Covaris
the pre-analytical advantage

Application Note

www.covarisinc.com

Active paraffin removal from FFPE tissues with Adaptive Focused Acoustics

SUMMARY

Covaris has developed a novel method of active paraffin removal from Formalin-Fixed, Paraffin Embedded (FFPE) tissues utilizing Adaptive Focused Acoustics (AFA) technology. When an AFA treatment is applied to a FFPE sample, the paraffin is separated from the tissue sample by emulsification. This is an active mechanical process eliminating the use of hazardous organic solvents, or oils. It results in a robust and easily automatable workflow, which generates consistent DNA yield sample to sample, and improves the quality of the extracted nucleic acids; two parameters essential for clinical research and diagnostics.

Edwin Rudd, Srikanth Kakumanu, Adrian Palmer, Hamid Khoja, Guillaume Durin, James Laugharn — Covaris, Inc., Woburn, MA, USA

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 in biobanks worldwide. Even today, clinical biopsies are stabilized using FFPE for histopathology and nucleic acid based diagnostic analysis.

Paraffin embedding is a key step to ensure proper preservation of the sample. Since paraffin is immiscible with water, tissues need to be dehydrated by progressively more concentrated ethanol baths. This is followed by a clearing agent, usually xylene, to remove the ethanol to enable molten paraffin wax to infiltrate the sample and replace the xylene. Unfortunately, due to tissue heterogeneity and variable techniques, the amount of paraffin can vary greatly inside a tissue sample, and even more greatly from sample to sample, operator to operator, laboratory to laboratory.

More and more clinical applications are based on the nucleic acids extracted from FFPE samples. To recover DNA or RNA, the paraffin must be disassociated from the tissue and the tissue rehydrated for enzymatic digestion. This is typically carried out by utilizing an organic solvent, such as xylene, CitriSolv or mineral oil, to dissolve the paraffin. These passive procedures result in an incomplete removal of the paraffin. The residual hydrophobic paraffin-solvent thin layer will subsequently constrain rehydration of the tissue. In a typical protocol, tissue is then digested by a hydrolytic protease, crosslinks are reversed by heating, and nucleic acids are purified. It is critical to both downstream recovery and quality to remove as much paraffin as possible for efficient enzyme digestion.

Here, we describe how the use of AFA technology to actively remove the paraffin from the tissue sample greatly simplifies the workflow while consequently increasing the reproducibility

ACTIVE PARAFFIN REMOVAL

Covaris focused-ultrasonicators (utilizing AFA technology) generate a highly repeatable acoustic energy field to mechanically process samples. When a focused acoustic burst is applied, numerous cavitations bubbles are generated within the sample. When each acoustic burst is ended, these microscopic bubbles collapse, creating high velocity jets of buffer. This process is repeated thousands of times per second, promoting rapid emulsification of the embedded paraffin in the tissue.

FIGURE 1: FFPE tissue processing with Covaris Focused-ultrasonicator

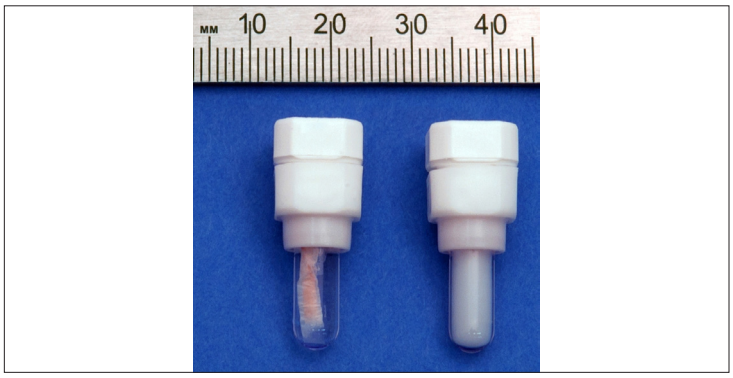

FIGURE 1: 10 µm FFPE tissue section before (left side) and after (right side) AFA processing.

FIGURE 2: Emulsified paraffin particles sizing

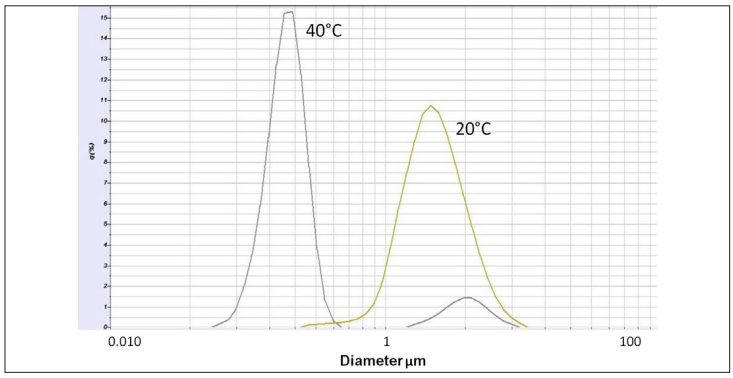

FIGURE 2: Particle size distributions of two paraffin emulsions. FFPE sample have been processed at two different temperatures: 40°C (blue trace) and 20°C (green trace)

After 5 minutes of AFA processing, the paraffin initially associated with the tissue sample has been emulsified and the solution appears milky (Figure 1). The white color observed is due to the presence of millions of tiny droplets of paraffin (in the 10 µm diameter range) that have been stripped from the tissue (Figure 2). They form a stable emulsion that can last for days. Since the paraffin has been removed from the tissue, it can then be efficiently rehydrated with lysis buffer. This complete rehydration is a key step in order to make it a substrate for protease digestion, and thus allowing complete extraction of the nucleic acids.

ACTIVE VS. PASSIVE PARAFFIN REMOVAL

To compare active paraffin removal with AFA to a passive removal with a chemical agent, three adjacent tissue sections were cut from the same tissue block. A first section was kept unprocessed as a negative control. A second section was processed with AFA technology and the third section was processed following QIAGEN QIAamp DNA FFPE Tissue Kit. Amount of paraffin presents in the tissue (Figure 3 upper figure) is proportional to intensity of the blue signal (paraffin has intrinsic auto-fluorescence properties).

Active paraffin removal with AFA results in a much more efficient paraffin removal compared to a passive treatment. On average, the autofluorescence intensity of left-over paraffin in the tissue is three times lower in samples treated with Covaris AFA than in samples treated with QIAGEN QIAamp DNA FFPE Tissue Kit. Active paraffin removal also results in a more homogenous paraffin removal. When studying the amount of paraffin left over in a 100 µm section of the tissue (Figure 3 lower figure), fluorescent intensity varies greatly (around 40% cv) in a sample treated with a QIAGEN kit. In a sample treated with AFA, fluorescent intensity is much more stable along the same 100 µm section (20% cv).

This homogenous removal of the paraffin of the entire sample allows for more efficient tissue rehydration. As the protease will only digest hydrated portions of the tissue, it allows for an unbiased digestion and extraction of nucleic acids representative of the entire sample.

FIGURE 3: Active vs. passive paraffin removal

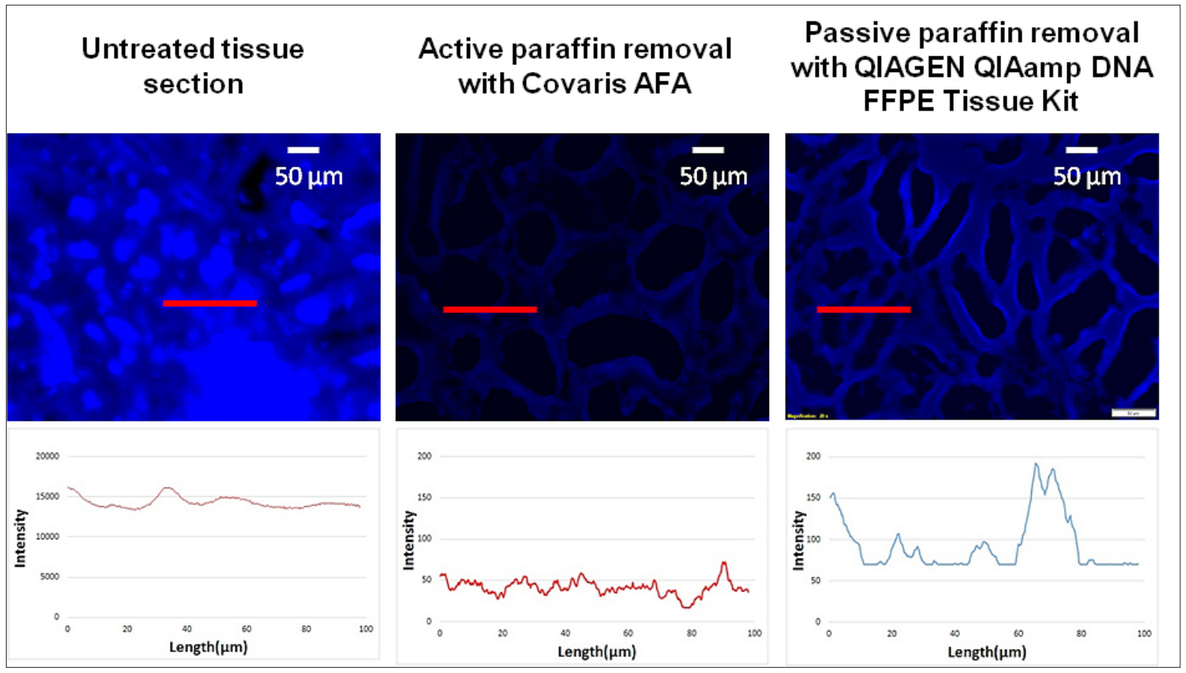

FIGURE 3: Comparison of active and passive paraffin removal. Upper figures: images of paraffin auto-fluorescence of tissue sections. Lower figures: paraffin auto fluorescence intensity plotted along a 100 µm section of the tissue (red line of the upper images).

MATERIALS AND METHODS

FFPE tissue samples

FFPE tissues were provided by CHTN (Cooperative Human Tissue Network, Eastern Division University of Pennsylvania). The FFPE samples used in this protocol were from normal human kidney tissue. The excess paraffin was trimmed away from the tissue. For each FFPE sample, a 25 µm section was cut using a microtome. For this study, all sections were adjacent.

Paraffin removal

Passive (solvent or oil based) removal with QIAGEN QIAamp DNA FFPE Tissue Kit (QIAGEN, Duesseldorf, Germany). Paraffin is removed following supplier instructions:

- Place one 25 µm scroll in a 1.5 ml micro centrifuge tube
- Add 160 µl of de-paraffinization solution and vortex for 10 seconds
- Incubate at 56°C for 3 min, and then cool to room temperature
- Add 180 µl of ATL buffer and vortex. Centrifuge 11,000 x g for 1 min
- Remove as much of the upper "oil" phase as possible
- Wash tissue three times with 1 ml of water. Removing "oil" phase between washes

Active (acoustic) removal with Covaris AFA. Paraffin is removed following Covaris DNA Extraction Kit instructions:

- Load FFPE tissue (25 µm section) into a Covaris microTUBE. Add Tissue SDS Buffer (110 µl)
- Affix Screw-Cap to the microTUBE
- Load the sample in a Covaris S220 Focused-ultrasonicator and process with AFA to dissociate the paraffin while simultaneously rehydrating the tissue. Instrument parameters are as follow : 10% DF, 175 PIP, 200 cycles per burst, 5 min at 20 °C
- During the AFA process the solution turns milky white as the paraffin is emulsified
- Spin transfer tissue to 1.5 ml micro centrifuge tube and wash three times with water (1 ml) to remove emulsified wax particles

Particles sizing

Sizing of the paraffin emulsion was realized after AFA treatment using a Horiba LA-950 Laser Particle Size Analyzer.

Fluorescence microscopy

After paraffin removal, samples were mounted on microscope slide (VWR) and let dried for at least 30 min.

A fluorescent microscope (Olympus Model IX73 with Fluorescence and EXIBLU Camera) was used to study auto-fluorescence of the paraffin remaining in the tissues after the removal treatment. Fluorescence was excited at 358 nm and all the samples were analyzed in DAP1 region (blue/cyan filter around 461 nm). Exposure time was manually set to 415 ms for all samples.

ACKNOWLEDGMENTS

FFPE Tissue Blocks were obtained from:

Theresa Kokkat, PhD and Diane McGarvey, Cooperative Human Tissue Network (CHTN), Eastern Division, University of Pennsylvania

USA: Covaris, Inc. • Tel: +1 781-932-3959 • Fax: +1 781-932-8705 • Email: customerservice@covarisinc.com • Web: www.covarisinc.com
EUROPE: Covaris Ltd. • Tel: +44 (0)845 872 0100 • Fax: +44 (0)845 384 9160 • Email: EUCustomerService@covarisinc.com • Web: www.covarisinc.com
PART NUMBER: M020017 REV A | EDITION MAY 2016