Quantitation of Peptides and Proteins by Mass Spectrometry

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Quantitation of peptides and proteins by Mass Spectrometry- Why?

- Just because we can?
- Quicker to develop than immunoassays- when no antibodies are required
- Multiplexing capabilities
- Allows us to do things that immunoassays don't
  - Better selectivity (vs. RIA)
  - Measure intact peptides and proteins (vs. presence of epitopes in immunoassays)
  - Confirm immunoassays results
- Cons:
  - Sensitivity?
  - Dynamic range?
  - Robustness/throughput?

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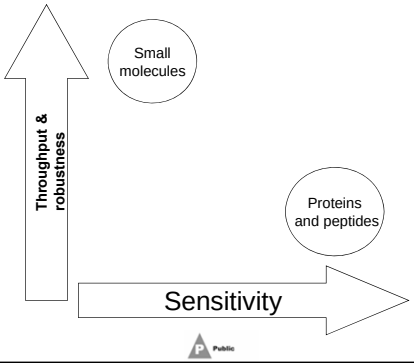
Sensitivity vs. Throughput and Robustness



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High vs. Low Flow LC Methods

	Nano	Cap	High
Flow rate (uL/min)	<1	5-20	300-1000
Speed	slow	medium	fastest
Robustness	variable	good	excellent
Sensitivity	best	good	least
Column availability	limited	poor	excellent

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How do we increase sensitivity in mass spec methods?

- Increase signal and reduce background

- More sensitive instruments (increase signal)
- Cleaner samples (reduce background)
- Better chromatographic separations (reduce background)

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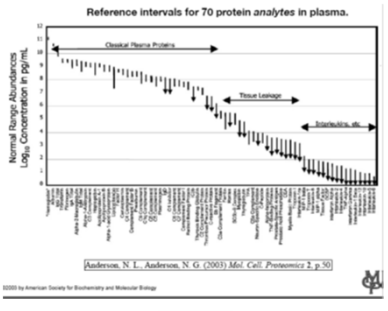
Dynamic range of plasma proteins



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Quantitation of Peptides

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Peptide analysis by mass spec

- <10 Kda
- Direct measurement: no digestion required
- Sample clean up: protein precipitation, SPE, IP
- Analysis: nano or capillary flow

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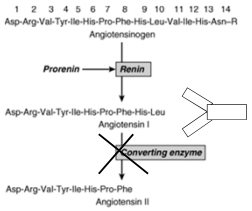
Plasma Renin Activity (PRA) assay



PRA= ng AngI/mL/h

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PRA: sample preparation

Protein Precipitation (PPT)

Reaction mix + I.S. (13C and 15N labeled Ang I)

↓

Protein ppt with 10% ACN, 0.1% FA

↓

Collect supernantant and Evaporate to dryness

↓

Resuspend in 50 uL 10% ACN w/ 0.1% formic acid

↓

LC-MS/MS analysis

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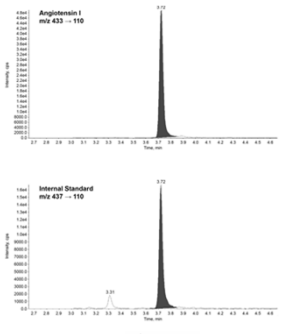
PRA: Ang I chromatograms



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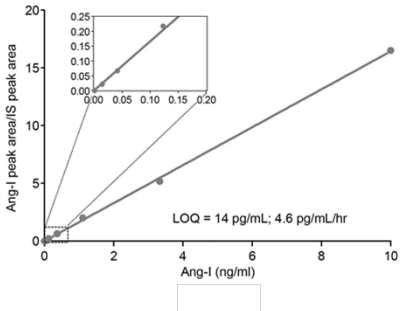
PRA: Standard curve



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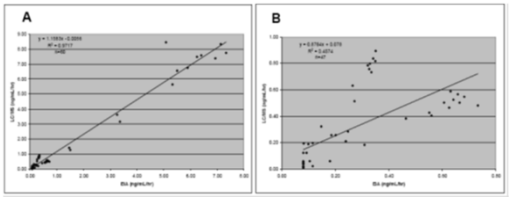
PRA: EIA vs. Mass Spec

- EIA LLOQ = 2 ng/ml
- Required a 24 h incubation
- Mass Spec-based assay LLOQ = 14 pg/ml (140x more sensitive)
- Allowed a 3 h incubation



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PRA: Precision

Inter-assay

Ang-I ng/mL/hr

	L	M	H
Mean	0.038	0.154	0.887
Std Dev	0.004	0.013	0.064
%CV	9.38	8.31	7.22
n	5	5	5

Intra-assay

Ang-I ng/mL/hr

	Sample A	Sample B
Mean	0.67	1.37
%CV	1.6	8.3
n	6	6

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PRA: Spike recovery

	+0.15 ng/mL Ang I		
Ctrl Plasma	**Theoretical**	**Observed**	**% Recovery**
0.747	0.897	0.905	105

	+0.90 ng/mL Ang I		
Ctrl Plasma	**Theoretical**	**Observed**	**% Recovery**
0.747	1.647	1.580	93

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PRA: Dilution Linearity

Dilution Factor	Ang I Concentration (ng/mL)	% Change from Undiluted
Undiluted	16.6	-
2	14.4	-13.3
4	16.3	-1.8
8	17.9	7.8
16	19.3	16.3
32	18.1	9.0
64	17.0	2.4

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PRA: Biological Variability

Subject	Endogenous Ang I (ng/mL)	3hr Generation Ang I (ng/mL)	% Endogenous Ang I	PRA (ng/mL/hr)
1	0.23	2.20	10.5	0.73
2	0.10	1.44	6.7	0.48
3	0.16	2.01	8.1	0.67
4	0.14	1.98	7.1	0.66
5	0.41	4.14	10.0	1.38
mean	0.2	2.4	8.5	0.8

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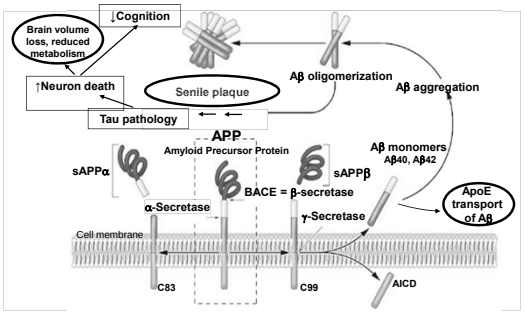
β amyloid proteins and Alzheimer's disease



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Mass Spec for β amyloid peptides

- Multiple immunoassays- Lack of standarization
- The Alzheimer's Association has established the Global Consortium for Biomarker Standardization to gather key researchers, clinicians, and industry, regulatory and government leaders in Alzheimer's disease to achieve consensus on the best ways to standardize and validate biomarker tests for use in clinical practices around the world.
- The Reference Materials Working Group
- The Reference Methods Working Group

- Mass spec-based method chosen as the gold standard
- 1 CRO, 2 academic centers and 1 mass spec manufacturer presented methods for the Aβ42
- LOQ as low as 50 pg/mL
- Run time as low as 8 minutes
- SPE, no immuno-purification involved in sample preparation

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Common issues with peptide quantitation

- Stickiness: binding to tubes, lines, etc.
- Chemical instability
- Optimization of sample extraction

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Quantitation of Peptides

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Analysis of proteins by mass spec

- Proteins >10 Kda
- Most common method using QQQ analysis is indirect measurement (digestion required)

- Digestion → direct measurement
- Immunocapture → digestion
- Digestion → immunocapture (SISCAPA)
- Immunodepletion of most abundant plasma proteins digestion →

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Protein Flux

- Patients are given an infusion of isotope labeled amino acid that is incorporated into newly synthesized proteins
- Requirements for protein flux:
  - Sensitivity: need to measure with high accuracy incorporation of label into only a small percentage of total protein
  - Robustness: need to measure 100s or 1000s of samples with reasonable throughput.

- In order to make these measurements we need:
  - Optimized sample preparation
  - Optimized capillary flow chromatography
  - Sensitive Instrumentation (mass spectrometers)

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Protein flux

- Protein A: ~ 1 μg/mL

- Protein B: ~ 100 ng/mL

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Protein flux sample preparation

0.5mL plasma in a 96-well deepwell plate using a TECAN liquid handling robot

↓

Immunoaffinity beads for both Protein A and B

↓

Overnight (18-20h) incubaion at 4°C

↓

3 washes (1x RIPA buffer, 2x pBS)

↓

Elution in 10% formic acid

↓

Trypsin digestion

↓

nanoAcquity UPLC system (Waters) coupled with a TSQ Vantage QQQ mass spectrometer (Thermo) with a CaptiveSpray ESI source (Michrom)

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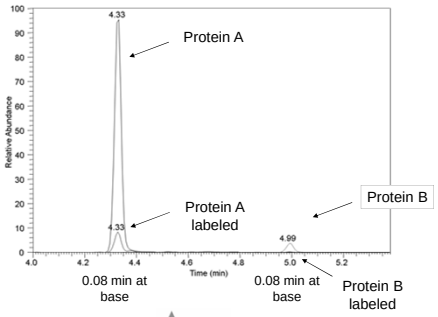
Protein Flux: IP from plasma



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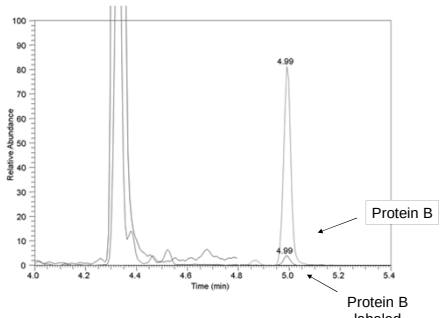
Protein flux: IP from plasma



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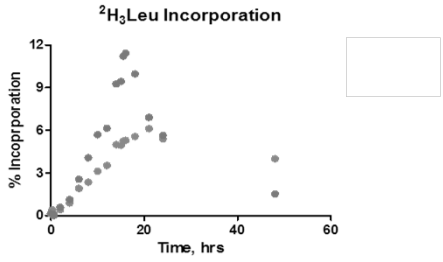
Incorporation of label into proteins



- The sensitivity achieved with this platform allows measurements of small changes in low abundant proteins

Protein flux: Precision

Protein A	Low	Medium	High
Mean (% labeled)	9.205	19.25	29.22
SD	0.315	0.434	0.998
%CV	3.42	2.25	3.42
n	6	6	4

Protein B	Low	Medium	High
Mean (% labeled)	3.99	19.74	36.3
SD	0.358	0.596	0.161
%CV	8.98	3.02	4.43
n	6	6	4

Protein flux: Biological variability

Protein A

Subject	Recovery (M3 peak height x10⁶)	Isotope ratio (M3/M0) %
1	13.6	9.1
2	23.0	9.6
3	13.9	8.9
4	17.2	9.0
5	20.9	8.7
6	38.2	8.7
7	30.3	9.6
8	24.2	9.3
9	64.2	9.1
10	37.7	8.8
11	39.0	9.3
12	33.4	8.6
13	20.4	9.0
14	37.5	9.4
15	39.7	9.0
16	47.7	9.0
17	21.3	9.2
18	43.1	9.1
19	34.7	8.9
20	24.8	8.8

Protein B

Recovery (M3 peak height x10⁶)	Isotope ratio (M3/M0) %
2.6	4.1
1.5	4.6
1.4	4.7
1.1	3.9
3.8	4.3
3.8	4.2
3.1	4.2
0.8	3.7
1.8	4.0
1.9	4.4
1.3	4.8
1.7	4.5
1.9	5.0
2.5	4.6
2.9	4.7
1.8	4.4
2.2	4.0
3.6	4.3
3.5	4.4
2.7	4.6

Protein flux Assay Statistics

- ~1500 samples and QCs
- <9 minutes per sample
- 10 days total analysis time
- 0 high pressure / column change / instrument clean

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Nano flow… an Art

- Nano flow columns and connections are often not optimized for usability
- Leaks are easy to achieve but often difficult to find/fix
- Small parts with very little room for error
- Set up is often difficult, especially for new users. Requires expert users

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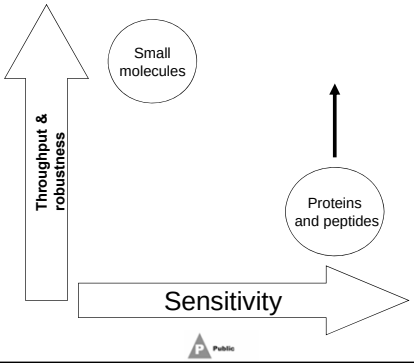
Sensitivity vs. Throughput and Robustness



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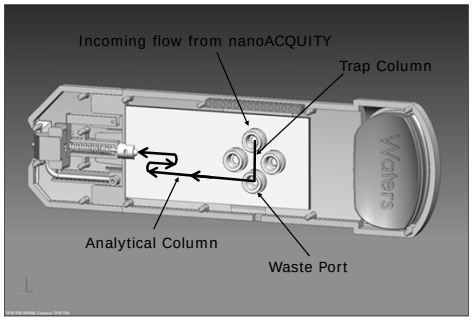
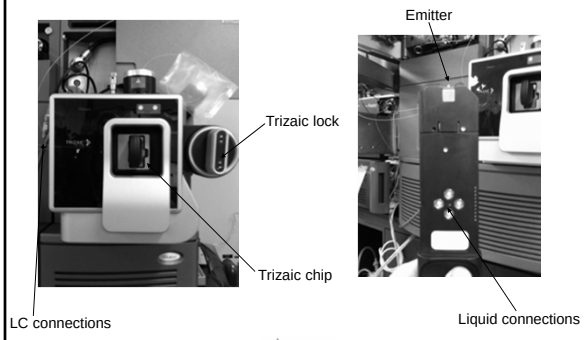
NanoTile device



Connections on TQS MS system



Conclusions:

- Mass Spectrometry is an important tool in the laboratory for the quantitation of peptides and proteins
- Technology still requires a hands-on approach
- Field is moving forward very rapidly
- Question:
  - Will mass spec eventually replace immunoassays?

Acknowledgements

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