PROTOCOL

truChIP® Chromatin Shearing Tissue Kit

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

PN 520237 and 520238

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INTENDED USE

The truChIP Chromatin Shearing Tissue 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 Chromatin Shearing Tissue Kit is designed and optimized for the efficient and reproducible shearing of chromatin from tissues using the Covaris Adaptive Focused Acoustics (AFA) technology.

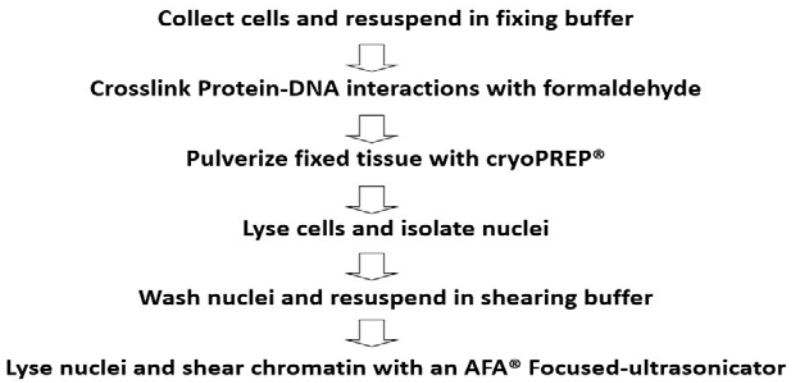
The following method was developed using mouse tissue (liver, brain, and muscle - **See Figure 4 in Appendix D**), but will work with a variety of tissue types.

truChIP is compatible with a variety of tissue types. The Covaris shearing buffer contains SDS, which may not be compatible with immunoprecipitations (IP) using antibodies against some commonly used protein tags (*e.g.,* FLAG tag). Therefore, Covaris recommends contacting the antibody manufacturer prior to using this kit. To start, Covaris recommends 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
010385	A	03/17	As released, based on document 010147 revC. Update template, include–2ml AFA tubes, include 16% methanol-free formaldehyde, and publish ME220 settings
010385	B	11/18	Add 3X IP Dilution Buffer and Insert CP01 cryoPREP Manual Dry Pulverizer Protocol
010385	C	1/19	Add wash buffer preparation instructions
010385	D	6/19	Add Water level clarification

PROCEDURE OVERVIEW



KIT CONTENTS

Buffer A	10 mL	10X Fixing Buffer
Buffer B	10 mL	5X Lysis Buffer
Buffer C	5 mL	10X Wash Buffer
Buffer D2	10 mL	10X SDS Shearing Buffer (Contains 0.25% SDS in 1X solution)
Buffer E	6 mL	1X Quenching Buffer
Buffer F	0.8 mL	100X Protease Inhibitor Cocktail
tissueTUBE	(10) TT05M XT tissueTUBE (PN 520140)	Extra Thick tissueTUBE designed to work with the Covaris milliTUBE
milliTUBE–2 mL	(10) milliTUBE–2 mL and cap (PN 520132)	For use with tissueTUBE
Dilution Buffer	25 mL	3X IP Dilution Buffer
Formaldehyde	5 x 1 mL ampules (PN 520237 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.
Note: Because certain cell lines may have more nuclease activity, Covaris recommends adding EDTA to a final concentration of 1 mM in the 1X Covaris Wash Buffer C.

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

STORAGE

The kit is shipped cold and should be stored at 2 to 8C. Buffers D2 and E may need to be warmed to 55C to dissolve precipitate and 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
- Liquid Nitrogen
- AFA tubes & holders/racks

Low Tissue Mass (~20-50mg) Consumables & Holders/Racks Required

Part Number	Description	M220 Holder & Insert	ME220 Holder & Insert	S-Series Holder	E220 Evolution Rack	E-Series Rack	L-Series 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	NA	500329

High Tissue Mass (50-120mg) Consumable & Holders/Racks Required

Part Number	Description	M220 Holder & Insert	ME220 Holder & Insert	S-Series Holder	E220Evo Rack	E220 Rack	LE220 Rack
520130	milliTUBE–1 mL with AFA Fiber	500414 & 500422	500520 & 500534	500371	500431	500368	500368

PROTOCOL

A. Tissue Preparation

The following protocol is designed for processing 20 - 120 mg of fresh or frozen tissue. For the initial experiment, Covaris recommends to process 120 mg of tissue using 6 time points to empirically determine the optimal processing times. Once the optimal processing time is determined, single samples with masses as low as ~20 mg can be processed without repeating the shearing time course optimization. If using an M220 or ME220 focused ultrasonicator, we recommend no more than ~80mg of tissue to be processed per AFA tube.

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

Buffer to be prepared	120 mg tissue protocol	20-50 mg tissue protocol
Cold 1X PBS	Reserve 5 mL of 1X PBS - Store on ice	- Reserve 2.5 mL of 1X PBS - Store on ice
1X Fixing Buffer A	**Final Volume: 1 mL** - Mix 100 µl of the 10X Fixing Buffer A with 0.9 mL of molecular biology grade water - Store on ice	**Final Volume: 500 µl** - Mix 50 µl of the 10X Fixing Buffer A with 450 µl of molecular biology grade water - Store on ice
Fresh 11.1% Formaldehyde	**Final Volume: 500 µl** - Mix 347 µl of 16% Formaldehyde with 153 µl of molecular biology grade water - Store on ice	**Final Volume: 250 µl** - Mix 173.5 µl of 16% Formaldehyde with 76.5 µl of molecular biology grade water - Store on ice
Quenching Buffer E	- Place in a 55C water bath to dissolve crystals, then place at ambient temperature	- Place in a 55C water bath to dissolve crystals, then place at ambient temperature

Step-by-Step Procedure

1. Weigh frozen or fresh tissue.

> **Note:** If processing tissue samples in batches, Covaris recommends that you fix, quench, wash with 1XPBS twice, remove 1XPBS and then store the samples at -80C. Alternatively, you may pulverize the tissue using the cryoPREP prior to storing the samples at -80C.

2. Cut tissue into small pieces (around 1 mm^3) using a razor blade or scalpel on ice.

3. Transfer the tissue into a 2 mL microcentrifuge tube.

4. Wash tissue sample with cold 1X PBS and spin for 5 minutes at 200 x g at 4C.

Reagent	Initial time course/120 mg of tissue	Single tube/20-50 mg of tissue
Cold 1X PBS	1.0 mL	400 µl

5. Aspirate the PBS and resuspend the tissue sample in cold 1X Covaris Fixing Buffer A.

Reagent	Initial time course/120 mg of tissue	Single tube/20-50 mg of tissue
1X Covaris Fixing Buffer A	1.0 mL	400 µl

Note: The use of fresh methanol-free formaldehyde solution is essential for reproducible crosslinking. The use of a fresh sealed ampoule is recommended. The use of a previously opened bottle is not recommended.

6. Fix tissue by adding freshly prepared 11.1% formaldehyde solution to a final concentration of 1%. Start timing the crosslinking reaction from the moment the formaldehyde is added.

Reagent	Initial time course/120 mg of tissue	Single tube/20-50 mg of tissue
Fresh 11.1% Formaldehyde Solution	100 µl	40 µl

7. Incubate the tube containing tissue on a rocker or a rotator at RT for 5 minutes to allow for efficient crosslinking.

Note: The optimal crosslinking time is tissue type and mass dependent. Therefore, we strongly advise for optimization of the crosslinking step. Excessive crosslinking may result in inefficient shearing. Alternatively, insufficient exposure to formaldehyde may result in failure to detect specific protein DNA interactions. **See Figure 1 in Appendix D.**

8. Quench the crosslinking reaction by adding Covaris Quenching Buffer E. Incubate on a rocker or rotator at RT for 5 minutes.

Reagent	Initial time course/120 mg of tissue	Single tube/20-50 mg of tissue
Covaris Quenching Buffer E	58 µl	24 µl

9. Spin down fixed tissue at 100-200 x g in a microcentrifuge tube at 4C for 5 minutes, and aspirate and discard the supernatant.

10. Wash the fixed tissue sample twice by resuspending in cold 1X PBS and centrifugation at 100-200 x g for 5 minutes at 4C.

Reagent	Initial time course/120 mg of tissue	Single tube/20-50 mg of tissue
Cold 1X PBS	1.0 mL	400 µl

11. Aspirate and discard the PBS, and transfer the fixed tissue using a spatula to a TT05M XT tissueTUBE.

Note: The tissue samples should be placed approximately in the middle of the tissueTUBE.
Note: If the fixed tissue is difficult to transfer and place in the middle of the tissueTUBE, then transfer the tissue anywhere inside of the tissueTUBE and place tissueTUBE on dry ice for 1 minute. The frozen tissue can then be easily moved to the middle of the tissue tube by pinching the bottom of the TT05M XT.

12. Screw the milliTUBE–2 mL to the TT05M XT tissueTUBE.

13. Unscrew the milliTUBE–2 mL by a quarter turn to vent the bag and prevent it from tearing during pulverization.

14. Attach the tissueTUBE assembly to the TT05 holder, and place on dry ice for 2 minutes.

Tissue Pulverization

1. Collect Sample
Insert the tissue sample in the center of the tissueTUBE.

2. Flash Freeze Sample
Submerge "pouch" portion LN_2 to flash freeze the tissue.

3. Dry Pulverization
Pulverize the frozen tissue sample.

4. Transfer Processed Sample
Cool the entire tissueTUBE assembly in a bed of dry ice. Invert the tissueTUBE assembly to transfer pulverized contents to glass tube.

Note: If you do not have access to a CP02 cryoPREP Automated Dry Pulverizer, you can use the CP01 cryoPREP Manual Dry Pulverizer (PN: 500230) to cryofracture the tissue sample in the TT05M XT tissueTUBE. See **Appendix B** for protocol.

15. Remove the tissueTUBE holder assembly from dry ice and submerge the bottom 2/3rd of the tissueTUBE into the liquid nitrogen for 45 seconds.

16. Rapidly remove the tissueTUBE holder assembly from the liquid nitrogen, and allow 2-3 seconds for the liquefied air to bubble out, and place in the cryoPREP. It is critical to minimize the time between the immersion in liquid nitrogen and dry pulverization.

17. Pulverize the tissue using a setting of 5 and remove the tube from the cryoPREP.

18. Cryofractured fixed tissue often forms a cake after impact. Using your finger nail only, quickly disperse the cake by rapid pinching of the tissueTUBE. If you notice large tissue chunks remaining, place the tissueTUBE back in the liquid nitrogen for 45 seconds and repeat the pulverization step using a setting of 5.

19. Invert the tissueTUBE/milliTUBE–2 mL assembly 180 degrees and quickly flick the tissueTUBE with your finger to transfer the pulverized tissue into the milliTUBE–2 mL.

20. Place the milliTUBE–2 mL containing the pulverized fixed tissue on dry ice, or transfer to a cryogenic storage vial for storage at -80C.

21. Proceed to nuclei preparation (next section).

B. Nuclei Preparation

The cell lysis and nuclei preparation require the use of all provided reagents with the Covaris AFA technology. Follow **ALL** steps of the protocol exactly to ensure 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.

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

Buffer to be prepared	120 mg tissue protocol	20-50 mg tissue protocol
1X Lysis Buffer B	**Final Volume: 1.5 mL** - Mix 300 µl of the 5X Lysis Buffer B with 1.2 mL of cold molecular biology grade water - Add 15 µl of Buffer F - Store on ice	**Final Volume: 0.75 mL** - Mix 150 µl of the 5X Lysis Buffer B with 0.6 mL of cold molecular biology grade water - Add 15 µl of Buffer F - Store on ice
1X Covaris Wash Buffer C	**Final Volume: 2.5 mL** - Mix 250 µl of the 10X Wash Buffer C with 2.25 mL of cold molecular biology grade water - Add 25 µl of Buffer F - Optional: Add EDTA to a final concentration of 1mM - Store on ice	**Final Volume: 1.25 mL** - Mix 125 µL of the 10X Wash Buffer C with 1.125 mL of cold molecular biology grade water - Add 12.5 µl of the Buffer F - Optional: Add EDTA to a final concentration of 1mM - Store on ice
1X Shearing Buffer D2 (1mM EDTA, 10mM Tris-HCl pH 7.6, 0.25% SDS)	**Final Volume: 1 mL** - D2 may need to be warmed to 55C to dissolve precipitate and cooled to room temperature before use - Mix 100 µl of the 10X SDS Shearing Buffer with 900 µl of cold molecular biology grade water - Add 10 µl of Buffer F - Store on ice	**Final Volume: 0.5 mL** - D2 may need to be warmed to 55C to dissolve precipitate and cooled to room temperature before use - Mix 50 µl of the 10X SDS Shearing Buffer with 450 µl of cold molecular biology grade water - Add 5 µl of Buffer F - Store on ice

Step-by-Step Procedure

1. Thaw crosslinked pulverized tissue on ice.

2. Using a razor blade or scissors, cut 1 mm from the tip of a P1000 pipette tip and use it for the next step.

3. Add cold 1X Covaris Lysis Buffer B containing protease inhibitors to the pulverized sample in the milliTUBE–2 mL and transfer the contents to a 2 mL microcentrifuge tube.

Reagent	Initial time course/120 mg of tissue	Single tube/20-50 mg of tissue
1X Covaris Lysis Buffer B	500 µl	200 µl

4. Rinse the milliTUBE–2 mL with another aliquot of cold 1X Covaris Lysis Buffer B containing protease inhibitor and add contents to the 2 mL microcentrifuge tube.

Reagent	Initial time course/120 mg of tissue	Single tube/20-50 mg of tissue
1X Covaris Lysis Buffer B	500 µl	200 µl

5. Incubate the 2 mL tube for a total of 20 minutes on a rocker or rotator at 4C with 3 second vortexing after 10 minutes.

6. Pellet nuclei by spinning at 1,700 x g in a 2 mL microcentrifuge tube for 5 minutes at 4C.

7. Aspirate and decant the supernatant and resuspend the pellet in 1X Covaris Wash Buffer C containing protease inhibitors. Incubate on a rocker for 10 minutes at 4C.

Reagent	Initial time course/120 mg of tissue	Single tube/20-50 mg of tissue
1X Covaris Wash Buffer C	1.0 mL	400 µl

8. Spin down nuclei at 1,700 x g in a 2 mL microcentrifuge tube for 5 minutes at 4C. Aspirate and decant the supernatant.

9. Wash pellet by resuspending in 1mL of 1X Covaris Wash Buffer C containing protease inhibitors and spinning down at 1,700 x g in a 2 mL microcentrifuge tube for 5 minutes at 4C.

Reagent	Initial time course/120 mg of tissue	Single tube/20-50 mg of tissue
1X Covaris Wash Buffer C	1.0 mL	400 µl

10. Resuspend nuclei in 1X Covaris Shearing Buffer D2 containing protease inhibitor and incubate on ice for 10 minutes with occasional vortexing.

Reagent	Initial time course/120 mg of tissue	Single tube/20-50 mg of tissue
1X Covaris Shearing Buffer D2	1.0 mL	130 µl

11. If optimizing for processing nuclei from 120 mg of tissue in 1 mL of shearing buffer, then transfer the nuclei to a milliTUBE–1 mL with AFA Fiber for conducting the 6-point time course.

12. If optimizing for processing nuclei from ~20 mg of tissue in 130 µl of shearing buffer, then transfer the nuclei to 6 microTUBEs with each tube containing 130 µl of sample suspended in shearing buffer.

C. Chromatin Shearing

Note: Carry out an initial time course shearing experiment to optimize the chromatin shearing parameters specific for your tissue. Covaris suggests a time course of 2, 4, 6, 8, 10 and 12 minutes if processing in 130 µl volume. If processing in 1.0 ml volume, we suggest a processing time of 2, 4, 8, 12, 16, and 20 minutes. **Refer to Figures 2 and 3 in Appendix D**

1. Shear chromatin with an AFA Focused-ultrasonicator with appropriate rack or holder; settings are provided in **Appendix A.**

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

2. If processing samples using the microTUBE–130, then individual microTUBEs will be required for each time point included.

3. If processing samples using the milliTUBE–1 mL with AFA fiber, you can process all the time points of the time course in the same tube according to the figure below. After each time point interval, remove 35 µl of the sample and place in in a microcentrifuge tube labeled with the total processing time.

Programmed Interval processing time (minutes)	2	2	4	4	3	5
Total processing time (minutes)	2	4	8	12	15	20

4. Replace the volume removed with 35 µl of 1x Covaris Shearing Buffer D2. Place the tube in the holder and process on the Covaris instrument for the next programmed interval processing time. 25 µl will be used for DNA shearing size range analysis. 10 µl will be used for epitope integrity analysis using a western blot protocol developed by Covaris.

5. Repeat steps 3 and 4 until all the time course points are completed.

6. Before proceeding to IP, dilute the chromatin 1:2 with the 3X Covaris IP Dilution Buffer.

7. Centrifuge sample at 10,000 x g for 5 minutes at 4C to prepare the supernatant for IP.

8. The supernatant is now ready for immunoprecipitation.

Note: Sheared chromatin must be diluted prior to IP to sequester the SDS in Shearing Buffer D2 and ensure antibodies perform optimally. Therefore, for homebrewed IP methods, dilute the chromatin with Covaris 1:2 with the provided 3X Dilution Buffer.

SUPPLEMENTAL MATERIAL

Appendix A: AFA Focused Ultrasonicator Operating Conditions

Low Tissue Mass 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)	2-20	2-20	2-12	2-12	2-12	2-12
Setpoint Temperature (C) [1]	7	9	6	6	6	6
Min/Max Temperature (C)	4/10	6/12	3/9	3/9	3/9	3/9
Degassing Mode	NA	NA	Continuous	Continuous	Continuous	Continuous
Max Cell Number (Million)	3M	3M	3M	3M	3M	3M
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

High Tissue Mass 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	140	140	140	420
Duty Factor (%)	10	15	5	5	5	30
CPB	200	1000	200	200	200	200
Treatment Time (minutes)	2-20	2-20	2-20	2-20	2-20	5-25
Setpoint Temperature (C) [1]	7	9	6	6	6	6
Min/Max (C)	4/10	6/12	3/9	3/9	3/9	3/9
Degassing Mode	NA	NA	Continuous	Continuous	Continuous	Continuous
Max Cell Number (Million)	30M	30M	30M	30M	30M	30M
AFA Intensifier Required	NA	NA	NA	NA	NA	NA
Water Level (run) [2]	Full	9	8	5	0	-4
Sample Volume (mL) [3]	1	1	1	1	1	1

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. Water level should always be 1mm below the neck of the milliTUBE–1 mL with AFA Fiber cap
3. Always fill the milliTUBE–1 mL with AFA Fiber with 1.0 mL of sample

Appendix B: Tissue Processing Protocol using the CP01 cryoPREP Manual Dry Pulverizer

1. Ensure the tissue sample is inserted and placed in the middle of the tissueTUBE by pitching the bottom of the bag.

2. While holding the TT05M XT tissueTUBE, insert the fixed sample specimen using forceps or tweezers through the orifice of the bag.

> **Note:** If the fixed tissue is difficult to transfer and place in the middle of the tissueTUBE, then transfer the tissue anywhere inside of the tissueTUBE and place on dry ice for 1 minute. The frozen tissue can then be easily moved to the middle of the tissueTUBE by pinching the bottom of the TT05M XT tissueTUBE.

3. After loading the sample into the TT05M XT tissueTUBE, attach the milliTUBE-2mL tube and place on dry ice for at least 2 minutes.

4. Using protective gloves, remove the pre-chilled CP01 cryoPREP Manual Dry Pulverizer from the freezer (-80C).

5. Place the pre-chilled portion (stainless steel block) of the CP01 cryoPREP Manual Dry Pulverizer into the holder (black piece) with the correct tissueTUBE Adapter.

6. Remove the tissueTUBE holder assembly from dry ice and submerge the bottom two-thirds of the tissueTUBE into liquid nitrogen (LN_2) for 45 to 90 seconds to flash freeze the sample.

7. With gloved hands, raise the cylinder on the CP01 cryoPREP Manual Dry Pulverizer and insert the chilled TT05M XT into the adapter (TT05 holder). For best pulverization results, dip the TT05M XT into liquid nitrogen (LN_2) immediately before loading into the adapter.

Note: The tissueTUBE assembly must be vented before striking it to prevent the tissueTUBE from breaking. To do this, unscrew the milliTUBE-2mL by a quarter of a turn prior to inserting into the TT05 holder.

8. Release the cylinder. Strike the top of the cylinder with the Covaris impactor (red) to pulverize the tissue sample.

9. After impact, raise the cylinder with a gloved hand and remove the TT05M XT tissueTUBE.

10. Invert the tissueTUBE/milliTUBE–2 mL assembly 180 degrees and quickly flick the tissueTUBE with your finger to transfer the pulverized tissue into the milliTUBE–2 mL.

11. Place the milliTUBE–2 mL containing the pulverized fixed tissue on ice, or transfer to a cryogenic storage vial for storage at -80C.

12. Visually inspect to ensure the tissue sample has been adequately impacted. If the sample was not completely pulverized, the sample may need to be repositioned in the TT05M XT tissueTUBE and struck again.

Note: If a second impact is required, flash freeze the sample in liquid nitrogen (LN_2) for 45 to 90 seconds before striking the tissue sample.

13. Proceed to nuclei preparation in Step B.

Note: If a second impact is required, inspect the tissueTUBE for punctures. If a punctures are observed, the tissueTUBE should not be impacted a second time.

Note: A quick immersion in liquid nitrogen (LN_2) after pulverization may aid in the transfer of the pulverized tissue.

Note: Prior to inversion, the processing tube should be chilled to prevent the cold contents from adhering to the inner walls of the tube as the pieces fall to the bottom.

Appendix D: Chromatin Shearing Efficiency Analysis Protocol

1. Take a 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 37C for 30 minutes.
3. Add 4 µl of Proteinase K (10 mg/mL) and reverse crosslink by heating at 65C overnight.
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 50 µl of elution buffer (10 mM Tris-HCl, pH 8.5).
6. We strongly advise the use of an Agilent Bioanalyzer DNA 12000 kit for assessing the chromatin shearing efficiency.
7. If you are using an agarose gel, add 1 volume of loading dye to 5 volumes of purified DNA.

Note: The use of loading dye without Bromophenol Blue is recommended. Bromophenol Blue migrates at ~300 bp and interferes with smear analysis. Running an agarose gel using a higher voltage and shorter time is not recommended as it will result in a false smear analysis.

a) Load 300 to 600 ng of purified DNA per lane.
b) Resolve on 1% agarose gel run at 30 V for 3.5 hours.
c) Stain gel with Ethidium Bromide after the gel is run.
d) Destain and view gel with a UV light source and record image.

Appendix E: Additional Notes

1. The treatment settings listed in this protocol are recommended guidelines. Actual results may vary depending on the cell type and mass input.
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.

Appendix F: Examples of DNA Fragments Size Distribution

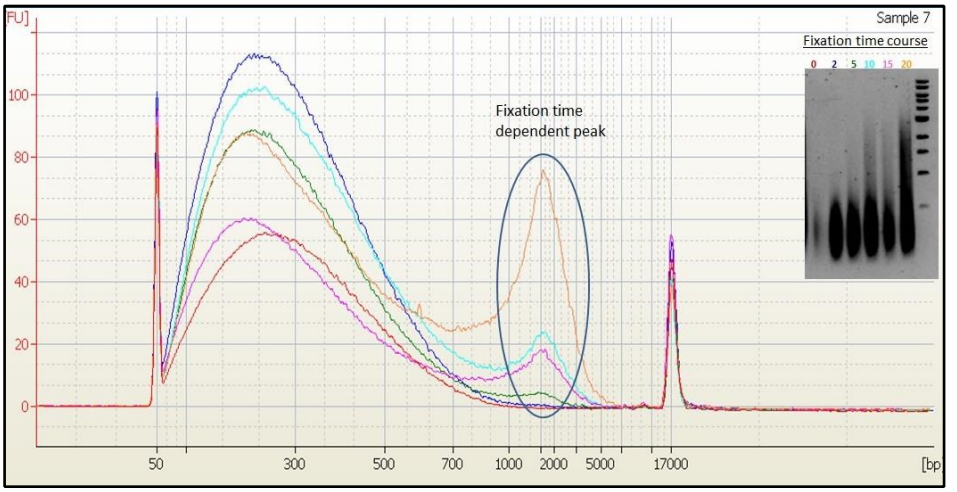
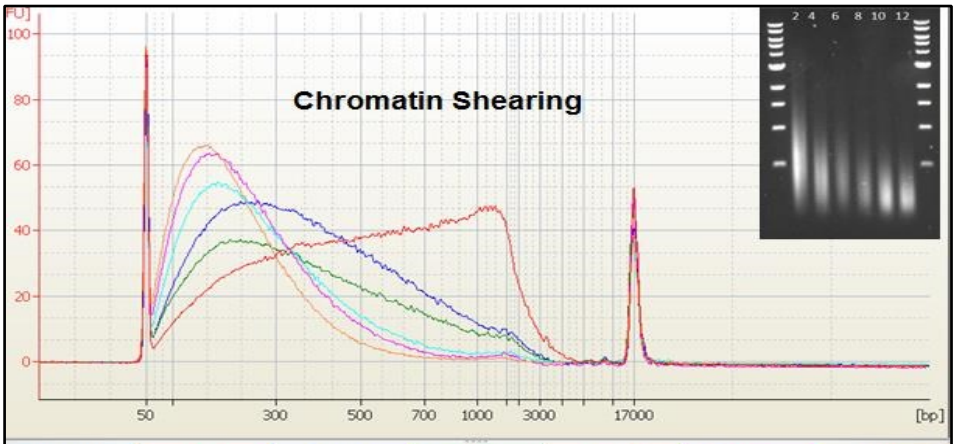
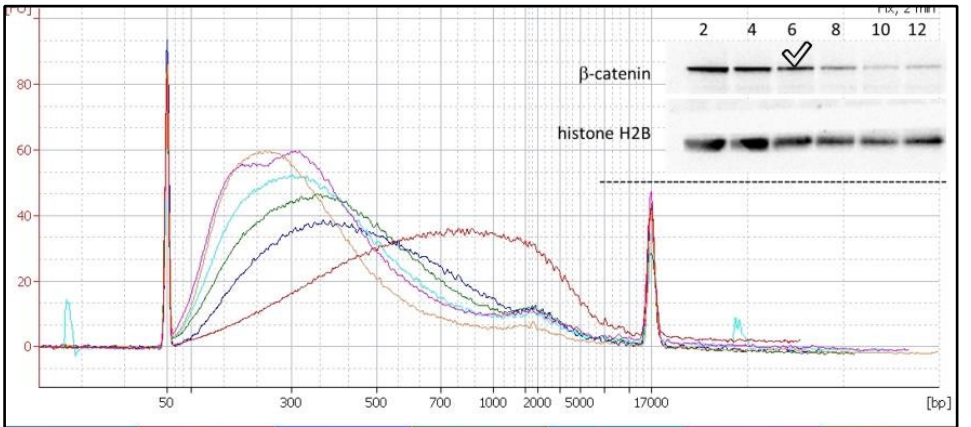


Figure 1. Effect of formaldehyde fixation time on shearing efficiency. 120mg of mouse liver tissues were fixed for 0, 2, 5, 10, 15, and 20 minutes; the nuclei were prepared following the protocol. Approximately 18mg of tissue equivalent nuclei from each fixation time point was sheared according to the setting in the protocol for 10 minutes. Note the increase in the higher molecular weight peak (~1.5kb) intensity with respect to fixation time



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 2. Chromatin shearing time course and fragment size distribution. 120mg of mouse liver tissue was fixed for 5 minutes; the nuclei were prepared following the protocol. Approximately 18mg tissue equivalent of nuclei were then processed for a time course of 2-12 minutes using the microTUBE-130 with the settings provided in this protocol. Note the change in fragment size and overall distribution as the processing time is increased.



Size range (bp)	2 min	4 min	6 min	8 min	10 min	12 min
1-150	1 %	2 %	4 %	5 %	7 %	8 %
151-700	51 %	74 %	76 %	77 %	77 %	81 %
701-7,000	46 %	22 %	18 %	13 %	12 %	9 %

Figure 3. Chromatin shearing time course and fragment size distribution. 120mg of mouse brain tissue was fixed for 5 minutes; the nuclei were prepared following the protocol. Approximately 18mg tissue equivalent of nuclei were then processed for a time course of 2-12 minutes using the microTUBE-130 with the settings provided in the protocol. Note the change in fragment size and overall distribution as the processing time is increased. The inset western blot depicts epitope integrity during chromatin shearing.

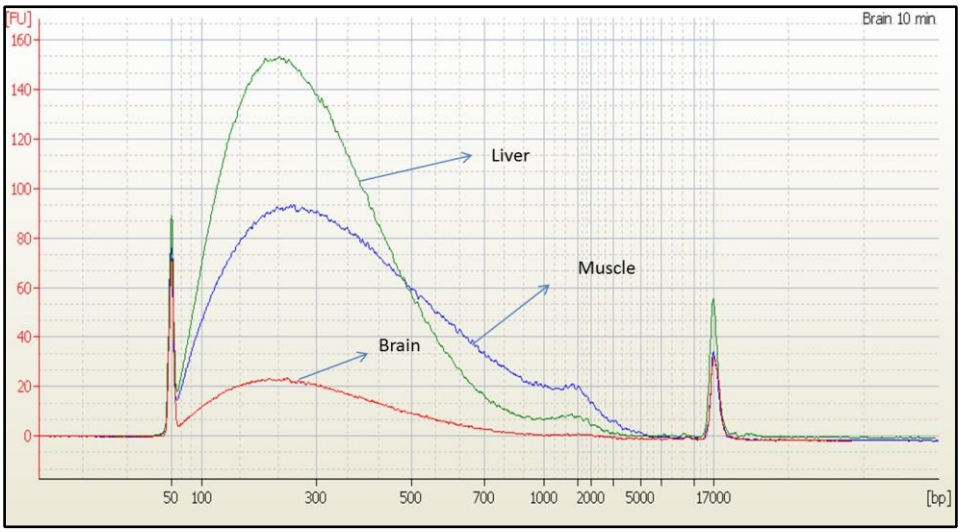


Figure 4. Chromatin shearing time course and fragment size distribution. Processing of three different tissue types using the optimized setting for 10 minutes generated consistent average fragment sizes and overall similar distribution profiles.

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