PROTOCOL

Covaris

truChIP™ Chromatin Shearing Reagent Kit

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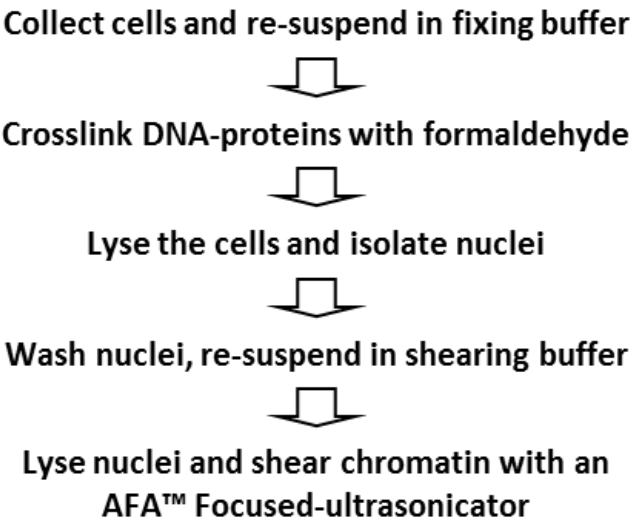
INTRODUCTION

The truChIP™ Chromatin Shearing Reagent Kit is optimized for the efficient and reproducible shearing of chromatin from adherent and suspension mammalian cell lines using Covaris AFA™ Focused-ultrasonicators. Focused-ultrasonicators provide a non-contact, isothermal method of shearing chromatin without compromising the structural integrity of the epitopes of interest for use in ChIP-qPCR (3), ChIP-Chip (1), and ChIP-Seq (2) applications.

This protocol will work with a variety of cell lines; however, additional optimization for your specific cell line may enhance the results. Initial optimization of fixation and chromatin shearing times are recommended.

PROCEDURE OVERVIEW



SAMPLE QUANTITY

The truChIP Chromatin Shearing Reagent it is compatible with a large range of cell number from less than 1 Million (1 x 10^6) cells up to 200 Million (2x 10^8). The Low Cell protocol has been optimized for chromatin shearing 1 to 3 Million Cells with the 130 µL the microTUBE with AFA Fiber; the High Cell protocol uses the 1 mL milliTUBE with AFA Fiber to process up to 30 Million cells; and the Batch protocol is optimized for preparing up to 200 Million cells for chromatin shearing in 6 separate milliTUBEs.

Single Sample	Low Cell	High Cell	Batch
Input cell number	1 to 3 Million (1-3 x 10^6) Cells	5 to 30 Million (0.5-3 x 10^7) Cells	50 to 200 Million (0.5-2 x 10^8) Cells
Number of samples sheared per kit	50	15	2
AFA Tube	microTUBE	milliTUBE	6 milliTUBEs
Shearing Volume	130 µL	1 mL	6 x 1 mL

KIT CONTENTS

Buffer A	7.5 mL
Buffer B	5 mL
Buffer C	2.5 mL
Buffer D3	6 mL
Buffer E	6 mL
Buffer F	0.8 mL
Formaldehyde	**5 x 1 mL ampoules (Kit 520154 only)**

Content Descriptions

Buffer A	10X Fixing Buffer
Buffer B	5X Lysis Buffer
Buffer C	10X Wash Buffer
Buffer D3	10X SDS Shearing Buffer (Contains 0.1% SDS in 1X solution)
Buffer E	1X Quenching Buffer
Buffer F	100X Protease Inhibitor cocktail
Formaldehyde	16% methanol-free formaldehyde **(**Kit 520154 only)

NOTE: MSDS information is available at http://covarisinc.com/resources/msds-sheets/

STORAGE

The kit is shipped cold and should be stored at 4-8 °C. Prior to use, kit reagent Buffers D3 and E may have to be warmed to 55 °C to dissolve precipitate and then cooled to room temperature before use. **NOTE: Mix solutions well before use to insure solutions are completely solubilized.**

SUPPLIED BY USER

- Molecular Biology Grade Water – Thermo Scientific (Cat. No. SH3053802), Mo Bio (Cat. No. 17012-200), or equivalent
- Fresh methanol-free 16% Formaldehyde – Thermo Scientific (Pierce) (Cat. No. 28908, 10 mL or 28906, 1mL ampules), or equivalent (unless provided in Covaris Kit)
- Phosphate Buffered Salt Solution (PBS) – Mo Bio (Cat. No. 17330-500), Thermo Scientific (Cat. No. SH30256.FS), or equivalent
- RNase A (DNase free) Thermo Scientific (Cat. No. EN0531) or equivalent
- Proteinase K (RNase and DNase free) Thermo Scientific (Cat. No. 17916), NEB (Cat. No. P8102S), or equivalent
- Covaris S-, E-, or L-Series instrument with chiller or Covaris M220
- Refrigerated centrifuge having 15,000 x g capability
- Rocker – Nutator® or equivalent
- M-Series, S-Series, E-Series, or L-Series AFA™ Focused-ultrasonicator with Chiller and appropriate holder or rack (see below).
- AFA Tubes

AFA Tubes and corresponding AFA Focused-ultrasonicator Holders and Racks:

Low Cell Protocol

Tube Part Number	Description	M-Series Holder and Insert	S-Series Holder	E-Series Rack	L-Series Rack
520045	microTUBE Snap-Cap	500358(*) 500414 (Holder) & 500421 (Insert)	500114	500111	NA
520052	microTUBE Crimp-Cap	NA	500114	500282	500282
520053	microTUBE strip	NA	NA	500191	500191
520078	microTUBE plate	NA	NA	No rack required	500329

High Cell Protocol

Tube Part Number	Description	M-Series Holder and Insert	S-Series Holder	E-Series Rack	L-Series Rack
520130	milliTUBE 1 mL	500348(*) 500414 (Holder) & 500422 (Insert)	500371	500368	500368

(*) These holders have been discontinued

PROTOCOL

A. Cell Preparation and Crosslinking

The truChIP protocol employs a two-step lysis method to prepare fix cells and prepare nuclei before shearing to ensure reproducible and efficient shearing of both suspension (non-adherent) and adherent cells. Follow the Cell Preparation and Crosslinking method (A.1 – Suspension and A.2 – Adherent) for your cell culture type.

NOTE: ChIP assays are sensitive to crosslinking and shearing conditions. Conducting optimization of these conditions by testing multiple fixation times and performing a shearing time course is strongly advised before conducting your ChIP experiments with any new cell line.

A.1 Suspension cells

1. Prepare solutions for the appropriate number of samples being processed fresh before starting.

	Low Cell	High Cell	Batch
1X cold PBS	**Final Volume: 2.0 mL per sample** -Store on ice	**Final Volume: 4 mL per sample** -Store on ice	**Final Volume: 40 mL per batch** -Store on ice
1X Fixing Buffer A	**Final Volume: 0.5 mL per sample** -Mix 50 µL of Fixing Buffer A with 0.450 mL Water	**Final Volume: 2 mL per sample** -Mix 200 µL of Fixing Buffer A with 1.8 mL Water	**Final Volume: 20 mL per batch** -Mix 2.0 mL of Fixing Buffer A with 18 mL Water
Fresh 11.1% Formaldehyde	**Final Volume: 1 mL per 1 to 20 samples** -Mix 690 µL of 16% Fresh Formaldehyde with 310 µL Water	**Final Volume: 1 mL per 1 to 5 samples** -Mix 690 µL of 16% Fresh Formaldehyde with 310 µL Water	**Final Volume: 2 mL per batch** -Mix 1.38 mL of 16% Fresh Formaldehyde with 0.62 mL Water
Quenching Buffer E	Place in a 55°C water bath to dissolve crystals, then place at ambient		
NOTE: The use of fresh methanol-free formaldehyde is essential to achieve reproducible results. The methanol-free formaldehyde ampoule is for one-time use. Once opened, its storage for later use is not recommended. **NOTE: Use Molecular Biology Grade Water for the preparation of all solutions.**			

2. Collect cells by centrifugation at 200 x g for 5 minutes, room temperature. Remove media and wash cells once with PBS and collect cells again by centrifugation. **NOTE: Some cells do not pellet well at 200 x g. If a "spongy" pellet is not visible, increase speed at 100 x g intervals until a pellet is visible.**

Reagent	Low Cell	High Cell	Batch
PBS	400 µL	1.5 mL	20 mL
Input cell number	1 – 3 x 10^6 Cells	1 – 3 x 10^7 Cells	0.5 – 2 x 10^8 Cells
Centrifuge Tube	2.0 mL	2.0 mL	50 mL

3. Re-suspend cells in room temperature Fixing Buffer A.

Reagent	Low Cell	High Cell	Batch
Fixing Buffer A	400 µL	1.5 mL	20 mL

4. Crosslink cells by adding freshly prepared 11.1% formaldehyde solution to a final concentration of 1% and start timing the crosslinking reaction. **NOTE: The use of fresh methanol-free formaldehyde is absolutely required to attain reproducible results.**

Reagent	Low Cell	High Cell	Batch
Fresh 11.1% Formaldehyde	40 µL	150 µL	2.0 mL

5. Place cells on a rocker at room temperature for the prescribed time (e.g., 5 minutes for most cell types) to allow for efficient crosslinking. **NOTE: Optimal crosslinking time and shearing conditions should be determined empirically for each cell type[4-6].**
6. Quench the crosslinking reaction by adding the appropriate volume of Buffer E to the fixed cells. Keep cells on a rocker at room temperature for an additional 5 minutes.

Reagent	Low Cell	High Cell	Batch
Quenching Buffer E	23 µL	87 µL	1.2 mL

7. Collect cells by centrifuging at 500 x g for 5 minutes at room temperature.
8. Aspirate the supernatant and wash twice with cold PBS.

Reagent	Low Cell	High Cell	Batch
Cold PBS	300 µL	1.0 mL	5.0 mL

9. Collect cells by centrifugation at 200 x g for 5 minutes, 4 °C.
10. Proceed to nuclei preparation and chromatin shearing steps. **NOTE: It may be possible to flash-freeze the fixed cells in liquid nitrogen at this time and store at -80 °C for short periods of time (e.g., 2 to 3 days). Longer-term storage may adversely affect shearing efficiency and reproducibility.**

A.2 Adherent cells

1. Grow the proper amount of cells to conduct a single ChIP assay or the initial time course until they are 80 to 90% confluent.

	35 mm Plate	60 mm Plate	100 mm Plate	150 mm Plate
Cell Density	~0.8×10^6	~2.0×10^6	~5.5×10^6	~15×10^6
Protocol	Low Cell	Low Cell	High Cell	High Cell
Number of Plates	1 to 3	1 to 2	2 to 5	1 to 2

NOTE: Cell densities provided above are estimates provided as a general guideline. Accurate cell densities for your cell lines should be determined.

2. Prepare solutions for the appropriate number of samples being processed fresh before starting.

	35 mm and 60 mm Dish	100 mm and 150 mm Plate
1X cold PBS	**Final Volume: 14 mL per Plate** -Store on ice	**Final Volume: 25 mL per Plate** -Store on ice
1X Fixing Buffer A	**Final Volume: 2 mL per Plate** -Mix 200 µL of Fixing Buffer A with 1.8 mL Water	**Final Volume: 5 mL per Plate** -Mix 500 µL of Fixing Buffer A with 4.5 mL Water
Fresh 11.1% Formaldehyde	**Final Volume: 300 µL per Plate** -Mix 208 µL of 16% Fresh Formaldehyde with 92 µL Water	**Final Volume: 1 mL per Plate** -Mix 0.69 mL of 16% Fresh Formaldehyde with 0.31 mL Water
Quenching Buffer E	Place in a 55°C water bath to dissolve crystals, then place at ambient	
NOTE: The use of fresh methanol-free formaldehyde is essential to achieve reproducible results. The methanol-free formaldehyde ampoule is for one-time use. Once opened, its storage for later use is not recommended.		

NOTE: Use Molecular Biology Grade Water for the preparation of all solutions.

3. Remove media and wash each plate one time with PBS.

Reagent	35 mm and 60 mm Plate	100 mm and 150 mm Plate
PBS	2 mL	5 mL

4. Remove PBS and add room temperature Buffer A to each dish.

Reagent	35 mm and 60 mm Plate	100 mm and 150 mm Plate
Fixing Buffer A	2 mL	5 mL

5. Crosslink cells by adding freshly prepared 11% formaldehyde solution to a final concentration of 1% and start timing the crosslinking reaction. **NOTE: The use of fresh methanol-free formaldehyde is absolutely required to attain reproducible results.**

Reagent	35 mm and 60 mm Plate	100 mm and 150 mm Plate
Fresh 11.1% Formaldehyde	200 µL	500 µL

6. Place cells on a shaking platform at room temperature for the prescribed time (e.g., 5 minutes for most cell types) to allow for efficient crosslinking. **NOTE: Optimal crosslinking time and shearing conditions should be determined empirically for each cell type[4-6].**
7. Quench the crosslinking reaction by adding the appropriate volume of Buffer E to fixed cells. Keep on a shaking platform at room temperature for an additional 5 minutes.

Reagent	35 mm and 60 mm Plate	100 mm and 150 mm Plate
Quenching Buffer E	120 µL	300 µL

8. Completely aspirate the solution from the plate.
9. Add cold PBS to each dish and scrape cells from the plate into a proper vessel.

Reagent	35 mm and 60 mm Plate	100 mm and 150 mm Plate
Cold PBS	450 µL	5 mL
Centrifuge Tube	2.0 mL tube	15 mL conical

10. Wash the plate with an additional volume of cold PBS to collect any remaining cells.

Reagent	35 mm and 60 mm Plate	100 mm and 150 mm Plate
Cold PBS	450 µL	5 mL
Centrifuge Tube	2.0 mL tube	15 mL conical

11. Collect cells at 200 x g for 5 minutes, 4 °C. **NOTE: Some cells do not pellet well at 200 x g. If a "spongy" pellet is not visible, increase speed at 100 x g intervals until a pellet is visible.**
12. Wash cells twice by resuspending in cold 1X PBS, and collecting by centrifugation at 200 x g, 4 °C.

Reagent	35 mm and 60 mm Plate	100 mm and 150 mm Plate
Cold PBS	450 µL	5 mL

13. Completely and carefully aspirate the supernatant from the tube(s), and place on ice.
14. Proceed to nuclei preparation and chromatin shearing steps. **NOTE: It may be possible to flash-freeze the fixed cells in liquid nitrogen at this time and store at -80 °C for short periods of time (e.g., 2 to 3 days). Longer-term storage may adversely affect shearing efficiency and reproducibility.**

B. Nuclei Preparation

1. Prepare the proper number of suspension or adherent cells according to the above protocol. Place the required number of AFA Tubes on ice to pre-chill while preparing samples to shear.

	Low Cell	High Cell	Batch
Number of fixed cells	1-3 x 10^6	1-3 x 10^7	0.5 – 2 x 10^8
AFA Tube	microTUBE	milliTUBE	6 milliTUBEs

1. Prepare the correct volume of fresh solutions for the nuclei preparation and chromatin shearing before use. **NOTE: Substituting any of the reagents or changing any steps will adversely affect shearing efficiency and reproducibility, which could be detrimental to your subsequent IP results.**

	Low Cell	High Cell	Batch
1X Lysis Buffer B	**Final Volume: 0.5mL per sample** -Mix 100 µL 5X Lysis Buffer B with 400 µL water. -Add 5 µL of 100X Buffer F. -Store on ice.	**Final Volume: 1.0 mL per sample** -Mix 200 µL 5X Lysis Buffer B with 0.8 mL water. -Add 10 µL of 100X Buffer F. -Store on ice.	**Final Volume: 10 mL per sample** -Mix 2 mL 5X Lysis Buffer B with 8 mL water. -Add 100 µL of 100X Buffer F. -Store on ice.
1X Wash Buffer	**Final Volume: 0.5 mL per sample** -Mix 50 µL 10X Wash Buffer C with 450 µL water. -Add 5 µL of 100X Buffer F. -Store on ice.	**Final Volume: 1.0 mL per sample** -Mix 100 µL 10X Wash Buffer C with 0.9 mL water. -Add 10 µL of 100X Buffer F. -Store on ice.	**Final Volume: 10mL per sample** -Mix 1.0 mL 10X Wash Buffer C with 9 mL water. -Add 100 µL of 100X Buffer F. -Store on ice.
Shearing Buffer D3	**Final Volume: 1 mL per sample** -Mix 100 µL 10X Shearing Buffer D3 with 0.9 mL water. -Add 10 µL of 100 X Buffer F. -Store on ice.	**Final Volume: 3 mL per sample** -Mix 300 µL 10X Shearing Buffer D3 with 2.7 mL water. -Add 30 µL of 100 X Buffer F. -Store on ice.	**Final Volume: 10 mL per sample** -Mix 1.0 mL 10X Shearing Buffer D3 with 9.0 mL water. -Add 100 µL of 100 X Buffer F. -Store on ice.
NOTE: Use Molecular Biology Grade Water for the preparation of all solutions.			

2. Add Lysis Buffer B containing 1X protease inhibitors to cross-linked cells to lyse plasma membrane, gently resuspend by aspirating/dispensing 4 times. If cells were frozen after formaldehyde fixation, thaw cells on ice first.

Reagent	Low Cell	High Cell	Batch
Lysis Buffer B	300 µL	1 mL	10 mL

3. If processing using Low Cell or High Cell volumes, then transfer to 1.5 mL microcentrifuge tube. If processing for Batch, then transfer to a 15 mL conical tube.
4. Incubate for 10 minutes on a rocker at 4 °C.
5. Collect intact nuclei by centrifugation at 1,700 x g for 5 minutes, 4 °C. Decant the supernatant without disturbing the nuclei pellet.

6. Gently resuspend pellet in Wash Buffer C containing protease inhibitor and incubate on a rocker for 10 minutes at 4 °C. **NOTE: The purpose of this wash is to significantly dilute the salts remaining from the Wash Buffer. Shearing in the presence of higher salt concentrations may lead to reversing of formaldehyde cross-links during processing.**

Reagent	Low Cell	High Cell	Batch
Wash Buffer C	300 µL	1 mL	10 mL

7. Collect nuclei by centrifugation at 1,700 x g for 5 minutes, 4 °C. Carefully remove and discard the wash solution, taking care not to disturb the nuclei pellet.
8. Gently rinse the sides of the tube with 1X Shearing Buffer D3 containing Protease inhibitor. Slowly dispense the buffer down the entire circumference of the upper-inside of the tube, taking care not to disturb the nuclei pellet.

Reagent	Low Cell	High Cell	Batch
Shearing Buffer D3	300 µL	1 mL	1.5 mL

9. Collect nuclei by centrifugation at 1,700 x g for 5 minutes, 4 °C. Decant the supernatant without disturbing the nuclei pellet.
10. Repeat steps 7 and 8 an additional time. Carefully remove and discard the supernatant, taking care not to disturb the nuclei pellet.

C. Chromatin Shearing

1. Resuspend nuclei pellet in the Shearing Buffer D3 and transfer to appropriate AFA Tube(s).

Reagent	Low Cell	High Cell	Batch
Shearing Buffer D3	130 µL	1 mL	6 mL
AFA Tube	1 microTUBE	1 milliTUBE	6 milliTUBE

2. Shear chromatin with an AFA Focused-ultrasonicator with appropriate rack or holder; settings are provided in Appendix A. **NOTE: Optimization of shearing time should be conducted whenever experimental parameters (e.g., cell type, cell number, or sample volumes) are changed.**
3. If processing samples for Low Cell Chromatin Shearing Optimization in microTUBEs, please aliquot 130ul of the nuclei preparation into 6 microTUBEs for carrying out the shearing time course of 2, 4, 6, 8, 10, and 12 minutes.
4. If processing samples for High Cell Chromatin Shearing Optimization, please aliquot 1 mL of the nuclei into one milliTUBE for carrying out the shearing time course of 2, 4, 8, 12, 15, and 20 minutes.
5. Using the milliTUBEs, you can process all the time points of the time course in the same tube according to the table below. After each time point interval, take out 35 µL of the sample and place in a microcentrifuge tube, and replace volume by adding 35 µL of shearing buffer to the milliTUBE.

Programmed Interval processing time (minutes)	2	2	4	4	3	5
Total processing time (minutes)	2	4	8	12	15	20

6. Place the milliTUBE in the holder/rack and process on the S or E series instrument for the next programmed interval processing time. 25 µL will be used for DNA shearing size range analysis, and 10 µL will be used for epitope integrity analysis using western.
7. After shearing, transfer samples into a pre-chilled microcentrifuge tube and place on ice until all tubes are processed. If batch processing in the high-throughput E220 system, samples are maintained in the instruments water bath at 4 °C before and after processing and are therefore stable.

NOTE: To check the efficiency of your shearing, reserve 25 µL of the sheared chromate, and see Appendix B for detailed instructions.

NOTE: Sheared chromatin can be stored at 4 °C for up to 2 days.

NOTE: Freezing sheared chromatin is not recommended. Freeze/thawing dramatically reduces IP efficiency and reproducibility of your ChIP assays.

NOTE: For subsequent immunoprecipitation, sheared chromatin can be diluted in the desired immunoprecipitation buffer. Alternatively, the composition of the shearing buffer can be adjusted appropriately for immunoprecipitation. The 1X SDS Shearing Buffer D3 composition is: 10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.1% SDS.

Appendix A: AFA™ Focused-ultrasonicator Operating Conditions

Low Cell Chromatin Shearing Protocol			
	M-Series	**S- and E-Series**	**L-Series**
Target BP (Range)	200 – 700	200 – 700	200 – 700
Duty Cycle	5%	2%	15%
Intensity Peak Incident Power	75 Watts	3 (S2/E210) 105 Watts (S220/E220)	300 Watts
Cycles per Burst	200	200	200
Processing Time	Empirical (2 to 12 min typical)	Empirical (2 to 12 min typical)	Empirical (15 to 30 min typical)
Bath Temperature	7 °C	6 °C	6 °C
Power mode	NA	Frequency sweeping (S2 and E210 only)	NA
Degassing mode	NA	Continuous	Continuous
Volume	130 µL	130 µL	130 µL
Max. cell number	3 x 10^6 cells	3 x 10^6 cells	3 x 10^6 cells
AFA Intensifer	NA	**E-Series - add AFA intensifier.** S-Series – intensifier is integrated with holder.	NA
Water level (RUN)*	Full	S2/S220 – level 12 E210/E220 – level 6	LE220 – level 6
NOTE			**Use of 96 microTUBE Plate (PN 520078) requires Rack-XT 96 microTUBE Plate**
*Water level should be ~1 mm below the bottom of the microTUBE cap			
IMPORTANT: Always fill microTUBEs with 130 µL of solution			

High Cell Chromatin Shearing Protocol			
	M-Series	**S- and E-Series**	**L-Series**
Target BP (Range)	200 – 700	200 – 700	200 – 700
Duty Cycle	10%	5%	15%
Intensity Peak Incident Power	75 Watts	4 (S2/E210) 140 Watts (S220/E220)	350 Watts
Cycles per Burst	200	200	200
Processing Time	Empirical (2 to 20 min typical)	Empirical (2 to 20 min typical)	Empirical (15 to 30 min typical)
Bath Temperature	7 °C	6 °C	6 °C
Power mode	NA	Frequency sweeping (S2 and E210 only)	NA
Degassing mode	NA	Continuous	Continuous
Volume	1.0 mL	1.0 mL	1.0 mL
Max. cell number	3 x 10^7 cells	3 x 10^7 cells	3 x 10^7 cells
AFA Intensifer	NA	NA	NA
Water level (RUN)*	Full	S2/S220 – level 8 E210/E220 – level 5	LE220 – level 5
*Water level should be ~1 mm below the bottom of the milliTUBE cap			
IMPORTANT: Always fill milliTUBEs with 1.0 mL of solution			

Appendix B – Chromatin Shearing Efficiency Analysis

1. Take a 25 µL aliquot of the sheared sample and transfer to 0.6 mL microcentrifuge tube.
2. Add 1 µL of RNase A (10 mg/mL) and incubate at 37 °C for 30 min.
3. Add 1 µL of Proteinase K (10 mg/mL) and reverse crosslink by heating at 65 °C overnight.
4. Purify DNA using either a commercial column based kit (e.g., Qiagen QIAquick PCR Purification Kit, Cat. No. 28104), or phenol-chloroform extraction and ethanol precipitation.
5. Elute from column, or resuspend pellet with 50 µL of elution buffer (10 mM Tris-HCl, pH 8.5).
6. Add 1 volume of loading dye to 5 volumes of purified DNA.
   NOTE: The use of loading dye without Bromophenol Blue is recommended. Bromophenol Blue migrates at ~300 bp and interferes with smear analysis.
7. Load 300 to 600 ng of purified DNA per lane.
8. Resolve on 1% agarose gel run at 30 V for 3.5 hours
9. Stain gel with Ethidium Bromide after the gel is run.
10. Destain and view gel with a UV light source and record image.
    NOTE: Alternatively, 1µL of purified DNA can be analyzed on an Agilent 2100 BioAnalyzer to provide a more accurate representation of the shearing size range and distribution.

Additional Notes:

1. The treatment settings listed in this document are recommended guidelines. Actual results may vary depending on the cell type and cell mass.
2. The Covaris process uses high frequency focused 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 scatter the acoustic energy from the sample. 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.
3. 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.

References:

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