

truChIP™ Tissue Chromatin Shearing Kit with SDS Shearing Buffer

Updated January 2015

INTRODUCTION

The *truChIP™* Tissue Chromatin Shearing Kit with SDS Shearing Buffer (PN 520083) is designed and optimized for the efficient and reproducible shearing of chromatin from tissue using Covaris AFA™ (Adaptive Focused Acoustics) technology.

The following method was developed using mouse tissue (liver, brain and muscle - see Figure 4), but will work with a variety of tissues.

Depending on the type of starting material, end-user optimization of crosslinking and shearing processing time will be required.

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 while using the reagents included in the kit, it is important to follow the procedure outlined in this document to generate reproducible and optimal data.

KIT CONTENTS

Formaldehyde	5x 1 mL ampoules
Buffer A	10 mL
Buffer B	10 mL
Buffer C	5 mL
Buffer D2	10 mL
Buffer E	6 mL
Buffer F	0.8 mL

Content Descriptions

Formaldehyde	16% methanol-free formaldehyde
Buffer A	10X Fixing Buffer
Buffer B	5X Lysis Buffer
Buffer C	10X Wash Buffer
Buffer D2	10X SDS Shearing Buffer (Contains 0.25% SDS in 1X solution)
Buffer E	1X Quenching Buffer
Buffer F	100X Protease Inhibitor cocktail
microTUBE	(12) 6 x 16mm glass tubes with AFA™ fiber and Snap-Cap
TT05XT/TC12x24	(6) tissueTUBE, Extra Thick/(6) TC12x24
milliTUBE	(6) 12 X 12 mm round bottom borosilicate tubes

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)
TT05XT, tissueTUBE Extra Thick are available in packages of 25 (PN 520072).
TT05P, plugs for tissueTUBE are available in packages of 25 (PN 520082)
TC12, 12x24mm glass tube & cap are available in packages of 100 (PN 520056)

Storage

The kit is shipped cold and should be stored at 4-8°C. Buffers D2 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.

SUPPLIED BY USER

- Molecular Biology Grade Water – Thermo Scientific (Cat. No. SH3053802), Mo Bio (Cat. No. 17012-200), or equivalent
- Fresh methanol-free 16% Formaldehyde – Thermo Scientific (Pierce) (Cat. No. 28908, 10 mL or 28906, 1mL ampules), or equivalent (unless provided in Covaris Kit)
- 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 tissue amount 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	500111	NA
520052	microTUBE Crimp-Cap	NA	500114	500282	500282
520053	microTUBE strip	NA	NA	500191	500191
520078	microTUBE plate	NA	NA	No rack required	500329

High tissue amount Protocol

Tube Part Number	Description	M-Series Holder and Insert	S-Series Holder	E-Series Rack	L-Series Rack
520130	milliTUBE 1 mL	500348(*) 500414 (Holder) & 500422 (Insert)	500371	500368	500368

(*) These holders have been discontinued

Sample Quantity

The kit contains enough reagents and microTUBES to process 6 different tissue samples.

Procedure Overview

Cut tissue in small pieces and resuspend in fixing buffer



Crosslink DNA-proteins with formaldehyde



Pulverize fixed tissue in CryoPrep™



Lyse cells and isolate nuclei



Wash nuclei, re-suspend in shearing buffer



Lyse nuclei and shear chromatin

PROTOCOLS

Tissue Preparation

The following protocol is designed for 20 - 120 mg of fresh or frozen tissue. For the initial experiment using your tissue sample, we suggest that you process 120 mg of tissue to allow for a 6 point time course to determine the optimal processing (shearing) time for your samples. Once the optimal processing (shearing) time is determined, single samples as low as 20 mg can be processed using the optimized processing time.

Solutions to prepare for this Section: (Sufficient for processing 20-120 mg of tissue)

NOTE: Reagent volumes below are for preparing **120 mg of tissue**. If preparing **20-50 mg of tissue** then prepare half the volume of the reagents.

- 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 1.0 ml of 1X Covaris Fixing Buffer by mixing 100µl of the 10X Fixing Buffer (A) with 0.9 ml of Molecular Biology grade Water and place on ice.
- Prepare fresh 11.1% formaldehyde solution by mixing 347 µl of 16 % HCHO with 153µl of Molecular Biology grade Water (500 µL final volume) and place on ice.
- Prepare 5 ml of 1X solution of PBS and place on ice

1. Weigh frozen or fresh tissue.
2. Cut tissue into small pieces (around 1 mm³) using a razor blade or scalpel on ice.
3. Transfer the tissue into a 2 ml microcentrifuge tube.
4. Wash tissue sample with cold PBS and spin for 5 min at 200xg.

Reagent	Initial time course/ 120 mg of tissue	Single tube/ 20-50 mg of tissue
1x PBS on ice	1.0 ml	400 µl

5. Aspirate the PBS and resuspend the tissue sample in cold Covaris fixing buffer (Buffer A).

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

6. Fix tissue by adding freshly prepared 11% formaldehyde solution to a final concentration of 1%. Start timing the crosslinking reaction from the moment the formaldehyde is added.

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.

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 room temperature (RT) for 5 minutes to allow for efficient crosslinking.

NOTE: The optimal crosslinking time is tissue type dependent, as well as tissue mass 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 and inefficient DNA shearing. See Figure 1.

8. Quench the crosslinking reaction by adding Covaris Quenching Buffer (E). Incubate on 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

- Spin down fixed tissue at 100-200xg in a microfuge at 4°C for 5 minutes, and aspirate the supernatant.
- Wash the fixed tissue sample twice by resuspension in cold PBS and centrifugation at 100-200xg for 5 minutes at 4°C.

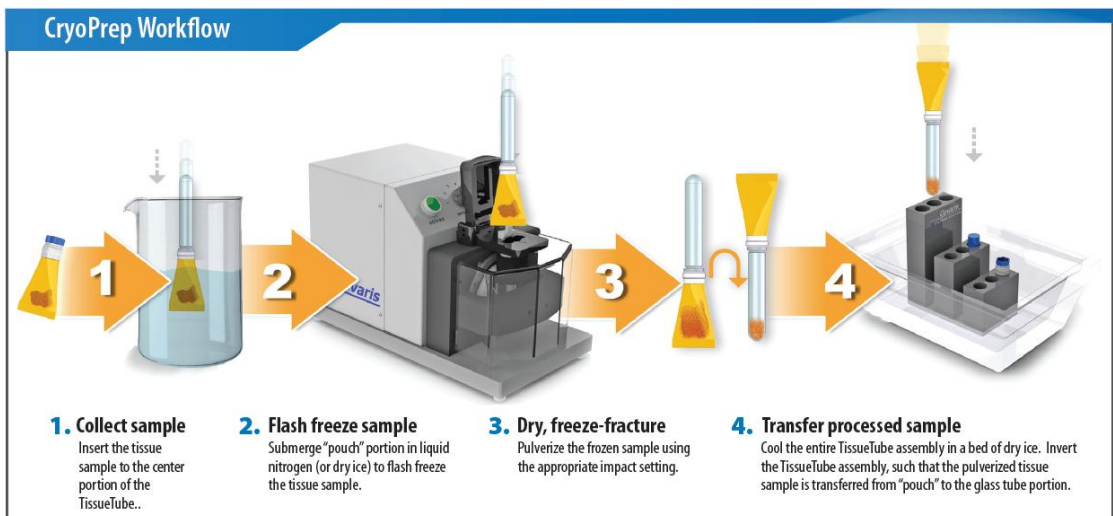
Reagent	Initial time course/ 120 mg of tissue	Single tube/ 20-50 mg of tissue
1x PBS on ice	1.0 ml	400 µl

- Aspirate the PBS, and transfer the fixed tissue using a spatula to a TT05XT tissue tube.

NOTE: The tissue samples should be placed approximately in the middle of the tissue tube.

- If the fixed tissue is difficult to transfer and place in the middle of the tissue tube, then transfer the tissue anywhere inside of the tissue tube, place tissue tube on dry ice for 1 minute. The frozen tissue can then be easily moved to the middle of the tissue tube.
- Screw a TC12x24 tube on the top of the TT05XT tissue tube.
- Attach the tissue tube assembly to the TT05XT holder, and place on dry ice for 2 minutes.

Tissue Pulverization



15. Remove the tissue tube-holder assembly from dry ice and submerge the bottom 2/3rd of the tissue tube into the liquid nitrogen for 45 seconds.
16. Quickly remove the tissue tube-holder assembly from the liquid nitrogen, and allow 2-3 seconds for the liquefied air to bubble out, and place in the CryoPrep™.
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 nails only, quickly disperse the cake by rapid pinching of the tissue tube. If you notice large tissue chunks remaining, place the tissue tube back in the liquid nitrogen for 45 seconds and repeat the pulverization step using a setting of 5.
19. Invert the tissue tube-TC12x24 assembly 180 degrees and quickly flick the tissue tube with your finger to transfer the pulverized tissue into the TC12x24 tube.
20. Place the TC12x24 tube containing the pulverized fixed tissue on dry ice.
21. 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: (Sufficient for processing 120 mg of tissue)

NOTE: Reagent volumes below are for preparing **120 mg of tissue**. If preparing **20-50 mg of tissue** then prepare half the volume of the reagents.

- Prepare 1.5 ml of 1X Covaris Lysis Buffer by mixing 300 µL of the 5X Lysis Buffer (B) with 1.2 ml of cold Molecular Biology grade Water. Add 15 µL of the 100x Halt Protease Inhibitor cocktail, and keep on ice.
- Prepare 2.5 ml of 1X Covaris Wash Buffer by mixing 250 µL of the 10X Wash Buffer (C) with 2.25 ml of cold Molecular Biology grade Water. Add 25 µL of the 100x Halt Protease Inhibitor cocktail, and keep on ice.
- Prepare 1 ml of 1X Covaris SDS Shearing Buffer by mixing 100 µL of the 10X SDS Shearing Buffer with 900 µL of cold Molecular Biology grade Water. Add 10 µL of the 100x Halt Protease Inhibitor cocktail, and keep on ice.

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 Covaris Lysis buffer containing protease inhibitors to the pulverized sample in the TC12x24 tube and transfer the contents to a 2 ml centrifugation tube.

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

4. Rinse the TC12x24 tube with another aliquot of cold Covaris Lysis buffer and add contents to the 2ml centrifugation tube.

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

5. Incubate the 2ml tube for a total of 20 minutes on a rocker or rotator at 4°C with 3 second vortexing every 10 minutes.
6. Pellet nuclei by spinning at 1,700xg for 5 minutes at 4°C.
7. Aspirate the supernatant and resuspend the pellet in Covaris Wash Buffer containing protease inhibitors. Incubate on a rocker for 10 minutes at 4°C.

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

8. Spin down nuclei at 1,700xg for 5 minutes at 4°C. Aspirate the supernatant.
9. Wash pellet by resuspending in 1ml Covaris Wash Buffer containing protease inhibitors and spinning down at 1,700xg for 5 minutes at 4°C.

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

10. Resuspend nuclei in Covaris SDS shearing buffer and incubate on ice for 10 min 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 μ l

- 11. If optimizing for the ultimate processing of nuclei from 120 mg of tissue in 1 ml of shearing buffer, then transfer the nuclei to a TC12x12 tubes for conducting the 6-point time course.**
- 12. If optimizing for the ultimate processing of nuclei from ~20 mg of tissue in 130 μ l of shearing buffer, then transfer the nuclei to 6 microTUBEs with each tube containing 130 μ l of nuclei in shearing buffer.**

AFA Chromatin Shearing

NOTE: Carry out an initial time course shearing experiment to optimize the chromatin shearing parameters specific for your tissue. We suggest 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, 15, and 20 minutes. Refer to **Figures 2 and 3** in the Example Data section.

1. If processing samples using the 130 µl microTUBEs, you will require a separate tube for each time point of the time course.
2. If processing samples using the 1 ml TC21x12 tubes, you can process all the time points of the time course in the same tube according to the table below. After each time point interval, take out 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

3. Replace the volume taken out with 35 µl of shearing buffer. Place the tube in the holder and process on the S or E series instrument for the next programmed interval processing time. 25 µl will be used for DNA shearing size range analysis, and 10 µl will be used for epitope integrity analysis using western.
4. Repeat steps 2 and 3 until all the time course points are completed.

Summary of Operating Conditions (130 µl volume in microTUBEs)

	M-Series	S- and E-Series	L-Series
Target BP (Range)	200 – 700	200 – 700	200 – 700
Duty Cycle	5%	2%	15%
Intensity		3 (S2/E210)	
Peak Incident Power	75 Watts	105 Watts (S220/E220)	300 Watts
Cycles per Burst	200	200	200
Processing Time	Empirical (2 to 12 min typical)	Empirical (2 to 12 min typical)	Empirical (15 to 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	3 x 10 ⁶ cells	3 x 10 ⁶ cells	3 x 10 ⁶ cells
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 96 microTUBE Plate (PN 520078) requires Rack-XT 96 microTUBE Plate
*Water level should be ~1 mm below the bottom of the microTUBE cap			
IMPORTANT: Always fill microTUBEs with 130 µL of solution			

Summary of Operating Conditions (1.0 ml volume in milliTUBEs)

	M-Series	S- and E-Series	L-Series
Target BP (Range)	200 – 700	200 – 700	200 – 700
Duty Cycle	10%	5%	15%
Intensity		4 (S2/E210)	
Peak Incident Power	75 Watts	140 Watts (S220/E220)	350 Watts
Cycles per Burst	200	200	200
Processing Time	Empirical (2 to 20 min typical)	Empirical (2 to 20 min typical)	Empirical (15 to 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	1.0 mL	1.0 mL	1.0 mL
Max. cell number	3 x 10 ⁷ cells	3 x 10 ⁷ cells	3 x 10 ⁷ cells
AFA Intensifier	NA	NA	NA
Water level (RUN)*	Full	S2/S220 – level 8 E210/E220 – level 5	LE220 – level 5
*Water level should be ~1 mm below the bottom of the milliTUBE cap			
IMPORTANT: Always fill milliTUBEs with 1.0 mL of solution			

After Covaris Treatment:

1. **The sheared chromatin should be used immediately for DNA shearing size range analysis, western, or IP.**

NOTE: If processing more than 1 sample on the S2/S220 system, transfer the sample into a microcentrifuge tube and place on ice as the subsequent samples are being

2. **If absolutely necessary, the sheared chromatin can be kept at 4C for up to two days.**
3. **Avoid freezing sheared chromatin since freeze/thawing will significantly reduce IP efficiency.**

NOTE: The Chromatin shearing buffer contains 0.25% SDS. Prior to IP, **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 37C for 30 min.**
3. **Add 4 µl of Proteinase K (10 mg/ml) and reverse crosslink overnight at 65C.**
4. **The sample is now ready for DNA purification for determining shearing size range analysis.**
5. **Please follow the Qiagen QIAquick PCR cleanup protocol for DNA purification.**

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.

6. **Add 30 µl of elution buffer to the column.**
7. **Incubate the column for 1 minute at RT and recover the DNA as described in the protocol.**
8. **Add 1 volume of loading dye to 5 volumes of purified DNA.**

NOTE: We strongly suggest the use of a single dye loading that does not contain bromophenol Blue. Since bromophenol Blue migrates along with the 300bp fragments, it prevents correct smear analysis.

9. We suggest loading 300-600 ng of the purified DNA per lane.
10. Resolve on 1% agarose gel run at 30V for 3.5 hours, and stain gel with Ethidium Bromide after the gel is run.
11. De-stain the gel and view gel with a UV light source and record image.
12. Alternatively staining dyes such as SYBR® Safe can be used directly in the agarose gel.
13. 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.

Example Data

Formaldehyde fixation Time course

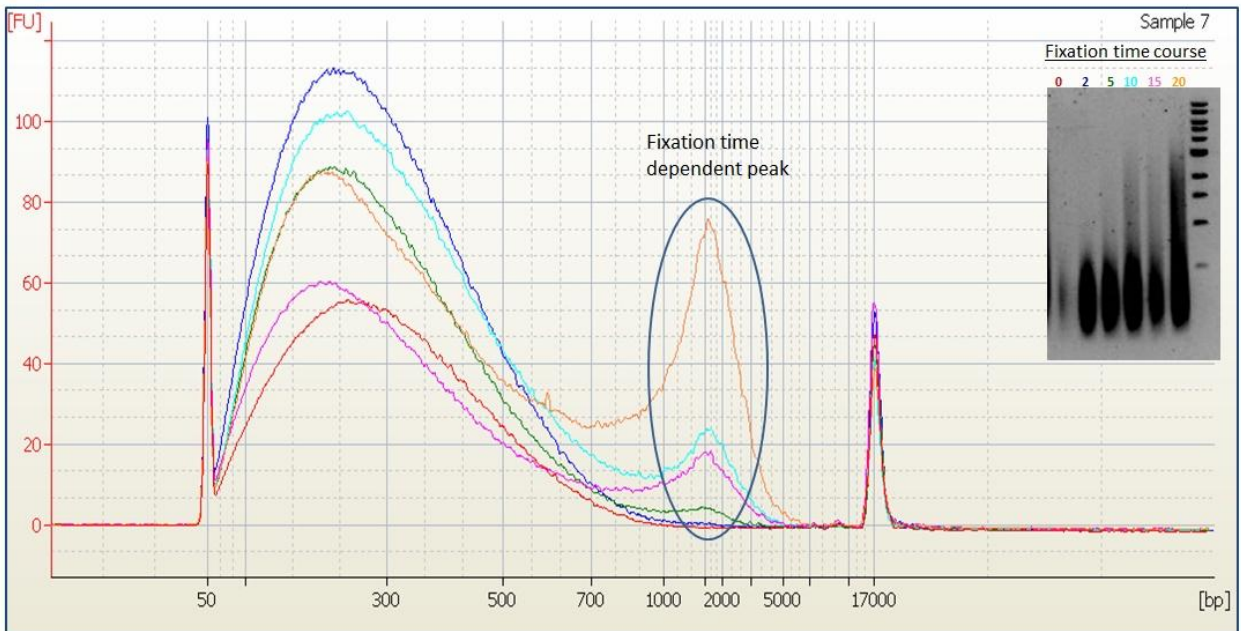


Figure 1: Effect of formaldehyde fixation time on shearing efficiency. 120mg of mouse liver tissue samples were fixed for 0, 2, 5, 10, 15, and 20 minutes, and nuclei prepared. Approximately 18mg of tissue equivalent nuclei from each fixation time point was sheared according to the setting in our protocol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

Chromatin shearing Time Course

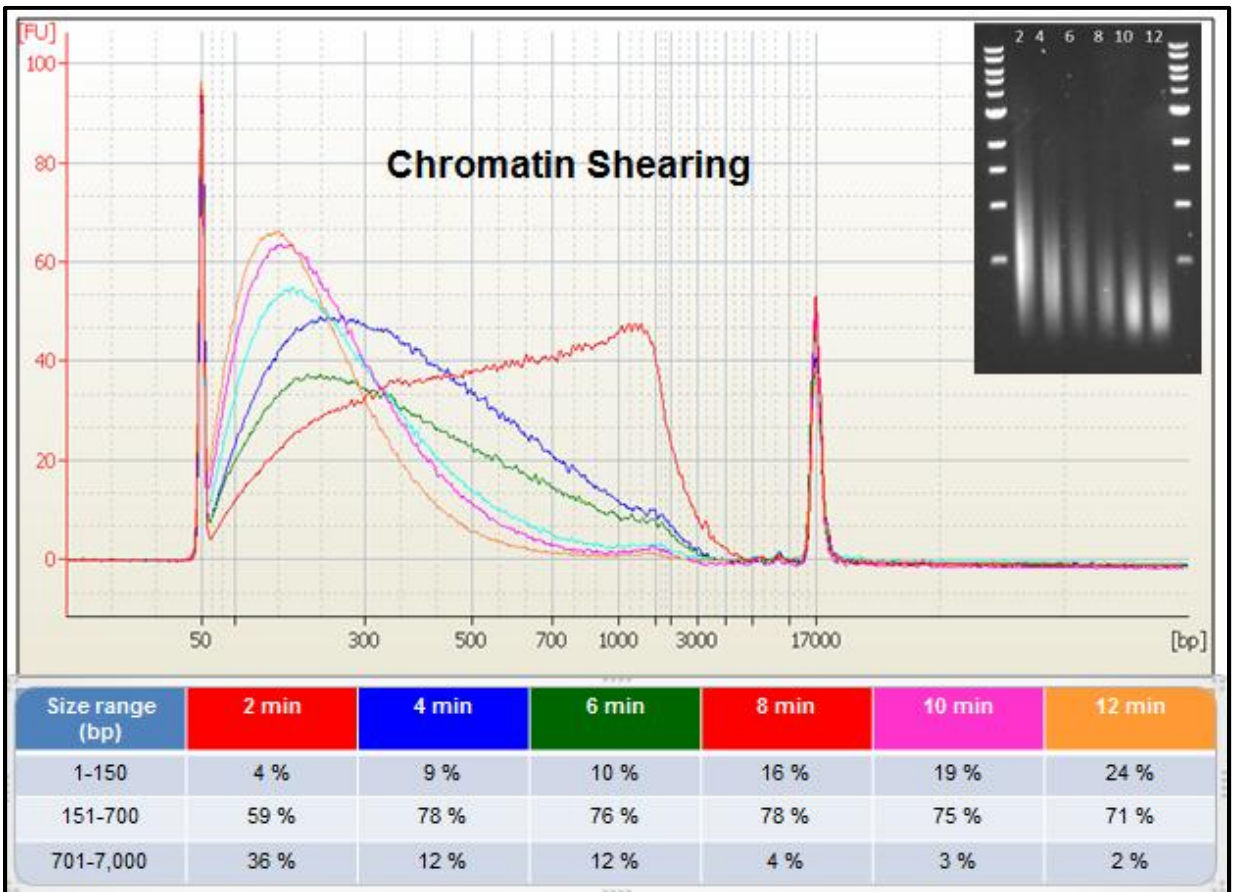


Figure 2: Chromatin shearing time course and fragment size distribution. 120mg of mouse liver tissue was fixed for 5 minutes, and nuclei prepared according to our protocol. Approximately 18mg tissue equivalent of nuclei were then processed for a time course of 2-12 minutes in 130uL volume according to the settings provided on our protocol. Note the change in fragment size, and distribution with increase in processing time.

Chromatin shearing Time Course

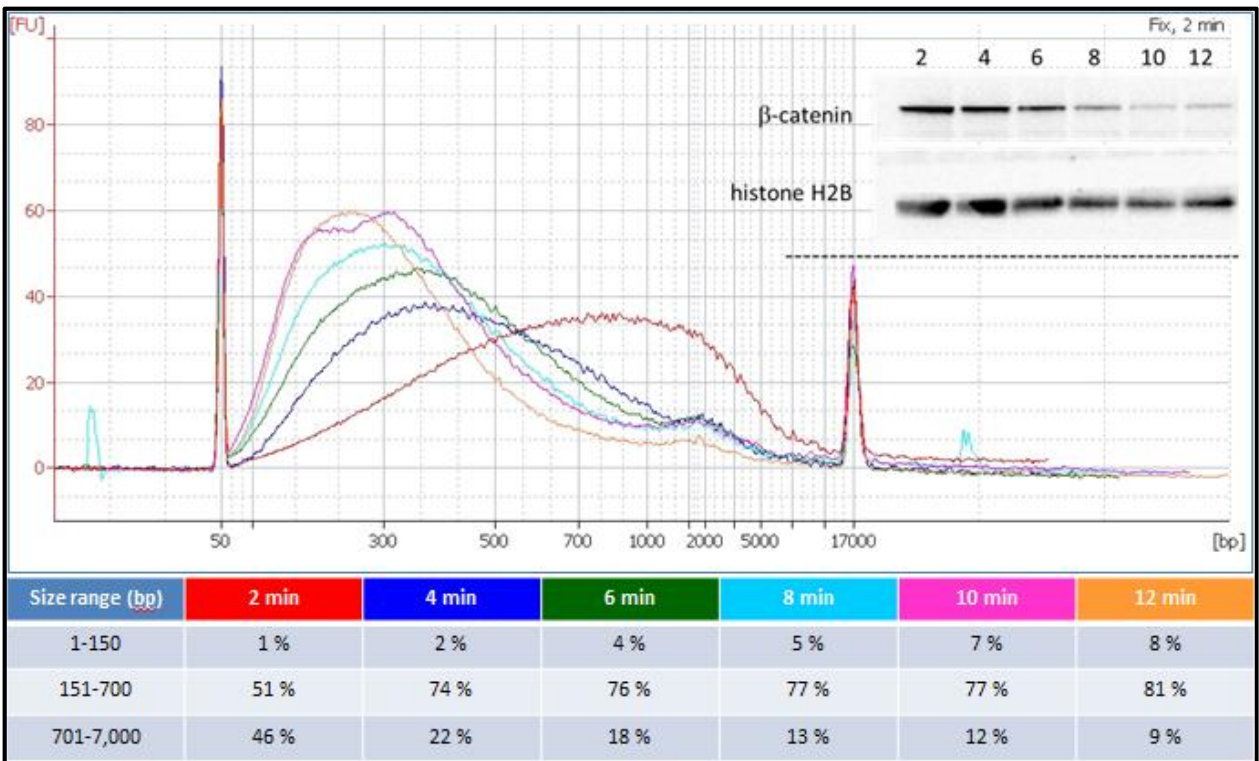


Figure 3: Chromatin shearing time course and fragment size distribution. 120mg of mouse brain tissue was fixed for 5 minutes, and nuclei prepared according to our protocol. Approximately 18mg tissue equivalent of nuclei were then processed for a time course of 2-12 minutes in 130uL volume according to the settings provided on our protocol. Note the change in fragment size, and distribution with increase in processing time while the inset western results indicate epitope integrity during the chromatin shearing process.

Highly Reproducible Across Tissue Types

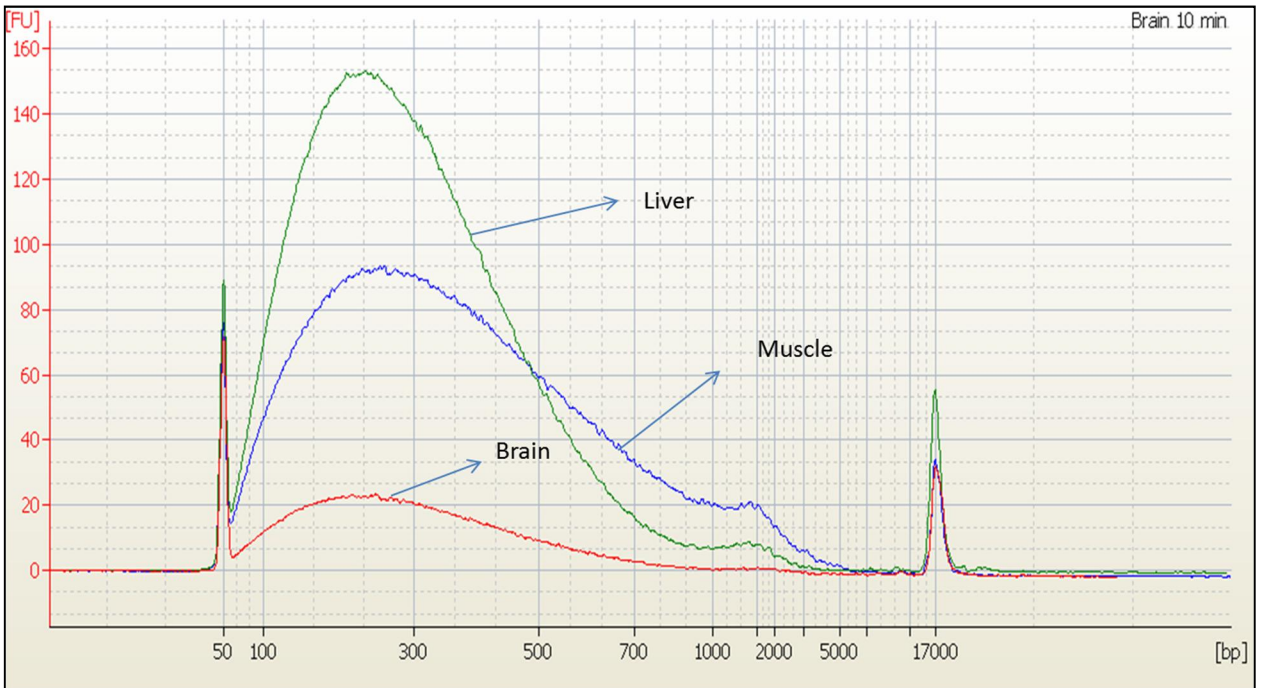


Figure 4: Processing of three different tissue types using the optimized setting for 10 minutes generated the same size average fragment size, and size distribution.

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_010147.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 AFA™ 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:

1. Lee T.I., Johnstone S.E., Young R.A., Chromatin immunoprecipitation and microarray-based analysis of protein location. *Nature Protocols* (2006) 1:729-748.
2. Dedon P.C., Soultis J.A., Allis C.D., Gorovsky M.A., A simplified formaldehyde fixation and immunoprecipitation technique for studying protein-DNA interactions. *Analytical Biochemistry* (1991) 197:8390.
3. Stewart D., Tomita A., Shi Y.B., Wong J., Chromatin immunoprecipitation for studying transcriptional regulation in *Xenopus* oocytes and tadpoles. *Methods Mol Biol* (2006) 322:165-182.
4. 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
5. Mukhopadhyay A, Deplancke B, Walhout AJM and Tissenbaum HA; Chromatin Immunoprecipitation (ChIP) coupled to detection by quantitative real-time PCR to study transcription factor binding to DNA in *Caenorhabditis elegans*. *Nature Protoc.* 2008, 3(4) 698-70.