Brain Tissue, Nuclei Isolation with Gradient

Contents

Equipment

Samples: 4

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

Equitment	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
MgCl2	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 ºC and store for 1 month max.

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

NIM (stock in fridge, can be kept for ~1 month, if keeping longer, freeze at -20 degrees)			
	Volume (µl)	**Final concentration (mM)**	**Conc.** (After adding DTT, RNAse inhib. etc) **(mM)**
1.5 M sucrose	3546.1	265.96	250
1 M KCl	531.9	26.60	25
1 M MgCl2	106.4	5.32	5
1 M Tris buffer	212.8	10.64	10
RNase free water	15602.8		
Total volume	20000		

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

Iodixanol diluent medium (IDM) (can be kept for ~1 month, if keeping longer, freeze at -20 degrees)			
	Volume (µl)	**Final concentration**	**Conc.** (After adding DTT, RNAse inhib. etc) **(mM)**
1 M KCl	4285.7	214.29	150
1 M MgCl2	857.1	42.86	30
1 M Tris buffer	1714.3	85.71	60
RNase free water	13142.9		
Total volume	20000		

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

1x PBS Mg+, RNAse- (frozen stock)		
	Volume (µl)	**Final conc. (mM)**
10x PBS	5000	1x
1 M MgCl2	125	2.5
RNase free water	44875	
Total volume	50000	

1x PBS , no Mg2+!, RNAse- (frozen stock)		
	Volume (µl)	**Final conc. (mM)**
10x PBS	5000	1x
RNase free water	45000	
Total volume	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 ºC 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	7567.0	1x
100 mM DTT	80.5	1 mM
Protease inhib.	161.0	1x
RNAse inhib.	80.5	0.4 U/µl
Superasin,	80.5	0.2 U/µl
Triton X-100	80.5	0.1% (v/v)
Total volume	8050.0	

- 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
Superasin,	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
Superasin	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

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

- Prepare BSA wash buffer

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

Step 2. Homogenization (1 hour)

ON ICE

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 ºC
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)

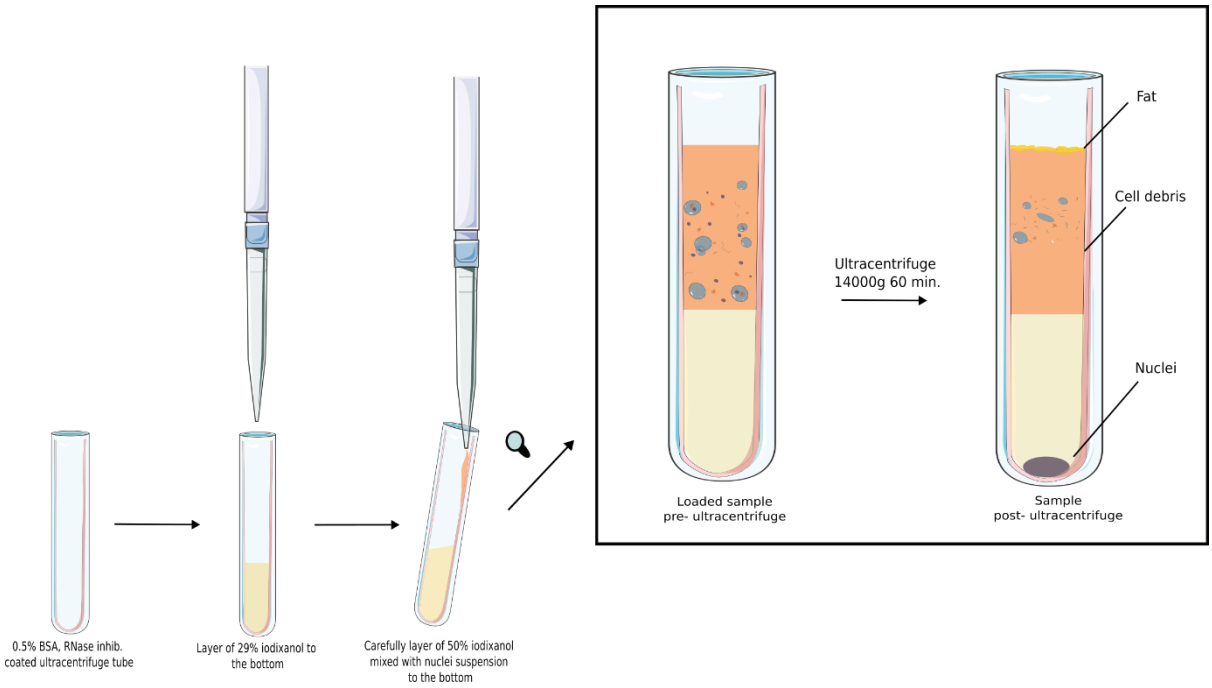

Figure 1 Gradient Isolation Methodology

ON ICE

7. Gently mix nuclei suspension with 250 µl of 50% iodixanol solution.
8. To precoated (see 1.) ultracentrifuge tube (Beckman, 343778) add 500 µl of 29% iodixanol solution.
9. Slowly layer 500 µl 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 ºC (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

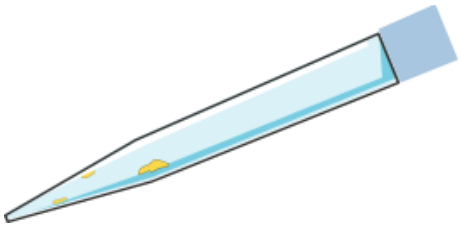

Figure 2 Change your pipette often, fat will be stuck on the sides of the tips!

TIP! Take your time removing the fat layer! Carefully try centering 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 ≥500 µl and transfer to new tube.
The point is to have NO fat.

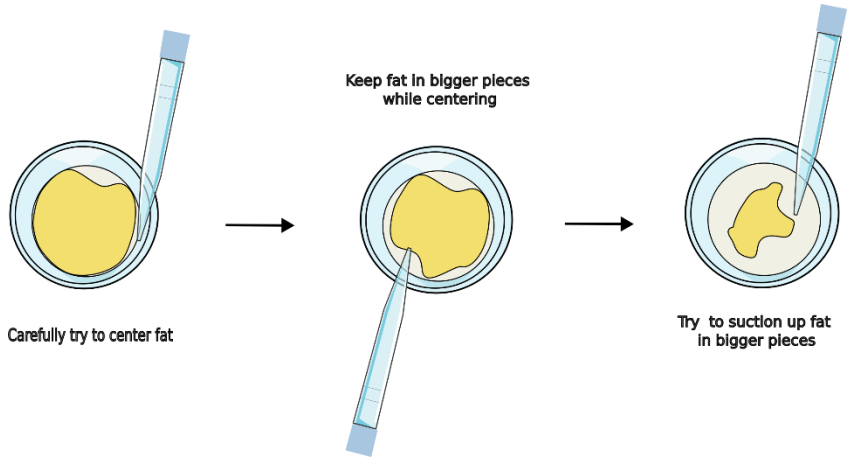

Figure 3 Center fatty layer before aspirating

12. Resuspend pellet in 500 µl **PBS, 1% BSA, no Mg+, 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