

DNA Extraction from FFPE samples with Covaris Focused-ultrasonicators

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Summary

This Application Note describes the use of Covaris Adaptive Focused Acoustic® (AFA®) technology to extract DNA from Formalin-Fixed, Paraffin Embedded (FFPE) tissues. When an AFA treatment is applied to a FFPE sample, it simultaneously deparaffinizes the sample, emulsifies the paraffin, and rehydrates the tissue. This is a mechanical process which eliminates the use of hazardous organic solvents. Using currently available methods, DNA extracted from FFPE tissues is not of sufficient quality for the sensitive, reproducible molecular analysis required for clinical research and diagnostics. The Covaris FFPE DNA Purification Kit provides a high yield and high quality solution for DNA extraction from FFPE tissue samples. Traditional quality control tests, such as DNA fragment size distribution and concentration, are not indicative of the quality required for successful assays. Here, the quality of the extracted DNA with the Covaris FFPE DNA Purification Kit is tested by a multiplex PCR-based quality control assay, showing the DNA is purified with yields more than 3 times higher than other commercially available extraction methods and is more readily available for PCR amplification. Overall, this new FFPE DNA extraction solution greatly enhances DNA availability, improves reproducibility, reduces hands on time, and ensures the seamless integration with Next Generation Sequencing (NGS) and other molecular analysis applications, making it a perfect solution for high value, often irreplaceable, clinical samples.

Introduction

Formalin fixation followed by paraffin embedding is the most widely used method of tissue preservation, and comprises the largest and best historical collection of patient material in pathology archives. FFPE has been the preferred method for preserving tissue samples because it allows for extended room temperature storage of tissues while maintaining histopathology integrity. It is estimated that there are more than 1 billion FFPE tissue samples available (1) in biobanks worldwide and these samples represent an invaluable source of information for molecular analysis. Even today, clinical biopsies and resected tissues are stabilized using FFPE for histopathology and nucleic acid based diagnostic analysis.

To recover nucleic acids from a FFPE sample, the paraffin must be disassociated from the tissue and the tissue rehydrated for enzymatic digestion. This is typically done by placing the sample in an organic solvent, such as xylene or CitriSolv, to dissolve the paraffin or by heating the sample in mineral oil. Generally, these procedures yield incomplete reversal of the formaldehyde crosslinks, mixtures of single and double stranded DNA, and low amounts of nucleic acid. Consequently, conventional methods are not suitable for high sensitivity analytical assays.

The protocol presented here dramatically changes these paradigms by using AFA technology to mechanically process the tissue sample. The purely mechanical process greatly simplifies the workflow while increasing the yield and reproducibility essential for clinical research and diagnostics.

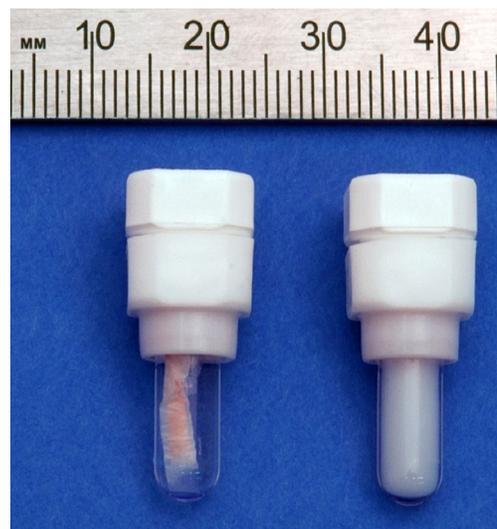


Figure 1: FFPE tissue processing with Covaris Focused-ultrasonicator. Sample before (left side) and after (right side) processing. Sample was a 10 μ m tissue section.

DNA Extraction from FFPE Samples

The acoustic energy provided by a Covaris Focused-ultrasonicator is used to simultaneously deparaffinize the sample and emulsify the paraffin (**Figure 1**). This is a mechanical process that does not rely on the use of organic solvents. During the acoustic treatment the tissue in the sample is rehydrated and homogenized thereby increasing substrate accessibility for Proteinase K digestion. The high frequency acoustic waves generated by AFA cause microstreaming, which enhances dissociation of biomolecules and improves DNA extraction yields.

The Covaris FFPE DNA Extraction Kit protocol offers 3 options and allows the user to select the size of the purified DNA. Depending on which option is chosen, the size can range from genomic DNA to small fragments suitable for NGS analysis. For all options, it takes less than 4 hours from FFPE samples to purified DNA, with minimal hands on time.

Option A	
Extract and fragment DNA for NGS	Subsequent to extraction, DNA is sheared to a size suitable for Next Gen Sequencing library construction.
Option B	
Extract large DNA fragments for qPCR analysis and microarray	An additional AFA step is used to enhance dissociation of biomolecules and improve DNA extraction yields. During this process DNA is also slightly sheared into fragments larger than 2 kb.
Option C	
Extract genomic DNA	DNA extraction with AFA. Fragments size will be the highest possible depending on the quality of the starting tissue.

Extract and Fragment DNA for NGS - Option A

DNA sequencing applications require controlled fragmentation of the DNA as part of the library preparation workflow. Covaris AFA technology, the standard DNA fragmentation method for NGS applications, can be used to fragment the DNA while still in the crude extract, thus reducing the number of downstream steps and simplifying the overall library preparation protocol. As in other AFA DNA shearing applications, the DNA fragment size is controlled by the amount of energy delivered by the Focused-ultrasonicator, making it tunable to specific sizes (**Figure 4**).

Extract and Fragment DNA for NGS - Option B & C

Most downstream analytical applications require the highest possible quality of DNA. The size of genomic fragments is highly dependent upon the handling of the tissue prior to its embedding in paraffin. These DNA extraction protocols permit the user to select for either maximum fragment size (**Option C**) or maximum yield with an additional AFA step (**Option B**) from a given sample (**Figures 2 and 3**).

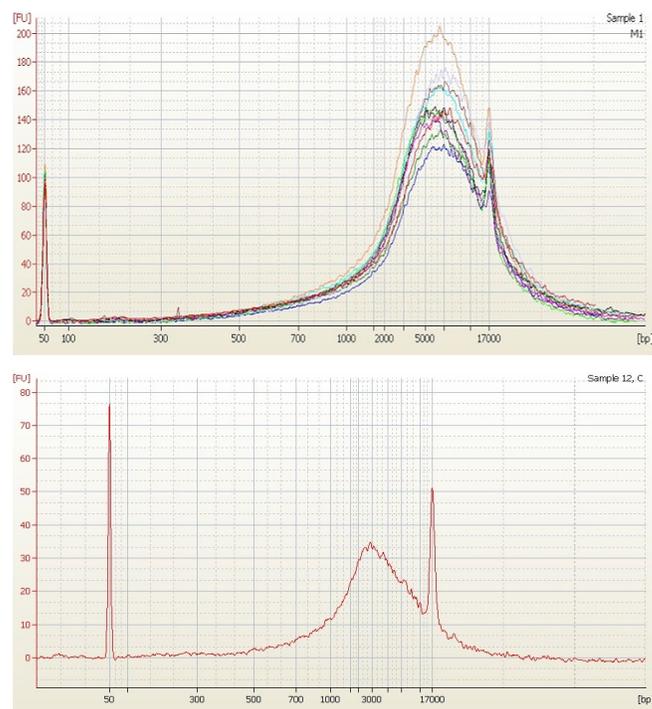


Figure 2: Extract Genomic DNA or large DNA Fragments (>2 kb). Fragments size distributions of DNA extracted from FFPE block. Top panel: Option C to extract genomic DNA. Ten 20 μ m sections from a kidney processed as replicates. Bottom panel: Option B to extract large DNA fragments (> 2 kb).

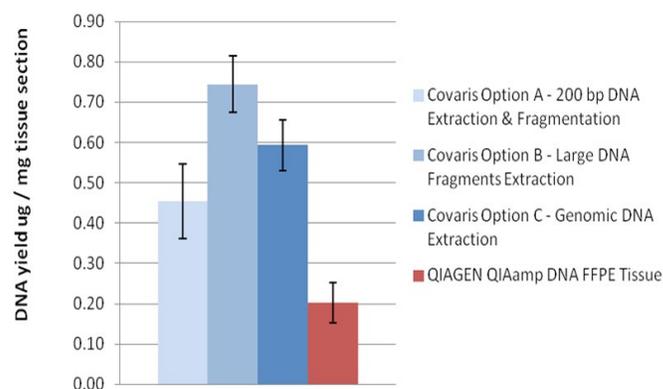


Figure 3: DNA Extraction yield. DNA quantification with Qubit Fluorometer. DNA was isolated from one 10 μ m slice for each sample. Adjacent slices were used and each condition was run with 6 replicates.

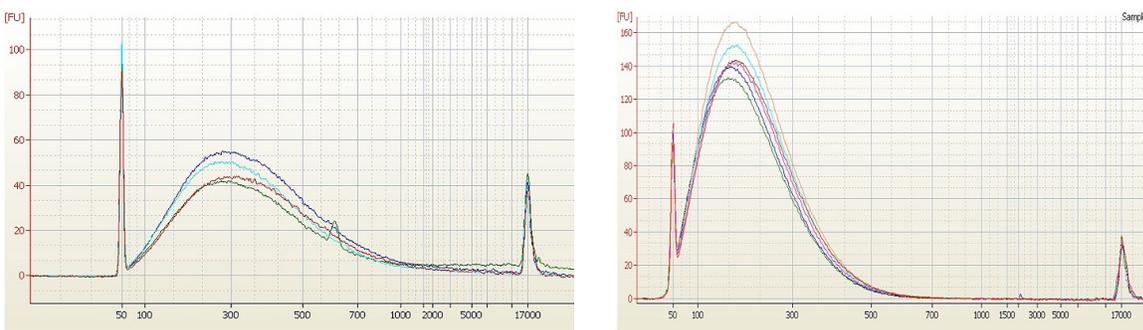


Figure 4: Fragment DNA for NGS. DNA fragments distribution profiles after direct fragmentation in the crude extract following option A. Left panel: 4 replicates fragmented at 300 bp. Right panel: 6 replicates fragmented at 200 bp. Samples were 10 μ m thick sections from FFPE kidney blocks.

Comparison of the Normalized DNA Yields of Various Extraction Methods

The DNA yields of samples extracted using each of the 3 Covaris options were compared to the yield from the commercially available QIAGEN kit following their standard protocol. The DNA yield was measured with Qubit (**Figure 3**). Covaris Option B yields the highest amount of DNA, the additional AFA step enhances dissociation of biomolecules and thus improves DNA extraction yield. Covaris Option C yields slightly less DNA than Option B, but still 3 times more than QIAGEN kit. The Covaris Option A appears to yield less DNA than method B or C, however this is an artefact. Qubit dye doesn't have the same binding efficiency for small and large DNA fragments and the standard curve is conducted with DNA > 1 kbp, so DNA yield appears artificially low for 200 bp DNA fragments.

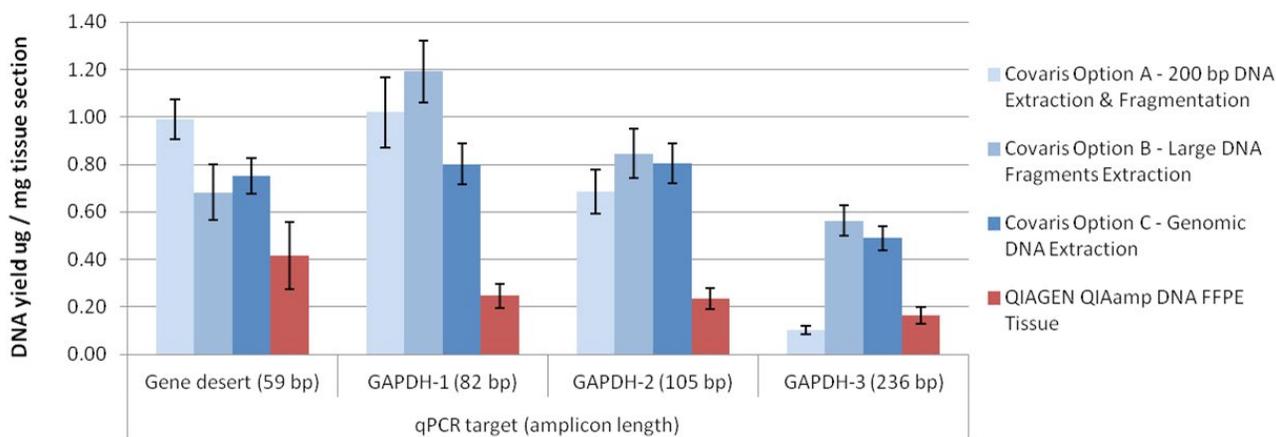


Figure 5: Quality Assessment Based on GAPDH Primer Sets. GAPDH gene: normalized DNA yields from Real-Time PCR of DNA extracted from FFPE Kidney samples. DNA was isolated from one 10 μ m slice for each sample. Adjacent slices were used and 6 samples were processed for each condition.

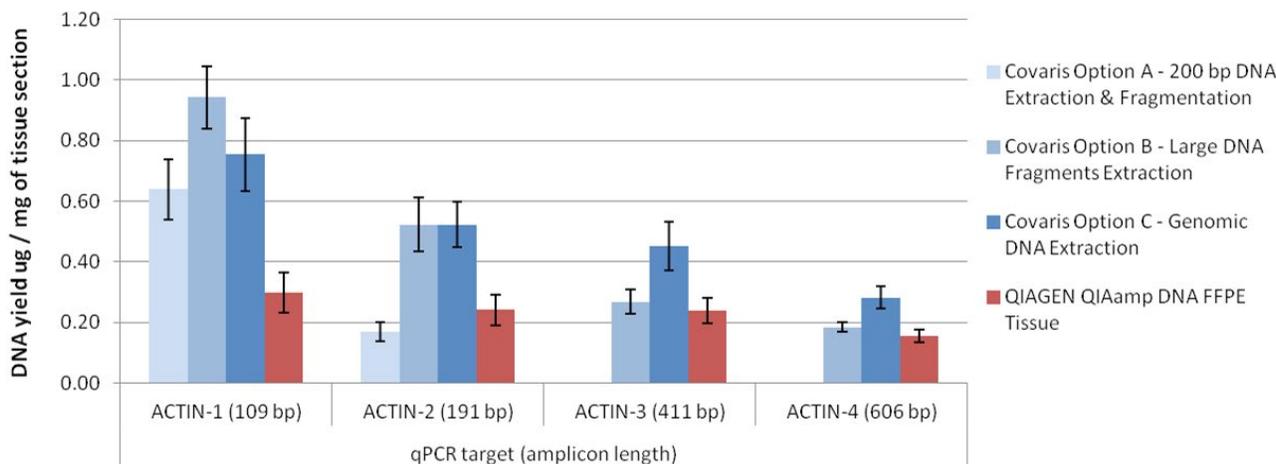


Figure 6: Quality Assessment Based on ACTIN Primer Sets. ACTIN gene: normalized DNA yields from Real-Time PCR of DNA extracted from FFPE Kidney samples. DNA was isolated from one 10 μ m slice for each sample. Adjacent slices were used and 6 samples were run for each condition.

Quality Control on Purified DNA

Fragments size distribution and concentration are not by themselves indicative of high quality DNA required for successful molecular analysis. However, PCR amplification of amplicons of various lengths is well correlated with analytical success (2) and can be used as a quality control measure. We tested the quality of the DNA Extracted with the Covaris DNA Purification Kit by running qPCR assays for two genes (GADPH and ACTIN), with amplicons of various lengths (from 59 to 606 bp) and compared it to the quality of the DNA extracted with QIAGEN QIAamp DNA FFPE Tissue kit. (Figures 5 and 6).

Covaris Option A allows for the extraction and fragmentation of DNA. DNA fragment size can be tuned; in this example, 200 bp fragments, the DNA fragment size most used in NGS, were generated. The target sequences in these fragments were readily amplifiable by qPCR for amplicon up to 200 bp in length, indicating that these fragments would be excellent substrates for NGS library preparation.

Covaris Option B and C protocols allow for extraction of high quality DNA containing target sequences that support the amplification of up to 606 bp amplicons at a yield higher than QIAGEN kit.

Materials and Methods

FFPE Tissue Samples

FFPE tissues blocks were obtained in collaboration with CHTN (Cooperative Human Tissue Network, Eastern Division University of Pennsylvania). The FFPE samples used in this protocol were from normal human kidney or endometrial tissue. Excess paraffin was trimmed away from the tissue. For each FFPE sample, a 10 or 20 µm section was cut using a microtome. The microtome was adjusted to generate scrolls as the tissue block was sectioned to facilitate loading in the microTUBE. Each section was weighed individually and placed in a microTUBE Screw-Cap.

DNA Isolation

Covaris FFPE DNA Purification Kit (PN 520117) protocols were followed to isolate the DNA from the FFPE tissue sections. A single 10 or 20 µm section was loaded into a microTUBE Screw-Cap with the appropriate volume of Covaris Lysis Buffer. A Covaris S220 Focused-ultrasonicator was used to simultaneously deparaffinize the sample, emulsify the paraffin and rehydrate the tissue. The tissue was then digested with Proteinase K at 56 °C for 1 hour.

Crosslink reversal was carried out at 80 °C for one hour. Then, depending on the downstream analytical application, 3 options are available: Option A – Extract and fragment DNA for NGS library construction, Option B – Extract large DNA fragments suitable for qPCR or microarray, or Option C – Extract large fragments of genomic DNA. The crude DNA extract was treated with RNase (Thermo Scientific, EN0531), and purified with included columns and eluted in 100 µl elution buffer. The purified DNA was then analyzed on a BioAnalyzer DNA using a 12000 DNA Kit (Agilent, Santa Clara, CA) to determine the fragments size range of the purified DNA.

QIAGEN QIAamp DNA FFPE Tissue (QIAGEN, Duesseldorf, Germany) was used following supplier instructions.

DNA Quantification

DNA quantification was carried out using Qubit Fluorometer (Invitrogen, USA) and dsDNA BR Assay. This assay is specific for double stranded DNA.

qPCR Analysis

qPCR was carried out using GADPH and ACTIN primer sets. Quantitative real time PCR amplifications and melting curve analyses were performed on Applied Biosystems 7500 Fast Real-Time PCR System using the SYBR® Green Real-Time PCR Master Mixes. The amplification mixture included 1X master mix, 0.5 µM primers, 5 µl of template DNA (100 µl FFPE DNA was diluted by 4 fold) in a final volume of 20 µl. Each run included positive standards consisting of dilutions of human genomic DNA (Promega, G1521, 2800 Woods Hollow Road, Madison, WI 53711 USA), and negative controls with all the elements of the reaction mixture except template DNA. All samples, standards and negative controls were run in triplicates. The cycling conditions consisted of an initial hold at 95 °C for 2 min and 40 cycles of 95 °C for 15 s, at specific T_m for 20 s, and 72°C for 30 s. The T_ms for each primer set may be different and are listed below. Following amplification, melting curves were acquired by a cycle of 95 °C for 15", cooling to 60 °C and collecting fluorescence continuously at a ramping rate of 1% until 95°C.

The non expressed (59 bp) primer set was the Human Negative Control Primer set 2, from Active Motif (1914 Palomar Oaks Way, Suite 150, Carlsbad, CA 92008), which amplifies a 59 base pair fragment from a gene desert on human chromosome 4.

The T_m of this set of primers is 58 °C. The GAPDH-1 (82 bp) primer set was the Human positive Control Primer set GAPDH also from Active Motif, which amplifies an 82 bp fragment from the human GAPDH gene promoter. The T_m of this primer set is 58°C. The primer sequences for both sets of primers are proprietary and not available from the supplier.

Two other primer sets amplifying amplicons in the coding region of GAPDH have been used.

Target	Primer Sequence	Amplicon Size (bp)	T _m (°C)
GAPDH-2	F : GGCTGAGAACGGGAAGCTTG	105	60
	R : ATCCTAGTTGCCTCCCCAAA		
GAPDH-3	F : CGGGTCTTTGCAGTCGTATG	236	60
	R : GCGAAAGGAAAGAAAGCGTC		

For the ACTIN gene, four primer sets amplifying amplicons in the coding region have been used.

Target	Primer Sequence	Amplicon Size (bp)	T _m (°C)
ACTIN-1	F : CACACTGTGCCCATCTACGAGG	109	64
	R : CGCGCTCGGTGAGGATCTTC		
ACTIN-2	F : CACACTGTGCCCATCTACGAGG	191	64
	R : TCGAAGTCCAGGGCGACGTAGC		
ACTIN-3	F : CACACTGTGCCCATCTACGAGG	411	64
	R : GAAGAAATGAGGGCGGACTTAG		
ACTIN-4	F : CACACTGTGCCCATCTACGAGG	606	64
	R : ACACCCACCTTGATCTTCATTG		

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References and Acknowledgements

FFPE Tissue Blocks were obtained from: Theresa Kokkat, PhD and Diane McGarvey, Cooperative Human Tissue Network (CHTN), Eastern Division, University of Pennsylvania

1. Tissue preparation: Tissue issues, Nature. 2007 August 23; 448 959-962
2. A multiplex PCR predictor for aCGH success of FFPE samples, Br J Cancer. 2006 January 30; 94(2): 333-337