PROTOCOL

Covaris

truChIP® Native Chromatin Shearing Kit

Adaptive Focused Acoustics™ (AFA)-based native chromatin shearing for ChIP-based Applications

Contents

INTENDED USE

The truChIP™ kit is intended for use in research applications (RUO). This product is not intended for the diagnosis, prevention, or treatment of disease.

INTRODUCTION

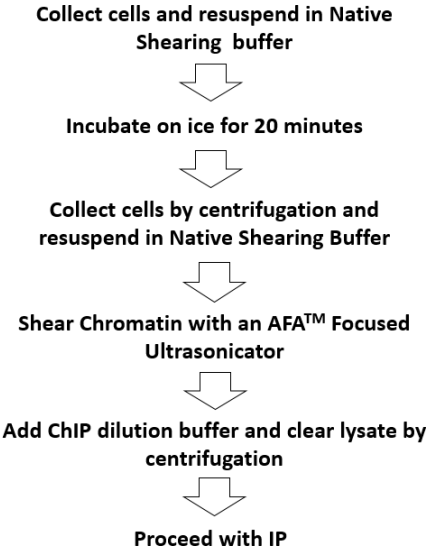
The truChIP™ Native Chromatin Shearing Kit is optimized for the efficient and reproducible shearing of chromatin from adherent and suspension mammalian cell lines using Covaris AFA™ Focused-ultrasonicators. This protocol provides highly reproducible, non-contact, and isothermal shearing to ensure a tight distribution of fragments without compromising the structural integrity of histone proteins is achieved. This truChIP kit can be used as an alternative to difficult to control enzymatic micrococcal nuclease (MNase) digestion of native chromatin (non-crosslinked) in preparation for ChIP-based applications.

truChIP™ is compatible with a variety of cell types including: primary cells, stem cells, and FACS sorted cells. To start, Covaris recommends users to perform a one-time shearing time course study to empirically determine the optimal treatment conditions.

REVISION HISTORY

Part Number	Revision	Date	Description of change
010427	A	08/17	As released

PROCEDURE OVERVIEW



SAMPLE INPUT REQUIREMENTS

The truChIP™ Native Chromatin Shearing Kit it is compatible with a range of cell inputs from less than 1 Million (1 x 10^6) cells and up to 200 Million (2x 10^8). The low cell protocol has been optimized for chromatin shearing of 1 to 3 Million Cells using the microTUBE-130 with AFA Fiber. The high cell has been optimized to process up to 30 Million cells using the milliTUBE-1mL with AFA fiber.

Single Sample	Low Cell	High Cell
Input cell number	1 to 3 Million (1-3 x 10^6) Cells	5 to 30 Million (0.5-3 x 10^7) Cells
AFA tube	microTUBE–130	milliTUBE–1 mL with AFA Fiber
Shearing volume	130 µl	1 mL

KIT CONTENTS

Buffer	Volume (mL)	Description
Buffer A	10	10X Native Shearing Buffer (NSB)
Buffer B	10	10X IP Dilution Buffer (IDB)
Buffer C	0.8	100X Protease Inhibitor Cocktail

Safety Data Sheets: http://covarisinc.com/resources/safety-data-sheets

STORAGE

The kit is shipped cold and should be stored at 2-8C.

Note: Mix solutions well before use to ensure solutions are completely solubilized

SUPPLIED BY USER

- Molecular Biology Grade Water – Thermo Scientific (Cat. No. SH3053802), Mo Bio (Cat. No. 17012-200), or equivalent
- 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 Focused-ultrasonicator (M220, ME220, S220, E220 Evolution, E220, or LE220)
- Refrigerated centrifuge having 15,000 x g capability
- Rocker – Nutator® or equivalent
- AFA tubes & holders/racks

Low Cell Consumables & Holders/Racks Required

Part Number	Description	M220 Holder & Insert	ME220 Holder & Insert	S-Series Holder	E220Evo Rack	E220 Rack	LE220 Rack
520045	microTUBE AFA Fiber Pre-Slit Snap-Cap	500414 & 500489	500514 & 500526	500114	500433	500111	NA
520052	microTUBE AFA Fiber Crimp-Cap	NA	500514 & 500526	500114	500433	500282	500282
520053	8 microTUBE Strip V1	NA	500514 & 500526	NA	500430	500191	500191
520217	8 microTUBE-130 AFA Fiber Strip V2	NA	500518 & 500526	NA	NA	NA	NA
520078	96 microTUBE Plate	NA	NA	NA	NA	NA	500329

High Cell Consumable & Holders/Racks Required

Part Number	Description	M220 Holder & Insert	ME220 Holder & Insert	S-Series Holder	E220Evo Rack	E220 Rack	LE220 Rack
520130	milliTUBE 1 mL with AFA Fiber	500414 & 500422	500520 & 500534	500371	500431	500368	500368

PROTOCOL

A. Cell Preparation and Chromatin shearing

The truChIP native chromatin shearing protocol uses a two-step lysis method to ensure reproducible and efficient shearing of both suspension and adherent cells. Follow the cell preparation method (**A.1 – Suspension and A.2 – Adherent**) for your cell culture type.

A.1 Suspension cells

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

Buffer	Low Cell	High Cell
1× cold PBS	**Final Volume: 2.0 mL per sample** - Store on ice	**Final Volume: 4 mL per sample** - Store on ice
1× Native Shearing buffer (NSB)	**Final Volume: 0.6 mL per sample** - Mix 60 µl of Buffer A with 0.534 mL of molecular biology grade water - Add 6 ul Buffer C	**Final Volume: 3 mL per sample** - Mix 300 µl of Buffer A with 2.67 mL of molecular biology grade water - Add 30 ul of Buffer C
2x IP Dilution Buffer (IDB)	**Final Volume: 0.5 mL per 1 to 20 samples** - Mix 100 µl of Buffer B with 0.40 mL of molecular biology grade water	**Final Volume: 1 mL per 1 to 5 samples** - Mix 200 µl of Buffer B with 0.80 mL of molecular biology grade water

2. Collect cells by centrifugation at 200 x g for 5 minutes at room temperature. Remove media and wash cells once with PBS and collect cells again by centrifugation.

Reagent	Low Cell	High Cell
PBS	400 µl	1.5 mL
Input cell number	1-3 x 10^6 Cells	1-3 x 10^7 Cells
Centrifuge Tube	2.0 mL	2.0 mL

Note 1: 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.

3. Re-suspend cells in cold 1X NSB.

Reagent	Low Cell	High Cell
1X NSB	400 µl	1.5 mL

4. Incubate on Ice for 20 minutes

5. Collect cells by centrifugation at 500 x g for 5 minutes at 4C.

6. Carefully remove all of the supernatant and resuspend in 1x NSB with protease inhibitors and proceed to chromatin shearing

Reagent	Low Cell	High Cell
1X NSB	130 µl	1.0 mL

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 x 10^6	~2.0 x 10^6	~5.5 x 10^6	~15 x 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 2: 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 and keep prepared solutions on ice.

Buffer	35 mm and 60 mm Dish	100 mm and 150 mm Plate
1× cold PBS	**Final Volume: 4 mL per Plate** - Store on ice	**Final Volume: 10 mL per Plate** - Store on ice
1× Native Shearing Buffer (NSB)	**Final Volume: 4 mL per Plate** - Mix 400 µl of Buffer A with 3.56 mL of molecular biology grade water - Add 40 ul of Buffer C	**Final Volume: 10 mL per Plate** - Mix 1000 µl of Buffer A with 8.9 mL of molecular biology grade water - Add 100 µl of Buffer C
2x IP Dilution Buffer (IDB)	**Final Volume: 300 µl** - Mix 60 µl of Buffer B with 240 µl of molecular biology grade water	**Final Volume: 1 mL** - Mix 200 µl of Buffer B with 0.8 mL of molecular biology grade water

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 Buffer NSB.

Reagent	35 mm and 60 mm Plate	100 mm and 150 mm Plate
1x NSB	2 mL	5 mL

5. Place plates on Ice for 20 minutes

6. Scrape cells from the plate into a proper vessel.

Reagent	35 mm and 60 mm Plate	100 mm and 150 mm Plate
Centrifuge Tube	15 mL conical	15 mL conical

7. Wash the plate with an additional volume of cold 1x NSB to collect any remaining cells.

Reagent	35 mm and 60 mm Plate	100 mm and 150 mm Plate
1x NSB	2 mL	5 mL

8. Collect cells at 200 x g for 5 minutes at 4C.

> **Note 3:** 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.

9. Carefully remove as much of the supernatant as possible, and resuspend in 1x NSB with protease inhibitors and proceed to chromatin shearing

Reagent	Low cell	High Cell
1x NSB	130 µl	1 mL

B. Chromatin Shearing

1. Shear chromatin with an AFA Focused-ultrasonicator with appropriate rack or holder; settings are provided in **Appendix A**.

> **Note 4:** Optimization of shearing time should be conducted whenever experimental parameters (*e.g.,* cell type, cell number, or sample volumes) are changed.

2. If processing samples for Low Cell Chromatin Shearing Optimization in microTUBEs, please aliquot 130 µl of the nuclei preparation into 6 microTUBEs for carrying out the shearing time course of 2, 4, 6, 8, 10, and 12 minutes.

Tube Number	1	2	3	4	5	6
Processing Time	2	4	6	8	10	12

If processing samples for High Cell Chromatin Shearing Optimization, please aliquot 1 mL of the nuclei into one milliTUBE–1 mL with AFA Fiber for carrying out the shearing time course of 2, 4, 8, 12, 15, and 20 minutes.

3. If processing samples using the milliTUBE–1 mL with AFA Fiber, you may process all time points of the time course study in the same tube according to the figure below. After each time point interval, remove 35 µl of the sample and place in in a pre-chilled microcentrifuge tube labeled with the total processing time–store on ice. Replace the removed volume with 35 µl of Native Shearing Buffer (NSB) before running the next time point.

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

4. Place the milliTUBE in the holder/rack and process on the ultrasonicator 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. Replace the removed volume with 35 µl of Native Shearing Buffer (NSB) before running the next time point.

5. After shearing, transfer samples into a pre-chilled microcentrifuge tube and place on ice until all tubes are processed. If batch processing multiple using a high-throughput ultrasonicator (*e.g.* E220), samples can be maintained in the instrument's water bath at 4C before and after processing.

6. For subsequent immunoprecipitation, dilute sheared chromatin 1:1 using the supplied 2X IP Dilution Buffer (IPD).

7. Vortex the sample for 5 seconds and centrifugation at 2000 x g at 4C to clear the lysate.

8. Transfer the cleared supernatant to a clean tube and proceed with IP.

Note 5: To check the efficiency of your shearing, reserve 25 µl of the sheared chromatin and see **Appendix B** for detailed instructions.
Note 6: Storing sheared chromatin is not recommended.
Note 7: Freezing sheared chromatin is not recommended. Freeze/thaw cycles reduce IP efficiency and reproducibility.

SUPPLEMENTAL MATERIAL

Appendix A: AFA: Focused-ultrasonicator Operating Conditions

Low Cell Native Chromatin Shearing Protocol						
Instrument	**M220**	**ME220**	**S220**	**E220 Evolution**	**E220**	**LE220**
Target Size (bp)	200-700	200-700	200-700	200-700	200-700	200-700
PIP	75	75	70	70	70	300
Duty Factor (%)	1	1	2	2	2	5
CPB	200	1000	200	200	200	200
Treatment Time (minutes)	2-20	2-20	2-12	2-12	2-12	15-30
Setpoint Temperature (C) [1]	7	9	6	6	6	6
Min/Max Temperature (C)	4/10	6/12	3/9	3/9	3/9	3/9
Max cell Number (Million)	3M	3M	3M	3M	3M	3M
AFA Intensifier Required [2]	NA	NA	Integrated	Yes	Yes	NA
Water Level (run) [3]	Full	9	12	6	6	6
Sample Volume (µl) [4]	130	130	130	130	130	130

Important Notes

1. If using the S220, E220 Evolution, E220, or LE220, set the temperature on the external chiller 3C below the setpoint temperature for the run. The min/max is set in SonoLab
2. If intensifier is required, please ensure PN 500141 is used
3. Water level should always be 1mm below the neck of the microTUBE–130 cap
4. Always fill the microTUBE-130 with 130 µl of sample

High Cell Native Chromatin Shearing Protocol						
Instrument	**M220**	**ME220**	**S220**	**E220 Evolution**	**E220**	**LE220**
Target Size (bp)	200-700	200-700	200-700	200-700	200-700	200-700
PIP	75	75	105	105	105	300
Duty Factor (%)	2	5	5	5	5	5
CPB	200	1000	200	200	200	200
Treatment Time (minutes)	2-20	2-20	2-20	2-20	2-20	15-30
Setpoint Temperature (C) [1]	7	9	6	6	6	6
Min/Max Temperature (C)	4/10	6/12	3/9	3/9	3/9	3/9
Max Cell Number (Million)	30M	30M	30M	30M	30M	30M
AFA Intensifier Required	NA	NA	NA	NA	NA	NA
Water Level (run) [2]	Full	9	8	5	0	-4
Sample Volume (mL) [3]	1	1	1	1	1	1

Important Notes

1. If using the S220, E220 Evolution, E220, or LE220, set the temperature on the external chiller 3C below the setpoint temperature for the run. The min/max is set in SonoLab
2. Water level should always be 1mm below the neck of the milliTUBE–1 mL with AFA Fiber cap
3. Always fill the milliTUBE–1 mL with AFA Fiber with 1.0 mL of sample

Appendix B: Chromatin Shearing Efficiency Analysis Protocol

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 37C for 30 min.
3. Add 1 µl of Proteinase K (10 mg/mL) and incubate at 56C for 2 hours in a PCR cycler with a heated lid.
4. Purify DNA using a commercial column based kit (*e.g.,* Qiagen QIAquick PCR Purification Kit, Cat. No. 28104)
5. Elute from column or resuspend pellet with 50 µl of elution buffer (10 mM Tris-HCl, pH 8.5).
6. Load 1µl of purified DNA onto the DNA 12000 chip and run on the Agilent 2100 BioAnalyzer.

Appendix C: Additional Note**s**

1. The treatment settings listed in this document are recommended guidelines. Actual results may vary depending on the cell type and mass.
2. The Covaris process uses high intensity focused ultrasonic (HIFU) 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. 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.