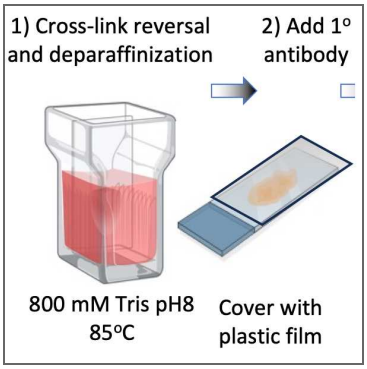

VERSION 2

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Version created by Steven Henikoff

CUTAC for FFPEs V.2

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Human Cell Atlas Method Development Community

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ABSTRACT

For more than a century, Formalin Fixed Paraffin Embedded (FFPE) sample preparation has been the preferred method for long-term preservation of biological material. However, the use of FFPE samples for epigenomic studies has been difficult because of chromatin damage from long exposure to high concentrations of formaldehyde. Previously, we introduced Cleavage Under Targeted Accessible Chromatin (CUTAC), an antibody-targeted chromatin accessibility mapping protocol based on CUT&Tag. Here we show that simple modifications of our single-tube CUTAC protocol are sufficient to produce high-resolution maps of paused RNA Polymerase II (RNAPII) at enhancers and promoters using FFPE samples. We find that transcriptional regulatory element differences produced by FFPE-CUTAC distinguish between mouse brain tumors and identify and map regulatory element markers with high confidence and precision, including microRNAs not detectable by RNA-seq. Our simple workflows make possible affordable epigenomic profiling of archived biological samples for biomarker identification, clinical applications and retrospective studies.

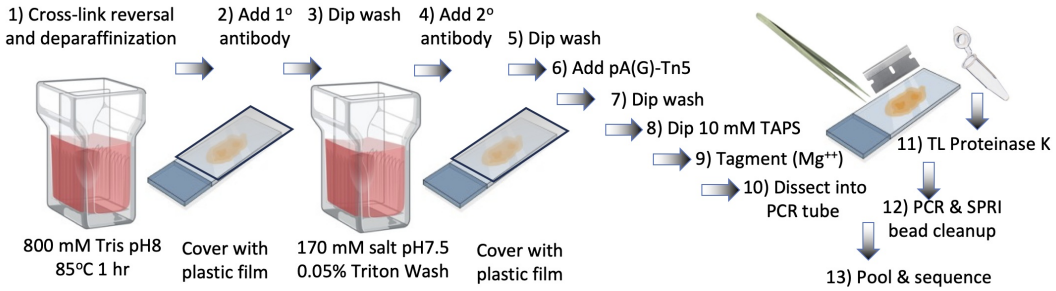

Figure 1: On-slide FFPE-CUTAC. Schematic of the protocol.

IMAGE ATTRIBUTION

Biorender.com

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We use this protocol and it's working

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Keywords: CUT&Tag, epigenetics, chromatin profiling, Formaldehyde-fixed paraffin-embedded sections

GUIDELINES

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

MATERIALS

A home workbench for CUT&Tag. Photo of the home workbench setup used for all experiments presented using this protocol.

- 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 and 2 mL microfuge tubes
- 0.5 ml maximum recovery PCR tubes (e.g. Fisher cat. no. 14-222-294)
- 10 micron section from a formaldehyde-fixed paraffin-embedded tissue block affixed to a *charged* glass slide
- 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)
- Ethanol (Decon Labs, cat. no. 2716)
- Distilled, deionized or RNAse-free H_2O (dH_2O e.g., Promega, cat. no. P1197)
- Roche Complete Protease Inhibitor EDTA-Free tablets (Sigma-Aldrich, cat. no. 5056489001)
- 1 M Tris-HCl pH 8.0
- 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)
- 10% Triton X-100 (Sigma-Aldrich, cat. no. X100)
- 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, CST #13523 (D9N5I)).
- 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.
- Thermolabile Proteinase K (NEB P8111S)
- 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)
- N,N-dimethylformamide (Sigma-Aldrich cat. no. D-8654-250mL)
- Bio-mag Plus amine magnetic beads (Polysciences cat. no. 86001-10
- 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)
- SPRI paramagnetic beads (e.g. HighPrep PCR Cleanup Magbio Genomics cat. no. AC-60500)

REAGENT SETUP (for up to 16 samples)

1 **Cross-link reversal buffer** Mix 800 µL 1 M Tris-HCl pH8.0, 200 µL dH_2O.

Rinse buffer Mix 1 mL 1 M HEPES pH 7.5 and 1.5 mL 5 M NaCl, 250 µl Triton-X100 and bring the final volume to 50 mL with dH_2O.

Triton-Wash buffer Mix 1 mL 1 M HEPES pH 7.5, 1.5 mL 5 M NaCl, 250 µl Triton-X100 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 the buffer at 4 °C for up to 2 days.

Primary antibody solution Mix 17 µl RNA Polymerase II-Ser5p: (Cell Signaling Technologies (D9N5I) mAb #13523) + 423 µl Triton-Wash buffer (1:25).

Secondary antibody solution Mix 17 µ guinea pig anti-rabbit (Antibodies Online) with 423 µL Triton-Wash buffer (1:25).

Protein A(G)-Tn5 solution Mix 21 µl Protein A(G)-Tn5 (Epicypher cat. no. 15-1117) with 419 µL Triton-Wash buffer (1:20).

CUTAC-DMF Tagmentation buffer Mix 17.7 mL dH_2O, 4 mL N,N-dimethylformamide, 220 µl 1 M TAPS pH 8.5, and 110 µl 1 M $MgCl_2$ (10 mM TAPS, 5 mM $MgCl_2$, 20% DMF). Store the buffer at 4 °C for up to 1 week.

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). Store at room temperature.

1% SDS/ProtK Release solution (For 16 samples) Mix 10 µl 10% SDS and 1 µl 1 M TAPS pH 8.5 in 79 µl dH_2O. Just before use add 10 µL Thermolabile Proteinase K (NEB cat. no. P8111S).

6% Triton Mix 600 µl 10% Triton-X100 + 400 µl dH_2O. Store at room temperature.

Deparaffinization in hot cross-link reversal buffer (1.5 hr).

2 Place slides in cross-link reversal buffer in a slide holder that is filled to completely cover the slides. Place the holder in a water bath at 85 ^{o}C and incubate for at least an hour. The paraffin will melt and float to the top. Remove slide holder to an ice-cold water bath to chill. Adding more solution to overfill will drain off any solid paraffin. 9m

Note

Overnight 85 ^{o}C incubations give similar results to 1 hr incubations. Be sure that the FFPE sections are affixed to a charged glass slide to avoid tissue loss during incubation.

Note

This protocol is for 16 samples but can be scaled up or down as needed. The example experiment shown in Figures 2-4 beginning with dry FFPE slides through sequencing-ready purified DNA libraries was accomplished in 1 day (~11 hours), but all of the steps can be lengthened with proper sealing to minimize evaporation. Overnight stopping points can be during any of the room temperature incubations by placing the plastic film-wrapped slides into a moist chamber and holding at 4 ^{o}C.

3 Remove slides to Rinse Buffer in a slide holder.

4 For Option 1 (on-slide), continue with Step 5. For Option 2 (Magnetic Beads), skip to Step 22.

Option 1: On-slide FFPE-CUTAC Incubation with primary antib...

5 For each slide, remove from slide holder, wick off excess liquid with a Kimwipe and place tissue-side up on a dark surface. Carefully pipette ~100 µl primary antibody solution over the tissue.

6 Cover the clear portion of the slide with a rectangle of plastic film using surface tension to spread the liquid, while excluding large bubbles and wrinkles. Place wrapped slides separated in a dry slide holder (Figure 2). 1h

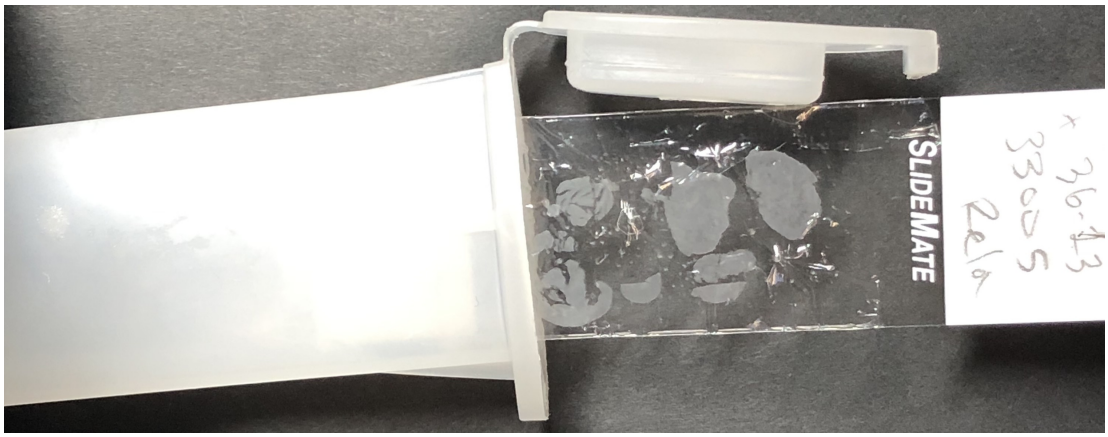

Figure 2: A small slide holder will hold two plastic film-wrapped slides without touching or disturbing the wrap. Closing the top will allow for long incubations without drying out.

Note

Any plastic wrap will seal adequately, but we recommend food service film on a heavy 2000 foot roll (e.g. Reynolds 912) for ease of pulling out wrap with both hands. Some kitchen wraps (Saran and Glad) are not as smooth and will be more difficult to work with. Before removing slides from the Rinse Buffer, use a razor to cut plastic film rectangles slightly wider and longer than the clear portion of the slide.

Note

Optionally, for incubating multiple slides with the same antibody or pA(G)-Tn5 solution, place the slides abutted against one another on the surface of a plastic box (Figure below). After adding the solution to the slide surfaces, lower stretched-out plastic film so that it makes contact with the liquid, then continue lowering so the meniscus moves over all of the samples.

Figure 3: Optional setup for incubating multiple slides with the same solution.

Expected result

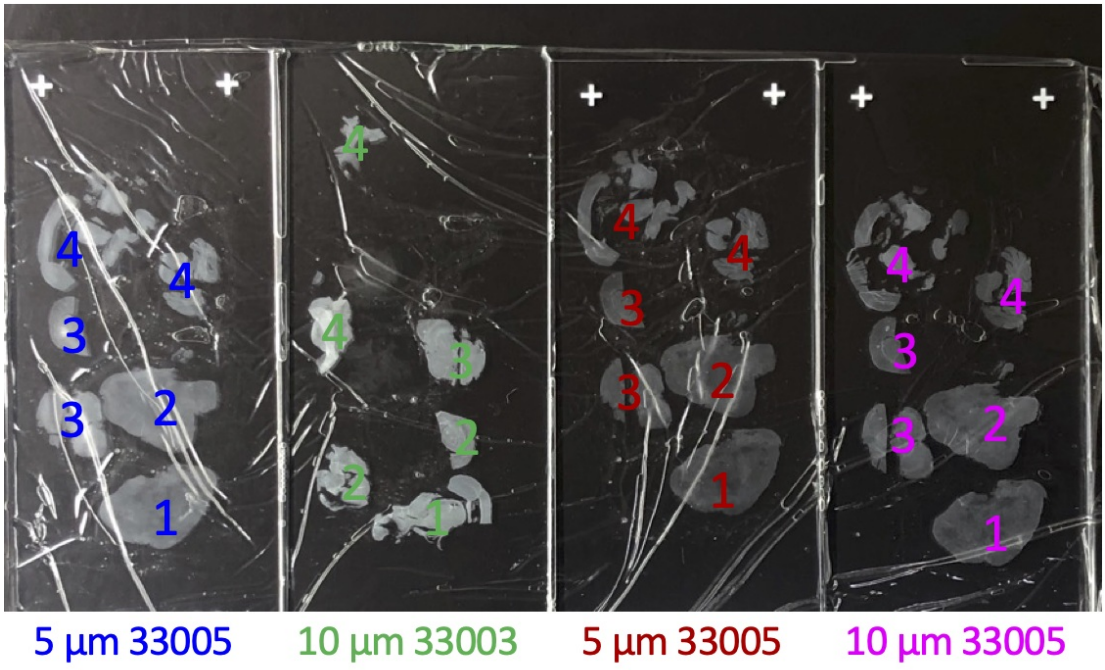

Figure 4: Example of an incubation step. On-slide FFPE-CUTAC was performed using a rabbit RNA Polymerase II Serine-5 monoclonal antibody (Cell Signaling Systems #13523). Four slides from two mouse RELA transgene-driven ependymoma FFPE blocks (5 and 10 µm from the 33005 block and 10 µm from the 33003 block) were processed in parallel. The slides were placed on top of plastic film over a black background for good visibility of tissue, slides were abutted and aligned for each incubation as indicated. About 100 µl antibody or pAG-Tn5 solution was added dropwise to cover the tissue, and the plastic film was slowly pulled over the top edge, minimizing bubbles and wrinkles. Photograph is of the samples during the pAG-Tn5 incubation. The 10 µm 33003 FFPE section was prepared on a standard microscope slide and shows partial loss of the sections with most of the tumor (pooled for tube 4), whereas the other three sections were prepared on charged slides and show full retention of samples throughout the protocol. Color-coded numbers indicate PCR tube sample.

Note

Any bubbles over tissue can be pushed to a section of tissue-free glass.

Note

Other antibodies that work with this protocol are H3K27ac (Abcam #4729) and RNA Polymerase II Serine-2,5p (Cell Signaling Technologies CST (D1G3K) mAb #13546.

7 Incubate horizontallly for at least 1 hr.

8 Remove plastic wrap and gently rinse slide with 1 mL Triton-Wash buffer.

Option 1: Incubation with secondary antibody (1.5 hr).

9 Wick off excess liquid with a Kimwipe and place tissue-side up on a dark surface. Carefully pipette ~100 µl primary antibody solution over the tissue.

10 Cover the clear portion of the slide with a rectangle of plastic film using surface tension to spread the liquid, while omitting bubbles and folds. Place wrapped slides separated in a dry slide holder.

11 Incubate horizontally for at least 1 hr.

12 Remove plastic wrap and gently rinse slide with 1 mL Triton-Wash buffer. Drain on paper towel or Kimwipe and place in a slide holder filled with Triton-Wash buffer for 10 min.

Option 1: Binding Protein A(G)-Tn5 adapter complex (1.5 hr)

13 Remove from slide holder and wick off excess liquid with a Kimwipe. Place tissue-side up on a dark surface. Carefully pipette ~100 µl pA(G)-Tn5 solution over the tissue.

14 Cover the clear portion of the slide with a rectangle of plastic film using surface tension to spread the liquid, while omitting bubbles and folds. Place wrapped slides separated in a dry slide holder.

Note

When using other commercial sources of Protein A-Tn5 or Protein AG-Tn5 use the concentration recommended by the manufacturer for CUT&Tag. If using homemade fusion protein use the concentration recommended in the protocol for CUT&Tag, where the stock concentration may be higher (*e. g.,* https://www.protocols.io/view/3xflag-patn5-protein-purification-and-meds-loading-j8nlke4e5l5r/v1).

15 Incubate horizontally for at least 1 hr.

16 Remove plastic wrap and gently rinse slide with 1 mL Triton-Wash buffer. Drain on paper towel or Kimwipe and place in a slide holder filled with Triton-Wash buffer for 10 min. Drain and place in a slide holder with Triton-Wash buffer for 10 min.

17 Drain on paper towel or Kimwipe and place in a slide holder filled with 10 mM TAPS pH 8.5 for 10 min.

Option 1: Tagmentation and dissection (1.5 hr)

18 Remove slides and drain on paper towel or Kimwipe and place in a slide holder containing cold Tagmentation buffer.

19 Incubate 1 hr in a water bath at 55 ^{O}C.

20 Remove each slide to a slide holder containing 10 mM TAPS pH 8.5 to hold.

21 Remove slide from slide holder, drain and use a Kimwipe to remove excess liquid from the top surface. Dissect or scrape using a total of no more than 5 µL 1% SDS/Thermolabile Proteinase K solution per PCR tube. For recovering all tissue from the slide dice and scrape with a safety razor blade. Proceed to Fragment release (Step 46).

Note

For dissection into a PCR tube, first add 2 µl to the tube, then 2 µl to the desired section of tissue using the pipette tip to spread the solution and loosen the tissue from the slide. Use a #3 jeweler's forceps and a scalpel to scrape each section into a pile and deposit it into the PCR tube. A 1 µl aliquot of the solution can be used to remove the remaining tissue from the slide into the tube.

Note

Working quickly reduces the chance that tissue will dry out during dissection. However, we have not noticed any loss of data quality when tissue dries before being wetted with SDS/Proteinase K solution.

Option 2: FFPE-CUTAC using Biomag-amine beads

22 Remove slide from slide holder, drain and use a Kimwipe to remove excess liquid from the top surface. For recovering all tissue from the slide use a safety razor blade, first dicing the tissue, then scraping into a 2 ml tube containing 1 ml Triton-wash buffer.

Note

For 10 micron sections, consistent results have been obtained with 1/4th of a slide per sample. Excellent results have also been obtained with 1/8th of a 10 micron section. We have also applied this protocol to 5 micron sections.

23 Add 1 µl Bio-mag Plus amine beads (48 mg/ml) per 8 final PCRs.

Note

Note the ~10-fold higher concentration of Bio-mag amine than ConA magnetic beads. Do not use ConA beads as they will bind bacteria contaminating FFPEs. Unlike ConA beads, Bio-Mag Plus amine beads are not activated and do not bind deparaffinized FFPE tissue shards as well. Amine beads require up-and-down full speed spins on a touch centrifuge (~3000xg) before placing on a magnet and decanting to avoid losses.

24 Pass through a 20-22 gauge 1" needle using a Luer-lock glass syringe ~20 times to break up tissue. Divide and transfer into PCR tubes.

Note

Use firm plunges but not so hard as to cause overflowing. This procedure may result in foaming. To clear the foam, spin 3000xg for 1 minute, then vortexing will disperse the small shards of 10 µm thick tissue.

Option 2: Incubation with primary antibody (1.5 hr)

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

Note

The protocol for FFPEs is similar to CUT&Tag-direct Version 4 and can be performed in parallel with native or lightly cross-linked nuclei or whole cells.

26 Resuspend beads in 25 µl primary antibody solution followed by vortexing.

27 Incubate at least 1 hr on Rotator or Nutator at room temperature.

Option 2: Incubation with secondary antibody (1.5 hr)

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

29 Resuspend beads in 25 µl secondary antibody solution followed by vortexing.

30 Incubate at least 1 hr on Rotator or Nutator at room temperature.

31 After a quick full spin, 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.

32 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.

33 Slowly withdraw 460 µl of supernatant with a 1 mL pipette tip without disturbing the beads.

Note

To remove the supernatant, set the pipettor to 460 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.

Note

Bead-bound shards from FFPEs stick to the sides of low-bind PCR tubes, which is especially conspicuous after Wash buffer removal and vortexing is not sufficient to wet them. Therefore, tubes should be mixed by inversion after vortexing.

34 After a quick full spin, place the tubes back into the magnet stand and remove the remaining supernatant with a 20 µl pipettor multiple times if necessary, to remove the entire supernatant without disturbing the beads. Proceed immediately to the next step.

Option 2: Binding Protein A(G)-Tn5 adapter complex

35 Mix pAG-Tn5 pre-loaded adapter complex in Triton-Wash buffer following the manufacturer's instructions (e.g. 1:20 for EpiCypher pAG-Tn5).

Note

This protocol is not recommended for "homemade" pA-Tn5 following our purification protocol, because the contaminating E. coli DNA will be preferentially tagmented relative to the less accessible FFPE DNA under the stringent 55 oC conditions used here. If homemade pA-Tn5 is used, it is important to minimize the amount added (≤1:200).

36 Pipette in 25 µl per sample of the pA(G)-Tn5 mix followed by vortexing.

37 After a quick spin, place the tubes on a Rotator or Nutator at room temperature for 1 hr or 4 °C overnight.

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

39 Carefully remove the supernatant using a 20 µl pipettor as in Step 31.

40 With the tubes still on the magnet stand, add 500 µl of the Triton-Wash buffer.

41 Slowly withdraw 460 µl with a 1 ml pipette tip without disturbing the beads as in Step 33.

42 After a quick full spin, place the tubes back on the magnet stand and remove and discard the supernatant with a 20 µL pipettor using multiple draws.

Option 2: Tagmentation (1.5 hr)

43 Resuspend the bead/FFPE pellet in 50 µl CUTAC-DMF tagmentation solution (5 mM MgCl2, 10 mM TAPS, 20% DMF, 0.05% Triton-X) while vortexing. Incubate 1 hr 55 °C in thermocycler.

Note

N,N-dimethylformamide is a dehydrating compound resulting in improved tethered Tn5 accessibility and library yield. Conditions used for FFPEs are the most stringent tested in PMID:33191916 (Figure 3 – figure supplement 2).

44 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 mix by vortexing.

45 Add 5 µL SDS/Proteinase K, vortex, spin, revortex and spin. Proceed to Fragment release (Step 46).

Fragment release (1.5 hr)

46 After a full speed spin, incubate at 37 ºC for 30 min and 58 ºC for 30 min (programmed in succession in a PCR cycler with a heated lid) to release pA-Tn5 from the tagmented DNA.

PCR (1 hr)

47 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.

Note

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.

48 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.

49 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 5 min
Cycle 4: 98 °C for 10 sec
Cycle 5: 63 °C for 30 sec
Cycle 6: 72 °C for 1 min
Repeat Cycles 4-6 11 times
Hold at 8 °C

Note

CUT&Tag uses short 2-step 10 sec cycles to favor amplification of nucleosomal and smaller fragments. However, after cross-link reversal, DNA in FFPEs are small and PCR amplicon sizes <120 bp are recommended (PMID: 25421801), which obviates the need to minimize the contribution of large DNA fragments. Insertion of a 1 min 72 °C extension and lengthening of the 63 °C annealing time from 10 sec to 30 sec results in better read-through of damaged DNA by Taq polymerase, resulting in a higher fraction of mappable reads than using the 2-step cycle favored for CUT&Tag and CUTAC.

Note

We recommend no more than 12 cycles. Do not add extra PCR cycles to see a signal by capillary gel electrophoresis (e.g. Tapestation). Extra PCR cycles reduce the complexity of the library and may favor contaminating bacterial DNA from the paraffin (Figure 2).

Post-PCR Clean-up (30 min)

50 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 µL of PCR product). Mix by pipetting up and down.

51 Let sit at room temperature 5-10 min.

52 Place on the magnet stand for a few minutes to allow the solution to clear.

53 Remove and discard the supernatant.

54 Keeping the tubes in the magnet stand, add 200 µL of 80% ethanol.

55 Completely remove and discard the supernatant.

56 Repeat Steps 55 and 56.

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

58 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.

59 Place on the magnet stand and allow to clear.

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

Tapestation analysis and DNA sequencing

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

Expected result

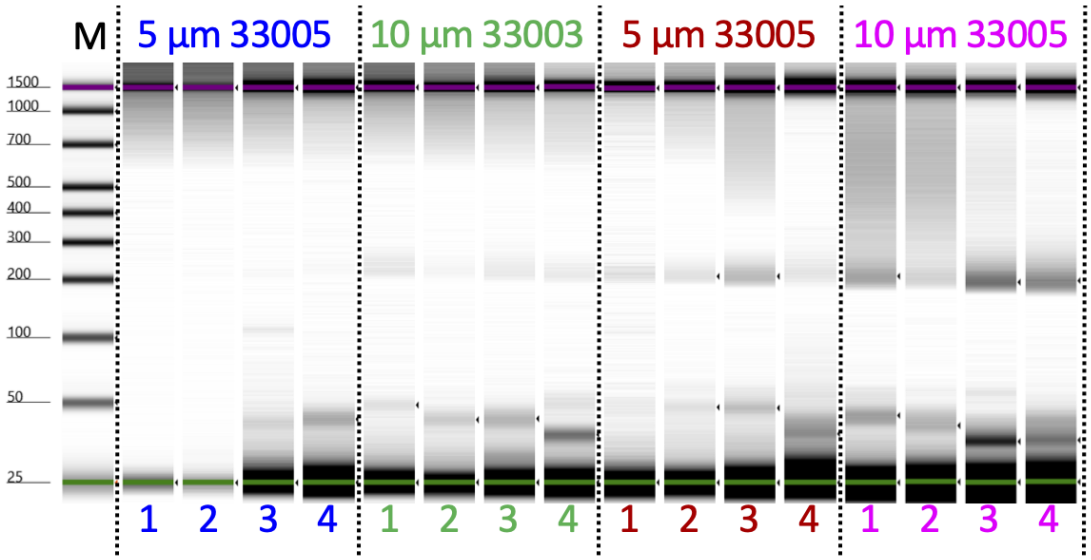

Figure : Tapestation gel image of 1/10th of each SPRI-bead purified DNA eluate.

Note

We use the quantification by Tapestation to estimate library concentration and dilute each library to 2 nM (or the concentration specified for Illumina library submission at the sequencing core that will process your sample) before pooling based on fragment molarity in the 175-500 bp range.

Note

Library samples from a single slide should be pooled using equal volumes to simplify comparisons between them. For direct comparisons between multiple slides processed in parallel using the same antibody, use equal volumes for all samples derived from them.

62 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.

63 Perform paired-end Illumina sequencing on the barcoded libraries following the manufacturer's instructions.

Note

We currently use paired-end 50x50 sequencing on an Illumina Next-Seq, obtaining ~400 million total mapped reads, or ~4 million per sample when there are 96 samples mixed to obtain approximately equal molarity.

Data processing and analysis

64 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.

Expected result

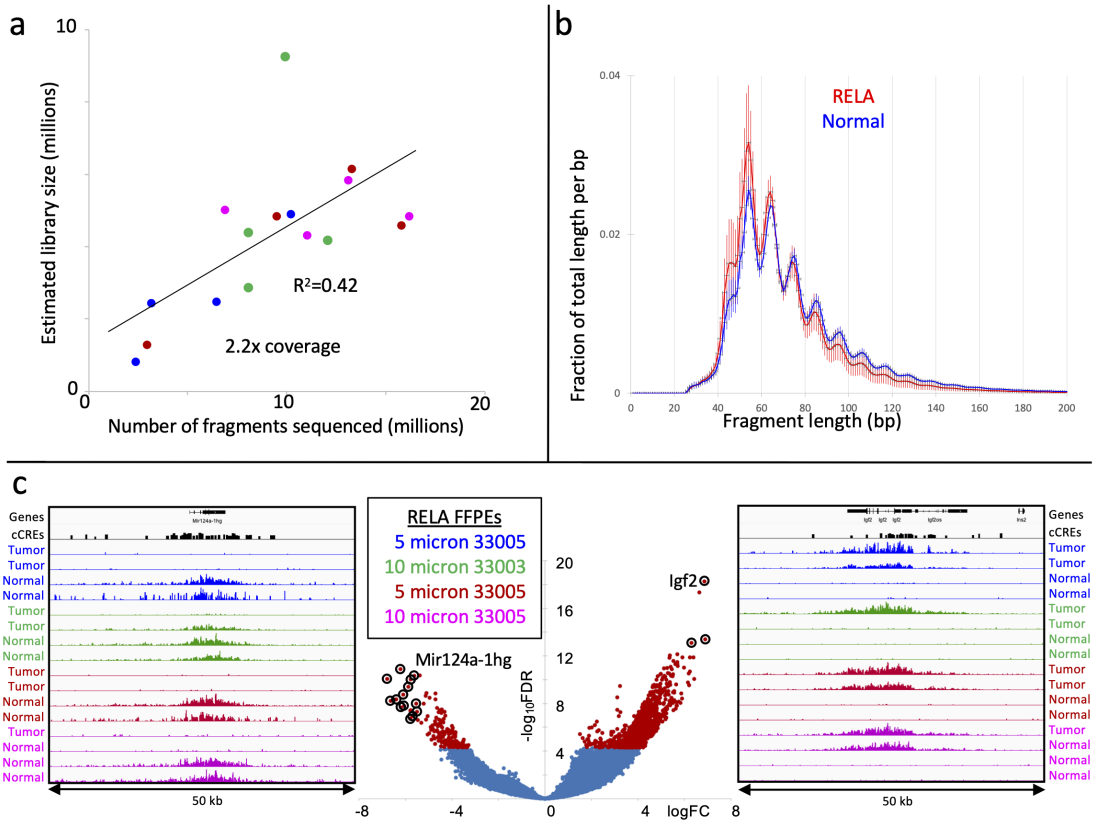

Figure 6: Analyses of the data produced in the experiment shown in Figures 2-3.
a) Remainder of each (barcoded) sample was pooled together with other barcoded samples and sequenced on a NextSeq 2000 PE50 flow cell and the library size was estimated based on Picard Tools Mark Duplicates (68,089,523 in total) and plotted against the total number of reads (149,314,057 in total) for each sample. Total unique fragment estimates were: 10,582,472 (5 µm blue), 20,708,800 (10 µm green), 16,833,815 (5 µm brown) and 19,964,436 (10 µm magenta). **b)** Fragment length distributions of tumor and normal sections from all slides. Mean with standard deviation error bars. **c)** Volcano plot (middle panel) produced using the Degust server with Voom/Limma option, comparing the RELA-driven tumor sections version normal sections for all four slides. The input table consisted of 343,731 rows of mouse candidate *cis*-regulatory elements (cCREs) from ENCODE with one column for each of the 16 samples. Tracks for the cCRE with the highest Fold-change up (Igf2) and down (Mir124a-1hg) are shown. Both Igf2 and Mir124a-1hg account for multiple of the highest scoring cCREs indicated by circles.

65 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.