PROTOCOL

Covaris

truXTRAC™ FFPE DNA microTUBE Kit for chemagen Technology (24)

For use with the Chemagen Prepito-D Automation Platform

Adaptive Focused Acoustics™ (AFA) -based DNA extraction and purification from Formalin-Fixed, Paraffin-Embedded (FFPE) Tissue

Contents

INTENDED USE

The truXTRAC FFPE DNA Kit is intended for use in molecular biology applications. This product is not intended for the diagnosis, prevention, or treatment of a disease.

INTRODUCTION

The truXTRAC FFPE DNA Kit for chemagen Technology is designed for the controlled and efficient extraction of DNA from Formalin Fixed, Paraffin Embedded (FFPE) tissue samples with Covaris Adaptive Focused Acoustics (AFA™) and subsequent purification on the Perkin Elmer chemagic Prepito®-D.

AFA enables the active removal of paraffin from FFPE tissue samples in aqueous buffer, allowing simultaneous tissue rehydration. Compared to traditional passive, chemical-based methods of paraffin removal, this mechanical process is not as limited by the thickness of FFPE tissue sections. The AFA process enables the use of thicker sections, which can increase DNA yield and minimize the impact of increased DNA degradation at the exposed surfaces of a section. The truXTRAC process results in high yields of high-quality DNA well suited for analytical methods such as next-generation sequencing or qPCR.

This protocol is optimized for sections up to 25 µm in thickness and cores up to 1.2 mm in diameter.

Important Notes on FFPE Samples:

The yield of DNA from FFPE tissue blocks is highly variable. Factors such as fixation time, size and thickness of the sections, the ratio of tissue to wax, the type of tissue, and the age of the FFPE block are the main causes for this variability.

The quality of DNA isolated from FFPE samples is also highly variable. During the fixation process, DNA is cross-linked to proteins and other nucleic acid molecules to varying degrees. Incomplete reversal of this crosslinking may cause the isolated DNA to perform less well in downstream applications such as PCR and qPCR. In addition, the size of DNA fragments isolated from FFPE samples is generally smaller than that of DNA isolated from fresh or frozen tissues. This is particularly evident in older FFPE sample blocks or sample blocks stored at elevated temperatures.

Note for first time users:

Given the highly variable yield of DNA from FFPE tissue blocks, we recommend using FFPE blocks that have been well characterized for yield and quality for initial testing of the truXTRAC FFPE kit. Ideally, samples should be extracted immediately after sectioning.

Please contact Covaris at Application Support (ApplicationSupport@covarisinc.com) if you have any questions.

KIT CONTENTS

Tissue SDS Buffer	3 ml
PK Solution	2x 275 µl
microTUBE-130 AFA Fiber Pre-Slit Screw-Cap	24

SDS INFORMATION IS AVAILABLE AT http://covarisinc.com/resources/safety-data-sheets/

STORAGE

This kit should be stored at room temperature (18 – 25 °C).

SUPPLIED BY USERS

Covaris Instruments and Parts

Required parts					
Focused-ultrasonicator	**LE220**	**E220 & E210**	**E220 evolution**	**S-Series**	**M220**
Rack/ Holder/ Insert	Rack-XT 24 Place microTUBE Screw-Cap (PN500388)	Rack 24 Place microTUBE Screw-Cap (PN 500308) and Intensifier (PN500141)	Rack E220e 4 Place microTUBE Screw Cap (PN500432)	Holder microTUBE Screw-Cap (PN500339)	Holder XTU PN500414 & Insert XTU PN500489 or Holder-XT PN500358 **(*)**
Accessories	Centrifuge and Heat Block microTUBE Adapter (PN500406)				
Optional parts					
Accessories	FFPE tissuePICK (PN520163)				
	FFPE sectionPICK (PN520149)				
	FFPE sectionWARMER (PN500403)				

(*) This holder has been discontinued

Perkin Elmer Chemagic Instruments and Parts

- chemagic Prepito-D
- Prepito truXTRAC DNA FFPE Kit (CMG-2037)

Other supplies:

- Dry heating block such as Eppendorf ThermoMixer or similar with either 1.5 or 2 mL heat block inserts. We recommend two heating blocks, preset at 56°C and 80°C respectively.
- RNase A (DNase free) at 10 mg/ml e.g., Thermo Scientific (PN EN0531)

PROCEDURE WORKFLOW OVERVIEW

Three different options are possible with Covaris truXTRAC FFPE DNA Kit. The three options differ in the workflows for DNA extraction.

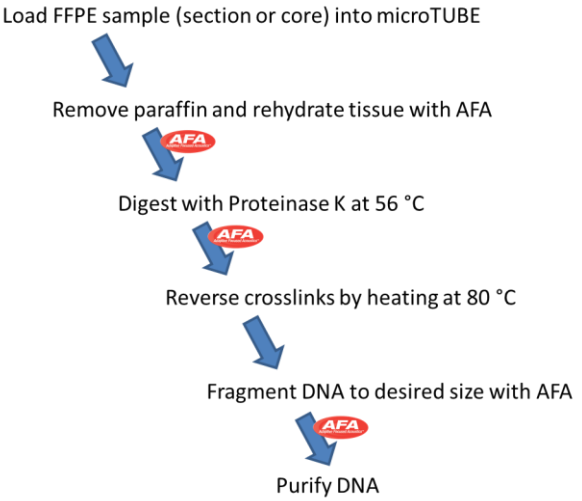
Option A: Shear DNA during extraction to a size suitable for next-generation sequencing library construction. Fragment size can be tuned between 200 and 400 bp.

Option B: Extract ~2kb DNA fragments. This protocol is recommended for most analytical applications, including PCR. Note that actual DNA fragment size will depend of the quality of the starting material.

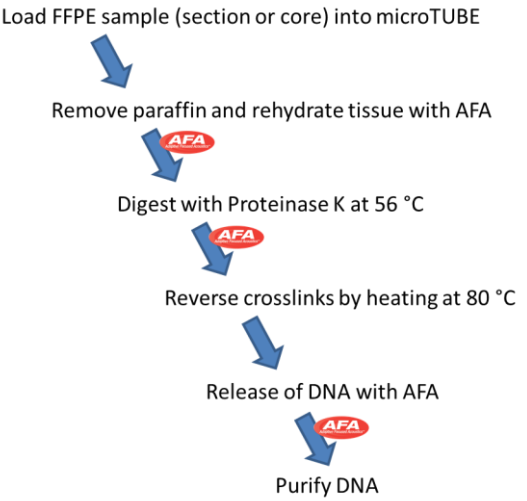
Option C: Extract large "genomic" DNA without any additional fragmentation. Actual DNA fragment size will depend on the quality of the starting material. For high-quality FFPE tissue blocks, we typically see an average fragment size of >= 8 kb.

Please refer to Appendix A for examples of final DNA fragment size distribution.

OPTION A – EXTRACT AND FRAGMENT DNA (FOR NGS)

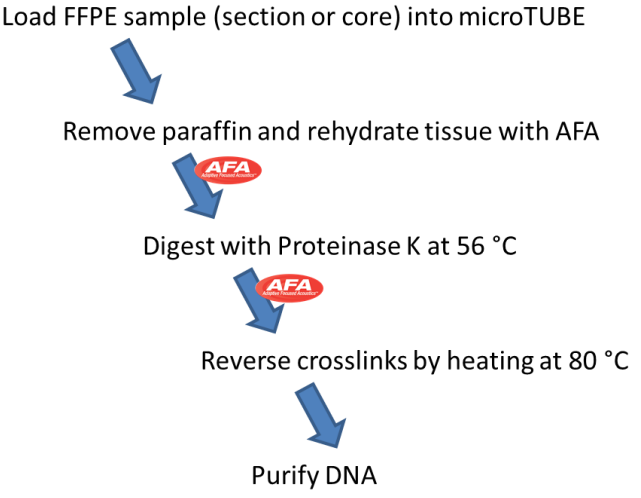


OPTION B - EXTRACT LARGE DNA FRAGMENTS (>2 KB)* WITH IMPROVED YIELD



* Actual DNA fragment size will depend of the quality of the starting tissue block

OPTION C - EXTRACT "GENOMIC" DNA*



* Actual DNA fragment size will depend of the quality of the starting tissue block

1 - PREPARATION

FFPE Tissue Sample

1. Sample Input requirements

The truXTRAC process is highly efficient at removing paraffin even from relatively thick FFPE sections while simultaneously rehydrating the tissue. Use of thicker sections is often desirable, both for increased yield and since DNA or RNA in the exposed surfaces of a section tends to degrade quickly. **We recommend using sections between 15 and 25 µm thick, or cores of 1.2 mm.**

NOTE: Excess paraffin will adversely affect the yield and quality of DNA and RNA extracted from FFPE. We strongly advise trimming off any excess of paraffin before sectioning a FFPE tissue block, or after the section has been cut from the FFPE block. A ratio of 80% tissue to 20% paraffin or higher is ideal.

	FFPE Sections Mounted on slide		FFPE Sections "scrolls" or "curls"		FFPE Cores
Size (thickness or diameter)	4 to 10 µm	7 to 10 µm	7 to 15 µm	16 to 25 µm	< 1.2 mm diameter
Size (length)	NA		<10 mm (A)		
Collection tool	tissuePICK (B)	sectionPICK (B)	NA		
Maximum number of samples Per Tube*	2x tissuePICK (200 mm^2 tissue for a 5 µm section)	2x sectionPICK	2	1	1

* Numbers represent trimmed sections only

NOTES

A. If the FFPE sample is longer than about 10 mm, cut it in half before loading.

B. For optimal tissuePICK and sectionPICK performances, tissue section should be mounted on uncoated slides. The tissuePICK and sectionPICK should always be used in conjunction with a sectionWARMER.

WARNING: The total mass of FFPE sample processed per extraction should be between 2 to 5 mg. Lower amounts may result in insufficient yield and higher amounts may cause spin columns to become partially or fully clogged.

2. Tissue Fixation Requirements

The yield and quality of DNA extracted from FFPE tissue blocks is highly dependent on tissue collection and paraffin embedding procedures. For good yields of high quality DNA:

- Use a maximum fixation time of 24 hours
- Use Formalin solution, neutral buffered, 4%
- Fix sample tissue sample as quickly as possible after collection

Buffer

1. **Check Tissue SDS Buffer**: A white precipitate may form during storage. Incubate the bottles at 50 – 70 °C before use to dissolve any precipitate.

Instruments

NOTE: For detailed instructions on how to prepare your particular instrument please refer to your instruments User Manual.

1. For S, E and LE-Series Focused-ultrasonicators, fill the water bath, set the chiller temperature as described in Table 1 and allow the system temperature to equilibrate and the water bath to degas.
   - E220 evolution: Load the plate definition "500432 Rack E220e 4 Place microTUBE Screw Cap" and check that the intensifier is in place.
   - E210 or E220: Load the plate definition "500308 Rack 24 Place microTUBE Screw-Cap" and check that the intensifier is in place.
   - LE-Series: Load the plate definition "500388 Rack-XT 24 Place microTUBE Screw-Cap".

NOTE: If you do not see a plate definition on your system, please contact Covaris technical support at TechSupport@covarisinc.com.

Table 1 - Focused-ultrasonicator setup

Instrument	Water level (Fill/RUN scale)	Chiller temperature
S-Series	**15**	**18°C**
E-Series & L-Series	**10**	**18°C**

2. For M-Series Focused-ultrasonicators, put the Holder XTU and the Insert XTU microTUBE 130 µl (or Holder-XT microTUBE) in place and fill the water bath until the water reaches the top of the holder. Allow system to reach temperature (**20°C**).
3. Insert the required number of Heat Block microTUBE Adapters into two heat blocks and heat them to 56 and 80**°**C.

Perkin Elmer chemagic Prepito®-D

Please refer to the chemagic Prepito-D instrument user manual (revision 2022-0020)

2 - DNA EXTRACTION FROM FFPE: TISSUE DE-PARAFFINIZATION, PROTEIN DIGESTION, AND DE-CROSSLINKING

Option A - Extract and fragment DNA (for NGS)

This protocol allows direct fragmentation of DNA to a size suitable for Next Generation Sequencing library construction during the extraction process.

1. Using table 2 below as a guide, generate a master mix of Processing Buffer by mixing Tissue Lysis Buffer and Proteinase K**.**

Table 2 – Processing Buffer master mix

Number of samples	Tissue SDS Buffer volume	Proteinase K volume
8	**704** µl	**176** µl
16	**1408** µl	**352** µl
x	**x * 88** µl	**x * 22** µl

2. Open microTUBE Screw-Cap, add 100 µl Processing Buffer master mix into each microTUBE and load FFPE tissue (section or core). Affix Screw-Cap back in place.

> **NOTE:** If the FFPE tissue samples are loose or broken the samples may be added to the microTUBE prior to Tissue SDS Buffer addition to facilitate easier loading.

3. Process the sample using the settings provided in Table 3 below to dissociate the paraffin while simultaneously rehydrating the tissue. (Please see the example in Appendix B.) During the AFA process it is normal for the solution to turn milky white as the paraffin is emulsified.

> **NOTE***:* Processed samples can be kept at room temperature for 2 hours while remaining samples are processed.

Table 3 - Paraffin removal and tissue rehydration settings

System	Duty Factor	Peak Incident Power	Cycles per burst	Treatment Time	Temperature (Instrument)
S220 or E220	**10%**	**175 Watts**	**200**	**300 sec**	**20 °C**
S2 or E210	**10%**	**5 (Intensity)**	**200**	**300 sec**	**20 °C**
M220	**20%**	**75 Watts**	**200**	**300 sec**	**20 °C**
LE220	**30%**	**450 Watts** [(1)]	**200**	**300 sec**	**20 °C**

(1) As Covaris LE220 process multiple samples at a time, its PIP is distributed across microTUBEs, and power received by individual microTUBE stays within the 200 W limit.

4. Protein digestion at 56°C
    a. Insert the required number of Heat Block microTUBE Adapters into a Heat Block and set the temperature to 56°C.
    b. Load the microTUBE into the adapter once the heat block has reached its set point.
    c. Incubate for 1 hour (sections <= 10 µm) or overnight (sections > 10 µm or cores) for protein digestion. If the digestion is incomplete after overnight incubation, add another 20 µl of Proteinase K solution, mix, and incubate for 1 more hour.

5. Incubate the samples at 80°C to reverse formaldehyde crosslinks:
    a. Insert the required number of Heat Block microTUBE Adapters into a Heat Block and set the temperature to 80°C.
    b. Load the microTUBE into the adapter once the heat block has reached its set point.
    c. Incubate for 1 hour.

NOTE: If you are using the same heat block for both the 56°C & 80°C incubations, the microTUBE should be stored at room temperature until the heat block reaches 80°C.

6. DNA fragments size can be tuned to the desired average fragment size by using the settings in Table 4 below.

NOTE: If the target size is not achieved then the treatment time should be adjusted.

Table 4 - DNA Shearing settings

E- and S-Series Focused-ultrasonicator			
Targeted fragment size	**200 bp**	**300 bp**	**400 bp**
Treatment Time	**300 sec**	**110 sec**	**80 sec**
PIP (S220 and E220)	**175 W**	**175 W**	**175 W**
Intensity (S2 and E210)	**5**	**5**	**5**
Duty Factor	**10%**	**10%**	**10%**
Cycle per Burst	**200**	**200**	**200**
Temperature	**20 °C**	**20 °C**	**20 °C**

M220 Focused-ultrasonicator			
Targeted fragment size	**200 bp**	**300 bp**	**400 bp**
Treatment Time	**450 sec**	**200 sec**	**120 sec**
PIP	**75 W**	**75 W**	**75 W**
Duty Factor	**20%**	**20%**	**20%**
Cycle per Burst	**200**	**200**	**200**
Temperature	**20 °C**	**20 °C**	**20 °C**

LE220 Focused-ultrasonicator			
Targeted fragment size	**200 bp**	**300 bp**	**400 bp**
Treatment Time	**300 sec**	**150 sec**	**80 sec**
PIP	**450 W** [(1)]	**450 W** [(1)]	**450 W** [(1)]
Duty Factor	**30%**	**30%**	**30%**
Cycle per Burst	**200**	**200**	**200**
Temperature	**20 °C**	**20 °C**	**20 °C**

7. Proceed to Section 3 – DNA Purification.

Option B - Extract large DNA fragments (>2 kb)* with improved yield

1. Using table 5 below as a guide, generate a master mix of Processing Buffer by mixing Tissue Lysis Buffer and Proteinase K**.**

Table 5 – Processing Buffer master mix

Number of samples	Tissue SDS Buffer volume	Proteinase K volume
8	**704** µl	**176** µl
16	**1408** µl	**352** µl
x	**x * 88** µl	**x * 22** µl

2. Open microTUBE Screw-Cap, add 100 µl Processing Buffer master mix into each microTUBE and load FFPE tissue (section or core). Affix Screw-Cap back in place.

> **NOTE:** If the FFPE tissue samples are loose or broken the samples may be added to the microTUBE prior to Tissue SDS Buffer addition to facilitate easier loading.

3. Process the sample using the settings provided in Table 6 below to dissociate the paraffin while simultaneously rehydrating the tissue. (Please see the example in Appendix B.) During the AFA process it is normal for the solution to turn milky white as the paraffin is emulsified.

> **NOTE***:* Processed samples can be kept at room temperature for 2 hours while remaining samples are processed.

Table 6 - Paraffin removal and tissue rehydration settings

System	Duty Factor	Peak Incident Power	Cycles per burst	Treatment Time	Temperature (Instrument)
S220 or E220	**10%**	**175 Watts**	**200**	**300 sec**	**20 °C**
S2 or E210	**10%**	**5 (Intensity)**	**200**	**300 sec**	**20 °C**
M220	**20%**	**75 Watts**	**200**	**300 sec**	**20 °C**
LE220	**30%**	**450 Watts** [(1)]	**200**	**300 sec**	**20 °C**

[(1)] As Covaris LE220 process multiple samples at a time, its PIP is distributed across microTUBEs, and power received by individual microTUBE stays within the 200 W limit.

4. Protein digestion at 56°C
    a. Insert the required number of Heat Block microTUBE Adapters into a Heat Block and set the temperature to 56°C.
    b. Load the microTUBE into the adapter once the heat block has reached its set point.
    c. Incubate for 1 hour (sections <= 10 µm) or overnight (sections > 10 µm or cores) for protein digestion. If the digestion is incomplete after overnight incubation, add another 20 µl of Proteinase K solution, mix, and incubate for 1 more hour.

5. Incubate the samples at 80°C to reverse formaldehyde crosslinks:
    a. Insert the required number of Heat Block microTUBE Adapters into a Heat Block and set the temperature to 80°C.
    b. Load the microTUBE into the adapter once the heat block has reached its set point.
    c. Incubate for 1 hour.

> **NOTE**: If you are using the same heat block for both the 56°C & 80°C incubations, the microTUBE should be stored at room temperature until the heat block reaches 80°C.

6. Process the sample using the settings in Table 7 below to release the DNA with AFA.

Table 7 – DNA release with AFA

System	Duty Factor	Peak Incident Power	Cycles per burst	Time	Temperature (Instrument)
S220 or E220	**10%**	**105 Watts**	**200**	**10 sec**	**20 °C**
S2 or E210	**10%**	**3 (Intensity)**	**200**	**10 sec**	**20 °C**
M220	**20%**	**75 Watts**	**200**	**10 sec**	**20 °C**
LE220	**30%**	**300 Watts** [(1)]	**200**	**10 Sec**	**20 °C**

7. Proceed to Section 3 – DNA Purification.

Option C - Extract genomic DNA*

1. Using table 8 below as a guide, generate a master mix of Processing Buffer by mixing Tissue Lysis Buffer and Proteinase K**.**

Table 8 – Processing Buffer master mix

Number of samples	Tissue SDS Buffer volume	Proteinase K volume
8	**704** µl	**176** µl
16	**1408** µl	**352** µl
x	**x * 88** µl	**x * 22** µl

2. Open microTUBE Screw-Cap, add 100 µl Processing Buffer master mix into each microTUBE and load FFPE tissue (section or core). Affix Screw-Cap back in place.

> **NOTE:** If the FFPE tissue samples are loose or broken the samples may be added to the microTUBE prior to Tissue SDS Buffer addition to facilitate easier loading.

3. Process the sample using the settings provided in Table 9 below to dissociate the paraffin while simultaneously rehydrating the tissue. (Please see the example in Appendix B.) During the AFA process it is normal for the solution to turn milky white as the paraffin is emulsified.

> **NOTE***:* Processed samples can be kept at room temperature for 2 hours while remaining samples are processed.

Table 9 - Paraffin removal and tissue rehydration settings

System	Duty Factor	Peak Incident Power	Cycles per burst	Treatment Time	Temperature (Instrument)
S220 or E220	**10%**	**175 Watts**	**200**	**300 sec**	**20 °C**
S2 or E210	**10%**	**5 (Intensity)**	**200**	**300 sec**	**20 °C**
M220	**20%**	**75 Watts**	**200**	**300 sec**	**20 °C**
LE220	**30%**	**450 Watts** [(1)]	**200**	**300 sec**	**20 °C**

[(1)] As Covaris LE220 process multiple samples at a time, its PIP is distributed across microTUBEs, and power received by individual microTUBE stays within the 200 W limit.

4. Protein digestion at 56°C
    a. Insert the required number of Heat Block microTUBE Adapters into a Heat Block and set the temperature to 56°C.
    b. Load the microTUBE into the adapter once the heat block has reached its set point.
    c. Incubate for 1 hour (sections <= 10 µm) or overnight (sections > 10 µm or cores) for protein digestion. If the digestion is incomplete after overnight incubation, add another 20 µl of Proteinase K solution, mix, and incubate for 1 more hour.

5. Incubate the samples at 80°C to reverse formaldehyde crosslinks:
    a. Insert the required number of Heat Block microTUBE Adapters into a Heat Block and set the temperature to 80°C.
    b. Load the microTUBE into the adapter once the heat block has reached its set point.
    c. Incubate for 1 hour.

> **NOTE**: If you are using the same heat block for both the 56°C & 80°C incubations, the microTUBE should be stored at room temperature until the heat block reaches 80°C.

6. Proceed to Section 3 – DNA Purification

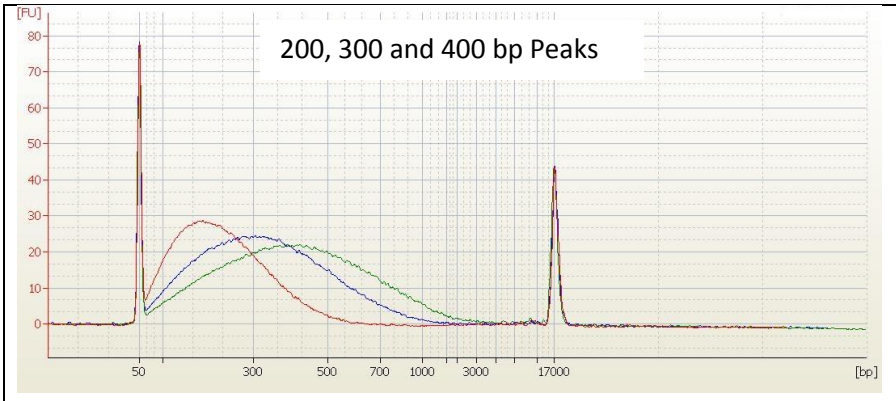
3 - DNA PURIFICATION WITH THE CHEMAGIC PREPITO

It requires the Prepito truXTRAC DNA FFPE Kit (CMG-2037) and associated script.

1. Transfer the FFPE lysate from the microTUBEs to the first row of a 96 well Deep Well Plate (DWP). DWP is included in the Prepito truXTRAC DNA FFPE Kit.
2. **Optional**: The sample can be treated with RNase A to remove RNA before DNA purification. Add 5 µl of RNase A solution to the FFPE lysate and incubate for 5 minutes at room temperature.
3. Follow Prepito truXTRAC DNA FFPE Kit instructions to automatically purify the DNA.

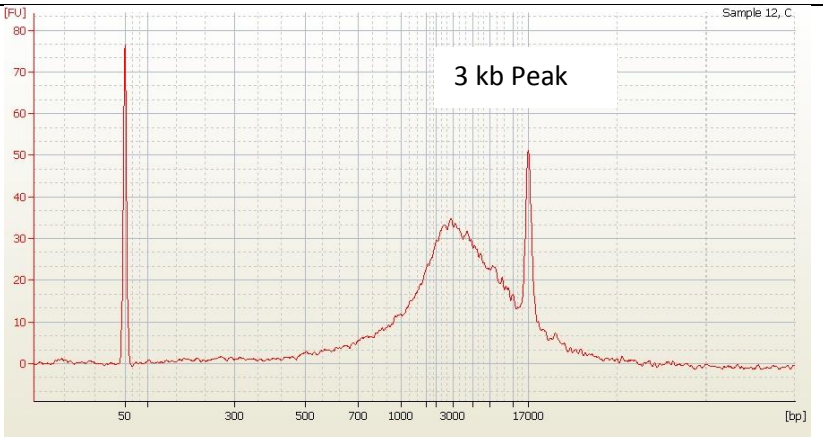
APPENDIX A – EXAMPLES OF DNA FRAGMENTS SIZE DISTRIBUTION

In these examples, DNA has been extracted from 10 µm sections off the same kidney tissue block with the Covaris FFPE kit following the 3 available options. The Bioanalyzer electropherograms below represent the fragment size distribution after purification. The size of the non-fragmented, genomic DNA (Option C) depends of the previous storage condition of the tissue block, including how it has been fixed and paraffin embedded.



Option A
Extract and fragment DNA (for NGS)

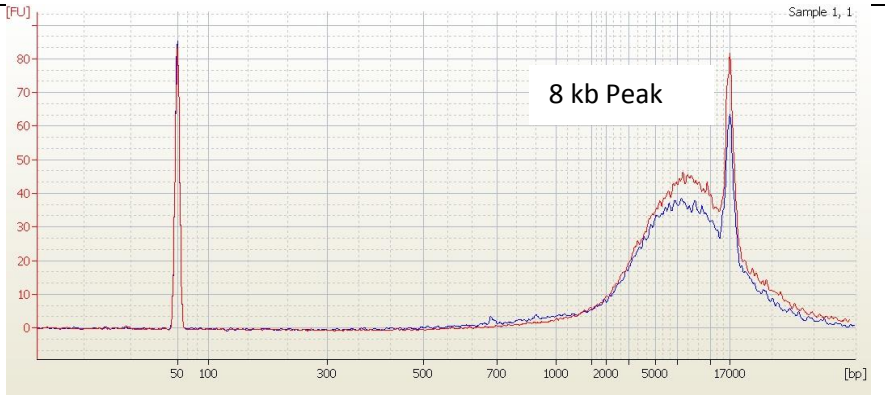
Subsequently to extraction, DNA is sheared to a size suitable for Next Gen Sequencing library construction. In this example, one sample has been sheared to 200 bp, one to 300 bp and one to 400 bp.



Option B
Extract large DNA fragments (>2 kb)*

AFA energy is used to release the DNA from the tissue. During this process the DNA is also sheared into fragments larger than 2 kb.

*Final size will depend of the quality of the starting tissue.



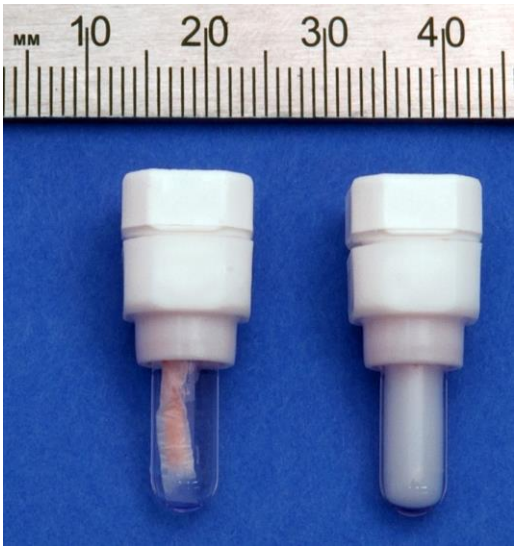
Option C
Extract genomic DNA*

DNA size will be the largest possible and will depend on the quality of the starting tissue.

*Final size will depend of the quality of the starting tissue

APPENDIX B – PARAFFIN EMULSIFICATION WITH AFA ENERGY

Paraffin is emulsified in microTUBE Screw-Cap using a Covaris S220 Focused-ultrasonicator. Sample before (left side) and after (right side) processing. Sample was a 10 µm kidney tissue section.



Additional Notes

1. Best Practices to determining the yield and purity of isolated DNA:
   - To determine DNA yield with the highest level of accuracy, a fluorometric assay such as the Qubit ™ (Life Technologies) should be used.
   - In addition, spectrophotometric analysis of DNA for A260/280 and A260/230 ratios will determine if protein or peptide contamination is present in the sample.
2. Tissue Blocks were obtained from: Theresa Kokkat, PhD and Diane McGarvey, Cooperative Human Tissue Network (CHTN), Eastern Division, University of Pennsylvania, USA
3. See following link: **http://covarisinc.com/wp-content/uploads/pn_010239.pdf** for updates to this document.
4. The treatment settings listed in this document are recommended guidelines. Actual results may vary depending on the tissue type, mass, and previous handling of FFPE samples.

APPENDIX C – TROUBLESHOOTING GUIDE

Issue	Cause	Solution	Comments / Suggestions
Low yield of DNA	Low tissue to wax ratio in FFPE section.	Repeat the procedure using additional sections until desired yield is achieved.	In your initial use of the truXTRAC FFPE kit use FFPE blocks that have been well characterized for yield and quality.
	Proteinase K stored above recommended temperature or expired.	Repeat the procedure using fresh Proteinase K.	Always store proteinase K solution at Room Temperature or 4°C
DNA does not perform well in downstream applications such as qPCR	DNA in FFPE sample blocks is severely cross-linked or degraded.	Design amplicons to be as small as possible (<100 bp).	DNA isolated using Covaris AFA technology is of the highest possible quality. Some FFPE sample blocks may be too degraded or cross-linked for some applications.
DNA fragments size too large when following Option A	Too much emulsified paraffin in the sample	Trim any excess paraffin from tissue blocks before proceeding with protocol. If it isn't possible to completely trim the paraffin from the FFPE block, we recommend running a time course at step 7 and to increase the treatment time by 30 seconds steps.	Too much emulsified paraffin absorbs some of the acoustic energy and will adversely affect DNA Shearing efficiency.