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Sample preparation for single nuclei sequencing of brain

Anna Martinez Casals^{1,2}, Nicholas Mitsios³

¹Science for life laboratory; ²KTH - Royal Institute of Technology;

³Karolinska Institute Stockholm

Human Protein Atlas

Tech. support email: contact@proteinatlas.org

Anna Martinez Casals

Science for life laboratory ; KTH - Royal Institute of Techn...

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*.

MATERIALS

MATERIALS

- ⊗ Optiprep (Iodixanol) **Sigma Aldrich Catalog #D1556-250ML**
- ⊗ Sucrose **Sigma Aldrich Catalog #S0389**
- ⊗ RNaseZap® RNase Decontamination Solution **Life Technologies Catalog #AM9780**
- ⊗ Triton X-100 **Sigma Aldrich Catalog #T8787**
- ⊗ Protector RNase Inhibitor **Sigma – Aldrich Catalog #3335402001**
- ⊗ LS Columns **Miltenyi Biotec Catalog #130-042-401**
- ⊗ Buffer Kit RNase-free **Thermo Fisher Scientific Catalog # AM9010**
- ⊗ Nuclease-Free Water **Thermo Fisher Scientific Catalog # AM9939**
- ⊗ Dithiothreitol (DTT) **Thermo Fisher Scientific Catalog #707265ML**
- ⊗ cOmplete™ EDTA-free Protease Inhibitor Cocktail **Sigma Aldrich Catalog #11873580001**

Protocol status: Working
We use this protocol in our group and it worked.

Created: Dec 04, 2018

Last Modified: Feb 26, 2019

PROTOCOL integer ID:
18300

Myelin Removal Beads II human mouse rat **Miltenyi Biotec Catalog #130-096-733**

Ultra pure BSA (50 mg/mL) **Thermo Fisher Scientific Catalog #cat# AM2616**

SUPERase• In™ RNase Inhibitor (20 U/μL) **Thermo Fisher Scientific Catalog #cat# AM2694**

PBS 1x without calcium & magnesium **VWR International Catalog #Cat# 21-040-CVR**

STEP MATERIALS

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 MgCl ₂	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
Supersasin 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 4°C or on ice for use. Afterwards, it should be discarded.

Nuclei Wash and Resuspension Buffer

1x PBS
1% BSA
0.2 U μl⁻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 MgCl ₂	450	30
1 M Tris buffer, pH 8.0	900	60
Nuclease-free water	8900	-
Total volume	15000	

The medium can be stored at 4°C 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 START INSTRUCTIONS

- 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).

Equipment

Dounce Tissue Grinder Set with Two Glass Pestles

NAME

Wheaton

BRAND

cat# 357538

SKU

<https://us.vwr.com/store/product/4832641/dounce-tissue-grinders-wheaton>

LINK

- 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.

Equipment

Falcon™ Test Tube with Cell Strainer Snap Cap

NAME

Corning

BRAND

cat# 352235

SKU

<https://www.fishersci.com/shop/products/falcon-tube-cell-strainer-cap-mesh-size-35um/0877123>

LINK

- 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.

Equipment

QuadroMACS Separator

NAME

MACS Separator - magnetic field

TYPE

Miltenyi biotec

BRAND

cat# 130-090-976

SKU

<https://www.miltenyibiotec.com/SE-en/products/macs-cell-separation/separators/quadromacstm-separator-and-starting-kits.html>

LINK

Equipment

MACS MultiStand

NAME

Miltenyi Biotec

BRAND

cat# 130-042-303

SKU

<https://www.miltenyibiotec.com/SE-en/products/macs-cell-separation/separators/macs-multistand.html>

LINK

- 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.