SOP: **Nuclei isolation from mouse tissue using a Dounce homogenizer and subsequent cryopreservation and DNaseI treatment**
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The following protocol describes the isolation of nuclei and subsequent cryopreservation and DNaseI treatment from tissue taken from mouse specimens using a Dounce homogenizer.

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 (100mL)	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
Dimethyl Sulfoxide (DMSO Hybri-Max (5 x 10mL)	D2650	Sigma-Aldrich
D-Sucrose	BP220-1	Fisher Scientific
EDTA 0.5M pH 8.0 (1 L)	AM9262	Ambion
EGTA 0.5M pH 8.0 (100mL)	BM-151	Boston BioProducts
Glycerol Redistilled (1 L)	03-117-502-001	Roche Applied Science
$MgCl_2$ 1M (100mL)	AM9530G	Ambion
Milli-Q or Molecular Biology Grade Sterile Water		
NaCl 5M solution (500mL)	46-032-CV	Mediatech, Inc.
PBS 1X (1 L)	21-040-CM	Mediatech, Inc.
Pefabloc SC Plus	11-873-601-001	Roche Applied Science
Potassium Chloride 1M (250mL)	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 (500mL)	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

500mL Corning 0.2 µm Filter System (Cat# 430758)
1 L Corning 0.2 µm Filter System (Cat# 430186)
15mL Corning Polypropylene Conical Centrifuge Tubes (Cat# 430766)
50mL Corning Polypropylene Conical Centrifuge Tubes (Cat# 430828)
Dounce 7mL Tissue Grinder with PYREX Pestles, Corning (VWR Cat# 22877-280)
70% Ethanol-rinsed Razor Blades
89x89x25mm Polystyrene Weighing Dish (Fisher Scientific Cat# 08-732-113)
Graduated pipets (5, 10, 25, 50mL)
Hemocytometer
Micropipet with P20 tips
Micropipet with P200 tips
Micropipet with P1000 tips
Micropipet with P2000 tips
Wide-bore pipet tips (1mL, 2mL) for nuclei pellet resuspension
Microscope (preferably phase contrast)
Eppendorf Refrigerated Centrifuge 5810R
0.22 µm Steriflip 50mL Disposable Vacuum Filter System (Millipore Cat# SCGP00525)
100 µm Steriflip 50mL Disposable Vacuum Filter System (Millipore Cat# SCNY00100)
20 µm Steriflip 50mL Disposable Vacuum Filter System (Millipore Cat# SCNY00020)
CryoTube Vials, 1.8mL (Nunc Cat# 368632)
Nalgene Cryo 1°C Freezing Container (Cat# 5100-0001)
Liquid Nitrogen Storage
37°C Water Bath
55°C Water Bath

Stock Reagents:

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

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.

Sucrose Buffer

Final concentration	*Stock concentration*	*Amount used from stock*
250mM D-Sucrose	0.5M D-Sucrose	250mL
10mM Tris-HCl, pH 7.5	1M Tris-HCl, pH 7.5	5mL
1mM $MgCl_2$	1M $MgCl_2$	0.5mL

Molecular Biology Grade sterile H_20 to 500mL

Filter sterilize with 500mL 0.2 µm Filter System. Store at 4°C. Add Complete Protease Inhibitor Tablet (1 per 50mL solution) just prior to use.

0.5M Spermine

Dissolve 5 grams Spermine Free Base in 49.43mL 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 13.77mL final volume Milli-Q or Molecular Biology Grade sterile dH_20.

Store at 4°C.

DNaseI 10X Digestion Buffer (per 50mL)

Final concentration	*Stock concentration*	*Amount used from stock*
60mM $CaCl_2$	1M $CaCl_2$	3mL
750mM NaCl	5M NaCl	7.5mL

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

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
0.1 mg/mL Pefabloc SC
50% Glycerol

Store in 250 µL aliquots at -20°C.

Buffer A (per Liter)

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

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.

1X DNaseI Digestion Buffer

Make day of use.

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

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	50mL
100mM NaCl	5.0M NaCl	20mL
0.10% SDS	10% SDS	10mL
100mM EDTA, pH 8.0	0.5M EDTA, pH 8.0	200mL
Molecular Biology Grade sterile H_2O		720mL

Combine stock solutions and add sterile dH_2O to a final volume of 1 liter. Dispense into 25mL 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 25mL 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 Sucrose Buffer and Buffer A (1 tablet per 50mL 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 15mL conical tubes, 1-5mL 1X DNaseI Digestion Buffer (1mL per 10.0 million 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 centrifuge 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. Cryo-preserved tissue samples may need lower levels of DNaseI than fresh tissues.

Nuclei isolation from solid mouse tissues

Tissue received for processing should be 1 square cm or smaller in size and collected in 5mL 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 3). All solutions (except DMSO) and tissue should be kept on wet ice. Note: a small portion of collected tissue is placed into 2mL RNA later Solution at time of dissection for subsequent RNA isolation.

1. Remove tissue from Belzer UW Cold Storage Solution and weigh quickly in a polystyrene weighing dish. Alternatively, for on-site tissues, weigh tissue quickly, bypassing the need for Belzer UW Cold Storage Solution.
2. Mince tissue with ethanol-rinsed razor blade or sterile scissors to ~2mm square pieces.
3. Transfer minced tissue into an ice-cold Dounce tissue grinder containing 3mL Mg Homogenization Buffer per gram tissue.
4. Slowly and smoothly dounce homogenize approximately 5-10 times with loose-fitting (A) pestle. Tissue should be reduced to ~90% homogenous small particles.
5. Filter homogenate using 100 µm Steriflip Vacuum Filter System.
6. Measure volume of filtered material.
7. Add 1/9 volume 100% DMSO to samples (10% final concentration), pipeting several times to adequately mix. Aliquot into cryotube vials. Freeze at -80°C overnight in Nalgene Cryo 1°C Freezing Container, then move to -135°C liquid nitrogen for long-term storage.

Day of DNaseI Treatment:

8. Thaw cryotube vials rapidly in 37°C water bath.
9. Bring volume to 15mL with Sucrose Buffer.
10. Centrifuge for 10 minutes at 600 x g at 4°C in an Eppendorf 5810R Centrifuge. Aspirate supernatant.
11. Resuspend pellet in 10mL Sucrose Buffer.
12. Filter solution using 20 µm Steriflip Vacuum Filter System.
13. Count nuclei using the hemacytometer. Centrifuge in 15mL Corning conical centrifuge tube for 10 minutes at 600 x g at 4°C. Aspirate supernatant.
14. Resuspend the pellet in 10mL Buffer A.
15. Count nuclei using the hemacytometer.
16. Aliquot into appropriate number of tubes for DNaseI treatment.
17. Centrifuge for 5 minutes at 500 x g at 4°C. Aspirate supernatant from all nuclei pellets.
18. 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 4mL 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 and qPCR, incubate the samples at 37°C for 30 minutes with 1.5 µL 30 mg/mL RNaseA per mL of DNased sample.