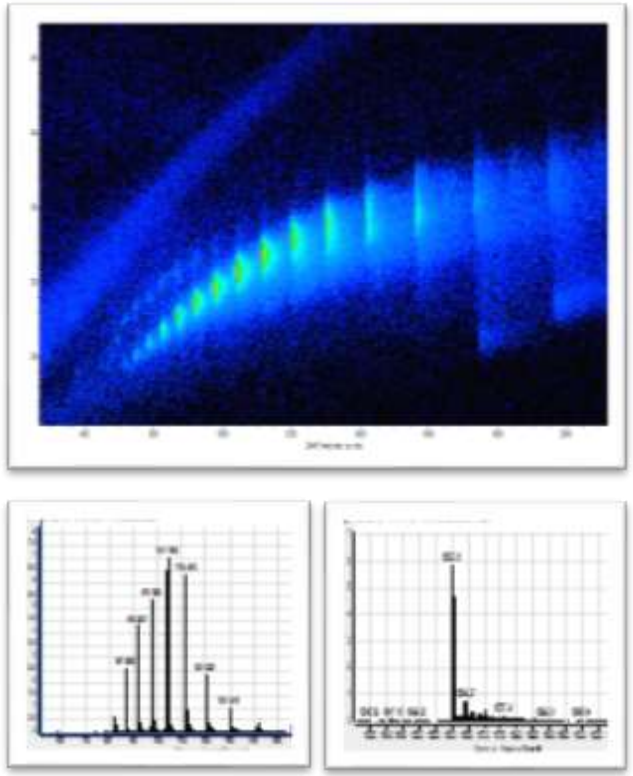
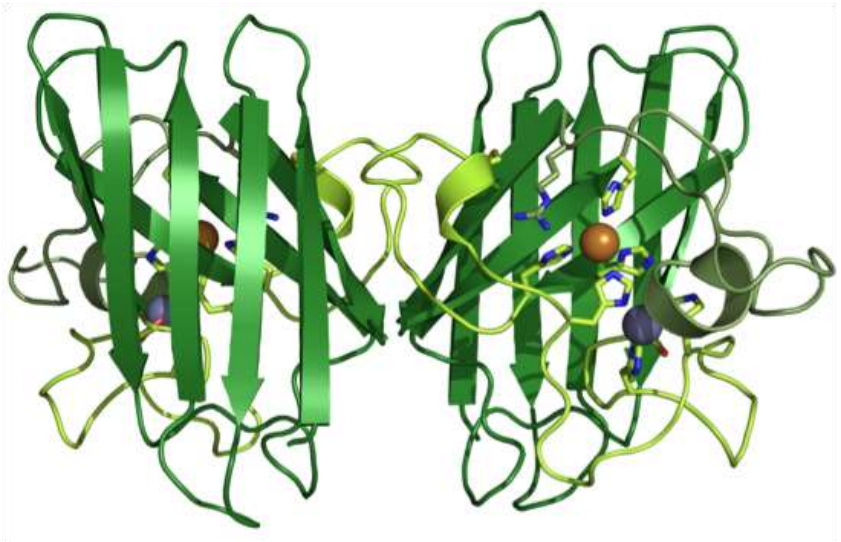
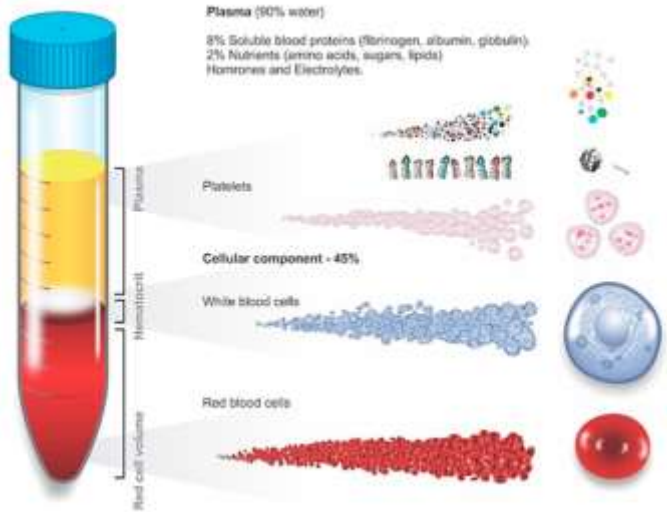
Comparison of Nano and Standard Flow Proteomics for Tissue and Plasma Samples







Blaine Roberts, PhD

Neuroproteomics Facility and Metalloproteomics Laboratory

Florey Institute of Neuroscience and Mental Health, University of Melbourne, Victoria, Australia

October 19th, 2017

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Disclosure

- My lab receives research support from Agilent for Australian Research Council Linkage grant (LP140100095)

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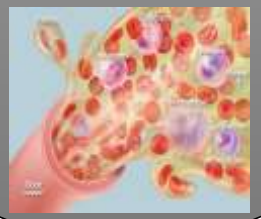
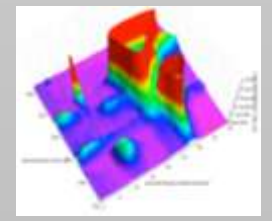
Blood Biomarkers

Alzheimer's, Parkinson's & Amyotrophic lateral sclerosis.



Metallo-proteomics

LC-ICP-MS
Q-TOF
QQQ



Functional Proteomics

Mechanism of Neurodegeneration

Amyloid beta
Alpha synuclein

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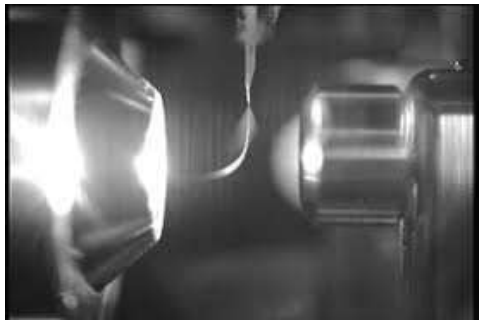
Compare Nano vs. Standard Flow for Bottom-up shotgun proteomics.

- Standard flow defined as LC column diameters of 1mm or greater with flow rates typically greater than 50µL/min.
- Nano column diameter <0.1mm and flow rates typically less than 1 µL/min

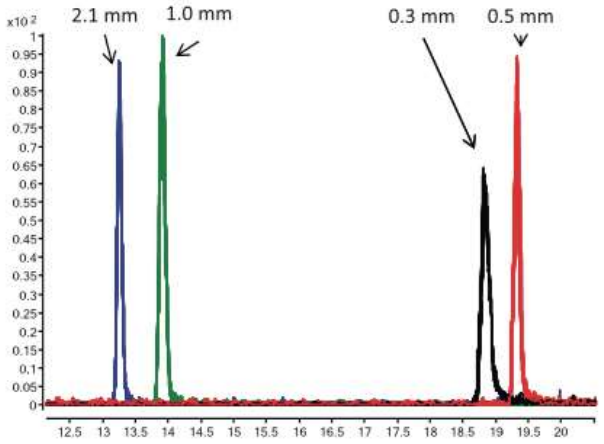
AJS

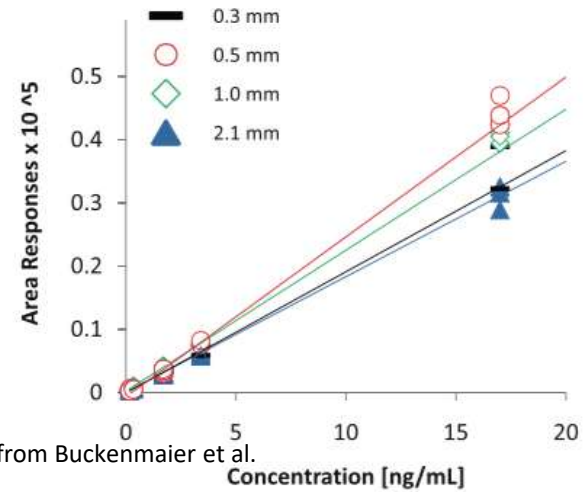
Chip cube



- Seminal work by Percy and Borchers systematically described the pros and cons of nano and std. flow HPLC for analysis of peptides.(1-3)

Agilent Jet Stream source is a mass biased detection system.





Copied from Buckenmaier et al.

With a concentration based detector the sensitivity increase is dependent on the square of the fold change in the the column diameter. (e.g. 2.1-> 1 mm (2.1/1)^2 = ~4) Thus the 1 mm will have a 4 x increase in response for the same mass injected on column. This is what makes nano so powerful (2.1/0.075mm)^2=784 times more sensitive!

1. **Percy, A. J., Chambers, A. G., Yang, J., Domanski, D., & Borchers, C. H. (2012).** ***Analytical and Bioanalytical Chemistry*****, *404*(4), 1089–1101**.
2. Percy, A. J., Yang, J., Chambers, A. G., Simon, R., Hardie, D. B., & Borchers, C. H. (2014). *Journal of Proteome Research*, *13*(8), 3733–3747.
3. Percy, A. J., Michaud, S. A., Jardim, A., Sinclair, N. J., Zhang, S., Mohammed, Y., et al. (2017). *Proteomics*, *17*(7). http://doi.org/10.1002/pmic.201600097
4. Buckenmaier, S., Miller, C. A., van de Goor, T., & Dittmann, M. M. (2015). *Journal of Chromatography A*, *1377*, 64–74.

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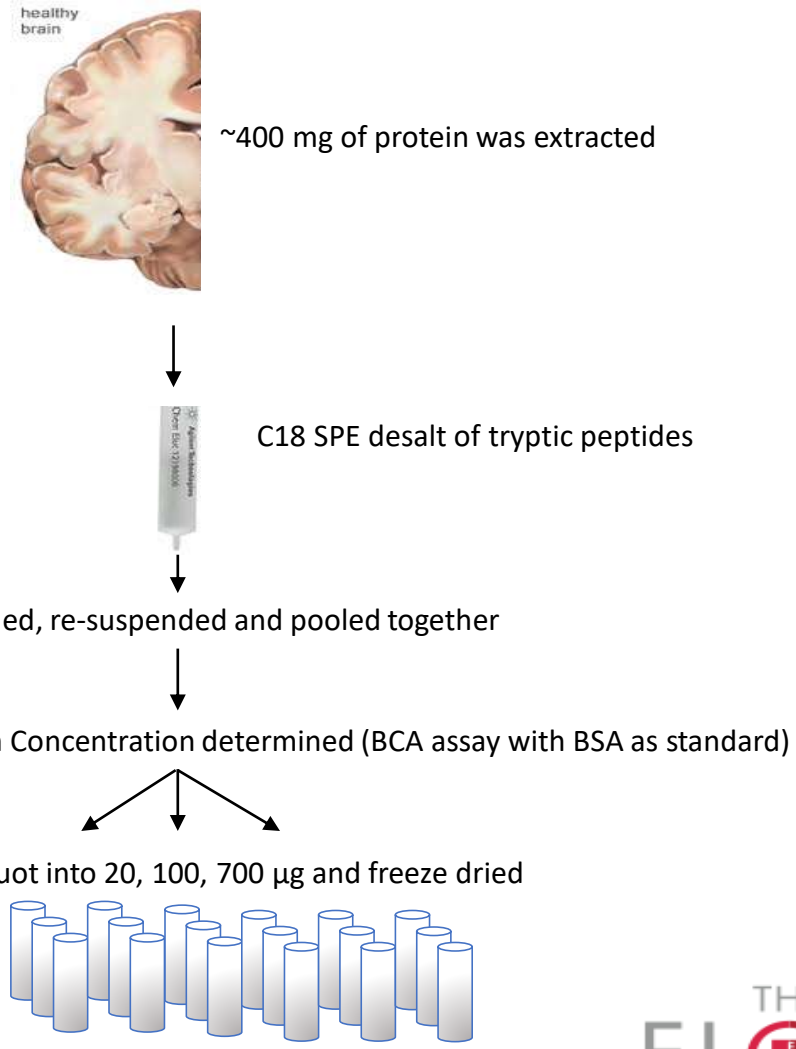
Generate a Quality Control Sample

- Generate a standard sample that we can use for analytical method development and QC within our lab.
- We wanted a complex sample and need to have 10's-100's of mg so we can use over the course of 1-2 years.
- Advantages of having a complex peptide QC
  1. Common sample type in our lab.
  2. Allows us to monitor the instrument performance over time.
  3. Identify instrument or LC performance issues.
  4. Provides a benchmark for comparing new methods.

Frontal cortex reference material

- Brain extract prepared as followed:

1. Brain homogenate was lysed
2. Proteins were precipitate and washed with acetone
3. Reduction with DTT
4. Alkylation with Iodoacetamide
5. Protein digestion with Trypsin
6. C18 SPE
7. Freeze dried
8. Pellet was dissolved in 5% acetonitrile in water
9. The protein concentration was determined
10. Replicate aliquots were made
11. Replicates were the freeze dried and stored at -80°C
12. For analysis samples were dissolved in 3% acetonitrile 0.1% TFA.



Instrument settings-Brain Ref. Standard Flow

Standard Flow dual spray AJS settings QTOF 6550	
Gas Temp./Sheath Gas temp. (˚C):	250˚C / 275 ˚C
Gas Flow/Sheath Gas flow:	14L/min : 12 L/min
MS range:	300-1700
MS rate:	8
MS/MS range:	50-1700 m/z
MS/MS rate:	3
Isolation width:	Narrow (1.3 m/z)
Collision Energy formula:	Slope 3.6 Offset –4.8
Nebulizer/Vcap/Nozzle:	35 psi/3500V/1000V
Auto MS/MS Max precursors per cycle	10
Threshold	1000 (0.001%)
Precursor abundance based scan speed	True
Target (counts/spectrum)	25,000 (accumulation limit=true)
Use dynamic precursor rejection	False
Purity Stringency/Cutoff	100/30 %
Active exclusion	0.25 min

Buffer A = Water 0.1% FA
Buffer B = Acetonitrile 0.1% FA
Advance Peptide Map 2.1x250 mm, 5mm guard
Column Temperature = 60˚C
Flow Rate 0.4 mL/min
Pressure Limit 600 Bar

Timetable

	Time	A	B
1	2.90 min	97.50 %	2.50 %
2	5.00 min	95.00 %	5.00 %
3	31.00 min	86.00 %	14.00 %
4	63.00 min	77.50 %	22.50 %
5	83.00 min	64.00 %	36.00 %
6	83.50 min	64.00 %	36.00 %
7	85.00 min	19.00 %	81.00 %
8	87.00 min	19.00 %	81.00 %
9	87.20 min	97.50 %	2.50 %

Post run time 4 min.

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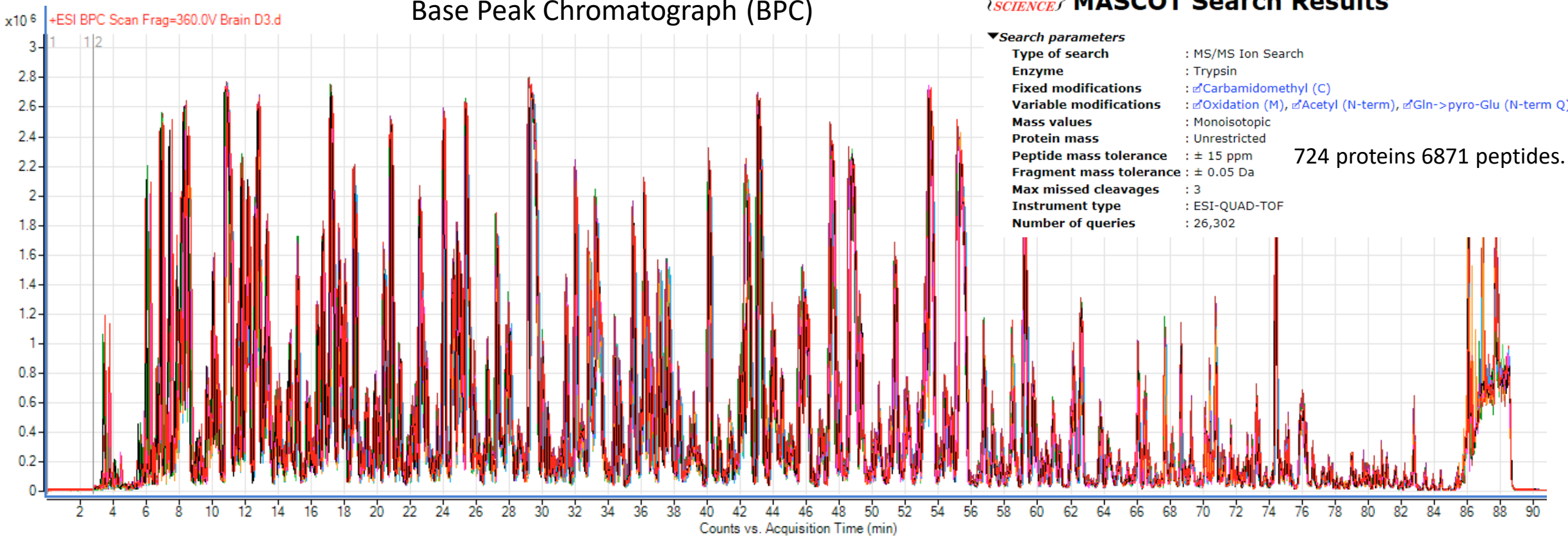
Analysis of Frontal Cortex Brain Reference Material

Base Peak Chromatograph (BPC)



MASCOT Search Results

Search parameters

Type of search	: MS/MS Ion Search
Enzyme	: Trypsin
Fixed modifications	: Carbamidomethyl (C)
Variable modifications	: Oxidation (M), Acetyl (N-term), Gln->pyro-Glu (N-term Q)
Mass values	: Monoisotopic
Protein mass	: Unrestricted
Peptide mass tolerance	: ± 15 ppm
Fragment mass tolerance	: ± 0.05 Da
Max missed cleavages	: 3
Instrument type	: ESI-QUAD-TOF
Number of queries	: 26,302

724 proteins 6871 peptides.

Overlay of 10 replicate injections of reference brain material, resolved chromatographically by a 2.1 x 250mm AdvanceBio Peptide Mapping column on a 1290 UHPLC system coupled to a 6550 QTOF.

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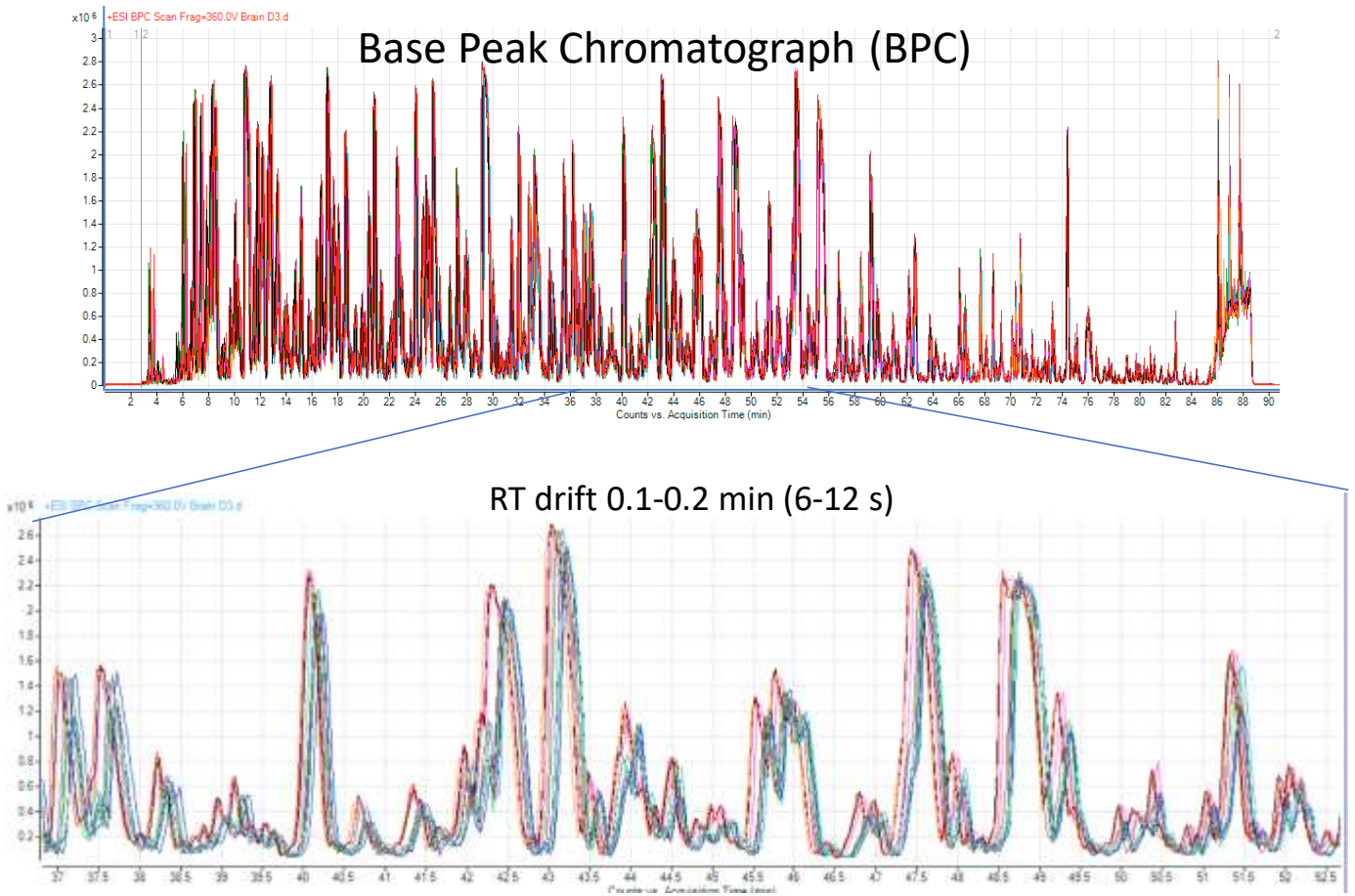
Stable Retention Time from Run to Run



Instrument settings-Brain Ref. Nano Flow

ChipCube settings QTOF 6550

Gas Temp. (˚C):	280˚C
Gas Flow:	11 L/min
MS range:	290-1700
MS rate:	8
MS/MS range:	50-1700 m/z
MS/MS rate:	3
Isolation width:	Narrow (1.3 m/z)
Collision Energy formula:	Slope 3.6 Offset –4.8
Vcap:	1900V
Auto MS/MS Max precursors per cycle	10
Threshold	1000 (0.001%)
Precursor abundance based scan speed	True
Target (counts/spectrum)	25,000 (accumulation limit=true)
Use dynamic precursor rejection	False
Purity Stringency/Cutoff	100/30 %
Active exclusion	0.25 min

Ref. Ion: 299,1221

Buffer A = Water 0.1% FA
Buffer B = Acetonitrile 0.1% FA
Polaris HR Chip 3C18 150 x 0.075 mm (360nL enrichment)
Flow Rate 300 nL/min
Pressure Limit 250 Bar

Timetable

	Time	A	B
1	2.90 min	97.50 %	2.50 %
2	5.00 min	95.00 %	5.00 %
3	31.00 min	86.00 %	14.00 %
4	63.00 min	77.50 %	22.50 %
5	83.00 min	64.00 %	36.00 %
6	83.50 min	64.00 %	36.00 %
7	85.00 min	19.00 %	81.00 %
8	87.00 min	19.00 %	81.00 %
9	87.20 min	97.50 %	2.50 %

Post run time 6 min.

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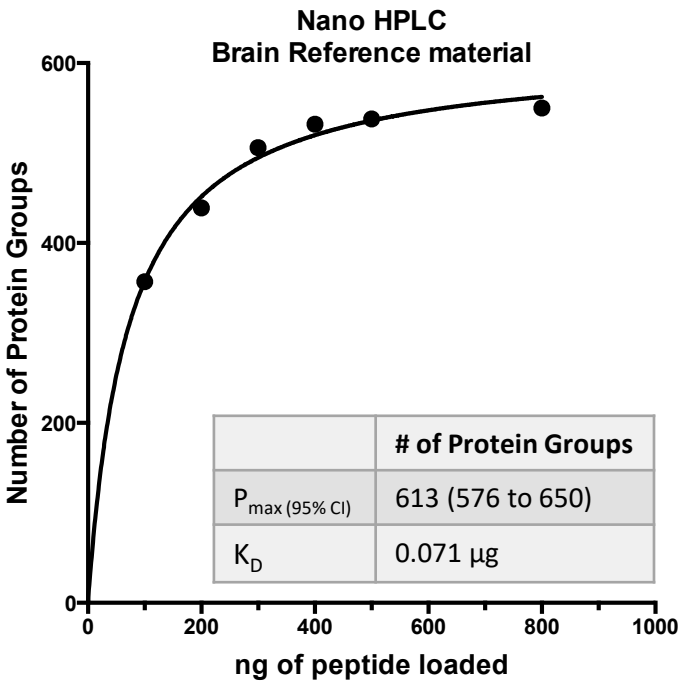
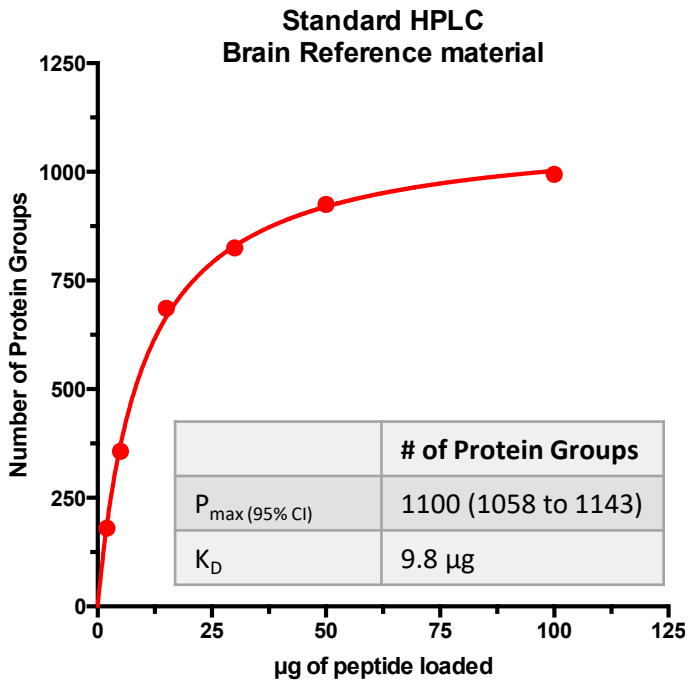
Comparing nano and standard flow

1.8x increase in ID's compared with nano



	# of Protein Groups
$P_{max\ (95\%\ CI)}$	613 (576 to 650)
K_D	0.071 µg



	# of Protein Groups
$P_{max\ (95\%\ CI)}$	1100 (1058 to 1143)
K_D	9.8 µg

- The data are fit to a hyperbola modeling a single site binding event.
- P_{max} is the theoretical maximum number of protein hits that can be achieved.
- K_D = Amount of protein to achieve ½ Pmax

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Summary of Nano vs Standard HPLC

- When sample is limited nano is still the best option with 20-50x increase in analytical sensitivity.
- Standard flow provides 1.8x increase in the number of proteins ID's .
- Standard flow also has superior RT stability 0.1-0.2 min vs. nano flow 0.5-0.9 min.

Future Directions

6560 Ion mobility-QTOF

- In addition to accurate mass, and retention time, ion mobility mass spectrometry provides a measurement of the collision cross section (CCS) area of peptides.
- The measurement of CCS and analytical sensitivity improvements (Baker et al.) greatly aids in the application of an accurate mass and retention time approach (e.g. RT, Gradient, CCS) AMRT-GC.
- Additionally a data independent collection scheme can be implemented.

http://www.agilent.com/en-us/training-events/eseminars/emerging-omics
" New developments in LC-IMS-MS proteomic measurements and informatic Analyses"

Baker, E. S., Burnum-Johnson, K. E., Ibrahim, Y. M., Orton, D. J., Monroe, M. E., Kelly, R. T., et al. (2015). Enhancing bottom-up and top-down proteomic measurements with ion mobility separations. *Proteomics*, *15*(16), 2766–2776. http://doi.org/10.1002/pmic.201500048

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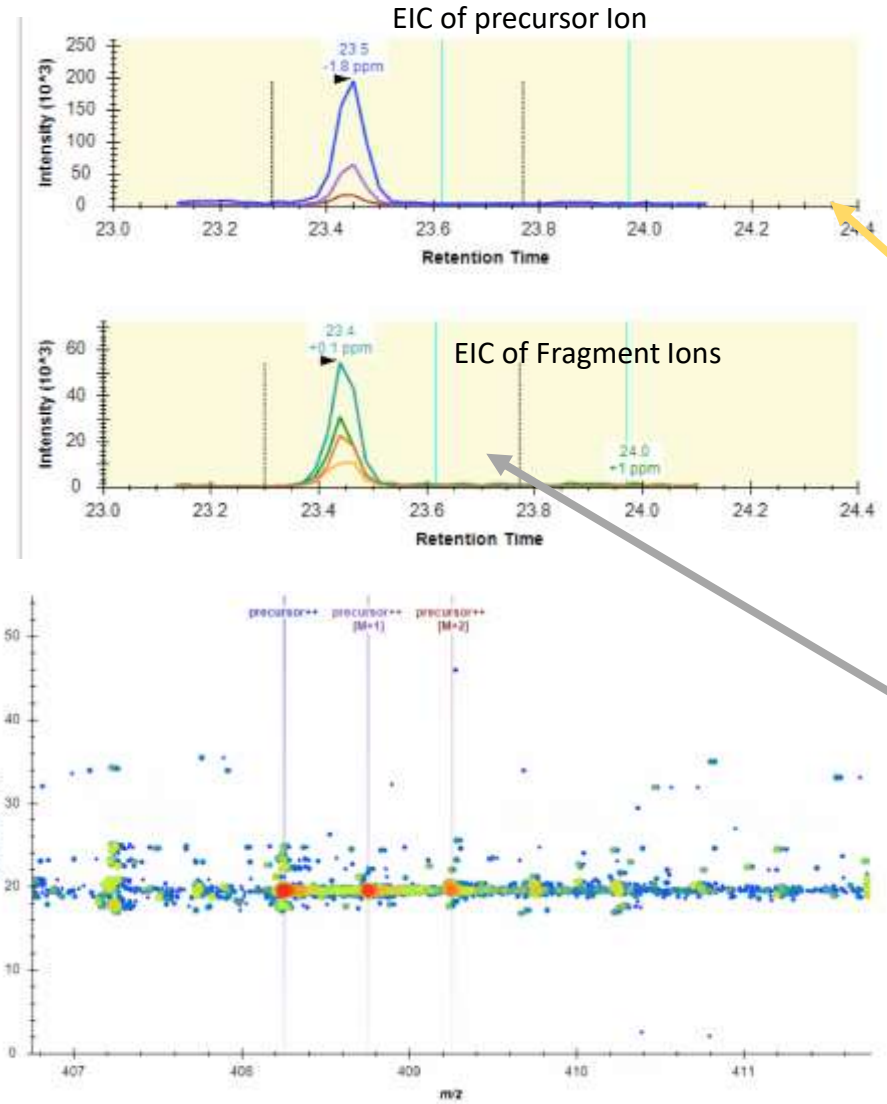
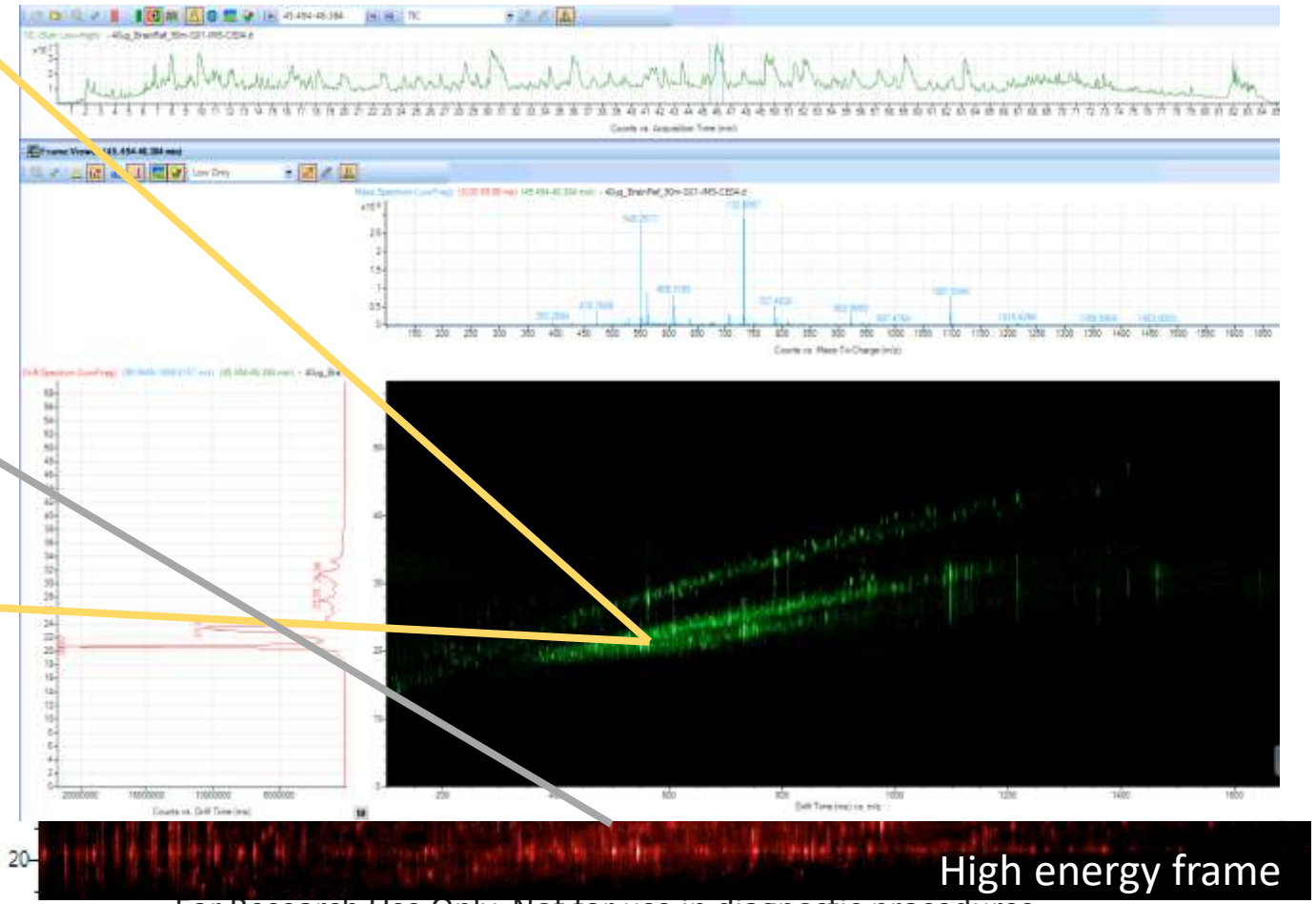
Example of DIA-IMS-QTOF



- Example of a data independent acquisition using IMS-QTOF.
- All precursors are fragmented by applying a fragmentation energy in the collision cell.
- Data analyzed using Skyline.



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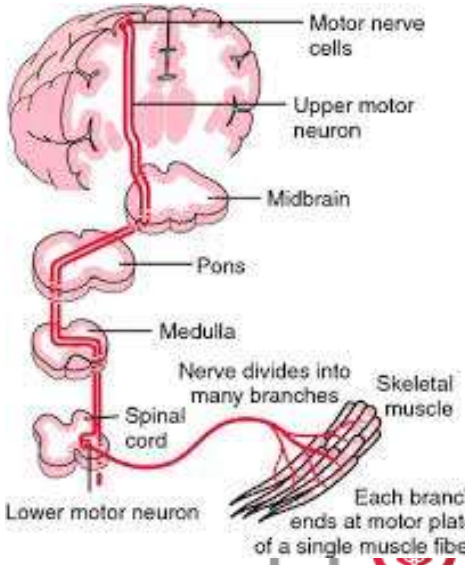
Amyotrophic Lateral Sclerosis (ALS)

- ALS is also referred to as motor neuron disease or Lou Gehrig's disease.
- It is a fatal neurodegenerative disease
  - Approximately 5,600 new case per year in the United States
  - As many as 30,000 living with the disease
  - It is a rapidly progressing disease with an average life expectancy of 2-5 years.
  - Difficult to diagnosis and can take 10-18 months.
  - Characterized by loss of motor function.
- The disease results in the progressive loss of motor neurons

www.MLB.com



My lab aims to understand what causes ALS and what are the molecular process that govern the disease ?
Further we aim to develop therapies and biomarkers for ALS.

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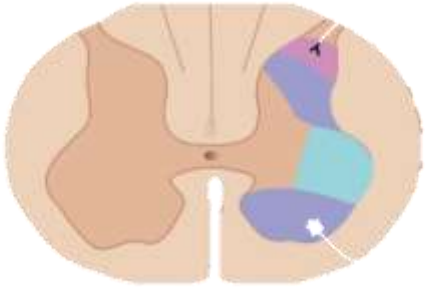
Discovery Proteomics in human spinal cord.

Human spinal cord samples from the Victorian Brain Bank



- Homogenize 50-100 mg of tissue Urea/deoxycholate
- Reduce, alkylate, digest with Trypsin
- Clean-up(Bravo, C18), and analyze by LC-MS/MS
- Protein concentration determined using BCA proteins Assay. BSA as standard.

Progenesis QI

- Data aligned, features detected, statistical analysis.
- MS/MS search conducted with MASCOT

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Instrument settings-Discovery QTOF

Standard Flow dual spray AJS settings QTOF 6550	
Gas Temp./Sheath Gas temp. (˚C):	250˚C / 275 ˚C
Gas Flow/Sheath Gas flow:	14L/min : 12 L/min
MS range:	300-1700
MS rate:	8
MS/MS range:	50-1700 m/z
MS/MS rate:	3
Isolation width:	Narrow (1.3 m/z)
Collision Energy formula:	Slope 3.6 Offset –4.8
Nebulizer/Vcap/Nozzle:	35 psi/3500V/1000V
Auto MS/MS Max precursors per cycle	10
Threshold	1000 (0.001%)
Precursor abundance based scan speed	True
Target (counts/spectrum)	25,000 (accumulation limit=true)
Use dynamic precursor rejection	False
Purity Stringency/Cutoff	100/30 %
Active exclusion	0.25 min

Buffer A = Water 0.1% FA
Buffer B = Acetonitrile 0.1% FA
Advance Peptide Map 2.1x250 mm, 5mm guard
Column Temperature = 60˚C
Flow Rate 0.4 mL/min
Pressure Limit 600 Bar

Timetable

	Time	A	B
1	2.90 min	97.50 %	2.50 %
2	5.00 min	93.70 %	6.30 %
3	29.00 min	86.50 %	13.50 %
4	31.00 min	86.20 %	13.80 %
5	58.00 min	77.50 %	22.50 %
6	73.00 min	59.50 %	40.50 %
7	74.00 min	59.50 %	40.50 %
8	75.00 min	19.00 %	81.00 %
9	77.00 min	19.00 %	81.00 %
10	77.20 min	97.50 %	2.50 %

Post run time 4 min.

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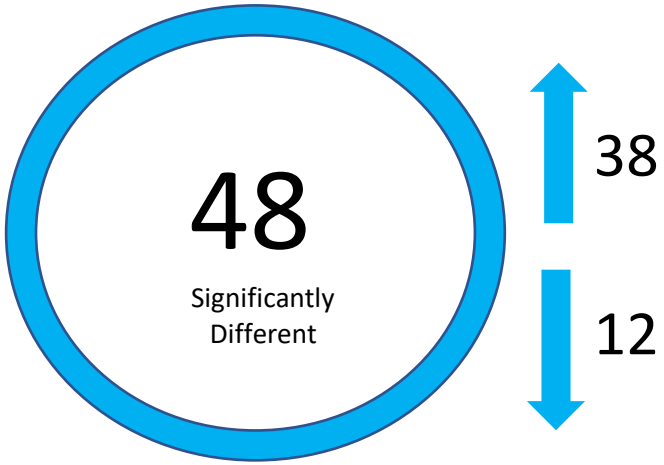
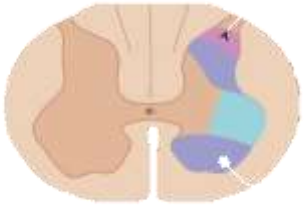
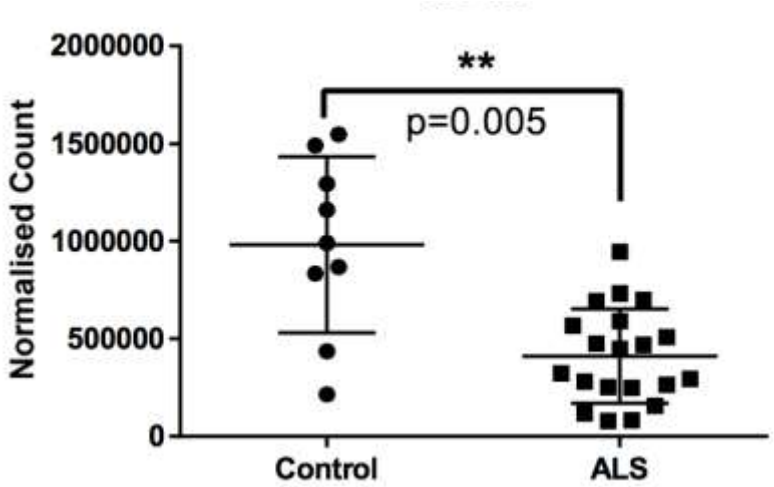
Results of Discovery Proteomics of human Amyotrophic lateral sclerosis (ALS) spinal cord

Control(n=11) vs ALS(n=23)





Decreased levels of protein A



Mean +/- STDEV ** p= 0.005

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Gunn, A and Roberts, B Unpublished

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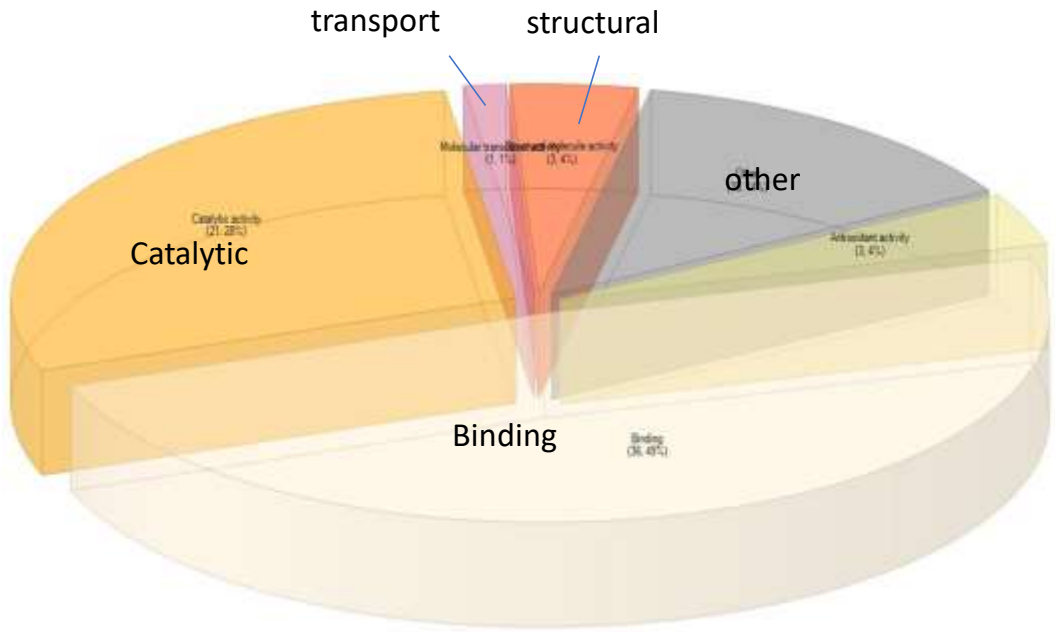
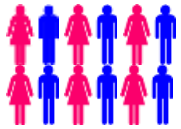
Pathway Analysis-Control vs ALS

pathway_name	num_overlapping_genes	overlapping_genes
Glial Cell Differentiation	3	MAG_HUMAN;MBP_HUMAN;CN37_HUMAN
Spinal Cord Injury	5	MAG_HUMAN;MBP_HUMAN;PGCB_HUMAN;LEG3_HUMAN;NOS2_HUMAN
Regulation of actin cytoskeleton - Homo sapiens (human)	5	PP12C_HUMAN;PI42A_HUMAN;MYH10_HUMAN;FGF1_HUMAN;ACTN1_HUM
Regulation of Actin Cytoskeleton	4	PI42A_HUMAN;MYH10_HUMAN;FGF1_HUMAN;ACTN1_HUMAN



Grouped by Function

Targeted Mass spectrometry based quantitation of CSF proteins



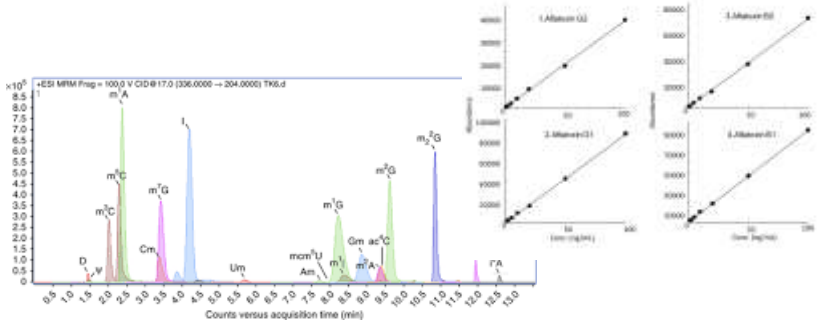
5-50 µL of CSF

Automated digestion and clean-up (SPE).

Bravo-system (Agilent)

LC-MS/MS

Absolution Quantitation of proteins



Alpha-1-antichymotrypsin
Alpha-1-antitrypsin
Alpha-synuclein

Amyloid beta A4 protein
Antithrombin-III
Apolipoprotein A-I
Apolipoprotein A-II
Apolipoprotein A-IV
Apolipoprotein C-I
Apolipoprotein C-II
Apolipoprotein D
Apolipoprotein E
Apolipoprotein L1
Apolipoprotein M
Beta-2-microglobulin
C-reactive protein
Ceruloplasmin

Cholinesterase
Chromogranin-A
Clusterin

Complement C3
Complement factor H
Cystatin-C
Fibrinogen alpha chain
Fibrinogen beta chain
Fibrinogen gamma chain
Gamma-enolase
Gelsolin
Glutathione peroxidase 3
Haptoglobin
Hemoglobin subunit alpha
Hemopexin
Histidine-rich glycoprotein
Ig kappa chain V-IV region

Ig mu chain C region
Ig mu heavy chain disease protein
IgGFc-binding protein

Insulin-like growth factor-binding protein 1
Lactotransferrin
Melanotransferrin
Osteopontin
Peroxiredoxin-2
Plasminogen
Protein AMBP
Serotransferrin
Transferrin receptor protein 1
Transthyretin
Vitamin D-binding protein
Zinc-alpha-2-glycoprotein

Developed by MRM-Proteomics Inc.

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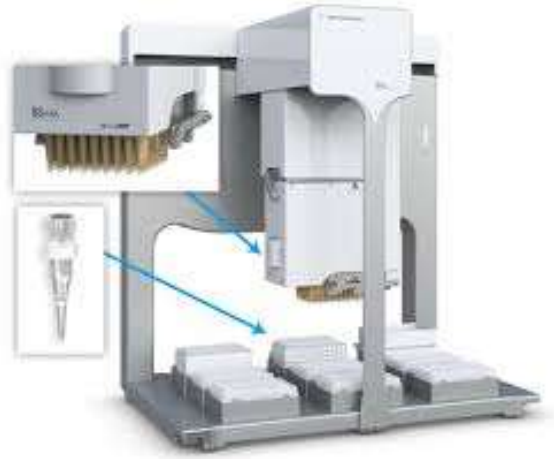
Comparison of Manual vs. Automated Workflow for Targeted Quantitation of CSF Proteins

1. Replicates of pooled CSF were prepared. 10µL CSF Diluted with 9 M urea +^{15}N labelled alpha-synuclein, reduce, alkylated and digest with Trypsin overnight at 37˚C.
2. Add heavy isotopically labelled lys and arg (^{15}N, ^{13}C) peptides and acidification of sample with formic acid. Peptides generated and quantitated by MRM-Proteomics Inc.
3. Solid phase extraction clean up.

HLB (Waters) with positive pressure manifold.

Reverse phase (RP-S) cartridges (Agilent) C_{18} cartridges



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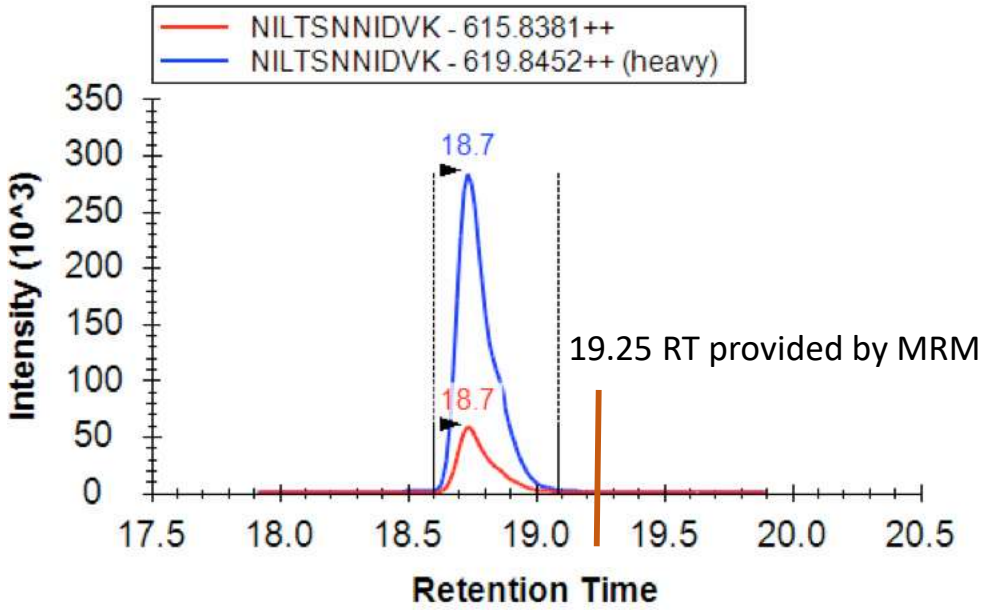
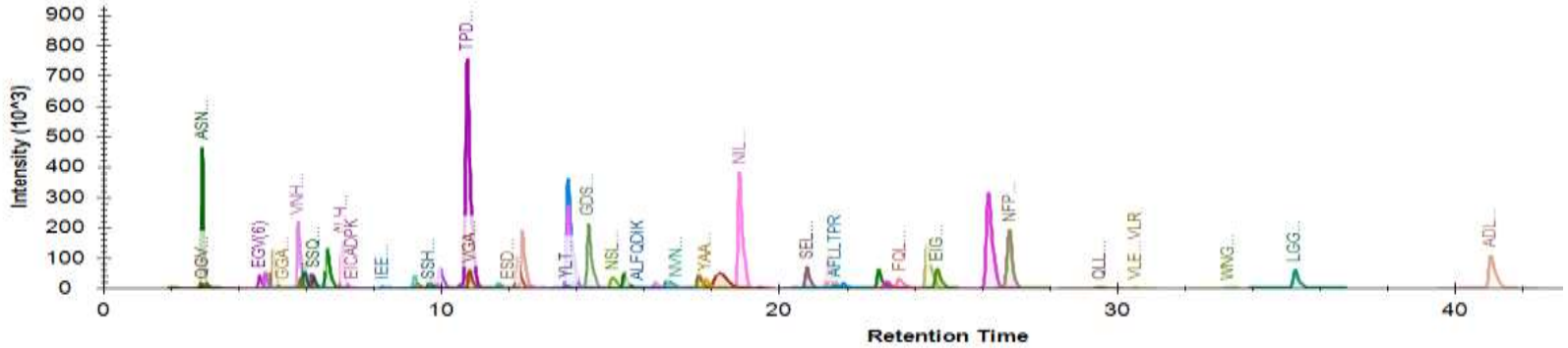
Comparison of Manual vs. Automated Workflow for Targeted Quantitation of CSF Proteins

Advanced Bio Peptide Map plus 2.1 x 150 mm (Agilent)
Gradient and SRM method developed by MRM-Proteomics Inc.

1290-6495 UHPLC-QQQ MS system (Agilent)

Standard Flow HPLC
2.1 x150mm





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Targeted QQQ-MS analysis setup for CSF measurment

Standard Flow dual spray AJS settings QQQ 6495

Gas Temp./Sheath Gas temp. (˚C):	150˚C / 250 ˚C
Gas Flow/Sheath Gas flow:	15 L/min : 11 L/min
High pressure RF	200 V
Low pressure RF	110 V
Nebulizer/Vcap/Nozzle:	30 psi/3500V/1500V
Dynamic MRM:	True
Retention Time window:	1.5 -2 min

Data analysis with QQQ quantitative analysis (B7.0.457.0)

Advance Peptide Map 2.1x 150 mm, 5mm guard
Column Temperature = 50˚C
Flow Rate 0.4 mL/min
Pressure Limit 600 Bar

Timetable

	Time	A	B
1	2.00 min	93.00 %	7.00 %
2	50.00 min	70.00 %	30.00 %
3	53.00 min	55.00 %	45.00 %
4	53.50 min	20.00 %	80.00 %
5	55.50 min	20.00 %	80.00 %
6	56.00 min	98.00 %	2.00 %

Post run time 4 min.

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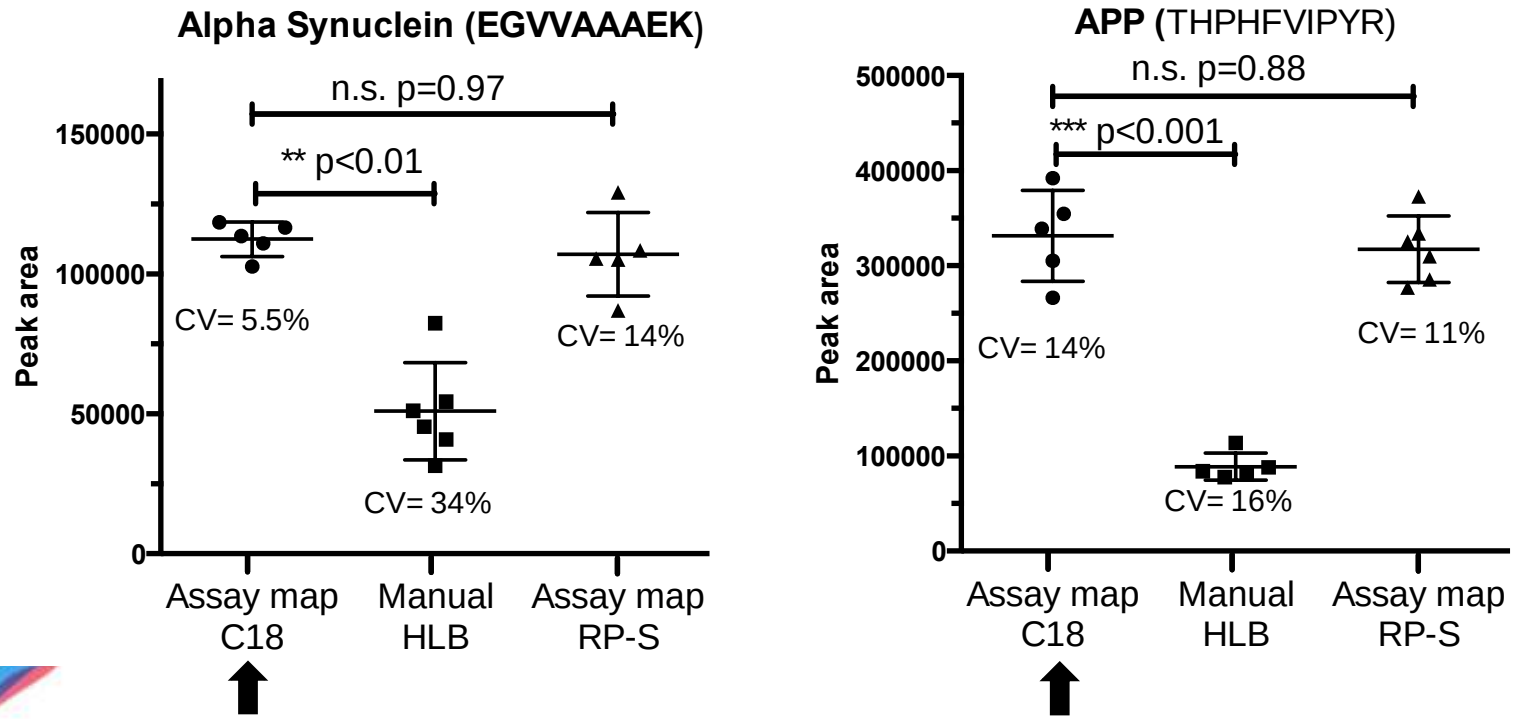
Quantitation of CSF Proteins Involved in Neurodegeneration

- Alpha synuclein and amyloid beta A4 precursor protein (APP) are of keen interest for there roles in Parkinson's and Alzheimer's disease, respectively.
- Measurement of alpha-syn and APP in biological fluids is of great interest do to their disease relevance and potential to be biological markers of disease process.

Comparison of raw peak area



We choose to use the AssayMAP C18 SPE clean up for our study.

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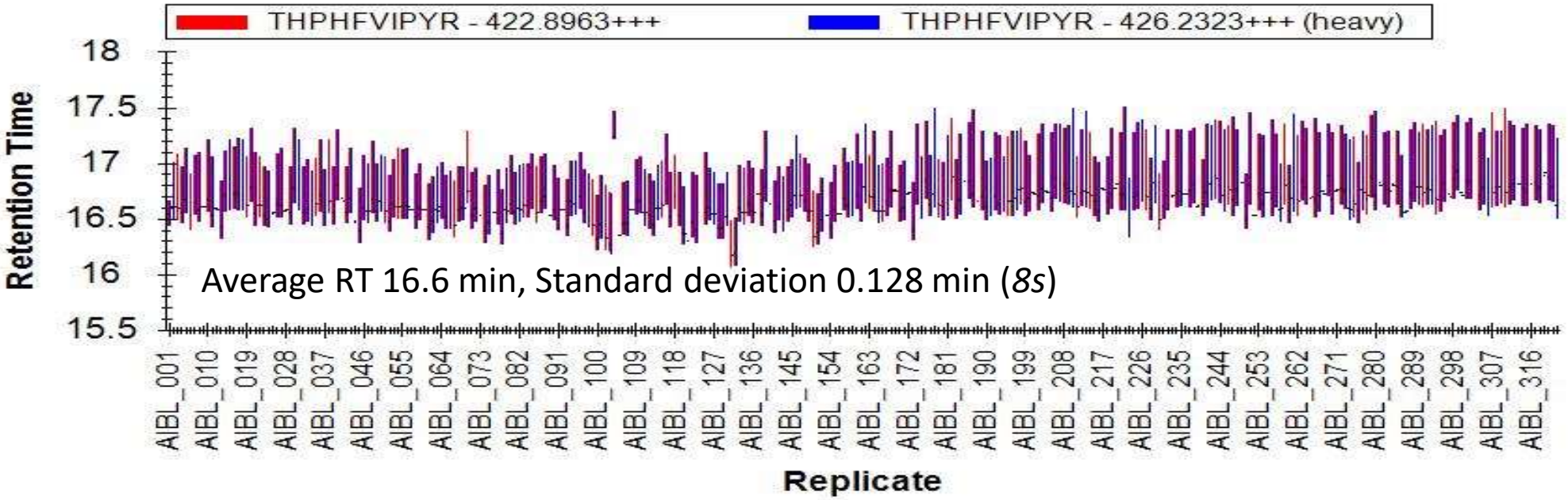
Measurement of 43 protein targets in over 300 CSF samples from the Australian Imaging and Lifestyle study of Ageing (AIBL)

- AIBL is one the largest, most well characterized longitudinal studies of Alzheimer's disease in the world. Over 1,100 participants with samples taken every 18 months. (https://aibl.csiro.au)

Example of retention time stability over the course of 400 hours of analysis time.

Amyloid A4 precursor protein (APP)



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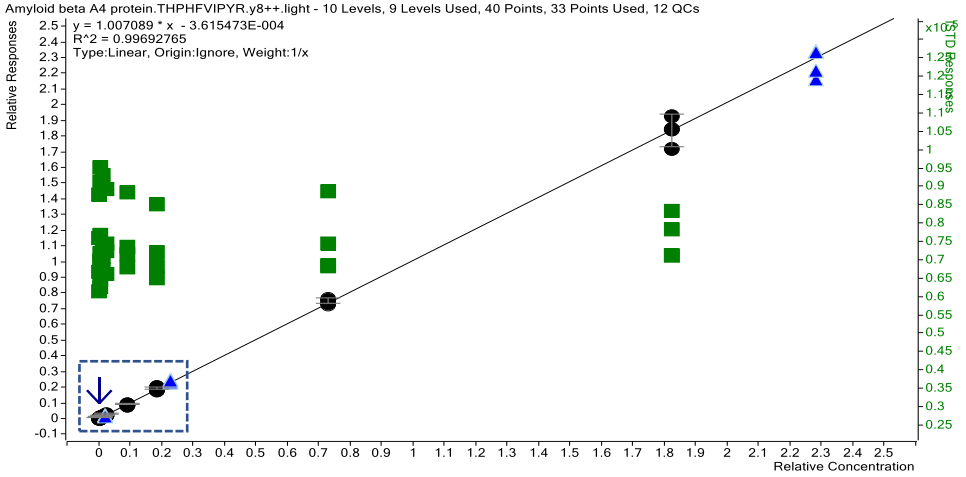
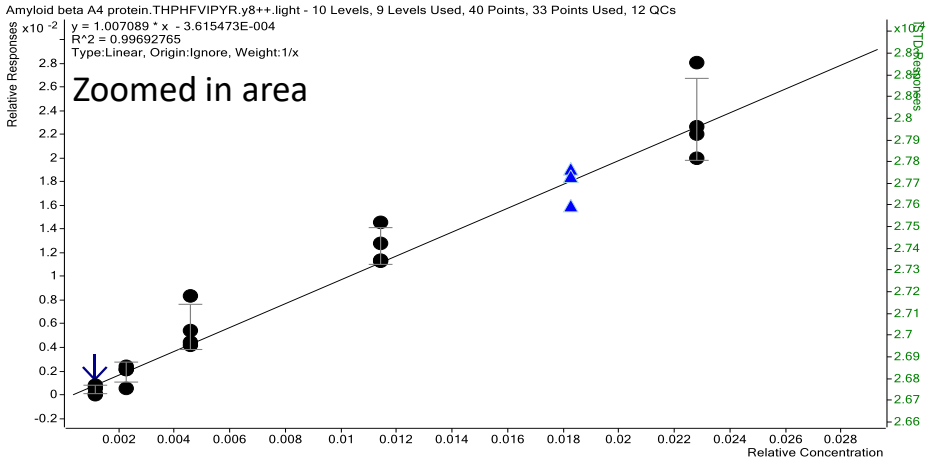
Excellent Limits of Detection allowed for Quantitation of low ng/mL range proteins

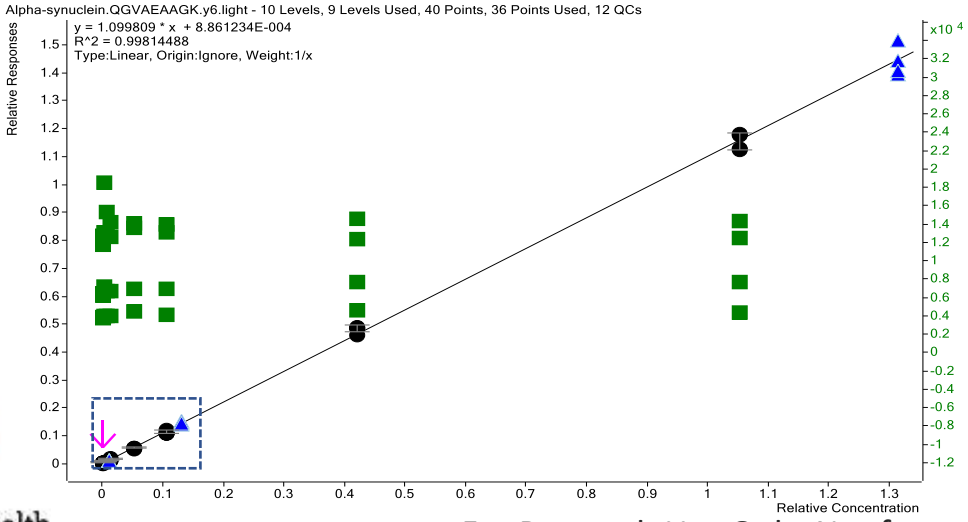
Heavy peptide response
Ratio L/H
QC material

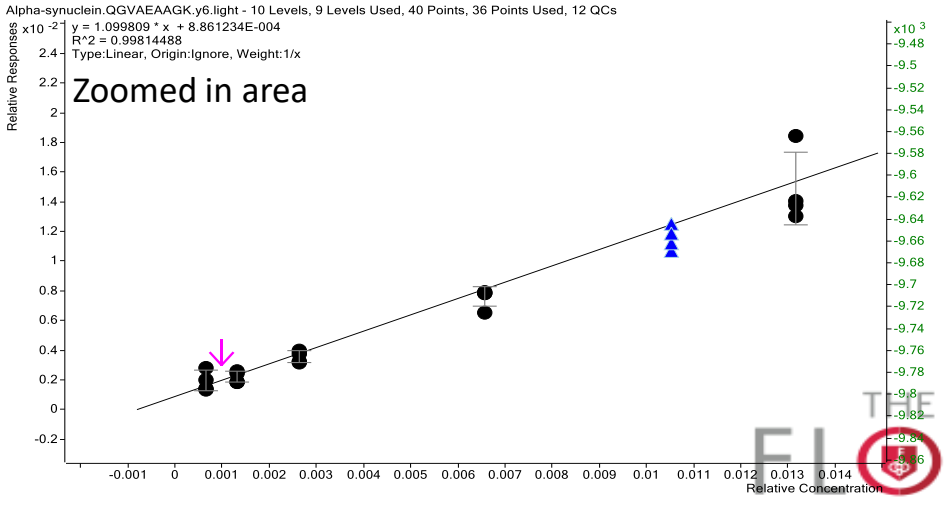
Standard Curve for APP





Standard Curve for alpha-syn





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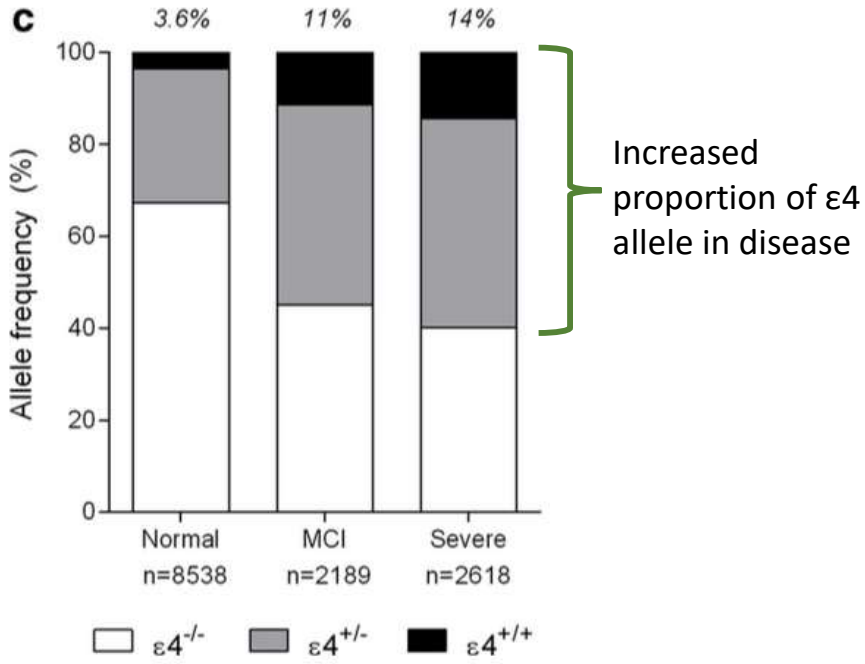
Genotyping Using Targeted Mass Spectrometry

- There are three major allels for the *APOE* gene in the population ε2, ε3, ε4.
- The *APOE* ε4 allele is associate with an increased risk of developing Alzheimer's disease (reviewed in Heffernan et al.)

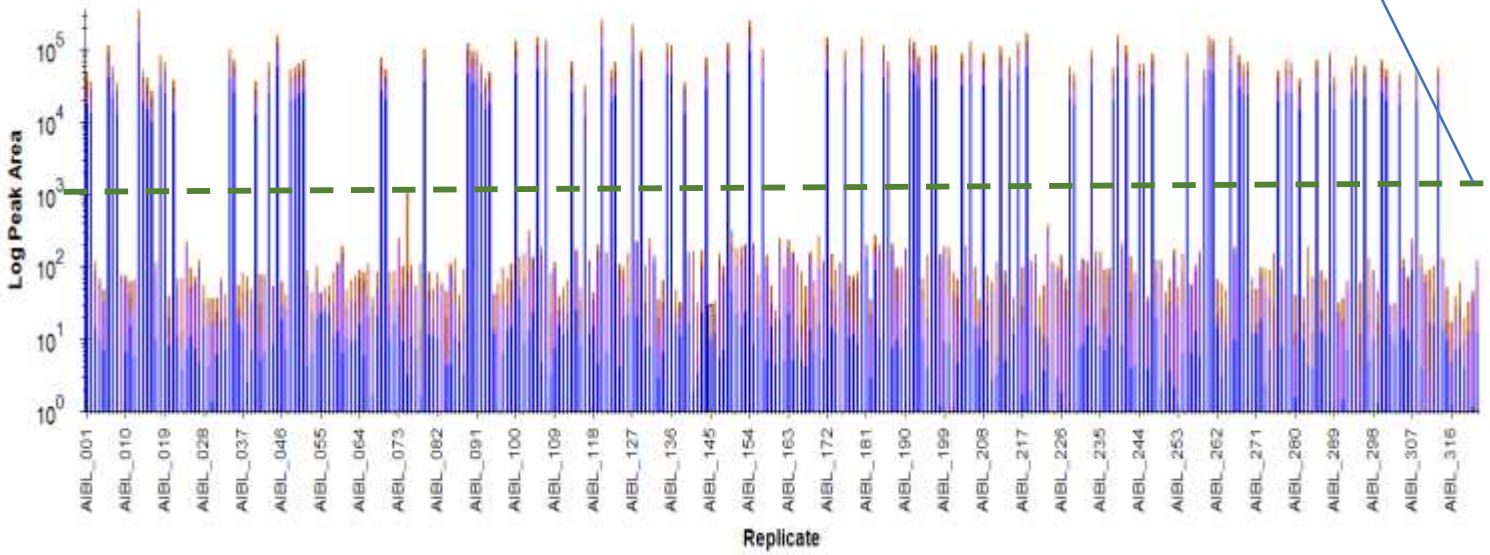


Increased proportion of ε4 allele in disease

Figure adapted from Heffernan et al.

Values above dashed line indicate positive for ε4 allele

ApoE genotyping using QQQ-MS

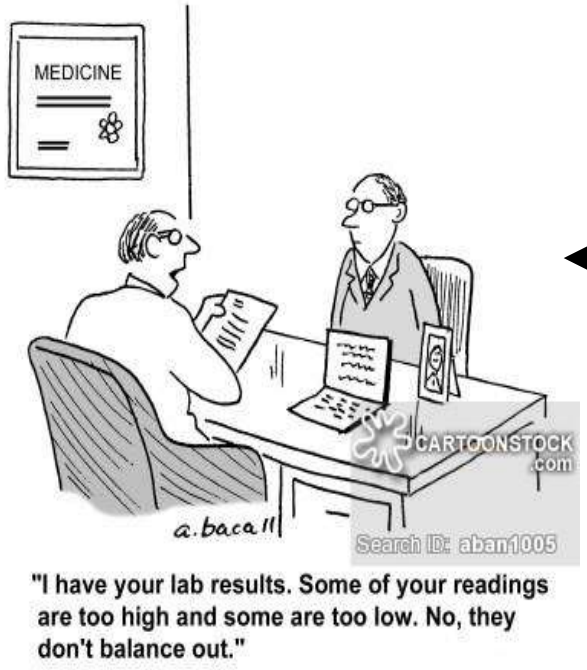


Heffernan, A. L., Chidgey, C., Peng, P., Masters, C. L., & Roberts, B. R. (2016). *Journal of Molecular Neuroscience : MN*, *60*(3), 316–324

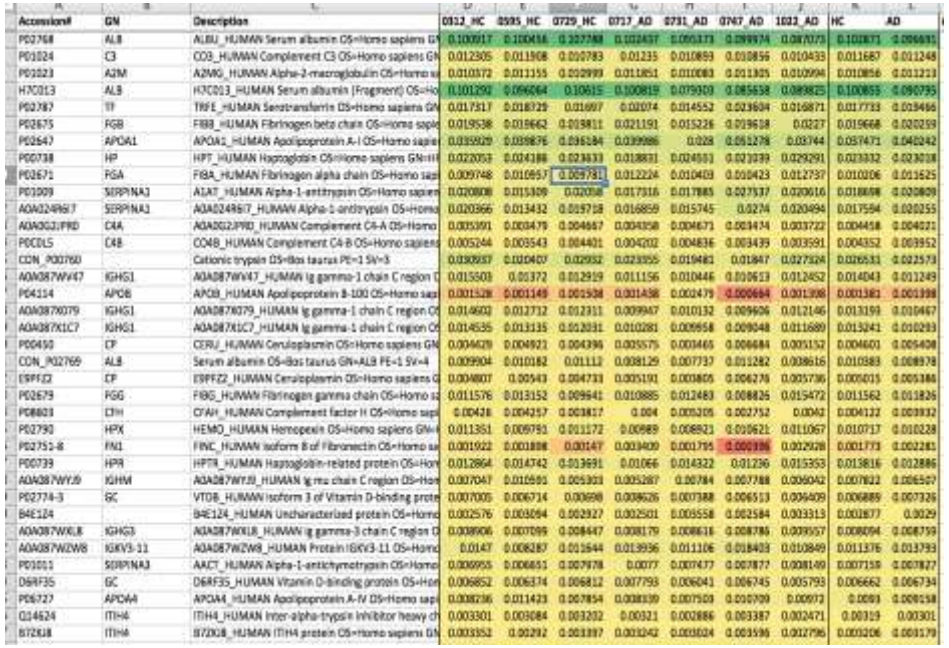
Summary of Targeted Analysis using Standard HPLC

- Standard flow provides excellent retention time reproducibility <0.2 minutes.
- Sensitivity low ng/mL for some protein targets. This makes QQQ a viable option to replace ELISA and western blot technologies.
- Multiplex measurement is a significant advantage over ELISA and westerns and make the best use of limited human samples like CSF.

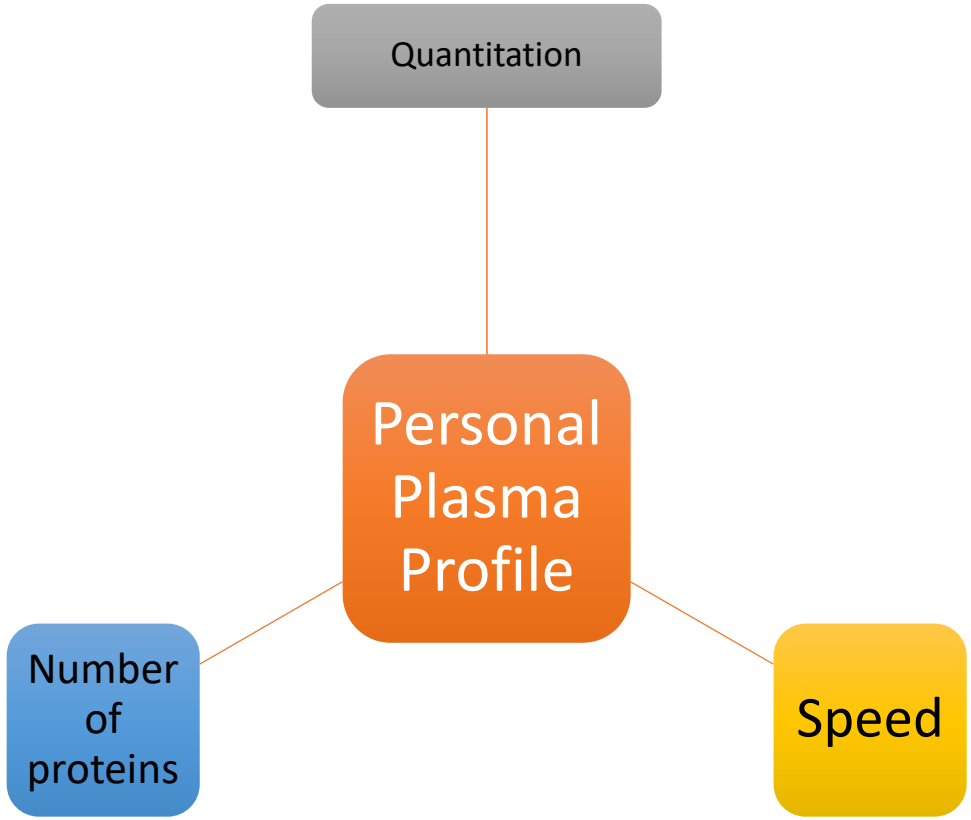
Goal of Personal Plasma Proteome Profile



"I have your lab results. Some of your readings are too high and some are too low. No, they don't balance out."



What are the tradeoffs between sample throughput and information content?

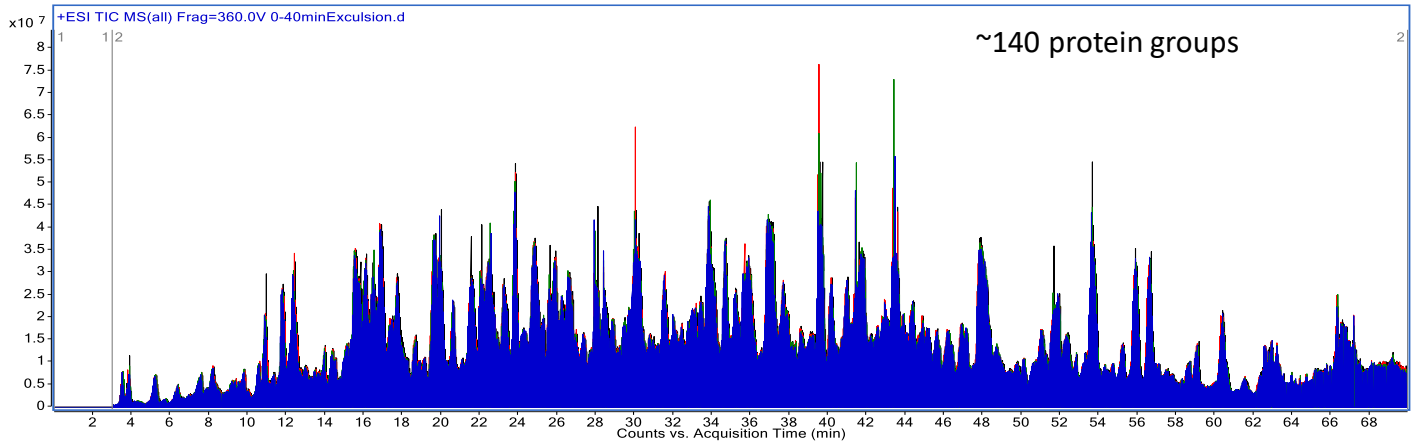


- Can we develop a plasma proteome profile using standard flow LC MS/MS?
  - Optimize sample throughput and information content
    - Time per sample (7 , 10, 15 , 30, 60, 120 min)
    - How many proteins can we quantify?

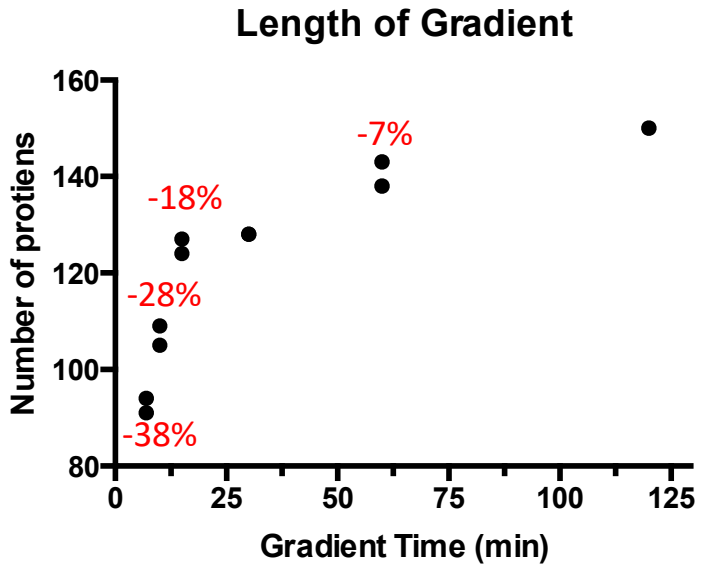
Geyer, P. E., Kulak, N. A., Pichler, G., Holdt, L. M., Teupser, D., & Mann, M. (2016) *Cell Systems*, *2*(3), 185–195. http://doi.org/10.1016/j.cels.2016.02.015

Plasma stats

Overlay of six injections of Neat plasma digest.



Maintain gradient decrease time



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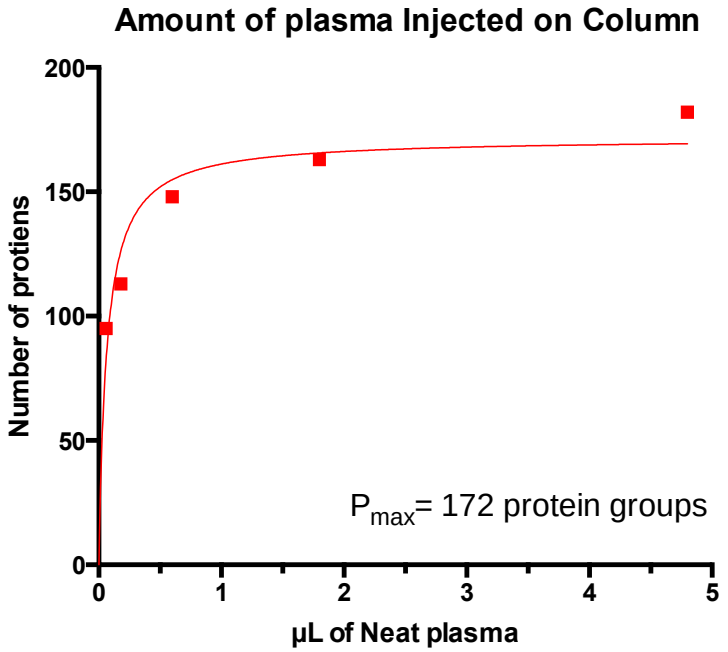
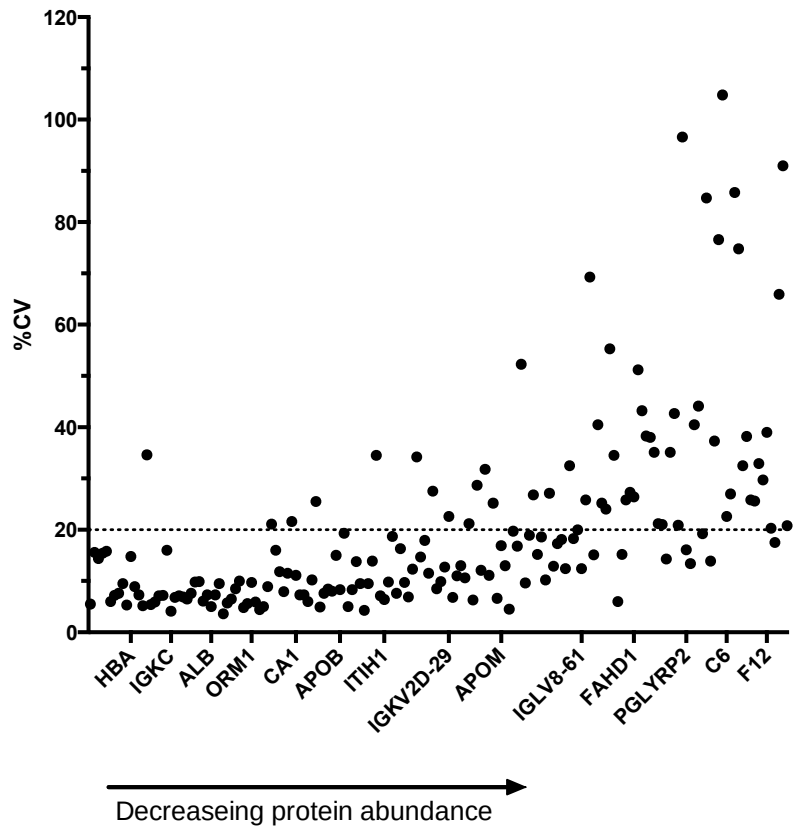
Sample load



Sample injected (µg)	Number of protein groups
3	95
9	113
30	148
90	163
240	182



- Using an accurate mass and retention time(AMRT) approach the number of protein groups can be expanded to 290 protein groups.

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Victorian Brain Bank
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