NicE-seq: a simple and quick method for accessible chromatin detection in fixed cells

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Abstract
Genome-wide accessible chromatin sequencing and identification has enabled deciphering the epigenetic information encoded in chromatin, revealing accessible promoters, enhancers, nucleosome positioning, transcription factor occupancy, and other chromosomal protein binding. The starting biological materials are often fixed using formaldehyde cross-linking. Here, we describe accessible chromatin library preparation from low numbers of formaldehyde fixed cells using a modified nick translation method, where a nicking enzyme nicks one strand of DNA and DNA polymerase incorporates biotin-conjugated dATP and methyl-dCTP. Once the DNA is labeled, it can be isolated for NGS library preparation. We also demonstrate a single tube method that enables direct NGS library preparation from low cell numbers without DNA purification.

Introduction

Nucleosome depleted regions on chromatin are accessible to transcription factor and other chromatin associated protein binding to drive cellular processes. Early studies identified these nucleosome-depleted regions being hypersensitive to DNase I and demonstrated an association of these protein-depleted regions with gene activation, and DNA methylation in eukaryotic organisms (1-4). This subsequently led to genome-wide mapping of DNase hypersensitive sites (DHS), also known as “open chromatin” by DNase-Chip or massive parallel DNA sequencing (5, 6). Following DNase-seq, another accessible chromatin determination method, FAIRE-Seq (Formaldehyde-Assisted Isolation of Regulatory Elements Sequencing) was used for isolation and sequencing of nucleosome-depleted regions of the genome. This method relied on formaldehyde crosslinked chromatin sheared by sonication and phenol-chloroform extraction of the accessible DNA in the aqueous phase that could be sequenced or hybridized to a DNA microarray (7). However, both methods were cumbersome and needed large numbers of cells. Recent improvements for DHS mapping include the addition of circular carrier DNA to perform single cell DNase I seq (scDNase I-seq), requiring an input of between one and 1000 cells. This technology revealed highly expressed genic regions with multiple active histone marks displaying constitutive DNase I hypersensitive sites among different single cell analysis data. However, in scDNase I-seq, the mappability of 1000 cells to the reference genome was low, ranging from 2-40% at the single cell level (8). Another complementary method, known as MNase-seq, uses non-specific endo and exonuclease activities of micrococcal nuclease to cleave protein-unbound regions of DNA on chromatin. Here, DNA bound to histones or other chromatin associated proteins remain undigested and sequencing of these DNA fragments yields genome wide maps of bound proteins (9-11). The undigested DNA displays a mirror image of accessible chromatin.

In 2013, ATAC-seq (Assay for Transposase-Accessible Chromatin using sequencing) was introduced to study chromatin accessibility genome-wide. The method was simpler and easier to use compared with MNase-seq (sequencing of micrococcal nuclease sensitive sites), FAIRE-seq and DNAse-seq. The basic principle relied on a prokaryotic Tn5 transposon, that is loaded with sequencing adapters creating an active dimeric transposome complex. The complex can provide the cut accessible chromatin and the simultaneous ligation of specific sequences. In the early days, ATAC-seq also generated non-specific amplification of non-nuclear DNA, such as the mitochondrial genome, accounting for ~50% of all reads (12). Subsequent improvements to the method have led to reduce mitochondrial DNA reads (13). This application of ATAC-seq to human cell lines and clinical samples has led to many landmark studies (14-19). Here we report an improved, robust, and sensitive method, nicking enzyme assisted sequencing (NicE-seq), for epigenetic profiling of the mammalian chromatin that can provide in-depth open versus closed chromatin sequence information from limited amounts of formaldehyde fixed cells (20-21). This method is suitable for identification of transcription factor occupancy and complementary to other accessible chromatin methods.

Materials

Prepare all solutions using ultrapure water (example: MilliQ water or equivalent by purifying deionized water, to attain a conductivity of 18 MΩ-cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature or at 4 ˚C (unless indicated otherwise).

1. Harvesting and Crosslinking Cells

1. HCT116 cells are cultured in McCoys 5A medium (Thermo Fisher Scientific #16600082) supplemented with 10% Fetal Bovine Serum (GemCell #100-500)
2. TrypLE (Thermo Fisher Scientific #12605028, store at R/T before use)
3. 50 ml conical falcon tubes and pipette tips for automatic pipet
4. Cell culture flasks
5. Trypan Blue Solution, 0.4% (Thermo Fisher Scientific #15250061)
6. Hemocytometer and inverted microscope
7. 1.5 mL Eppendorf tube for cell harvest; 1.5 mL DNA LoBind tube (Eppendorf AG #022431021)
8. 16% formaldehyde (Thermo Fisher Scientific #28908)
9. 1X PBS (from 10X PBS, Gibco #70011-044)
10. 2.5 M Glycine (Sigma #G7126)
11. End-over-end bench top rotator (VWR #10136-084)

2. Accessible Chromatin Labelling

1. Prepare Cytosolic Buffer: 15 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 60 mM KCl, 15 mM NaCl, 1% NP40 and 300 mM sucrose. Add 0.5 mM fresh DTT before use (NEB #B7705S). Note: Use freshly made cytosolic buffer, Cytosolic buffer can be stored up to 2-3 week at 4˚C without the addition of DTT. For microbial stability, it may be sterile filtered through 0.22 µM membrane).

2. Prepare a 10x dNTP mix: 240 µM dATP, 240 µM mdCTP (NEB #N0356S), 60 µM biotin-14-dCTP (Thermo Fisher Scientific #19518018), 300 µM dGTP, 300 µM dTTP and 60 µM of biotin-14-dATP (Thermo Fisher Scientific #19524016).

3. Prepare accessible chromatin labelling buffer (for one labeling reaction): 20 µl of NEB Buffer2 10x (NEB #B7002S), 1 U of Nt.CviPII (NEB #R0626S), 10 U of DNA Polymerase I (NEB #M0209S) with 30 µM of each dNTP including 6 µM of biotin-dCTP and 6 µM of biotin dATP (Thermo Fisher Scientific #19518018) and adjust the final volume with water to 100 µl.

3. Other material and equipment for chromatin labeling

1. 37 ˚C Incubator, 65 ˚C heat-block and bench-top centrifuge

2. 0.5 M EDTA (Invitrogen #15575-038) and RNase A (Invitrogen #12091021)
3. Proteinase K (NEB #P8107S) and 20% SDS (TEKNOVA #S0295)
4. Thermolabile Proteinase K (NEB #P8111S)
5. Phenol:Chloroform:Isoamyl Alcohol (Invitrogen #15593-031)
6. Isopropanol (Pharmco-Aaper #231HPLC99)
7. Glycogen (Sigma-Aldrich #10901393001)
8. 80% ethanol in nuclease free water (Thermo Fisher Scientific #AM9932)
9. 1X TE buffer

4. Material for Quality Control

1. Heat block at 95 ˚C
2. Ice-water bath
3. Positively charged nylon membranes (Amersham #RPN119B)
4. UV light
5. Blotting-grade blocker (Bio-Rad #170-6404)
6. 1X PBS with 0.1% Tween20
7. Goat anti-biotin-HRP antibody (CST #7075)
8. LumiGLO reagent (CST #7003)

5. Material of the library construction

1. Covaris S2 sonicator and Covaris microtubes (Covaris #500330)
2. NEB Ultra II DNA library prep Kit for Illumina (NEB #E7645)
3. Index primers set (NEB #7335S or E7500S)
4. Prepare 2X High Salt Buffer: 10 mM Tris-HCl pH8.0, 2 M NaCl, 1 mM EDTA, adjust with nuclease-free water
5. Prepare High Salt Buffer with 0.05% Triton-X100
6. DNA LoBind Eppendorf tubes (Eppendorf AG #022431021)
7. Gelatin from cold water fish skin (Sigma-Aldrich #G7765)
8. Magnetic Streptavidin C1 beads (Thermo Fisher Scientific #65002)
9. Blocked Streptavidin beads (blocked in 0.1% fish gelatin in 1X PBS at 4 ˚C for O/N)
10. NEBNext Sample Purification Beads (E7104) or AMPure XP beads (Beckman Coulter #A63881)
11. Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific #Q32854)
12. End-over-end rotator (VWR #10136-084)
13. Bioanalyzer (Agilent 2100 Bioanalyzer with Agilent High Sensitivity DNA Kit, #5067-4526)
14. Monarch Genomic DNA Purification Kit (NEB #T3010S)
15. PCR machine

Methods

Carry out all procedures at room temperature unless otherwise specified.

1. Harvesting and Crosslinking Cells

1. Take cells from the incubator and visually check their health under the microscope. Remove old medium in the flask and transfer 50 ml to a sterile conical centrifuge tube and then add 5-10 mL TrypLE to the adherent cells in the flask, and incubate for 5 min at 37 ˚C.
2. Gently tap to detach cells, pipet cells to the old medium containing sterile conical centrifuge (the old medium/serum will inhibit the activity of the trypsin). Spin down 5 min at room temp, 1500 rpm and remove supernatant (decant)
3. Wash in 5 ml 1X PBS, spin down (5 min at room temp, 1500 rpm), remove supernatant and resuspend in 5 ml 1X PBS.
4. Count cells: Dilute small amounts of cells (1:1) with Trypan Blue Stain (gives you a dilution factor of 2) (eg: take 100 μl cells and 100 μL stain). Pipet into small edge of the hemocytometer at counting chamber. Calculate the number of cells per ml.
5. Calculate how many cells in totals. Take 10e6 cells and transfer to 1.5 ml DNA LoBind Eppendorf tube.
6. Add 62.5 μl of 16% formaldehyde and adjust with 1X PBS up to 1 ml (final concentration would be 1% formaldehyde), incubate cells 10 min at room temp by the end-over-end rotator.
7. Quench the reaction by adding 125 mM Glycine (for a volume of 1 ml, add 52 μl of 2.5 M stock) and incubate 5 min at room temp by the end-over-end rotator.
8. Wash cells twice by resuspending in 1 ml of 1X PBS and spinning down 1500 rpm, 1 min at 4˚C. Remove supernatant and cells may be stored at -80 ˚C for later use. For immediate use, resuspend cells in 1X PBS (0.5 ml) and counter the number of cells and make aliquots depending on how many cells will be needed for downstream work (ideally 400 μl for 1 million cells, that will give 250, 000 in 0.1 ml). Note: Cells may be lost during centrifuge steps. Therefore, the above counting step is crucial for universal NicE-seq. In our experience, cell loss up to ~40% during the crosslink step may occur, if we count cells before adding formaldehyde. This is a critical step when cell numbers are limited.

2. Accessible Chromatin Labelling & Decrosslinking

1. Start with 250-250,000 crosslinked cells suspended in 1X PBS (25 μl)
2. Add 400 μl Cytosolic buffer to the cells and incubate for 10 min on ice with occasional tapping for mixing. The nuclei can be visualized under the microscope at this point (circular with smooth edges).
3. Spin the nuclei down 10 min at 4 ˚C, 3000 rpm. Discard the supernatant.

4. Wash the nuclei pellet with 1X PBS 5 min at 4 ˚C, 3000 rpm. Discard the supernatant. If cell number is small <2000, this step can be skipped. Please make it sure that you don't lose the nuclei, leave a small amount (10-20 μl) of supernatant in the tube.
5. Add 100 μl of cold 1X PBS buffer and tap/pipet gently to dissolve the cells
6. Add 100 μl of Accessible Chromatin labeling buffer (at this point, total volume is 200 μl). Incubate for 2 hours at 37 ˚C. Occasionally tap the reaction to mix.
7. Add 20 μl of 0.5 M EDTA and 2 μg of RNase A to each sample. Incubate 30 min at 37 ˚C to digest RNA.
8. Add 20 μl of 20% SDS and 20 μl of Proteinase K. Incubate for O/N at 65 ˚C

3. Labeled genomic DNA Extraction using phenol chloroform/spin column method

1. Add 250 μl of phenol/chloroform. Vortex 3 times, 5-10 sec each time.
2. Centrifuge 10 min at 4 ˚C, 14000 rpm. Transfer aqueous phase (upper part) to Eppendorf tube (will be maximum 250 μl but starting from a lot of cells you don't need to take so much as this will increase the chances of collecting the interphase that contains proteins).
3. Add 0.7 volume of isopropanol and 2 μl of glycogen. Incubate for 2 hours at -80 ˚C or O/N at -20 ˚C
4. Centrifuge 10 min at 4 ˚C, 14000 rpm
5. Carefully wash DNA pellet with 500 μl of 80% ethanol. Centrifuge 10 min at 4 ˚C, 14000 rpm
6. Remove ethanol solution completely. Air dry sample and resuspend DNA in 50 μl of 1X TE buffer.
   Optional: Extract biotin labeled genomic DNA by column purification using NEB Monarch Genomic DNA Purification Kit.
7. Measure DNA concentration by Qubit High Sensitivity Kit with reading ds DNA HS mode (in case concentration too high: dilute 10X, when starting with high number of cells (~250,000) concentration will be high making a dilution is standard).

4. Quality Control (Optional)

1. Denature genomic DNA by heating for 3 min at 95 ˚C and incubate 3 min in an ice-water bath
2. Make a serial dilution (1, 0.5, 0.25, 0.125 μg) of genomic DNA in MQ water on ice. Never exceed a total volume of 5 μl per spot).
3. Prepare a positively charged nylon membrane (Amersham #RPN119B) by marking with a pencil the circles where the spot will be with DNA. Spot the dilution on the membrane and let dry.
4. Cut mark the upper left corner (for orientation).
5. Wet the membrane by dripping MQ water on top of it so that it is fully hydrated.
6. DNA crosslinking by UV: put membrane in UV crosslinker machine.

7. Wash the membrane with 1X PBST. Transfer the membrane to a square protein gel box (with lid).
8. Block the membrane with 5% skimmed milk in 1X PBST for 30-60 min at room temp on a shaker.
9. Add HRP-conjugated goat anti-biotin antibody (1/2000 dilution) for 1 h
10. Detect biotin signal with LumiGLO reagent.

5. UniNicE-seq library construction using sonication for DNA fragmentation

1. For fragmentation of genomic DNA, take 200 ng of genomic DNA, transfer to Covaris microtube and add up to 50 μl of 1X TE buffer (If genomic DNA is less than 200 ng, the entire DNA can be used for the sonication). Use the following setting to obtain 150 bp fragments (Intensity: 5 Duty Cycle: 10%; cycles per burst: 200; treatment time: 2 min).
2. After the sonication step is done, transfer the content to 1.5 ml DNA LoBind Eppendorf tube.

6. UniNicE-seq library construction

1. *For DNA pull-down,* add 1 ml of 1X High Salt Buffer to the fragmented DNA tube and add 30 μl of blocked beads. If DNAs quantity (below 50 ngs) or number of starting cell (below 2K), the bead amount can be reduced to 15-20 μl.
2. Incubate for 2 hours at 4 ˚C on the end-over-end rotator.
3. Place the tube on magnetic rack. When the solution is clear, remove the liquid using a pipette and resuspend the beads for 5 min with 1 ml of 1X High Salt Buffer containing 0.05% Triton-X 100 at room temp.
4. Repeat wash steps 3 times more (4 times in total).
5. Wash the beads once with 1 ml of 1X TE buffer by inverting 5 times.
6. Resuspend the beads in 50 μl of 1X TE buffer. These beads will be used for PCR amplification step for library making. Therefore, care must be taken not to lose the beads.
7. *For end-repair/dA tailing*: To the above beads, add 7 μl of NEB Next Ultra II End Prep Reaction Buffer, 3 μl of NEB Next Ultra II Prep Enzyme Mix (the final volume would be 60 μl).
8. Mix well, incubate for 30 min at 20 ˚C and follow the incubation for 30 min at 65˚C.
9. Wash the bead with 1 ml of 1X High Salt Buffer with 0.05% Triton-X100 and incubate it on end-over-end rotator for 5 min at room temp. Use magnetic rack to collect the beads.
10. Repeat the above wash step. Use magnetic rack to collect the beads.
11. Add 1 ml of 1X TE and incubate on the end-over-end rotator for 5 min at room temp.
12. Re-suspend the bead in 60 μl of 1X TE buffer.
13. *For adaptor Ligation,* Add 30 μl of NEB Next Ultra II ligation Master Mix, 1 μl of NEB Next Ligation Enhancer, 1 μl of 1:10 diluted of NEB Next Adaptor for

Illumina. (Note: If start material of DNA is very low <50 ng, the adaptor can be diluted up to 1:30).

14. Incubate for O/N or 2-16 hours at room temp.
15. Remove the solution and collect the magnetic beads using a magnetic rack.
16. Wash the bead with 1 ml of 1X High Salt Buffer with 0.05% Triton-X100 and incubate it on the end-over-end rotator for 5 min at room temp.
17. Repeat once more the wash step.
18. Add 1 ml of 1X TE and incubate on the end-over-end rotator for 5 min at room temp.
19. Capture the bead and remove the solution, re-suspend the bead in 16 μl of 1X TE buffer.
20. Add 3 μl of USER enzyme and incubate for 20 min at 37 ˚C.
21. *For PCR amplification*, transfer all the 19 μl into the PCR tube.
22. Add 3 μl of Index Primer (10 μM), 3 μl of Universal primer (10 μM), and 25 μl of NEB Ultra II Q5 Master Mix. The total volume of the final reaction is 50 μl.
23. Set up PCR: 8 cycles for sonication method and 12 cycles for enzymatic digestion at 30 sec 98 ˚C; initial denaturation; 10 sec 98 ˚C; denaturation; 30 sec 65 ˚C; annealing; 45 sec 65 ˚C; extension; 5 min 72 ˚C; final extension; Hold 4 ˚C.
    Note: Depending on the amount pf DNA, PCR cycles can be modified. If starting material of DNA is below 50 ng, PCR cycles can be increased up to 12-13 cycles.

7. PCR Clean up using AMPure beads

1. Bring the AMPure beads solution to room temp before the clean-up steps (If PCR clean-up conduct with cold beads, it may affect the efficiency for the recovery of DNA). After PCR reaction is over, PCR tubes can be vortex for 2-3 sec followed by quick spin down. Put PCR reaction tubes on the magnetic rack for 1 min, transfer the solution (containing library) to a new DNA LoBind tube, and add 0.9 volume (so here, 45 μl) AMPure beads.
2. Incubate 10-15 min at room temp.
3. Put samples on magnetic rack. When the solution looks clear, remove it and wash the beads twice with 200 μl of freshly prepared 80% ethanol by slowly pipetting the ethanol on the beads without removing the Eppendorf tube off of the rack. Wait 10 seconds, remove the liquid from the beads and repeat. After removing the ethanol for a second time, quickly spin down the tubes, put them back on magnetic rack and remove the remaining ethanol at the bottom of the tube.
4. Resuspend the bead in 10 μl of 0.1X TE and incubate for 10 min at room temp.
5. Put back on the magnetic rack and transfer the library containing solution into the new DNA LoBind tube. It is the final library DNA.
6. Measure the amount of the library DNA using the Qubit HS DNA Kit set at ds High Sensitivity mode. If the concentration is > 1 ng/μl, the yield of library is acceptable for the NextGen-sequencing.

7. Analyze the library DNA on the Bioanalyzer with DNA High Sensitivity ChIP to check the actual library quantity. (Note: In case, the ligated adaptor is still visible in Bioanalyzer, the library pool can be re-purified using AMPure bead after the libraries are combined).

Optional method A: Sonication free labeled DNA enrichment for NicE-seq library preparation using nicking enzyme digestion

1. Take 200 ng of genomic DNA into 1.5 ml of DNA LoBind Eppendorf tube, add 10 μl of 10X NEB Buffer #2, 1 U of Nt.CviPII, and adjust with MQ water up to 100 μl.
2. Incubate O/N at 37 ˚C.
3. After O/N digestion, heat-inactivate Nt.CviPII by incubating the reaction tube at 65 ˚C for 10 min.
   Note: The enzyme amounts can be adjusted for digestion. For 100 ng of genomic DNA from HCT116 cells, 0.5 U of Nt.CviPII can be used for accessible chromatin enrichment.
4. For the library preparation, use steps of 6 and 7.

Optional Method B: One tube NicE-seq

This method is sonication-free, one tube NicE-seq library from cultured cells, and is recommended for low cell number ie < 1000 cells. However, starting materials between 250 – 5K cells are adapted to one tube NicE-seq method.

1. Harvesting and Crosslinking Cells

Harvest and Crosslink cells by the same procedure that described in 1 of the Harvesting and Crosslinking Cells Section. Care must be taken to wash cells with 1X PBS before 1% formaldehyde for fixation, followed by two washes with 1X PBS. Note: residual formaldehyde and glycine may inhibit downstream reaction.

2. Accessible Chromatin Labelling and Decrosslinking

1. Start with 250-100,000 crosslinked cells suspended in 25 μl of 1X PBS.
2. Add 400 μl Cytosolic buffer to the cells and incubate for 10 min with occasional mixing by tapping. The nuclei can be visualized under the microscope (circular with smooth edges).
3. Spin the nuclei down for 10 min at 4 ˚C, 3000 rpm. Discard the supernatant. Wash the nuclei with 1X PBS for 5 min at 4 ˚C, 3000 rpm. Discard the supernatant. Note: If cell number is small < 2000 cells, this step can be skipped. To avoid the loss of the nuclei, leave a small amount (10-20 μl) of supernatant in the tube.
4. Add 100 μl of cold 1X PBS buffer and tap/pipet gently to resuspend the nuclei.

5. Add 100 μl of Accessible Chromatin labeling buffer (at this point, total volume is 200 μl). Incubate for 2 hours at 37 ˚C. Occasionally tap to mix the reaction.
6. Add 2 μl of 0.5 M EDTA, 18 μl of MQ water, and 2 ug of RNase A to each sample. Incubate for 30 min at 37 ˚C to digest RNA.
   Note: the high concentration of EDTA can be inhibitory for nicking enzyme digestion of DNA. Hence, the amount of 0.5 M EDTA is reduced to prevent the undigested DNA for this method compared with the method, 2. Accessible Chromatin Labelling and Decrosslinking section.
7. Add 2.2 μl of 10% SDS and 2 μl of Thermolabile Proteinase K (TLPK). The final SDS concentration would be 0.1%.
8. Incubate for O/N at 37 ˚C.
9. Incubate for 15 min at 55 ˚C to inactivate TLPK.
10. Add 22 μl of 10% TritonX-100, mix well, and leave the tube for 5-10 min at room temp. The final Triton X-100 concentration would be 1%.

3. Fragmentation of labeled chromatin by the nicking enzyme digestion

1. Add 75 μl of 10X NEB Buffer #2, 670 μl of MQ water, 5 U of Nt.CviPII in the reaction tube and mix well to the a final volume of 1 ml.
2. Incubate for 4 hours at 37 ˚C by the end-over-end rotator.
3. Incubate for 10 min at 65 ˚C to inactivate Nt.CviPII.

4. DNA pull down

1. Add 30 μl of Streptavidin bead, 300 μl of 5M NaCl and MQ water up to 1.5 ml.
2. Incubate for 2 hours at 4 ˚C.
3. Wash the bead with 1X High Salt Buffer containing 0.05% TritonX-100 for 5 min at the room temp by the end-over-end rotator and remove the solution carefully.
4. Repeat the above step 3 times more (4 times in total)
5. Wash the bead once with 1 ml of 1X TE buffer by in inverting a couple of times.
6. Resuspend the bead in 50 μl of 1X TE buffer. These beads will be used for the library making and PCR amplification steps.

5. End-repair/dA tailing

Follow the same procedure that described in *Method 6. UniNicE-seq library construction section.*

6. Adaptor ligation

Follow the same procedure that described in *Method 6. UniNicE-seq library construction section.* If the staring material is < 1000 cells, the adaptor can be diluted up to 1:30.

7. PCR amplification and PCR Clean up by AMPure Beads

Follow the same procedure that described in Method 6, UniNicE-seq library construction section and in *Method 7, PCR Clean up using AMPure beads.* PCR cycle can be modified up to 13 cycles while avoiding high PCR duplication. For very low number cells < 250 cells, PCR cycle can be modified up to 20 cycles.

A) Onetube NicE-seq of the Human FFPE Samples from 5-10 μm tissue section

1. Removal of paraffin from the tissue section slide

Start with 5-10 μm FFPE section on the slide

1. Add 500 μl of Mineral oil (Sigma-Aldrich # 330760) to the area of tissue on the slide.
2. Incubate for 20 min at 52 ˚C.
3. Remove the mineral oil carefully, transfer the slide to coupling jars/plate with the ethanol 100%, and incubate for 5 min at R/T.
4. Transfer the slide to gradually 90% ethanol, 80% ethanol, 70% ethanol, each step for 5 min at R/T and hydrate in MQ water for 5 min.
5. Transfer the slide in 1X PBST buffer and incubate for 1 hour at 65 ˚C. Gradually cool the slide down to RT.
6. Cooling it down at R/T.
7. Transfer the slide to 1X PBST buffer containing Proteinase K (45 μl of Proteinase K in 50 ml of 1X PBST) in a square petri dish and incubate for 15 min at R/T.
8. Exchange the slide in 1X PBS buffer for 2 min at R/T and repeat it once more.
9. Incubate the slide with 1X PBS buffer for 2 min at R/T.
10. Incubate the slide with Cytosolic buffer for 20 min at 4 ˚C.
11. Incubate the slide with 1X PBS buffer for 5 min at R/T.

2. Accessible Chromatin Labelling and Decrosslinking

1. Mark the circle the area of tissue by the liquid-repellent slide maker pen and carefully drop it off with 200 μl of Accessible Chromatin Labelling solution onto the circle.
2. Incubate for 2 hours at 37 ˚C in a humidified chamber.
3. Transfer the slide in 1X PBS containing 0.5 M EDTA and incubate for 10 min at R/T.
4. Collect the tissue carefully into 1.5 ml DNA LoBind Eppendorf tube by using a surgical scalpel and add 200 μl of ATL buffer (QIAGEN, QIAamp DNA FFPE Tissue Kit #56404).
5. Add 2 ug of RNase A and incubate for 30 min at 37 ˚C.
6. Add 20 μl of Proteinase K and incubate for O/N at 65 ˚C.
7. Incubate for 2 min at 95 ˚C for heat-inactivation of Proteinase K.

8. Cooling it down for 15 min at R/T.

3. Enrichment of labeled chromatin by the nicking enzyme digestion

1. Add 50 μl of 10X NEB Buffer #2, 2.5 U of Nt.CviPII to the tube and adjust with MQ water up to 500 μl, and mix well.
2. Incubate for O/N at 37 ˚C.
3. Incubate for 15 min at 65 ˚C for heat-inactivation of Nt.CviPII.

4. DNA pull down

1. Add 30 μl of Streptavidin C1 beads and add 1 ml of 1X High Salt Buffer and incubate for 2 hours at 4 ˚C by end-over-end rotator.
2. Wash the bead with 1 ml of 1X High Salt Buffer containing 0.05% TritonX-100 for 5 min at R/T.
3. Repeat the above wash step 3 times more (4 times in total).
4. Wash the bead with 1X TE for 5 min at R/T.
5. Resuspend the bead in 50 μl of 1X TE.

5. End-repair/dA tailing

Follow the same procedure that described in *Method 6. UniNicE-seq library construction section.*

6. Adaptor ligation

Follow the same procedure that described in *Method 6. UniNicE-seq library construction section.*

7. PCR amplification and PCR Clean up by AMPure Beads

Follow the same procedure that described in Method 7. PCR amplification and PCR Clean up by AMPure Beads section.

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