SOP: **Human hematopoietic cells: DNaseI treatment, crosslinking, and preserving cells for RNA**
Date modified: **02/07/2011**
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Summary

Human hematopoietic cells were provided as a service by the S. Heimfeld Laboratory at the Fred Hutchinson Cancer Research Center, Seattle, WA and include CD3+, CD4+, CD8+, CD14+, CD19+/CD20+, CD34+, and CD56+ cells, from both mobilized and non-mobilized donors. Cells were obtained from human leukapheresis product using standard procedures. Briefly, the lymphocyte subclasses were isolated by immunomagnetic separation using the CliniMACS affinity-based technology (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's recommendations. Reagents, tubing sets, and buffers are purchased from Miltenyi Biotec. The cells are provided either as freshly isolated or as cryopreserved (using the protocol entitled "SOP: Cryopreservation of hematopoietic cells from human leukapheresis product 02/07/2011 R.S. Hansen, T.K. Canfield (UW)"). Cryopreserved hematopoietic cells were thawed using the protocol entitled "SOP: Slow thawing of cryopreserved hematopoietic cells from human leukapheresis product 02/07/2011 R.S. Hansen, T.K. Canfield (UW)" prior to further processing. Both freshly prepared and cryopreserved hematopoietic cells were then processed further (e.g., for DNaseI treatment, crosslinked for ChIP, and/or preserved for RNA) as detailed in the following protocol.

Chemicals Ordering Information

Item	Catalog Number	Manufacturer
1,4-Dithioerythritol (1 g)	D9680	Sigma-Aldrich
AG501-X8 (D) 20-50 Mesh Resin, Molecular Biology Grade	143-6425	BioRad
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
EDTA 0.5M pH 8.0 (1 L)	AM9262	Ambion
EGTA 0.5M pH 8.0 (100mL)	BM-151	Boston BioProducts
Formaldehyde 37 wt. % solution in water (25mL)	252549	Sigma-Aldrich
Glycerol Redistilled (1 L)	03-117-502-001	Roche Applied Science
Glycine (250 g)	50046	Fluka
IGEPAL CA-630	I8896	Sigma-Aldrich
MEM Medium (1 L)	10-010-CM	Cellgro Mediatech
$MgCl_2$ 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
RPMI 1640 Medium (1 L)	10-040-CM	Cellgro Mediatech
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 List

Becton Dickinson 5mL syringe (Cat# 309646)
Becton Dickinson 18 gauge 1 inch PrecisionGlide needle (Cat# 305195)
15mL Corning Polypropylene Conical Centrifuge Tubes (Cat# 430766)
50mL Corning Polypropylene Conical Centrifuge Tubes (Cat# 430828)
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
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.

Deionized IGEPAL CA-630

Warm stock bottle of IGEPAL CA-630 to 37°C as it is quite viscous at room temperature.

Make a 10% solution by adding 4mL of warmed IGEPAL (dispensed via a 5mL syringe attached to an 18 gauge needle) to 36mL of Milli-Q or Molecular Biology Grade sterile dH_20. Vortex extensively until solubilized.

Add 2 grams AG501-X8 resin to the 40mL 10% IGEPAL solution. This resin will "deionize" the solution. "Spent" resin will be golden in color; the solution is still deionized if the resin is a combination of blue and golden beads.

Store solution in a tinfoil-wrapped conical tube at 4°C.

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.

2X IGEPAL CA-630 Solution

Buffer A supplemented with IGEPAL CA-630 at a final concentration of 0.02%-0.06%.

Make day of use.

Add 80µL-240µL 10% IGEPAL CA-630 to 40mL final volume of Buffer A. Note: the final concentration will have to be determined, depending on the particular hematopoietic cell.

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 H2O		720mL

Combine stock solutions and add sterile dH2O 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)

1M Glycine Solution (50mL)

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

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

Formaldehyde Solution

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

3.5mL Formaldehyde Master Mix
1.5mL 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	2.5mL
142.9mM NaCl	5.0M NaCl	1.0mL
1.43mM EDTA, pH 8.0	0.5M EDTA, pH 8.0	0.1mL
Molecular Biology Grade sterile dH_2O		31.4mL

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

Nuclei Preparation

Prior to Nuclei Isolation:

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

2. Prepare fresh 2X IGEPAL CA-630 solution. Keep on ice.

3. 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).

4. Prepare fresh 1X DNaseI Digestion Buffer:
   (Dilute 10X DNaseI Digestion Buffer 1:9 with Buffer A).

5. 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.

6. Warm Stop Buffer and 1X DNaseI Digestion Buffer (minus DNaseI) in 37°C water bath. Allow solutions to equilibrate for 60 minutes prior to use.

7. 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 80, 120, and 160 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 hematopoietic cells

1. Count cell suspension using a hemocytometer and aliquot the number of cells necessary for experimentation into a conical tube.
2. Pellet cells for 5 minutes at 500 x g at 4°C.
3. Aspirate off media and resuspend the cells in ice-cold PBS at 5 million cells per mL. Recount cell suspension using a hemocytometer. **Note: Cells/nuclei should be kept on ice for remaining steps until DNaseI treatment. In addition, cells/nuclei should be resuspended always with a wide-bore pipet or pipet tip!**
4. Aliquot portion of cells for DNaseI treatment, crosslinking, and RNA into separate conical tubes.
5. Again, pellet cells for 5 minutes at 500 x g at 4°C.
6. Aspirate off PBS wash. For RNA aliquot, resuspend cell pellet in RNA later Solution and store overnight at 4°C. Transfer the next day to long-term storage at -20°C. For the crosslinking aliquot, immediately proceed with crosslinking protocol (see below). For DNaseI treatment aliquot, resuspend cell pellet **gently** and **thoroughly** with ice-cold Buffer A at 5 million cells per mL. **From this step onward, work quickly**.
7. Add an equal volume of ice-cold 2X IGEPAL CA-630 solution (ranging from 0.02%-0.06%) to the DNaseI treatment aliquot. The final concentration of IGEPAL CA-630 in the solution will therefore range from 0.01%-0.03%. Mix well by inversion and incubate on ice 5-6 minutes.
8. Monitor progress of cells releasing nuclei under the microscope and stop when nuclei are released.
9. Pellet nuclei for 5 minutes at 500 x g at 4°C.
10. Carefully aspirate supernatant as pellet is quite “slippery.”
11. Wash nuclei pellet with fresh ice-cold Buffer A at 5 million nuclei per mL.
12. Count nuclei using the hemocytometer. Nuclei counts should be equivalent or slightly lower than initial cell count. Nuclei should be free-floating with little visible clumping.
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.

Crosslinking Protocol

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

1. Resuspend cell pellet in 10mL tissue culture media without fetal bovine serum (RPMI or MEM) at room temperature in an orange-capped 50mL Corning conical centrifuge tube.
2. Add 1mL 11% Formaldehyde Solution (made fresh) to a final concentration of 1%. Incubate on a rocker platform for 10 min at room temperature.
3. Add 1.57mL 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 10mL 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 20mL ice-cold PBS. Centrifuge for 5 min at 300 x g at 4ºC.
6. Repeat rinse with 12mL 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 of Hematopoietic Nuclei

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 1 hr 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.