SOP: **Nuclei isolation from fresh mouse tissues and DNaseI treatment**
Date modified: **01/12/2011**
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The following protocol describes the isolation of nuclei and subsequent DNaseI treatment from tissue taken from fresh mouse specimens.

Chemicals Ordering Information

Item	Catalog Number	Manufacturer
1,4-Dithioerythritol (1 g)	D9680	Sigma-Aldrich
Belzer UW Cold Storage Solution (1 L)		Bridge to Life, Ltd.
Calcium Chloride 1M (100 mL)	MT-140	Boston BioProducts
Complete EDTA-free Protease Inhibitor Tablets, Mini	04-693-132-001	Roche Applied Science
Deoxyribonuclease I (Type II from bovine pancreas 200 kU)	D4527	Sigma-Aldrich
D-Sucrose	BP220-1	Fisher Scientific
EDTA 0.5M pH 8.0 (1 L)	AM9262	Ambion
EGTA 0.5M pH 8.0 (100 mL)	BM-151	Boston BioProducts
Glycerol Redistilled (1 L)	03-117-502-001	Roche Applied Science
$MgCl_2$ 1M (100 mL)	AM9530G	Ambion
Milli-Q or Molecular Biology Grade Sterile Water		
NaCl 5M solution (500 mL)	46-032-CV	Mediatech, Inc.
OptiPrep (250 mL)	1114542	Axis-Shield, Oslo, Norway
Pefabloc SC Plus	11-873-601-001	Roche Applied Science
Potassium Chloride 1M (250 mL)	R-250	Boston BioProducts
Proteinase K >800 u/mL	P4850	Sigma-Aldrich
Ribonuclease A 30 mg/mL	R4642	Sigma-Aldrich
RNA later Solution	AM7021	Ambion
SDS 10% Solution (500 mL)	AM9822	Ambion
Spermidine Free Base (1 g)	0215206801	MP Biomedicals Inc.
Spermine Free Base (5 g)	0215207001	MP Biomedicals Inc.
Tricine	T5816	Sigma-Aldrich
Tris-HCl 1M pH 7.5 (1 L)	46-030-CM	Mediatech, Inc.
Tris-HCl 1M pH 8.0 (1 L)	46-031-CM	Mediatech, Inc.

Materials List

500 mL Corning 0.2 µm Filter System (Cat# 430758)
1 L Corning 0.2 µm Filter System (Cat# 430186)
15 mL Corning Polypropylene Conical Centrifuge Tubes (Cat# 430766)
50 mL Corning Polypropylene Conical Centrifuge Tubes (Cat# 430828)
15 mL MIDSCI Polypropylene Conical Centrifuge Tubes (Cat# C15B)
Dounce 7 mL Tissue Grinder with PYREX Pestles, Corning (VWR Cat# 22877-280)
Graduated pipets (5, 10, 25, 50 mL)
Hemocytometer
Micropipet with P20 tips
Micropipet with P200 tips
Micropipet with P1000 tips
Micropipet with P2000 tips
Wide-bore pipet tips (1 mL, 2 mL) for nuclei pellet resuspension
Microscope (preferably phase contrast)
Beckman Coulter Avanti J-E Refrigerated Floor Centrifuge
Eppendorf Refrigerated Centrifuge 5810R
0.22 µm Steriflip 50 mL Disposable Vacuum Filter System (Millipore Cat# SCGP00525)
100 µm Steriflip 50 mL Disposable Vacuum Filter System (Millipore Cat# SCNY00100)
20 µm Steriflip 50 mL Disposable Vacuum Filter System (Millipore Cat# SCNY00020)
CryoTube Vials, 1.8 mL (Nunc Cat# 368632)
Nalgene Cryo 1°C Freezing Container (Cat# 5100-0001)
Liquid Nitrogen Storage
37°C Water Bath
55°C Water Bath
Rocker Platform

Stock Reagents:

Unless otherwise noted, all buffers and stock solutions should be pre-chilled to 4°C (on ice) prior to use.

0.5M Spermine

Dissolve 5 grams Spermine Free Base in 49.43 mL final volume Milli-Q or Molecular Biology Grade sterile dH_20.

Store in convenient aliquots at -20°C.

0.5M Spermidine

Dissolve 1 gram Spermidine Free Base in 13.77 mL final volume Milli-Q or Molecular Biology Grade sterile dH_20.

Store at 4°C.

Mg Homogenization Buffer (per 50 mL)

Final concentration	*Stock concentration*	*Amount used from stock*
25mM D-Sucrose	0.5M D-Sucrose	2.5 mL
20mM Tricine, pH 7.8	0.5M Tricine, pH 7.8	2.0 mL
15mM NaCl	5M NaCl	0.15 mL
60mM KCl	1M KCl	3.0 mL
2mM $MgCl_2$	1M $MgCl_2$	0.1 mL
0.5mM Spermidine	0.5M Spermidine	0.05 mL
Molecular Biology Grade sterile H_20 to 50 mL		

Combine indicated amounts of stock solutions and add sterile H_20 to a final volume of 50 mL. Filter sterilize with 50 mL 0.22 µm Filter System and store at 4°C. Use within 1 week. Add one Complete Protease Inhibitor Tablet just prior to use.

EDTA Homogenization Buffer (per 50 mL)

Final concentration	*Stock concentration*	*Amount used from stock*
25mM D-Sucrose	0.5M D-Sucrose	2.5 mL
20mM Tricine, pH 7.8	0.5M Tricine, pH 7.8	2.0 mL
15mM NaCl	5M NaCl	0.15 mL
60mM KCl	1M KCl	3.0 mL
1mM EDTA, pH 8.0	0.5M EDTA, pH 8.0	0.1 mL
0.5mM EGTA, pH 8.0	0.5M EGTA, pH 8.0	0.05 mL
0.5mM Spermidine	0.5M Spermidine	0.05 mL
Molecular Biology Grade sterile H_20 to 50 mL		

Combine indicated amounts of stock solutions and add sterile H_2O to 50 mL. Filter sterilize with 50 mL 0.22 µm Filter System and store at 4°C. Use within 1 week. Add one Complete Protease Inhibitor Tablet just prior to use.

OptiPrep Dilution Buffer (per 50 mL)

Final concentration	*Stock concentration*	*Amount used from stock*
120mM Tricine, pH 7.8	0.5M Tricine, pH 7.8	12.0 mL
90mM NaCl	5M NaCl	0.9 mL
360mM KCl	1M KCl	18.0 mL
6mM EDTA, pH 8.0	0.5M EDTA, pH 8.0	0.6 mL
3mM EGTA, pH 8.0	0.5M EGTA, pH 8.0	0.3 mL
3mM Spermidine	0.5M Spermidine	0.3 mL
Molecular Biology Grade sterile H_20 to 50 mL		

Combine indicated amounts of stock solutions and add sterile H_20 to a final volume of 50 mL. Filter sterilize with 50 mL 0.22 µm Filter System and store at 4°C. Use within 1 week. Add one Complete Protease Inhibitor Tablet just prior to use.

50% OptiPrep Solution (per 50 mL)

Mix 5 volumes OptiPrep with 1 volume of OptiPrep Dilution Buffer.
Prepare just prior to use and store on ice.

Buffer A (per Liter)

Final concentration	*Stock concentration*	*Amount used from stock*
Sterile MilliQ Water		918 mL
15mM Tris-HCl, pH 8.0	1M Tris-HCl, pH 8.0	15 mL
15mM NaCl	5M NaCl	3 mL
60mM KCl	1M KCl	60 mL
1mM EDTA, pH 8.0	0.5M EDTA, pH 8.0	2 mL
0.5mM EGTA, pH 8.0	0.5M EGTA, pH 8.0	1 mL
0.5mM Spermidine	0.5M Spermidine Free Base	1 mL

Combine indicated amounts of stock solutions and sterile dH_2O to a final volume of 1 liter. Store at 4°C. Use within 1 week.

DNaseI 10X Digestion Buffer (per 50 mL)

Final concentration	*Stock concentration*	*Amount used from stock*
60mM CaCl2	1M CaCl2	3 mL
750mM NaCl	5M NaCl	7.5 mL

Combine stock solutions and 39.5 mL Milli-Q or Molecular Biology Grade sterile dH_20
Can be stored at room temperature up to 1 year.

1X DNaseI Digestion Buffer

Make day of use.

For 50 mL: add 5 mL 10X DNaseI Digestion Buffer to 45 mL Buffer A.
Allow to equilibrate to 37°C for 60 minutes prior to use.

Stock DNaseI

Solubilize on ice **with no vortexing** an entire bottle of DNaseI Type II from Bovine Pancreas in the following storage buffer at a final concentration of 10U/µL:

20mM Tris-HCl, pH 7.6
50mM NaCl
2mM $MgCl_2$
2mM $CaCl_2$
1mM Dithioerythritol
0.1mg/mL Pefabloc SC
50% Glycerol

Store in 250 µL aliquots at -20°C.

Stop Buffer (per Liter)

Final concentration	*Stock concentration*	*Amount used from stock*
50mM Tris-HCl, pH 8.0	1.0M Tris-HCl, pH 8.0	50 mL
100mM NaCl	5.0M NaCl	20 mL
0.10% SDS	10% SDS	10 mL
100mM EDTA, pH 8.0	0.5M EDTA, pH 8.0	200 mL
Molecular Biology Grade sterile H_2O		720 mL

Combine stock solutions and add sterile dH_2O to a final volume of 1 liter. Dispense into 25 mL aliquots and store at 4°C. (SDS will precipitate upon storage at 4°C but will go back into solution upon warming to 37°C).

On day of use, add the following to a 25 mL aliquot:
50 µL 0.5M Spermidine Free Base (final concentration: 1mM)
15 µL 0.5M Spermine Free Base (final concentration: 0.3mM)

Nuclei Preparation

Prior to Nuclei Isolation:

1. Add protease inhibitor tablets to Mg Homogenization Buffer, EDTA Homogenization Buffer, and Buffer A (1 tablet per 50 mL solution) and solubilize. Keep on ice.

2. Add spermine free base and spermidine free base to Stop Buffer. (If SDS has precipitated out of solution, warm to 37°C to resuspend SDS **prior** to adding supplements).

3. Prepare fresh 1X DNaseI Digestion Buffer: (Dilute 10X DNaseI Digestion Buffer 1:10 with Buffer A).

4. Aliquot 1X DNaseI Digestion Buffer: In 15 mL conical tubes, 1-5 mL 1X DNaseI Digestion Buffer (1mL per 10.0 E+6 expected nuclei); the number of tubes is determined by the number of DNaseI treatments to be done.

4. Warm Stop Buffer and 1X DNaseI Digestion Buffer (minus DNaseI) in 37^{o}C water bath. Allow to equilibrate for 60 minutes prior to use.

5. Pre-cool centrifuges to 4°C. All centrifugations should be done at 4°C.

Notes:

Work quickly using reagents maintained at appropriate temperatures.

Using DNaseI at 60, 80, and 120 units/mL, we observe high levels of cutting in HS sites with little cutting in non-HS regions. This difference in cutting can easily be measured using qPCR. Variation with DNaseI stock lots should be verified by individual lab empirically.

Nuclei isolation from solid mouse tissues

Tissue received for processing should be 1 square cm or smaller in size and collected in 5 mL Belzer UW (University of Wisconsin) Cold Storage Solution. Tissue dissected and prepared on site (same size as above) in collaborator's laboratory can be placed directly into Mg Homogenization Buffer (step 2). All solutions and tissue should be kept on wet ice. Note: a small portion of collected tissue is placed into 2 mL RNA later Solution at time of dissection for subsequent RNA isolation.

1. Add 5 mL Mg Homogenization Buffer to Dounce tissue grinder on ice.
2. Remove tissue from Belzer UW Cold Storage Solution and cut into ~2mm square pieces with sterile scissors or razor blade. Proceed with similar-dimension cutting for on-site tissues, bypassing need for Belzer UW Cold Storage Solution. Transfer tissue to Dounce tissue grinder.
3. Slowly and smoothly dounce homogenize approximately 5-10 times with loose-fitting (A) pestle. Tissue should be reduced to ~90% homogenous small particles.
4. Filter homogenate using 100µm Steriflip Vacuum Filter System to remove large material and transfer into second Dounce tissue grinder.
5. Bring volume back to 5 mL with Mg Homogenization Buffer.
6. Slowly and smoothly dounce homogenize approximately 5-10 times, depending on tissue consistency, with tight-fitting (B) pestle.
7. Filter solution using 20µm Steriflip Vacuum Filter System.
8. Bring volume again back to 5 mL with Mg Homogenization Buffer.
9. Add 3 mL 50% OptiPrep Solution and mix by inversion until homogenous (approximately 10 times).
10. Layer 8 mL tissue homogenate with 1 mL wide-bore pipet tip carefully onto 4 mL 25% - 1 mL 35% two-step OptiPrep gradient made in 15 mL MIDSCI polypropylene conical centrifuge tube.
11. Centrifuge 20 minutes 4°C at 6100 x g in a swinging bucket rotor using Beckman Coulter Avanti J-E centrifuge or equivalent.
12. Upon tube removal from the centrifuge, nuclei should be visible at the 25%-35% OptiPrep interface.
13. Aspirate off upper layer of homogenate and 1 mL of the 25% OptiPrep layer.
14. Transfer nuclei at the 25%-35% OptiPrep interface with 1 mL wide-bore pipet tip to a clean 15 mL Corning conical centrifuge tube containing 12 mL EDTA Homogenization Buffer on ice. Approximately 2 mL of the nuclei-containing interface will be transferred. Mix by inversion.
15. Count nuclei using the hemacytometer.
16. Pellet nuclei by centrifugation for 10 minutes 4°C at 250 x g in an Eppendorf Refrigerated Centrifuge 5810R.
17. Aspirate off supernatant and resuspend nuclei pellet in 10 mL Buffer A.
18. Count nuclei using the hemacytometer.
19. Aliquot into appropriate number of tubes for DNaseI treatment.
20. Pellet nuclei by centrifugation for 5 minutes 4°C at 250 x g in an Eppendorf Refrigerated Centrifuge 5810R.

21. Aspirate supernatant from all nuclei pellets and gently tap each tube to loosen nuclei pellet. Place tubes on ice.
22. Proceed with DNaseI treatment.

DNaseI Treatment

1. Stop Buffer and 1X DNaseI Digestion Buffer should be equilibrated to 37°C in water bath prior to starting nuclei isolation. (Buffers should be allowed to equilibrate 60 minutes at 37°C).

2. Just prior to starting DNaseI reaction with the nuclei pellet, add 5 µL proteinase K per mL Stop Buffer.

3. Also just prior to starting DNaseI I reaction with the nuclei pellet, add the appropriate amount of DNaseI enzyme to the 1X DNaseI Digestion Buffer aliquots (For example: For an 80 unit/mL digestion, add 32 µL of 10 units/µL stock DNaseI enzyme to 4 mL of 1X DNaseI Digestion Buffer). Mix thoroughly but gently by pipeting (**DO NOT VORTEX**) as the enzyme denatures easily with aeration.

   Remaining steps should be timed carefully:

4. Gently tap nuclei pellets a few times on the side of the ice bucket to loosen. Place tubes with loose nuclei pellets in 37°C water bath and allow temperature to equilibrate for 1 minute.

5. Gently resuspend nuclei with 1X DNaseI Digestion Buffer plus enzyme. Pipet several times gently using wide-bore tips to ensure homogenous suspension.

6. Incubate for 3 minutes at 37°C in water bath.

7. Add equal volume of Stop Buffer to DNaseI reaction tube and mix by inverting tube several times. Transfer tube to 55°C water bath.

8. Digest sample 1hr in the 55°C water bath.

9. Store treated samples at 4°C. Samples have been found to be stable for up to 2 years at 4°C.

10. Anytime prior to gel electrophoresis, incubate the samples at 37°C for 30 minutes with 1.5 µL 30 mg/mL RNaseA per mL of DNased sample.