PROTOCOL

Covaris

TITLE: Chromatin shearing with SDS detergent buffers

Summary of Operating Conditions

Target Base Pair (Range)	300-700
Duty Cycle	20%
Intensity	8
Cycles per Burst	200
Processing Time	We suggest an **initial** time course of 5-30 minutes to determine optimal shearing time for your sample.
Temperature (bath)	4°C
Power mode	Frequency Sweeping
Degassing mode	Continuous
Volume	300ul-3ml (tube selection specified below)
Starting material	$2x10^7$- 10^8 cells
Water level (FILL/RUN)	S2 – level 15 E210 – level 13 *Water level should be 1mm below the bottom of the TC12 tube cap
AFA Intensifier	**No Intensifier (remove from E transducer)**

Supplies

	Description		Part Number
Sample Vessel	<1.0ml of cells	Covaris TC12 tubes (12x24mm)	520056
	<2ml of cells	Covaris TC13 tubes (13x65mm)	520010
	3ml of cells	Covaris TC16 tubes (16x100mm)	520011
Holders for S2	TC12 tubes	THQ12X24	500199
	TC13 tubes	THQ13	500011
	TC16 tubes	THQ16	500012
Holder for E210	24 tube rack:	TR12X24 for TC12 tubes	500203
		TR2413 for TC13 tubes	500033
	12 tube rack:	TR1216 for TC16 tubes	500031

Buffers

Fixing buffer	50mM Hepes-KOH, pH 7.5, 100mM NaCl, 1mM EDTA, pH 8.0, 0.5mM EGTA, pH 8.0
Formaldehyde solution	prepare fresh from 37% HCHO by mixing 6ml of HCH0 with 14 ml water
LB1 buffer	50mM Hepes-KOH, pH 7.5, 140mM NaCl, 1mM EDTA, 10% glycerol, 0.5%NP-40, 0.25%Triton X-100
LB2 buffer	10mM Tris-HCl, pH 8.0, 200mM NaCl, 1mM EDTA, pH 8.0, 0.5mM EGTA, pH 8.0
Shearing buffer	1% Sodium Dodecyl Sulfate (SDS), 10mM EDTA and 50mM Tris, pH 8.1

Protease inhibitor	Dissolve one Complete Protease Inhibitor Cocktail Tablet (Roche Cat# 11697498 001) in 1ml H_2O to make a 50X solution
Quenching Solution	2.5M Glycine

METHOD

Cross linking Suspension cells:

1. Spin down ~10^8 cells at 1000 rpm for 5 minutes. Remove media and add 20ml of fixing buffer.
2. Fix cells by adding 2ml of the freshly prepared formaldehyde solution.
3. Keep cells on rocker at RT for 10 minutes to allow for efficient cross-linking.
4. Quench the cross linking reaction by adding 2.5M Glycine to a final concentration 125mM glycine. Keep on rocker at RT for 5 minutes.
5. Spin down cells at 1000 rpm for 5 minutes, and aspirate the supernatant.
6. Wash the cells in 5ml of cold PBS. Spin down at 1000 rpm for 5 minutes at 4°C.
7. Aspirate the PBS, flash freeze, and keep the cell pellet frozen at -80°C until needed.

Cross linking adherent cells:

1. Grow cells in ten 15cm^2 plates. This should generate ~10^8 cells.
2. Remove media, and add 20ml of 1X PBS per plate.
3. Fix cells by adding 2ml of the freshly prepared formaldehyde solution per plate.
4. Keep on rocker at RT for 10 minutes to for efficient allow cross linking.
5. Quench the cross linking reaction by adding 2.5M Glycine to a final concentration 125mM glycine. Keep on rocker at RT for 5 minutes.
6. Completely aspirate the solution from each plate.
7. Add 5 ml cold 1X PBS to each plate, and scrape cells from the plate.
8. Collect the scraped cells into a 50ml conical tube. Spin down cells at 1000 rpm for 5 minutes at 4°C.
9. Wash by resuspending the cells with 5ml of cold 1X PBS and spin to pellet cells.
10. Completely aspirate PBS, and snap freeze and store at -80C until needed

Nuclei preparation:

1. Thaw 10^8 cross-linked cells on ice.
2. Add 10ml lysis buffer **LB1** containing protease inhibitors.
3. Incubate for 10 min on a rocker at 4°C.
4. Pellet cells by spinning at 1000 rpm for 5 minutes at 4°C.
5. Resuspend pellet in 10ml **LB2** containing protease inhibitors, and incubate for 10 min 4°C on a rocker.

6. Spin down nuclei at 1000 rpm for 5 minutes at 4°C.
7. Resuspend pellet in 1ml **Shearing Buffer** containing protease inhibitors.
8. Repeat steps 6 and 7 twice more.
9. Resuspend pellet in 3ml of **Shearing Buffer** containing protease inhibitors.
10. For processing on the Covaris please use the following suggested sample tubes:
    a. 300µl-1000µl of cells - use Covaris TC12 (12mm X 24mm) tubes.
    b. 1.5-2ml of cells - use Covaris TC13(13mmX 65mm) tubes, and
    c. 3ml of cells - use Covaris TC16 (16mm X 100mm) tubes.

Chromatin Shearing:

When operating an E210, please verify that any 500141 Intensifier has been removed from the transducer prior to running a method. The Intensifier may be removed by following the instruction in Covaris document 010111.

It is recommended to carry out a time course shearing using your cell line to optimize the chromatin shearing parameters specific for your cell line, cell mass, and sample volume. We suggest conducting a time course of 5, 10, 15, 20, 25, and 30 minutes.

1. Transfer the sheared samples into microcentrifuge tubes, and centrifuge at 14000 rpm at 4°C for 1 minute.
2. Transfer the supernatant to new microcentrifuge tubes, and discard the cell debris.
3. Aliquot 100µl of the supernatant for chromatin shearing efficiency analysis.
4. Freeze the sheared chromatin containing supernatant at -70°C, for shearing efficiency analysis.

Chromatin shearing efficiency analysis:

1. Take the 100µl aliquot of the sheared sample and reverse-cross link overnight at 65°C.
2. We strongly advise using Qiagen purification columns to clean up the reverse-cross linked sample. Use 50ul per column so as not to exceed column capacity.
3. *Alternatively, if no purification columns are available, you can Phenol/Chloroform extract the sample, ethanol precipitate, and resuspend in TE.*
4. Elute the sample from the column twice using 30ul of elution buffer.
5. Add 5µl of loading dye to 25µl of the purified sample.
6. Load varied amounts on a 1% agarose gel. We suggest loading, 5µl, 10µl, and 15µl.
7. Resolve on 1% agarose gel, and stain with Ethidium Bromide after gel run.

SUPPLEMENTARY DATA

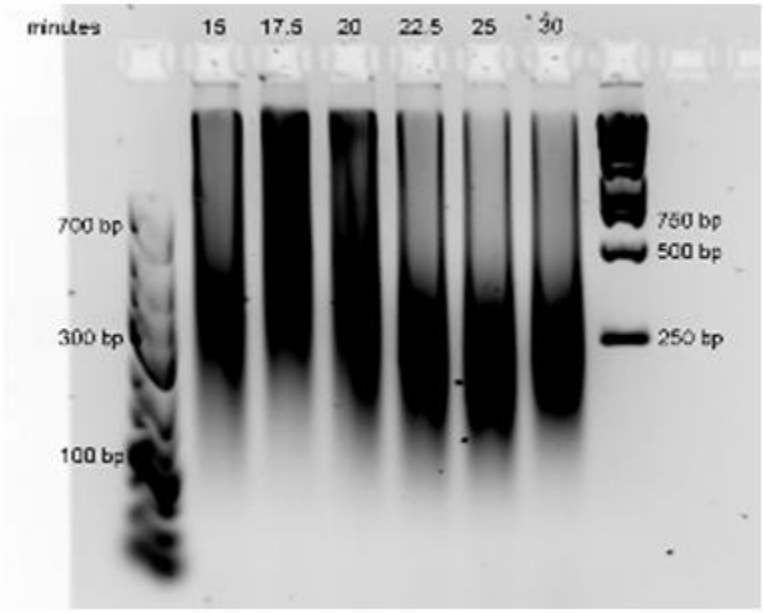

Figure 1- Time course chromatin shearing of 2x10^8 MCF10A cells in 1ml using TC12 tubes.

Gel image courtesy of Tamara Tjitrowirjo, and Sofia Gruvberger Saal Department of Oncology, Lund University.

NOTES:

1. Methods are transferable between the S2 system and the automated E210 (batch) system. Recommended settings are subject to change without notice. See following link: www.covarisinc.com/pdf/pn_010298 for updates to this document.

2. The treatment settings listed in this document are recommended guidelines. Actual results may vary depending on the cell type and cell mass.

3. The Covaris process uses high frequency acoustic energy and as such is influenced by objects in the acoustic path from the transducer surface to the fluid sample. For example, particles and bubbles in the water bath may inhibit the response. Please replace the bath water on a daily basis and ensure that appropriate time has been allowed for degassing and water bath temperature to stabilize prior to use of the instrument.

4. Bubbles in the sample fluid in the tube may diminish the acoustic dose effectiveness. Be sure to fill the tubes slowly with the recommended volumes and avoid the use of additional detergents that may induce foaming.

Reference:

Nelson Lau, Ph.D.
Department of Molecular Biology
Massachusetts General Hospital
Boston, Massachusetts, USA

Dr. Seongho Ryu
Mittal Laboratory
Lehman Brothers Lung Cancer Institute
Cornell University
New York, New York, USA

Subhashini Sadasivam, Ph.D.
Dana-Farber Cancer Institute & Harvard Medical School,
Department of Medical Oncology
Boston, Massachusetts, USA