# Sample preparation for single nuclei sequencing of brain

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Feb 26, 2019

Working

dx.doi.org/10.17504/protocols.io.v44e8yw

Human Protein Atlas

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ABSTRACT

Sample preparation for single nuclei sequencing using fresh frozen tissue from human postmortem brain. The protocol is a combination of an already existing and published protocol in Nature protocols *Using single nuclei for RNA-seq to capture the transcriptome of postmortem neurons* and the 10x Genomics handbook *Sample preparation, isolation of nuclei for single nuclei RNA sequencing*.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Krishnaswami SR, Grindberg RV, Novotny M, Venepally P, Lacar B, Bhutani K, Linker SB, Pham S, Erwin JA, Miller JA, Hodge R, McCarthy JK, Kelder M, McCorrison J, Aevermann BD, Fuertes FD, Scheuermann RH, Lee J, Lein ES, Schork N, McConnell MJ, Gage FH, Lasken RS. Using single nuclei for RNA-seq to capture the transcriptome of postmortem neurons. Nat Protoc. 2016 Mar;11(3):499-524. doi: 10.1038/nprot.2016.015. Epub 2016 Feb 18. https://www.ncbi.nlm.nih.gov/pubmed/26890679

PROTOCOL STATUS

**Working**

We use this protocol in our group and it worked.

MATERIALS

| NAME | CATALOG # | VENDOR |
|---|---|---|
| Optiprep ( Iodixanol) | D1556-250ML | Sigma Aldrich |
| Sucrose | S0389 | Sigma Aldrich |
| RNaseZap® RNAse Decontamination Solution | AM9780 | Life Technologies |
| Triton X-100 | T8787 | Sigma Aldrich |
| Protector RNase Inhibitor | 3335402001 | Sigma – Aldrich |
| LS Columns | 130-042-401 | Miltenyi Biotec |
| Buffer Kit RNase-free | AM9010 | Thermo Fisher Scientific |
| Nuclease-Free Water | AM9939 | Thermo Fisher Scientific |
| Dithiothreitol (DTT) | 707265ML | Thermo Fisher Scientific |
| cOmplete™ EDTA-free Protease Inhibitor Cocktail | 11873580001 | Sigma Aldrich |
| Myelin Removal Beads II human mouse rat | 130-096-733 | Miltenyi Biotec |
| Ultra pure BSA (50 mg/mL) | cat# AM2616 | Thermo Fisher Scientific |

| NAME | CATALOG # | VENDOR |
|---|---|---|
| SUPERase• In™ RNase Inhibitor (20 U/µL) | cat# AM2694 | Thermo Fisher Scientific |
| PBS 1x without calcium & magnesium | Cat# 21-040-CVR | VWR International |

MATERIALS TEXT

Buffers:

### Nuclei isolation medium 1 (NIM1)

| Component | Volume (µl) | Final concentration (mM) |
|---|---|---|
| 1.5 M sucrose | 2500 | 250 |
| 1 M KCl | 375 | 25 |
| 1 M MgCl2 | 75 | 5 |
| 1 M Tris buffer | 150 | 10 |
| Nuclease-free water | 11900 | - |
| Total volume | 15000 | - |

Important! This buffer should be prepared in advance, it can be stored in 50-ml tube at 4C for up to 6 months.

### Nuclei isolation medium 2 (NIM2)

| Component | Volume (µl) | Final concentration (mM) |
|---|---|---|
| NIM1 | 4895 | |
| 1 mM DTT | 5 | 1 µM |
| 50x protease inhibitor | 100 | 1x |
| Total volume | 5000 | |

Important! This buffer should be prepared fresh and placed at 4C or on ice for use. Afterwards, it should be discarded

### Homogenization buffer

| Component | Volume (µl) | Final concentration (mM) |
|---|---|---|
| NIM2 | 1455 | 1 x |
| RNaseIn 40 U µl−1 | 15 | 0.4 U µl−1 |
| Superasin 20 U µl−1 | 15 | 0.2 U µl−1 |
| Triton X-100 10% (v/v) | 15 | 0.1% (v/v) |
| Total volume | 1500 | |

Important! This buffer should be prepared fresh, protected from the light and placed at 4C or on ice for use. Afterwards, it should be discarded.

### Nuclei Wash and Resuspension Buffer

1x PBS
1% BSA
0.2 U ul-1 Rnase inhibitor

### LS Column Calibration Buffer

1x PBS
0.5% BSA

### Iodixanol medium (IDM)

| Component | 1× volume (µl) | Final concentration (mM) |
|---|---|---|
| 1.5 M sucrose | 2500 | 250 |

| | | |
|---|---|---|
| 1 M KCl | 2250 | 150 |
| 1 M MgCl2 | 450 | 30 |
| 1 M Tris buffer, pH 8.0 | 900 | 60 |
| Nuclease-free water | 8900 | - |
| Total volume | 15000 | |

The medium can be stored at 4C for up to 6 months.

### Iodixanol dilutions

| Component | 1× volume (µl) | Final concentration |
|---|---|---|
| Iodixanol 60% (vol/vol) | 12500 | 50% vol/vol |
| IDM | 2500 | - |
| Total volume | 15000 | |
| Component | 1× volume (µl) | Final concentration |
| Iodixanol 60% | 7250 | 29% vol/vol |
| IDM | 7750 | - |
| Total volume | 15000 | |

The reagents can be stored at 4C for up to 6 months.

SAFETY WARNINGS

Regulations about working with tissue samples may vary between institutions, it is important to be aware about the guidelines before to start any experiment.

BEFORE STARTING

- Run Bioanalyser to check RNA integrity number (RIN) and evaluate the quality of the tissue.
- Clean workstation, pipettes and tools with RNaseZap solution before to start the experiment.
- Precool the Dounce homogenizer and pestles on ice. Once they are cooled, fill the homogenizer with 1ml of cold homogenization buffer and keep it on ice.

## Nuclei isolation

1 Place a Petri dish on dry ice and transfer the tissue there, cut it using a chilled scalpel. Transfer the tissue into the precooled Dounce homogenizer and keep the homogenizer on ice during the rest of the protocol ( to reduce heat caused by friction).

Dounce Tissue Grinder Set with Two Glass Pestles

**Wheaton cat# 357538**

2 Homogenize the tissue with five strokes of the loose pestle, followed by 10–15 strokes of the tight pestle.

3 Filter the homogenate through a BD Falcon tube with a cell strainer cap.

Falcon™ Test Tube with Cell Strainer Snap Cap

Corning cat# 352235

4 Transfer the volume to a chilled 1.5 ml eppendorf and centrifuge at 1000 rcf for 00:08:00 at 4 °C .

5 Remove the supernatant without disrupting the nuclei pellet.

## Myelin removal

6 Add 1800 µl nuclei wash and resuspension buffer to the pelleted nuclei and, using a regular-bore pipette tip, gently pipette mix 8-10 times.

7 Add 200 µl Myelin Removal Beads II to the resuspended nuclei. Mix with a wide-bore pipette tip (no vortex). Incubate for 00:15:00 at 4 °C .

8 Meanwhile, place LS columns in the magnetic field of a MACS separator each with 3 ml LS Column Calibration Buffer to rinse the columns.

QuadroMACS Separator

MACS Separator - magnetic field

Miltenyi biotec cat# 130-090-976

MACS MultiStand

Miltenyi Biotec cat# 130-042-303

9 Using a 10 ml serological pipette, dilute the nuclei suspension with 10 ml Nuclei Wash and Resuspension buffer, gently pipette mix 5 times.

10 Centrifuge the nuclei at 500 rcf for **00:10:00** at **4 °C** .

11 Remove the supernatant without disrupting the nuclei pellet and add **2 ml** Nuclei Wash and Resuspension buffer.

12 Apply **1 ml** nuclei suspension in each column.

13 Wash each of the columns with **1 ml** Nuclei Wash and Resuspension buffer **go to step #13 once more.**

14 Collect the effluent into two 5ml Eppendorf tubes.

15 Centrifuge the two vials with nuclei at 500 rcf for **00:05:00** at **4 °C** .

16 Remove the supernatant from the two vials without disrupting the nuclei pellet and add **500 µl** Nuclei Wash and Resuspension buffer.

17 Pool both resuspended nuclei in a new pre-chilled 1.5 ml Eppendorf.

## Density gradient centrifugation

18 Centrifuge at 1000 rcf for **00:08:00** at **4 °C** .

19 Meanwhile, prepare the iodixanol dilution mix (IDM) and iodixanol dilutions to place them on ice.

20 Aspirate the supernatant (~1,000 µl) and gently resuspend the pellet in 250 µl of homogenization buffer.

21 Strain the mixture through a BD Falcon tube with a cell strainer cap to remove any remaining aggregates, and place it on ice.

22 Gently mix the nuclei with 250 µl of 50% (vol/vol) iodixanol, and, to a new Eppendorf tube, add 500 µl of 29% iodixanol. Slowly layer 500 µl of the nuclei mixture over the 29% iodixanol.

23 Centrifuge at 13.500 rcf for **00:20:00** at **4 °C** .

24 Remove and discard the supernatant without disrupting the nuclei pellet.

25 Resuspend the final pellet in 60 µl Nuclei Wash and Resuspension buffer. The sample is ready for QC-nuclei counting.