Scalable FFPE Sample Preparation Method for Clinical Proteomics LC-MS Assays

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Overview

We present a high-throughput workflow for the preparation of FFPE tissue samples for downstream mass spectrometry-based proteomics analysis. Tissue homogenization through digestion are performed in single well on a 96-well plate in approximately 8 hours allowing for same day analysis.

Introduction

Immunohistochemistry (IHC) is the dominant method for detection of protein biomarkers in clinical tissue specimen, which are primarily formalin fixed paraffin embedded (FFPE) tissue. However, IHC is dependent on the sensitivity and specificity of antibodies which can lead to high background. IHC assays are not often quantitative, and interpretation is based on comparison of intensity between independent tests. Multiplexing IHC assays can be difficult and are not widely utilized in clinical settings. Mass spectrometry (MS) based proteomics enables direct detection and multiplexing through targeted or untargeted methods.

Figure 1. Bone marrow biopsy with plasma cell neoplasm showing H&E stain, IgG stain, and IgA immunohistochemical stains. High background staining on IgG and IgA complicate interpretation.

FFPE tissue is compatible with MS based analysis, however, adoption of MS-based proteomic assays in clinical environments requires robust, fast, scalable, high throughput pre-analytical workflows for extraction of protein. This study highlights development and optimization of a robust, reliable, fast, and scalable protein extraction method for FFPE samples including a rapid trypsin digestion protocol.

Methods

General Methods for FFPE Sample Preparation

Deparaffinized healthy human liver, spleen, and myometrium unstained tissue sections (6 µm) were transferred to 0.5 mL tubes for the standard protocol or 96 AFA-TUBE TPX plate (PN 520291) wells containing 0.002% zwitterionic, Tris-EDTA buffer at pH 7.8 with sterile scalpels. These control tissues were used for method comparison and optimization. Four replicates were utilized for each conditions in all experiments. The total amount of extracted protein was quantified using a BCA assay and a DIGESTIF labelled standard (Promise Advanced Proteomics) was spiked into all samples prior to reduction, alkylation, and trypsin digestion. Samples were desalted, concentrated, and approximately 0.5 µg of total protein were analyzed by LCMS to normalize for differences in protein extraction efficiency. Raw files were searched using Byonic with Proteome Discoverer and/or analyzed using Skyline.

Immunoglobulin Relative Abundance Assay Methods

A selection of 8 FFPE biopsies from a larger set of 44 samples used for validation of a clinical assay were selected for repeat preparation but using the newly optimized FFPE sample preparation method (*Figure 8*). All cases were diagnosed as plasma cell neoplasms with 30% or greater disease burden as previously determined by CD138 IHC. Approximately 1 µg of total protein with 10 fmole of immunoglobulin stable isotope labeled peptides were analyzed by LCMS using a scheduled PRM method on a Q Exactive Plus (Thermo) and data was analyzed with Skyline Software.

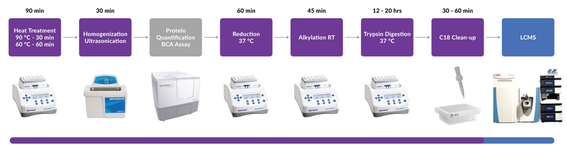


Figure 2. Previously validated FFPE tissue preparation method for clinical mass spectrometry-based assays.

Optimization Steps

1. Homogenization using an ultrasonic cleaner was compared to homogenization with the Covaris LE220Rsc Focused-ultrasonicator using Adaptive Focused Acoustics® (AFA®) method.
2. Heating protocols for formalin crosslink reversal was optimized.
3. Shorter reduction and alkylation procedures were tested.
4. Trypsin digestion in the Covaris LE220Rsc Focused-ultrasonicator was compared to conventional approaches with overnight incubation at 37 °C .

Results and Discussion

Homogenization Method Comparison & Optimization

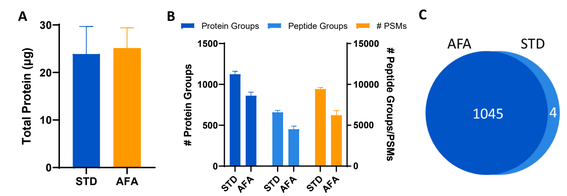
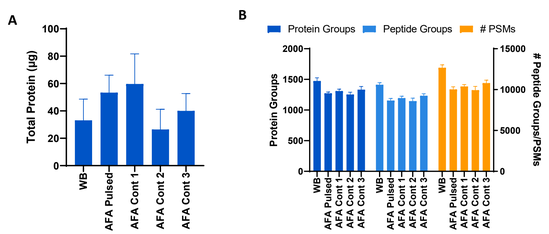


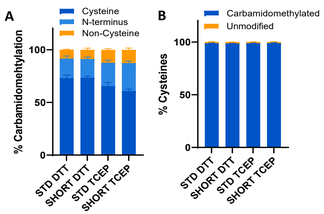
Figure 3. Homogenization of FFPE in the Covaris LE220Rsc extracts similar amounts of total protein and a similar protein profile. A) The total protein extracted was quantified by a BCA assay and the average of four replicates per condition are plotted. **B)** Protein groups, peptide groups, and peptide spectral matches (PSM) reported in the search results are averaged for the four replicates per condition. **C)** A Venn diagram of proteins identified in at least 3 out of 4 replicates of a condition displays nearly complete overlap in proteins identified.



- **KEY:** ***AFA pulsed:*** AFA time = (30 x 10 s) 300 s , PIP = 100W, DP = 25%, CPB = 200
- ***AFA Cont 1:*** AFA time = 300 s, PIP = 100W, DP = 25%, CPB = 200
- ***AFA Cont 2:*** AFA Cont 1 w/ PIP = 200W; ***AFA Cont 3*** = AFA Cont 2 w/ DF = 50%

Figure 4. Homogenization using a continuous AFA method is efficient and reproducible. A) Four different AFA methods were compared to the standard protocol and the total protein extracted is plotted. Results using AFA pulsed or continuous are similar. **B)** Protein, peptide, and PSMs identified are similar across methods. AFA continuous was selected as the total time is less than pulsed.

Reduction and Alkylation Procedure Optimization



KEY: STD = 60 min red, 45 min alk;
SHORT = 25 min red., 20 min alk.

Figure 5. A shortened reduction and alkylation procedure results in complete modification of cysteines. A) Percentages of carbamidomethylations identified on cysteines, peptide n-terminus, and other amino acid side chains are plotted. Results are comparable between the standard and short procedure. **B)** Percentages of unmodified and modified cysteine residues are plotted and almost all cysteine containing peptides are detected with a modified side chain. The short procedure will be used.

Heating Protocol Optimization

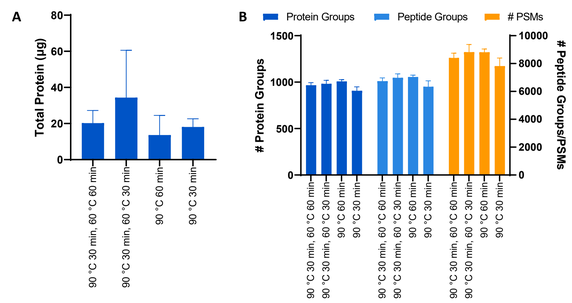


Figure 6. Two step heating protocols result in more protein extracted from the FFPE tissue than a single temperature process. The process can be shortened to a total of 1 hour without impacted protein extraction and the number of peptides identified. **A)** The total protein extracted was quantified by a BCA assay and the average of four replicates per condition are plotted. **B)** Protein groups, peptide groups, and peptide spectral matches (PSM) reported in the search results are averaged for the four replicates per condition.

AFA Rapid Trypsin Digestion Testing

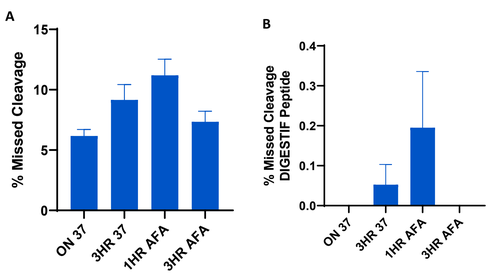


Figure 7. Trypsin digestion for 3 hours with AFA results in a total missed cleavage rate of 7%. A) Missed cleavage results as reported by the search results are plotted and indicate that the overnight digestion at 37 °C had the lowest missed cleavages at approximately 6%, but 3 hours with AFA was only 7%. **B)** Multiple pairs of fully tryptic peptides and single missed cleavage species were measured using a parallel reaction monitoring LCMS method. Only one of the peptides with a missed cleavage was detected in any samples and it was only detected in the 3 hour at 37 °C and the 1 hour with AFA condition.

Optimized FFPE Sample Preparation Method

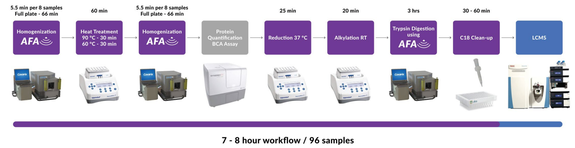


Figure 8. Optimized FFPE tissue preparation method for clinical mass spectrometry-based assays. Total time for sample processing has been shortened to 8 hours which allows for same day LCMS analysis.

Application to Immunoglobulin Relative Abundance Assay

	Sample	1	2	3	4	5	6	7	8
Morphology	Tissue	RPIC	LPIC	clot	LPIC	RPIC	LPIC	LPIC	RPIC
	% Plasma Cells	60-70	60	100	70-80	90	50	80-90	40-50
	IHC/ISH	λ	λ	κ	κ	λ	κ	κ	κ
LCMS Assay Results	IgG	95.1	89.7	99.4	98.9	96.5	91.9	95.9	96.0	2.7	1.1	13.3	4.9	100.0	99.1	15.5	5.9
	IgA	3.9	9.0	0.4	0.8	2.9	7.1	2.4	0.0	97.1	98.7	86.6	95.0	0.0	0.0	83.7	93.2
	IgM	0.9	1.3	0.2	0.3	0.6	1.1	1.7	4.0	0.2	0.2	0.1	0.1	0.0	0.9	0.9	0.8
	IgD	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	IgE	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	Kappa	93.3	95.7	0.5	0.8	95.5	97.3	85.0	92.2	81.5	86.2	94.3	96.8	95.6	93.7	94.7	96.8
	Lambda	6.7	4.3	99.5	99.2	4.5	2.7	15.0	7.8	18.5	13.8	5.7	3.2	4.4	6.3	5.3	3.2
	IgG1	11.3	17.5	99.8	99.9	88.8	99.2	92.2	93.6	65.8	86.5	67.6	94.9	64.0	95.4	53.5	81.3
	IgG2	1.6	0.0	0.1	0.0	0.0	0.0	7.2	0.0	21.9	0.0	27.6	0.0	29.9	0.0	41.2	13.0
	IgG3	86.8	82.2	0.0	0.0	1.0	0.7	0.6	0.4	8.5	8.5	2.4	1.9	3.6	2.1	3.4	3.3
	IgG4	0.3	0.3	0.0	0.0	0.2	0.1	0.0	0.0	3.8	5.0	2.4	3.2	2.5	2.4	1.9	2.4
Serum Studies	FLC Kappa	105.0		0.8		76.4		1.1		0.3		8.0		232.3		2.0	
	FLC Lambda	1.0		8.8		0.3		0.8		268.7		0.6		0.2		1.2	
	IFE	IgG K/L bicional		IgG L		IgG K		IgG K		IgA L		IgA K		Free Kappa		IgA K	

Table 1. Results of the immunoglobulin relative abundance assay are concordant regardless of the FFPE sample preparation method utilized. A selection of 8 plasma cell neoplasm cases analyzed are shown, including morphology and serum study data showing the correlation with targeted LCMS tissue analysis. First column of LCMS results are from the standard method (*Figure 2*) and the second column contains the results when another section was prepared using the optimized method shown in *Figure 8*. A heat map coloration is used for each sample and protein category in which red is the most abundant and white the least abundant.

Conclusions

1. AFA facilitates workflow simplification by allowing homogenization through digestion in a single well with an automation compatible high-throughput (HTP) workflow.
2. Immunoglobulin relative abundance assay results are concordant when the sections of the same tissue samples are processed using the more rapid sample preparation method.
3. Increased throughput and decreased turn-around-time makes FFPE tissue proteomics compatible with the clinical setting.

References and Acknowledgements

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3. Dressler FF, Schoenfeld J, Revyakina O, Vogele D, Kiefer S, Kirfel J, Gemoll T, Perner S. Systematic evaluation and optimization of protein extraction parameters in diagnostic FFPE specimens. Clin Proteomics. 2022 May 2;19(1):10.

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