Candidate-Based Plasma Proteomics

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ABRF Longbeach
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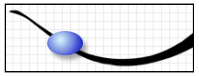
PPI 

Clinical Measurement of Proteins in Plasma is Very Well Established

Since when?	Decades
How many tests?	> 10 million/yr
What instruments?	50-100,000 machines in hospitals etc
How accurate?	CV~5-10% worldwide at 100pg/ml

Cardiac damage	TnI, CK-MB, Mb, MPO, BNP
Cancer	PSA, CA-125, Her-2
Inflammation	CRP, SAA, cytokines, RF
Liver Damage	ALT, ALP, AST, GGT (enzyme assays)
Coagulation	AT-III, proteins C&S, fibrinogen, VWF
Allergy	IgE against various antigens
Infectious disease	HIV-1, Hepatitis BsAg

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There Are More Existing Candidate Disease Markers Than You Might Think:

177 Candidate Cardiovascular Disease Marker Proteins

	Name	Accession	Normal Concentration (pg/ml)	Source for concentration	Reason
1	activin A	P08476	6.0E+02	(Eldar-Geva *et al.*, 2001)	Released by heparin from vascular endothelium (Phillips *et al.*, 2000)
2	adiponectin (ADPN)	Q15848	4.8E+06	(Mallamaci *et al.*, 2002)	Higher levels in essential hypertensives (Mallamaci *et al.*, 2002)
3	albumin	P02768	4.1E+10	(Laboratories, 2001)	Negative acute phase reactant, lower levels associated with increased risk of cardiovascular mortality (Shaper *et al.*, 2004)
4	aldolase C	P09972	4.0E+03	(Asaka *et al.*, 1990)	A more specific and sensitive marker of cerebrovascular diseases than aldolase A (Asaka *et al.*, 1990)
5	alpha 2 antiplasmin (alpha 2 AP)	P08697	7.0E+07	Progen test insert	An important regulator of the fibrinolytic system
6	alpha 2 macroglobulin (alpha 2 M)	P01023	1.8E+09	(Laboratories, 2001)	Major plasma protease inhibitor
7	alpha(1)-antichymotrypsin (ACT)	P01011	4.2E+07	(Putnam, 1975)	Major plasma protease inhibitor
8	alpha1 acid-glycoprotein (AAG)	P02763	6.9E+08	(Laboratories, 2001)	Acute phase reactant
9	alpha1-antitrypsin (AAT)	P01009	1.4E+09	(Laboratories, 2001)	Major plasma protease inhibitor
10	angiotensin-converting enzyme (ACE)	P12821			Lower in stroke patients than controls (Catto *et al.*, 1996)
11	angiotensinogen	P01019	1.5E+06	(Bloem *et al.*, 1995)	Precursor of major blood pressure control peptide
12	antithrombin III (AT III)	P01008	2.0E+08	(Kalafatis *et al.*, 1997)	Major inhibitor of thrombin
13	apolipoprotein A-I	P02647	1.4E+09	(Glowinska *et al.*, 2003)	Low level associated with mortality and myocardial infarction five years after CABG(Skinner *et al.*, 1999)
14	apolipoprotein A-II	P02652	2.4E+08	(Luo & Liu, 1994)	Lipoprotein
15	apolipoprotein A-IV	P06727	1.6E+08	(Kondo *et al.*, 1989)	A relatively independent risk factor for CHD(Warner *et al.*, 2001)
16	apolipoprotein B	P04114	7.3E+08	(Glowinska *et al.*, 2003)	Major component of LDL
17	apolipoprotein C-I	P02654	6.1E+07	(Riesen & Sturzenegger, 1986)	Lipoprotein
18	apolipoprotein C-II	P02655	3.3E+07	(Bury *et al.*, 1986)	Lipoprotein
19	apolipoprotein CIII	P02656	1.2E+08	(Onat *et al.*, 2003)	marker of CHD independent of cholesterol(Onat *et al.*, 2003)
20	apolipoprotein D	P05090			Lipoprotein
21	apolipoprotein E	P02649	4.0E+07		presence of epsilon4 allele a strong independent predictor of adverse events (Brscic *et al.*, 2000)
22	apolipoprotein L1	O14791			Lipoprotein
23	aspartate aminotransferase, mitochondrial (m-type)	P00505			diagnostic for early detection of myocardial infarction (Yoneda *et al.*, 1992)
24	basic fibroblast growth factor (bFGF)	P09038	6.0E+03	(Song *et al.*, 2002)	sICAM-1level increases in acute cerebral infarction (Song *et al.*, 2002)
25	beta(2)-glycoprotein I, nicked	P02749			may control extrinsic fibrinolysis via a negative feedback pathway loop (Yasuda *et al.*, 2004)
26	B-type neurotrophic growth factor (BNGF)	P01138	7.0E+02	(Reynolds *et al.*, 2003)	Candidate stroke marker (Reynolds *et al.*, 2003)

From: Candidate-Based Proteomics in the Search for Biomarkers of Cardiovascular Disease, Leigh Anderson, J. Physiol., 563.1:23-60 (2005).

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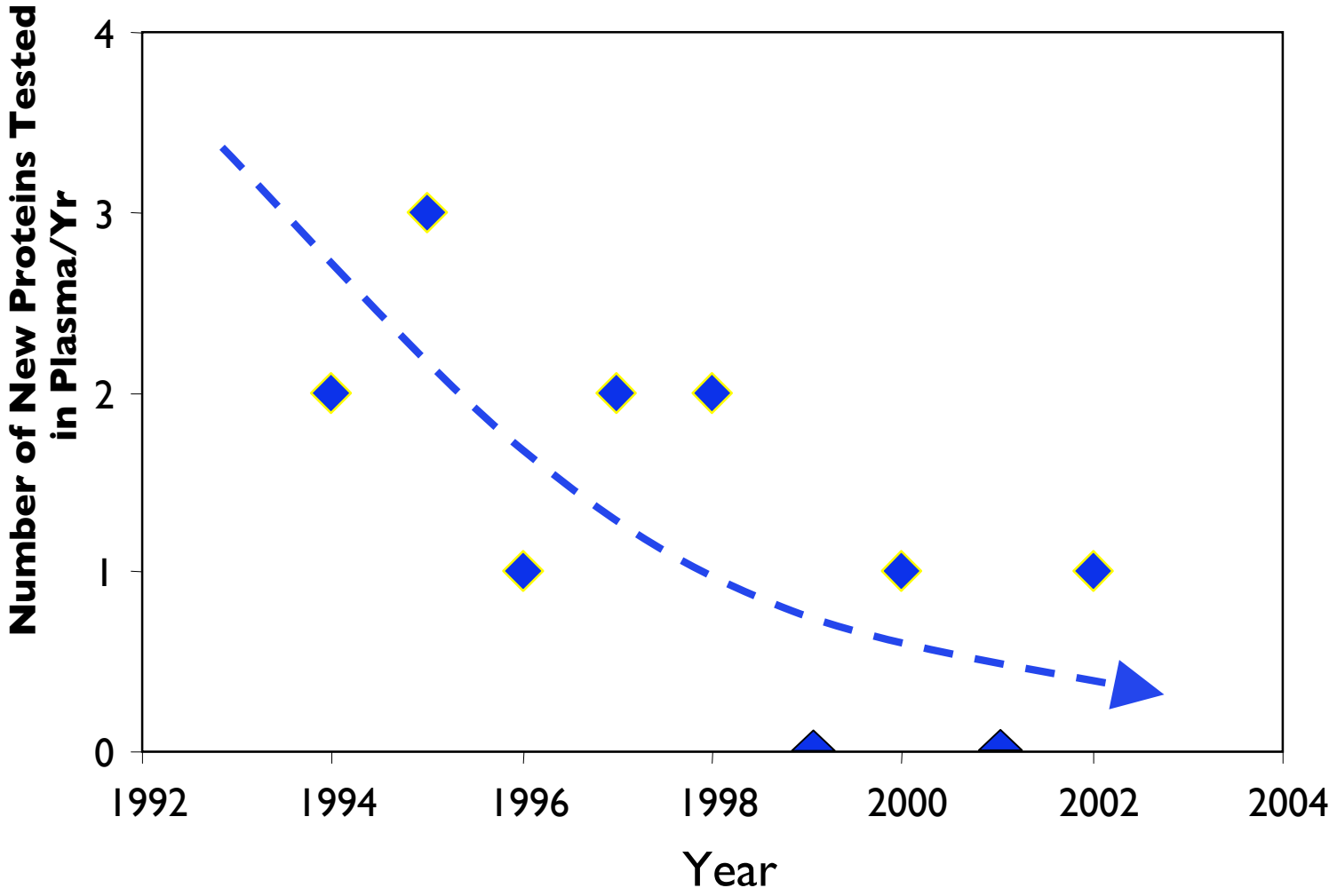
However: Introduction of New Clinical Biomarkers is Slowing

New FDA-Approved (CLIA) Diagnostic Protein Tests in Serum/Plasma
Declined for the Last Decade



From:The human plasma proteome: history, character, and diagnostic prospects. Anderson, N. L. Anderson, N. G., Mol Cell Proteomics (2002) 1:845-67.

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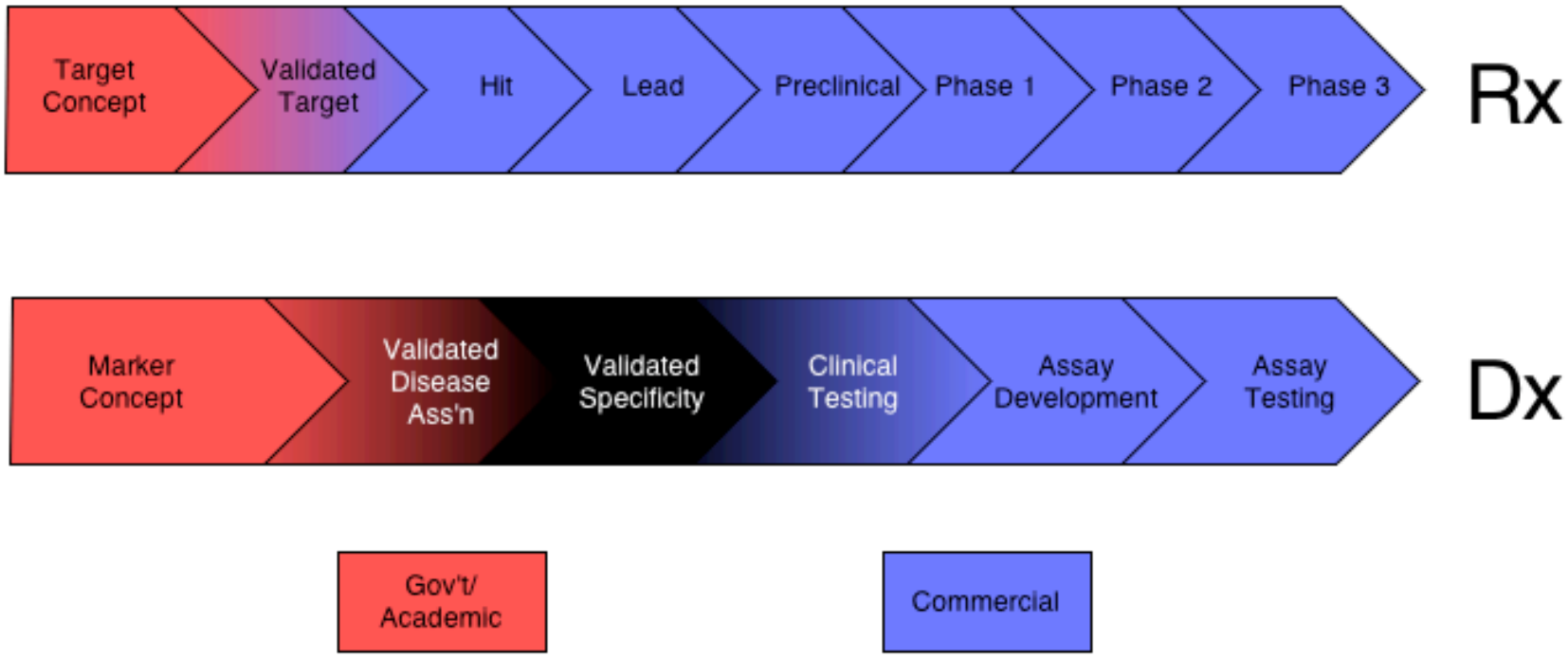
A Major Part of the Problem: Lack of a Biomarker Pipeline



- Dx industry does not cover discovery or validation as Rx does (Dx scale = Rx * 0.05)
- NIH has not funded marker validation on a par with discovery
- A major resource gap has developed in the Dx pipeline at the point of marker validation

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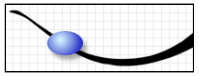
Milestones for Biomarker Proteomics

Growing funding for biomarker discovery	Yes
Candidates in at least one commercial pipeline	Yes?
Consensus on best experimental approach to biomarker discovery	No
Generally-accessible pipeline for translating candidates to tests	No
Clinical tests in general use	No

PPI 

The plasma proteome is a hard one…

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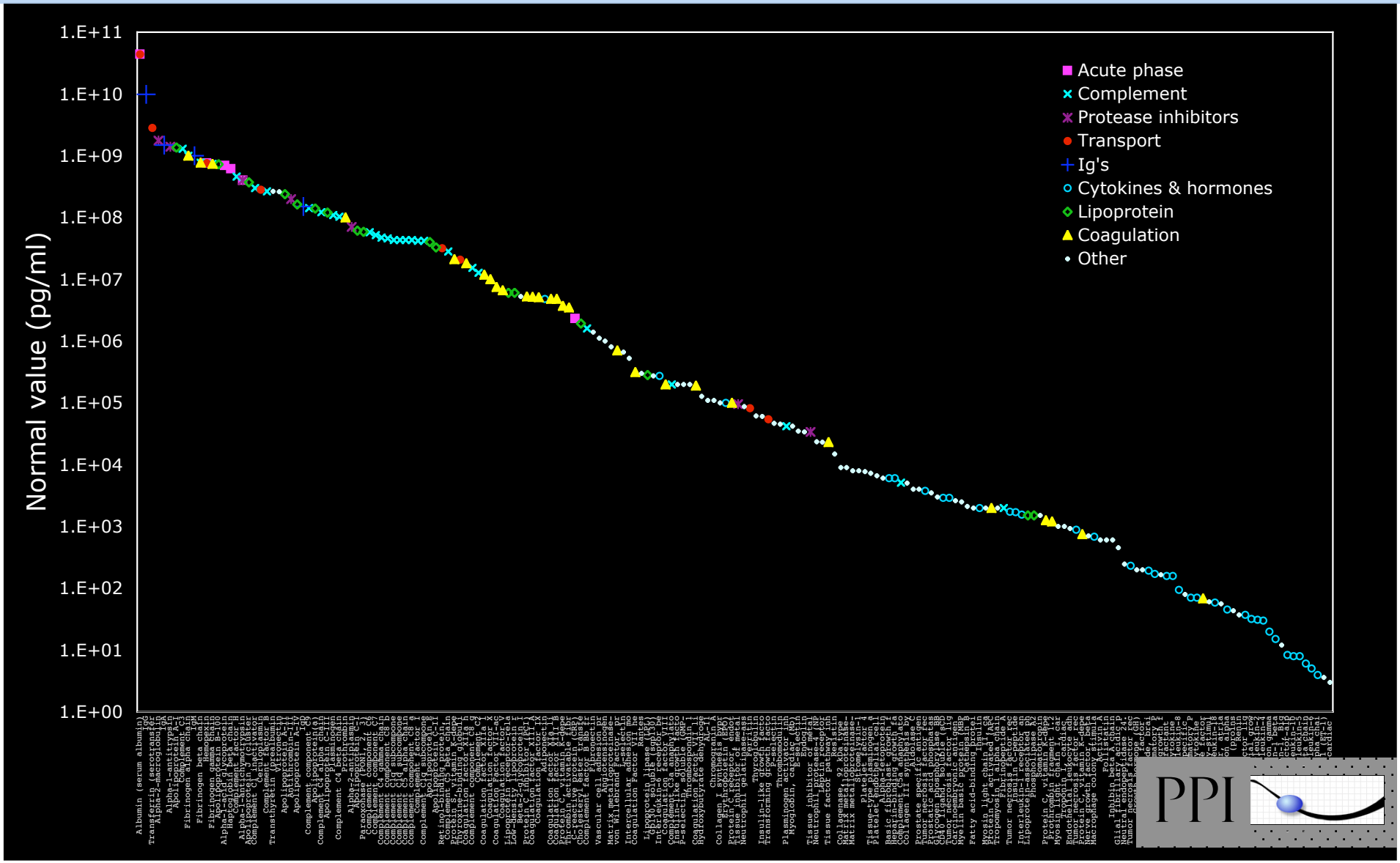
Proteins Measured Clinically in Plasma Span > 10 Orders of Magnitude in Abundance

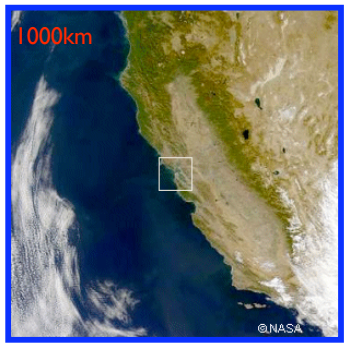
(199 proteins, literature values)



10^{10} Really Is Wide Dynamic Range

(Here on a linear scale)

10



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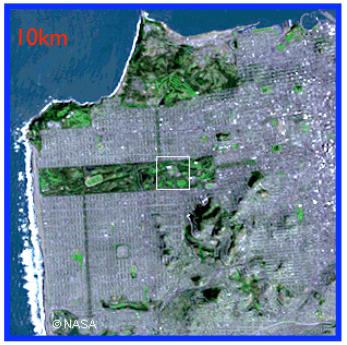

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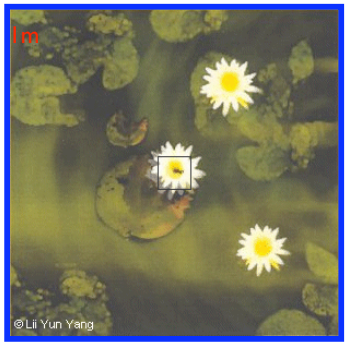

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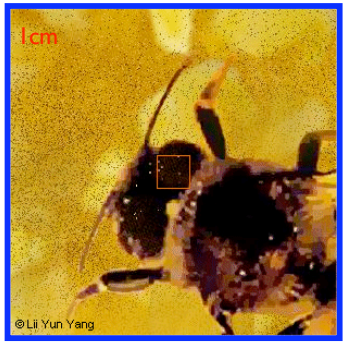

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Slide courtesy Bruno Domon, ETH Zurich

Detection Limits in Clinical Diagnostics

for a 50 kdal protein analyte

Biomarker concentration	pg/ml	amol/ml	Molecules/ml
50 mg/ml	50,000,000,000	1,000,000,000	6.02E+17
10 mg/ml	10,000,000,000	200,000,000	1.20E+17
1 mg/ml	1,000,000,000	20,000,000	1.20E+16
100 ug/ml	100,000,000	2,000,000	1.20E+15
10 ug/ml	10,000,000	200,000	1.20E+14
1 ug/ml	1,000,000	20,000	1.20E+13
100 ng/ml	100,000	2,000	1.20E+12
10 ng/ml	10,000	200	1.20E+11
1 ng/ml	1,000	20	1.20E+10
100 pg/ml	100	2	1.20E+09
10 pg/ml	10	0.2	1.20E+08
1 pg/ml	1	0.02	1.20E+07

Immunoassays

QqQMS given 1000-fold enrichment

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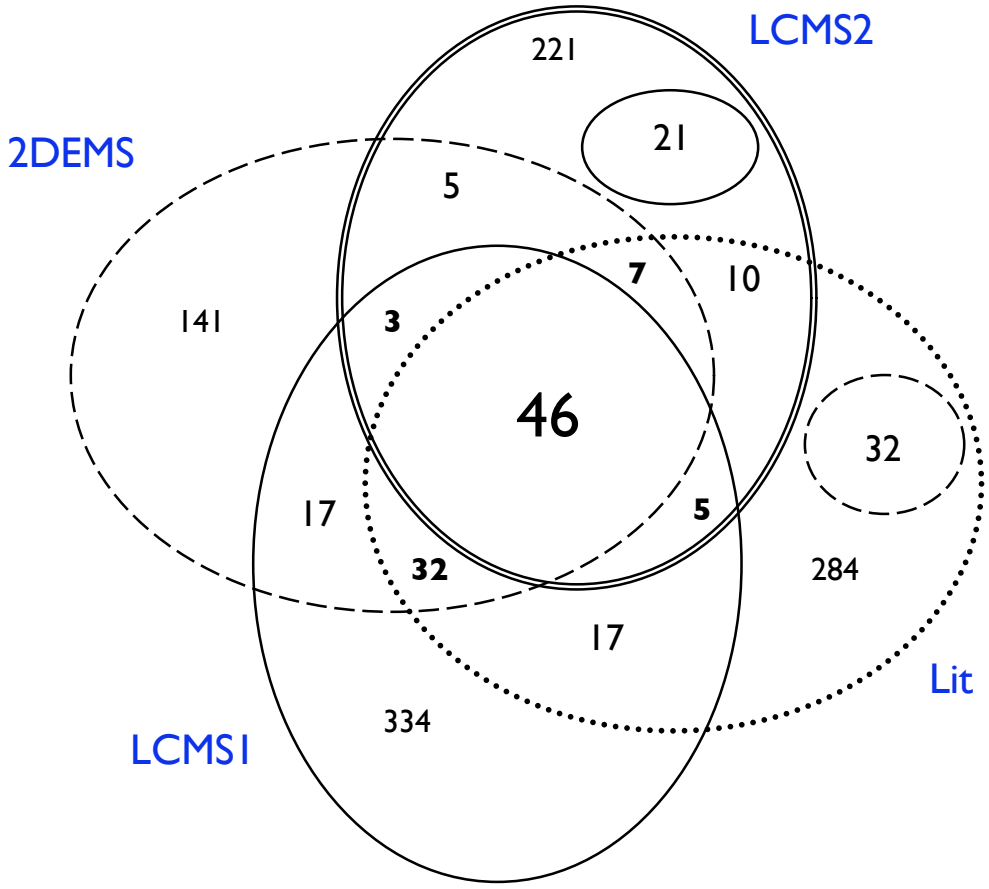
Different Platforms See Different Plasma Proteomes: Small Overlap of Four Plasma Proteome Datasets

(Number of NR proteins)



- 46 proteins in all four lists
- 195 proteins in 2 or more lists
- 1175 NR proteins total

From: The Human Plasma Proteome: A Non-Redundant List Developed by Combination of Four Separate Sources, N. L. Anderson et al, Molec. Cell Proteomics, 3: 311-326 (2004).

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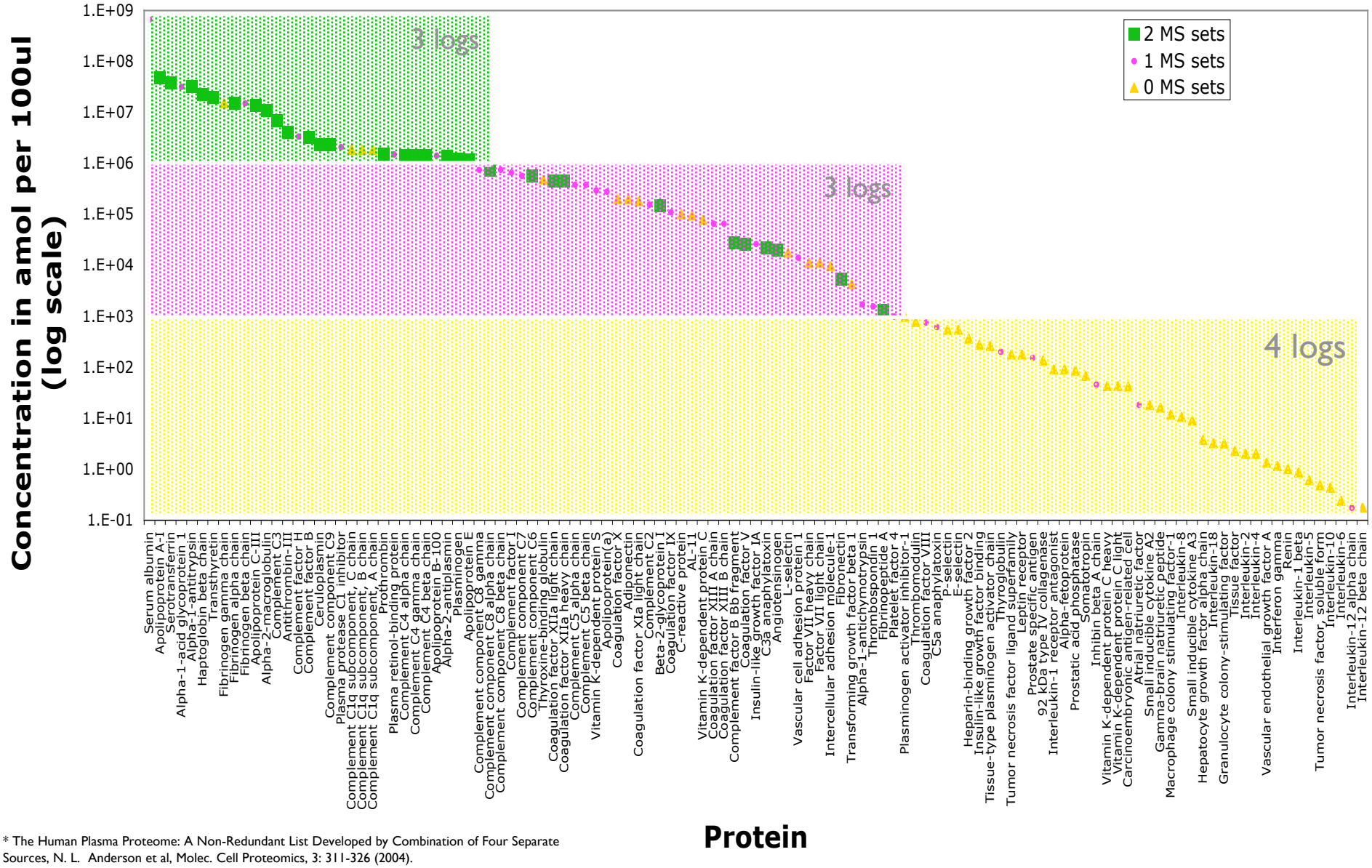
Plasma Proteome Surveys Detect Primarily Higher Abundance Proteins



* The Human Plasma Proteome: A Non-Redundant List Developed by Combination of Four Separate Sources, N. L. Anderson et al, Molec. Cell Proteomics, 3: 311-326 (2004).

Challenges and Limitations of Biomarker Discovery in Plasma

- So far, there is no comprehensive exploratory proteomics platform for plasma
- Multi-dimensional fractionation is crucial to penetrate deeper into the abundance distribution
  - However this multiples analytical effort, increasing cost and decreasing sample numbers
- Adapting/confirming candidates between platforms is challenging
- Use of multiple platforms significantly enhances chance of finding new markers
- Where possible, it may make more sense to discover the markers in tissues or non-plasma fluids where they occur at higher concentrations… but we ultimately need to measure them in plasma

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What kind of pipeline could translate the results of disparate plasma biomarker discovery efforts into In Vitro Diagnostics (IVD)?

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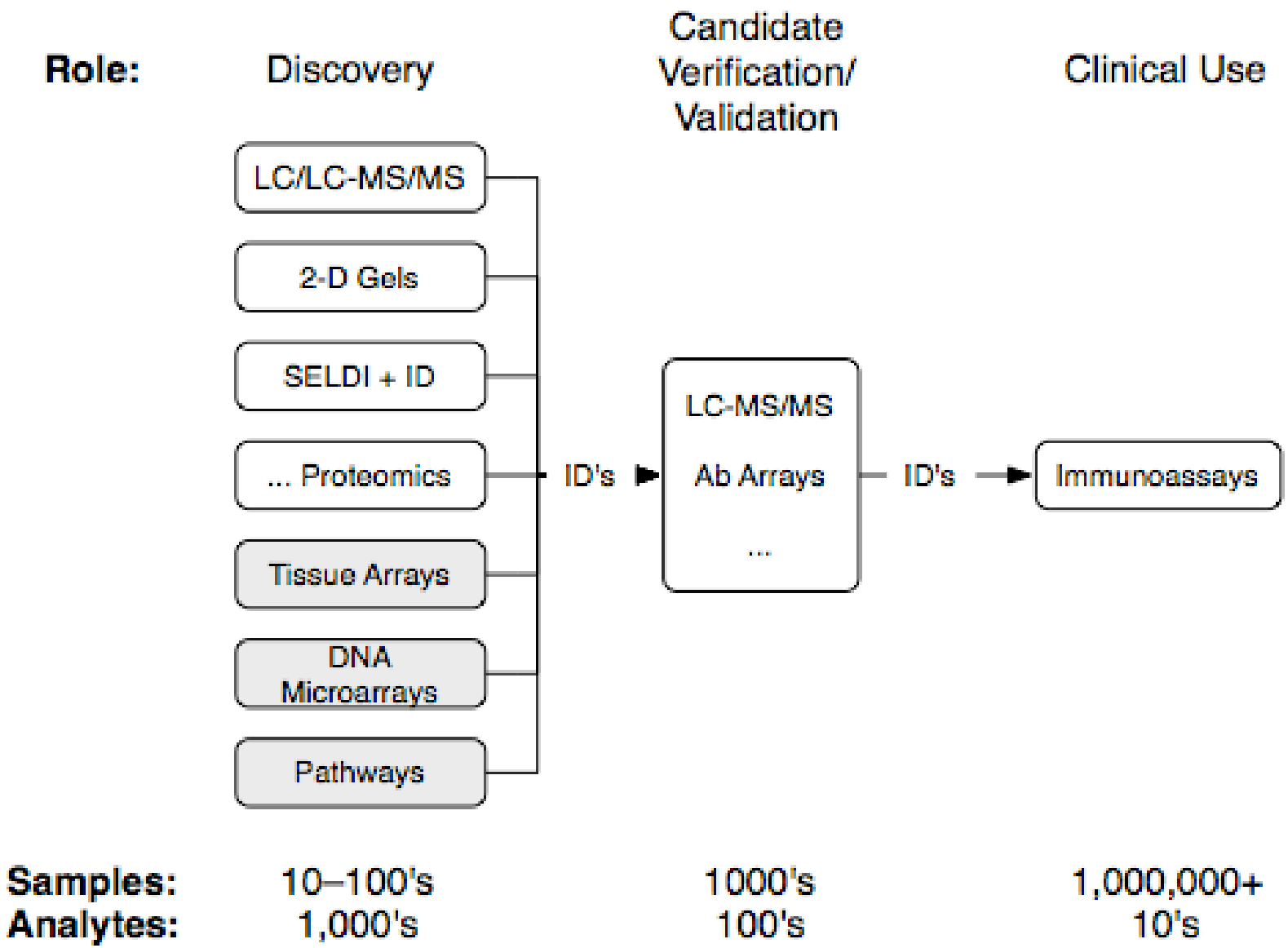
A Three-Stage Dx Pipeline



Biomarker validation is where the rubber meets the road

- Is your marker really associated with the target disease in real-world samples?
- Is the signal strong enough relative to underlying biological variation, including all the other diseases (i.e. the "noise")?
- Sadly, one of these answers is "No" most (>>90%) of the time.

- A focus on validation allows us to face these tests early, and to find the needles in the required large haystacks.
- Validation is a candidate-based business

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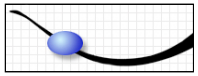
How do we interface between biomarker discovery platforms and a platform for biomarker validation?

Do we have to?

PPI 

A Major Technology Gulf Exists Between Discovery Proteomics and Routine Diagnostic Platforms: Neither Is Well-Suited for Validation Phase

Discovery Proteomics		Routine "In Vitro Diagnostics" (IVD)
50-700	# proteins	1-20
$1,000-$10M	$ per analysis	$2-100
25-50%	CV	3-5%
4-52 wks	time required	~15 min
2-50	# samples	100-1,000,000

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Technology Alternatives for Candidate-Based Proteomics

- Immunoassays (likely clinical test implementation)
  - Very sensitive
  - Expensive: IVD-quality assays cost $2-4 million
  - Specificity issues with less well-developed assays
  - Multiplexing limits in a single assay volume

- Hybrid MS-based assays
  - Peptide MS for quantitation and identification
  - Specific enrichment for sensitivity
  - Absolute analyte specificity
  - Multiplex 25-200 assays/analysis

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SISCAPA: Proteomics Meets Analytical Chemistry

Stable Isotope-labeled Standards with Capture on Anti-Peptide Antibodies

- Objective: high-throughput quantitative measurement of identified proteins
  - a rapid, sensitive, specific and multiplexable method for quantitative measurement of identified proteins in complex samples
- Target application: the validation of candidate biomarkers in plasma
  - overcomes the sensitivity limitations of current proteomics platforms
  - avoids the cost/time/multiplex limitations of current specific (e.g., sandwich) immunoassays
- SISCAPA employs existing technologies for immunocapture, chromatography, and quantitative mass spectrometry in a new combination with novel reagents
  - works with existing LC and QqQ-MS instrument platforms
- Mindset borrows from analytical chemistry and clinical diagnostics, and focuses exclusively on identified candidates - a significant evolution from the "survey everything" tradition of proteomics

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SISCAPA Steps

1. **Assay design**: A tryptic peptide (the "monitor" peptide) is selected from the sequence of the target protein and used as a quantitative surrogate. MRM assay parameters for the peptide determined.

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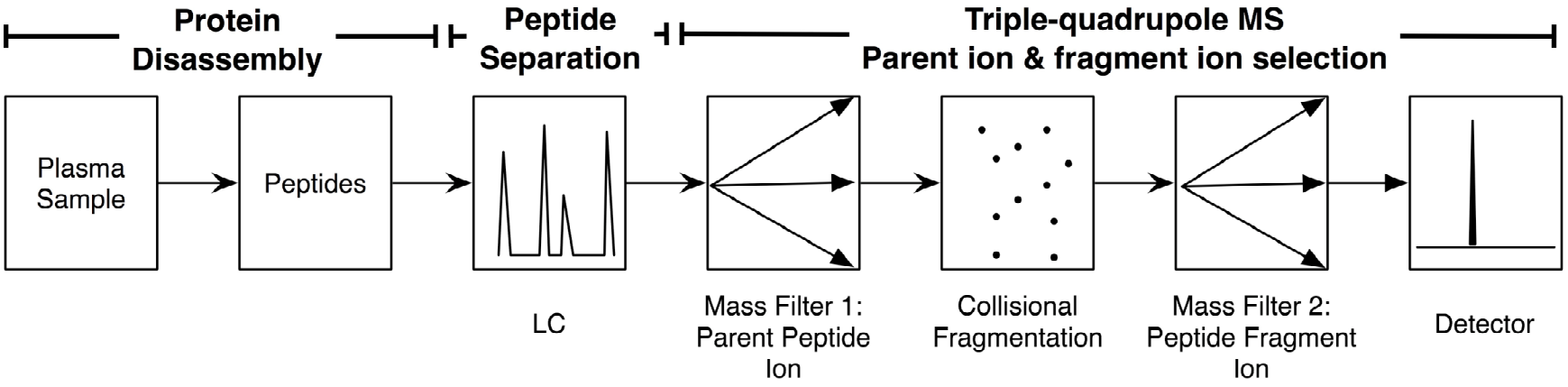
Multiple-Reaction Monitoring (MRM): Specific Mass Spectrometric Assay for Peptides

- MRM
  - is a 2-stage MS assay capable of absolute analyte specificity with high precision (CV < 10%).
  - measures selected peptides in a sample digest as quantitative surrogates for the proteins from which they derive
  - assays for peptides can be designed directly from protein sequence
  - are implemented using triple-quadrupole mass spectrometers (QqQMS), very widely used for small molecular assays in plasma (drug metabolites, inborn errors, pesticides)
  - assays can be multiplexed (100+ assays per run)



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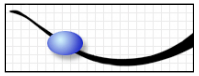
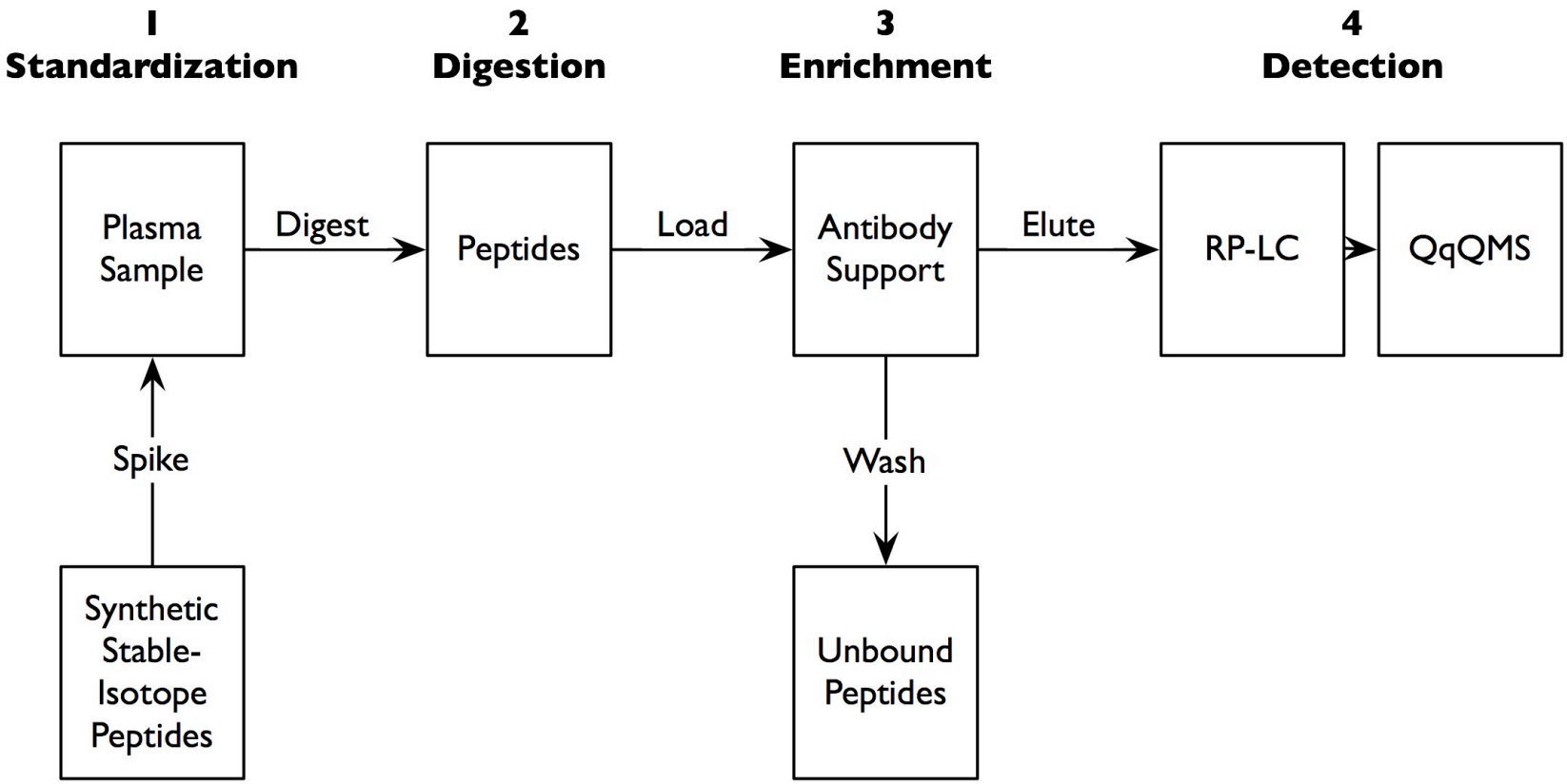
SISCAPA Steps

1. **Assay design**: A tryptic peptide (the "monitor" peptide) is selected from the sequence of the target protein and used as a quantitative surrogate. MRM assay parameters for the peptide determined.
2. **Sample treatment**: The protein sample is digested to peptides, releasing monitor peptides
3. **Addition of internal standard**: A synthetic, stable-isotope-labeled version of the monitor peptide is added to the sample (before or after digestion) at known concentration
4. **Analyte enrichment**: Immobilized antibodies specific for the monitor peptide are used to capture both natural and labeled forms from the digest
5. **Analyte measurement**: A mass spectrometer system (typically LC-QqQMS) is used to measure the relative amounts of natural and labeled peptides
6. **Result calculation**: The concentration of the target protein is calculated from the ratio of natural:labeled monitor peptide

PPI 

SISCAPA Process Schematic Diagram



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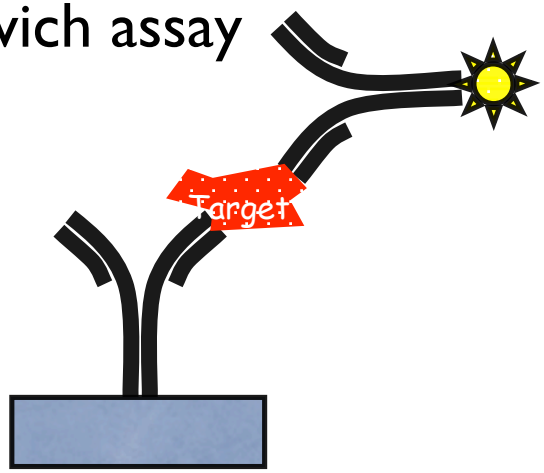
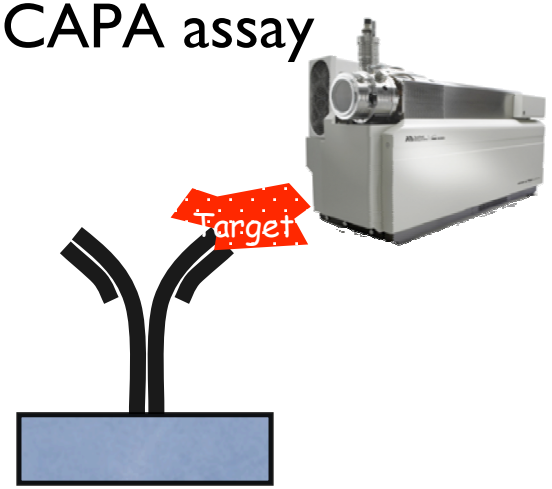
LC-MS/MS as Universal Antibody

- SISCAPA is effectively a sandwich immunoassay for a target peptide, in which the second antibody is replaced by a mass spectrometer (acting as a generic second antibody applicable to all analytes and with absolute structural specificity).

Sandwich assay



SISICAPA assay



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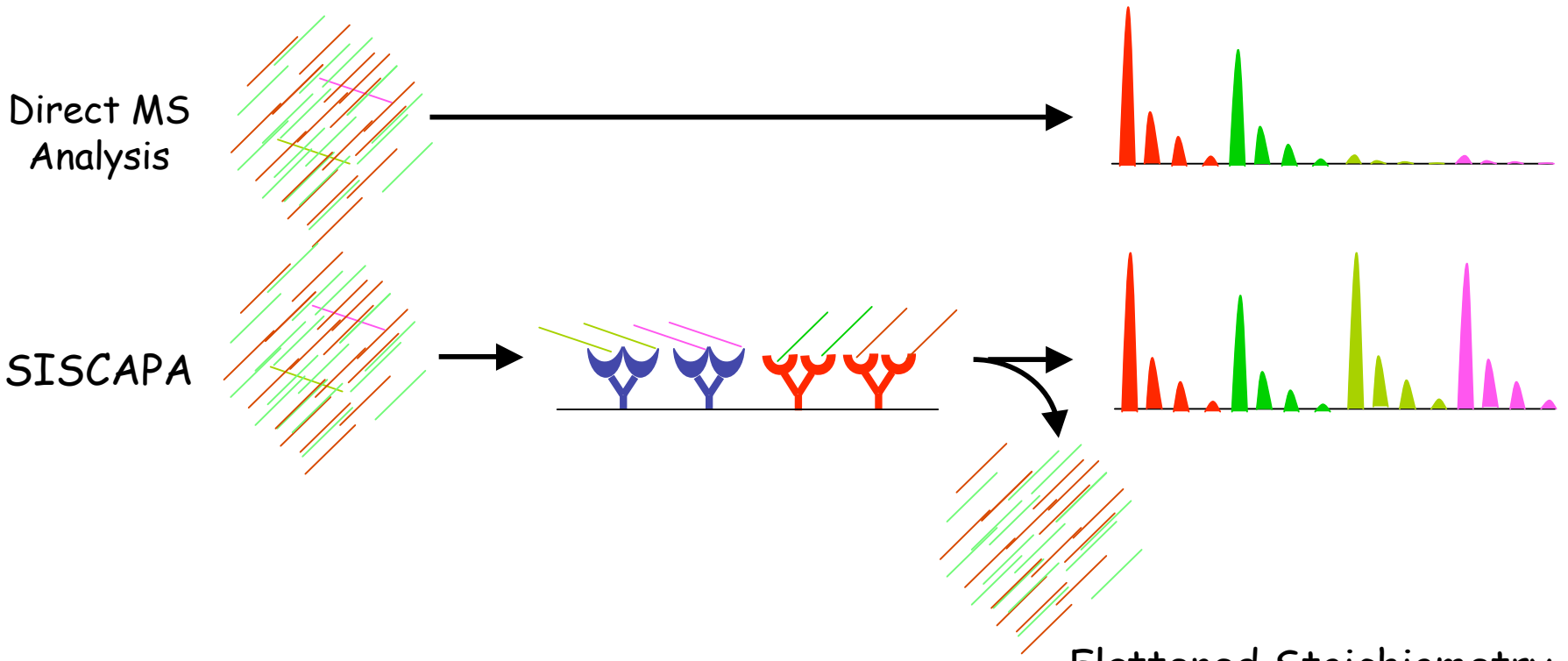
Multiplex Assays: SISCAPA Brings Multiple Peptides Into a Compressed Dynamic Range for MS

Captures Similar Amounts of High- and Low-Abundance Peptides From a Digest, Flattening the Sample Dynamic Range to Fit MS



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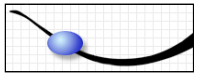
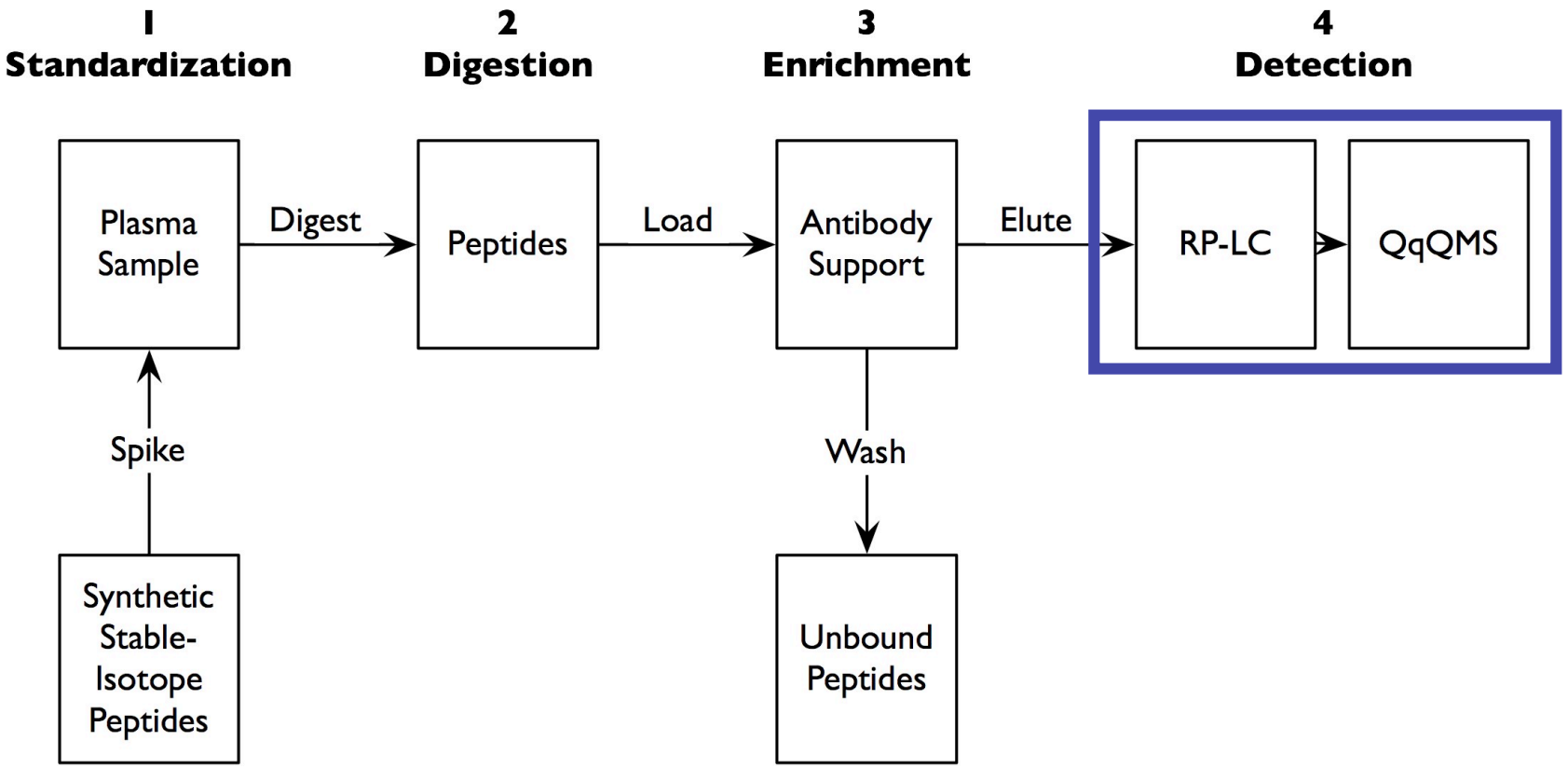
SISCAPA Proof-of-Concept Studies

- MRM measurement of peptide abundance
  - *Collaboration with Christie Hunter, ABI*
- Efficient production of labeled SIS peptides
  - *Collaboration with Jerry Becker, Roche Protein Expression Group*
- Enrichment of target peptides using immobilized anti-peptide antibodies
  - *Collaborations with Terry Pearson, Derek Smith, U. Victoria, BC*

PPI 

MRM Measurement of Peptide Abundance



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MRM Measurement of Peptide Abundance

- MRM measurement (QqQ-MS) is the most accurate quantitative MS mode
- Peptide MRM's work well even in complex digests
- Monitor peptides from >50 high/medium abundance plasma proteins can be measured in digests without antibody enrichment
- Demonstrates robust MS measurement technology for SISCAPA

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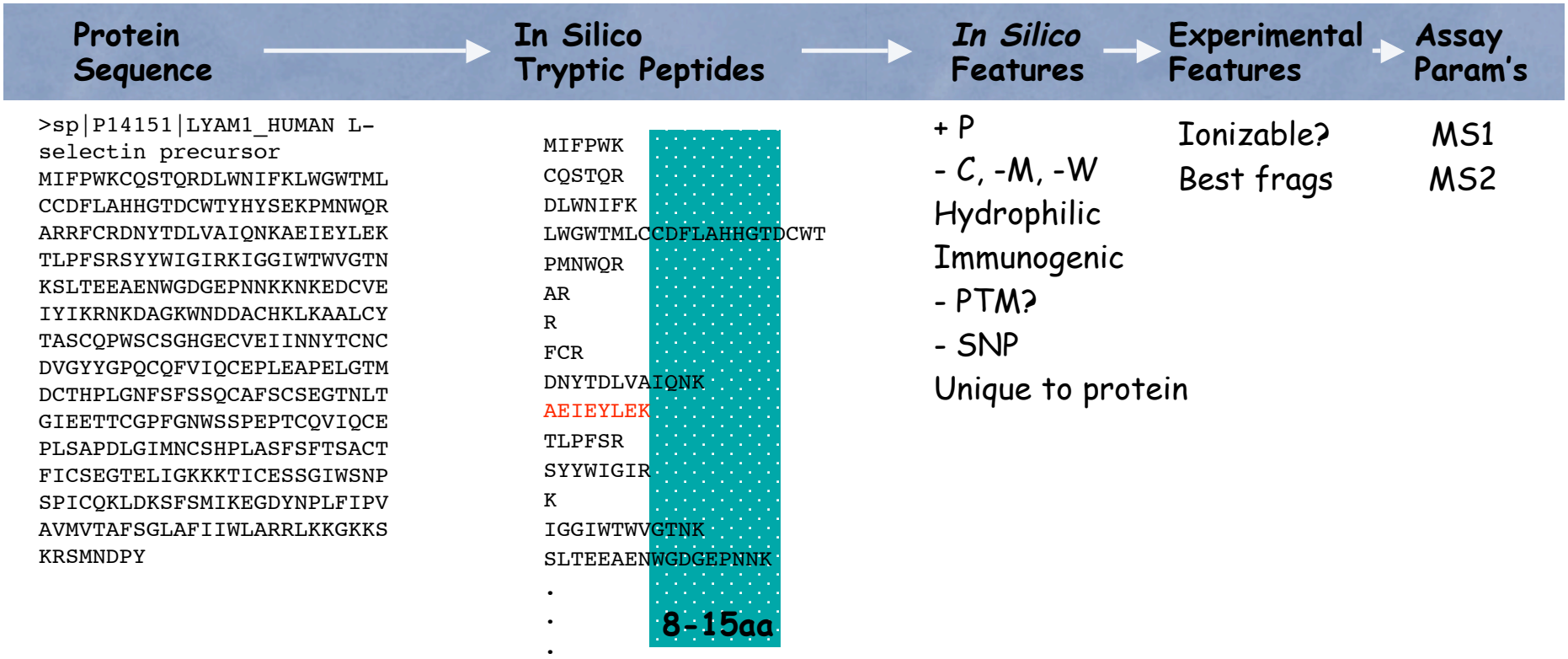
SISCAPA Peptide Selection:
In Silico MS Assay Design via Bioinformatics



- Pure in silico yields a low proportion of good peptides
- Addition of experimental data yields >90% success

- Improvements needed in prediction of ionization and fragmentation

Quantitative Mass Spectrometric MRM Assays for Major Plasma Proteins,
Leigh Anderson and Christie Hunter, Mol Cell Proteomics, in press 2005

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Initial Selection of 30 Targets

Protein	peptide_compound_accession	fmol_per_ml	peptide_sequence	occurrences_in_protein	variants	mod_res	conflicts	carbohyd
Apolipoprotein A-I	P02647_25_267_trypsin_32_196_206	4.8E+07	ATEHLSTLSEK	1	1	0	0	0
Alpha-1-acid glycoprotein 1	P02763_19_201_trypsin_12_121_135	3.2E+07	NWGLSVYADKPETTK	1	0	0	0	0
Haptoglobin beta chain	P00737_162_406_trypsin_1_1_9	2.3E+07	ILGGHLDAK	1	0	0	0	0
Fibrinogen gamma chain	P02679_27_453_trypsin_17_141_151	1.5E+07	DTVQIHDITGK	1	0	0	0	0
Fibrinogen alpha chain	P02671_36_640_trypsin_56_433_441	1.5E+07	TVIGPDGHK	2	0	0	0	0
Fibrinogen beta chain	P02675_45_491_trypsin_31_257_269	1.5E+07	QGFGNVATNTDGK	1	0	0	0	0
Alpha-1-antichymotrypsin	P01011_24_423_trypsin_31_284_292	8.8E+06	EIGELYLPK	1	0	0	0	0
Complement C3	P01024_23_1663_trypsin_105_883_891	7.1E+06	TGLQEVEVK	4	0	0	0	0
Antithrombin-III	P01008_33_464_trypsin_48_360_370	4.1E+06	DDLYVSDAFHK	1	0	0	0	0
Ceruloplasmin	P00450_20_1065_trypsin_17_159_168	2.3E+06	IYHSHIDAPK	1	0	0	0	0
Prothrombin	P00734_44_622_trypsin_46_444_455	1.5E+06	ETAASLLQAGYK	2	0	0	1	0
Complement C4 gamma chain	P01028_1454_1744_trypsin_26_194_202	1.4E+06	ITQVLHFTK	1	0	0	1	0
Apolipoprotein B-100	P04114_28_4563_trypsin_416_3764_3772	1.4E+06	FPEVDVLTK	1	0	0	0	0
Alpha-2-antiplasmin	P08697_40_491_trypsin_2_13_25	1.4E+06	LGNQEPGGQTALK	1	0	0	0	0
Plasminogen	P00747_20_810_trypsin_73_652_661	1.2E+06	LSSPAVITDK	2	0	0	0	0
Apolipoprotein E	P02649_19_317_trypsin_46_275_282	1.2E+06	QWAGLVEK	1	0	0	0	0
Coagulation factor XIIa heavy chain	P00748_20_372_trypsin_1_1_8	4.6E+05	IPPWEAPK	1	0	0	0	0
Apolipoprotein(a)	P08519_20_4548_trypsin_249_4385_4398	2.8E+05	LFLEPTQADIALLK	1	0	0	0	0
Coagulation factor X	P00742_41_488_trypsin_56_436_448	2.0E+05	SHAPEVITSSPLK	3	0	0	0	0
Adiponectin	Q15848_19_244_trypsin_13_117_131	2.0E+05	IFYNQQNHYDGSTGK	1	0	0	0	0
Beta-2-glycoprotein I	P02749_20_345_trypsin_34_309_317	1.5E+05	EHSSLAFWK	1	1	0	0	0
Coagulation factor IX	P00740_47_461_trypsin_16_135_142	1.1E+05	VSVSQTSK	2	0	0	0	0
C-reactive protein	P02741_19_224_trypsin_4_14_23	1.0E+05	ESDTSYVSLK	1	0	0	0	0
Vitamin K-dependent protein C	P04070_43_461_trypsin_32_234_241	7.8E+04	WELDLDIK	2	1	0	0	0
Coagulation factor XIII A chain	P00488_38_731_trypsin_62_587_598	6.6E+04	STVLTIPEIIIK	1	0	0	0	0
Coagulation factor XIII B chain	P05160_21_661_trypsin_25_198_208	6.6E+04	LIENGYFHPVK	1	0	0	0	0
Cholesteryl ester transfer protein	P11597_18_493_trypsin_5_38_47	3.6E+04	ASYPDITGEK	2	0	0	0	0
Coagulation factor V	P12259_29_2224_trypsin_92_898_907	2.7E+04	DPPSDLLLLK	1	0	0	0	0
Angiotensinogen	P01019_34_485_trypsin_6_50_61	2.0E+04	ALQDQLVLVAAK	1	0	0	0	0
L-selectin	P14151_39_372_trypsin_6_33_40	1.8E+04	AEIEYLEK	1	0	0	0	0

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MRM Assays Are Easily Implemented for High-Abundance Plasma Proteins

Alpha-1-acid glycoprotein 1 peptide EQLGEFYEALDBLR mass1742.8

Detected ions passing MS1 and MS2 (871.9/1186.6) over course of 180 minute LC peptide separation: single sequence-specific peak

MS1: 871.9 ± 1 amu
Whole peptide, +2 charge

↓

Peptide fragmented

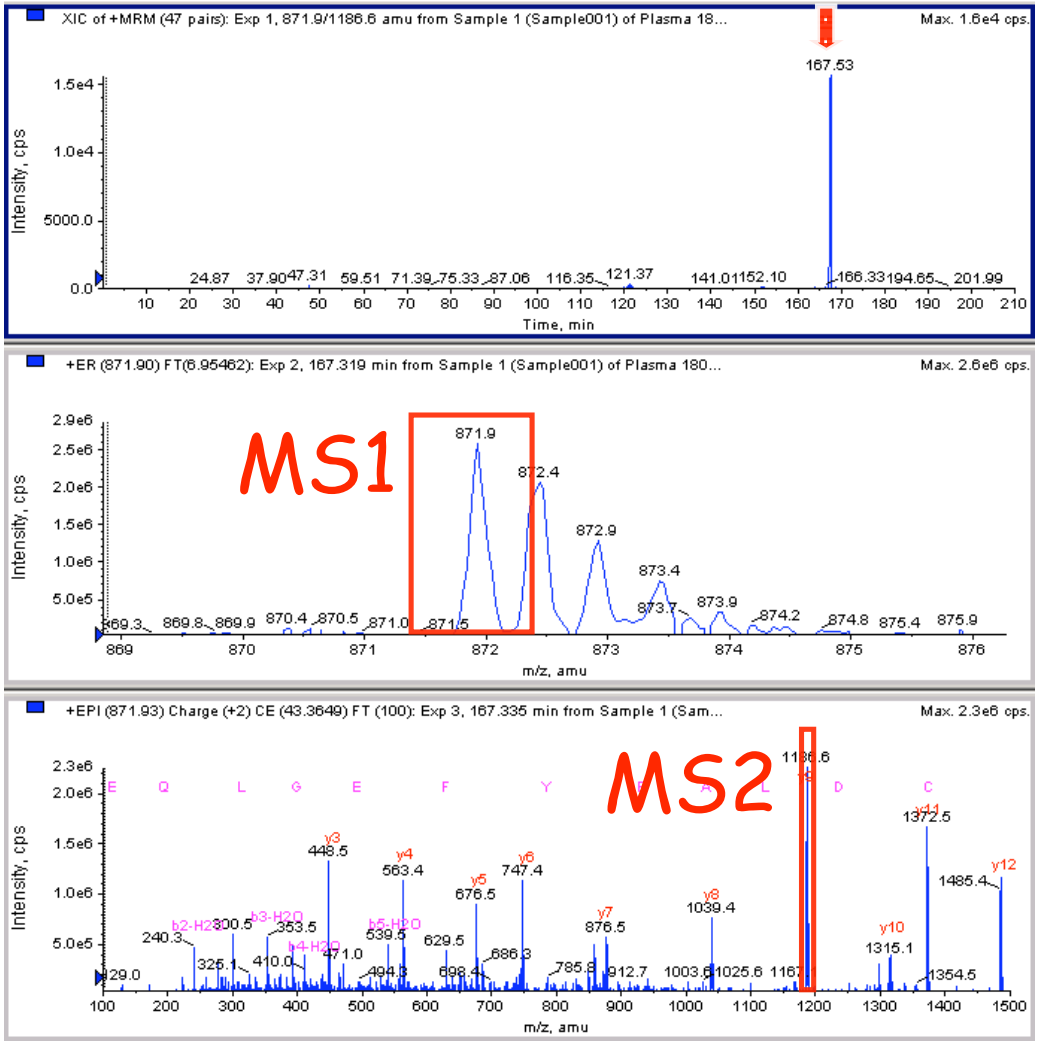
↓

MS2: 1186.6 ± 1 amu
Fragment ion, +1 charge

↓

Detector

This defines an MRM assay, referring to "multiple reaction monitoring"



Quantitative Mass Spectrometric MRM Assays for Major Plasma Proteins, Leigh Anderson and Christie Hunter, Mol Cell Proteomics, in press 2005

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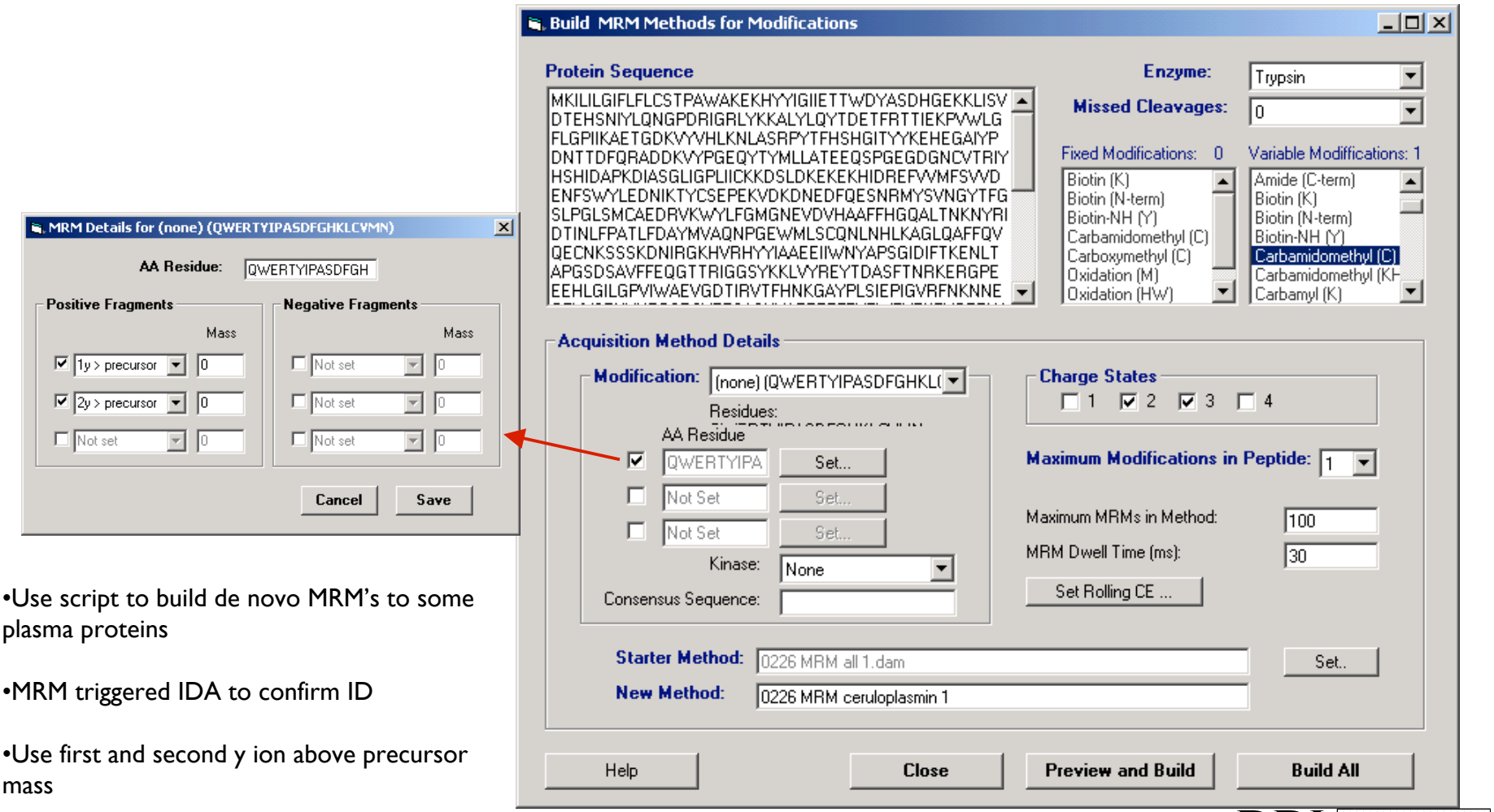
MRM-Triggered IDA to Develop Peptide MRM Transitions

(workflow now called MIDAS: MRM-Initiated Detection and Sequencing)



- Use script to build de novo MRM's to some plasma proteins
- MRM triggered IDA to confirm ID
- Use first and second y ion above precursor mass

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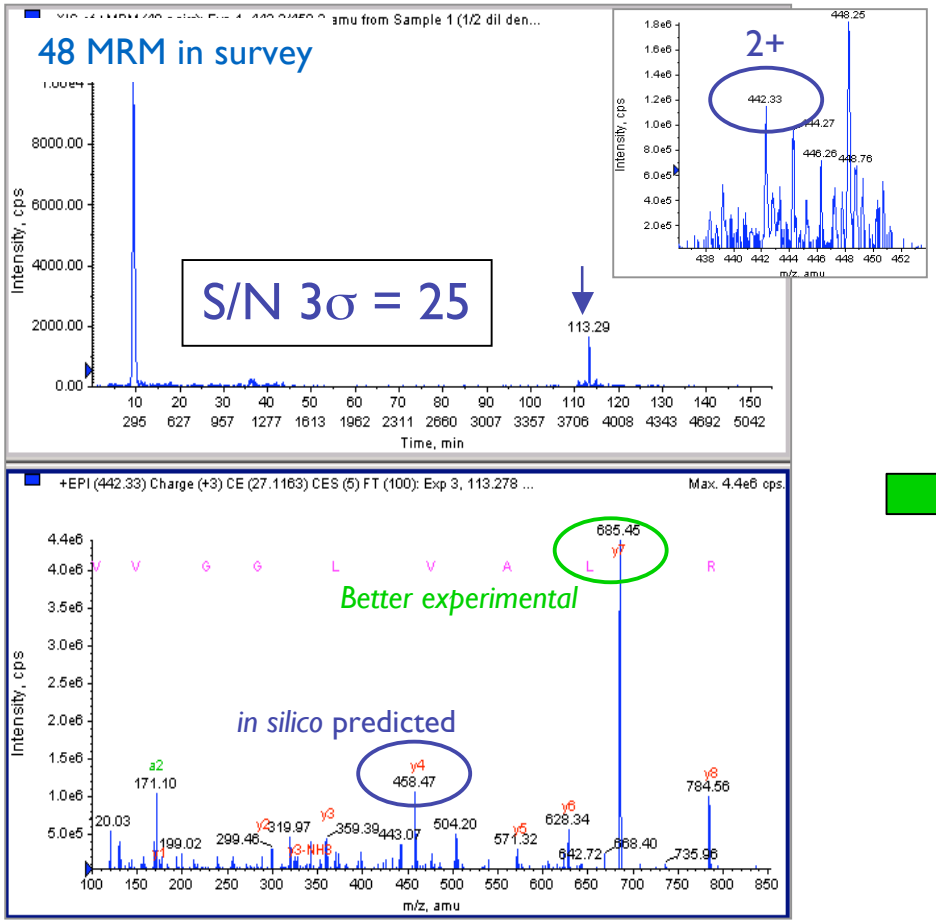
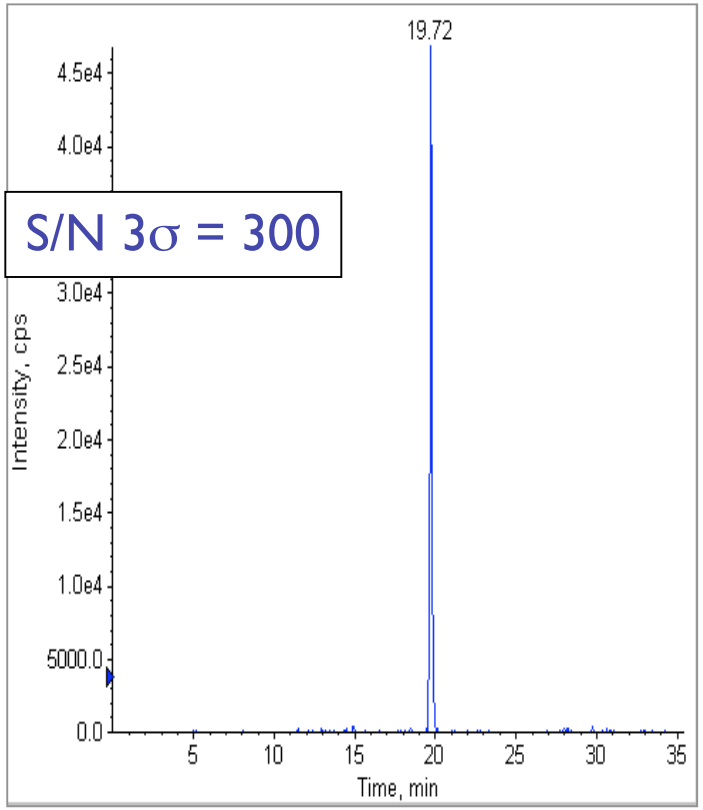
Experimental Data Improves MRM Design

Coagulation Factor XIIa light chain – 456 fmol/uL plasma – 4 fmol on column





Final MRM peak in Assay
- clean background, better S/N

MRM's Were Successfully Designed for 47 High-to-Medium Abundance Plasma Proteins

Afamin	DADPDTFFAK	Complement factor B	EELLPAQDIK
Albumin	LVNEVTEFAK	Complement factor H	SPDVINGSPISQK
α-1-acid glycoprotein 1	NWGLSVYADKPETTK	Fibrinogen α	GSESGIFTNTK
α-1-antichymotrypsin	EIGELYLPK	Fibrinogen β	QGFGNVATNTDGK
α-1B-glycoprotein	LETPDFQLFK	Fibrinogen γ	DTVQIHDITGK
α-2-antiplasmin	LGNQEPGGQTALK	Fibronectin	DLQFVEVTDVK
α-2-macroglobulin	LLIYAVLPTGDVIGDSAK	Gelsolin, isoform 1	TGAQELLR
Angiotensinogen	PKDPTFIPAPIQAK	Haptoglobin β	VGYVSGWGR
Antithrombin-III	DDLYVSDAFHK	Hemopexin	NFPSPVDAAFR
Apolipoprotein A-I	ATEHLSTLSEK	Heparin cofactor II	TLEAQLTPR
Apolipoprotein A-II	SPELQAEAK	Histidine-rich glycoprotein	DSPVLIDFFEDTER
Apolipoprotein A-IV	SLAPYAQDTQEK	Inter-α-trypsin inhibitor HC	AAISGENAGLVR
Apolipoprotein B-100	FPEVDVLTK	Kininogen	TVGSDTFYSFK
Apolipoprotein C-I	TPDVSSALDK	L-selectin	AEIEYLEK
Apolipoprotein C-III	DALSSVQESQVAQQAR	Plasma retinol-binding protein	YWGVASFLQK
Apolipoprotein E	LGPLVEQGR	Plasminogen	LFLEPTR
β-2-glycoprotein I	ATVVYQGER	Prothrombin	ETAASLLQAGYK
Ceruloplasmin	EYTDASFTNR	Serum amyloid P	VGEYSLYIGR
Clusterin	LFDSDPITVTVPVEVSR	Transferrin	EDPQTFYYAVAVVK
Coagulation factor XIIa HC	VVGGLVALR	Transthyretin	AADDTWEPFASGK
Complement C3	TGLQEVEVK	Vitamin D-binding protein	THLPEVFLSK
Complement C4 γ	ITQVLHFTK	Vitronectin	FEDGVLDPDYPR
Complement C4 β	VGDTLNLNLR	Zinc-α-2-glycoprotein	EIPAWVPFDPAAQITK
Complement C9	AIEDYINEFSVR		

Quantitative Mass Spectrometric MRM Assays for Major Plasma Proteins,
Leigh Anderson and Christie Hunter, Mol Cell Proteomics, in press 2005

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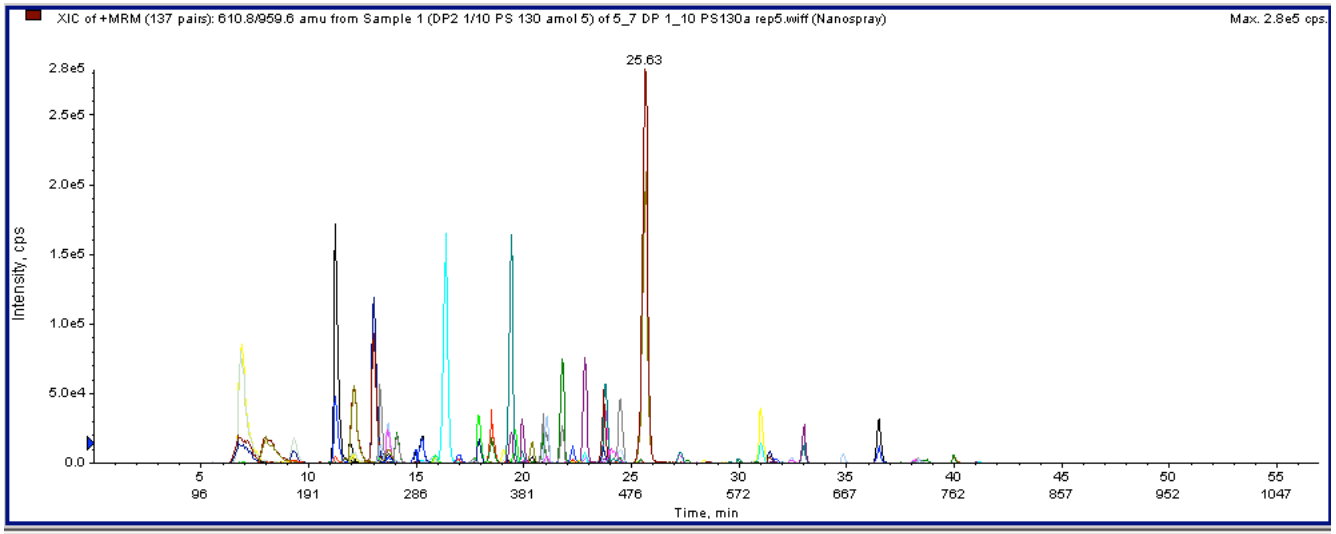
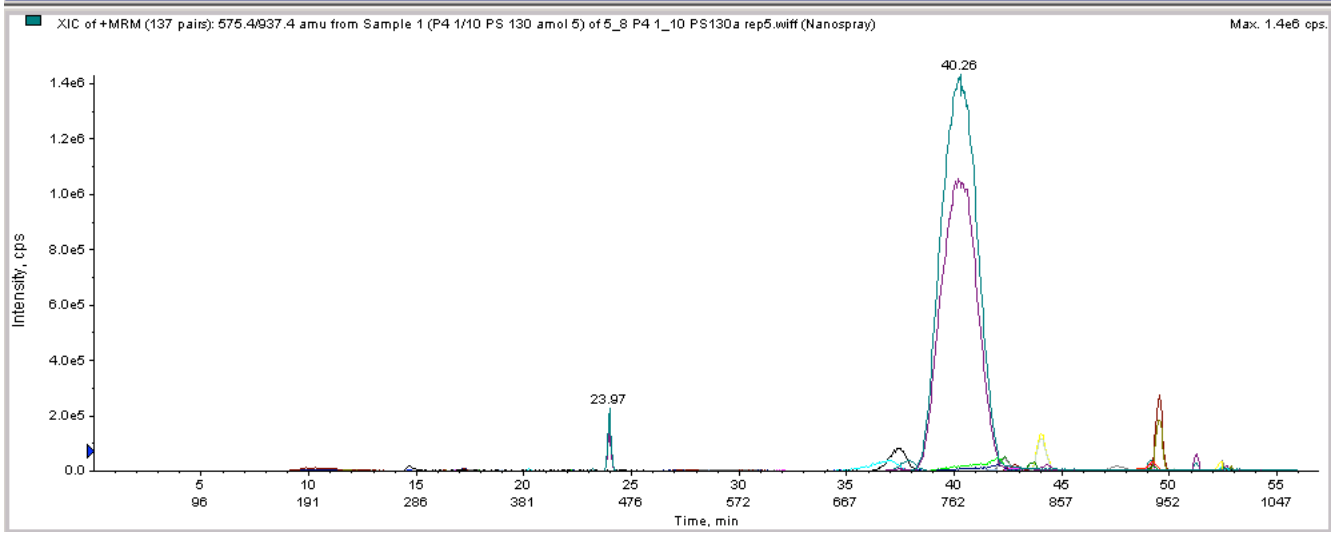
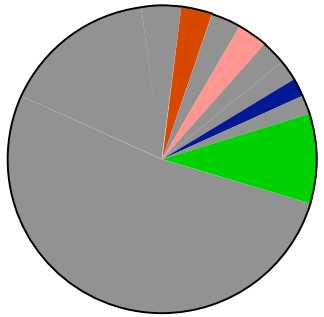
Subtraction of Top 6 Proteins

(albumin, IgG, IgA, haptoglobin,transferrin and antitrypsin)
Using Agilent MARS Column

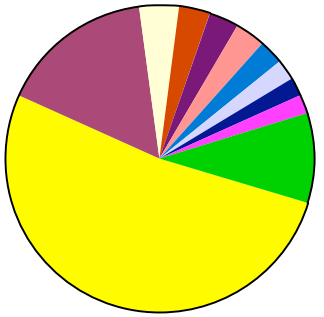




Depleted Plasma Digest of 0.01 uL plasma on column



Whole Plasma Digest of 0.01 uL plasma on column



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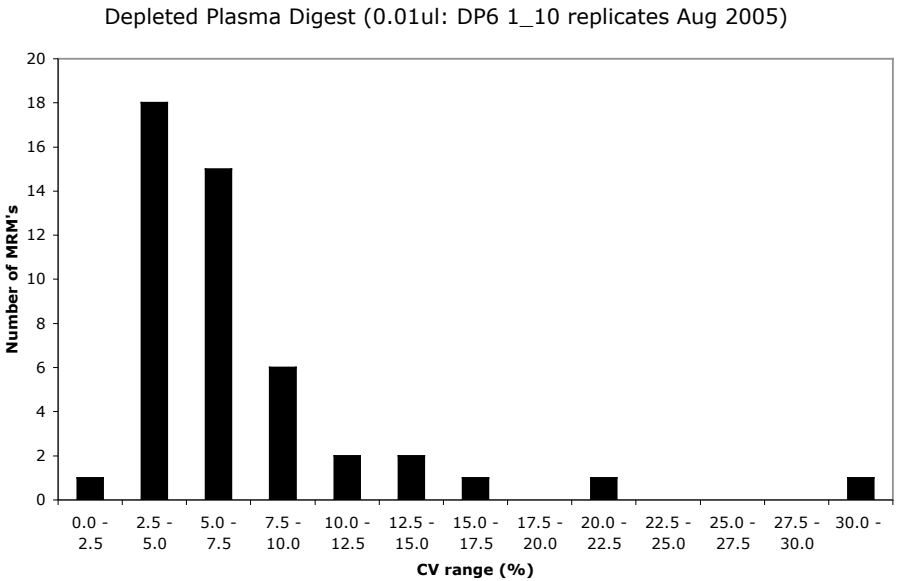
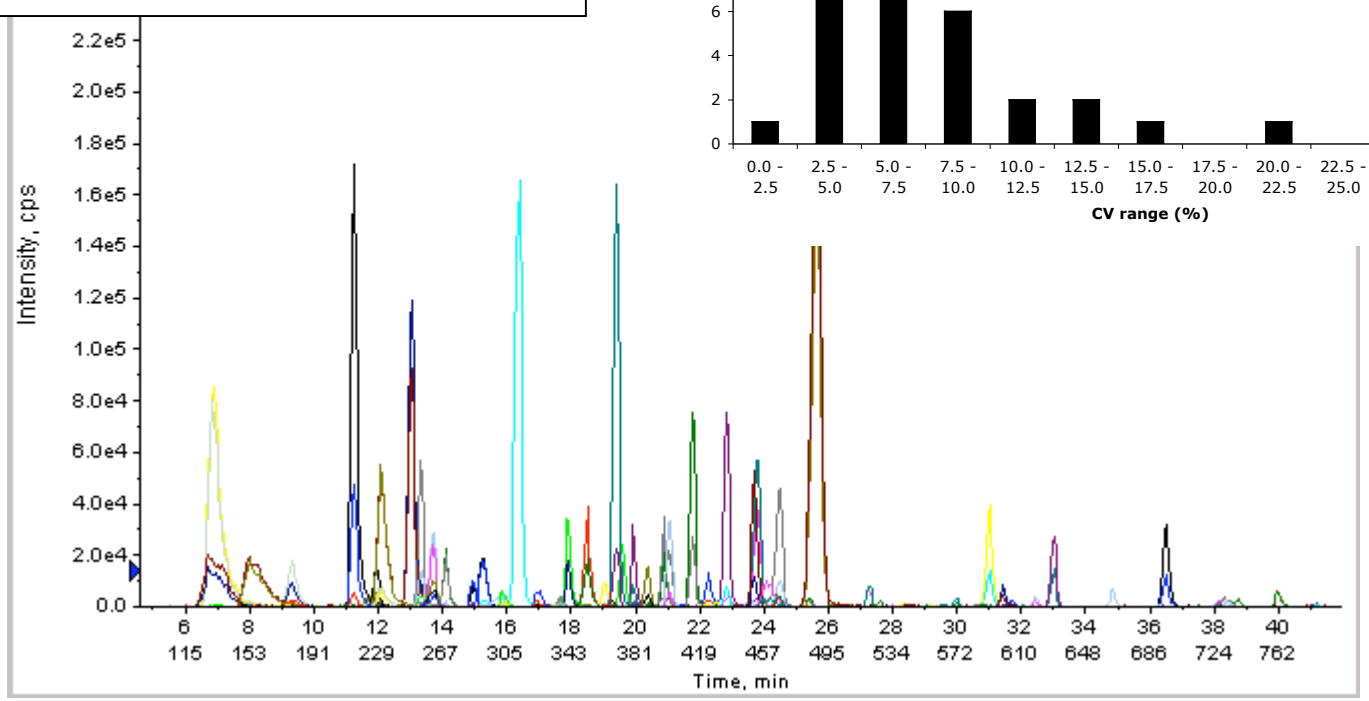
MRM Assays for Plasma Proteins Give Low CV's

52 proteins monitored
40 cardiovascular markers
137 MRMs
17 stable isotope peptides as IS
60 peptides, two MRM transitions each





Quantitative Mass Spectrometric MRM Assays for Major Plasma Proteins,
Leigh Anderson and Christie Hunter, Mol Cell Proteomics, in press 2005

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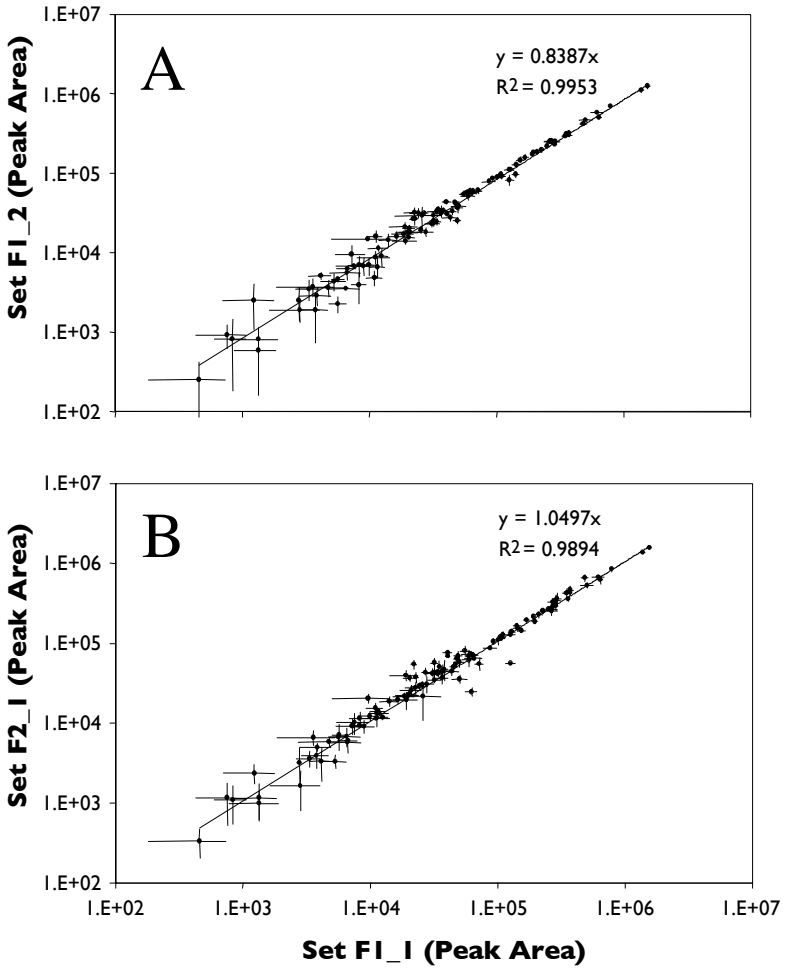
MRM Assays Show High Reproducibility of Depletion (MARS) and Digest Steps



Quantitative Mass Spectrometric MRM Assays for Major Plasma Proteins, Leigh Anderson and Christie Hunter, Mol Cell Proteomics, in press 2005

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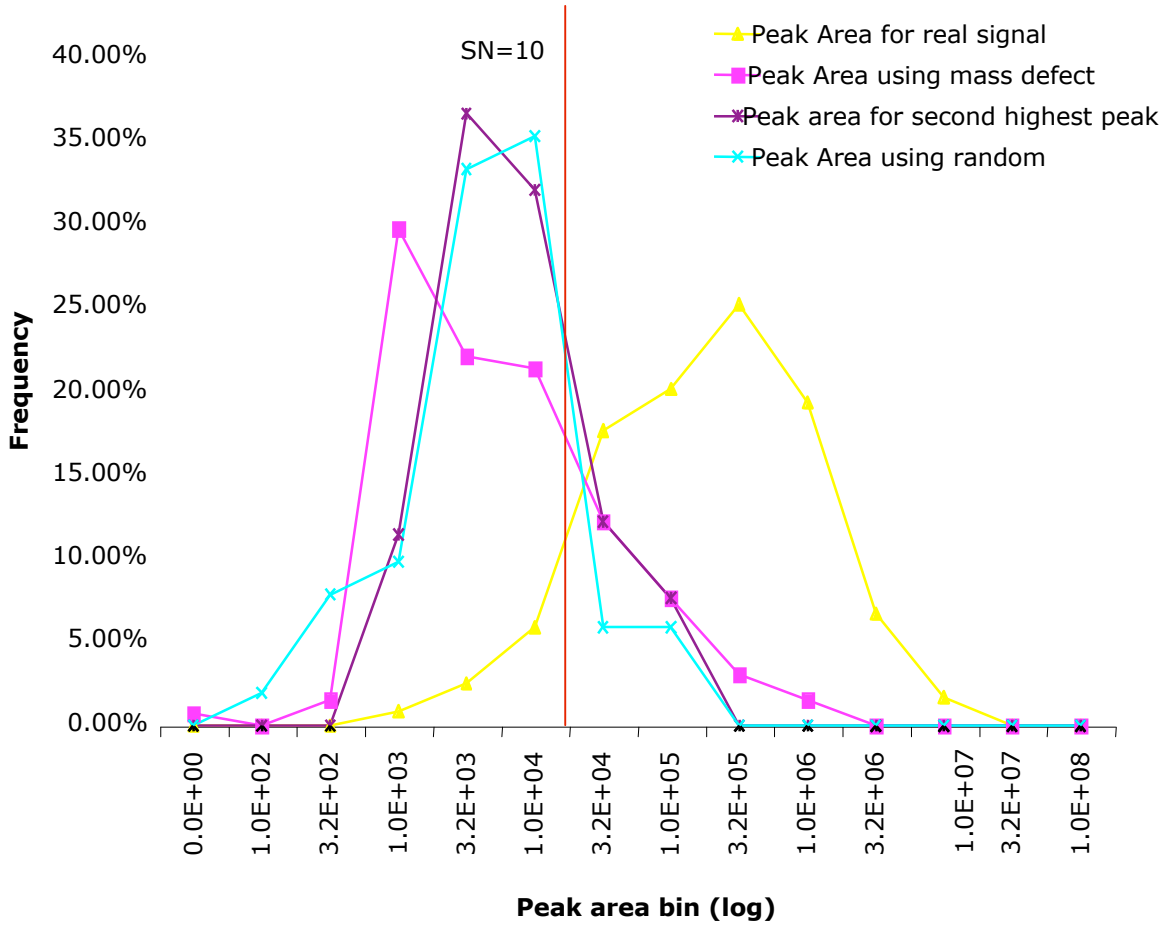
Designed vs "Random" MRM's

Occupancy of Plasma MRM Space Is Low

- Apex of the random vs real distributions are >2 orders of magnitude apart
- If we use a cutoff of peak areas for real signal at 2e4, then only 10% of MRM channels will contain random signal



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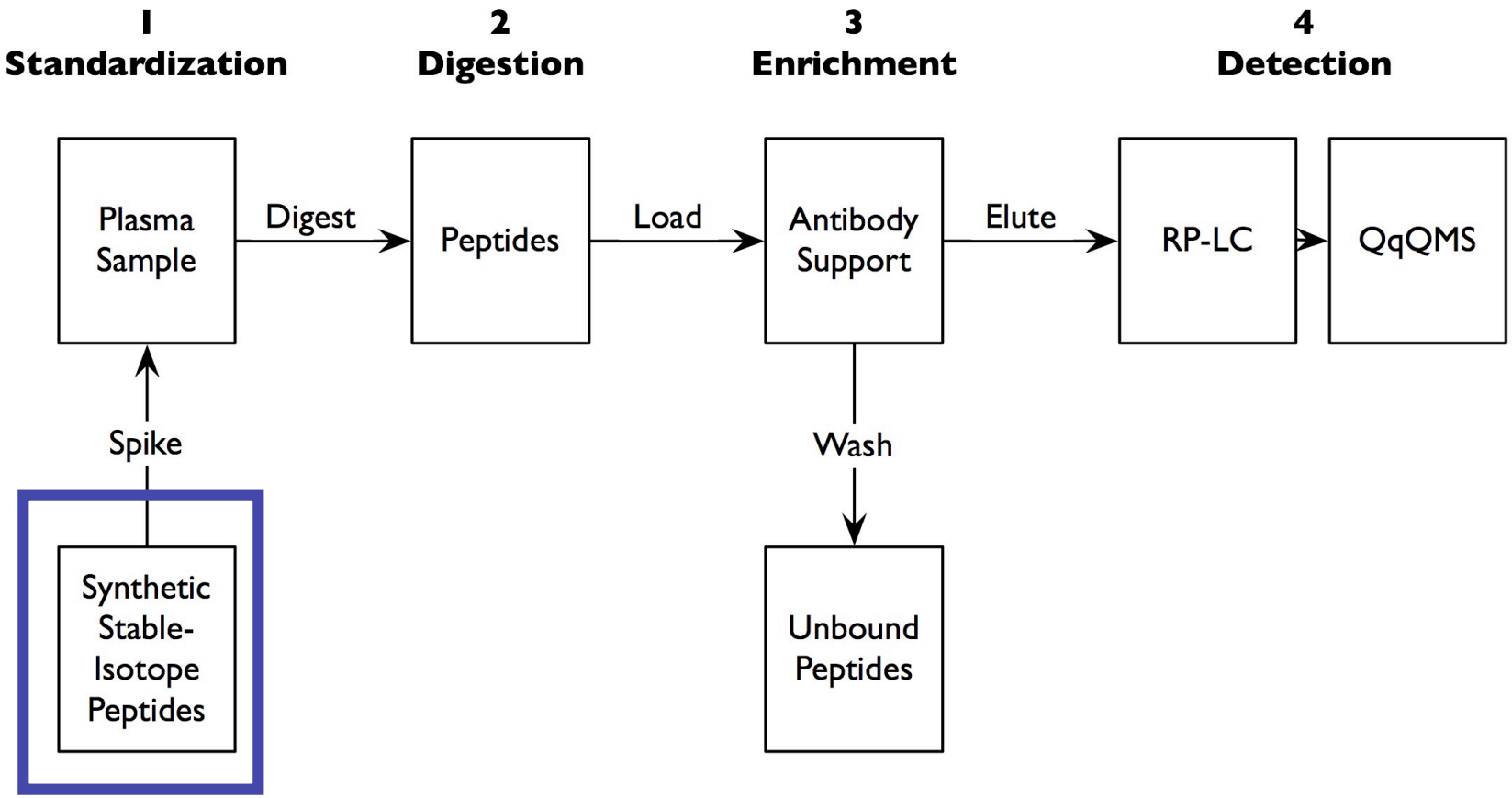
Stable Isotope-labeled Standard (SIS) Peptides



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Stable Isotope-labeled Standard (SIS) Peptides

- Provide internal standardization for MRM measurements (SIS and Nat measured via 2 related MRM's)
- Can be made by standard solid-phase synthesis methods, followed by purification and amino acid analysis (for absolute quantitation)
- Typically incorporate one labeled amino acid (a U-^{13}C, U-^{15}N lysine or arginine at C-terminus, as appropriate in tryptic peptide sequence)
- Sets of SIS can be made more cheaply as polyproteins using cell-free synthesis from synthetic gene constructs (polySIS)

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Efficient Production of Stable Isotope-labeled Standards (SIS) as polySIS*

Apolipoprotein A-I	ATEHLSTLSEK
Alpha-1-acid glycoprotein 1	NWGLSVYADKPETTK
Haptoglobin beta chain	ILGGHLDAK
Fibrinogen gamma chain	DTVQIHDITGK
Fibrinogen alpha chain	TVIGPDGHK
Fibrinogen beta chain	QGFGNVATNTDGK
Alpha-1-antichymotrypsin	EIGELYLPK
Complement C3	TGLQEVEVK
Antithrombin-III	DDLYVSDAFHK
Ceruloplasmin	IYHSHIDAPK
Prothrombin	ETAASLLQAGYK
Complement C4 gamma chain	ITQVLHFTK
Apolipoprotein B-100	FPEVDVLTK
Alpha-2-antiplasmin	LGNQEPGGQTALK
Plasminogen	LSSPAVITDK
Apolipoprotein E	QWAGLVEK
Coagulation factor XIIa heavy chain	IPPWEAPK
Apolipoprotein(a)	LFLEPTQADIALLK
Coagulation factor X	SHAPEVITSSPLK
Adiponectin	IFYNQQNHYDGSTGK
Beta-2-glycoprotein I	EHSSLAFWK
Coagulation factor IX	VSVSQTSK
C-reactive protein	ESDTSYVSLK
Vitamin K-dependent protein C	WELDLDIK
Coagulation factor XIII A chain	STVLTIPEIIIK
Coagulation factor XIII B chain	LIENGYFHPVK
Cholesteryl ester transfer protein	ASYPDITGEK
Coagulation factor V	DPPSDLLLLK
Angiotensinogen	ALQDQLVLVAAK
L-selectin	AEIEYLEK

KATEHLSTLSEKNWGLSVYADKPETTKILG
GHLDAKDTVQIHDITGKTVIGPDGHKQGF
GNVATNTDGKEIGELYLPKTGLQEVEVKDD
LYVSDAFHKIYHSHIDAPKETAASLLQAGY
KITQVLHFTKFPEVDVLTKLGNQEPGGQTA
LKLSSPAVITDKQWAGLVEKIPPWEAPKLF
LEPTQADIALLKSHAPEVITSSPLKIFYNQQ
NHYDGSTGKEHSSLAFWKVSVSQTSKES
DTSYVSLKWELDLDIKSTVLTIPEIIIKLIEN
GYFHPVKASYPDITGEKDPPSDLLLLKALQ
DQLVLVAAKAEIEYLEK

Synthesize gene CVD_1a (codon optimized)

Clone into pIVEX2.4d expression vector

Express in Roche RTS E coli-based cell-free system (^{15}N,^{13}C-Lys)

polySIS protein standard

PPI

* Patent Pending

polySIS* Peptide Standards Provide Absolute Protein Quantitation

polySIS CVD_1 (lys-labeled standard)

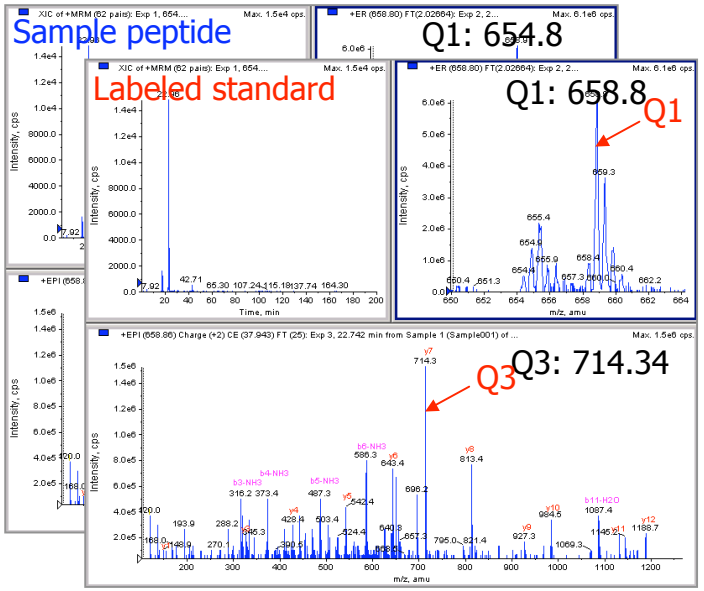
ATEHLSTLSEKNWGLSVYADKPETTKILGGHLDAKD
TVQIHDITGKTVIGPDGHK**QGFGNVATNTDGK**EI
GELYLPKTGLQEVEVKDDLYVSDAFHKIYHSHIDAPK
ETAASLLQAGYKITQVLHFTKFPEVDVLTKLGNQEPG
GQTALKLSSPAVITDKQWAGLVEKIPPWEAPKLFLE
PTQADIALLKSHAPEVITSSPLKIFYNQQNHYDGSTG
KEHSSLAFWKVSVSQTSKESDTSYVSLKWELDLDIK
STVLTIPEIIIKLIENGYFHPVKASYPDITGEKDPPSDL
LLLKALQDQLVLVAAKAEIEYLEK

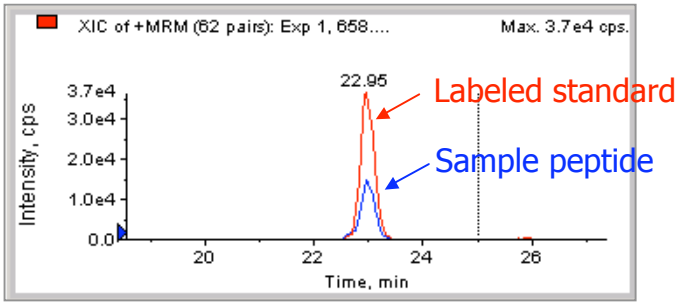
Fibrinogen β chain (in sample)

MKRMVSWSFHKLKTMKHLLLLLLCVFLVKSQGVND
NEEGFFSARGHRPLDKKREEAPSLRPAPPPISGGGYR
ARPAKAAATQKKVERKAPDAGGCLHADPDLGVLCPT
GCQLQEALLQQERPIRNSVDELNNNVEAVSQTSSSS
FQYMYLLKDLWQKRQKQVKDNENVVNEYSSELEKH
QLYIDETVNSNIPTNLRVLRSILENLRSKIQKLESDVS
AQMEYCRTPCTVSCNIPVVSGKECEEIIRKGGETSE
MYLIQPDSSVKPYRVYCDMNTENGGWTVIQNRQDG
SVDFGRKWDPYK**QGFGNVATNTDGK**NYCGLPGE
YWLGNDKISQLTRMGPTELLIEMEDWKGDKVKAHY
GGFTVQNEANKYQISVNKYRGTAGNALMDGASQLM
GENRTMTIHNGMFFSTYDRDNDGWLTSDPRKQCSK
EDGGGWWYNRCHAANPNGRYYWGGQYTWDMAK
HGTDDGVVWMNWKGSWYSMRKMSMKIRPFFPQQ

Digest

***LC-TQMS of* QGFGNVATNTDGK**



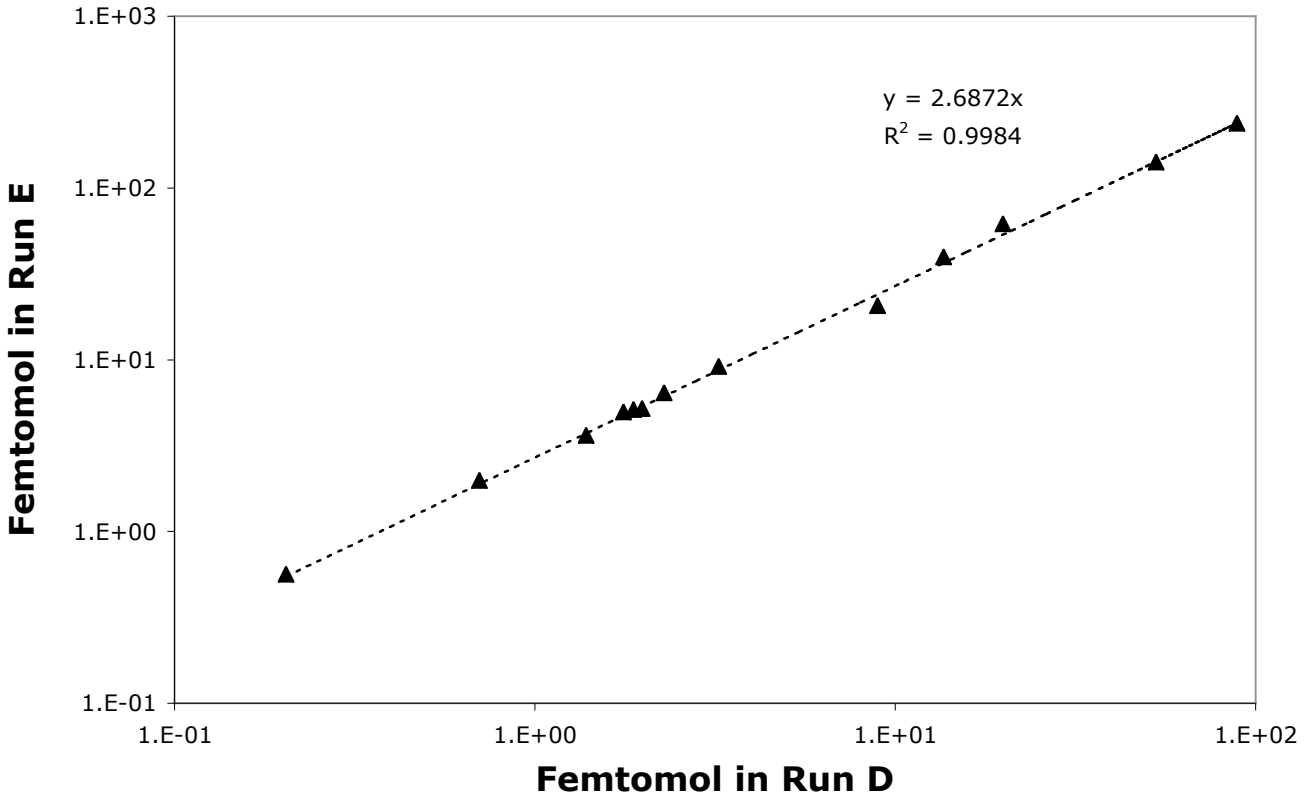


MRM Chromatogram

* Patent Pending

PPI

Reproducibility of MRM Measurements of Nat:SIS Ratios for 13 Peptides in Two Experiments (n=10) Across 2.7 Logs



Quantitative Mass Spectrometric MRM Assays for Major Plasma Proteins, Leigh Anderson and Christie Hunter, Mol Cell Proteomics, in press 2005

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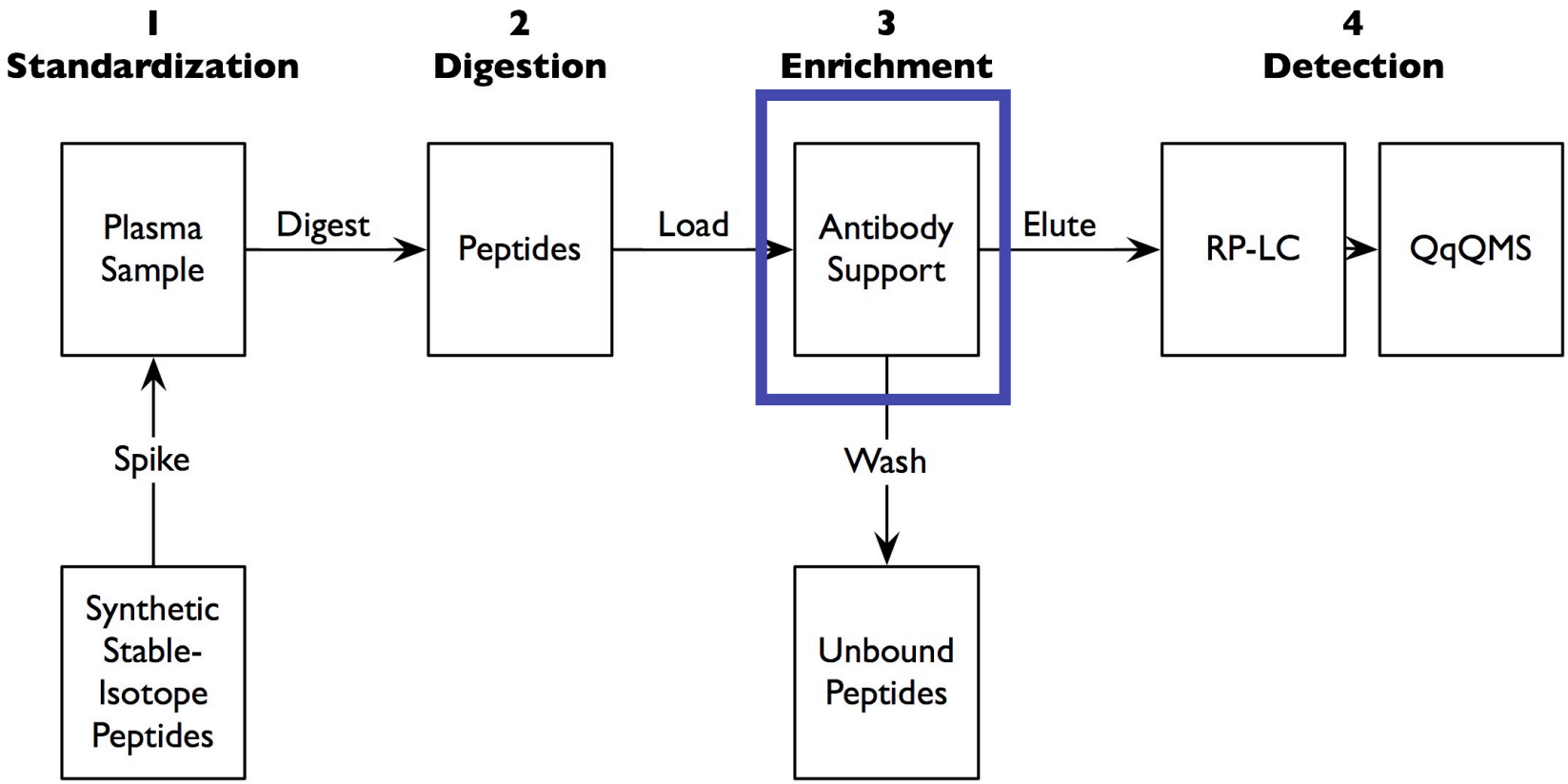
Enrichment of Target Peptides Using Immobilized Anti-peptide Antibodies



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Enrichment of Target Peptides Using Immobilized Anti-peptide Antibodies

- Rabbit polyclonal antibodies (short immunization protocol) made to four peptides coupled to BSA carrier and affinity purified on peptide-agarose
- Antibodies immobilized at high density on POROS support, QC'ed by flow cytometry of POROS beads with fluorescently-labeled peptides
- SISCAPA using nanoaffinity column format (100nl column) in nanoflow LC
- Bound & eluted proteins resolved by C18 reversed phase chromatography and MS
- Four antibodies yielded average >100-fold enrichments in column format
- Subsequent tests using magnetic bead carriers yielded up to 10,000-fold enrichment using same antibodies (Paulovich group, paper submitted)

No.	Protein	Tryptic Peptide
2	IL-6	EALAENNLNLPK
3	Hemopexin	NFPSPVDAAFR
4	Alpha-1-Antichymotrypsin	EIGELYLPK
5	TNF-alpha	DLSLISPLAQAVR

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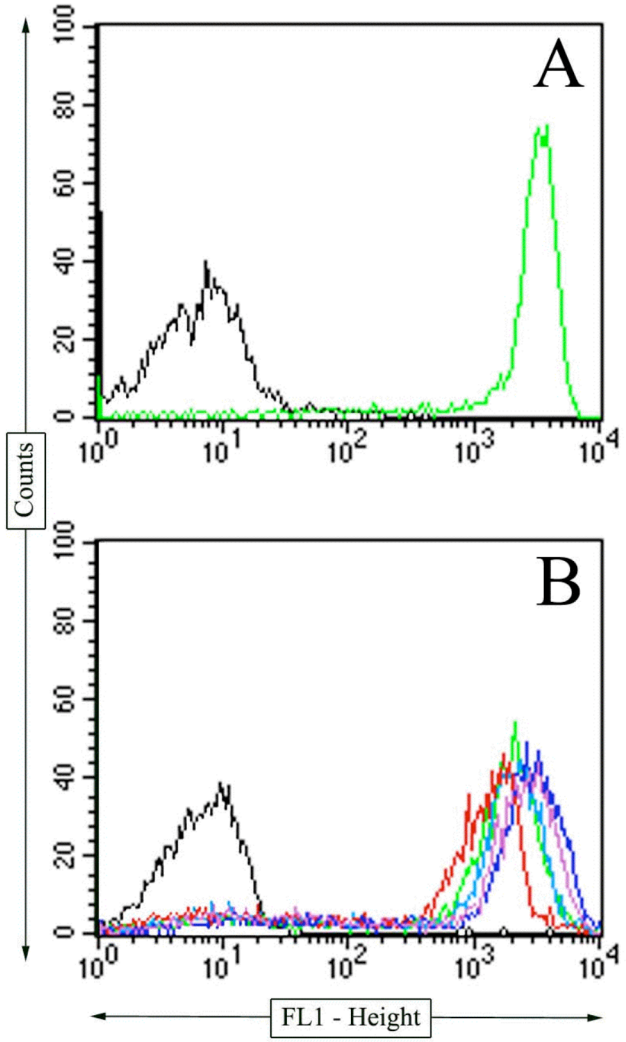
Flow Cytometric QC of Rabbit Anti-peptide Antibodies Coupled to POROS® Protein G Beads



A: Black profile; POROS® protein G beads incubated with biotinylated protein L and fluorescein-labeled streptavidin (negative control). Green profile; POROS® Streptavidin beads incubated with biotinylated Protein L and detected with fluorescein-conjugated streptavidin (positive control).

B: Detection of covalently coupled rabbit anti-peptide antibodies on POROS® Protein G beads. Beads covalently coupled with 5 rabbit affinity-purified Ab's incubated first with biotinylated Protein L, followed by detection with fluorescein-conjugated streptavidin.

An effective and rapid method for functional characterization of immunoadsorbents using POROS® beads and flow cytometry. N. Leigh Anderson, N.L., Haines, L.R. and Pearson, T.W. Journal of Proteome Research 3:228-34 (2004).

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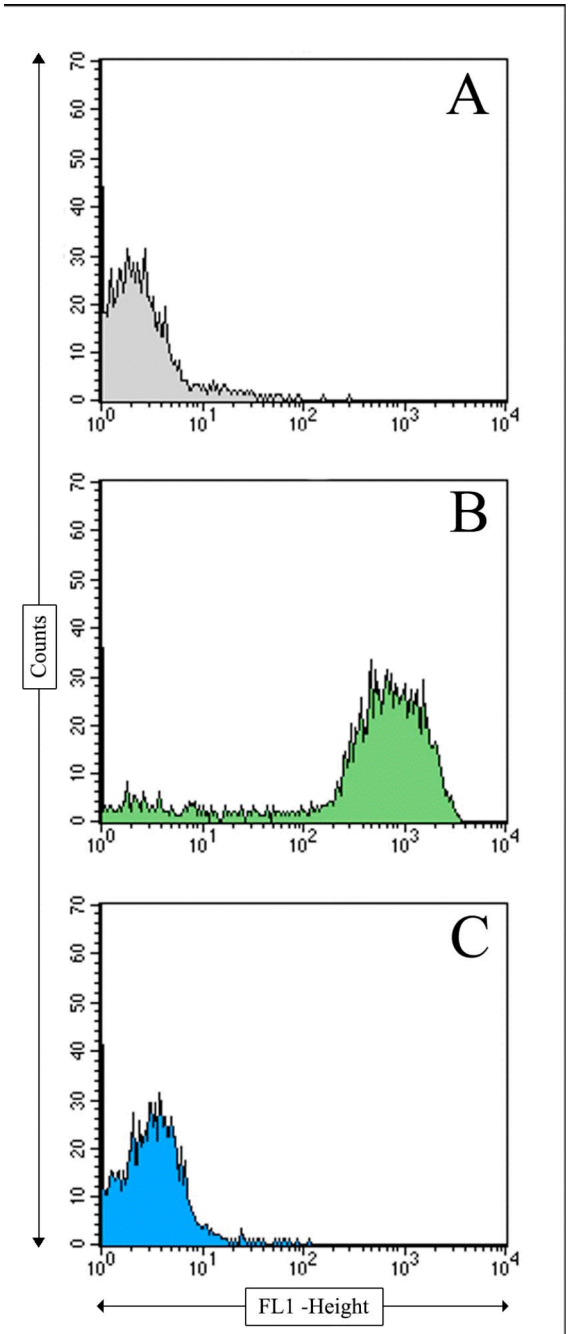

Rapid Characterization of Specific Peptide Binding to Immobilized Antibody Supports

Detection of peptide antigens (fluorescently labeled with Alexa Fluor 488) bound to antibody-coupled POROS® Protein G beads by flow cytometry

A: Ab 4 beads plus dye only (negative control: Alexa Fluor 488, no peptide)

B: Ab 4 beads plus fluorescent homologous peptide (ALX4)

C: Ab 4 beads plus fluorescent heterologous peptide (ALX6)

For each set of beads, 2500 events were analyzed

An effective and rapid method for functional characterization of immunoadsorbents using POROS® beads and flow cytometry. N. Leigh Anderson, N.L., Haines, L.R. and Pearson, T.W. Journal of Proteome Research 3:228-34 (2004).

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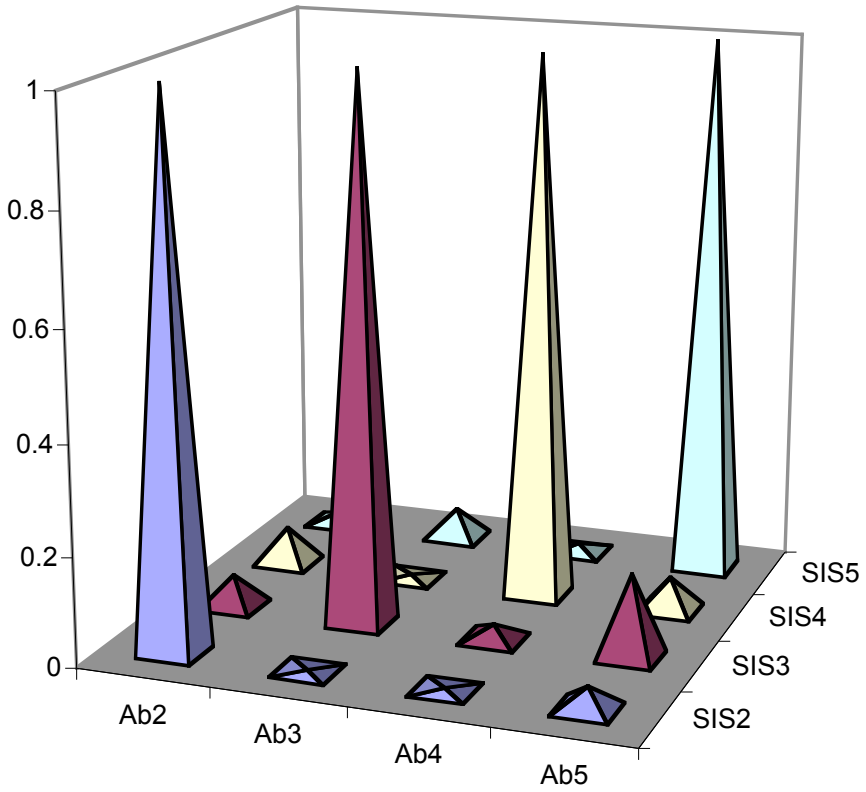
Relative Quantities of Four SIS Peptides Bound by Four Anti-Peptide Antibodies Measured by MRM: Average Correct Peptide Enriched > 100-fold



MRM measurements of specific vs off-target binding of all pairs of 4 peptides and their 4 specific antibodies

SIS2-5: stable-isotope labeled version of peptides 2-5

Ab2-5: antibodies raised against peptides 2-5

MRM signals (vertical axis) are normalized to the largest signal for that antibody

No.	Protein	Tryptic Peptide
2	IL-6	EALAENNLNLPK
3	Hemopexin	NFPSPVDAAFR
4	Alpha-1-Antichymotrypsin	EIGELYLPK
5	TNF-alpha	DLSLISPLAQAVR

Mass Spectrometric Quantitation of Peptides and Proteins Using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). Anderson, N.L., et al, Journal of Proteome Research, 3: 235-44 (2004).

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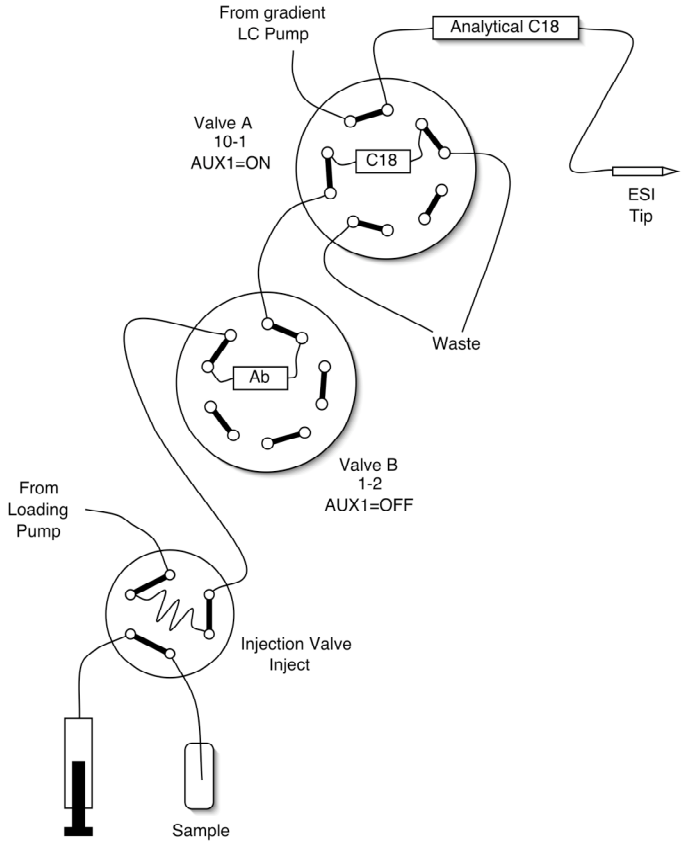
SISCAPA: LC Setup for Initial Enrichment Studies



- Anti-peptide Ab column (100nl) packed in 100u capillary tubing with frit (100u ID x 1cm POROS support)
- Acid-eluted peptide (from Ab column) captured on C18 trap and resolved by nanoflow RP-LC into ESI-MS/MS

Mass Spectrometric Quantitation of Peptides and Proteins Using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). Anderson, N.L., et al, Journal of Proteome Research, 3: 235-44 (2004).

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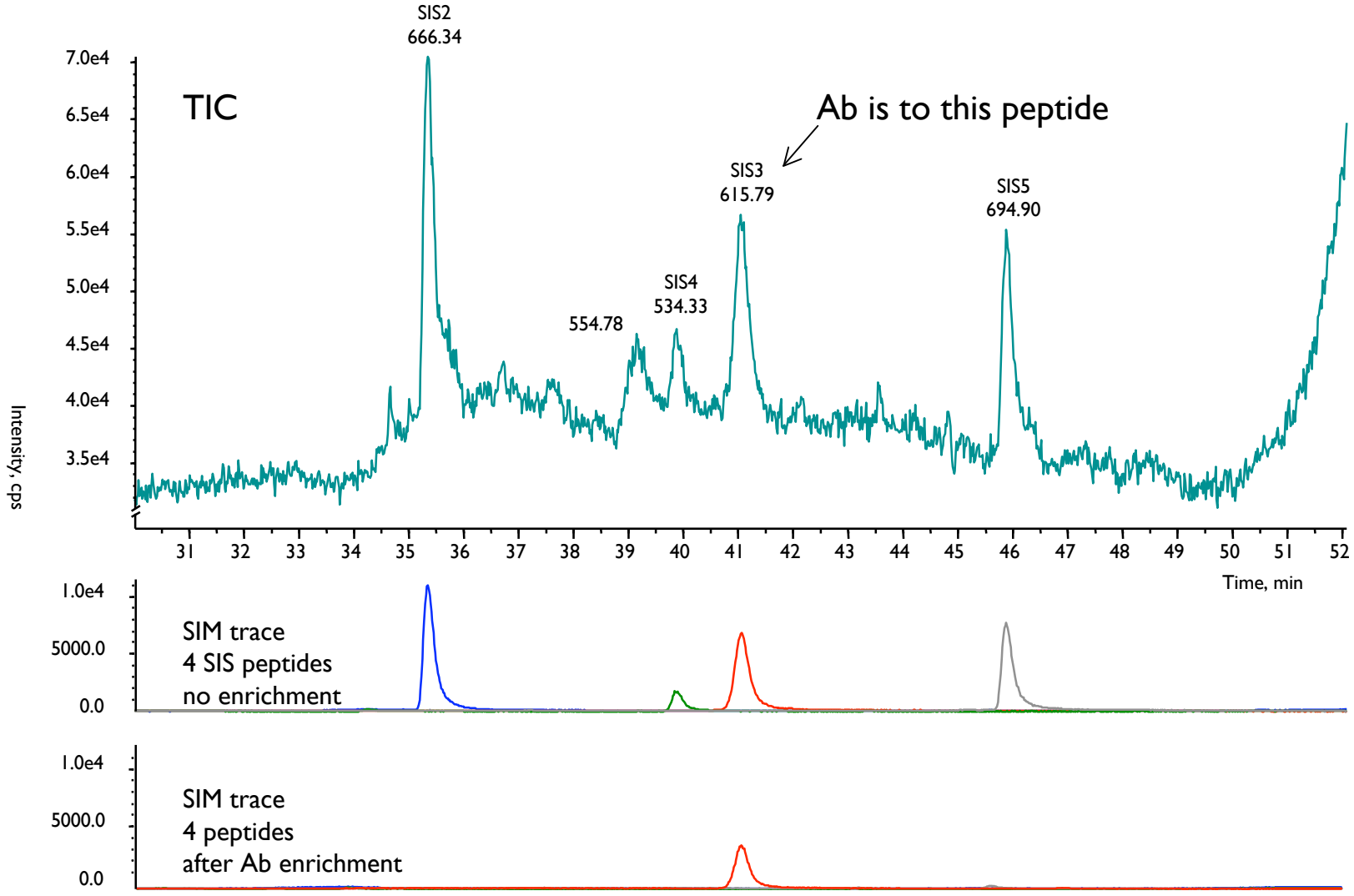
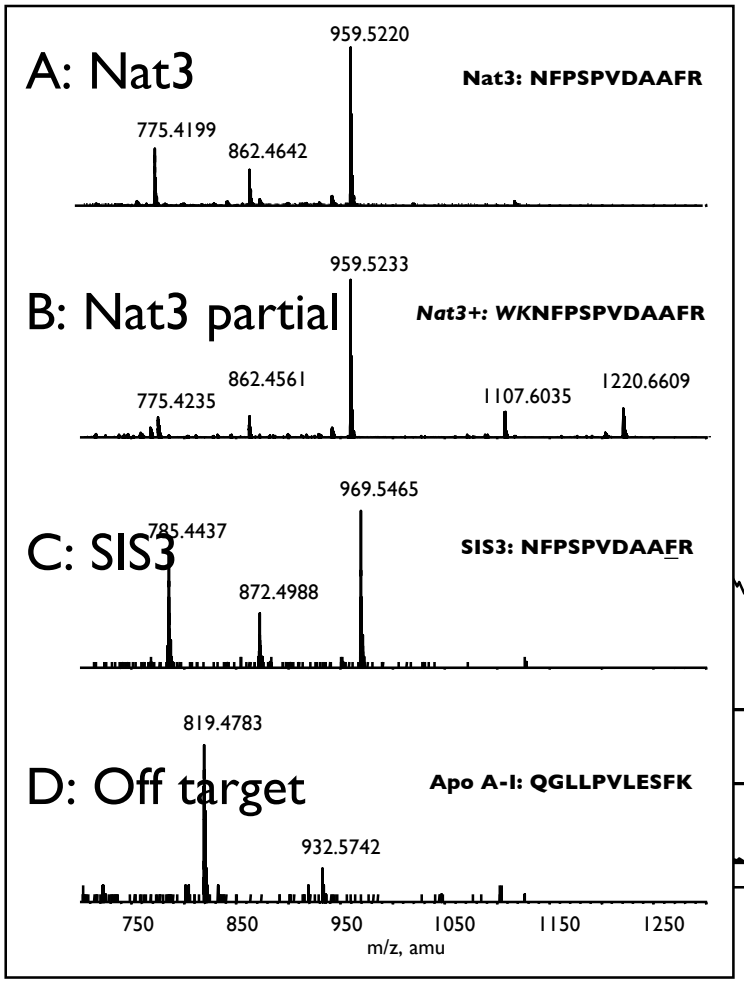
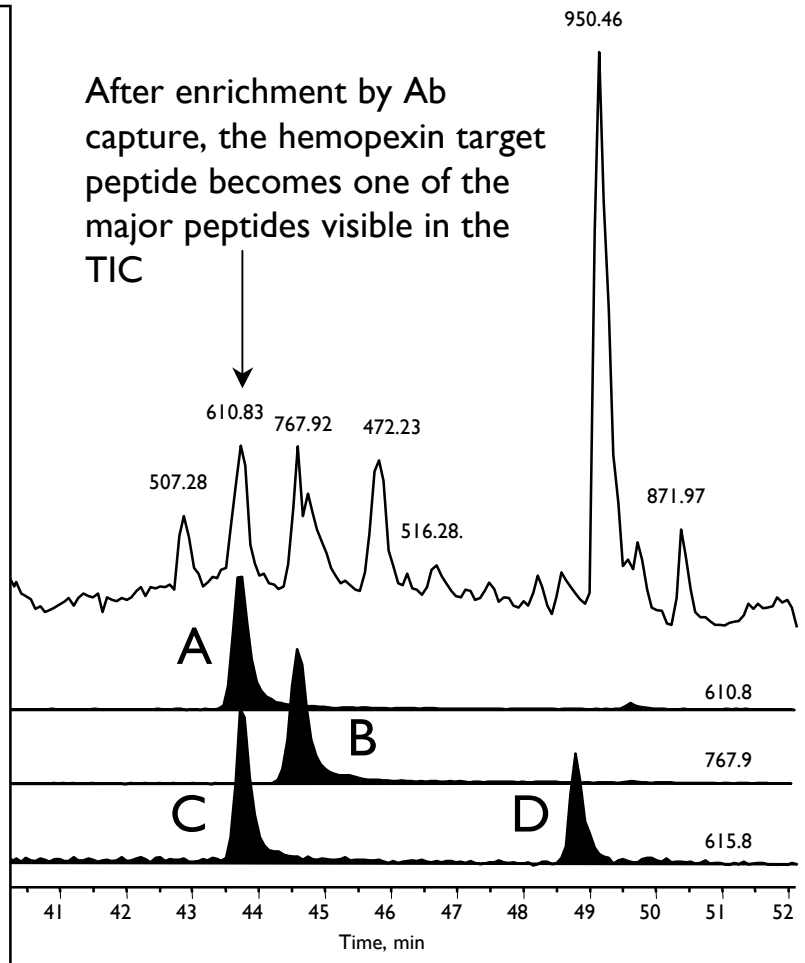
Ab Capture from SIS Peptide Mixture with Selected Ion Monitoring



SISCAPA Enrichment of a Hemopexin Monitor Peptide from a Whole Plasma Digest





Mass Spectrometric Quantitation of Peptides and Proteins Using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). Anderson, N.L., et al, Journal of Proteome Research, 3: 235-44 (2004).

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Conclusions

- Planning for validation is critical in biomarker studies
- MS-based specific assays for biomarker candidates have characteristics required for validation studies
  - assay parameters easily generated
  - good CV's (<10%)
  - multiplexable (100-200)
  - implementable on conventional QqQMS platforms
- Assay parameters now in hand for ~50 abundant plasma proteins
- Specific peptide enrichment with antibodies (SISCAPA) appears to extend sensitivity downwards into the region of 1ng/ml
- Candidate-based methods, necessary for biomarker validation, may offer a more sensitive and shareable approach for certain "discovery" problems as well ("only" 22,000 targets?)

PPI

Acknowledgments

- SISCAPA Experiments
  - Derrick Smith, Bob Olafson, Derryl Hardy, UVIC-Genome B.C. Proteomics Centre
  - Terry Pearson, Lee Haines, Angela Jackson, Department of Biochemistry and Microbiology, University of Victoria, B.C, Canada
  - John Rush, Cell Signaling
- Plasma Proteome Database
  - Malu Polanski (PPI)
  - Richard Fagan, Anna Lobley, Inpharmatica Ltd., London
  - Rembert Pieper, Tina Gatlin, The Institute for Genomic Research
  - Radhakrishna S. Tirumalai, Timothy D. Veenstra, Mass Spectrometry Center, U. S. National Cancer Institute
  - Joshua N. Adkins, Joel G. Pounds, Biological Sciences Department, Pacific Northwest National Laboratory
- polySIS labeled peptide standards
  - Jerry Becker, Andrew Breite, Roche Protein Expression Group, Indianapolis
- MRM Assay Development
  - Christie Hunter, Tina Settineri, Applied Biosystems, Foster City
- Magnetic Bead Processing
  - Ian Jardine, Thermo Electron Corp.
- Graphics
  - Arkitek Studios, Seattle
- Grant Funding
  - National Cancer Institute (contract # 23XS144A)

www.plasmaproteome.org

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SISCAPA Publications

- **High sensitivity quantitation of peptides by mass spectrometry.** Anderson, Norman L., United States Patent Application 20040072251. The basic SISCAPA patent application.
- **Mass Spectrometric Quantitation of Peptides and Proteins Using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA).** Anderson, N.L., Anderson, N.G., Haines, L.R., Hardie, D.B., Olafson. R.W., and Pearson, T.W. Journal of Proteome Research, 3: 235-44 (2004). Initial proof of concept results with SISCAPA using 4 peptides & Ab's.
- **An effective and rapid method for functional characterization of immunoadsorbents using POROS® beads and flow cytometry.** N. Leigh Anderson, N.L., Haines, L.R. and Pearson, T.W. Journal of Proteome Research 3:228-34 (2004). Methods for characterizing SISCAPA immobilized Ab's.
- **Candidate-Based Proteomics in the Search for Biomarkers of Cardiovascular Disease,** Leigh Anderson, J. Physiol., 563.1:23-60 (2005). Explanation of the role of SISCAPA in biomarker validation and a list of 177 interesting candidate biomarkers in CVD.
- **The Roles of Multiple Proteomics Platforms in a Pipeline for New Diagnostics**, N. Leigh Anderson, Mol Cell Proteomics, 4: 1441 - 1444 (2005). Role of SISCAPA assays in the Dx pipeline.
- **Quantitative Mass Spectrometric MRM Assays for Major Plasma Proteins**, Leigh Anderson and Christie Hunter, Mol Cell Proteomics, in press 2005. Capabilities of QqQMS as the MS measurement platform for use in quantitating peptides/proteins in plasma digests.

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