A Highly Sensitive FFPE Tissue Workflow by Coupling the Micro Pillar Array Column with High Resolution Mass Spectrometry

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ABSTRACT

Purpose: This workflow enables comprehensive proteomics analysis of large-scale patient samples with limited starting material.

Methods: 50cm PharmaFluidics µPAC™ columns were coupled to a Thermo Scientific™ Orbitrap Fusion™ Lumos™ Tribrid™ mass spectrometer using an Thermo Scientific™ EASY-Spray™ ion source to analyze 10ng to 50ng of protein digests.

Results: We found good column to column reproducibility of 50cm µPAC columns for large-scale studies. The setup is capable of delivering >1000 injections of complex samples with 5% CV or less for retention time drift and protein identification. More than 1,500 proteins were detected from 10ng of HeLa Protein Digest Standard. For real sample applications, more than 1,000 proteins were quantified from 5ng of PDX tumor lysates, and more than 1,500 proteins were quantified from 50ng of FFPE tissue lysate. Proteomics analyses from the PDX tumors and FFPE breast cancer tissues demonstrate the feasibility of identifying pathologically relevant biomarkers from limited sample material. More importantly, this limited material workflow is automatable, reproducible, and scalable for large cohort studies.

INTRODUCTION

Biologically relevant patient samples such as archival formalin fixed and paraffin embedded (FFPE) tissues and tumor biopsies are valuable material for investigation of diseases. Unlike cultured cells, patient samples are often collected in limited quantities and used for disease diagnostics before they are available for research. Furthermore, FFPE tissues have lower protein yield than fresh frozen tissues due to formalin-induced cross-linking of proteins. Therefore, disease relevant patient samples present a particular challenge for proteomics analysis. For this application, we explored the feasibility of coupling the micro pillar array column with a high resolution mass spectrometer to establish a standardized protocol for limited material samples and to provide a robust, sensitive, and scalable proteomics workflow for clinical research.

MATERIALS AND METHODS

Material

Reagents and HPLC grade buffers used for proteomics analyses are from Thermo Fisher Scientific. Thermo Scientific™ Pierce™ Peptide Retention Time Calibration Mixture (PRTC), Pierce™ HeLa protein digest standard and Pierce™ EasyPep™ MS sample Prep kit are from Thermo Fisher Scientific (Rockford, IL).

Sample Preparation

Patient-derived xenograft (PDX) tumors were obtained through collaborations with MIT and Mayo clinics (see acknowledgements). FFPE tissues were purchased from BioIVT (Hicksville, NY) and Zyagen (San Diego, CA). The EasyPep MS Mini Sample Prep kit was used to extract proteins and generate tryptic peptides from 5mg of cryo-pulverized PDX tumors for LC-MS/MS analysis. Protein concentration was determined by the Invitrogen™ Qubit™ Protein Assay kit. Protein yield (µg) per mg tissue were as follows: GR1 = 69.8µg protein/mg tissue, GR2 = 65.6µg/mg, GR4 = 73.7g/mg, and Gr5 = 70µg/mg. Two 10µM sections per patient case were deparaffinized and rehydrated prior to protein extraction. Proteins from FFPE tissues were extracted in 5% SDS in 50mM TEAB buffer using Adaptive Focused Acoustics (AFA) ultrasonication on the Covaris M220 system and digested with the S-trap column (Protifi, NY). Peptide yields (µg) per mg tissue were measured by the Pierce™ Quantitative Fluorometric Peptide Assay and listed as follows: Normal breast = 0.40µg/mg tissue, 7C = 1.07µg/mg, 8C = 0.87µg/mg, 12C = 0.58µg/mg, and 15C = 0.86µg/mg.

Test Method(s)

A 50cm µPAC™ column (PharmaFluidics, Belgium) was coupled to a Orbitrap Fusion Lumos Tribrid mass spectrometer to determine the loading capacity and sensitivity of analyzing low ng HeLa digest standard (QC). The column was connected to a EASY-Spray emitter. The Thermo Scientific™ Dionex™ UltiMate™ UHPLC was used to deliver the linear gradient for peptide separation. The HeLa digest standard was also used to evaluate and optimize LC and mass spectrometry data acquisition parameters. The Thermo Scientific™ Pierce™ Peptide Retention Time Calibration mixture was used to monitor LC conditions over time (QC). Data-dependent acquisition (DDA) and CID fragmentation were used to generate MS and MS2 spectra. Home-made C18 column was packed in 75µm x 25 cm fused silica of Dr. Maisch chromatographic material (ReproSil-Pur 120 C18-AQ, 1.9 µm).

Data Analysis

Skyline software was used for retention time analysis and results were exported for plotting. Thermo Scientific™ Proteome Discoverer™ 2.3 software was used for searching acquired MS2 spectra against human protein database (UniProt reviewed, December 2018) and post-data analysis (LFQ, heatmap and PCA plots). 1% FDR was set as the filtering threshold for protein and peptide identification.

RESULTS

1. WORKFLOW DEVELOPMENT:

Figure 1 – An illustration of the limited sample workflow with the PharmaFluidics µPAC column coupled to a Orbitrap Fusion Lumos mass spectrometer.

A 50cm PharmaFluidics µPAC column was connected to the UltiMate 3000 UHPLC and to an EASY-Spray source-equipped Orbitrap Fusion Lumos mass spectrometer through an EASY-Spray transfer line. See methods for sample preparation, LC gradient, and MS parameters used for the workflow.

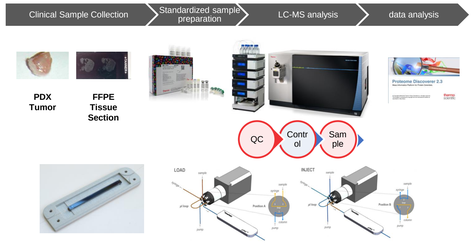


Figure 2 – Characterization of the limited sample workflow with HeLa digest standard and the PharmaFluidics µPAC column coupled to a Orbitrap Fusion Lumos mass spectrometer.

Peptides from 10ng to 100ng of HeLa protein digest standard were separation on the 50cm PharmaFluidics µPAC column using UltiMate 3000 UHPLC and an EASY-Spray ion source over 45 minute gradient at 750nl/min flow rate. Box-and-whisker plots show median and maximum 1.5 interquartile range (IQR) of protein (1% FDR) and peptides (1% FDR) identified from each concentration and 5 technical replicating runs.

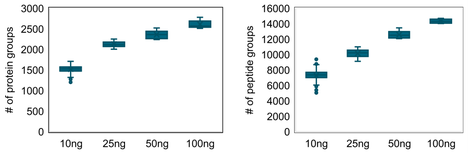


Figure 3 – Comparison of the column performance between the PharmaFluidics µPAC column and home-made C18 column with 10ng HeLa digest standard and coupled to a Orbitrap Fusion Lumos mass spectrometer.

Peptides from 10ng of HeLa digest standard were analyzed either on the 50cm PharmaFluidics µPAC column or a home-made C18 column (see method for column dimension) using the UltiMate 3000 UHPLC with either an EASY-Spray source-equipped or Flex source-equipped Orbitrap Fusion Lumos mass spectrometer. Bar graphs show means of protein (1% FDR) and peptides (1% FDR) identified from each concentration and standard deviations from at least 3 runs.

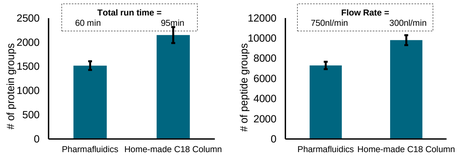


Figure 4 – Chromatographic reproducibility of the PharmaFluidics µPAC column.

Over 300 runs of Pierce peptide retention standard (10fmol) were evaluated for mass accuracy, averaged peak widths (FWHM), retention time drift, and area under the curve (MS1 total area).

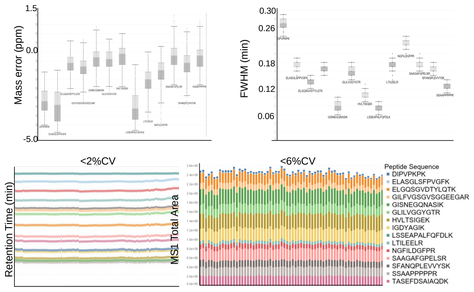


Figure 5 – HPLC gradient and the PSM distribution.

LC gradient for the limited material workflow using the Dionex UltiMate 3000 UHPLC is shown in (A). Distribution of PSM (annotated spectra) is binned by time and shown in the chromatographic scale (B).

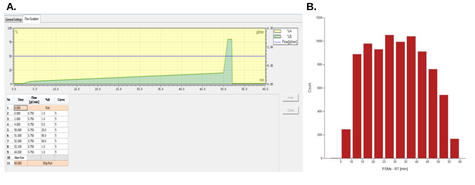
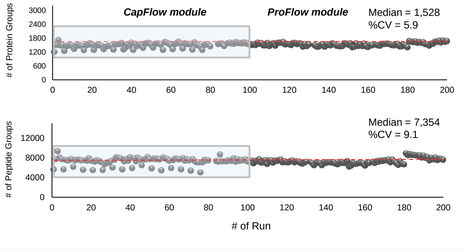


Figure 6 – Reproducibility and robustness of protein identifications using the limited sample workflow.

Average numbers of identified protein and peptide groups from 200 runs of 10 ng HeLa digest standard using the 60 minute gradient on a 50cm PharmaFluidics µPAC column at 750nl/min flow rate. First 100 runs were run with the CapFlow flow meter module and the next 100 runs were run with the ProFlow flow meter module.



2. WORKFLOW APPLICATIONS:

Figure 7 – Analysis of patient-derived xenograft (PDX) tumors using the limited material workflow.

A) Patient-derived Glioblastoma tumor cells were injected in the flanks of immunocompromised mice. B) Violin plots showed distributions, median, and maximum 1.5 interquartile range of proteins (1% FDR) detected in 5ng to 100ng of digested tumor lysates from four different PDX tumors. C&D) Violin plots showed distributions, median, and maximum 1.5 interquartile range of proteins (1% FDR) and peptides (1%FDR) detected in individual PDX tumors (GR1,2,4, 5) from 5ng of tumor lysates.

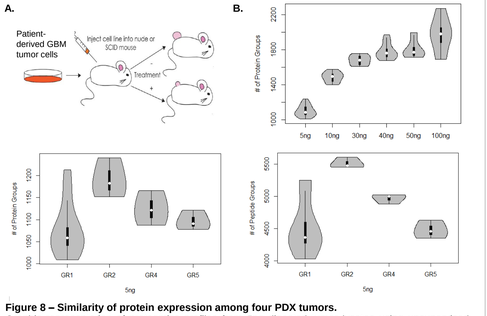


Figure 8 – Similarity of protein expression among four PDX tumors.

Graphic representation of proteomics profiles from 5ng digested tumor lysates using unsupervised hierarchical clustering showed that the closest similarity is between vehicle controls (GR1 and GR4). Manhattan distance function (the sum of the differences) with a complete linkage method were used to generate the heatmap (left graph). PCA with a reduced dimension is shown on right graph.

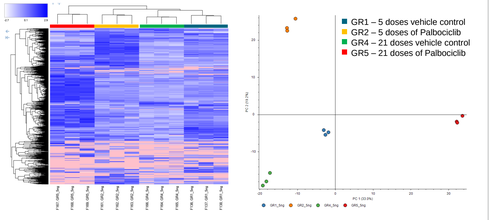


Figure 9 – Analysis of normal and breast cancer FFPE tissues using the limited material workflow.

Peptides from 50ng of FFPE digested lysates were analyzed. Bar graphs show means of protein (1% FDR) and peptides (1% FDR) identified from each concentration and standard deviations from at least 3 runs.

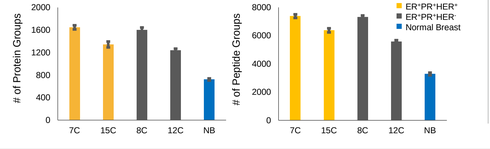
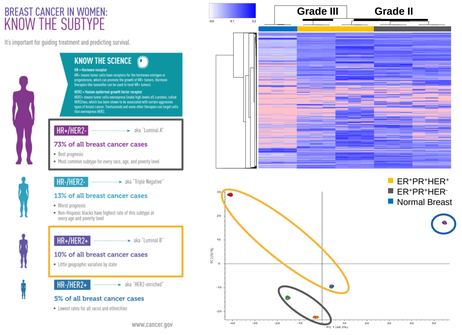


Figure 10 – Clustering of normal breast and hormone positive breast cancer based on protein expression.

Graphic representation of proteomics profiles from 50ng digested FFPE tissue lysates using unsupervised hierarchical clustering showed that the closest similarity is between ER+PR+HER- samples. Manhattan distance function (the sum of the differences) with a complete linkage method were used to generate the heatmap (right top graph). PCA with a reduced dimension is shown on right bottom graph.



CONCLUSIONS

- Rigorous QC parameters were established using peptide and protein digest standards for the limited material workflow.
- Reproducible distribution of PSM were achieved within 60 minutes of total run time for over 1,000 injections. Very little carryover (<0.1%) was observed from run to run.
- Excellent peak width (6s – 15s FWHM) and robust retention time were observed from peptide standard and HeLa protein digest standard. The analytical workflow is suitable for both untargeted and targeted analysis.
- A standardized protocol established from this study allows automation of sample preparation for limited clinical research material.
- Results generated from the limited material workflow presented in this study support the feasibility of larger-scale population studies for biomarker discovery using limited clinical research material.
- High quality quantitative proteome analysis is crucial to decipher pathological differences from patient samples.

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