

Store at
4°C & -20°C

#86652

CUT&RUN Assay Kit

1 Kit
(8 or 24 assays)Support: +1-978-867-2388 (U.S.)
cellsignal.com/supportOrders: 877-616-2355 (U.S.)
orders@cellsignal.com

For Research Use Only. Not for Use in Diagnostic Procedures.

Components Shipped as 91931	Item #	Mini Kit (P) Quantity	Standard Kit (S) Quantity	Storage Temp
Concanavalin A Bead Activation Buffer	91275	1.7 mL	5 mL	4°C
Antibody Binding Buffer (CUT&RUN, CUT&Tag)	15338	800 µl	2.5 mL	4°C
10X Wash Buffer (CUT&RUN, CUT&Tag)	31415	4.5 mL	15 mL	4°C
CUT&RUN DNA Extraction Buffer	42015	3.6 mL	7 mL	4°C
Calcium Chloride	55676	25 µl	100 µl	4°C
Concanavalin A Magnetic Beads	82307	80 µl	240 µl	4°C

Components Shipped as 72917	Item #	Mini Kit (P) Quantity	Standard Kit (S) Quantity	Storage Temp
pAG-MNase Enzyme	57813	20 µl	40 µl	-20°C
Digitonin Solution	16359	1.2 mL	2 x 1.2 ml	-20°C
100X Spermidine	27287	400 µl	1.3 mL	-20°C
Sample Normalization Spike-In DNA (1 ng/µl)	36598	40 µl	120 µl	-20°C
Sample Normalization Primer Set	85756	64 µl	150 µl	-20°C
CUT&RUN 4X Stop Buffer	48105	300 µl	1 mL	-20°C
Protease Inhibitor Cocktail (200X)	7012	200 µl	750 µl	-20°C
Proteinase K (20 mg/ml)	10012	20 µl	100 µl	-20°C
RNase A (10 mg/ml)	7013	20 µl	50 µl	-20°C
Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb	9751	20 µl	20 µl	-20°C
Rabbit (DA1E) mAb IgG XP® Isotype Control (CUT&RUN)	66362	20 µl	100 µl	-20°C
SimpleChIP® Human RPL30 Exon 3 Primers	7014	50 µl	150 µl	-20°C
SimpleChIP® Mouse RPL30 Intron 2 Primers	7015	50 µl	150 µl	-20°C

Description: The CUT&RUN Assay Kit is designed to conveniently provide reagents needed to perform up to 8 (P size) or 24 (S size) digestion reactions from cells and is optimized for 5,000-100,000 cells per reaction. The kit has been optimized to work for all types of DNA binding proteins, including histones, transcription factors and cofactors. This kit has also been validated for fixed cells, primary cells, and tissues. A complete assay can be performed in as little as one day.

The CUT&RUN Assay Kit also provides important controls to ensure a successful CUT&RUN experiment. The kit contains a positive control Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751 and a negative control Rabbit (DA1E) mAb IgG XP® Isotype Control (CUT&RUN) #66362, both of which can be used for qPCR or Next Generation sequencing (NG-seq) analysis. PCR primer sets are provided for the human (#7014) and mouse (#7015) RPL30 gene locus to be used in conjunction with the control antibodies. Tri-methyl-histone H3 Lys4 is found at the active RPL30 gene promoter, so digestion targeted by this antibody will enrich for the RPL30 gene, while digestion targeted by the normal rabbit IgG mouse mAb will not enrich for the RPL30 gene. This kit is compatible with both qPCR and NG-seq.

Specificity/Sensitivity: The CUT&RUN Assay Kit can be utilized with any CUT&RUN-validated antibody to detect endogenous levels of protein-DNA interactions and histone modifications in mammalian cells and tissues (see Figures 1–5). The kit is compatible with multiple species of antibodies, including rabbit and mouse. The positive control Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751 detects multiple species of tri-methyl histone H3 Lys4 protein, including human, mouse, rat, and monkey. Primer sets are included for the human (#7014) and mouse (#7015) positive control RPL30 gene locus; however, the use of other species with the kit requires the design of additional control primer sets.

Storage: All components in this kit are stable for at least 12 months when stored at the recommended temperature.

Reagents not supplied:

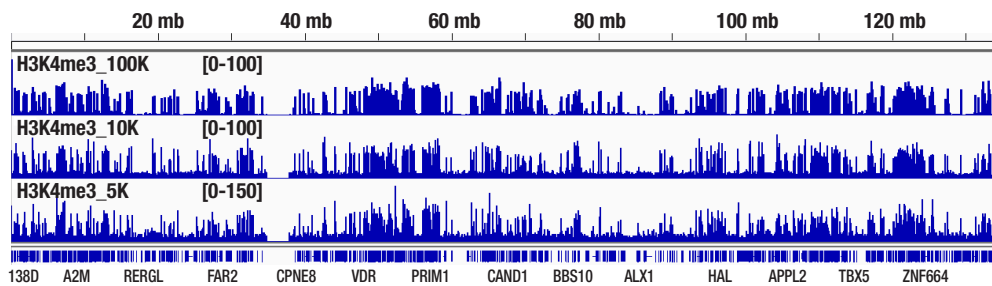
1. Nuclease-Free Water #12931
2. Trypan Blue Stain (0.4%)
3. DNA Purification Buffers and Spin Columns (ChIP, CUT&RUN, CUT&Tag) #14209 (Optional)
4. Phenol/chloroform/isoamyl alcohol (25:24:1) (Optional)
5. Chloroform/isoamyl alcohol (24:1) (Optional)
6. Glycogen (20 mg/ml) (Optional)
7. 3 M Sodium Acetate (Optional)
8. 100% Ethanol
9. 16% Formaldehyde, Methanol-Free #12606
10. SimpleChIP® Universal qPCR Master Mix #88989
11. DNA Library Prep Kit for Illumina® (ChIP-seq, CUT&RUN) #56795
12. Multiplex Oligos for Illumina® (ChIP-seq, CUT&RUN) #29580 or #47538
13. Safe-lock 1.5 ml tubes
14. Safe-lock 2 ml tubes
15. 10% SDS Solution #20533
16. Glycine Solution (10X) #7005
17. Phosphate Buffered Saline (PBS-1X) pH7.2 (Sterile) #9872

Please visit www.cst-science.com/CUT-RUN for more information about the benefits of CUT&RUN.

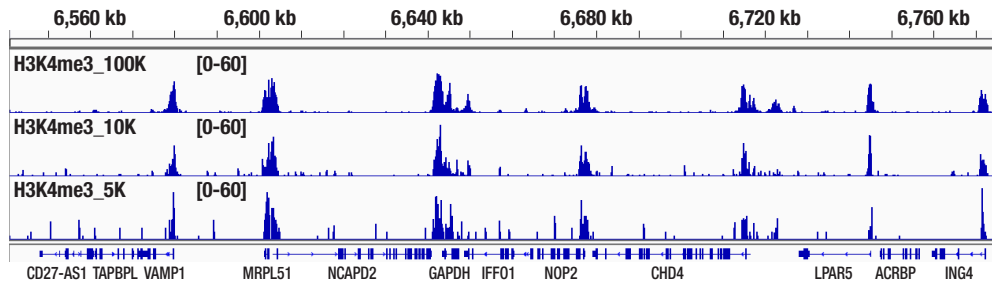
U.S. Patent No. 5,675,063.

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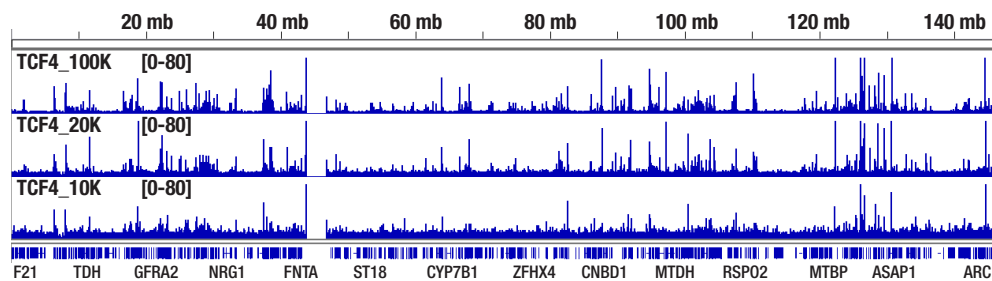
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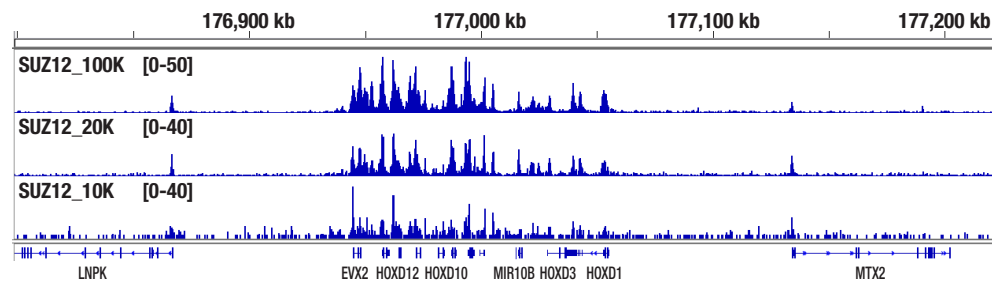
◀ Figure 1. CUT&RUN was performed with 100,000, 10,000, or 5,000 HCT 116 cells (as indicated) and Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751, using the CUT&RUN Assay Kit. DNA Libraries were prepared using DNA Library Prep Kit for Illumina® (ChIP-seq, CUT&RUN) #56795. This figure shows binding of H3K4me3 across chromosome 12.



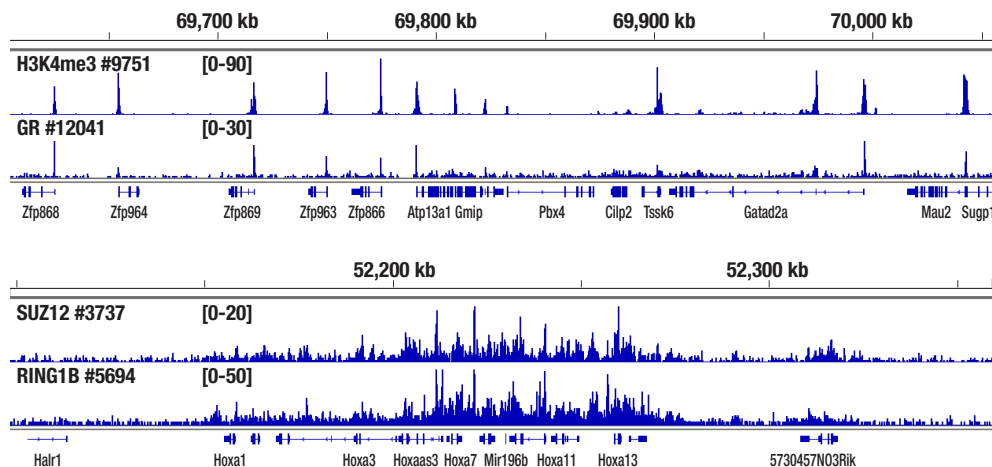
◀ Figure 2. CUT&RUN was performed with 100,000, 10,000, or 5,000 HCT 116 cells (as indicated) and Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751, using the CUT&RUN Assay Kit. DNA Libraries were prepared using DNA Library Prep Kit for Illumina® (ChIP-seq, CUT&RUN) #56795. This figure shows enrichment around the GAPDH gene, a known target of H3K4me3.



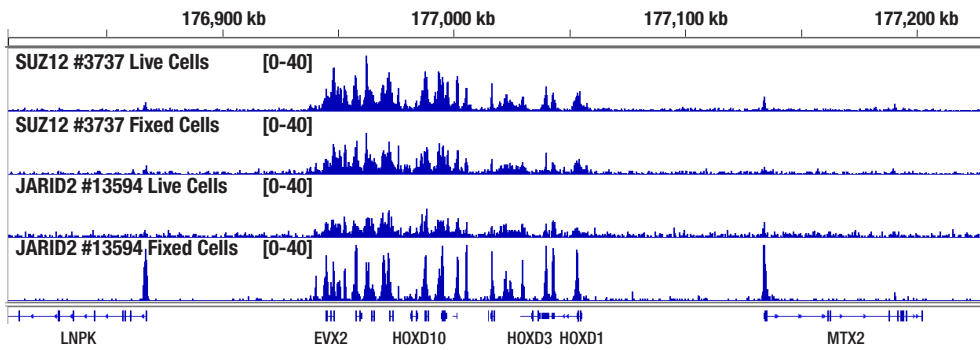
◀ Figure 3. CUT&RUN was performed with 100,000, 20,000, or 10,000 HCT 116 cells (as indicated) and TCF4/TCF7L2 (C48H11) Rabbit mAb #2569, using the CUT&RUN Assay Kit. DNA Libraries were prepared using DNA Library Prep Kit for Illumina® (ChIP-seq, CUT&RUN) #56795. This figure shows binding of TCF4/TCF7L2 across chromosome 8.



◀ Figure 4. CUT&RUN was performed with 100,000, 20,000, or 10,000 NCCIT cells (as indicated) and SUZ12 (D39F6) XP® Rabbit mAb #3737, using the CUT&RUN Assay Kit. DNA Libraries were prepared using DNA Library Prep Kit for Illumina® (ChIP-seq, CUT&RUN) #56795. The figure shows binding across the Hoxd gene cluster, a known target of SUZ12.



◀ Figure 5. CUT&RUN was performed with 2.5 mg of medium-fixed mouse liver tissue (0.1% formaldehyde, 10 min) and Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751, Glucocorticoid Receptor (D6H2L) XP® Rabbit mAb #12041, SUZ12 (D39F6) XP® Rabbit mAb #3737, or RING1B (D22F2) XP® Rabbit mAb #5694, using the CUT&RUN Assay Kit. DNA Libraries were prepared using DNA Library Prep Kit for Illumina® (ChIP-seq, CUT&RUN) #56795. The upper panel shows binding of H3K4me3 and Glucocorticoid Receptor around the Zfp866 gene, while the lower panel shows binding of SUZ12 and RING1B across the HoxA gene cluster.



◀ Figure 6. CUT&RUN was performed with 100,000 live or fixed NCCIT cells and SUZ12 (D39F6) XP[®] Rabbit mAb #3737 or JARID2 (D6M9X) Rabbit mAb #13594, using the CUT&RUN Assay Kit. DNA Libraries were prepared using DNA Library Prep Kit for Illumina[®] (ChIP-seq, CUT&RUN) #56795. The figure shows binding of SUZ12 and JARID2 across the HOXD gene cluster. Fixation is not required for SUZ12 (a core component of PRC2 complex) but increases the enrichment for JARID2 (an accessory component of PRC2 complex).

CUT&RUN Intro and Method Overview

Introduction:

Like the chromatin immunoprecipitation (ChIP) assay, Cleavage Under Targets & Release Using Nuclease (CUT&RUN) is a powerful and versatile technique used for probing protein-DNA interactions within the natural chromatin context of the cell (1-4). This assay can be used to identify multiple proteins associated with a specific region of the genome, or the opposite, to identify the many regions of the genome associated with a particular protein. In addition, the CUT&RUN assay can be used to define the spatial and temporal relationship of a particular protein-DNA interaction. For example, the CUT&RUN assay can be used to determine the specific order of recruitment of various protein factors to a gene promoter or to “measure” the relative amount of a particular histone modification across an entire gene locus during gene activation. In addition to histone proteins, the CUT&RUN assay can also be used to analyze binding of transcription factors and cofactors, DNA replication factors, and DNA repair proteins (Figures 1-5).

CUT&RUN provides a rapid, robust, and true low cell number assay for detection of protein:DNA interactions in the cell. Unlike the ChIP assay, CUT&RUN is free from formaldehyde cross-linking, chromatin fragmentation, and immunoprecipitation, making it a much faster and more efficient method for enriching protein:DNA interactions and identifying target genes. CUT&RUN can be performed in less than one day, from cells or tissue to purified DNA, and has been shown to work with as few as 5,000-10,000 cells per assay (Figures 1-4). Instead of fragmenting all of the cellular chromatin as done in ChIP, CUT&RUN utilizes an antibody-targeted digestion of chromatin, resulting in much lower background signal than seen in the ChIP assay. As a result, CUT&RUN requires only 1/10th the sequencing depth that is required for ChIP-seq assays (1,2). Finally, the inclusion of simple spike-in control DNA allows for accurate quantification and normalization of target-protein binding that is not possible with the ChIP method. This provides for effective normalization of signal between samples and between experiments.

Background References:

1. Skene, J.S. and Henikoff, S. (2017) eLIFE 6, e21856.
2. Skene, J.S. et al. (2018) Nature Protocols 13, 1006-1019.
3. Meers, M.P. et al. (2019) eLIFE 8, e46314.
4. Meers, M.P. et al. (2019) Molecular Cell 75, 1-14.

Method Overview:

Ab & pAG-MNase binding:

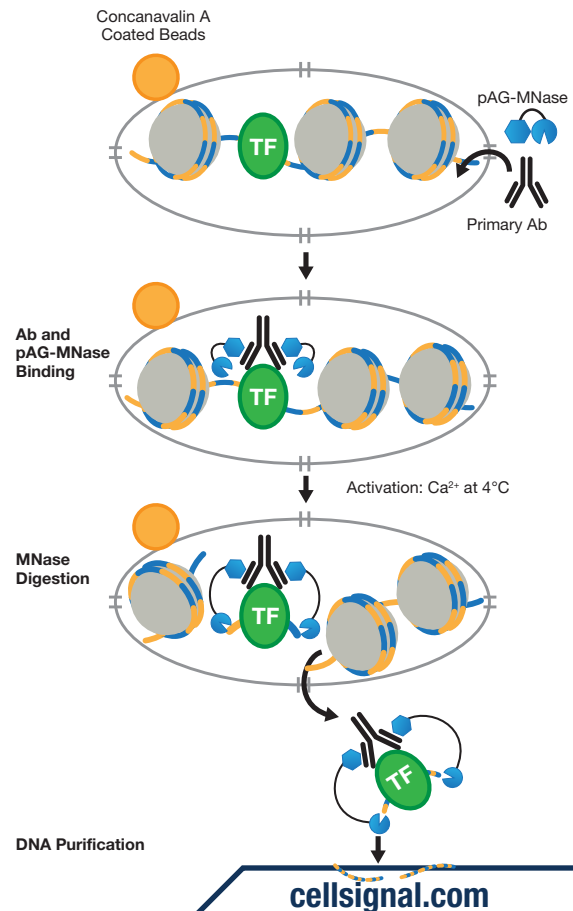
1. Either live or fixed cells are immobilized on Concanavalin A Magnetic Beads to allow for subsequent buffer and reagent exchanges.
2. Cell membranes are then permeabilized with digitonin to facilitate the entry of primary antibody and pAG-MNase fusion enzyme into the cell nuclei.
3. The target-specific primary antibody recruits the pAG-MNase to the chromatin through protein-protein interactions between the antibody and the pAG domain of the fusion enzyme.

MNase Digestion:

4. The addition of Ca²⁺ activates the pAG-MNase, which gently cleaves and liberates the desired chromatin fragments, allowing them to diffuse away from the genomic chromatin, out of the cell, and into the supernatant.

DNA Purification:

5. DNA is purified using DNA purification spin columns or phenol/chloroform extraction followed by ethanol precipitation. The purified, enriched DNA is then identified and quantified using qPCR or NG-seq.



CUT&RUN Protocol

!	This ! signifies an important step in the protocol regarding volume changes based on the number of CUT&RUN reactions being performed.
!!	This !! signifies an important step to dilute a buffer before proceeding.
SAFE STOP	This is a safe stopping point in the protocol, if stopping is necessary.

I. Cell and Tissue Preparation

For most cell types, live cells can be used in the CUT&RUN assay to generate robust enrichment of histones, transcription factors, and cofactors. For certain cell types that are fragile or sensitive to Concanavalin A, a light cell fixation helps to preserve the cells and keep them intact. In addition, fixation may help to boost enrichment of low abundance and/or weak binding transcription factors and cofactors if robust signal is not observed using fresh cells. Please note that over-fixation of cells will inhibit the CUT&RUN assay.

Our CUT&RUN assay works with a wide range of cell or tissue inputs. As defined in the protocol, one CUT&RUN reaction can contain between 5,000 to 250,000 cells or 1 to 5 mg of tissue. Buffer volumes used throughout the protocol do not need to be adjusted based on the amount of cells or tissue per reaction, as long as it is within this range. When indicated, buffer volumes do need to be increased proportionally based on the number of reactions being performed. If possible, we recommend using 100,000 cells or 1 mg of tissue per reaction. If cells are limiting, we recommend using at least 5,000 to 10,000 cells per reaction for histone modifications and 10,000 to 20,000 cells per reaction for transcription factors or cofactors.

NOTE: The amount of digitonin recommended for cell permeabilization is in excess and should be sufficient for permeabilization of most cell lines and tissue types. However, not all cell lines and tissues exhibit the same sensitivity to digitonin. If your specific cell line or tissue does not work with the recommended digitonin concentration, you can optimize conditions by following the protocol provided in Appendix A. Digitonin treatment should result in permeabilization of >90% of the cell population.

A. Live Cell Preparation

Before Starting:

! All buffer volumes should be increased proportionally based on the number of CUT&RUN reactions being performed.

- Remove and warm 200X Protease Inhibitor Cocktail #7012 and 100X Spermidine #27287. Make sure both are completely thawed. Please note that the Protease Inhibitor Cocktail #7012 will refreeze when placed on ice due to containing DMSO.
- Prepare 1X Wash Buffer (2 mL for each cell line and additional 100 µL for each reaction or input sample). For example, to prepare 2.5 mL of 1X Wash Buffer, add 250 µL 10X Wash Buffer #31415 + 25 µL 100X Spermidine #27287 + 12.5 µL 200X Protease Inhibitor Cocktail #7012 + 2212.5 µL Nuclease-free Water #12931. Equilibrate it to room temperature to minimize stress on the cells.

NOTE: Steps for live cell (no fixation) preparation should be performed in succession at room temperature to minimize stress on the cells. To minimize DNA fragmentation, avoid vigorous vortexing and cavitation of samples during resuspension. When preparing live cells for CUT&RUN, we recommend preparing the Concanavalin A Beads (Section II, Steps 1 to 5) prior to preparing the cells as to minimize the amount of time the cells sit around during bead preparation. Activated beads can be stored on ice until ready to use.

- Harvest fresh cell cultures at room temperature to minimize stress on the cells. Collect 5,000 to 100,000 cells for each reaction and an additional 5,000 to 100,000 cells for the input sample. Be sure to include reactions for the positive control Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751 and the negative control Rabbit (DA1E) mAb IgG XP® Isotype Control (CUT&RUN) #66362.

NOTE: For adherent cells, the cells first need to be detached from the dish using Trypsin and neutralized with at least 3 volumes of tissue culture medium. We do not recommend scraping the cells from the dish because this can stress and even lyse the cells. Cells should be counted using a hemocytometer or other cell counter to ensure the proper number of cells are being used for the experiment.

- Centrifuge cell suspension for 3 min at 600 x g at room temperature and remove the liquid.

NOTE: The challenge of working with low cell numbers (<100,000 total cells) is that the centrifuged cell pellet is not always visible by eye, making it easy to lose cells during the wash steps. Therefore, when working with low cell numbers, we recommend skipping the wash steps 3 to 5 below. The binding of the Concanavalin A beads to cells is tolerant to having 40% cell medium in the binding reaction. Therefore, after the initial centrifugation of the cell suspension in Step 2, one can remove most of the supernatant, leaving behind ≤40 µL cell medium per reaction. Then in Step 6 add enough 1X Wash Buffer (+ spermidine + PIC) to the cell suspension to achieve a total volume of 100 µL per reaction.
- Resuspend cell pellet in 1 mL of 1X Wash Buffer (+ spermidine + PIC) at room temperature by gently pipetting up and down.
- Centrifuge for 3 min at 600 x g at room temperature and remove the liquid.
- Wash the cell pellet a second time by repeating steps 3 and 4 one time.
- For each reaction or input sample, add 100 µL of 1X Wash Buffer (+ spermidine + PIC) and resuspend the cell pellet by gently pipetting up and down.
- Transfer 100 µL of cells to a new tube and store at 4°C until Section V. This is the input sample.

NOTE: The input sample will be incubated at 55°C later in the protocol, so it is recommended to use a safe-lock 1.5 mL tube to reduce evaporation during the incubation.
- Immediately proceed to Section II.

B. Fixed Cell Preparation

NOTE: The following reagents are required for fixed cell preparation and are not included in this kit: 37% formaldehyde or 16% Formaldehyde Methanol-Free #12606, Glycine Solution (10X) #7005, and 10% SDS Solution #20533.

Before Starting:

! All buffer volumes should be increased proportionally based on the number of CUT&RUN reactions being performed.

- Remove and warm 200X Protease Inhibitor Cocktail #7012 and 100X Spermidine #27287. Make sure both are completely thawed. Please note that the Protease Inhibitor Cocktail #7012 will refreeze when placed on ice due to containing DMSO.
- Prepare 1X Wash Buffer (2 mL for each cell line and additional 100 µL for each reaction or input sample). For example, to prepare 2.5 mL of 1X Wash Buffer, add 250 µL 10X Wash Buffer #31415 + 25 µL 100X Spermidine #27287 + 12.5 µL 200X Protease Inhibitor Cocktail #7012 + 2212.5 µL Nuclease-free Water #12931. Equilibrate it to room temperature to minimize stress on the cells.
- Prepare 2.7 µL of 37% formaldehyde or 6.25 µL of 16% Formaldehyde Methanol-Free #12606 per 1 mL of cell suspension to be processed and keep at room temperature. Use fresh formaldehyde that is not past the manufacturer's expiration date.

1. Collect 5,000 to 100,000 cells for each antibody/MNase reaction and an additional 5,000 to 100,000 cells for the input sample. Be sure to include reactions for the positive control Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751 and the negative control Rabbit (DA1E) mAb IgG XP[®] Isotype Control (CUT&RUN) #66362.

NOTE: With adherent cell lines, cells first need to be detached from the dish using Trypsin and neutralized with at least 3 volumes of medium. We don't recommend scraping the cells from the dish because this can stress and even lyse the cells. Cells should be counted using a hemocytometer or other cell counter to ensure the proper number of cells are being used for the experiment.

2. Add 2.7 μ L 37% formaldehyde or 6.25 μ L 16% Formaldehyde Methanol-Free #12606 per 1 mL of cell suspension to achieve a final concentration of 0.1% formaldehyde. Swirl tube to mix and incubate at room temperature for 2 min.
3. Stop cross-linking by adding 100 μ L of Glycine Solution (10X) #7005 per 1 mL of fixed cell suspension. Swirl the tube to mix and incubate at room temperature for 5 min.
4. Centrifuge cell suspension for 3 min at 3,000 x g at 4°C and remove the liquid. Immediately proceed to Step 5. (**SAFE STOP**) Alternatively, fixed cell pellets may be stored at -80°C before using for up to 6 months.

NOTE: The challenge of working with low cell numbers (<100,000 total cells) is that the centrifuged cell pellet is not always visible by eye, making it easy to lose cells during the wash steps. In this case we do NOT recommend freezing down cell pellets. In addition, when working with these low cell numbers, we recommend skipping the wash steps 5 to 7 below. The binding of the Concanavalin A beads to cells is tolerant to having 40% cell medium in the binding reaction. Therefore, after the initial centrifugation of the cell suspension in Step 4, one can remove most of the supernatant, leaving behind \leq 40 μ L cell medium per reaction. Then in Step 8 add enough 1X Wash Buffer (+ spermidine + PIC) to the cell suspension to achieve a total volume of 100 μ L per reaction.

5. Resuspend cell pellet in 1 mL of 1X Wash Buffer (+ spermidine + PIC) by gently pipetting up and down.
6. Centrifuge for 3 min at 3,000 x g at 4°C and remove the liquid.
7. Wash the cell pellet a second time by repeating steps 5 and 6 one time.
8. For each reaction or input sample, add 100 μ L of 1X Wash Buffer (+ spermidine + PIC) and resuspend the cell pellet by gently pipetting up and down.
9. Transfer 100 μ L of cells to a new tube and store at 4°C until Section V. This is the input sample.

NOTE: The input sample will be incubated at 55°C later in the protocol, so it is recommended to use a safe-lock 1.5 mL tube to reduce evaporation during the incubation.

10. Immediately proceed to Section II.

C. Tissue Sample Preparation

For most tissue types, 1 mg of lightly fixed tissue (0.1% formaldehyde for 2 min) can generate robust enrichment of histones, transcription factors and cofactors. Formaldehyde fixation is not required for enrichment of histone modifications. However, many transcription factors and cofactors do require light fixation of the tissue for optimal results. Some low abundance and/or weak binding transcription factors and cofactors may require a medium fixation (0.1% formaldehyde for 10 min) for optimal results. In addition, medium fixation may improve results when using difficult tissue types, like fibrous tissues. Please note that over-fixation will inhibit the CUT&RUN assay. Fixed tissue samples can be frozen at -80°C for up to 6 months before using.

NOTE: When preparing fresh tissue (no fixation) for CUT&RUN, we recommend preparing the Concanavalin A Beads (Section II, Steps 1 to 5) prior to preparing the tissue as to minimize the amount of time the cells sit around during bead preparation. Activated beads can be stored on ice until ready to use.

NOTE: The following reagents are required for fixed tissue preparation and are not included in this kit: 37% formaldehyde or 16% Formaldehyde Methanol-Free #12606, Phosphate Buffered Saline (PBS) #9872, Glycine Solution (10X) #7005, and 10% SDS Solution #20533.

Before Starting:

! All buffer volumes should be increased proportionally based on the number of CUT&RUN reactions being performed.

- Remove and warm 200X Protease Inhibitor Cocktail #7012 and 100X Spermidine #27287. Make sure both are completely thawed. Please note that the Protease Inhibitor Cocktail #7012 will refreeze when placed on ice due to containing DMSO.
- Prepare 1X Wash Buffer (3 mL for each tissue type and additional 100 μ L for each reaction or input sample). For example, to prepare 3.5 mL of 1X Wash Buffer, add 350 μ L 10X Wash Buffer #31415 + 35 μ L 100X Spermidine #27287 + 17.5 μ L 200X Protease Inhibitor Cocktail #7012 + 3097.5 μ L Nuclease-free Water #12931. Equilibrate it to room temperature to minimize stress on the cells.
- Prepare the following buffers if tissue fixation is needed:
 - ◊ Prepare 1 mL fixation buffer for each tissue type by adding 2.7 μ L of 37% formaldehyde or 6.25 μ L of 16% Formaldehyde Methanol-Free #12606 and 5 μ L 200X Protease Inhibitor Cocktail (PIC) #7012 into 1 mL of Phosphate Buffered Saline (PBS) #9872. Use fresh formaldehyde that is not past the manufacturer's expiration date.
 - ◊ Prepare 1 mL of PBS #9872 + 5 μ L Protease Inhibitor Cocktail (PIC) #7012 for each tissue type and place on ice.
 - ◊ Prepare 100 μ L of Glycine Solution (10X) #7005 per 1 mL of fixation buffer.

1. Weigh 1 mg fresh tissues for each antibody/MNase reaction and an additional 1 mg of tissue for the input sample. Be sure to include reactions for the positive control Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751 and the negative control Rabbit (DA1E) mAb IgG XP[®] Isotype Control (CUT&RUN) #66362.

NOTE: For some transcription factors or cofactors, or for difficult tissue types like fibrous tissues, up to 5 mg tissue per reaction can be used without scaling up reagents.

2. Place tissue sample in a dish and finely mince using a clean scalpel or razor blade. Keep dish on ice. It is important to keep the tissue cold to avoid protein degradation.

NOTE: We recommend light fixation of tissues because this condition works optimally for most tissue types and protein targets. However, if fresh tissues are desired, skip Steps 3 to 8 and immediately proceed to Step 9.

3. Immediately transfer minced tissue to 1 mL of fixation solution and swirl tube to mix.

NOTE: This volume of fixation solution is sufficient for up to 50 mg of tissue. If processing >50 mg, scale up fixation solution and 1X PBS + PIC solution in Step 7 accordingly.

4. Incubate at room temperature for 2 min.

NOTE: For difficult tissue types (like fibrous tissues) or low abundance and/or weak binding transcription factors or cofactors, extending the formaldehyde fixation to 10 min may improve results.

5. Stop cross-linking by adding 100 μ L of Glycine Solution (10X) #7005 per 1 mL of fixation buffer. Swirl the tube to mix and incubate at room temperature for 5 min.

6. Centrifuge tissue for 5 min at 2,000 x g at 4°C and remove the liquid.

7. Resuspend tissue with 1 mL of 1X PBS + PIC.

8. Centrifuge for 5 min at 2,000 x g at 4°C and remove the liquid and proceed to step 9. (**SAFE STOP**) Alternatively, fixed tissue pellets may be stored at -80°C before disaggregation for up to 6 months.

9. Resuspend tissue in 1 mL of 1X Wash Buffer (+ spermidine + PIC) and transfer the sample to a Dounce homogenizer.

10. Disaggregate tissue pieces into single-cell suspension with 20-25 strokes until no tissue chunks are observed.

11. Transfer cell suspension to a 1.5 mL tube and centrifuge at 3,000 x g for 3 min at room temperature, remove supernatant from cells.

12. Resuspend cell pellet in 1 mL of 1X Wash Buffer (+ spermidine + PIC).

13. Centrifuge cell suspension for 3 min at 3,000 x g at room temperature and remove the liquid.

14. Wash the cell pellet a second time by repeating steps 12 and 13 one time.

15. For each reaction, add 100 μ L of 1X Wash Buffer (+ spermidine + PIC) and resuspend the cell pellet by gently pipetting up and down.

16. Transfer 100 μ L of cells to a new tube and store at 4°C until Section V. This is the input sample.

NOTE: The input sample will be incubated at 55°C later in the protocol, so it is recommended to use a safe-lock 1.5 mL tube to reduce evaporation during the incubation.

17. Immediately proceed to Section II.

II. Binding of Concanavalin A Beads and Primary Antibody

Before Starting:

! All buffer volumes should be increased proportionally based on the number of CUT&RUN reactions being performed.

- Remove and warm Digitonin Solution #16359 to 90-100°C for 5 min and make sure it is completely thawed and in solution. Immediately place the thawed Digitonin Solution #16359 on ice.

NOTE: Digitonin Solution #16359 should be stored at -20°C. Please keep on ice during use and store at -20°C when finished for the day.

- Remove and warm 200X Protease Inhibitor Cocktail #7012 and 100X Spermidine #27287. Make sure both are completely thawed. Please note that the Protease Inhibitor Cocktail #7012 will refreeze when placed on ice due to containing DMSO.
- Place Concanavalin A Bead Activation Buffer on ice.
- For each reaction, prepare 1 μ L 100X Spermidine #27287 + 0.5 μ L 200X Protease Inhibitor Cocktail #7012 + 2.5 μ L Digitonin Solution #16359 + 96 μ L Antibody Binding Buffer #15338 and place on ice (100 μ L per reaction).

1. Carefully resuspend Concanavalin A Magnetic Beads by gently pipetting up and down, making sure not to splash any bead suspension out of the tube. Transfer 10 μ L of the bead suspension per each CUT&RUN reaction to a new 1.5 mL microcentrifuge tube.

NOTE: Avoid vortexing the Concanavalin A Magnetic Bead suspension as repeated vortexing may displace the Concanavalin A from the beads.

2. Add 100 μ L Concanavalin A Bead Activation Buffer per 10 μ L beads. Gently mix beads by pipetting up and down.

3. Place tube on a magnetic rack until solution becomes clear (30 sec to 2 min) and then remove the liquid.

NOTE: To avoid loss of beads, remove liquid using a pipet. Do not aspirate using a vacuum.

4. Remove tubes from the magnetic rack. Wash the beads a second time by repeating steps 2 and 3 one time.

5. Add a volume of Concanavalin A Bead Activation Buffer equal to the initial volume of bead suspension added (10 μ L per sample) and resuspend by pipetting up and down.

NOTE: If Concanavalin A Beads are prepared prior to cell or tissue preparation, as recommended for live cells and fresh tissue, the activated beads can be stored on ice until use.

6. Make sure Concanavalin A Beads are mixed well into solution. Add 10 μ L of activated bead suspension per reaction to the washed cell suspension prepared in Section I-A Step 8, Section I-B Step 10, or Section I-C Step 17.

7. Mix the samples well by pipetting up and down. Incubate for 5 min at room temperature.

NOTE: Concanavalin A Magnetic Beads may clump or stick to the sides of the tube. Beads can be resuspended by pipetting up and down. Rocking or shaking of sample tubes is not necessary.

8. Place the tube on the magnetic rack until the solution turns clear (30 sec to 2 min), then remove and discard the liquid.

9. Remove tube from the stand. Add 100 μ L of Antibody Binding Buffer (+ spermidine + PIC + digitonin) per reaction and place on ice.

10. Aliquot 100 μ L of the cell:bead suspension into separate 1.5 mL tubes for each reaction and place on ice.

11. Add the appropriate amount of antibody to each reaction and mix gently by pipetting up and down.

NOTE: The amount of antibody required for CUT&RUN varies and should be determined by the user. For the positive control Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751, add 2 μ L of antibody to the sample. For the negative control Rabbit (DA1E) mAb IgG XP® Isotype Control (CUT&RUN) #66362, add 5 μ L to the sample. We strongly recommend using the negative

control antibody and NOT a no-antibody control, because the latter results in high levels of non-specific MNase digestion and high background signal. We recommend using the input sample for comparison with both qPCR and NG-seq analysis, when possible.

12. Incubate tubes at 4°C for 2 hr. This step can be extended to overnight.

NOTE: Concanavalin A Magnetic Beads may clump or stick to the sides of the tube. Beads can be resuspended by pipetting up and down. Rocking or shaking of sample tubes is not necessary.

III. Binding of pAG-MNase Enzyme

Before Starting:

! All buffer volumes should be increased proportionally based on the number of CUT&RUN reactions being performed.

- Remove and warm Digitonin Solution #16359 to 90-100°C for 5 min and make sure it is completely thawed and in solution. Immediately place the thawed Digitonin Solution #16359 on ice.

NOTE: Digitonin Solution #16359 should be stored at -20°C. Please keep on ice during use and store at -20°C when finished for the day.

- Remove and warm 200X Protease Inhibitor Cocktail #7012 and 100X Spermidine #27287. Make sure both are completely thawed. Please note that the Protease Inhibitor Cocktail #7012 will refreeze when placed on ice due to containing DMSO.

- For each reaction, prepare 3.2 mL of Digitonin Buffer (320 μ L 10X Wash Buffer #31415 + 32 μ L 100X Spermidine #27287 + 16 μ L 200X Protease Inhibitor Cocktail #7012 + 80 μ L Digitonin Solution #16359 + 2.752 mL Nuclease-free Water #12931).

NOTE: The Digitonin Buffer prepared here will be used for both Section III and IV.

- For each reaction, make a pAG-MNase pre-mix by adding 50 μ L of Digitonin Buffer (described above) and 1.5 μ L of pAG-MNase Enzyme to a new tube. For example, for 10 reactions, transfer 500 μ L of Digitonin Buffer to a new tube and add 15 μ L of pAG-MNase Enzyme. Mix by gently pipetting up and down and place on ice.

1. Place the tubes from Section II, Step 12 on the magnetic rack until the solution turns clear (30 sec to 2 min) and then remove the liquid.

2. Remove tubes from the magnetic rack and add 1 mL of Digitonin Buffer (+ spermidine + PIC + digitonin). Resuspend beads by gently pipetting up and down, make sure to collect all beads that are stuck to the tube wall.

3. Place the tubes on the magnetic rack until the solution turns clear (30 sec to 2 min) and then remove the liquid.

4. Remove tubes from magnetic rack. Add 50 μ L of pAG-MNase pre-mix to each tube and gently mix the sample by pipetting up and down.

5. Incubate tubes at 4°C for 1 hr.

NOTE: Concanavalin A Magnetic Beads may clump or stick to the sides of the tube. Beads can be resuspended by pipetting up and down. Rocking or shaking of sample tubes is not necessary.

6. Immediately proceed to Section IV.

IV. DNA Digestion and Diffusion

Before Starting:

! All buffer volumes should be increased proportionally based on the number of CUT&RUN reactions being performed.

- Remove and warm Digitonin Solution #16359 to 90-100°C for 5 min and make sure it is completely thawed and in solution. Immediately place the thawed Digitonin Solution #16359 on ice.

NOTE: Digitonin Solution #16359 should be stored at -20°C. Please keep on ice during use and store at -20°C when finished for the day.

- Place Calcium Chloride on ice.

- If starting with fixed materials in Section I, make sure the 10% SDS Solution #20533 is completely in solution. Warming it up to 37°C will help to dissolve the SDS precipitates.

- For each reaction, prepare 150 μ L of 1X Stop Buffer (37.5 μ L 4X Stop Buffer #48105 + 3.75 μ L Digitonin Solution #16359 + 0.75 μ L RNase A #7013 + 108 μ L Nuclease-free Water #12931).

Optional: Sample Normalization Spike-In DNA can be added into the 1X Stop Buffer if sample normalization is desired (for example, see Figure 8 in Section VII). For qPCR analysis, we recommend adding 5 μ L (5 ng) of Spike-In DNA to each reaction. For NG-seq analysis, we recommend diluting the Sample Normalization Spike-In DNA 100-fold into Nuclease-free Water #12931 and then adding 5 μ L (50 pg) of Spike-In DNA to each reaction. When using 100,000 cells or 1 mg of tissue per reaction this ensures that the normalization reads are around 0.5% of the total sequencing reads. If more or less than 100,000 cells or 1 mg of tissue are used per reaction, proportionally scale the volume of Sample Normalization Spike-In DNA up or down to adjust normalization reads to around 0.5% of total reads.

- Place the tubes from Section III, Step 6 on the magnetic separation rack until the solution turns clear (30 sec to 2 min) and then remove the liquid.
- Remove tubes from the magnetic separation rack. Add 1 mL of Digitonin Buffer (+ spermidine + PIC + digitonin) prepared in Section III and resuspend beads by gently pipetting up and down.
- Place the tubes on the magnetic rack until the solution turns clear (30 sec to 2 min) and then remove the liquid.
- Repeat steps 2 and 3 one time.
- Remove tubes from magnetic rack. Add 150 μ L of Digitonin Buffer (+ spermidine + PIC + digitonin) prepared in Section III to each tube and mix by pipetting up and down.
- Place tubes on ice for 5 min to cool before digestion.
- Activate pAG-MNase by adding 3 μ L cold Calcium Chloride to each tube and mix by pipetting up and down.
- Incubate samples at 4°C for 30 min.

NOTE: Digestion should be performed in a 4°C cooling block or refrigerator. The temperature of ice can get as low as 0°C, which can limit digestion and decrease signal. Rocking or shaking of sample tubes is not necessary.

NOTE: Fixed samples will be incubated at 65°C later in the protocol, so it is recommended to use a safe-lock 2 mL tube to reduce evaporation during the incubation.

- Add 150 μ L of 1X Stop Buffer (+ digitonin + RNase A + spike-in DNA [optional]) to each sample and mix by pipetting up and down. Rocking or shaking of sample tubes is not necessary.
 - Incubate the tubes at 37°C for 10 min without shaking to release DNA fragments into the solution.
 - Centrifuge at 4°C for 2 min at 16,000x g and place the tubes on a magnetic rack until the solution is clear (30 sec to 2 min).
 - Transfer the supernatants to new 2 mL microcentrifuge tubes. These are your enriched chromatin samples.
- NOTE:** If live cells or fresh tissues (not fixed) are used for the CUT&RUN assay, skip Steps 13-14 and immediately proceed to Step 15.
- NOTE:** SDS may precipitate out of solution if samples are not pre-warmed to room temperature.
- To reverse the crosslinks in fixed cell or tissue samples, allow samples to warm to room temperature and add 3 μ L of 10% SDS Solution #20533 (0.1% final concentration) and 2 μ L of proteinase K (20 mg/mL) #10012 to each sample.
 - Vortex each sample and incubate at 65°C for at least 2 hr. This incubation can be extended overnight. After incubation, quickly spin samples at 10,000 x g for 1 sec to collect evaporation from the cap of tubes.
 - Equilibrate samples to room temperature and proceed to Section VI. (**SAFE STOP**) Alternatively, samples can be stored at -20°C for up to 1 week. However, be sure to warm samples to room temperature before DNA purification (Section VI).

V. Preparation of the Input Sample

Fragmentation of input DNA is required for compatibility with downstream NG-Sequencing but is not necessary for downstream qPCR analysis. If a sonicator is not available, we recommend using the unfragmented input DNA for qPCR analysis; however, the input DNA should be purified using phenol/chloroform extraction and ethanol precipitation because the size of unfragmented input DNA is too large to be purified using DNA spin columns. If a sonicator is not available and downstream NG-Sequencing analysis is desired, one can use the CUT&RUN normal IgG antibody sample as the negative control, although this is not ideal because the normal IgG-enriched sample may show non-specific DNA enrichment. Alternatively, an input DNA fragmentation protocol using MNase is available at <https://cst-science.com/CUT-RUN-input-digestion>.

! All buffer volumes should be increased proportionally based on the number of input samples being prepared.

Before Starting:

- Remove and warm DNA Extraction Buffer #42015. Make sure it is completely thawed.
 - For each input sample, prepare 2 μ L Proteinase K #10012 + 0.5 μ L RNase A #7013 + 197.5 μ L DNA Extraction Buffer #42015 (200 μ L total per input sample).
- Add 200 μ L of DNA Extraction Buffer (+ Proteinase K + RNase A) to the 100 μ L input sample from Section I-A Step7, Section I-B Step 9, or Section I-C Step 16. Mix by pipetting up and down.
 - Place the tube at 55°C for 1 hr with shaking.
 - Place the tubes on ice for 5 min to completely cool the sample.
 - Lyse the cells and fragment the chromatin by sonicating the input samples. Incubate samples on ice for 30 seconds between pulses.

NOTE: Sonication conditions may need to be determined empirically by testing different sonicator power settings and/or durations of sonication, following the protocol in Appendix B. Optimal sonication conditions will generate chromatin fragments ranging in size from 100-600 bp. Sonication for 5 sets of 15-sec pulses using a VirTis Virsonic 100 Ultrasonic Homogenizer/Sonicator at setting 6 with a 1/8-inch probe sufficiently fragments the input chromatin.

- Clarify lysates by centrifugation at 18,500 x g for 10 min at 4°C. Transfer supernatant to a new 2 mL microcentrifuge tube.
- Immediately proceed to Section VI DNA Purification. (**SAFE STOP**) Alternatively, samples can be stored at -20°C for up to 1 week. However, be sure to warm samples to room temperature before DNA purification procedures (Section VI).

VI. DNA Purification

DNA can be purified from input and enriched chromatin samples using DNA spin columns, as described in Section VI - A, or phenol/chloroform extraction followed by ethanol precipitation as described in Section VI - B. Purification using DNA spin columns is simple and fast, providing good recovery of DNA fragments above 35 bp (Figure 7A, Lane 2). Phenol/chloroform extraction followed by ethanol precipitation is more difficult, but provides better recovery of DNA fragments below 35 bp (Figure 7A, Lane 3); however, as shown in Figure 7B, the majority of DNA fragments generated in the CUT&RUN assay are larger than 35 bp. Therefore, DNA spin columns provide a quick and simple method for purification of > 98% of the total CUT&RUN DNA fragments.

Purified DNA can be quantified prior to NG-seq analysis using a picogreen-based DNA quantification assay. For CUT&RUN reactions containing 100,000 cells, the expected DNA yield for a CUT&RUN reaction ranges from 0.5 to 10 ng per reaction for transcription factors and cofactors, and 1 to 20 ng per reaction for histone modifications.

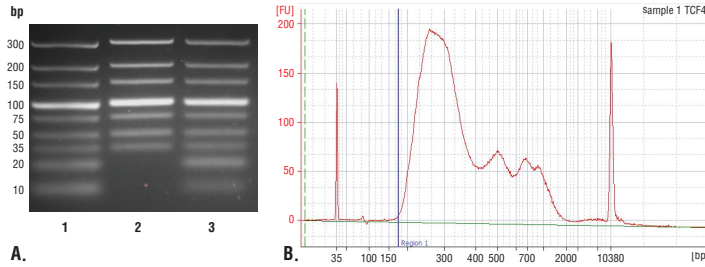


FIGURE 7. Comparison of DNA purification using spin columns or phenol/chloroform extraction followed by ethanol precipitation. (A) A low range DNA ladder mix (lane 1, unpurified) was purified using either DNA Purification Buffers and Spin Columns (ChIP, CUT&RUN, CUT&Tag) #14209 (lane 2) or phenol/chloroform extraction followed by ethanol precipitation (lane 3) and separated by electrophoresis on a 4% agarose gel. As shown, phenol/chloroform extraction followed by ethanol precipitation efficiently recovers all DNA fragment sizes, while DNA spin columns recover DNA fragments ≥ 35 bp. (B) DNA was purified using phenol/chloroform extraction followed by ethanol precipitation from a CUT&RUN assay performed using TCF4/TCF7L2 (C48H11) Rabbit mAb #2569. The size of the DNA fragments in the library was analyzed using a Bioanalyzer (Agilent Technologies). The adaptor and barcode sequences added to the library during construction account for 140 bp in fragment length. Therefore, starting 35 bp DNA fragments would be 175 bp in length after library preparation (indicated with blue vertical line in figure). As shown, less than 2% of the total CUT&RUN enriched DNA fragments are less than 175 bp (starting length of 35 bp), suggesting that DNA purification spin columns are sufficient for capture of > 98% of the total CUT&RUN DNA fragments.

A. DNA Purification Using Spin Columns

NOTE: DNA can be purified from input and enriched chromatin samples using the DNA Purification Buffers and Spin Columns (ChIP, CUT&RUN, CUT&Tag) #14209 (not included in this kit) and the modified protocol below. Steps 1 through 5 are modified to reflect the requirement for adding 5 volumes (1.5 mL) of DNA Binding Buffer to the 300 μ L of input and enriched chromatin samples.

Before starting:

!! Add 24 mL of ethanol (96-100%) to DNA Wash Buffer before use. This step only has to be performed once prior to the first set of DNA purifications.

- Remove one DNA Purification collection tube for each enriched chromatin sample or input sample to be purified.
- 1. Add 1.5 mL DNA Binding Buffer to each input and enriched chromatin sample and mix by pipetting up and down.

NOTE: 5 volumes of DNA Binding Buffer should be used for every 1 volume of sample.
- 2. Transfer 600 μ L of each sample from Step 1 to a DNA spin column in collection tube.
- 3. Centrifuge at 18,500 x g in a microcentrifuge for 30 sec.
- 4. Remove the spin column from the collection tube and discard the liquid. Replace the spin column in the empty collection tube.
- 5. Repeat steps 2-4 until the entire sample from Step 1 has been spun through the spin column. Replace the spin column in the empty collection tube.
- 6. Add 750 μ L of DNA Wash Buffer to the spin column in collection tube.
- 7. Centrifuge at 18,500 x g in a microcentrifuge for 30 sec.
- 8. Remove the spin column from the collection tube and discard the liquid. Replace spin column in the empty collection tube.
- 9. Centrifuge at 18,500 x g in a microcentrifuge for 30 sec.
- 10. Discard collection tube and liquid. Retain spin column.
- 11. Add 50 μ L of DNA Elution Buffer to each spin column and place into a clean 1.5 mL tube.
- 12. Centrifuge at 18,500 x g in a microcentrifuge for 30 sec to elute DNA.
- 13. Remove and discard DNA spin column. Eluate is now purified DNA. **(SAFE STOP)** Samples can be stored at -20°C for up to 6 months.

B. DNA Purification Using Phenol/Chloroform Extraction and Ethanol Precipitation

NOTE: The following reagents are required for the phenol/chloroform extraction and ethanol precipitation and are not included in this kit: phenol/chloroform/isoamyl alcohol (25:24:1), chloroform/isoamyl alcohol (24:1), 3M Sodium Acetate (pH 5.2), 20mg/ml glycogen, 100% ethanol, 70% ethanol, and 1X TE buffer or Nuclease-free Water #12931.

- 1. Add 300 μ L of phenol/chloroform/isoamyl alcohol (25:24:1) to each input and enriched chromatin sample and mix thoroughly by vortexing for 30 sec.

- 2. Separate layers by centrifugation at 16,000 x g for 5 min in a microcentrifuge. Carefully transfer most of the top aqueous layer (avoiding the interphase) to a new tube.
- 3. Add 300 μ L of chloroform/isoamyl alcohol (24:1) to the aqueous sample and mix thoroughly by vortexing for 30 sec.
- 4. Separate layers by centrifugation at 16,000 x g for 5 min in a microcentrifuge. Carefully transfer most of the top aqueous layer (avoiding the interphase) to a new tube.
- 5. Add 25 μ L of 3M Sodium Acetate (pH 5.2), 1 μ L 20mg/mL glycogen, and 600 μ L of 100% ethanol to each aqueous sample and mix by vortexing for 30 sec.
- 6. Incubate samples at -80°C for 1 hr or -20°C overnight to precipitate DNA.
- 7. Pellet DNA by centrifugation at 16,000 x g for 5 min at 4°C in a microcentrifuge.
- 8. Carefully remove supernatant and wash pellet with 70% ethanol.
- 9. Pellet DNA by centrifugation at 16,000 x g for 5 min at 4°C in a microcentrifuge.
- 10. Decant supernatant and air dry pellet.
- 11. Resuspend pellet in 50 μ L of 1X TE buffer or Nuclease-free Water #12931. This is the purified DNA. **(SAFE STOP)** Samples can be stored at -20°C for up to 6 months.

VII. Quantification of DNA by qPCR

Recommendations:

- The Sample Normalization Primer Set included in the kit is specific for the *S. cerevisiae* ACT1 gene and can be used to quantify the signal from the Sample Normalization Spike-In yeast DNA for sample normalization (optional).
- The additional control primers included in the kit are specific for the human or mouse RPL30 gene (#7014 or #7015) and can be used for quantitative real-time PCR analysis of the Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751 sample. If the user is performing CUT&RUN on another species, the user needs to design the appropriate control primers and determine the optimal PCR conditions for that species.
- PCR primer selection is critical. For CUT&RUN, PCR amplicon sizes should be approximately 60 to 80 bp in length. Primers should be designed with optimum melting temperature around 60°C and GC content around 50%.
- 2 μ L of purified DNA is sufficient for qPCR-mediated quantification of target genes for histones, transcription factors, and cofactors.
- A Hot-Start Taq polymerase is recommended to minimize the risk of nonspecific PCR products.
- Use Filter-tip pipette tips to minimize risk of contamination.

- 1. Label the appropriate number of PCR tubes or PCR plates compatible with the model of PCR machine to be used. PCR reactions should include the positive control tri-methyl-histone H3 Lys4 sample, the negative control rabbit IgG sample, a tube with no DNA to control for DNA contamination, and an input DNA sample. If desired, a serial dilution of the input DNA (undiluted - 100% input, 1:5 - 20% input, 1:25 - 4% input, 1:125 - 0.8% input) can be used to create a standard curve and determine the efficiency of amplification and quantify the amount of DNA in each immune-enriched sample.

NOTE: If sample normalization is performed, only the CUT&RUN samples are to be analyzed using the Sample Normalization Primer Set. The input DNA does not contain the Normalization Spike-In DNA.

- 2. Add 2 μ L of the appropriate DNA sample to each tube or well of the PCR plate.
- 3. Prepare a master reaction mix as described below. Set up 2-3 replicates for each PCR reaction. Add enough reagents to account for loss of volume (1-2 extra reactions). Add 18 μ L of reaction mix to each PCR reaction tube or well.

Reagent	Volume for 1 PCR Reaction (18 μ L)
Nuclease-free H ₂ O #12931	6 μ L
5 μ M Primers	2 μ L
SimpleChIP® Universal qPCR Master Mix #88989	10 μ L

- 4. Start the following PCR reaction program:
 - a. Initial Denaturation 95°C for 3 min
 - b. Denature 95°C for 15 sec
 - c. Anneal and Extension 60°C for 60 sec
 - d. Repeat steps b and c for a total of 40 cycles.

- Analyze quantitative PCR results using the software provided with the real-time PCR machine. Alternatively, one can calculate the IP efficiency manually using the Percent Input Method and the equation shown below. With this method, signals obtained from antibody reaction are expressed as a percent of the total input chromatin. If a serial dilution of the input DNA sample is used, plot and utilize the standard curve against the Log(10) of % Input (100%, 20%, 4%, 0.8%) to calculate the signals obtained from each antibody reaction.

$$\diamond \text{ Percent Input} = 100\% \times 2^{-(C[T]_{100\% \text{ Input Sample}} - C[T]_{\text{ IP Sample}})}$$

$$\diamond C[T] = C_T = \text{Average threshold cycle of PCR reaction}$$

- For sample normalization, choose the sample that has the lowest C[T] value for the Sample Normalization Primer Set as the selected sample (e.g. Sample 1 in the example table below) and calculate the normalization factor of other samples using the below equation. Adjust the signals from the test primer sets using the respective normalization factors.

	C[T] value of Sample Normalization Primer Set	** Normalization Factor for qPCR	Signal Before Normalization (% Input Calc'd from Step 5)	Signal After Normalization
Sample 1	23.31	$2^{(23.31-23.31)}=1.00$	24.4%	24.4%/1.00=24.4%
Sample 2	24.24	$2^{(23.31-24.24)}=0.52$	12.0%	12.0%/0.52=23.1%
Sample 3	25.08	$2^{(23.31-25.08)}=0.29$	6.28%	6.28%/0.29=21.7%
Sample 4	26.30	$2^{(23.31-26.30)}=0.13$	2.72%	2.72%/0.13=20.9%

**Normalization Factor for qPCR = $2^{(C[T]_{\text{ Selected Sample}} - C[T]_{\text{ the Other Sample}})}$

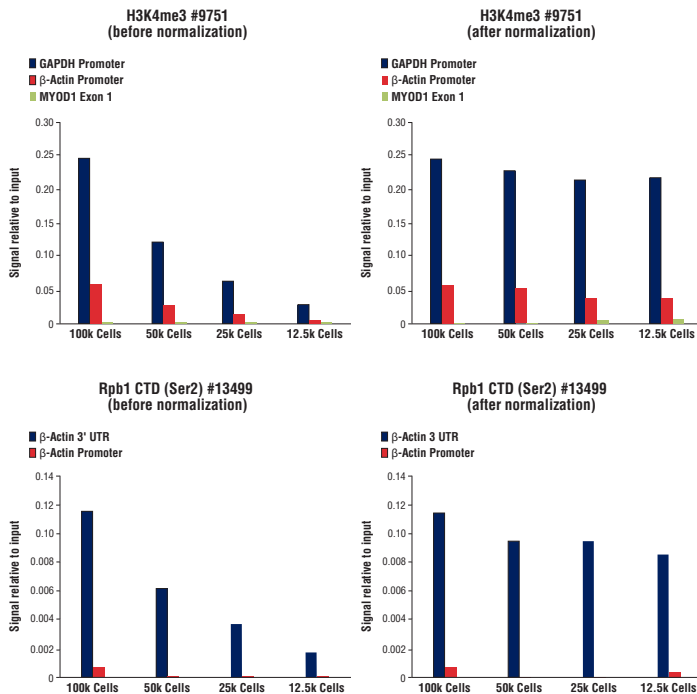


FIGURE 8. Normalization of CUT&RUN signals using spike in DNA for qPCR analysis. CUT&RUN was performed with a decreasing number of HCT116 cells and either Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751 (upper panels) or Phospho-Rpb1 CTD (Ser2) (E1Z3G) Rabbit mAb #13499 (lower panels). Enriched DNA was quantified by real-time PCR using SimpleChIP® Human GAPDH Exon 1 Primers #5516, SimpleChIP® Human β-Actin Promoter Primers #13653, SimpleChIP® Human β-Actin 3' UTR Primers #13669, and SimpleChIP® Human MyoD1 Exon 1 Primers #4490. The amount of immunoprecipitated DNA in each sample is represented as signal relative to the total amount of input chromatin for 100,000 cells. Non-normalized enrichments are depicted in the left panels. The Sample Normalization Spike-In DNA was added into each reaction proportionally to the starting cell number. Based on the difference of qPCR signals from spike in DNA in each sample, CUT&RUN signals were normalized to the sample containing 100,000 cells. Normalized enrichments are depicted in the right panels.

VIII. NG-Sequencing Library Construction

The immuno-enriched DNA samples prepared with this kit are directly compatible with NG-seq. For downstream NG-seq DNA library construction, use a DNA library preparation protocol or kit compatible with your downstream sequencing platform. For sequencing on Illumina® platforms, we recommend using the DNA Library Prep Kit for Illumina® (ChIP-seq, CUT&RUN) #56795 with Multiplex Oligos for Illumina® (ChIP-seq, CUT&RUN) #29580 or #47538, following the Protocol for CUT&RUN DNA.

- Because of the very low background signal generated in CUT&RUN, a sequencing depth of 5 million reads per sample is usually sufficient for histone modifications and transcription factors. The duplication rate of reads significantly increases if the sequencing depth is greater than fifteen million per sample. The signal to noise ratio decreases if the sequencing depth is lower than two million per sample.
- For less than 20,000 starting number of cells, it is common to obtain lower mapping rates or higher duplication rates in the NGS reads. If this happens, we recommend increasing the sequencing depth to obtain a sufficient amount of unique mapped reads for downstream data analysis.
- When performing sample normalization, map CUT&RUN sequencing data for all samples to both the test reference genome (e.g. human) and the sample normalization *S. cerevisiae* yeast genome. Choose the sample that has the least number of unique yeast reads as the selected sample (e.g. Sample 1 in table below) and calculate the normalization factor of other samples using the equation below. Downsize the number of unique reads aligned to test reference genome for each sample using the respective normalization factors. Use the downsized dataset for further NGS analysis.

An Example of Sample Normalization for NGS Assay

	The Number of Unique Reads Aligned to Yeast	Normalization Factor for NGS	The Number of Unique Reads Aligned to Test Reference Genome Before Normalization	The Number of Unique Reads Aligned to Test Reference Genome After Normalization
Sample 1	219,275	219,275/ 219,275 = 1.00	5,077,747	5,077,747 X 1.00 = 5,077,747
Sample 2	411,915	219,275/ 411,915 = 0.53	9,896,671	9,896,671 X 0.53 = 5,268,306
Sample 3	816,235	219,275/ 816,235 = 0.27	17,842,773	17,842,773 X 0.27 = 4,793,320
Sample 4	1,120,826	219,275/ 1,120,826 = 0.20	23,836,679	23,836,679 X 0.20 = 4,663,339

Normalization Factor for NGS = the number of unique yeast reads from Selected Sample / the number of unique yeast reads from the other sample

APPENDIX A: Determination of Cell Sensitivity to Digitonin

In the CUT&RUN protocol, the addition of digitonin to the buffers facilitates the permeabilization of cell membranes and entry of the primary antibody and pAG-MNase enzyme into the cells and nuclei. Therefore, having an adequate amount of digitonin in the buffers is critical to the success of antibody and enzyme binding and digestion of targeted genomic loci. Different cell lines exhibit varying sensitivities to digitonin cell permeabilization. While the amount of digitonin recommended in this protocol should be sufficient for permeabilization of most cell lines or tissues, you can test your specific cell line or tissue using this protocol. We have found that the addition of excess digitonin is not deleterious to the assay, so there is no need to perform a concentration curve. Rather, a quick test to determine if the recommended amount of digitonin works for your cell line is sufficient.

Before starting:

- Remove and warm Digitonin Solution #16359 to 90-100°C for 5 min. Make sure it is completely thawed. Immediately place the thawed Digitonin Solution #16359 on ice.

NOTE: Digitonin Solution #16359 should be stored at -20°C. Please keep on ice during use and store at -20°C when finished for the day.
 - For each cell or tissue sample, prepare 100 µL of Wash Buffer (10 µL 10X Wash Buffer #31415 + 90 µL Nuclease-free Water #12931). It is not necessary to add spermidine or Protease Inhibitors for this test.
- In a 1.5 mL tube, collect 10,000 - 100,000 cells. For tissue, collect disaggregated cells from 1 mg of tissue (Section I-C Step 1-13).
 - Centrifuge for 3 min at 600 x g at room temperature and withdraw the liquid.

NOTE: If the cell pellet is not visible by eye, we recommend removing as much cell medium as possible without disturbing the cell pellet after the initial centrifugation of the cell suspension in Step 2 and leave behind some cell medium per reaction. Then in Step 3 add enough 1X Wash Buffer to the cell suspension to achieve a total volume of 100 µL.
 - Resuspend cell pellet in 100 µL of Wash Buffer.
 - Add 2.5 µL Digitonin Solution #16359 to each reaction and incubate for 10 min at room temperature.
 - Mix 10 µL of cell suspension with 10 µL of 0.4% Trypan Blue Stain.
 - Use a hemocytometer or cell counter to count the number of stained cells and the total number of cells. Sufficient permeabilization results in > 90% of cells staining with Trypan blue.
 - If less than 90% of cells stain with Trypan blue, then increase the amount of Digitonin Solution #16359 added to the Digitonin Buffer and repeat steps 1-5 until > 90% cells are permeabilized and stained. Use this amount of Digitonin Solution #16359 in Sections I - IV.

APPENDIX B: Sonication Optimization for the Input Sample

Sonication of the input DNA sample is recommended because only fragmented genomic DNA (<10 kb) can be purified using DNA purification spin columns. Additionally, the fragmented genomic DNA (<1kb) may be used as the negative control in NG-seq analysis. Sonication should be optimized so that the input DNA is 100-600 bp in length.

We recommend using the input sample for NG-seq because it provides a convenient and unbiased representation of the cell genome. While the IgG sample can also be used as a negative control for NG-seq, it may show enrichment of specific regions of the genome due to non-specific binding. Unfragmented input DNA can be used for qPCR analysis. However, unfragmented DNA must be purified using phenol/chloroform extraction followed by ethanol precipitation.

Before starting:

- All buffer volumes should be increased proportionally based on the number of input samples being prepared.
- Remove and warm DNA Extraction Buffer #42015 at room temperature, making sure it's completely thawed and in solution.
- For each input sample, prepare 2.1 mL 1X Wash Buffer (210 µL 10X Wash Buffer #31415 + 1.89 mL Nuclease-free Water #12931) and equilibrate it to room temperature to minimize stress on the cells. It is not necessary to add spermidine or Protease Inhibitor Cocktail #7012 to this Wash Buffer.
- For each input sample, prepare 2 µL Proteinase K #10012 + 0.5 µL RNase A #7013 to 197.5 µL DNA Extraction Buffer #42015 (200 µL per input sample).

- In a 1.5 mL tube, collect the same number of cells you use for the input in your CUT&RUN experiment (5,000 to 100,000 cells) for each sonication condition being tested. For tissue, collect disaggregated cells from the same amount of tissue you use for the input in your CUT&RUN experiment (Section I-C Step 1-13) for each sonication condition being tested. .
- Centrifuge for 3 min at 600 x g at room temperature and remove the liquid.

NOTE: If the centrifuged cell pellet is not visible by eye when working with low cell numbers (<100,000 cells), we recommend skipping the wash steps 3-5 below. Remove as much cell medium as possible without disturbing the cell pellet after the initial centrifugation of the cell suspension in Step 2 and leave behind some cell medium per reaction. Then in Step 6 add enough 1X Wash Buffer to the cell suspension to achieve a volume of 100 µL per sonication condition being tested.
- Resuspend cell pellet in 1 mL of 1X Wash Buffer by gently pipetting up and down.
- Centrifuge for 3 min at 600 x g at room temperature and remove the liquid.
- Wash the cell pellet again by repeating steps 3 and 4 one time.
- Add 100 µL of 1X Wash Buffer per sonication condition being tested and resuspend the cell pellet by gently pipetting up and down.
- Aliquot 100 µL of the cell suspension into a new tube for each sonication condition.

NOTE: Samples will be incubated at 55°C in Step 9, so it is recommended to use a safe-lock 1.5 mL tube to reduce evaporation during the incubation.
- Add 200 µL DNA Extraction Buffer (+ Proteinase K + RNase A) to each sample and mix by pipetting up and down.
- Place the tubes at 55°C for 1 hr with shaking.
- Place the tubes on ice for 5 min to completely cool down the samples.
- Determine optimal sonication conditions for your sonicator by setting up a time-course experiment with increasing numbers of 15 sec pulse sonication cycles. Be sure to incubate samples on ice for 30 sec between pulses.
- Clarify lysates by centrifugation at 18,500 x g in a microcentrifuge for 10 min at 4°C. Transfer supernatant to a new 2 mL microcentrifuge tube.
- Purify the DNA samples with DNA Purification Spin Columns or phenol/chloroform extraction followed by ethanol precipitation, following the directions in Section VI.
- Elute the DNA from the column or resuspend DNA pellet in 30 µL of 1X TE buffer or Nuclease-free Water #12931.
- Determine DNA fragment sizes by electrophoresis. Load > 15 µL sample on a 1% agarose gel with a 100 bp DNA marker. A dye-free loading buffer (30% glycerol) is recommended to better observe the DNA smear on gel.
- Choose the sonication conditions that generate the optimal DNA fragment size of 100-600 bp and use for Preparation of the Input Sample in Section V, Step 4. If optimal sonication conditions are not achieved, increase or decrease the power setting of the sonicator or number of sonication cycles and repeat the sonication time course experiment.

APPENDIX C: Troubleshooting Guide

For a detailed troubleshooting guide, please go to <https://cst-science.com/troubleshooting-CUT-RUN>