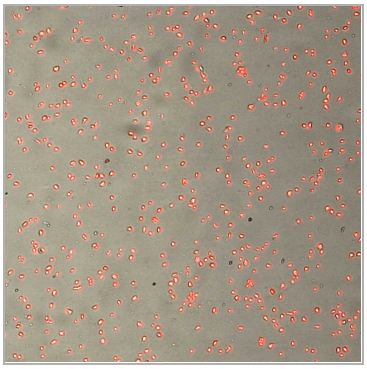

APR 13, 2023



**DOI:**
dx.doi.org/10.17504/protocols.io.yxmvm25xng3p/v1

**Protocol Citation:** Jonathan W Brenton, Carlo Sala Frigerio, Mina Ryten, Regina H Reynolds, e.gustavsson, Kylie Montgomery 2023. Nuclear Isolation of Post-Mortem Brain Tissue for snRNAseq. **protocols.io** https://dx.doi.org/10.17504/protocols.io.yxmvm25xng3p/v1



**Protocol status:** Working
We use this protocol and it's working

**Created:** Jan 30, 2023

**Last Modified:** Apr 13, 2023

**PROTOCOL integer ID:** 76084

**Keywords:** ASAPCRN

# Nuclear Isolation of Post-Mortem Brain Tissue for snRNAseq

Jonathan W Brenton[1,2], Carlo Sala Frigerio, Mina Ryten[1,2], Regina H Reynolds[1,2], e.gustavsson[1,2], Kylie Montgomery[1]

[1]Genetics & Genomic Medicine Dept, UCL GOS Institute of Child Health, 30 Guilford St, London WC1N 1EH;
[2]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD, 20 815, USA

Jonathan W Brenton: jonathan.brenton.14@ucl.ac.uk;
Carlo Sala Frigerio: csalafrigerio@gmail.com
Mina Ryten: mina.ryten@ucl.ac.uk
Regina H Reynolds: regina.reynolds.16@ucl.ac.uk
e.gustavsson: e.gustavsson@ucl.ac.uk
Kylie Montgomery: kylie.montgomery@ucl.ac.uk

Ryten Laboratory

Jonathan W Brenton

## ABSTRACT

This protocol is used to isolate nuclei from frozen brain tissue for single nuclear RNA sequencing using 10x Genomics GEM isolation using the Chromium accessory and Single Cell 3' Reagent Kits.

It is adapted from a protocol designed by Carlo Sala Frigerio.

The nuclei are homogenised by hand, run through a density gradient, resuspended, counted and diluted to an appropriate concentration to run in the Chromium.

This protocol is designed to be continued with the Chromium Single Cell 3' Reagent Kits User Guide (v3.1 Chemistry Dual Index) to isolate nuclei in GEMs.

## MATERIALS

Equipment

| | A | B | C | D | E |
|---|---|---|---|---|---|
| | Manufacturer | Instruments | code | notes | website |

| | A | B | C | D | E |
|---|---|---|---|---|---|
| | Beckman coulter | Optima XPN-90 | | | |
| | Beckman coulter | SW41Ti rotor | | swing-out, 6 slots | |
| | 10X Genomics | Chromium | | Firmware 4.00 at least | |
| | Logos bio | Luna-FL dual fluorescence cell counter | | | https://logosbio.com/automated-cell-counters/fluorescence/luna-fl |
| | Biorad | C1000 Touch with 96-Deep Well Reaction Module | 1851197 | | https://www.bio-rad.com/en-uk/product/c1000-touch-thermal-cycler?ID=LGTW9415 |
| | Any | Scale measuring to 0.01g or 0.001g | | | |

List of Items

| | A | B | C |
|---|---|---|---|
| | Item | Code | Manufacturer |
| | Tissue grinders, Potter-Elvehjem type, glass vessel, 2 mL | 432-0200 | VWR |
| | Tissue grinders, Potter-Elvehjem type, plain plunger, 2 mL | 432-0206 | VWR |
| | Tube, Thinwall, Ultra-Clear™, 13.2 mL, 14 x 89 mm (qty. 50) | 344059 | Beckman Coulter |
| | Eppendorf PCR Tubes, 0.2 mL, PCR clean, 8-tube strips, 120 pcs. (960 tubes) | 0030124359 | Eppendorf |
| | DNA LoBind Tube Microcentrifuge Tube, 0.5 mL | 0030108035 | Eppendorf |
| | DNA LoBind Tube Microcentrifuge Tube, 1.5 mL | 0030108051 | Eppendorf |
| | DNA LoBind Tube Microcentrifuge Tube, 2.0 mL | 0030108078 | Eppendorf |
| | Reagent reservoir, white PS, 50 mL, sterile | 613-1184 | VWR |
| | Falcon® 5 mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap | 352235 | Corning |
| | Protein LoBind Tubes 2mL | 0030108132 | Eppendorf |
| | Protein LoBind Tubes 1.5mL | 0030108116 | Eppendorf |

| | A | B | C |
|---|---|---|---|
| | Nuclease-Free Water (Ambion) | AM9937 | Thermo Fisher |
| | 10% Tween-20 | 166-2404 | Bio-Rad |
| | Low TE Buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA) | 12090-015 | Thermo Fisher |
| | Glycerol 50% | G5516-500ML | Sigma-Aldrich |
| | Reagent, SPRIselect, 60 mL | B23318 | Beckman Coulter |
| | Ethanol, Pure (200 Proof, anhydrous) | 459836-1L | Sigma-Aldrich |
| | Buffer EB | 19086 | Qiagen |
| | | | |
| | Sucrose, BioUltra, for Molecular Biology | 84097-1KG | Sigma-Aldrich |
| | Calcium Chloride | C5670-100G | Sigma-Aldrich |
| | Magnesium acetate tetrahydrate | M5661-50G | Sigma-Aldrich |
| | Ethylenediaminetetraacetic acid solution | 03690-100ML | Sigma-Aldrich |
| | Phenylmethanesulfonyl fluoride | 78830-5G | Sigma-Aldrich |
| | 2-Mercaptoethanol | 63689-25ML-F | Sigma-Aldrich |
| | Magnesium chloride hexahydrate | M2670-500G | Sigma-Aldrich |
| | Potassium chloride | P9333-500G | Sigma-Aldrich |
| | Digitonin 5% | BN2006 | Thermo Fisher/Invi |
| | Nonidet P40 substitute Bioxtra | 74385-1L | Sigma-Aldrich |
| | Trizma HCl solution | T2194-1L | Sigma-Aldrich |
| | OptiPrep™ Density Gradient Medium | D1556-250ML | Sigma-Aldrich |
| | phosphate-buffere saline 1X w/o Ca and Mg | 21-040-CV | Corning Cellgro |
| | RNasin Plus Rnase Inhibitor, 10,000 units | N2615 | Promega |
| | MACS BSA stock solution | 130-091-376 | Miltenyi Biotec GmbH |
| | | | |
| | High Sensitivity D5000 Ladder | 5067-5594 | Agilent |
| | High Sensitivity D5000 ScreenTape | 5067-5592 | Agilent |

| | A | B | C |
|---|---|---|---|
| | High Sensitivity D5000 Reagents | 5067-5593 | Agilent |
| | High Sensitivity D1000 Ladder | 5067-5587 | Agilent |
| | High Sensitivity D1000 Sample Buffer | 5067-5603 | Agilent |
| | High Sensitivity D1000 ScreenTape | 5067-5584 | Agilent |
| | High Sensitivity D1000 Reagents | 5067-5585 | Agilent |
| | LightCycler® 480 Multiwell Plate 384, white | 4729749001 | Roche Lifescience |
| | KAPA library quantification kit for Roche Lightcycler LC480 (KK4854) | 7960298001 | Roche Lifescience |
| | Falcon tubes, 50 mL | | |
| | Falcon tubes, 15 mL | | |
| | | | |
| | Foreceps | | |
| | 70% ethanol | | |

Stock Tables

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| | | g | stock (M) | FW (g/mol) | Volume (L) | |
| | sucrose | 85.575 | 0.5 | 342.3 | 0.5 | |
| | CaCl2 | 8.400 | 0.5 | 110.98 | 0.151378627 | |
| | Mg Acetate*4H2O | 26.806 | 0.5 | 214.45 | 0.25 | |
| | PMSF | 0.120 | 0.1 | 174.19 | 0.006889029 | in 2-propanol, store at 4C |

Everything filter using 0.2 um filters

Beta-Mercaptoethanol

| | A | B | C |
|---|---|---|---|
| | | Dilution | Final Concentration (M) |

| | A | B | C |
|---|---|---|---|
| | 2-Mercaptoethanol | 175 uL in 50 mL | 0.05 |

Glycerol

mix 1:1 99% Glycerol and water, filter with 0.2um filter. Can be aliquoted and stored at -20C (equilibrate to room temperature before use). Or store in a 50mL falcon at room temperature.

## Buffer Making for 6 samples (7x made to allow for waste)

1 Prepare buffers from stocks, keep On ice .

### 1.1 Homogenisation Buffer (HB)

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| | HB (Homogenisation Medium) | V (µl) | Stock conc | | Final conc | |
| | Sucrose | 17920.0 | 500 | mM | 320 | mM |
| | CaCl2 | 140.0 | 1000 | mM | 5 | mM |
| | Mg Acetate | 168.0 | 500 | mM | 3 | mM |
| | Tris pH 7,4 (Sigma) | 280.0 | 1000 | mM | 10 | mM |
| | EDTA | 5.6 | 500 | mM | 0.1 | mM |
| | NP40 | 280.0 | 10 | % | 0.1 | % |

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| | Digitonin | 56.0 | 5 | % | 0.01 | % |
| | PMSF | 56.0 | 50 | mM | 0.1 | mM |
| | β-mercaptoethanol | 560.0 | 50 | mM | 1 | mM |
| | Nuclease free water | 8534.4 | | | | |
| | Total Volume | 28000.0 | 4000 | per sample | | |

Resuspend Digitonin first.

For Digitonin: If a precipitate forms in the 5% Digitonin solution, we performed the recommend steps in the manual. This requires heating the solution at 95 degrees C for 5 mins and vortexing slowly to dissolve the precipitate. Cool to room temperature prior to use. The 5% Digitonin solution stays in solution at room temperature for up to 1 week.Although in practice this tends to precipitate after a day so most likely this will need reheating after a day or two.

## 1.2 Gradient Medium

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| | GM (Gradient Medium) | V (µl) | Stock conc | | Final conc | |
| | CaCl2 | 105 | 1000 | mM | 5 | mM |
| | Optiprep | 17500 | 60 | % | 50 | % |
| | MgCl2 | 63 | 1000 | mM | 3 | mM |
| | Tris pH 7,4 | 210 | 1000 | mM | 10 | mM |
| | PMSF | 42 | 50 | mM | 0.1 | mM |
| | β-mercaptoethanol | 420 | 50 | mM | 1 | mM |
| | Nuclease free water | 2660.0 | | | | |
| | Total Volume | 21000 | 3000 | per sample | | |

1.3 ODN

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| | ODN | V (µl) | Stock conc | | Final conc | |
| | KCl | 1342.6875 | 2000 | mM | 150 | mM |
| | MgCl2 | 537.075 | 1000 | mM | 30 | mM |
| | Tris pH 7,4 (Sigma) | 1074.15 | 1000 | mM | 60 | mM |
| | Sucrose | 8951.25 | 500 | mM | 250 | mM |
| | Nuclease free water | 5997.3375 | | | | |
| | Total Volume | 17902.5 | | | | |

1.4 29% Cushion

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| | 29% Cushion | V (µl) | Stock conc | | Final conc | |
| | Optiprep | 15225 | 60 | % | 29 | % |
| | ODN | 16275 | | | | |
| | Total Volume | 31500 | 4500 | per sample | | |

## Homogenisation

2 Rinse douncers with some HB buffer – leave these On ice too.

3 Add 1 mL HB to douncer.

4 Put douncer on scale in any support (falcon tube holder was used here), zero the scale and add

sample to douncer. Aim for between 80-150mg tissue. ~ 100 mg of tissue works well.

5 Homogenise:~20 strokes with pestle A and then~80 strokes with pestle B; try to keep On ice as much as possible. If meninges present following 10 strokes with pestle B remove with forceps as this can add a lot of resistance. Clean forceps in dH20 water, wipe clean and then spray with 70% ethanol and wipe and air dry.

6 Keep douncer with homogenised sample On ice for  and repeat steps 3-6 for other samples.

7 For each sample transfer ~ 1 mL homogenate (everything in douncer) to 15mL falcon and add 1650 µL HB (total V = 2.65 mL). Mix by inverting as well as pipetting. All On ice.

8 Add and mix 2.65 mL ( 0.883 mL x 3) Gradient medium to sample (total V = 5.30 mL). Mix by inverting gently as well as pipetting. All On ice.

## Gradient Preparation and Isolation by Centrifugation

9 Add 4 mL of optiprep cushion to an ultracentrifuge tube On ice for each sample.

10 Layer 5.25 mL sample on top of cushion. To do this slowly add homogenate/gradient medium mixture just above the top of the optiprep cushion and keeping the pipette tip against the wall of the ultracentrifuge tube. One way to do this is to use a P200 first to layer 200 µL - 400 µL of sample on top of the cushion and the proceed with a P1000 to layer additional mLs of sample. Keep thumb under plunger of pipette to make sure addition of sample is gradual and is not pushed to the bottom of the gradient. Try and keep the bottom of the tube touching the ice as much as possible to keep the tube cool.

11 Check that sample's weight in ultracentrifuge tube is balanced (do in pairs – ie samples facing

each other in ultracentrifuge should be equal in weight), in case adjust weight with HB.

12 Add samples to ultracentrifuge buckets.

13 Spin samples in SW41Ti 7700 rpm 00:30:00 4 °C - keep all tube holders in the machine even if empty and keep positions matched, ie holder 1 is in position 1. The ultracentrifuge should be pre-cooled to 4 °C prior to adding the samples. 30m

## During Ultracentrifugation

14 Take out 10X beads from -80 °C and GEM kit from -20 °C. Prepare Template Switch Oligo as per 10x instructions if needed.

15 Prepare the resuspension buffer:

15.1 RNAseq Resuspension Buffer (for 6 samples + 1 for waste). Prepare and keep on ice.

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| | RNAseq Resuspension Buffer | | | | | |
| | | V (uL) | stock | | final | |
| | 1x PBS ( w/o Ca and Mg) | 9464 | | | | |
| | BSA | 1680 | 10 | % | 1.5 | % |
| | RNase IN (Promega Purple cap) | 56 | 40 | u/uL | 0.2 | u/uL |
| | final volume | 11200 | | | | |

## Resuspension and filtering

16 Gently take away supernatant using plastic Pasteur or P1000, then P200 Gilson. Nuclei will be a white smear at the bottom of the tube, the tube will gain condensation quickly so can wipe it to get a better look at the nuclei at the end and try and hold up the tube to a dark surface to get a look at the nuclei (although not all can be seen). Keep tube steady and try not to mix and shake, which may resuspend the nuclei. Remove as much supernatant as possible by pipetting from the surface of the liquid and following the layer down to the lip of the ultracentrifuge tube. At this point remove as much as possible before nuclei start to come along with supernatant. Usually 200 µL - 500 µL remains at the bottom of tube.

When in doubt leave supernatant as not to decrease yield or keep supernatant in a separate tube and filter as a separate sample that could be combined later in case of low yield. However, the vast majority of nuclei will be present at the bottom of the tube and this typically doesn't add many nuclei.

17 Add in 200 µL of suspension buffer, transfer to chilled 1.5 or 2mL LoBind tube (either protein or DNA) On ice. All further steps should also be done On ice and nuclei/samples should be kept cool as much as possible to prevent nuclei clumping.

18 Wash out ultracentrifuge tube with further 200 µL of suspension buffer, pool in the LoBind tube On ice.

19 Add a further 400 µL of suspension buffer to the LoBind tube On ice (800 µL total suspension buffer).

20 Mix gently by pipetting and filter through a filter cap Falcon tube and add to a new 1.5 or 2ml LoBind tube (protein or DNA) On ice.

21 Keep On ice until the other samples have been resuspended and filtered.

22 Centrifuge 500 rcf for 00:05:00 and at 4 °C. 5m

23 Nuclei can now be seen, after wiping away condensation, as a white smear on the side of the tube in the DNA LoBind tubes or more of a pellet if using protein LoBind tubes. Remove supernatant and resuspend nuclei in 400 µL resuspension buffer by pipetting.

24 Filter through a filter-cap Falcon tube.

25 Transfer to LoBind Eppendorf On ice and centrifuge 500 rcf for 00:05:00 and at 4 °C. 5m

26 Remove supernatant and resuspend nuclei in 400 µL (200 µL if nuclei pellet looks small in Lo Bind tube) resuspension buffer by pipetting.

27 Filter through a filter-cap Falcon tube. Keep On ice.

## Nuclei Counting

28 Count each aliquot with Luna FL (18 µL sample + 2 µL Acridine Orange/Propidium Iodide). Add 10 µL to slide. Save images and counts.

29 Create a new RNAseq aliquot of 1000 nuclei/uL in 80 µL by diluting original sample.

30 Count diluted sample and record number (save files if needed but adds time for each sample).

31 Round count to nearest 100 and use this when loading the chip. Ie if one sample has concentration of 1100 and another 900 use two different cell concentrations in 10x protocol to load rather than rediluting sample to achieve 1000 cells.