MOLECULAR & CELLULAR PROTEOMICS

MCP

Title

Large-scale inter-laboratory study to develop, analytically validate and apply highly multiplexed, quantitative peptide assays to measure cancer-relevant proteins in plasma

Authors

Susan E. Abbatiello*[1], Birgit Schilling*[2], D. R. Mani*[1], Lisa J. Zimmerman[3], Steven C. Hall[4], Brendan MacLean[5], Matthew Albertolle[4,16], Simon Allen[4,17], Michael Burgess[1], Michael P. Cusack[2,18], Mousumi Ghosh[6,19], Victoria Hedrick[7], Jason M. Held[2,20], H. Dorota Inerowicz[7], Angela Jackson[8], Hasmik Keshishian[1], Christopher R. Kinsinger[9], John Lyssand[10,21], Lee Makowski[11], Mehdi Mesri[9], Henry Rodriguez[9], Paul Rudnick[12], Pawel Sadowski[10,22], Nell Sedransk[13], Kent Shaddox[3], Stephen J. Skates[14], Eric Kuhn[1], Derek Smith[8], Jeffery R. Whiteaker[15], Corbin Whitwell[3], Shucha Zhang[15,23], Christoph H. Borchers[8], Susan J. Fisher[4], Bradford W. Gibson[2], Daniel C. Liebler[3], Michael J. MacCoss[5], Thomas A. Neubert[10], Amanda G. Paulovich[15], Fred E. Regnier[7], Paul Tempst[6], and Steven A. Carr[1#]

Affiliations

[1]Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02142, USA

[2]Buck Institute for Research on Aging, Novato, California 94945, USA

[3]Department of Biochemistry, Vanderbilt University School of Medicine, and the Jim Ayers Institute for Precancer Detection and Diagnosis, Vanderbilt-Ingram Cancer Center, Nashville, Tennessee 37232, USA.

[4]Department of Obstetrics, Gynecology, and Reproductive Sciences, University of California, San Francisco, San Francisco, California 94143, USA

[5]Department of Genome Sciences, University of Washington School of Medicine, Seattle, Washington 98195, USA

[6]Memorial Sloan-Kettering Cancer Center, New York, New York 10065, USA

[7]Purdue University, West Lafayette, Indiana 47907, USA

[8]University of Victoria-Genome BC Proteomics Centre, Victoria, British Columbia V8Z 7X8 CAN

[9]National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA

[10]Kimmel Center for Biology and Medicine at the Skirball Institute, New York University School of Medicine, New York, New York 10016, USA

[11]Argonne National Laboratory (currently at Northeastern University, Boston Massachusetts 02115, USA)

[12]National Institute of Standards and Technology, Gaithersburg, Maryland 20899, USA

[13]National Institute of Statistical Sciences, Research Triangle Park, North Carolina 27709, USA

[14] Biostatistics Center, Massachusetts General Hospital, Boston, Massachusetts 02114, USA

[15]Fred Hutchinson Cancer Research Center, Seattle, Washington 98109, USA

[16]Current Address: Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146, USA

[17]Current Address: Genentech, 1 DNA Way, South San Francisco, California 94080, USA

[18]Current Address: Google Inc., Mountain View, CA 94043, USA

[19]Current Address: West Campus Analytical Core, Yale University, West Haven, Connecticut 06516, USA

[20]Current Address: Washington University School of Medicine, St. Louis, Missouri 63110, USA

[21]Current Address: LI-COR Inc., Lincoln, Nebraska 68504-5000, USA

[22]Current Address: Institute for Future Environments, Queensland University of Technology Brisbane QLD 4001, Australia

[23]Current Address: Gillings School of Global Public Health, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7400, USA

*These authors contributed equally to the study

Communications regarding this manuscript should be addressed to S.A.C.

scarr@broad.mit.edu

Running Title:

Highly multiplexed assays for cancer-relevant proteins (54)

Abstract (298/300 words)

There is an increasing need in biology and clinical medicine to robustly and reliably measure tens-to-hundreds of peptides and proteins in clinical and biological samples with high sensitivity, specificity, reproducibility and repeatability. Previously, we demonstrated that LC-MRM-MS with isotope dilution has suitable performance for quantitative measurements of small numbers of relatively abundant proteins in human plasma, and that the resulting assays can be transferred across laboratories while maintaining high reproducibility and quantitative precision. Here we significantly extend that earlier work, demonstrating that 11 laboratories using 14 LC-MS systems can develop, determine analytical figures of merit, and apply highly multiplexed MRM-MS assays targeting 125 peptides derived from 27 cancer-relevant proteins and 7 control proteins to precisely and reproducibly measure the analytes in human plasma. To ensure consistent generation of high quality data we incorporated a system suitability protocol (SSP) into our experimental design. The SSP enabled real-time monitoring of LC-MRM-MS performance during assay development and implementation, facilitating early detection and correction of chromatographic and instrumental problems. Low to sub-nanogram/mL sensitivity for proteins in plasma was achieved by one-step immunoaffinity depletion of 14 abundant plasma proteins prior to analysis. Median intra- and inter-laboratory reproducibility was <20%, sufficient for most biological studies and candidate protein biomarker verification. Digestion recovery of peptides was assessed and quantitative accuracy improved using heavy isotope labeled versions of the proteins as internal standards. Using the highly multiplexed assay, participating laboratories were able to precisely and reproducibly determine the levels of a series of analytes in blinded samples used to simulate an inter-laboratory clinical study of patient samples. Our study further establishes that LC-MRM-MS using stable isotope dilution, with

appropriate attention to analytical validation and appropriate quality c`ontrol measures, enables sensitive, specific, reproducible and quantitative measurements of proteins and peptides in complex biological matrices such as plasma.

Introduction

Biology and clinical medicine are increasingly in need of methods to robustly and reliably measure many tens to hundreds of peptides and proteins in a given sample with high sensitivity, specificity and reproducibility. Targeted mass spectrometry (MS) methods offer biologists and clinical researchers an ever-increasing suite of experimental approaches and data analysis tools to accomplish this task without the need for immunoassays (1-4). With rapid advances in sample processing, instrument hardware and data acquisition software over the past 10 years, liquid chromatography multiple reaction monitoring mass spectrometry (LC-MRM-MS) using stable isotope labeled peptide standards has matured into a robust approach for peptide-based protein quantification. This approach is available to any laboratory with access to a triple quadrupole mass spectrometer interfaced with a high performance LC system. Ever increasing refinement of targeted LC-MS methods has positioned this technique as an attractive workflow for verification of candidate protein biomarkers in the clinical arena, as well as biology (5-26). Achievable limits of quantification (LOQs) can be in the ng/mL to low µg/mL range with coefficients of variation (CVs) <20% which are suitable for verification studies in clinical or biological contexts (5, 9, 14, 18, 26, 27). Furthermore, coupling peptide immunoaffinity enrichment with LC-MRM-MS allows for limits of detection (LODs) that approach those of ELISA assays (low pg/mL) (28-30) and intra- and interlaboratory CVs of <15% (31).

Despite numerous reports describing the application of LC-MRM-MS for quantification of target peptides, questions remain about the sensitivity, specificity, reproducibility, quantitative precision and accuracy of the measurements as well as the transferability of the methods and assays across laboratories. These questions are driven, in part, by the lack of methodological detail or rigorous analytical validation of targeted MS measurements in many published studies, preventing readers from understanding how well the assays work or to be able to implement the described assays in their own laboratories (10). Since 2005 the Clinical Proteomics Technology Assessment for Cancer (CPTAC) network of the National Cancer Institute has had, as one area of focus, the evaluation, refinement and application of LC-MRM-MS methodology for peptide-based verification of proteins and their modifications in biofluids and tissue. Our efforts have focused on making these assays more precise, accurate, reproducible and transferable between different laboratories, expertise levels, and LC-MS instrument platforms with the goal of widespread adoption initially by the proteomics community, but ultimately also by the clinical laboratory and biology communities. Previously, we demonstrated the reproducibility and transferability of peptide-based MRM assays across eight laboratories (5) by measuring levels of 10 signature peptides representing seven proteins that were spiked across a defined concentration range (1-500 fmol/µL) into neat human plasma. The study was performed in three phases whereby each phase introduced additional sources of variability in sample preparation and instrumental analyses. In the final phase, which included all sources of variability including proteolytic digestion, the median interlaboratory CV of the eight peptides consistently detected was ≤20% across the concentration range tested. This study demonstrated the implementation of a targeted, quantitative and multiplexed LC-MRM-MS assay across multiple laboratories to reproducibly measure a small number of

proteins present at moderate to high abundance (≥2-6 µg/mL in plasma) yielding CVs in an acceptable range for biomarker verification studies (10, 27, 32).

Here we significantly expand upon our previous work, detailing critical steps in the assay development phase essential for successful development of highly multiplexed MRM assays, including the use of a system suitability protocol (SSP) (33) to monitor LC-MRM-MS performance during assay development to detect and correct problems early. We also highlight key advances in hardware and software that we have incorporated into the current design that became available since our initial study. The present study utilized 8 different LC-MS instrument configurations in 11 separate laboratories on a total of 14 individual systems to target and quantitatively measure >100 peptides from a total of 34 proteins, including 27 that are cancer relevant (**Table 1**). Similar considerations on a smaller scale have been recently discussed using protein and peptide standards as part of quality control for large quantitative studies (34). In our study, sensitivity for proteins in plasma was increased into the low-to-sub nanogram/mL level by one-step immunoaffinity depletion as well as gradient optimization to maximize the chromatographic resolution in the sample matrix. Use of heavy-labeled protein internal standards added to samples prior to processing greatly improved the accuracy of protein-level quantification. Intra- and inter-laboratory reproducibility sufficient for most biological studies as well as for candidate protein biomarker verification was achieved. Overall, this study demonstrates that highly multiplexed MRM-MS based assays can, with appropriate attention to experimental design, analytical validation, and suitable quality control measures, be implemented by multiple laboratories to provide sensitive, specific, reproducible and quantitative measurements of proteins and peptides of clinical and biological interest in complex biological matrices, specifically plasma.

METHODS

Materials

A tryptic digest of 6 bovine proteins in equamolar mix (P/N PTD/00001/63) was purchased from Bruker-Michrom, Inc. (Auburn, CA). Picofrit columns (75 µm ID, 10 µm tip ID) pre-packed with ReproSil-Pur C18-AQ resin (3 µm particle size, Dr. Maisch) were purchased from New Objective (Woburn, MA). Synthetic unlabeled (light) peptides and the corresponding stable-isotope labeled (heavy) versions were purchased from Thermo Fisher Scientific (San Jose, CA). Twenty-seven proteins were expressed in *E. coli* in both unlabeled (light) and uniformly ^{15}N-labeled forms and purified for use in this study at Argonne National Laboratories (**Table 1** and **Supplemental Table 1**). Pooled and filtered (0.2 µm) human K_2EDTA plasma was obtained from the National Institute of Standards and Technology (NIST). Plasma delipidation and depletion of the 14 most abundant proteins using the Multiple Affinity Removal System (MARS-14) depletion column (Agilent, Santa Clara) was performed at Caprion Proteomics, Menlo Park, CA (formerly PPD Biomarker Discovery Sciences). Mass spectrometry grade Trypsin Gold was obtained from Promega (Madison, WI). Iodoacetamide, dithiothreitol and urea were purchased from Sigma Chemical Company.

Synthetic peptides and proteins. Peptides were selected from proteins detected in data-dependent experiments in breast cancer samples conducted at the Broad Institute (data not shown) and supplemented with publicly available data in GPM and the *in silico* prediction program, ESP Predictor (35). Between 1 and 5 peptides per protein were selected for synthesis, based on the common rules for peptide stability, length, and unique sequence. One hundred and twenty-five synthetic peptides were purchased in their unlabeled and labeled form, the latter containing C-terminal arginine and lysine residues as $^{13}C_6\,^{15}N_4$ (R10) and $^{13}C_6\,^{15}N_2$ (K8) analogs, respectively (Table 1). Of the 125

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synthetic peptides used in this study, 115 peptides represented new, potentially cancer relevant peptide targets (derived from 27 proteins), while 10 peptides (derived from 7 proteins) were previously assayed in Addona *et al.* (5). Peptide and isotopic purity of the synthetic peptides was estimated to be >98% as determined by LC-UV and MALDI-MS by Thermo Fisher Scientific (San Jose, CA). The concentrations of synthetic peptides and target proteins were determined by amino acid analysis. 1:1 mixtures of the heavy and light versions of each of the proteins were evaluated in two of the study centers by LC-MS/MS following reduction, alkylation and digestion using the same digestion protocol used for the plasma samples. The observed ratios of released peptides were close to the expected 1:1 ratios.   Of course, all peptide and protein LODs, LOQs and determined amounts of peptides or proteins present are subject to potential inaccuracies of amino acid analysis.   Isotopic purity of heavy U-15N-labeled proteins was assessed after tryptic digestion at the Buck Institute for Age Research by ESI-MS/MS on a hybrid quadrupole time-of-flight QSTAR Elite mass spectrometer (AB SCIEX, Concord, Canada). For the resulting tryptic U-^{15}N-labeled peptides, comparisons between the observed isotope distributions obtained in the acquired spectra and simulated distributions suggested that the isotopic peptide purity was typically >98%. This process was automated using the Isotope Pattern Calculator (IPC) available at PNNL (http://omics.pnl.gov/software/IPC.php), that was customized further in-house.

Study Phases: Samples, Sample Preparation, and Experimental Setup

Study Phase I: This phase (see **Figure 1**) consisted of MRM assay development including selection, testing and optimization of peptide transitions, nano-chromatography conditions and LC-MRM operating conditions. Details are found in sections immediately below. Assay development was carried out at six of the participating sites using

instrument platforms from four vendors (AB SCIEX, ThermoFisher Scientific, Waters and Agilent) prior to distribution of peptide reagents and plasma samples for use in Phases II and III. A range of heavy spike concentrations were evaluated by these groups using a 16 point response curve. From the results obtained, the 9 best concentrations for Phases II and III were selected. Conditions for plasma digestion were chosen based on digestion studies of 34 protein standards used in the study. Proteins were spiked into depleted plasma and digested under various conditions including denaturation with deoxycholate (DOC), trifluroethanol (TFE), and urea, as well as assessing the subsequent use of two enzymes for digestion (Lys-C/trypsin). The resulting data were evaluated for missed cleavage products and maximized peptide recovery. Digestion with Lys-C in 2M urea (2 hours) followed by dilution of urea to <1 M and addition of trypsin (16 hours) was the most reproducible of the methods evaluated, yielding the lowest median % CV for all peptides quantified by SID-MRM-MS. More complete results are described elsewhere (36). Reduction and alkylation conditions were as previously reported (5). All sample kits were centrally prepared at Vanderbilt University and subsequently distributed to each participating site. Reagents and samples are described in **Supplemental Document 1**.

MRM Assay Method Development: MRM-MS transition lists were developed and applied to all fourteen participating triple quadrupole mass spectrometers representing four different vendors (AB SCIEX, ThermoFisher Scientific, Waters and Agilent). Skyline (37) MRM transition selection for each peptide was performed independently for each instrument configuration. For AB SCIEX and Waters instruments, spectral libraries were built in Skyline from data dependent acquisitions (on a 4000 QTRAP, a QSTAR Elite, and a QTOF Premier instrument) and peptide search engine results using the BiblioSpec library builder (38). Then, MRM transitions were selected based on the most abundant

fragment ions from these discovery platform data. Alternatively, for ThermoFisher and Agilent platforms, the SRM Refinement approach (39) was employed on the triple quadrupole MS to determine optimal MRM transitions for each peptide. For each vendor platform the best 5 transitions per peptide based on extracted ion current chromatogram peak intensity were selected. Evaluation of interferences from the plasma and from the exogenous peptide spikes was conducted by preparing 3 L:H peptide mixtures in 0.5 µg/µL plasma: 10:1, 1:1 and 1:10, where the concentrations of the light heavy peptides was 1 fmol/µL or 10 fmol/µL to generate the L:H ratios. Each transition was evaluated to ensure the L:H peak area ratio for that sample matched the theoretical ratio in the prepared sample and if the ratio deviated by more than 10%, the transition was removed from the list. The final MRM assay culminated in the 3 most abundant and interference-free transitions per peptide (transitions were selected for the unlabeled, $^{13}C/^{15}N$-isotopically labeled, and U-^{15}N-isotopically labeled version of each peptide). Collision energy values used were based upon linear regression equations provided in Skyline for each vendor platform (40). The final list of MRM transitions for each platform is listed in **Supplemental Table 2**. Finally, four different Skyline "Instrument Method templates" documents (transition lists only) were generated, including all specific MRM transition information and were distributed to instrument operators.

Optimization of reversed phase nanoflow high performance liquid chromatography (nanoHPLC): Peptide mixtures were separated by on-line reversed phase nanoHPLC systems equipped with autosamplers: specifically, two NanoLC-1D Plus systems, seven NanoLC-2D systems and two NanoLC_Ultra systems (i.e., one NanoLC_Ultra 1D Plus and one NanoLC_Ultra 2D Plus) from Eksigent Technologies (Dublin, CA), one Ultimate 3000 system from Dionex (Sunnyvale, CA), one nanoAquity system (Waters, Milford, MA), and two 1100 series systems (Agilent). Peptide separations were performed on

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PicoFrit® (New Objective, Woburn, MA) pre-packed columns (75 μm ID x 120 mm, 10 μm ID tip) packed with ReproSil-Pur C18-AQ (3 μm particle size and 120 Å pore size) using a flow rate of 300 nL/min. Mobile phase compositions were 0.1% (v/v) formic acid in water (Solvent A) and 90% (v/v) acetonitrile with 0.1% (v/v) formic acid (Solvent B). One microliter injections of the peptide digestion mixtures were separated using a binary gradient of 3-7% B in 3 min, 7-25% B in 27 min, 25-40% B in 7 min, 40-90% B in 3 min, and at 90% B for 4 min. All instrument configurations acquired data using a direct injection configuration set-up, with the exception of the Agilent ChipCube-LC instruments that used an HPLC Chip containing both precolumn (160 nL) and analytical column (75 μm ID x 150 mm, Zorbax 5 μm or Polaris 3 μm beads). Additional details including plumbing configurations and autosampler injection routines are described in the SOP (see **Supplemental Documents 1 and 2**). Mass Spectrometer Operating Parameters are described in detail in **Supplemental Document 3**. All MRM transitions are listed in **Supplemental Table 2** for each instrument platform. A total of 750 MRM transitions were monitored for Phase II and 1095 for Phase III.

Study Phase II: The samples analyzed in this phase of the study (see Figure 1) were prepared centrally at Vanderbilt University. Briefly, samples used to generate a nine-point response curve were prepared in human MARS-14 depleted K_2EDTA plasma. The depleted plasma was denatured, reduced and alkylated, digested with Lys-C and trypsin and desalted according to a standard operating procedure (see **Supplemental Document 1**). The resulting digested, depleted plasma was spiked with 125 synthetic $^{12}C/^{14}N$ and corresponding $^{13}C/^{15}N$-isotopically labeled internal standard (IS) peptides. Nine concentration point samples were prepared by serial dilution to generate calibration curves spanning a concentration range of 1 amol/μL to 100 fmol/μL (with 1 μL volume on-column) of all light peptides spiked into a 0.5 μg/μL background of the depleted

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plasma digestion with a constant concentration of 10 fmol/µL of all ^{13}C/^{15}N-isotopically labeled IS peptides. The individual concentrations of the light peptides were 100.00, 23.71, 5.62, 1.33, 0.316, 0.075, 0.018, 0.004, 0.001 fmol/µL (loading 1 µL of sample on-column). The four calibration curves were generated individually. In addition, six samples, referred to as blinded samples, with light peptide concentrations unknown to the instrument operator were provided to each site. The blinded samples were analyzed at the end of each of the four singlicate response curves (see **Supplemental Table 3**). Phase II sample kits were shipped to the 9 participating laboratories (representing 14 LC MSMS instrument configurations). Of the original 11 laboratories involved in Phase II, 2 were unable to continue with Phase III and were removed from the study. Additional details regarding sample kits, reagents and sample preparation, including details for digestion, desalting, sample acquisition order etc. are described in the Phase II SOP (see **Supplemental Document 1**).

Study Phase III: For Phase III (see Figure 1), response curves and blinded samples were generated by spiking 27 unlabeled undigested ANL cancer relevant target proteins (Table 1) and 6 unlabeled undigested previously characterized proteins (5) into depleted, undigested human K_2EDTA plasma. The 9-point response curve for the 27 unlabeled cancer relevant proteins spanned a concentration range of 10 amol/µL to 100 fmol/µL ((loading 1 µL of sample on-column) in a background of depleted human K_2EDTA plasma (0.5 µg/µL). The six additional unlabeled proteins, used as a digestion reproducibility control, were spiked at a constant concentration such that following digestion and dilution, a final on-column amount of 2.5 fmol equivalent was achieved. In addition, 27 U-^{15}N-labeled proteins were spiked into depleted plasma at a constant concentration, so that after digestion and dilution yielded 115 U-^{15}N target peptides at 25

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fmol equivalent on-column. Samples were prepared such that after protein digestion the individual concentrations of the proteolytically generated light peptides were calculated to be 100, 24, 5.6, 1.3, 0.82, 0.32, 0.075, 0.018, and 0.010 fmol/µL (1 µL injection volume). In addition, six blinded samples with light protein concentrations unknown to the instrument operator at each site were provided and analyzed at the end of each of the four singlicate response curves (see **Supplemental Table 3**). Phase III sample kits with undigested light and heavy proteins spiked into undigested depleted plasma were prepared at Vanderbilt University and sent to the 7 participating laboratories (representing 11 LC-MRM-MS instrument configurations). The samples were digested and desalted at the individual sites. MS operators digested 3 independent protein calibration sets and blinded samples. Data for the third protein calibration curve were acquired as a technical MS duplicate, so that as in Phase II, there was a total of 4 LC-MRM-MS acquisition replicates. All instruments operators spiked the ^{13}C/^{15}N-isotopically labeled peptides, post-desalt, to yield a final concentration of 10 fmol/µL. At 3 participating sites the ^{13}C/^{15}N-isotopically labeled peptides were spiked in pre- and post-desalt as independent experiments to assess sample loss during desalting. Protein digestion efficiencies were estimated using U-^{15}N-labeled proteins that had been spiked into depleted plasma. Additional details are described in the Phase III SOP (see **Supplemental Document 2**).

System Suitability Monitoring. Stock solutions (1 pmol/µL per protein) of the commercial predigested "Bovine 6 Protein Mix," referred to as 6 ProteinMix-QC (33), was prepared at Vanderbilt University. As described in detail in the SOP (see **Supplemental Documents 1 and 2**), prior to analysis, individual CPTAC sites further diluted the stock solution to a working solution of 50 fmol/µL 6 ProteinMix-QC and monitored prior to and during Phases II and III.

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LC-MRM-MS Data Acquisition. Mass spectrometric data were acquired following a comprehensive and well-defined run order of all calibration curves and blinded samples in quadruplicates per study. Run orders also included definition of system suitability acquisitions to track instrument performance throughout the studies. Sample setup, sample and file naming nomenclature, etc. are described in the accompanying SOP (**Supplemental Documents 1 and 2**). Scheduled, multiplexed LC-MRM-MS acquisition was employed based on the retention times of each peptide monitored. In preparation for the scheduled response curve LC-MRM-MS runs, in which 750 transitions were monitored in one run (Phase II), participating sites monitored all synthetic isotopically labeled peptides with ~ 375 transitions (125 peptides with 3 transitions each) in 6-7 unscheduled LC-MRM-MS runs (~60 transitions per run, keeping cycle times ~ 1 sec). Retention times for scheduling were determined empirically using these six/seven runs and were verified by performing a single scheduled LC-MRM-MS run prior to analyzing the response curve samples. Retention times of all peptides were used to generate a single scheduled method with 2 minute retention time windows. All method building and data analysis was performed using Skyline.

To minimize instrument-to-instrument variability in chromatography, pre-packed PicoFrit columns (New Objective, Woburn, MA) were purchased for all sites except for those using ChipCube ion sources (Agilent, Santa Clara, CA), Nanoflex cHiPLC dual column systems (AB SCIEX, Foster City, CA) chip-based column plumbing, and NanoAcquity UHPLC systems (Waters, Milford, MA). Sites with alternate hardware configurations were set up to take advantage of best current available technologies suited to their LC systems (see **Supplemental Documents 1-3**).

Data Analysis. Skyline was used as a common data analysis platform across sites that fostered sharing of data during acquisition as well as creating final reports from each

laboratory. Raw data files were imported into Skyline which uses the ProteoWizard Data

Access Library. The MRM transitions for each peptide were integrated with Skyline to

generate extracted ion chromatograms which were then manually adjusted, if necessary.

Skyline peak area calculations based on the final, adjusted peak boundaries were used

as the primary measure or peptide abundance. Each site performed their own data

analyses and Skyline peak boundary adjustments. Skyline custom reports were used to

export results from processed MRM data, such as peak areas, peak heights,

chromatographic parameters, etc. These data results reports were further processed in

R statistical programming language (41) and our own cross-site validation tools to

generate further statistical results and graphics.

Statistical and Graphical Methods. Data from MRM-MS experiments were

preprocessed and integrated in Skyline, and exported as a table in csv format. A

uniform processing and Skyline export template was used for all MS instruments, which

resulted in an output that was instrument independent. Specific data fields were

extracted from Skyline and include Sample name, peptide sequence, replicate name,

retention time, area light, area heavy, full-width-half-maximum (FWHM) peak width,

fragment ion, precursor charge and product *m/z*. These extracted fields were then

processed using custom developed code written in the R statistical programming

language (41). QuaSAR, an open source software algorithm was used to generate

calibration curve regressions and a variety of different plots for each site

(http://genepattern.broadinstitute.org/gp/pages/index.jsf?lsid=QuaSAR). This link

prompts the user to login at GenePattern, it also provides free registration at the

GenePattern website upon choosing 'click to register'; then under modules browse to

'Proteomics' then to 'Quasar' or search for the 'Quasar' module directly. Comprehensive

plots were generated to analyze not only the overall statistics, *e.g.* CV, but to provide a

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more detailed understanding of the LC and MS parameters and measurements from replicate to replicate, between sites and across peptides.

Metrics for Assessing the Performance of the Quantitative MRM Assays. The metrics used for assessing reproducibility of the MRM assays were intra- and inter-Lab precision. Intra-Lab precision was defined as the median CV calculated from replicates of each concentration point for a particular peptide for each site study. Inter-Lab precision was defined as the CV calculated at each concentration point for a particular peptide across all replicates and sites and for each study. Values for CVs were calculated as the ratio of the standard deviation to the mean of the replicate measurements at a given concentration. Both of these assessment metrics were determined based on quadruplicate measurements for a single transition used to calculate limit of detection (LOD) and limit of quantification (LOQ), see below.

Determination of LOD and LOQ: Limit of detection for all monitored peptides from the 9-point response curve in 0.5 µg/µL depleted plasma were determined as previously described (5, 42). Once the LOD was determined separately for each peptide transition, the LOQ was calculated using the customary relation: LOQ = 3 x LOD (43). The LOD was based on the variance of the blank sample (sample A, digested depleted plasma with the heavy isotope peptides and no analyte spiked in) and the variance of the lowest level spike-in sample (sample B, with analyte at 1 amol/µL). Assuming a type I error rate α=0.05 for deciding that the analyte is present when it is not, and a type II error rate β=0.05 for not detecting the analyte when it is present, the LOD was derived as:

$$LOD = LOB + c_\beta \times SD_S$$

LOB (limit of blank) was defined as the 95th percentile of the blank A1 samples (44). This was estimated as the mean plus $t_{1-\beta} \times SD_b$, where SD_b was the standard deviation in

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the blank samples, and SD_S was the standard deviation of the low-level spike in sample B. For a relatively small number of repeated measurements for sample B, c_β was approximated as $t_{1-\beta}$ where $t_{1-\beta}$ is the (1-β) percentile of the standard t distribution on f degrees of freedom, where f is the number of replicates minus 1. Detailed calculations of the various components of the LOD are listed in the QuaSAR LOD/LOQ output tables for each site, which are available on the Panorama webserver for the manuscript.

LOD values are initially calculated for all three transitions monitored for each peptide. The transition with the minimum LOD is chosen as the "best" transition. This transition is used to report LOD and LOQ for inter- and intra-lab CV calculations.

Digestion and desalt losses are calculated using Phase III data from the three sites where the SIS peptides were spiked in both before and after desalting (pre- and post-desalt, respectively). Using the slope of the calibration curve as a representation of overall recovery for a given peptide, we calculate:

Digestion loss = Recovery using U15N standard - Recovery using pre-desalt SIS

Desalt loss = Recovery using pre-desalt SIS – Recovery using post-desalt SIS

Percentage loss is calculated by normalizing the respective loss to corresponding total peptide loss (= digestion loss + desalt loss). The results are averaged over the three sites for tabulation (**Supplemental Table 8**) and visualization (**Supplemental Figure 2**).

Public Access to the Data. An ftp server at the National Institute of Standards (NIST) was used by the CPTAC teams for initiating uploads and downloads of all data files. Currently, all raw data associated with this manuscript is uploaded at Chorus (https://chorusproject.org). The processed, quantitative data associated with this manuscript resides at the interactive Panorama webserver: 'http://proteome.gs.washington.edu/software/panorama/cptac_study9.html'. Posted

information includes processed data results and downloadable Skyline documents from all participating sites.

Results

Overview of Experimental Design: The experimental design incorporated three discrete phases (**Figure 1**). Phase I consisted of assay development prior to distribution of peptide reagents and plasma samples for use by the participating laboratories. Assay development included gradient optimization, peptide transition selection, optimization of depleted plasma digestion conditions, determination of the heavy peptide spike amount, and a 16 point response curve to select the 9 concentrations spanning the linear range and below the LOD for Phases II and III. Assay development was conducted at 6 of the participating sites and employed instrument platforms from all four vendors.

In Phase II, samples consisting of depleted plasma spiked with increasing amounts of 125 peptides (Table 1) from 1 amol to 100 fmol and constant 10 fmol amounts of the heavy-labeled versions of each peptide were distributed in quadruplicate to each participating site as "ready-to-analyze" kits, together with a detailed analysis protocol (**Supplemental Document 1**). In addition, six samples with four different analyte concentrations (**Supplemental Table 3**) blinded to the participants were also provided in quadruplicate. Some of the 11 participating sites had multiple instruments in the study resulting in a total of 14 instruments and 8 distinct LC-MS platforms (see methods and Supplemental Document 3). Each site used a pre-defined and instrument-specific MRM-MS transition list to establish the retention times of the target peptides in their systems. Response curves were generated on each instrument for each of the quadruplicate sample sets by analyzing the samples from low to high concentration followed by wash and blank runs and then six blinded samples (**Supplemental Documents 1 and 2**). In

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addition, all groups also acquired data for a common quality control sample using a system suitability protocol [SSP, (33)] before starting analyses and after every 6-8 subsequent LC-MRM-MS runs. Phase II primarily evaluated instrument variability as all sample preparation, including digestion of depleted plasma, was performed centrally in a single laboratory prior to sample distribution.

In Phase III, variability caused by sample processing and handling, and determination of LOD/LOQ were evaluated by having each site reduce, alkylate and trypsin digest samples spiked with proteins (vs. spiked peptides) as the source of the analyte peptides (**Figure 1**). Twenty-seven proteins were expressed in *E. coli* in both unlabeled (light) and uniformly ^{15}N-labeled forms and purified for use in this study at Argonne National Laboratories (**Supplemental Table 1**). To generate response curves, light proteins were spiked into depleted plasma (0.01 – 100 fmol/μL) while the U^{15}N-labeled proteins were added as internal standards to evaluate increased accuracy of protein-level quantification when using labeled peptides released from labeled proteins during enzymatic digestion. The labeled proteins were also used to assess the extent of peptide loss during enzymatic digestion (see below and **Supplemental Figure 2** and **Supplemental Table 8**). Synthetic ^{13}C/^{15}N-labeled versions of all peptide analytes were introduced post-desalt of the digest and immediately prior to LC-MRM-MS analysis (analogous to Phase II). The heavy synthetic peptides contain only a single labeled amino acid and have different and readily distinguishable masses and transition ions compared to the peptides derived from the U^{15}N-proteins in which every nitrogen atom in the peptide has been replaced with ^{15}N (**Supplemental Table 1**).

While peptide standards are typically spiked in prior to the desalting step to account for losses during desalting, (31, 45-49) in this experiment the peptide standards were spiked in post-desalt to allow for measurement of percent recovery of targeted peptides from

the digested proteins and to assess variability in the reduction, alkylation and digestion steps within and across laboratories. Therefore, we also calculated the loss and effect on CV for addition of stable isotope-labeled peptides pre-desalt vs. post-desalt (see below and Supplemental Figure **2** and **Supplemental Table 8**). Six additional unlabeled proteins were added at constant levels to all Phase III plasma samples to serve as process controls (**Supplemental Table 1**). A separate set of depleted plasma samples containing 3 different concentrations of the 27 cancer relevant proteins were also generated and the spike concentrations of the proteins in these samples were blinded to the participants.

All sample sets were prepared centrally, in triplicate, and shipped to the participating sites. Following the SOP (**Supplemental Document 2**), each site denatured, reduced, alkylated, trypsin digested and desalted each sample and then added $^{13}C/^{15}N$-labeled peptide standards prior to LC-MRM-MS analysis. The SSP was again used as described, above. All LC-MRM-MS data were integrated at the individual sites using Skyline. Reports were exported from Skyline in a pre-defined format and further processed in QuaSAR

(http://genepattern.broadinstitute.org/gp/pages/index.jsf?lsid=QuaSAR) to determine Limits of Detection and Quantitation, CVs for replicate samples, to construct response curves and to evaluate data for interferences. All data (raw and processed) were submitted for review using a central ftp site with controlled access at the National Institute of Standards and Technology (NIST).

Assay Development and Ongoing Monitoring of Site Performance. Purified light ($^{12}C/^{14}N$) and heavy ($^{13}C/^{15}N$) peptide forms of 125 peptides were used to optimize LC gradient and triple quadrupole transition selection (Q1/Q3) for all instrument platforms (**Supplemental Table 2**). The sequences of 115 of these peptides were derived from

the 27 cancer-relevant proteins while 10 peptides were the same as used in our earlier

study (5). Selection of specific peptides to target from each protein to target was based

on a combination of empirical data from discovery proteomics experiments (Broad

Institute, data not shown), Peptide Atlas, The Global Proteome Machine (GPM), and,

when empirical data were not available, by using the peptide selection algorithm

ESPPredictor [(35),

http://www.broadinstitute.org/cancer/software/genepattern/modules/ESPPredictor.html].

In Phase I, three to five transitions per peptide were evaluated for interference in

depleted plasma using AuDIT (50), and the three most abundant transitions with the

least interference for each peptide were selected to monitor. Protein digestion was

optimized to reduce missed cleavage products and achieve maximum peptide recovery

(see Methods). The final digestion protocol used both Lys-C and trypsin in tandem

(**Supplemental Documents 1 and 2**). The missed cleavage rate for proteins using the

double digestion protocol was determined to be ca. 20% (36).

Not all peptides initially selected and analyzed by LC-MRM-MS were used in the final

analyses. Peptide performance was assessed by the following criteria. The peak area

of the analyte peptide at the concentration above the calculated LOQ (determined in

Phase II) was defined as the minimum acceptable peak area, and varied by peptide and

instrument. Peptides were excluded if the peak areas of the $U^{15}N$-labeled peptides

derived from the labeled protein standards (Phase III) were smaller than the minimum

acceptable peak area. This criterion was applied to maintain consistency in comparing

Phase II and Phase III data across the same sub-set of peptides, and to ensure that

reliable quantification could be maintained such that the internal standard area was

above the LOQ. This resulted in a total of 96 peptides compared across the two study

phases (peptides marked with an asterisk in Table 1). Data from all peptides are available in Chorus.

In order to be included in the cross-instrument comparison, each data set had to be generated following the SOP (**Supplemental Documents 1 and 2**) with only minor deviations permitted. In addition, data sets from each instrument were evaluated using the SSP data to ensure instruments were in good working order during each study Phase prior to inclusion in data analysis.   Thirteen of fourteen instruments completed Phase II while eight instruments completed Phase III. Instruments were excluded from a specific phase of the study based on failing the SSP due to large retention time shifts, retention time scheduling problems, or unacceptably high peak area CVs. In addition to these objective criteria, three instruments did not continue to Phase III because the sites had other commitments and/or lacked funding to continue the studies. Complete datasets for all instruments, regardless of inclusion in data analysis, can be found on Panoramaweb.org

(http://proteome.gs.washington.edu/software/panorama/cptac_study9.html).

Limits of Detection and Quantification. The LODs and LOQs were determined from the response curve data generated at each site for the peptides monitored in Phase II and Phase III (**Figure 2, Supplemental Tables 4 and 5**). The median peptide LOD ranged from 0.053-0.162 fmol/μL for the thirteen instruments in Phase II. For Phase III, the median LODs determined using the synthetic heavy-labeled peptides as internal standards ranged from 0.037 to 0.186 fmol/μL for the 8 participating instruments. This corresponds to LODs between 17 ng/mL to 83 ng/mL of protein assuming 100% release efficiency of the analyte peptide from the protein (using average protein MW of 50 kDa). When U^{15}N-labeled proteins were used as internal standards in Phase III, LODs ranged from 0.096 to 0.51 fmol/μL across sites (44 – 230 ng/mL protein concentration in

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plasma). This increase in LOD is attributed to higher variability exhibited by the $U^{15}N$-labeled peptides due to a combination of losses related to proteolytic digestion and losses during desalting.

The LOD and LOQ achieved in Phase II of this study were compared to our previous SID-MRM-MS interlaboratory study in which ten peptides were monitored in neat plasma digests [(5), Figure 3, **Supplemental Table 6**]. Two of the 10 peptides were derived from C-reactive protein, a moderately abundant plasma protein, and are readily detected in all samples (including the depleted plasma blanks) in both studies. Therefore these peptides were not used for this comparison. The LOD and LOQ of the remaining 8 peptides were improved between 3 and 5-fold in the current study (Phase II) compared to our previous study (5). The improvement in detection sensitivity is likely due to a combination of the increased relative concentration of the monitored analytes in the depleted plasma, decreased interference/ion suppression from removal of peptides from highly abundant proteins that were depleted and optimization of the chromatographic conditions.

Use of U-^{15}N-labeled Protein Standards for Improved Quantitative Accuracy. The response curves generated in Phase III exhibited less than 100% recovery due to incomplete release of analyte peptides from the light proteins and peptide loss from desalting post digestion (21, 31). To evaluate the potential to compensate for idiosyncrasies in peptide release and recovery post desalt, and to improve quantitative accuracy, we added $U^{15}N$-labeled proteins as internal standards for 27 of the target proteins (Figure 1, Phase III). Labeled proteins were spiked into all samples (i.e., response curve concentration points, blank samples containing no added light proteins and blinded samples) at 25 fmol/µL prior to digestion. Synthetic $^{13}C/^{15}N$ peptides were introduced post-desalt at 10 fmol/µL to monitor for analyte peptide recovery from both

the light and heavy versions of the proteins. When the U^{15}N-heavy peptides derived from the U^{15}N-labeled proteins were used to normalize the analyte peptide response (arising from the unlabeled protein spikes), the measured concentration more accurately reflected the protein amounts spiked into the original samples (**Supplemental Figure 1**). Using heavy protein internal standards, the median peptide recoveries now approached 100% (97% median value across all sites, all peptides, **Supplemental Table 7**), reflecting improved quantitative accuracy in the calculation of protein concentration in the samples. In contrast, median peptide recovery using only labeled peptide standards was 34% across the 9 instruments, due to losses occurring during proteolytic digestion as well as loss of peptide during the desalting step (see below, Supplemental Figure **2** and **Supplemental Table 8**).

Evaluation of Peptide Loss Related to Digestion and Desalting and Effects on CV and Determined LOD. Apparent loss of peptides during the proteolytic digestion step can arise by incomplete digestion of the corresponding proteins or by loss of fully released peptides by precipitation, adsorption on surfaces, etc. The loss of peptides during proteolysis was assessed using the peak area ratios of ^{13}C/^{15}N-labeled peptides spiked pre-desalt to U^{15}N-protein-derived peptide (see Methods). The median peptide loss was ca. 70% (i.e., 30% recovery), with a range in loss from 30% to 99% (i.e., 1% to 70% recovery) across >96 peptides used in the measurement (**Supplemental Figure 2** and **Supplemental Table 8**).

To determine peptide loss due to desalting post digestion, labeled standard peptides were added pre- and post-desalt and analyzed by three participating instruments in Phase III. Comparing peptide recovery in these sample pairs with the peptide derived from the U^{15}N-labeled protein standard enabled estimation of peptide loss due to desalting (see Methods for details). The median loss due to desalting was ca. 27%, with

an interquartile range of 13% to 40% (**Supplemental Figure 2** and **Supplemental Table 8**).

The effects of SIS peptide addition pre- or post-desalt on CV and LOD were also evaluated. Addition of peptides post desalt resulted in higher CVs than for pre-desalt addition (Supplemental Figures 2a and 2c, respectively). The overall precision is better above the LOQ when the SIS peptide is added pre-desalt as analyte and SIS peptide are affected equivalently during the desalt step. As expected, the determined median peptide LOD was artificially lower (more sensitive) when SIS peptides were added post-desalt because losses occurring due to desalt are not taken into account.

Analysis of Blinded Sample Performance. Sets of samples spiked with peptide (125 peptides in Phase II) and protein (27 proteins in Phase III) analytes at concentrations blinded to the study participants were analyzed at the sites after each response curve replicate in Phases II and III. The blinded sample concentrations were chosen to span a range similar to the response curve (0.1 – 75 fmol/μL) and were run in pseudo-random order to mimic the analysis of unknown samples in a biomarker verification study. Results are shown in **Figure 4 and Supplemental Table 9** and allow for direct comparison of quantitative accuracy and peptide recovery across the participating laboratories and instruments. In Phase II, the blinded levels of spiked peptides were determined using heavy synthetic peptides (Figure 4, panel A), with a median concentration of 1.6 fmol/μL and a range of 1.4 - 2.9 fmol/μL across the 13 instruments for the 1.8 fmol/μL concentration point (**Supplemental Table 9**). The median CVs of these measurements were below 20% for 11 out of 13 instruments (**Supplemental Figure 3**), similar to the CVs achieved in the response curve data for samples with concentrations ≥ 1.8 fmol/μL (see below). Twelve out of thirteen instruments

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demonstrated CVs well below 15% for the 20 fmol/µL blinded samples and twelve out of thirteen had CVs 12% or lower for the 72 fmol/µL point.

In Phase III, the blinded levels of spiked proteins were determined using both heavy synthetic peptides and U[15]N-labeled protein standards (Figure 4, panels B and C, respectively). The lowest concentration point was removed based on lack of robust detection at this level in preliminary studies, and the remaining concentrations were adjusted to the three values shown (Figure 4B, C). The median concentrations in Phase III determined at each of the sites for the spiked proteins using synthetic peptides added post-desalt were significantly lower than their actual concentrations, and lower than the concentrations determined in Phase II (**Figure 4** and **Supplemental Table 10**. This was expected since the peptides being measured were derived from digestion of proteins (Phase III) in the sample rather than spiked synthetic peptides (Phase II). When peptides derived from the U-^{15}N-labeled proteins were used to calculate concentration, peptide recovery (synonymous with accuracy in this context) improves from 30% to 101% for the 75 fmol/µL sample. For the three sites that also added the heavy isotope-labeled peptides pre-desalt, the median recovery calculated using the SIS peptides was 42%. The range of median determined concentrations using protein standards (Figure 4, Panel C) was narrower using heavy protein standards and similar to the peptide spikes alone (Figure 4, Panel A). The CVs for Phase III are significantly higher for all instruments relative to Phase II, reflecting the greater variability introduced by incomplete digestion and/or loss of peptides released from the protein spikes as well as variable losses from desalting (**Supplemental Figure 2**).

Inter- and Intra-laboratory Reproducibility. The median CV for all peptides at each concentration point for each of the participating laboratories and instruments in Phases II and III of the study are shown in **Figure 5 and Supplemental Table 11**. The CV was

calculated for 96 peptides monitored in the assay) from the process replicates analyzed (n = 4 for Phase II, n = 4 for Phase III, where 3 replicates were process replicates and 1 was a technical replicate). Overall, Phase II (**Figure 5A**) showed a steep decrease (improvement) in intralaboratory CV as the analyte peptide concentrations increased above 0.018 fmol/µL, with nine out of thirteen sites having median CVs below 20% at 0.316 fmol/µL. In Phase III (**Figure 5B**), where the majority of the sample processing was conducted at the individual sites and protein-level spike-ins were used (with $^{13}C/^{15}N$-labeled peptides as internal standards), the variability was higher at the same protein concentrations, most likely due to incomplete digestion and peptide recovery. The median intralaboratory CV values did not fall below the 20% mark until the analyte protein concentration exceeded 1.3 fmol/µL. When the Phase III data were processed using the $U^{15}N$ peptides derived from the $U^{15}N$-labeled proteins vs. the synthetic $^{13}C/^{15}N$ peptides as internal standards, both intra-lab and inter-lab CVs improved above the LOQ, indicating the variability observed in the analyte was also observed in the $U^{15}N$ peptide standard, further supporting addition of an internal standards as far upstream in the workflow as possible (data not shown).

Interlaboratory CV, which was calculated for each peptide across all sites (n = 52 for Phase II and n = 32 for Phase III) were considerably higher. The median interlaboratory CV by peptide was 18% at the 1.3 fmol/ µL concentration point, while in Phase III for the same concentration, it was 45% when using $^{13}C/^{15}N$ peptide spikes post-desalt, and 36% when using $U^{15}N$-labeled proteins. This calculation of CV takes into account the variability between sites, which was found to be much larger in general, particularly when more sample handling was involved.

Monitoring Digestion and Assay Variability with Protein and Peptide Controls. To better assess variability due to protein digestion and sample handling, six unlabeled

proteins were spiked into all Phase III samples above the anticipated peptide LOQs (2.5 fmol/μL) to serve as digestion controls. The six proteins were well-characterized in our previous study (5). In addition eight synthetic ^{13}C/^{15}N-labeled peptides (selected tryptic sequences from the six proteins) were added to the samples post-desalt (10 fmol/μL). To assess process variability encompassing the digestion and desalt protocol, the CV of the raw peak areas of the unlabeled digestion control peptides were determined across each sample monitored on each instrument (n = 100-150 sample injections). **Figure 6** shows the process variability (red bars) for the 8 peptides at one representative site. The majority of the peptides (5 out of 8) have raw peak area CVs less than 30% with all peptides having CVs of 35% or less. It is important to note that these CVs are calculated with raw peak area, not peak area ratios as are used for quantification and do not reflect precision of quantification. Variability of raw peak areas tends to be higher when assessed over an experiment due to variations in the LC and MS, whereas use of an internal standard normalizes for this type of variation. Technical variability, reflecting the LC-MRM-MS system performance during the Phase III study, is represented by the blue bars in which the raw peak area CVs of the ^{13}C/^{15}N-labeled peptides are plotted. All eight peptides had CVs less than 25%, indicating good system performance over the course of the study.

Discussion

The current study builds upon the prior work of Addona *et al*. (5), and focuses on defining and addressing the issues encountered in the development and execution of

large-scale SID-MRM-MS assays. Here we designed the largest inter-laboratory

investigation to date, targeting 125 peptides derived from 27 cancer-relevant proteins

and 6 control proteins to precisely and reproducibly measure the analytes in human

plasma at 11 laboratories using 8 distinct instrument platforms and a total of 14 LC-MS

instrument configurations. We evaluated additional aspects of SID-MRM-MS assay

development and application, including the use of $U^{15}N$-labeled protein standards for

improved quantitative accuracy, use of internal digestion controls to monitor intra- and

inter-lab reproducibility, the benefits of immuno-depletion of abundant plasma proteins to

increase sensitivity for quantification of protein analytes, use of pre-packed columns and

column heaters to improve assay reproducibility, uniform data processing using vendor

neutral Skyline and QuaSAR software, and use of a SSP for monitoring instrument

performance throughout assay development and study phases.

Successful Assay Development Requires Optimization of Multiple Experimental

Parameters. Substantial work was performed to develop the highly multiplexed assay

described herein. Digestion studies were performed to select the best digestion

conditions for the target proteins in plasma, minimizing missed cleavage products and

maximizing peptide recovery. The precursor/product ion pairs used for each of the 8

different LC-triple quadrupole configurations were selected and prioritized by ion

intensity and lack of interferences. Importantly, the selected transitions were tested in

the presence of digested, depleted plasma to account for interferences from the sample

matrix or from the standards themselves. Collision energy values were based on

previously determined linear regression equations for each vendor platform in Skyline,

based on charge and m/z of the analyte peptides (40). Of note, different vendor

platforms had different lists of transitions for each target peptide, based on their

empirical performance. A highly detailed SOP was written and circulated with the

sample kits to ensure uniform sample handling, chromatography and data acquisition across sites.

Peptide selection was based on both empirical data and predictive algorithms. Between 1 and 5 peptides were selected for each protein, adhering to the usual selection rules (see Methods and 2, 35). However, not all peptides had the same level of performance by LC-MRM-MS, as was revealed during Phase I (method development). We found that we could not rely solely on historic targeted data or predictive algorithms which necessitated obtaining empirical data on the peptides in the targeted assay. One peptide containing an N-terminal carboxyamidomethyl cysteine (CGTGIVGVFVK, PDLI1) was found to spontaneously cyclize at the N-terminus with associated loss of hydrogen [observed in synthetic standard, data not shown, (51). Other peptides had poor chromatographic peak shapes or were found to elute over several minutes rather than the 7-15 second FWHM elution time observed for the majority of the peptides. Additionally, and important in Phase III, not all peptides were efficiently recovered from the protein during digestion and/or post-desalting, resulting in a wide range of recoveries of the U-^{15}N peptides generated from the U-^{15}N-protein standards, ranging from "not detected" to ca. 70% recovered. These effects were observed despite having selected proteotypic peptides for MRM assay development based largely on prior observation from discovery proteomics data in the literature. However, neither prior observation of peptides or prediction tools for selecting the best responding peptides from proteins are predictors of completeness of digestion or recovery of peptides post digestion. In the present study, the largest source of loss of target peptides occurred during the digestion step, with a median loss of ca. 70% across the nearly 100 peptides. Losses due to the digestion step were over 2-times greater than losses due to desalting for the set of peptides studies here. Unfortunately, no predictive tools are available to score peptide

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release/recovery efficiency during protein digestion, which is the dominant reason for the lower success rate in Phase III.

Assessment of the data and comparing multiple peptides per protein allowed the option to select the best performing peptides for subsequent calculations and site-to-site comparisons for assay metrics. Only those peptides found to have detectable signal in the U-^{15}N-labeled form in Phase III were used for calculations. In Phase III, the peptide-level success rate for detection was 83%, while the success rate for detection and quantification of proteins was 93%, with peptides for 25 out of 27 protein spikes detected and quantified. This likely reflects average success rates for detection in targeted peptide quantification when peptide prediction tools are used and empirical data are not always available.

System Suitability Standards and Rigorous SOPs Can Greatly Reduce Problems in Assay Construction. Highly multiplexed MRM-MS assays require an elevated level of system performance in order to target >100 peptides in a single LC run. Through development and utilization of SOPs and a SSP we have demonstrated here that highly multiplexed (100's of analytes), quantitative assays having high sensitivity, well defined specificity and good reproducibility within and across labs can be developed and implemented. Each site was required to acquire SSP data before and throughout the study to track system performance. Instruments that did not maintain good retention time stability, such that peaks were cut off during acquisition or missed entirely and could not provide data for the 4 process replicates were removed from subsequent data analysis. In addition, instruments with elevated peak area CVs for the SSP, either before (>25%) or throughout the study (>45%), were also eliminated because peak area CV of a SSP has been shown to correlate with assay sensitivity (33). The use of a SSP was evaluated as a proof-of-concept for data evaluation in this study and was new to some of the

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participating laboratories. As a result, some of the sites failed to examine their SSP data in real-time leading to deterioration in performance that was not identified and corrected during the study, but instead was identified post-acquisition. In this study, the most common reasons for repeated sample injections were related to shifting retention times in the chromatography, or decreased MS signal due to a dirty ion source. Sites that followed the SOP and used the SSP produced superior results.

Use of Peptide and Protein Controls Allows for Monitoring of Technical and Process Variation. Phase III of our study incorporated two additional controls to monitor both technical and process variability. Technical variation throughout the assay was monitored by determining the raw peak area CV for 8 synthetic $^{13}C/^{15}N$-labeled peptides that were spiked into each sample at 10 fmol/µL. This approach allowed for monitoring variability introduced through the LC or MS and could be considered analogous to the SSP. Use of Skyline to visualize the data allowed for a quick assessment of variability at the individual sample level based on raw peak area as well as across the entire study (peak area CV, **Figure 6**). Technical variability in LC-MS instrument performance between the peptide controls and the SSP were compared for Phase III for the 9 different instruments and showed good agreement in general. Sample to sample deviations could be caused by either LC-MS issues or fluctuations in the sample background, which could affect the overall signal intensities of the technical control peptides. Any discord between the technical peptide controls and the SSP is likely sample related. Process variability was tracked through the use of 6 unlabeled protein standards spiked into each sample at low concentrations. Monitoring the peak area ratio between the light, protein-derived peptides and the heavy synthetic peptides, as well as the raw peak area of the protein-derived peptides, provided an assessment of the variability introduced due to digestion and desalting for each data set. In this study,

the same information could be obtained by monitoring the peptides derived from the

U^{15}N -labeled proteins, which were compared to the unlabeled process control proteins.

Limits of Quantification are Substantially Improved by Immunoaffinity Depletion and to a Lesser Extent by Use of Newer LC-MS Technology Significant improvements in sensitivity were achieved in the present study as compared to our previous work. The principal, but not sole difference, was the use of immunoaffinity depletion of plasma for these studies. Depletion of the top 14 most abundant human plasma proteins decreased sample complexity and allowed a higher effective concentration of monitored analytes to be loaded on the column. LOQs were improved 3-5 fold (at the peptide level) relative to our earlier study while simultaneously increasing assay multiplex level more than 10-fold. The increase in sensitivity afforded by depletion is best captured at the protein level, which was over 20-fold for the 7 proteins compared between the studies. This value agrees well with previously published data in which the effects of immuno-depletion were evaluated (19). Optimization of the reversed phase gradient to improve peptide separation likely also contributed to the improvement observed in the LOQ.

The use of newer technologies such as latest generation triple quadrupole mass spectrometers with faster dwell times and brighter ion sources, as well as UHPLC systems for improved chromatographic resolution did enhance sensitivity, but less than we initially anticipated (Figure 2). The differences in determined LODs between different generations of instruments from different vendors that were operating properly and that met SSP performance criteria generally varied by less than 2-fold (**Figure 2;** instruments not meeting performance standards were excluded from these results). The largest differences (for example, between sites 56C, 65A and the rest of the sites/instruments) were likely due to small differences in chromatographic configurations. For example, the

AB SCIEX 4000 QTRAPs used PicoFrits while the AB SCIEX 5500 QTRAPs used chip columns in parallel with post-column attachment to ion source which introduced some band broadening. Another example is the Waters Xevo TQ that employed a trap column while the Waters TQS system did not. We conclude that while use of the newest technology has the potential to improve sensitivity, signal to background biological noise remained the principal limiter on assay sensitivity. The two methods that have been clearly demonstrated to decrease biological noise while retaining high analyte signal are fraction MRM (fMRM) and SISCAPA also referred to as immunoMRM (9, 19, 28-31)

Reproducibility of Assays Within and Across Sites. The variability observed at each site (intralaboratory CV) was found to range from 13 to 39% (median of 15% across sites) at the 0.316 fmol/ μL concentration point for the 13 sites in Phase II. The interlaboratory CV, calculated according to Hoofnagle (52) was 31% at the 1.3 fmol/ μL concentration point when using $U^{15}N$-labeled protein standards. The variability observed in Phase II is a measurement of technical variability in the LC-MS platforms used at each site, and was found to be improved over that found in our previous study. This improvement can be attributed to several factors implemented in this study, including a more rigorous SOP, the use of pre-packed columns and column heaters, and close monitoring of instrument performance using a SSP, which when combined will minimize technical variability. Phase III variability was higher (58% at 0.316 fmol/ μL and 46% at 1.3 fmol/ μL) than in Phase II, which was not surprising considering that reduction, alkylation, digestion and other sample handling steps were performed at each instrument site rather than centrally. Use of the $U^{15}N$-labeled proteins improved variability for many peptides as compared to the $^{13}C/^{15}N$-labeled peptides (42% at 0.316 fmol/ μL and 31% at 1.3 fmol/ μL), further supporting their use for both precise and accurate quantification by LC-MRM-MS.

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Samples were immunoaffinity depleted of abundant plasma proteins at a single site prior to further analysis (see Methods). Therefore, variability that could be introduced by the abundant protein depletion step was not directly measured. Assessing the contribution of this step to the variability would have required additional experiments involving depletion of all process replicates in Phase III samples at each protein concentration at each of the sites. While time and funding were limiting factors preventing us from evaluating this step in the current study, several published studies have evaluated the reproducibility of column-based abundant protein depletion and have found it to be robust and reproducible. The Smith laboratory at PNNL evaluated protein recovery and depletion efficiency using the IgY-12 column from Sigma, alone (53) or in combination with a second column that removes an additional ca. 50-60 proteins (so-called "Supermix" strategy (54). In both cases the reproducibility of process replicates was <20% based upon the number of proteins identified and spectral counting (n=5). The Carr laboratory (55) used IgY-12 column depletion and peptide fractionation prior to SID-MRM-MS to quantify low-level candidate cardiovascular biomarkers in plasma from multiple patients and multiple timepoints/patient. They found the total process variability (%CV), including that introduced by SCX-based peptide fractionation prior to MRM, to range from <1% to a maximum of 35% across three process replicates of 24 samples. This degree of variability is similar to, and no higher than, the intra- and interlaboratory CVs presented here as well as that reported in our previous interlaboratory study (5). Together these prior studies suggest that minimal additional variation is introduced within laboratories by use of column-based abundant protein depletion methods. Factors that could affect inter-laboratory reproducibility include incomplete wash and regeneration steps and lot-to-lot variability of the depletion columns. One impact of incomplete depletion could be a change in the LOD/LOQ for some analytes caused by ion suppression from peptides derived from abundant proteins that were not adequately

depleted. However, unless detection of the analyte was near the noise level in the MS, such variation in depletion would not likely result in either a failure to be able to detect the analyte or in the ability to quantify that analyte relative to the heavy internal standard peptide as both analyte and labeled standard would be affected equally by any ion suppression. Protein analytes partially bound to proteins targeted by the depletion column could also have their levels change in the depleted plasma sample. In such a case, the values measured for peptides from that protein could be lower than for a properly operating column.

Use of Labeled Internal Standard Proteins Improves Quantitative Accuracy. This is the largest study to date utilizing $U^{15}N$-labeled proteins as internal standards for protein quantification. The $U^{15}N$-labeled proteins were expressed and purified using the same process as the unlabeled proteins used in this study and, in all regards, should behave quite similarly. An added benefit to using heavy-labeled proteins as internal standards is the ability to monitor multiple peptides from each protein. In cases where digestion is incomplete, resulting in missed cleaved peptides or in cases where additional peptides from the protein would strengthen the statistics of the measurement, as long as the heavy-labeled protein behaves the same way as the endogenous protein, the additional peptide forms can be readily monitored. Such experiments are especially tractable using instruments with high resolution, accurate mass capabilities where the accurate mass measurement of the precursor and the fragments significantly improves the confidence of the analyses and selection and optimization of transitions is not necessarily required (15, 56).

Blinded samples were incorporated in our study to model real-world sample analysis such as would be encountered in a biomarker verification study and to evaluate consistency of results across laboratories. The accuracy obtained in Phase III of the

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study, where laboratories did all sample processing themselves, was 101% when using the $U^{15}N$-labeled proteins for normalization. While use of heavy-labeled proteins in the present study greatly improved quantitative accuracy for measurement of the light versions of the same proteins also expressed in *E. coli*, their use in general for quantification of endogenous proteins may not be as accurate. Discrepancies could arise as a result of modifications (e.g., cleavage forms, posttranslational modifications, etc.) present in the endogenous protein that are not present in the heavy-labeled protein standard. In studies where accuracy is a requirement, demonstrations of parallelism of the internal standard to the endogenous protein in the sample matrix should be carried out (10).

When heavy-labeled proteins are not available, using synthetic $^{13}C/^{15}N$-labeled peptides is the next best option, and is likely a necessity for quantification of most posttranslational modifications, which are difficult or impossible to recapitulate accurately in a recombinant protein. However, SIS peptides added post-digestion and pre-desalt cannot account for variability in peptide recovery from the digestion step. Peptide recovery from the digestion step ranged from 1% to 70% for the 96 peptides in the present study. While it makes sense to avoid peptides that are predicted to have a higher mis-cleavage propensity (i.e., double basics at either end of the peptide or acidic residues in close proximity to the cleavage sites), such mis-cleaved peptides are not necessarily recovered to a lesser extent from the digest than peptides that are predicted to be fully tryptic (57). A mis-cleaved form may be the dominant form of the peptide released from the protein and/or it may have solubility properties that are superior to that of the fully tryptic. Adding SIS peptides or "wing peptides" to the sample pre-digest (47, 58) may improve accuracy when using heavy labeled peptides as substitutes for labeled proteins.

While uniformly ^{15}N-labeled proteins are commonly synthesized for NMR (59), they are not the ideal choice for heavy-labeled protein reference standards for mass spectrometry. The mass shift relative to the unlabeled peptide varied depending on the number of nitrogen atoms per peptide. Shorter peptides with lysine at the C-terminus tend to have precursor masses that are close (<2 amu) to the ^{13}C/^{15}N-labeled synthetic peptides. This makes the internal standard peptide difficult to distinguish from the analyte peptide on instruments like triple quadrupole MS systems that use relatively wide (0.4 to >1.0 mass unit) precursor selection windows. In addition, the monoisotopic (M+H)[+] peptide masses were typically not the dominant precursor ions derived from the intact proteins that had isotopic purities of >98% (**Supplemental Figure 4 and Supplemental Table 12**). In future studies, the proteins would ideally be produced with only specific amino acid residues labeled (e.g., ^{13}C/^{15}N-labeled lysine and arginine) at very high isotopic purity to minimize under-labeled forms of each peptide as well as allow for fixed mass shifts for each peptide.

In conclusion, this study explored the feasibility of developing and implementing a highly multiplexed SID-MRM-MS assay targeting 125 peptides in depleted plasma on 14 different triple quadrupole instrument platforms. Improved LOQs were achieved through the use of depleted plasma, LC gradient optimization, and reduced sample load per injection. Reproducibility was improved over our previous study due to addition of a SSP, the use of pre-packed columns and column heaters as well as protein internal standards. Improved quantitative accuracy was achieved through the use of U[15]N-labeled proteins and addition of the synthetic ^{13}C/^{15}N-labeled peptide standards earlier in the sample handling workflow. The use of Skyline made it possible to develop targeted assays on all instrument platforms and integrated data for further processing and analyses in QuaSAR. We demonstrated the ability to use proteins and synthetic peptides as

process and technical controls that can be used in any type of targeted experiment to assess and track variability from sample to sample throughout a study. The evaluation and rigorous testing, in addition to development of tools and technologies to improve the precision and accuracy of SID-MRM-MS resulted in a plethora of data for benchmarking targeted MRM-MS workflows as well as developing additional computational tools for analysis.

AUTHOR CONTRIBUTIONS: The CPTAC Network contributed collectively to this study. The following CPTAC Network investigators made significant intellectual contributions to the work described:

Study design and SOP development: S. E. Abbatiello, S. A. Carr, S. C. Hall, H. Keshishian, B. Schilling, N. Sedransk, L. J. Zimmerman.

Central sample preparation: C. Whitwell, K. Shaddox, L. J. Zimmerman.

Data acquisition and processing: S. E. Abbatiello, M. Albertolle, S. Allen, M. Burgess, M. Ghosh, J.M. Held, V. Hedrick, H. D. Inerowicz, A. Jackson, H. Keshishian, J. Lyssand, P. Rudnick, P. Sadowski, B. Schilling, S. J. Skates, D. Smith, J. R. Whiteaker, , S. Zhang, L. J. Zimmerman

Bioinformatics and statistical analysis: M. P. Cusack, B. MacLean, D.R. Mani, N. Sedransk.

Manuscript writing and preparation: S. E. Abbatiello, S. A. Carr, S. C. Hall, D.R. Mani, B. Schilling, L. J. Zimmerman.

General: C. H. Borchers, S. J. Fisher, B. W. Gibson, C. R. Kinsinger, E. Kuhn, D. Liebler, M. MacCoss, L. Makowski, M. Mesri, T. Neubert, A. G. Paulovich, F. E. Regnier, H. Rodriguez, P. Tempst.

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Figure Legends

Figure 1. Schematic of the experimental design of the three phases of the study. Phase I consisted of method development and optimization of the sample handling, LC, and MS parameters for peptide detection. Phase II was generation of the peptide-level response curve in which 125 light peptides were spiked into depleted, digested plasma at 9 concentrations and 125 ^{13}C/^{15}N peptides were spiked in as internal standards and 750 transitions were monitored on the different LC-MRM-MS platforms. Phase III introduced unlabeled (light) and uniformly ^{15}N-labeled proteins into the workflow, which were spiked into depleted plasma to generate a 9-point response curve. Samples were further processed at the individual sites to denature, reduce, alkylate, desalt and reconstitute the samples with ^{13}C/^{15}N peptide standards for LC-MRM-MS analysis, resulting in a total of 1095 transitions for each method. Skyline was integral from Phase I through Phase III for transition selection, method building, retention time scheduling, and data integration across the different vendor platforms.

Figure 2. Limit of Detection distributions for the peptides monitored at each site. The black bar in each box represents the median peptide LOD at that site, the box represents the interquartile range and the whiskers represents 3x the interquartile range.

Outlier peptides are shown as black dots. Panel A represents data from Phase II for the 13 instruments completing the study. Panel B shows the LOD distribution for the 8 instruments that completed Phase III, with the synthetic $^{13}C/^{15}N$ peptides used as internal standards. Panel C represents the same Phase III data, except the $U^{15}N$-peptides, derived from the $U^{15}N$-proteins, were used as internal standards.

Figure 3. Comparison of the Limits of Detection (LODs) for the eight peptides from the Addona *et al*., 2009 Study and the current study (Phase II). The box and whisker chart represents the distribution of LODs from the participating sites in both studies for the peptide-level spike experiment.

Figure 4. Evaluation of the accuracy of determined concentrations for 125 peptides in the blinded samples: Sets of samples were spiked with peptide (125 peptides in Phase II) and protein (27 proteins in Phase III) analytes at concentrations blinded to the study participants were analyzed at the sites after each response curve replicate in Phases II and III. Panel A shows the four blinded sample concentrations and the range of peptide concentrations detected at each site in Phase II. Panels B and C represent the Phase III blinded sample concentrations determined when using the $^{13}C/^{15}N$ peptides (panel B) or the $U^{15}N$-proteins (panel C) as internal standards. The light blue lines represent the actual concentrations of spiked proteins. Note that in Panel B all measured concentrations are well below the actual concentrations when calculating concentration based on spiked heavy peptides. Concentration values are much closer to the actual values in Panel C where concentration values were relative to peptides derived from the digestion of $U^{15}N$-labeled internal standard proteins.

50

Figure 5. Reproducibility plots for Phases II and III at each sample concentration. The median peak area %CV for 115 peptides is shown for Phase II (panel A) and Phase III (panel B) for all sites.

Figure 6. Technical and Process Variability Assessed from Digestion Controls and SIS Peptide Spikes for Phase III. Six unlabeled (light) proteins were spiked into all samples pre-digestion at a fixed concentration (2.5 fmol/μL). The red bars represent the CV of the raw peak areas arising from the light peptides, and reflect the process variability (due to digestion, desalt, and sample handling) of the assay for 40 individual samples. Eight $^{13}C/^{15}N$ peptides were spiked into all samples post-desalt at 10 fmol/μL. The blue bars represent the CV of the raw peak areas from the $^{13}C/^{15}N$ peptides, and reflect the technical variability of the LC-MRM-MS measurements. Here, we see the process variability exceeds the technical variability for all peptides and is 35% or less, based on raw peak area. The technical variability is 25% or less for all peptides over the measurement of 40 different samples, and is 20% or less for 6 of the 8 peptides. This is an example from Phase III, site 56B90, plotted in Skyline.

Figure 1

Figure 2

LOD distribution for all peptides at a site

Phase II,
Using 13C/15N
peptides as IS

LOD distribution for all peptides at a site
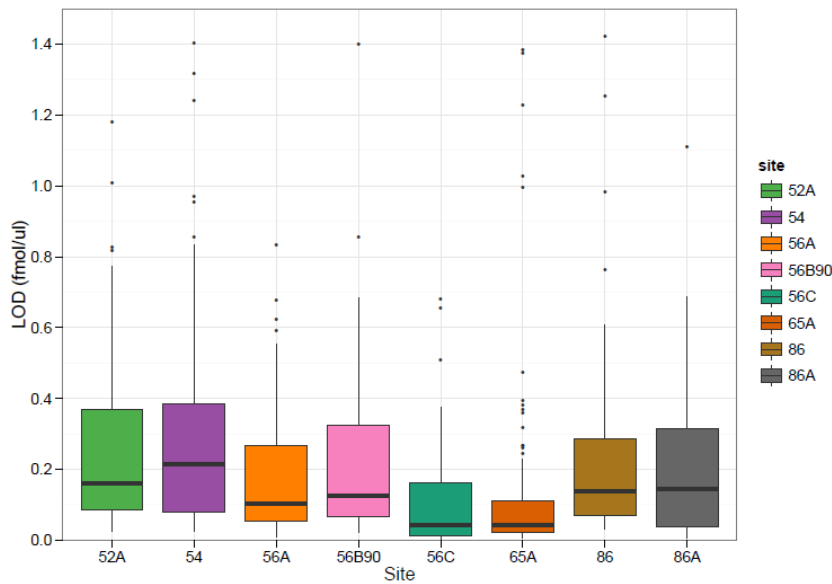
Phase III,
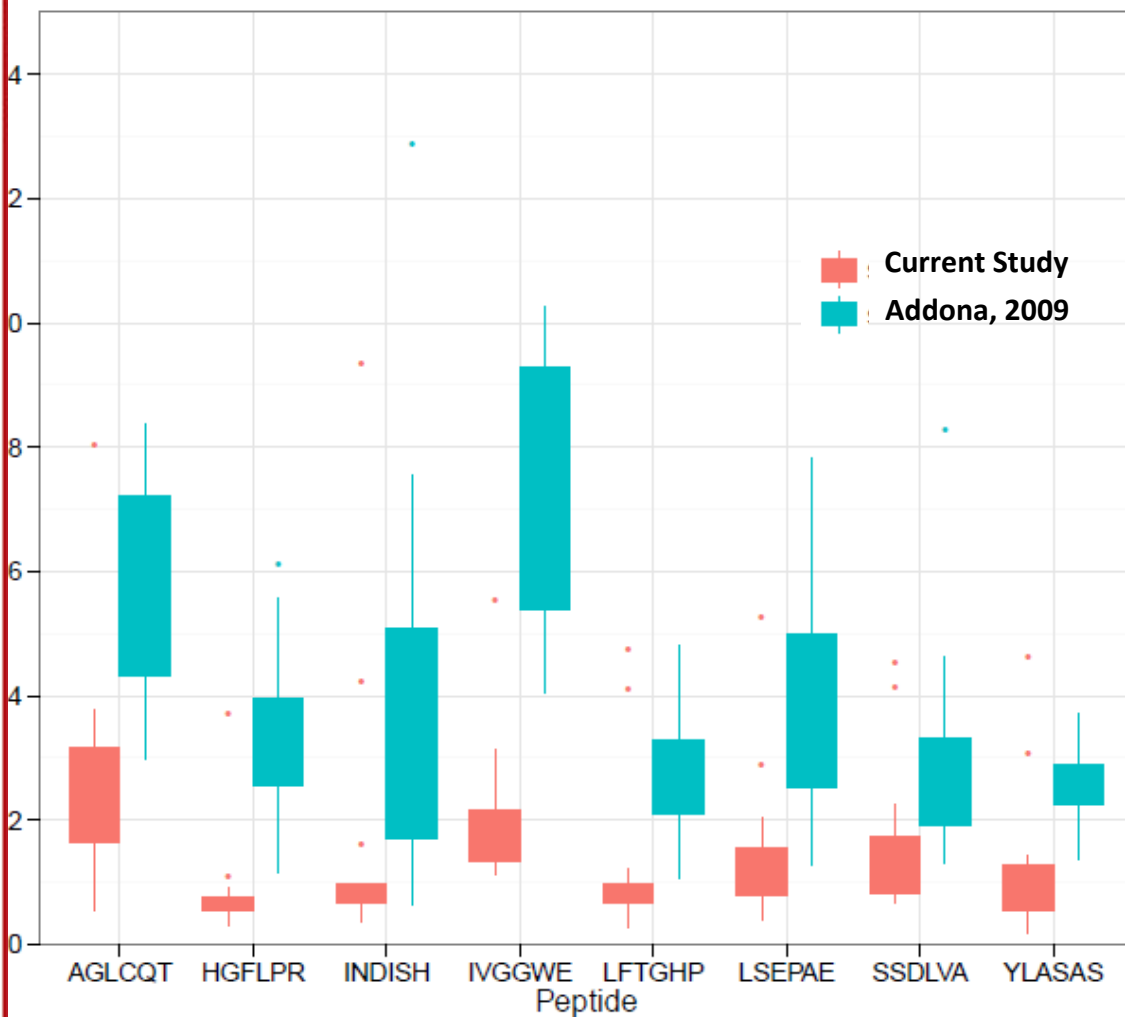Using 13C/15N
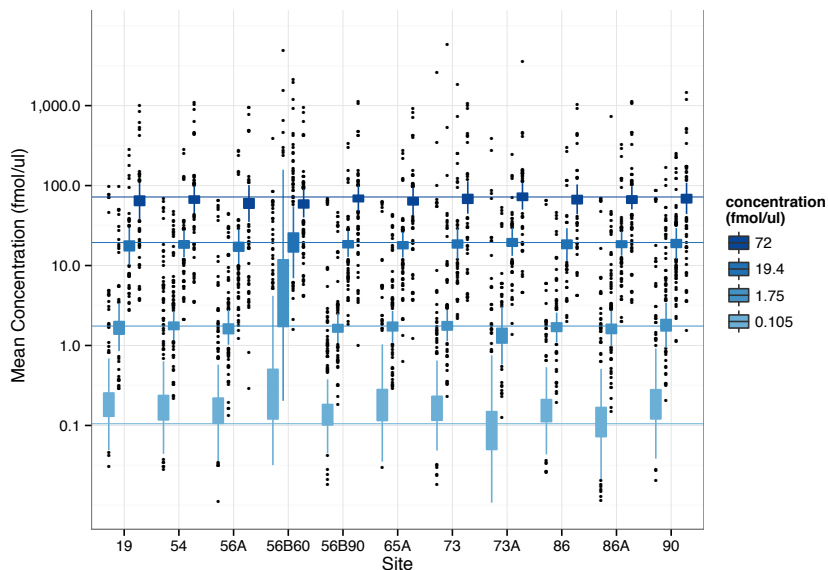peptides as IS

LOD distribution for all peptides at a site
N15 Standard
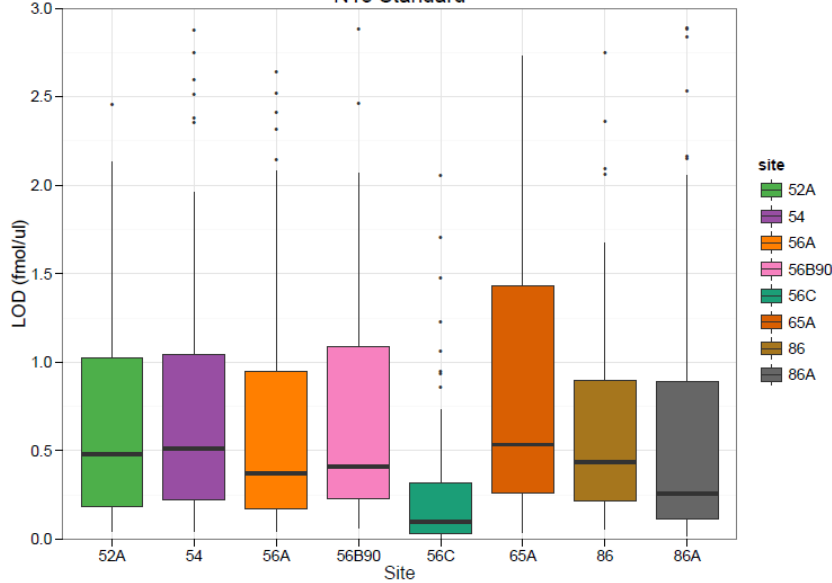
Phase III,
Using 15N
proteins as IS

Figure 3. Comparison of LODs in Study 7.1 and 9.1

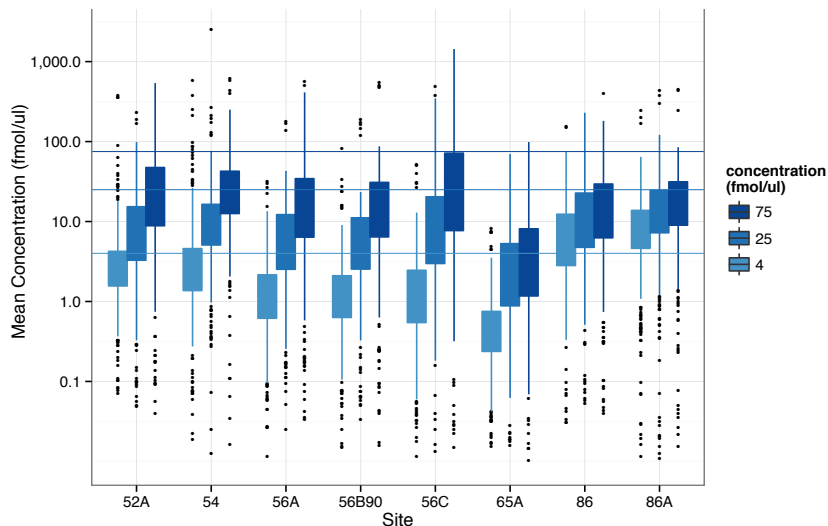# A. Measured concentrations relative to synthetic peptide standard, Phase II

Blinded sample measured concentration

B. Measured concentrations relative to synthetic peptide standard, Phase III

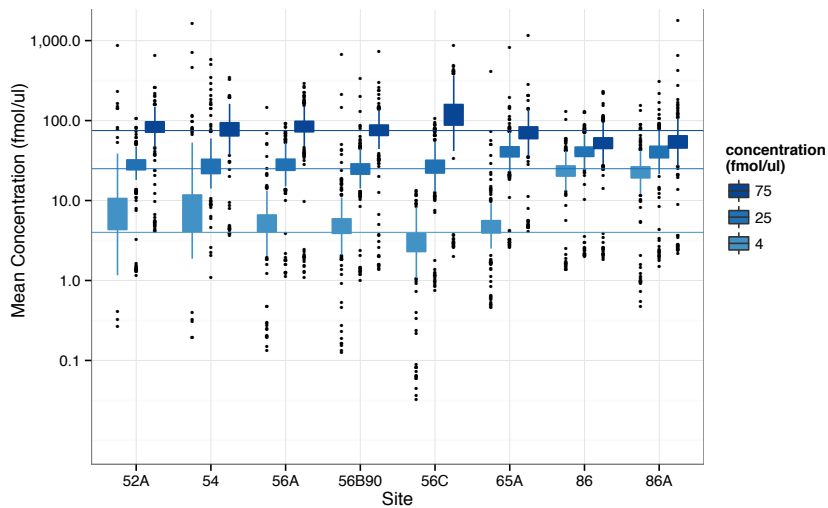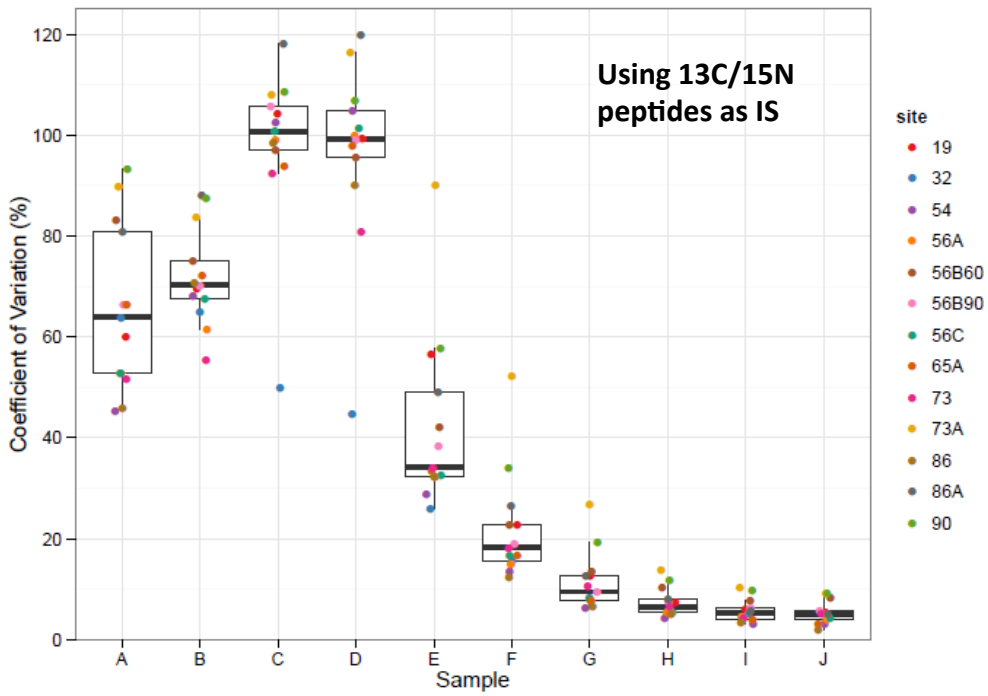# C. Measured concentrations relative to U15N protein standards, Phase III

ure 4

A

Median CV across all peptides

Using 13C/15N peptides as IS
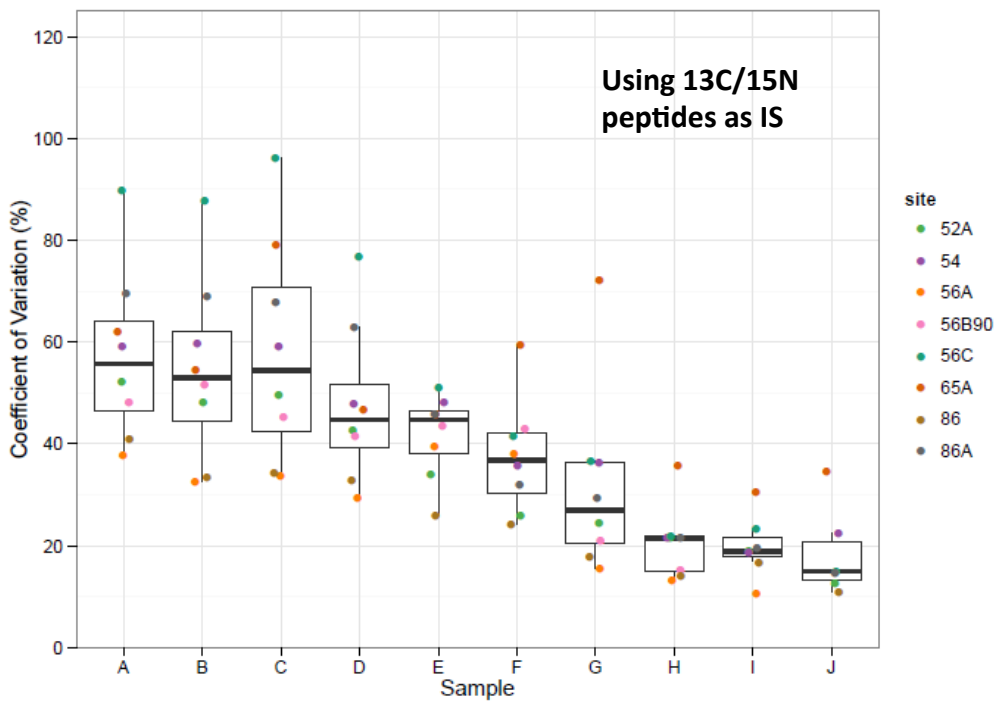
B

Median CV across all peptides

Using 13C/15N peptides as IS

Technical and Process Variability Assessed From Digestion Controls and SIS Peptide Spikes