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## Bench top CUT&amp;Tag V.3

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1 Works for me dx.doi.org/10.17504/protocols.io.bcuihw6

Human Cell Atlas Method Development Community



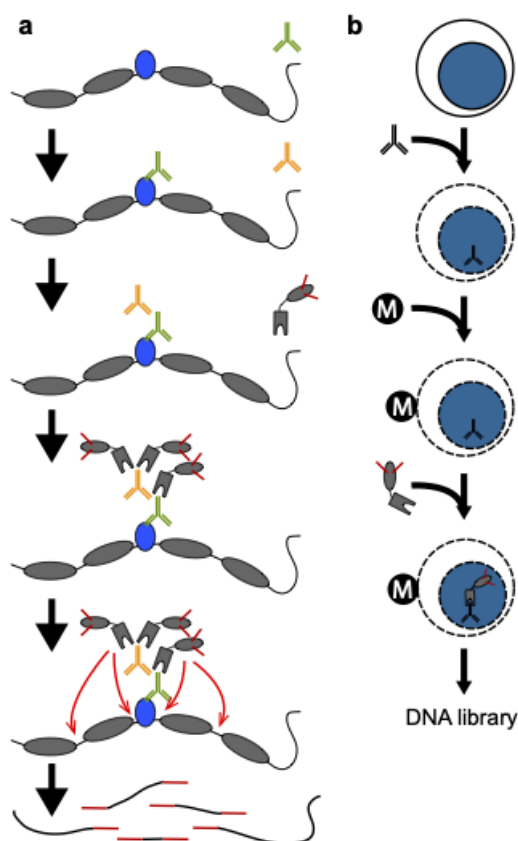
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## ABSTRACT

Here we describe a bench top protocol for Cleavage Under Targets and Tagmentation (CUT&Tag), an enzyme-tethering strategy that provides efficient high-resolution sequencing libraries for profiling diverse chromatin components. In CUT&Tag, a chromatin protein is bound in situ by a specific antibody, which then tethers a proteinA-Tn5 transposase fusion protein. Activation of the transposase efficiently generates fragment libraries with high resolution and exceptionally low background. All steps from live cells to sequencing-ready libraries can be performed in one day. We have demonstrated the utility of CUT&Tag by profiling histone modifications, RNA Polymerase II and transcription factors on low cell numbers and single cells. Version 3 includes an optional light formaldehyde treatment of cells or nuclei prior to antibody addition. Light fixation reduces the tendency of cells or nuclei to clump, which can also be reduced for fixed and unfixed nuclei by omitting digitonin from all steps.



In situ tethering for CUT&Tag chromatin profiling. **a)** The steps in CUT&Tag. Added antibody (green) binds to the target chromatin protein (blue) between nucleosomes (gray ovals) in the genome, and the excess is washed away. A second antibody (orange) is added and enhances tethering of pA-Tn5 transposome (gray boxes) at antibody-bound sites. After washing away excess transposome, addition of  $Mg^{++}$  activates the transposase and integrates adapters (red) at chromatin protein binding sites. After DNA purification genomic fragments with adapters at both ends are enriched by PCR. **b)** CUT&Tag is performed on a solid support. Unfixed cells or nuclei (blue) are permeabilized and mixed with antibody to a target chromatin protein. After addition and binding of cells to Concanavilin A-coated magnetic beads (M), all further steps are performed in the same reaction tube with magnetic capture between washes and incubations, including pA-Tn5 tethering, integration, and DNA purification.

## EXTERNAL LINK

<https://www.nature.com/articles/s41467-019-09982-5>

Kaya-Okur HS, Wu SJ, Codomo CA, Pledger ES, Bryson TD, Henikoff JG, Ahmad K, Henikoff S: CUT&Tag for efficient epigenomic profiling of small samples and single cells. *Nature Communications* 2019 10:1930 (PMID:31036827).

## GUIDELINES

### CUT&Tag for efficient epigenomic profiling of small samples and single cells

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## MATERIALS TEXT

- Standard (not 'lobind') microfuge tubes (0.5 ml, 1.5 ml and 2 ml)
- 0.5 ml PCR tubes (LabSource T54-252)
- Cell suspension. We have used human K562 cells and H1 hESCs
- Distilled, deionized or RNase-free H<sub>2</sub>O (dH<sub>2</sub>O e.g., Promega, cat. no. P1197)
- 1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.9 (HEPES (K+); Sigma-Aldrich, cat. no. H3375)
- 1 M Potassium Chloride (KCl; Sigma-Aldrich, cat. no. P3911)
- 10% Triton X-100 (Sigma-Aldrich, cat. no. X100)
- 2 M Spermidine (Sigma-Aldrich, cat. no. S0266)
- Glycerol
- Roche Complete Protease Inhibitor EDTA-Free tablets (Sigma-Aldrich, cat. no. 5056489001)
- Phosphate-buffered saline (1X PBS, 10X stock solution from Fisher cat. no. BP3994)
- 16% (w/v) formaldehyde (10 x 1 ml ampules, Thermo-Fisher ca. no. 28906)
- 2.5 or 1.25 M glycine
- Dimethyl sulfoxide (DMSO; Sigma-Aldrich cat. no. D4540)
- Concanavalin A (ConA)-coated magnetic beads (Bangs Laboratories, ca. no. BP531).
- Antibody to an epitope of interest. Some commercial antibodies are marketed as "ChIP-grade", but antibody binding for ChIP occurs in solution whereas antibody binding for CUT&RUN and CUT&Tag occurs in situ. Because in situ binding conditions are more like those for immunofluorescence (IF) than those for ChIP, we suggest choosing IF-tested antibodies if CUT&RUN-tested antibodies are not available.
- Positive control antibody to an abundant epitope, *e.g.*  $\alpha$ -H3K27me3 rabbit monoclonal antibody (Cell Signaling Technology, cat. no. 9733)
- Secondary antibody, *e.g.* guinea pig  $\alpha$ -rabbit antibody (Antibodies online cat. no. ABIN101961) and rabbit  $\alpha$ -mouse antibody (Abcam cat. no. ab46540).
- 5% Digitonin (EMD Millipore, cat. no. 300410)
- Protein A–Tn5 (pA–Tn5) fusion protein. Store at -20 °C.
- Double-stranded adapters with 19mer Tn5 mosaic ends (Sequence information was derived from Picelli, S. et al. *Genome Res* 24, 2033-2040 (2014), and ordered through Eurofins, 100  $\mu$ M in TE buffer)
- Mosaic end\_reverse [PHO]CTGTCTCTTATACACATCT
- Mosaic end\_Adapter A TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
- Mosaic end\_Adapter B GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG
- 1 M Manganese Chloride (MnCl<sub>2</sub>; Sigma-Aldrich, cat. no. 203734)
- 1 M Calcium Chloride (CaCl<sub>2</sub>; Fisher, cat. no. BP510)
- 1 M Potassium Chloride (KCl; Sigma-Aldrich, cat. no. P3911)
- 100 mM Magnesium Chloride (MgCl<sub>2</sub>; Sigma-Aldrich, cat. no. M8266-100G)
- 1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.5 (HEPES (Na+); Sigma-Aldrich, cat. no. H3375)

- 5 M Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S5150-1L)
- 0.5 M Ethylenediaminetetraacetic acid (EDTA; Research Organics, cat. no. 3002E)
- 30% Bovine Serum Albumen (BSA, Sigma-Aldrich, cat. no. A8577)
- 10% Sodium dodecyl sulfate (SDS; Sigma-Aldrich, cat. no. L4509)
- Proteinase K (20 mg/ml Thermo Fisher Scientific, cat. no. E00492)
- Phase-lock tubes (Qiagen MaXtract High Density cat. no. 129046)
- Phenol-chloroform-isoamyl alcohol 25:24:1 (PCI) Invitrogen - Thermo Fisher, cat. no. 15593049)
- Chloroform 366919-1L Sigma
- SPRI paramagnetic beads (e.g. Agencourt AMPure XP, Beckman Coulter, cat. no. A63880)
- 1 M Tris-HCl pH 8.0
- Ethanol (Decon Labs, cat. no. 2716)
- NEBNext HiFi 2x PCR Master mix
- PCR primers: 10  $\mu$ M stock solutions of a universal i5 primer and 16 i7 primers with unique barcodes [Buenrostro, J.D. et al. Nature 523:486 (2015)] in 10 mM Tris pH 8. Standard salt-free primers may be used. Do not use Nextera primers.

#### SAFETY WARNINGS

Digitonin is toxic and care should be taken especially when weighing out the powder. Use full PPE including a mask, lab coat and gloves while handling any amount of digitonin.

#### BEFORE STARTING

Prepare reagents (STEP 1)

## REAGENT SETUP (for up to 16 samples)

- 1 **NE1 buffer (for preparing nuclei)** Mix 1 ml 1M HEPES-KOH pH 7.9, 500  $\mu$ L 1M KCl, 12.5  $\mu$ L 2 M spermidine, 500  $\mu$ L 10% Triton X-100, and 10 ml glycerol in 38 ml dH<sub>2</sub>O, and add 1 Roche Complete Protease Inhibitor EDTA-Free

**Digitonin (5%)** Dissolve 50 mg digitonin in 1 ml DMSO. Hold at room temperature for up to 1 week, or freeze at -20 °C. When using native or fixed nuclei prepared in Triton-X100, digitonin may be omitted from all solutions.

**Caution:** Digitonin is toxic and care should be taken especially when weighing out the powder. Use full PPE including a mask, lab coat and gloves while handling any amount of digitonin. Be aware that DMSO can penetrate through the skin.

**Binding buffer** Mix 200  $\mu$ L 1M HEPES pH 7.9, 100  $\mu$ L 1M KCl, 10  $\mu$ L 1M CaCl<sub>2</sub> and 10  $\mu$ L 1M MnCl<sub>2</sub>, and bring the final volume to 10 mL with dH<sub>2</sub>O. Store the buffer at 4 °C for 6 months.

**Wash buffer** Mix 1 mL 1 M HEPES pH 7.5, 1.5 mL 5 M NaCl, 12.5  $\mu$ L 2 M spermidine, bring the final volume to 50 mL with dH<sub>2</sub>O, and add 1 Roche Complete Protease Inhibitor EDTA-Free tablet. Store the buffer at 4 °C for up to 1 week.

**Dig-wash buffer (optional for nuclei)** Mix 400  $\mu$ L 5% digitonin with 40 mL Wash buffer. Store the buffer at 4 °C for up to 2 days.

**Antibody buffer** Mix 8  $\mu$ L 0.5 M EDTA and 6.7  $\mu$ L 30% BSA with 2 mL Dig-wash buffer and chill on ice.

**Dig-300 buffer** Mix 1 mL 1 M HEPES pH 7.5, 3 mL 5 M NaCl and 12.5  $\mu$ L 2 M spermidine, bring the final volume to 50 mL with dH<sub>2</sub>O, and add 100  $\mu$ L 5% digitonin (0.01%) and 1 Roche Complete Protease Inhibitor EDTA-Free tablet. Buffer without digitonin may be held for 1 week, but Digitonin should only be added the same day to minimize precipitation.

**Tagmentation buffer** Mix 5 mL Dig-300 buffer and 50  $\mu$ L 1 M MgCl<sub>2</sub>.

### Tn5-adapter complex formation:

1. Anneal each of Mosaic end - adapter A (ME-A) and Mosaic end - adapter B (ME-B) oligonucleotides with Mosaic end – reverse oligonucleotides.
2. To anneal, dilute oligonucleotides to 200  $\mu$ M in annealing buffer (10mM Tris pH8, 50mM NaCl, 1 mM EDTA). Each pair of oligos, ME-A+ME-Reverse and ME-B+ME-Reverse, is mixed separately resulting in 100  $\mu$ M annealed product.
3. Place the tubes in a 90-95°C hot block and leave for 3-5 minutes, then remove the hot block from the heat source allowing for slow cooling to room temperature (~45 minutes).
4. Mix 8  $\mu$ L 100  $\mu$ M preannealed ME-A and 8  $\mu$ L 100  $\mu$ M preannealed ME-B with 100  $\mu$ L 5.5  $\mu$ M pA-Tn5.
5. Incubate the mixture on a rotating platform for 1 hour at room temperature and then store at -20 °C.

## Optionally prepare and lightly fix cells or nuclei and cryopreserve (1 hr)

- 2 Harvest fresh culture(s) in a conical centrifuge tube (15 ml or 50 ml) at room temperature and count cells. The same protocol can be used for up to ~500,000 mammalian (e.g., human K562 or H1 embryonic stem cells) cells per sample to be sequenced.



For fresh or frozen tissues, we recommend adapting tissue preparation procedures developed for CUT&RUN:

<https://www.protocols.io/view/cut-run-with-drosophila-tissues-umfeu3n> and

<https://epigeneticsandchromatin.biomedcentral.com/articles/10.1186/s13072-018-0243-8> (Figures 5-6). Scraping or Accutase solution (Stem Cell Technologies cat. no. 07920) can be used to detach adherent cultured cells.

- 3 Centrifuge 3 min 600 x g in a swinging bucket rotor at room temperature and drain liquid.
- 4 Resuspend in 1 volume PBS at room temperature while pipetting. For fixed cells skip to Step 9.
- 5 Centrifuge 3 min 600 x g in a swinging bucket rotor at room temperature and drain liquid.

- 6 Resuspend in 1/2 volume ice-cold NE1 buffer with gentle vortexing. Let sit on ice 10 min.
- 7 Centrifuge 4 min 1300 x g at 4 °C in a swinging bucket rotor and drain liquid by pouring off and inverting onto a paper towel for a few seconds.
- 8 Resuspend in 1/2 volume of PBS (relative to starting culture). For unfixed nuclei, skip to Step 11.
- 9 While gently vortexing add 16% formaldehyde to 0.1% (e.g., 62 µL to 10 ml) and incubate at room temperature for 2 minutes.



Based on: Oh KS, Ha J, Baek S, Sung MH. XL-DNase-seq: improved footprinting of dynamic transcription factors. *Epigenetics Chromatin* 2019;12(1):30.

- 10 Stop cross-linking by addition of 1.25 M glycine to twice the molar concentration of formaldehyde (e.g., 600 µL to 10 ml).
- 11 Centrifuge 4 min 1300 x g at 4 °C and drain liquid by pouring off and inverting onto a paper towel for a few seconds.
- 12 Resuspend in Wash buffer to a concentration of ~1 million cells per ml. Check nuclei using ViCell or cell counter slide.
- 13 Nuclei may be slowly frozen by aliquoting 900 µL into cryogenic vials containing 100 µL DMSO, mixed well, then placed in a Mr. Frosty container filled to the line with isopropanol and placed in a -80 °C freezer overnight then stored at -80 °C.
- 14 To begin the CUT&Tag protocol, thaw frozen cell or nuclei (native or X-linked) aliquots at room temperature, for example by placing in a 20 ml beaker of water. Skip to Step 19.



Light fixation reduces the tendency of cells or nuclei to clump in the Dig-300 buffer, but can interfere with binding of some antibodies, reducing yield. Clumping can also be reduced for fixed and unfixed nuclei prepared using this protocol by omitting digitonin from all steps. We have not observed any difference in CUT&Tag results for nuclei prepared using Triton-X100 with or without digitonin.

**CRITICAL STEP:** When using unfixed cryopreserved cells or nuclei, we recommend that cavitation during resuspension and vigorous vortexing be avoided.

#### Prepare fresh cells (30 min)

- 15 Harvest fresh culture(s) at room temperature and count cells.

**CRITICAL STEP:** When using fresh cells, all steps prior to the cell permeabilization are performed at room temperature to minimize stress on the cells. We recommend that cavitation during resuspension and vigorous vortexing be avoided.



For fresh or frozen tissues, we recommend adapting tissue preparation procedures developed for CUT&RUN: <https://www.protocols.io/view/cut-run-with-drosophila-tissues-umfeu3n> and <https://epigeneticsandchromatin.biomedcentral.com/articles/10.1186/s13072-018-0243-8> (Figures 5-6). Scraping or Accutase solution (Stem Cell Technologies cat. no. 07920) can be used to detach adherent cultured cells.

- 16 Centrifuge 3 min 600 x g at room temperature and withdraw liquid.

- 17 Resuspend in at least 1 volume Wash buffer at room temperature, centrifuge 3 min 600 x g at room temperature and withdraw liquid.
- 18 Resuspend in 1.5 mL Wash buffer and transfer to a 2 mL tube and hold on ice until beads are ready.

#### Bind cells or nuclei to Concanavalin A-coated beads (30 min)

- 19 Gently resuspend and withdraw enough of the ConA bead slurry such that there will be 10  $\mu$ L for each final sample of up to 500,000 cells. The following is for 16 samples.
- 20 Transfer 170  $\mu$ L ConA bead slurry into 1.6 mL Binding buffer in a 2 mL tube and mix by pipetting. Place the tube on a magnet stand to clear (30 s to 2 min).
- 21 Withdraw the liquid completely, and remove from the magnet stand. Add 1.5 mL Binding buffer, mix by pipetting, remove liquid from the cap with a quick pulse on a microcentrifuge.
- 22 Place on magnet stand to clear, withdraw liquid, and resuspend in 170  $\mu$ L Binding buffer (10  $\mu$ L per sample) and hold the bead slurry at room temperature until fresh cells are ready or the frozen aliquot is thawed.
- 23 Retrieve the tubes with cells or nuclei, and while vortexing gently (1100 rpm), slowly add the bead slurry. Place on an end-over-end rotator for 5-10 min.

#### Bind primary antibody (2 hr to overnight)

- 24 After a quick spin to remove liquid from the cap (<100 x g), place the tubes on a magnet stand to clear and withdraw the liquid.
- 25 Resuspend cells in 800  $\mu$ L ice-cold Antibody buffer with gentle vortexing, place on ice, and divide into 16 1.5 mL tubes, 50  $\mu$ L each (for 16 samples; scale up or down as needed).
- 26 Add 0.5-1  $\mu$ L primary antibody to each sample with gentle vortexing.

CRITICAL STEP: We use 1:50 - 1:100 by default or the manufacturer's recommended concentration for immunofluorescence.

- 27 Nutate (or rotate) at room temperature for 2 hr or nutate overnight to several days at 4 °C. Liquid should remain in the bottom and on the side of the tube while rocking.

**CRITICAL STEP:** To evaluate success of the procedure without requiring library preparation, include in parallel a positive control antibody (e.g.  $\alpha$ -H3K27me3), and optionally a negative control antibody (e.g. rabbit  $\alpha$ -mouse IgG).



We have not noticed any difference between the efficiency of a 2 hr room temperature incubation and an overnight 4 °C incubation. We have successfully performed CUT&RUN and CUT&Tag after 4 °C incubation for up to 5 days.

#### Bind secondary antibody (1 hr)

- 28 After a quick spin to remove liquid from cap (<100 x g), place each tube on the magnet stand to clear and pull off the liquid.

- 29 Mix the secondary antibody 1:100 in Dig-wash buffer and squirt in 100  $\mu$ L per sample while gently vortexing to allow the solution to dislodge the beads from the sides.



Although not needed for CUT&RUN, the secondary antibody step is required for CUT&Tag to increase the number of Protein A binding sites for each bound antibody. We have found that without the secondary antibody the efficiency is very low.



Protein A (and Protein G) do not bind well to some antibodies, so if intending to use a different secondary antibody from those recommended (guinea pig anti-rabbit and rabbit anti-mouse for Protein A), check [here](#) first.

- 30 Place the tubes on a nutator at room temperature for 30–60 min. ⌚ 00:30:00

- 31 After a quick spin, place the tubes on a magnet stand to clear and withdraw the liquid.

- 32 Add 1 mL Dig-wash buffer. Invert 10x or gently vortex to allow the solution to dislodge most or all of the beads.

- 33 Repeat Steps 31-32 twice.

#### Bind pA-Tn5 adapter complex (1.5 hr)

- 34 Mix pA-Tn5 adapter complex in Dig-300 buffer to a final concentration of 1:250 for 100  $\mu$ L per sample.

**CRITICAL STEP:** pA-Tn5 aliquots received from the CUT&RUN team are pre-loaded with adapters suitable for single- or dual-indexing on a paired-end Illumina flow-cell platform.

- 35 After a quick spin, place the tubes on the magnet stand to clear and pull off the liquid.

- 36 Squirt in 100  $\mu$ L of the pA-Tn5 mix while gently vortexing to allow the solution to dislodge most or all of the beads.



The increased NaCl is necessary to avoid pA-Tn5 binding to accessible sites in chromatin, but can result in clumping, and in the presence of 0.05% digitonin can cause cell lysis. By reducing the digitonin concentration to 0.01% (from 0.05%) in the 300 mM NaCl buffer these problems are minimized. Barely visible clumps may appear when beads are suspended, but this does not appear to affect the efficiency of incubations or washes. Light fixation of cells or nuclei reduces or eliminates clumping, and digitonin can be omitted for nuclei for nuclei prepared in Triton-X100.

- 37 Place the tubes on a nutator at room temperature for 1 hr. ⌚ 01:00:00

- 38 After a quick spin, place the tubes on a magnet stand to clear and pull off the liquid.

- 39 Add 1 mL Dig-300 buffer. Invert 10x or gently vortex to allow the solution to dislodge most or all of the beads.

- 40 Repeat steps 38-39 twice.

## Tagmentation (1 hr)

- 41 After a quick spin, place the tube on the magnet stand to clear and pull off the liquid.
- 42 Add 300  $\mu$ L Tagmentation buffer while gently vortexing.
- 43 Incubate at 37 °C for 1 hr in a water bath or incubator. ⌚ 01:00:00

## DNA extraction (1 hr)

- 44 To stop tagmentation and solubilize DNA fragments, add 10  $\mu$ L 0.5M EDTA, 3  $\mu$ L 10% SDS and 2.5  $\mu$ L 20 mg/mL Proteinase K to each sample.
- 45 Mix by full-speed vortexing ~2 s, and incubate 1 hr 55 °C to digest (and reverse cross-links). ⌚ 01:00:00



It is typical for the beads to form a large clump during incubation with Proteinase K and SDS owing to the viscoelasticity of DNA. However, for abundant genome-wide epitopes, large-scale fragmentation of the genome will normally result in reduced clumping and release of beads into suspension, turning the liquid brownish relative to negative controls.



Step 45) Left: H3K27me3; Right: IgG.

- 46 Add 300  $\mu$ L PCI and mix by full-speed vortexing ~2 s.



Do not separate the liquid from the beads, but rather add the PCI directly to the aqueous mix and extract the whole mixture.

- 47 Transfer to a phase-lock tube, and centrifuge 3 min room temperature at 16,000 x g.
- 48 Add 300  $\mu$ L Chloroform and invert ~10x to mix (do not vortex). Centrifuge 3 min room temperature at 16,000 x g.
- 49 Remove the aqueous layer by pipetting to a fresh 1.5 mL tube containing 750  $\mu$ L 100% ethanol, pipetting up and down to mix.



Do not add glycogen, which will inhibit the PCR. No carrier is needed owing to high concentration of endogenous RNA and DNA relative to tagmented fragments.

- 50 Chill on ice and centrifuge at least 10 min at 4 °C 16,000 x g.



- 51 Carefully pour off the liquid and drain on a paper towel. There is typically no visible pellet.
- 52 Rinse in 1 mL 100% ethanol and centrifuge 1 min at 4 °C 16,000 x g.
- 53 Carefully pour off the liquid and drain on a paper towel. Air dry.
- 54 When the tube is dry, dissolve in 25-30 µL 1 mM Tris-HCl pH 8, 0.1 mM EDTA and vortex on full to dissolve.

#### PCR (1 hr)

- 55 21 µL DNA + 2 µL of 10 µM Universal or barcoded i5 primer + 2 µL of 10 µM uniquely barcoded i7 primer. In a thin-wall 0.5 ml PCR tube, using a different barcode for each sample\*. Save remaining DNA as a backup.

\*Indexed primers described by Buenrostro, J.D. et al. Single-cell chromatin accessibility reveals principles of regulatory variation. Nature 523:486 (2015).

- 56 Add 25 µL NEBNext HiFi 2x PCR Master mix.
- 57 1. Mix, quick spin and place in Thermocycler and begin cycling program with heated lid.

Cycle 1: 72 °C for 5 min (gap filling)

Cycle 2: 98 °C for 30 sec

Cycle 3: 98 °C for 10 sec

Cycle 4: 63 °C for 10 sec

Repeat Cycles 3-4 13 times

72°C for 1 min and hold at 8 °C

**CRITICAL STEP:** To minimize the contribution of large DNA fragments and excess primers, PCR should be performed for 12-14 cycles, preferably with a 10 s 60-63°C combined annealing/extension step.



The cycle times are based on using conventional Peltier cycler (e.g., BioRad/MJ Tetrad), in which the ramping times are sufficient for annealing to occur as the sample cools from 98 °C to 60 °C. Therefore, the use of a rapid cycler with a high ramping rate will require adjustment to assure annealing.



Do not add extra PCR cycles to see a signal by capillary gel electrophoresis (e.g. TapeStation). If there is no nucleosomal ladder for the H3K27me3 positive control, you may assume that CUT&Tag failed, but observing no signal for a sparse chromatin protein such as a transcription factor is normal (see the CTCF example in Steps 66-69), and the barcoded sample can be concentrated for mixing with the pool of barcoded samples for sequencing. Extra PCR cycles reduce the complexity of the library and may result in an unacceptable level of PCR duplicates.

### Post-PCR Clean-up (30 min)

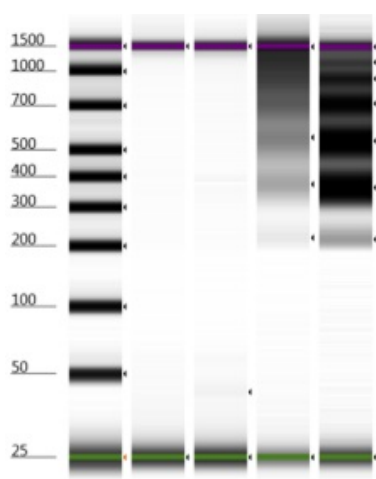
- 58 After tubes have cooled, remove from the cyclor and add 1.3 volume (65  $\mu$ L) SPRI bead slurry, mixing by pipetting up and down.
- 59 Quick spin and let sit at room temperature 5-10 min. ⌚ 00:10:00
- 60 Place on magnet and allow to clear before carefully withdrawing liquid. On magnet and without disturbing the beads, add 200  $\mu$ L 80% ethanol.
- 61 Withdraw liquid with a pipette to the bottom of the tube and add 200  $\mu$ L 80% ethanol.
- 62 Withdraw liquid and after a quick spin remove the remaining liquid with a 20  $\mu$ L pipette. Do not air-dry the beads, but proceed immediately to the next step.
- 63 Remove from magnet stand, add 25  $\mu$ L 10 mM Tris-HCl pH 8 and vortex on full.
- 64 After 5 min place on magnet stand and allow to clear. ⌚ 00:05:00
- 65 Remove liquid to a fresh 0.5 ml tube with a pipette.

### DNA sequencing and data processing

- 66 Determine the size distribution and concentration of libraries by capillary electrophoresis using an Agilent 4200 TapeStation with D1000 reagents or equivalent.



A representative TapeStation image



Human K562 cells. Left to right: Markers, IgG (guinea pig anti-rabbit negative control), CTCF, H3K4me3, H3K27me3. In the rightmost 2 lanes, the band at  $\sim$ 350 represents mononucleosomes with oligonucleosomes above, and the band at 200 include fragments that are released from nucleosome-depleted regions adjacent to the antibody-tethered nucleosome. The entire CTCF sample was concentrated and used for sequencing, resulting in a satisfactory profile (Step 69).

- 67 Mix barcoded libraries to achieve equal representation as desired aiming for a final concentration as recommended by the manufacturer. After mixing, perform an SPRI bead cleanup if needed to remove any residual PCR primers.
- 68 Perform paired-end Illumina sequencing on the barcoded libraries following the manufacturer's instructions (e.g. PE25x25 on a HiSeq 2500 with 1% Phi-X spike-in).



Using paired-end 25x25 sequencing on a HiSeq 2-lane rapid run flow cell we obtain ~300 million total mapped reads, or ~3 million per sample when there are 96 samples mixed to obtain approximately equal molarity.

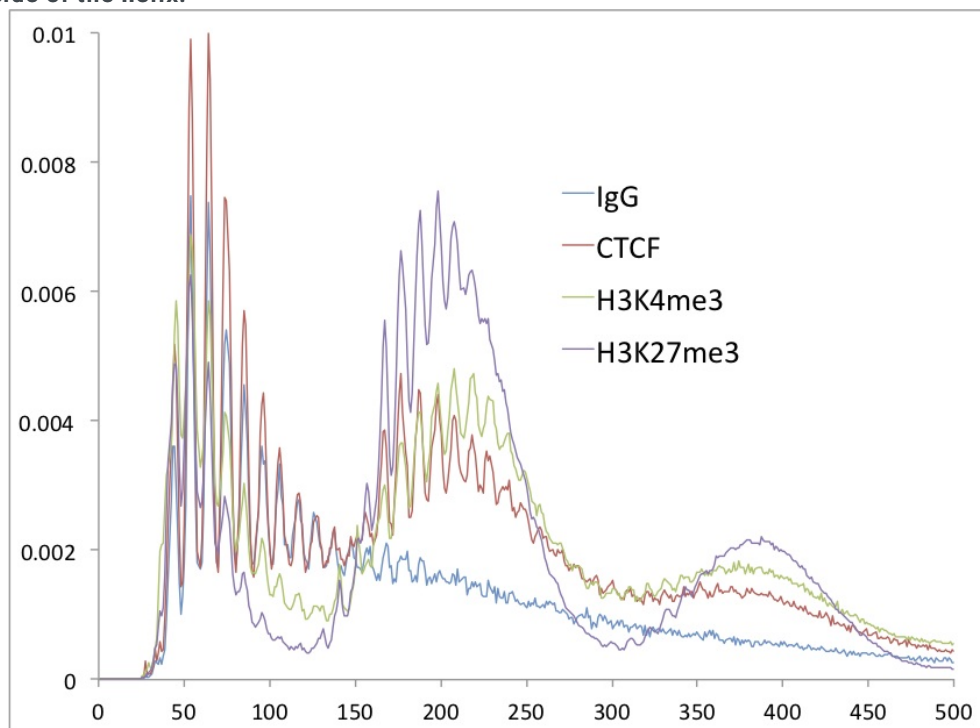
- 69 We align paired-end reads using Bowtie2 version 2.3.4.3 with parameters: `--end-to-end --very-sensitive --no-mixed --no-discordant --phred33 -l 10 -X 700`.

We align E. coli carry-over fragments to the NCBI Ecoli genome (Escherichia coli str. K12 substr. MG1655 U00096.3) with `--no-overlap --no-dovetail` options (`--end-to-end --very-sensitive --no-overlap --no-dovetail --no-mixed --no-discordant --phred33 -l 10 -X 700`) to avoid possible cross-mapping of the experimental genome to that of the carry-over E. coli DNA that is used for calibration.

To calibrate samples in a series for samples done in parallel using the same antibody we use counts of E. coli fragments carried over with the pA-Tn5 the same as one would for an ordinary spike-in. Our [sample script in Github](#) can be used to calibrate based on either a spike-in or E. coli carry-over DNA.



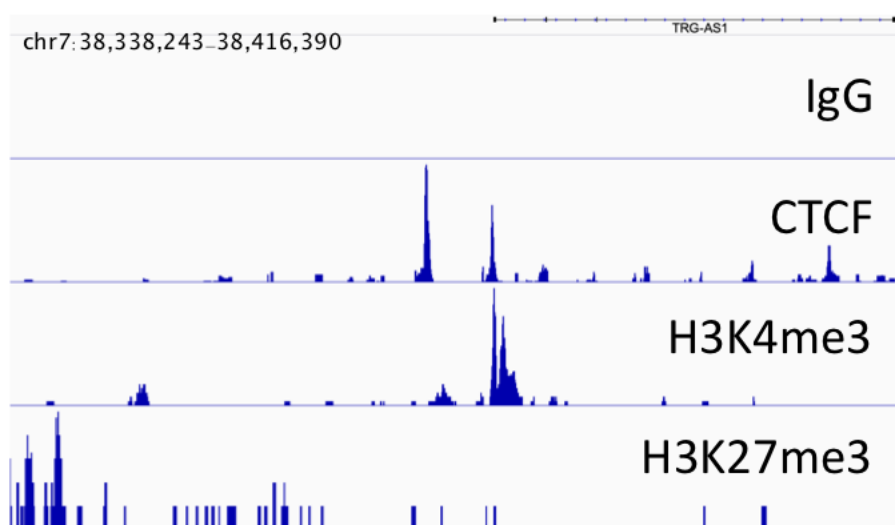
**Fragment lengths show a 10-bp periodicity suggestive of preferential cut-and-paste events on one side of the helix.**



Fraction of sequenced fragments plotted as a function of fragment length for the samples shown in Step 65.



Because of the low backgrounds with CUT&Tag, 2-3 million paired-end reads is sufficient to effectively profile histone modifications and transcription factors, even for the human genome. For maximum economy, we mix up to 96 barcoded samples per 2-lane flow cell, and perform paired-end 25x25 bp sequencing.

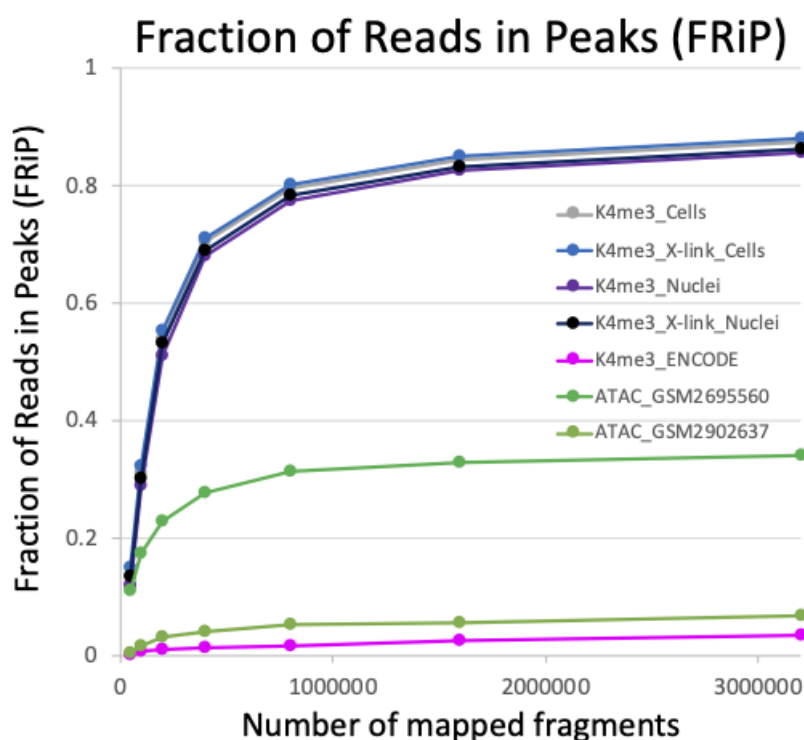


IGV tracks from the same four samples showing a promoter region. Average 2.1 million reads/sample, autoscaled. Despite the low sequencing depth for all of the samples, strong peaks are detected above the sparse background.

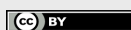
- 70 Most data analysis tools used for ChIP-seq data, such as [bedtools](#), [Picard](#) and [deepTools](#), can be used on CUT&Tag data. Analysis tools designed specifically for CUT&RUN/Tag data include the [SEACR peak caller](#) also available as a [public web server](#), [CUT&RUNTools](#) and [henipipe](#).



CUT&Tag provides high signal-to-noise and reproducibility for native and lightly cross-linked cells and nuclei.



H3K4me3 CUT&Tag was performed on cryopreserved aliquots of native and cross-linked human K562 cells and nuclei (500,000 starting cells per sample). Aliquots were thawed for H3K4me3 CUT&Tag (Active Motif cat# 39159, lot 22118006) on two different occasions. The 8 barcoded libraries were mixed with 70 others and sequenced. Pairs of biological replicates were pooled and MACS2 was used to call peaks. The Fraction of Reads in Peaks (FRiP) was calculated following [ENCODE recommendations for evaluating data quality](#) (PMID:22955991). The ENCODE ChIP-seq standard is to sequence at least 10 million unique reads until  $\geq 1\%$  FRiP is achieved, however, Bench top CUT&Tag reaches 80% FRiP with only ~800,000 sequenced read pairs for both native and cross-linked cells and nuclei. This high information content of CUT&Tag reflects the low background and high reproducibility of antibody-targeted tagmentation relative to K562 cell ChIP-seq data (ENCODE, magenta) or untargeted chromatin tagmentation data from two different groups (ATAC-seq, green).



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