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CUTAC for FFPEs V.3

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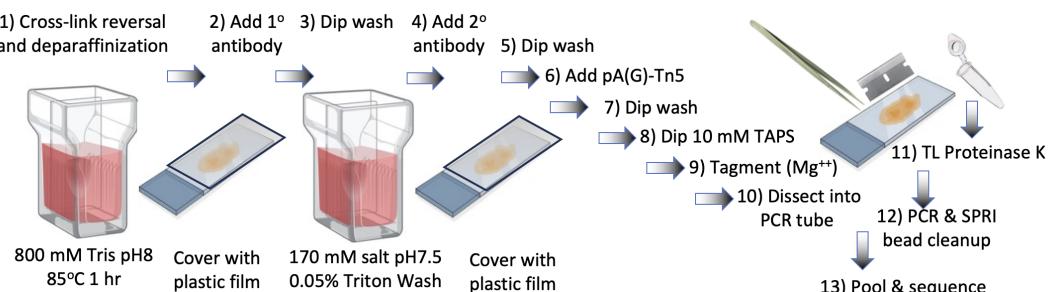


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ABSTRACT

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. 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

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, Jorja G. Henikoff, Ronald M. Paranal, Jacob E. Greene, Zachary R. Russell, Frank Szulzewsky, Sita Kugel, Eric C. Holland & Kami Ahmad (2024) **Direct measurement of RNA Polymerase II hypertranscription in cancer FFPE samples** *bioRxiv* doi: <https://doi.org/10.1101/2024.02.28.582647>.

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We use this protocol and it's working

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GUIDELINES

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

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 µL, 20 µL, and 10 µL)
- 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 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)
- 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 (PolIIIS5P, 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)
- 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 μ l 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 μ l 10% Triton-X100, 12.5 μ l 2 M spermidine and 20 μ l 0.5 M EDTA, 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 long incubations and storage of Triton-wash buffer, add Sodium azide to 0.02% (100 μ l 10% \rightarrow 50 mL), but handle this toxic chemical carefully, and wear a mask when weighing it out.

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

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

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

CUTAC-DMF Tgmentation 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 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).

6% Triton Mix 600 µl 10% Triton-X100 + 400 µl dH₂O. Store at room temperature.

Option 1: On-slide FFPE-CUTAC deparaffinization in hot cross-link reversal

- 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 85-90 °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 85-90 °C incubations 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.

- 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 (continued): On-slide FFPE-CUTAC Incubation with primary antib...

- 5 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.
- 6 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.

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

Expected result

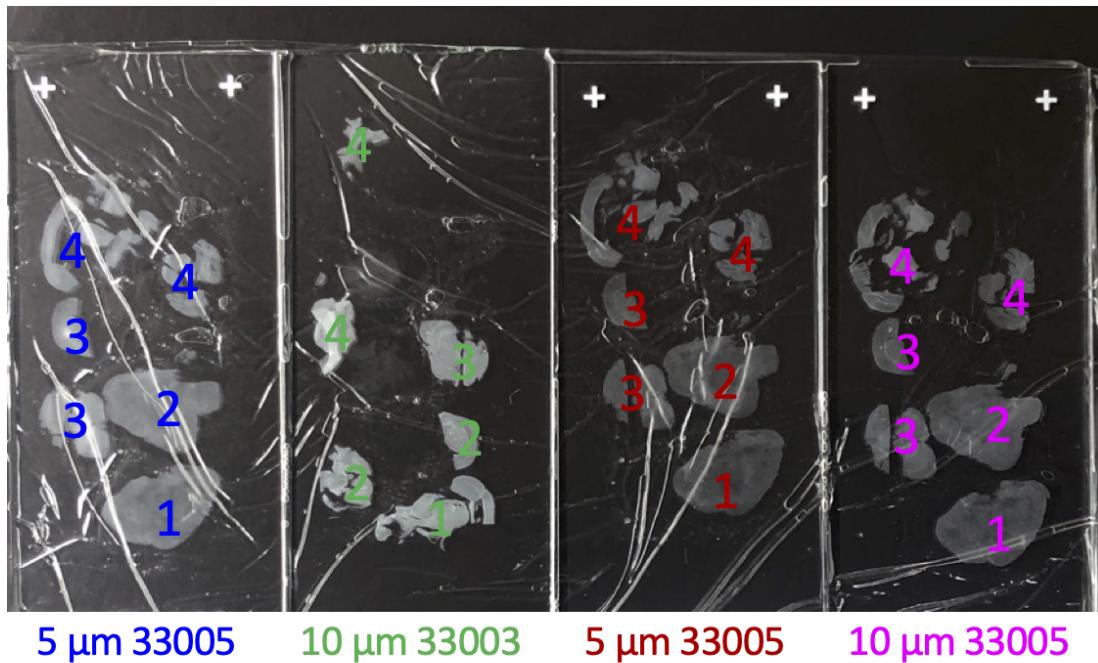


Figure 4: Example of an incubation step. On-slide FFPE-CUTAC was performed using a rabbit RNA Polymerase II Serine-5 monoclonal antibody (Cell Signaling Systems #13523). Four slides from two mouse RELA transgene-driven ependymoma FFPE blocks (5 and 10 μ m from the 33005 block and 10 μ m from the 33003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

- 8 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).

- 9 Wick off excess liquid with a Kimwipe and place tissue-side up on a dark surface. Carefully pipette ~50 µl secondary 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 at room temperature for at least 1 hr.
- 12 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)

- 13 Remove from slide holder and wick off excess liquid with a Kimwipe. Place tissue-side up on a dark surface. Carefully pipette ~50 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.

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 at room temperature for at least 1 hr.
- 16 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.
- 17 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 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°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 larger tissue amounts, use more SDS/TLProtK solution and divide up into PCR tubes such that no more than 5 µL is deposited into each tube. To recover all tissue from the slide dice and scrape with a safety razor blade. Vortex and centrifuge to compact beads in the bottom of the tube and proceed to Fragment Release (Step 52).

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 #3-5 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 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 beads: Deparaffinization in mineral oil and c...

- 22 FFPE slide or curl: Scrape all or part of a 10 µm FFPE slide (**Figures 2-4**) or a "curl" (**Figure 5**) into a 1.7 ml tube (e.g. MCT-175-C), add 200 µL mineral oil. Vortex, spin, and place in a 90°C water bath for 5 min. While still warm vortex to fully suspend the paraffin and spin on full.

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 curl up to form a tight rod.



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

- 23 Using a blue pestle attached to a pestle motor place the pestle into the bottom of the tube, start the motor and homogenize with short up-and-down motions for ~20 sec.
- 24 Add 200 µl warm Cross-link reversal buffer, then 6 µl 1:10-diluted Biomag Plus amine beads into the bottom (aqueous) layer, vortex and homogenize ~20 sec with the motorized pestle.
- 25 Add 800 µl warm Cross-link reversal buffer and vortex to mix, spin on full and replace in the 90°C water bath. Incubate ≥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.

- 26 Remove from water bath, mix by hard vortexing and spin on full. Very carefully remove the top (oil) layer without disturbing the interface, where there will be trapped tissue and beads, leaving behind a thin oil layer above the meniscus.
- 27 Add 500 µl mineral oil, mix by inversion (do not vortex), spin on full and carefully remove the mineral oil layer leaving behind a thin oil layer. Repeat with a second 500 µl mineral oil wash. Respin to clear tube sides and pipet off excess oil, leaving behind a thin oil layer above the meniscus.
- 28 Add 2.4 µL (undiluted) Pierce glutathione beads to bottom of tube avoiding the mineral oil on the surface. Mix by inversion.
- 29 Do a quick spin and place on the magnet stand. When clear carefully remove the supernatant using a 200 µL low-bind pipette tip.

Note

Bio-Mag Plus amine magnetic beads are ~1.5 micron in diameter and have a rough hydrophilic surface that sticks weakly to deparaffinized tissue shards (**Figure 6**). Pierce glutathione magnetic agarose beads are 10-40 micron but are inert and don't appear to stick, although they trap the tissue as they migrate in a magnetic field. In a magnetic field, the combination rapidly forms a tight pellet that is not disrupted by the pipette when decanting the supernatant.

- 30 Add 1 ml Triton-Wash buffer and vortex followed by a quick spin, and divide into two or more PCR tubes. The following assumes two PCR tubes per scrape or curl, one for RNAPII-Ser5p and one for H3K27ac, but for smaller aliquots volumes should be adjusted to maintain the concentration of reagents.

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 using this one-tube protocol.

- 31 Place tubes on magnet stand and carefully remove supernatant using a low-bind 200 µL pipette tip.

Option 2 (continued): Incubation with primary antibody

- 32 Resuspend beads in 100 µl primary antibody solution followed by vortexing.

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.

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

Option 2 (continued): Incubation with secondary antibody

- 34 After a quick spin, place the tubes on the magnet stand to clear and withdraw and discard the antibody supernatant using a 200 µL low-bind pipette tip.

Note

Incubation solutions may become cloudy because Triton-X100 forms micelles around dissolved mineral oil molecules to form a colloidal suspension. This is normal.

- 35 Resuspend beads in 100 µl secondary antibody solution followed by vortexing.

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

- 37 After a quick spin, place the tubes on the magnet stand and withdraw and discard the antibody supernatant using a 200 µL low-bind pipette tip.

- 38 While on the magnet stand, slowly drip in 500 µl of Triton-Wash buffer. Carefully withdraw and discard the wash supernatant using a 200 µL low-bind pipette tip. Proceed immediately to the next step.

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

- 39 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 fragmented 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 ($\leq 1:200$).

- 40 Add 100 µl pA(G)-Tn5 mix followed by vortexing. Place the tubes on a Rotator or Nutator at room temperature for ≥ 1 hr.
- 41 After a quick spin, place the tubes on the magnet stand and withdraw and discard the pA(G)-Tn5 supernatant using a 200 µl low-bind pipette tip.
- 42 While on the magnet stand, slowly drip in 500 µl of Triton-Wash buffer. Carefully withdraw and discard the wash supernatant using a 200 µl low-bind pipette tip.
- 43 While on the magnet stand, add 200 µl TAPS wash. Withdraw and discard the TAPS wash supernatant using a 200 µL low-bind pipette tip. Proceed immediately to the next step.

Option 2 (continued): Tagmentation

- 44 Resuspend the bead/FFPE pellet in 100 μ l CUTAC-DMF fragmentation solution (5 mM MgCl₂, 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).

- 45 After a quick full centrifugation, place the tubes on a magnet stand and withdraw and discard the Fragmentation buffer supernatant using a 200 μ l low-bind pipette tip.
- 46 While on the magnet stand, add 100 μ l TAPS wash. Withdraw and discard the TAPS wash supernatant using a 200 μ l low-bind pipette tip.
- 47 Add 10 μ l 1% SDS/Thermolabile Proteinase K solution per PCR tube. Vortex, quick spin and proceed to Fragment Release (Step 48).

Fragment Release and PCR

- 48 Incubate at 37°C for 30 min and 58°C for 30 min (programmed in succession in a PCR cycler with a heated lid) to release pA-Tn5 from the fragmented DNA. Open the tubes and add 15 μ L 6% 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.

- 49 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.

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

- 51** 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 14 cycles. Extra PCR cycle reduce the complexity of the library, although for very limited samples (e.g. 1 cm² 5 micron thick sections) up to 15 cycles might be needed to obtain sufficient material.

Post-PCR Clean-up (30 min)

- 52 After the PCR program ends, remove tubes from the thermocycler, vortex to resuspend, and add 130 µL of SPRI beads (ratio of 1.3 µL of SPRI beads to 1 µL of PCR product). Mix by pipetting up and down.
- 53 Let sit at room temperature 5-10 min.
- 54 Place on the magnet stand for a few minutes to allow the solution to clear.
- 55 Remove and discard the supernatant.
- 56 Keeping the tubes in the magnet stand, add 400 µL of 80% ethanol.
- 57 Completely remove and discard the supernatant.

- 58** Repeat Steps 56 and 57.
- 59** Perform a quick spin and remove the remaining supernatant, avoiding air drying the beads by proceeding immediately to the next step.
- 60** Remove from the magnet stand, add 22 µl 10 mM Tris-HCl pH 8, vortex and quick spin. Let sit for at least 5 min to elute the DNA.
- 61** Place on the magnet stand and allow to clear.
- 62** Remove the liquid to a fresh 1.5 mL tube with a pipette, avoiding transfer of beads.

Tapestation analysis and DNA sequencing

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

Expected result

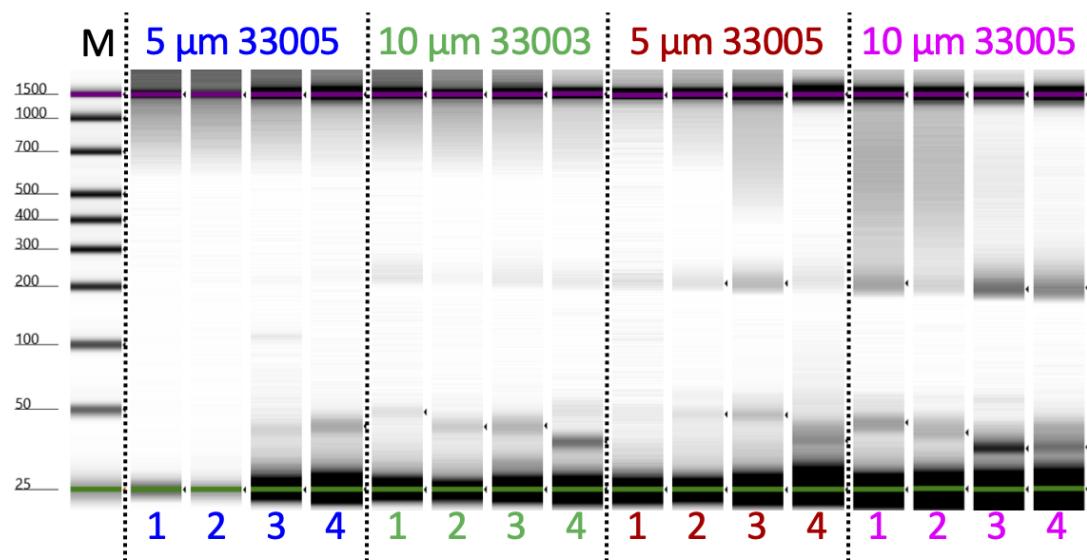


Figure 6: Tapestation gel image of 1/10th of each SPRI-bead purified DNA eluate from an on-slide experiment.

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.

64

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.

- 65 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

- 66 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

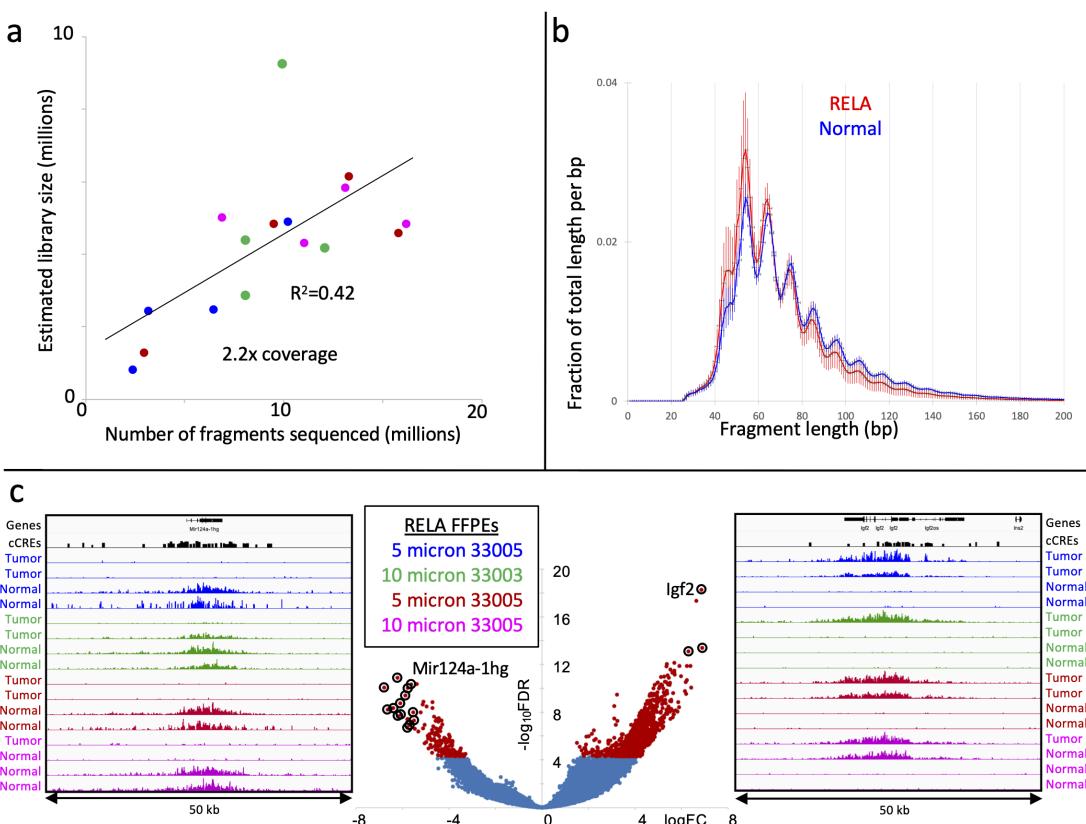


Figure 7: Analyses of the data produced in the experiment shown in Figures 4 and 6.

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 (Igf2) and down (Mir124a-1hg) are shown. Both Igf2 and Mir124a-1hg account for multiple of the highest scoring cCREs indicated by circles.

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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](#).