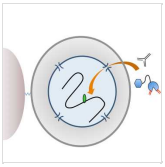

Version 3

Jun 05, 2021

CUT&Tag-direct with CUTAC V.3

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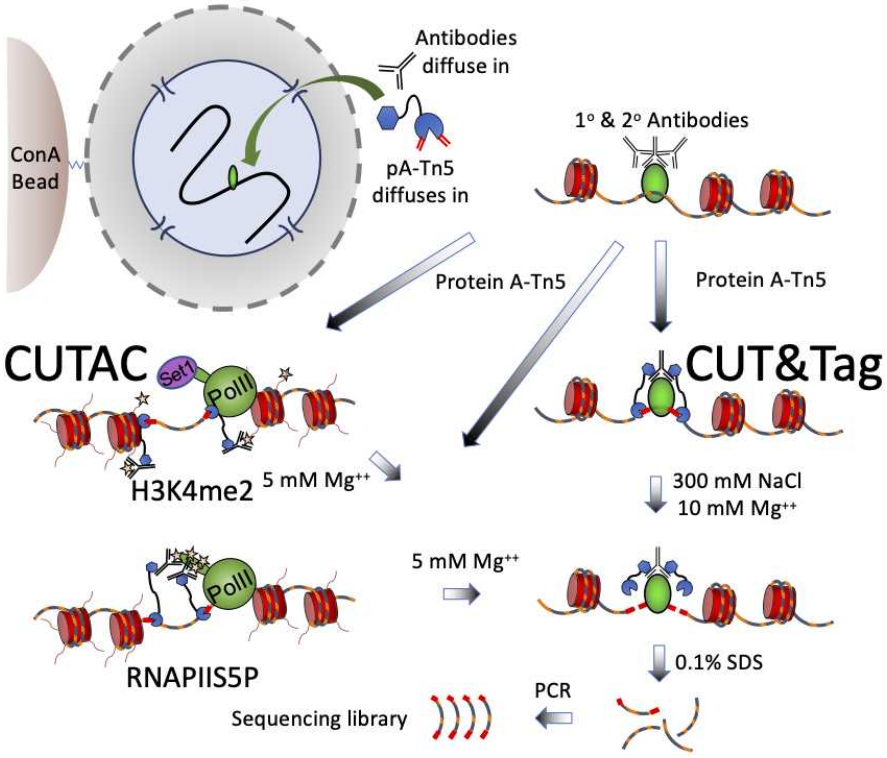
Steven Henikoff
Fred Hutchinson Cancer Research Center

Citation: Steven Henikoff, Jorja Henikoff, Kami Ahmad (06/05/2021). CUT&Tag-direct with CUTAC. https://dx.doi.org/10.17504/protocols.io.bqwvmxe6

ABSTRACT



Scheme for simultaneous CUT&Tag and (H3K4me2 or RNAPIIS5P) CUTAC. CUT&Tag-direct is performed *in situ* in single PCR tubes with Concanavalin A (ConA) bead-bound nuclei that remain intact throughout the protocol during successive liquid changes, incubations and washes, 12 cycles of PCR amplification and one SPRI bead clean-up. CUTAC is performed identically except that low-salt conditions are used for tagmentation. H3K4me2 CUTAC maps accessible sites near H3K4me2/3-marked (starred) nucleosome tails, which are methylated by the conserved Set1 lysine methyltransferase. The complex that includes Set1 associates with the initiation form of RNAPII, which is heavily phosphorylated on Serine-5 of the heptameric C-terminal domain repeat units on the largest RNAPII subunit (RNAPIIS5P). For RNAPIIS5P CUTAC, pA-Tn5 is anchored directly to RNAPIIS5 phosphates (starred). Whereas CUT&Tag is suitable for any chromatin epitope, CUTAC is specific for H3K4me2, H3K4me3 and RNAPIIS5P. The only other difference between the protocols is that tagmentation is performed in the presence of 300 mM NaCl for CUT&Tag and in a low ionic strength buffer for CUTAC.

We previously introduced Cleavage Under Targets & Tagmentation (CUT&Tag), an epigenomic profiling method in which antibody tethering of the Tn5 transposase to a chromatin epitope of interest maps specific chromatin features in small samples and single cells.With CUT&Tag, intact cells or nuclei are permeabilized, followed by successive addition of a primary antibody, a secondary antibody, and a chimeric Protein A-Transposase fusion protein that binds to the antibody. Addition of Mg++ activates the transposase and inserts sequencing adapters into adjacent DNA*in situ*.We have since adapted CUT&Tag to also map chromatin accessibility by simply modifying thetransposaseactivation conditions when using histone H3K4me2, H3K4me3 or Serine-5-phosphorylated RNA Polymerase II antibodies. Using these antibodies, tagmentation of accessible DNA sites is redirected to produce chromatin accessibility maps with exceptionally high signal-to-noise and resolution. All steps from nuclei to amplified sequencing-ready libraries are performed in single PCR tubeswith non-toxic reagents using inexpensive equipment, making our simplified strategy for simultaneous chromatin profiling and accessibility mapping suitable for the lab, home workbench or classroom.

EXTERNAL LINK

https://doi.org/10.7554/eLife.63274

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** *eLife* 9:e63274, DOI: 10.7554/eLife.63274.

Citation: Steven Henikoff, Jorja Henikoff, Kami Ahmad (06/05/2021). CUT&Tag-direct with CUTAC. https://dx.doi.org/10.17504/protocols.io.bqwvmxe6

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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** *eLife* 9:e63274, DOI: 10.7554/eLife.63274.

KEYWORDS

CUT&Tag, CUTAC, epigenetics, chromatin profiling

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GUIDELINES

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

MATERIALS TEXT

A home workbench for CUT&Tag. Photo of the home workbench setup used for all experiments presented using this protocol. A typical experiment begins by mixing cells with activated ConA beads in 32 single PCR tubes, with all liquid changes performed on the magnet stands. The only tube transfer is the removal of the purified sequencing-ready libraries from the SPRI beads to fresh tubes for Tapestation analysis and DNA sequencing. The total time from thawing frozen nuclei until elution from SPRI beads is ~8 hr.

- 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, and 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 native or cross-linked nuclei suspension (e.g. human K562 or H1 cells) prepared as described in the Appendix (Steps 48-60, cite: Kaya-Okur et al. (2020) *Nature Protocols*).
- 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 H_2O (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)
- 200X Bovine Serum Albumen (BSA, NEB, cat no. B9001S)
- 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 RNA Polymerase II Phospho-Rpb1 CTD Serine-5 phosphate (PolIIS5P) or histone H3K4me2. We have obtained excellent results with these rabbit monoclonal antibodies: Cell Signalling Technology Phospho-Rpb1 CTD (Ser5), CST #13523 (D9N5I) and Epicypher H3K4me2 #13-0027.
- 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 [tris(hydroxymethyl)methylamino]propanesulfonic acid (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 (2015)] in 10 mM Tris pH 8. Standard salt-free primers may be used. We do not recommend Nextera or NEBNext primers.
- 10% Sodium dodecyl sulfate (SDS; Sigma-Aldrich, cat. no. L4509)

Citation: Steven Henikoff, Jorja Henikoff, Kami Ahmad (06/05/2021). CUT&Tag-direct with CUTAC. https://dx.doi.org/10.17504/protocols.io.bqwvmxe6

- 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 and cryopreserve nuclei (STEPS 50-62 at the lab)
Prepare reagents (STEP 1, OK at the lab, at home or in the classroom)

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. *HEPES-NaOH pH 7.5 is OK.

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 2 days.

Antibody buffer Mix 5 µl 200X BSA with 1 ml Wash buffer and chill on ice. BSA is present in some but not all antibody solutions, and 0.1% BSA in this buffer helps to prevent bead loss during later steps.

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 2 days.

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

CUTAC Tagmentation buffer 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 $MgCl_2$). Store the buffer at 4 °C for up to 1 day.

CUTAC-hex Tagmentation buffer 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 MgCl2 (10 mM TAPS, 5 mM $MgCl_2$, 10% 1,6-hexanediol). Store the buffer at 4 °C for up to 1 day.

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, ensuring that there will be 3.5µL for each final sample of up to ~50,000 mammalian cells, which yield ≥50% K562 nuclei using this protocol. Transfer the ConA bead slurry into 1 mL of Binding buffer in a 1.5 mL tube.

This protocol has been used for up to 16 samples (60 µL beads) in 1 mL or 32 samples (120 µL beads) in 2 mL Binding buffer (in a 2 mL tube).

3 Mix by pipetting. Place the tube on a magnet stand to clear (~1 min).

Citation: Steven Henikoff, Jorja Henikoff, Kami Ahmad (06/05/2021). CUT&Tag-direct with CUTAC. https://dx.doi.org/10.17504/protocols.io.bqwvmxe6

4 Withdraw the supernatant completely, and remove the tube from the magnet stand. Add 1 mL Binding buffer and mix by pipetting up and down.

5 Place on the magnet stand to clear, remove and discard the supernatant, and resuspend in 60 µL Binding buffer (3.5 µL per sample).

Bind nuclei to ConA beads (15 min)

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

The CUTAC control can use either native or lightly cross-linked nuclei, preferably prepared as described in the Appendix (Steps 52-64). Do not use whole cells, which require a detergent and may also inhibit the PCR.

7 Transfer the thawed nuclei suspension in aliquots of no more than ~50,000 starting mammalian cells to each thin-wall PCR tube and mix with 3.5 µL ConA beads. Attach to tube Rotator and rotate at room temperature for 10 min.

Nuclei prepared according to the protocol in the Appendix (Steps 52-64) have been resuspended in Wash buffer. Beads can be added directly to the aliquot for binding and then transferred to PCR tubes ensuring 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 the 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 at this step.To avoid beads being aspirated with the supernatant, set the pipette to a volume that is 5 µL less than the total volume to be removed. Use a careful second draw with a 20 µL pipette tip and remove as much supernatant as possible, without aspirating beads.

Bind primary antibody (1 hr)

9 For each CUT&Tag and CUTAC sample, mix the primary antibody 1:50-1:100 with Antibody buffer. Resuspend beads in 25 µL per sample with gentle vortexing.

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

Citation: Steven Henikoff, Jorja Henikoff, Kami Ahmad (06/05/2021). CUT&Tag-direct with CUTAC. https://dx.doi.org/10.17504/protocols.io.bqwvmxe6

CUTAC works best using either an RNA Polymerase II CTD-phosphorylated antibody (Ser5P > Ser2P/Ser5P > Ser2P) or an α-H3K4me2 antibody. α-H3K4me3 also works but is less efficient and is depleted at enhancer sites. Several antibodies to other histone epitopes have been tested, including α-H3K4me1, α-H3K36me3, α-H3K27ac and α-H2A.Z, but all have failed.

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, avoiding the need for a quick spin before the next step.

After incubation, the tubes can be stored overnight at 4 °C.

The α-RNA PolIISer5P and α-H3K4me2 antibodies used for both CUT&Tag and CUTAC serve as controls to evaluate success of the CUTAC procedure. 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 and discard the supernatant.

Protein in the antibody solution improves bead adherence to the tube wall, allowing for complete removal of the liquid without dislodging the beads by doing two successive draws with a 20 µL pipettor set for maximum volume while being careful not to dislodge the beads by surface tension during the second draw.

12 Mix the secondary antibody 1:100 in Wash buffer and squirt in 25 µL per sample while gently vortexing to allow the solution to dislodge the beads from the sides.

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 0.5-1 hr. 00:30:00

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14 After a quick spin (<500 x g or just enough to remove the liquid from the sides of the tube), place the tubes on the magnet stand to clear and remove and discard the supernatant with two successive draws, using a 20 µL tip with the pipettor set for maximum volume.

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

16 Slowly remove 470 µL of supernatant with a 1 mL pipette tip without disturbing the beads.

To remove the supernatant, 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. During liquid removal, the surface tension will drag the beads down the tube. A small drop of liquid that is left behind will be removed in the next step.

17 After a quick spin (< 500 x g or just enough to remove the liquid from the sides of the tube), place the tubes back into the magnet stand and remove the remaining supernatant with a 20 uL pipettor multiple times if necessary, to remove the entire supernatant without disturbing the beads. Proceed immediately to the next step.

Bind pA-Tn5 adapter complex (1.5 hr)

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

19 Pipette in 25 µL per sample of the pA-Tn5 mix while vortexing and invert by rotation to ensure that beads adhering to the sides near the top of the top are resuspended.

When using the recommended Macsimag magnet stand, dislodging the beads after resuspending in pA-Tn5 can be done by removing the plexiglass tube holder from the magnet and with fingers on top to prevent the tubes from opening or falling out, inverting by rotating sharply a few times.

1h

20 After a quick spin, place the tubes on a rotator at room temperature for 1-2 hr. **01:00:00**

21 After incubating in the rotator, perform a quick spin and place the tubes in the magnet stand.

22 Carefully remove the supernatant using a 20 µL pipettor twice to avoid disturbing the beads as in Step 16.

23 1.With the tubes still on the magnet stand, add 500 µl of the 300-wash buffer.

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24 Slowly withdraw 470 µl with a 1 ml pipette tip without disturbing the beads as in Step 16.

25 After a quick spin, place the tubes back on the magnet stand and remove and discard the supernatant with a 20 µL pipettor using multiple draws. Proceed immediately to Step 27. For a CUTAC sample proceed immediately to Step 26.

CUTAC Tagmentation (performed in parallel with other samples)

26 Resuspend the bead/nuclei pellet in 50 µL CUTAC (5 mM $MgCl_2$, 10 mM TAPS) or CUTAC-hex tagmentation solution (5 mM $MgCl_2$, 10 mM TAPS, 10% 1,6-hexanediol) while vortexing or inverting by rotation to allow the solution to dislodge most or all the beads as in Step 19. Place the tube(s) in a cold block and proceed to Step 28.

10% 1,6-hexanediol or N,N-dimethylformamide compete for hydrophobic interactions and result in improved tethered Tn5 accessibility and library yield, at the expense of slightly increased background.

CUT&Tag tagmentation and particle release (2.5 hr)

27 Resuspend the bead/nuclei pellet in 50 µL tagmentation solution while vortexing or inverting by rotation to allow the solution to dislodge most or all the beads as in Step 19.

28 After a quick spin (<500 x g), incubate at 37 ºC for 1 hr (20 min for CUTAC) in a PCR cycler with a heated lid. Hold at 8 ºC. **01:00:00** **00:20:00** 1h 20m

29 Place tubes on a magnet stand and remove and discard the supernatant with a 20 µL pipettor using multiple draws then resuspend the beads in 50 µL TAPS wash and invert by rotation as in Step 19.

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

31 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.

Rolling the tube back and forth rapidly between thumb and forefinger while brushing the pipette tip along the sides of the tube will effectively wet the beads – follow by a quick spin to bring most of the beads to the bottom.

32 After a quick spin, incubate at 58 ºC for 1 hr in a PCR cycler with a heated lid to release pA-Tn5 from the tagmented DNA. **01:00:00**

PCR (1 hr)

33 To the PCR tube containing the bead slurry add 15 µL of 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 speed and place tubes in the metal tube holder on ice.

Citation: Steven Henikoff, Jorja Henikoff, Kami Ahmad (06/05/2021). CUT&Tag-direct with CUTAC. https://dx.doi.org/10.17504/protocols.io.bqwvmxe6

Indexed primers are described by Buenrostro, J.D. et al. Single-cell chromatin accessibility reveals principles of regulatory variation. Nature 523:486 (2015). We do not recommend Nextera or NEB primers, which might not anneal efficiently using this PCR protocol.

34 Add 25 µL NEBnext (non-hot-start), vortex to mix, and perform a quick spin. Place the tubes immediately in the thermocycler and proceed immediately with the PCR.

35 Begin the cycling program with a heated lid on the thermocycler:
Cycle 1: 58 °C for 5 min (gap filling)
Cycle 2: 72 °C for 5 min (gap filling)
Cycle 3: 98 °C for 30"
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, the PCR should be performed for no more than 12-14 cycles, preferably with a 10 s 60-63 °C combined annealing/extension step (Cycle 5).

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 unacceptably high level of PCR duplicates.

Cycle 3 (98 °C) can be extended from 30 sec to 5 min for cross-linked samples (preferred for CUTAC) to ensure complete cross-link reversal and fragment release.

Post-PCR Clean-up (30 min)

36 After the PCR program ends, remove tubes from the thermocycler and add 65 µL of SPRI beads (ratio of 1.3 µL of SPRI beads to 1 L of PCR product). Mix by pipetting up and down.

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37 Let sit at room temperature 5-10 min. **00:05:00**

38 Place on the magnetic stand for a few minutes to allow the solution to clear.

39 Remove and discard the supernatant.

40 Keeping the tubes in the magnetic stand, add 200 uL of 80% ethanol.

41 Completely remove and discard the supernatant.

42 Repeat Steps 40 and 41.

43 Perform a quick spin and remove the remaining supernatant with a 20 uL pipette, avoiding air drying the beads by proceeding immediately to the next step.

44 Remove from the magnet stand, add 22 µL 10 mM Tris-HCl pH 8 and vortex at full speed. Let sit for 5 min to 1 hr. **00:05:00**

45 Place on the magnet stand and allow to clear.

46 Remove the liquid to a fresh 1.5 ml tube with a pipette, avoiding transfer of beads.

Tapestation analysis and DNA sequencing (outsource)

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

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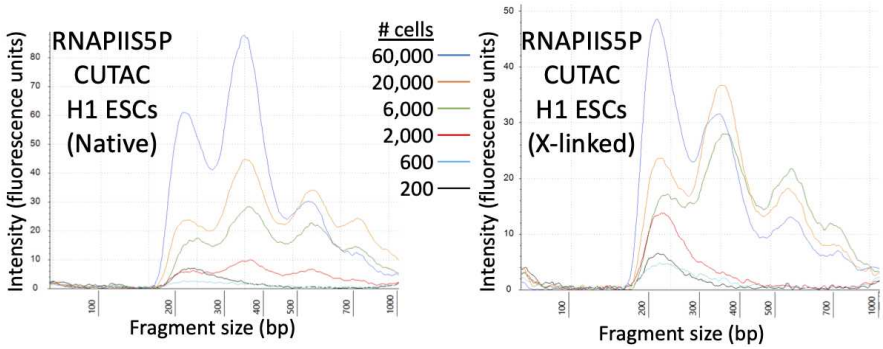


Figure 3. Tapestation profiles for a low-cell number RNAPIIS5P CUTAC experiment. Tagmentation was performed for 20 min at 37ºC in CUTAC-hex buffer. Representative tracks for these samples are shown below Step 50.

We use the quantification by Tapestation to estimate library concentration and dilute each library to 2 nM before pooling based on fragment molarity in the 175-1,000 bp range. The concentration 2 nM has been determined empirically as the optimal library concentration used in the HiSeq by the Fred Hutch Genomics Shared Resource.

48 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.

49 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

50 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 as 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, Kami Ahmad (06/05/2021). CUT&Tag-direct with CUTAC. https://dx.doi.org/10.17504/protocols.io.bqwvmxe6

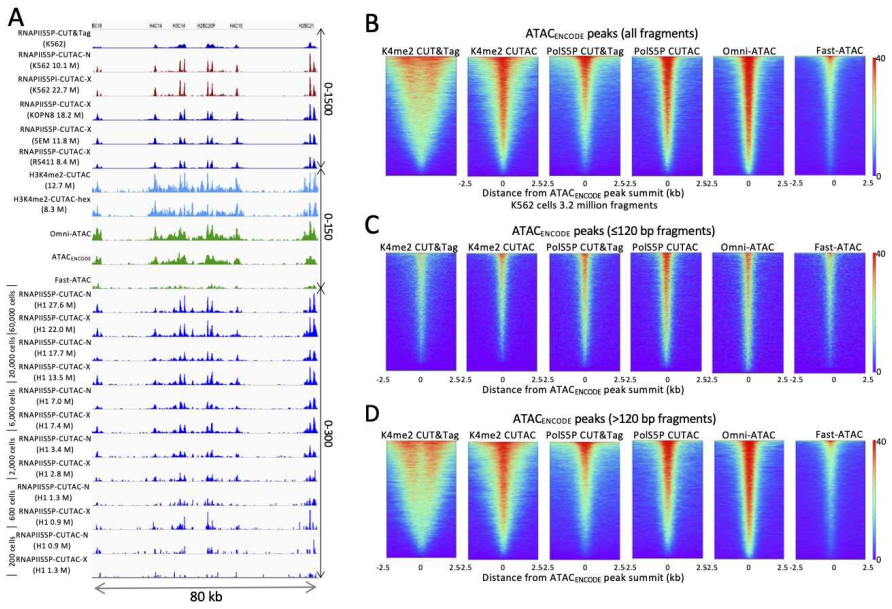
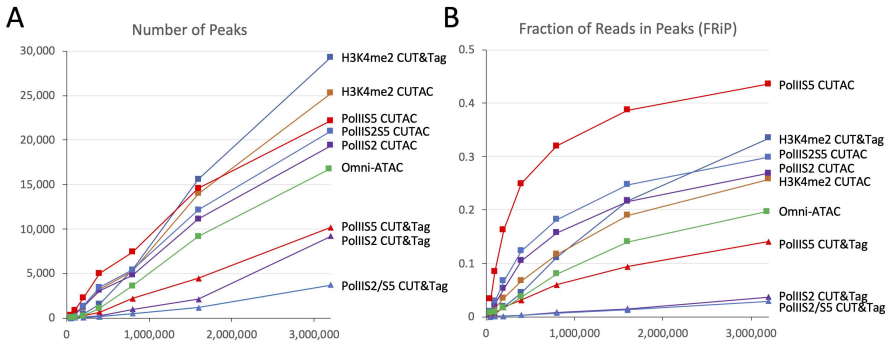


Figure 4. Accessible DNA corresponds to binding sites of the initiating form of RNA Polymerase II (RNAPII). A) Tracks show profiles of the Chromosome 1 histone gene cluster, with 12 small intronless genes expressed at high levels in all dividing cells. Whereas RNAPIIS5P CUT&Tag shows broad enrichment over each of the genes, the CUTAC protocol applied to the RNAPIIS5P epitope, either native (RNAPIIS5P CUTAC-N) or cross-linked (RNAPIIS5P CUTAC-X), yields sharp promoter delineation, better than H3K4me2 CUTAC ± 1,6-hexanediol (turquoise) or the best K562 ATAC-seq datasets (green), all downsampled to 3.2 million mapped fragments. Note the 10-fold difference in scale between RNAPIIS5P CUTAC (0-1500 and K4me2-CUTAC/ATAC (0-150). Similar results were obtained for three mixed-lineage leukemia cell lines (KOPN8, SEM and RS411) and H1 embryonic stem cells down to ~2,000 cells. No changes were made to the protocol for low cell numbers. Numbers in parentheses are estimated library sizes in millions of mapped paired-end reads. B-D) RNAPIIS5P occupies sites of accessible chromatin in K562 cells. B) Left to right: K4me2 CUT&Tag, K4me2 CUTAC, RNAPIIS5P CUT&Tag, RNAPIIS5P CUTAC, Omni-ATAC and Fast-ATAC datasets were downsampled to 3.2 million fragments and aligned over ATAC-seq peaks called using MACS2 on data generated by the ENCODE project (ATACENCODE). C) Same as (A) except using only subnucleosome-sized fragments (≤120 bp). CUTAC RNAPIIS5P sites are virtually indistinguishable from high-quality ATAC-seq data, directly demonstrating that ATAC-seq maps sites of the initiation form of RNA Pol II. D) Same as (A) except using only > 120 bp fragments. ENCODE ATAC-seq fragments were downsampled to 3.2 million, ChrM (mitochondrial DNA) was removed and MACS2 was used to call peaks. Heatmaps are centered over ENCODE ATAC-seq peak summits and ordered by occupancy over the 5 kb span displayed. Fast-ATAC is an improved version of ATAC-seq that reduces mitochondrial reads (Corces et al., Nat. Genet. 48: 1193-1203, 2016), and Omni-ATAC is an improved version that additionally improves the signal-to-noise ratio (Corces et al., Nat Methods 14: 959-962, 2017). ATAC_ENCODE is the current ENCODE standard (Moore et al., Nature 583: 699-710, 2020). Datasets are available from GEO (GSE158327)



Pol-CUTAC shows high sensitivity and specificity. Mapped fragments from the indicated K562 datasets were sampled, and peaks were called using MACS2. **A**) Number of peaks and **B**) fraction of reads in peaks for CUT&Tag (triangles) and CUTAC (squares) profiles for H3K4me2, PolIISer5 phosphate (initiation form), PolIISer2 phosphate (elongation form), PolIIS2P/S5P, and Omni-ATAC (green). Pol-CUTAC for PolIIS5 phosphate shows the best sensitivity (most peaks at low sampling) and the best signal-to-noise (highest FRiP at all sampling levels). Tagmentation was for 10 min at 37 °C in CUTAC-tag buffer. Datasets are available from GEO (GSE158327).

51 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 and CUT&RUNTools.

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

See Efficient chromatin accessibility mapping *in situ* by nucleosome-tethered tagmentation for examples of CUT&Tag@home applications.

Appendix: Prepare and (optionally) lightly fix nuclei and cryopreserve (1 hr in the lab)

52 **Prepare NE1 buffer:** Mix 1 ml 1M HEPES-KOH pH 7.9, 500 µL 1M KCl, 12.5 µL 2 M spermidine, 500 µL 10% Triton X-100, and 10 ml glycerol in 38 ml dH_2O, and add 1 Roche Complete Protease Inhibitor EDTA-Free.

Prepare 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.

Other reageants:
- 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)
- 1.25 M glycine
- Dimethyl sulfoxide (DMSO; Sigma-Aldrich cat. no. D4540)

53 Harvest fresh culture(s) in a conical centrifuge tube (15 ml or 50 ml) at room temperature and count cells.

54 Centrifuge for 3 min at 600 x g in a swinging bucket rotor at room temperature and drain liquid.

55 Resuspend in 1 volume of PBS at room temperature by pipetting up and down.

56 Centrifuge for 3 min at 600 x g in a swinging bucket rotor at room temperature and drain the liquid.

57 Resuspend in 1/2 volume (relative to starting culture) ice-cold NE1 buffer with gentle vortexing. Let sit on ice for 10 min.

58 Centrifuge for 4 min at 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.

59 Resuspend in 1/2 volume of PBS. For unfixed nuclei, skip to Step 62.

60 While gently vortexing, add 16% formaldehyde to 0.1% (e.g., 62 µL to 10 ml) and incubate at room temperature for 2

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min.

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.

Light fixation reduces the tendency of cells or nuclei to clump in the Dig-300 buffer, but can interfere with the binding of some antibodies, reducing yield.

61 Stop cross-linking by adding 1.25 M glycine to twice the molar concentration of formaldehyde (e.g., 600 µL to 10 ml).

62 Centrifuge for 4 min at 1300 x g at 4 °C and drain liquid by pouring off and inverting onto a paper towel for a few seconds.

63 Resuspend in Wash buffer to a concentration of ~1 million cells per ml. Check nuclei using ViCell or a cell counter slide.

64 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 and stored at -80 °C long term.

We have found that good results are obtained using native or cross-linked cells even after being stored in the freezer compartment of a side-by-side refrigerator for >6 months.

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