Developing Peptide MRM-based Assays for Cardiovascular Biomarker Proteins in Plasma Using a Hybrid Triple Quadrupole Linear Ion Trap Mass Spectrometer

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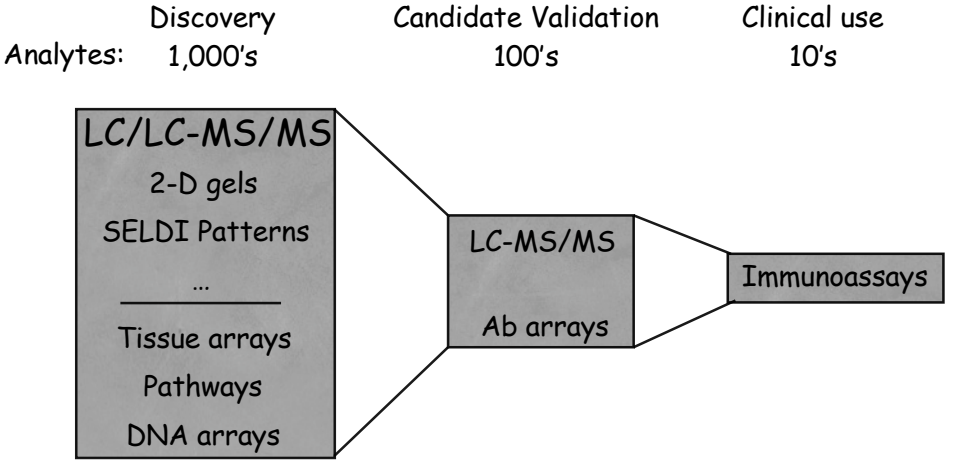
Biomarker Pipeline



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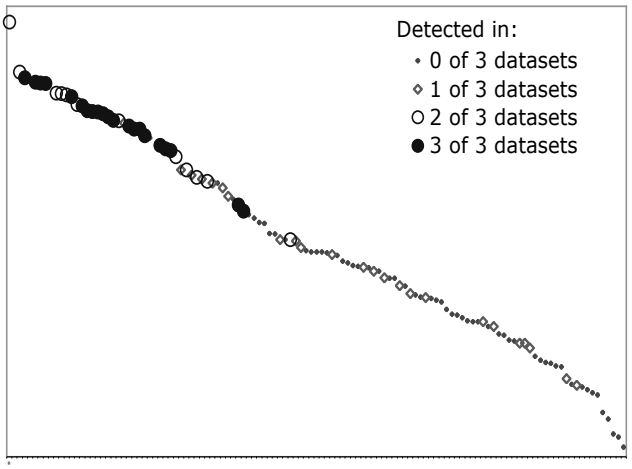
Plasma Proteome Discovery Platforms Have Limited Sensitivity and Lack Comprehensiveness



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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Role for Candidate-Based Proteomics

- Multiplexed specific assays (candidate approach) can address three important drawbacks of biomarker discovery platforms for the middle pipeline stage (verification/validation):
  - Throughput
  - Sensitivity
  - Coverage (range of analytes)
- Candidate-based approaches forego most of the potential for discovery of new biomarkers, but retain some ability to "discover" optimal multi-analyte panels.
- Our purpose is to develop an MS-based candidate approach for high-throughput biomarker verification/validation in human plasma and serum

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Published List of 177 CVD Candidates

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

Table 1. A table of 177 candidate markers of cardiovascular disease (CVD) and stroke, assembled through literature search

	Name	Accession	Normal concentration (pg ml^{-1})	Source for concentration	Reason	Coagu-lation	Lipo-protein	Acute Phase
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	(Specialty 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	(Specialty Laboratories, 2001)	Major plasma protease inhibitor			
7	alpha(1)- anti-chymotrypsin (ACT)	P01011	4.2E +07	(Putnam, 1975)	Major plasma protease inhibitor			+

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In Silico Selection of MRM Peptides

One or more tryptic peptides used as quantitative surrogates for the protein ("monitor" peptide concept)

"Inside every bad protein there is at least one good peptide"

Began with 29,155 peptides from "mature" protein forms (21,609 unique)

Downloaded SP annotation & computed parameters

Looked for occurrence in Pounds exptl data set

Ranked peptides on composite index of desirable properties

Term	Explanation
asn * -2 +	Susceptible to deamidation, which would change the mass of a pe containing it.
gln * -2 +	"
met * -3 +	Susceptible to oxidation, which would change the mass of a peptid containing it.
trp * -3 +	"
cys * -10 +	"
pro * 5 +	Produces enhanced peptide structure and can improve immunoge
kp * 2 +	Results in an additional positive charge in a peptide
rp * 2 +	"
dp * 5 +	Introduces a site of efficient gas-phase fragmentation and improve detection
chymc_sites * -3 +	Peptide more likely to be degraded by chymotryptic activity
occurrences_in_protein * 2 +	Multiply occurring peptide would give better detection
carbohyd * -50 +	Glycosylation of a peptide changes its mass and decreases MS de
mod_res * -50 +	Any documented chemical modification of a residue in the peptide changes its mass
conflicts * -20 +	Any potential sequence errors in the peptide decrease its usefulne MS analyte
variants * -10 +	Any genetic variants in the peptide decrease its usefulness as an M analyte by restricting its use to a subset of patients
sign(calc_mass-800)*100 +	Applies a negative value to mass less than 800
sign(1800-calc_mass)*100 +	Applies a negative value to mass greater than 2,000
sign(detect - 0.0001) * 10 + detect * 20 +	Gives 10 points for detection in the Pounds experimental MS/MS d 30 if the peptide is the most frequently detected for its protein (dete aforementioned index of detectability)

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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	carbahyd
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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Additional MRM's Developed Based on Experimental Data (35 Additional Proteins)

- Beavis GPM website (historical LC-MS/MS data on plasma)
- Classical IDA analysis of plasma digest
- MRM-triggered IDA to find additional proteins on CVD panel that were not found by normal IDA

Final Method

- 137 MRM's
- 17 stable isotope peptides as IS
- 52 proteins monitored
- 60 peptides, two transitions each
- Includes 40 cardiovascular markers

Analytical Platform

- ABI/Sciex 4000 QTRAP
- 75u LC Packings C18 column, 250nl/min

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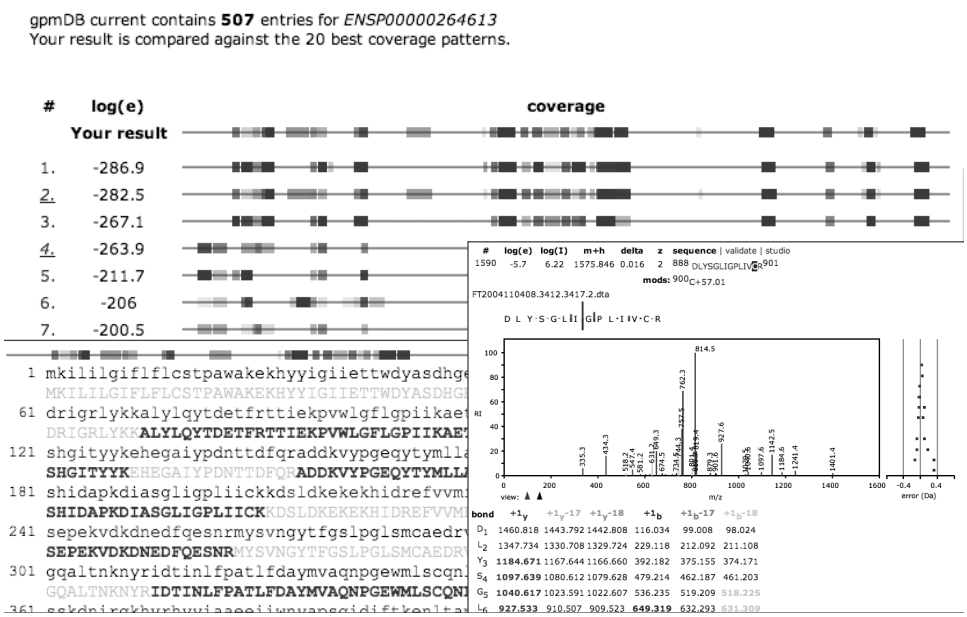
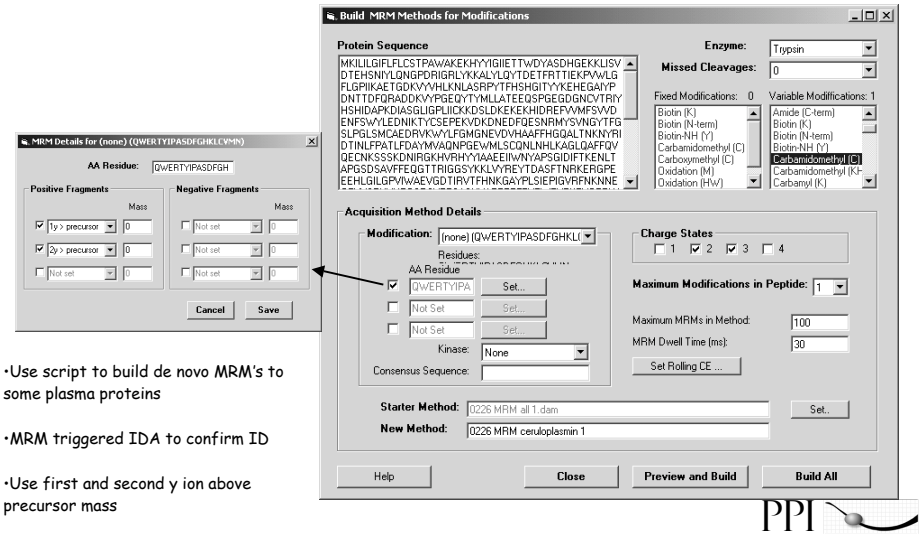
Beavis' GPM Useful in MRM Design



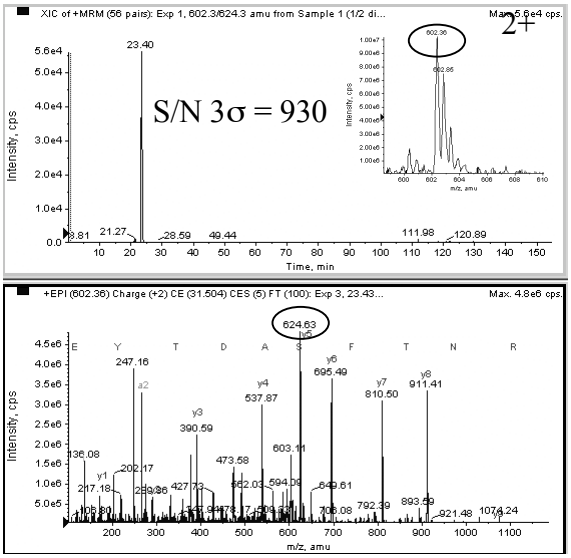
MRM-Triggered IDA to Develop Peptide MRM Transitions

(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

Example Evidence for Ceruloplasmin (8 peptides found)



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MRM's Selected for polySIS CVD_1 Peptides

Order	Protein	Peptide	Unlabeled Q1	Unlabeled Q3	Labeled Q1	Labeled Q3	CE
1	Apolipoprotein A-I	ATEHLSTLSEK	405.88	664.35	408.54	672.35	25
2	Alpha-1-acid glycoprotein 1	NWGLSVYADKPETTK	570.29	1052.53	575.61	1068.53	35
3	Haptoglobin beta chain	ILGGHLDAK	462.26	697.36	466.26	705.36	31
4	Fibrinogen gamma chain	DTVQIHDITGK	409.54	670.35	412.21	678.35	23
5	Fibrinogen alpha chain	TVIGPDGHK	462.25	723.38	466.24	731.38	25
6	Fibrinogen beta chain	QGFGNVATNTDGK	654.80	706.34	658.80	714.34	30
7	Alpha-1-antichymotrypsin	EIGELYLPK	531.29	819.46	535.29	827.46	26
8	Complement C3	TGLQEVEVK	501.77	731.39	505.77	739.39	25
9	Antithrombin-III	DDLYVSDAFHK	437.21	803.40	439.87	811.40	27
10	Ceruloplasmin	IYHSHIDAPK	394.20	767.40	396.90	775.40	25
11	Prothrombin	ETAASLLQAGYK	626.33	879.49	630.33	887.49	32
12	Complement C4 gamma chain	ITQVLHFTK	362.88	645.37	365.55	653.37	30
13	Apolipoprotein B-100	FPEVDVLTK	524.28	803.45	528.28	811.45	25
14	Alpha-2-antiplasmin	LGNQEPGGQTALK	656.84	771.44	660.84	779.44	40
15	Plasminogen	LSSPAVITDK	515.79	743.43	519.79	751.43	25
16	Apolipoprotein E	QWAGLVEK	465.75	616.37	469.75	624.37	28
17	Coagulation factor XIIa heavy chain	IPPWEAPK	469.25	727.38	473.25	735.38	27
18	Apolipoprotein(a)	LFLEPTQADIALLK	786.45	1069.63	790.45	1077.63	33
19	Coagulation factor X	SHAPEVITSSPLK	455.92	632.36	458.58	640.36	28
20	Adiponectin	IFYNQQNHYDGSTGK	591.27	727.33	593.94	735.33	30
21	Beta-2-glycoprotein I	EHSSLAFWK	552.77	838.45	556.77	846.45	23
22	Coagulation factor IX	VSVSQTSK	418.22	736.38	422.22	744.38	24
23	C-reactive protein	ESDTSYVSLK	564.77	696.39	568.77	704.39	29
24	Vitamin K-dependent protein C	WELDLDIK	516.27	716.42	520.27	724.42	25
25	Coagulation factor XIII A chain	STVLTIPEIIIK	663.91	712.46	667.91	720.46	26
26	Coagulation factor XIII B chain	LIENGYFHPVK	439.57	847.45	442.23	855.45	25
27	Cholesteryl ester transfer protein	ASYPDITGEK	540.76	759.39	544.76	767.39	30
28	Coagulation factor V	DPPSDLLLLK	555.82	898.56	559.82	906.56	30
29	Angiotensinogen	ALQDQLVLVAAK	634.88	956.58	638.88	964.58	34
30	L-selectin	AEIEYLEK	497.75	794.43	501.75	802.43	22

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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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Absolute Protein Quantitation in Relation to polySIS* Peptide Standards

polySIS CVD_1 (lys-labeled standard)

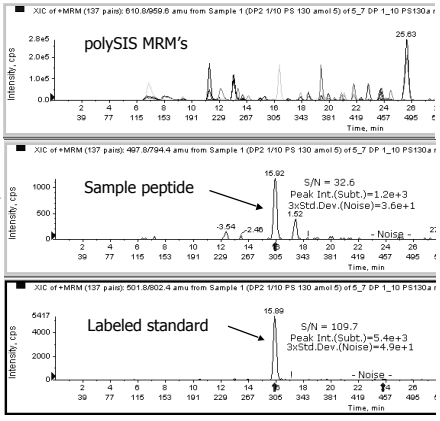
ATEHLSTLSEKNWGLSVYADKPETTKILGG
HLDAKDTVQIHDITGKTVIGPDGHKQGFG
NVATNTDGKEIGELYLPKTGLQEVEVKDDL
YVSDAFHKIYHSHIDAPKETAASLLQAGYKI
TQVLHFTKFPEVDVLTKLGNQEPGGQTALK
LSSPAVITDKQWAGLVEKIPPWEAPKLFLE
PTQADIALLKSHAPEVITSSPLKIFYNQQNH
YDGSTGKEHSSLAFWKVSVSQTSKESDTS
YVSLKWELDLDIKSTVLTIPEIIIKLIENGYF
HPVKASYPDITGEKDPPSDLLLLKALQDQL
VLVAAK**AEIEYLEK**

sL-Selectin (in sample)

WTYHYSEKPMNWQRARRFCRDNYTDLVAI
QNK**AEIEYLEK**TLPFSRSYYWIGIRKIGGI
WTWVGTNKSLTEEAENWGDGEPNNKKNK
EDCVEIYIKRNKDAGKWNDDACHKLKAAL
CYTASCQPWSCSGHGECVEIINNYTCNCD
VGYYGPQCQFVIQCEPLEAPELGTMDCTHP
LGNFSFSSQCAFSCSEGTNLTGIEETTCGPF
GNWSSPEPTCQVIQCEPLSAPDLGIMNCSH
PLASFSFTSACTFICSEGTELIGKKKTICESS
GIWSNPSPICQKLDKSFSMIKEGDYN

Digest

***LC-TQMS of* AEIEYLEK**



* Patent Pending

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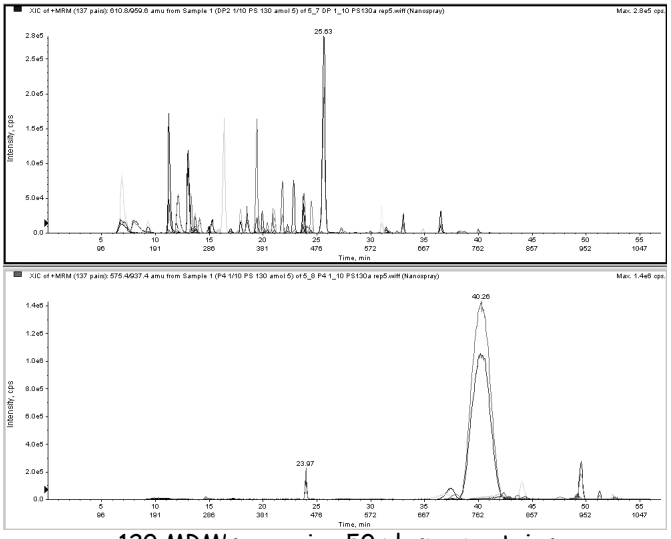
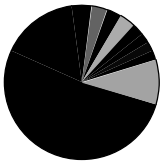
Subtraction of Top 6 Proteins (albumin, IgG, IgA, haptoglobin,transferrin and antitrypsin) Using Agilent MARS Column

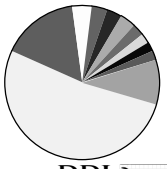


120 MRM's covering 50 plasma proteins

Depleted Plasma Digest of 0.01 uL plasma on column



Whole Plasma Digest of 0.01 uL plasma on column



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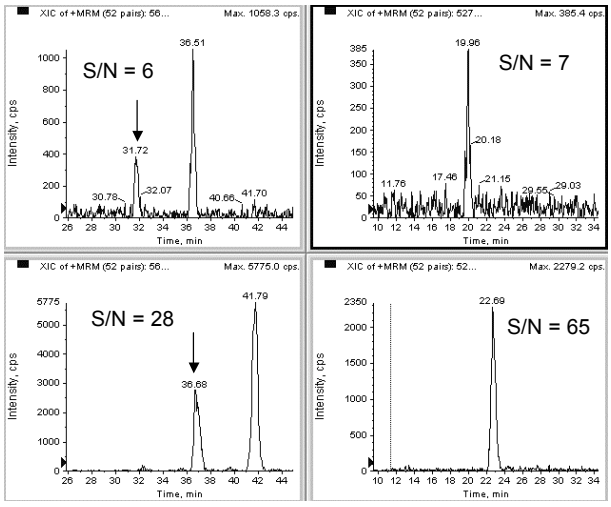
Immunosubtraction of Top 6 Proteins Yields 5-9-fold Improved S/N

Afamin peptides

LPNNVLQEK : 527.8/844.5 DADPDTFFAK : 563.8/825.4



Plasma

Depleted Plasma

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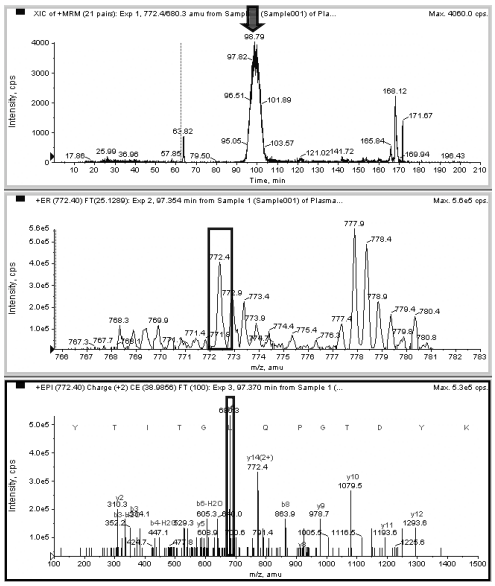
Fibronectin
SYTITGLQPGTDYK
1543.8

Fibronectin, a protein of much lower normal abundance (1.4μg/ml) could be measured using peptide SYTITGLQPGTDYK (selected *in silico*) using the transition 772.4/680.3 with S/N of 170, suggesting an LLOQ of ~100ng/ml.



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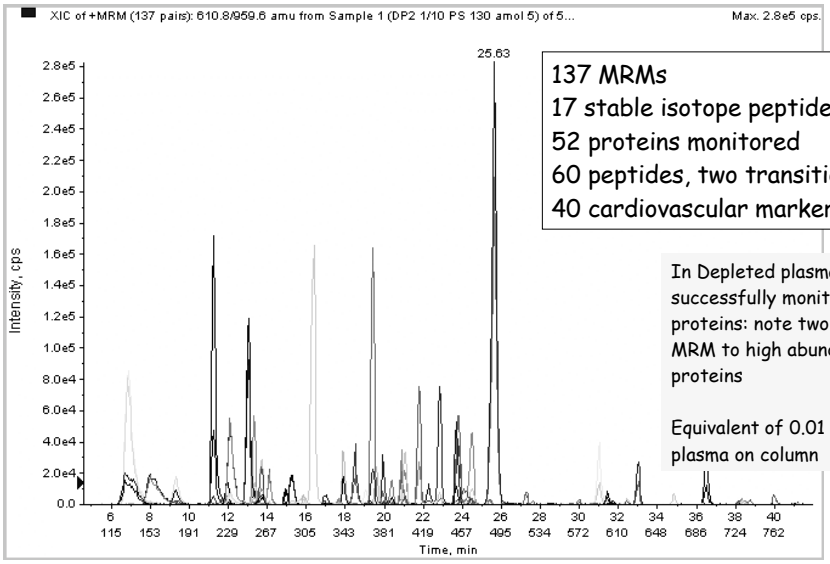
Current MRM Method



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

In Depleted plasma, successfully monitored 48 proteins: note two were MRM to high abundance proteins

Equivalent of 0.01 uL of plasma on column

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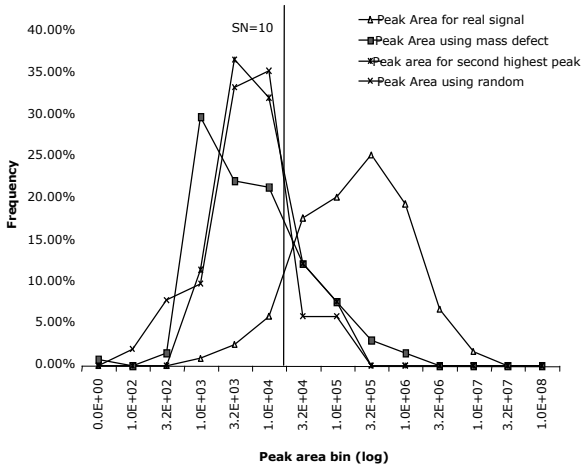
Designed vs "Random" MRM's

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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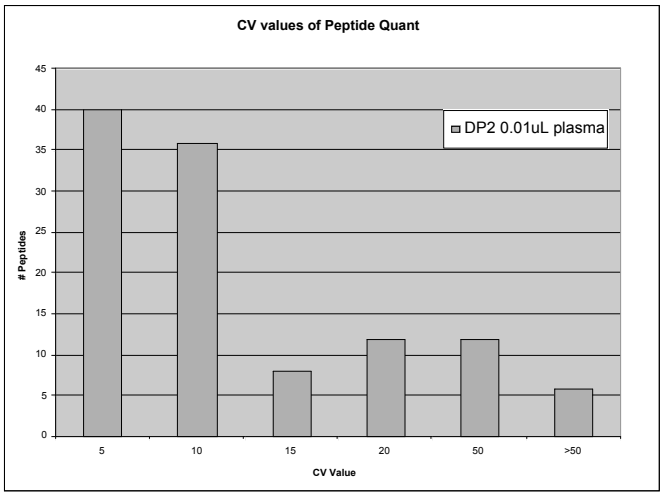
Reproducibility of MRM Panel in Depleted Plasma



Avg CV with IS 7%
Avg CV overall for good peaks 9.5%

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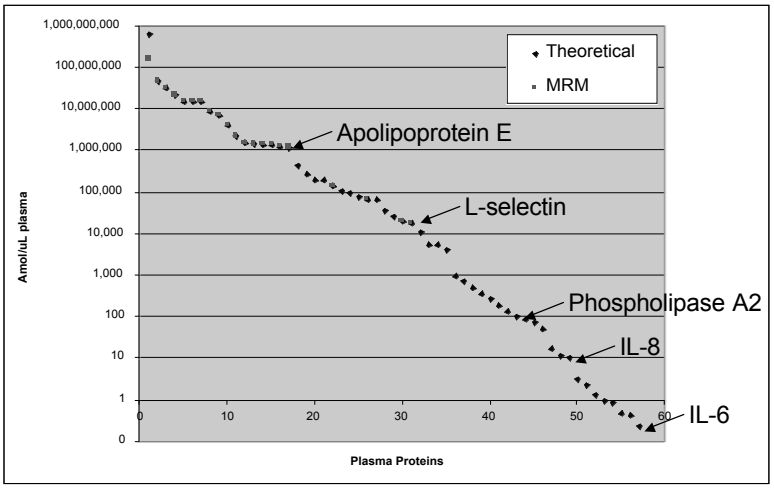
Dynamic Range of Current Method in Depleted Plasma: ~4.5 Orders



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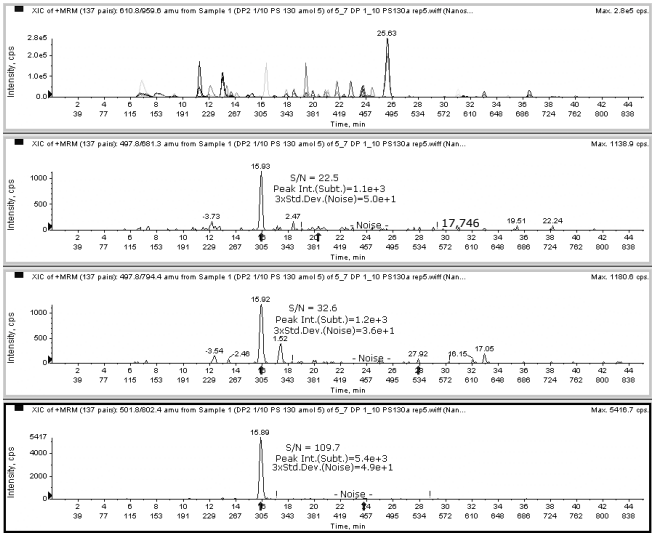
Dynamic Range of Current Method Detection of L-selectin



Peak area ratio for the L-selectin monitor peptide (AEIEYLEK 497.8/794.4) and SIS (501.8/802.4) standard was 0.216.

Given 1,300 amol SIS loading (one copy of this peptide per molecule of intact L-selectin) = 280 amol L-selectin per 0.01ul injection.

Molecular weight of plasma L-selectin is ~35,000, giving a measured concentration of 980 ng/ml vs published normal value of 670 ng/ml.

The dynamic range between albumin (~55mg/ml), and L-selectin is ~4.5 orders of magnitude

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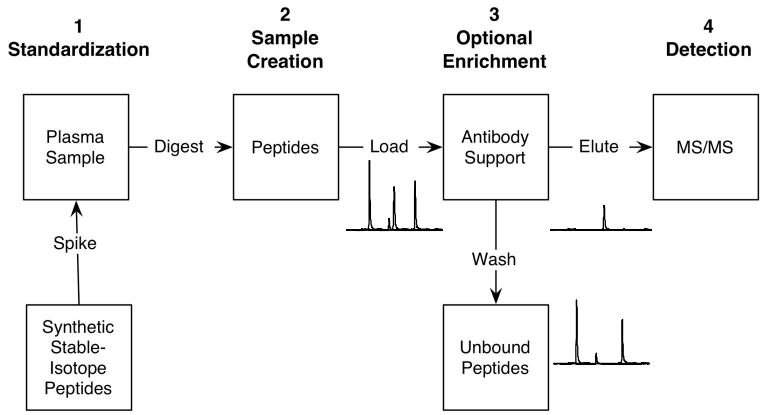
SISCAPA*: A Method Combining The Specificity of MS Detection with Sensitivity of Antibody Capture

(SISCAPA = Stable Isotope Standards with Capture by Anti-Peptide Antibody)



* patent pending

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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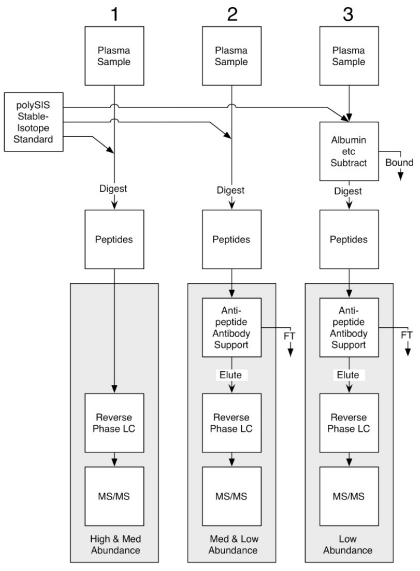
Strategies for Progressive Increases in Sensitivity of LC-MS Peptide Quantitation



Our approach generates a coherent, layered series of methods based on a single analytical platform (TQMS), with an explicit sensitivity *vs* cost tradeoff

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Conclusions

- Large numbers of candidate biomarkers already exist to jumpstart verification/validation, and from which improved panels could be constructed
- MRM assays of monitor peptides offer a potentially rapid path to verification/validation with less cost and effort than sandwich immunoassays
- Optimal MRM design makes use of both *in silico* and experimental data
- Current MRM's appear able to access the top 5 logs of plasma protein abundance, and cover everything visible on a plasma 2-D gel
- Novel paths to creation of internal standards (e.g., polySIS proteins) can facilitate assays development
- Marker panel development and validation in large sample sets (e.g., epidemiological studies) now appears feasible

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SISCAPA Experiments
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www.plasmaproteome.org PPI

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)

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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