PROTOCOL

Covaris

truChIP® Ultra-Low Chromatin Shearing Kit

Adaptive Focused Acoustics (AFA)-based chromatin shearing for ChIP-based applications

Products PN 520156 and PN 520158

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

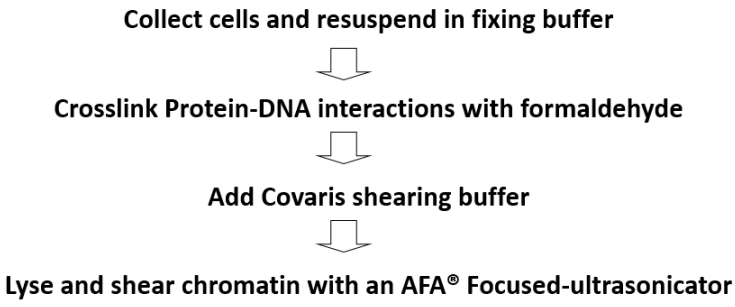
The truChIP Ultra-Low Chromatin Shearing Kit is optimized for the efficient and reproducible shearing of chromatin from adherent and suspension mammalian cell lines using Covaris AFA Focused-ultrasonicators. Focused-ultrasonicators provide a non-contact and isothermal method of shearing chromatin without compromising the structural integrity of the target epitopes of interest. This kit can be used to prepare sample for ChIP-qPCR, ChIP-ChiP, and ChIP-seq applications.

truChIP is compatible for use with a variety of cell lines, however, additional optimization for your specific cell line may improve results. To start, Covaris recommends for users to perform a one-time fixation and shearing time course study to empirically determine the optimal treatment conditions.

REVISION HISTORY

Part Number	Revision	Date	Description of change
010255	C	04/17	Update template and publish ME220 settings
010255	D	7/17	Remove specific content information for Buffer D3
010255	E	7/17	Correct procedure overview
010255	F	11/17	Added step in chromatin shearing protocol
010255	G	1/19	Update LE220 chromatin shearing settings

PROCEDURE OVERVIEW



SAMPLE INPUT REQUIREMENTS

The truChIP Ultra-Low Chromatin Shearing Kit is designed to perform efficient chromatin shearing of 100,000 cells or less.

Single Sample	Low Cell
Input cell number	<100,000
Number of samples sheared per kit	50
AFA tube	microTUBE–130
Shearing volume	130 µl

KIT CONTENTS

Buffer A	4 mL	10X Fixing Buffer
Buffer D3	6 mL	10X SDS Shearing Buffer
Buffer E	6 mL	1X Quenching Buffer
Buffer F	0.8 mL	100X Protease Inhibitor Cocktail
Formaldehyde	2x1 mL ampules (PN 520156 only)	16% methanol-free formaldehyde

Note: Certain mammalian cell lines may have more proteases (nucleases), therefore, end-users can substitute **Buffer F** with other commercially available protease inhibitor cocktails if required.

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

STORAGE

The kit is shipped cold and should be stored at 2-8C. Prior to use, kit reagent Buffers D3 and E may have to be warmed to 55C to dissolve precipitate and then cooled to room temperature before use.

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

Ultra-Low Cell Protocol (<100K) 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
520216	microTUBE-130 AFA Fiber Screw-Cap	500414 & 500489	500522 & 500534	500339	NA	NA	NA
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	No rack required	500329

PROTOCOL

A. Cell Preparation and Crosslinking

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. Therefore, Covaris recommends users to include multiple fixation and shearing time points to empirically determine the optimal treatment conditions.

A.1 Suspension cells

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

Buffer	Instructions
1X Cold PBS	**Final Volume: 2.0 mL per sample** - 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 of molecular biology grade water
Fresh 5% Formaldehyde	**Final Volume: 1 mL per 1 to 20 samples** - Mix 312.5 µl of 16% Fresh Formaldehyde with 687.5 µl of molecular biology grade water
Quenching Buffer E	Place in a 55C water bath to dissolve crystals, then place at ambient

Note: The use of fresh methanol-free formaldehyde is required to achieve reproducible results. The methanol-free formaldehyde ampule is for one-time use only–storage for later use is not recommended

2. Collect cells by centrifugation at 200 x g for 5 minutes at room temperature. Remove media and wash cells once with 1X Cold PBS and collect cells again by centrifugation.

Reagent	Ultra-Low Cell
1X Cold PBS	400 µl
Input cell number	$1x10^5$ Cells
Centrifuge Tube	1.5 mL

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.

3. Resuspend cells in room temperature 1X Fixing Buffer A.

Reagent	Ultra-Low Cell
Fixing Buffer A	400 µl

4. Crosslink cells by adding freshly prepared 5% formaldehyde solution to a final concentration of 0.25% and set timer.

Reagent	Ultra-Low Cell
Fresh 5% Formaldehyde	20 µl

> **Note:** The use of fresh methanol-free formaldehyde is required to achieve reproducible results.

5. Place cells on a rocker at room temperature for the recommended time.

> **Note:** We recommend including two fixation times. Typically, **2.5 and 5 minutes** for stem and primary cells, and **5 and 10 minutes** for all other cell types.

6. Quench the crosslinking reaction by adding the appropriate volume of Quenching Buffer E to the fixed cells. Keep cells on a rocker at room temperature for an additional 5 minutes.

Reagent	Ultra-Low Cell
Quenching Buffer E	12 µl

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	Ultra-Low Cell
1X Cold PBS	300 µl

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

10. Proceed to nuclei preparation and chromatin shearing steps.

> **Note:** You may flash-freeze the fixed cells in liquid nitrogen at this point and store at -80C for short periods of time (*e.g.,* 2 to 3 days). Longer-term storage is not recommended.

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.

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

Buffer	Instructions
1X cold PBS	**Final Volume: 14 mL** - Store on ice
1X Fixing Buffer A	**Final Volume: 2 mL** - Mix 200 µl of Fixing Buffer A with 1.8 mL of molecular biology grade water
Fresh 5% Formaldehyde	**Final Volume: 1 mL** - Mix 312.5 µl of 16% Fresh Formaldehyde with 687.5 µl of molecular biology grade water
Quenching Buffer E	Place in a 55C water bath to dissolve crystals, then place at ambient
Important Notes • The use of fresh methanol-free formaldehyde is required to achieve reproducible results • The methanol-free formaldehyde ampule is for one-time use only–storage for later use is not recommended	

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

Reagent	Volume
1X PBS	2 mL

4. Remove PBS and add room temperature 1X Fixing Buffer A to each dish.

Reagent	Volume
Fixing Buffer A	2 mL

5. Crosslink cells by adding freshly prepared 5% formaldehyde solution to a final concentration of 0.25% and start timing the crosslinking reaction.

Reagent	Volume
Fresh 5% Formaldehyde	100 µl

> **Note:** We recommend including two fixation times. Typically, **2.5 and 5 minutes** for stem and primary cells, and **5 and 10 minutes** for all other cell types.

6. Place cells on a rocker at room temperature for the recommended time.

> **Note:** The use of fresh methanol-free formaldehyde is required to achieve reproducible results.

7. Quench the crosslinking reaction by adding the appropriate volume of Quenching Buffer E to the fixed cells. Keep cells on a rocker at room temperature for an additional 5 minutes.

Reagent	Ultra-Low Cell
Quenching Buffer E	60 µl

8. Completely aspirate the solution from the plate.

9. Add cold PBS to each dish and scrape cells from the plate into the proper vessel.

Reagent	Ultra-Low Cell
1X Cold PBS	450 µl
Centrifuge Tube	1.5 mL tube

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

Reagent	Ultra-Low Cell
1X Cold PBS	450 µl
Centrifuge Tube	1.5 mL tube

11. Collect cells by centrifuging at 500 x g for 5 minutes, 4C.

> **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 PBS and collecting by centrifugation at 500 x g, 4C.

Reagent	Ultra-Low Cell
1X Cold PBS	450 µl
Centrifuge Tube	1.5 mL tube

13. Completely and carefully aspirate the supernatant from the tube(s) and place tube(s) on ice.

14. Proceed to chromatin shearing steps.

> **Note:** You may flash-freeze the fixed cells in liquid nitrogen at this point and store at -80C for short periods of time (*e.g.,* 2 to 3 days). Longer-term storage is not recommended.

B. Chromatin Shearing

1. Using the table below, prepare a sufficient volume of 1× Shearing Buffer D3 using the Buffer D3 and Buffer F stocks. A 15% excess volume is recommended when preparing this buffer.

Total Number of Samples	Buffer D3 – 10X SDS Shearing Buffer	Buffer F – 100X Protease Inhibitor Cocktail	Molecular biology grade water
1	15 µl	1.5 µl	133.5 µl
6	90 µl	9 µl	801 µl
12	180 µl	18 µl	1.6 mL
24	360 µl	36 µl	3.2 mL
X	X 15 µl	X 1.5 µl	X 133.5 µl

* Calculations include 15% excess

2. Resuspend nuclei pellet in the Shearing Buffer D3 and transfer to appropriate AFA Tube(s). If conducting a shearing time course experiment, aliquot 130 µl of ≤100,000 fixed cells into 6 microTUBEs.

Reagent	Ultra-Low Cell
Shearing Buffer D3	130 µl
AFA Tube	microTUBE-130

3. Shear chromatin with an AFA Focused-ultrasonicator with appropriate rack or holder; settings are provided in **Appendix A.** For the shearing time course, use processing times of 2, 4, 6, 8, 10, and 12 minutes.

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

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

5. After processing, add 2-3 volumes of your IP buffer, and centrifuge samples at 10,000 x g, 4C for 5 minutes to pellet insoluble material

6. Transfer the supernatant to a new pre-chilled microcentrifuge tube.

Note: To check the efficiency of your shearing, reserve 130 µl of the sheared chromatin and see **Appendix B** for detailed instructions
Note: Sheared chromatin can be stored at 4C for up to 2 days
Note: Freezing sheared chromatin is not recommended. Freeze/thaw cycles reduce IP efficiency and reproducibility
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 1× SDS Shearing Buffer D3 composition is: 10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.1% SDS**

SUPPLEMENTAL MATERIAL

Appendix A: AFA Focused-ultrasonicator Operating Conditions

Ultra-Low Cell 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 (%)	5	5	2	2	2	15
CPB	200	1000	200	200	200	200
Treatment Time (minutes)	2-12	2-12	2-12	2-12	2-12	2-20
Setpoint Temperature (C) [1]	7	7	6	6	6	6
Min/Max Temperature (C)	4/10	6/12	3/9	3/9	3/9	3/9
Sample Input	<100,000	<100,000	<100,000	<100,000	<100,000	<100,000
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

Appendix B: Chromatin Shearing Efficiency Analysis Protocol

1. Take a 130 µl aliquot of the sheared sample and transfer to 0.6 mL microcentrifuge tube.
2. Add 5 µl of RNase A (10 mg/mL) and incubate at 37C for 30 min.
3. Add 5 µl of Proteinase K (10 mg/mL) and reverse crosslink by heating at 65C overnight in a PCR cycler with a heated lid.
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 25 µl of elution buffer (10 mM Tris-HCl, pH 8.5).
6. 1µL of purified DNA can be analyzed on an Agilent 2100 BioAnalyzer 12K chip to provide a more accurate representation of the shearing size range and distribution.
7. Alternatively, an aliquot of the sample can be run on a High Sensitivity Agilent 2100 chip.

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.

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