SOP: **Nuclei isolation from human tissue using a Dounce homogenizer and subsequent DNaseI treatment and crosslinking**
Date modified: **08/11/2010**
Modified by: **E. Giste/ T. Canfield (UW)**

The following protocol describes the isolation of nuclei and subsequent DNaseI treatment and crosslinking from tissue taken from human specimens using a Dounce homogenizer. The Dounce homogenizer is used on soft tissues such as adrenal, brain, kidney, liver, lung, ovary, renal cortex, renal pelvis, spleen, testis, and thymus.

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
Dimethyl Sulfoxide (DMSO Hybri-Max (5 x 10 mL)	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 (100 mL)	BM-151	Boston BioProducts
Formaldehyde 37 wt. % solution in water (25 mL)	252549	Sigma-Aldrich
Glycerol Redistilled (1 L)	03-117-502-001	Roche Applied Science
Glycine (250 g)	50046	Fluka
MEM Medium (1 L)	10-010-CM	Cellgro Mediatech
$MgCl_2$ 1M (100 mL)	AM9530G	Ambion
Milli-Q or Molecular Biology Grade Sterile Water		
NaCl 5M solution (500 mL)	46-032-CV	Mediatech, Inc.
PBS 1X (1 L)	21-040-CM	Mediatech, Inc.
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
RPMI 1640 Medium (1 L)	10-040-CM	Cellgro Mediatech
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.
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)
Dounce 7mL 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)
Eppendorf Refrigerated Centrifuge 5810R
100 µm Steriflip 50mL Disposable Vacuum Filter System (Millipore Cat# SCNY00100)
20 µm Steriflip 50mL Disposable Vacuum Filter System (Millipore Cat# SCNY00020)
CryoTube Vials, 1.8 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.

Sucrose Buffer

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

Molecular Biology Grade sterile H_20 to 500 mL

Filter sterilize with 500 mL 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 49.43 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.

DNaseI 10X Digestion Buffer (per 50 mL)

Final concentration	*Stock concentration*	*Amount used from stock*
60mM $CaCl_2$	1M $CaCl_2$	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.

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		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.

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.

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)

1M Glycine Solution (50 ml)

Final concentration	*Stock concentration*	*Amount used from stock*
1.0 M	Glycine	3.76 g

Add Molecular Biology Grade sterile H_2O to 50 mL.
Store at 4°C.

Formaldehyde Solution

(11% Formaldehyde, 50mM Tris-HCl, pH 8.0, 0.1M NaCl, 1mM EDTA)

3.5 mL Formaldehyde Master Mix
1.5 mL 37% Formaldehyde —stored in flammable cabinet

Make fresh just prior to use. Keep for duration of experiment at room temperature.

Formaldehyde Master Mix (35mL)

Final concentration	*Stock concentration*	*Amount used from stock*
71.4mM Tris-HCl, pH 8.0	1.0M Tris-HCl, pH 8.0	2.5 mL
142.9mM NaCl	5.0M NaCl	1.0 mL
1.43mM EDTA, pH 8.0	0.5M EDTA, pH 8.0	0.1 mL
Molecular Biology Grade sterile dH_2O		31.4 mL

Combine stock solutions and add sterile dH_2O to a final volume of 35 mL.
Store at 4°C.

Nuclei Preparation

Prior to Nuclei Isolation:

1. Add protease inhibitor tablets to Sucrose 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 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 human 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. All solutions (except DMSO) 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. Weigh tissue.
2. Mince tissue with razor blade or scissors.
3. Homogenize tissue with 3 mL Sucrose Buffer per gram tissue in Dounce tissue grinder.
4. Dounce approximately 5 times slowly and smoothly with loose (A) pestle.
5. Filter homogenate using 100 µm Steriflip Vacuum Filter System.
6. Bring volume to 15 mL with Sucrose Buffer.
7. Centrifuge for 10 minutes at 600 x g at 4°C in an Eppendorf 5810R Centrifuge. Aspirate supernatant.
8. Resuspend pellet in 10 mL Sucrose Buffer.
9. Filter solution using 20 µm Steriflip Vacuum Filter System.
10. Count nuclei using the hemacytometer. If enough material is available, aliquot a portion of the nuclei for crosslinking. Centrifuge in 15 mL Corning conical centrifuge tube(s) for 10 minutes at 600 x g at 4°C. Aspirate supernatant(s). Note: proceed with crosslinking protocol immediately on the appropriate pellet.
11. Resuspend the pellet portioned for DNaseI treatment in 10 mL Buffer A.
12. Count nuclei using the hemacytometer.
13. Aliquot into appropriate number of tubes for DNaseI treatment.
14. Centrifuge for 5 minutes at 500 x g at 4°C. Aspirate supernatant from all nuclei pellets.
15. Proceed with DNaseI treatment.

To Cryo-Preserve Samples:

1. Weigh tissue.
2. Mince tissue with razor blade or scissors.
3. Homogenize tissue with 3 mL Sucrose Buffer per gram tissue in Dounce tissue grinder.
4. Dounce approximately 5 times slowly and smoothly with loose (A) pestle.
5. Filter homogenate using 100 µm Steriflip Vacuum Filter System.
6. Bring up to 2.7 mL with Sucrose Buffer.
7. Add 0.3 mL 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 15 mL 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 10 mL Sucrose Buffer.
12. Filter solution using 20 µm Steriflip Vacuum Filter System.
13. Count nuclei using the hemacytometer. If enough material is available, aliquot a portion of the nuclei for crosslinking. Centrifuge in 15 mL Corning conical centrifuge tube(s) for 10 minutes at 600 x g at 4°C. Aspirate supernatant(s). Note: proceed with crosslinking protocol immediately on the appropriate pellet.
14. Resuspend the pellet portioned for DNaseI treatment in 10 mL 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.

Crosslinking Protocol

Note: Perform steps# 1-5 as soon as possible after obtaining cell pellet

1. Resuspend cell pellet in 10 mL tissue culture media without fetal bovine serum (RPMI or MEM) at room temperature in an orange-capped 50 mL Corning conical centrifuge tube.
2. Add 1 mL 11% Formaldehyde Solution (made fresh) to a final concentration of 1%. Incubate on a rocker platform for 10 min at room temperature.
3. Add 1.57 mL 1.0M Glycine Solution (0.125M final concentration) to quench the reaction. Incubate on rocker platform for 5 min at room temperature.
4. Centrifuge for 5 min at 300 x g at 4ºC in an Eppendorf 5810R Centrifuge.
5. Remove supernatant with 10 mL pipet and discard into a formaldehyde waste receptacle (1 liter plastic bottle) for later neutralization. Note: a quenched cell pellet can stay on ice at this point until all samples are gathered. Rinse pellet with 20 mL ice-cold PBS. Centrifuge for 5 min at 300 x g at 4ºC.
6. Repeat rinse with 12 mL ice-cold PBS, transferring to an orange-capped 15mL Corning conical centrifuge tube. Centrifuge for 5 min at 300 x g at 4ºC.
7. Remove supernatant then store pellet at -80ºC.

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 and qPCR, incubate the samples at 37°C for 30 minutes with 1.5 µL 30 mg/mL RNaseA per mL of DNased sample.