughly to completely resuspend.
18) Slowly add 47.75 µL SPRIselect reagent (1X reaction volume) to each reaction.
19) Gently vortex 8-strip tubes and quick spin. Incubate at RT for 5 minutes.
20) Place 8-strip tubes on magnetic separation rack at RT for 2 minutes. Pipette to remove and discard supernatant.
21) Keeping 8-strip tubes on magnet, add 180 µL 85% EtOH directly onto beads. Pipette to remove and discard supernatant.
22) Repeat previous step for a total of two washes, without removing 8-strip tubes from the magnet.
23) Remove 8-strip tubes from magnet and briefly spin to collect liquid. Return to magnetic rack, and pipette to remove any residual supernatant (EtOH).
24)Remove tubes from magnet, leave caps open, and air dry beads for 2 minutes.
25) Add 12 µL of 0.1X TE Buffer to each reaction to elute target DNA.
26) Gently vortex 8-strip tubes until beads are fully resuspended and then quick spin to collect liquid in tube bottom. Incubate at RT for 2 minutes.
*IMPORTANT: Do not discard supernatant! Adapter-ligated DNA is now in the supernatant.
27) Place on magnetic rack at RT for 2 minutes.
28) Transfer 10.5 µL of eluted DNA to new 8-strip PCR tubes. Discard beads.

13 **4. Indexing PCR**

29) Following the primer selection instructions in Appendix II, assign a unique pair of i5 & i7 indexing primers to each reaction. Mark the combination as consumed on the Primer Tracking Table (see the Protocol Quick-Start Card).
30) To each 10.5 µL reaction, add the following reagents individually and in order. Visually inspect tips to confirm that the correct volume was aspirated and change tips between each addition to prevent cross-contamination.
1 µL assigned i7 primer 1 µL assigned i5 primer
12.5 µL of High Fidelity 2X PCR Master Mix (mix well before using)
*IMPORTANT:Discard i7 primer tube caps and replace using fresh caps provided with kit.

This is an important step to avoid i7 primer cross-contamination.
31) Mix reactions by vortexing, and then quick spin to collect liquid.
32) Place reactions in a thermocycler with a heated lid set to 105°C and perform PCR using the following parameters:

Step #	Temperature	Time	Cycles	Notes
1	98°C	45 sec	1	Hot start activation of DNA Polymerase
2	98°C	15 sec	14	DNA melting
3	60°C	10 sec	Hybrid annealing/extension	
4	72°C	60 sec	1	Final extension
5	4°C	∞		Hold temperature

*NOTE:The PCR cycling parameters are designed to enrich 200-700 bp DNA fragments.

14 **5. PCR Cleanup**

If performing library prep in a single day: proceed to the next step.

36) Gently vortex 8-strip tubes and quick spin. Incubate at RT for 5 minutes.
* NOTE:PCR-amplified DNA binds to beads.
37) Place 8-strip tubes on magnetic separation rack at RT for 2 minutes. Pipette to remove and discard supernatant.
38) Keeping 8-strip tubes on magnet, add 180 µL 85% EtOH directly onto beads. Pipette to remove and discard supernatant.
39) Repeat previous step for a total of two washes, without removing 8-strip tubes from the magnet.
40) Remove 8-strip tubes from magnet and briefly spin to collect liquid. Return tomagnetic rack, and pipette to remove any residual supernatant (EtOH).
Remove tubes from magnet, leave caps open, and air dry the beads for 2 minutes.
* NOTE: Over drying the beads may result in poor recovery. 0.1X TE Buffer should be added when all visible liquid has evaporated, but the beads are still dark brown and glossy. If the beads turn light brown and crackly, then they are too dry.
42) Add 12 µL of 0.1X TE Buffer to each reaction to elute target DNA.
43) Gently vortex 8-strip tubes until beads are fully resuspended and then quick spin to collect liquid in tube bottom. Incubate at RT for 2 minutes.
* IMPORTANT: Do not discard supernatant! PCR-amplified DNA is now in the supernatant.

6. PCR cleanup, continued

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44) Place on magnetic rack at RT for 2 minutes.
45) Transfer 10.5 µL of eluted DNA to new 8-strip PCR tubes. Discard beads.
*NOTE:Safe pause point. Libraries can be stored at -20oC overnight.

16 **7. Analysis of library fragment size**

46) Use 1 µL to quantify CUT&RUN libraries using the QubitTM fluorometer with the 1X dsDNA HS Assay Kit as per the manufacturer's instructions.
*NOTE:Typical yield for a purified CUT&RUN sequencing library is ~300-500 ng; see FAQs for more information.
47) For each library, prepare 5 µL at 10 ng/µL for loading onto the Agilent Bioanalyzer®or TapeStation®system.
*NOTE:Record the dilution factor, which will be needed to calculate library molarity from theresults (reported as DNA concentrations in nM for the desired 200 - 700 bp region).
48) Load and analyze 1 µL diluted sequencing library using the High Sensitivity DNA Kit (Bioanalyzer®) or the D1000 ScreenTape System & Reagents (TapeStation®) as per the manufacturer's instructions.
*NOTE:Typical concentration for final library (200 – 700 bp region) is 100-200 nM. If library concentrations fall below 0.5 nM, see FAQs for guidance.
49) The final traces should show predominant enrichment of mononucleosomefragments, such as those yielded by the H3K4me3 and CTCF antibodies inFigure 5(~300 bp: ~170 bp + sequence adapter length).
*NOTE:Fragment distributions for positive (e.g.H3K4me3) and negative (e.g.IgG) control reactions can be used to validate library prep workflows. See FAQs for more information.

17 **8. Illumina sequencing**

*NOTE:Paired-end sequencing (2 x 50 cycles minimum) is recommended for CUT&RUN toidentify both ends of MNase cleavage for target footprinting analysis.
50) Select appropriate Illumina®sequencing platform (see Appendix I) based on the number of CUT&RUN libraries and desired sequencing depth.
*IMPORTANT:In contrast to ChIP-seq, only 3-8 million paired-end reads per reaction areneeded for adequate CUT&RUN coverage. For low abundance targets (e.g.H3K4me3), aim for 3-5 million reads. For high abundance targets (e.g.H3K27me3), aim for 5-8 million.
51) Pool libraries at desired ratios using the molarity calculations from Section VI (200-700 bp region) and load onto Illumina®sequencer. General steps:
a. Dilute each library to the same nM concentration, depending on final yields.For NextSeq 2000 and NextSeq 500/550, dilute to 1-4 nM.
b. Pool equimolar libraries into one tube.
c. Dilute pooled libraries to appropriate concentration and in the volume required for Illumina®platform. Follow guidelines from specific Illumina®kit to load onto sequencer (support.illumina.com).

d. When setting up a multiplexed sequencing run, make sure dual i5 & i7 barcodes are correctly assigned for each library (seeImportant, below). For a full list of NGS indexes in an easy-to-copy format, see the CUTANATM Library Prep Multiplexing Primers Excel spreadsheet atepicypher.com/14-1001.
*IMPORTANT:Make sure that each library in a sequencing run has a unqiue pair of barcodes.Samples with the same pair of indexes must be sequenced in separate lanes/flow cells.