truChIP™ High Cell Chromatin Shearing Kit with SDS Shearing Buffer
INTRODUCTION

The trueChIP™ High Cell Chromatin Shearing Kit with SDS Shearing Buffer (PN 520076) 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

<table>
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<tr>
<th>Buffer</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer A</td>
<td>10 ml of 10X Covaris Fixing Buffer</td>
</tr>
<tr>
<td>Buffer B</td>
<td>10 ml of 5X Covaris Lysis Buffer</td>
</tr>
<tr>
<td>Buffer C</td>
<td>5 ml of 10X Covaris Wash Buffer</td>
</tr>
<tr>
<td>Buffer D1</td>
<td>10 ml of 10X Covaris SDS Shearing Buffer (Contains 0.1% SDS in 1X solution)</td>
</tr>
<tr>
<td>Buffer E</td>
<td>10 ml of 1X Covaris Quenching Buffer</td>
</tr>
<tr>
<td>Buffer F</td>
<td>0.8 ml of 100X Halt Protease Inhibitor cocktail (Thermo Scientific Cat#78438)</td>
</tr>
<tr>
<td>TC12x12 AFA Tubes</td>
<td>(12) 12x12 mm round bottom borosilicate tubes with AFA fiber and screw caps</td>
</tr>
</tbody>
</table>

NOTE: MSDS information is available at [www.covarisinc.com/chromatin-shearing.html](http://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
Crosslink DNA-proteins with formaldehyde
Lyse the cells and isolate nuclei
Wash nuclei, re-suspend in shearing buffer
Lyse nuclei and shear chromatin
PROTOCOLS

Cross linking of Suspension Cells

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

This method is for the effective cross linking 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 cross linking 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.

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

5. Quench the cross linking 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. At this point, either store the cell pellet by snap freezing and storing at -80°C until needed, or proceed to nuclei preparation (next section).

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

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

Cross linking of Adherent Cells

NOTE: The cross linking 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 µl 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 cross linking reaction.

6. Place plate on a rocker at RT for 5 minutes to allow efficient cross linking.

7. Quench the cross linking reaction by adding 0.3ml 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. At this point, either store the cell pellet by snap freezing and storing at -80°C until needed, or proceed to nuclei preparation (next section)
**Nuclei Preparation**

**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 SDS Shearing Buffer** by mixing 1.3 ml of the 10X SDS Shearing Buffer (D1) 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.

**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.
1. Thaw cross linked 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 SDS Shearing Buffer (D1) containing protease inhibitors by slowly dispensing the wash buffer down the inside of the tube so as not to disturb the nuclei pellet.

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 SDS Shearing Buffer (D1) containing protease inhibitors. Use 1.0 ml of the buffer per maximum of 1-3 x 10⁷ cell equivalents.

11. For the initial time course experiment, we suggest that you process enough cells for six 1.0ml aliquots containing 1 x 10⁷ cells each.

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 cross links during processing.

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

<table>
<thead>
<tr>
<th>Target Base Pair (Range)</th>
<th>200-700</th>
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</thead>
</table>

<table>
<thead>
<tr>
<th>Duty Cycle</th>
<th>5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intensity</td>
<td>4 for (S2 or E210)</td>
</tr>
<tr>
<td>Peak Incident Power</td>
<td>140 Watts for (S220/E220)</td>
</tr>
<tr>
<td>Cycles per Burst</td>
<td>200</td>
</tr>
<tr>
<td>Processing Time</td>
<td>Run an <strong>initial</strong> time course between 2 and 20 minutes to determine optimal shearing time for your sample.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Temperature (bath)</th>
<th>4°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Power mode</td>
<td>Frequency Sweeping (S2 and E210 only)</td>
</tr>
<tr>
<td>Degassing mode</td>
<td>Continuous</td>
</tr>
<tr>
<td>Volume</td>
<td>1.0 ml in TC12x12 AFA tubes</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Maximum cells equivalent per tube</th>
<th>1-3 x 10^7 cells</th>
</tr>
</thead>
</table>

*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

**No Intensifier (remove from E instrument) (See Insert)**

### Supplies

<table>
<thead>
<tr>
<th>Supplies</th>
<th>Description</th>
<th>Part Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample Vessel</td>
<td>1.0 ml Covaris TC12x12 AFA tubes (12 x 12 mm) with AFA fiber</td>
<td>520081</td>
</tr>
<tr>
<td>Holder- S2/S220</td>
<td>Holder for TC12x12 AFA tubes</td>
<td>500274</td>
</tr>
<tr>
<td>Rack-E210/E220</td>
<td>12 tube rack: for TC12x12 AFA tubes</td>
<td>500276</td>
</tr>
</tbody>
</table>
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 cross linked 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 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 cross linked 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.
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_010128.pdf](http://www.covarisinc.com/pdf/pn_010128.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:


2. Ralph M Bernstein, Ph.D. and Frederick C. Mills, Ph.D., Laboratory of Immunology, Division of Therapeutic Proteins, CDER, FDA, NIH Campus, Bethesda, MD. We very much appreciate their contribution to the shearing buffer SDS concentration titration experiment, formaldehyde fixation reduction time, and initial evaluation of our protocols and reagents.


5. Haring M, Offerman S, Danker T, Horst I, Peterhansel C and Stam M; Chromatin immunoprecipitation: optimization, quantitative analysis and data normalization, Plant Methods 2007, 3:11