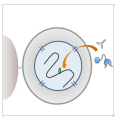

Apr 17, 2020

CUT&Tag@home

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1 Works for me dx.doi.org/10.17504/protocols.io.bd26i8he

Human Cell Atlas Method Development Community

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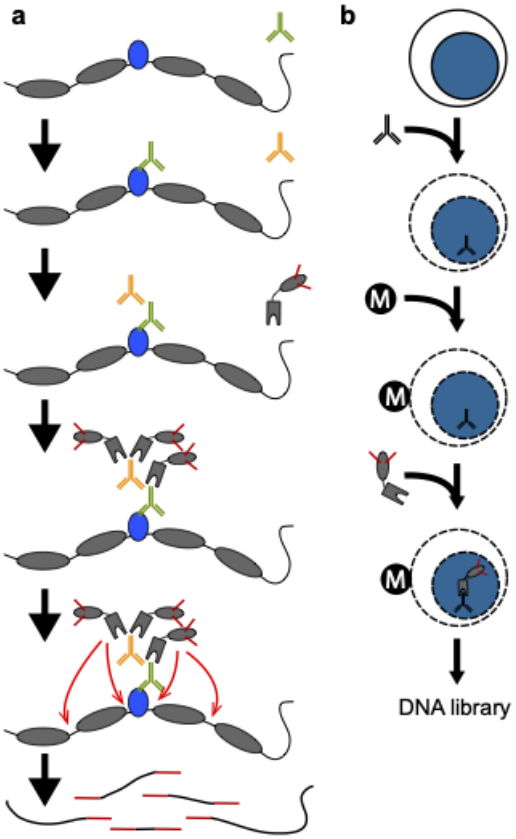
ABSTRACT

CUT&Tag@home 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.



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.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

CUT&Tag-Direct: Kaya-Okur, H.S., Janssens, D.H., Henikoff, J.G., Ahmad, K., and Henikoff, S. (2020). Efficient low-cost chromatin profiling with CUT&Tag. Submitted for publication; CUT&Tag@home: Henikoff, S. & Henikoff, J.G. (2020) Profiling the epigenome at home. *bioRxiv* https://www.biorxiv.org/content/10.1101/2020.04.15.043083v1.full.pdf

Citation: Steven Henikoff, Derek Janssens, Hatice Kaya-Okur, Jorja Henikoff, Kami Ahmad (04/17/2020). CUT&Tag@home. https://dx.doi.org/10.17504/protocols.io.bd26i8he

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@home. 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 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 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)
- 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

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- 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 cat. no. ABIN101961) or rabbit α-mouse antibody (Abcam cat. no. ab46540)
- Protein A–Tn5 (pA-Tn5) fusion protein loaded with 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 µM in TE buffer)
- 1 M Magnesium Chloride ($MgCl_2$; Sigma-Aldrich, cat. no. M8266-100G)
- 1 M TAPS pH 8.5 (with NaOH)
- NEBNext 2X PCR Master mix (ME541L)
- PCR primers: 10 µM 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 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 4 µL 0.5 M EDTA and 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.

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

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 ~100,000 mammalian cells. The following is for 16 samples.

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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).

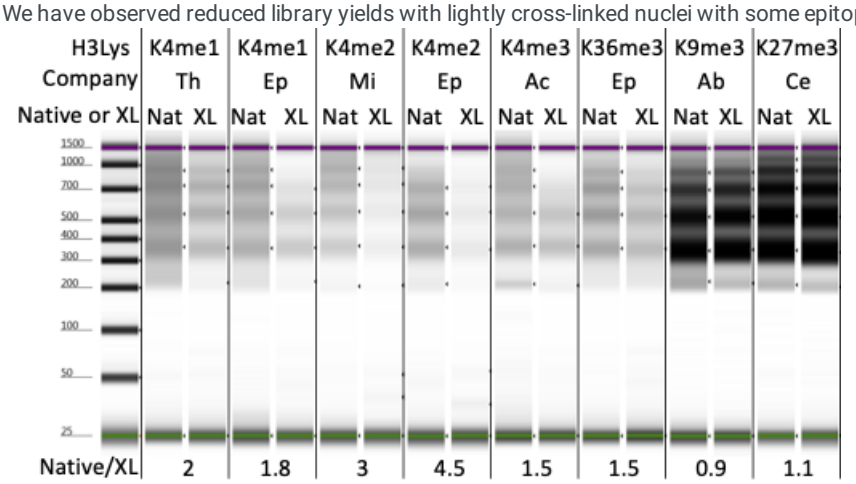
4 Withdraw the liquid completely, and remove from the magnet stand. Add 1 mL Binding buffer and mix by pipetting.

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 native or lightly cross-linked nuclei aliquot at room temperature, for example by placing in a 20 ml beaker of water.

We have observed reduced library yields with lightly cross-linked nuclei with some epitopes and antibodies.



Yields of CUT&Tag@home libraries from lightly cross-linked nuclei vary depending on the epitope and antibody. In the lab, a nuclei prep was split and prepared as either native (Nat) or cross-linked (XL), then aliquoted and frozen. At home, aliquots were thawed and libraries were prepared from 50,000 starting cells using this protocol with the following rabbit antibodies: H3K4me1-Th (Thermo #710795 lot 1998633); H3K4me1-Ep (Epicypher 13-0026 lot 28344001); H3K4me2-Mi (Millipore 07-030 lot 3229364); H3K4me2-Ep (Epicypher 13-0027); H3K4me3-Ac (Active Motif 39159 lot 22118006); H3K36me3-Ep (Epicypher Rabbit monoclonal #13-0031, lot 18344001); H3K9me3-Ab (Abcam ab8898 lot GR3302452-1); H3K27me3-Cs (CST #9733). The Tapestation image for 1/10th of each library is shown, where Native/XL is the molar ratio of yields over a 175-1000 bp range.

7 Mix 25-200
µL of nuclei suspension with 3-5 µL ConA beads in thin-wall 0.5 ml PCR tubes and let sit at room temperature for 10 min.

Using more than ~100,000 nuclei or >5 µL Con A beads per sample may inhibit the PCR.

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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.

Bind primary antibody (1 hr)

9 Resuspend cells in 50 µL Antibody buffer then 0.5µL antibody (1:100) with gentle vortexing.

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.

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.

To evaluate success of the procedure without requiring library preparation, include in parallel a positive control antibody (e.g.α-H3K27me3), and an optional negative control 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.

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

30m

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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.

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 the liquid with a 1 mL pipette tip without disturbing the beads.

To withdraw the liquid, set the pipettor to 600 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 all the liquid is withdrawn, and remove the pipettor. This will leave behind at most a small drop of liquid.

18 After a quick spin, place the tubes on a magnet stand to remove the last drop with a 20 µL pipette tip and proceed immediately to the next step.

Bind pA-Tn5 adapter complex (1.5 hr)

19 Mix pA-Tn5 adapter complex in 300-wash buffer to a final concentration of 1:200.

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.

20 Squirt in 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.

1h

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

22 Place the tubes on a magnet stand to clear and pull off the liquid.

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

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

25 Squirt in 50 µL per sample of 300-wash buffer while vortexing and invert by rotation to allow the solution to dislodge most or all of the beads.

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26 After a quick spin, 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.

27 After a second quick spin, place the tubes on a magnet stand to remove the last drop with a 20 µL pipette tip and proceed immediately to the next step.

Tagmentation and particle release (2.5 hr)

28 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 of the beads as in Step 20.

29 After a quick spin (<500 x g), incubate at 37 ºC for 1 hr in a PCR cycler with heated lid. **01:00:00**

30 Place tubes on a magnet stand, and withdraw the liquid, with the pipettor set to 5 µL less than the volume to be removed, followed by a quick spin.

31 Place the tubes on a magnet stand and remove any remaining liquid using a 20 µL pipette tip, then resuspend the beads in 50 µL TAPS wash and invert by rotation to mix.

32 Place tubes on a magnet stand, and withdraw the liquid with the pipettor set to 5 µL less than the volume to be removed, followed by a quick spin.

33 Place the tubes on a magnet stand and remove any remaining liquid using a 20 µL pipette tip, and proceed immediately to the next step.

34 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.

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

PCR (1 hr)

36 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.

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

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

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

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

41 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.

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

43 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.

44 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**

45 Place on the magnet stand and allow to clear.

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

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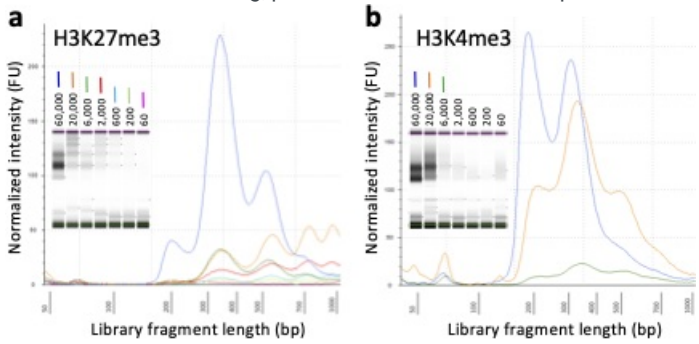
Tapestation analysis and DNA sequencing (outsource)

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

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.



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.

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.

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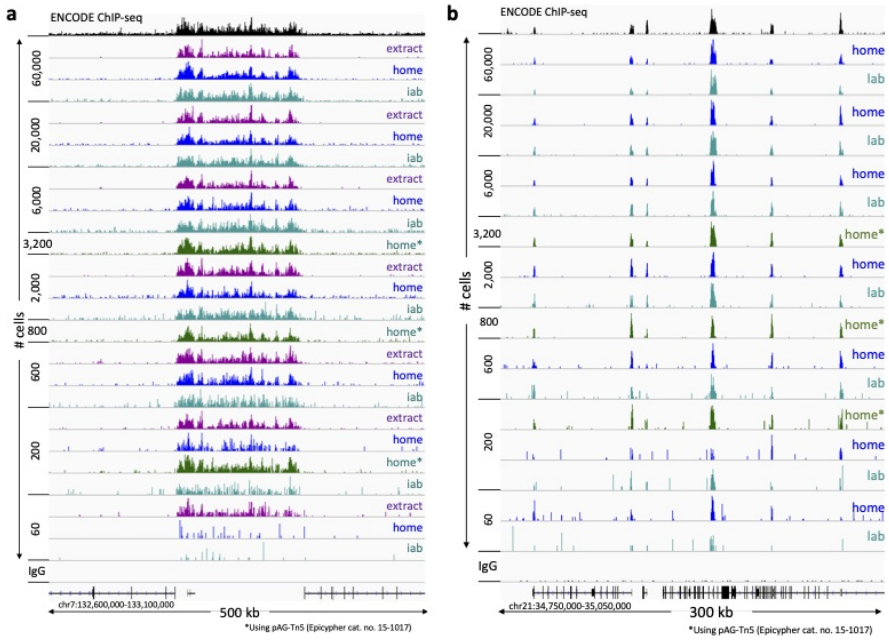
Data processing and analysis

50 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.



CUT&Tag@home data 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.

51 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.

See https://www.biorxiv.org/content/10.1101/2020.04.15.043083v1.full.pdf for examples of CUT &Tag@home applications

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