

High Throughput Sample Preparation for Stroke Biomarker Discovery using the Digital Proteome Chip

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Overview

Purpose: To develop an efficient and high-throughput quantitative workflow incorporating Tandem Mass Tags (TMT) coupled with a pl-based protein separation.

Methods: Sample preparation: Top 12 abundant protein depletion, intact protein labeling with TMT, followed by separation using the Digital Proteome Chip (dPC).

Mass Spectrometry: LTQ Orbitrap XL nanospray LC-MS/MS.

Bioinformatic analysis: SEQUEST™ for protein identification, Dynamite, (an in-house developed algorithm for analysis of TMT data) and MSrat™ for pl fractionation analysis.

Results: The pl separation resulted in the identification of 180 proteins and post-translationally modified isoforms. TMT quantification demonstrated that several proteins and peptides were differentially expressed across the clinical sample pools. The demonstrated workflow allowed for rapid and comprehensive analysis of numerous complex samples.

Introduction

Stroke is the number one cause of long-term disability and the third leading cause of mortality in the United States. In an aging population, the incidence of stroke doubles every decade after 55 years. However, no biomarker exists to help triage high-yield and high-risk therapy such as endovascular retrieval of blood clots from cerebral vasculature, or administration of tPA, the only FDA approved medical therapy for acute ischemic stroke. Plasma is the biological sample type most feasible to obtain in the clinical setting. However, the high dynamic range of proteins in plasma presents a challenge for proteomic analysis. Automating the discovery process would allow for a reproducible, in-depth view of stroke patient plasma during the acute and sub acute stages and could provide quantitative analysis of acute injury and remodeling. In this study, we applied a combination of two powerful separation and labeling technologies. In order to reduce the complexity of the plasma samples, we used digital isoelectric focusing (dPC). In addition, to allow efficient quantification of numerous proteins across many clinical samples, isobaric tandem mass tags (TMT) were used to label the samples. TMT isobaric mass tags (1,2) enable quantitative labeling of proteins extracted from cells and tissues. Each isobaric tagging reagent is composed of an amine-reactive NHS-ester group, a spacer arm and an MS/MS reporter (Figure 1). The reagents are used to label peptides or proteins prepared from distinct samples. For each sample, a unique reporter mass results in a unique MS/MS spectrum. These reporter ions are in the low mass region of the MS/MS spectrum and are used to report relative protein expression levels during peptide fragmentation. This approach may also unveil promising and important prognostic and therapeutic targets.

Methods

Sample preparation and IEF on dPC

Plasma samples were collected under IRB from stroke patients at multiple time points into K₂EDTA tubes, and spun at 2500RCF for 30min. Aliquots (250 µL) were frozen to -80°C until use. Samples were thawed on ice and 125 µL diluted with 1.2mL of 150mM NaCl 25mM Na₂HPO₄ 2.5mM EDTA 2% n-octylglucopyranoside 1X HALT protease inhibitor cocktail (pH 7.4) (ThermoFisher) mixed and filtered through a 0.45µm spin filter. Using an automated method on an HPLC, the flow-through from each sample was run through an IgY Top12 depletion column (Beckman Coulter), collected and concentrated using 2mL Vivaspin (Sartorius) MW cutoff filters. Proteins were removed from the filter using 250 µL of 8M GuHCl 150mM Tris-HCl 10mM DTT after incubation at 37°C for 1 hour. After alkylation with iodoacetic acid, the samples were loaded into 0.1-0.5mL 3500MWCO Slide-A-Lyzer cassettes (ThermoFisher), and dialyzed against sodium bicarbonate (50mM), then with 2 exchanges against 200mM triethylammonium bicarbonate (pH 8.5). After removal from the cassettes, 100 µL (100µg) aliquots of the dialyzed protein were mixed with an appropriate TMT label dissolved in 40 µL of acetonitrile and allowed to react for 2 hours. Residual TMT reagent was quenched with the addition of ammonium bicarbonate and allowed to react for another 2 hours. Sample sets were pooled in 126-131 tag groups and lyophilized in 300µg total pools (50µg each).

Digital protein Chip (dPC) Method

Lyophilized Pools of samples were resuspended in dPC cathode buffer for the 4.2-6.2 chip and run according to the standard protocol.

Processing of dPC plugs

Fixed sample plugs were ejected in pairs into 200 µL PCR (AB1300) plates using the dPC plug harvester filled with destain (50% ethanol 7.5% acetic acid (180µL) for 1 hour. Plugs are then subjected to 2 x 180µL rinses of acetonitrile. Trypsin (15µL of 15mg/L) was added to each well. Plugs were allowed to digest overnight at 37°C and then 50µL of 100mM ammonium bicarbonate in 30% acetonitrile was added. After 1 hour at 37°C the supernatant was removed and saved to another plate. Next, 60µL of 50% acetonitrile 0.1% formic acid water was added to the plugs and incubated 37°C for 1 hr. The supernatant was removed and pooled with the first and subsequently lyophilized. The plugs were then discarded. The lyophilized samples were dissolved in 11µL of 97/3/0.2% water/acetonitrile/formic acid. Empty wells in the plate were filled with column regeneration solution, (5% formic acid in acetonitrile) and peptide standards and the plate was sealed with an Easy Peel heat seal (ThermoFisher).

LC-MS/MS

Samples were loaded onto a MicroAS (ThermoFisher) using 10µL no waste injection (qualitative analysis). The LC configuration employed a Surveyor MS pump equipped with static split that results in 80:1 splitting at 210µL/min at 95% A (0.2% formic acid in water), 5% B (0.2% formic acid in acetonitrile) with a 75µm X 18cm column packed with Hypersil Gold 3µm C18 media (ThermoFisher). An LTQ-Orbitrap XL (ThermoFisher) was used in a "Dual top 3" configuration with a full scan intensity trigger of 8e⁴, used to trigger up to 3 paired data dependent MS/MS events. The first MS/MS was an HCD scan set at a high normalized collision energy in order to enhance the lower MW ions including the TMT reporters, while the second scan was a simple CID scan performed in the trap and used for identification in cases in which HCD energy was too high for efficient fragmentation. Target values and maximum injection times for the HCD scan were 2e5 and 1500ms and for the LTQ scan they were 3e4 and 400ms.

Data Analysis

Raw files from the LTQ were searched using SEQUEST through Bioworks 3.3.1 SR1 against the Refseq Human database (09-17-08), with static carboxymethyl modified cysteines and TMT modified lysine and differentially modified oxidized methionines and n-terminal peptide TMT modification. TMT ratios were extracted using Dynamite, an in-house algorithm that integrates TMT masses for each individual MS2 scan allowing the absolute intensity measurements and quantitative ratios to be derived, and merged with the exported Bioworks Result tables.

FIGURE 1. Schematic Workflow .

1) Top 12 depletion of the most abundant plasma proteins, 2) Reduction and alkylation in GuHCl, 3) Dialysis into triethylammonium bicarbonate, 4) Addition of TMT labeling reagent, quenching of TMT labeling reagent, 5) Lyophilization and 6) Dissolving dried protein into freshly prepared TMT cathode buffer.

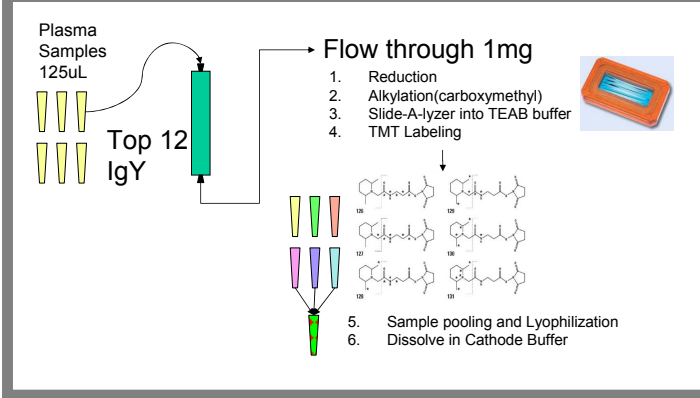


FIGURE 2. dPC Chip workflow.

Steps in running a dPC, total time 1.5hour.

The assembly and running protocol for the dPC chips is highly simplified and user friendly. Components, reagents and running conditions are prepackaged and aliquoted to ensure high reproducibility.

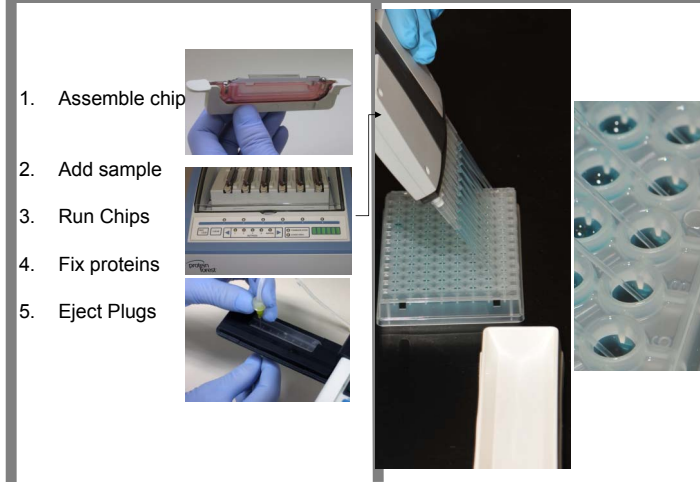


FIGURE 3. Multichannel rinsing and extraction of plugs.

Using a 12 channel pipettor with small orifice gel loading tips allows for a highly controlled removal of destain, acetonitrile and peptide extraction solutions from gel plugs. Total hands-on processing time for 3 chips (with 18 samples) is less than 20min

FIGURE 4. TMT reporter ions and MS2 from HCD scan along with adjacent CID scan of Afamin peptide T1NPAVDHCCK(TMT)TNFAFR

Both fragmentation methods yield highly identifiable peptides, but the HCD scan energy is tuned to favor reporter ions whereas the CID scan is more universally useful for MS2 based identifications.

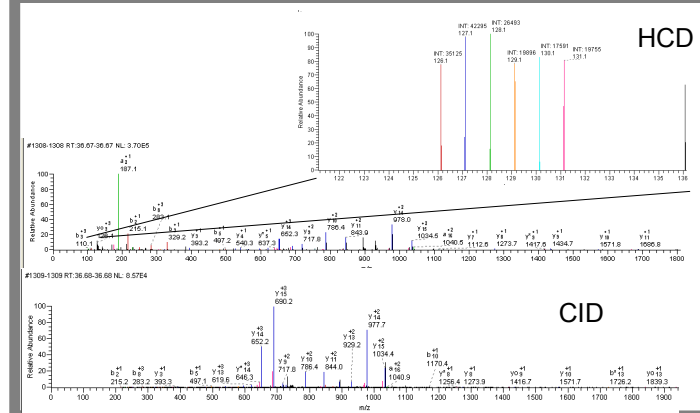


FIGURE 5. Protein example: Quantification of protein Apo A-IV isoform differences across clinical sample pools.

Three clinical sample pools were separated on dPC chips (1-3 below). In pool 1, the most abundantly observed Apo A-IV isoform had an observed pl of 5.21 as compared with the sequence calculated pl of 5.3. In addition low level non-differentially expressed isoforms were found in pools 1 and 3. This demonstrates the power of isoelectric focusing at the protein level for fractionation of post translationally modified proteins in a high dynamic range mixture such as plasma.

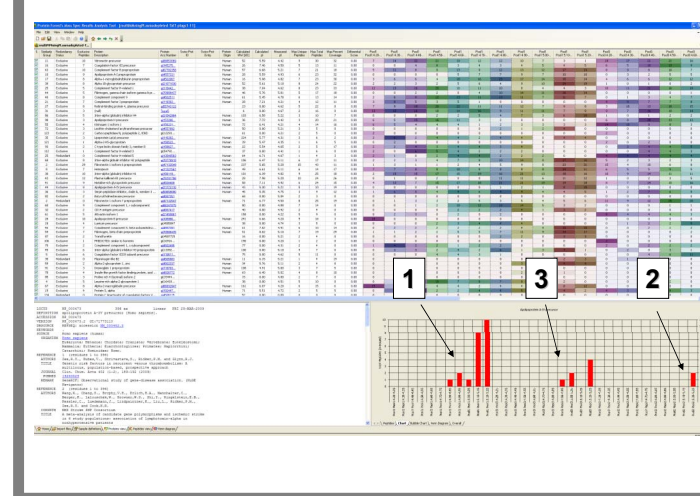
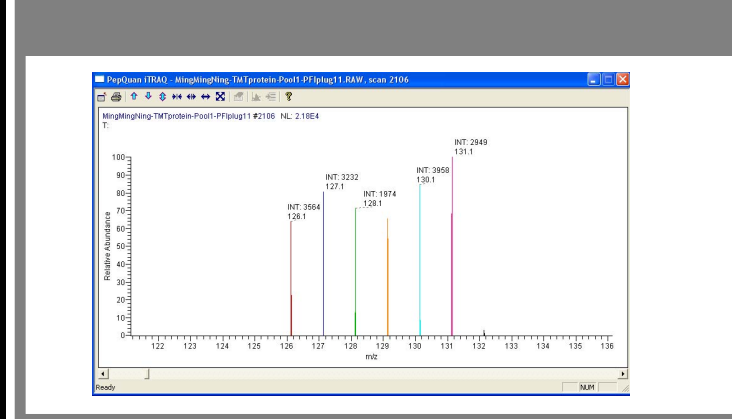


FIGURE 6. Quantification of peptide LLPHANEVSQK(TMT)IGDNLN from Apo A-IV across 6 clinical samples from dPC chip pool 1. The calculated area ratios were: 1.21 : 1.10 : 0.67 : 0.80 : 1.34 : 1.00, demonstrating that this peptide is differentially expressed in these samples.



Conclusions

- Combining the enhanced fractionation ability of the dPC with depleted samples and the highly parallel quantification ability of the TMT reporters allowed the in-depth analysis of 183 proteins from 18 clinical samples.
- The pl based fractionation allowed for additional information on several protein isoforms.

References

- Dayon, L., et al. (2008). Relative quantification of proteins in human cerebrospinal fluids by MS/MS using 6-plex isobaric tags. *Anal.Chem.* 80(8):2921-31.
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Acknowledgements

We would like to thank Helen Byers and Malcolm Ward of Proteome Sciences for their help with the TMT labeling protocol for intact proteins.

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