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Bench top CUT&Tag V.2

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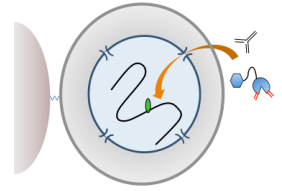
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Human Cell Atlas Metho...



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We use this protocol and it's working

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Abstract

Here we describe a single-tube 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 a single tube on the benchtop or a microwell in a high-throughput pipeline, and the entire procedure 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.

Guidelines

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

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Materials

- Standard (not 'lobind') microfuge tubes (0.5 ml, 1.5 ml and 2 ml).
- Cell suspension. We have used human K562 cells and H1 hESCs.
- Concanavalin A (ConA)-coated magnetic beads (Bangs Laboratories, ca. no. BP531).
- Antibody to an epitope of interest.
- Positive control antibody to an abundant epitope, *e.g.* α -H3K27me3 rabbit monoclonal antibody (Cell Signaling Technology, cat. no. 9733)
- Secondary antibody, *e.g.* guinea pig α -rabbit antibody (Antibodies online ABIN101961)
- 5% Digitonin (EMD Millipore, cat. no. 300410)
- Dimethyl sulfoxide (DMSO; Sigma-Aldrich cat. no. D4540)
- 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 μ M in TE buffer)
- Mosaic end_reverse [PHO]CTGTCTCTTATACACATCT
- Mosaic end_Adapter A TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
- Mosaic end_Adapter B GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG
- Distilled, deionized or RNase-free H₂O (dH₂O *e.g.*, Promega, cat. no. P1197)
- 1 M Manganese Chloride (MnCl₂; Sigma-Aldrich, cat. no. 203734)
- 1 M Calcium Chloride (CaCl₂; Fisher, cat. no. BP510)
- 1 M Potassium Chloride (KCl; Sigma-Aldrich, cat. no. P3911)
- 100 mM Magnesium Chloride (MgCl₂; 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)
- 2 M Spermidine (Sigma-Aldrich, cat. no. S2501)
- 30% Bovine Serum Albumen (BSA, Sigma-Aldrich, cat. no. A80577)
- Roche Complete Protease Inhibitor EDTA-Free tablets (Sigma-Aldrich, cat. no. 5056489001)
- 10 mg/ml RNase A (Thermo, cat. no. EN0531)
- 10% Sodium dodecyl sulfate (SDS; Sigma-Aldrich, cat. no. L4509)
- Proteinase K (20 mg/ml Thermo Fisher Scientific, cat. no. EO0492)
- 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
- Agencourt AMPure XP paramagnetic beads (Beckman Coulter, cat. no. A63880)
- 1 M Tris-HCl pH 8.0
- Ethanol (Decon Labs, cat. no. 2716)
- 0.5 ml PCR tubes (LabSource T54-252)
- NEBNext HiFi 2x PCR Master mix
- PCR primers: A universal i5 primer and 16 i7 primers with unique barcodes [Buenrostro, J.D. et al. Nature 523:486 (2015)].

Troubleshooting

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 start

Prepare reagents (STEP 1)

REAGENT SETUP (for 16 samples)

- 1 Digitonin (5%)** Dissolve 50 mg digitonin in 1 ml DMSO. Hold at room temperature for up to 1 week, or freeze at -20 °C.
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 µL 1M HEPES pH 7.5, 100 µL 1M KCl, 10 µL 1M CaCl₂ and 10 µL 1M MnCl₂, and bring the final volume to 10 mL with dH₂O. 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 µL 2 M spermidine, bring the final volume to 50 mL with dH₂O, and add 1 Roche Complete Protease Inhibitor EDTA-Free tablet. Store the buffer at 4 °C for up to 1 week.

Dig-wash buffer Mix 400 µL 5% digitonin with 40 mL Wash buffer. Store the buffer at 4 °C for up to 2 days.

Antibody buffer Mix 8 µL 0.5 M EDTA and 6.7 µL 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 µL 2 M spermidine, bring the final volume to 50 mL with dH₂O, and add 100 µL 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 µL 1 M MgCl₂.

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. Mix 16 µL of 100 uM equimolar mixture of preannealed ME-A and ME-B oligonucleotides with 100 µL of 5.5 µM protein A - Tn5 fusion protein.
 3. Incubate the mixture on a rotating platform for 1 hour at room temperature and then store at -20 °C.

**Note**

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. In early May, 2019, the 3XFlag-pA-Tn5-FI (pA-Tn5) plasmid will be available from AddGene (# 124601).

Bind cells to Concanavalin A-coated beads (0.5-1 hr)

- 2 Gently resuspend and withdraw enough of the ConA bead slurry such that there will be 10 μ L for each final sample of 100,000 cells. The following is for 16 samples.
- 3 Transfer 170 μ 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).
- 4 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.
- 5 Place on magnet stand to clear, withdraw liquid, and resuspend in 170 μ L Binding buffer (10 μ L per sample) and hold until cells are washed and ready.
- 6 **CRITICAL STEP:** 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.

Harvest fresh culture(s) at room temperature and count cells. The same protocol can be used for up to ~100,000 mammalian cells per sample to be sequenced.

Note

If necessary, cells can be cryopreserved in 10% DMSO using a Mr. Frosty isopropyl alcohol chamber. 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).

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

- 8 Resuspend in at least 1 volume Wash buffer at room temperature, centrifuge 3 min 600 x g at room temperature and withdraw liquid.
- 9 Resuspend in 1.5 mL Wash buffer and transfer to a 2 mL tube. While vortexing gently (1100 rpm) add bead slurry dropwise. Place on end-over-end rotator for 5-10 min.
- 10 After a quick spin to remove liquid from cap (<100 x g), place the tubes on a magnet stand to clear and withdraw the liquid.

Bind primary antibody (2 hr to overnight)

- 11 (Resuspend cells in 800 µL ice-cold Antibody buffer with gentle vortexing, place on ice, and divide into 16 1.5 mL tubes, 50 µL each (for 16 samples; scale up or down as needed).
- 12 Add 0.5-1 µL 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.

- 13 Place on nutator at 4 °C and incubate at overnight to several days at 4 °C. Alternatively, nutate for 2 hr at room temperature. Liquid should remain in the bottom and 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. α -H3K27me3), and optionally a negative control antibody (e.g. rabbit α -mouse IgG).

Note

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)


- 14 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.



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

Note

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.

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

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

- 18 Add 0.8 - 1 mL Dig-wash buffer. Invert 10x or gently vortex to allow the solution to dislodge most or all of the beads.

- 19 Repeat Steps 17–18 twice.

Bind pA-Tn5 adapter complex (1.5 hr)

- 20 Mix pA-Tn5 adapter complex in Dig-300 buffer to a final concentration of 1:250 for 100 μ 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.

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


- 22 Squirt in 100 μ L of the pA-Tn5 mix while gently vortexing to allow the solution to dislodge most or all of the beads.

**Note**


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.

- 23 Place the tubes on a nutator at room temperature for 1 hr.  01:00:00
- 24 After a quick spin, place the tubes on a magnet stand to clear and pull off the liquid.
- 25 Add 0.8 - 1 mL Dig-300 buffer. Invert 10x or gently vortex to allow the solution to dislodge most or all of the beads.
- 26 Repeat steps 24-25 twice.

Tagmentation (1 hr)

- 27 After a quick spin, place the tube on the magnet stand to clear and pull off the liquid.
- 28 Add 300 μ L Tagmentation buffer while gently vortexing.
- 29 Incubate at 37 °C for 1 hr.  01:00:00

DNA extraction (1 hr)

- 30 To stop tagmentation and solubilize DNA fragments, add 10 μ L 0.5M EDTA, 3 μ L 10% SDS and 2.5 μ L 20 mg/mL Proteinase K to each sample.
- 31 Mix by full-speed vortexing ~2 s, and incubate 1 hr 50 °C or 37 °C overnight to digest.
 01:00:00

Note

It is typical for the beads to form a large clump during incubation 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 30) Left: H3K27me3; Right: IgG.

- 32 Add 300 μ L PCI and mix by full-speed vortexing ~2 s.

Note

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

- 33 Transfer to a phase-lock tube, and centrifuge 3 min room temperature at 16,000 x g.
- 34 Add 300 μ L Chloroform and invert ~10x to mix (do not vortex). Centrifuge 3 min room temperature at 16,000 x g.
- 35 Remove aqueous layer by pipetting to a fresh 1.5 mL tube containing 750 μ L 100% ethanol, pipetting up and down to mix.
- 36 Chill on ice and centrifuge 15 min at 4 °C 16,000 x g.
- 37 Carefully pour off the liquid and drain on a paper towel. There is typically no visible pellet.
- 38 Rinse in 1 mL 100% ethanol and centrifuge 1 min at 4 °C 16,000 x g.




- 39 Carefully pour off the liquid and drain on a paper towel. Air dry.
- 40 When the tube is dry, dissolve in 25-30 μ L 10 mM Tris-HCl pH8 1 mM EDTA containing 1/400 RNase A.

Note

The large excess of RNA over DNA in the purified nucleic acid acts as a carrier during purification, but this RNA can skew estimates of DNA concentrations in the final libraries, so is best digested away prior to PCR and Ampure bead clean-up.

- 41 Incubate 10 min 37 °C and store at 4 °C or proceed directly to the next step.

 00:10:00

PCR (1 hr)

- 42 21 μ L DNA + 2 μ L Universal i5 primer (10 μ M) + 2 μ L uniquely barcoded i7 primers (10 μ M) In a thin-wall 0.5 ml PCR tube, using a different barcode for each sample*. Save remaining DNA as a backup.

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

- 43 Add 25 μ L NEBNext HiFi 2x PCR Master mix.
- 44 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 at least 12-14 cycles, preferably with a 10 s 60-63°C combined annealing/extension step.

Post-PCR Clean-up (30 min)


45

Note

Section timing: 30 min

After tubes have cooled, remove from the cycler and add 1.1 volume (55 μ L) Ampure XP beads, mixing by pipetting up and down.

46

Quick spin and let sit at room temperature 10 min.  00:10:00


47

Place on magnet and allow to clear before carefully withdrawing liquid. On magnet and without disturbing the beads, add 200 μ L 80% ethanol.

48

Withdraw liquid with a pipette to the bottom of the tube and add 200 μ L 80% ethanol.

49

Withdraw liquid and after a quick spin remove the remaining liquid with a 20 μ L pipette and allow to dry for 4-5 min.  00:05:00

50

Remove from magnet stand, add 25 μ L 10 mM Tris-HCl pH 8 and vortex on full.

51

After 5 min place on magnet stand and allow to clear.

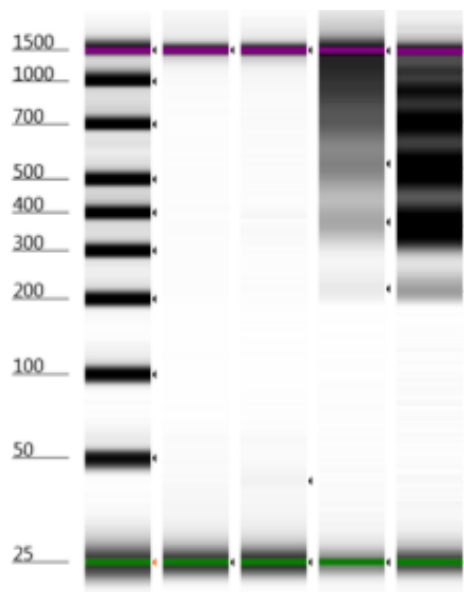
52

Remove liquid to a fresh 0.5 ml tube with a pipette.

DNA sequencing and data processing

53

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

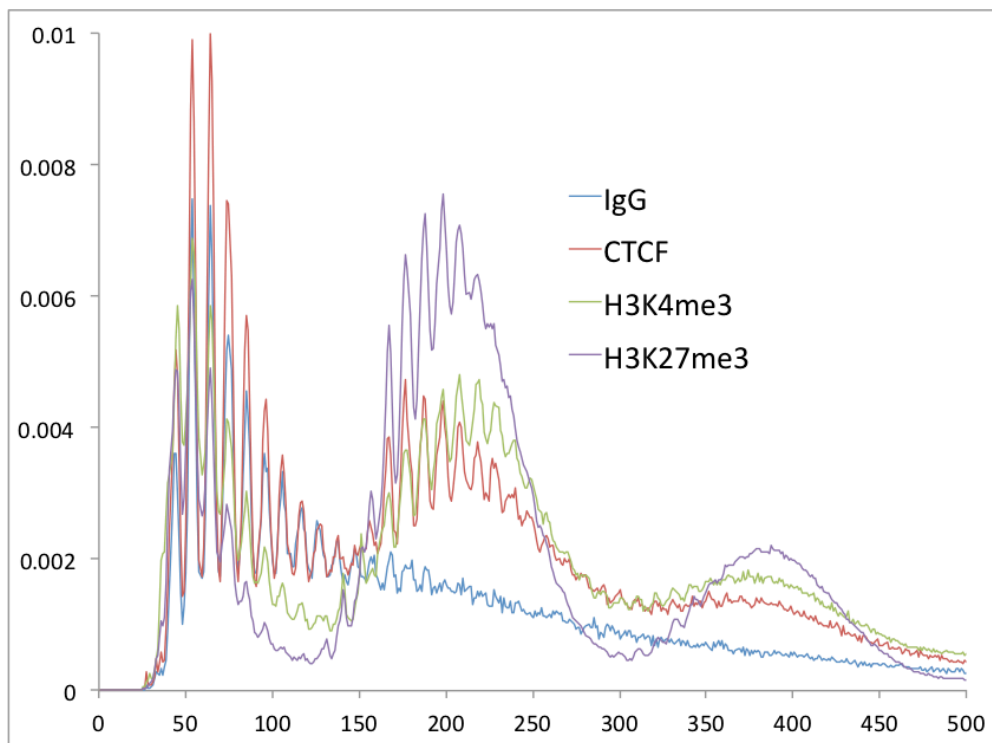
Note**A representative Tapestation image**

Human K562 cells. Left to right: Markers, IgG, CTCF, H3K4me3, H3K27me3.

- 54 Mix barcoded libraries to achieve equal representation as desired aiming for a final concentration as recommended by the manufacturer. After mixing, perform an Ampure bead cleanup if needed to remove any residual PCR primers.
- 55 Perform paired-end Illumina sequencing on the barcoded libraries following the manufacturer's instructions.
- 56 We align paired-end reads using Bowtie2 version 2.2.5 with options: `--local --very-sensitive-local --no-unal --no-mixed --no-discordant --phred33 -l 10 -X 700`. For mapping *E. coli* carry-over fragments, we also use the `--no-overlap --no-dovetail` options to avoid possible cross-mapping of the experimental genome to that of the carry-over *E. coli* DNA that is used for calibration.

Note

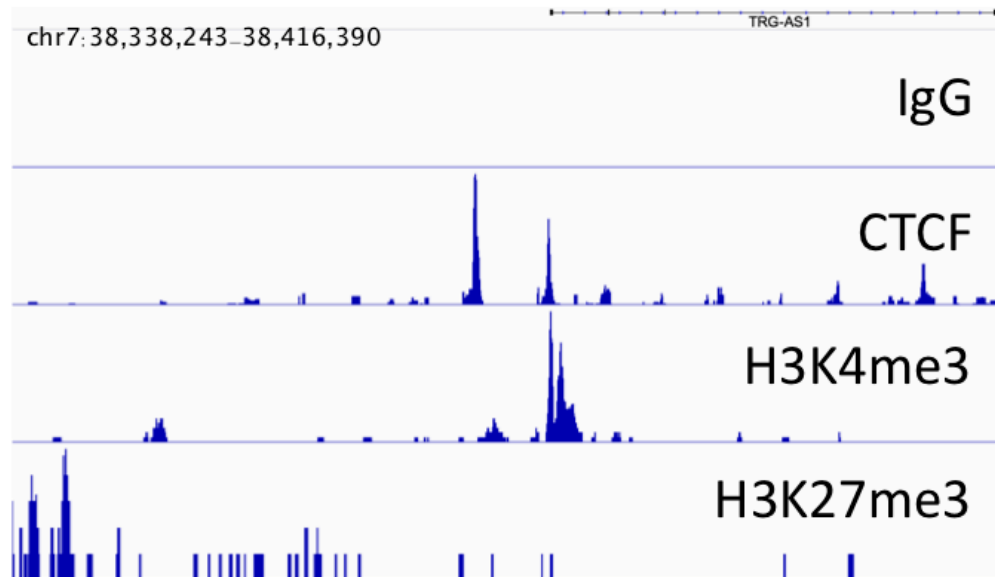
Fragment lengths show a 10-bp periodicity suggestive of preferential cleavages on one side of the helix.



Fraction of sequenced fragments plotted as a function of fragment length for the samples shown in Step 52.

Note

Because of the low backgrounds with CUT&Tag, 2-3 million paired-end reads may be sufficient to 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 low sequencing depth, strong peaks are detected above the sparse background. For calling peaks, we recommend SEACR (Sparse Enrichment Analysis for Cut&Run), which is designed for CUT&RUN and CUT&Tag datasets: <https://www.biorxiv.org/content/10.1101/569129v1>.