A B R F
Proteomics Standards Research Group

ABRF-sPRG2009 Study: Development of Quantitative Standards for Plasma Proteomics

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Abstract

Blood plasma is a highly complex physiological fluid reflecting a unique signature of the physiological state of all tissues of an organism. Plasma is abundant, rich with proteins shed by all tissues, readily obtainable by standardized procedures, and well preserved for prolonged periods of storage. Despite its complexity and overwhelming dynamic range of at least 10 orders of magnitude, plasma is considered as a source of protein biomarkers comprehensibly sampling the phenotype of the human body. Various MS-based proteomic approaches have been developed for plasma analysis and potential applications in clinical diagnostics, yet enabling a successful proteomic assay is still a formidable challenge. Availability of a plasma proteomics standard covering a broad dynamic range would facilitate method development, optimization and validation for efficient plasma analysis.

The Proteomics Standards Research Group (sPRG) has designed a 2-year study, sPRG2009 to develop such standard consisting of depleted plasma spiked with stable-isotope labeled peptides of ~50 human proteins covering ~5 orders of magnitude dynamic range.

Considerations in Sample Design

Experimental Option	Advantages	Disadavantages	Our Choice
Serum vs. Plasma	Simpler protein mixture Less sample handling	Potential protein losses Higher complexity	*Serum*
Deplete abundant proteins vs. non-depletion	Expand dynamic range Less sample handling	Potential protein losses Dynamic range problems	*Deplete "top 20"*
Fractionate at protein level vs. no fractionation	Expand dynamic range Less sample handling	Multiplies analysis time Dynamic range problems	*no fractionation*
Analyze at protein level by biological assay, ELISA or similar, or mass spectrometry?	Sensitivity speed, sensitivity selectivity, info. gained	few examples cross-reactivity, need antibody serum is beyond current technol.	*No analysis of intact proteins*
Or digest sample for mass spectrometry?	instruments and methods widely available; removes digestion as a variable in the study.	multiplies sample complexity; may lose isoform discrimination	*Digest with a combination of Lys-C, trypsin*

Aims and Objectives

•The aims of the 1st year of the sPRG2009 study were:

- to develop requirements for selection of candidate proteins and peptides;
- to explore practical aspects for preparing plasma samples and achieving complete digestion;
- to explore analytical platforms for efficient quantitative proteomic plasma analysis;
- to assemble a list of peptides for synthesis of stable-isotope labeled standards.

The tentative goals for the 2nd year of the sPRG2009 study are:

- to synthesize stable-isotope labeled peptide standards for selected human plasma proteins;
- to create a publicity domain (sPRG2009 forum and email account) to collect feedback on the study design and recruit future participants of the study;
- to test the sPRG2009 standard using alternative platforms in the laboratories of sPRG members;
- to develop a guideline for conducting the study;
- to distribute the sPRG standard among proteomic labs actively involved in quantitative plasma studies;
- to collect data from participants of the sPRG2009 study and evaluate:
  - abilities to quantitate plasma protein standards;
  - performance of the alternative methodological, instrumental and bioinformatics platforms.

Peptide Selection Criteria

Synthesis Difficulties	• Peptide Length: $8 \leq N \leq 20$ amino acid residues • Imide Formation: [DN].[GASN][1] • DKP Formation: C-terminal acid peptide with G or P at C1 or C2 position. • Repeats: XXX • Hydrophobic: multiple aliphatic aa's such as A, L, I, V • Incomplete side chain removal: tBu and Trt
Peptide Stability	• No Potential Imide Formation Residues [DN].[GASN][2] • No Potential Deamidation Residues: [NG] • No Acid Labile Residues: [DP] • No Oxidation Senstive: Residues - Met, Cys Trp • No *N*-terminal Gln Residues (converts to pyroglutamic acid)
Heterogeneity	• Problems with Peptide Synthesis and Stability may lead to two or more species. • No missed cleavages allowed • Enzyme Cleavage Specificity: – No *N*- or *C*-terminal K↓K, K↓R, R↓K, or R↓R cleavages; no K↓P or R↓P cleavages [3] • Pro-Pro cis-trans isomers may separate during chromatography • No known PTMs (e.g., phosphorylation) or consensus recognition sequences (e.g., *N*-glycosylation)
Detectability	• 3 peptides per protein • Predicted proteotypic peptides are preferred • Chromatography: Peptide should be retained by C18 column and eluted in by reasonable percentage of acetonitrile buffer (not too hydrophilic or hydrophobic). • Peptide Length: $8 \leq N \leq 20$ amino acid residues

Potential Approaches to Peptide Identification and Quantitation

a non-exclusive survey of methods using stable isotope labeled (SIL) internal standards

	Non-targeted detection methods	Targeted detection methods
Based primarily on MS/MS	• Perform undirected MS/MS on as many peptides as possible and identify them by database search. • Inspect protein list for those of interest. • Extract quantitative information from MS scans of peptide and SIL standard corresponding to the identified MS/MS spectra. • Example: data-dependent LC-MS/MS or MudPIT with an ion trap instrument. • Advantages: simplicity, generality. • Disadvantages: Biased towards high-abundance proteins; element of chance in peptides selected by the instrument for MS/MS; interference of isobaric peptides in quantitation.	• Perform MS/MS on all ions with precursor masses of selected peptides and their SIL standards. • *Either* detect all product ions (e.g. ion trap or Q-TOF) • *or* Detect only selected product ions (triple quadrupole SRM – discussed in detail below) • Extract quantitative information from ion chromatograms of selected product ions. • Advantages: sensitivity, specificity, wide dynamic range for SRM method. • Disadvantages: More work to create experiment definition; limits on number of ions monitored in one LC-MS experiment; dynamic range limitations in trapping instruments.
Based primarily on high resolution MS	• Perform LC-MS or LC-MS/MS, collecting high resolution MS scans • Find desired peptides and SIL standards by "exact" mass and retention times. • Extract quantitative information from MS scans. • Example: LC-MS or MS/MS on FT-ICR or orbitrap. • Advantages: simplicity, specificity from ppm mass accuracy. • Disadvantages: potential isobaric interferences remain in highly complex samples; dynamic range limitations in trapping instruments; might still need MS/MS to confirm identifications.	• SIM scans of masses corresponding to peptides of interest. • Find desired peptides and SIL standards by "exact" mass and retention times. • Extract quantitative information from MS scans. • Example: LC-MS or MS/MS on FT-ICR or orbitrap. • Advantages: higher sensitivity/dynamic range than full scan MS experiment. • Disadvantages: potential isobaric interferences remain in highly complex serum sample; dynamic range limitations in trapping instruments; might still need MS/MS to confirm identifications.

References

1. Tam, et al., Techniques Prot. Chem.,1995, VI, 555-562.
2. Stephenson and Clarke, J Biol Chem., 1989, 264, 6164-70 .

J. Proteome Res. 2007; 7; 300-305.)

Mallick, et al., Nat Biotechnol, 2007, 1, 125-31.

- Anderson, N.L.; The roles of multiple proteomic platforms in a pipeline for new diagnostics. *Mol Cell Proteomics*. 2005 4(10):1441-4.
- Haab, B.B. et al., Immunoassay and antibody microarray analysis of the HUPO Plasma Proteome Project reference specimens: Systematic variation between sample types and calibration of mass spectrometry data. *Proteomics* (2005) **5** 3278–3291.
- Anderson, L., Candidate-based proteomics in the search for biomarkers of cardiovascular disease. *J Physiol*. (2005) **263** 23-69.
- Polanski, M., & Anderson, L., A list of candidate cancer biomarkers for targeted proteomics. *Biomarker Insights* 2006 **2** 1-48.
- Mallick et al., Computational prediction of proteotypic peptides for quantitative proteomics *Nature Biotechnol.* (2007) **25**: 125-131.
- Tam, et al., Techniques Prot. Chem.,1995, VI, 555-562.
- Stephenson, R.C., Clarke, S., Succinimide formation from aspartyl and asparaginyl peptides as a model for the spontaneous degradation of proteins. J Biol Chem., 1989, 264, 6164-70.
- Rodriguez, J., Gupta, N., Smith, R.D., Pevzner, P.A., Does Trypsin Cut Before Proline? J. Proteome Res. 2007; 7; 300-305.
- Bairoch A., *et al.*, The Universal Protein Resource (UniProt). *Nucleic Acids Res*. (2005) **33**: D154-159.

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