Chapter 6

Low-Input CUT&RUN for Mouse Oocytes and Preimplantation Embryos

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Abstract

Cleavage Under Target & Release Using Nuclease (CUT&RUN) enables the detection of DNA regions that are bound by a protein of interest. This method is suitable for low-input materials because of the absence of an immunoprecipitation step. However, it sometimes fails when applying it to fragile cells, such as mouse oocytes. Here we describe our low-input CUT&RUN protocol optimized for mouse oocyte and preimplantation embryo samples in which the primary antibody and protein A-MNase binding steps are completed before the cells are bound to Concanavalin A-coated magnetic beads. This modification prevents crush of oocytes and early embryos and unwanted loss of chromatin during CUT&RUN procedures.

Key words Epigenetics, Histone modification, Oocytes, Preimplantation embryo

1 Introduction

Cleavage Under Target & Release Using Nuclease (CUT&RUN) is a chromatin profiling method in which protein A- or G-conjugated micrococcal nuclease (MNase) cleaves DNA regions that are bound by an antibody against a protein of interest [1, 2]. Purification of shorter DNA fragments followed by PCR amplification and next-generation sequencing allows genome-wide profiling of the chromatin localization of the protein of interest. This method is suitable for small numbers of in vivo cell materials because of the absence of an immunoprecipitation step which often causes DNA loss and high background noise.

To study epigenetic regulation in mouse oocytes and preimplantation embryos, we have used CUT&RUN for the recent few years [3–5]. However, we have realized that the amount of DNA recovered from oocytes and early preimplantation embryos varies greatly from experiment to experiment even by a protocol that stably works well for embryonic stem cells (ESCs) and late stages

Izuho Hatada and Takuro Horii (eds.), *Epigenomics: Methods and Protocols*, Methods in Molecular Biology, vol. 2577,
https://doi.org/10.1007/978-1-0716-2724-2_6,

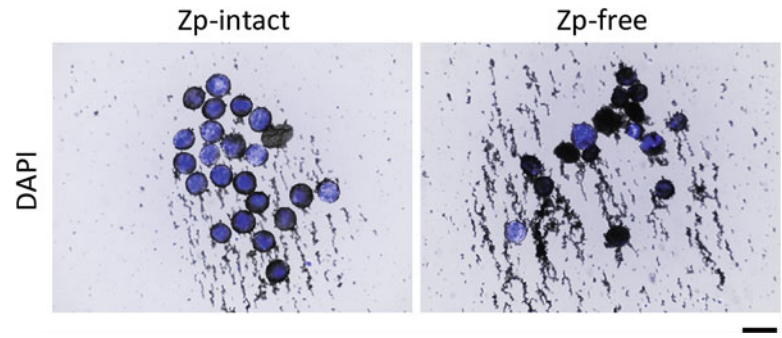

Fig. 1 Detection of DNA in zona pellucida (ZP)-intact and ZP-free oocytes after multiple washes in the standard CUT&RUN procedure. Twenty-two ZP-intact and ZP-free GV oocytes were captured by Concanavalin A-coated (ConA) beads, respectively, and washed several times with Dig-wash buffer. After ConA beads clumps were recovered by a glass capillary under a microscopy, they were stained with DAPI (blue). Note that DNA is undetectable in many of the clumps in the ZP-free oocyte sample, indicating loss of chromatin during wash steps. Scale bars, 100 μm

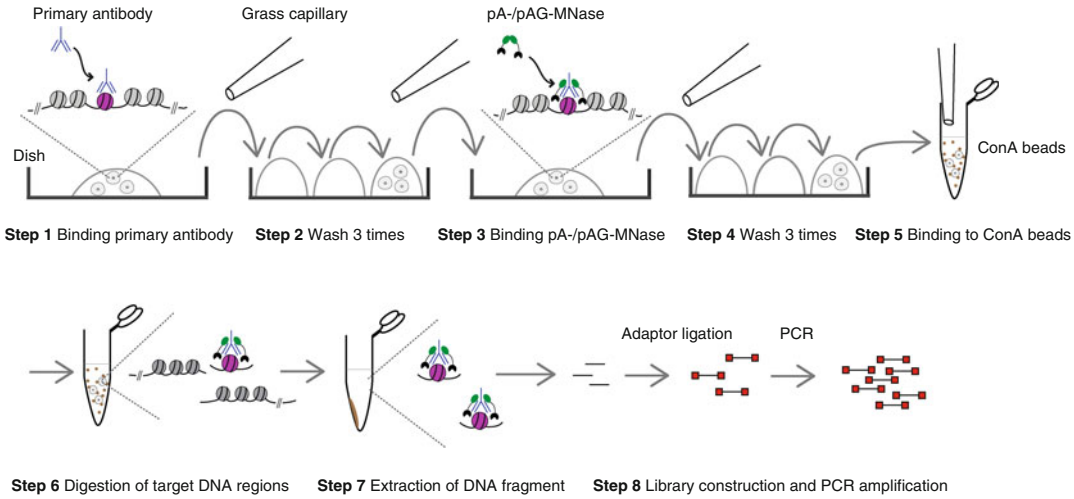

Fig. 2 Flowchart of the steps of the modified CUT&RUN method

of preimplantation embryos. By careful examination of this cause, we found that oocytes and embryos after removal of zona pellucida (ZP) (ZP-free oocytes/embryos) were fragile and frequently crushed during repeated wash steps of the CUT&RUN procedure (Fig. 1). We thought that cells with large sizes (~80 μm in mouse oocytes) tend to be easily crushed when the cells captured by Concanavalin A-coated magnetic beads are retained in a small amount of liquid after the wash buffer is removed. Consequently, we guessed that the chromatin is leaked out and lost by wash steps. To prevent this, we modified the procedure (Fig. 2). This modified

method minimizes the variation between experiments and yields relatively constant amounts of DNA from a smaller number of oocytes and early preimplantation embryos.

2 Materials

Prepare all solutions using molecular biology grade water. To reduce the risk of DNA contamination, purchase ready-to-use reagents if commercially available. Store them in aliquots so that reagents can be discarded when contamination is found.

2.1 Reagents for Sampling of Oocytes and Preimplantation Embryos

1. Acidic Tyrode's solution.
2. 10% PVP (Irvine Scientific) or homemade: Dissolve PVP powder in M2 media overnight and filtrate it with 0.45-μm pores.
3. 5-M NaCl.
4. 0.25% trypsin/0.1-mM EDTA.
5. M2 media.
6. PBS containing 0.2% BSA.
7. Cytochalasin B.
8. Colcemid.
9. 50-mm plastic dish.

2.2 Reagents

1. M HEPES, pH 7.5.
2. 5-M NaCl.
3. 1-M spermidine.
4. Roche complete Mini EDTA-free tablet.
5. 5% digitonin (*see* **Note 1**).
6. 0.5-M EDTA.
7. 0.5-M EGTA.
8. 10-mg/mL RNase A.
9. 20-mg/mL glycogen.
10. Concanavalin A-coated beads (Bangs Laboratories).
11. Primary antibody (*see* **Note 2**).
12. pA−/pAG-MNase (*see* **Note 3**).
13. 10% SDS solution.
14. Carrier RNA.
15. 20-mg/mL Proteinase K.
16. Phenol/Chloroform/Isoamyl alcohol, pH 8.0.
17. 100-mM $CaCl_2$.

18. 3-M NaOAC.
19. 100% EtOH.
20. SPRIselect (Beckman).
21. NEBNext Ultra II DNA Library Prep Kit (NEB).
22. NEBNext Multiplex Oligos for Illumina (NEB).

2.3 Buffers

1. Binding buffer: 20-mM HEPES, pH 7.5, 10-mM KCl, 1-mM $CaCl_2$, 1-mM $MnCl_2$. Prepare 20 mL per experiment; store at 4 °C for 6 months.
2. Wash buffer: 20-mM HEPES, pH 7.5, 150-mM NaCl, 0.5-mM spermidine, one mini Roche complete EDTA-free tablet. Prepare 10 mL per experiment; store at 4 °C for 1 week.
3. Dig-wash buffer: 0.02% digitonin in wash buffer. Prepare 1.5 mL per sample; use within a day (*see* **Note 1**).
4. Antibody incubation buffer: 2-mM EDTA in Dig-wash buffer. Prepare 100 μL per sample; use within a day.
5. 10× STOP buffer: 1700-mM NaCl, 100-mM EDTA, 20-mM EGTA, 0.02% digitonin, 250-μg/mL RNase A, 500-μg/mL glycogen. Prepare 200 μL per ten samples; store at 4 °C for 1 week.
6. Elution buffer: 10-mM Tris–HCl, pH 8.5.

3 Methods

Keep environment clean to avoid contamination of DNA. Wipe pipettes, pens, and tube racks with DNA AWAY (Thermo Fisher). Work on a plastic wrap sheet on the bench. All Subheadings 3.1, 3.2, and 3.3 are performed on disposable plastic dish, and a glass capillary attached to a mouth pipette is used for transferring cells (*see* **Note 4**).

3.1 Removal of Zona Pellucida and Polar Bodies (See Note 5)

1. Remove zona pellucida (ZP) by brief treatment of acidic Tyrode's solution supplemented with 0.5% PVP and 50-mM NaCl (this supplementation allows gentle removal of ZP and reduces the frequency of cell lysis during the procedures), and wash three times in M2 media. Note that metaphase II (MII)-stage oocytes are melted in Ab buffer and cannot be handled by a glass capillary. Therefore, ZP should not be removed from MII oocytes.
2. When collecting one-cell stage zygotes, incubate ZP-free zygotes in M2 medium containing 5-μg/mL cytochalasin B and 0.1-μg/mL colcemid for 10–15 min at 37 °C. After incubation, remove polar bodies (PBs) by pipetting with a narrow

glass capillary. After PBs are removed, transfer the zygotes back to a culture medium without cytochalasin B and colcemid, and incubate at least 30 min so that cytoskeleton of zygotes is restored. This restoration step is critical for not being melted in Ab buffer.

3. When collecting two-cell and four-cell embryos, incubate ZP-free embryos in 0.25% trypsin/1-mM EDTA supplemented with 0.5% PVP and 50-mM NaCl for ~1 min at room temperature. Remove polar bodies by pipetting with a narrow glass capillary and wash three times in M2 media.
4. (Optional) Wash oocytes/embryos three times in 200-μL drops of PBS containing 0.2% BSA. From this step, use new petri dish (*see* **Note 6**).
5. Transfer and wash oocytes/embryos two times in 200-μL drops of Ab buffer with a thick glass capillary (a diameter of >200 μm). Handle the cells gently from this step, because they are fragile in Ab buffer.

3.2 Bind Primary Antibody

1. Transfer oocytes/embryos in a 100-μL drop of Ab buffer containing 1 μL of primary antibody (*see* **Note 7**).
2. Close the lid of the petri dish and incubate overnight at 4 °C (*see* **Note 8**).

3.3 Bind pA-/pAG-MNase

1. Wash oocytes/embryos twice in 200-μL drops of Dig-wash buffer. Use a newly prepared Dig-wash buffer on the day of use (*see* **Note 9**).
2. Close the lid and incubate for 20 min at room temperature.
3. Transfer oocytes/embryos in a 100-μL drop of Dig-wash buffer containing 500 ng/μL of pA-/pAG-MNase.
4. Close the lid and incubate for 3 h at 4 °C.
5. Wash oocytes/embryos twice in 200-μL drops of Dig-wash buffer.
6. Close the lid and incubate for 20 min at room temperature.

3.4 Prepare Magnetic Beads

1. Thoroughly resuspend Bio-Mag Plus Concanavalin A-coated beads (ConA beads). Transfer 10 μL of ConA beads per sample to 1 mL of binding buffer in a 1.5-mL tube and mix by vortexing.
2. Place the tube on a magnetic stand and wash once in 1 mL of binding buffer.
3. Place the tube on a magnetic stand and remove the supernatant. Add 50 μL (per sample) of wash buffer and resuspend ConA beads by vortexing. Split 50 μL into new 1.5-mL tubes (*x* the number of samples).

3.5 Digestion

1. Transfer oocytes/embryos in the tube prepared at Subheading 3.4, **step 3** and incubate for 10 min at room temperature (*see* **Note 10**).
2. Place the tube on a magnetic stand and remove the supernatant (*see* **Note 11**). Add 180 μL of ice-cold Dig-wash buffer and mix by vortexing at weak setting for a few seconds.
3. Incubate for 2 min on ice.
4. Add 3.6 μL of 100-mM $CaCl_2$ and mix by vortexing at weak setting for 5–10 s. Place the tubes on ice.
5. Incubate for 20 min on ice.
6. Stop the digestion reaction by adding 20 μL of 10× STOP and vortexing at weak setting for 5–10 s.
7. Stand on ice for 5 min.

3.6 DNA Purification

1. Incubate the tubes at 37 °C for 20 min at 1000 rpm.
2. Place the tube on a magnetic stand, and transfer the supernatant to a new 1.5-mL LoBind tube.
3. Add 2 μL of 10% SDS and 2.5 μL of 20-mg/mL Proteinase K, and mix by tapping.
4. Incubate at 60 °C for 45 min followed by 72 °C for 20 min.
5. Add 20-ng carrier RNA.
6. Transfer the sample into a phase-lock tube, add 200 μL of Phenol/Chloroform/Isoamyl alcohol (pH 8.0), and mix by vortexing at maximum setting for 15 s.
7. Stand the tube at room temperature for 3 min and centrifuge for 5 min at 16,000 × *g* at room temperature.
8. Take the supernatant to a new 1.5-mL LoBind tube, and add 20 μL of 3-M NaoAC, 2 μL of 20-mg/mL glycogen, and 750 μL of 100% EtOH. Mix by vortexing at maximum setting for 15 s.
9. Incubate overnight at −20 °C (*see* **Note 12**).
10. Centrifuge the samples for 30 min at 16,000 × *g* at 4 °C and remove the supernatant. Wash the pellet once with 800 μL of 80% EtOH (*see* **Notes 13** and **14**).
11. Centrifuge for 10 min at 16,000 × *g* at 4 °C and completely remove the supernatant (*see* **Note 15**). Dry in air for 5–10 min until the pellet is turned to semitransparent.
12. Dissolve the precipitates in 50-μL water thoroughly by tapping (*see* **Note 16**).

3.7 End Repair and Adaptor Ligation (NEBNext Ultra II DNA Library Prep Kit)

1. Transfer the sample to a PCR tube. Add 7.0 μL of end repair buffer (*see* **Note 17**) and 3.0 μL of End prep enzyme. Mix thoroughly by pipetting 15–20 times.
2. Incubate at 20 °C for 30 min followed by 65 °C for 30 min.
3. Add 2.5 μL of NEB adaptor (1:12 diluted), 1.0 μL of ligation enhancer, and 30 μL of ligase master mix. Pipette the entire volume up and down 15–20 times to mix thoroughly.
4. Incubate at 20 °C for 2 h.
5. Add 3.0 μL of USER enzyme, and mix thoroughly by pipetting 15–20 times.
6. Incubate at 37 °C for 15 min.

3.8 Clean Up the Adaptor-Ligated DNA

1. Transfer the DNA sample to a new 1.5-mL LoBind tube containing ×1.8 (173.7 μL) of SPRI beads. Vortex thoroughly at maximum setting for 10–20 s, spin briefly, and leave it for 5 min at room temperature.
2. Place the tube on a magnetic stand for 5 min and remove the supernatant.
3. Add 180 μL of freshly prepared 80% ethanol and stand for >30 s. Then, remove the supernatant. Repeat three times.
4. Remove the supernatant completely (*see* **Note 18**); air-dry the beads for ~5 min (*see* **Note 19**). Add 25 μL of elution buffer (EB), and mix thoroughly by vortexing at maximum setting for 10 s.
5. Spin briefly and leave it for 5 min. Place the tube on a magnetic stand for 1 min and take the supernatant to a new PCR tube.

3.9 PCR Amplification and Library Purification

1. (Optional) Determine the PCR cycle used in the next step. Dilute 0.5 μL of the sample in 0.5-mL water (×1/1000 dilution). Keep the rest of the sample (~24.5 μL) on ice. Amplify with Illumina universal primer and Illumina indexed primer (any index is allowed) according to a standard qPCR protocol. Use 1 μL of the 1/1000 diluted sample as a template in a 10-μL reaction (e.g., DW 3.4 μL, primer mix 0.6 μL, template 1 μL, 2×Mix 5 μL). The optimal PCR cycle is [Ct value −11] (*see* **Note 20**).
2. Amplify the rest of the samples with indexed primers in a 52-μL reaction: Primer mix 2 μL, template 24.5 μL, and 2× KAPA HiFi Mix 26 μL. PCR condition; 95 °C, 2′; (98 °C, 20″; 60 °C, 30″; 72 °C, 60″); repeat for total [Ct value −11]; 72 °C, 5′; 12 °C forever.

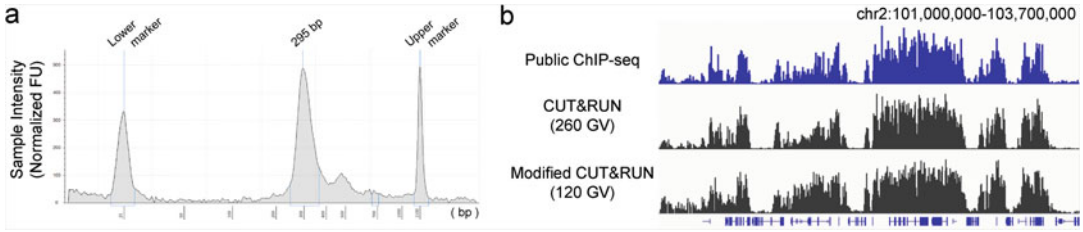

Fig. 3 Evaluation of the library quality. (**a**) An example of the size distribution of DNA fragments measured by Tapestation. (**b**) Genome browser view of H3K27me3 distribution in GV oocytes in ChIP-seq [6], a standard CUT&RUN method [5], and the modified CUT&RUN method

3. After PCR amplification, add ×0.9 (46.8 μL) of SPRI beads. Then, follow the same procedure as **steps 1–5** in Subheading 3.8. At last, elute with 20 μL of EB.
4. Check the library quality by Qubit and Tapestation (Fig. 3).

4 Notes

1. Dissolve digitonin powders in ultrapure water at 75 °C and make 20–50-μL aliquots. On the day of use, incubate at 75 °C for 15 min to dissolve it completely.
2. It is critical to find an antibody suitable for low-input CUT&RUN. As positive controls, we recommend anti-H3K4me3 (Active Motif, #39159) and anti-H3K27me3 (Diagenode; #C15410069) antibodies, which work well in less than 200 oocytes.
3. When using mouse-raised antibodies, pG- or pAG-MNase should be used. Commercial products of pAG-MNase are available (Cell Signaling Technology; #40366S or Epicypher; #15-1016).
4. When handling hundreds of oocytes by a glass capillary, make sure to minimize the carry-over amount of solution.
5. The presence of zona pellucida did not affect the results of H3K27me3 CUT&RUN in our trial. However, to remove polar bodies, ZP must be removed. Furthermore, ZP removal allows avoiding contamination of cumulus cells and sperm.
6. Wash samples carefully under a microscopy to make sure that no cumulus cells or polar bodies are contaminated. Also, keep environment around a microscopy clean to avoid contamination of DNA.
7. We recommend 1:100 dilution of antibody as a default. A lower concentration of antibody often yields lower amount of DNA recovered.

8. We recommend overnight at 4 °C in low-input CUT&RUN.
9. Dig-wash buffer should be prepared just before use. Occasionally, digitonin may be precipitated. This precipitate may interfere with the handling of the samples by glass capillary.
10. Minimize the carryover of buffer solutions. Make sure that no cell remains in the glass capillary.
11. Before the supernatant is discarded, observe it under a microscope to make sure there is no cell dropped from the ConA beads.
12. If the centrifugation is started after 18 h of incubation at −20 °C, the pellet is occasionally bigger than usual potentially due to salt precipitation, which causes low efficiency in library construction. Therefore, we do not leave the samples at −20 °C more than 18 h but start centrifugation on the morning.
13. Samples after replacement with 80% ethanol can be stored at −20 °C up to a week.
14. Prepare 80% ethanol freshly. Store at RT.
15. Be careful that the precipitates are fragile. Remove supernatant carefully. We use a 1-mL tip to remove most of the supernatant, and then use a fin tip to completely remove it.
16. We recommend doing the following end repair and adaptor ligation steps in a 50-μL scale according to the commercial instruction, because library construction occasionally fails if the scale is halved. This kind of failure at half usage is only seen in CUT&RUN, but not in other library constructions in our hands, for unknown reasons.
17. Thaw the buffer at 37 °C water bath particularly at the first use, and make sure that white small precipitates are completely dissolved.
18. We use a 1-mL tip to remove most of the supernatant, and then use a fin tip to completely remove it.
19. Dry until the bead clumps lose their luster and show tiny cracks.
20. The Ct value is expected to be less than 27. Over 27 often results in failure of the experiment. Such libraries will have many PCR duplicates and yield poor-quality data. In this case, increase the number of cells and/or change the antibody. Selection of good antibodies is important for low-input CUT&RUN.

Acknowledgments

We thank Dr. Steven Henikoff for providing the protein A-MNase and Drs. Weikun Xia and Wei Xie for helpful discussion on CUT&RUN. This project was partly supported by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) Leading Initiative for Excellent Young Researchers Grant to A.I., Japan Society for the Promotion of Science (JSPS) Grants-in-Aid for Scientific Research (KAKENHI) (18H02359 to A.I.), Grant-in-Aid for Scientific Research on Innovative Areas (19H05754 to A.I.), Grant-in-Aid for JSPS Fellows (20J21541 to R.H.), Japan Agency for Medical Research and Development PRIME (JP18gm6110012 and 21gm6310022h0001 to A.I.), Uehara Memorial Foundation to A.I., and RIKEN Pioneering Project "Genome Building from TADs" to A.I.

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