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Supplementary Protocol 2 – Isolation of nuclei from frozen tissues for ATAC-seq

Protocol Notes

1. All steps should be performed on ice or at 4C. Pre-chill a centrifuge to 4C.
2. It is important to perform the density gradient centrifugation in a swinging bucket centrifuge to avoid collapse of the gradient layers. The original iodixanol gradient protocol calls for a high-speed centrifugation (10,000 RCF for 20 minutes). However, such centrifuges are uncommon so we adopted a slower speed centrifugation which still works well. However, higher-speed centrifugation may further improve the purity of nuclei.
3. Make sure to add the correct amount of protease inhibitor tablets to the 6x HB unstable solution.
4. See the order list at the end of the protocol for suppliers and catalog numbers.

Protocol

1. Place frozen tissue into a pre-chilled 2 ml Dounce with 2 ml cold 1x Homogenization Buffer (HB). If you have little tissue, you can homogenize in 1ml or less HB buffer.
2. Allow frozen tissue to thaw for 5 minutes. Often times the tissue chunk will sink to the bottom of the Dounce once thawed.
3. Dounce with A pestle until resistance goes away (~ 10 strokes).
4. Dounce with B pestle for 20 strokes.
5. Pre-clear large chunks by filtration via a 100 um CellTrics.
6. Pre-clear larger chunks by pelleting at 100 RCF for 1 min in a pre-chilled centrifuge.
7. Avoiding pelleted chunks of connective tissue, transfer 400 ul to a round bottom 2 ml Lo-Bind eppendorf tube.
8. Add 1 volume (400 ul) of 50% Iodixanol solution to give a final concentration of 25% Iodixanol and mix by pipetting.
9. Layer 600 ul of 29% Iodixanol solution under the 25% mixture. Avoid mixing of layers.

10. Layer 600 ul of 35% Iodixanol solution under the 29% mixture. Avoid mixing of layers.
    This step requires gradual removal of the pipette tip during pipetting to avoid excessive volume displacement.
11. In a swinging bucket centrifuge, spin for 20 min at 3,000 RCF with the brake off. (we used the maximum speed of our centrifuge 4,816 RCF)
12. Aspirate the top layers down to within 300 ul of the nuclei band. (nuclei are present at the interface of the 29% and 35% iodixanol solutions)
13. Using 200 ul volume, collect the nuclei band and transfer to a fresh tube containing cold ATAC-RSB+0.1% Tween-20
14. Centrifuge nuclei for 5 minutes at 500 RCF in a pre-chilled 4C fixed-angle centrifuge.
15. Aspirate supernatant using two pipetting steps. (900 ul of supernatant was aspirated, the remaining 100ul of supernatant was carefully aspirated by pipetting with a p200 pipette, avoiding the cell pellet)
16. Add 1 ml of cold ATAC-RSB+0.1% Tween-20 to the tube and invert the tube several times to wash the nuclei pellet.
17. Centrifuge nuclei for 5 minutes at 500 RCF at 4C in the fixed-angle centrifuge.
18. Aspirate supernatant as before using two pipetting steps.
19. Resuspend the pellet in 25 ul 2x TD buffer by pipetting up and down 6 times.
20. Count nuclei using Trypan blue staining and transfer 50,000 nuclei into a new tube. If the nuclei are too concentrated to accurately count, dilute in 2x TD buffer. (e.g. use 2ul nuclei suspension, add 8ul 2x TD buffer and 10 ul Trypan Blue solution)
21. Adjust 50,000 nuclei to 25 ul total using 2x TD buffer.
22. Add 25 ul of Omni-ATAC ATAC-seq reaction mix (2.5 ul Tagment DNA Enzyme 1, 16.5 ul PBS, 0.5 ul 1% digitonin, 0.5 ul 10% Tween-20, 5 ul H2O) to the 25 ul of 50,000 nuclei. Pipet up and down 6 times.
23. Incubate reaction at 37°C for 30 minutes in a thermomixer with 1000 RPM mixing. (we did it in a heat block, mix by taping the tube every 10 min during the incubation)
24. Cleanup reaction with a Zymo DNA Clean and Concentrator-5 Kit. Make sure to use a different kit for pre- and post-amplification so as to not cross contaminate post-amplification product into pre-amplification samples.
    If you don't have time or don't feel like doing the cleanup immediately following transposition, resuspend the ATAC reaction in 250 ul (5 volumes) of DNA Binding Buffer and store at -20°C. The DNA is stable for at least 2

weeks in this buffer at -20°C. Allow to warm back to room temperature and mix thoroughly before loading onto the column. (we always continue the next step)

25. Elute DNA in 21 ul elution buffer and store at -20°C until ready to amplify. This elution typically results in ~20 ul of product. Use all 20 ul of product in the following PCR.
26. Amplify for 8 cycles using NEBNext 2x MasterMix:

8 cycle Amplification	
10 uM Nextera Primer1	2.5 ul
10 uM Nextera primer2 with index code	2.5 ul
2x NEBNext Master Mix	25 ul
Transposed Sample	20 ul
Cycling Conditions	
72°C	5 min
98°C	30 sec
Then 8 cycles of:	
98°C	10 sec
63°C	30 sec
72°C	1 min
Hold at 4°C	

27. Size selection with Ampure XP beads: use our lab PEG (8.25%/19.5%) buffer or follow the next steps.
28. Add 150 µl EB to the PCR reaction, then add 110 µl beads (0.55 x sample volume) and mix 10 times by pipetting.
29. Incubate 5 min at room temperature. Separate on magnetic stand.
30. Transfer supernatant (310 µl) to a new tube.
31. Add 190 µl beads (1.5 x sample volume).
32. Incubate 5 min at room temperature. Separate on magnetic stand.
33. Wash beads 2 times with 200 µl ethanol (85 %).
34. After the second wash, remove ethanol completely and dry beads on a 37°C heat block for ~1min, then resuspend beads in 10 µl EB by pipetting 10 times.
35. Separate on magnetic stand and transfer the final library to a new tube.
36. Quantify final libraries using Qubit (1 µl/sample, Qubit dsDNA HS Assay Kit) and check for library size distribution using 4200 TapeStation (High Sensitivity D1000 ScreenTape and Reagents).

37. Samples are now ready for sequencing on Illumina platform e.g. NextSeq (75 bp PE).

Stock Buffer Preparations

Prepare these buffers in advance. These buffers are stable at room temperature. Sterile filtration is recommended, especially for sucrose.

6x Homogenization Buffer Stable Master Mix

Reagent	Final Conc.	Fold Dilution (x)	Vol for 100ml
1M CaCl2	30 mM	33.33	3 ml
1M Mg(Ac)2	18 mM	55.56	1.8 ml
1M Tris pH 7.8	60 mM	16.67	6 ml
H2O			89.2 ml

1M Sucrose

Add 34.23 g sucrose to 78.5 ml water.

Same Day Buffer Preparations

Prepare these buffers on the day of processing.

6x Homogenization Buffer Unstable Solution (650 ul per sample)

Reagent	Final Conc.	Fold Dilution (x)	Vol per sample
6x Homogenization Buffer Stable	6x	1.00	92.36 ul
cOmplete protease inhibitor 7x	6x	1.17	557.14 ul
1.43 M β-mercaptoethanol	1 mM	1430.00	0.5 ul

Prepare 7x cOmplete protease inhibitor by dissolving one tablet in 1.5 ml 6x Homogenization Buffer Stable.

Prepare 1.43 M β-mercaptoethanol by diluting ten folds of the 14.3 M β-mercaptoethanol in water.

1x Homogenization Buffer Unstable Solution (2 ml per sample)

Reagent	**Final Conc.**	**Fold Dilution (x)**	**Vol per sample**
6x Homogenization Buffer Unstable	1x	6.00	333.33 ul
1M Sucrose	320mM	3.13	640.00 ul
500 mM EDTA	0.1mM	5000.00	0.40 ul
10% NP40	0.1%	100.00	20.00 ul
H2O			1006.27 ul

50% Iodixanol Solution (400 ul per sample)

Reagent	**Final Conc.**	**Fold Dilution (x)**	**Vol per sample**
6x Homogenization Buffer Unstable	1x	6.00	66.67 ul
60% Iodixanol Solution	50%	1.20	333.33 ul

29% Iodixanol Solution (600 ul per sample)

Reagent	Final Conc.	Fold Dilution (x)	Vol per sample
6x Homogenization Buffer Unstable	1x	6.00	100.00 ul
1M Sucrose	160 mM	6.26	96.00 ul
60% Iodixanol Solution	29%	2.07	290.00 ul
H2O			114.00 ul

35% Iodixanol Solution (600 ul per sample)

Reagent	Final Conc.	Fold Dilution (x)	Vol per sample
6x Homogenization Buffer Unstable	1x	6.00	100.00 ul
1M Sucrose	160 mM	6.25	96.00 ul
60% Iodixanol Solution	35%	1.71	350.00 ul
H2O			54.00 ul

ATAC-RSB

Reagent	Final Conc.	Vol for 50 ml
1M Tris-HCl pH 7.4	10 mM	500 ul
5M NaCl	10 mM	100 ul
1M $MgCl_2$	3 mM	150 ul

H2O		49.25 ml

Detergents - All detergents are resuspended as 100x stock solutions

- *Digitonin:* Digitonin is supplied at 2% in DMSO. Dilute 1:1 with water to make a 1% (100x) stock solution. Avoid more than 5 freeze thaw cycles. Can be kept at -20°C for up to 6 months.
- *Tween-20:* Tween-20 is supplied at 10%. Use at this concentration (100x stock). Store at 4°C.
- *NP40:* NP40 is supplied at 10%. Use at this concentration (100x stock). Store at 4°C.

2x TD Buffer

Reagent	**Final Conc.**	**Vol for 100 ml**
1M Tris-HCl pH 7.6	20 mM	2 ml
1M $MgCl_2$	10 mM	1 ml
Dimethyl Formamide	20%	20 ml
H2O		77 ml

Aliquot and store in -20C

Order List

- Eppendorf 2 ml Lo-Bind tubes (Sigma, Z666556-250EA)
- Iodixanol (aka Optiprep, Sigma, D1556-250ML)
- Sucrose (Sigma, S0389)
- 100 um CellTrics (Sysmex, 04-004-2328)
- Trypan Blue (Thermo, 15250061)
- NP40 (Roche/Sigma, 11332473001)
- EDTA (Ambion/Thermo, AM9260G)
- β-mercaptoethanol (Sigma, M3148-100ML)
- CaCl2 (Sigma, 21115-100ML)

- Mg(Ac)2 (Boston BioProducts, MT-190)
- Tris pH 7.8 (Sigma, T2569-1L)
- cOmplete protease inhibitor (Sigma/Roche, 11836170001)
- Tris pH 7.4 (Boston BioProducts, BBT-74)
- NaCl (Invitrogen/Thermo, AM9759)
- MgCl2 (Ambion/Thermo, AM9530G)
- Digitonin (Promega, G9441)
- Tween-20 (Sigma/Roche, 11332465001)
  Our lab has Sigma-P9416-100ML (100%) which needs to be diluted to 10%
- PBS (Corning, 21-031-CM)
- Tris pH 7.6 (Boston BioProducts, BBT-76)
- Dimethyl Formamide (Sigma, D4551-250ML)
- H2O (Corning, 46-000-CM)
- 2 ml Dounce Tissue Grinder Set (Sigma, D8938-1SET)
- 250 ml, 0.2 um PES filters (VWR, 10040-464)
- Tagment DNA Enzyme 1 (Illumina, FC-121-1030)
- 2x NEBNext Master Mix (NEB, M0541)
- DNA Clean and Concentrator-5 Kit (Zymo, D4014)
- Ampure XP beads (Beckman Coulter, A63880)
- Ethanol (Sigma, E7023-500ml)
- Qubit dsDNA HS Assay Kit (Thermo, Q32851)
- 4200 TapeStation High Sensitivity D1000 ScreenTape (Agilent, 5067-5584)
- 4200 TapeStation High Sensitivity D1000 Reagents (Agilent, 5067-5583)