SOP: Nuclei isolation from fresh or frozen human tissue and subsequent DNaseI treatment: Pseudo-nano scale

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Purpose

This protocol describes nuclei isolation from fresh and frozen tissues and subsequent DNaseI treatment under controlled conditions for optimal release of DNA fragments from accessible chromatin regions.

Chemicals Ordering Information

Item	Catalog Number	Manufacturer
AG501-X8 (D) resin	143-6425	Bio-Rad
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
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
Envirocide	13-3324	Metrex
Glycerol Redistilled (1 L)	03-117-502-001	Roche Applied Science
Liquid Nitrogen	N230LT35	Airgas
$MgCl_2$ 1M (100mL)	AM9530G	Ambion
Molecular Biology Grade Sterile Water	46-000-CM	Mediatech, Inc.
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
SDS 10% Solution (500mL)	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 Required

Item	Catalog Number	Manufacturer
15mL Conical Tubes	430766	Corning
100 µm Steriflip 50mL	SCNY00100	Millipore
20 µm Steriflip 50mL	SCNY00020	Millipore
50mL Conical Tubes	430828	Corning
500mL 0.2 µm Filter	430758	Corning
89x89x25mm Weighing Dish	08-732-113	Fisher Scientific
Bessman Tissue Pulverizer, Medium	189475	Spectrum Laboratories
GentleMACS C Tube	130-093-237	Miltenyi Biotec
Graduated pipets (5, 10, 25, 50mL)		VWR
Hemacytometer	UX-79001-00	Fisher Scientific
Micropipet with P20 tips		Rainin
Micropipet with P200 tips		Rainin
Micropipet with P1000 tips		Rainin
Wide-bore pipet tips (1mL)		Rainin

Equipment Required

Item	Manufacturer
37°C Water Bath	Fisher Scientific
55°C Water Bath	Fisher Scientific
GentleMACS Dissociator	Miltenyi Biotec
Microscope (preferably phase contrast)	Nikon
Refrigerated Centrifuge 5810R	Eppendorf

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	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.

5X Stop Buffer **(per 50mL)**

Final concentration	*Stock concentration*	*Amount used from stock*
125mM Tris-HCl, pH 8.0	1.0M Tris-HCl, pH 8.0	6.25mL
0.25% SDS	10% SDS	1.25mL
250mM EDTA, pH 8.0	0.5M EDTA, pH 8.0	25mL
Molecular Biology Grade sterile H_2O		17.5mL

Combine stock solutions and add sterile dH_2O to a final volume of 50mL. 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 1mL aliquot:

5µL 0.5M Spermidine Free Base (final concentration: 1mM)

Nuclei Preparation

Prior to Nuclei Isolation:

- Add protease inhibitor tablets to Sucrose Buffer and Buffer A (1 tablet per 50mL solution) and solubilize. Keep on ice.
- 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).
- Prepare fresh 1X DNaseI Digestion Buffer:
  (Dilute 10X DNaseI Digestion Buffer 1:10 with Buffer A).
- 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.
- Warm Stop Buffer and 1X DNaseI Digestion Buffer (minus DNaseI) in 37°C water bath. Allow to equilibrate for 60 minutes prior to use.
- 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. Variation with DNaseI stock lots should be verified empirically. Cryo-preserved tissue samples may need lower levels of DNaseI than fresh tissues.

Nuclei isolation from fresh human 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. All solutions (except DMSO) and tissue should be kept on wet ice.

- Weigh tissue.
- Mince tissue with razor blade or scissors in a polystyrene weighing dish.
- Transfer minced tissue into a gentleMACS C tube with 10mL Sucrose Buffer.
- Homogenize tissue using gentleMACS Dissociator Program “E.01 C Tube.”
- Filter homogenate using 100 µm Steriflip Vacuum Filter System.
- Bring volume to 15mL with Sucrose Buffer.
- Centrifuge for 10 minutes at 600 x g at 4°C in an Eppendorf 5810R Centrifuge. Aspirate supernatant.
- Resuspend pellet in 10mL Sucrose Buffer.
- Filter solution using 20 µm Steriflip Vacuum Filter System.

- Count nuclei using the hemacytometer. Centrifuge in 15mL Corning conical centrifuge tube(s) for 10 minutes at 600 x g at 4°C. Aspirate supernatant(s). Resuspend the pellet portioned for DNaseI treatment in 10mL Buffer A.
- Count nuclei using the hemacytometer.
- Aliquot into appropriate number of tubes for DNaseI treatment. (Nuclei/ tube should be between 300,000 and 2 Million)
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- Centrifuge for 5 minutes at 500 x g at 4°C. Aspirate supernatant from all nuclei pellets.
- Proceed with DNaseI treatment.

Nuclei isolation from flash frozen human tissues

Tissue received for processing are usually flash frozen and transferred to cryovials or are flash frozen in a sample cage and placed in a plastic bag. These are stored at -80n until use. All solutions should be kept on wet ice.

- Cut a piece of flash frozen tissue and weigh on a LN2 cooled metal plate.
- If soft (eg. Brain, thymus, pancreas), tissue is transferred into a gentleMACS C tube with 4mL Sucrose Buffer
- Homogenize tissue using gentleMACS Dissociator Program “E.01 C Tube.”
- If hard (eg, heart, muscle, kidney), tissue is “cold-smashed” in LN2, using a weigh boat on a LN2 cooled metal plate and the pestle from the Bessman pulverizer.
- Transfer smashed tissue into a gentleMACS C tube with 4mL Sucrose Buffer.
- Homogenize tissue using gentleMACS Dissociator Program “E.01 C Tube.”
- For both soft and hard tissues, filter homogenate using 100 µm Steriflip Vacuum Filter System.
- Bring volume to 8mL with Sucrose Buffer.
- Centrifuge for 10 minutes at 600 x g at 4°C in an Eppendorf 5810R Centrifuge. Aspirate supernatant.
- Resuspend pellet in 10mL Sucrose Buffer.
- Filter solution using 20 µm Steriflip Vacuum Filter System.
- Count nuclei using the hemacytometer. Centrifuge in 15mL Corning conical centrifuge tube(s) for 10 minutes at 600 x g at 4°C. Aspirate supernatant(s). Resuspend the pellet portioned for DNaseI treatment in 10mL Buffer A.
- Count nuclei using the hemacytometer.
- Aliquot into appropriate number of tubes for DNaseI treatment. (Nuclei/ tube should be between 300,000 and 2 Million)
- Centrifuge for 5 minutes at 500 x g at 4°C. Aspirate supernatant from all nuclei pellets.
- Proceed with DNaseI treatment.

DNaseI Treatment

- 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).
- Just prior to starting DNaseI reaction with the nuclei pellet, add 40 µL RNAse per mL Stop Buffer.
- 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:

- 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.
- Gently resuspend nuclei with 200ul 1X DNaseI Digestion Buffer plus enzyme. Pipet several times gently using wide-bore tips to ensure homogenous suspension.
- Incubate for 3 minutes at 37°C in water bath.
- Add 50ul 5X Stop Buffer to DNaseI reaction tube and mix by inverting tube several times.
- Digest sample 30 min at 37°C.
- Store treated samples at 4°C. Samples have been found to be stable for up to 2 years at 4°C.
- Any time prior to gel electrophoresis and fragment analysis, incubate the samples at 55°C for 1 hour with 1 µL ProteinaseK per 100 µL of DNased sample.

Next steps: QC by gel electrophoresis and fragment analysis