**Protocol for nuclear isolation from rodent brain tissue**

**(with option for subsequent RNA isolation)**

Reagents List:

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| DTT | Sigma D0632 |
| Spermine tetrahydrochoride | Sigma S1141 |
| Sermidine trihydrochloride | Sigma S2501 |
| IGEPAL CA-630 | Sigma I8896 |
| OptiPrep Density Gradient Medium (60% Iodixanol) | Sigma D1556 |
| Cell Strainer (40µm, blue) | BD Falcon 352340 |
| RNasin Plus RNase Inhibitor | Promega N2611 |
| Complete, EDTA-free Protease Inhibitor Cocktail | Sigma, Roche 11873580001 |
| Tricine | Sigma T0377 |
| 7mL Dounce Homogenizer |  |
| Nuclease Free Water (not DEPC-Treated) |  |
| Centrifuge Tubes, DNA LoBind |  |
| Ultrapure BSA |  |
| Sucrose (filtered) |  |

In the days before:

Order all reagents and prepare all stock solutions / stock buffers:

* 1M DTT (1000X) (154mg/mL H2O)
* 0.15M spermine (1000X) (52mg/mL H2O)
* 0.5M spermidine (1000X) (127mg/mL H2O)
* Buffer HB: 0.25M sucrose + 25mM KCl + 5mM MgCl2 + 20mM Tricine-KOH, pH to 7.8. (Store in 5mL aliquots at -20ºC)
* Diluent: 150mM KCl + 30mM MgCl2 + 120mM Tricine-KOH, pH to 7.8. (Store in 1mL aliquots at -20ºC)
* 5% IGEPAL CA-630: 0.95mL Buffer HB + 50µL IGEPAL CA-630

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| **Buffer HB:** | | **Diluent:** | |
| **Stock (100mL)** | **Volume** | **Stock 10mL** | **Volume** |
| 0.5M sucrose (171g/L) | 50mL |  |  |
| 1M KCl | 2.5mL | 1M KCl | 1.5mL |
| 1M MgCl2 | 0.5mL | 1M MgCl2 | 0.3mL |
| 0.75M Tricine-KOH, pH 7.8 | 2.66mL | 0.75M Tricine-KOH, pH 7.8 | 1.6mL |
| RNase clean H2O | ≤ 100 mL | RNase clean H2O | ≤ 10mL |

Day-of Preparation:

1. Turn on the centrifuge and fast-temp to 4ºC. Reduce the break to level 1 or OFF. Reduce the ramp up speed to level 5.
2. Make the following solutions and subsequently maintain on ice. The following volumes prepare enough for two samples:
   1. 3mL working solution (50% Iodixanol):
      * 2.5mL Optiprep
      * 0.5mL Diluent
      * 3.6µL 35% ultrapure BSA
      * 4.8µL RNAsin
   2. 3mL Complete Buffer HB
      * 3mL Buffer HB
      * 4.5µL RNAsin
      * 3.6µL 35% ultrapure BSA
      * 3µL spermine (of 1000X stock solution)
      * 3µL spermidine (of 1000X stock solution)
      * 3µL DTT (of 1000X stock solution)
      * 0.3 Protease tablet
   3. Gradient solutions
      * 0.75mL 30% Iodixanol:   
        0.45mL Working solution + 0.3mL Complete Buffer HB
      * 0.75mL 40% Iodixanol:  
        0.6mL Working solution + 0.15mL Complete Buffer HB

Nuclei Isolation Protocol:

1. Make all working solutions and maintain on ice. Do not proceed until solutions are prepared and chilled.
2. Take frozen tissue punches from -80ºC storage.
3. Transfer the brain region with a p1000 pipette with 200µL Complete Buffer HB and add to a clean Dounce with 300uL Complete Buffer HB (on ice); the total liquid volume should be 500uL after transfer.
4. Homogenize the tissue with a tight pestle (B) 10 times.
5. Add 16µL 5% IGEPAL CA-630 to the Dounce and homogenize 5 more times.
6. Filter the homogenate through a mini 40µm strainer into a 2mL conical tube.
7. Add 0.5mL Working solution to homogenate and mix by pipetting. This should result in a 25% Iodixanol solution with sample.
8. Add 100µL 40% Iodixanol to the bottom of a 1.5mL DNA LoBind tube. Mark the top of this volume on the outside of the tube with a permanent marker. Carefully layer 100µL 30% Iodixanol on top of the 40% Iodixanol layer.
9. Gently mix the 25% Iodixanol solution with sample by inversion and add the 1mL volume by drop pipetting along the side of the tube to create a layer on top of the 30% Iodixanol.
10. Carefully transfer the samples to a swinging-bucket centrifuge and spin at 4500 rcf (g) for 40-mintues at 4ºC. The ramp up speed should be moderate (level 5) and the slow down speed should be very slow (level 1) or OFF (level 0).
11. Retrieve the samples and carefully transfer them on ice to the bench. Collect 60µL of the sample band (“@ layer”) and the 30%-40% Iodixanol interface (marked by permanent marker) and transfer into an Eppendorf tube. Repeat this, if desired, to collect 60µL volumes from immediately above and below the initial aliquot.
12. Gently mix the sample by pipetting and mix a 6µL aliquot with 6µL Trypan blue in a new 200mL tube. Add 10µL of this 1:1 mixture to a hemocytometer and visualize on a brightfield microscope at 10X to calculate nuclei concentration.
    1. Count nuclei in all four corners
    2. Average the four counts
    3. Multiply the average by 2 (to account for the 1:1 dilution with Trypan blue)
    4. Multiply by 10,000 to get the number of nuclei per mL.
13. Transfer sample to genomics core for 10X Genomics protocol OR move on to homogenization and RNA extraction. If the latter, samples can be frozen at -80ºC.

**Date:**

**Samples:** #1. #2. #3. #4.

Time Nuclei Isolation Start: Time Submitted to Core:

**Individual Sample Information:**

**#1: Species:**

**Sample Concentration**: nuclei / mL (or) nuclei / µL

**Volume provided to Core**: µL **Volume to reach 20,000 nuclei**: µL

**Sample Description**:

**Sample Notes:**

**#2: Species:**

**Sample Concentration**: nuclei / mL (or) nuclei / µL

**Volume provided to Core**: µL **Volume to reach 20,000 nuclei**: µL

**Sample Description**:

**Sample Notes:**

**#3: Species:**

**Sample Concentration**: nuclei / mL (or) nuclei / µL

**Volume provided to Core**: µL **Volume to reach 20,000 nuclei**: µL

**Sample Description**:

**Sample Notes:**

**#4: Species:**

**Sample Concentration**: nuclei / mL (or) nuclei / µL

**Volume provided to Core**: µL **Volume to reach 20,000 nuclei**: µL

**Sample Description**:

**Sample Notes:**

**Sample:**

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A \_\_\_\_\_\_ + \_\_\_\_\_\_ + \_\_\_\_\_\_ + \_\_\_\_\_\_ = \_\_\_\_\_\_

B \_\_\_\_\_\_ /4 = \_\_\_\_\_\_

C \_\_\_\_\_\_ \* 2 = \_\_\_\_\_\_

D \_\_\_\_\_\_ \* 10,000 = \_\_\_\_\_\_ nuclei / mL

E \_\_\_\_\_\_ \* 0.001 = \_\_\_\_\_\_\_ nuclei / µL

F 20,000 nuclei ÷ \_\_\_\_\_\_\_ nuclei / µL = \_\_\_\_\_\_\_ µL

**Sample:**

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A \_\_\_\_\_\_ + \_\_\_\_\_\_ + \_\_\_\_\_\_ + \_\_\_\_\_\_ = \_\_\_\_\_\_

B \_\_\_\_\_\_ /4 = \_\_\_\_\_\_

C \_\_\_\_\_\_ \* 2 = \_\_\_\_\_\_

D \_\_\_\_\_\_ \* 10,000 = \_\_\_\_\_\_ nuclei / mL

E \_\_\_\_\_\_ \* 0.001 = \_\_\_\_\_\_\_ nuclei / µL

F 20,000 nuclei ÷ \_\_\_\_\_\_\_ nuclei / µL = \_\_\_\_\_\_\_ µL

**Sample:**

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B \_\_\_\_\_\_ /4 = \_\_\_\_\_\_

C \_\_\_\_\_\_ \* 2 = \_\_\_\_\_\_

D \_\_\_\_\_\_ \* 10,000 = \_\_\_\_\_\_ nuclei / mL

E \_\_\_\_\_\_ \* 0.001 = \_\_\_\_\_\_\_ nuclei / µL

F 20,000 nuclei ÷ \_\_\_\_\_\_\_ nuclei / µL = \_\_\_\_\_\_\_ µL

**Sample:**

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A \_\_\_\_\_\_ + \_\_\_\_\_\_ + \_\_\_\_\_\_ + \_\_\_\_\_\_ = \_\_\_\_\_\_

B \_\_\_\_\_\_ /4 = \_\_\_\_\_\_

C \_\_\_\_\_\_ \* 2 = \_\_\_\_\_\_

D \_\_\_\_\_\_ \* 10,000 = \_\_\_\_\_\_ nuclei / mL

E \_\_\_\_\_\_ \* 0.001 = \_\_\_\_\_\_\_ nuclei / µL

F 20,000 nuclei ÷ \_\_\_\_\_\_\_ nuclei / µL = \_\_\_\_\_\_\_ µL

**Optional: RNA isolation from isolated nuclei**

[modified from the Quick-Start Protocol for Qiagen RNeasy Micro Kit]

Required reagents and equipment:

* Trizol
* Chloroform
* EtOH (70% and 80% solutions in ultrapure water)
* Ultrapure (RNase-free) water
* Qiagen RNeasy Micro Kit and contents
* RNase Away or 10% Bleach
* Fumehood
* Benchtop centrifuge (in fume hood) with max speed ≥ 8000 rcf (g)

1. Add 200µL Trizol to remaining nuclei (~54µL) and homogenize.
2. Add an additional 500µL Trizol in each sample.
3. Let samples sit at RT for 5 minutes.
4. Add 140µL chloroform and shake vigorously for 30 seconds.
5. Let samples sit at RT for 3 minutes.
6. Spin samples at 4ºC at 12,000 rcf (g) for 15 minutes.
7. Remove the upper layer to new tubes.
8. Add 1 volume of 70% EtOH to the lysate and mix well by pipetting. Do not centrifuge. Proceed immediately to the next step.
9. Transfer the sample, with any precipitate, to an RNeasy MinElute spin column in a 2 mL collection tube (supplied). Close the lid and centrifuge for 15 seconds at ≥ 8000 x rcf (g). Discard the flow-through.
10. Add 350µL Buffer RW1 to the RNeasy MinElute spin column. Close the lid. Centrifuge for 15 seconds at ≥ 8000 x rcf (g). Discard the flowthrough.
11. Add 10µL DNase I stock solution to 70µL Buffer RDD. Mix by inverting the tube. Add the DNase I incubation mix (80µL) directly to the RNeasy MinElute spin column membrane. Place on the benchtop (20-30ºC) for 15 minutes. Add 350 µL Buffer RW1 to the RNeasy MinElute spin column. Close the lid, and centrifuge for 15 seconds a ≥ 8000 x rcf (g). Discard the collection tube.
12. Place the RNeasy MinElute spin column in a new 2 mL collection tube (supplied). Add 500 µL Buffer RPE to the spin column. Close the lid, and centrifuge for 15 seconds at ≥ 8000 x rcf (g). Discard the flow-through.
13. Add 500 µL of 80% EtOH to the RNeasy MinElute spin column. Close the lid, and centrifuge for 2 minutes at ≥ 8000 x rcf (g). Discard the collection tube.
14. Place the RNeasy MinElute spin column in a new 2 mL collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 minutes to dry the membrane. Discard the flow-through and collection tube.
15. Place the RNeasy MinElute spin column in a new 1.5 mL collection tube (supplied). Add 14µL RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 minute at full speed to elute the RNA.