Novel, Automated Co-extraction of High-quality DNA and RNA from a Single FFPE Sample

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Abstract

Formalin-fixed, paraffin-embedded (FFPE) tissue preservation is the preferred method to archive clinical tissue biopsy samples for histopathological diagnosis. As advances in clinical molecular pathology continue to grow, the importance of reliable methods of extraction from FFPE tissue specimens become vital to ensure that patients receive timely and accurate reports. However, nucleic acid extraction from FFPE samples can be challenging and labor intensive, often resulting in degraded and fragmented DNA and RNA. Given the precious and limited availability of these clinical samples, the ability to differentially co-extract high-yield and high quality DNA and RNA from a single sample input provides a tremendous advantage. Coupling the Covaris LE220R-plus Focused-ultrasonicator with liquid handling automation and the truXTRAC® FFPE kits for high-yield co-extraction, in this poster we demonstrates a standardized clinical FFPE extraction workflow providing downstream result confidence (higher yields and corresponding higher DV200 scores), increased efficiency, decreased sample variability, and reduction of manual "touch points" throughout the process. Furthermore, it is shown that the automated DNA and RNA workflows yield similar results as compared to manual methods using our truXTRAC FFPE kits.

Introduction: Covaris Adaptive Focused Acoustics® (AFA®) Technology

- Advanced acoustic technology enabling non-contact mechanical processing of samples
- Controlled bursts of focused high-frequency acoustic energy for efficient, reproducible, and isothermal sample processing (***Figure 1***)
- Enables the acoustic energy to be focused into a discrete focal zone within a sample vessel
- Minimal energy input, avoiding the adverse effects heat and sample over-processing typical of ordinary sonicators

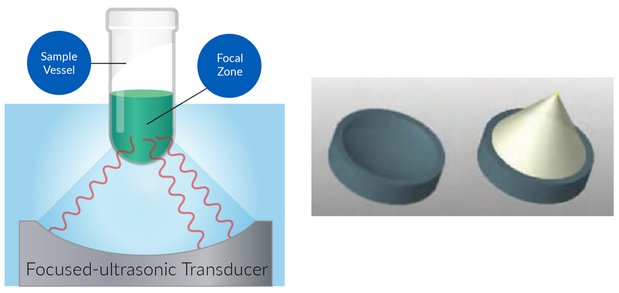


Figure 1: AFA-energetics® reproducibly processes samples in a temperature-controlled and non-contact environment. The concave nature of the transducer enables the precise control of the acoustics waves to the focal zone.

Fundamentals of AFA: Cavitation Process

- Acoustic waves pass through a solution cause localized pressure fluctuations
- Fluctuations cause dissolved gases to form microscopic bubbles
- Bubbles grow, oscillate, and collapse
- These processes generate shear forces
- AFA-energetics enables precise control of the generated shear forces
  - Cumulative effect of hundreds of thousands of cavitation bubbles

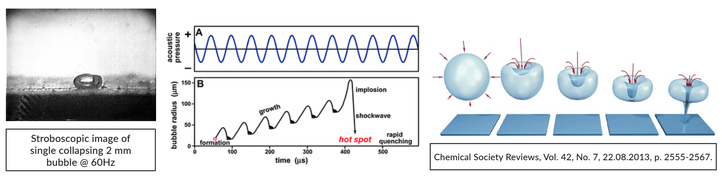


Figure 2: Cavitation Process. The acoustics waves induce localized fluctuations in pressure as the energy passes through the aqueous medium forming microscopic cavitation bubbles (from dissolved gases) in the regions of relative low pressure. The cavitation bubbles grow and oscillate to a critical size and then collapse. The oscillation and collapse of the bubbles generates acoustic microstreaming which creates hydrodynamic shear stress in the sample. Covaris AFA technology and instrumentation enables the precise control of these generated shear forces.

Covaris truXTRAC FFPE total Nucleic Acid Kit

- Designed for efficient and simultaneous extraction of total nucleic acids (DNA and RNA) from FFPE tissue samples using Covaris AFA technology platform (*Figure 3*)
- Enables the active, organic solvent-free removal of paraffin from FFPE tissue samples in an aqueous buffer
- Active tissue rehydration
- Reversal of formaldehyde crosslinks to improve extraction/purification of longer transcripts (increased DV_{200} scores)

Streamlined and Standardized Workflow

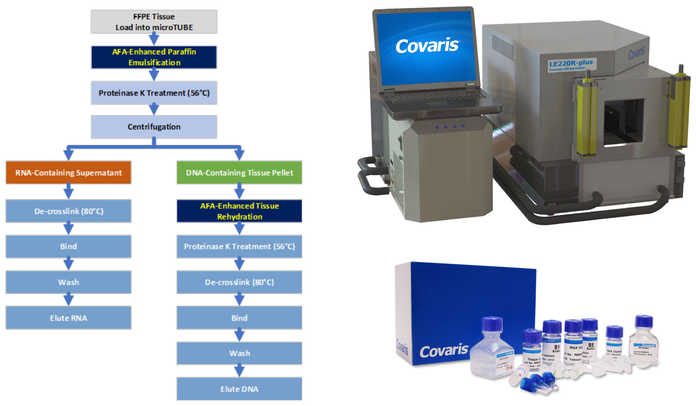


Figure 3: (Pictured) Workflow diagram of the truXTRAC FFPE total NA Kit, LE220R-plus Focused-ultrasoncator, and truXTRAC FFPE total NA Kit. The technology enables the sequential de-paraffinization, tissue rehydration, and co-extraction of DNA and RNA from the same sample material; thus, preserving the limited source material.

RNA and DNA Yield & Quality

As it has been well documented, FFPE sample storage and difficulty in processing can lead to nucleic acid degradation, often resulting in fragmented RNA transcripts. The assessment of RNA quality has traditionally been performed using the RNA Integrity Number (RIN) from Agilent, but recent studies have shown that mean RNA fragment size is a better and more reliable quality determinant for RNA quality. The DV_{200} metric (percentage of RNA fragments >200 nt in size) was developed by Illumina® to more accurately and reproducibly assess the quality of RNA extracted from FFPE tissue. These studies have also indicated a positive direct correlation (R2 = 0.99) between the DV_{200} metric and downstream sequencing results. Illumina notes that RNA samples with a DV_{200} score of <30% are not recommended for further downstream processing and subsequent sequencing (Evaluating RNA Quality from FFPE Samples, Illumina Tech Note, 2016).

In this study, RNA and DNA were extracted from three 10 µm thick scrolls each per specimen following the truXTRAC FFPE total NA Plus (magnetic bead) kit protocol and the Competitor Q FFPE kit in parallel (***Figures 4 through 6***). Nucleic acid concentrations were quantified fluorimetrically (Qubit™, Invitrogen™ by Thermo Fisher Scientific) and RNA and DNA fragment size distributions were verified via capillary electrophoresis (Fragment Analyzer Automated CE System, Advanced Analytical, a part of Agilent).

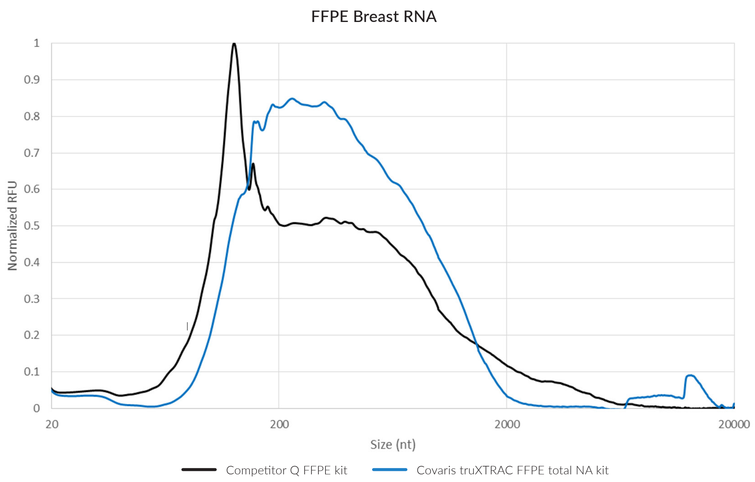
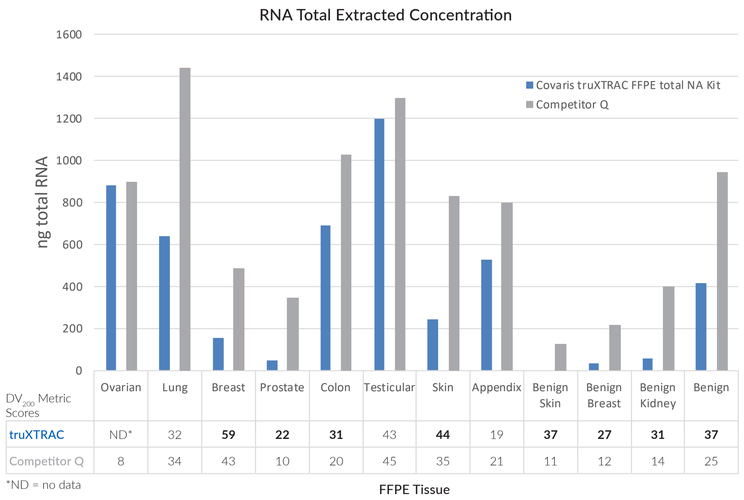


Figure 4: Representative electropherogram illustrating the fragment size distribution of FFPE extracted RNA from RNA extracted from FFPE breast tissue with using the Covaris truXTRAC FFPE total NA Kit with – column purification and Competitor Q kit. It was observed that Competitor Q extracts lower molecular weight RNA, while the Covaris truXTRAC FFPE total NA Kit recovers a higher molecular weight distribution with the mean of >200 nt. The extracted RNA was analyzed on a Fragment Analyzer Automated CE System (Advanced Analytical, a part of Agilent).



DV_{200} Metric Scores	Ovarian	Lung	Breast	Prostate	Colon	Testicular	Skin	Appendix	Benign Skin	Benign Breast	Benign Kidney	Benign
truXTRAC	ND*	32	**59**	**22**	**31**	43	**44**	19	**37**	**27**	**31**	**37**
Competitor Q	8	34	43	10	20	45	35	21	11	12	14	25

*ND = no data

Figure 5: RNA extraction yield; DV and Competitor Q FFPE kit. While the total RNA yields obtained from Competitor Q FFPE kit were higher in 10 of the 12 samples as compared to the Covaris truXTRAC FFPE total NA kit, the DV_{200} scores for the truXTRAC FFPE total NA kit significantly outperformed Competitor Q across the sample types. Data is provided courtesy of Shawn Levy, Director of the Genomic Services Lab at HudsonAlpha Institute for Biotechnology, Huntsville, AL., USA.

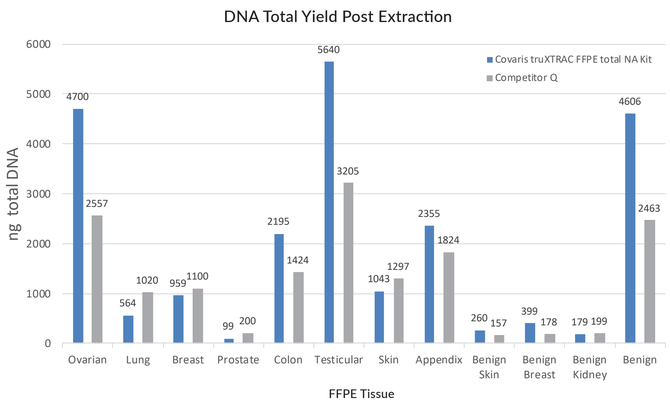


Figure 6: Comparison of yields from DNA extraction using the Covaris truXTRAC FFPE total NA kit and Competitor Q FFPE kit. In 6 of the 12 FFPE sample types tested, the DNA yields obtained with the truXTRAC FFPE total NA kit were significantly higher (up to 100% greater) as compared to those obtained with Competitor Q FFPE kit. The remainder of the samples showed similar yields between the kit methods. Data is provided courtesy of Shawn Levy, Director of the Genomic Services Lab at HudsonAlpha Institute for Biotechnology, Huntsville, AL., USA.

truXTRAC FFPE total NA Kit Manual vs. Automated Clinical Workflow Comparison

Through a collaboration with OmniSeq®, the LE220R-plus Focused-ultrasonicator was integrated with liquid handling automation for scalability and throughput, sample tracking and workflow robustness, and reproducibility. As an added benefit, OmniSeq did not observe lower DNA and RNA extraction yields when comparing the manual and automated workflow. As a result, similar cDNA and DNA library preparation yields were obtained using the truXTRAC FFPE total NA kit. Furthermore, comparable downstream sequencing results were obtained (***Tables 1 through 3***).

		OmniSeq Immune Report Card℠ Assay Sequencing Performance Results							
		DNA QC Metrics			DNA QC Metrics				
Sample (DNA)	Manual vs. Automation	Mapped Reads	Valid Reads (%)	Positively Expressed HK Genes	Mapped Reads	On Target %	Mean Depth	Uniformity %	Exonic Bases ≥ 20x
A1	Manual	2,735,879	92%	10	5,635,030	96%	347	96%	1,149,009
A1	Automation	2,787,396	89%	10	3,993,898	96%	242	95%	1,142,259
B1	Manual	4,547,629	93%	10	5,876,299	97%	374	97%	1,150,403
B1	Automation	5,263,349	91%	10	5,298,795	96%	334	97%	1,150,570
C1	Manual	4,925,479	92%	10	4,953,057	97%	318	96%	1,146,896
C1	Automation	5,874,845	92%	10	4,234,211	96%	272	97%	1,150,150
D1	Manual	3,636,183	89%	10	5,590,907	95%	346	95%	1,145,758
D1	Automation	4,354,899	89%	10	5,371,301	96%	334	96%	1,147,159
Threshold Values (RNA and DNA-seq)		≥ 200,000	≥ 67	≥ 6	N/A	> 94%	N/A	> 92.6%	≥ 850,000

Table 1: OmniSeq Immune Report Card assay sequencing performance results for manual and automated DNA and RNA extraction of the Covaris truXTRAC FFPE kits. This assay provides clinicians with a comprehensive immune profile of their patient, greatly improving their ability to select a personalized immunotherapy treatment based on their patient's unique gene expression (OmniSeq website, https://www.omniseq.com/irc/).

		OmniSeq Comprehensive® Assay Sequencing Performance Results			
		DNA QC Metrics	DNA QC Metrics		
Sample	Manual vs. Automation	Reference Gene Count	On Target %	Mean Depth	Uniformity %
A2	Manual	5	98%	658	95%
A2	Automation	5	97%	594	97%
B2	Manual	5	97%	596	94%
B2	Automation	5	97%	564	97%
C2	Manual	5	97%	575	96%
C2	Automation	5	97%	450	96%
D2	Manual	5	97%	648	97%
D2	Automation	5	97%	490	95%
Threshold Values (RNA and DNA-seq)		>3 genes	>89%	>450	>82%

Table 2: OmniSeq Comprehensive Assay sequencing performance results for manual and automated DNA and RNA extraction of the Covaris truXTRAC FFPE kits. This assay is a next generation assay that tests tumor DNA and RNA to identify somatic mutations (SNVs, CNVs and fusions) in 144 genes for solid tumors to help guide targeted therapeutic management for patients with cancer (OmniSeq website, https://www.omniseq.com/comprehensive/).

Gene Fusions (RNA-seq)		Read Count	
Sample	Gene fusion	Manual	Automation
A2	EML4-ALK	165,477	728,245
A4	MET-MET.M13M15	3,442	2,500
Copy Number Variations (CNV)		**Mean Copy Number**	
Sample	Gene	Manual	Automation
A2	BAP1	1.3	1.5
A2	TET2	1.3	1.2
A2	FBXW7	1.2	1.3
A2	CDKN2A	1.2	1.2
A2	BRCA2	1.2	1.2
A4	BRCA1	1.3	1.3
SNV/Indels		**Variant Allele Frequency (VAF)**	
Sample	Gene	Manual	Automation
A2	ATM	0.500	0.541
A3	CDKN2A	0.331	0.350
A3	PTEN	0.672	0.705
A3	TP53	0.525	0.539
A4	EGFR	0.499	0.426
A4	CDKN2A	0.266	0.204

Table 3: OmniSeq Comprehensive Assay somatic variant detection reporting accuracy for manual and automated DNA and RNA extraction of the Covaris truXTRAC FFPE total NA kit. For both workflow methods, similar copy number of somatic variants were detected.

Conclusion

In this poster, we have demonstrated the use of Covaris AFA technology for de-paraffinization and tissue rehydration in combination with the Covaris truXTRAC FFPE kits for DNA and RNA co-extraction from FFPE tissue samples. Both the manual and automated methods are amenable to clinical workflows where the most challenging FFPE sample types are being examined. The truXTRAC FFPE total NA kit provides co-extraction of high-quality DNA and RNA yields similar or better than alternative methods. More specifically for RNA extraction, the DV_{200} scores for Covaris truXTRAC processed samples are higher for the majority of the FFPE sample types studied and above the recommended threshold level for high-quality downstream sequencing, as compared to Competitor Q. The integration of the Covaris LE220R-plus instrument with liquid-handling automation in concert with a unique co-extraction process based on the truXTRAC FFPE kits enables the standardization of a clinical FFPE extraction workflow that provides downstream result confidence (higher yields and corresponding higher DV_{200} scores), increased efficiency, and decreased sample variability; thus, allowing better clinical analysis and more robust outcomes.

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