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SPLiT-seq Nuclei isolation for Micro-dissected Mouse Brain Tissue V.1

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Protocol status: Working

We use this protocol and it's working

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Abstract

Protocol for nuclei isolation prior to SPLiT-seq library preparation. Specifically optimized for the storage of nuclei from micro-dissected brain regions from mice or human (50-100mg).

Guidelines

Tissue collection for this protocol requires prior approval by the users' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee.

Materials

Dounce Homogenizers:

2mL x 60 mm KIMBLE Dounce tissue grinder set (Sigma-Aldrich, D8938)

Consumables:

DNA LoBind® Tubes, 2.0 mL, PCR clean, colorless (Eppendorf, 022431048)

DNA LoBind® Tubes, 1.5 mL, PCR clean, colorless (Eppendorf, 022341021)

SureStrain™ Premium Cell Strainers, 40 µM (MTC Bio, C4040)

Reagents:

1M CaCl₂ in H₂O (Sigma-Aldrich, 21115)

Mg(Ac)₂ (Sigma-Aldrich, M5661)

0.5M EDTA, pH 8 (Invitrogen Ambion, AM9260G)

1M Tris-HCL, pH 8, Molecular Biology Grade, Ultrapure (ThermoFisher Scientific, J22638)

Triton X-100, laboratory grade (Sigma-Aldrich, X100)

Bovine Serum Albumin Fraction V, 7.5% solution (ThermoFisher Scientific, 15260037)

SUPERase•In™ RNase Inhibitor, 20 U/µL (Invitrogen, AM2696)

RNaseOUT™ Recombinant Ribonuclease Inhibitor, 5,000 units (Invitrogen, 10777019)



Setup

30m

- 1 Chill dounce homogenizers and tubes at 4°C in wet ice bucket

Per sample:

2mL x 60 mm KIMBLE Dounce tissue grinder set
(2) DNA LoBind® Tubes, 1.5 mL

- 2 Prepare stock lysis buffer (can be stored at 4°C for 1 week)

For 15 mL stock:

 1.641 g Sucrose

 75 µL 1M CaCl₂

 45 µL 1M Mg(Ac)₂

 3 µL 0.5M EDTA

 150 µL Tris-HCl, pH 8.0

 15 µL Triton X-100

Fill to  15 mL with UltraPure H₂O (DNase-free, RNase-free)

- 3 Prepare wash & resuspension buffer

For 12 mL:

 1.2 mL Bovine Serum Albumin Fraction V, 7.5% solution

 120 µL SUPERase•In™ RNase Inhibitor, 20 U/µL

 120 µL RNaseOUT™ Recombinant Ribonuclease Inhibitor, 5,000 units

Fill to  12 mL with 1X PBS

Dounce Homogenization

- 4 Add DTT and RNase inhibitors to lysis buffer

5m



Aliquot  1 mL of stock lysis buffer per sample (~100 mg)

Per 1 mL (per sample)

Add **1:1000** 1,4-Dithiothreitol (DTT)

Add  5 μ L SUPERase•In™ RNase Inhibitor, 20 U/uL per mL

Add  5 μ L RNaseOUT™ Recombinant Ribonuclease Inhibitor, 5,000 units

- 5 Add  500 μ L of lysis buffer (+ DTT & RNase inhibitors) to each sample (in a 1.5 mL eppendorf tube or cryovial) 5m
- 6 Incubate for 10 minutes on ice. 10m
 - Incubation time may need to be optimized depending on tissue
- 7 With a wide-pore P1000 barrier pipet tip homogenize the tissue by pipetting up and down (3-5X) until the tissue readily passes through the pipet tip. 5m
- 8 Transfer tissue homogenate to the dounce homogenizer with a wide-pore P1000 barrier pipet tip. 5m
- 9 Wash the sample tube with of lysis buffer (+ DTT & RNase inhibitors) and transfer buffer to the dounce homogenizer. 5m
 - This step ensures optimal retention of semi-homogenized tissue for dounce homogenization
- 10 Dounce 10-30 times with Pestle A 10m
 - The # of dounces should be optimized by tissue
- 11 Dounce 5-10 times with Pestle B 10m
 - The # of dounces should be optimized by tissue

Washing

- 12 Pass  500 μ L at a time through a 40 μ M strainer into a 1.5 mL LoBind Eppendorf tube (pre-chilled) using a wide-pore P1000 barrier pipet tip.

**Note**

When pipetting nuclei suspension through the strainer, make a seal between the pipet tip and the mesh, slowly pipet to pass nuclei through. (add picture)

- 13 Centrifuge at 600 x g for 10 min. @ 4°C.
 - The spin speed can be adjusted between 300 - 800 x g, faster spins risk damage to the nuclear membrane.
- 14 Remove the supernatant and add  500 µL of wash & resuspension buffer. Let the pellet sit for 2 min. on ice prior to re-suspending.
- 15 Gently re-suspend the nuclei pellet with a wide-pore P1000 barrier pipet tip.
- 16 Centrifuge at 600 x g for 5 min. @ 4°C.
- 17 Repeat steps 14-16.  [go to step #14](#)

Note

The number of washes can be optimized by tissue type. Increased washes = less debris, however too many washes will negatively impact the final nuclei yield.

Start with 2-3 washes.

- 18 Remove the supernatant and add  500 µL of wash & resuspension buffer. Let the pellet sit for 2 min. on ice prior to re-suspending.
- 19 Gently re-suspend the nuclei pellet with a wide-pore P1000 barrier pipet tip.
- 20 Pass the resuspended nuclei through a 40 µm Flow cell strainer into a 1.5 mL LoBind Eppendorf tube (pre-chilled).



- 21 Centrifuge at 600 x g for 5 min. @ 4°C.
- 22 Remove the supernatant and add  750 µL of wash & resuspension buffer. Let the pellet sit for 2 min. on ice prior to re-suspending.



Note

At this point you can pause and check the quality of the nuclei under a microscope at 40-60X magnification.

Fixation and Permeabilization

- 23 Prepare solutions for fixation & permeabilization and place on ice:

Fixation Solution:

 750 µL 16% Formaldehyde

 2.25 mL 1X PBS

Permeabilization Solution:

 2 mL 5% Triton X-100

 2 µL SUPERase•In™ RNase Inhibitor, 20 U/uL

Neutralization Buffer:

 500 µL 1M Tris-HCl, pH 8.0

 200 µL 5% Triton X-100

 4.8 mL 1X PBS

- 24 Filter nuclei through a 40 µM strainer into a 1.5 mL LoBind Eppendorf tube (pre-chilled) using a wide-pore P1000 barrier pipet tip to a 15 mL conical tube.

**Note**

This filter step is to ensure that only singlet nuclei are taken into the fixation. If there are a large presence of doublet nuclei in the sample this will lead to a high prevalence of doublets in the downstream dataset.

- 25 Add  250 μL of pre-chilled Fixation Solution and immediately mix gently by pipetting up and down 3X with a wide-pore P1000 barrier pipet tip.

**Note**

Caution should be taken when pipetting nuclei in the fixation solution, too forceful or frequent pipetting will risk shearing of nuclear membrane. Only pipet enough to suspend nuclei in solution (3X recommended)

- 26 Incubate for 10 minutes on ice.
- 27 Add  40 μL of Permeabilization Solution and mix gently by pipetting up and down 3X with a wide-pore P1000 barrier pipet tip.
- 28 Incubate for 3 minutes on ice.
- 29 Add 1 mL cold nuclei neutralization buffer and gently invert the tube 3X to mix
- 30 Centrifuge at 500 x g for 5 min. @ 4°C in the swinging bucket rotator.

Nuclei Storage

- 31 Prepare nuclei storage buffer and place on ice:

Nuclei Storage Buffer:



 6 mL 1X PBS

 15 μ L SUPERase•In™ RNase Inhibitor, 20 U/ μ L

 7.5 μ L RNaseOUT™ Recombinant Ribonuclease Inhibitor, 5,000 units

- 32 Remove the supernatant and add  300 μ L of Nuclei Storage Buffer. Let the pellet sit for 2 min. on ice prior to re-suspending.
- 33 Gently re-suspend the nuclei pellet with a wide-pore P1000 barrier pipet tip.
- 34 Filter nuclei through a 40 μ M strainer into a 1.5 mL LoBind Eppendorf tube (pre-chilled) using a wide-pore P1000 barrier pipet tip.
- 35 Add  5 μ L of DMSO and gently flick the tube 3X, wait 1 minute on ice. Repeat 3X to add a total of  15 μ L DMSO.
- 36 Mix the final suspension 5X with a P200 barrier pipet tip and aliquot for future experiments.
- 37 Place aliquoted nuclei suspensions in a Mr. Frosty to cool at $-1^{\circ}\text{C}/\text{minute}$ in a -80°C freezer overnight.
- 38 Fixed nuclei suspensions can be stored at -80°C for up to 6 months.

