Internal standard triggered-parallel reaction monitoring mass spectrometry enables multiplexed quantification of candidate biomarkers in plasma

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Expanded Materials and Methods

Reagents and materials. Ammonium bicarbonate (A6141), EDTA (E7889), Hydroxylamine solution (438227), Iodoacetamide (IAM; A3221), Phosphatase Cocktail 2 (P5726), Phosphatase Cocktail 3 (P0044), Protease Inhibitor (P8340), Tris (T2694), and Urea (U0631) were obtained from Sigma (St. Louis, MO). Acetonitrile (A955), TCEP (77720), TMT10plex™ isobaric label reagent set (TMT, #90110), and water (MS-grade; W6) were obtained from ThermoFisher Scientific (Waltham, MA). Other reagents obtained were HEPES (Alfa Aesar #J63002), EGTA (Bioworld #40520008-1), formic acid (Millipore #111670), Trypsin (Promega, V5113), Lys-C (WAKO #125-05061), and Rapigest (Waters #186002123). Heavy stable isotope-labeled standards (SIS) were synthesized by Vivitide (Gardner, MA) at a scale between 0.4-0.7 nmol and 25%-75% purity per peptide. The heavy peptides incorporated a fully atom labeled 13C and 15N isotope at the C-terminal K or R position of each peptide, resulting in a mass shift of +8 or +10 Da, respectively (if available; otherwise, the incorporation occurred at another amino acid residue (leucine (L), isoleucine (I), proline (P), valine (V), phenylalanine (F) or aspartic acid (D)).

PDX plasma sample depletion for biomarker discovery. All animal experiments were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee (IACUC, Protocol AN-2289) and performed in compliance with the Guide for the Care and Use of Laboratory Animals of the NIH. Human tumor tissue was transplanted into epithelium-free “cleared” fat pads of 4-week-old SCID/Beige (Envigo) female mice as ~1 mm³ fragments and allowed to grow to ~500 mm³. Successful transplantation of human tissue was verified by H&E staining. Blood was collected from the mouse via the inferior vena cava using a syringe filled with 50 µL 0.5M EDTA. To avoid red blood cell lysis, blood samples were immediately centrifuged at 2000 x g for 10 minutes. The plasma was collected into 100uL aliquots and stored at -80°C. Immuno-depletion columns coupled to an ÄKTA HPLC system were used to deplete plasma.
samples of high-abundant proteins (Agilent Multiple Affinity Removal Column MARS3 (Agilent 5188-5218) or Seppro mouse LC10 IgY7 column (Sigma S5699)) and mid-abundant proteins (Seppro mouse LC5 SuperMix column (Sigma S5824)). The MARS3 column was available for the first discovery experiment but not for subsequent experiments. Protein concentrations before and after depletion were measured by BCA Protein Assay Kit (ThermoFisher, 23235). Each sample was depleted three times in succession with the depletion columns stripped, neutralized, and equilibrated between each depletion. 67 µL of mouse plasma was diluted with 433 µL of Seppro Dilution Buffer and passed through a 0.45 µm spin filter before loading onto the ÄKTA HPLC system. The depleted samples was collected in a 20 mL flowthrough fraction and the three independent depletions were pooled and concentrated in an Amicon Ultra Centrifugal Filter Unit (3 kDa cutoff; Millipore UFC900324) and buffer exchanged three times with 1x Urea buffer (6 M Urea, 25 mM Tris (pH 8.0), 1 mM EDTA, 1 mM EGTA).

**Digestion, desalting, TMT-labeling and fractionation of depleted PDX plasma samples for biomarker discovery.** Depleted plasma samples were reduced in 24 mM TCEP 30 minutes at 37 °C with shaking, followed by alkylation with 43 mM IAM in the dark at room temperature for 30 minutes. Lysates were then diluted to 2 M Urea with 200 mM Tris (pH 8.0). Lys-C was dissolved in 25 mM Tris (pH 8.0) at 200 µg/mL and added to lysates at 1:100 (enzyme:protein) ratio by mass and incubated for 2 hours at 37 °C with shaking. 200 mM Tris (pH 8.0) was added to achieve 1 M Urea final concentration and trypsin added at a 1:50 trypsin:protein ratio and incubated for 2 hours at 37°C with shaking. After 2 hours, a second trypsin aliquot was added at a 1:100 trypsin:protein ratio and incubated overnight at 37 °C with shaking. The reaction was quenched with formic acid (FA) to a final concentration 1% by volume. Samples were desalted using Oasis HLB 96-well plates (Waters 186000309) and a positive pressure manifold (Waters). The plate wells were washed with 3 x 400 µL of 50% acetonitrile (MeCN)/0.1% FA, and then equilibrated
with 4 x 400 μL of 0.1% FA. The digests were applied to the wells, washed 4 x 400 μL 0.1% FA before being eluted drop by drop with 3 x 400 μL of 50% MeCN/0.1% FA. The eluates were lyophilized, followed by storage at -80 °C until use. A portion of samples were TMT-labeled according to manufacturer’s instructions prior to mass spectrometry analysis. Digested plasma samples were fractionated using a previously described basic reverse phase chromatography workflow4.

**Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis of depleted, digested and fractionated PDX plasma samples for biomarker discovery.** The samples were separated using a nanoACQUITY UPLC system (Waters) by reversed-phase HPLC prior to LC-MS/MS analysis on a Thermo Scientific Orbitrap Fusion Lumos Tribrid mass spectrometer operated in positive mode. The analytical column was manufactured in-house using ReproSil-Pur 120 C18-AQ 1.9 um stationary phase (Dr.MaischGmbH) and slurry packed into a 25-cm length of 360 μm o.d.x75 μm i.d. fused silica picofrit capillary tubing (New Objective). The analytical column was heated to 50 °C using an AgileSLEEVE column heater (Analytical Sales and Services) and equilibrated to 98% Mobile Phase A (MP A, 3% MeCN/0.1% FA) and 2% Mobile Phase B (MP B, 90% MeCN/0.1% FA) and maintained at a constant column flow of 200 nL/min. The sample was injected into a 5-μL loop placed in-line with the analytical column which initiated the gradient profile (min:%MP B): 0:2, 1:6, 85:30, 94:90, 100:90, 101:50, 110:50. A spray voltage of 1800 V was applied to the nanospray tip. MS/MS analysis consisted of 1 full scan MS from 350-1800 m/z at resolution 60,000 followed by data dependent MS/MS scans using 30% normalized collision energy of the 20 most abundant ions. Selected ions were dynamically excluded for 45 seconds.

**Data analysis of LC-MS/MS results from PDX plasma samples for biomarker discovery.** Raw MS/MS spectra from the analysis were searched against reviewed Mouse Universal Protein
Resource (UniProt) sequence database and a combined mouse and human UniProt sequence database, release 2018_08 using MaxQuant\textsuperscript{5} v1.5.5.1. The search was performed with tryptic enzyme constraint set for up to two missed cleavages, oxidized methionine set as a variable modification, and carboxamidomethylated cysteine set as a static modification. Peptide MH+ mass tolerances were set at 20 ppm. The overall FDR was set at ≤1%. Results from the search against the combined human/mouse database allowed categorization of peptides into 3 classes: i. human-specific, ii. mouse-specific, and iii. ambiguous (mouse or human). All spectra that were categorized as human specific in this search that also returned an identification in the search against the mouse-only database were filtered out to ensure candidate biomarker proteins were human in origin.

**Candidate biomarker and heavy standard peptide selection from the analysis of PDX plasma samples.** 5,498 unique human-specific peptides to 1,314 candidate biomarker proteins were identified from amongst peptides empirically observed in the PDX plasma biomarker discovery data (Fig. 1). These peptides were filtered based on length (between 7 - 25 amino acids), missed tryptic cleavages (< 2 missed cleavages) and hydrophobicity (SSRCalc\textsuperscript{6} between 10 - 40), resulting in 2,852 peptides to 1,263 proteins. This list was supplemented by additional peptides from Peptide Atlas\textsuperscript{7} (http://www.peptideatlas.org/) and/or SRMAAtlas\textsuperscript{8} (http://www.srmatlas.org/) (n = 2,116) to ensure all non-keratin and IgG candidate biomarkers were represented by at least three peptides. 4,968 SIS peptides were synthesized by Vivitide (Gardner, MA). Peptides were solubilized in 1 mL of 3% MeCN/0.1% FA, an equal volume of each SIS peptide was mixed together and stored at -80 °C until use. Heavy standards to another 208 peptides were already available\textsuperscript{9} and were also included in the SIS mix, for a total of 5,176 peptides, with ≥3 peptides identified for 1,303 (99%) candidate biomarker proteins (Fig. 2). Target sequences with the heavy amino acid noted can be found in Table S1.
Cell lysate preparation for response curve for IS-PRM method characterization. Yeast cells (Saccharomyces cerevisiae) were harvested and lysed using a previously described method. MCF10A cells were obtained from American Type Culture Collection (ATCC, CRL-10317) and authenticated by short tandem repeat (STR) DNA profile. Cells were grown in DMEM:F12 (ThermoFisher 11320-033), supplemented with 5% horse serum (ThermoFisher 16050-122), 0.5 μg/mL hydrocortisone (Sigma H-0888), 20 ng/ml hEGF (ThermoFisher PHG0311), 10 μg/mL insulin (Sigma I-0516), 100 ng/mL cholera toxin (Sigma C-8052), and 100 units/mL penicillin and 100 μg/mL streptomycin (ThermoFisher 15140148) at 37 °C, 5% CO₂. Cells were harvested at ~80% confluence by trypsinization, washed 2x with PBS and lysed in 1x Urea buffer (6 M Urea, 25 mM Tris (pH 8.0), 1 mM EDTA, 1 mM EGTA plus 1% each of Protease Inhibitor (Sigma P8340), Phosphatase Inhibitor Cocktail 2 (Sigma P5726), and Phosphatase Inhibitor Cocktail 3 (Sigma P0044) at 5x10⁷ cells / mL. Chromatin was disrupted by sonication, cleared by centrifugation (20,000 RCF, 10 minutes, 4°C) and lysates transferred to cryovials (ThermoFisher 374081) and stored in vapor phase of an LN2 tank.

High-pH reverse phase (bRP) liquid chromatography for fractionation of IS-PRM method development, characterization and evaluation. Peptide digest was loaded onto a LC system consisting of an Agilent 1200 HPLC (Agilent, Santa Clara, CA) with mobile phases of 5 mM NH4HCO3, pH 10 (A) and 5 mM NH4HCO3 in 90% MeCN, pH 10 (B). The peptides were separated by a 2.1 mm x 250 mm Zorbax Extend- C18, 3.5 μm, column (Agilent Cat. #773700-902) over 60 minutes at a flow rate of 1.0 mL/min by the following timetable: hold 1% B for 5 minutes, gradient from 1 to 40% B for 30 minutes, 40 to 90% B for 5 minutes, hold at 90% B for 5 minutes, 90 to 1% B for 1 minute, re-equilibrate at 1% B for 14 minutes. 0.5 minute fractions were collected from 2 – 50 minutes by the shortest path by row in a 1 mL deep well plate (Thermo Cat. #95040450). Fractions were concatenated accordingly to produce 6-24 fractions for the studies described.
**LC-MS/MS analysis of samples for IS-PRM method development, characterization and evaluation.** IS-PRM and directed DDA methods were implemented by LC-MS/MS on an Easy-nLC 1000 (Thermo Scientific) coupled to an Orbitrap Eclipse mass spectrometer (Thermo Scientific) operated in positive ion mode. The LC system consisted of a fused-silica nanospray needle (PicoTip™ emitter, 75 µm ID x 27 cm, New Objective) packed in-house with ReproSil-Pur C18-AQ, 3 µm and a trap (IntegraFrit™ Capillary, 100 µm ID x 2 cm, New Objective) packed with Magic C18 AQ, 5 µm, 200 Å (Bruker) with mobile phases of 0.1% FA in water (A) and 0.1% FA in 80% MeCN (B). The peptide sample was diluted in 20 µL of 0.1% FA, 3% MeCN and 4 µL was loaded onto the column and separated over 210 minutes at a flow rate of 300 nL/min with a gradient from 4 to 9% B for 2 minutes, 9 to 25% B for 78 minutes, 25 to 44% B for 60 minutes, hold 63% B for 9 minutes, 63 to 90% B for 10 minutes, hold 90% B for 1 minute. HPLC separations were carried out at 40 °C using a column heater. A spray voltage of 2200 V was applied to the nanospray tip.

**Directed DDA mass spectrometry.** Directed DDA MS/MS analysis occurred over a 1 second cycle time consisting of 1 full scan MS from 300-1500 m/z at resolution 240,000 (at m/z 200), a target AGC value of 1.2 X 10^6, and maximum fill times of 50 ms followed by data dependent MS/MS scans using HCD activation with 27% normalized collision energy of the most abundant ions. The targeted mass lists consisted of 7775 entries based on +2 and +3 charge states for each SIS peptide with m/z in the full scan MS range. Selected ions were dynamically excluded for 60 seconds after a repeat count of 1. Raw MS/MS spectra from the analysis were searched against reviewed human Universal Protein Resource (UniProt) sequence database, release 2021_01 using MSFragger\(^11\). The search was performed with tryptic enzyme constraint set for up to two missed cleavages, oxidized methionine and heavy labeled K, R, L, I, P, V, F and D set as
a variable modification, and carbamidomethylated cysteine set as a static modification. Peptide MH+ mass tolerances were set at 20 ppm. The overall FDR was set at ≤1%. Spectral library was built from the search results using SpectraST\textsuperscript{12}.

**IS-PRM parameter optimization.** The heavy SIS mix (~500 fmol) was spiked into 200 µg of trypsin/LysC-proteolyzed yeast lysate, bRP fractionated into 12 fractions, and then subjected to directed DDA using the method described above. The directed DDA results and spectral library were analyzed in Skyline\textsuperscript{13} and the height of the MS1 peak and the six most intense transitions for each precursor were identified. For each of the 5,176 target peptides, the precursor charge state that had the most intense sum of these six transitions was selected as the target. These targets were incorporated into a single IS-PRM method that was used to analyze every bRP fraction in the assay characterization and biomarker quantification experiments. From these results, the target precursor, six transitions and intensity thresholds (2% of the height of the MS1 peak) were exported from Skyline.

**IS-PRM mass spectrometry.** IS-PRM was adapted from the SureQuant native implementation in the instrument control software of the Orbitrap-Eclipse. The analysis occurred over a 3.5 second cycle time consisting of 6 full scan MS events from 300-1500 m/z at resolution 120,000 (at m/z 200), with each full scan corresponding to independent precursor targeted mass lists with different isolation offsets. The PRM event targeting the precursor ions selected for the heavy isotope-containing standard (“heavy”) peptides employed an Orbitrap resolution of 7,500 (at m/z 200), a target AGC value of 5 X 10\textsuperscript{5}, and maximum fill times of 10 ms. The PRM event targeting the precursor ions selected for the endogenous (“light”) peptides triggered with the detection of least 5 transitions from a group included in a transition inclusion list corresponding to the precursor ion entry in the precursor inclusion list and employed an Orbitrap resolution of 60,000 (at m/z 200), a target AGC value of 5 X 10\textsuperscript{5}, and maximum fill times of 116 ms.
**Peptide identification and quantification by IS-PRM.** Identification was considered successful if the ratio dot product of the transition intensities between the heavy and light peptides was > 0.98. Quantification was considered successful if the PRM results contained peak areas from at least 4 transitions (light endogenous peptides) or 5 transitions (heavy SIS peptides), had at least 5 points across the peak and had a peak area greater than 5,000. All quantifications were manually checked and any one transition with interference in either the heavy or light peptide was removed from the analysis. Peptide concentrations are reported as the peak area ratio (PAR) of the light and heavy peptides.

**Verification of candidate biomarkers.** For each peptide, a z-score was calculated by standardizing the mean intensity difference between the cancer subtype pool and the 2 control pools with the empirical standard deviation of the peptide abundances across normal pools. If there were missing results in the control pools, the standard deviation across all pools was used and the peptide z-score was weighted by 0.5. The weighted z-score for a protein was calculated as weighted sum of z-scores of peptides mapping to the protein, approximated weighted z-scores by a normal distribution and p-values were obtained from a right tailed test. The proteins were screened for markers by fitting a regression model. Relative PAR for each protein was calculated by first normalizing the peptide PAR to the median value across all plasma pools, summed together for all peptides from a given protein and regressed on the ordinal sample categories coded as: 1---non-proliferative, 2---proliferative, 3---atypia, and 4---cancer to obtain the regression coefficient and p-value of the trend for all the candidate biomarkers. Student t-tests were used to compare the mean difference between cases and controls of each of the 22 verified candidate biomarkers. The combined p-value of the 22 p-values was estimated using the meanp function of the metap R package. Permutations (n = 1000) were performed using random sets of 22 proteins.
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Figure S1: A targeted MS workflow based on IS-PRM applied to prioritization of breast cancer biomarkers for validation studies. Proteins in a biological sample are converted to peptides by enzymatic digestion and synthetic heavy isotope-labeled standards for each target peptide are spiked into the mixture. In step 1, the peptide mixture is analyzed by liquid chromatography-mass spectrometry, using an inclusion list containing one precursor (peptide) and six transition (fragment) m/z associated with each heavy standard. An MS1 scan is performed to look for precursor m/z on the inclusion list. Once a heavy precursor m/z has been observed above a given intensity threshold, step 2 is initiated, where a low-resolution MS2 scan is triggered to identify transition fragment ions associated with the targeted heavy peptide. If 5 out of 6 transitions are detected, then a high-resolution MS2 scan is initiated for the light version of the peptide in step 3. Finally, in step 4, parallel reaction monitoring (PRM) using the high resolution MS2 scans of light and heavy peptides allows for conclusive identification of the peptide sequence and relative quantification of the endogenous “light” peptide, which is reported as the peak area ratio (PAR) of the light peptide intensity over the heavy peptide intensity. Created with BioRender.com.
Figure S2: The workflow for the characterization of the analytical performance of the IS-PRM assay. (a) Digested MCF10A cell lysate was serially diluted in digested yeast lysate to concentration points of 100%, 10%, 1% and 0.1% MCF10A. A blank consisting of 0% MCF10A, 100% yeast lysate was also included. 50 µg of each point was proteolytically digested, spiked with heavy standard peptides to 1,314 proteins, and fractionated into six samples, each of which were analyzed in triplicate by the optimized IS-PRM method. Created with BioRender.com. (b) The number of peptides successfully quantified in each of 6 fractions for the five concentration points analyzed in triplicate. For box plots, bold line shows median, box shows inner quartile, vertical line shows 5-95 percentile, outliers are shown as individual points, density of measurements is indicated by the thin line.
Figure S3: A summary of the results applying the IS-PRM assay to plasma of human breast cancer patients. (a) 138 plasma samples were depleted of high- and mid-abundance proteins and pooled into 6 samples (3 breast cancer subtype pools and 3 benign breast lesion controls), proteolytically digested, spiked with heavy standard peptides to 1,314 proteins, and fractionated into 24 samples, each of which were analyzed by the optimized IS-PRM method. Created with BioRender.com. (b) The number of peptides successfully quantified in each of 24 fractions for the six plasma pools. For box plots, bold line shows median, box shows inner quartile, vertical line shows 5-95 percentile, outliers are shown as individual points, density of measurements is indicated by the thin line.
The IS-PRM method relies on consistent signal intensities and mass accuracies to ensure that the internal standard triggers are applied consistently throughout the course of a study. Two quality control samples were analyzed regularly to ensure instrument stability: digested HeLa lysate analyzed every week to confirm the mass accuracy was less than 5ppm and digested yeast lysate analyzed daily to confirm signal sensitivity. For the yeast QC samples, the (a) intensities and (b) retention times of 11 selected peptides were compared to historical values using a Levey-Jennings plot to ensure that they passed Westgard rules. This vigorous QC program avoided any system degradation during the six months in which samples from this study were analyzed. 200 ng of a commercially available yeast lysate digest was analyzed every day that the study was run on the Thermo Eclipse instrument and the intensities of 11 selected peptides were compared to historical values using a Levey-Jennings plot to ensure that they passed Westgard rules.

Figure S4. Levey-Jennings plot of peak areas and retention times from a representative yeast QC LC-MS/MS analysis. The IS-PRM method relies on consistent signal intensities and mass accuracies to ensure that the internal standard triggers are applied consistently throughout the course of a study. Two quality control samples were analyzed regularly to ensure instrument stability: digested HeLa lysate analyzed every week to confirm the mass accuracy was less than 5ppm and digested yeast lysate analyzed daily to confirm signal sensitivity. For the yeast QC samples, the (a) intensities and (b) retention times of 11 selected peptides were compared to historical values using a Levey-Jennings plot to ensure that they passed Westgard rules. This vigorous QC program avoided any system degradation during the six months in which samples from this study were analyzed. 200 ng of a commercially available yeast lysate digest was analyzed every day that the study was run on the Thermo Eclipse instrument and the intensities of 11 selected peptides were compared to historical values using a Levey-Jennings plot to ensure that they passed Westgard rules.