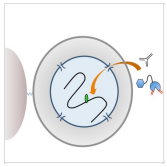

Version 2

Sep 18, 2020

CUT&Tag-direct with CUTAC V.2

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1 Works for me dx.doi.org/10.17504/protocols.io.bmbfk2jn

Human Cell Atlas Method Development Community

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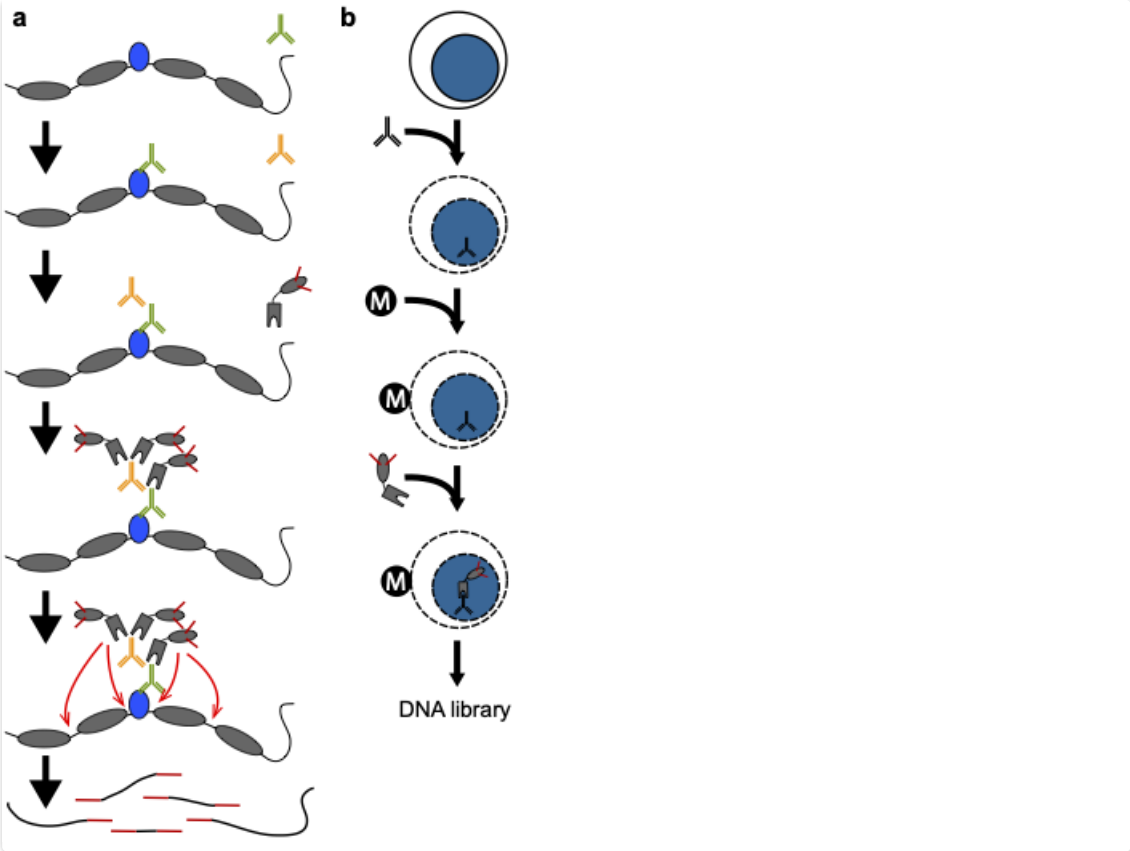
ABSTRACT

CUT&Tag@direct uses a modification of Bench-top CUT&Tag which includes incubation in 0.1% SDS post-tagmentation for quantitative release of targeted fragments, followed directly by PCR with Triton-X100 to neutralize the SDS. This protocol is performed in single PCR tubes from nuclei to sequencing-ready libraries and should be suitable for high throughput. The protocol has been enhanced by the addition of hyperaccessibility mapping by Cleavage Under Targeted Accessible Chromatin (CUTAC), where H3K4me2 CUT&Tag samples are tagmented in low salt for mapping of the hyperaccessible site close to the H3K4me2-labeled nucleosomes.



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 transposome 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 Concanavalin 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.

Citation: Steven Henikoff, Jorja Henikoff, Hatice S Kaya-Okur, Kami Ahmad (09/18/2020). CUT&Tag-direct with CUTAC. https://dx.doi.org/10.17504/protocols.io.bmbfk2jn

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Steven Henikoff, Jorja G. Henikoff, Hatice S Kaya-Okur, Kami Ahmad (2020) **Efficient transcription-coupled chromatin accessibility mapping in situ** bioRxiv
https://www.biorxiv.org/content/10.1101/2020.04.15.043083v2

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PROTOCOL CITATION

Steven Henikoff, Jorja Henikoff, Hatice S Kaya-Okur, Kami Ahmad 2020. CUT&Tag-direct with CUTAC. **protocols.io**
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Version created by Steven Henikoff

MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

Steven Henikoff, Jorja G. Henikoff, Hatice S Kaya-Okur, Kami Ahmad (2020) **Efficient transcription-coupled chromatin accessibility mapping in situ** bioRxiv
https://www.biorxiv.org/content/10.1101/2020.04.15.043083v2

KEYWORDS

CUT&Tag, bench top protocol, epigenetics, formaldehyde cross-linking

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42055

GUIDELINES

Based on https://www.protocols.io/view/bench-top-cut-amp-tag-z6hf9b6.

MATERIALS TEXT

100X Bovine Serum Albumen (BSA, NEB, cat no. B9001S)

Equipment, supplies, reagents and solutions for CUT&Tag-direct. All experiments were performed on a counter in a home laundry/utility room using stock solutions and frozen nuclei aliquots prepared in the lab. There are no hazardous materials or dangerous equipment used in this protocol, however appropriate lab safety training is recommended.

- Chilling device (e.g.metal heat blocks on ice or cold packs in an ice cooler)
- Pipettors (e.g. Rainin Classic Pipette 1 mL, 200 µL, 20 µL, 10 µL)
- Disposable tips (e.g. Rainin 1 mL, 200 µL, 20 µL)
- Disposable centrifuge tubes for reagents (15 mL or 50 mL)
- Standard 1.5 ml microfuge tubes
- 0.5 ml maximum recovery PCR tubes (e.g. Fisher cat. no. 14-222-294)
- Frozen uncross-linked nuclei suspension (e.g. human K562 cells) prepared as described in: https://www.protocols.io/view/bench-top-cut-amp-tag-bcuhiwt6.
- Concanavalin A (ConA)-coated magnetic beads (Bangs Laboratories, ca. no. BP531)
- Strong magnet stand (e.g. Miltenyi Macsimag separator, cat. no. 130-092-168)
- Vortex mixer (e.g. VWR Vortex Genie)
- Mini-centrifuge (e.g. VWR Model V)
- PCR thermocycler (e.g. BioRad/MJ PTC-200)
- Distilled, deionized or RNAse-free H2O (dH_2O e.g., Promega, cat. no. P1197)
- 1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.9 (HEPES (K+); Sigma-Aldrich, cat. no. H3375)
- 1 M Manganese Chloride ($MnCl_2$; Sigma-Aldrich, cat. no. 203734)
- 1 M Calcium Chloride ($CaCl_2$; Fisher, cat. no. BP510)
- 1 M Potassium Chloride (KCl; Sigma-Aldrich, cat. no. P3911)
- Roche Complete Protease Inhibitor EDTA-Free tablets (Sigma-Aldrich, cat. no. 5056489001)
- 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)
- 2 M Spermidine (Sigma-Aldrich, cat. no. S0266)
- 0.5 M Ethylenediaminetetraacetic acid (EDTA; Research Organics, cat. no. 3002E)
- 100X Bovine Serum Albumen (BSA, 10 mg/ml)
- Antibody to an epitope of interest. 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/Tag-tested antibodies are not available
- CUTAC control antibody to histone H3K4me2. We have obtained excellent results with Epicypher 13-0027 and Millipore 07-030.
- Secondary antibody, e.g. guinea pig α-rabbit antibody (Antibodies online cat. no. ABIN101961) or rabbit α-mouse antibody (Abcam cat. no. ab46540)
- Protein A/G–Tn5 (pAG-Tn5) fusion protein loaded with double-stranded adapters with 19mer Tn5 mosaic ends Epicypher cat. no. 15-1117.
- 1 M Magnesium Chloride ($MgCl_2$; Sigma-Aldrich, cat. no. M8266-100G)
- 1 M TAPS pH 8.5 (with NaOH)
- 1,6-hexanediol (Sigma-Aldrich cat. no. 240117-50G)
- N,N-dimethylformamide (Sigma-Aldrich cat. no. D-8654-250mL)
- NEBNext 2X PCR Master mix (ME541L)
- PCR primers: 10 µM stock solutions of i5 and i7 primers with unique barcodes [Buenrostro, J.D. et al. Nature 523:486

Citation: Steven Henikoff, Jorja Henikoff, Hatice S Kaya-Okur, Kami Ahmad (09/18/2020). CUT&Tag-direct with CUTAC. https://dx.doi.org/10.17504/protocols.io.bmbfk2jn

(2015)] in 10 mM Tris pH 8. Standard salt-free primers may be used. Do not use Nextera or NEBNext primers.
- 10% Sodium dodecyl sulfate (SDS; Sigma-Aldrich, cat. no. L4509)
- 10% Triton X-100 (Sigma-Aldrich, cat. no. X100)
- SPRI paramagnetic beads (e.g. HighPrep PCR Cleanup Magbio Genomics cat. no. AC-60500)
- 10 mM Tris-HCl pH 8.0
- Ethanol (Decon Labs, cat. no. 2716)

SAFETY WARNINGS

There are no hazardous materials or dangerous equipment used in this protocol, however appropriate lab safety training is recommended.

BEFORE STARTING

Prepare reagents (STEP 1)

REAGENT SETUP (for up to 16 samples)

1 **Binding buffer** Mix 200 µL 1M HEPES-KOH pH 7.9, 100 µL 1M KCl, 10 µL 1M $CaCl_2$ and 10 µL 1M $MnCl_2$, and bring the final volume to 10 mL with dH_2O. Store the buffer at 4 °C for up to several 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 dH_2O, and add 1 Roche Complete Protease Inhibitor EDTA-Free tablet. Store the buffer at 4 °C for up to several months.

Antibody buffer Mix 10 µL 100X BSA with 1 mL Wash buffer and chill on ice.

300-wash 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 dH_2O and add 1 Roche Complete Protease Inhibitor EDTA-Free tablet. Store at 4 °C for up to several months.

CUT&Tag Tagmentation solution Mix 1 mL 300-wash buffer and 10 µL 1 M $MgCl_2$ (to 10 mM).

CUTAC Tagmentation solution Mix 197 µL dH_2O, 2 µL 1 M TAPS pH 8.5 and 1 µL 1 M $MgCl_2$ (10 mM TAPS, 5 mM $MgCl_2$).

CUTAC-dilution Tagmentation solution Mix 15 mL dH_20, 33 µL 1 M $MgCl_2$ (2 mM $MgCl_2$) and preheat to 37^{O}C.

CUTAC-hex Tagmentation solution Mix 97 µL dH_2O, 100 µL 20% (w/v) 1,6-hexanediol, 2 µL 1 M TAPS pH 8.5 and 1 µL 1 M $MgCl_2$ (10 mM TAPS, 5 mM $MgCl_2$).

CUTAC-DMF Tagmentation solution Mix 177 µL dH_2O, 20 µL N,N-dimethylformamide, 2 µL 1 M TAPS pH 8.5 and 1 µL 1 M $MgCl_2$ (10 mM TAPS, 5 mM $MgCl_2$).

TAPS wash buffer Mix 1 mL dH_2O, 10 µL 1 M TAPS pH 8.5, 0.4 µL 0.5 M EDTA (10 mM TAPS, 0.2 mM EDTA)

0.1% SDS Release solution Mix 10 µL 10% SDS and 10 µL 1 M TAPS pH 8.5 in 1 ml dH_2O

0.67% Triton neutralization solution Mix 67 µL 10% Triton-X100 + 933 µL

Prepare Concanavalin A-coated beads (15 min)

2 Resuspend and withdraw enough of the ConA bead slurry such that there will be 3-5 µL for each final sample of up to ~50,000 mammalian cells. The following is for 16 samples.

3 Transfer 85 µL ConA bead slurry into 1 mL Binding buffer in a 1.5 mL tube and mix by pipetting. Place the tube on a magnet stand to clear (30 s to 2 min).

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4 Withdraw the liquid completely, and remove from the magnet stand. Add 1 mL Binding buffer and mix by pipetting.

5 Place on magnet stand to clear, withdraw liquid, and resuspend in 85 µL Binding buffer (for 5 µL per sample).

Bind nuclei to ConA bead (15 min)

6 Thaw a frozen aliquot of native nuclei at room temperature, for example by placing in a 20 ml beaker of water.

The CUTAC control requires uncrosslinked nuclei.

7 Transfer the thawed nuclei suspension in aliquots of no more than 50,000 starting mammalian cells to each PCR tube and mix with 3-5 µL ConA beads in thin-wall 0.5 ml PCR tubes and let sit at room temperature for 10 min.

Nuclei prepared according to the Benchtop CUT&Tag Version 3 protocol have been resuspended in Wash buffer. Beads can be added directly to the aliquot for binding and then transferred to PCR tubes such that no more than 5 µL of the original ConA bead suspension is present in each PCR tube for single-tube CUT&Tag.

Using more than ~50,000 mammalian nuclei or >5 µL Con A beads per sample may inhibit the PCR.

8 Place the tubes on a magnet stand to clear and withdraw the liquid.

In low-retention PCR tubes, surface tension will cause bead-bound cells to slide down to the bottom of the tube, so to avoid losses here and below, set the pipettor to 5 µL less than the liquid volume to be removed and use multiple draws to remove the last liquid without losing beads.

Bind primary antibody (1 hr)

9 Resuspend cells in 25-50 µL Antibody buffer then 0.5 µL antibody (1:50-1:100) with gentle vortexing. For each CUTAC control sample (dilution, removal or post-wash), prepare a separate H3K4me2 tube (or split the H3K4me2 sample at the appropriate step).

For bulk processing, resuspend in Antibody buffer containing antibody (1:100) with gentle vortexing.

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

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The CUTAC control will only work using a α-H3K4me2 antibody. α-H3K4me3 also works but is less efficient and is depleted of enhancer peaks. Several antibodies to other histone epitopes have been tested, including α-H3K4me1, α-H3K27ac and α-H2A.Z but all have failed.

For any of the three CUTAC options we recommend a range of tagmentation times from 5 min to 1 hr, as results vary depending on cell number, (H3K4me2) antibody and pA(G)-Tn5 and possibly other variables.

10 Place on a Rotator at room temperature and incubate 1-2 hr.

01:00:00

Volumes up to 50 µL will remain in the tube bottom by surface tension during rotation.

The α-H3K4me2 antibody used for both CUT&Tag and CUTAC serves as a control evaluate success of the procedure without requiring library preparation. An optional negative control is performed by omitting the primary antibody.

Bind secondary antibody (1 hr)

11 Place tubes on the magnet stand to clear. Withdraw the liquid with the pipettor set to 5 µL less than the volume to be removed.

12 Mix the secondary antibody 1:100 in Wash buffer and squirt in 50 µL 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.

30m

13 Place the tubes on a Rotator and rotate at room temperature for 30 min. 00:30:00

14 After a quick spin (<500 x g), place the tubes on a magnet stand to clear and withdraw the liquid with the pipettor set to 5 µL less than the volume to be removed.

Surface tension causes beads slide down the side of low-retention PCR tubes, and removing the last drop

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Surface tension causes beads slide down the side of low retention PCR tubes, and removing the last drop can result in loss of beads. To avoid this, remove the 50 µL volume with 3 successive draws using a 20 µL tip with the pipettor set for maximum volume.

15 After a quick spin, replace on the magnet stand and withdraw the last drop with a 20 µL pipette tip.

16 With the tubes still on the magnet stand, carefully add 500 µL Wash buffer. The surface tension will cause the beads to slide up along the side of the tube closest to the magnet.

17 Slowly withdraw 470 µL with a 1 mL pipette tip without disturbing the beads, followed by complete liquid removal using multiple draws with a 20 µL pipettor.

To withdraw the liquid, set the pipettor to 470 µL, and keep the plunger depressed while lowering the tip to the bottom. The liquid level will rise to near the top completing the wash. Then ease off on the plunger until the liquid is withdrawn, and remove the pipettor. This will leave behind a small drop of liquid that is removed with a 20 µL pipettor, avoiding significant bead loss.

18 Replace on magnet and withdraw the liquid with a 20 µL pipettor using multiple draws. Proceed immediately to the next step.

19 Remove tubes from the magnet and squirt in 50 µL Wash buffer, vortex gently followed by a quick spin.

Bind pA-Tn5 adapter complex (1.5 hr)

20 Mix pAG-Tn5 pre-loaded adapter complex in 300-wash buffer following the manufacturer's instructions.

For CUT&Tag using Epicypher pAG-Tn5 (cat. no. 15-1117) dilute 1:20 as recommended, except for CUTAC, which is optimal at 1:40.

21 Squirt in 25-50 µL per sample of the pA-Tn5 mix while vortexing and invert by rotation to allow the solution to dislodge most or all of the beads.

When using the recommended Macsimag magnet stand, dislodging the beads can be done by removing the plexiglass tube holder from the magnet, and with fingers on top to prevent the tubes from opening up or falling out, invert by rotating sharply a few times.

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https://dx.doi.org/10.17504/protocols.io.bmbfk2jn

1h

22 After a quick spin (<500 x g), place the tubes on a Rotator at room temperature for 1 hr. **01:00:00**

23 For a CUTAC by dilution H3K4me2 sample, hold at RT until Step 27. Continue with other tubes.

24 After a quick spin place the tubes on a magnet stand to clear and and withdraw the liquid with a 20 µL pipettor using multiple draws. For a CUTAC by removal sample, hold on ice until Step 29.

25 With the tubes still on the magnet stand, add 500 µL 300-wash buffer.

26 Slowly withdraw the liquid with a 1 mL pipette tip as in Step 17.

27 After a quick spin, place the tubes on a magnet stand to clear and withdraw the liquid with a 20 µL pipettor using multiple draws. Proceed immediately to Step 32. For a CUTAC post-wash sample proceed immediately to Step 31.

CUTAC by dilution (performed in parallel with other samples)

28 Prewarm CUTAC by dilution tube at 37^{o}C in a 0.5 ml PCR tube heating block and squirt in 500 µL prewarmed 2 mM $MgCl_2$. Incubate 20 min. **00:20:00**

The degree of tagmentation will vary depending on the number of nuclei and other factors, but can be controlled by varying tagmentation times. See the the figure in Step 50 for guidance (panel A-B for dilution), where it is shown that longer tagmentation will increase yield, but reduce signal-to-noise.

29 Chill the tube and skip to Step 34.

CUTAC by removal (performed in parallel with other samples)

30 Add 50 µL 10 mM TAPS 5 mM $MgCl_2$ with gentle vortexing and incubate at 37^{o}C for 20 min. **00:20:00**

This step is critical to remove most but not all of the excess pA-Tn5 and avoid high background levels of cleavage (Step 50 figure panels C-D for CUTAC by removal). Washing will over-deplete the remaining unbound enzyme, and reduce recovery of small CUTAC fragments.

31 Chill the tube and skip to Step 34.

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CUTAC post-wash (performed in parallel with other samples)

32 Resuspend the bead/nuclei pellet in 25-50 µL CUTAC tagmentation solution (5 mM $MgCl_2$, 10 mM TAPS) while vortexing or inverting by rotation to allow the solution to dislodge most or all of the beads as in Step 20. Proceed to Step 33.

Tagmentation and particle release (2.5 hr)

33 Resuspend the bead/nuclei pellet in 25-50 µL tagmentation solution while vortexing or inverting by rotation to allow the solution to dislodge most or all of the beads as in Step 20.

34 After a quick spin (<500 x g), incubate at 37 ºC for 1 hr in a PCR cycler with heated lid. Hold at 8^{O}C. **01:00:00**

35 Place tubes on a magnet stand and withdraw the liquid with a 20 µL pipettor using multiple draws then resuspend the beads in 50 µL TAPS wash and invert by rotation as in Step 20.

36 After a quick spin, replace tubes on the magnet stand, and withdraw the liquid with a 20 µL pipettor using multiple draws.

37 Resuspend the beads in 5 µL 0.1% SDS Release solution using a fresh 20 µL pipette tip to dispense while wetting the sides of the tubes to recover the fraction of beads sticking to the sides.

Twirling the tube back and forth rapidly between thumb and finger will effectively wet the sides of the tube, followed by a quick spin to bring most of the beads to the bottom.

38 Incubate at 58 ºC for 1 hr in a PCR cycler with heated lid to release pA-Tn5 from the tagmented DNA. **01:00:00**

PCR (1 hr)

39 To the PCR tube containing the bead slurry add 15 µL Triton neutralization solution + 2 µL of 10 µM Universal or barcoded i5 primer + 2 µL of 10 µM uniquely barcoded i7 primers, using a different barcode for each sample. Vortex on full and place tubes in metal tube holder on ice.

Indexed primers are described by Buenrostro, J.D. et al. Single-cell chromatin accessibility reveals principles of regulatory variation. Nature 523:486 (2015). Do not use Nextera or NEB primers.

40 Add 25 µL NEBnext (non-hot-start), vortex to mix, followed by a quick spin.

41 Mix, quick spin and place in Thermocycler and begin cycling program with heated lid:
Cycle 1: 58 °C for 5 min (gap filling)

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Cycle 2: 72 °C for 5 min (gap filling)
Cycle 3: 98 °C for 30 sec
Cycle 4: 98 °C for 10 sec
Cycle 5: 60 °C for 10 sec
Repeat Cycles 4-5 11 times

72°C for 1 min and hold at 8 °C

To minimize the contribution of large DNA fragments and excess primers, PCR should be performed for no more than 12 cycles, preferably with a 10 s 60-63 °C combined annealing/extension step.

The cycle times are based on using a conventional Peltier cycler (e.g., BioRad/MJ PTC200), in which the ramping times (3 °C/sec) 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 higher ramping rate will require either reducing the ramping time or other adjustments 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, 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)

42 After tubes have cooled, remove from the cycler and add 1.3 volume (65 µL) SPRI bead slurry, mixing by pipetting up and down.

43 Quick spin and let sit at room temperature 5-10 min. **00:05:00**

44 Place on magnet 5 min to allow the beads to clear before withdrawing the liquid. While still on the magnet stand add 200 µL 80% ethanol.

45 Withdraw the liquid with a pipette to the bottom of the tube, and add 200 µL 80% ethanol.

46 Withdraw the liquid and after a quick spin, remove the remaining liquid with a 20 µL pipette. Do not air-dry the beads, but proceed immediately to the next step.

47 Remove from the magnet stand, add 22 µL 10 mM Tris-HCl pH 8 and vortex on full. Let sit at least 5 min. **00:05:00**

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https://dx.doi.org/10.17504/protocols.io.bmbfk2jn

48 Place on the magnet stand and allow to clear.

49 Remove the liquid to a fresh 1.5 ml tube with a pipette.

Tapestation analysis and DNA sequencing (outsource)

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

CAUTION: Minor differences in bead losses during solution changes and in efficiency of removal and wash steps are exponentially amplified during PCR (A, C), but nevertheless may result in high data quality and acceptable yields (B, D). Best results are obtained when the CUTAC library yield is no higher than the CUT&Tag library yield, as higher CUTAC than CUT&Tag yields indicate background tagmentation, inflating library size estimates.

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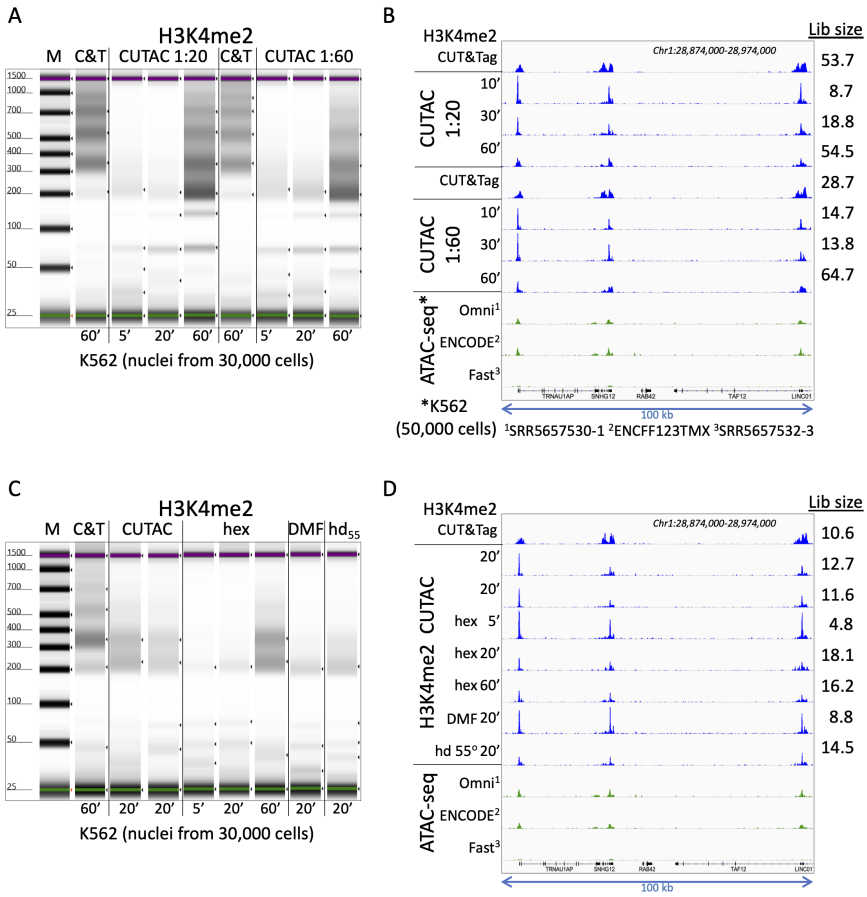

Low-salt tagmentation reduces fragment size, increasing peak resolution: A-B) H3K4me2 CUT&Tag (C&T) and low-salt tagmentations using the Dilution protocol were performed at 37°C using Epicypher 13-0027 antibody and Epicypher 15-1117 pAG-Tn5 for the times indicated using pA/G-Tn5 at either 1:20 (Manufacturer's recommendation) or 1:60 showing a bigger effect of tagmentation time than amount of pA/G-Tn5, and improvement in yield but reduction in signal-to-noise with longer tagmentations. A) Tapestation gel images showing time of tagmentation and yields based on loading 2 µL of each 20 µL sample and integrating over the 175-1000 bp range. M = markers, C&T = CUT&Tag; B) Group-autoscaled tracks showing fragment normalized count densities for sequenced libraries resolved in (A) and for published ATAC-seq data. **C-D**) Same as A-B using the Removal protocol with no additive (CUTAC), 10% 1,6-hexanediol (hex), 10% N,N-dimethylformamide (DMF) or 10% of both at 55°C (hd55) for the times indicated. All datasets were sampled down to 3.2 million and mapped to hg19. A representative 100-kb region was group-autoscaled using IGV. Estimated library size (Lib size, millions of fragments) was calculated by the Mark Duplicates program in Picard tools.

51 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.

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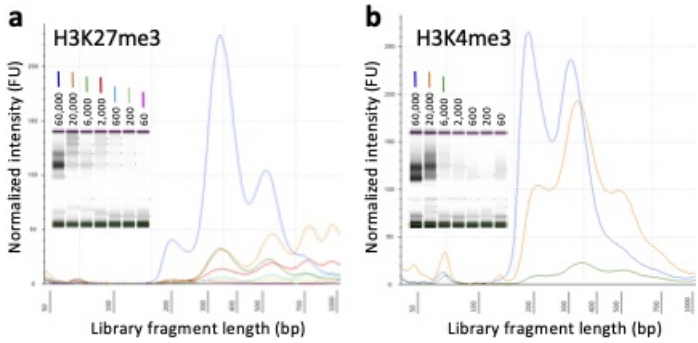


CUT&Tag@home for low cell numbers: At home: Frozen native K562 cell nuclei (~50% recovery) were thawed and diluted into 50 µL volumes of Wash buffer in PCR tubes, and 3 µL of activated ConA beads was added to each. Successive incubations with primary antibody, secondary antibody, pA-Tn5 and Mg++, followed by SDS release, amplification for 12 cycles, and SPRI bead cleanup in the same PCR tubes produced ready-to-sequence libraries from thawed nuclei in 8 hours. (**a**) H3K27me3 and (**b**) H3K4me3. **At the lab:** 1/10th of each sample was analyzed by Tapestation. Nucleosomal ladders were detectable for intermediate and low numbers of cells. The barcoded libraries from this experiment were mixed with others, totalling 53 libraries for paired-end PE25 sequencing on an Illumina 2-lane HiSeq2500 flowcell.

52 Perform paired-end Illumina sequencing on the barcoded libraries following the manufacturer's instructions. For maximum economy, paired-end PE25 is more than sufficient for mapping to large genomes.

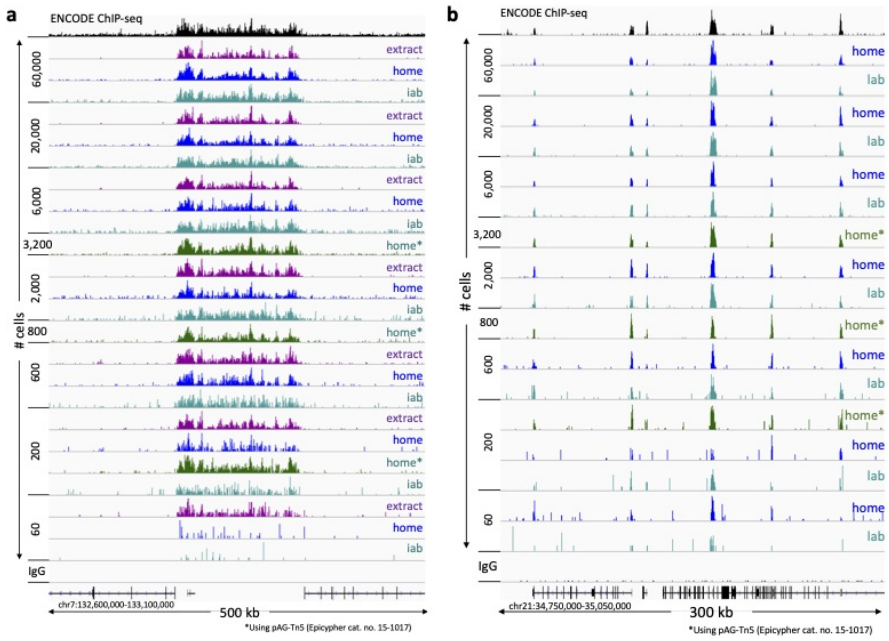
Using paired-end 25x25 sequencing on a HiSeq 2-lane rapid run flow cell we obtain ~300 million total mapped reads, or ~3 million per sample when there are 96 samples mixed to obtain approximately equal molarity.

Data processing and analysis

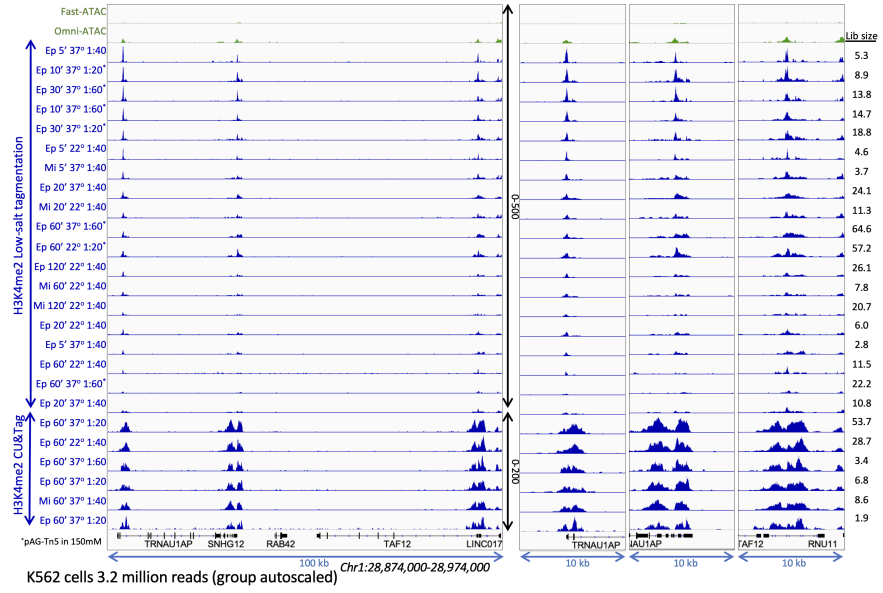
53 We align paired-end reads to hg19 using Bowtie2 version 2.3.4.3 with options: --end-to-end --very-sensitive --no-unal --no-mixed --no-discordant --phred33 -I 10 -X 700. For mapping E. coli carry-over fragments, we also use the --no-overlap --no-dovetail options to avoid possible cross-mapping of the experimental genome to that of the carry-over E. coli DNA that is used for calibration. Tracks are made as bedgraph files of normalized counts, which are the fraction of total counts at each basepair scaled by the size of the hg19 genome.

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.

Citation: Steven Henikoff, Jorja Henikoff, Hatice S Kaya-Okur, Kami Ahmad (09/18/2020). CUT&Tag-direct with CUTAC. https://dx.doi.org/10.17504/protocols.io.bmbfk2jn



CUT&Tag-direct produces high-quality datasets with low cell numbers. (a) The H3K27me3 CUT&Tag@home libraries from human K562 cells analyzed by Tapestation in Step 48 were sequenced and tracks were compared to an ENCODE dataset produced using the original extraction protocol and the single-tube protocol in the lab for a representative region centered over a prominent Polycomb-silenced domain. Asterisks indicate CUT&Tag@home datasets produced using a commercial pAG-Tn5 preparation (Epizyme cat. no. 15-1017). **(b)** Same as (a) for H3K4me3 comparing results from CUT&Tag@home to those produced using the single-tube protocol in the lab for a 300-kb region centered over the GART-SON bidirectional promoter. Tracks are autoscaled for clarity, except for the IgG negative control tracks, which were scaled the same as that for the 60-cell CUT&Tag@home sample.



Optimization of CUTAC conditions: H3K4me2 CUT&Tag and low-salt tagmentation were performed using either a rabbit polyclonal [Millipore 07-030 lot 3229364 (Mi)] or rabbit monoclonal [Epicypher 13-0027 (Ep)] antibody with pAG-Tn5 (Epicypher 15-1117 lot #20142001-C1) at the indicated dilutions. Dilution tagmentation in 2 mM $MgCl_2$ was used at either 22°C or 37°C. Raw paired-end reads were sampled down to 3.2 million and mapped to hg19. A representative 100 kb region is shown (left) and expanded (right) around active promoters and group-autoscaled separately for low-salt tagmentation and standard CUT&Tag using IGV. Estimated library size (Lib size) was calculated by the Mark Duplicates program in Picard tools.

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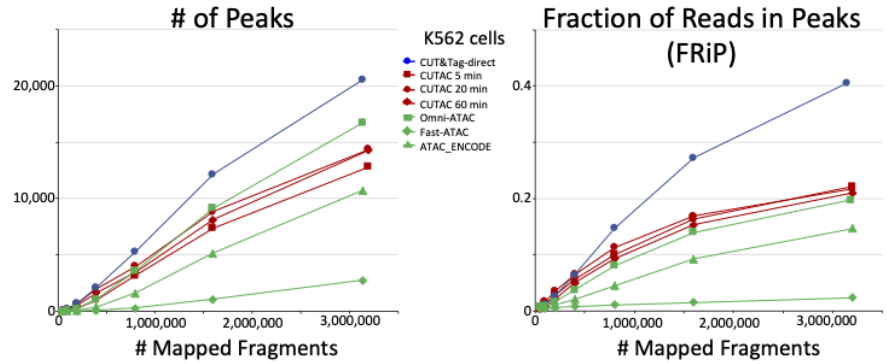
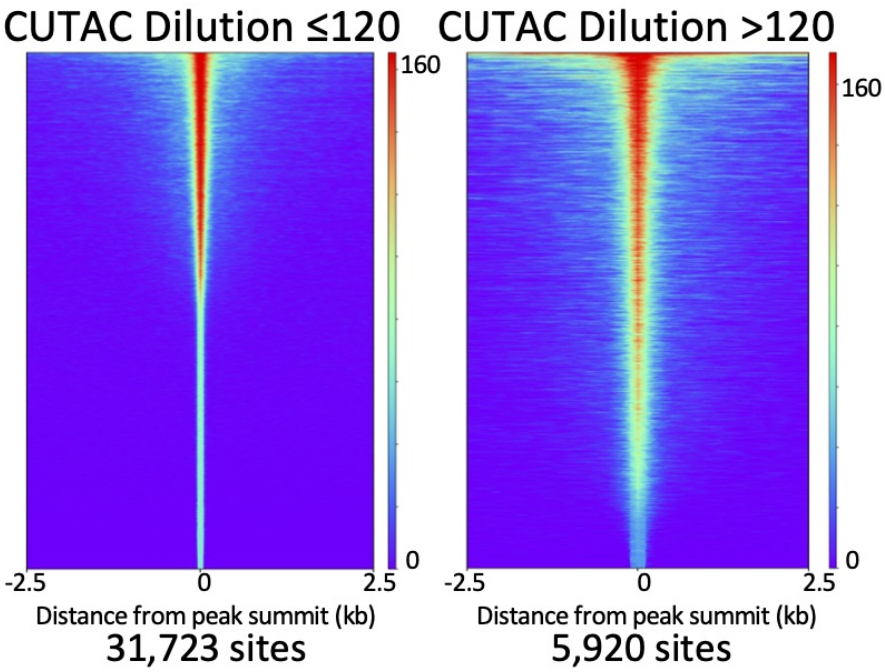


CUTAC data quality is similar to the best available ATAC-seq K562 cell data: Raw reads from the indicated datasets were sampled and mapped to hg19 using bowtie2, and peaks were called using MACS2. A) Number of peaks (left) and fraction of reads in peaks for CUT&Tag (blue), CUTAC Removal (red) and ATAC-seq (green). MACS2 was used to call peaks after mitochondrial DNA removal. Peak numbers and FRiP values respectively estimate relative sensitivity and specificity for peak-calling.

54 Our CUT&Tag Data Processing and Analysis Tutorial on Protocols.io provides step-by-step guidance for mapping and analysis of CUT&Tag sequencing data. 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.

For best CUTAC results, map ≤ 120 bp fragments, which enrich for fragments confined to accessible DNA and improve peak-calling.



Small fragments dominate NDRs. Comparison of small (≤120 bp) and large (>120) fragments from a representative CUTAC dataset showsnarrowing for small fragments around their summits. For each dataset a 3.2 million fragment random sample was split into small and large fragment groups, MAC2 was used to call peaks and heatmaps were ordered by density over the peak midpoints (sites).

See Efficient transcription-coupled chromatin accessibility mapping in situ for examples of CUT&Tag@home

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