**Before you start the protocol:**

1. All steps should be performed on ice or at 4°C. Pre-chill a swinging bucket centrifuge and a fixed angle centrifuge to 4°C.
2. Pre-chill all Dounces and pestles to 4°C in a fridge.
3. Pre-chill all tubes. Tubes should already be labeled as per above. This should include:
   1. One 2 ml round-bottom LoBind tube for gradient separation, hand-labeled with Sample ID
   2. One 1.5 ml LoBind tube for RNA homogenate with printed cryolabel, hand-labeled on top with Box-Location such as A601-A1 plus the Sample ID such as #1
   3. One 2 ml Nunc Cryotube for extra nuclei with printed cryolabel
   4. One 50 ml conical for filtration step, unlabeled but pre-chilled
4. Prepare all buffers. For faster dissolution, crush protease inhibitor tablets prior to addition to 1x Homogenization Buffer Unstable Solution. (this solution has to be made first and given enough time for the protease to completely dissolved) DTT, Spermidine, and Spermine are stored at -20°C. All detergents, ATAC-RSB, and other buffers are stored at 4°C. Do not prepare transposition mix ahead of time.
5. Fill up a 2 L beaker with 500 ml Milli-Q water to soak the used Dounces and pestles.

**Isolation of Nuclei via Dounce Homogenization and Density Gradient Centrifugation:**

1. Remove samples from LN2 storage if using frozen tissues or cells
2. Place 20 mg frozen tissue into a pre-chilled 2 ml Dounce with 1 ml cold 1x HB and let thaw for 3-5 minutes -> 5 min.
   1. For >30 mg tissue, use 2 ml 1x HB. For 10-20 mg tissue, use 1 ml 1x HB. For 50 um tissue sections, use 0.5 ml 1x HB.
3. If you would like to collect RNA from the same sample, add 10 ul RiboLock per ml of 1x HB and mix well.
4. Dounce with “A” loose pestle until resistance goes away (~10 strokes).
5. Place “A” pestle into beaker with Milli-Q water to soak for cleaning later.
6. Dounce with “B” tight pestle for 20 strokes.
7. Place “B” pestle into beaker with Milli-Q water to soak for cleaning later.
8. Filter during transfer using a Flowmi strainer and transfer homogenate to a pre-chilled 2 ml LoBind tube.
9. Place Dounce into beaker with Milli-Q water to soak for cleaning later.
10. Pellet nuclei by spinning 5 min at 4°C at 350 RCF
11. Remove all but 50 ul of supernatant containing cytoplasmic RNAs and transfer to a pre-chilled and pre-labeled 1.5 ml LoBind tube. If the pellete is not clearly seen, you can leave more supernatant in the tube, up to 400 ul and add less amount of 1X HB buffer
12. Gently resuspend nuclei in 350 ul 1x HB. Make sure nuclei are fully resuspended without clumps.
13. Add 1 volume (400 ul) of 50% Iodixanol Solution and mix well by pipetting
14. Slowly layer 600 ul of 30% Iodixanol solution under the 25% mixture. Avoid mixing of layers by wiping the side of the pipette tip with a Kimwipe to remove Iodixanol solutions from the external surfaces of the pipette tip.
15. Layer 600 ul of 40% Iodixanol solution under the 30% mixture. Avoid mixing of layers by wiping the side of the pipette tip with a Kimwipe to remove Iodixanol solutions from the external surfaces of the pipette tip.
    1. During this step, you will need to gradually draw your pipette tip up to avoid overflowing the tube.
16. In a pre-chilled swinging bucket centrifuge, spin for 20 min at 4°C at 3,000 g with the brake off. Handle tubes gently so as to not disturb the gradient.
17. Using a vacuum, aspirate the top layers down to within 200-300 ul of the nuclei band at the 30%-40% interface.
18. Using a 200 ul volume, collect the nuclei band and transfer to a fresh tube. Do not aspirate more than 200 ul at this step.
19. If necessary, dilute nuclei by adding up to 1 ml of RSB-T Buffer and mix gently by pipetting

**Transposition of Nuclei:**

1. Count nuclei using Trypan blue staining (1:1 ration of Trypan to sample)
2. Label and chill 1.5 ml LoBind tubes according to how many will be needed for transpositions. All samples should be transposed in technical replicate assuming sufficient nuclei are available.
3. Transfer 50,000 nuclei into a 1.5 ml tube containing 500-1000 ul of RSB-T Buffer
4. Centrifuge nuclei for 10 minutes at 500 RCF at 4°C. At this point, the pellet should be visible if 50,000 nuclei were used.
5. Using a p1000 pipette, remove all but the last 100 ul of supernatant. Remove last 100 ul with p200 pipette.
6. Add 50 ul ATAC-seq Reaction Mix to each tube and pipette up and down 6 times to resuspend nuclei pellet.
7. Incubate reactions at 37C for 30 min in a thermoshaker with 1000 rpm constant shaking.
8. Add 250 ul of Binding Buffer from the Zymo DNA Clean and Concentrator 5 kit. Mix well by vortexing and inverting.
9. Pulse centrifuge to collect volume in the bottom of the tube.
10. Transfer to -20°C for short term storage.

**Cleanup and Freezing Down Tubes:**

1. Pellet remaining nuclei by centrifugation for 10 min at 500 RCF at 4°C
2. During spin, write the number of nuclei on the pre-printed label
3. Carefully aspirate supernatant using two pipetting steps.
4. Gently resuspend pellet in 100 ul of cold BAM Banker media and transfer to a pre-chilled 2 ml Nunc cryovial.
5. Slow-freeze nuclei in a freezing container
6. Move homogenate to -80°C storage.
7. Clean all Dounces and pestles with Milli-Q water followed by 70% ethanol.
8. Once dry, replace the kimwipes in the plastic boxes to avoid mold and return the Dounces and pestles. Place back at 4°C.
9. **The next day, once the nuclei have frozen, move them to permanent storage at -80°C.**

**Stock Buffers**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| ***1.034x Homogenization Buffer Unstable Solution*** | | For 200 ml stock solution | | 200000 |
| *Stock* | *Name* | *Final Conc.* | *Fold Dilution (x)* | *Total Volume (ul)* |
| 1 | M Sucrose | 0.26 | 3.87 | 51706.50 |
| 2 | M KCl | 0.03 | 77.36 | 2585.33 |
| 1 | M MgCl2 | 0.01 | 193.40 | 1034.13 |
| 0.75 | M Tricine-KOH pH 7.8 | 0.02 | 36.26 | 5515.36 |
| - | Water | - | - | 139158.69 |
|  |  |  | Total Volume (ul) | 200000.00 |
|  |  |  |  |  |
| ***Diluent Buffer*** | | For 100 ml stock solution | | 100000 |
| *Stock* | *Name* | *Final Conc.* | *Fold Dilution (x)* | *Total Volume (ul)* |
| 2 | M KCl | 0.15 | 13.33 | 7500.00 |
| 1 | M MgCl2 | 0.03 | 33.33 | 3000.00 |
| 0.75 | M Tricine-KOH, pH 7.8 | 0.12 | 6.25 | 16000.00 |
| - | Water | - | - | 73500.00 |
|  |  |  | Total Volume (ul) | 100000.00 |
|  |  |  |  |  |
| ***50% Iodixanol Solution*** | | For 50 ml stock solution | | 50000 |
| *Stock* | *Name* | *Final Conc.* | *Fold Dilution (x)* | *Total Volume (ul)* |
| - | Diluent Buffer | 1 | - | 8333.33 |
| 60 | % Iodixanol | 50 | 1.20 | 41666.67 |
| \*\*Remake monthly for stability | |  | Total Volume (ul) | 50000.00 |
|  |  |  |  |  |
| ***ATAC-RSB Buffer*** | | For 500 ml stock solution | | 500000 |
| *Stock* | *Name* | *Final Conc.* | *Fold Dilution (x)* | *Total Volume (ul)* |
| 1 | M Tris-HCl pH 7.5 | 0.01 | 100.00 | 5000.00 |
| 5 | M NaCl | 0.01 | 500.00 | 1000.00 |
| 1 | M MgCl2 | 0.003 | 333.33 | 1500.00 |
| - | Water | - | - | 492500.00 |
|  |  |  | Total Volume (ul) | 500000.00 |

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| ***1M Sucrose*** | | For 300 ml stock solution | | 300000 |
| *Stock* | *Name* | *Final Conc.* | *Fold Dilution (x)* | *Total* |
| - | g Sucrose (Powder) | 1000 | - | 102.69 g |
|  | H2O |  |  | 235.5 ml |

**Order List**

|  |  |  |
| --- | --- | --- |
| **Item** | **Supplier** | **Cat Number** |
| Eppendorf 2 ml Lo-Bind tubes | Sigma | Z666556-250EA |
| Iodixanol | Sigma | D1556-250ML |
| Sucrose | Sigma | S7903-250G |
| NP40 | Roche (Sigma) | 11332473001 |
| Tricine | Sigma | T0377-25G |
| Potassium Hydroxide (KOH) | Sigma | P5958-250G |
| cOmplete Protease Inhibitors | Roche | 11697498001 |
| MgCl2 | Ambion (Thermo) | AM9530G |
| KCl | Ambion (Thermo) | AM9640G |
| DTT | Thermo | R0861 |
| Spermidine | Sigma | S2501 |
| Spermine | Sigma | S3256-1G |
| Flowmi cell strainers | Fisher | 03-421-228 |
| Tris-HCl pH 7.5 | Invitrogen | 15567-027 |
| NaCl | Ambion (Thermo) | AM9759 |
| Tween 20 | Roche (Sigma) | 11332465001 |
| H2O | Invitrogen | 10977-015 |
| Dounce Tissue Grinder Set | Sigma | D8938-1SET |
| RiboLock | Thermo | EO0384 |