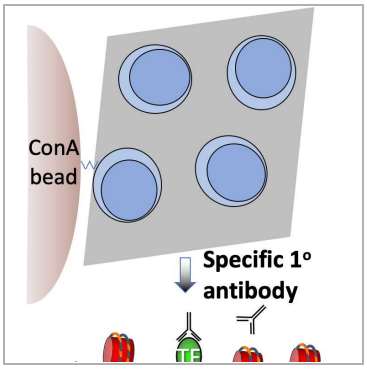

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MANUSCRIPT CITATION:
Steven Henikoff, Jorja G. Henikoff, Kami Ahmad, Ronald Paranal, Zach R. Russell, Frank Szulzewsky, Derek H. Janssens, Eric C. Holland (2023) **Epigenomic analysis of Formalin-Fixed Paraffin-Embedded samples by CUT&Tag** *bioRxiv.*

Protocol status: Working
We use this protocol and it's working

CUTAC for FFPEs

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Human Cell Atlas Method Development Community

Steven Henikoff
Fred Hutchinson Cancer Research Center

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. Recently, we showed 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 found that transcriptional regulatory element differences produced by FFPE-CUTAC distinguish between tumor specimens and identify regulatory element markers with high confidence and precision. Our simple step-by-step workflow makes possible affordable epigenomic profiling of archived biological samples for biomarker identification, clinical applications and retrospective studies.

GUIDELINES

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

MATERIALS

Created: Dec 12, 2022

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PROTOCOL integer ID: 73851

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

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.

- 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 glass slide
- 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)
- Tube Rotator or Nutator
- PCR thermocycler (e.g. BioRad/MJ PTC-200)
- 1 ml syringe + 1" 22 gauge and 3/16" 26 gauge needles
- Xylenes (Histology grade)
- Mineral Oil (Sigma cat. no 330779)
- Ethanol (Decon Labs, cat. no. 2716)
- 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)
- 10% Triton X-100 (Sigma-Aldrich, cat. no. X100)
- 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, 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)
- 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)
- SPRI paramagnetic beads (e.g. HighPrep PCR Cleanup Magbio Genomics cat. no. AC-60500)
- 10 mM Tris-HCl pH 8.0

SAFETY WARNINGS

Deparaffinization uses xylene, a toxic aromatic compound, and should be performed in a fume hood. There are no hazardous materials or dangerous equipment used in other steps of this protocol, however appropriate lab safety training is recommended.

REAGENT SETUP (for up to 16 samples)

1 **Cross-link reversal buffer** Mix 800 µL Tris-HCl pH8.0, 195 µL dH_2O and 5 µL Triton-X100.

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.

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.

Antibody buffer Mix 5 µl 200X BSA with 1 ml Triton-Wash buffer and chill on ice.

CUTAC-DMF Tagmentation buffer Mix 780 µl dH_2O, 200 µl N,N-dimethylformamide, 10 µl 1 M TAPS pH 8.5, 5 µl Triton-X100 and 5 µl 1 M $MgCl_2$ (10 mM TAPS, 5 mM $MgCl_2$, 20% DMF, 0.05% Triton-X100). 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 32 samples) Mix 20 µl 10% SDS and 2 µl 1 M TAPS pH 8.5 in 158 µl dH_2O. Just before use add 20 µL Thermolabile Proteinase K (NEB cat. no. P8111S).

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

Option 1: Deparaffinize FFPE section affixed to slide using ...

2 In a fume hood, immerse slide(s) in xylene for 10 min, then transfer to fresh xylene for 5 min. 9m

Safety information

Xylene vapor is a flammable and when inhaled can depress the central nervous system. It should be used in a fume hood and handled with gloves.

Note

For a non-toxic deparaffinization/processing protocol, skip to Option 2: Deparaffinize FFPE section affixed to microscope slide with mineral oil.

3 Transfer slide(s) to a 50:50 mixture of xylene and 100% ethanol for 3 min. This can be reused or discarded in toxic waste container. 3m

4 Transfer slide(s) to 100% ethanol for 3 min. Repeat once. 6m

5 Immerse slide(s) 95% ethanol for 3 min. 3m

6 Immerse slide(s) in 70% ethanol for 3 min. 3m

7 Immerse slide(s) in 50% ethanol for 3 min. 3m

8 Rinse slide(s) with tap water or tap-distilled water with change(s). 2m

Option 1: Process deparaffinized FFPE sample for CUT&Tag...

9 Dice with a razor blade, scrape and pick up the 10 µm section and deposit it into a 1.5 mL tube containing 400 µL Cross-link reversal buffer (800 mM Tris-HCl pH8.0, 0.05% Triton-X100).

Note

We have stored deparaffinized slides or scrapes in 0.005% sodium azide + 100 µg/ml Ampicillin for up to a few weeks.

10 Incubate 8-16 hours at 85 °C in a heating block. 1h

Note

- The rate of formaldehyde cross-link reversal increases with temperature (PMID: 24848408), where 1 hr at 65 °C is calculated to be sufficient for near-complete reversal. For FFPEs, we use higher temperatures up to 90-95 °C, which denatures contaminating DNA (**Figure 2**) so it is no longer a substrate for Tn5, but spares chromatin in situ. We have found that mappability improves with incubations at 80-90 °C overnight (8-16 hr).

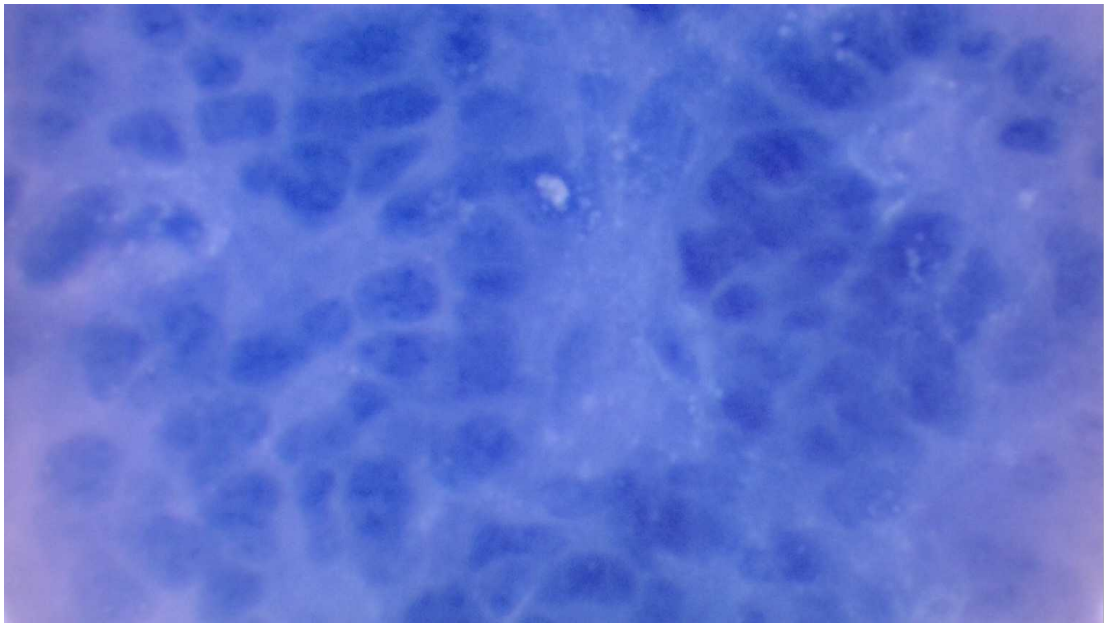

Figure 1. Part of an FFPE mouse brain tumor 10 µm shard after needle dispersion and 90 °C pre-treatments, stained with Trypan blue.

Note

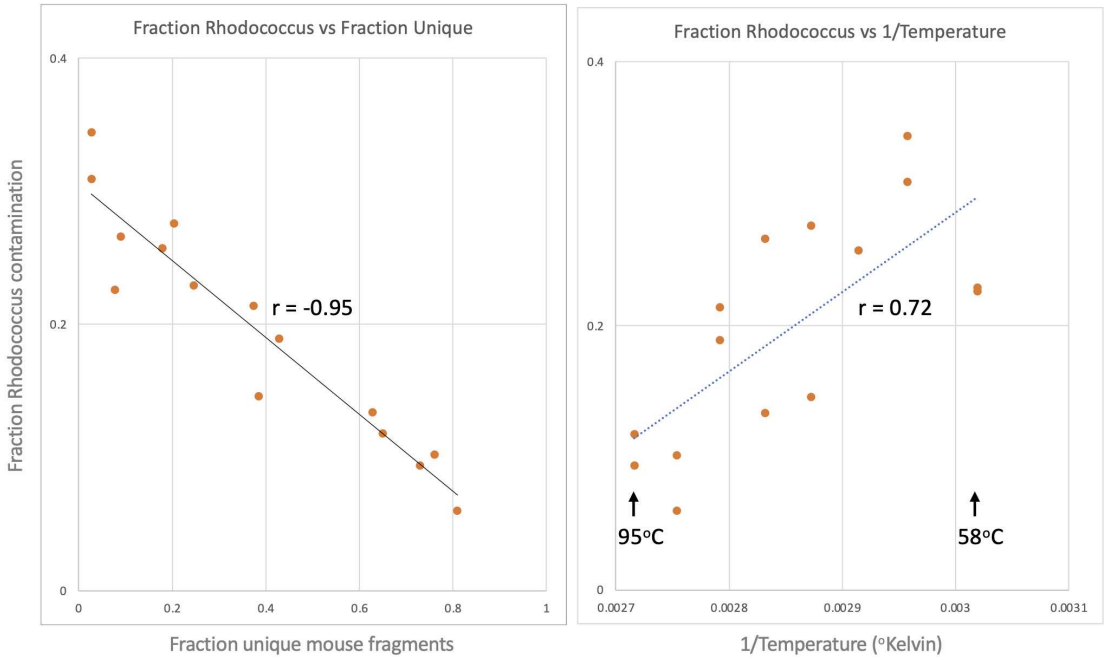

Figure 2. Left: Reducing DNA contamination increases yields. Right: Arrhenius plot illustrates how high temperatures decrease the fraction of contaminating DNA, which when denatured is not a substrate for Tn5.

Rhodococcus is a soil bacteria that metabolizes petroleum products, including waxes, and we find Rhodocccus DNA to be a prominent (but not the only) bacterial contaminant of FFPEs.

11 Resuspend and withdraw enough of the ConA bead slurry, ensuring that there will be ~5 µl for each final sample. For example, we add 160 µl ConA bead slurry to 1.5 mL of Binding buffer for 32 samples.

Note

Prepare beads shortly before use. We have used 7.5 µL bead slurry per sample with excellent results.

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

13 Withdraw the supernatant completely, and remove the tube from the magnet stand. Add 2 mL Binding buffer (for 32 samples) and mix by vortexing.

14 Add 240 µl to each tube while vortexing, where one section will be used for 4 replicate samples (60 µL per sample). Place on Rotator 10-20 min.

Note

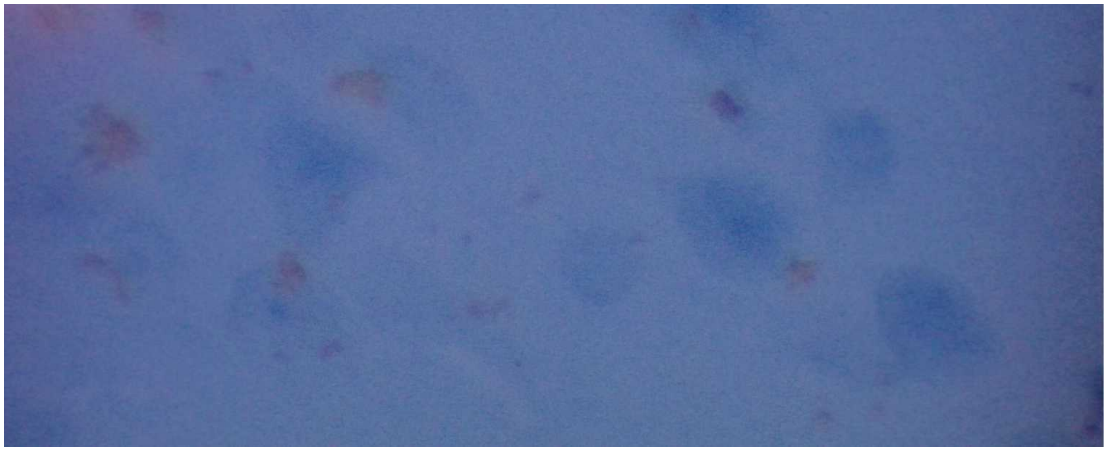

Figure 4: Beads bound to tissue shards.

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.

15 Pass through a 22 gauge 1" needle using a Luer-lock glass syringe 20 times to break up tissue.

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: Deparaffinize FFPE section affixed to slide with m..

16 Scrape off excess paraffin and continuously scrape all of part of the paraffin-embedded 10-µm section. Using tweezers (*e.g.* #3 watchmaker's forceps) carefully lift off the curls and plunge into mineral oil in a 2 ml tube using 100 µl per final sample.

Note

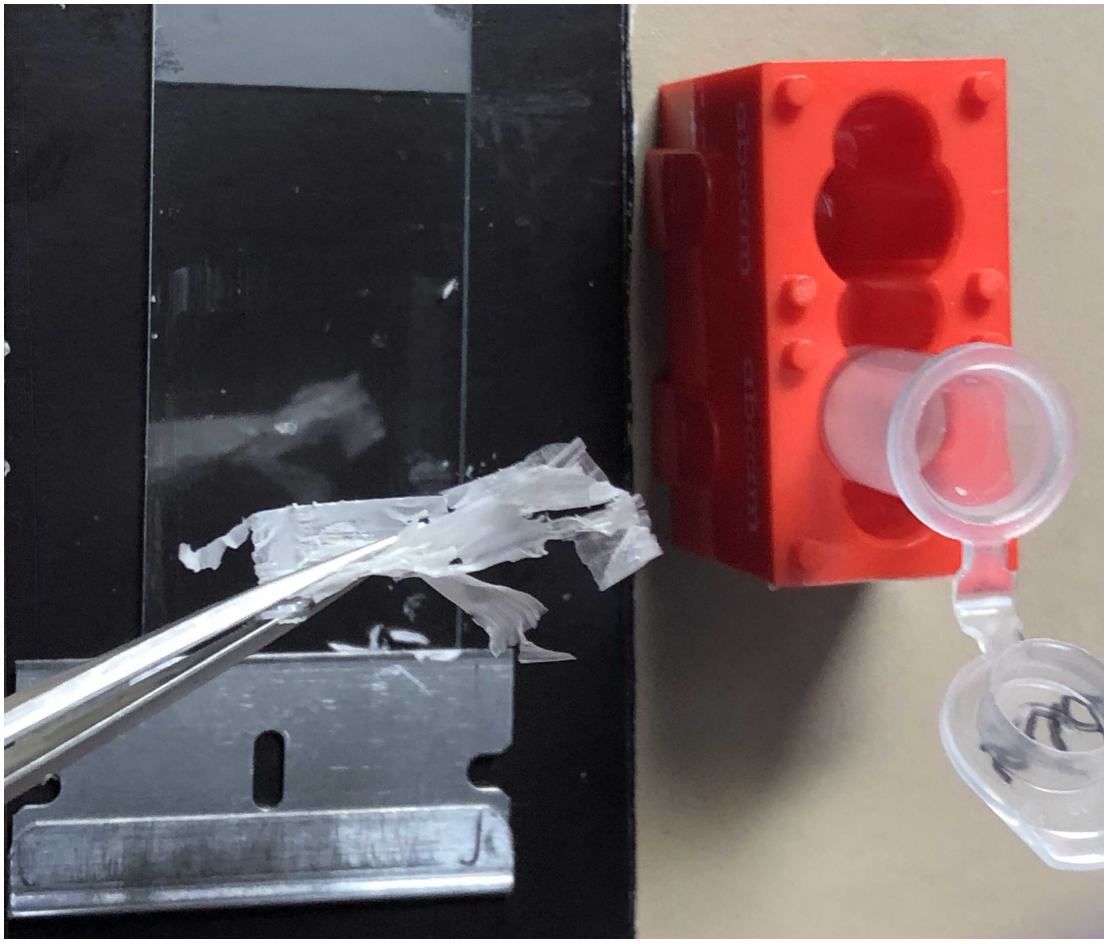

Figure 3: Transferring paraffinized curls to mineral oil.

17 Heat 10 min 80^{o}C to dissolve the curls. Add 1 volume Cross-link reversal buffer (800 mM Tris-HCl pH8.0, 0.05% Triton-X100. Vortex 10 sec, centrifuge briefly, and pass 20 times through a 22 gauge needle on a 1 ml Luer Lock syringe. Centrifuge at 3000xg 1 min. The paraffin will partially solidify in the upper layer while the tissue partitions to the lower (aqueous) layer.

18 Heat briefly to melt the upper layer and remove without disturbing the lower layer using a wide-bore or cut-off 200 µl low-bind pipette tip. Add 1 volume mineral oil, vortex, centrifuge, heat and decant the upper layer.

19 Repeat Step 18 until the interface is clear or nearly so. Using a wide-bore 200 µl pipette tip transfer 100 µl @ to PCR tubes.

Note

It is not necessary to remove the remaining thin layer of mineral oil, which could result in loss of tissue adhering to the meniscus. This small amount of oil is eventually lost during bead washes.

20 Place tubes in a thermocycler and incubate at 85 °C for at least 2 hours. We have found that mappability improves with incubations at 80-90 °C overnight (8-16 hr).

21 Resuspend and withdraw enough of the ConA bead slurry, ensuring that there will be ~5 µl for each final sample. For example, we add 160 µl ConA bead slurry to 1.5 mL of Binding buffer for 32 samples. Place the pipette tip below the meniscus to avoid coating the beads with oil and discharge the beads while mixing by pipetting.

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

23 Withdraw the supernatant completely, and remove the tube from the magnet stand. Add 2 mL Binding buffer (for 32 samples) and mix by vortexing.

24 Resuspend in 160 µL Binding buffer. Add 5 µl to each sample while vortexing. Place on Rotator 10-20 min.

Note

For this option, insert the pipette tip beneath the oil-water interface before expelling the beads. It is important that the beads do not make contact with the mineral oil, which may prevent their binding to the FFPE tissue shards.

Bind primary antibody (2 hr)

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

Note

n The protocol for FFPEs is similar to CUT&Tag-direct Version 3 and can be performed in parallel with native or lightly cross-linked nuclei or whole cells. Although whole cells are not appropriate with that version, including 0.05% Triton-X100 from antibody binding to tagmentation stabilizes the bead pellet and permeabilizes cells such that by the time of tagmentation the remaining cellular material is no longer inhibitory for PCR. We now add 0.05% Triton-X100 by default for all CUT&Tag and CUTAC protocols, including for single cells. We find that best results are obtained adding 1:10 Thermo-labile Proteinase K to the fragment-release solution and incubating as in this protocol pre-PCR.

26 For each CUT&Tag and CUTAC sample, mix the primary antibody 1:25 with Antibody buffer. Resuspend beads in 25 µl per sample followed by vortexing.

Note

For FFPEs we use 1:25 antibody dilutions and incubate 1-2 hr to overnight at room temperature to maximize antibody penetration. For long RT incubations we add sodium azide to a final concentration of .005% as a precaution to prevent microbial growth. We have used Pol2Ser5 (CST (D9N5I) mAb #13523), Pol2Ser2,5 (CST (D1G3K) mAb #13546), Pol2Ser5+Ser2,5 (mixed) and H3K27ac (Abcam ab4729) with success. Pol2Ser5 results in the sharpest peaks but typically with reduced yield relative to the other single antibodies.

27 Place on a rotator at room temperature and incubate at least 1 hr on Rotator at room temperature.

01:00:00

Note

We have found that 1 hr incubations at room temperature suffice for primary and secondary antibodies and pAG-Tn5. For overnight RT incubations, add 0.005% sodium azide as a precaution to inhibit bacterial growth.

Bind secondary antibody (1.5 hr)

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

29 Mix the secondary antibody 1:100 in Wash buffer and squirt in 25 µl per sample followed by vortexing.

> Note
>
> 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.

30 Place the tubes on a rotator or nutator and rotate or nutate at room temperature for 1 hr. 1h

01:00:00

31 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.

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 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 µl 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)

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 ^{o}C 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-Tn5 mix followed by vortexing.

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

01:00:00

38 After incubating in the rotator, perform a quick 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 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 43.

Tagment (1.5 hr, performed in parallel with standard CUT&...

43 Resuspend the bead/FFPE pellet in 50 µl CUTAC-DMF tagmentation solution (5 mM $MgCl_2$, 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.

Fragment Release (2.5 hr)

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

46 Resuspend the beads by squirting in 5 µL 1% SDS/ProtK Release solution followed by vortexing.

47 After a quick spin, incubate at 37 ºC for 1 hr and 58 ºC for 1 hr (programmed in succession in a PCR cycler with a heated lid) to release pA-Tn5 from the tagmented DNA. 02:00:00 2h

PCR (1 hr)

48 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.

49 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.

50 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 contaminatirg bacterial DNA from the paraffin (Figure 2).

Post-PCR Clean-up (30 min)

51 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.

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

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

54 Remove and discard the supernatant.

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

56 Completely remove and discard the supernatant.

57 Repeat Steps 55 and 56.

58 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.

59 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

60 Place on the magnet stand and allow to clear.

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

Tapestation analysis and DNA sequencing

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

Expected result

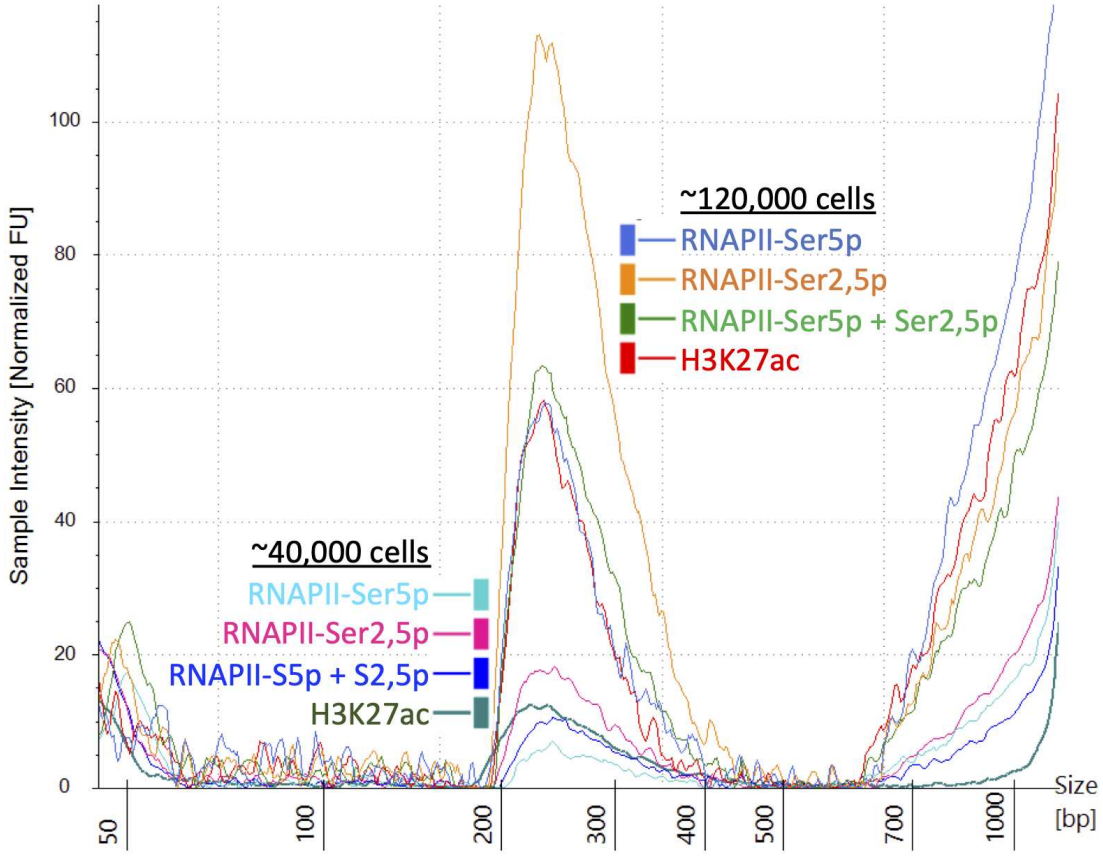

Figure 5. Tapestation profiles for FFPE CUTAC samples pre-incubated 85 ^{O}C 12 hr using four different antibodies on samples. Each sample was divided 3/4-1/4 in the TAPS-wash before fragment release. Antibodies (1:25): RNAPII-Ser5p Cell Signaling Technology #13523, RNAPII-Ser2,5 Cell Signaling Technology #13546, H3K27ac: Abcam #4729. A 10 µm section of a mouse brain tumor FFPE was deparaffinized using Option 1 (xylene). Note that both the CUTAC peaks the high-molecular weight smears scale with the amount of sample. Use a 175-500 bp range for estimating molar concentration. There is no need to remove the high molecular weight smear, which is not tagmented and does not interfere with the flow cell run.

Note

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 175-500 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.

63 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.

64 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

65 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

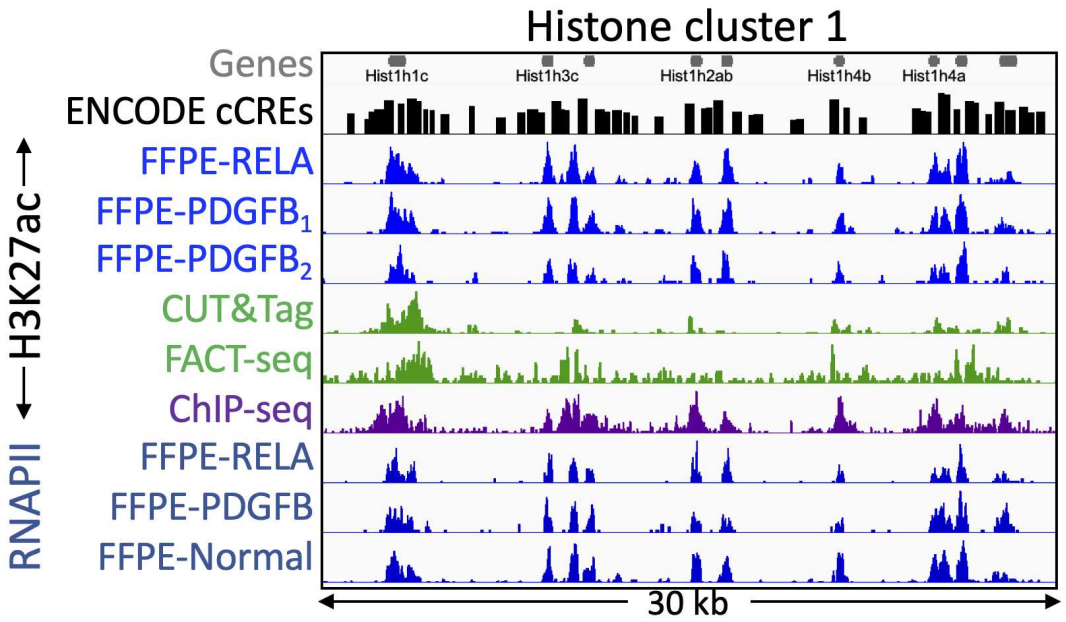

Figure 6. A gene-rich housekeeping gene region was chosen to minimize the effect of cell-type differences between FFPE-CUTAC (A RelA-driven and two replicates of a PDGFB-driven brain tumor) and FACT-seq and CUT&Tag (kidney data from Zhao et al. (2021) Nucleic Acids Res.). A forebrain H3K27ac ChIP-seq sample from the ENCODE project is shown for comparison, using the same number of fragments (10 million) for each sample. Also shown are tracks from FFPE-CUTAC samples using an antibody to RNAPII-Ser2,5p. A track for Candidate *cis*-Regulatory Elements (cCREs) from the ENCODE project is shown above the data tracks, which are autoscaled for clarity.

Note

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.

66 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.