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Enabling Epigenetics Research

ChIC/CUT&RUN Assay Kit

Catalog No. 53180

(Version A4)

Revision	Date	Description of Change
A2	July, 2023	Updated Kit photo
		Corrected typo on page 2
A3	October, 2023	Updated storage temperature range for 0.1 M $CaCl_2$ and Updated SPRI volume from 1.1 to 1.2 step 3 page 9
A4	December, 2023	Corrected DNA Binding Buffer to DNA Purification Binding Buffer on page 4

Contents

Overview

CUT&RUN (Cleavage Under Targets & Release Using Nuclease) is an epigenetic method used to investigate the genome-wide distribution of various chromatin-associated proteins and their modifications[1]. CUT&RUN is similar to chromatin immunoprecipitation (ChIP), in that it utilizes an antibody to target chromatin-associated marks and proteins, but requires less sample material and less sequencing depths than ChIP[1-3].

In CUT&RUN, a protein of interest is tagged with an antibody and bound to the chromatin in intact cells. Then, a micrococcal nuclease (MNase) is used to cleave the DNA specifically at the binding sites of the protein of interest. The MNase is fused to Protein A and Protein G, and the Protein AG portion will attach to the antibody already bound to the protein of interest. Next, the MNase is activated by the addition of calcium, which in turn fragments the DNA, allowing the DNA to enter the supernatant. The released fragments are purified, sequenced, and mapped to the reference genome to determine the protein's binding sites.

The advantages of CUT&RUN over ChIP-Seq are a simplified protocol without sonication of samples, robust data from fewer cells (as few as 5,000 for histone modifications or 25,000 for some transcription factors) and shallow sequencing depth (as few as 2,000,000 reads for robust marks or 25 reads on average for transcription factors)[3].

CUT&RUN is a valuable tool for studying chromatin-associated proteins because it is sensitive, specific, and requires fewer cells than ChIP, making it ideal for identifying binding patterns of chromatin-associated proteins such as transcription factors or histone modifications genome-wide. Chromatin-associated proteins play critical roles in regulating various cellular processes such as gene expression, DNA replication, DNA repair, and cell differentiation. Understanding the binding patterns of these proteins can provide insight into how these cellular processes are regulated.

Product	Format	Catalog No.
ChIC/CUT&RUN Assay Kit	24 rxns	53180

Kit Components and Storage

Please store each component at the temperature indicated in the table below. Kit and components are guaranteed for 6 months after receipt when stored properly.

Reagents	Quantity	Storage
5% Digitonin	600 µL	-20°C
RNase A (10 µg/µL)	40 µL	-20°C
Protease Inhibitor Cocktail, 500 µL	2 x 500 µL	-20°C
ChIC/CUT&RUN pAG-MNase	70 µL	-20°C
Glycogen 20 mg/mL	12 µL	-20°C
Histone H3K4me3 pAb, 10 µg, 1 mg/mL	8 µL	-20°C
Negative Control IgG, Rabbit	8 µL	-20°C
1 M Spermidine	28 µL	-20°C
Nuclei Isolation Buffer	2.7 mL	4°C
Concanavalin A Beads	300 µL	4°C
0.1 M $CaCl_2$	45 µL	RT or 4°C
Stop Solution	1.1 mL	4°C
1X Binding Buffer	6 mL	4°C
Dig-Wash Buffer	55 mL	4°C
0.5 M EDTA	250 µL	RT
DNA Purification Wash Buffer*	10 mL	RT
DNA Purification Elution Buffer	5 mL	RT
DNA Purification Binding Buffer**	6 mL	RT
3M Sodium Acetate	500 µL	RT
DNA Purification Columns SF	24	RT
0.2 mL Strip Tubes, Attached Caps	7 strips	RT

*DNA Purification Wash Buffer must be reconstituted to a final concentration of 80% ethanol prior to use. Add 40 mL of 100% ethanol to the DNA Purification Wash Buffer prior to use.

**DNA Purification Binding Buffer must be reconstituted to a final concentration of 60% isopropyl alcohol prior to use. Add 9 mL of 100% isopropanol to the DNA Purification Binding Buffer bottle prior to use.

Additional Materials Required

- Antibody to protein of interest
- Ethanol, 100%
- Isopropanol, 100%
- Microcentrifuge
- Vortexer
- Magnetic bar or magnetic plate rack for 0.2 mL tubes
- 0.2 mL PCR tubes
- 1.5 or 2 mL microcentrifuge tubes
- Wet ice and ice bucket
- Nutator or orbital shaker
- Nutator or orbital shaker that can be placed at 4°C
- NGS Library Preparation kit compatible with small fragments such as NEBNext® Ultra™ II DNA Library Kit for Illumina: E7645L
- Indexing primers such as NEBNext Multiplex Oligos for Illumina (Index Primers Set 1): E7335S
- Indexing primers such as NEBNext Multiplex Oligos for Illumina (Index Primers Set 2): E7500S

Prepare Buffers

Prepare DNA Purification Binding Buffer: add 9 mL 100% isopropanol to the DNA Purification Binding Buffer bottle to a final concentration of 60% isopropanol.

Prepare DNA Purification Wash Buffer: add 40 mL of 100% ethanol to the DNA Purification Wash Buffer bottle, the final concentration of ethanol is 80%.

Prepare Stop Solution: Add 11 µL Glycogen to the vial of Stop Solution. Add 22 µL of RNase A to Stop Solution. You will need 40 µL Stop Solution per reaction. Solution will be stable for 6 months at 4°C.

Before beginning, prepare Complete Wash Buffer, Cell Permeabilization Buffer and Antibody Buffer. Some of each buffer will be the base component of the next buffer that follows in sequence in the table below, and the remaining portions of each buffer will be stored as indicated in this table and used as indicated in the protocol.

Buffer Name	Components	1 Reaction	8 Reactions	24 Reactions	Storage
Complete Dig-Wash Buffer	Dig-Wash Buffer	1.8 mL	14.4 mL	43.2 mL	4°C, for use on Day 1
	Protease Inhibitor Cocktail	18 µL	144 µL	432 µL	
	1 M Spermidine	0.9 µL	7.2 µL	21.6 µL	
Cell Permeabilization Buffer	Complete Dig-Wash Buffer	1.4 mL	11.2 mL	33.6 mL	4°C, for use on Day 2
	5% Digitionin	2.8 µL	22.4 µL	67.2 µL	
Antibody Buffer	Cell Permeabilization Buffer	100 µL	800 µL	2.4 mL	Ice, for use on Day 1
	0.5 M EDTA	0.4 µL	3.2 µL	9.6 µL	

For nuclei isolation, (Step 13, optional but recommended) prepare Complete Nuclei Isolation Buffer: You will need 100 µL per sample. Add 1.4 µL 1 M Spermidine to the Nuclei Isolation Buffer bottle. When ready to use in the assay you will need to Add 10 µL 100X Protease Inhibitior Cocktail (PIC) per 1 mL Nuclei Isolation Buffer.

Day 1 (approximately 3 hours)

Prepare Concanavalin A Beads (20 - 30 minutes)

Note: When preparing beads, cells or nuclei, the buffers are kept on ice but the samples are handled on the bench at room temperature for convenience.

1. Resuspend Concanavalin A Beads by vortexing.
2. Dispense 10 µL resuspended Concanavalin A Beads per sample into 0.2 mL tubes with attached caps. Beads should be resuspended periodically so the proper amount of bead slurry is pipetted into each sample if processing multiple samples at the same time.
3. Place tubes on magnetic plate rack or magnet bar.
4. Remove supernatant from beads and discard.
5. Add 100 µL 1X Binding Buffer per sample.
6. Resuspend Concanavalin A Beads with 1X Binding Buffer by gently pipetting up and down.
7. Place tubes back on magnet to capture beads. Remove supernatant and discard.
8. Repeat steps 5 - 7 for a total of 2 washes.
9. Add 10 µL of Binding Buffer to each sample and store on ice until ready for next steps.

Prepare Cells (10 minutes)

10. Prepare 5,000 to 500,000 cells, freshly grown, or thaw cryopreserved cells (5,000 to 500,000 per reaction). If you are starting with adherent cells, we recommend using TrypLE Express for cell-dissociation. Cryopreserved cells can be thawed on the bench or at 37°C water bath or heat block.
11. Centrifuge cells at 600 x *g* for 3 minutes at room temperature.
12. Remove and discard supernatant.

Prepare Nuclei (optional but recommended) (15 minutes)

13. Prepare 100 µL per reaction of Complete Nuclei Isolation Buffer by adding 10 µL Protease Inhibitor Cocktail and 0.5 µL 1 M Spermidine per 1 mL Nuclei Isolation Buffer needed.

Note: You may add 1.4 µL 1 M Spermidine to the full bottle of Nuclei Isolation Buffer. The Nuclei Isolation Buffer with the added Spermidine can be stored for 6 months at 4°C. Then, when ready to perform the assay, add 10 µL fresh Protease Inhibitor Cocktail per 1 mL Complete Nuclei Isolation Buffer per sample. You will need 100 µL of the Complete Nuclei Isolation Buffer per sample.

14. Gently resuspend cell pellets in 100 µL Complete Nuclei Isolation Buffer per up to 500,000 cells. This volume does not need to scale down for less than 500,000 cells. Gently pipette or flick tube to ensure pellet is fully resuspended.
15. Place nuclei samples on ice for 10 minutes.
16. Centrifuge nuclei at 600 x *g* for 3 minutes at room temperature.
17. Remove and discard supernatant.

Wash Cells/Nuclei (30 minutes)

18. Gently resuspend cell or nuclei pellets in 100 µL Complete Dig-Wash Buffer per sample (500,000 cells or nuclei per sample). Gently pipette or flick tube to ensure pellet is fully resuspended.
19. Centrifuge cells or nuclei at 600 x *g* for 3 minutes at room temperature.
20. Remove and discard supernatant.
21. Repeat steps 18 - 20 for a total of 2 washes.

Note: When cell number is limited or pellet is not easily visible the second wash can be omitted.

22. Add 100 µL of Complete Dig-Wash Buffer per sample and gently resuspend by pipetting or flicking tube until fully resuspended.
23. Transfer 100 µL of washed cells/nuclei to prepared Concanavalin A Beads.
24. Resuspend beads and cells/nuclei by gently pipetting up and down.
25. Allow to sit at room temperature for 10 minutes.
26. Place tubes on magnet or magnetic rack.
27. Remove and discard supernatant.
28. Add 50 µL of Antibody Buffer to each sample.

Antibody Binding (overnight)

29. Add 1 µg or 1 µL of appropriate antibody to each sample.

Note: We recommend including samples for positive control Histone H3K4me3 and Negative Control IgG, Rabbit.

30. Resuspend by gently pipetting up and down.
31. Place samples on Nutator or orbital shaker overnight at 4°C at speed of 20-30 RPM. Ensure caps are slightly elevated to avoid beads getting stuck in the lid.

Day 2 (approximately 5 hours)

Washes (10 minutes)

1. Quick spin tubes and place tubes on magnet or magnetic rack at room temperature.
2. Remove and discard supernatant.
3. Add 200 µL Cell Permeabilization Buffer to each sample without disturbing pellet.
4. Remove and discard supernatant.
5. Repeat steps 3 - 4 for a total of 2 washes.

ChIC/CUT&RUN pAG-MNase Binding (20 minutes)

6. Add 50 µL Cell Permeabilization Buffer to each sample.
7. Add 2.5 µL of ChIC/CUT&RUN pAG-MNase to each sample.
8. Remove tubes from magnet. Resuspend beads and mix by gently pipetting up and down.
9. Allow to sit at room temperature for 10 minutes.
10. Place tubes on magnet or magnetic rack.
11. Remove and discard supernatant.
12. Add 200 µL Cell Permeabilization Buffer to each sample without disturbing pellet.
13. Remove and discard supernatant.
14. Repeat steps 12 - 13 for a total of 2 washes.
15. Add 50 µL Cell Permeabiliaztion Buffer to each sample.
16. Place tubes on ice and pause for 2 minutes to allow samples to cool before proceeding to the next steps.

Targeted Chromatin Digestion (3 hours)

17. Add 1 µL of 0.1 M $CaCl_2$ to each sample.
18. Mix by gently pipetting up and down to resuspend beads and place back on ice.
19. Place on Nutator or orbital shaker at 4°C for 2 hours at speed of 20-30 RPM.
20. Remove from Nutator or orbital shaker and place samples on ice.
21. Add 40 µL of Stop Solution to each sample.
22. Mix samples by gently pipetting up and down.
23. Incubate samples at 37°C for 10 minutes in a thermal cycler. Set lid to 65°C. This step releases the chromatin fragments into the supernatant.
24. Place tubes on magnet or magnetic rack for at least 30 seconds.

ChIC/CUT&RUN DNA Isolation and Purification (45 minutes)

25. Carefully transfer samples to fresh 1.5 mL microcentrifuge tubes, avoiding any beads.

Note: It is recommended to transfer the supernatant to a fresh tube and place on magnet or magnetic rack to ensure no beads/cells are transferred in the next steps.

26. Be sure that fresh isopropanol has been added to the bottle of DNA Purification Binding Buffer to a final percentage of 60% to DNA Purification Binding Buffer before this step. To each sample tube, add 450 µL of DNA Purification Binding Buffer.

27. Vortex briefly and let stand 1 minute at room temperature.

28. Transfer sample and buffer to DNA Purification Columns SF.

29. Centrifuge at 16,000 x *g* for 1 minute.

30. Pour off flow through.

31. Add 200 µL DNA Purification Wash Buffer to each column.

32. Centrifuge columns at maximum speed (16,000 x *g*) for 1 minute.

33. Repeat steps 31 - 32 for a total of 2 washes.

34. Pour off flow through.

35. Centrifuge at maximum speed (16,000 x *g*) for 1 minute to ensure all traces of DNA Purification Wash Buffer are removed.

36. Transfer column to new collection tube (collection tubes can be 1.5 mL microcentrifuge tubes with the lids removed).

37. To each column, add 55 µL DNA Purification Elution Buffer.

38. Let stand at room temperature for 1 minute.

39. Centrifuge at maximum speed (16,000 x *g*) for 1 minute.

40. Transfer eluted ChIC/CUT&RUN DNA to new tubes. Store at -80°C if not being used immediately for NGS Library Preparation. Samples can be stored for up to 1 week. Your samples are now ready for NGS Library Preparation.

NGS Library Preparation (3 hours)

1. For NGS Library Preparation, we recommend a kit such as NEBNext® Ultra™ II for DNA Library Preparation for Illumina. These instructions were developed with the NEBNext® Ultra™ II for DNA Library Preparation for Illumina. Follow the NEBNext® Ultra™ II for DNA Library Preparation for Illumina instructions with the additional steps below. You will also need the NEBNext Multiplex Oligos for Illumina.
2. Dilute ligation adapter to 1:25 concentration (0.6 µM). Proceed to Step 2 of the NEBNext® Ultra™ II for DNA Library Preparation instructions.
3. For Step 3, SPRI bead cleanup, perform a double-sided cleanup with 0.4X bead/sample ratio followed by a 1.5X bead/sample ratio. For the first SPRI beans cleanup, multiply sample volume x 0.4. For the second SPRI cleanup multiply sample volume x 1.2. Perform Steps 3.A1 through 3.A9. Do not perform Step 3B.

IMPORTANT! Measure sample volumes before SPRI cleanup as high viscosity can lead to deviation from expected volume.

4. For Step 3A.10 perform a quick spin.
5. For Step 3A.12 elute the beads in 17 µL of DNA Purification Elution Buffer.
6. Follow Step 4 of the NEBNext® Ultra™ II for DNA Library Preparation instructions and perform 12 PCR cylces for PCR enrichment of CUT&RUN libraries. Follow the instructions for your Multiplex Oligos Primer Set.
7. Following PCR enrichment, perform a double-sided SPRI bead cleanup on the PCR product. Perform a double-sided cleanup with 0.4X bead/sample ratio followed by a 1.2X bead/sample ratio.

    A. Add 20 µL SPRI beads for 50 µL PCR product (0.4X bead/original sample ratio) and pipette up and down 10 times to mix.

    B. Incubate at Room Temperature for 5 minutes.

    C. Place on magnetic stand for 5 minutes to pellet.

    D. Move supernatant to new tubes and discard beads from previous tubes.

    E. Perform a second cleanup by adding 40 µL SPRI beads to the tubes with samples (1.2X bead/original sample ratio) and pipette up and down 10 times to mix.

    F. Incubate for 5 minutes at Room Temperature and place on magnetic stand for 5 minutes to pellet beads.

    G. Remove supernatant and discard.

    H. Without removing the tubes from the magnetic stand, gently wash beads with 200 µL of 80% Ethanol.

    I. Aspirate and discard supernatant.

    J. Perform a second wash by repeating steps 7H - 7I.

    K. Using a P10 pipette, remove any leftover Ethanol from the tubes and let air dry for approximately 5 minutes at Room Temperature, without over-drying (look for beads going from shiny to matte).

L. Remove tubes from magnet and reconstitute beads with 22 µL of DNA Purification Elution Buffer.

M. Incubate for 1 minute at Room Temperature.

N. Move tubes back to magnetic stand and allow 5 minutes to pellet beads at Room Temperature.

O. Transfer 20 µL of libraries to fresh strip tubes. Store at -20°C or proceed to Illumina NGS sequencing.

References

1. Schmid, M. *et al.* (2004) *Mol. Cell.*, 16(1): 147-157
2. Skene, P.J. *et al.* (2017) *Elife 6*, e21856
3. Skene, P.J. *et al.* (2018) *Nat. Protoc.,* 13, 1006-1019

Troubleshooting Guide

Problem/Question	Recommendation
No visible nuclei pellet	The pellet may not be visible.
	Be sure to position tubes with caps oriented in centrifuge so that you know where the pellet should be in the tube, and proceed carefully with next steps.
What is the recommended sequencing depth?	25 million reads for transcription factors. For some histone marks you may go down to 2 million reads.
How many cells should I use for transcription factor targets?	We recommend 500,000 cells and a CUT&RUN validated antibody.
High background	Be sure to start with viable cells.
	Check that cells/nuclei are intact by an automated cell counting method such as the Countess II or by Trypan Blue staining and a hemocytometer.
Will the negative control produce a library?	Yes. The negative control IgG will produce a library.
SPRI beads are not pelleting towards the bottom or taking too long to pellet.	Using a bar magnet, start by pelleting the SPRI beads towards the top of the tube and gradually work your way down to the bottom of the tubes. After, place the tubes on a magnetic plate.

Technical Services

If you need assistance at any time, please call or send an e-mail to Active Motif Technical Service at one of the locations listed below.

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