

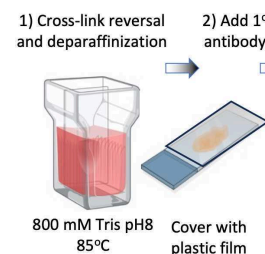
Feb 25, 2025

Version 4

CUTAC for FFPEs V.4

DOI

<https://dx.doi.org/10.17504/protocols.io.14egn292zg5d/v4>



Steven Henikoff¹

¹Fred Hutchinson Cancer Center



Steven Henikoff

Fred Hutchinson Cancer Center

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.14egn292zg5d/v4>

External link: <https://doi.org/10.7554/eLife.63274>

Protocol Citation: Steven Henikoff 2025. CUTAC for FFPEs. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.14egn292zg5d/v4> Version created by **Steven Henikoff**



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** *Nature Communications* 14:5930. <https://www.nature.com/articles/s41467-023-41666-z>.

Steven Henikoff, Ye Zheng, Ronald M Paranal, Yiling Xu, Jacob E Greene, Jorja G Henikoff, Zachary R Russell, Frank Szulzewsky, H Nayanga Thirimanne, Sita Kugel, Eric C Holland, Kami Ahmad (2025) **RNA polymerase II at histone genes predicts outcome in human cancer** *Science* 387(6735):737-743. <https://www.science.org/doi/10.1126/science.ads2169>.

License: This is an open access protocol distributed under the terms of the **Creative Commons Attribution License**, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working

Created: May 11, 2024

Last Modified: February 25, 2025

Protocol Integer ID: 99608

Keywords: CUT&Tag, epigenetics, chromatin profiling, Formaldehyde-fixed paraffin-embedded sections, targeted chromatin accessibility mapping protocol, chromatin accessibility mapping protocol, targeted accessible chromatin, cleavage under targeted accessible chromatin, possible affordable epigenomic profiling, regulatory element markers with high confidence, regulatory element marker, epigenomic study, biological samples for biomarker identification, biomarker identification, cutac distinguish between mouse brain tumor, paused rna polymerase ii, chromatin damage, difficult because of chromatin damage, cutac for ffpe, transcriptional regulatory element difference, including microrna, use of ffpe sample, using ffpe sample, ffpe sample, detectable by rna, ffpe, rna polymerase ii, rna

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. Version 2 includes improvements in both the on-slide and bead options and extension of the improved bead option to curls.

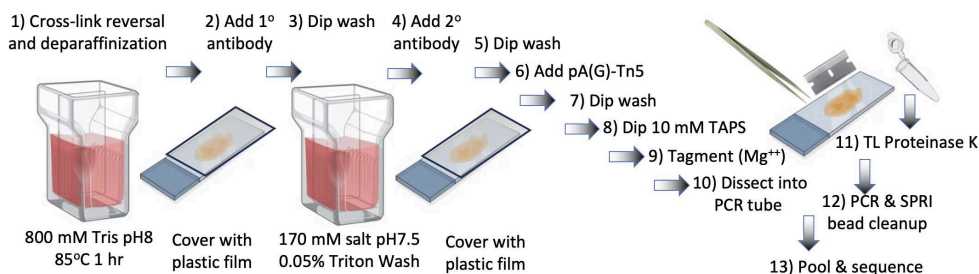


Figure 1: On-slide FFPE-CUTAC. Schematic of the protocol.

Image Attribution

Biorender.com

Guidelines

Based on <https://www.protocols.io/view/cut-amp-tag-direct-for-whole-cells-with-cutac-x54v9mkmzg3e/v5>.

Materials

- 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 μ L, 20 μ L, and 10 μ L)
- Kimble Kontes Pellet Pestle Motor (DWK Life Sciences cat no. 749540-0000)
- Disposable pestles (Fisher cat. no. 12-141-364)
- Disposable tips (e.g. Rainin 1 mL, 200 μ L, 20 μ L)
- 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)
- 4-10 micron section from a formaldehyde-fixed paraffin-embedded tissue block either as a curl or 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)
- Safe Clear II (Fisher cat. no. 23-044192)
- Bio-Mag Plus amine magnetic beads (48 mg/mL, Polysciences cat. no. 86001-10). Dilute 1:10 with 10 mM Tris pH8/1 mM EDTA for use.
- Pierce glutathione magnetic beads (Fisher cat. no. 88822).
- Ethanol (Decon Labs, cat. no. 2716)
- Distilled, deionized or RNase-free H₂O (dH₂O 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)
- 0.5 M EDTA pH 8
- 10% Sodium azide (caution: toxic)
- 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 (PolII S5P, 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 Epiccypher cat. no. 15-1117.
- Thermolabile Proteinase K (NEB P8111S)
- 1 M Magnesium Chloride (MgCl₂; 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)
- NEBNext 2X PCR Master mix (ME541L)
- PCR primers: 10 μ M 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 8 ml 1 M Tris-HCl pH8.0, 2 ml dH₂O and 4 µl 0.5 mM EDTA.

Rinse buffer (Option 1) Mix 1 mL 1 M HEPES pH 7.5 and 1.5 mL 5 M NaCl, and bring the final volume to 50 mL with dH₂O.

Triton-Wash buffer Mix 1 mL 1 M HEPES pH 7.5, 1.5 mL 5 M NaCl, 250 µl 10% Triton-X100, 12.5 µl 2 M spermidine, bring the final volume to 50 mL with dH₂O, and add 1 Roche Complete Protease Inhibitor EDTA-Free tablet. Store the buffer at 4 °C for up to 2 days.

Note

To completely prevent bacterial contamination during storage of Triton-wash buffer, add 0.2 mM EDTA or sodium azide to 0.02% (100 µl 10% -> 50 mL) or both. Handle sodium azide carefully, and wear a mask when weighing it out.

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 µl 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 (Epiccypher cat. no. 15-1117) with 419 µl Triton-Wash buffer (1:20).

CUTAC-DMF Tagmentation buffer Mix 17.7 mL dH₂O, 4 mL N,N-dimethylformamide, 220 µl 1 M TAPS pH 8.5, and 110 µl 1 M MgCl₂ (10 mM TAPS, 5 mM MgCl₂, 20% DMF). Store the buffer at 4 °C for up to 1 week.

TAPS-EDTA wash buffer Mix 1 mL dH₂O, 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 dH₂O. Just before use add 10 µL Thermolabile Proteinase K (NEB cat. no. P8111S).



5% Triton Mix 1 mL 10% Triton-X100 + 1 mL dH₂O. Store at room temperature.

Option 1: On-slide FFPE-CUTAC: Deparaffinization in Safe Clear II and cross-link reversal.

- 2 Place slides in a Coplin jar containing Safe Clear II and hold for 10 min at room temperature.
- 3 Transfer slides to a Coplin jar containing 100% ethanol and hold for 3-5 min.
- 4 Transfer slides to a Coplin jar containing 50% ethanol and hold for 3-5 min.
- 5 Transfer slides to a Coplin jar containing 10% ethanol and hold for 3-5 min.
- 6 Transfer slides to a plastic Coplin jar containing cross-link reversal buffer and incubate at 85°C for at 1 hr.

Note

Incubations as long as overnight at 80-90 °C in cross-link reversal buffer have yielded high-quality results similar to results using 1 hr incubations. Be sure that the FFPE sections are affixed to a charged glass slide to avoid tissue loss during incubations.

Note

The Option 1 protocol is for 16 samples but can be scaled up or down as needed. The example experiment shown in Figures 4, 6 and 7 beginning with dry FFPE slides through sequencing-ready purified DNA libraries was accomplished in one long 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 4-8 °C.

Option 1 (continued): On-slide FFPE-CUTAC Incubation with primary antibody.

- 7 For each slide, remove from slide holder, wick off excess liquid from the glass surface with a Kimwipe (without touching the tissue) and place tissue-side up on a dark surface

for visibility. Carefully pipette ~50 µl primary antibody solution over the tissue.

- 8 Cover the clear portion of the slide with a rectangle of plastic film (or a square for small tissue sections) using surface tension to spread the liquid, while excluding large bubbles and wrinkles. Place wrapped slides separated in a dry slide holder (**Figure 2**) or in the rack of a staining dish, which can be used as a "moist chamber" (**Figure 3**).

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. Antibodies to histone methylations have failed, and unsatisfactory results have been obtained using an antibody to CTCF.

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.



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. For small tissue sections (e.g. 1 cm²), using small plastic wrap squares that cover the sample but do not wrap around the slide will require proportionally less volume, saving on reagent costs.

Note



Figure 3: Examples of moist chambers using wet paper towels in a tupperware tray and staining dish. When covered slides stay wet under plastic wrap rectangles or squares (for small tissue sections and reduced volumes). Slides are placed in the rack for incubation, and afterwards are placed face up on the wet paper towel in the tupperware tray to wash the bottom before removing the plastic wrap and rinsing the top.

- 9 Incubate at room temperature for at least 1 hr.

Expected result

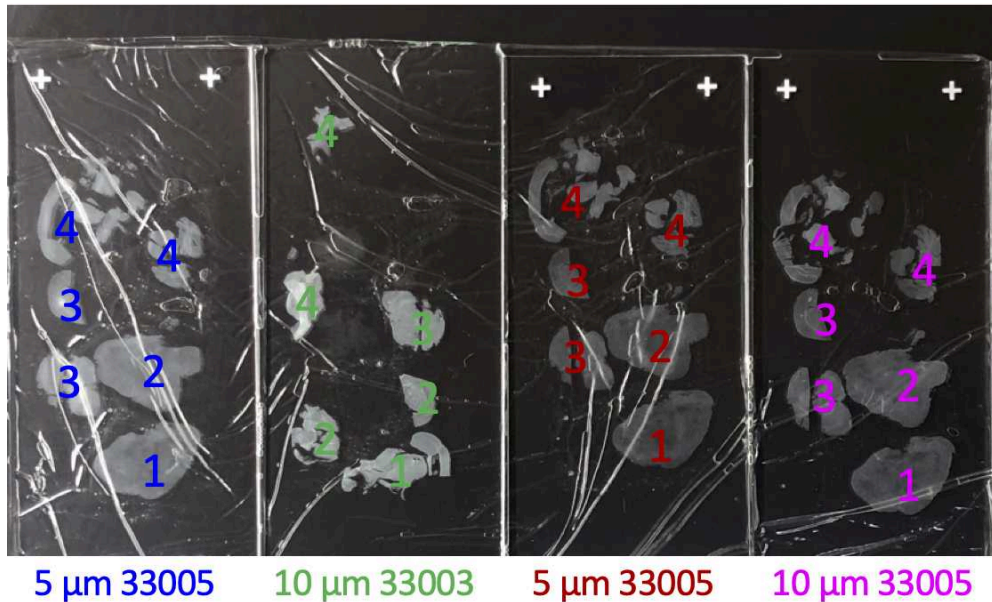


Figure 4: Example of an incubation step. On-slide FFPE-CUTAC was performed using a rabbit RNA Polymerase II Serine-5 monoclonal antibody (Cell Signaling Systems #13523). Four slides from two mouse RELA transgene-driven ependymoma FFPE blocks (5 and 10 μ m from the 33005 block and 10 μ m from the 33003 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 μ l 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 μ m 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.

- 10 Remove plastic wrap and gently rinse slide by pipetting 1 mL Triton-Wash buffer dropwise over the top of the slide.

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

- 11 Wick off excess liquid with a Kimwipe and place tissue-side up on a dark surface. Carefully pipette ~50 μ l secondary antibody solution over the tissue.



- 12 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.
- 13 Incubate at room temperature for at least 1 hr.
- 14 Remove plastic wrap and gently rinse slide 1-2 times with 1 mL Triton-Wash buffer.

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

- 15 Remove from slide holder and wick off excess liquid with a Kimwipe. Place tissue-side up on a dark surface. Carefully pipette ~50 μ l pA(G)-Tn5 solution over the tissue.
- 16 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.

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>).

- 17 Incubate at room temperature for at least 1 hr.
- 18 Remove plastic wrap and gently rinse slide 1-2 times 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.
- 19 Drain on paper towel and wick off excess liquid with a Kimwipe and place in a slide holder filled with 10 mM TAPS pH 8.5 for 10 min.

Option 1 (continued): Tagmentation, dissection and fragment release (2.5 hr)



- 20 Remove slides and drain on paper towel or Kimwipe and place in a slide holder containing cold Tagmentation buffer.
- 21 Incubate 1 hr in a water bath at 55°C.
- 22 Remove each slide to a slide holder containing TAPS-EDTA wash buffer to hold.
- 23 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 μ L 1% SDS/Thermolabile Proteinase K solution per PCR tube. To recover all tissue from the slide dice and scrape with a safety razor blade. For larger tissue amounts, use more SDS/TLProtK solution and divide up into PCR tubes such that no more than 5 μ L is deposited into each tube.

Note

For dissection into a PCR tube, first add 2 μ L to the tube, then 2 μ L to the desired section of tissue using the pipette tip to spread the solution and loosen the tissue from the slide. Use a #3-5 jeweler's forceps and a scalpel or razor blade to scrape each section into a pile and deposit it into the PCR tube. A 1 μ L 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.

- 24 Incubate at 37°C for 30 min and 58°C for 30 min to release pA-Tn5 from the tagmented DNA. Open the tubes and add 15 μ L 5% Triton-X100, close and incubate at 37°C for 30 min on the cyclor. Proceed to Step 48.

Option 2: FFPE-CUTAC using beads: Deparaffinization in Safe Clear II.

- 25 FFPE slide or curl: Scrape all or part of a 5-10 μ m FFPE slide (**Figures 2-4**) or a "curl" (**Figure 5**) into a 1.5-2 ml tube (e.g. MCT-175-C). Add 320 μ L Safe Clear II. Vortex, spin, and place in a 56°C water bath for 3 min. Cool and centrifuge on full for 2 min.

Note

The Option 2 protocol is for 16 samples but can be scaled up or down as needed. Sequencing-ready purified DNA libraries can be obtained in one long day (~10 hours), but any of the 1 hr antibody or pAG-Tn5 incubations can be extended to a few hours at room temperature or at 4-8°C overnight.

Note

Vortex hard to mix, but in some steps a "quick vortex" is used. With a touch mini-centrifuge, "spin on full" is just up to full speed then down, whereas "quick spin" is only to remove liquid from the cap and down from the sides.

Note

Curls are thin sections that are released from the microtome without being affixed to slides and either curl up to form a tight rod (10 μ m) or fold (5 μ m). Best permeabilization is obtained with 5 μ m curls.



Figure 5. A curl (white) in a 1.5 mL Eppendorf tube.

Note

Using more than half of a curl from a 10 micron section equivalent to the amount of tissue on the slides in Figures 2-4 might result in inhibition of PCR when using a one-tube protocol, and tube transfer before PCR is recommended.



- 26 Remove liquid avoiding the pellet. Quick spin and remove final liquid with low-bind pipette tip. Place in 37°C heating block with caps open for 10 min.
- 27 Add 1 mL Cross-link reversal buffer and place in 85°C water bath for 1 hr.

Note

90°C incubations can be extended for several hours or overnight without noticeable consequences. Likewise, room temperature incubations with affinity reagents can be extended up to overnight by performing at 4-8°C. We have not noticed differences for longer room temperature or cold incubation times and have not tested times less than 1 hr, which might be OK for shortening this protocol to fit into a single day.

- 28 When cool add 10 µl 1:10 Biomag beads (final concentration 4.8 mg/ml) and vortex. Place on Rotator (or Nutator) for 10-20 min.
- 29 Do a quick spin and place on the magnet stand. When clear carefully remove ~850 µl with a 1 ml pipettor, quick spin followed by using a 200 µL low-bind pipette tip. Proceed immediately to antibody addition.

Note

- Bio-Mag Plus amine magnetic beads are ~1.5 µm in diameter and have a rough hydrophilic surface that sticks weakly to deparaffinized tissue shards (**Figure 6**). We also have found that 0.8 µm Streptavidin-coated magnetic microsphere beads (Bangs Streptavidin Magnefy™ cat#MFYS1N) work equally well using 10-20 µL. Do not use ConA- or WGS-coated beads, which result in capture of wax-eating bacteria (*Rhodococcus erythropolis*, PMID: 37739938), which dominate the PCR.

Option 2 (continued): Incubation with primary antibody

- 30 Resuspend beads in 50 µl primary antibody solution followed by vortexing, quick spin and transfer to a 0.5 mL PCR tube using a wide-bore low-bind 200 µL pipette tip being careful not to leave behind any tissue sections.

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.



- 31 Incubate 1-2 hr on Rotator (or Nutator) at room temperature.

Option 2 (continued): Incubation with secondary antibody

- 32 After a quick spin, place the tubes on the magnet stand to clear and carefully remove supernatant using a 200 μ L low-bind regular pipette tip.
- 33 Resuspend beads in 50 μ L secondary antibody solution followed by vortexing.
- 34 Incubate 1-2 hr on Rotator (or Nutator) at room temperature.
- 35 After a quick spin, place the tubes on the magnet stand to clear and carefully remove supernatant using a 200 μ L low-bind regular pipette tip.
- 36 While on the magnet stand, slowly drip in 500 μ L of Triton-Wash buffer. Carefully remove ~460 μ L with a 1 mL pipettor and a quick spin followed by using a 200 μ L low-bind pipette tip. Proceed immediately to the next step.

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

- 37 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 tagged relative to the less accessible FFPE DNA under the stringent 55°C conditions used here. If homemade pA-Tn5 is used, it is important to minimize the amount added ($\leq 1:200$).

- 38 Add 50 μ L pA(G)-Tn5 mix followed by vortexing. Place the tubes on a Rotator or Nutator at room temperature for 1-2 hr.
- 39 After a quick spin, place the tubes on the magnet stand to clear and carefully remove supernatant using a 200 μ L low-bind regular pipette tip.



- 40 While on the magnet stand, slowly drip in 500 μ L Triton-Wash buffer. Carefully remove ~460 μ L with a 1 mL pipettor and a quick spin followed by using a 200 μ L low-bind regular pipette tip.
- 41 While on the magnet stand, slowly drip in 500 μ L 10 mM TAPS pH8.5. Carefully remove ~460 μ L with a 1 mL pipettor and a quick spin followed by using a 200 μ L low-bind regular pipette tip. Proceed immediately to the next step.

Option 2 (continued): Tagmentation

- 42 Resuspend the bead/FFPE pellet in 50 μ L CUTAC-DMF tagmentation solution (5 mM $MgCl_2$, 10 mM TAPS, 20% DMF, 0.05% Triton-X100) while vortexing. Incubate at 55°C for 1 hr in a thermocycler.

Note

N,N-dimethylformamide is a dehydrating compound resulting in improved tethered Tn5 accessibility and library yield. A 55°C incubation used for FFPEs is the most stringent tested in PMID:33191916 (Figure 3 – figure supplement 2).

- 43 After a quick full centrifugation, place the tubes on a magnet stand and withdraw and discard the Tagmentation buffer supernatant using a 200 μ L low-bind pipette tip.
- 44 While on the magnet stand, slowly drip in 500 μ L TAPS-EDTA wash. Withdraw and discard the TAPS wash supernatant using a 200 μ L low-bind regular pipette tip.
- 45 Add 5 μ L 1% SDS/Thermolabile Proteinase K solution per PCR tube. Vortex, quick spin and proceed to Fragment Release (Step 46).

Option 2 (continued): Fragment Release

- 46 Incubate at 37°C for 30 min and 58°C for 30 min to release pA-Tn5 from the tagmented DNA. Open the tubes and add 15 μ L 5% Triton-X100, close and incubate at 37°C for 30 min on the cycler.

Note

Volumes here and below are calculated based on assuming that the tissue amount is equivalent to half that of a 10 micron FFPE slide or curl. Except for the sequencing primers, volumes may be scaled accordingly for different amounts of tissue.



- 47 Spin on full and remove supernatant to one or more PCR tubes for amplification.

Note

Previous versions of this protocol used the CUT&Tag-direct procedure without tube transfer, however, depending on the size and thickness of a curl or scrape, there is the risk of too much "gunk" inhibiting the PCR, and we have increased the PCR volume from 50 μ l to 100 μ l. Owing to the very high efficiency of this protocol, where even 5% of a curl may be enough for a single library, we recommend either using the supernatant or splitting the bead slurry into multiple aliquots for PCR technical replicates.

Note

Yields using the protocol are high and it might be worthwhile to split the sample into replicates and/or reduce the number of cycles from the recommended 12-13.

PCR

- 48 Add 2 μ l of 10 μ M Universal or barcoded i5 primer + 2 μ l of 10 μ M barcoded i7 primers, using a different barcode pair for each sample. Vortex on full 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 μ l NEBnext (non-hot-start), vortex to mix, and perform a quick spin. Place the tubes 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 11-12 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 13 cycles, and fewer than 12 cycles may be optimal for larger curl samples. Extra PCR cycle reduce the complexity of the library.

Post-PCR Clean-up (30 min)

- 51 After the PCR program ends, remove tubes from the thermocycler, vortex to resuspend, and add 65 μ L of SPRI beads (ratio of 1.3 μ L of SPRI beads to 1 μ L of PCR product). Mix by pipetting up and down.
- 52 Let sit at room temperature 5-10 min.
- 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 μ L 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, avoiding air drying the beads by proceeding immediately to the next step.
- 59 Remove from the magnet stand, add 22 μ l 10 mM Tris-HCl pH 8, vortex and quick spin. Let sit for at least 5 min to elute the DNA.
- 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

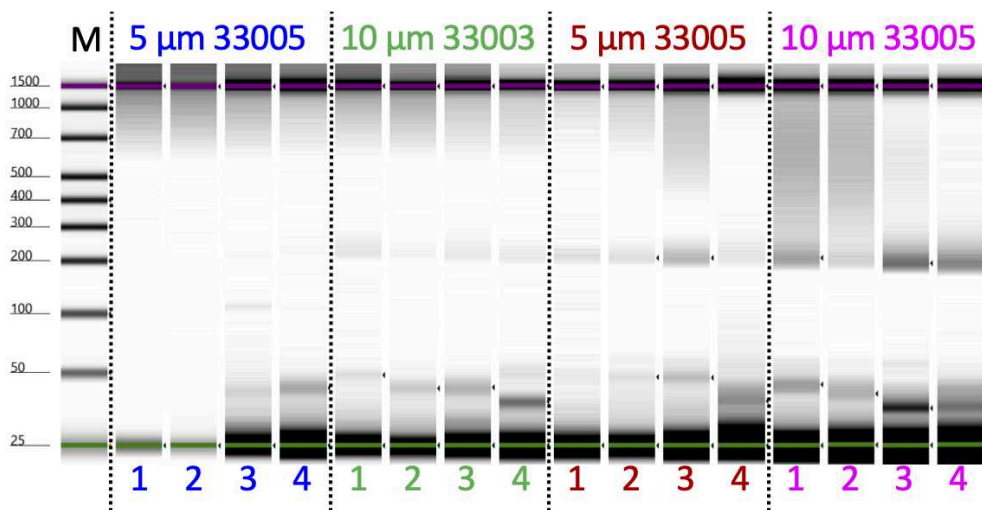


Figure 6: Tapestation gel image of $1/10^{\text{th}}$ of each SPRI-bead purified DNA eluate from an on-slide experiment.

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.

- 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

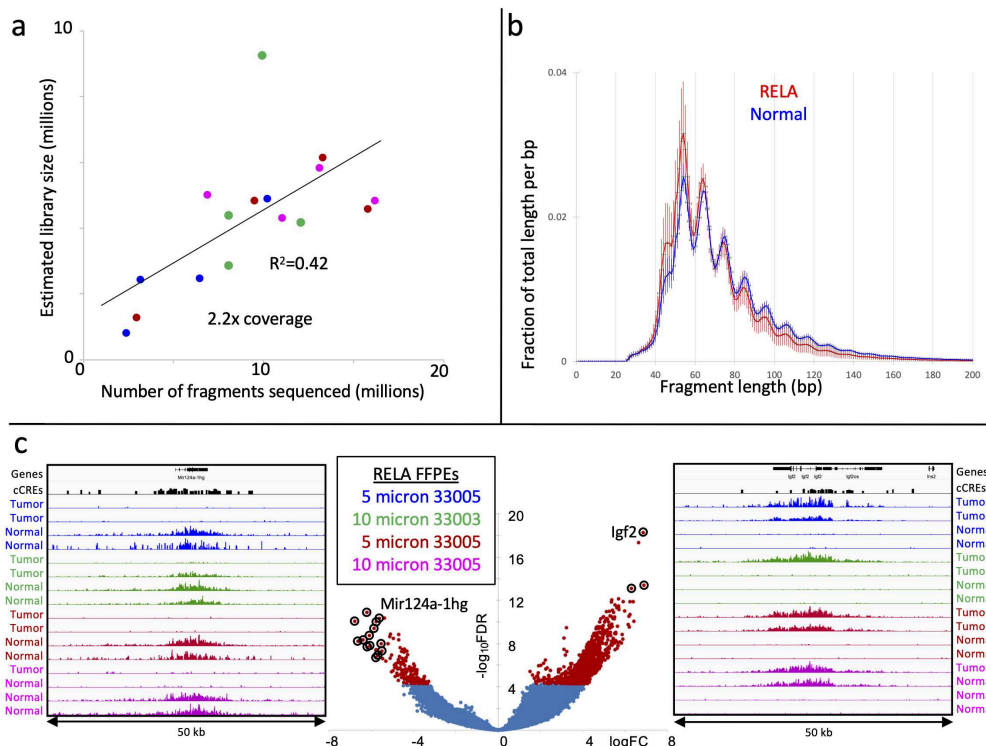


Figure 7: Analyses of the data produced in the experiment shown in Figures 4 and 6.

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 μ m blue), 20,708,800 (10 μ m green), 16,833,815 (5 μ m brown) and 19,964,436 (10 μ m 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 (Igfb2) and down (Mir124a-1hg) are shown. Both Igfb2 and Mir124a-1hg account for multiple of the highest scoring cCREs indicated by circles.

- 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**.

Protocol references

Steven Henikoff*, Jorja G Henikoff, Kami Ahmad, Ronald Paranal, Zachary R Russell, Frank Szulzewsky, Derek H Janssens, Eric C Holland (2023) **Epigenomic analysis of Formalin-Fixed Paraffin-Embedded samples by CUT&Tag** *Nature Communications* 14:5930. <https://www.nature.com/articles/s41467-023-41666-z>.

Steven Henikoff+*, Ye Zheng+, Ronald M Paranal, Yiling Xu, Jacob E Greene, Jorja G Henikoff, Zachary R Russell, Frank Szulzewsky, H Nayanga Thirimanne, Sita Kugel, Eric C Holland, Kami Ahmad (2025) **RNA polymerase II at histone genes predicts outcome in human cancer** *Science* 387(6735):737-743. <https://www.science.org/doi/10.1126/science.ads2169>.

Acknowledgements

S. H. is an Investigator of the Howard Hughes Medical Institute