

## **Brain Tissue, Nuclei Isolation with Gradient**

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## Equipment

### Samples: 4

Swinging-bucket rotor centrifuge, fixed rotor centrifuge will lead to substantial loss of nuclei.

Equipment	Cat. no	Company
Optima™ MAX-XP tabletop ultracentrifuge		Beckman Coulter
1 mL, Open-Top Thickwall Polycarbonate Tube, 11 x 34mm - 100Pk	343778	Beckman Coulter
1.5 ml DNA LoBind tubes	0030108051	Eppendorf SE
40µm strainer	542140	Greiner
Biomasher II® Disposable Micro Tissue Homogenizer (Non-sterile)	25534-100	Polysciences

## Reagents

Reagent	Cat.no.	Company
Sucrose	A2211	Applichem
KCl	60142	Sigma-Aldrich
MgCl <sub>2</sub>	M1028	Sigma-Aldrich
Tris Buffer, pH 8.0	T-3038	Sigma-Aldrich
Triton X-100 100%	648466	Merck Biosciences
10x PBS	AM9625	Ambion
20% BSA	0332-25G	VWR
Nuclease free water		
OptiPrep™ (Iodixanol 60%)	7820	Stemcell™ Technologies
RNase inhibitor 40 U/µl	2313B	Takara
SUPERase In™ RNase Inhibitor (20 U/µL) 10.000units	W4502-VWR	Invitrogen
cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail	11873580001	Roche
DTT	Y00147	Invitrogen

## Stock solutions

Stock solutions can be prepared months in advance and frozen. Thaw for 24 hours at 4 °C and store for 1 month max.

<b>Sucrose 1.5M (frozen stock)</b>				
	<b>Mw g/mol</b>	<b>1000 ml</b>	<b>100 ml</b>	<b>50 ml</b>
<b>Sucrose,</b>	342.3	513.45	51.345	25.6725
<b>RNasefree water</b>		Till 1000 ml	Till 100 ml	Till 50 ml

<b>NIM (stock in fridge, can be kept for ~1 month, if keeping longer, freeze at -20 degrees)</b>			
	<b>Volume (µl)</b>	<b>Final concentration (mM)</b>	<b>Conc. (After adding DTT, RNase inhib. etc) (mM)</b>
<b>1.5 M sucrose</b>	3546.1	265.96	250
<b>1 M KCl</b>	531.9	26.60	25
<b>1 M MgCl<sub>2</sub></b>	106.4	5.32	5
<b>1 M Tris buffer</b>	212.8	10.64	10
<b>RNase free water</b>	15602.8		
<b>Total volume</b>	20000		

<b>10% Triton X100 (frozen stock)</b>		
	<b>Volume (µl)</b>	<b>Final concentration</b>
Triton X-100 100%	1000	10% (v/v)
<b>RNase free water</b>	9000	

<b>Iodixanol diluent medium (IDM) (can be kept for ~1 month, if keeping longer, freeze at -20 degrees )</b>			
	<b>Volume (µl)</b>	<b>Final concentration</b>	<b>Conc. (After adding DTT, RNase inhib. etc) (mM)</b>
<b>1 M KCl</b>	4285.7	214.29	150
<b>1 M MgCl<sub>2</sub></b>	857.1	42.86	30
<b>1 M Tris buffer</b>	1714.3	85.71	60
<b>RNase free water</b>	13142.9		
<b>Total volume</b>	20000		

<b>Protease inhib., 50x cOmplete (frozen stock)</b>		
	<b>Volume (μl)</b>	<b>Final conc.</b>
<b>cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail</b>	1 tablet	50x
<b>RNase free water</b>	1000	

<b>1x PBS Mg<sup>+</sup>, RNase- (frozen stock)</b>		
	<b>Volume (μl)</b>	<b>Final conc. (mM)</b>
<b>10x PBS</b>	5000	1x
<b>1 M MgCl<sub>2</sub></b>	125	2.5
<b>RNase free water</b>	44875	
<b>Total volume</b>	50000	

<b>1x PBS , no Mg<sup>2+</sup>!, RNase- (frozen stock)</b>		
	<b>Volume (μl)</b>	<b>Final conc. (mM)</b>
<b>10x PBS</b>	5000	1x
<b>RNase free water</b>	45000	
<b>Total volume</b>	50000	

## **Tissue Preparation**

Very carefully cut approximately 50 mg (0.05g) of frozen, or fresh, tissue into DNA 1.5 ml tube on dry ice and store at, -70 /-80 °C till protocol is run. Please keep in mind, that any kind of thawing will affect results.

## Step 1. Preparation (1 hour)

NOTE! Entire protocol is standardized to only account for **4 samples**.

1. Precool all centrifuges and keep BioMasher tubes on ice.

Prepare all solutions, and keep, on ice

- Prepare Homogenization buffer

Homogenization buffer (always prepare fresh!)		
	Volume (μl)	Final concentration
NIM	5645.03	1x
100 mM DTT	60.38	1 mM
Protease inhib.	120.75	1x
RNase inhib.	60.38	0.4 U/μl
Supersin,	60.38	0.2 U/μl
Triton X-100	60.38	0.1% (v/v)

- Prepare Iodixanol 50% solution from Optiprep™ (Iodixanol 60%)

50% Iodixanol (always prepare fresh!)		
	Volume (μl)	Final concentration (mM)
Optiprep (Iodixanol 60%)	2275.1	50% vol/vol
IDM	318.5	
Protease inhib.	54.6	1x
RNase inhib.	27.3	0.4 U/μl
Supersin,	27.3	0.2 U/μl
100 mM DTT	27.3	1 mM
Total volume	2730.1	

- Prepare Iodixanol 29% solution from the above mentioned 50% solution

29% iodixanol (always prepare fresh!)		
	Volume (μl)	Final conc. (mM)
50% Iodixanol	1334	29% (vol/vol)
NIM	908.04	
Protease inhib.	19.32	1x
RNase inhib.	9.66	0.4 U/μl
Supersin	9.66	0.2 U/μl
100 mM DTT	9.66	1 mM
RNase free water	9.66	
Total volume	2300	

- Prepare Pre-coat solution

<b>PBS, 0.5% BSA, Mg+ RNase inhibitors- (coating of centrifuge tubes) (always prepare fresh!)</b>		
	<b>Volume (μl)</b>	<b>Final conc. (mM)</b>
<b>1x PBS</b>	3860.0	
<b>20% BSA</b>	100.0	0.50%
<b>100 mM DTT</b>	40	
<b>Total volume</b>	4000.0	

- Prepare BSA wash buffer

<b>PBS, 1% BSA, no Mg+, RNase inhibitors+ (resuspension and washing of pellet after gradient centrifugation) (always prepare fresh!)</b>		
	<b>Volume (μl)</b>	<b>Final conc. (mM)</b>
<b>1x PBS</b>	1890.0	
<b>20% BSA</b>	100.0	1.00%
<b>RNase inhib.</b>	10.0	0.2 U/μl
<b>Total volume</b>	2000.0	



## Step 2. Homogenization (1 hour)

1. Pre-coat ultracentrifuge tubes (Beckman Coulter, 343778) with 1 ml of Pre-coat solution per tube and leave on ice. These will be used again in step 8.
2. Transfer 0.5 ml cold Homogenization buffer into pre-cooled 1.5 ml tube containing tissue.
3. Homogenize the tissue with pestle until no pieces are visible (~10-15 strokes)

**TIP!** Homogenize by gently rotating pestle in downwards spiral.

4. Transfer homogenate into pre-cooled 1.5 ml tube (DNA LoBind) by passing it through 40µm cell strainer
  - a. Flush walls of tube, in which tissue was homogenized, with 500 µl Homogenization buffer and pass-through filter into the same pre-cooled 1.5 ml tube
  - b. Wash the filter with an additional 200 µl homogenization buffer
5. Centrifuge at 1000 g for 8 minutes at 4 °C
6. Gently aspirate and discard supernatant and resuspend pellet in 200 µl Homogenization buffer. Adjust volume to a final total of 250 µl by adding more, or less, Homogenization buffer.

### Step 3. Gradient Centrifugation (1 hour)

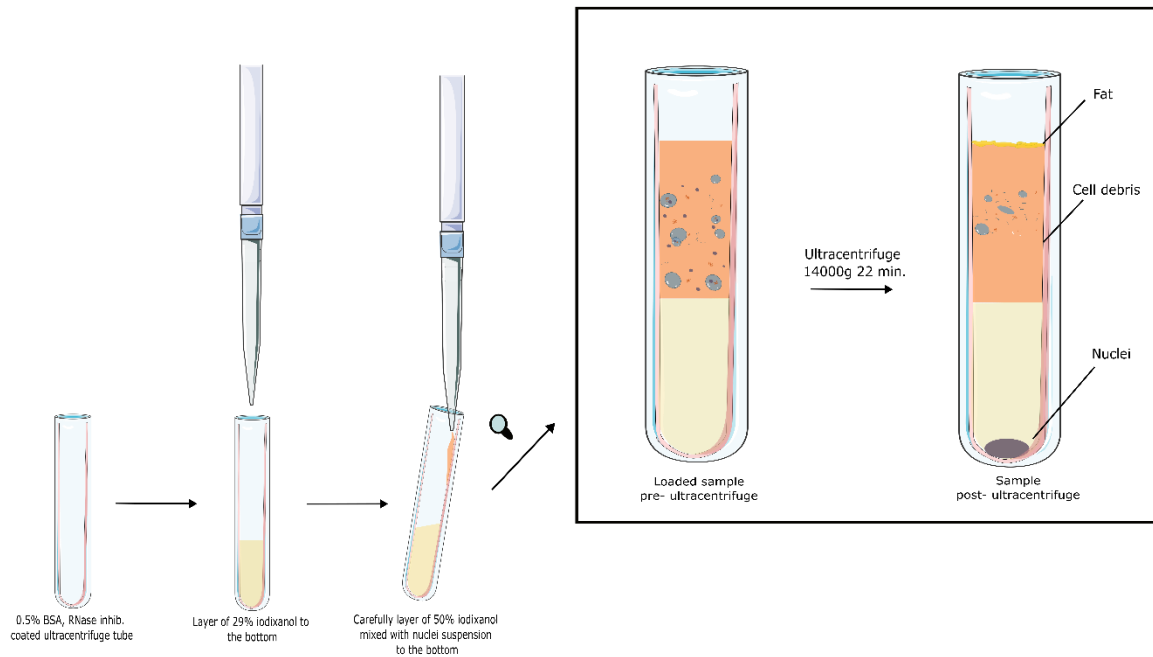


Figure 1 Gradient Isolation Methodology

7. Gently mix nuclei suspension with 250  $\mu$ l of 50% iodixanol solution.
8. To precoated (see Figure 1.) ultracentrifuge tube (Beckman, 343778) add 500  $\mu$ l of 29% iodixanol solution.
9. Slowly layer 500  $\mu$ l of the nuclei mixture over the 29% iodixanol solution in the ultracentrifuge tube on ice.
10. Centrifuge in ultracentrifuge using swing-bucket rotor at 14000g max for 22 min at 4  $^{\circ}$ C (TLS-55 rotor, MAX-XP ultracentrifuge, Accel 3, Decel 2).
11. Very carefully remove supernatant by aspirating individual layers, and discard. Top layer of fat should ALWAYS be removed first.

**TIP!** Change pipette VERY often. Fat will be stuck to the sides of pipette tip and risk being pushed back into suspension if pipette is not changed

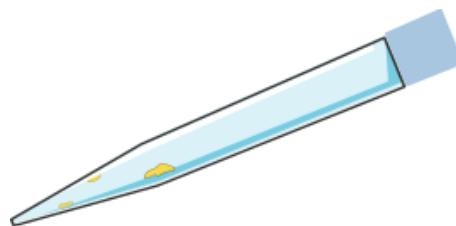


Figure 2 Change your pipette often, fat will be stuck on the sides of the tips!

**TIP!** Take your time removing the fat layer! Carefully try centring the fat in the middle and “detach” it from the tube walls before aspirating. If fat is stuck to the sides of the ultracentrifuge tube: carefully resuspend aspirate as much supernatant as possibly before resuspending the pellet in volume  $\geq 500 \mu\text{l}$  and transfer to new tube.

The point is to have NO fat.

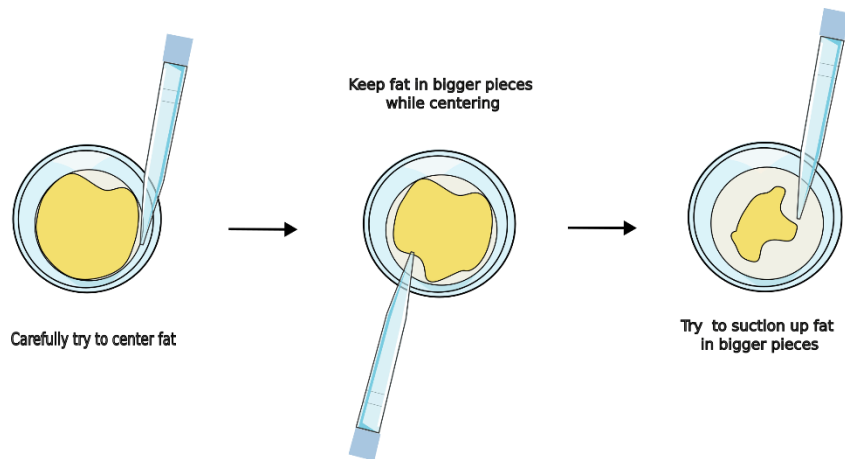


Figure 3 Centre fatty layer before aspirating

12. Resuspend pellet in  $500 \mu\text{l}$  **PBS, 1% BSA, no Mg<sup>+</sup>, with RNase inhibitor**. Determine the concentration of your nuclei and check sample under microscope to assess the presence of debris and clumping. Keep sample on ice while counting and assessing debris level.
13. Directly move on to fixation protocol