

truCHIP™ Ultra Low Cell Chromatin Shearing Kit

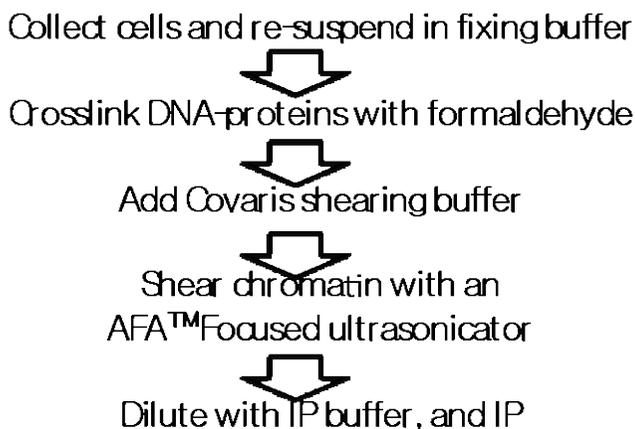
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INTRODUCTION

The truChIP™ Ultra Low Cell 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 Ultra Low Chromatin Shearing Kit is designed to carry out efficient chromatin shearing of 100,000 cells or less

Single Sample	Ultra Low Cell
Input cell number	≤100000
Number of samples sheared per kit	50
AFA Tube	microTUBE
Shearing Volume	130 µL

KIT CONTENTS

Formaldehyde	2 x 1 mL ampoules (Kit 520156 only)
Buffer A	4 mL
Buffer D3	6 mL
Buffer E	6 mL
Buffer F	0.8 mL

Content Descriptions

Formaldehyde 16% methanol-free formaldehyde (ThermoFisher part number 28906)
Remaining formaldehyde from the ampoules should not be stored, and discarded. If the kit is going to be used with multiple experiments, then additional ampoules should be ordered.

Buffer A 10X Fixing 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

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
- 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	500282	NA
520053	8 microTUBE strip	NA	NA	500191	500191
520078	96 microTUBE plate	NA	NA	No rack required	500329

(*) This holder has been discontinued

PROTOCOL

A. Cell Preparation and Crosslinking

For suspension cells (non adherent) proceed to step A.1 and for adherent cells proceed to step A.2..

NOTE: ChIP assays are sensitive to crosslinking and shearing conditions. Before conducting ChIP experiments with this kit it is strongly advised to optimize these conditions by testing multiple fixation times and performing a shearing time course study.

A.1 Suspension cells

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

Reagent	Prepared Volume
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 Water
Fresh 5% Formaldehyde	Final Volume: 1 mL per 1 to 20 samples -Mix 312.5 µL of Fresh 16% Formaldehyde with 687.5 µL 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. 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	Volume
PBS	400 µL
Input cell number	1 x 10 ⁵ Cells
Centrifuge Tube	1.5 mL

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

Reagent	Volume
Fixing Buffer A	400 µL

4. Crosslink cells by adding freshly prepared 5% formaldehyde solution to a final concentration of .25% and start timing the crosslinking reaction. **NOTE: The use of fresh methanol-free formaldehyde is absolutely required to attain reproducible results.**

Reagent	Volume
Fresh 5% Formaldehyde	20 μ L

- Place cells on a rocker at room temperature for the desired time (e.g., 5 minutes for most cell types) to achieve efficient crosslinking. **NOTE: Optimal crosslinking time and shearing conditions should be determined empirically for each cell type⁴⁻⁶.**
- Quench the crosslinking reaction by adding Buffer E to the fixed cells. Place the cell suspension back on the rocker at room temperature for an additional 5 minutes.

Reagent	Volume
Quenching Buffer E	12 μ L

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

Reagent	Volume
Cold PBS	300 μ L

- Collect cells by centrifugation at 500 x g for 5 minutes, 4 °C.
- Proceed to 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

- Grow the required number of cells needed for the initial time course or ChIP experiment until they are 80 to 90% confluent.
- Prepare solutions for the appropriate number of samples being processed fresh before starting.

Reagent	Prepared Volume
1X cold PBS	Final Volume: 14 mL -Store on ice
1X Fixing Buffer A	Final Volume: 2 mL -Mix 200 μ L of Fixing Buffer A with 1.8 mL Water
Fresh 5% Formaldehyde	Final Volume: 1000 μL - Mix 312.5 μ L of Fresh 16% Formaldehyde with 687.5 μ L 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.	
NOTE: Use Molecular Biology Grade Water for the preparation of all solutions.	

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

Reagent	Volume
PBS	2 mL

- Remove PBS and add room temperature 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. **NOTE: The use of fresh methanol-free formaldehyde is absolutely required to attain reproducible results.**

Reagent	Volume
Fresh 5% Formaldehyde	100 μ L

6. Place cells on a shaking platform at room temperature for the desired time (e.g., 5 minutes for most cell types) to achieve efficient crosslinking. **NOTE: Optimal crosslinking time and shearing conditions should be determined empirically for each cell type⁴⁻⁶.**
7. Quench the crosslinking reaction by adding the appropriate volume of Buffer E to fixed cells. Continue mixing on a shaking platform at room temperature for an additional 5 minutes.

Reagent	Volume
Quenching Buffer E	60 μ L

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	Volume
Cold PBS	450 μ L
Centrifuge Tube	1.5 mL tube

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

Reagent	Volume
Cold PBS	450 μ L
Centrifuge Tube	1.5 mL tube

11. Collect cells at 500 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 PBS, and collecting by centrifugation at 500 x g, 4 °C.

Reagent	Volume
Cold PBS	450 μ L

13. Completely and carefully aspirate the supernatant from the tube(s), and place tube(s) on ice.
14. Proceed to 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. Chromatin Shearing

1. Resuspend fixed cells in the Shearing Buffer D3 and transfer to AFA Tube(s) If conducting a shearing time course experiment aliquot 130ul of $\leq 100,000$ fixed cells into 6 microTUBEs.

Reagent	Volume
Shearing Buffer D3	130 μ L
AFA Tube	1 microTUBE

2. Shear chromatin with an AFA Focused-ultrasonicator with appropriate rack or holder (refer to table in supplies section) with the settings provided in Appendix A. For a 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.**
3. 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.
4. After processing, add 2-3 volumes of your IP buffer, and centrifuge samples at 10,000 x g, 4 °C for 5 minutes to pellet insoluble material.
5. Transfer the supernatant to a new pre-chilled microcentrifuge tube; this is your sheared chromatin to proceed with the immunoprecipitation step of your CHIP protocol.

NOTE: To check the efficiency of the chromatin shearing in the initial time course, reserve 130 μ L of the sheared chromatin, 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 has to be diluted in the desired immunoprecipitation buffer. 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

Ultra Low Cell Chromatin Shearing Protocol			
	M-Series	S- and E-Series	L-Series
Target BP (Range)	200-700	200-700	200-700
Duty Factor	5%	2%	15%
Intensity Peak Incidence Power	N/A 75 Watts	3 (S2 and E210) 105 Watts (S220 and E220)	N/A 300 Watts
Cycles per Burst	200	200	200
Processing Time	Empirical (2-12 min typical)	Empirical (2-12 min typical)	Empirical (15-30 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	100,000	100,000	100,000
AFA Intensifier	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 microTUBE plate (PN 520078) requires Rack-XT for 96 microTUBE Plate (PN 500329)
*Water level should be 1mm below the bottom on the microTUBE cap			
IMPORTANT: Always fill microTUBES with 130 µL of sample			

Appendix B – Chromatin Shearing Efficiency Analysis

1. Take a 130 μ L aliquot of the sheared sample and transfer to 0.6 mL microcentrifuge tube.
2. Add 5 μ L of RNase A (10 mg/mL) and incubate at 37 °C for 30 min.
3. Add 5 μ L 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., QIAquick PCR Purification Kit, QIAGEN Cat. No. 28104), or phenol-chloroform extraction and ethanol precipitation.
5. Elute from column, or resuspend pellet with 25 μ L of elution buffer (10 mM Tris-HCl, pH 8.5).
6. 1 μ L 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.
Please note that the size range will seem larger, and the distribution wider when using the High Sensitivity chip.

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.

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