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*Proteomics Standards Research Group (sPRG)*

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# Using Skyline to analyze the SPRG2013-2014 Targeted Proteomics Assay (TPA) standard

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# What is the ABRF?

- ❖ ABRF members are from over 300 international core laboratories in academia, government, and industry, in a [broad spectrum of biomolecular technologies](#)
- ❖ The ABRF promotes the [research, technology, communication and education](#)
- ❖ ABRF has [Research Groups](#) and Committees, Affiliates and Chapters, [annual conferences](#) with educational courses, a quarterly journal, a [Newsletter](#), [Research Group publications](#), and Listserves
- ❖ The ABRF is unique for providing [benchmarking studies](#) by its Research Groups and has efficient mechanisms for networking and sharing



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# Proteomics Standards Research Group (sPRG) 2013-2014

## **Christopher Colangelo (Chair)**

Craig Dufresne

Alexander R. Ivanov (Ad hoc)

Toni Koller

Brett Phinney (EB Liaison)

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## **Yale University**

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Northeastern University

Stony Brook University

University of California, Davis

Vanderbilt University

National Institute of Standards and Technology

Proteome Software

University of Massachusetts Medical School

University of Washington SOM

UT M.D. Anderson



# Study Rationale / Design

- Design a comprehensive standard mixture of heavy-labeled tryptic peptides that can be used as internal standards for proteomics applications.
- Widely applicable across human, mouse, and rat proteomes.
- Proteotypic in nature.
- Utility towards both discovery and targeted proteomics applications.



# Study Rationale / Benefits

- Heavy/light peptide ratios will allow for normalization and evaluation for both inter- and intra- sample comparisons.
- System suitability of LC-MS/MS performance including benchmarking and reproducibility of LC peptide elution profiles, evaluation of MS sensitivity and dynamic range.
- Provide additional control for longitudinal proteomics studies and can be used in interlaboratory studies.
- Peptides can be converted into absolute quantitation standards via cleanup, purification, and amino acid analysis.



# Study Design

- 1000 stable isotope labeled tryptic peptides conserved across *Homo sapiens*, *Mus musculus* and *Rattus norvegicus*.
- Peptide mixture will be spiked at fixed concentration to a HEK digest at 3 different dilutions (3 orders of magnitude).
- Participants will analyze the samples using LC-MS instrument platform of their choice, report ratios, return the questionnaire and raw data to the sPRG (via study anonymizer or possibly Panorama).
- Spectral library will be constructed, data searched, and peptide ratios determined for all data sets.
- Comparison of ratios, normalization of data, and peptide retention/elution order will be compared across data sets.



# Peptide Selection

## Peptides selected via databases at Yale, NIST, Stony Brook

YPED; 115 LC-MS/MS HEK experiments

- MASCOT SCORE > identity
- BLAST search common to human, mouse, and rat
- K or R tryptic cleavage (C-terminal)

6750 peptides, <1% to 67% observed in the samples

- Removed peptides with upstream proline to K, R
- Removed peptides with upstream KK, RR
- Removed peptides with M
- Removed peptides w/ miscleavages

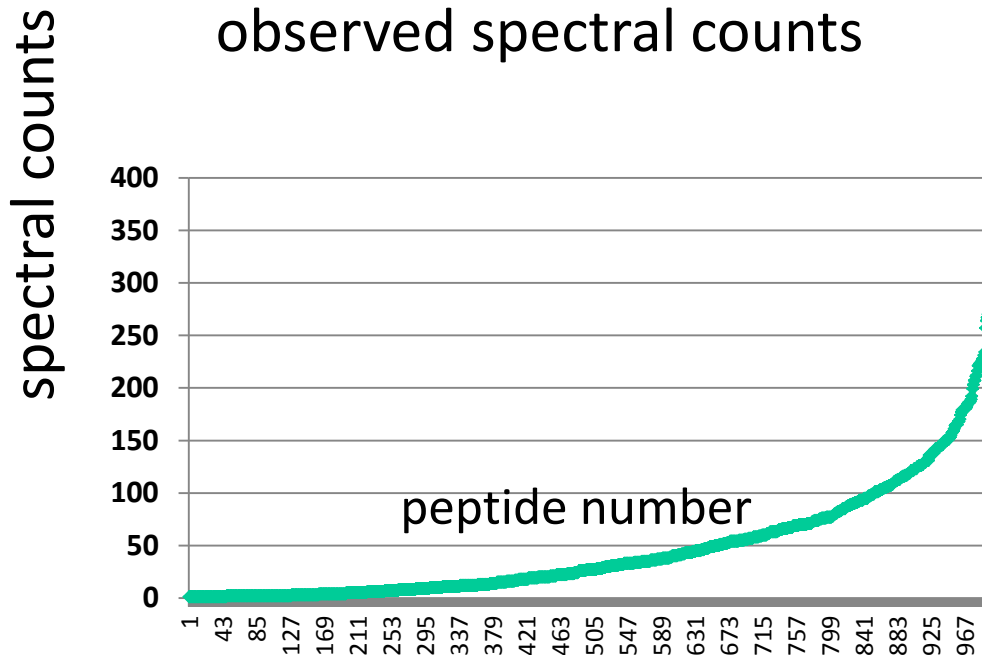
1500 peptide candidates to send to JPT Peptide Technologies

- JPT selected **1000** peptides for synthesis

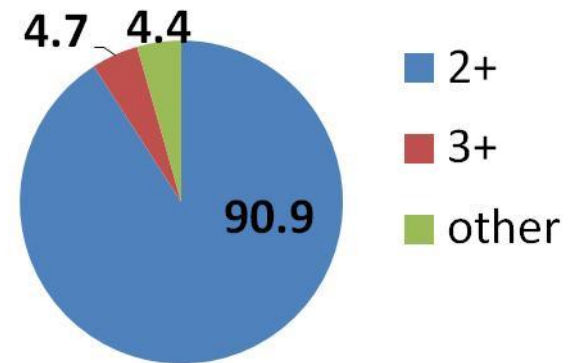


# Peptide Selection and Other Criteria

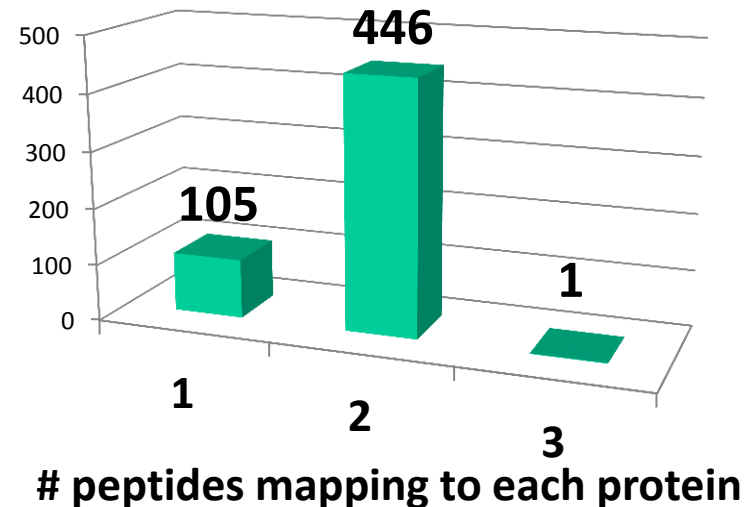
## Dynamic Range (YPED) observed spectral counts



## Charge State (NIST)



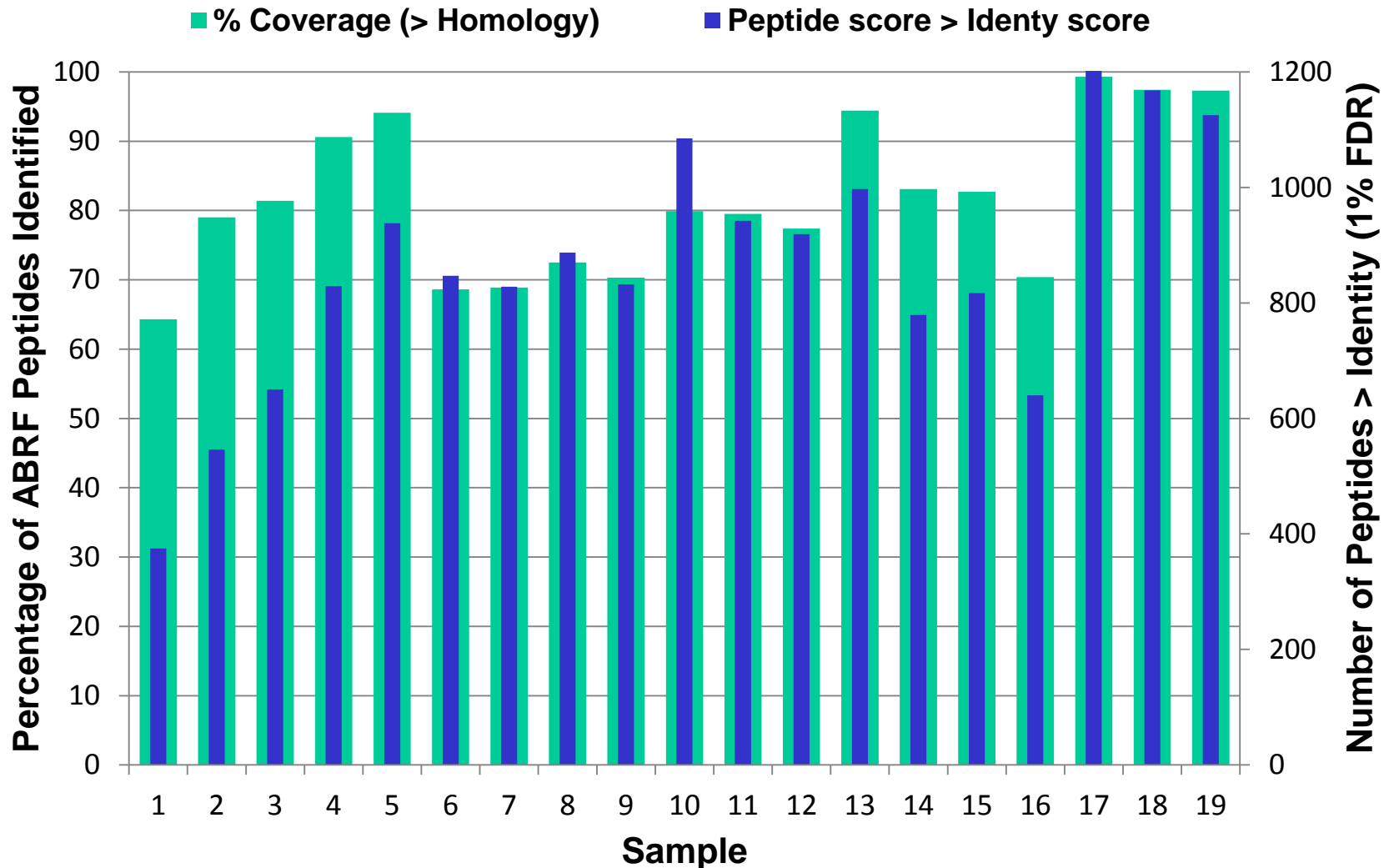
## Peptides Map to 552 proteins







# Peptide Identification in ABRF Labeled Peptide Standard Mix





# Using Skyline for AUC analysis

- 1000 peptides + 5 ug HEK lysate
  - We decided on AUC analysis as we did not have enough time to develop a scheduled MRM method
  - Still possible for someone out there to develop a scheduled MRM method if you want
    - Might be a good test for RT prediction algorithms



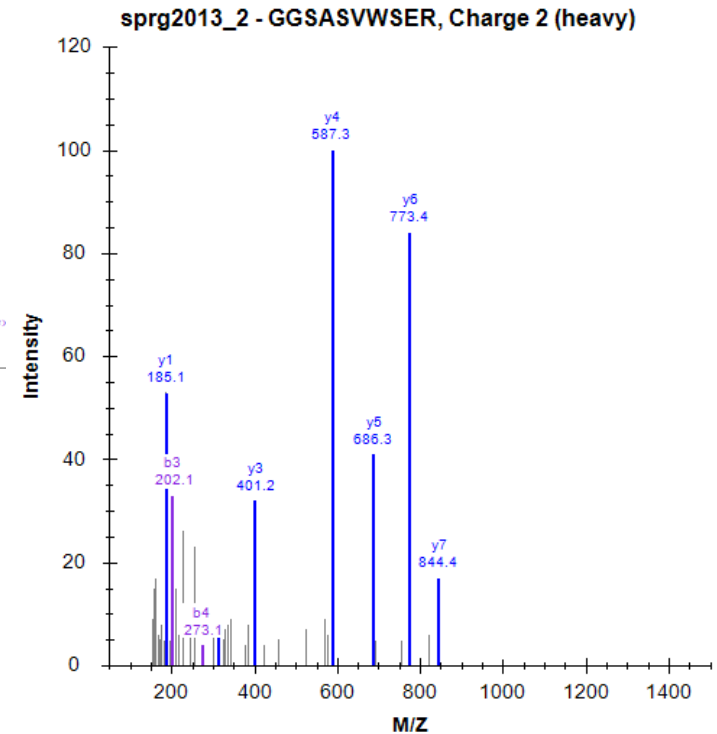
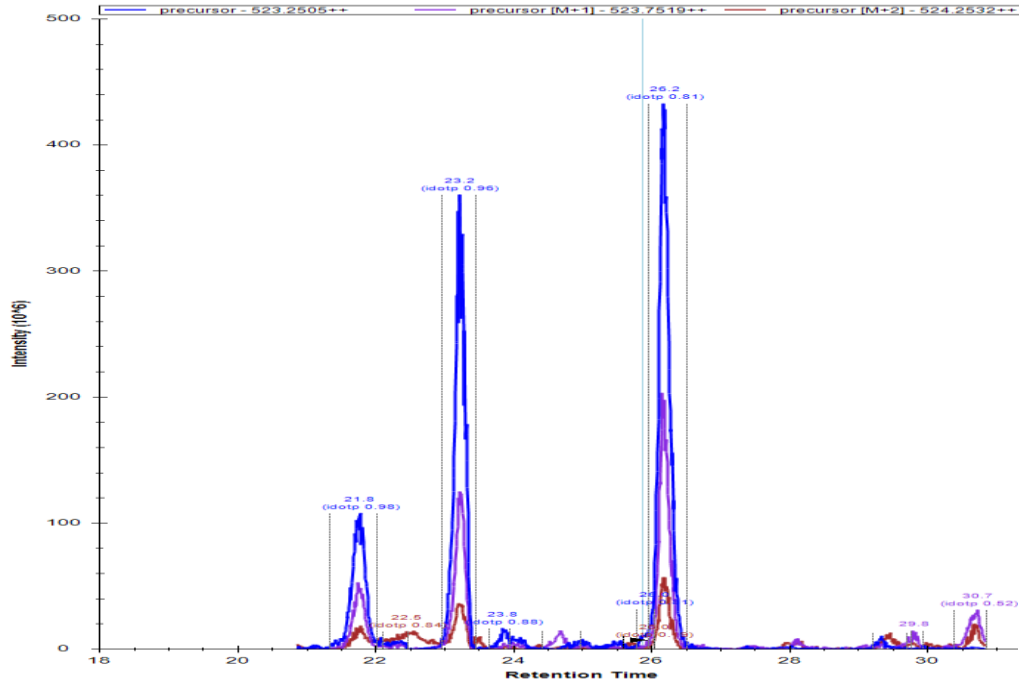
# Using Skyline for AUC analysis

- 1 pmol peptide mix spiked into 5 ug HEK 293 lysate
- Set MS1 resolving power at 70K
  - Q-Exactive Data

The screenshot shows the 'Transition Settings' dialog box in Skyline software. The 'Full-Scan' tab is selected. The 'MS1 filtering' section includes: 'Isotope peaks included' set to 'Count', 'Precursor mass analyzer' set to 'Orbitrap', 'Peaks' set to '3', 'Resolving power' set to '70,000', and 'At' set to '400 Th'. The 'Isotope labeling enrichment' is set to 'Default'. The 'MS/MS filtering' section includes: 'Acquisition method' set to 'None', 'Product mass analyzer' is empty, 'Isolation scheme' is empty, and 'Resolution' is empty. The 'Retention time filtering' section has three radio buttons: 'Include all matching scans' (unselected), 'Use only scans in retention time scheduling windows' (unselected), and 'Use only scans within 5 minutes of MS/MS IDs' (selected). 'OK' and 'Cancel' buttons are at the bottom.

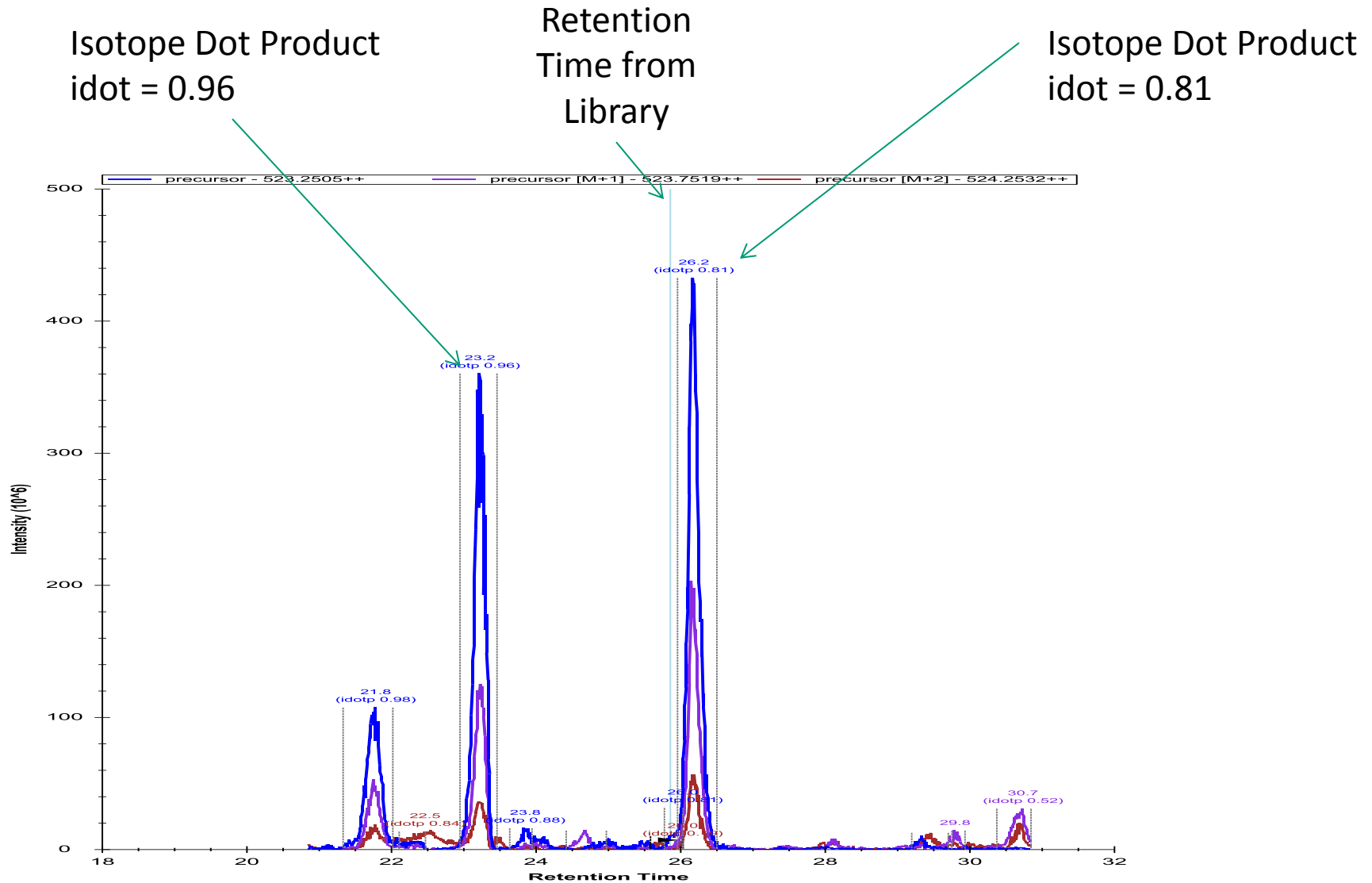


# Example Peptide GGSASVWSER (523.2505, 2+)



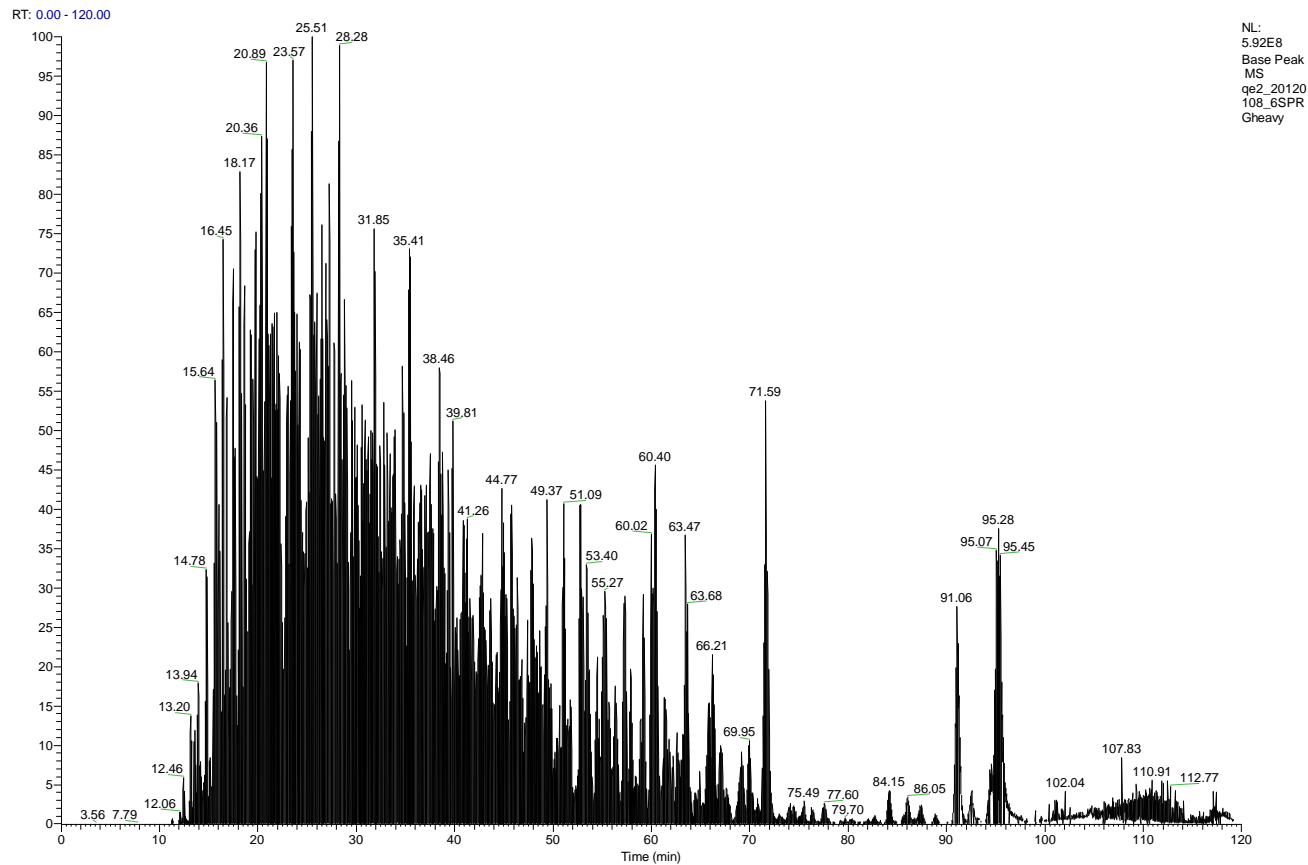


# Example Peptide GGASVWSER (523.2505, 2+)





# Lets look at raw data

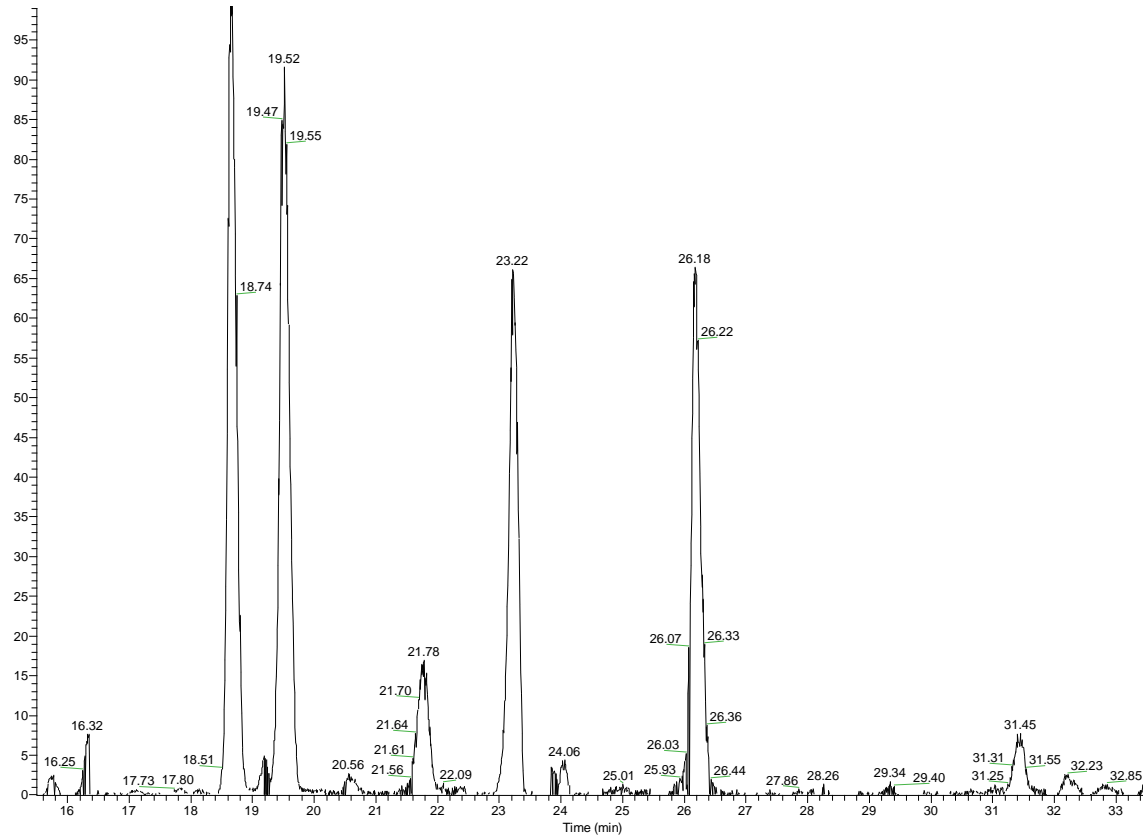




# XIC

## 523.25-523.27 m/z

RT: 15.50 - 33.54



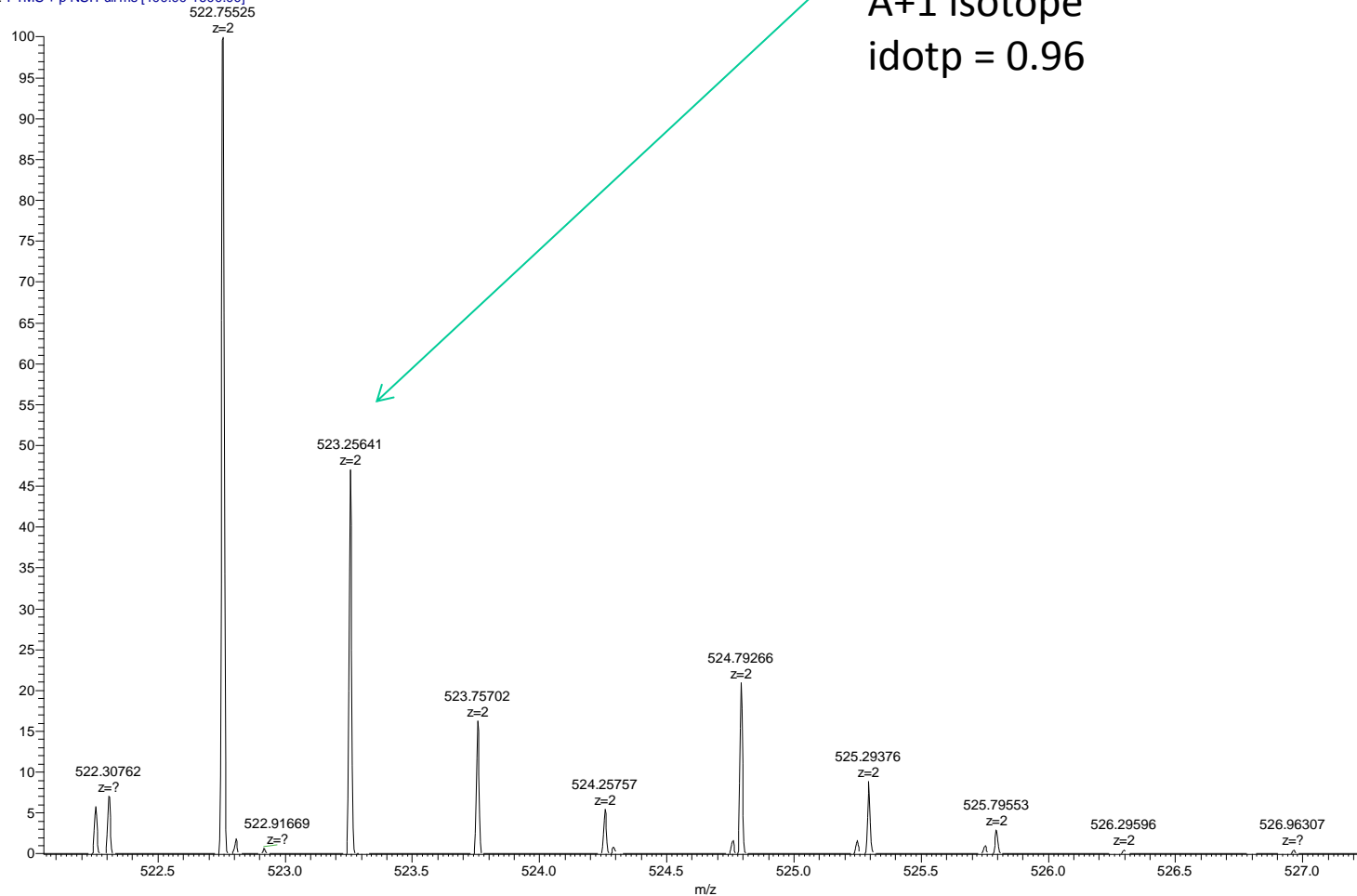
NL: 1.70E8  
Base Peak m/z=  
523.25-523.27 F: FTMS  
+ p NSI Full ms  
[400.00-1600.00] MS  
qe2\_20120108\_6SPRGh  
eavy



# MS Spectra (522-527 m/z) Peak at 23 min

523.25641  
A+1 isotope  
idotp = 0.96

qe2\_20120108\_6SPRGheavy #6572 RT: 23.20 AV: 1 NL: 1.87E8  
T: FTMS + p NSI Full ms [400.00-1600.00]

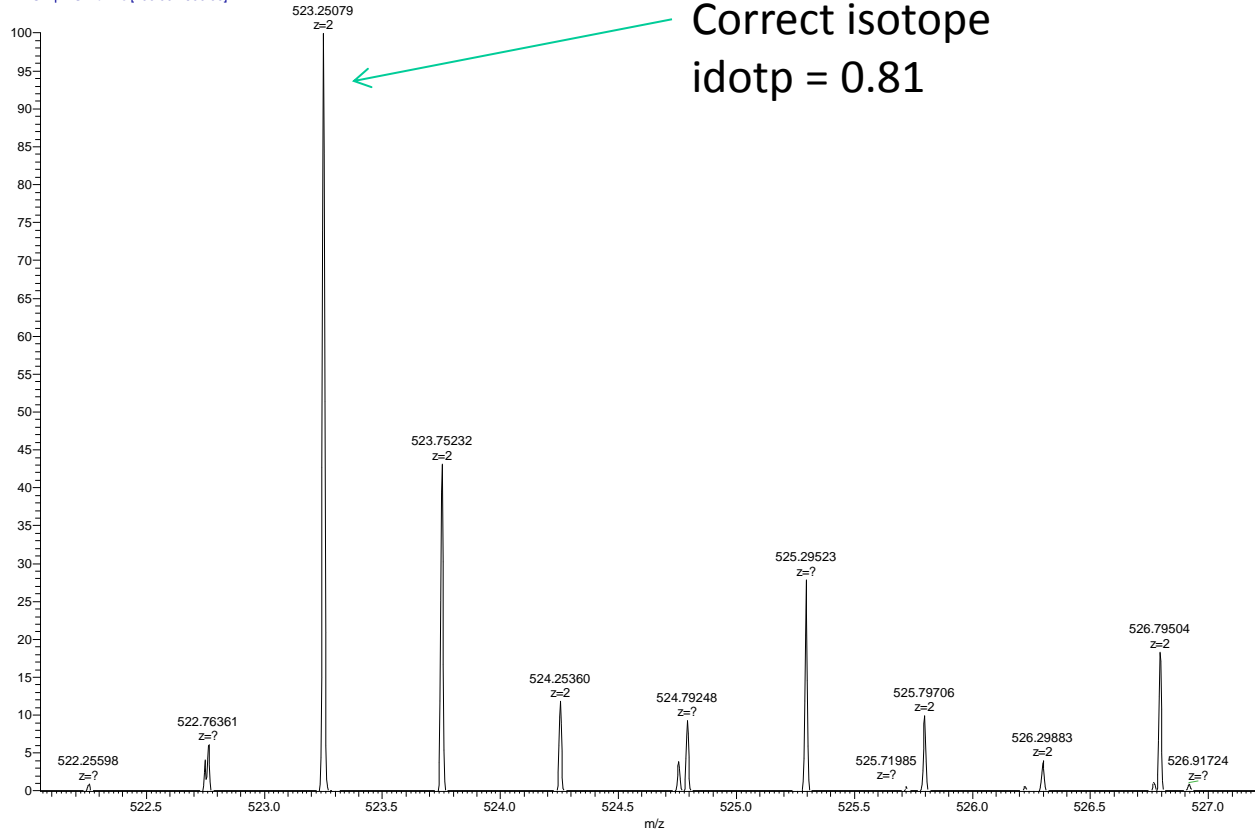






# MS Spectra (522-527 m/z) Peak at 26 minutes

qe2\_20120108\_6SPRGheavy#7683 RT: 26.16 AV: 1 NL: 1.11E8  
T: FTMS + p NSI Full ms [400.00-1600.00]



523.25079  
Correct isotope  
idotp = 0.81



# Precautions

- Skyline's Isotope Dot Product (idotp) is actually better for the wrong analyte
  - This can get a bit tricky when you are analyzing 1000 peptides
  - Probably best to not rely on just idotp
  - We have found that getting the RT's correctly in the library really is critical



# Making a library that has proper RT's

- This has been very finicky in our hands
- Some workflows work, some don't
  - Different combinations of software all seem to affect this
    - Peak List generator (mzML...mzXML....)
    - Searching software (x!tandem, Mascot...)
    - Meta file has to have the same name as the raw data file (except for the extension)

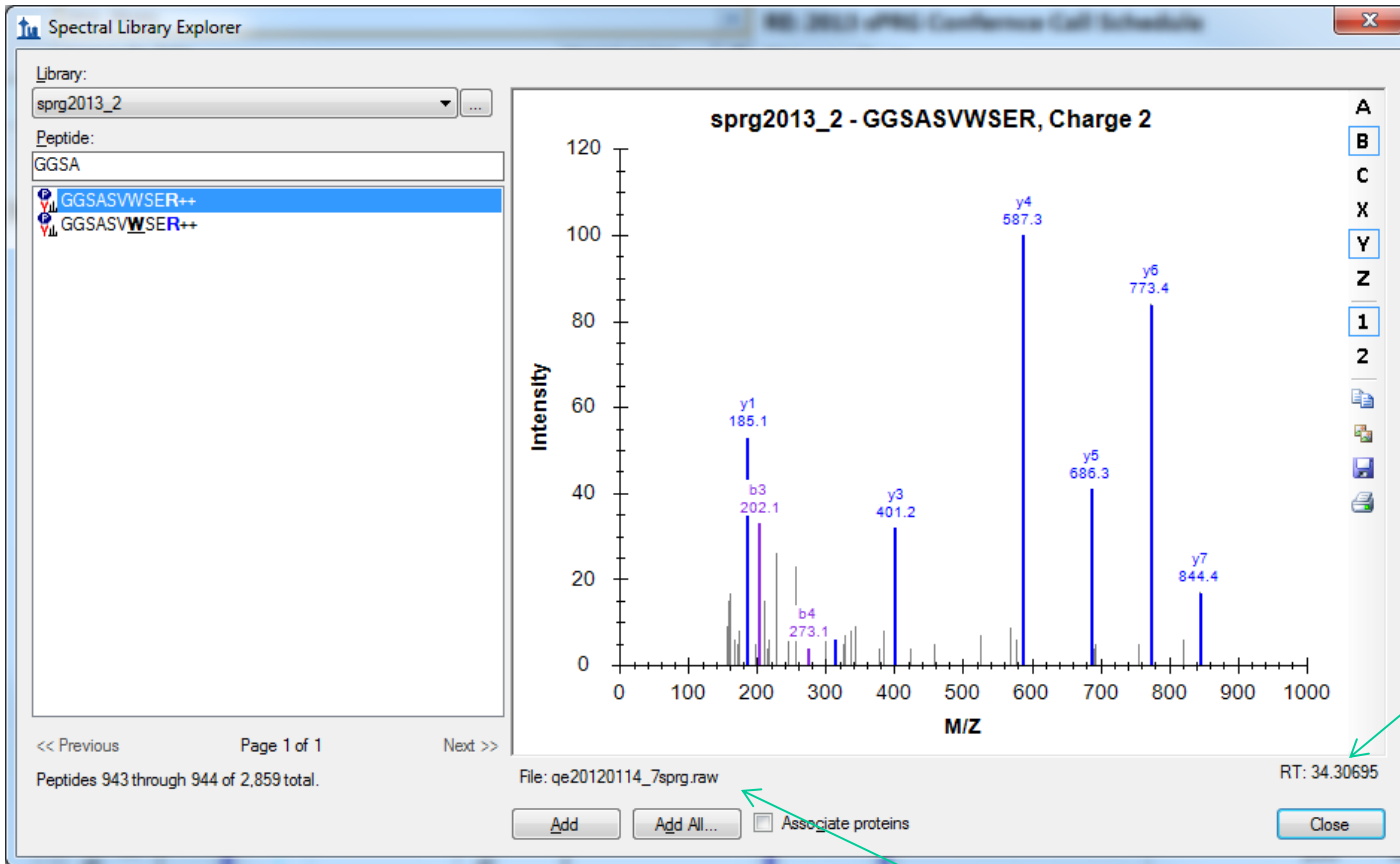


## Generating libraries with correct RT's (our workflow)

- Generate mzML using proteome discoverer
- Combine all peptides into an artificial protein
- Search with x!tandem (turning on the skyline option!)
- Rename the x!tandem output files to extension \*.xtan.xml
- Make Library in skyline



# Correct library

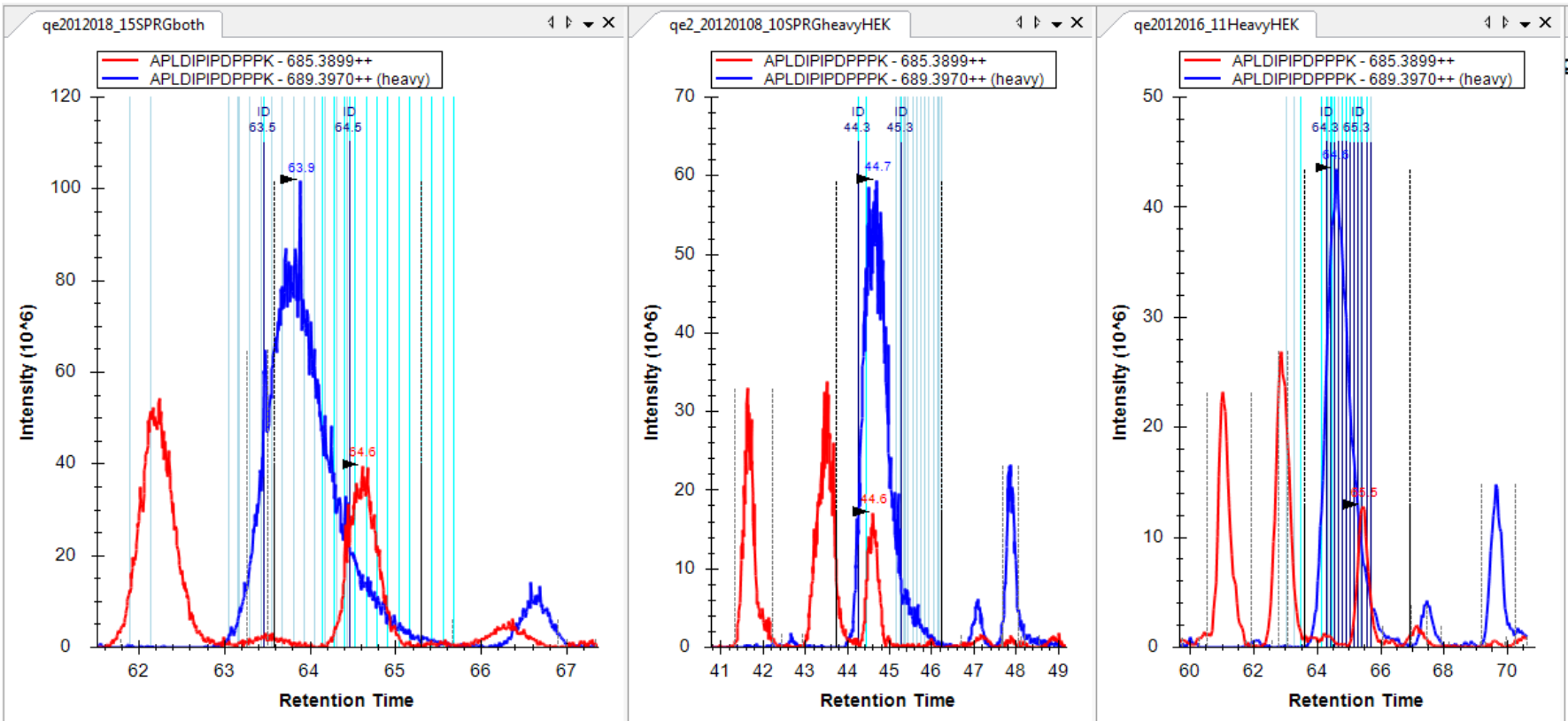


RT

File Name

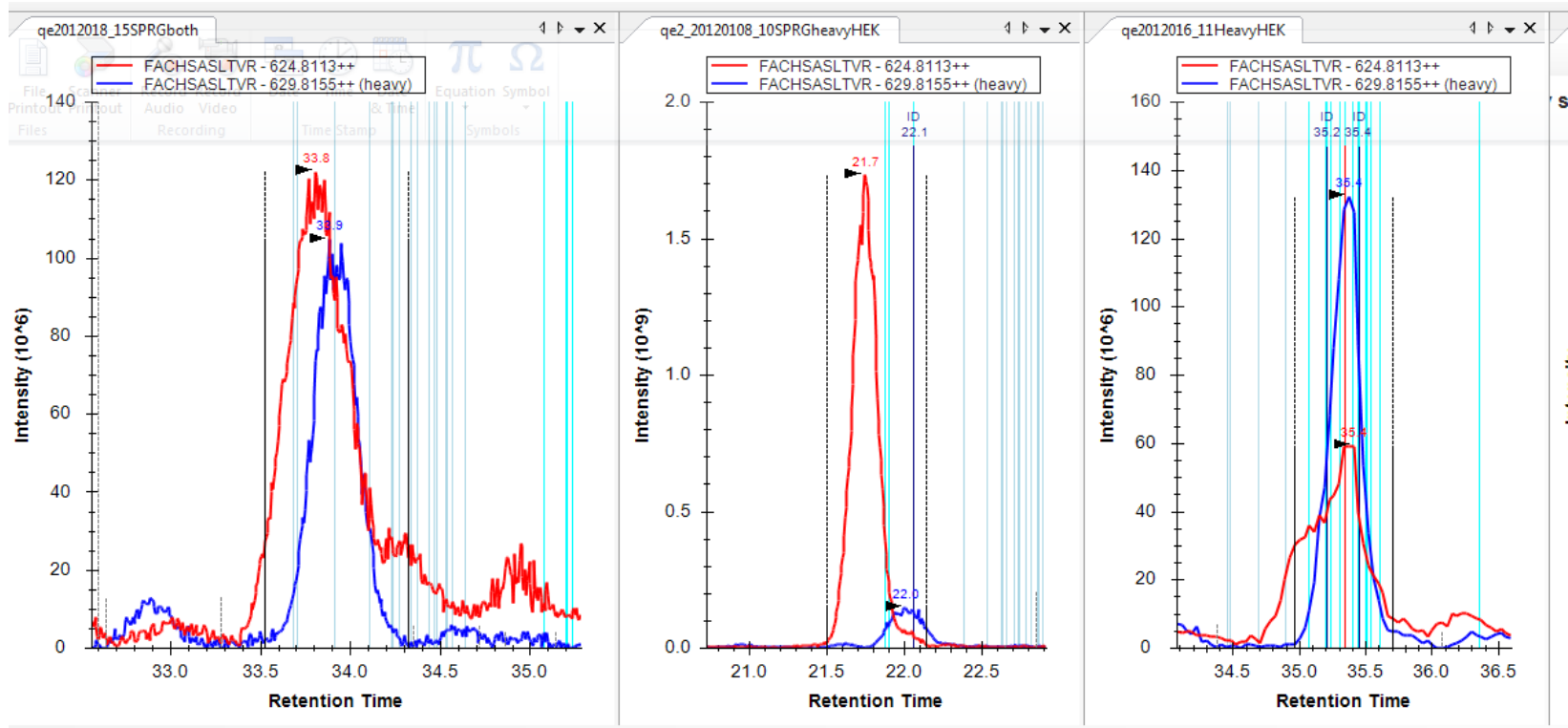


# HEK lysate Non-Co-eluting labeled peptides??



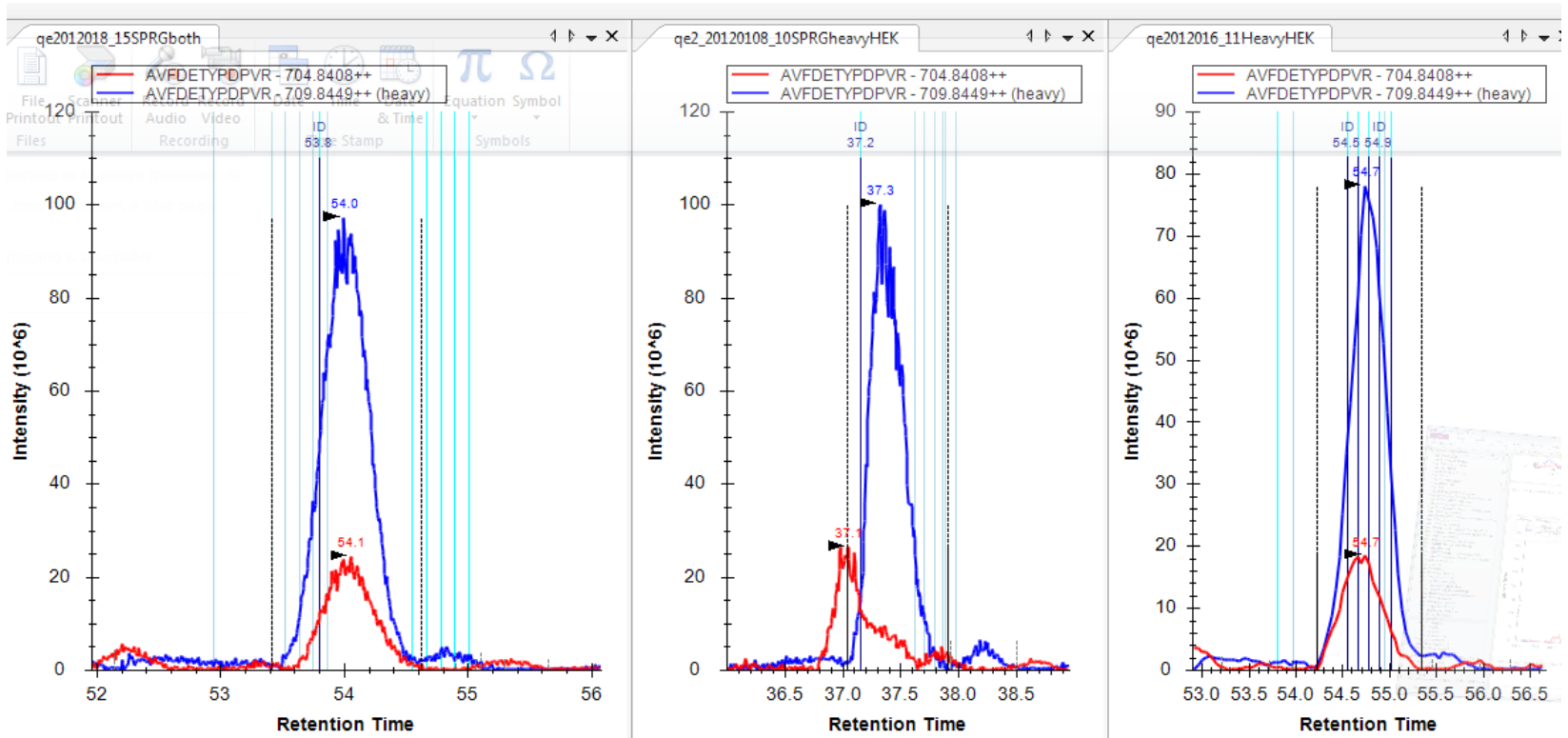


# More non co-eluting pairs





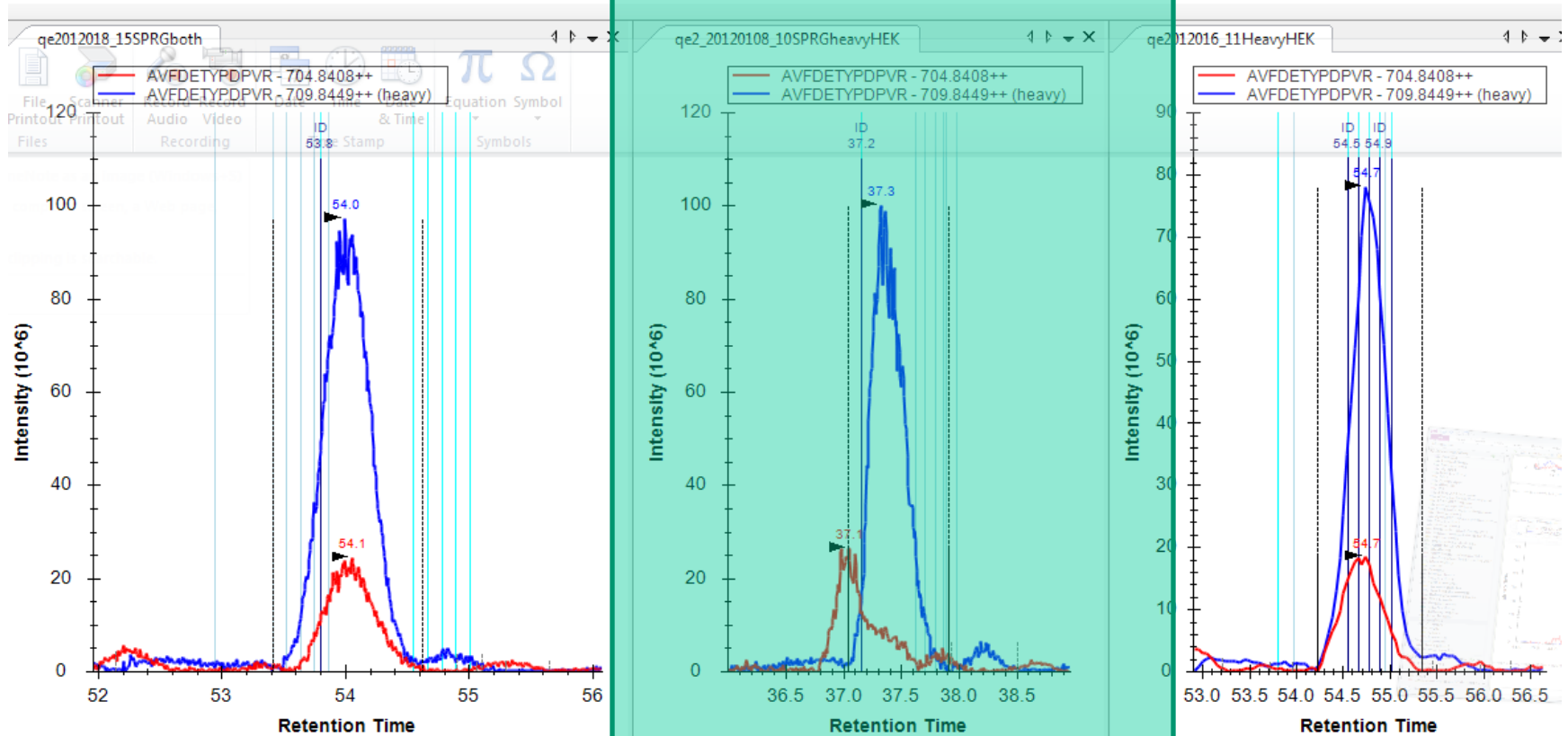
# And more







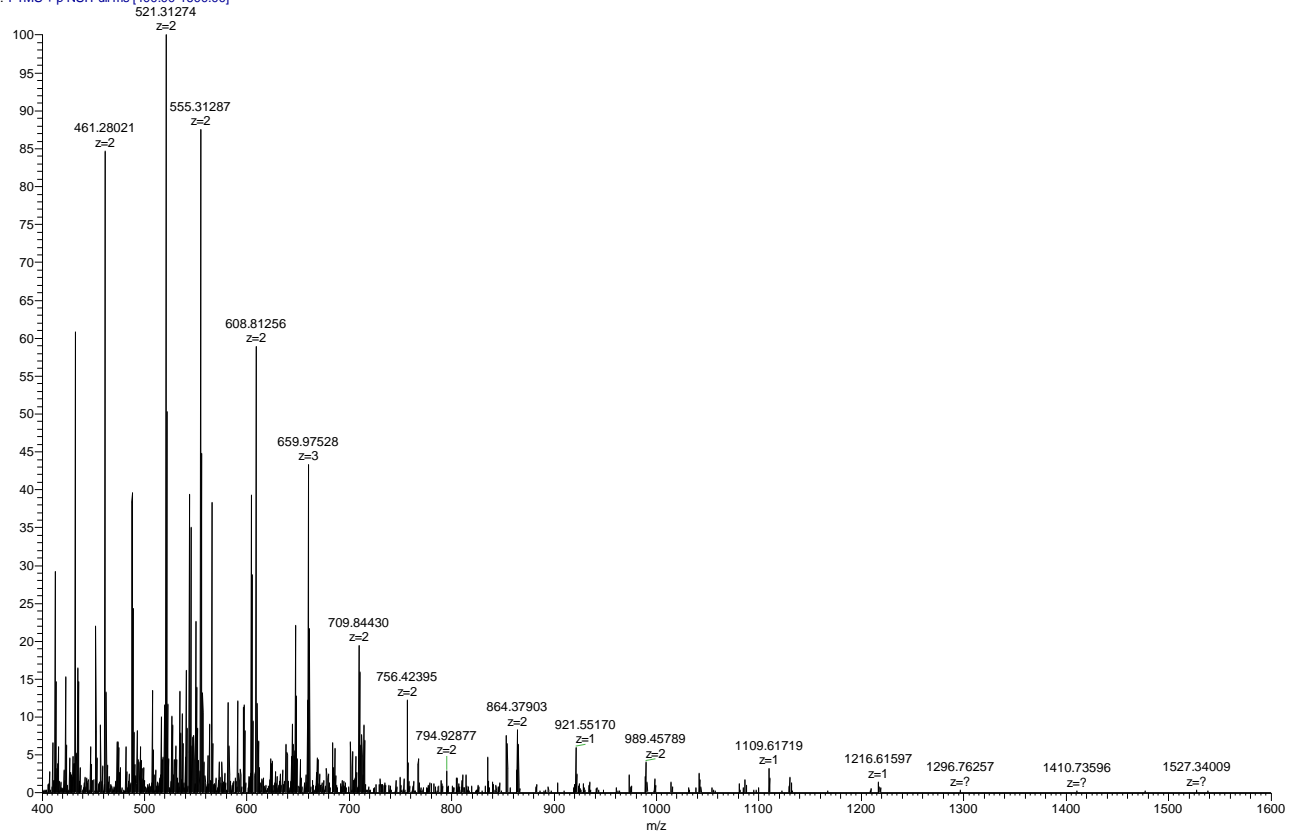
# Lets look at this one





# MS spectra very complex

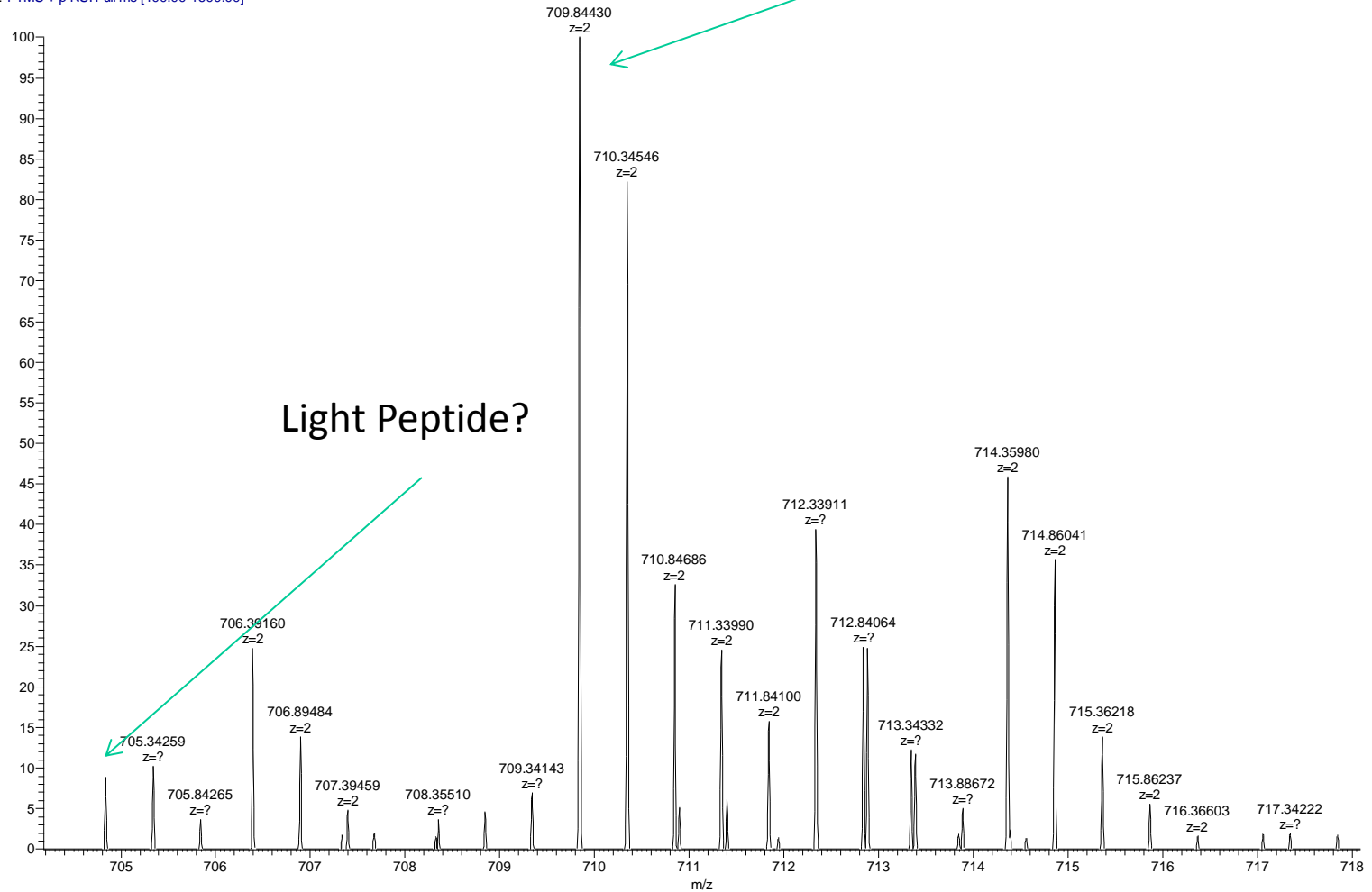
qe2\_20120108\_10SPRGheavyHEK #13810 RT: 37.50 AV: 1 NL: 3.97E7  
T: FTMS + p NSI Full ms [400.00-1600.00]





# Very complex

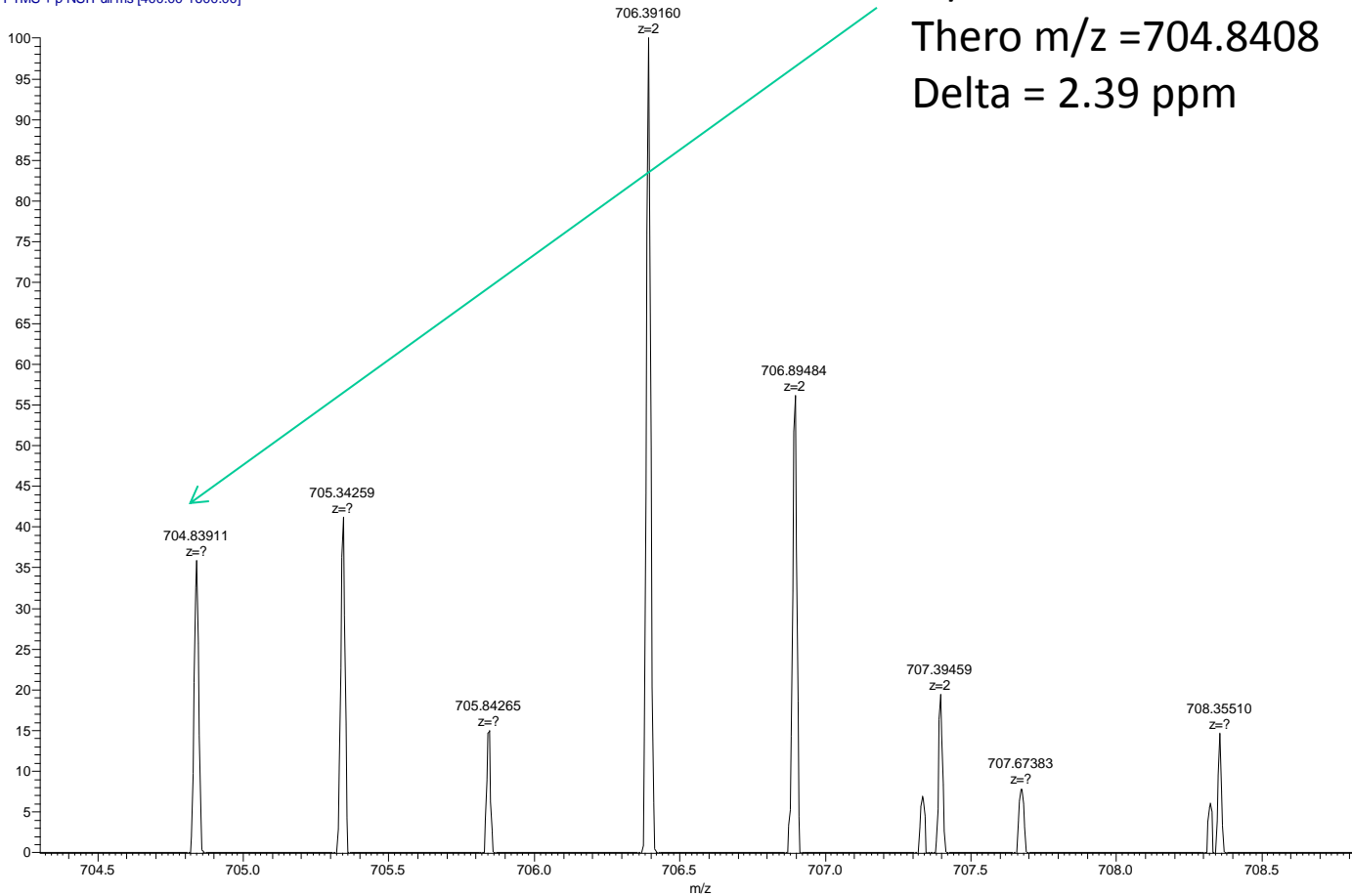
qe2\_20120108\_10SPRGheavyHEK #13810 RT: 37.50 AV: 1 NL: 7.73E6  
T: FTMS + p NSI Full ms [400.00-1600.00]





# Light HEK peptide?

qe2\_20120108\_10SPRGheavyHEK #13810 RT: 37.50 AV: 1 NL: 1.91E6  
T: FTMS + p NSI Full ms [400.00-1600.00]



m/z = 704.83911  
Thero m/z = 704.8408  
Delta = 2.39 ppm



# MS/MS spectra

- None Acquired for light peptide
- Is this the correct light peptide?
  - Probably not
- Next steps
  - Need to acquire with an isolation list or a MRM or Data Independent method??? Maybe use a much longer gradient or LC-LC-MS/MS



# Skyline analysis

- Can take a lot of analysis time to go over 1000 peptides in skyline in full scan mode
- Need to consider adding additional data metrics to reduce need to manually check each integration
- Figuring out if the light peptide of a heavy/light pair is real or interference is a real problem. Especially the more complex your data is.
- sPRG sample is probably too complex to analyze by Full scan in one 2 hour LC-MS/MS run



# Conclusions

- Progress has been made for the 1<sup>st</sup> year in development of an innovative proteomics standard.
- Study sample is designed to represent proteins spanning three orders of magnitude in concentration.
- 1,000 isotope labeled peptides were synthesized and analyzed. Greater than 99% of the peptides were identified during the validation runs.
- In peptide dilution experiments, the majority of peptides behaved as expected with a linear response; however, a few peptides had a non-linear response.
- The peptides were spiked into a HEK digest and showed minimal variation in both retention time and peak area.
- The sPRG is hopeful that the designed formulation will become a valuable resource in various mass spectrometry-based proteomic applications, including quantitative and differential protein profiling, interlaboratory studies, as well as general LC-MS/MS benchmarking.



# How to Get a sample

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- E-mail
  - [sprg2013@googlegroups.com](mailto:sprg2013@googlegroups.com)
- Samples are limited so be certain you can analyze the sample if you ask for one.





# Acknowledgments

- SPRG

## Current Membership

[Dr. Christopher Colangelo](#) (Chair) - *Yale University*

[Dr. Craig P. Dufresne](#) - *Thermo Fisher Scientific*

[Dr. Alexander R. Ivanov](#) - *Northeastern University*

[Dr. Antonius Koller](#) - *Stony Brook University*

[Brendan MacLean](#) - *University of Washington*

[Dr. Kristie L. Rose](#) - *Vanderbilt University Medical Center*

[Dr. Paul A Rudnick](#) - *NIST*

[Brian C. Searle](#) - *Proteome Software Inc.*

[Dr. Scott A. Shaffer](#) - *University of Massachusetts Medical School*

[Dr. Brett S Phinney](#) (EB Liaison) - *Proteomics Core UC Davis Genome Center*

[Dr. David Hawke](#) – *MD Anderson Cancer Center*



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# Acknowledgments part 2

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- JPT Peptide
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