

Oct 30, 2023

Village Nuclei Isolation With Optiprep

DOI

[dx.doi.org/10.17504/protocols.io.36wgq3bmxlk5/v1](https://doi.org/10.17504/protocols.io.36wgq3bmxlk5/v1)

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Protocol Citation: liv_spina, Tara McDonald, Nora Reed, Alyssa Lutservitz 2023. Village Nuclei Isolation With Optiprep. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.36wgq3bmxlk5/v1>

Manuscript citation:
Wells et al., PMID [36796362](https://pubmed.ncbi.nlm.nih.gov/36796362/)

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Protocol status: Working

We use this protocol and it's working

Created: October 30, 2023

Last Modified: October 30, 2023

Protocol Integer ID: 90142

Keywords: fresh-frozen nuclei, nuclei extraction, snRNAseq, RNAseq, human tissue, brain tissue, multiple donors

Abstract

Isolation of nuclei from fresh-frozen brain tissue from sets of multiple (typically 2-20) human donors for analysis as a “cell village” (**Wells et al., PMID 36796362**) in which nuclei from all donors are analyzed together. Adapted from dx.doi.org/10.17504/protocols.io.bs99nh96, Luciano G Martelotto, with modifications to optimize for human brain tissue and allow the “cell village” approach.

Materials

Supplies

- Scalpels
- Glass slides
- Dounce
- 20 μm vacuum filter
- Eppendorf tubes (1.5 mL and 5 mL)
- Eppendorf or Ranin pipette tips
- Dry ice
- Metal plate
- OCT (Optimal cutting temperature compound)
- RNase free water
- Cell counting supplies (LUNA-FL)

Other Reagents:

- PBS
- BSA
- RNase inhibitor (i)
- Nuclei EZ lysis buffer (NUC201-1KT)
- OptiPrep Density Gradient Medium (60% Iodixanol - G60) (ab286850)

Stock solutions:

- **500 mM tricine:** 8.96 g of tricine in 100 mL water
- **1 M KCl:** 7.45 g of KCl in 100 mL water
- **1 M MgCl₂·6H₂O:** 20.3 g of MgCl₂·6H₂O in 100 mL water

Before start

- Turn on and chill centrifuge to 4°C
- Prepare all the reagents needed on ice
- Clean glass slides with ethanol
- Gather the dounce, pestles, and scalpels on ice

Solutions to make fresh before starting experiment:

- Nuclei EZ lysis buffer -- Pour 12 mL of buffer into the dounce and reserve 1 mL for wetting down the sides of the dounce
- PBSA + i (1% BSA and 1 U/ μ L RNase inhibitor in 1X PBS) -- For powder BSA, add 0.01 g of BSA per 1 mL of 1X PBS
- G30: (Mix the G60 and GD together thoroughly before adding the GH)

Before Starting

1 Gather Supplies

- Scalpels
- Glass slides
- 14 mL Dounce
- 20 µm vacuum filter
- Eppendorf tubes (1.5 mL and 5 mL)
- Eppendorf or Rainin pipette tips
- Dry ice
- Metal plate
- OCT (Optimal cutting temperature compound)
- RNase free water
- Cell counting supplies (LUNA-FL)

Other Reagents:

- PBS
- BSA
- RNase inhibitor (i)
- Nuclei EZ lysis buffer (NUC201-1KT)
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Stock solutions:

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1.1 Optiprep Buffers (can be made in advance and kept on hand)

A	B	C	D
GD		GH	
Component	Volume	Component	Volume
500 mM Tricine	24 mL	Sucrose	8.5 g
1M KCl	15 mL	Water	50 mL
1M MgCl ₂ x 6H ₂ O	3 mL	500 mM Tricine	4 mL
Water	50 mL	KCl	2.5 mL
1M KOH	Adjust to pH 7.8	1M MgCl ₂ ·6H ₂ O	0.5 mL
	Bring volume up to 100 mL with water (keep at room temp)	1M KOH	Adjust to pH 7.8
			Bring volume up to 100 mL with water (keep at 4°C)

- 1.2
 - Turn on and chill centrifuge to 4°C
 - Prepare all the reagents needed on ice
 - Clean glass slides with ethanol
 - Gather the dounce, pestles, and scalpels on ice

1.3 Solutions to make fresh before starting experiment

Nuclei EZ lysis buffer

- Pour 12 mL of buffer into the dounce and reserve 1 mL for wetting down the sides of the dounce

PBSA + i (1% BSA and 1 U/μL RNase inhibitor in 1X PBS)

- For powder BSA, add 0.01 g of BSA per 1 mL of 1X PBS

G30: (Mix the G60 and GD together thoroughly before adding the GH)

A	B	C	D	E	F
G30		PBSA + i		Nuclei EZ Lysis Buffer	
Component	Volume	Component	Volume	Component	Volume
G60 (OptiPrep)	6.0 mL	PBS (pH 7.4) (1X)	1 mL of 10X PBS	EZ Lysis Buffer	12,975 μL
GD	1.2 mL	BSA (100X)	1 mL of 10% BSA	RNase Inhibitor	325 μL
GH	4.8 mL	RNase Inhibitor (200X)	50 μL		
		RNase free water	7,950 μL		

Tissue Homogenization

1h 30m

- 2 On a glass slide **on dry ice**, shave off pieces of tissue with a scalpel until you have 50±3 mg of tissue
- 2.1 For managing tissue, use OCT to adhere tissue to glass slide



- 3 Place tissue pieces on a boat on dry ice. Hold the tissue on dry ice until all tissue has been cut
- 4 Transfer all tissue to dounce filled with Nuclei EZ Lysis Buffer
- 4.1 Use 1 mL of the reserved Nuclei EZ Lysis Buffer to wet down the side of the dounce if tissue is stuck on the side of the dounce
- 5 Gently dounce tissue on ice 20 times with pestle "A" then 20 times with pestle "B" until the tissue is homogenized
- 6 Incubate on ice for 10 minutes

Nuclei Isolation

1h 30m

- 7 Wet a 20 μ m vacuum filter with 1 mL of PBSA + i 1h
- 8 Vacuum filter the homogenized tissue
- 8.1 If the filter clogs, remove any unfiltered volume with a pipette, replace the filter, and continue filtering
- 9 Transfer the homogenized tissue to (3) 5 mL tubes
- 10 Centrifuge the samples at 4°C for 5 min (500 x g)
- 11 Remove and discard the supernatant (be careful not to disturb cell pellets) and resuspend each pellet in 900 μ L G30
- 11.1 Alternatively, split pellet into (9) 1.5 mL tubes (total), resuspend each pellet in 300 μ L of G30, then underlay each sample with 1 mL of G30



12 Gently underlay each 5mL tube with 3 mL G30

Place the pipette tip filled with G30 at the bottom of the 5mL tube. Slowly dispense G30 underneath the cell suspension. A clear separation will form between homogenate and G30 (cloudy on top, clear on the bottom). Do not disturb the separation between the layers

13 Centrifuge the samples at 4°C for 10 min (4,255 x g)

13.1 For capturing smaller cell types, spin at 8,000 x g for 20 minutes

14 Remove and discard the supernatant

14.1 Remove the top (very viscous) layer from the tube first- this layer contains fats and must be removed before removing the rest of the supernatant

Nuclei Wash, Resuspension, and Quantification

20m

15 Resuspend the pellets in 1 mL PBSA + i

16 Centrifuge the samples at 4°C for 5 min (500 x g) (single wash)

17 Remove the supernatant and resuspend the pellets in 200 - 1,000 μ L of PBSA + i (depending on the size of the pellet)

18 Count nuclei using LUNA-FL counter

Preserving Leftover Nuclei

15m

19 Optional if you have excess nuclei, or if you need to pause before continuing.

20 Centrifuge the nuclei at 4°C for 5 min (500 x g)

- 21 Remove the supernatant and resuspend the nuclei in CryoStor (CryoStor® CS10), mix by pipetting
- 21.1 $\leq 1\text{k}$ nuclei / μL of CryoStor
- 22 Transfer the nuclei to cryotubes and store them in a Mr Frosty in a -80°C freezer until frozen (1 day)
- 23 For long term storage, transfer cryotubes to regular storage in a -80°C freezer

Thawing Cryo-Stored Nuclei

30m

- 24 Thaw the CryoStored nuclei
- 25 Centrifuge the nuclei at 4°C for 10 min ($1200 \times g$)
- 26 Remove the supernatant and resuspend in 1mL PBSA + i
- 27 Centrifuge the nuclei at 4°C for 5 min ($500 \times g$) (single wash)
- 28 Remove the supernatant and resuspend the pelleted nuclei in PBSA + i
- 28.1 Count the nuclei before proceeding to next steps

Protocol references

Wells et al., PMID 36796362

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