Store at RT, 4°C, & -20°C

# CUT&Tag Assay Kit

Cell Signaling TECHNOLOGY®

#77552

1 Kit
(24 assays)

**Support:** +1-978-867-2388 (U.S.)
cellsignal.com/support

**Orders:** 877-616-2355 (U.S.)
orders@cellsignal.com

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

| Components Ship As: 44284 | Item # | Kit Quantity | Storage Temp |
|---|---|---|---|
| Concanavalin A Magnetic Beads | 82307 | 1 x 240 µL | 4°C |
| Concanavalin A Bead Activation Buffer | 91275 | 1 x 5 mL | 4°C |
| Antibody Binding Buffer (CUT&RUN, CUT&Tag) | 15338 | 1 x 2.5 mL | 4°C |
| 10X Wash Buffer (CUT&RUN, CUT&Tag) | 31415 | 1 x 6 mL | 4°C |
| Magnesium Chloride | 86963 | 1 x 40 µL | 4°C |
| 0.5 M EDTA, pH 8.0 | 7011 | 1 x 200 µL | 4°C |
| 10X High Salt Wash Buffer (CUT&Tag) | 18878 | 1 x 3 mL | 4°C |
| 10% SDS Solution | 20533 | 1 x 200 µL | RT |
| DNA Binding Buffer | 10007 | 1 x 30 mL | RT |
| DNA Wash Buffer (add 4x volume ethanol before use) | 10008 | 1 x 6 mL | RT |
| DNA Elution Buffer | 10009 | 1 x 1 mL | RT |
| DNA Purification Columns and Collection Tubes | 10010 | 24 Pack | RT |
| **Components Ship As: 65086** | **Item #** | **Kit Quantity** | **Storage Temp** |
| CUT&Tag pAG-Tn5 (Loaded) | 79561 | 1 x 50 µL | -20°C |
| Digitonin Solution | 16359 | 2 x 1.2 mL | -20°C |
| 100X Spermidine | 27287 | 1 x 900 µL | -20°C |
| Protease Inhibitor Cocktail (200X) | 7012 | 1 x 500 µL | -20°C |
| Proteinase K (20 mg/ml) | 10012 | 1 x 40 µL | -20°C |
| Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb | 9751 | 1 x 20 µL | -20°C |
| Normal Rabbit IgG | 2729 | 1 x 20 µL | -20°C |
| Goat Anti-Rabbit IgG (H+L) Antibody | 35401 | 1 x 24 µL | -20°C |
| Donkey Anti-Mouse IgG (H+L) Antibody | 52885 | 1 x 24 µL | -20°C |
| Normal Mouse IgG | 68860 | 1 x 20 µL | -20°C |

**Description:** The CUT&Tag Assay Kit is designed to conveniently provide reagents needed to perform up to 24 reactions. The kit has been optimized to work in fresh or lightly fixed cells for all types of DNA-binding proteins, including histones, transcription factors, and cofactors. In addition, the kit has been optimized to work for histones in fresh or lightly fixed tissues. For analysis of transcription factors and cofactors in tissues, we recommend using the CUT&RUN Assay Kit #86652. If possible, we recommend using 100,000 cells or 1 mg of tissue per CUT&Tag reaction. If starting cell number is limited, this kit is validated to work with as few as 5,000 to 10,000 cells per reaction for histone modification targets and as few as 20,000 cells per reaction for transcription factors and cofactors. This kit is compatible with both whole cells and nuclei as starting material. We have not found that using nuclei generates a stronger signal or a higher signal-to-noise reaction compared to whole cells. A complete assay can be performed in as little as one day. The CUT&Tag Assay Kit also provides important controls to ensure a successful CUT&Tag experiment, including positive control Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751 and negative controls Normal Rabbit IgG #2729 and Normal Mouse IgG #68860. The negative control Normal Rabbit IgG #2729 or Normal Mouse IgG #68860 is optional for the experiments, depending on the requirements of the peak calling algorithm used. This kit is compatible with downstream Next Generation sequencing (NG-seq) analysis, but not qPCR.

**Background:** Similar to Cleavage Under Targets and Release Using Nuclease (CUT&RUN), Cleavage Under Targets and Tagmentation (CUT&Tag) is a powerful technique used for probing protein-DNA interactions within the natural chromatin context of the cell (1-3). CUT&Tag has many of the same

**Storage:** All components in this kit are stable for 6 months when stored at the recommended temperature.

**Reagents Not Supplied:**

1. Safe-lock 1.5 mL tubes
2. Nuclease-Free Water #12931
3. Trypan Blue Stain (0.4%)
4. 100% Ethanol
5. 16% Formaldehyde, Methanol-Free #12606 (Optional)
6. Glycine Solution (10X) #7005 (Optional)
7. Phosphate Buffered Saline (PBS-1X) pH7.2 (Sterile) #9872 (Optional)
8. CUT&Tag Dual Index Primers and PCR Master Mix for Illumina Systems #47415

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

**Background References:**

(1) Kaya-Okur, H.S. et al. (2019) *Nat Commun* 10, 1930.
(2) Kaya-Okur, H.S. et al. (2020) *Nat Protoc* 15, 3264-3283.
(3) Henikoff, S. et al. (2021) *Bio Protoc* 11, e4043.







**Applications:** **W**—Western **IP**—Immunoprecipitation **IHC**—Immunohistochemistry **ChIP**—Chromatin Immunoprecipitation **IF**—Immunofluorescence **F**—Flow cytometry **FC-FP**— Flow cytometry-Fixed/Permeabilized **FC-L**— Flow cytometry-Live **E-P**—ELISA-Peptide
**Species Cross-Reactivity:** **H**—human **M**—mouse **R**—rat **Hm**—hamster **Mk**—monkey **Mi**—mink **C**—chicken **Dm**—D. melanogaster **X**—Xenopus **Z**—zebrafish **B**—bovine **Dg**—dog **Pg**—pig **Sc**—S. cerevisiae **Ce**—C. elegans **Hr**—Horse
**All**—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

advantages as the CUT&RUN assay in that it provides a rapid, robust, and true low cell number protocol for detection of protein-DNA interactions in the cell. In addition, the CUT&Tag assay adds an *in situ* adaptor DNA ligation step carried out by the pAG-Tn5 enzyme, in which an adaptor DNA is ligated directly to antibody-targeted chromatin DNA fragments in the cell. As a result, subsequent DNA library preparation is much faster and easier than library preparation following the CUT&RUN assay, free from DNA end repair, A-tailing, and adaptor ligation *in vitro*. CUT&Tag works very well for analyzing histone modifications, in addition to mapping some transcription factor and cofactor binding.

**Specificity/Sensitivity:** The CUT&Tag Assay Kit can be utilized with any CUT&Tag-validated antibody to detect endogenous levels of protein-DNA interactions and histone modifications in mammalian cells (see Figures 1–3). One CUT&Tag reaction can use between 5,000 to 250,000 cells or 1 to 5 mg of tissue of starting material (see Figures 5-7). The kit is compatible with multiple species of antibodies, including rabbit and mouse (see Figure 4). 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.

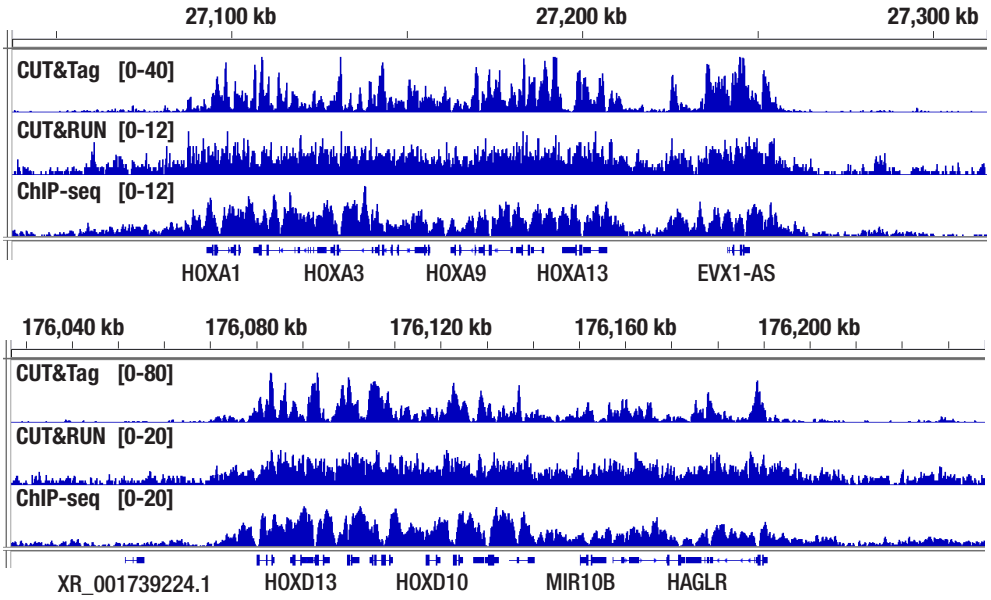



*Figure 1. CUT&Tag, CUT&RUN, and ChIP-seq assays were performed with NCCIT cells and Tri-Methyl-Histone H3 (Lys27) (C36B11) Rabbit mAb #9733, using this CUT&Tag Assay Kit, the CUT&RUN Assay Kit #86652, or the SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads) #9005. DNA libraries were prepared using CUT&Tag Dual Index Primers and PCR Master Mix for Illumina Systems #47415 for CUT&Tag samples and DNA Library Prep Kit for Illumina Systems (ChIP-seq, CUT&RUN) #56795 for ChIP-seq and CUT&RUN samples. The upper panel compares enrichment around HoxA genes, while the lower panel compares enrichment around HoxD genes, both are known target genes of H3K27me3.*

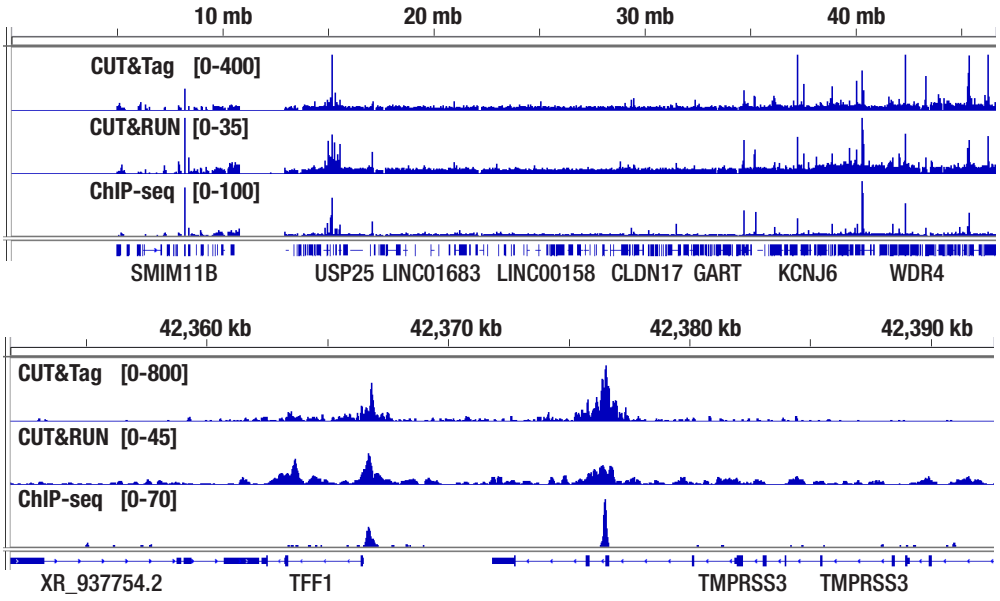
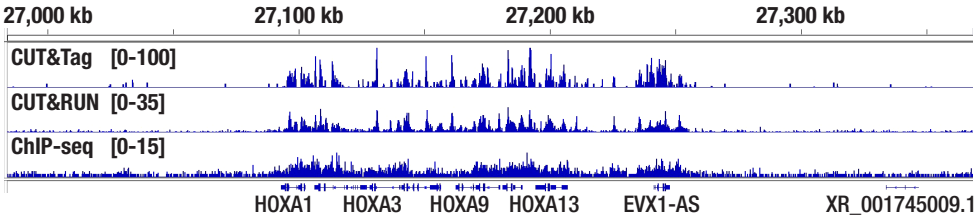



*Figure 2. CUT&Tag, CUT&RUN, and ChIP-seq assays were performed with MCF7 cells grown in phenol red free medium and 5% charcoal stripped FBS for 4 d, then treated with β-estradiol (10 nM) for 45 min and Estrogen Receptor α (D8H8) Rabbit mAb #8644, using this CUT&Tag Assay Kit, the CUT&RUN Assay Kit #86652, or the SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads) #9005. DNA libraries were prepared using CUT&Tag Dual Index Primers and PCR Master Mix for Illumina Systems #47415 for CUT&Tag samples and DNA Library Prep Kit for Illumina Systems (ChIP-seq, CUT&RUN) #56795 for ChIP-seq and CUT&RUN samples. The upper panel compares enrichment of Estrogen Receptor α across chromosome 21, while the lower panel compares enrichment around TFF1, a known target gene of Estrogen Receptor α.*



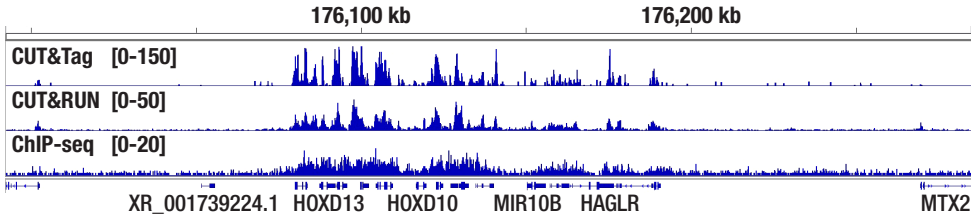
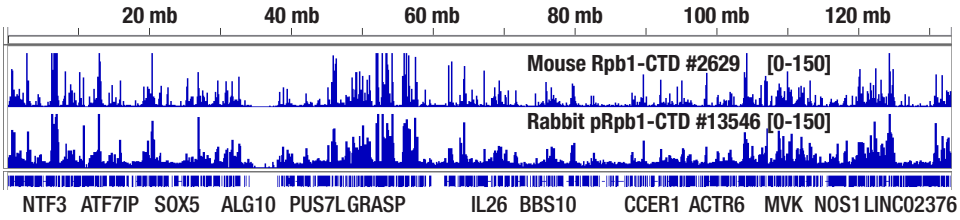




*Figure 3. CUT&Tag, CUT&RUN, and ChIP-seq assays were performed with NCCIT cells and JARID2 (D6M9X) Rabbit mAb #13594, using this CUT&Tag Assay Kit, the CUT&RUN Assay Kit #86652, or the SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads) #9005. DNA libraries were prepared using CUT&Tag Dual Index Primers and PCR Master Mix for Illumina Systems #47415 for CUT&Tag samples and DNA Library Prep Kit for Illumina Systems (ChIP-seq, CUT&RUN) #56795 for ChIP-seq and CUT&RUN samples. The upper panel compares enrichment around HoxA genes, while the lower panel compares enrichment around HoxD genes, both are known target genes of JARID2.*



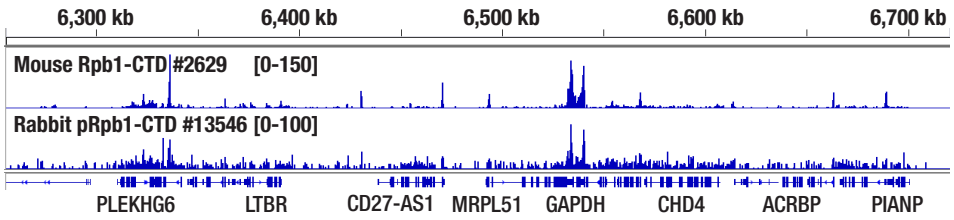
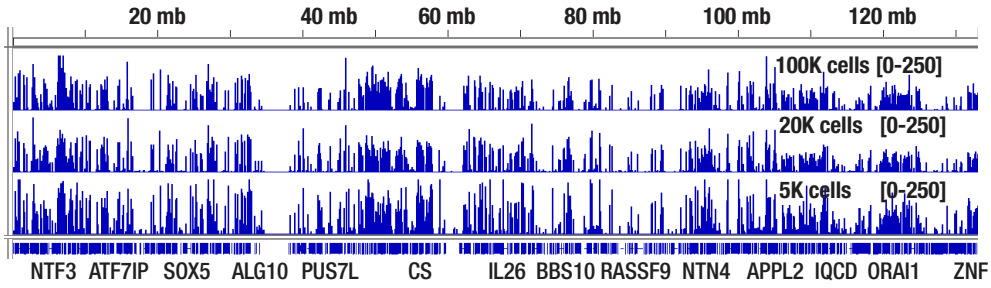




*Figure 4. CUT&Tag assay was performed with HeLa cells and either Rpb1 CTD (4H8) Mouse mAb #2629 or Phospho-Rpb1 CTD (Ser2/Ser5) (D1G3K) Rabbit mAb #13546, using this CUT&Tag Assay Kit. DNA libraries were prepared using CUT&Tag Dual Index Primers and PCR Master Mix for Illumina Systems #47415. The upper panel compares enrichment of Rpb1 across chromosome 12, while the lower panel compares enrichment around GAPDH, a known target gene of Rpb1.*



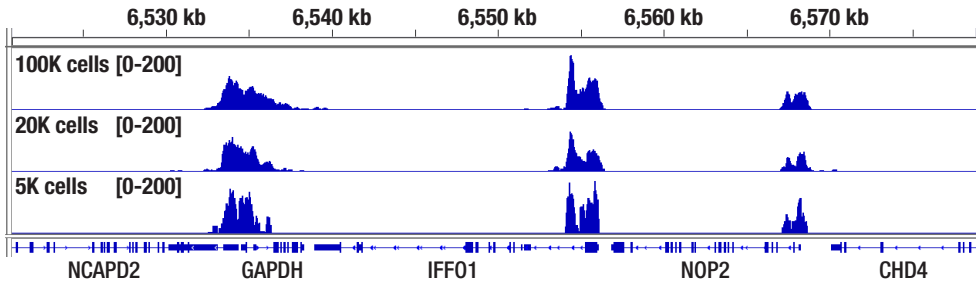




*Figure 5. CUT&Tag assay was performed with 100,000, 20,000, or 5,000 NCCIT cells (as indicated) and Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751, using this CUT&Tag Assay Kit. DNA libraries were prepared using CUT&Tag Dual Index Primers and PCR Master Mix for Illumina Systems #47415. The upper panel compares enrichment of H3K4me3 across chromosome 12, while the lower panel compares enrichment around GAPDH, a known target gene of H3K4me3.*

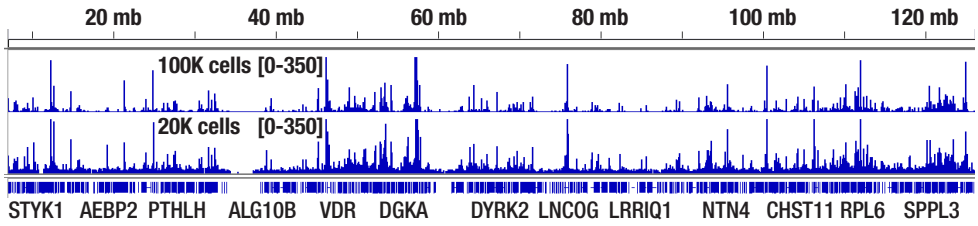



*Figure 6. CUT&Tag assay was performed with 100,000 or 20,000 Hep G2 cells treated with Thapsigargin #12758 (300 nM) for 4h (as indicated) and ATF-4 (D4B8) Rabbit mAb #11815, using this CUT&Tag Assay Kit. DNA libraries were prepared using CUT&Tag Dual Index Primers and PCR Master Mix for Illumina Systems #47415. The upper panel compares enrichment of ATF-4 across chromosome 12, while the lower panel compares enrichment around DDIT3/CHOP, a known target gene of ATF-4.*

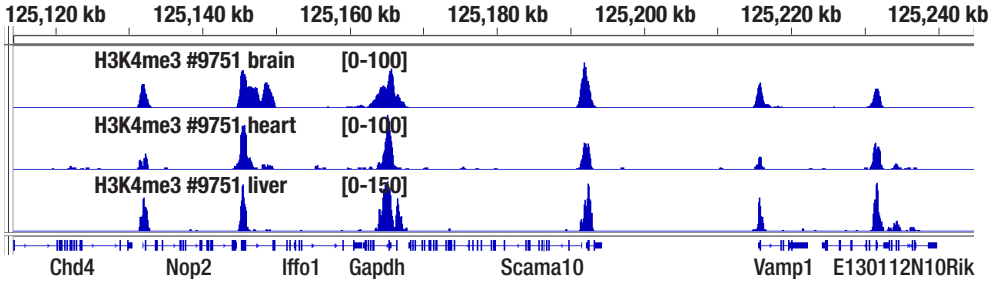
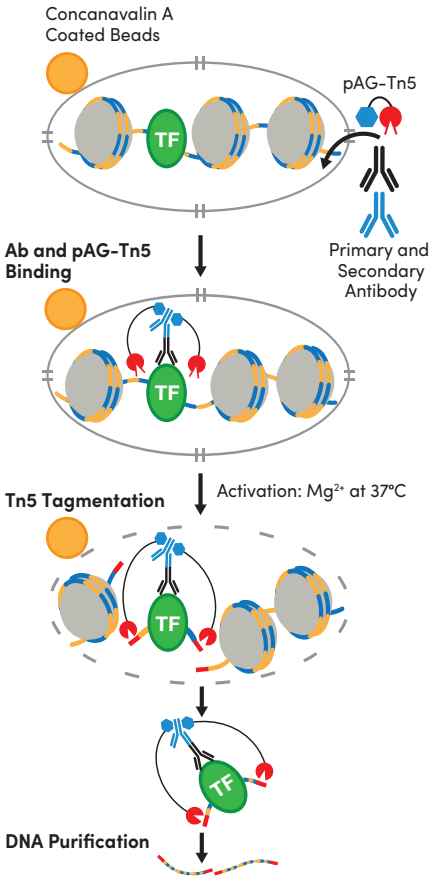



*Figure 7. CUT&Tag assay was performed with 1 mg of fresh mouse brain, heart, or liver tissues (as indicated) and Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751, using this CUT&Tag Assay Kit. DNA libraries were prepared using CUT&Tag Dual Index Primers and PCR Master Mix for Illumina Systems #47415. The figure shows enrichment of H3K4me3 around its known target gene GAPDH.*

# CUT&Tag Method Overview




**Primary and Secondary Antibody Binding:**

1. 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 and secondary antibodies and pAG-Tn5 into the cell nuclei.

**pAG-Tn5 Binding:**

3. The pAG-Tn5 fusion transposase is recruited to the chromatin through protein-protein interactions between the antibodies and the pAG domain of the fusion enzyme.

**Tn5 Tagmentation:**

4. The addition of $Mg^{2+}$ activates the pAG-Tn5, which cuts targeted genomic DNA and ligates adaptor DNA on site. A further incubation with SDS at 58°C breaks open the cellular and nuclear membranes, releasing genomic and adaptor bound desired chromatin fragments into the supernatant.

**DNA Purification:**

5. DNA is purified using DNA purification spin columns. The small, adaptor bound, desired DNA is then selectively amplified by PCR and analyzed by NG-seq.

# CUT&Tag 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. Activation of Concanavalin A Beads

### Before Starting:

! All buffer volumes should be scaled proportionally to the number of CUT&Tag reactions being performed.

- Place Concanavalin A Bead Activation Buffer on ice.

1. Determine the number of CUT&Tag reactions to be run. We strongly suggest including a reaction for the positive control Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751. The negative control Normal Rabbit IgG #2729 or Normal Mouse IgG #68860 is optional, depending on the requirements of the peak calling algorithm used.
2. Carefully resuspend Concanavalin A Magnetic Beads into a homogeneous slurry by gently pipetting up and down, making sure not to splash any bead suspension out of the tube.

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

3. Transfer 10 µl of the bead suspension for each CUT&Tag reaction to a new 1.5 ml microcentrifuge tube. If planning to do more than 14 CUT&Tag reactions at one time, use two or more 1.5 ml microcentrifuge tubes. No more than 140µl of Concanavalin A beads should be added to each 1.5 ml microcentrifuge tube.
4. Add 100 µl of Concanavalin A Bead Activation Buffer per 10 µl of beads. Gently mix beads by pipetting up and down.
5. Place tube on a magnetic rack for 30 sec to 2 min and then remove the supernatant using a pipette.

**NOTE:** To avoid loss of beads, do NOT aspirate using a vacuum throughout the protocol.

6. Remove tubes from the magnetic rack. Wash the beads a second time by repeating steps 4 and 5.
7. Add a volume of Concanavalin A Bead Activation Buffer equal to the initial volume of bead suspension added (10 µl per reaction) and resuspend by pipetting up and down.

**NOTE:** The activated beads can be stored on ice for up to 8 hrs.

## II. Cell and Tissue Preparation

For most cell types, live cells can be used in the CUT&Tag assay to generate robust enrichment of histones, transcription factors, and cofactors. We strongly recommend using live cells whenever possible. For certain cell types that are fragile or sensitive to Concanavalin A, please refer to Appendix A for a light fixation protocol of cells prior to CUT&Tag. Please note that cell fixation does not increase CUT&Tag signals and over-fixation can be detrimental to the tagmentation reaction.

Fresh tissues can also be used in the CUT&Tag assay to generate robust enrichment of histones. However, non-histone targets such as transcription factors and cofactors are not well enriched in the CUT&Tag assay. For analysis of transcription factors and cofactors in tissues, we recommend using the CUT&RUN Assay Kit #86652. Fresh tissues typically generate comparable or stronger CUT&Tag signals than fixed tissues. If fixation is necessary, refer to Appendix B for a light fixation protocol of tissues prior to the CUT&Tag experiment.

Our CUT&Tag assay works with a variety of different cell and tissue types and a wide range of starting material amounts. We recommend using 100,000 cells or 1 mg of tissue per reaction. If starting cell number is limited, histone modification targets may work with as few as 5,000 to 10,000 cells per reaction, and transcription factor and cofactors may work with as few as 20,000 cells per reaction. Success of low input reactions depends on target abundance and antibody sensitivity. An adequate amount of starting material is critical for desired CUT&Tag signal, especially for transcription factors and cofactors. Up to 250,000 cells or 5 mg tissue can be used per reaction. Buffer volumes throughout the protocol used in one reaction do not need to be adjusted based on the cell number or tissue mass, as long as the values fall within the designated range (5,000-250,000 cells or 1-5 mg of tissue). When indicated, buffer volumes do need to be scaled proportionally to the number of reactions being performed.

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 C. Digitonin treatment should result in permeabilization of > 90% of the cell population.

### A. Live Cell Preparation

### Before Starting:

! All buffer volumes should be scaled proportionally to the number of CUT&Tag reactions being performed.

- Thoroughly thaw 200X Protease Inhibitor Cocktail #7012 and 100X Spermidine #27287 before use and store them at -20°C when finished for the day. Please note that the Protease Inhibitor Cocktail #7012 will refreeze when placed on ice due to containing DMSO.
- Prepare Complete Wash Buffer (2 ml for each cell preparation and an additional 100 µl for each CUT&Tag reaction) and keep at room temperature. For example, if using both untreated and drug-treated cells (2 cell preparations) and testing with 4 antibodies (positive control H3K4me3 #9751, negative control IgG #2729, and two experimental antibodies; 8 reactions), a total of 4.8 ml of Complete Wash Buffer will be needed.

| Complete Wash Buffer | Volume (per cell preparation) | Volume (per reaction) | Total volume |
|---|---|---|---|
| 10X Wash buffer (CUT&RUN, CUT&Tag) #31415 | 200 µl | 10 µl | Add both columns together for total volume needed for each reagent. |
| 100X Spermidine #27287 | 20 µl | 1 µl | |
| Protease Inhibitor Cocktail (200X) #7012 | 10 µl | 0.5 µl | |
| Nuclease free water #12931 | 1770 µl | 88.5 µl | |

# CUT&Tag Protocol (continued)

**NOTE:** All steps for live cell preparation should be performed in succession at room temperature to minimize stress on the cells. Do not vortex cell samples to avoid DNA fragmentation and cavitation of cells.

1. Harvest 100,000 live cells for each reaction at room temperature to minimize stress on the cells.

**NOTE:** For adherent cells, detach them from the dish using Trypsin and neutralize with at least 3 volumes of tissue culture medium. We do not recommend scraping the cells from the dish to prevent cell lysis. Cells should be counted accurately using a hemocytometer or a cell counter to ensure that an accurate number of cells are being used for the experiment.

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

**NOTE:** If working with fewer than 100,000 total cells and the centrifuged cell pellet is not visible by eye, we recommend skipping the wash steps 3 to 5 below and moving directly to Step 6. After the initial centrifugation of the cell suspension in Step 2, remove most of the supernatant, leaving behind ≤40 µl of supernatant per reaction. Then in Step 6, add enough Complete Wash Buffer to the cell suspension to achieve a total volume of 100 µl per reaction.

3. Resuspend cell pellet in 1 ml of Complete Wash Buffer at room temperature by gently pipetting up and down.
4. Centrifuge for 3 min at 600 x g at room temperature and remove the supernatant.
5. Wash the cell pellet a second time by repeating steps 3 and 4 one time.
6. Add 100 µl of Complete Wash Buffer per reaction and resuspend the cell pellet by gently pipetting up and down.
7. Immediately proceed to Section III.

### B. Fresh Tissue Sample Preparation

**Before Starting:**

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

- Thoroughly thaw 200X Protease Inhibitor Cocktail #7012 and 100X Spermidine #27287 before use and store them at -20°C when finished for the day. Please note that the Protease Inhibitor Cocktail #7012 will refreeze when placed on ice due to containing DMSO.
- Prepare Complete Wash Buffer (3 ml for each tissue type and additional 100 µl for each reaction) and keep it at room temperature to minimize stress on the cells. For example, if using wild type and transgenic liver as starting material (2 tissue types) and testing 4 antibodies (positive control H3K4me3 #9751, negative control IgG #2729, and two experimental antibodies; 8 reactions), a total of 6.8 mL of Complete Wash Buffer is needed.

| Complete Wash Buffer | Volume (per tissue type) | Volume (per reaction) | Total volume |
|---|---|---|---|
| 10X Wash buffer (CUT&RUN, CUT&Tag) #31415 | 300 µl | 10 µl | Add both columns together for total volume needed for each reagent. |
| 100X Spermidine #27287 | 30 µl | 1 µl | |
| Protease Inhibitor Cocktail (200X) #7012 | 15 µl | 0.5 µl | |
| Nuclease free water #12931 | 2655 µl | 88.5 µl | |

1. Weigh 1 mg fresh tissue for each reaction.
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.
3. Resuspend tissue in 1 ml of Complete Wash Buffer and transfer the sample to a Dounce homogenizer.
4. Disaggregate tissue pieces into a single-cell suspension with 20-25 strokes until no tissue chunks are observed.
5. Transfer cell suspension to a 1.5 ml tube and centrifuge at 3,000 x g for 3 min at room temperature, and pipette to remove supernatant from cells.
6. Resuspend the cell pellet in 1 ml of Complete Wash Buffer.
7. Centrifuge cell suspension for 3 min at 3,000 x g at room temperature and remove the supernatant.
8. Wash the cell pellet a second time by repeating steps 6 and 7 one time.
9. Add 100 µl of Complete Wash Buffer per reaction and resuspend the cell pellet by gently pipetting up and down.
10. Immediately proceed to Section III.

## III. Binding of Concanavalin A Beads and Primary Antibody

**NOTE:** For all incubation steps in Sections III-V, it is not necessary to mix samples by rocking or rotation. Instead, we recommend simply placing sample tubes in a rack at the designated temperatures. Mixing the samples during the incubation steps does not increase the performance of the assay. Instead, rotation or rocking the samples may lead to increased bead clumping and bead loss due to potential sticking on the tube walls and caps.

**Before Starting:**

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

- Warm Digitonin Solution #16359 at 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 during use. Store at -20°C when finished for the day.
- Thoroughly thaw 200X Protease Inhibitor Cocktail #7012 and 100X Spermidine #27287 before use and store them at -20°C when finished for the day. Please note that the Protease Inhibitor Cocktail #7012 will refreeze when placed on ice due to containing DMSO.
- Prepare 100 µl of Complete Antibody Binding Buffer per reaction and place on ice.

| Complete Antibody Binding Buffer | Volume (per reaction) |
|---|---|
| Antibody Binding Buffer (CUT&RUN, CUT&Tag) #15338 | 96 µl |
| 100X Spermidine #27287 | 1 µl |
| Protease Inhibitor Cocktail (200X) #7012 | 0.5 µl |
| Digitonin Solution #16359 | 2.5 µl |

1. Thoroughly mix the activated Concanavalin A Beads prepared in Section I Step 7 by gently pipetting up and down. Add the beads suspension to the washed cell suspension prepared in Section II-A Step 6, or Section II-B Step 9.
2. Incubate the sample for 5 min at room temperature.
3. Place the tube on the magnetic rack for 30 sec to 2 min, then remove and discard the supernatant.

# CUT&Tag Protocol (continued)

4. Remove tube from the magnet. Add 100 µl of Complete Antibody Binding Buffer per reaction and mix gently by pipetting.
5. Aliquot 100 µl of the cell:bead suspension into separate 1.5 ml tubes for each reaction and place on ice.
6. Add the appropriate amount of primary antibody to each reaction and mix gently by pipetting up and down.

**NOTE:** The amount of antibody required for CUT&Tag varies and should be determined by the user. For the positive control Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751, or the negative control Normal Rabbit IgG #2729 or Normal Mouse IgG #68860, add 2 µl of antibody to the reaction. If possible, we highly recommend using CUT&Tag-validated antibodies in the assay. CST offers a selection of CUT&Tag validated antibodies with supporting data and appropriate dilution ratios available on www.cellsignal.com.

7. Incubate samples at room temperature for 1 hour. This step can be extended to overnight at 4°C.
8. Immediately proceed to Section IV.

## IV. Binding of Secondary Antibody

### Before Starting:

**!** All buffer volumes should be scaled proportionally to the number of CUT&Tag reactions being performed.

- Warm Digitonin Solution #16359 at 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 during use. Store at -20°C when finished for the day.
- Thoroughly thaw 200X Protease Inhibitor Cocktail #7012 and 100X Spermidine #27287 before use and store them at -20°C when finished for the day. Please note that the Protease Inhibitor Cocktail #7012 will refreeze when placed on ice due to containing DMSO.
- Freshly prepare 1.05 ml of Digitonin Buffer per reaction and place on ice. Please note that the Digitonin Buffer prepared here will be used for both Section IV and V.

| Digitonin Buffer | Volume (per reaction) |
|---|---|
| 10X Wash buffer (CUT&RUN, CUT&Tag) #31415 | 105 µl |
| 100X Spermidine #27287 | 10.5 µl |
| Protease Inhibitor Cocktail (200X) #7012 | 5.25 µl |
| Digitonin Solution #16359 | 26.25 µl |
| Nuclease free water #12931 | 903 µl |

1. Make secondary antibody pre-mix. For each reaction, dilute 1 µl of Anti-Rabbit IgG (H+L) Antibody #35401 or 1 µl of Anti-Mouse IgG (H+L) Antibody #52885 into 50 µl Digitonin Buffer. Proportionally scale up the secondary antibody pre-mix based on the number of reactions. Mix by gently pipetting up and down and place on ice.
2. Place the sample tubes containing the primary antibody incubation solution from Section III Step 7 on the magnetic rack for 30 sec to 2 min and then remove the supernatant.
3. Add 50 µl of secondary antibody pre-mix to each sample tube and gently mix the sample by pipetting up and down.
4. Incubate samples at room temperature for 30 min.
5. Immediately proceed to Section V.

## V. Binding of pAG-Tn5 Enzyme and Tagmentation

### Before Starting:

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

- Make sure the 10% SDS Solution #20533 is completely in solution. Warming it up at 37°C can help to dissolve the SDS precipitates.
- Warm Digitonin Solution #16359 at 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 during use. Store at -20°C when finished for the day.
- Thoroughly thaw 200X Protease Inhibitor Cocktail #7012 and 100X Spermidine #27287 before use and store them at -20°C when finished for the day. Please note that the Protease Inhibitor Cocktail #7012 will refreeze when placed on ice due to containing DMSO.
- Prepare 1.2 ml of High Salt Digitonin Buffer per reaction and place on ice.

| High Salt Digitonin Buffer | Volume (per reaction) |
|---|---|
| 10X High Salt Wash Buffer (CUT&Tag) #18878 | 120 µl |
| 100X Spermidine #27287 | 12 µl |
| Protease Inhibitor Cocktail (200X) #7012 | 6 µl |
| Digitonin Solution #16359 | 30 µl |
| Nuclease free water #12931 | 1032 µl |

- Prepare 150 µl of Tagmentation Buffer per reaction and place on ice.

| Tagmentation Buffer | Volume (per reaction) |
|---|---|
| High Salt Digitonin Buffer (described above) | 148.5 µl |
| Magnesium Chloride #86963 | 1.5 µl |

1. For each reaction, make a pAG-Tn5 pre-mix by diluting 2 µl of CUT&Tag pAG-Tn5 (Loaded) #79561 into 50 µl of High Salt Digitonin Buffer. Proportionally scale up the pAG-Tn5 pre-mix based on the number of reactions. Mix by gently pipetting up and down and place on ice.
2. Place the sample tubes containing the secondary antibody incubation solution from Section IV Step 4 on the magnetic rack for 30 sec to 2 min and then remove the supernatant.
3. Remove tubes from the magnetic rack and add 500 µl of Digitonin Buffer prepared in Section IV. Resuspend beads by gently pipetting up and down.
4. Place the tubes on the magnetic rack for 30 sec to 2 min and then remove the supernatant.
5. Repeat steps 3 and 4 one time for a second wash.
6. Remove tubes from magnetic rack. Add 50 µl of pAG-Tn5 pre-mix to each tube and gently mix the sample by pipetting up and down.
7. Incubate samples at room temperature for 1 hr.
8. Place the tubes on the magnetic separation rack for 30 sec to 2 min and then remove the supernatant.
9. Remove tubes from the magnetic separation rack. Add 500 µl of High Salt Digitonin Buffer and resuspend beads by gently pipetting up and down.
10. Place the tubes on the magnetic rack for 30 sec to 2 min and then remove the supernatant.
11. Repeat steps 9 and 10 one time for a second wash.

# CUT&Tag Protocol (continued)

12. Remove tubes from magnetic rack. Add 150 µl of Tagmentation Buffer to each tube and mix by pipetting up and down.
13. Incubate samples at 37°C for 1 hr.
14. To stop tagmentation, add 6.75 µl of 0.5 M EDTA #7011, 8.25 µl of 10% SDS #20533 and 1.5 µl of 20 mg/mL Proteinase K to each sample and mix by a quick vortex.
15. Incubate samples at 58°C for 1 hr to release tagmented chromatin fragments into solution. This incubation can be extended overnight. If incubating overnight, use safe-lock tubes to prevent sample evaporation.

**NOTE:** If starting with fixed cells or tissues, incubate samples at 65°C for 2 hr in safe-lock tubes to sufficiently reverse cross-links. This incubation can be extended overnight.

16. Centrifuge tubes at room temperature for 2 min at 16,000x g and place the tubes on a magnetic rack for 30 sec to 2 min.
17. Transfer the supernatants to new 1.5 ml tubes. These are your CUT&Tag DNA samples to be purified.
18. 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).

## VI. DNA Purification

### Before starting:

- Please equilibrate DNA Purification Columns, DNA Binding Buffer, DNA Wash Buffer, and DNA Elution Buffer to room temperature before use.
- **!!** Add 24 ml of ethanol (96-100%) to DNA Wash Buffer #10008 before use. This step only has to be performed once prior to the first set of DNA purifications.
- Use one DNA Purification collection tube #10010 for each CUT&Tag DNA sample to be purified.

1. Add 833 µl DNA Binding Buffer to each CUT&Tag DNA 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 µl 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 µl 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 30 µl of DNA Elution Buffer to each spin column and place into a clean 1.5 ml tube.

**NOTE:** To fully elute DNA from the columns, a minimum volume of 30 µl of DNA Elution Buffer is required.

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.

**NOTE:** Considering the typical low yield of CUT&Tag DNA, we strongly recommend using all of the 30 µl of DNA sample for library amplification.

## VII. 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 Systems platforms, we recommend using the CUT&Tag Dual Index Primers and PCR Master Mix for Illumina #47415. Please note that the DNA Library Prep Kit for Illumina Systems (ChIP-seq, CUT&RUN) #56795 and Multiplex Oligos for Illumina Systems (ChIP-seq, CUT&RUN) #29580 or #47538 are not compatible with CUT&Tag DNA samples.

## Additional Recommendations for DNA Library Preparation:

- The yield of the amplified CUT&Tag DNA library can vary based on the DNA quantification method used. If using the Nanodrop or QIAxpert Systems, the expected reading is 10-20 ng/µl for histone targets and 5-12 ng/µl for non-histone targets. If the library concentration is lower than 3 ng/µl with the Nanodrop or QIAxpert Systems, please refer to the troubleshooting guide before sequencing your samples. If using the Qubit Fluorometric Quantification system or the Picogreen assay, the expected reading is 3-10 ng/µl for histone targets and could be lower than 1 ng/µl for non-histone targets. Because of these low concentrations, the Bioanalyzer or TapeStation systems may generate a profile with very weak or even no visible peaks, especially for targets that are not abundant in cells. In these cases, we still recommend continuing with NGS if the positive control Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751 generates the expected library yield and/or Bioanalyzer peaks, indicative of an overall successful experiment. Please refer to our CUT&Tag FAQ web page for supporting data or if extra guidance is needed to pool together samples with a variety of yields.
- A sequencing depth of 2 million reads per sample is usually sufficient for CUT&Tag assay, regardless of target types. 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 one million reads per sample.
- If starting with less than 20,000 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 enough unique mapped reads for downstream data analysis.

## APPENDIX A: Fixed Cell Preparation

We strongly recommend using live cells whenever possible. For certain cell types that are fragile or sensitive to concanavalin A, please refer to the protocol below to lightly fix cells prior to the CUT&Tag experiment. Please note that cell fixation does not significantly increase CUT&Tag signals. In fact, over-fixation may lead to weaker CUT&Tag signals. Refer to the description in Section II to determine the appropriate cell number in each reaction.

**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 and Glycine Solution (10X) #7005.

### Before Starting:

**!** All buffer volumes should be scaled proportionally to the number of CUT&Tag reactions being performed.

- 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







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# CUT&Tag Protocol (continued)

manufacturer's expiration date.

- Thoroughly thaw 200X Protease Inhibitor Cocktail #7012 and 100X Spermidine #27287 before use and store them at -20°C when finished for the day. Please note that the Protease Inhibitor Cocktail #7012 will refreeze when placed on ice due to containing DMSO.
- Prepare Complete Wash Buffer (2 ml for each cell preparation and an additional 100 µl for each CUT&Tag reaction) and keep it at room temperature. For example, if using both untreated and drug-treated cells (2 cell preparations) and testing with 4 antibodies (positive control H3K4me3 #9751, negative control IgG #2729 or #68860 and two experimental antibodies; 8 reactions), a total of 4.8 ml of Complete Wash Buffer will be needed.

| Complete Wash Buffer | Volume (per cell preparation) | Volume (per reaction) | Total volume |
|---|---|---|---|
| 10X Wash buffer (CUT&RUN, CUT&Tag) #31415 | 200 µl | 10 µl | Add both columns together for total volume needed for each reagent. |
| 100X Spermidine #27287 | 20 µl | 1 µl | |
| Protease Inhibitor Cocktail (200X) #7012 | 10 µl | 0.5 µl | |
| Nuclease free water #12931 | 1770 µl | 88.5 µl | |

1. Harvest 100,000 live cells for each reaction.

**NOTE:** For adherent cells, detach them from the dish using Trypsin and neutralize with at least 3 volumes of tissue culture medium. We do not recommend scraping the cells from the dish to prevent cell lysis. Cells should be counted accurately using a hemocytometer or other cell counter to ensure the proper number of cells are being used for the experiment.

2. Add 2.7 µl of 37% formaldehyde or 6.25 µl of 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 µl 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 supernatant. 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:** If working with fewer than 100,000 total cells and the centrifuged cell pellet is not visible by eye, we do NOT recommend freezing down cell pellets. Instead, we recommend continuing on with the protocol and skipping the wash steps 5 to 7 below. After the initial centrifugation of the cell suspension in Step 4, remove most of the supernatant, leaving behind ≤40 µl cell medium per reaction. Then in Step 8 add enough Complete Wash Buffer to the cell suspension to achieve a total volume of 100 µl per reaction.

5. Resuspend cell pellet in 1 ml of Complete Wash Buffer by gently pipetting up and down.
6. Centrifuge for 3 min at 3,000 x g at 4°C and remove the supernatant.
7. Wash the cell pellet a second time by repeating steps 5 and 6 one time.
8. For each reaction, add 100 µl of Complete Wash Buffer and resuspend the cell pellet by gently pipetting up and down.
9. Immediately proceed to Section III.

## APPENDIX B: Fixed Tissue Sample Preparation

For most tissue types, 1 mg of fresh tissue is sufficient to generate robust enrichment of histones. If fresh tissue is not accessible, lightly fixed tissue (0.1% formaldehyde for 2 min) can be used. Fixed tissue samples can be frozen at -80°C up to 6 months before using. Over-fixation may lead to weaker CUT&Tag signals. The CUT&Tag assay does not work well for enrichment of transcription factors and cofactors from tissues. For analysis of transcription factors and cofactors, we recommend using the CUT&RUN Assay Kit #86652.

**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, and Glycine Solution (10X) #7005.

### Before Starting:

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

- 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.
- Prepare 100 µl of Glycine Solution (10X) #7005 per 1 ml of fixation buffer.
- Thoroughly thaw 200X Protease Inhibitor Cocktail #7012 and 100X Spermidine #27287 before use and store them at -20°C when finished for the day. Please note that the Protease Inhibitor Cocktail #7012 will refreeze when placed on ice due to containing DMSO.
- Prepare Complete Wash Buffer (3 ml for each tissue type and additional 100 µl for each reaction) and keep it at room temperature to minimize stress on the cells.

| Complete Wash Buffer | Volume (per tissue type) | Volume (per reaction) | Total volume |
|---|---|---|---|
| 10X Wash buffer (CUT&RUN, CUT&Tag) #31415 | 300 µl | 10 µl | Add both columns together for total volume needed for each reagent. |
| 100X Spermidine #27287 | 30 µl | 1 µl | |
| Protease Inhibitor Cocktail (200X) #7012 | 15 µl | 0.5 µl | |
| Nuclease free water #12931 | 2655 µl | 88.5 µl | |

- Prepare 1 ml Fixation Buffer for each tissue type. Use fresh formaldehyde that is not past the manufacturer's expiration date.

| Fixation Buffer | Volume (per tissue type) |
|---|---|
| Formaldehyde | 2.7 µl of 37% or 6.25 µl of 16% |
| Protease Inhibitor Cocktail (200X) #7012 | 5 µl |
| Phosphate Buffered Saline (PBS) #9872 | 992.3 µl |

- Prepare 1 ml of Fixation Wash Buffer for each tissue type and place on ice.

# CUT&Tag Protocol (continued)

| Fixation Wash Buffer | Volume (per tissue type) |
|---|---|
| Protease Inhibitor Cocktail (200X) #7012 | 5 µl |
| Phosphate Buffered Saline (PBS) #9872 | 995 µl |

1. Weigh 1 mg fresh tissues for each reaction.
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.
3. Immediately transfer minced tissue to 1 ml of Fixation Buffer 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 the amount of Fixation Buffer used in Step 3 and Fixation Wash Buffer used in Step 7 accordingly.

4. Incubate at room temperature for 2 min.
5. Stop cross-linking by adding 100 µl 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 supernatant.
7. Resuspend tissue with 1 ml of Fixation Wash Buffer.
8. Centrifuge for 5 min at 2,000 x g at 4°C and remove the supernatant 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 Complete Wash Buffer 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 Complete Wash Buffer.
13. Centrifuge cell suspension for 3 min at 3,000 x g at room temperature and remove the supernatant.
14. Wash the cell pellet a second time by repeating steps 12 and 13 one time.
15. For each reaction, add 100 µl of Complete Wash Buffer and resuspend the cell pellet by gently pipetting up and down.
16. Immediately proceed to Section III.

## APPENDIX C: Determination of Cell Sensitivity to Digitonin

In the CUT&Tag protocol, the addition of digitonin to the buffers facilitates the permeabilization of cell membranes and entry of the primary antibody, secondary antibody, and pAG-Tn5 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 show differing 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:

- Warm Digitonin Solution #16359 at 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 during use. Store at -20°C when finished for the day.
- Prepare 100 µl of 1X Wash Buffer per reaction. It is not necessary to add spermidine or Protease Inhibitors in the buffer for this test.

| 1X Wash Buffer | Volume (per reaction) |
|---|---|
| 10X Wash Buffer (CUT&RUN, CUT&Tag) #31415 | 10 µl |
| Nuclease-free Water #12931 | 90 µl |

1. In a 1.5 ml tube, collect 100,000 cells (from Section II-A, Step 1), centrifuge for 3 min at 600 x g at room temperature and withdraw the supernatant. For tissue, collect disaggregated cells from 1 mg of tissue (from Section II-B, Steps 1-8).
2. Resuspend cell pellet in 100 µl of 1X Wash Buffer.
3. Add 2.5 µl Digitonin Solution #16359 to each reaction and incubate for 10 min at room temperature.
4. Mix 10 µl of cell suspension with 10 µl of 0.4% trypan blue stain.
5. 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.
6. If less than 90% of cells stain with Trypan blue, then increase the amount of Digitonin Solution #16359 added to each reaction and repeat steps 1-5 until > 90% cells are permeabilized and stained. Use this amount of Digitonin Solution #16359 in Sections I - V.

## APPENDIX D: Troubleshooting Guide

For a detailed troubleshooting guide, please go to cst-science.com/troubleshooting-CUT-Tag.