

An Optimized Sample Preparation Method of Formalin-Fixed Paraffin-Embedded Tissues for Mass Spec Applications

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ABSTRACT

Purpose: To optimize a protocol for extracting proteins from FFPE tissues compatible with MS sample prep and LC-MS/MS analysis.

Method: We evaluated different paraffin removal protocols and lysis/homogenization techniques using FFPE sections from normal and tumor breast, lung, and colon tissues. We assessed protein yield to identify the most optimized protocol and verified compatibility with Thermo Scientific™ EasyPep™ MS sample prep kits, Thermo Scientific™ TMT10plex™ reagents, Thermo Scientific™ Pierce™ High pH Reversed-Phase Peptide Fractionation Kit and subsequent LC-MS/MS analysis. Fresh frozen tissues were compared to FFPE samples.

Results: Higher protein yield was observed using the EasyPep lysis buffer and micro pestle-based tissue homogenization. This optimized method coupled to EasyPep MS sample prep kits allowed identification and quantitation of 2,000-3,500 proteins from breast/lung/colon tissue samples. TMT10plex reagents were used for relative quantification of 5,000 proteins to identify differentially expressed proteins.

INTRODUCTION

Formalin-Fixed Paraffin-Embedded Tissues (FFPE) are an underappreciated resource in the field of proteomics. Hospitals and tissue banks worldwide have large archives of biologically relevant information trapped in these little blocks. Mass spectrometry (MS) analysis of FFPE tissue proteomes gives valuable data about uniquely expressed proteins, differences in protein abundance, and post-translational modifications. This data can be used to further understand patient samples and the factors that influence their medical condition. Currently, there is no standardized or optimized protocol for extracting proteins from FFPE tissues for MS analysis. The objective of this study is to optimize a protocol for extracting proteins from FFPE tissues that was compatible with EasyPep MS sample prep method and subsequent LC-MS/MS analysis.

MATERIALS AND METHODS

FFPE Sample Preparation

Fresh frozen and FFPE tissue samples were obtained from BioIVT. FFPE blocks from fresh frozen tissues (Colon, Lung, Breast) were prepared at Mercy Health Rockford, Rockford IL. Tissue sections from FFPE blocks were generated using manual or automatic Microtome and collected 2 to 3 sections in a 1.5mL low-protein bind tube. Protein extraction was done using EasyPep kit lysis buffer. Samples were homogenized using micro pestle grinding for at least 30 sec followed by probe sonication for 10, 1 sec, 70-amp pulses. Samples were heated at 95° C for 2 hours (New protocol). The samples were centrifuged for 10 mins at 14,000xg to pellet debris and supernatant was placed in a new 1.5mL low-protein bind tubes. Protein concentration and yield was calculated using Thermo Scientific™ Pierce™ Rapid-Gold BCA Protein Assay (PN: A53225).

MS Sample Preparation & TMT Labeling

10 to 100ug of samples were processed using Thermo Scientific™ EasyPep™ Mini MS Sample Prep Kit (PN: A40006). Thermo Scientific™ Pierce™ Quantitative Colorimetric Peptide assay (PN: 23275) was performed on digested samples to determine peptide concentration before LC-MS loading and TMT experiments. Normalized digested samples were labeled with Thermo Scientific™ TMT10plex reagents (PN: 90110). After labeling and quenching, the combined samples were fractionated using Thermo Scientific™ Pierce™ High pH Reversed-Phase Peptide Fractionation Kit (PN: 84868) and analyzed by LC-MS/MS.

LC-MS Analysis

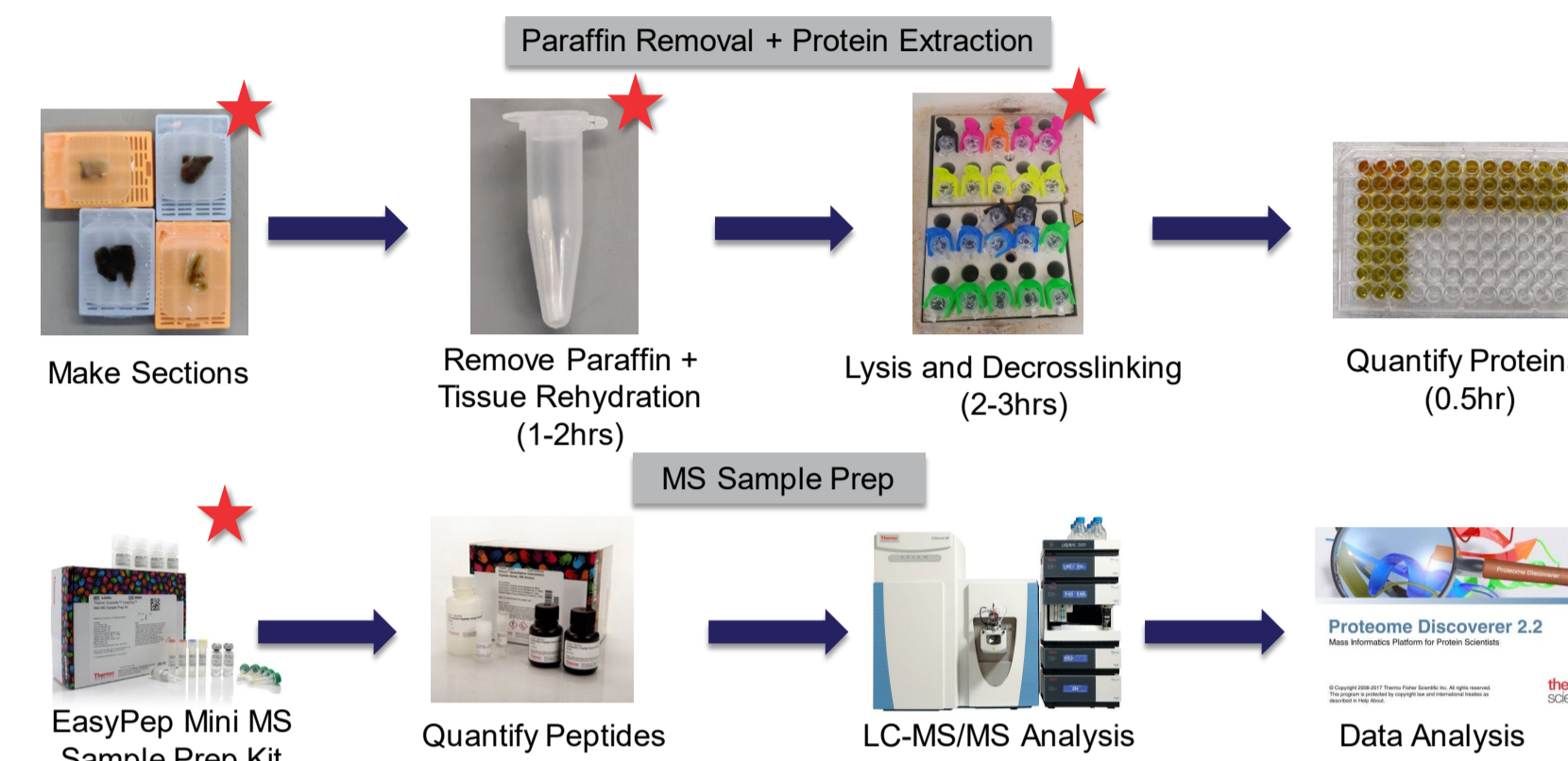
Digested samples (1µg per injection) were separated using a Thermo Scientific™ Dionex™ Ultimate™ 3000 Nano LC system using a 50cm C18 Thermo Scientific™ EASY-Spray™ column with an acetonitrile gradient from 5% to 25% over 80 min, 25% to 50% over 40 min, at a flow rate of 300 nL/min. A Thermo Scientific™ Q Exactive™ HF Hybrid Quadrupole-Orbitrap™ mass spectrometer was used for acquiring the top 20 MS/MS spectra in DDA mode.

MS Data Analysis

For DDA data analysis, Thermo Scientific™ Proteome Discoverer™ 2.2 software was used to search MS/MS spectra with the Sequest™ HT search engine with a precursor mass tolerance of 10 ppm and fragment mass tolerance of 0.02 Da. Static modification included carbamidomethylation (C). Dynamic modifications included methionine oxidation and deamidation (N, Q). Data were searched against the Uniprot human protein database and results were filtered using a 1% FDR threshold.

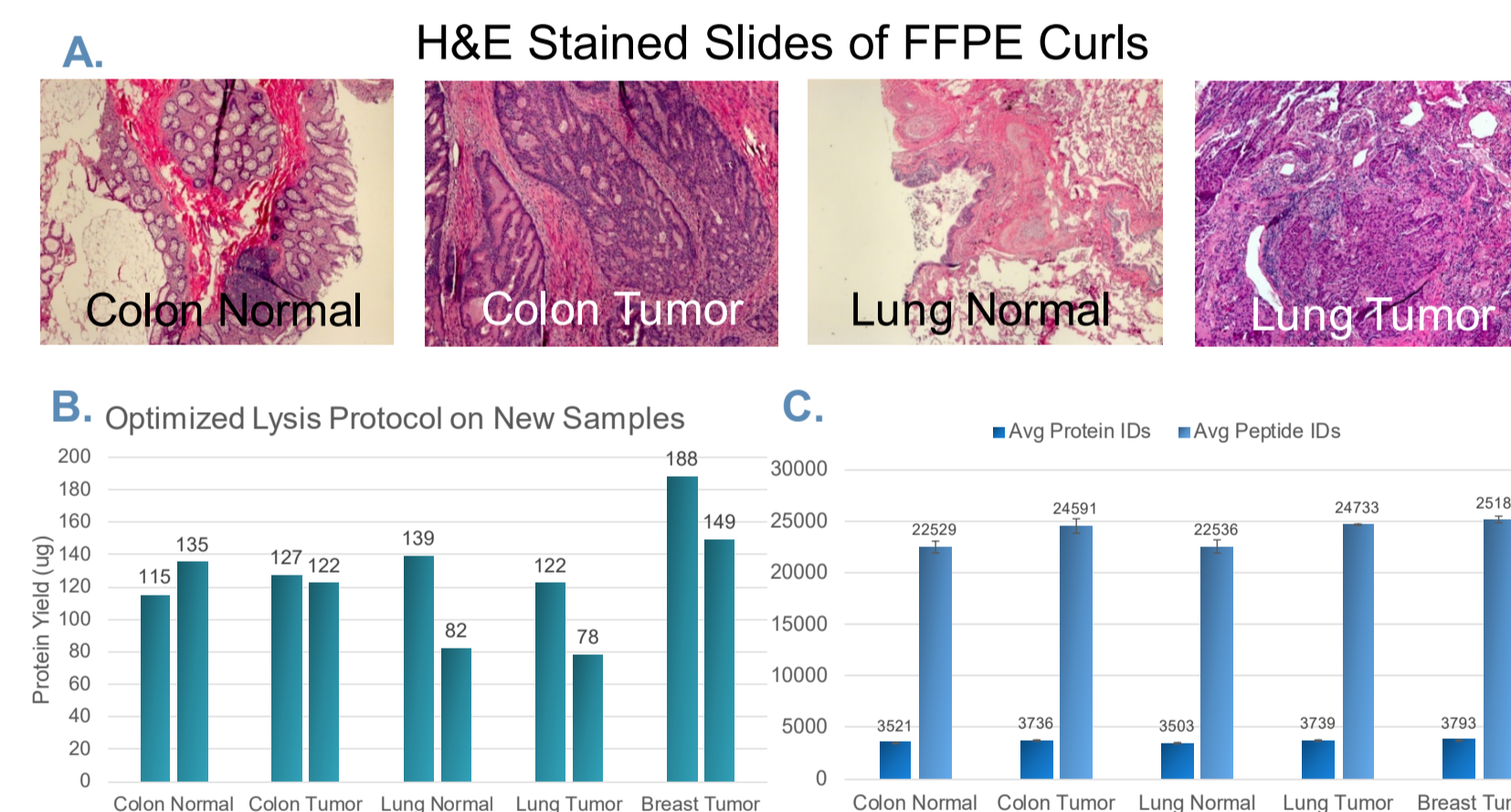
RESULTS

Figure 1. FFPE to MS Application Workflow



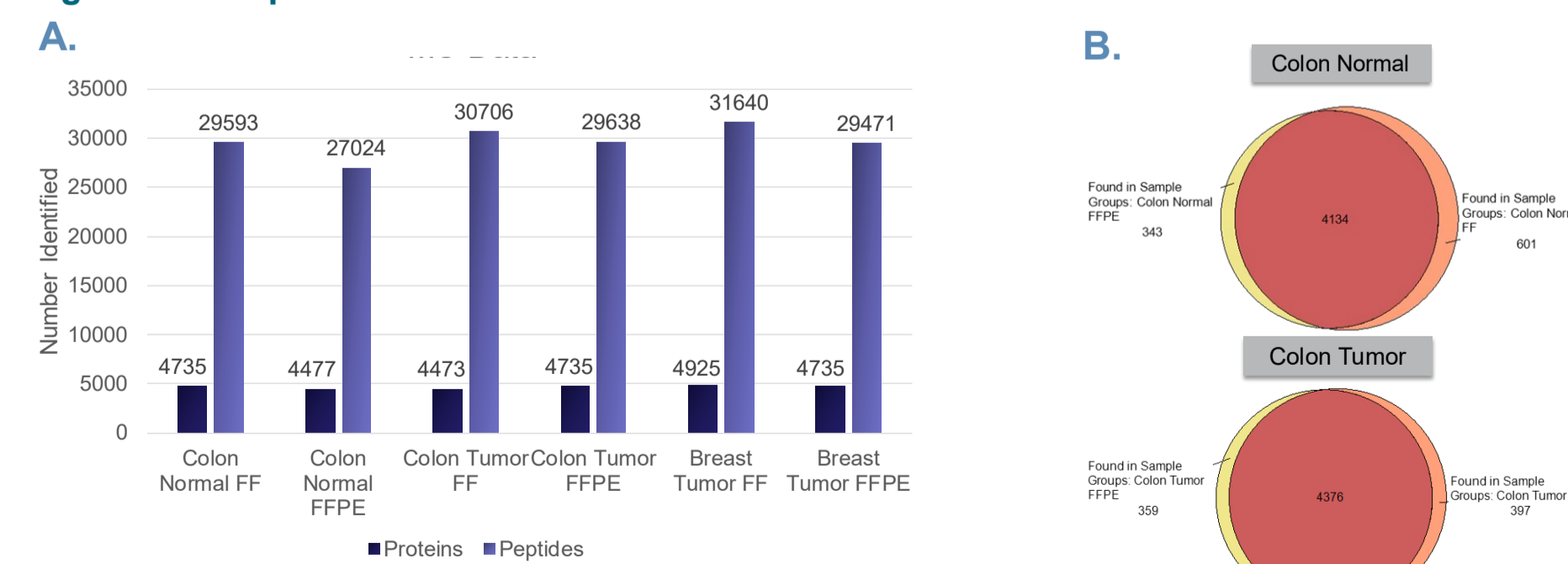
We developed a simplified method for FFPE to MS workflows. First, FFPE sample are prepared for paraffin removal, tissue lysis, and protein quantification. Second, the newly developed EasyPep Mini MS sample prep kit is used to prepare samples for MS analysis. The red stars symbolize areas of our workflow where we modified our standard procedure for optimal compatibility with downstream sample prep and subsequent LC-MS analysis.

Figure 2. Evaluate Robustness of Optimized Protocol for Different Tissue Types



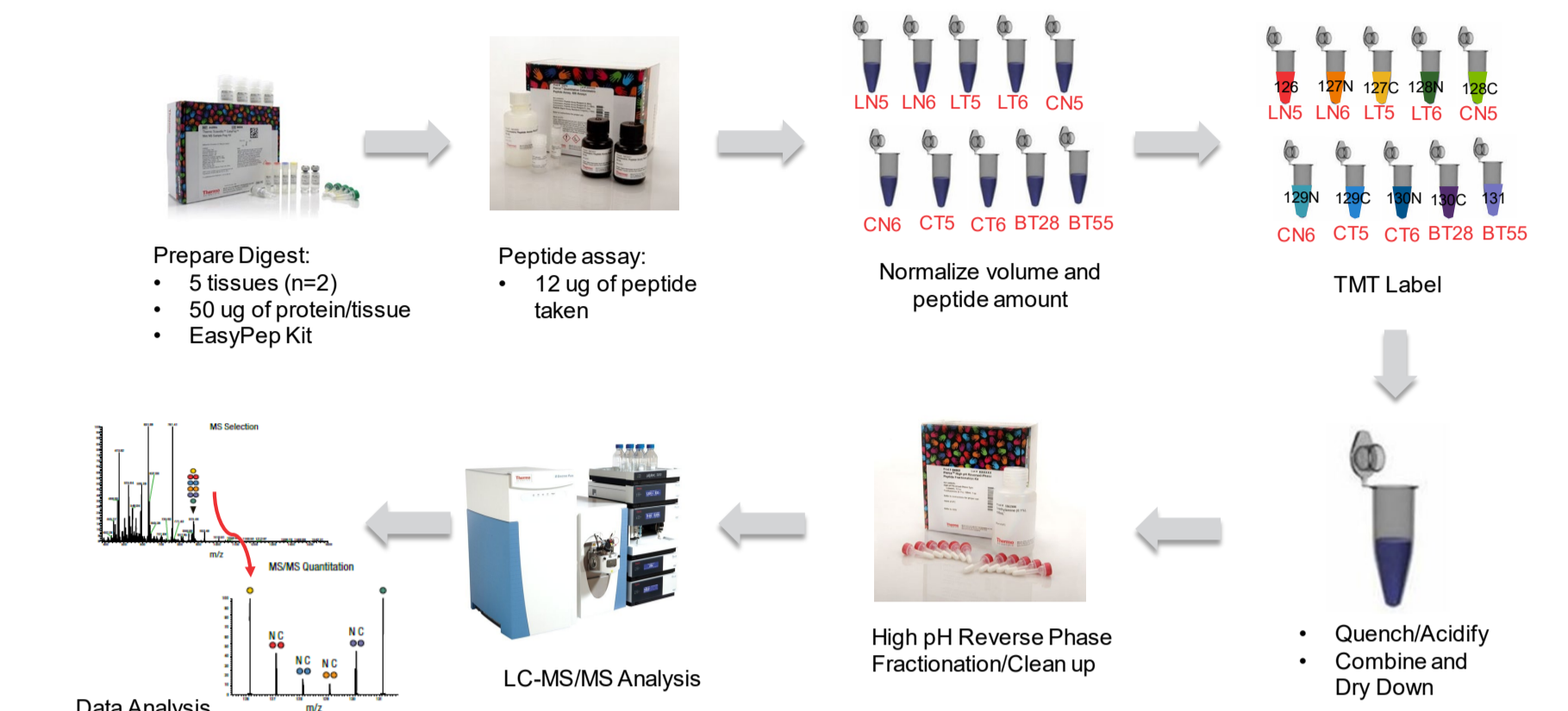
Our optimized protocol resulted in high yields for all sample types, with slightly lower average yields in lung tumor and lung normal samples (Graph B). Samples used for lysis optimization included fresh, frozen tissues made into FFPE blocks at Mercy Health Rockford hospital (Image A). Curles and slides were made from blocks and H&E stained to assess quality and disease phenotype. (Image A). MS analysis from our optimized sample prep method resulted in >3,500 proteins identified in each sample with high reproducibility between biological replicates (Graph C).

Figure 3. Comparison of Fresh Frozen to Matched FFPE Tissue Extraction



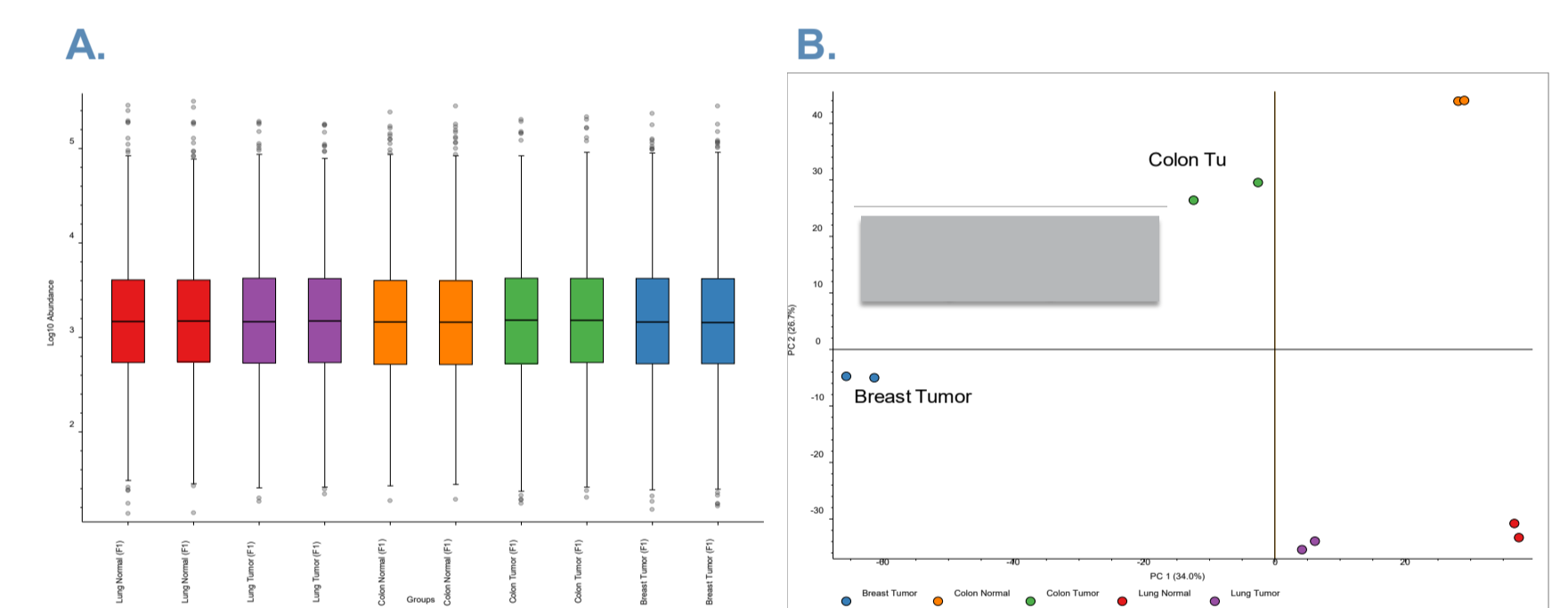
Comparison of fresh frozen (FF) and matched FFPE samples by LC-MS analysis. Graph A shows protein and peptide identification across different FF and matched FFPE samples (>4,450 proteins identified for each sample). Overall similar number of proteins were identified between FF and FFPE for each tissue type as shown in graphs A and B. However, Volcano plot analysis of colon tumor and breast tumor showed differences in protein abundance between FF and matched FFPE samples (Data not shown).

Figure 4. TMT10plex FFPE Experimental Workflow



The TMT10plex FFPE workflow begins with EasyPep MS sample prep of deparaffinized FFPE tissue samples. The concentrations of the eluted, clean peptides was determined using a colorimetric peptide assay and normalized before for TMT labeling. Each sample is labeled with a unique isobaric tag, quenched, and combined into a single sample. The combined sample separated into 8 fractions using high pH reversed phase fractionation spin columns. Each fraction is analyzed by LC-MS/MS.

Figure 5. TMT10plex Experimental Data Analysis



A box-whisker plot (Graph A) shows normalized abundance of all proteins for each of 10 samples. Similar median was observed for each sample, which shows proper normalization of peptides/proteins before TMT labeling and fractionation. Principal component analysis (PCA) (Graph B) distinguishes all major tissue types and grouping of the biological replicates based on similar proteins/peptide abundances.

CONCLUSIONS

- Successfully optimized a protocol for protein extraction from FFPE tissues that provided compatibility and reproducibility with EasyPep Mini MS sample prep kit, TMT reagents, and LC-MS/MS analysis.
- Fresh frozen and matched FFPE tissues provided similar number of identified proteins/peptide groups. However, some differences in protein abundance was observed for some FF and matched FFPE tissues.
- The TMT10plex reagent workflow coupled to high pH reversed phase fractionation enabled differential proteome analysis of FFPE normal and tumor samples for >3,500 proteins.

ACKNOWLEDGEMENTS

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TRADEMARKS

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