*tru*ChIP™ Low Cell Chromatin Shearing Kit with SDS Shearing Buffer

INTRODUCTION

The *tru*ChIP™ Low Cell Chromatin Shearing Kit with SDS Shearing Buffer (PN 520085) 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 cross linking 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 D1	10 ml of 10X Covaris SDS Shearing Buffer (Contains 0.1% SDS in 1X solution)
Buffer E	6 ml of 1X Covaris Quenching Buffer
Buffer F	0.8 ml of 100X Halt Protease Inhibitor cocktail (Thermo Scientific Cat#78438)
microTUBE	(12) 6 x 16 mm glass tubes with AFA fiber and Snap-Cap

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

> NOTE: microTUBE, AFA Fiber with Snap-Cap is available in packages of 25 (PN 520045)

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 28906, 1.0 ampules), 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 15,000 x g capability
- Rocker - Nutator® or equivalent

Sample Quantity

2 x 10^7 cells starting material

1. Process 2 x 10^7 cells for a 6 time point shearing optimization experiment necessary for determining optimal shearing conditions. Each time point sample will contain 3 x 10^6 cells equivalent nuclei.
2. Process 3 independent 2 x 10^7 cell experiments including nuclei preparation, and a total of 6 shearing experiments in 130µl volume using our snap cap microTubes (Covaris Cat# 520045).

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

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 ~2 x 10^7cells for use with the Covaris Chromatin Shearing Kit. Please note that the equivalent of 1-3 x 10^6 cells can be sheared in a single microTUBE. To establish the optimal shearing conditions, the nuclei from 2 x 10^7 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 **2 ml of 1X Covaris Fixing Buffer** by mixing 200 µl of the 10X Fixing Buffer (A) with **1.8 ml of Molecular Biology grade Water**. Store on ice.
- Prepare fresh **11.1% formaldehyde solution** by mixing **0.69 ml of 16% formaldehyde** with **0.31 ml of Molecular Biology grade Water** (final volume 1.0 ml).
- Prepare **5 ml of 1X PBS** and keep on ice.

1. **Spin cells down at 100-200 x g for 5 minutes at room temperature (RT). Remove media and wash cells once with 1.5 ml of PBS. Spin cells down at 100-200 x g for 5 min. Remove PBS carefully.**
2. **Re-suspend cells in 1.5 ml of Covaris Fixing Buffer (A).**
3. **Using a wide-bore pipette tip, transfer the cells to a 2.0 ml microcentrifuge tube.**
4. **Crosslink cells by adding 150 µl of 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 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.

5. **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.

6. **Quench the crosslinking reaction by adding 87 µl of Covaris Quenching Buffer (E) to the fixed cells. Keep on rocker at RT for 5 minutes.**
7. **Spin cells down at 100-200 x g for 5 minutes at RT, and aspirate the supernatant.**
8. **Wash the cells twice with 1.0 ml of cold PBS. Spin cells down at 100-200 x g for 5 minutes at 4°C, and completely aspirate the PBS.**
9. **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 **4.5 ml of 1X Covaris Fixing Buffer** by mixing **450 µl** of the 10X Fixing Buffer (A) with **4.05 ml of Molecular Biology grade Water**. Store on ice.
- Prepare fresh **11.1% formaldehyde solution** by mixing **1.39 ml of 16% formaldehyde** with **610 µl of Molecular Biology grade Water** (final volume 2.0 ml).
- Prepare **20 ml of 1X solution of PBS** and 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.**
2. **Remove media, and wash with 5.0 ml of PBS.**
3. **Remove PBS.**
4. **Fix cells by adding 5 ml of 1X Fixing Buffer (A) solution to the culture dish.**
5. **Add 0.5 ml of fresh 11% formaldehyde to a final concentration of 1% 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 ampule 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 crosslinking 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 300 µl 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 2.0 ml cold PBS to each dish and scrape cells from the plate.**
10. **Collect the scraped cells into a 15 ml conical tube.**
11. **Add an additional 2.0 ml volume of cold PBS to collect remaining cells in the flask.**
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, and completely aspirate the PBS.**
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 **2 ml of 1X Covaris Lysis Buffer** by mixing **400 µl** of the 5X Lysis Buffer (B) with **1600 µl of cold Molecular Biology grade Water**. Add **20 µl** of the **100X Protease inhibitor** stock solution and keep on ice.
- Prepare **2 ml** of **1X Covaris Wash Buffer** by mixing **200 µl** of the 10X Wash Buffer (C) **with 1.8 ml of cold Molecular Biology grade Water. Add 20 µl of the 100X Protease inhibitor** stock solution and keep on ice.
- Prepare 4 ml of **1X Covaris Shearing Buffer D1** by mixing **400 µl** of the 10X Shearing Buffer (D1) with 3.6 ml of cold Molecular Biology grade Water. Add 40 µl of the **100X Protease inhibitor** stock solution and keep on ice.
- Remove 6 of the Snap cap microTUBEs from the box, and place the tubes on the microTUBE prep station on ice.

1. **Thaw crosslinked cells on ice.**
2. **Add 1.0 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. Carefully remove and discard the supernatant so as not to disturb the pellet.**
5. **Resuspend pellet in 1.0 ml of Covaris Wash Buffer (C) containing protease inhibitors per 1 x 10^7 cells, 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 1.0 ml Covaris Shearing Buffer (D1) containing protease inhibitors by slowly dispensing the 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 910 µl of Covaris SDS Shearing Buffer (D1) containing protease inhibitors. Always use 130 µl of the buffer per maximum of 3 x 10^6 cell equivalents.**
11. **For the initial time course experiment, we suggest that you process enough cells for six 130 µl aliquots containing 1- 3 x 10^6 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, cell mass, and sample volume. We suggest a time course of 2, 4, 6, 8, 10 and 12.

Chromatin AFA Shearing

Summary of Operating Conditions

Target Base Pair (Range)	**200-700**
Duty Cycle	2%
Intensity	3 for (S2 or E210)
Peak Incident Power	105 Watts for (S220/E220)
Cycles per Burst	200
Processing Time	Run an **initial** time course between 2 and 12 minutes to determine optimal shearing time for your sample. Refer to Figures 1 and 2.
Temperature (bath)	4°C
Power mode	Frequency Sweeping (S2 and E210 only)
Degassing mode	Continuous
Volume	130 µl in microTUBE, AFA Fiber with Snap-Cap

IMPORTANT: Always fill the microTUBES with 130 µl of solution for AFA treatment

Maximum cells equivalent per tube	3 x 10^6 cells
*Water level (RUN)	S2/S220 – level 12 E210/E220 – level 6

*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	130 µl Covaris microTUBE, AFA Fiber with Snap-Cap	520045
Holder- S2/S220	Holder for Micro Tubes	500114
Rack-E210/E220	24 tube rack for microTUBES	500111
Note: Rack for E210/E220 requires Intensifier		500141

After Covaris Treatment:

1. **Transfer the sheared samples into cold 0.6 ml 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.

3. **Transfer the supernatant containing sheared chromatin to a new cold 0.6 ml microcentrifuge tube.**
4. **Remove 25 µl of the supernatant for chromatin shearing efficiency analysis described in the next section.**
5. **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 0.1% SDS. 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

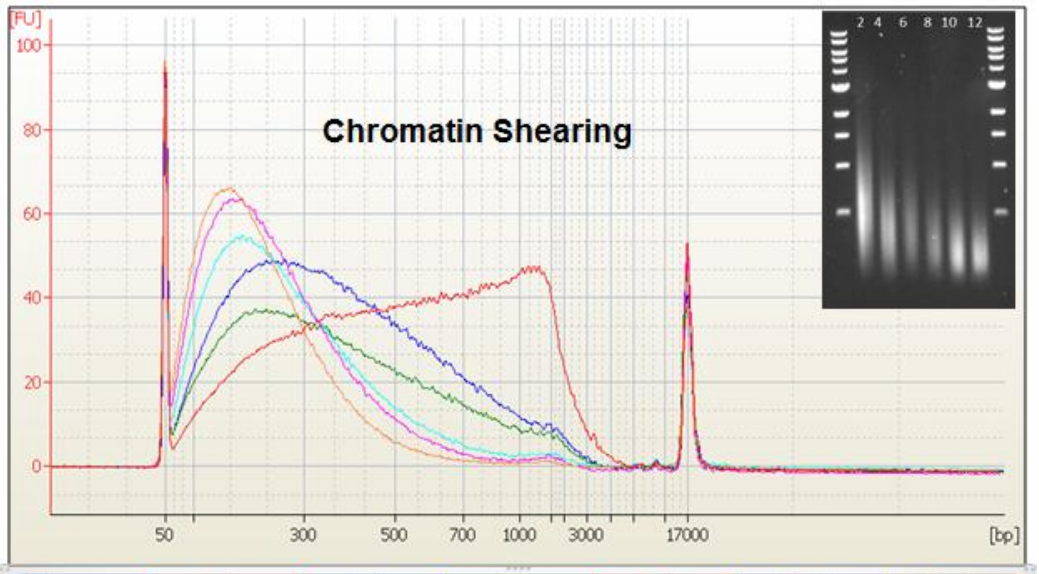
1. **Take the 25 µl aliquot of the sheared sample and transfer to 0.6 ml microcentrifuge tube.**
2. **Add 1 µl of RNase A (10 mg/ml) and incubate at 37°C for 30 min.**
3. **Add 1 µl of Proteinase K (10 mg/ml) and reverse crosslink overnight at 65°C.**

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% agarose gel. We suggest loading, 5 µl, 10 µl, and 15 µl.**
5. **Resolve on 1% 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.



Size range (bp)	2 min	4 min	6 min	8 min	10 min	12 min
1-150	4 %	9 %	10 %	16 %	19 %	24 %
151-700	59 %	78 %	76 %	78 %	75 %	71 %
701-7,000	36 %	12 %	12 %	4 %	3 %	2 %

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

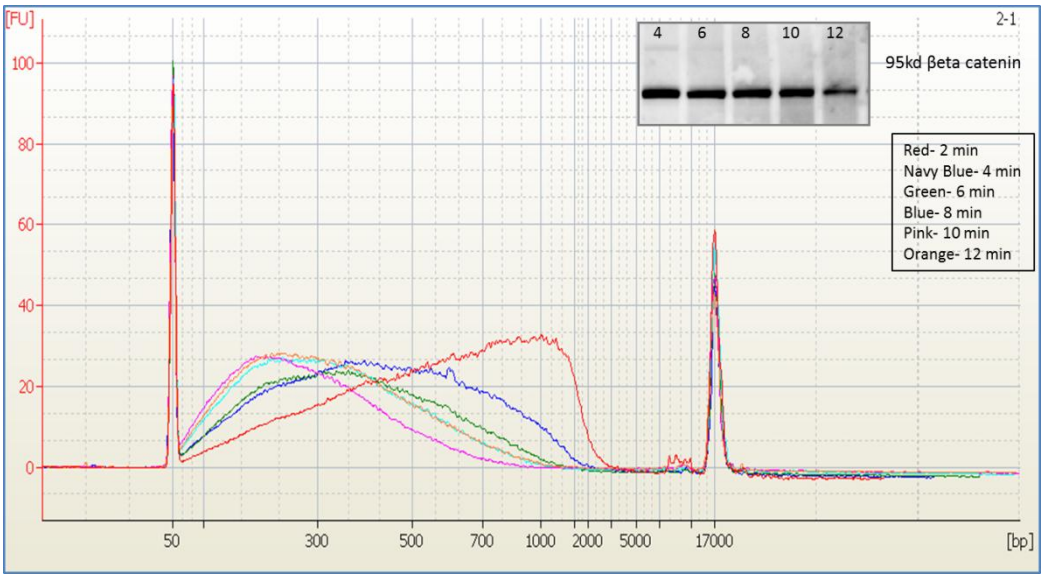


Figure 2: Time course chromatin shearing of $3x10^6$ HeLa cells equivalent in 130 µl of shearing buffer.

Please note that epitope integrity is preserved during the shearing.

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_010145.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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