*tru*ChIP™ High Cell Chromatin Shearing Kit with Non-ionic Shearing Buffer

INTRODUCTION

The *tru*ChIP™ High Cell Chromatin Shearing Kit with Non-ionic Shearing Buffer (PN 520075) is designed and optimized for the efficient and reproducible shearing of chromatin from adherent and suspension cell lines specifically using Covaris AFA™ (Adaptive Focused Acoustics) technology.

Depending on the type of starting material, this kit may require the end-user to optimize crosslinking and shearing steps.

AFA technology allows for a non-contact, isothermal method of shearing chromatin without compromising the structural integrity of the epitopes of interest for use in ChIP-qPCR, ChIP-Chip, and ChIP-Seq applications.

> **Important**: The reagents, consumables, and every step of the included protocol in this kit are designed and optimized specifically for Covaris AFA technology. Therefore, it is important to follow the procedure outlined in this document while using the reagents included in the kit to generate reproducible and optimal data.

KIT CONTENTS

Buffer A	10 ml of 10X Covaris Fixing Buffer
Buffer B	10 ml of 5X Covaris Lysis Buffer
Buffer C	5 ml of 10X Covaris Wash Buffer
Buffer D	10 ml of 10X Covaris Non-ionic Shearing Buffer
Buffer E	6 ml of 1X Covaris Quenching Buffer
Buffer F	0.8 ml of 100X Halt Protease Inhibitor cocktail (Thermo Scientific Cat#78438)
TC12x12 AFA Tubes	(12) 12x12 mm round bottom borosilicate tubes with AFA fiber and screw caps

> NOTE: MSDS information is available at www.covarisinc.com/chromatin-shearing.html.

> NOTE: TC12x12 AFA tubes are available in packages of 100 (PN 520081)

Storage

The kit is shipped cold and should be stored at 4-8°C. Prior to use, kit reagent Buffers D1 and E may have to be warmed to 55°C to dissolve precipitate and cooled to room temperature before use.

> NOTE: Mix buffers well to insure uniformity before use.

Reagents Supplied By User

- Molecular Biology Grade Water – Thermo Scientific (Cat. No. SH3053802), Mo Bio (Cat. No. 17012-200), or equivalent
- 16% Formaldehyde, Methanol-free – Thermo Scientific (Pierce) (Cat. No 28908, 10 ml), 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# EN0531) or equivalent
- Proteinase K (RNase and DNase free) Thermo Scientific (Cat#17916) or equivalent

Equipment Supplied By User

- Covaris S- or E-series instrument with chiller
- Refrigerated centrifuge with 10,000 x g capability
- Rocker - Nutator® or equivalent

Sample Quantity

The kit contains enough reagents and TC12X12 tubes (Covaris Cat# 520081) for:

1. Processing ~$5x10^7$-$2x10^8$cells for the initial time course utilizing 6 TC12X12 tubes.
2. Additional reagents and 6 extra TC12X12 tubes for processing another ~$5x10^7$-$2x10^8$cells once the processing conditions have been determined from the time course experiment.

Reagents in the kit are sufficient to process five 150 mm culture dishes for a total of 5 x 10^7 cells.

Procedure Overview

Collect cells and re-suspend in fixing buffer

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Crosslink DNA-proteins with formaldehyde

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Lyse the cells and isolate nuclei

⇩

Wash nuclei, re-suspend in shearing buffer

⇩

Lyse nuclei and shear chromatin

PROTOCOLS

Crosslinking of Suspension Cells

Efficient crosslinking without over crosslinking the chromatin is essential for optimal shearing. It is strongly advised that you carry out a crosslinking time course experiment to determine the optimal crosslinking time for your cells. Effective crosslinking time of cell lines can vary from as low as 20 seconds to as high as 5 minutes.

This method is for the effective crosslinking of 1.2 x 10^8 cells for use with the Covaris Chromatin Shearing Kit. Please note that the equivalent of 1-3 x 10^7 cells can be sheared in a single TC12X12 tube. To establish the optimal shearing conditions, the nuclei from ~10^8 cells should be prepared for carrying out the initial six time-point shearing time course.

> Important: The crosslinking steps and reagents are specifically designed for use with Covaris AFA technology. Follow all steps of the protocol accordingly in order to insure efficient preparation of your cells for chromatin shearing.

Solutions to prepare for this section:

- Place **Covaris Quenching Buffer** (Buffer E) in a 55°C water bath to dissolve crystals, and then place at room temperature prior to use.
- Prepare **20 ml of 1X Covaris Fixing Buffer** by mixing **2 ml** of the 10X Fixing Buffer (A) with **18 ml of Molecular Biology grade Water**.
- Prepare **20 ml** fresh **11.1% formaldehyde solution** by mixing **13.875 ml of 16% HCHO** with **6.125 ml of Molecular Biology grade Water**.
- Prepare **40 ml of 1X solution of 1X PBS**. Store on ice.

1. **Spin down ~10^8 cells at 100-200 x g for 5 minutes at room temperature (RT). Remove media and wash cells once with 20.0 ml of PBS. Spin cells down at 100-200 x g for 5 min. Remove PBS carefully.**
2. **Resuspend cells in 20 ml of Covaris Fixing Buffer (A).**
3. **Crosslink cells by adding 2 ml of the freshly prepared formaldehyde solution and start timing the crosslinking reaction.**

NOTE: The use of fresh methanol-free formaldehyde solution is essential in reproducible crosslinking of cells. The use of a sealed ampoule is recommended. The use of a previously opened bottle or ampoule is not recommended.

4. **Place cells on a rocker at room temperature (RT) for 5 minutes to allow for efficient crosslinking.**

NOTE: Optimal crosslinking time is cell line dependent, as well as cell concentration dependent. We strongly advise optimization of the crosslinking step. Excessive crosslinking or insufficient exposure to formaldehyde may result in failure to detect specific protein DNA interactions.

5. **Quench the crosslinking reaction by adding 1.2 ml of Covaris Quenching Buffer (E) to the fixed cells. Keep on rocker at RT for 5 minutes.**
6. **Spin cells down at 100-200 x g for 5 minutes at RT, and aspirate the supernatant.**
7. **Wash the cells twice with 5.0 ml of cold PBS. Spin cells down at 100-200 x g for 5 minutes at 4°C, and completely aspirate the PBS.**
8. **Proceed to nuclei preparation (next section).**

Crosslinking of Adherent Cells

NOTE: The crosslinking steps and reagents are specifically designed for use with Covaris AFA technology. Follow all steps of the protocol accordingly to insure efficient preparation of your cells for chromatin shearing.

Solutions to prepare for this section:

- Place **Covaris Quenching Buffer** (Buffer E) in a 55°C water batch to dissolve crystals, and then place at room temperature prior to use.
- Prepare **50 ml of 1X Covaris Fixing Buffer** by mixing **5 ml** of the 10X Fixing Buffer (A) with **45 ml of Molecular Biology grade Water**.
- Prepare **20 ml of** fresh **11.1% formaldehyde solution** by mixing **13.875 ml of 16% HCHO** with **6.125 ml of Molecular Biology grade Water**.
- Prepare **200 ml of 1X solution of PBS**. Store on ice.

1. **Grow cells to 80-90% confluency in a 150 mm culture dish containing 20 ml of growth media. This should generate ~1-2x10^7 cells/dish.**
2. **Remove media, and wash with 10.0 ml of PBS.**
3. **Remove PBS.**
4. **Add 5 ml of Covaris Fixing Buffer to each culture dish.**
5. **Fix cells by adding 0.5 ml of the freshly prepared formaldehyde solution to each plate, and start timing the crosslinking reaction.**

NOTE: The use of fresh methanol-free formaldehyde solution is essential in reproducible crosslinking of cells. The use of a fresh sealed ampoule is recommended. The use of a previously opened bottle or ampoule is not recommended.

6. **Place plate on a rocker at RT for 5 minutes to allow efficient crosslinking.**

NOTE: Optimal crosslinking time is cell line dependent, as well as cell concentration dependent. We strongly advise optimization of the cross linking step. Excessive crosslinking or insufficient exposure to formaldehyde may result in failure to detect specific protein DNA interactions.

7. **Quench the crosslinking reaction by adding 0.3 ml of Covaris Quenching Buffer (E) to each dish. Keep on rocker at room temperature (RT) for an additional 5 minutes.**
8. **Completely aspirate the solution from the plate.**
9. **Add 5.0 ml cold PBS to each dish and scrape cells from the plate.**
10. **Add an additional 2.0 ml volume of cold PBS to collect remaining cells from the plate.**
11. **Collect the scraped cells into a 50 ml conical tube.**
12. **Spin cells down at 100-200 x g for 5 minutes at 4°C.**
13. **Wash the pellet twice by resuspending the cells in 5.0 ml of cold PBS. Spin cells down at 100-200 x g for 5 minutes at 4°C.**
14. **Proceed to nuclei preparation (next section)**

Nuclei Preparation

IMPORTANT: The cell lysis and nuclei preparation steps and reagents are specifically designed for use with the Covaris AFA technology. Follow **ALL** steps of the protocol exactly to insure efficient and reproducible chromatin shearing. Substituting any of the reagents or any of the steps will adversely affect the efficient shearing of the chromatin, and subsequent IP efficiency.

Solutions to prepare for this section:

- Prepare **10 ml of 1X Covaris Lysis Buffer** by mixing **2 ml** of the 5X Lysis Buffer (B) with **8 ml of cold Molecular Biology Grade Water.** Add **100 µl** of the **100X Protease inhibitor** stock solution, and keep on ice.
- Prepare **11 ml of 1X Covaris Wash Buffer** by mixing **1.1 ml** of the 10X Wash Buffer (C) with **9.9 ml of cold Molecular Biology Grade Water. Add 110 µl of the 100X Protease inhibitor** stock solution, and keep on ice.
- Prepare **13 ml of 1X Covaris Shearing Buffer** by mixing **1.3 ml** of the 10X Shearing Buffer (D) with 11.7 ml of cold Molecular Biology Grade Water. Add 130 µl of the **100X Protease inhibitor** stock solution, and keep on ice.
- Remove 6 of the TC12X12 tubes from the box, and place the tubes on ice.

1. **Thaw crosslinked cells on ice.**
2. **Add 10.0 ml Covaris Lysis Buffer (B) containing protease inhibitors.**
3. **Incubate for 10 minutes on a rocker (or equivalent) at 4°C.**
4. **Pellet nuclei by spinning at 1,700 x g for 5 minutes at 4°C.**
5. **Resuspend pellet in 10.0 ml of Covaris Wash Buffer (C) containing protease inhibitors and incubate for 10 minutes at 4°C on rocker.**
6. **Spin nuclei down at 1,700 x g for 5 minutes at 4°C.**
7. **Gently rinse the sides of the tube with 1.5 ml Covaris Shearing Buffer (D) containing protease inhibitors by slowly dispensing the wash buffer down the inside of the tube so as not to disturb the nuclei pellet.**

NOTE: The purpose of this wash is to significantly dilute any remaining salts from the Covaris Wash Buffer in step 5. Shearing in presence of high salt may lead to reversing the crosslinks during processing.

8. **Spin nuclei down at 1,700 x g for 5 minutes at 4°C.**
9. **Repeat steps 7 and 8 one more time. Carefully remove and discard the supernatant so as not to disturb the pellet. Continue the procedure as described in step 10 or alternatively, the nuclei pellet can be flash frozen and stored at -80°C.**
10. **Resuspend pellet in Covaris Shearing Buffer (D) containing protease inhibitors. Use 1.0 ml of the buffer per maximum of 1-3 x 10^7 cell equivalents.**
11. **For the initial time course experiment, we suggest that you process enough cells for six 1.0ml aliquots containing 1- 3 x 10^7 cells each.**

NOTE: Carry out a time course shearing experiment using your cell line to optimize the chromatin shearing parameters specific for your cell line and cell mass. We suggest a time course of 2, 4, 8, 12, 15 and 20 minutes.

Chromatin AFA Shearing

Summary of Operating Conditions

Target Base Pair (Range)	**200-700**
Duty Cycle	5%
Intensity	4 for (S2 or E210)
Peak Incident Power	140 Watts for (S220/E220)
Cycles per Burst	200
Processing Time	Run an **initial** time course between 2 and 20 minutes to determine optimal shearing time for your sample.
Temperature (bath)	4°C
Power mode	Frequency Sweeping (S2 and E210 only)
Degassing mode	Continuous
Volume	1.0 ml in TC12x12 AFA tubes

IMPORTANT: Always fill the TC12x12 tubes with 1 ml of solution for AFA treatment

Maximum cells equivalent per tube	1 x 10^7 cells
*Water level (RUN)	S2/S220 – level 8 E210/E220 – level 5

*Water level should be ~1mm below the bottom of the TC12x12 AFA tube cap

AFA Intensifier	The S2 instrument intensifier is built into the holder. The E instrument **requires the Intensifier (See Insert)**

Supplies	**Description**	**Part Number**
Sample Vessel	1.0 ml Covaris TC12x12 AFA tubes (12 x 12mm) with AFA fiber	520081
Holder- S2/S220	Holder for TC12x12 AFA tubes	500274
Rack-E210/E220	12 tube rack: for TC12x12 AFA tubes	500276
Note: Rack for E210/E220 requires Intensifier		500141

After Covaris Treatment:

1. **Transfer the sheared samples into cold 1.5ml microcentrifuge tubes and centrifuge at 10,000 x g at 4°C for 5 minutes to pellet insoluble material.**

NOTE: If processing samples on the S2/S220 system, transfer the sample into a microcentrifuge tube and place on ice as the subsequent samples are being processed.

2. **Transfer the supernatant containing sheared chromatin to a new cold microcentrifuge tube.**
3. **Remove 50 µl of the supernatant for chromatin shearing efficiency analysis described in the next section.**
4. **The remaining sheared chromatin can be used in accordance with your immunoprecipitation protocol or flash frozen and stored at -80°C. Sheared crosslinked chromatin can be stored at -80°C for up to 3 months.**

NOTE: The Chromatin shearing buffer contains (NOT yet determined). You will have to equilibrate the salt and detergent in the sheared chromatin in accordance with the requirements of your immunoprecipitation protocol.

Chromatin Shearing Efficiency Analysis

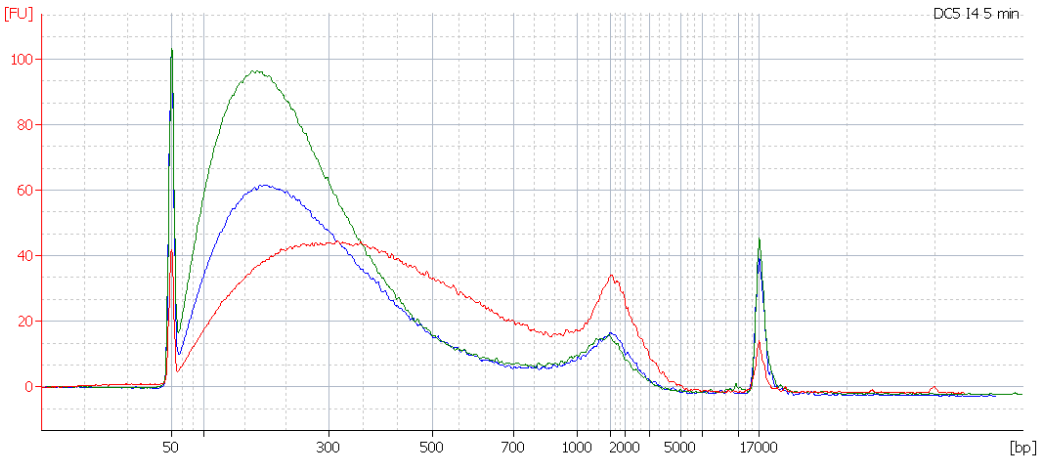
1. **Take the 50 µl aliquot of the sheared sample and transfer to 1.5 ml microcentrifuge tube.**
2. **Add 1 µl of 10 mg/ml RNase A (DNase free) and incubate at 37°C for 30 min.**
3. **Add 1 µl of 10 mg/ml Proteinase K and reverse crosslink overnight at 65°C.**

We recommend using the Qiagen QIAquick PCR Purification Kit (Cat. No. 28104) to clean up the reverse crosslinked sample.

NOTE: Alternatively, if no purification columns are available, you can perform phenol/chloroform extraction and ethanol precipitate the sample.

1. **Add 50 µl of elution buffer to the column.**
2. **Incubate the column for 1 minute at RT and recover the DNA as described in the protocol.**
3. **Add 1 volume of loading dye to 5 volumes of purified DNA.**
4. **Load varying amounts of sample on a 1.5% agarose gel. We suggest loading, 5 µl, 10 µl, and 15 µl.**
5. **Resolve on 1.5% agarose gel, and stain gel with Ethidium Bromide after the gel is run.**
6. **View gel with a UV light source and record image.**
7. **Since the DNA has been RNase, and proteinase K treated as the IP'd material will be, it can be saved and used as the input sample for possible qPCR analysis.**

NOTE: Alternatively, you can run 1µl of purified DNA on an Agilent 2100 BioAnalyzer 12k chip which provides a much more accurate representation of the shearing size range and distribution.



25 KD H2B

Size Range (bp)	5 minutes	10 minutes	15 minutes
1-150	8%	15%	19%
151-700	70%	73%	73%
701-7,000	21%	11%	8%

Figure 1: Chromatin shearing time course and fragment size distribution. Note the change in fragment size and distribution with increase in processing time.

The sheared chromatin samples were separated on the gel, transferred to the membrane which was probed with Anti-Ubiquityl-Histone H2B Antibody. Note the strong signal indicating that epitopes haven't been damaged during AFA treatment.

Additional Notes:

1. Methods are transferable between the S2 and S220 systems and the automated E210 (batch) system. Recommended settings are subject to change without notice. See following link: http://www.covarisinc.com/pdf/pn_010127.pdf 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 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.
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.

References:

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