The Diagnostic Potential of the Human Plasma Proteome

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Protein Biomarkers

- Closer to function than DNA or mRNA (mRNA:protein correlation often < 0.5)
- Accessible in blood (plasma) - the primary clinical specimen
- Already widely used in clinical practice (e.g., definitive role of heart muscle troponin in diagnosing acute myocardial infarct)
- Potentially capable of defining all disease states (in a multivariate sense): the strong biomarker hypothesis
- Valuable in drug and device development to confirm efficacy (mechanism), detect toxicity, define disease state, select and stratify patient populations

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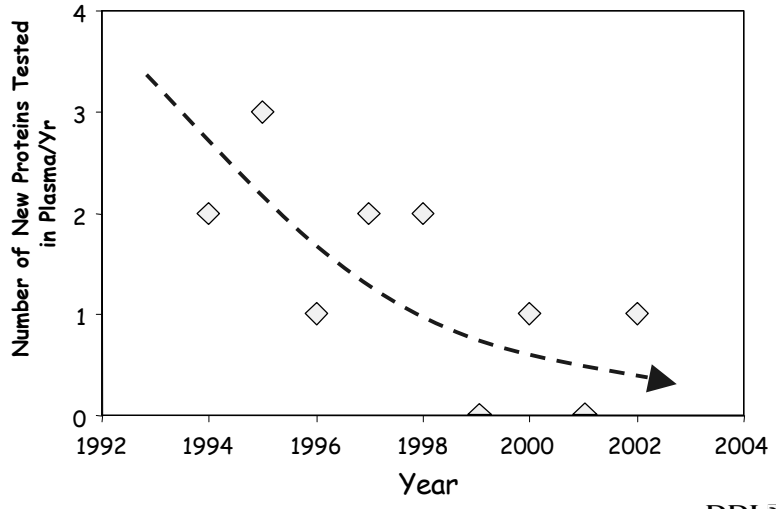
Introduction of New Clinical Biomarkers is Slowing

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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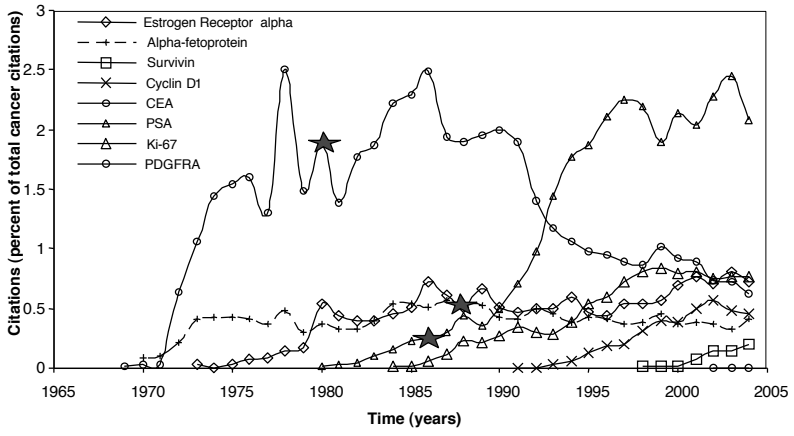
Timescale for Diagnostic Marker Development Has Been Long: Cancer Markers



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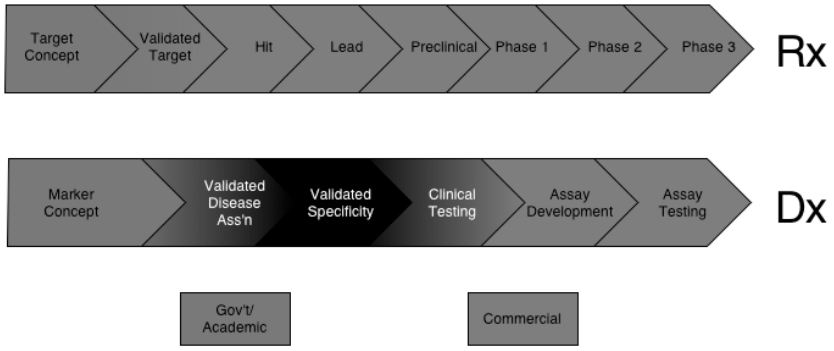
Integrated Dx Pipelines Are Rare



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

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"The appealing notion that research advances travel from bench to bedside is laudable, but conceptually flawed. Even though the U.S. Congress fully anticipates that funding to the National Institutes of Health (NIH) will result in advances in clinical medicine and that other forces, presumably non-governmental, will translate the latest in exciting science into health technologies, under the system of healthcare we have today, this advancement is not likely to happen."

Floyd Bloom
President, AAAS
Science 300:1680-1685 (2003)

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Plasma Biomarker Discovery: Major Challenge for Proteomics

- Plasma is:
  - the largest (most proteins) and deepest (widest dynamic range) representation of the human proteome in one sample
  - in communication with essentially all tissues and physiological processes (Venetian canal effect)
  - the most universally available clinical sample
  - the source of >100,000 kg of pure therapeutic protein/yr
- Thus for proteomics plasma is:
  - the most difficult sample
  - the most important sample

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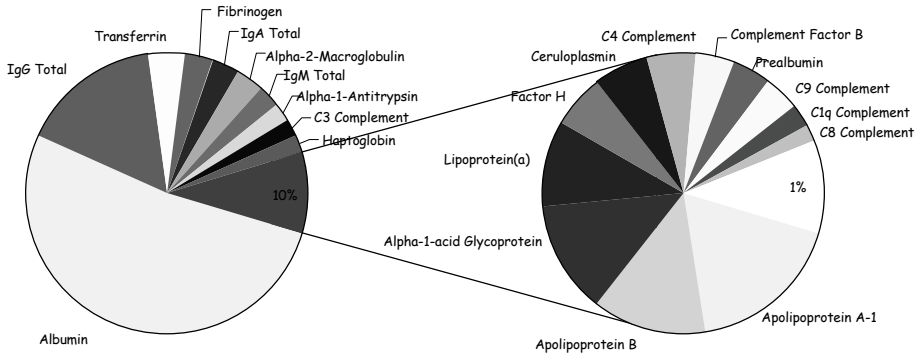
A Small Number of Proteins Make Up the Top 99% of Plasma by Mass



0 - 90%

(10 proteins)

90 - 99%

(12 proteins)

The human plasma proteome: History, character, and diagnostic prospects. Anderson, N.L. and Anderson, N.G., Molecular and Cellular Proteomics, 1.11, 845-867 (2002)

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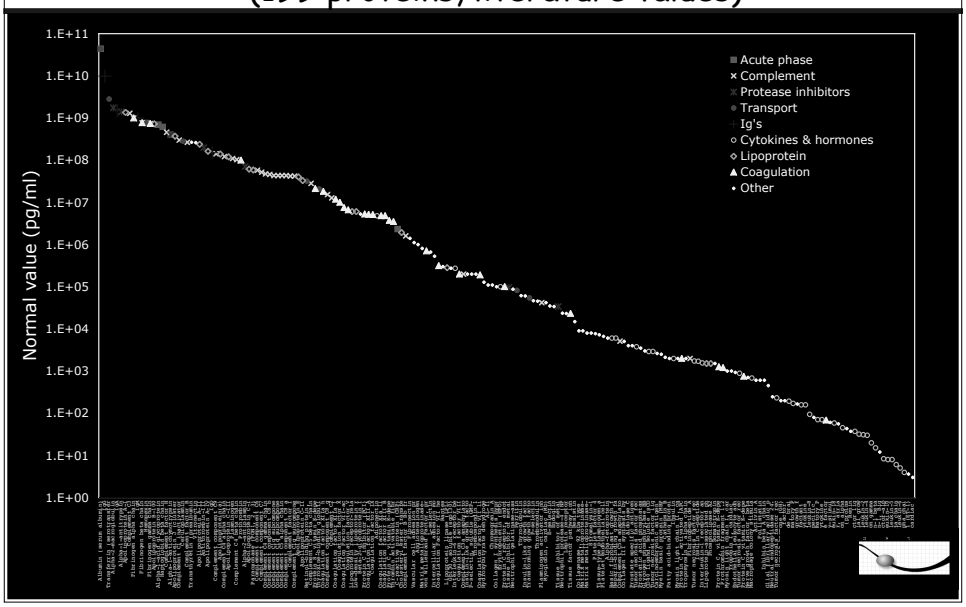
Proteins Measured Clinically in Plasma Span > 10 Orders of Magnitude in Abundance

(199 proteins, literature values)



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

Immuno assays

QqQMS given 1000-fold enrichment

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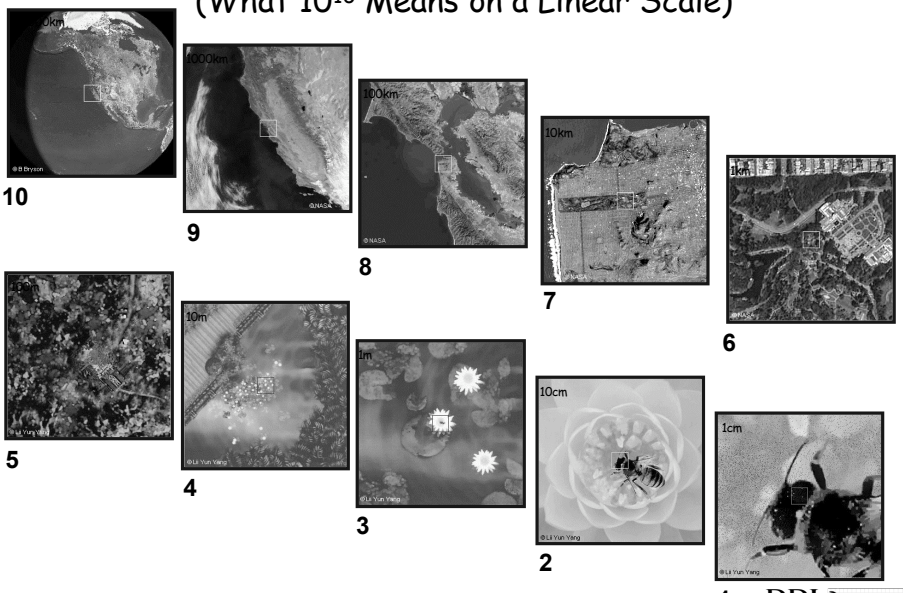
Wide Dynamic Range

(What 10^{10} Means on a Linear Scale)



Slide courtesy Bruno Domon, ETH Zurich

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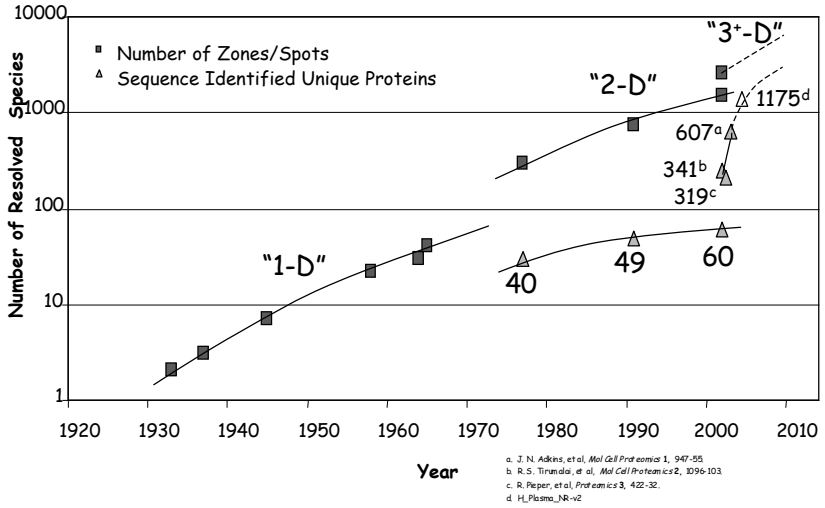
History of Plasma Proteomics



a. J. N. Adkins, et al, *Mol Cell Proteomics* **1**, 947-55.
b. R. S. Tirumalai, et al, *Mol Cell Proteomics* **2**, 1096-103.
c. R. Pieper, et al, *Proteomics* **3**, 422-32.
d. H_Plasma_NR-v2

The human plasma proteome: History, character, and diagnostic prospects. Anderson, N.L. and Anderson, N.G., Molecular and Cellular Proteomics, 1.11, 845-867 (2002)

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The State of the Art in Plasma Proteomics c. 1976: 2-D Gels

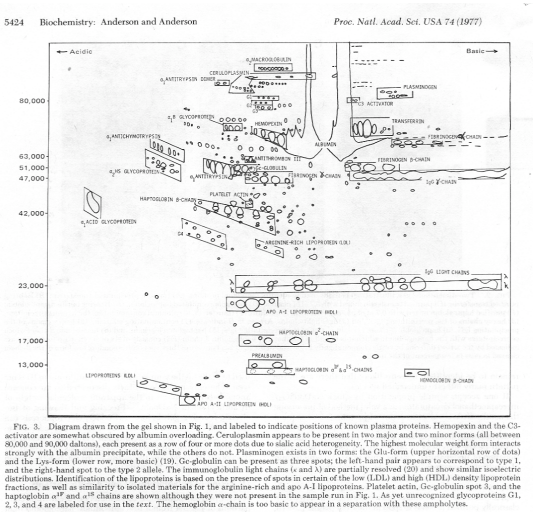

FIG. 3. Diagram drawn from the gel shown in Fig. 1, and labeled to indicate positions of known plasma proteins. Hemopexin and the C3-activator are somewhat obscured by albumin overloading. Ceruloplasmin appears to be present in two major and two minor forms (all between 80,000 and 90,000 daltons), each present as a row of four or more dots due to sialic acid heterogeneity. The highest molecular weight form interacts strongly with the albumin precipitate, while the others do not. Plasminogen exists in two forms: the Glu-form (upper horizontal row of dots) and the Lys-form (lower row, more basic) (19). Gc-globulin can be present as three spots; the left-hand pair appears to correspond to type 1, and the right-hand spot to the type 2 allele. The immunoglobulin light chains (κ and λ) are partially resolved (20) and show similar isoelectric distributions. Identification of the lipoproteins is based on the presence of spots in certain of the low (LDL) and high (HDL) density lipoprotein fractions, as well as similarity to isolated materials for the arginine-rich and apo A-I lipoproteins. Platelet actin, Gc-globulin spot 3, and the haptoglobin α^{1F} and α^{1S} chains are shown although they were not present in the sample run in Fig. 1. As yet unrecognized glycoproteins G1, 2, 3, and 4 are labeled for use in the *text*. The hemoglobin α-chain is too basic to appear in a separation with these ampholytes.

Anderson,L., Anderson,N. G. High resolution two-dimensional electrophoresis of human plasma proteins. (1977) PNAS 74, 5421-5

2-D Electrophoresis

300+ resolved spots

40 identified proteins

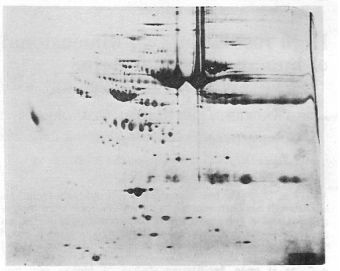

FIG. 1. Two-dimensional gel of human plasma proteins. The sample was 10 μl of fresh heparinized plasma denatured in Na-DodSO$_4$/mercaptoethanol.

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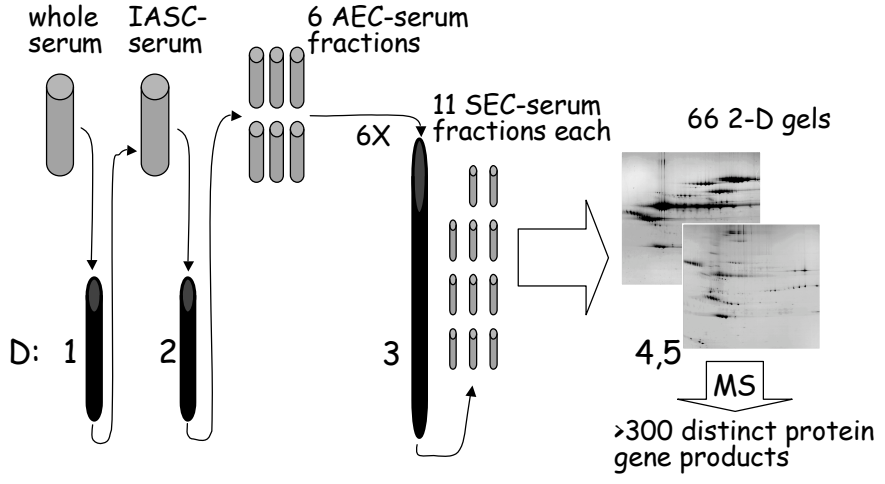
Multi-Dimensional 2DE-Based Plasma Biomarker Discovery Proteomics

(e.g., 3-D Chromatography + 2-DE + LC/MS)



The human serum proteome: Display of nearly 3700 chromatographically separated protein spots on two-dimensional electrophoresis gels and identification of 325 distinct proteins. Pieper, R., et al Proteomics 3(7): 1345-64. (2003).

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Different Technologies Provide Different Views of the Human Plasma Proteome

- Four datasets compared:
  - Base list of ~450 proteins reported in "non-proteomics" literature as measured/detected in plasma or serum
  - Three sets of 300-600 proteins each from proteomics surveys (2-D gels + MS/MS; LC/LC-MS/MS)
- Made non-redundant using methods of genomics
  - Redundancy definition: >95% homology over >=15 amino acid subsequence
- Questions:
  - What is the overlap between different methods?
  - Can low-abundance proteins be detected in surveys?
  - Can a useful plasma database be collected?

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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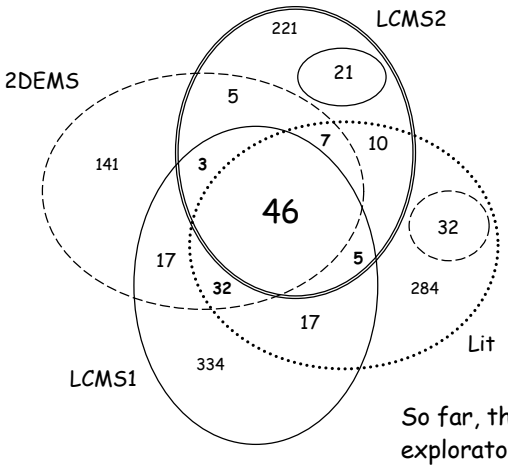
Overlap of Four Plasma Proteome Datasets

(Number of NR proteins)



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

So far, there is no comprehensive exploratory proteomics platform for plasma

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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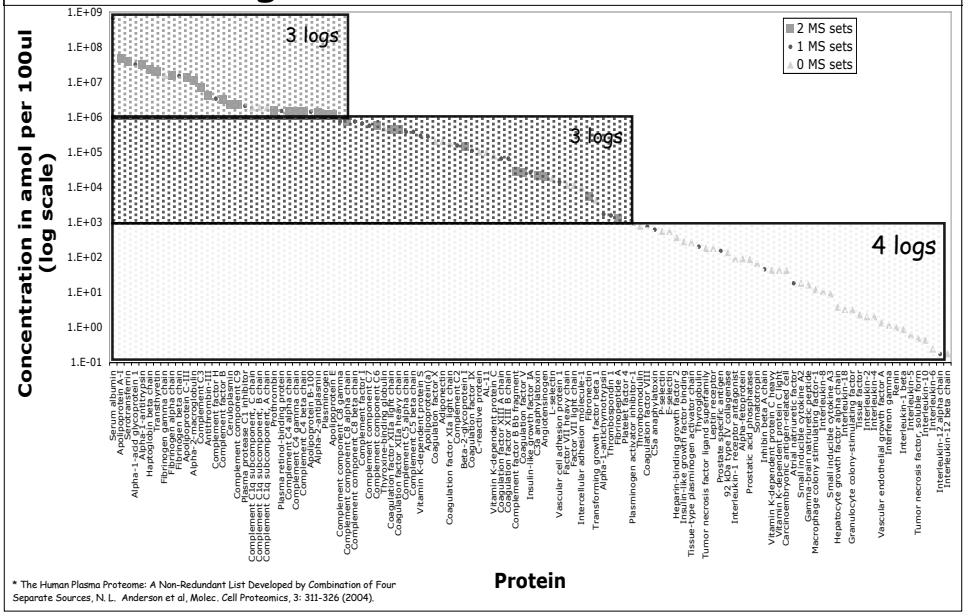
Plasma Proteome Surveys Detect Primarily Higher Abundance Proteins



* 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).

Strategic Directions for Plasma Biomarker Discovery

- Pursue biomarker discovery at the site of biology (typically within tissue) where concentration and differential are highest
- Extend fractionation technologies for deeper plasma exploration, especially for PTM's
- Use specific assays to investigate candidates from proteomics and other approaches in plasma (directed approach: validation platform)

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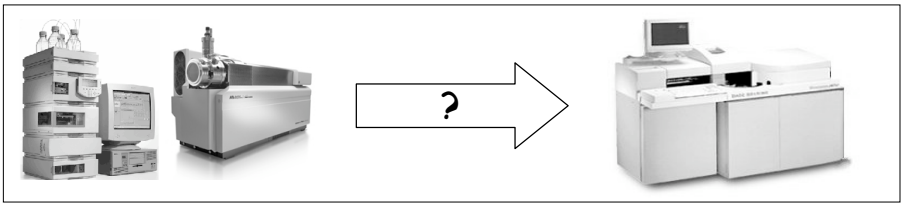
A Major Technology Gap Exists Between Discovery and Routine Diagnostic Proteomics



Discovery		Routine 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	100-10,000

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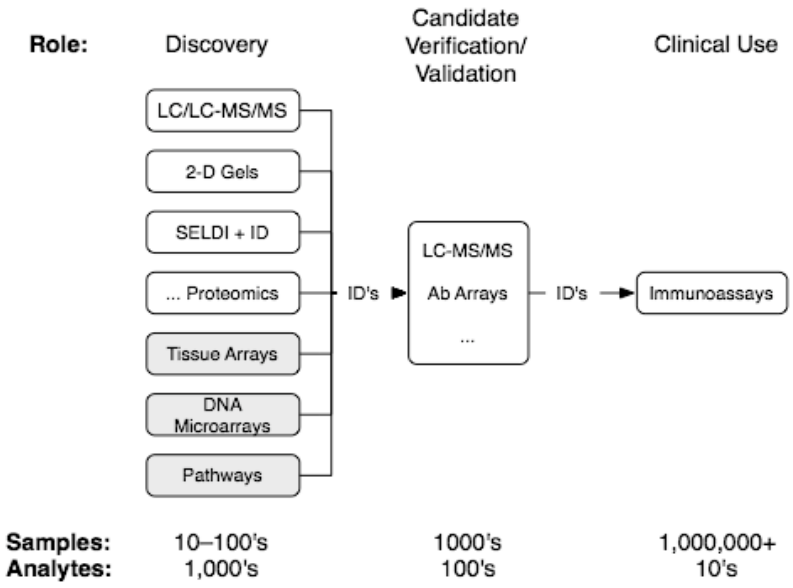
Three-Stage Dx Pipeline



A Candidate-Based Approach: Specific Assays for Identified Candidates

- Specific assays (e.g., immunoassays) can have much greater sensitivity than survey methods
- Rich sets of biomarker candidates are emerging:
  - 177 cardiovascular
    - **Candidate-Based Proteomics in the Search for Biomarkers of Cardiovascular Disease,** Leigh Anderson, J. Physiol., in press 2004
  - ~1200 cancer
    - Manuscript submitted
- The challenge is to construct a specific assay for any sequence-defined protein candidate quickly, at low cost

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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., in press 2004

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Technology Alternatives for Targeted 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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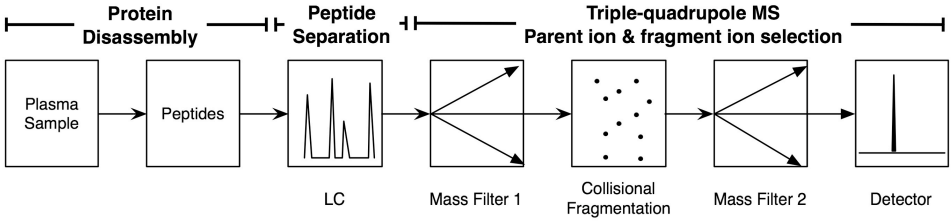
A General Approach to Protein Measurement via MS/MS

Objective: direct design of protein assays from sequence, using peptides as measurement surrogates:

- Variation of protein physical properties make them hard to address comprehensively
- Postulate: within every protein (good,bad) there is ≥1 good tryptic peptide
- Absolute quantitation using isotope dilution (stable isotope labeled peptide internal standards)



- The triple-quadrupole MS (LC-MS/MS) platform is very widely used for small molecular assays in plasma (drug metabolites, inborn errors, pesticides)
- Multiplex 100-200 assays per run

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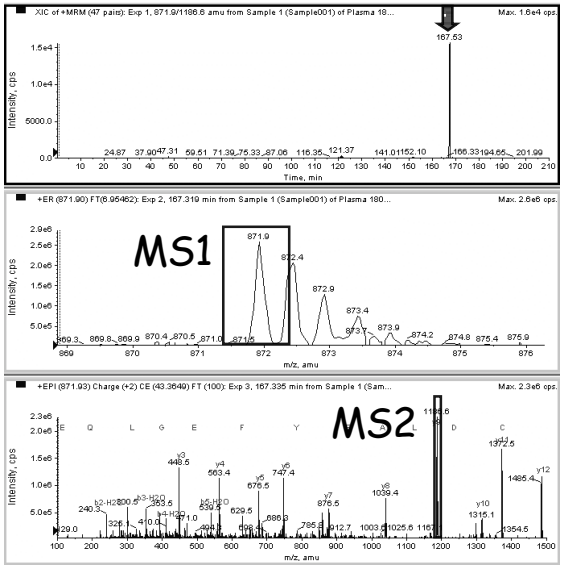
Alpha-1-acid glycoprotein 1 peptide EQLGEFYEALDBLR 1742.8

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"



Collaboration with Christie Hunter (ABI) using 4000 Q TRAP MS

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In Silico MS Assay Design via Bioinformatics

Protein Sequence	→ In Silico Tryptic Peptides	→ *In Silico* Features	→ Experimental Features	→ Assay Param's
>sp|P14151|LYAM1_HUMAN L-selectin precursor MIFPWKCQSTQRDLWNIFKLWGWTML CCDFLAHHGTDCWTYHYSEKPMNWQR ARRFCRDNYTDLVAIQNKAEIEYLEK TLPFSRSYYWIGIRKIGGIWTWVGTN KSLTEEAENWGDGEPNNKKNKEDCVE IYIKRNKDAGKWNDDACHKLKAALCY TASCQPWSCSGHGECVEIINNYTCNC DVGYYGPQCQFVIQCEPLEAPELGTM DCTHPLGNFSFSSQCAFSCSEGTNLT GIEETTCGPFGNWSSPEPTCQVIQCE PLSAPDLGIMNCSHPLASFSFTSACT FICSEGTELIGKKKTICESSGIWSNP SPICQKLDKSFSMIKEGDYNPLFIPV AVMVTAFSGLAFIIWLARRLKKGKKS KRSMNDPY	MIFPWK CQSTQR DLWNIFK LWGWTMLCCDFLAHHGTDCWT PMNWQR AR R FCR DNYTDLVAIQNK AEIEYLEK TLPFSR SYYWIGIR K IGGIWTWVGTNK SLTEEAENWGDGEPNNK . . . **8-15aa**	+ P - C, -M, -W Hydrophilic Immunogenic - PTM? - SNP Unique to protein	Ionizable? Best frags	MS1 MS2

- 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

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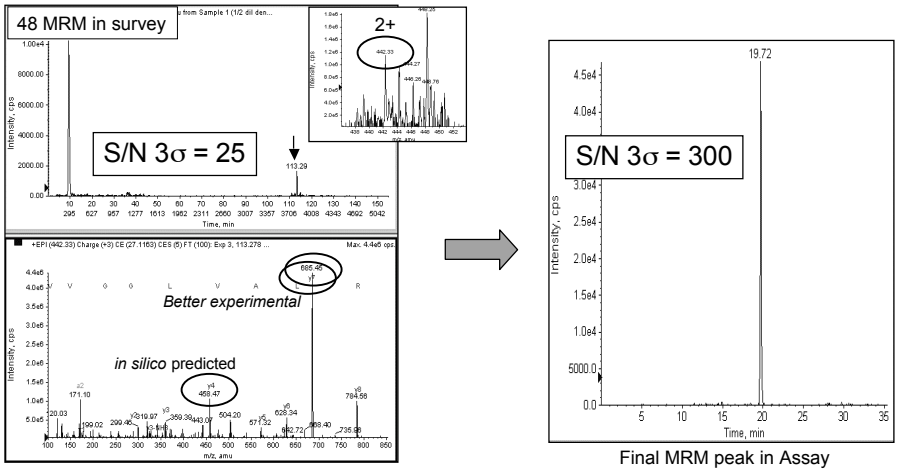
MIDAS™ Workflow for Better MRM Design

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



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

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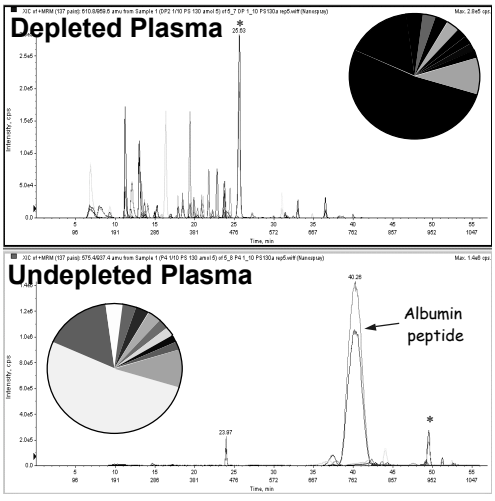
Depletion of Major Proteins Improves S/N for Most MRM's



- Equivalent amounts of plasma on column (0.01 uL)
- Depletion of six of the most abundant proteins in plasma (albumin, IgG, IgA, transferrin, haptoglobin, and antitrypsin) improves chromatography and detection of lower abundance proteins.
- S/N improves ~5-10x for signals of lower abundance proteins in depleted plasma

Collaboration with Christie Hunter (ABI) using 4000 Q TRAP MS

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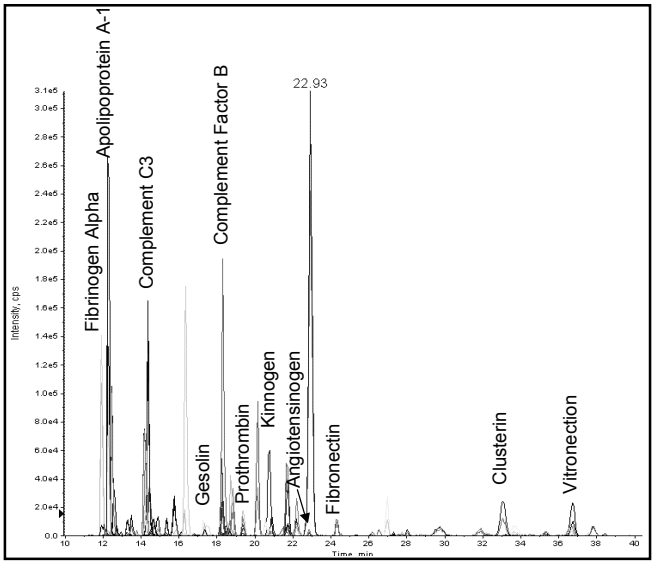
Peptide MRMs to Protein Biomarkers



- Method monitors 137 MRM transitions
- Data generated in depleted/digested plasma, 0.01μL loaded directly on nanocolumn
- Signal / Noise good even in very complex mixture in a short (30 mins) nanoLC run

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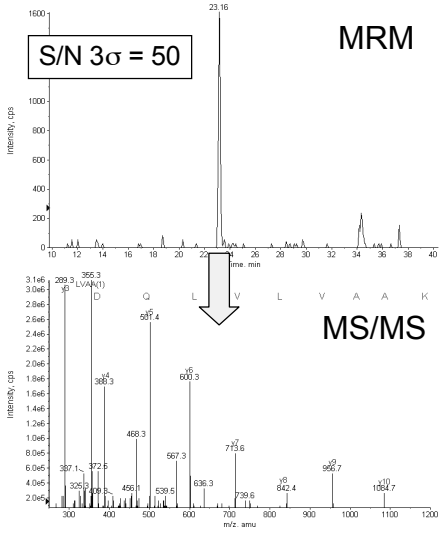
Absolute Structural Confirmation of Peptide ID

Full MS/MS scan triggered by MRM peak



- MIDAS™ workflow to increase confidence in peptide MRM, confirm retention time
- Survey experiment consists of all MRM transitions, detection of signal drives acquisition of MS/MS to confirm ID
- ALQDQLVLVAAK of Angiotensin identified
  - Present at 20116 amol/uL in plasma
  - 200 amol on column as 0.01uL plasma equivalent loaded

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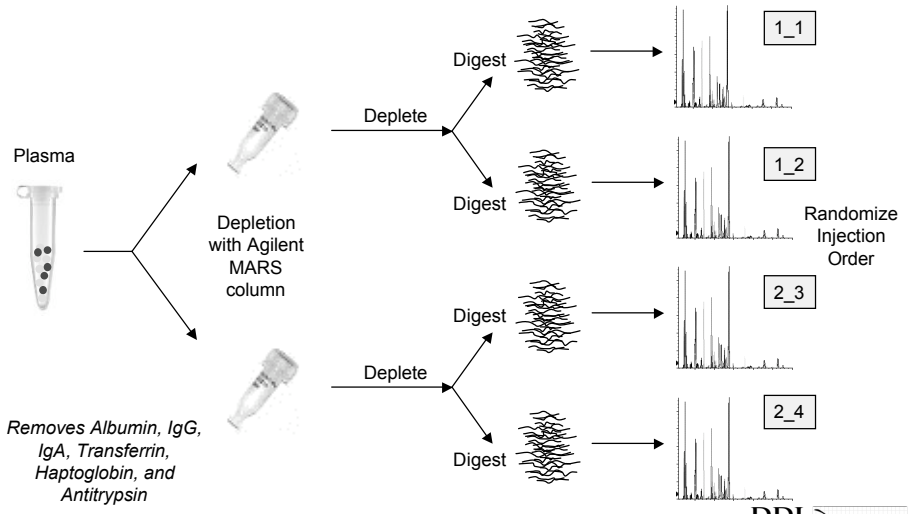
Collaboration with Christie Hunter (ABI) using 4000 Q TRAP MS

Evaluating the Reproducibility of Depleted and Digestion



Collaboration with Christie Hunter (ABI) using 4000 Q TRAP MS

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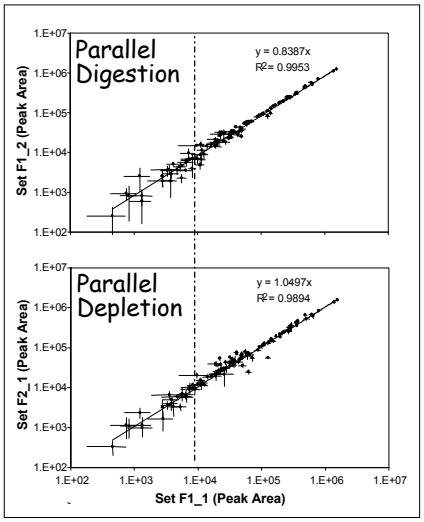
Correlation Between Parallel Depletion and Digestion



Excellent correlation between parallel digests, R^2=0.995 and 0.998

Parallel depletions (which include the effects of parallel digestions) also show good correlation, R^2=0.989 and 0.991

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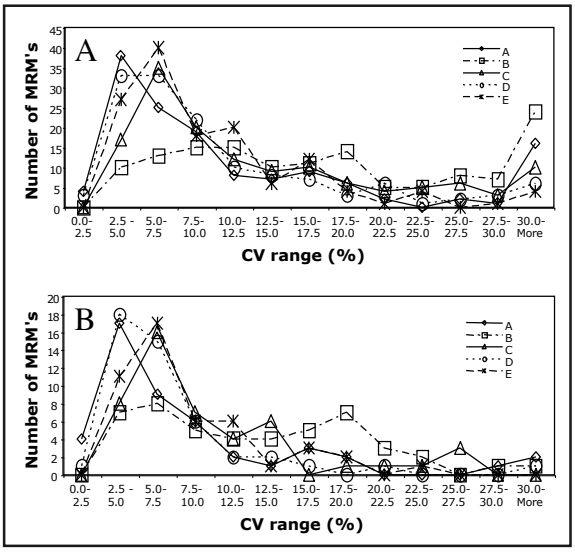
CV's of MRM Protein Assays



A) 137 MRM's

- CV's from 5 experiments, each of 10 replicate runs

B) 47 "best" MRM's

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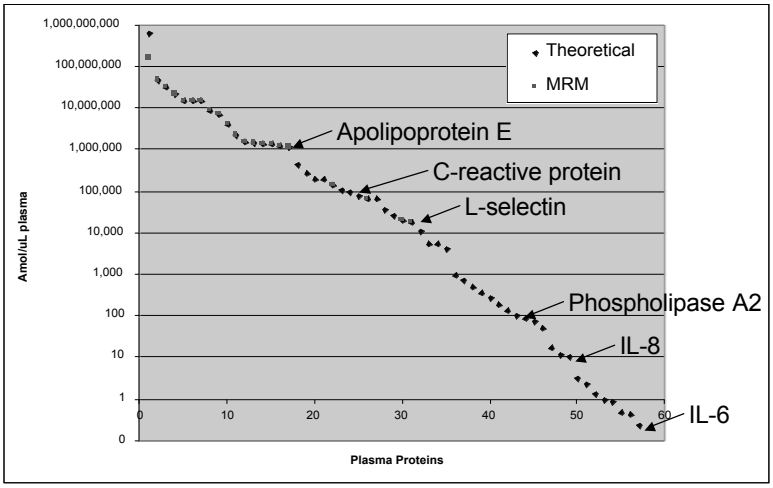
Dynamic Range of Current MRM Validation Method – Depleted Plasma



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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

KATEHLSTLSEKNWGLSVYADKPETTKI
LGGHLDAKDTVQIHDITGKTVIGPDGH
KQGFGNVATNTDGKEIGELYLPKTGLQ
EVEVKDDLYVSDAFHKIYHSHIDAPKET
AASLLQAGYKITQVLHFTKFPEVDVLTK
LGNQEPGGQTALKLSSPAVITDKQWAG
LVEKIPPWEAPKLFLEPTQADIALLKSHA
PEVITSSPLKIFYNQQNHYDGSTGKEHS
SLAFWKVSVSQTSKESDTSYVSLKWEL
DLDIKSTVLTIPEIIIKLIENGYFHPVKAS
YPDITGEKDPPSDLLLLKALQDQLVLVA
AKAEIEYLEK

↓

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

* Patent Pending

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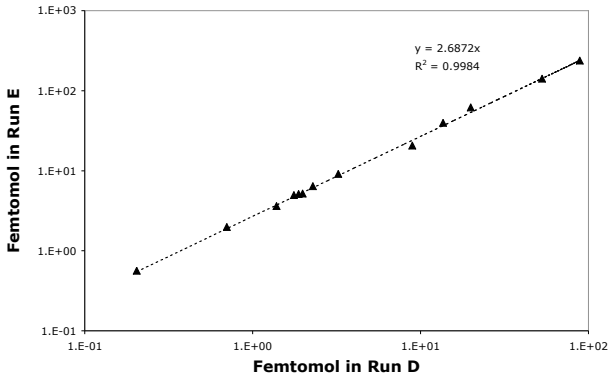
Comparing Peptide:Internal Standard Ratios for 14 Proteins in Two Experiments



Expt D
N=10 reps
10nl load

Expt E
N=10 reps
33nl load

Collaboration with Christie Hunter (ABI) using 4000 Q TRAP MS

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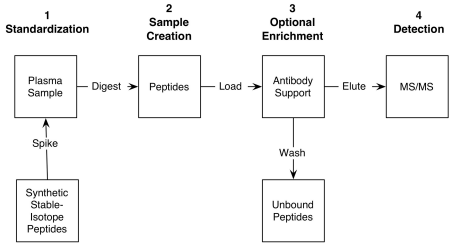
Sensitivity Enhancement via Specific Capture

Employ MS/MS assay as "2nd antibody" with absolute specificity
Add specific capture step, e.g., using anti-peptide antibody
- Demonstrated 10^2-fold sensitivity improvement with polyclonals
- Up to 10^5-fold enhancement expected with monoclonal Ab's

Forgiving of Ab performance
- Complete specificity not required (MS does this)
- Ab does not quantitate (stable isotope standards + MS do this)

SISCAPA is an immunoassay in which the second (detection) antibody is replaced by a mass spectrometer. This provides:
- •Increased assay specificity (absolute structural specificity of MS/MS)
- •High sensitivity (low fmol- high amol at peptide level)
- •Lower cost (one Ab instead of two)
- •Lower analyte-analyte interference (and greater sample stability) by digesting proteins to peptides



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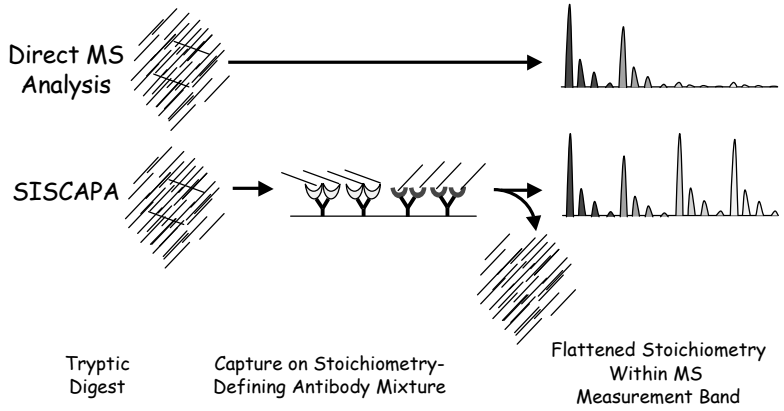
SISCAPA Captures Similar Amounts of Peptides, Flattening the Abundance Distribution to Accommodate MS Dynamic Range



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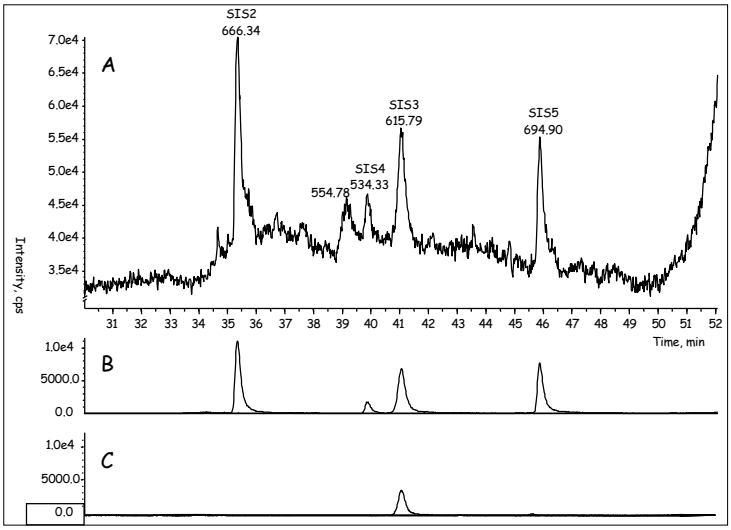
Ab Capture from SIS Peptide Mixture with Selected Ion Monitoring (SIM)



Total ion current from a sample of mixed peptides

Ion current in 4 SIM channels (4 SIS peptide masses)

Ion current in 4 SIM channels after binding and elution from Ab3

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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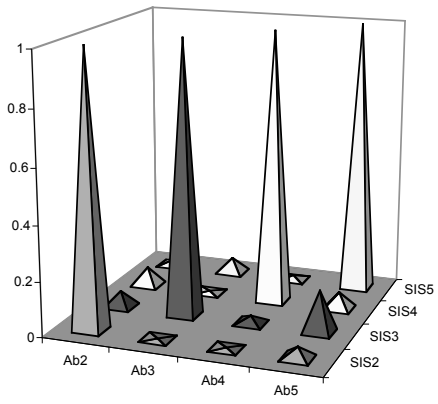
Relative Quantities of Four SIS Peptides Bound by Four Anti-Peptide Antibodies, Using Two-stage MS Selection (SRM)
Average Peptide Enrichment by Ab > 100-fold



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

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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Critical Objectives for Biomarker Proteomics

- Progress candidate biomarkers into large-scale validation testing
  - Bring reliable, quantitative proteomics to the clinical and epidemiology communities
- Generate unequivocal successes: biomarkers that transform clinical treatment of patients
- Answer basic biological questions:
  - Are there plasma biomarkers defining any given disease state? (i.e., resolve the biomarker hypothesis)
  - If panels are required in the general Dx case, how many protein components are usually required?
- Transform healthcare economics by providing fast, cheap, accurate diagnosis

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Acknowledgements

SISCAPA Experiments
Bob Olafson, Derryl Hardy, UVIC-Genome B.C. Proteomics Centre
Terry Pearson, Lee Haines, 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, present address: 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, Applied Biosystems, Foster City
MALDI TOF/TOF
Glen Hortin, Alan Remaley, Steve Drake, Gerry Hoehn, Clinical Chemistry Laboratory, NIH Clinical Center
Graphics
Arkitek Studios, Seattle

Grant Funding
National Cancer Institute (contract # 23XS144A)

www.plasmaproteome.org

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Recent Relevant Papers

The human plasma proteome: History, character, and diagnostic prospects. Anderson, N.L. and Anderson, N.G., Molecular and Cellular Proteomics, 1.11, 845-867 (2002)

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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).

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