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more capabilities.
new possibilities.

Research to Routine Workflows for Large Molecules using Proteomic Tools and the Q Exactive HR/MS

European Bioanalytical Forum 16 November 2011

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Agenda

- Research to Routine
  - Small Vs. Large Molecule
  - Physicochemical
  - Types of instruments/technology and experiments
  - Small molecule regulations vs Large molecule regulations
- Application of HR/MS
  - Why HR/MS
  - Q Exactive
- Proven Proteomic workflow tools applied to routine quantitation

RESEARCH TO ROUTINE

CONTRAST OF LARGE VS. SMALL

Biologics Development: Research to Routine

Unregulated

- Range of traditional "Proteomics" experiments through to high throughput screening.
- Research focus, minor component interest, many analytes, same type of test.
- Internal based procedures rather than regulated SOP's

Regulated

- Routine and Semi-Routine experiments supporting safety and efficacy studies.
- Few drugs, many samples, high expense
- GLP & GMP environment – strict SOP's

Activities				
	Traditional Proteomics, Protein-protein interactions Pathway analysis Activity, PD Biomarker dev.	PTM Analysis Disulfide Mapping Protein Characterization Structural Analysis Immunogenicity Neutralization	PK/PD, LBA's, Cell based assays, PTM Optimization, Protein Characterization, Safety, Efficacy, Metabolism, DDI	PK/PD, CQA/QbD, Production Characterizations, Drug monitoring

Small vs Large Chemical Characteristics

Characteristic	Small	Large
Molecular Weight	<800	>5000
Endogenous	No	Often Yes
Solubility	Hydrophobic	Hydrophilic
Purity	Homogeneous	Heterogeneous
Immunogenic	No	Yes
Conjugated	Yes	No
Valence	Monovalent	Divalent
Kinetics	Fast	Slow
Detection	Isotopic	Activity/functional
Stability	Chemical/enzymatic	Immunologic/enzymatic
Metabolic interference	Yes/knowledge	Unknown
Protein Binding	Yes	No

B. De Silva, Bristol-Myers Squibb

Small vs Large Analytical Methodology

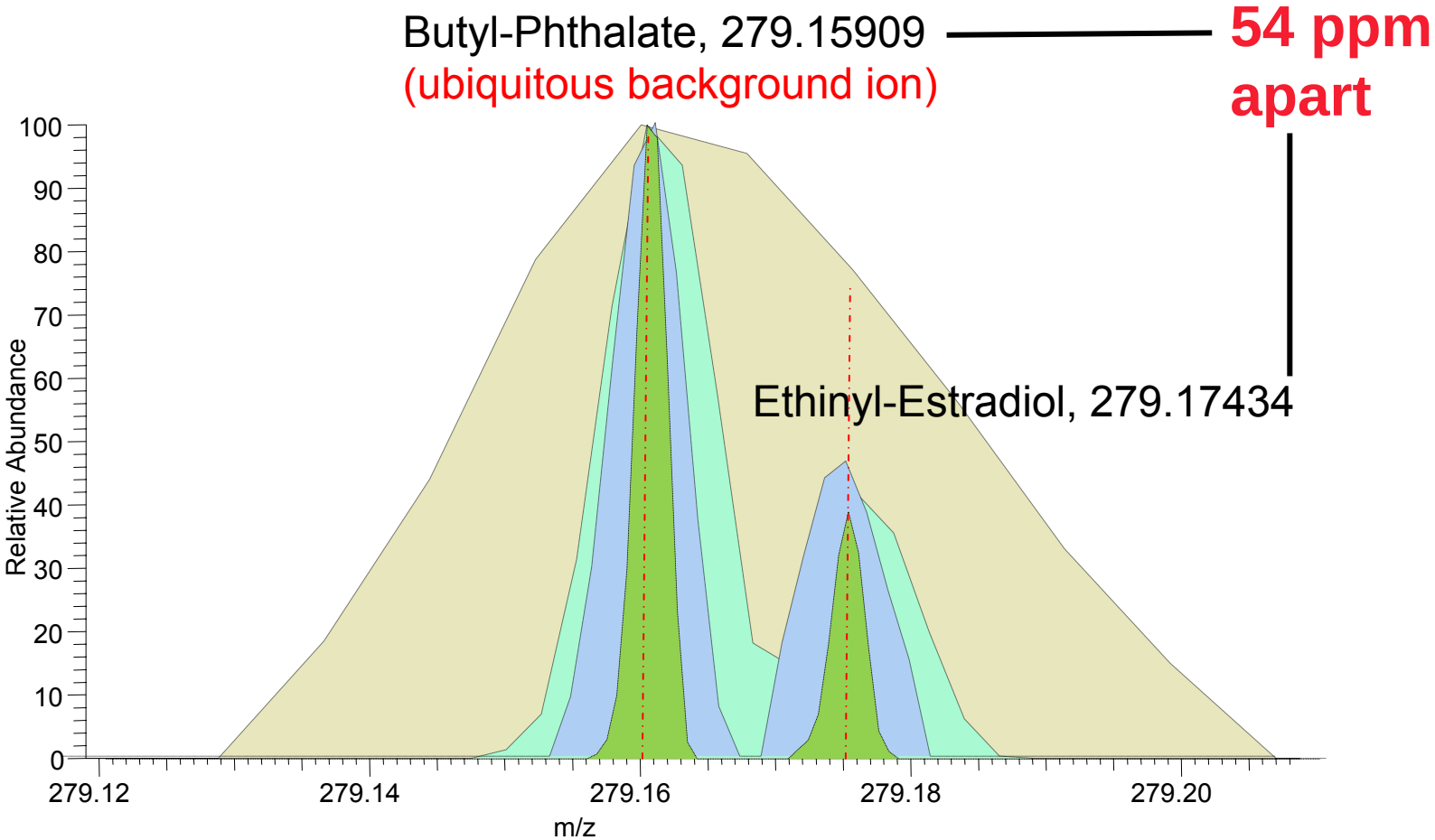
Characteristic	Small	Large
Basis of Measurement	Analyte	Antigen-Ab reaction
Detection	Direct	Indirect
Reagents	Common and available	Unique, not commercial or available
Analytes	Small	Small and macromolecule
Sample Preparation	Yes	No
Calibration Curves	Linear	Non-Linear
Assay Environment	Organic	Aqueous
Development Time	Weeks	Months
Technology	LC/MS (Affinity based Extraction)	LBA: ELISA, RIA, ECL, Multiplexing
Stability	Drug	Drug + Reagents
Regulations	Very defined and rigid	Some flexibility

B. De Silva, Bristol-Myers Squibb

APPLICATION OF HR/MS

Specificity = Resolution + Mass Accuracy

Resolution: 10k, 30k, 50k, 100k



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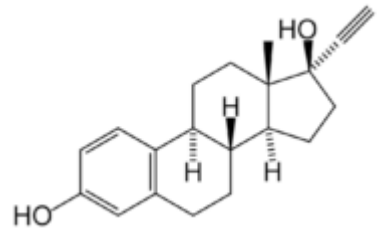
100 ppb Ethinyl-Estradiol – 100k vs 10 K Res

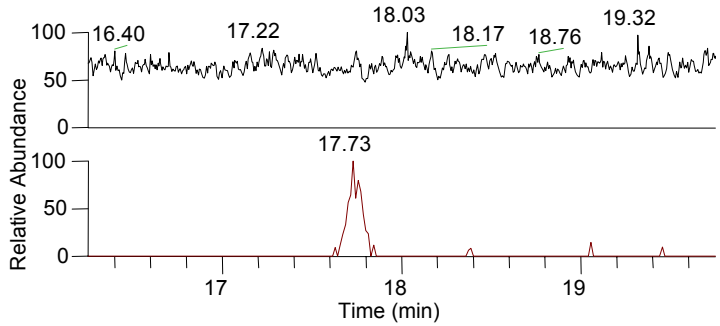
h Resolution and Mass Accuracy Esse

Ethinyl-Estradiol



Phthalate

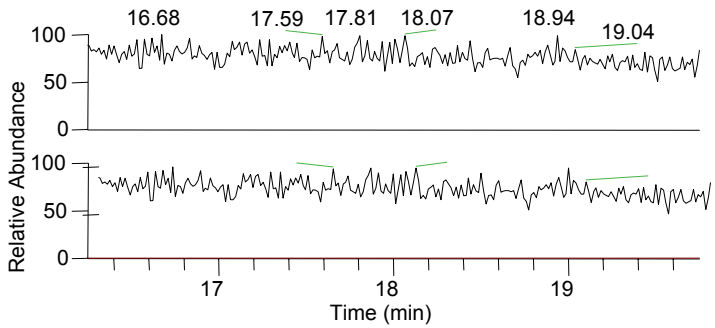
E Estradiol



Res = 100,000

Phthalate

E Estradiol



Res = 10,000

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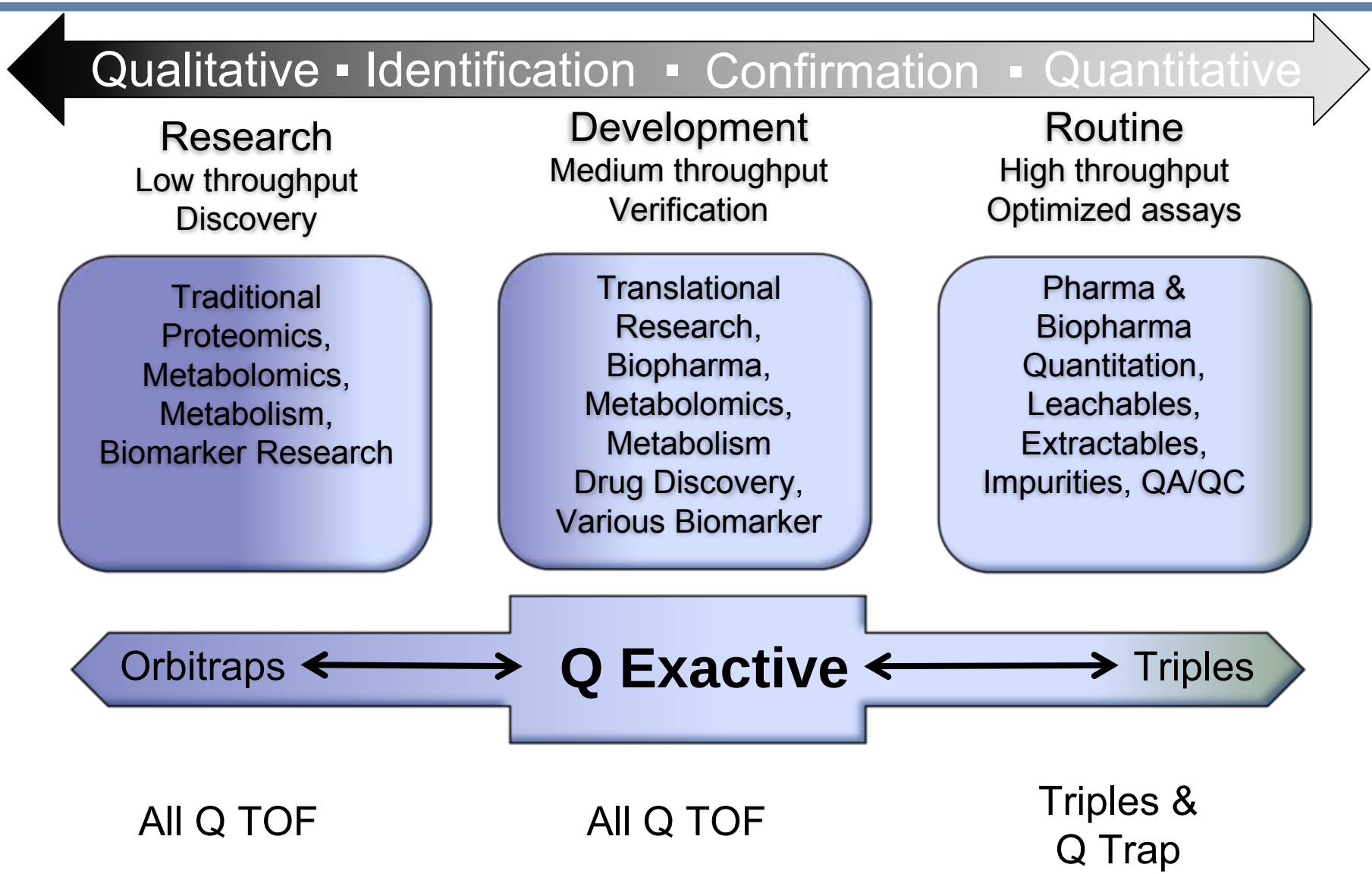
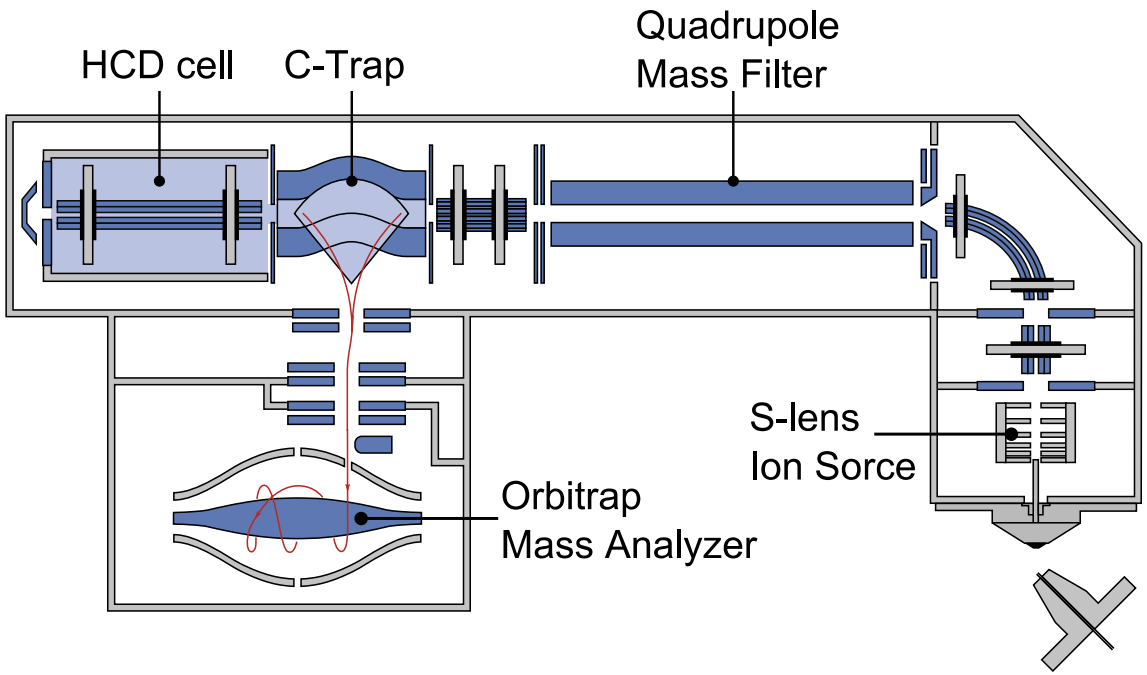
When does HR/MS make sense?

- Research phase
- Early to late discovery phases
- Pre-clinical phase
- QA/QC - characterization
- Ligand binding assay development
  - Specificity testing
  - Stability testing
- Assay troubleshooting, unexpected results
- Reagents unavailable, difficult to produce, expensive
- Bridging methods, reagents
- Biomarker research, multiple biomarkers

Research to Routine: Range of Experiments for LC/MS



Q Exactive ™ Hardware Innovations



- S-Lens ion source
- Quadrupole mass filter
- Advanced signal processing

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Specifications/Details

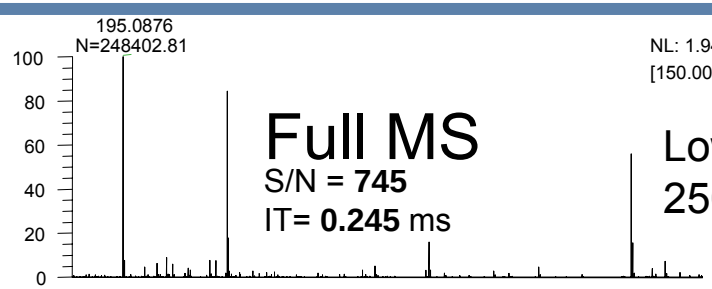
- Thermo Scientific HyperQuad mass filter
- Mass range: 50-4000 m/z
- Linear range: 4-5 orders of magnitude
- Variable precursor isolation width selection from 0.4 Da to full mass range
- Resolution : up to 140,000
  - 17k, 35k, 70k, 140k at m/z 200
  - scan speed dependent on resolution setting
- Sources:
  - ESI probe compatible with liquid flow rates of < 1 µL/min to 1 mL/min without splitting
  - APCI source compatible with liquid flow rates of 50 µL/min to 2 mL/min without splitting
  - Nanospray/microspray

Scan Speed and Operation Modes

- Compatibility for UHPLC up to 70k resolution
- Compatibility for >5 second wide peaks at 140,000 resolution
- **Full MS** with high resolution accurate mass detection
- Selected Ion Monitoring (**SIM**) and Multiplex SIM (mSIM)
- **MS/MS** of isolated ions with high resolution accurate mass detection
- 'All Ion Fragmentation' in the HCD collision cell
- Source fragmentation of all ions in the source region
- Positive/negative ion switching
- Data Dependent on-the-fly decision making
- Timed SIM for scheduled data

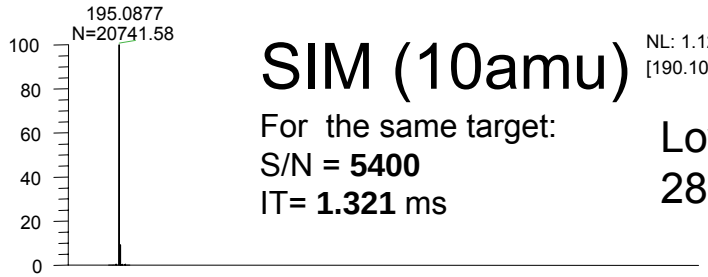
What Do We Gain by Selected Ion Monitoring?

- In Full MS, total C-trap charge capacity is shared between multiple signals of different intensity
- Signal-to-noise ratio becomes dependent on the ratio of compound of interest to other analytes-much less so in SIM!
- In Orbitrap instruments, SIM could become MRM without any additional overhead!



Full MS
S/N = **745**
IT= **0.245** ms

Lowest signal 250330

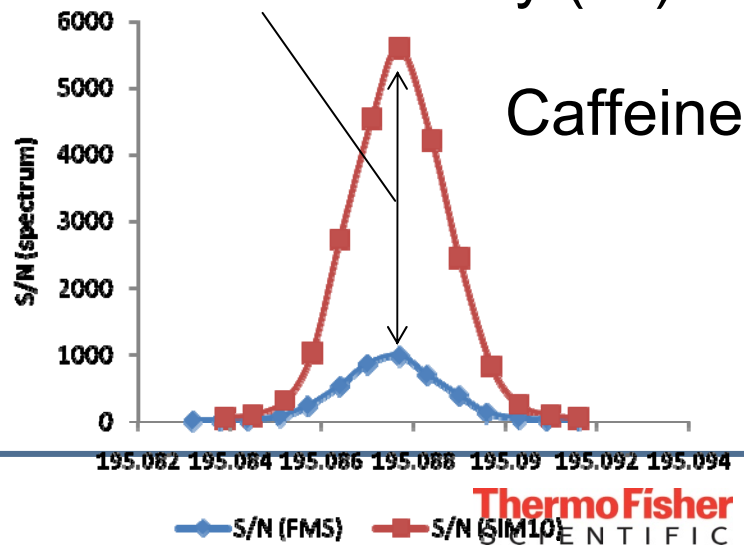


SIM (10amu)
For the same target:
S/N = **5400**
IT= **1.321** ms

Lowest signal 28240

- Sensitivity gain 5 – 10 x with SIM mode
- The gain will be higher in more complex matrices

Gain in sensitivity (7x)



Caffeine

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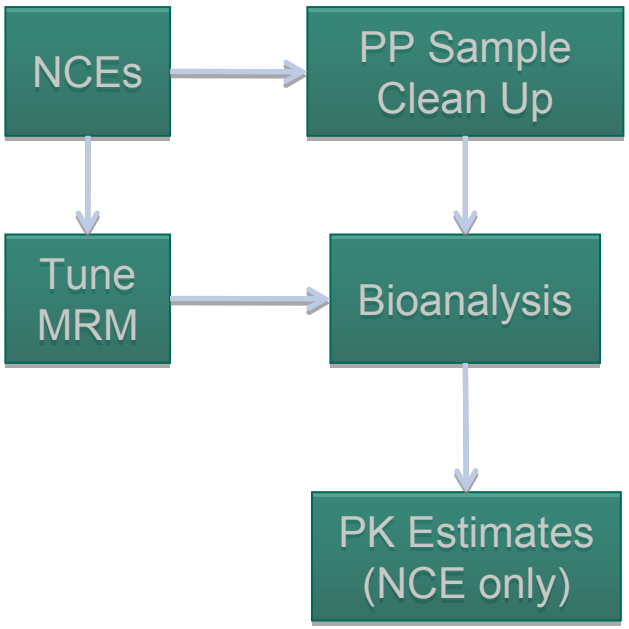
QUANTITATION

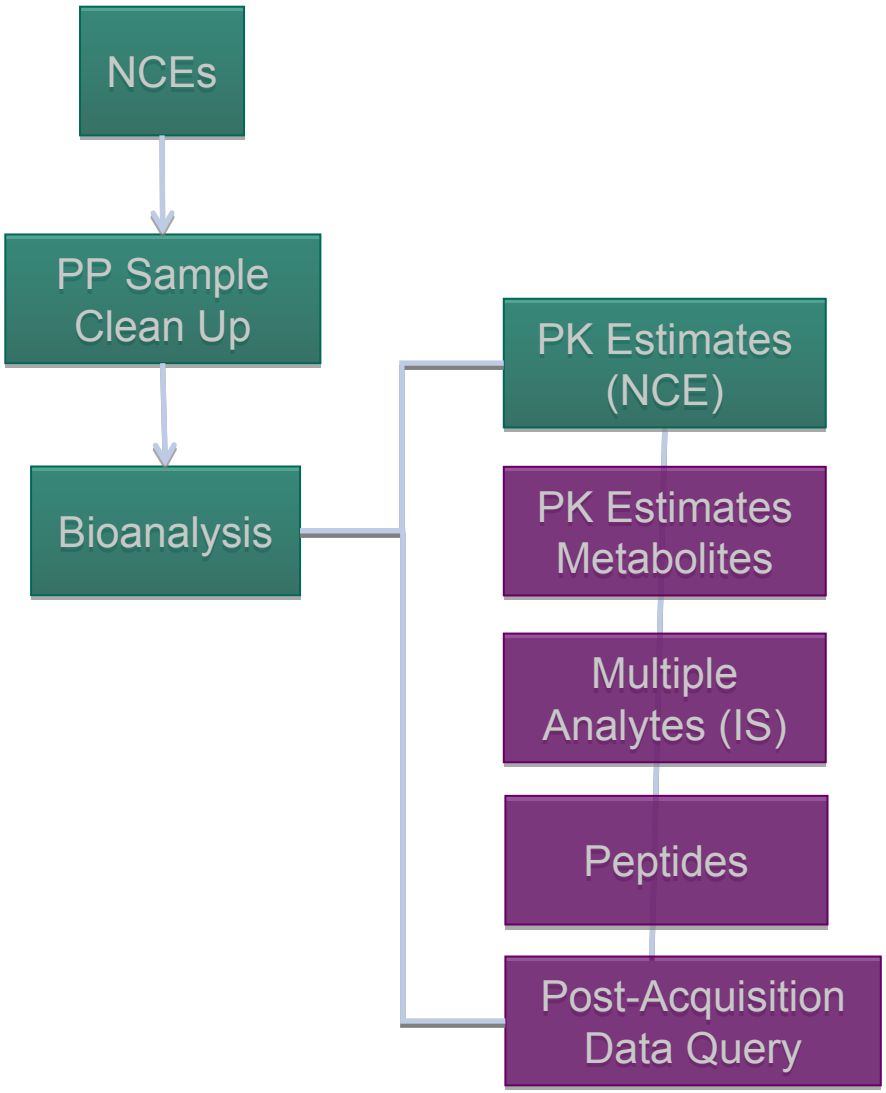
Traditional Workflow (Triple Quadrupoles)



LC-SRM Analysis

- Tuning MRM takes time & requires some level of expertise
- MRM methods are not easily transferable between platforms from different vendors, which makes scalability difficult.
- Peptides require determination of charge state and the optimal SRM transition, which is again platform dependent. Expertise required, even for a basic assay.
- Limits the number of transitions (duty cycle & no. of scans per analyte).
- Difficult to automate set-up to get sequence information – expertise required.

HRMS Workflow (Q Exactive)



LC-HRMS

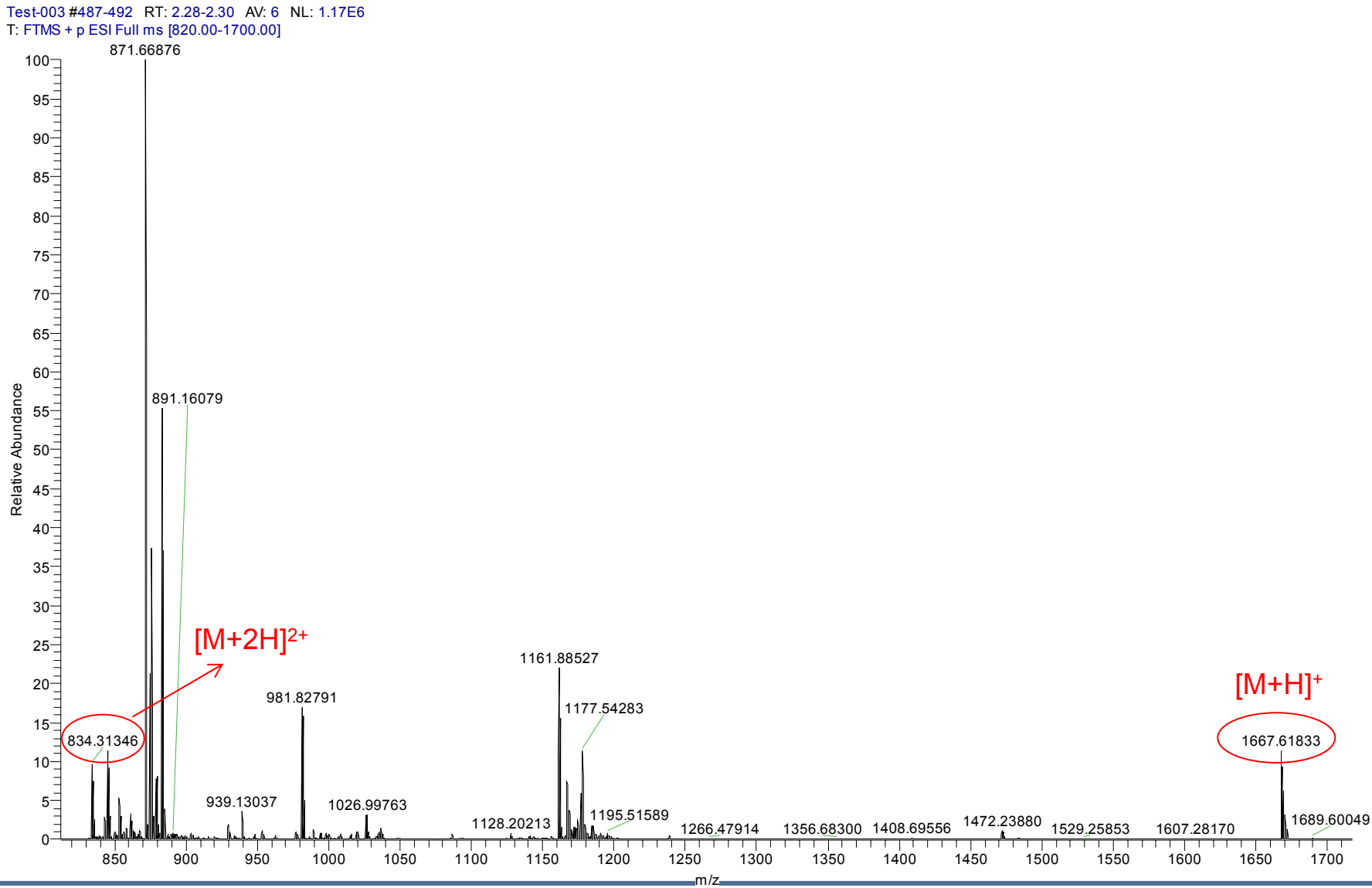
- No compound dependent tuning required – easier to use/faster to set-up
- Post-acquisition data analysis
- Providing PK data as well as *critical new information (metabolites, biomarkers)*
- More value in terms of the Fail Fast paradigm

Quantitation Summary of Small Molecules on Q-Exactive

Compound	TSQ		QE Full Scan (70K)		QE tSIM (70K)	
	LOD	LLOQ	LOD	LLOQ	LOD	LLOQ
Oxycodone	1	**5**	5	10	1	**5**
Buprenorphine	50	100	50	**50**	50	**50**
Paroxetine	1	10	1	**5**	10	10
Ketoconazole	50	100	1	**50**	1	**50**
Clonazepam	1	**10**	5	50	10	10
Verapamil	1	**1**	5	5	1	5
Alprazolam	1	**5**	5	10	10	50
Reserpine	1	**10**	10	50	5	**10**
Clopidogrel	1	**5**	50	50	5	10

All Regression linear $1/x^2$ or quadratic $1/x^2$

Uroguanylin in Glucagon Peptide Matrix (Full Scan) – Q Exactive



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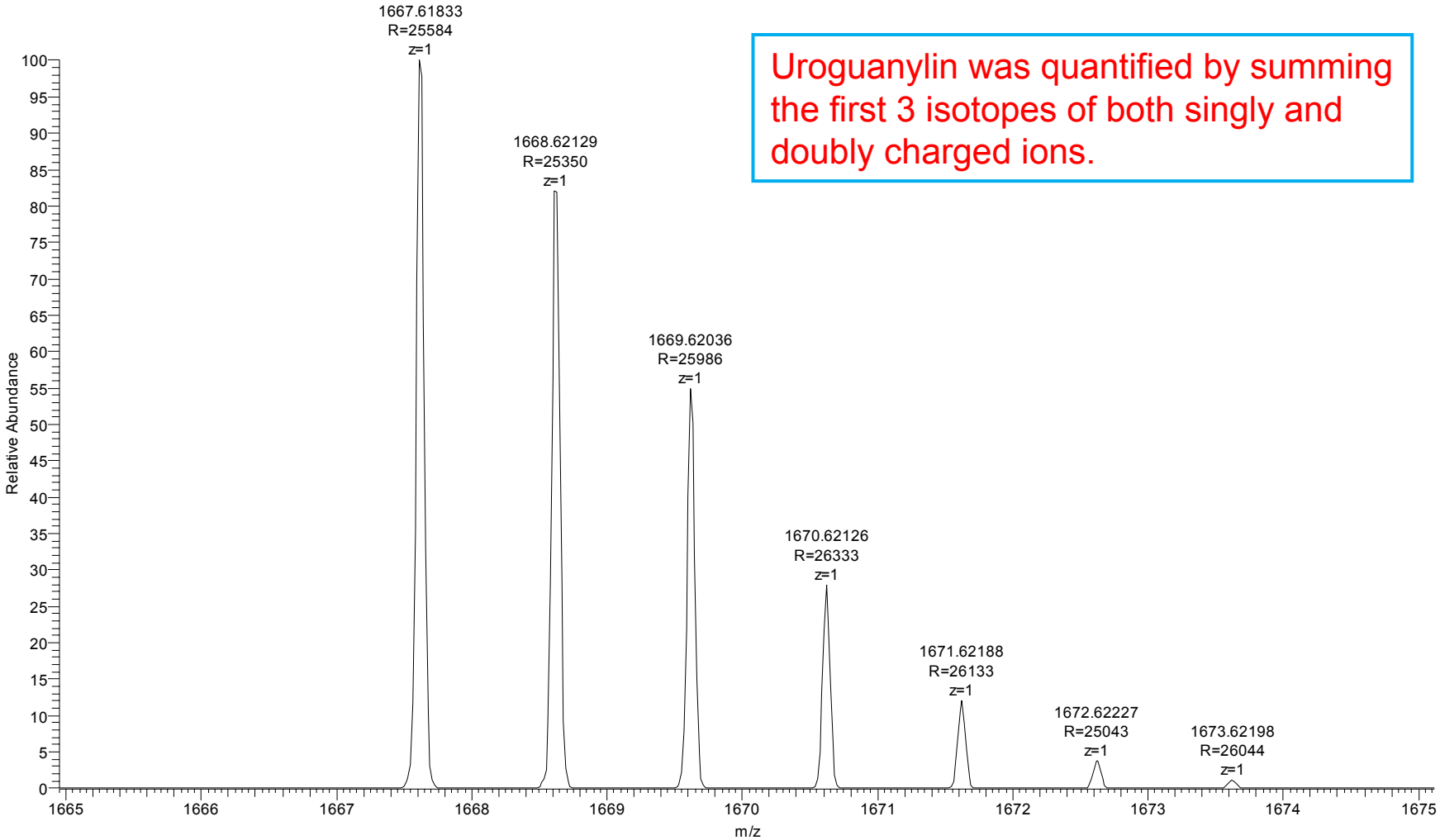
Uroguanylin Singly Charged Species

Test-003 #487-492 RT: 2.28-2.30 AV: 6 NL: 1.32E5
T: FTMS + p ESI Full ms [820.00-1700.00]



Uroguanylin was quantified by summing the first 3 isotopes of both singly and doubly charged ions.

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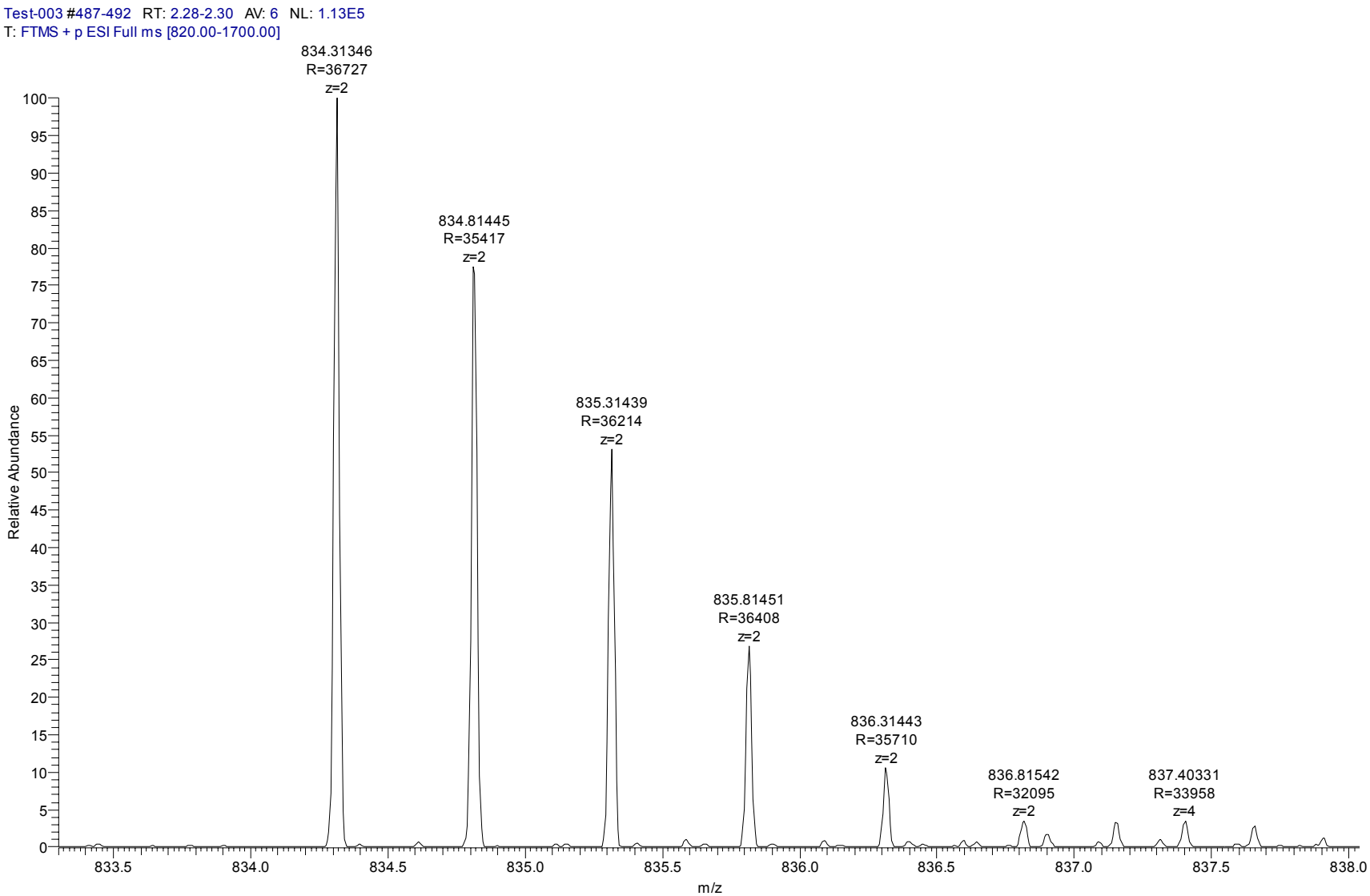
Uroguanylin Doubly Charged Species



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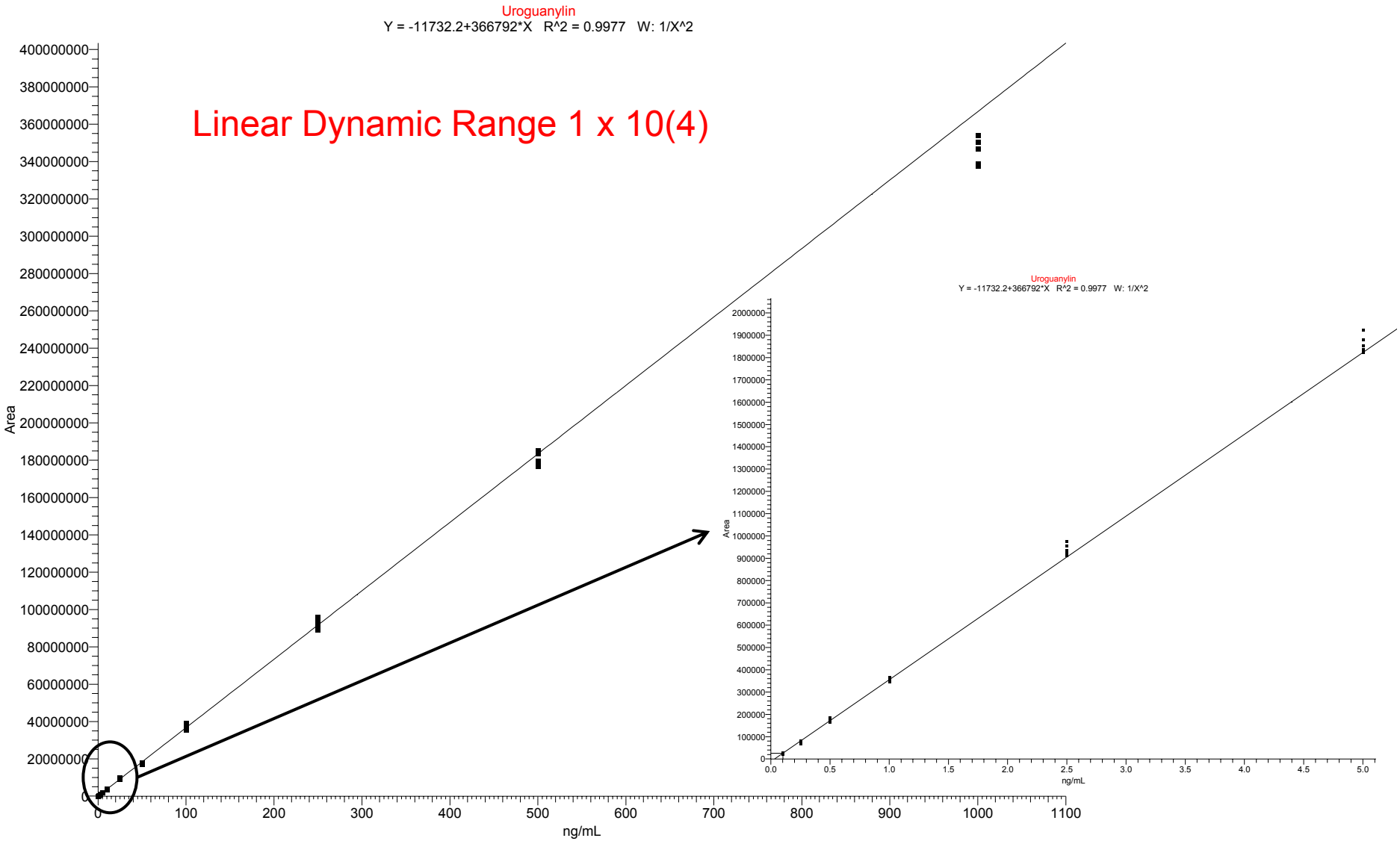
Q Exactive: 100 pg/mL to 10,000 pg/mL – Uroguanylin in Glucagon Peptide Matrix (t-SIM) R^2=0.9977, Linear $1/x^2$



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Insulin Quantitation Results

Nominal Concentration (ng/mL)	Replicate #	Mean Calculated Concentration	Stdev	% CV
0.25	4	0.260	0.0300	11.5
0.5	4	0.434	0.0501	11.5
1	4	0.906	0.0540	5.96
2.5	4	2.81	0.0568	2.02
5	4	4.79	0.112	2.33
10	4	10.3	0.284	2.77
25	4	27.9	0.247	0.89
50	4	44.0	1.35	3.08
100	4	92.8	4.29	4.62
250	4	276	9.99	3.62
1000	4	979	36.7	3.75

Exendin Quantitation Results

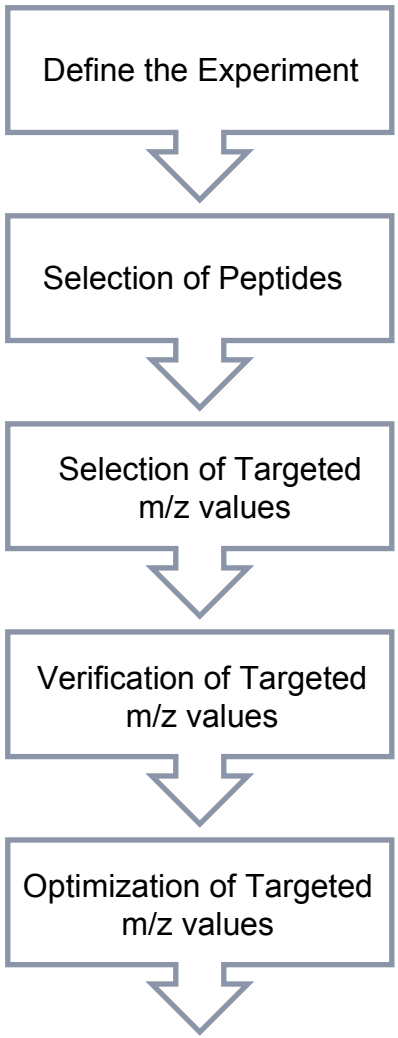
Nominal Concentration (ng/mL)	Replicate #	Mean Calculated Concentration	Stdev	% CV
5	4	5.143	0.418	8.13
10	4	9.693	0.520	5.36
25	4	24.37	1.22	5.00
50	4	45.46	0.705	1.55
100	4	92.83	13.0	14.0
250	4	261.7	5.31	2.03
500	4	507.3	2.03	0.40
1000	4	966.0	25.2	2.61
2500	4	2675.6	60.7	2.27
5000	4	5205	169	3.24
10000	4	9740	150	1.54

GLP-1 Quantitation Results

Nominal Concentration (ng/mL)	Replicate #	Mean Calculated Concentration	Stdev	% CV
0.1	4	0.099	0.0098	9.91
0.25	4	0.251	0.0205	8.16
0.5	4	0.534	0.0337	6.31
1	4	0.905	0.0226	2.50
2.5	4	2.69	0.0578	2.15
5	4	5.36	0.126	2.36
10	4	9.99	0.120	1.20
25	4	26.6	0.664	2.50
250	4	50.6	0.562	1.11
100	4	80	0.692	0.86
250	4	243	14.2	5.86
500	4	462	15.5	3.35
1000	4	860	7.90	0.92
5000	4	5383	62.8	1.17
10000	4	9796	164	1.67

PROTEOMICS RESEARCH TO ROUTINE

Workflow Steps



Identify the protein sequence under study

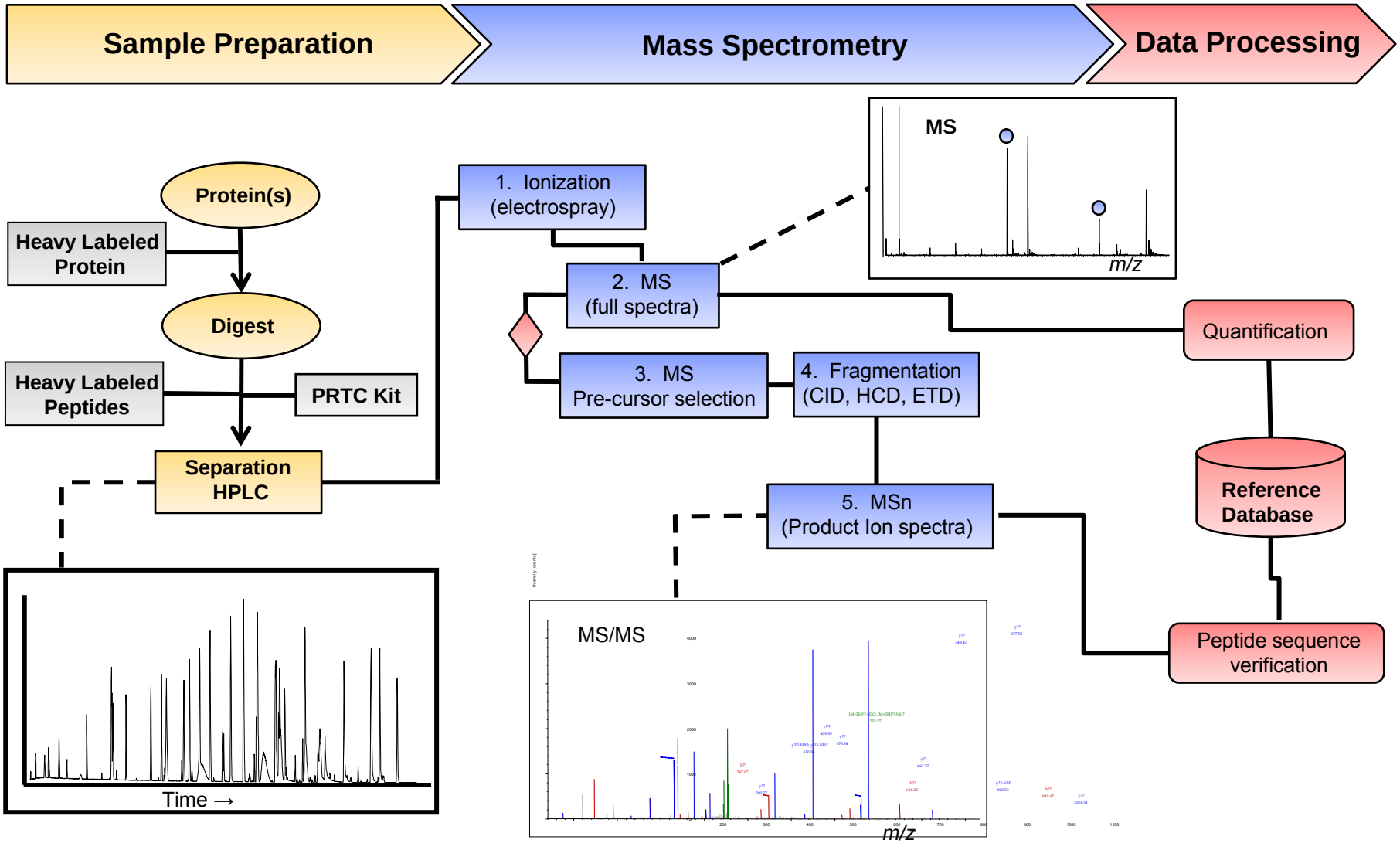
Literature search, in silico digestion, BLAST searchers to ensure sequence specificity WRT to the background matrix

Sensitivity based on intense fragmentation, selectivity WRT background

Comparison of experimental LC-MS and MS/MS parameters to references (incorporation of recombinant protein analysis and/or heavy labeled synthetic peptides

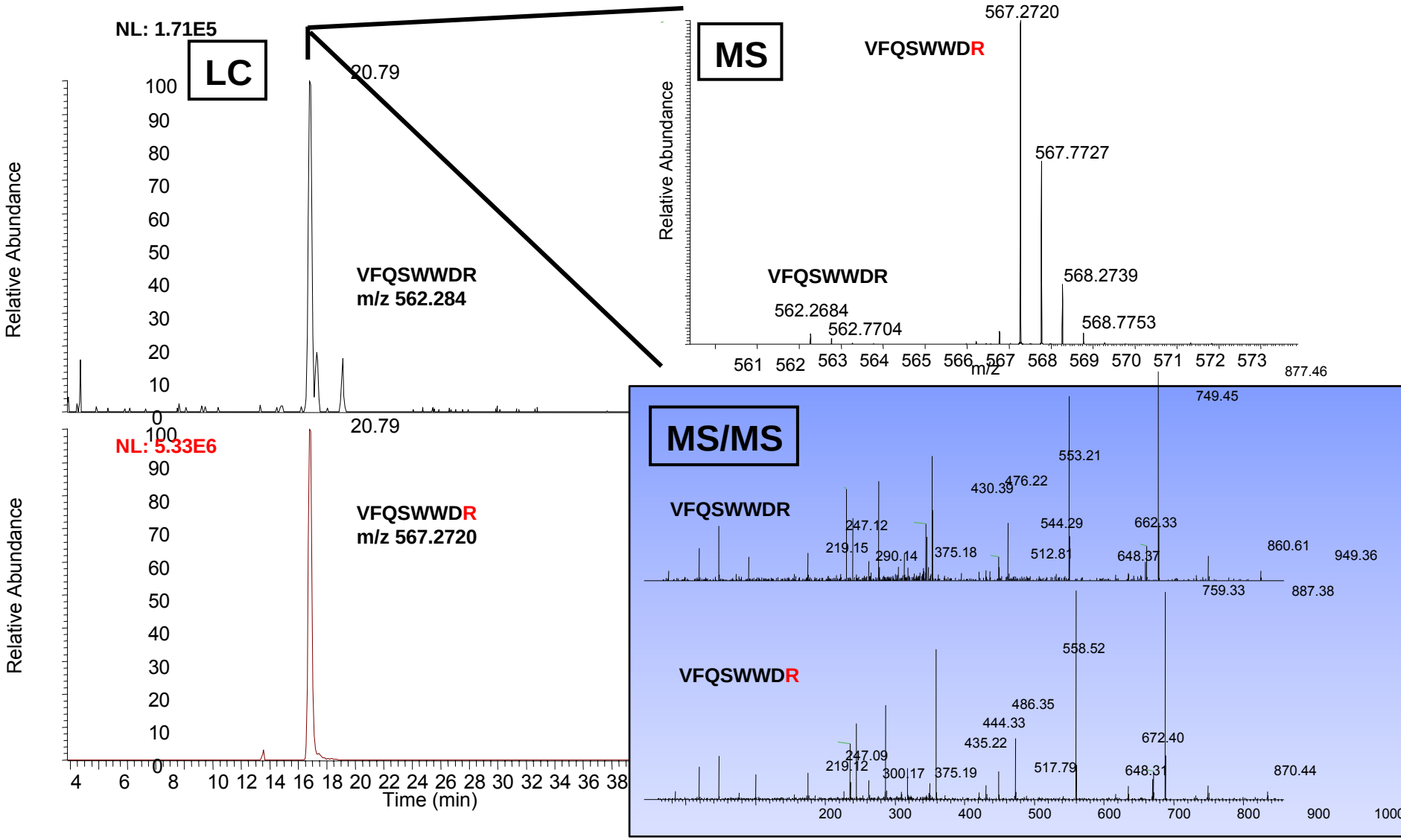
Keeping only the best peptides/product ions, collision energies, scheduled time windows

Workflow for Targeted Protein Quantitation Development



Domon and Aebersold Nature Biotechnology 2010, 7(28), 710-721

Target Verification Using Heavy Labeled Peptides



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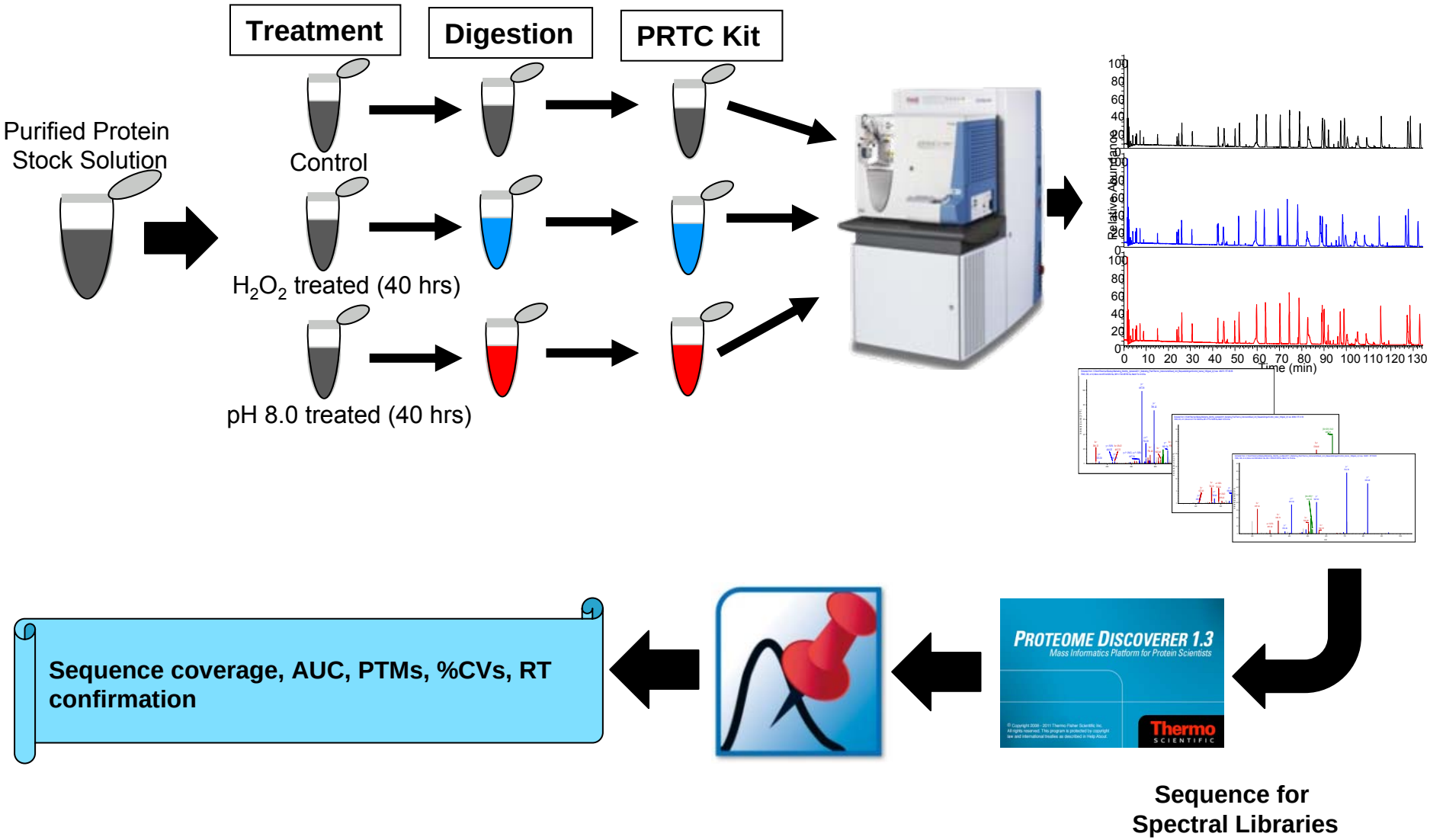
HeavyPeptide™ AQUA™ Standards

Amino acid	Mass difference to standard AA	Isotope
Alanine / A	+ 4 Da	U-^{13}C3; ^{15}N
Arginine / R	+ 10 Da	U-^{13}C6; ^{15}N4
Isoleucine / I	+ 7 Da	U-^{13}C6; ^{15}N
Leucine / L	+ 7 Da	U-^{13}C6; ^{15}N
Lysine / K	+ 8 Da	U-^{13}C6; ^{15}N2
Phenylalanine / F	+ 10 Da	U-^{13}C9; ^{15}N
Proline / P	+ 6 Da	U-^{13}C5; ^{15}N
Valine / V	+ 6 Da	U-^{13}C5; ^{15}N
Other amino acids on request		

HeavyPeptide™ AQUA™ Benefits

- HeavyPeptide™ AQUA™ products enable absolute quantification of all proteins in a sample.
- The HeavyPeptide AQUA kits can now be prepared with covalent modifications, such as phosphorylation, which are chemically identical to naturally occurring post-translational modifications (PTMs). As a result, the HeavyPeptide AQUA kits are an extremely cost-effective solution, enabling researchers to identify and quantify peptides of interest much faster, with significantly increased precision.
- This answers the need for relative and absolute quantification of the expression levels for all proteins in complex samples. This is essential since PTMs significantly increase the size of proteomes over their corresponding genomes.
- The HeavyPeptide AQUA product range produces a clear and consistent gain in efficiency, transparency and reproducibility of experiments.

Experimental



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Pierce Peptide Retention Time Calibration Kit + Pinpoint 1.2 Software

- Peptide Retention Time Calibration Kit
- Pinpoint 1.2 Software for Targeted Protein Quan

- Application:
  - Quickly assess and optimize chromatography and MS instrument performance
  - Predict peptide retention times using calculated hydrophobicity factors
  - Predict peptide elution across multiple instrument platforms
  - Improve quantification and increase multiplexing with optimized scheduled SRM windows
  - **Incorporation of the PRTC kit provides a system QC, normalization, and RT correlation across experiments and instrumental platforms.**

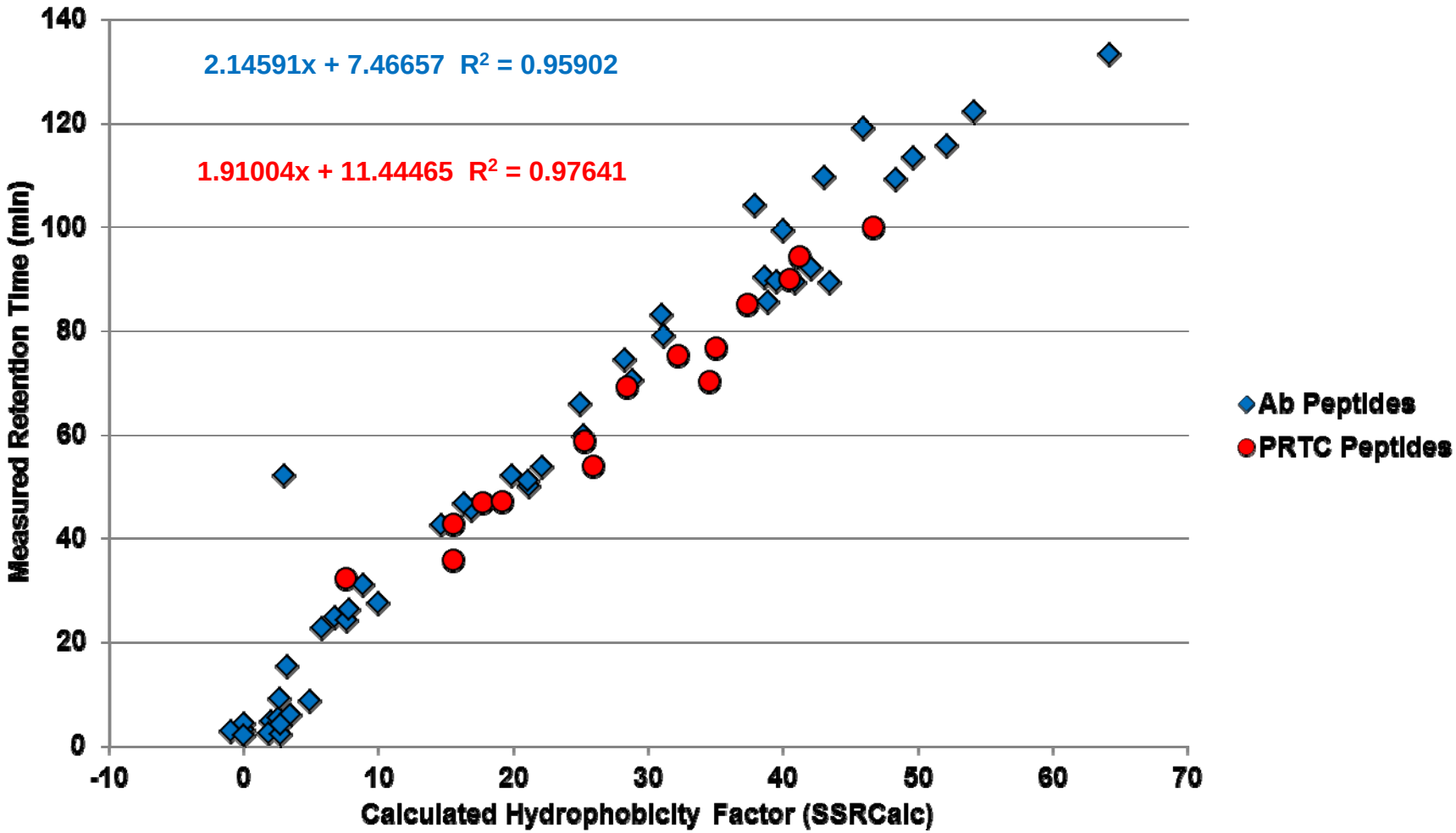
Pinpoint

- Key features
  - **High-purity** 15 synthetic heavy peptides mixed at equimolar ratio
  - **Elutes** across entire chromatographic gradient
  - **Fully automated** using Pinpoint 1.2 QC page

Peptide Retention Time Calibration (PRTC) Kit (Heavy Peptides)

#	Sequence	Observed Mass (Z=2)	Hydrophobicity Factor (HF)
1	SSAAPPPPPR	493.7	7.5681
2	GISNEGQNASIK	613.3	15.50003
3	HVLTSIGEK	496.3	15.52207
4	DIPVPKPK	451.3	17.65144
5	IGDYAGIK	422.7	19.15385
6	TASEFDSAIAQDK	695.8	25.8834
7	SAAGAFGPELSR	586.8	25.23967
8	ELGQSGVDTYLQTK	773.9	28.36797
9	GLILVGGYGTR	558.3	32.17702
10	GILFVGSGVSGGEEGAR	801.4	34.51977
11	SFANQPLEVVYSK	745.4	34.96488
12	LTILEELR	498.8	37.30326
13	NGFILDGFPR	573.3	40.41916
14	ELASGLSFPVGFK	680.4	41.18506
15	LSSEAPALFQFDLK	787.4	46.66305

Retention Time Analysis – Comparison with Internal Standards



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Conclusions

- Quanfirmation enabled characterization and quantification are performed in a single experiment
- Separating quantification (MS-level) and qualitative analysis enables reprocessing data for targeted peptide expansion
- Introduction of PRTC kit enabled method reproducibility for AS, LC, and MS methods
- PRTC kit provides direct relationship of calculated hydrophobicity factors to measured retention times and scalability
- Absolute and/or relative quantitation using heavy peptides
- Entire method is integrated with Proteome Discoverer and Pinpoint

Acknowledgments

- Kevin Cook
- Scott Peterman, Ph.D.
- Zhiqi Hao, Ph.D.

THANK YOU

Targeted Peptide Verification using HR/AM MS-Level Data

Experimental

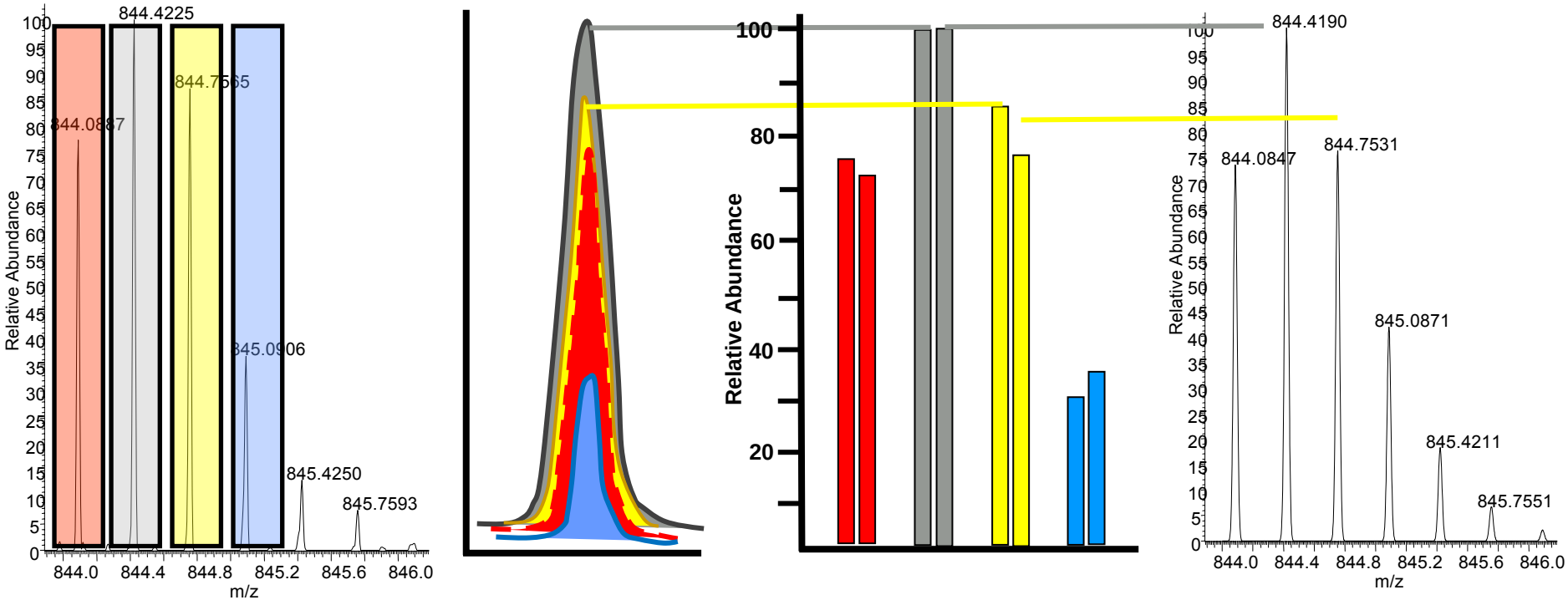
SMGGKEDLIWELLNQAQEHFGK
+3 CS = $C_{112}H_{175}N_{30}O_{35}S_1$

Theoretical

Mass Spectral Data

4.7 4.1 4.0 4.1

XICs



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