

IPD Project Details

Project ID: IPD4200

Project Title: A comprehensive comparison of Rapid and Deep Plasma Proteomics workflows to identify and quantify biomarkers of Sickle Cell Anaemia

Description: Plasma serves as a rich source of protein biomarkers but in-depth proteomic analysis is challenging due to the vast dynamic range of protein abundance. Pre-fractionation of plasma proteins is commonly practiced to improve the proteome coverage but the protocols are time-expensive, suffer from flowchart complexity, and often less reproducible. Here, we explore multiple strategies of shotgun proteomics to optimize biomarker discovery workflows for Sickle Cell Anaemia (SCA) patients from remote India. A deep proteomics workflow via off-line reverse phase Ultra High-Pressure Liquid Chromatography based fractionation of tryptic digested plasma peptides followed by optimized pooling of peptides based on charge and hydrophobicity yielded the best depth of plasma proteome with a trade-off of significantly long experimental time. Alternatively, a rapid analysis of tryptic digested plasma peptides via a shorter gradient mass spectrometry run saves time but quantifies only ~ 50% of the proteins than the deep workflow. Intriguingly, despite the difference in proteome coverage, more than 80% of known FDA and SCA biomarkers quantified in the deep workflow are also quantified in the rapid workflow. Given the practical difficulties of sample collection and plasma preservation in rural India, we propose the deep proteomics workflow for biomarker discovery in smaller cohorts and the rapid workflow for biomarker validations in larger cohorts. Additional targeted proteomics based strategies may be designed for the validation of missing biomarkers in the rapid workflow.

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Sample Preparation: Workflow#1 8M urea in 7.5 mM Dithiothreitol was added to 100 µg of plasma protein in 10:1 (V/V) ratio and incubated at room temperature (RT) for 10 min followed by addition of 45mM DTT in 50 mM ammonium bi-carbonate and incubation for 20 min. Next, 100mM Iodoacetamide in 50mM ammonium bi-carbonate was added to the tube and incubated for 30 min at RT. Urea concentration in sample was diluted 10 fold using 50mM ammonium bi-carbonate buffer and digestion of proteins was performed using mass spectrometry grade trypsin (Thermo Scientific) in 1:20 ratio

(15ng/μl in 25mM ammonium bi-carbonate/1mM calcium chloride) for 16 hours at 37°C. After 16 hours, Trifluoroacetic acid (10%) was added to stop the trypsin activity and samples were vacuum dried. Dried peptides were dissolved in 5% Acetonitrile/0.1% Trifluoroacetic acid, desalted and enriched using 10μl Pierce C18 column tips (Thermo Scientific) according to the protocol in Rappsilber et al. (J Rappsilber et al. 2003). An 3μg equivalent of peptides were used for the LC-MS/MS.

Peptide Separation: Workflow#2 Immunodepletion of 10μl plasma equivalent to ~500μg of estimated protein was done using Pierce Top 12 Abundant Protein (?1-Acid Glycoprotein, Fibrinogen, ?1-Antitrypsin, Haptoglobin, ?2-Macroglobulin, Immunoglobulin A, Albumin, Immunoglobulin G, Apolipoprotein A-I, Immunoglobulin M, Apolipoprotein A-II, Transferrin) depletion spin columns (Thermo Scientific) as per the manufacturer provided protocol. Depleted plasma was digested using In-solution digestion procedure explained above for Workflow#1 and 4μg equivalent of digested peptides were used for the LC-MS/MS. Workflow#3 To 150μg of the estimated plasma protein, 4X SDS-PAGE loading dye (250mM Tris-Cl pH 6.8, 10% SDS, 20% ?-mercaptoethanol, 0.1% bromophenol blue, 40% glycerol) was added. After boiling the samples at 95°C for 5 min, it was loaded on a Nu-PAGE 4%–12% Bis–Tris Protein Gel (Invitrogen). Electrophoresis was done using MES buffer (100 mM MES, 100 mM Tris–Cl, 2 mM EDTA, 7 mM SDS) as gel running buffer at 200V for 40 min. The gel was stained and fixed with Coomassie Brilliant Blue R-250 stain solution (Coomassie R-250 0.1%, methanol 50%, glacial acetic acid 10%) followed by destaining. Protein bands were excised into in 9 pieces as marked in Fig.3A. In-Gel reduction, alkylation and digestion were done as described by Shevchenko et al. (A Shevchenko et al. 1996). Finally, the peptides were desalted and enriched using 10μl Pierce C18 column tips (Thermo Scientific) as per Rappsilber et al. (J Rappsilber et al. 2003). An equivalent to 4μg of peptides were used for the LC-MS/MS. Workflow#4 For the Workflow#4, peptides obtained from In-solution digestion procedure as described in Workfolow#1 from 150μg plasma were fractionated by an off-line Reverse Phase chromatography using C18 column (Zorbax 300SB-C18 4.6*250mm,5micron) coupled to an Ultimate-3000 UHPLC (ThermoScientific). Trifluoroacetic acid (0.1%) in deionized water and 100% Acetonitrile were used as buffer A and buffer B, respectively, for gradient generation in dual buffer system. Peptide separation and elution were achieved with a gradient change of 10% solvent B to 60% solvent B from 15 min – 90 min followed by 80% solvent B for 10 min and 100% solvent B for further 20 min; total run time – 120 min and 125 fractions (300μl) were collected using an automated fraction collector coupled to the UHPLC system. First 96 fractions contained protein peaks with absorbance at 215 nm and 280nm. These fractions were concatenated into 8 pools. Pooling scheme:
1+9+17+25+33+41+49+57+65+73+81+89,
2+10+18+26+34+42+50+58+66+74+81+90,.... and so on). Approximately 2.5 μg peptide equivalent from each pool were injected for LC-MS/MS. For the basic pH strategy (Table 2), the solvent system A and B (without trifluoroacetic acid) was adjusted to pH 10 with 5N ammonium hydroxide (PE Geyer et al. 2016). LC-MS/MS Peptides were then analysed on Q-Exactive mass spectrometer (Thermo Scientific) interfaced with nano-flow LC

system (EASY-nLC 1200, Thermo Scientific). EasySpray Nanocapillary Column PepMapTM RSLC C18 (Thermo Fisher) (75 μ m $\times$ 15 cm; 3 μ m; 100 μ) was used to separate the peptides. Gradient time was set at 60 min (workflow#3), 120 min (workflow#1 and #2) and 150 min (workflow#4), mobile phase (5% Acetonitrile, 0.1% formic acid: solvent A and 90% Acetonitrile, 0.1% formic acid: solvent B) at flow rate of 200 nL/min for workflow#4, 300 nL/min flow rate was used for the other workflows. Full scan of MS spectra (from 400 to 1650 m/z for workflow #1 and 400 to 1750 m/z for other workflows) were acquired followed by MS/MS scans of top 10 peptide with charge state 2 or higher.

Protein Characterization: For peptide identification and quantification, raw spectra files were loaded onto Proteome Discoverer version PD2.20.388 (Thermo Scientific) and searched against SwissProt Human Fasta database (version January 2020) and a database of known contaminants (SequestHT search engine). The search parameters included static and dynamic modification of cysteine by carbamidomethylation and dynamic modification of methionine by oxidation, respectively, enzyme specificity of trypsin allowing for up to 2 missed cleavages. Precursor mass tolerance was set to 5 ppm and fragment mass tolerance 0.02 Da was used. Other parameters included minimum 1 peptide for identification with 6 amino acid length. Percolator (q-value) was used for validating peptide spectrum matches and peptides, accepting only the top-scoring hit for each spectrum, satisfying the cut off values for FDR 1%. Label Free Quantification (LFQ) was performed using Minora Feature Detector node in the processing workflow, and the Precursor Ions Quantifier node and the Feature Mapper in the consensus workflow.

Experiment Type: Shotgun proteomics

Species: Homo sapiens-9606

Tissue: Blood plasma (bto:0000131)

Cell Type: Blood cell (cl:0000081)

Disease: Unknown

Instrument Details: Q Exactive (MS:1001911)

Protein Modifications: acetylated residue, iodoacetamide derivatized residue

PubMed ID: