Skyline Tutorial Webinar #12

Isotope Labeled Internal Standards in Skyline

With

Christina Ludwig (Proteomics Researcher)

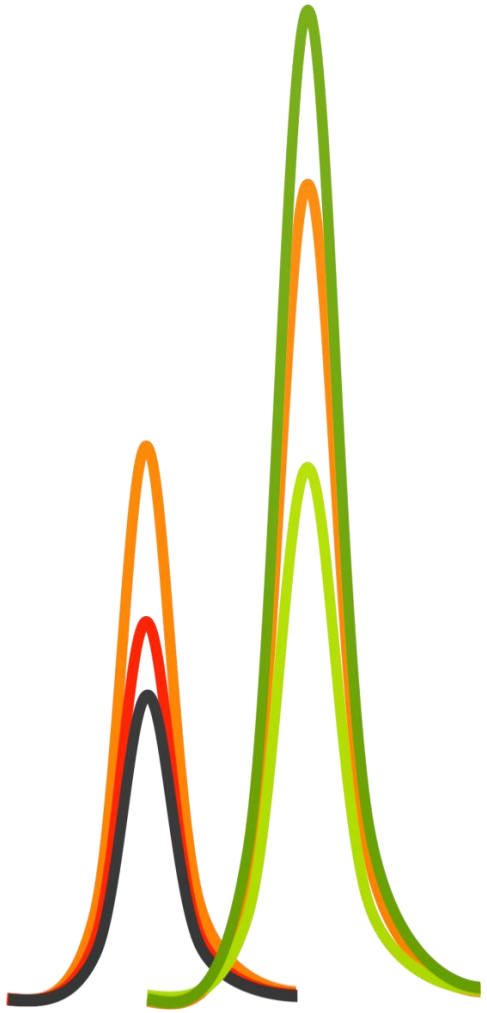
Ariel Bensimon (Proteomics Researcher)

Agenda

- Welcome from the Skyline team!
- **Isotope Labeled Standards in Skyline**
- Quick intro with Brendan MacLean
- with Ariel Bensimon

- with Tina Ludwig

- Audience Q&A – submit questions to Google Form:
  https://skyline.gs.washington.edu/labkey/qa4skyline.url



Lecture:
Isotope Labeled Standards in Skyline

Webinar , 1 December 2015

Christina Ludwig
TU Munich
Germany

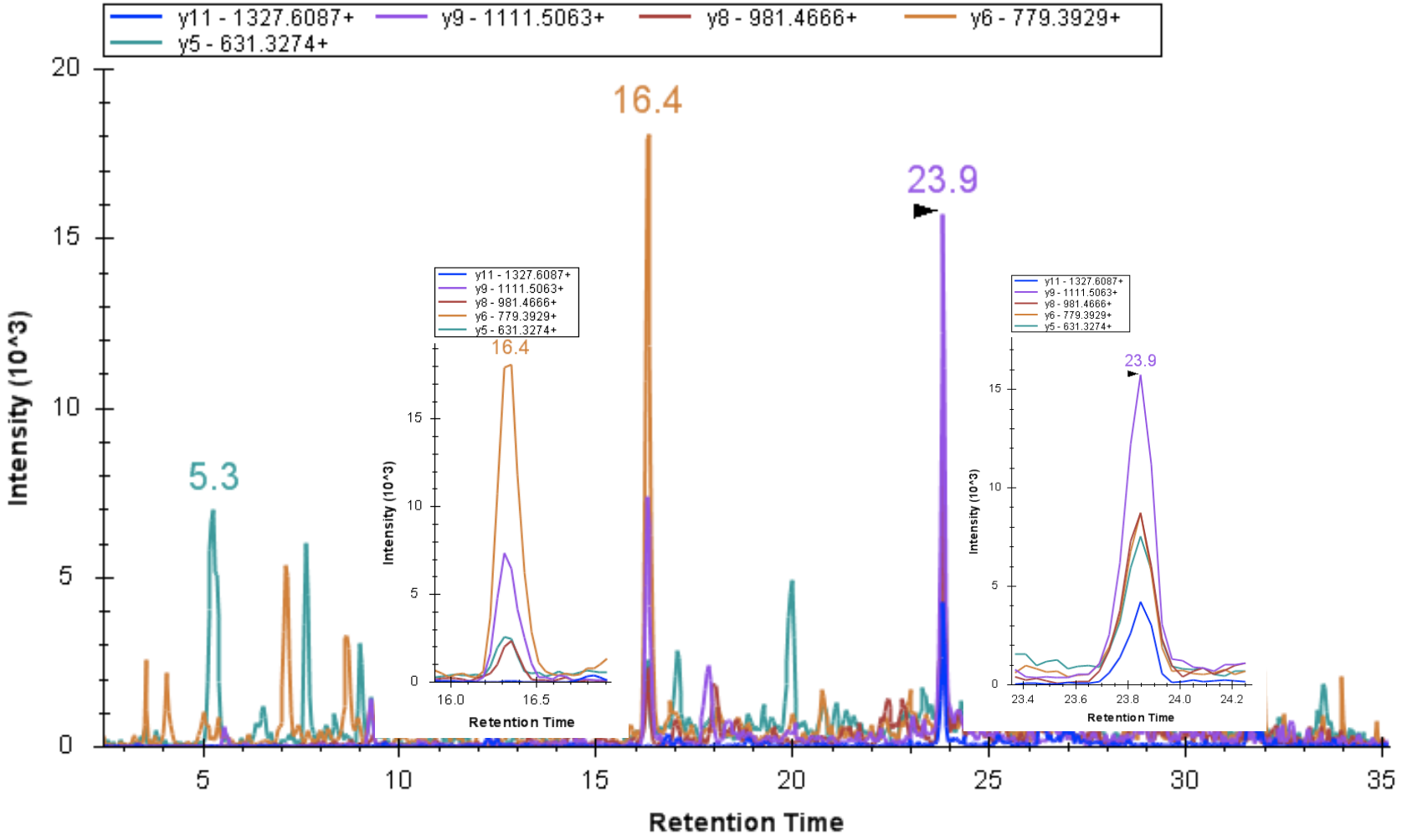
Ariel Bensimon
ETH Zürich
Switzerland

Technische Universität München

ETH Zürich

Motivation – why use isotope labeled standards ?

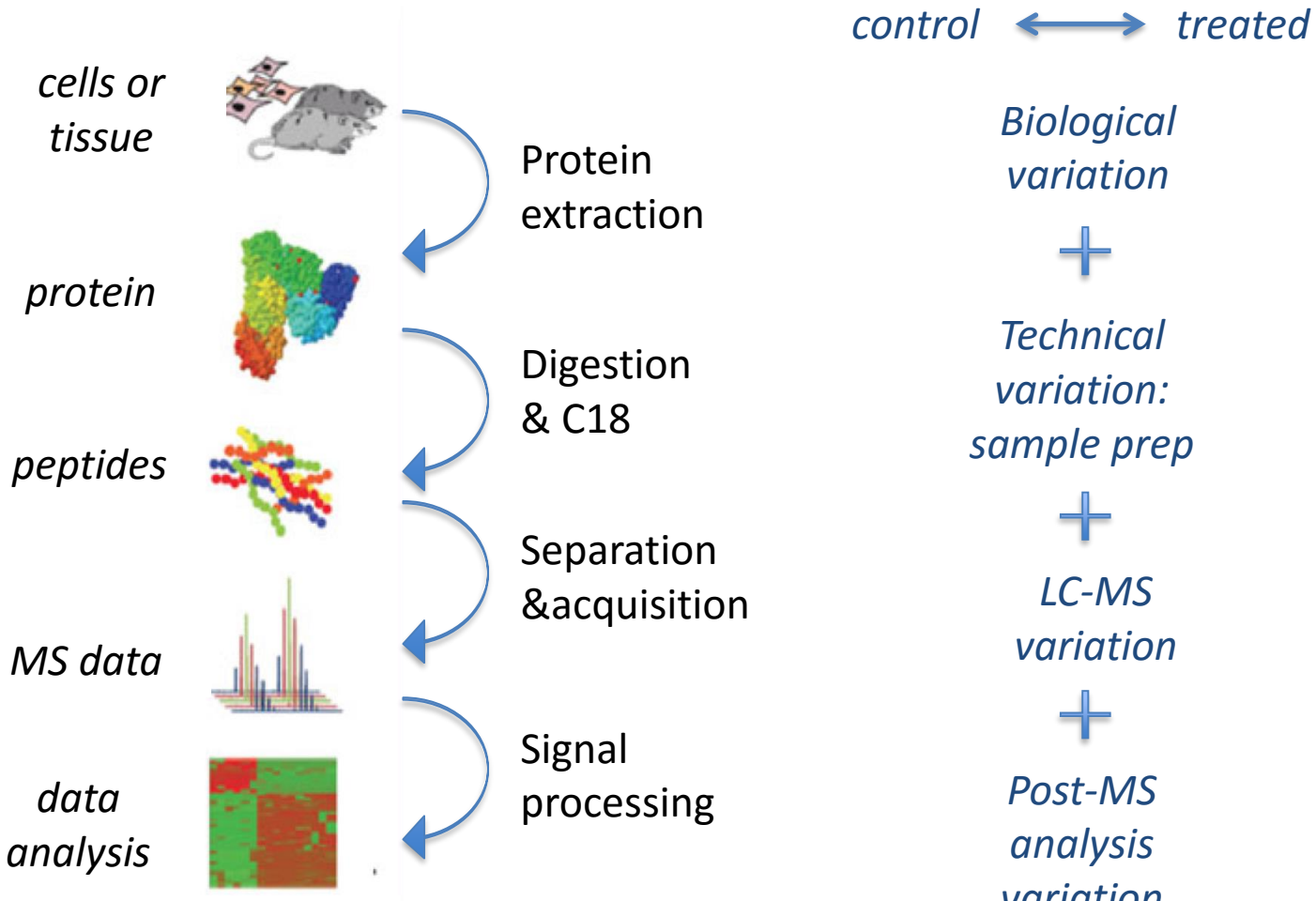
For the correct identification of a peptide: selecting the correct peak



Motivation – why use isotope labeled standards ?

For the accurate quantification of a peptide: accounting for sources of variation

- We assume that extra sample handling does not introduce extra variation



Bantscheff, M., Lemeer, S., Savitski, M. M. & Kuster, B., Anal Bioanal Chem 404, 939–965 (2012).

Outline

Introduction - Ariel

- How to get stable-isotope labeled information into a Skyline ?

Improve confident peptide identification - Ariel

- Generating a reference for identification
- Using a reference for peak selection
- Using a reference for optimal quantification

Improve quantitative precision and accuracy - Tina

- Label-free versus label-based quantification
  - Metabolic, chemical, enzymatic and spike-in labeling
- Relative versus absolute quantification
  - Single and multiple point calibration

Stable-isotope labeling

- **Heavy** reference standards are generated such that they carry one or several isotope-labeled atoms.
  - typically used isotope-labeled atoms: **^{13}C, ^{15}N, ^{18}O**
  - most common amino acids: K, R but also A, L, I, F, P, V.
  - use of ^{2}H less favorable due to chromatographic elution differences
- Be aware of the purity of isotope labeling.
- These **heavy** references are **chemically identical** to the endogenous (**light**) targets and hence we assume they show the same behavior in terms of
  - sample preparation biases
  - Chromatography
  - ionization
  - fragmentation

Modifications tab

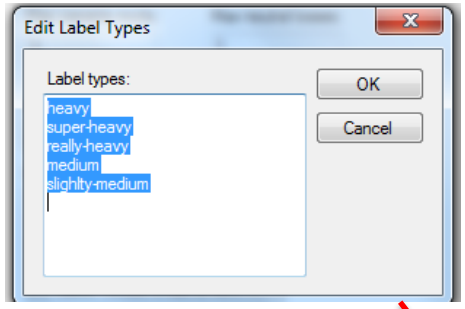
Settings>>peptide senttings>>modifications (see also webinar 10)

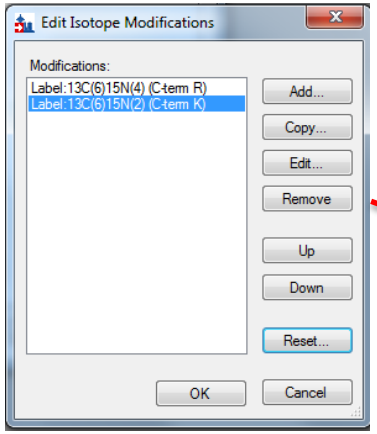
You can define and name labels ; select those relevant for your experiment. Some appear in default

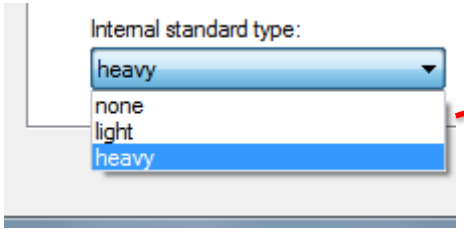
You edit a set of possible isotope modifications; select those relevant for each of the labels

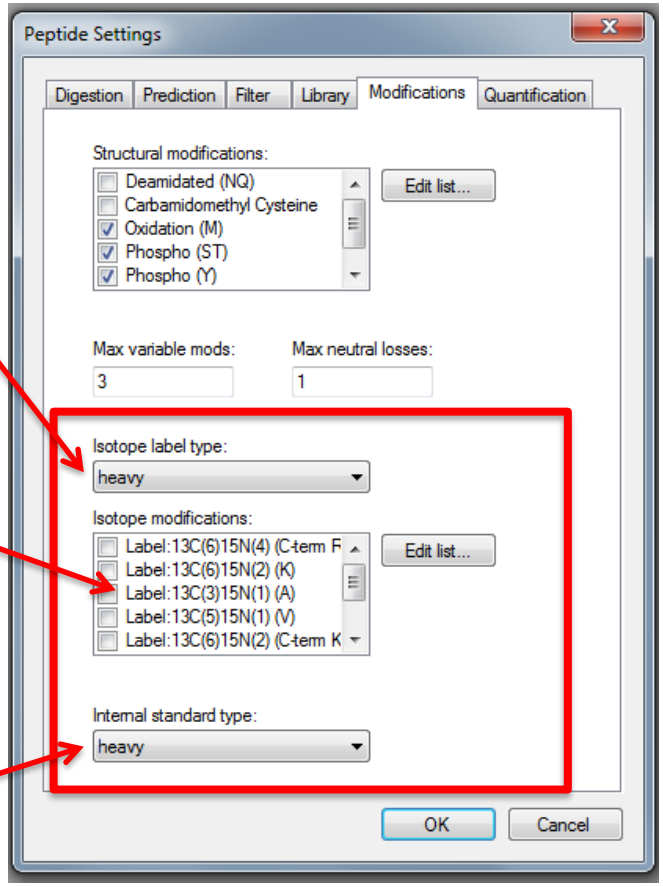
You can select which (if any) label is the internal standard

You assume the standard is present; Reverse is possible





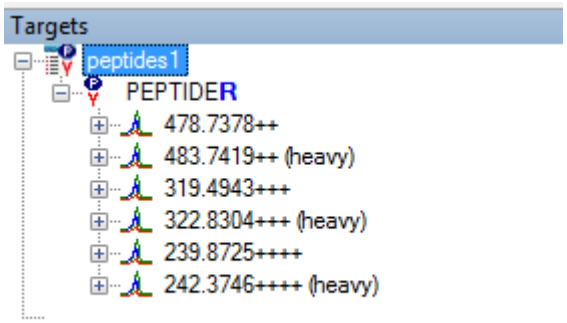




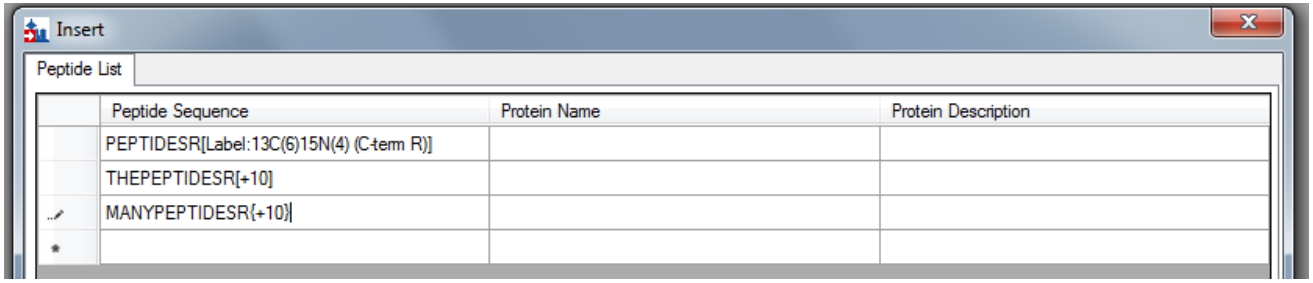
Insert peptides

Edit>>Insert>>peptides (see also webinar 10)

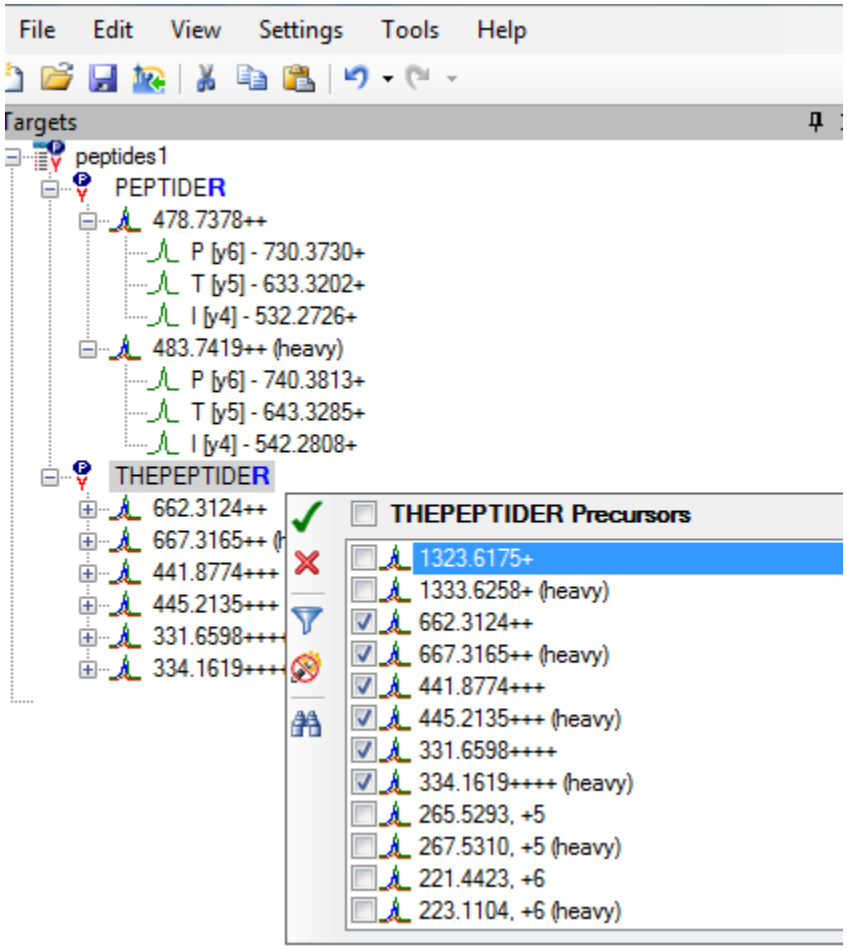
- Insert a light peptide sequence

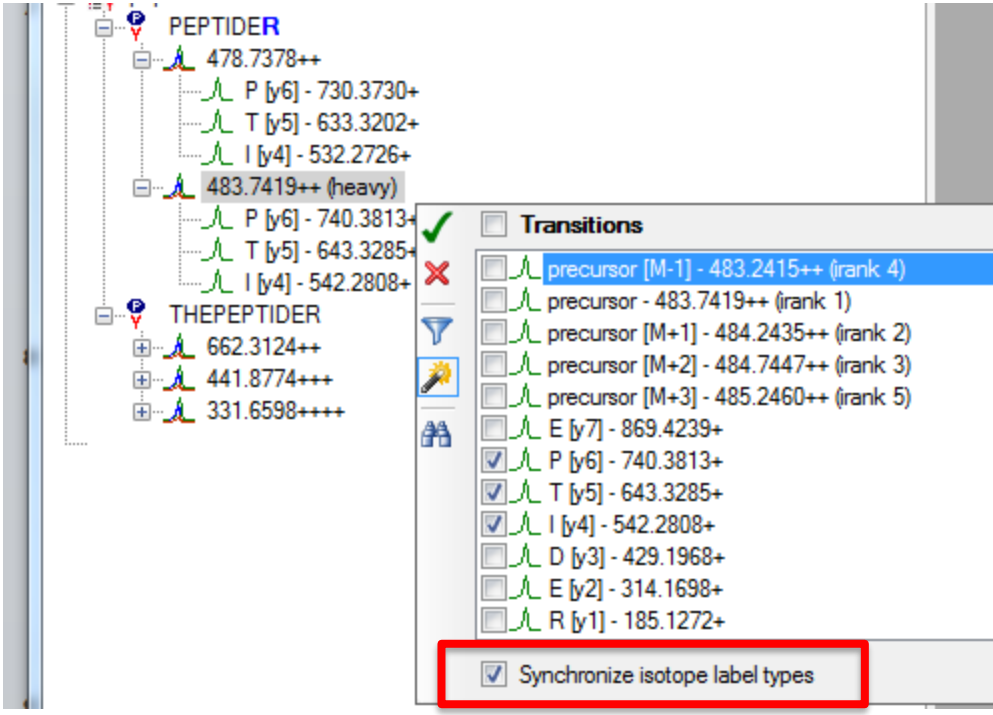


- Insert a modified peptide sequence
  - Full name
  - Mass in []
  - Correct: Mass in {}

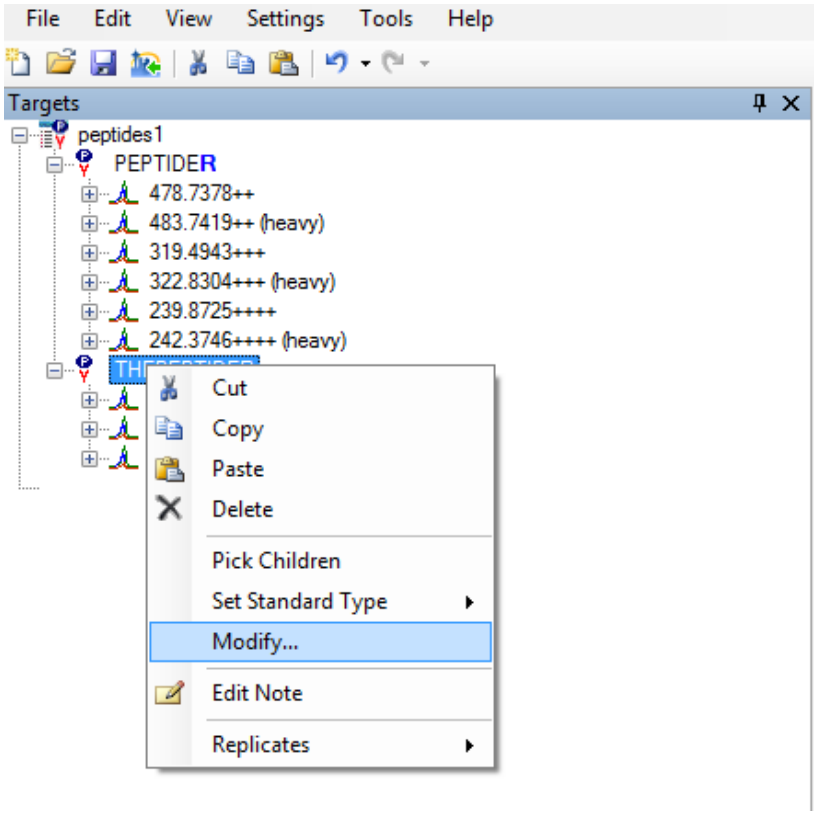


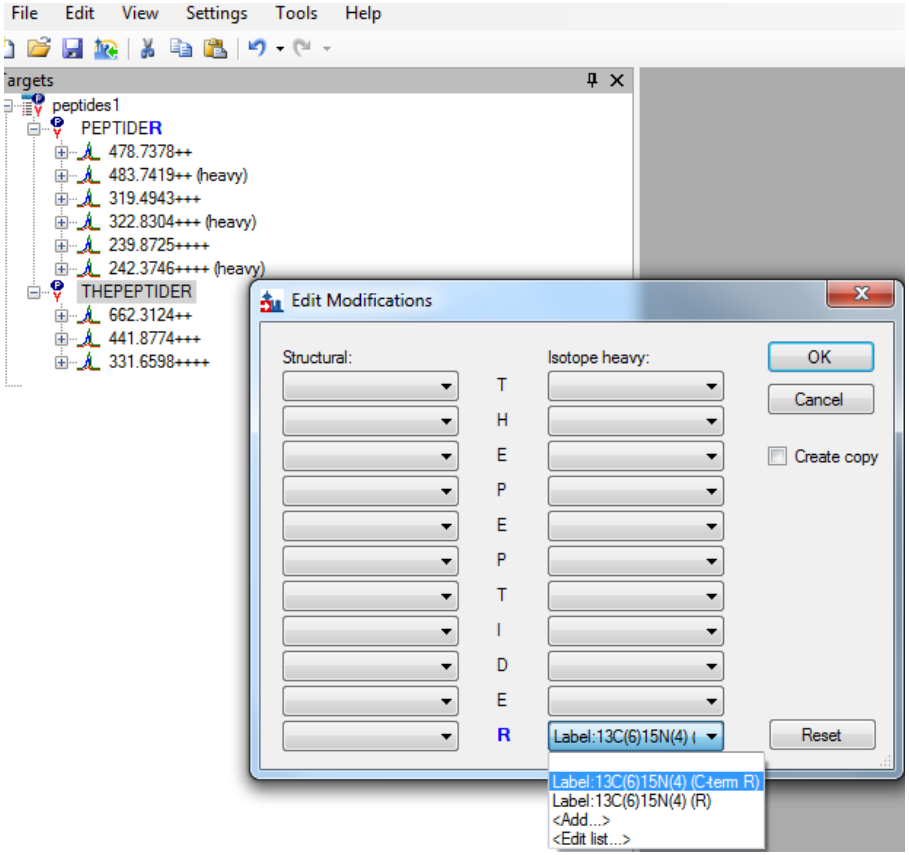
Select precursors & transitions





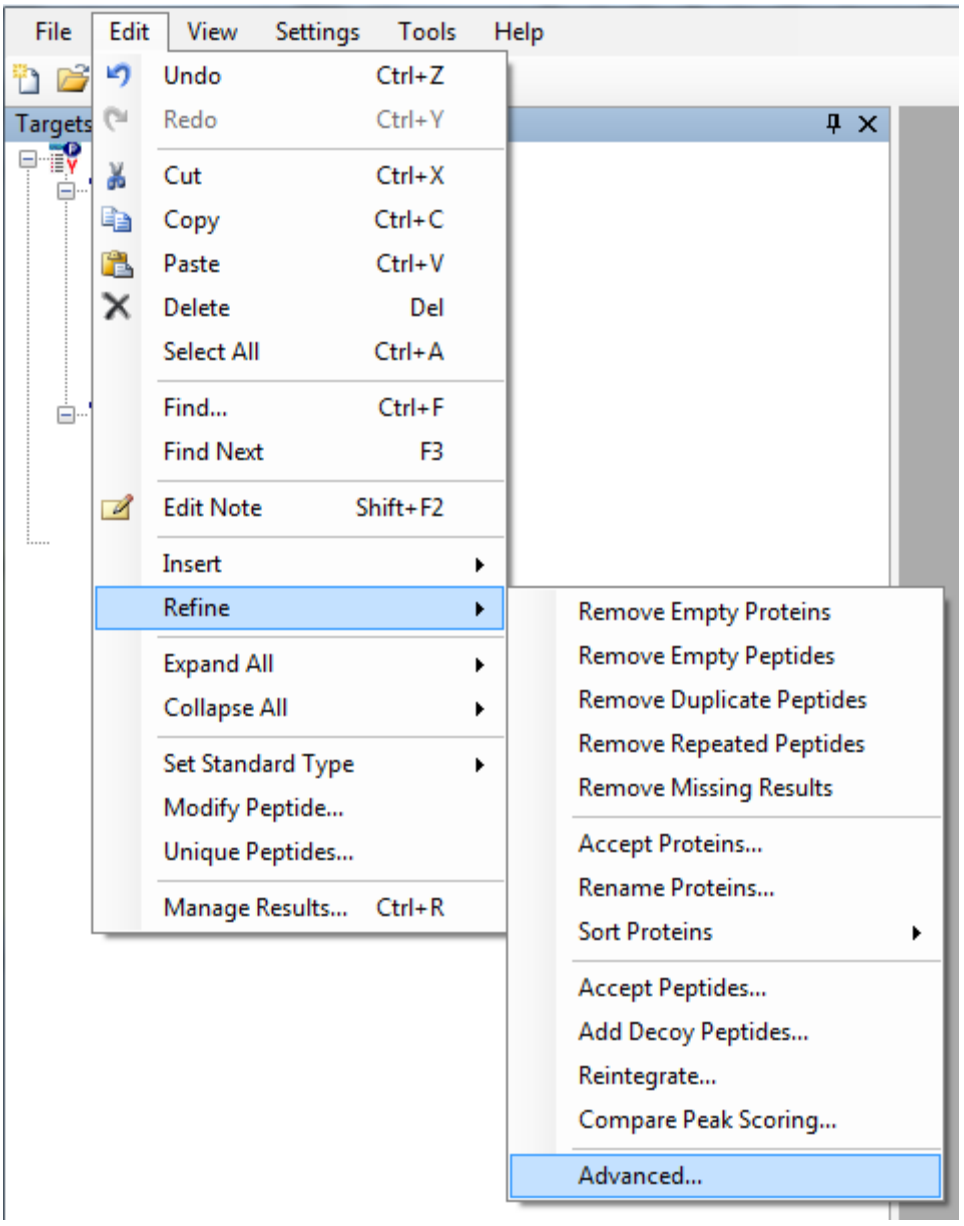
Modify peptides

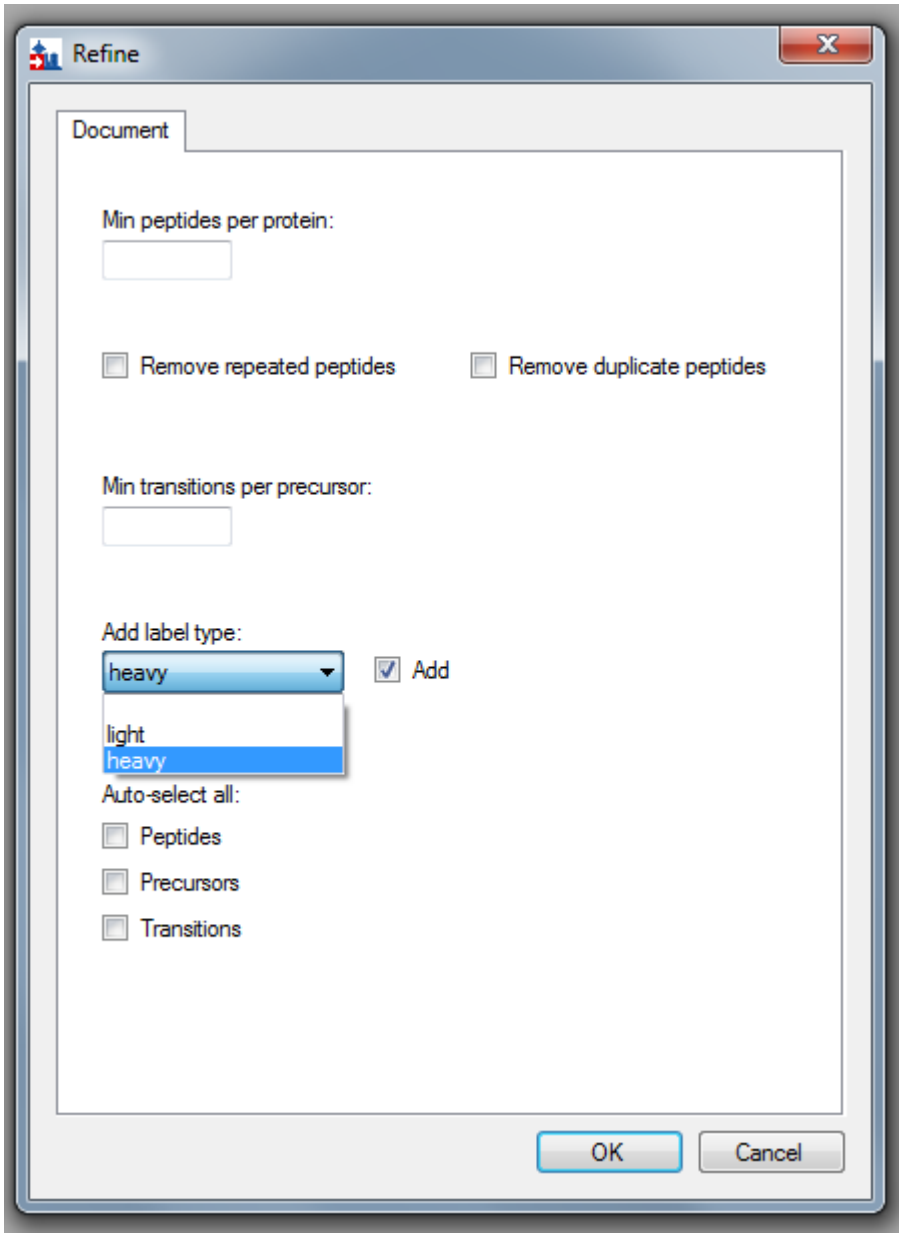




Make sure these are first set in Modifications tab

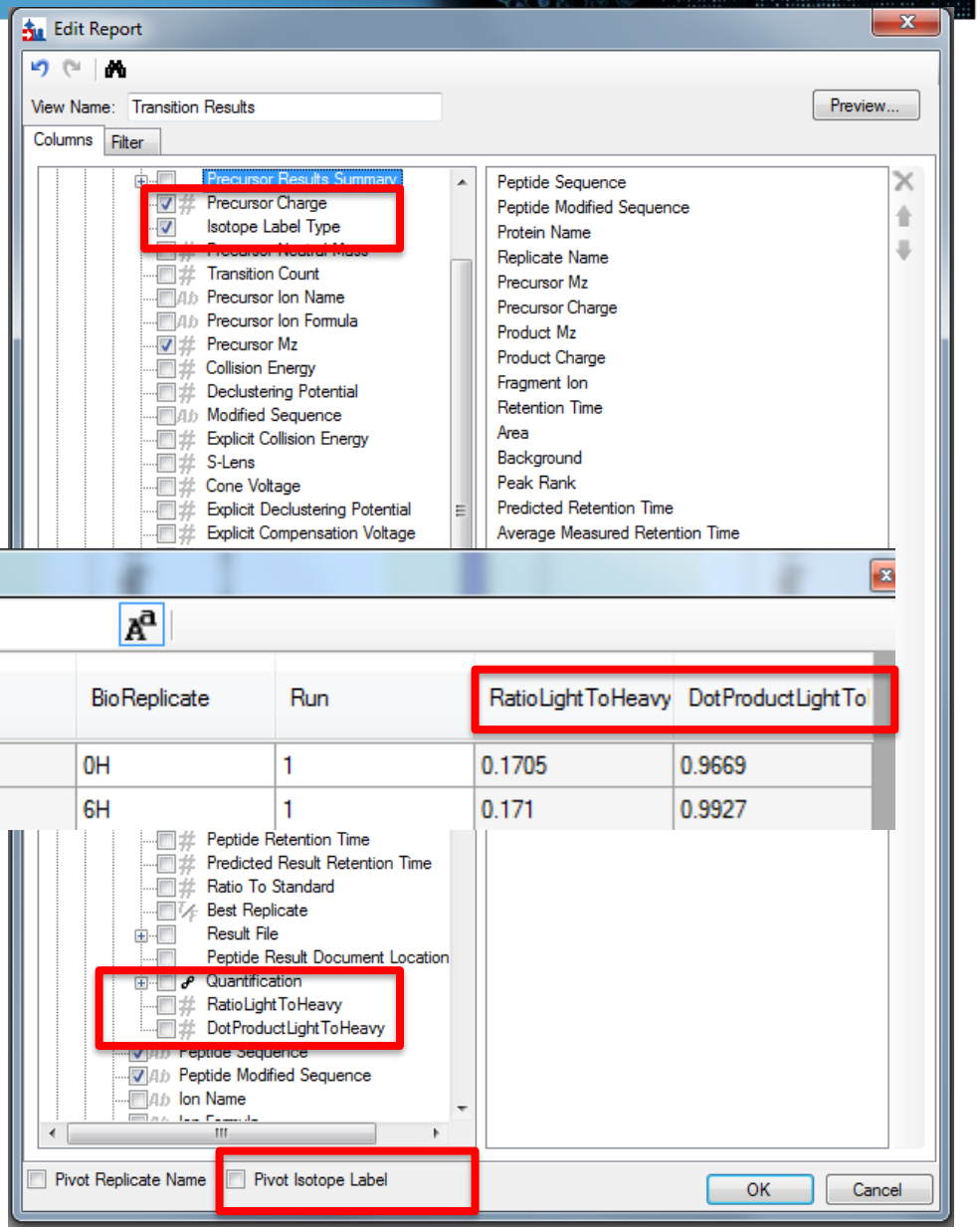
Add label


File
Edit
View
Settings
Tools
Help
Undo Ctrl+Z
Redo Ctrl+Y
Cut Ctrl+X
Copy Ctrl+C
Paste Ctrl+V
Delete Del
Select All Ctrl+A
Find... Ctrl+F
Find Next F3
Edit Note Shift+F2
Insert
Refine
Expand All
Collapse All
Set Standard Type
Modify Peptide...
Unique Peptides...
Manage Results... Ctrl+R
Remove Empty Proteins
Remove Empty Peptides
Remove Duplicate Peptides
Remove Repeated Peptides
Remove Missing Results
Accept Proteins...
Rename Proteins...
Sort Proteins
Accept Peptides...
Add Decoy Peptides...
Reintegrate...
Compare Peak Scoring...
Advanced...
Targets


Refine
Document
Min peptides per protein:
Remove repeated peptides
Remove duplicate peptides
Min transitions per precursor:
Add label type:
heavy
Add
light
heavy
Auto-select all:
Peptides
Precursors
Transitions
OK
Cancel

Export & Results

- Isotope label information can be selected in any report exported:
- Pivot based on the isotope label
- In results grid:



Results tutorial :
https://skyline.gs.washington.edu/labkey/_webdav/home/software/Skyline/@files/tutorials/CustomReports-1_2.pdf

Outline

Introduction - Ariel

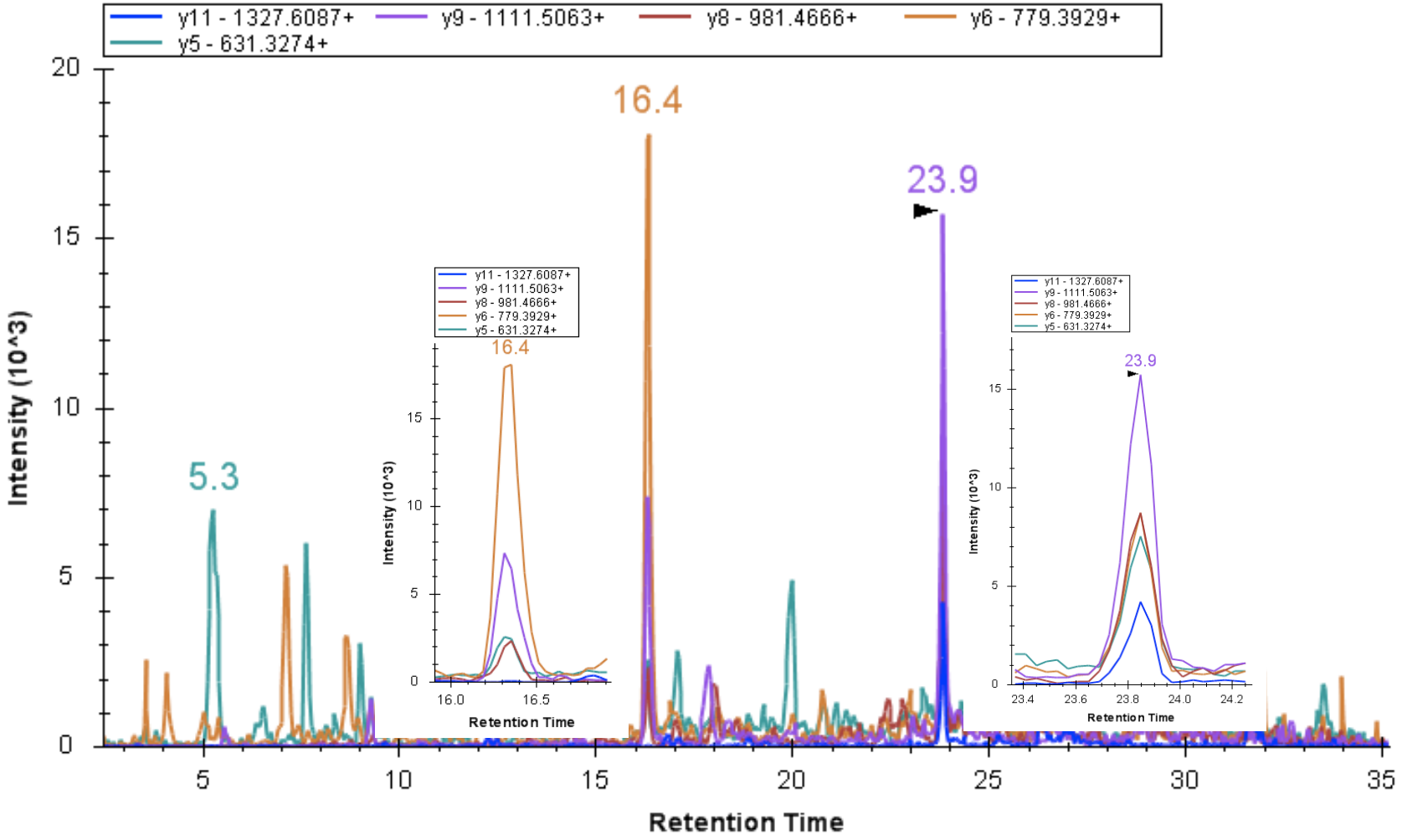
- How to get stable-isotope labeled information into a Skyline ?

Improve confident peptide identification - Ariel

- Generating a reference for identification
- Using a reference for peak selection
- Using a reference for optimal quantification

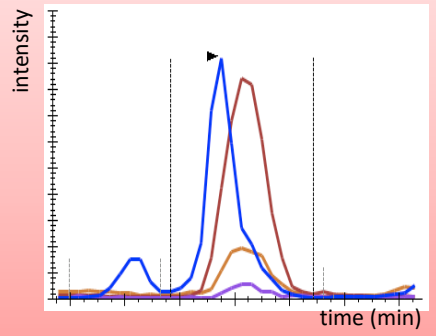
Motivation – why use isotope labeled standards ?

- For the correct identification of a peptide: selecting the correct peak

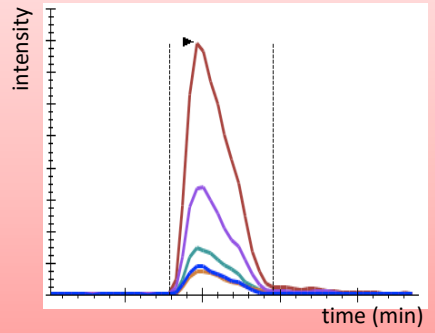


Criteria for reliable peak identification

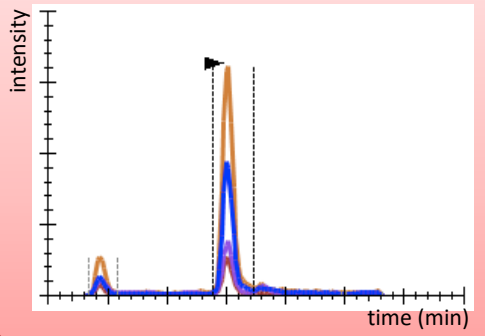
1. Co-elution



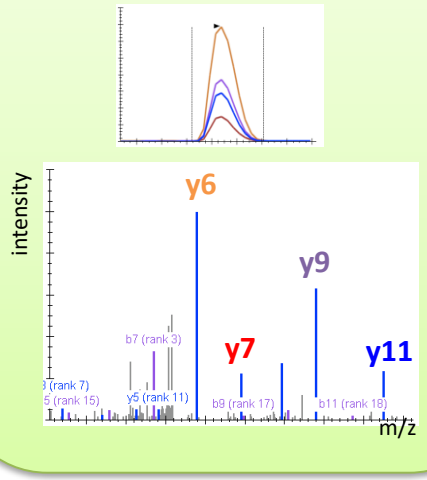
2. Peak shape



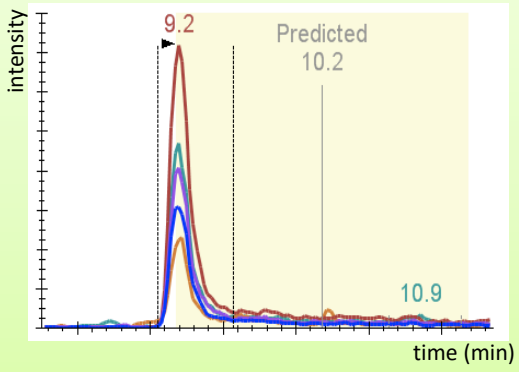
3. Signal intensity



4. Correlation peak intensities to library spectrum

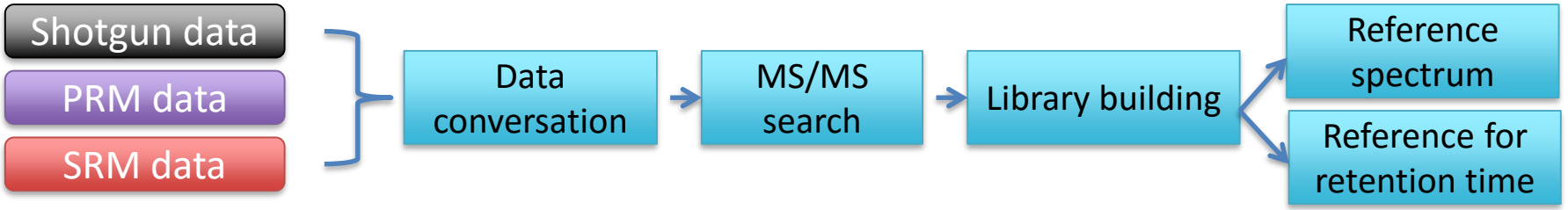


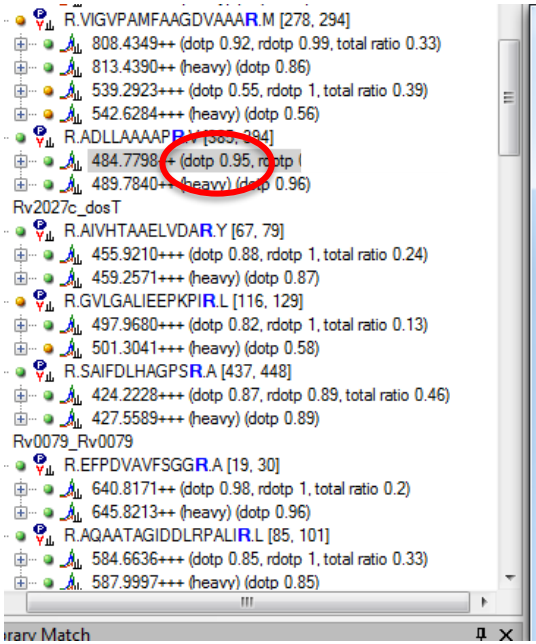
5. Correlation retention time (empirical)

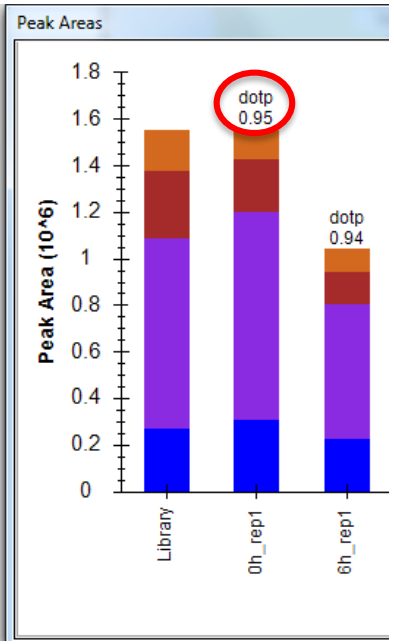


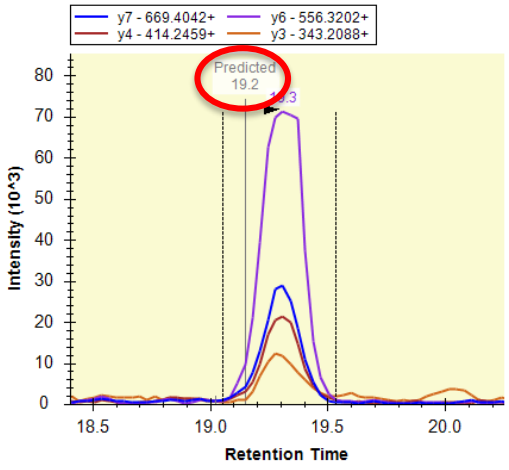
Generating a reference for identification

- We can use a synthetic standard to generate :
  - A reference spectrum for the spectral library (see also webinar 4, tutorials)
  - A reference retention time value , for the RT library (see webinar 7, tutorials)







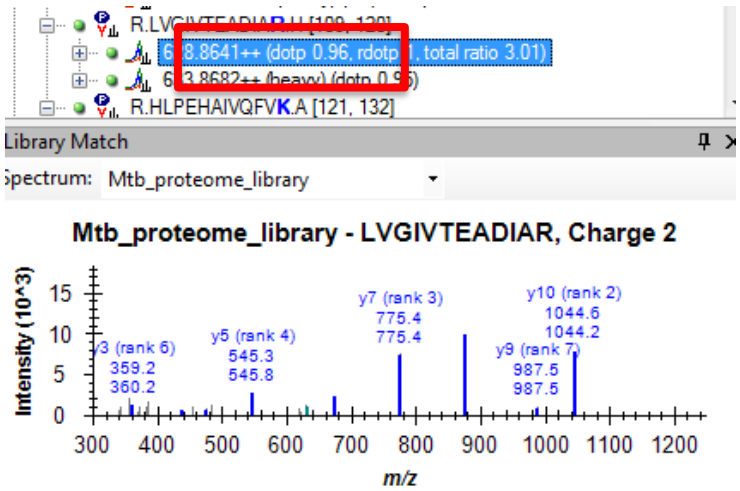


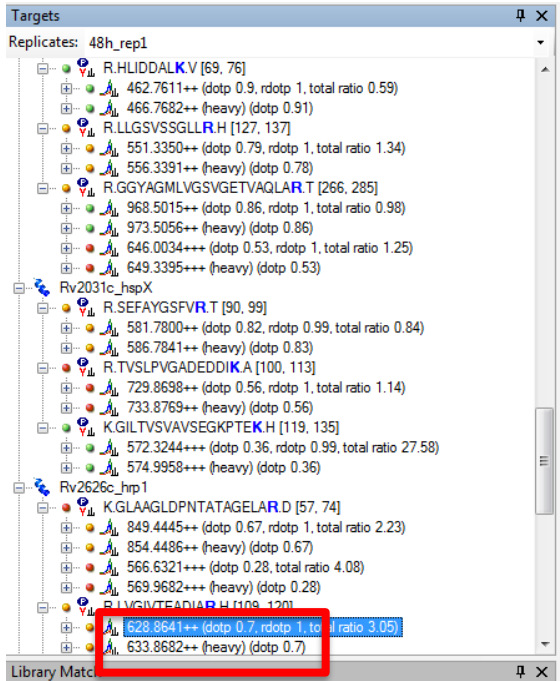
To read how is dotp calculated in Skyline: https://skyline.gs.washington.edu/labkey/announcements/home/support/thread.view?rowId=20003

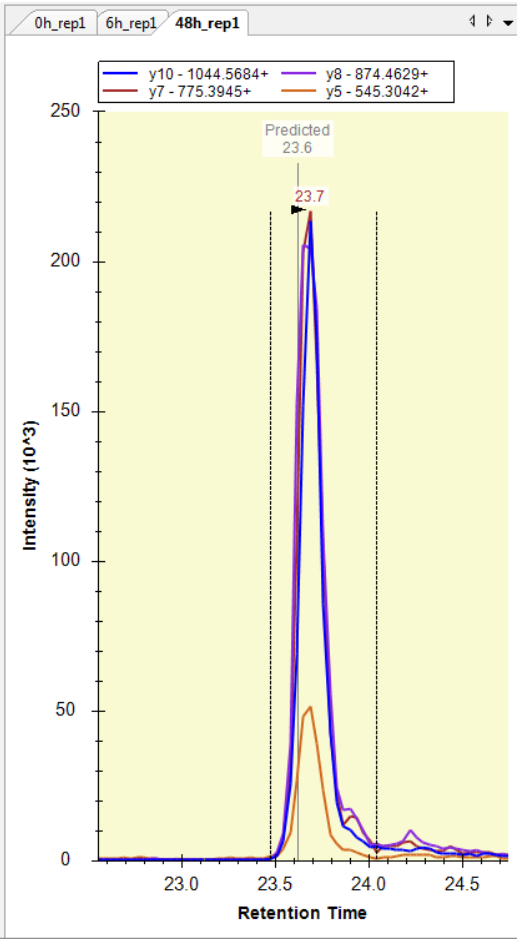
Generating a reference for identification

- We can use a synthetic standard to generate a reference:
  - peptide or protein standard.
  - heavy or light standard (chemically identical).
  - Skyline transfers information, if you activate the heavy label in Modifications.

- To ensure a good dotp: Perform an identification experiment with the same MS setup (CE etc) , as in the targeted experiment.

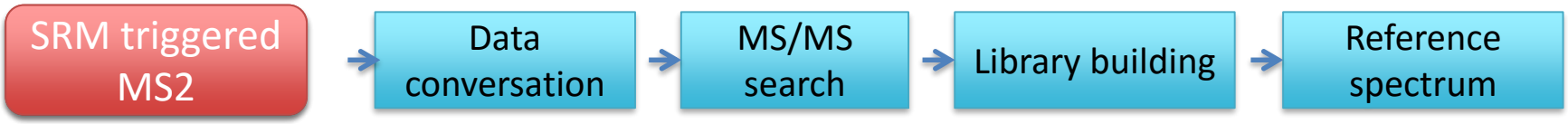






Generating a reference for identification

- We can use a synthetic standard to generate a reference spectrum for the spectral library in SRM:



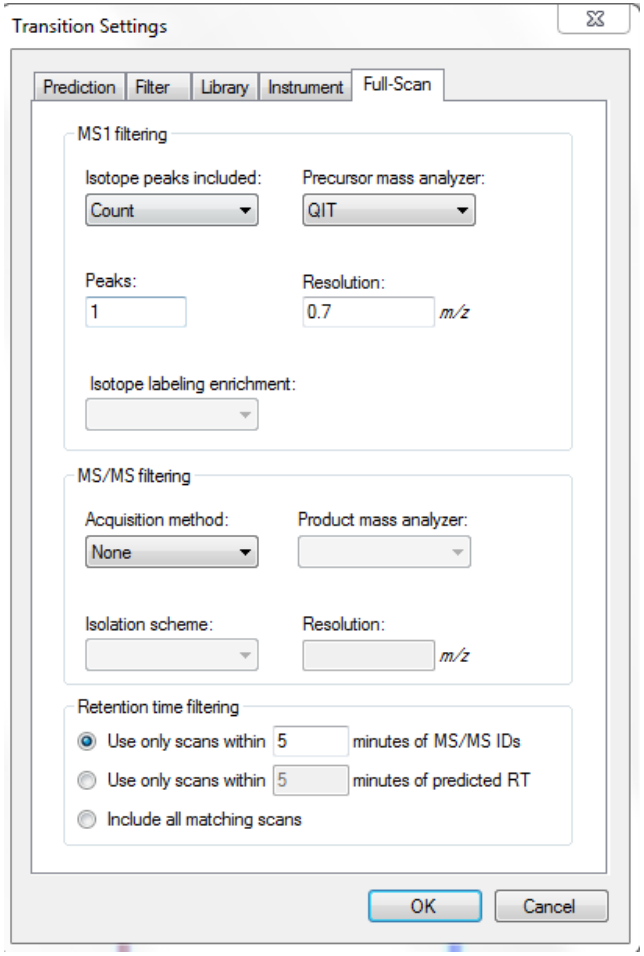
- SRM triggered MS2 on QTRAP: if a product ion is monitored above a threshold, switch to Enhanced Product Ion (switch Q3 to LIT, acquire full fragment scan).
  - For assay generation (using synthetic standards).
    - http://targetedproteomics.ethz.ch/downloads.html (tutorials 2013)
  - For validation (of any endogenous peptide peak).

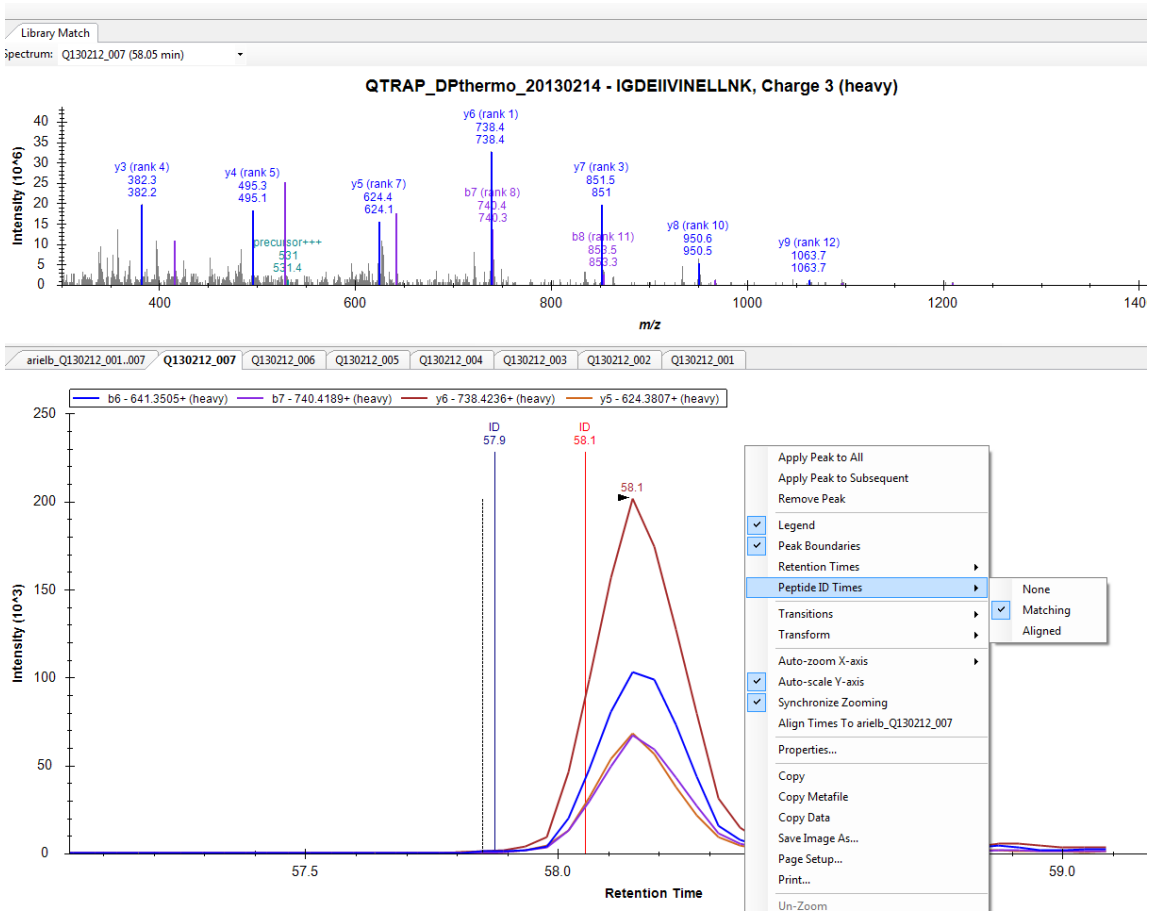
- Targeted MS2: SRM triggered MS2 as well as PRM. In both modes, one can view the peptide MS/MS events using Skyline.

Picotti, P., et al. (2010). High-throughput generation of selected reaction-monitoring assays for proteins and proteomes. Nat Methods *7*, 43–46.

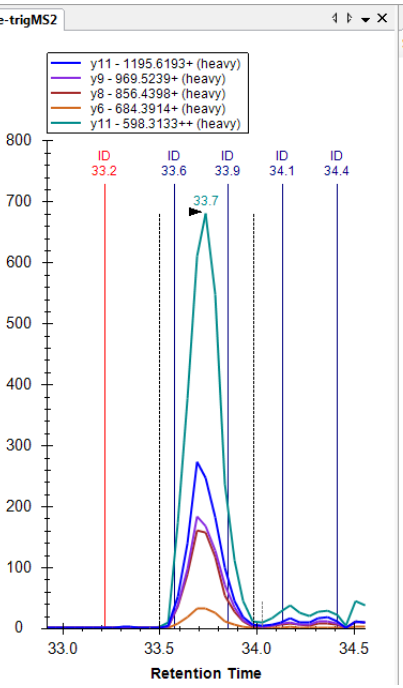
Targeted MS2 in Skyline

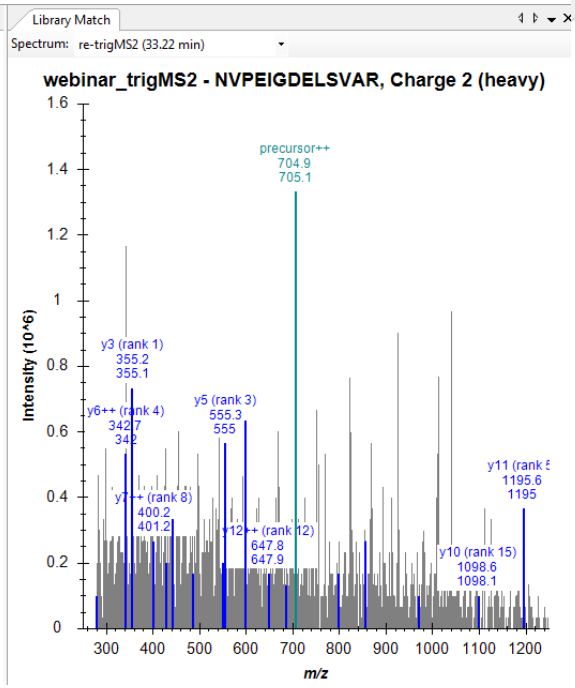
For SRM trig-MS2 files: Enable the Full-scan Enable ID matching in the view
Note: for PRM the parameters are different (webinar 3)

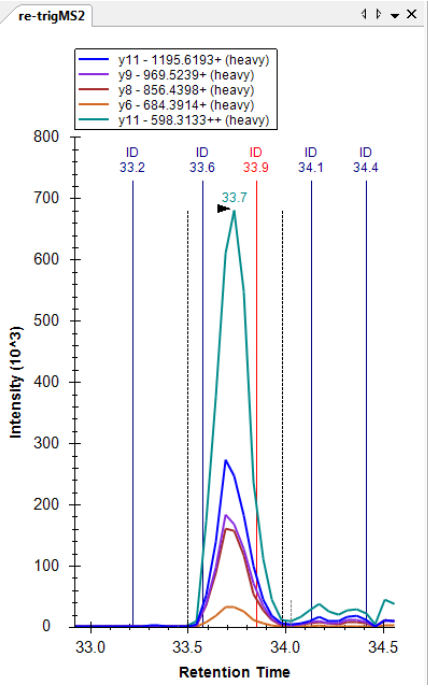


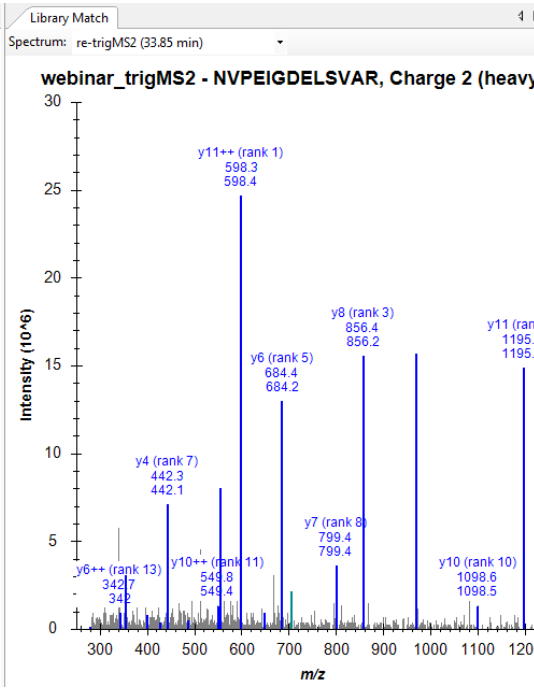


Targeted MS2 in Skyline







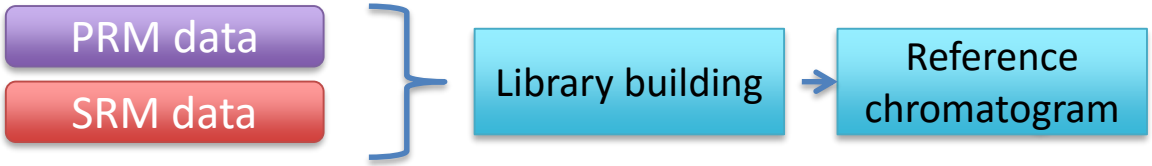


- When working with with synthetic standards for assay generation : ensure the quality of your spectra

  https://**skyline**.gs.washington.edu/labkey/tutorial_**library_explorer**.url

Generating a reference for identification

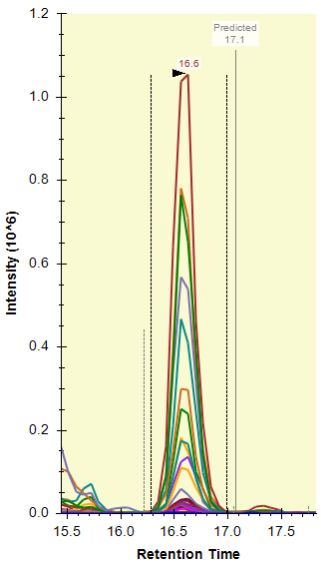
- We can use a synthetic standard to generate a reference for the chromatogram library: heavy or light standard; peptide or protein standard.



Panorama

Webinar 11; Tutorial (chromatogram libraries)

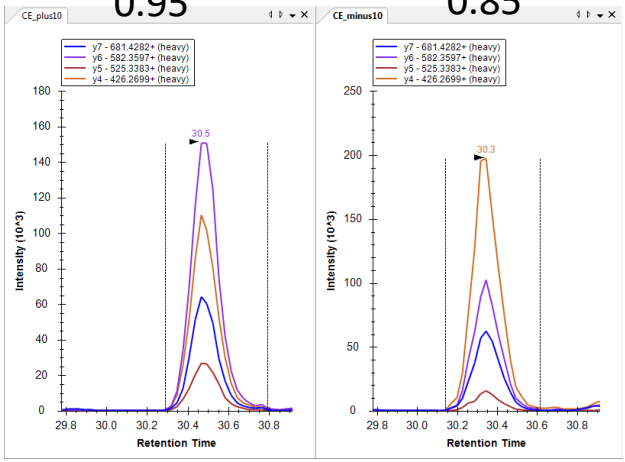
All b,y ions



- Notes:
  - In SRM: you would need to measure all the desired transitions.
  - You will still get a dotp from a chromatogram library.
  - Ensure similarity in MS setup (CE etc).

Dotp: 0.95

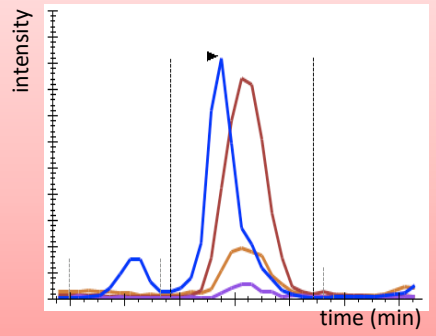
Dotp: 0.85



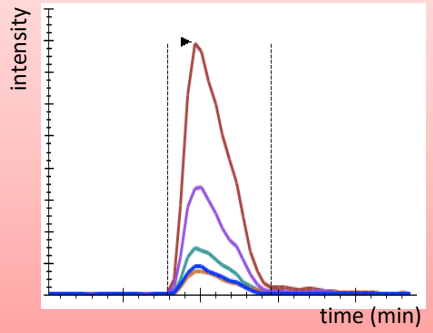
Same peptide with different CEs

Criteria for reliable peak identification

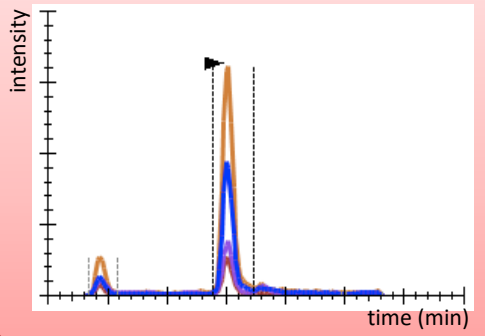
1. Co-elution



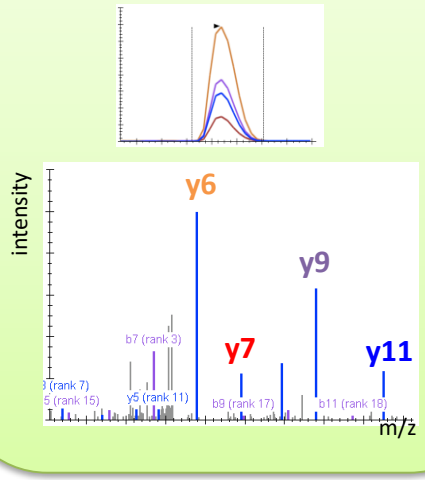
2. Peak shape



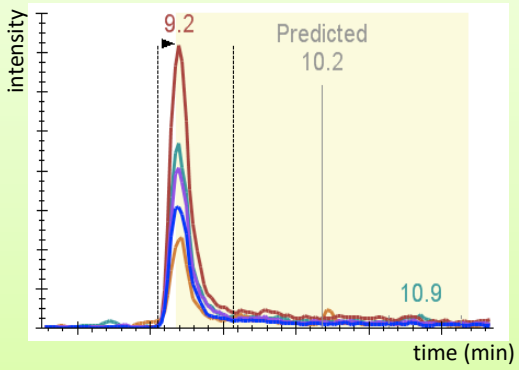
3. Signal intensity



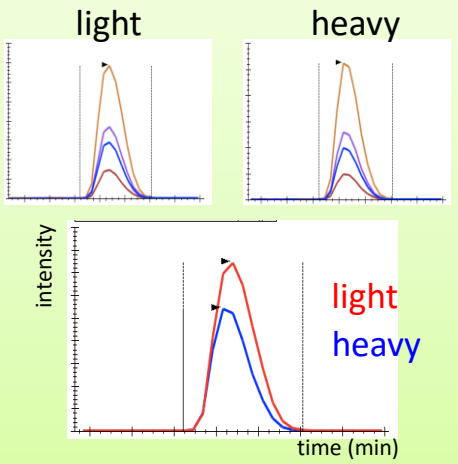
4. Correlation peak intensities to library spectrum



5. Correlation retention time (empiric or predicted*)

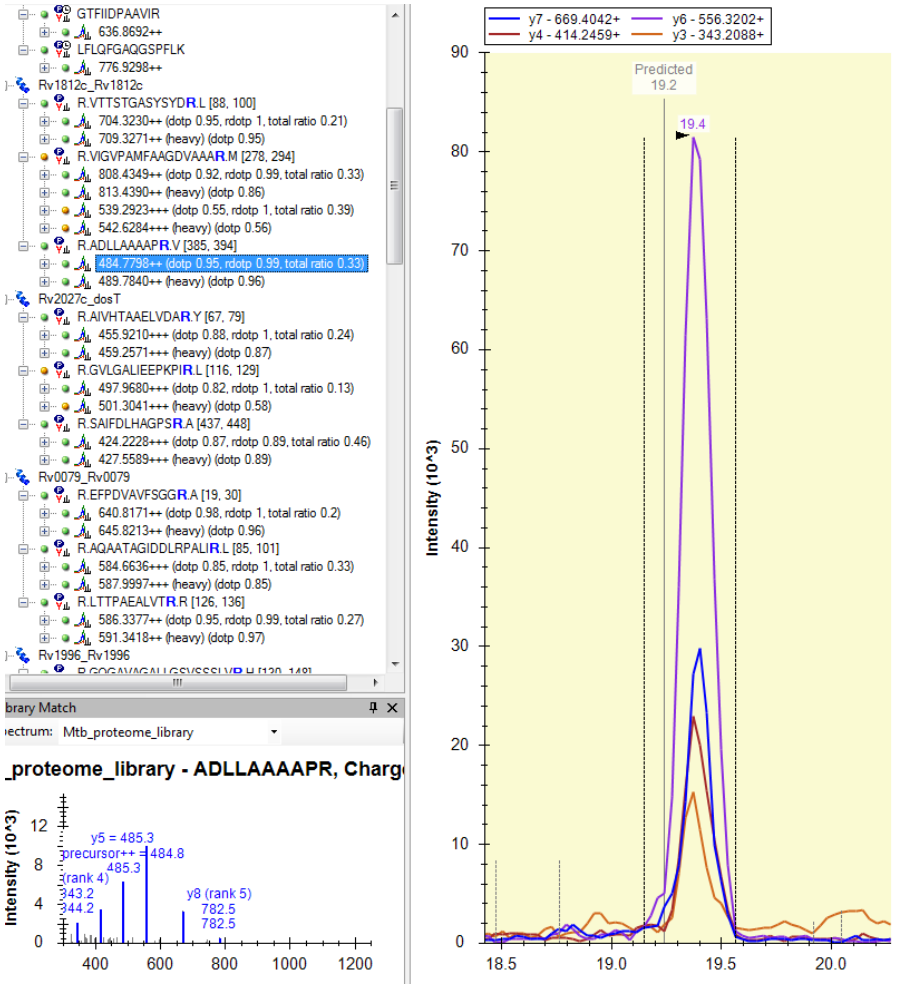


6. Correlation with heavy-labeled standard

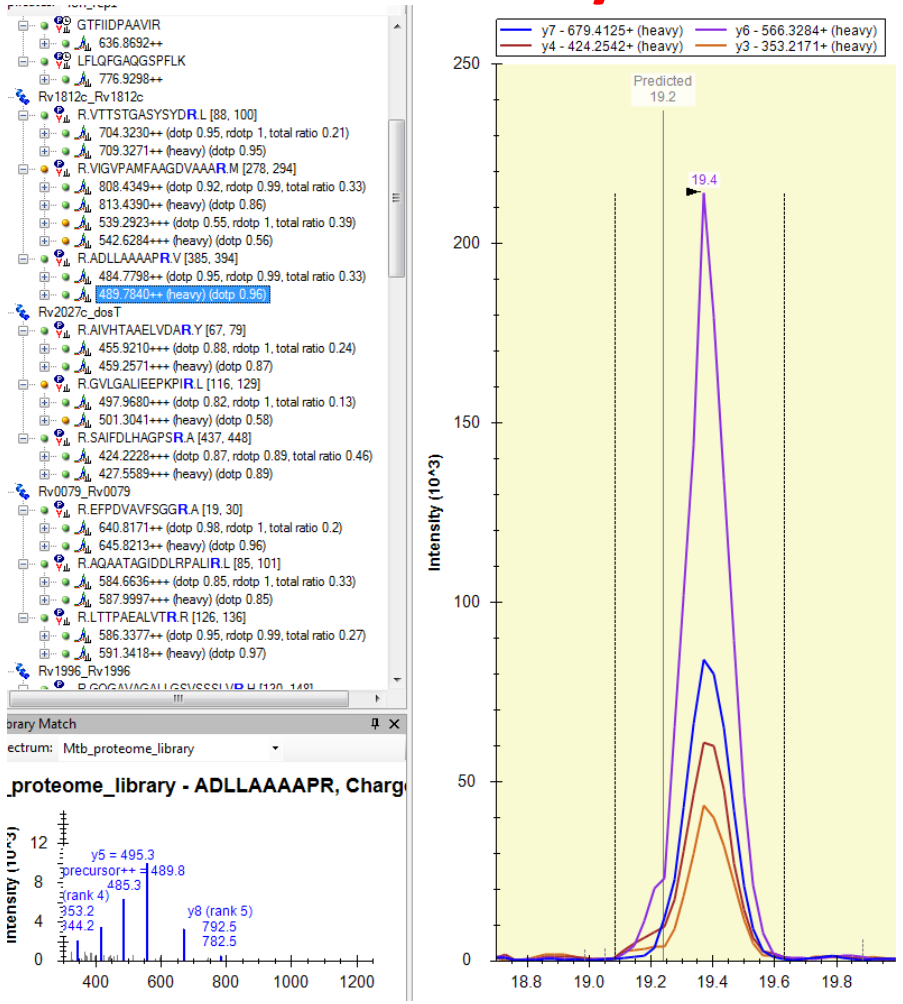


View individual precurosors

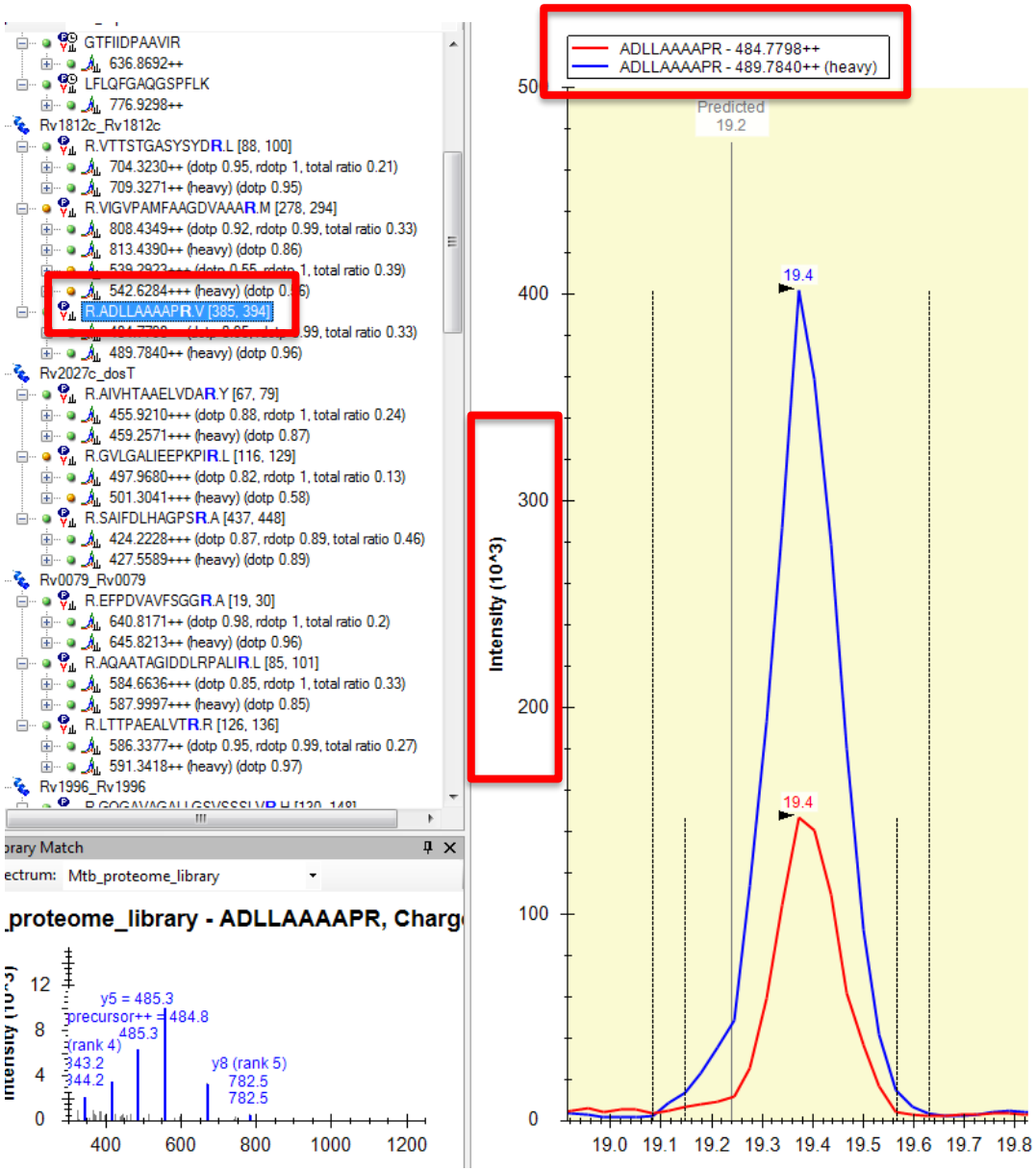
light



heavy



View pairs



View:

Total sum, no transition info

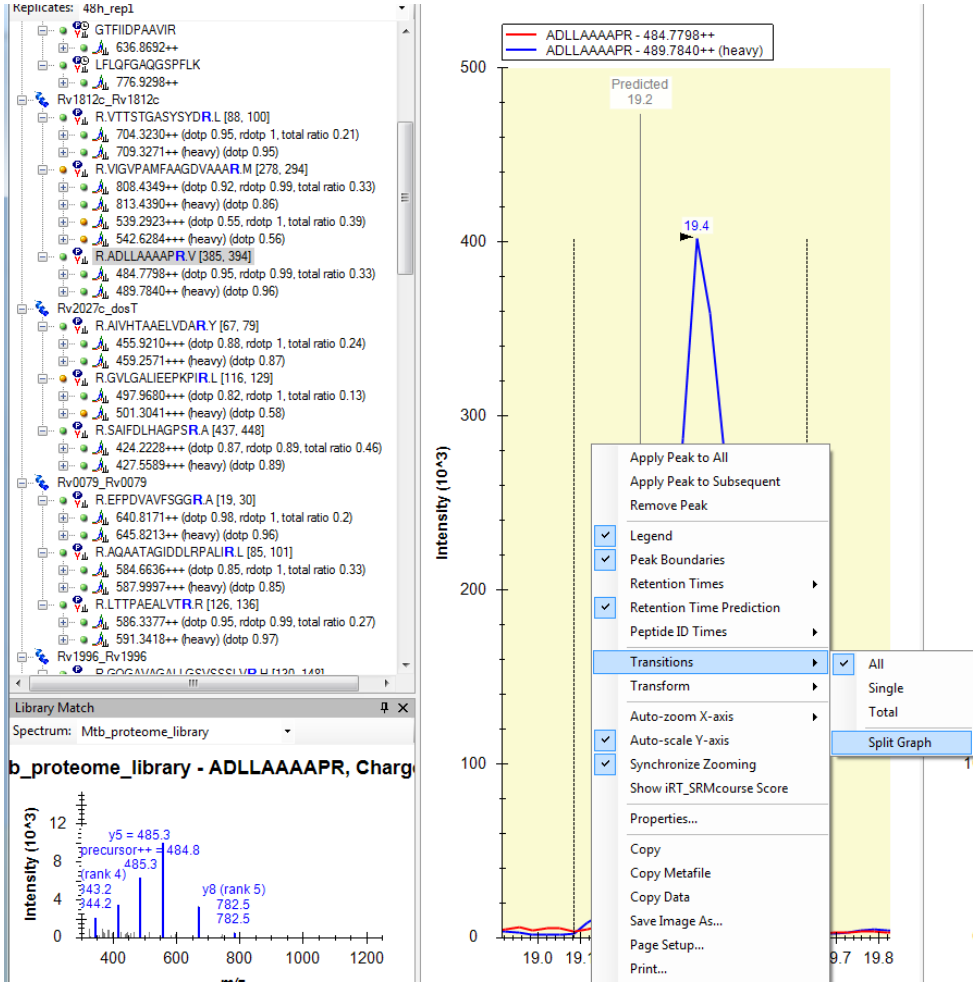
Same boundries

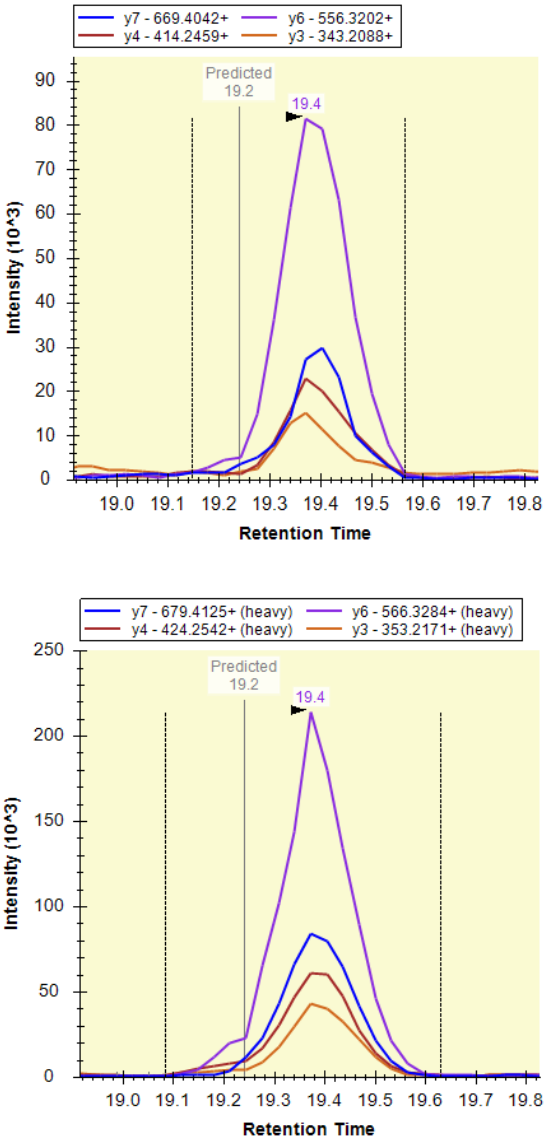
These **heavy** references are **<u>chemically identical</u>** to the endogenous (**light**) :

Co-elution

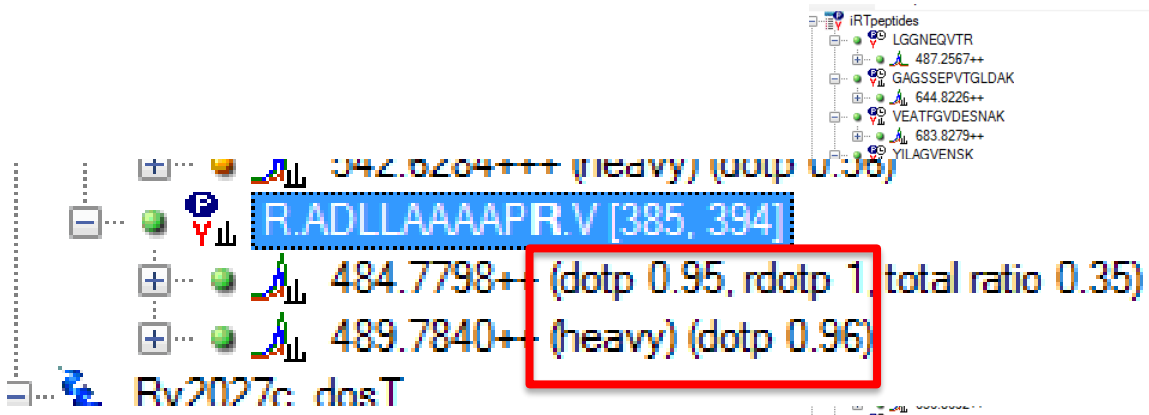
Same peak boundries

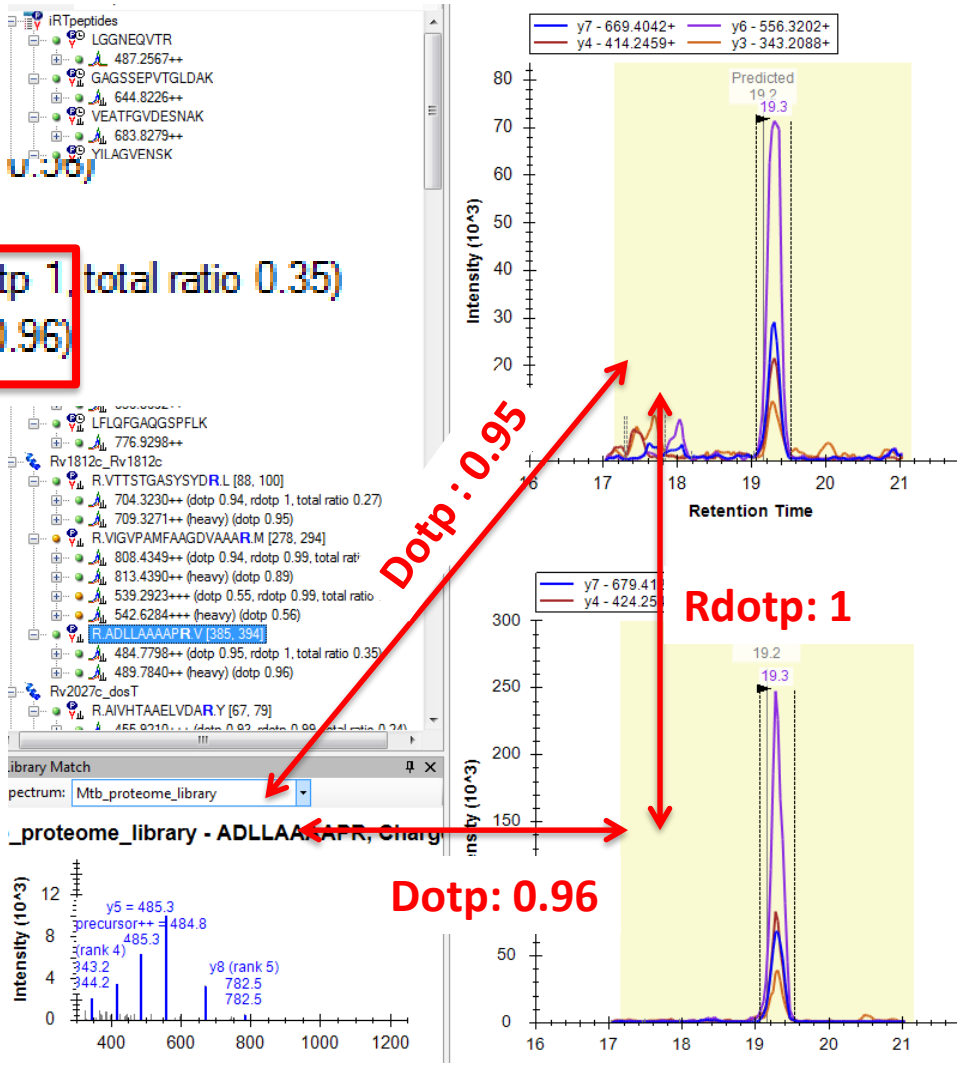
Split graph


Replicates: 48h_rep1
GTFIIDPAAVIR
636.8692++
LFLQFGAQGSPFLK
776.9298++
Rv1812c_Rv1812c
R.VTTSTGASYSYDR.L [88, 100]
704.3230++ (dotp 0.95, rdotp 1, total ratio 0.21)
709.3271++ (heavy) (dotp 0.95)
R.VIGVPAMFAAGDVAAAR.M [278, 294]
808.4349++ (dotp 0.92, rdotp 0.99, total ratio 0.33)
813.4390++ (heavy) (dotp 0.86)
539.2923+++ (dotp 0.55, rdotp 1, total ratio 0.39)
542.6284+++ (heavy) (dotp 0.56)
R.ADLLAAAAPR.V [385, 394]
484.7798++ (dotp 0.95, rdotp 0.99, total ratio 0.33)
489.7840++ (heavy) (dotp 0.96)
Rv2027c_dosT
R.AIVHTAAELVDAR.Y [67, 79]
455.9210+++ (dotp 0.88, rdotp 1, total ratio 0.24)
459.2571+++ (heavy) (dotp 0.87)
R.GVLGALIEEPKPIR.L [116, 129]
497.9680+++ (dotp 0.82, rdotp 1, total ratio 0.13)
501.3041+++ (heavy) (dotp 0.58)
R.SAIFDLHAGPSR.A [437, 448]
424.2228+++ (dotp 0.87, rdotp 0.89, total ratio 0.46)
427.5589+++ (heavy) (dotp 0.89)
Rv0079_Rv0079
R.EFPDVAVFSGGR.A [19, 30]
640.8171++ (dotp 0.98, rdotp 1, total ratio 0.2)
645.8213++ (heavy) (dotp 0.96)
R.AQAATAGIDDLRPALIR.L [85, 101]
584.6636+++ (dotp 0.85, rdotp 1, total ratio 0.33)
587.9997+++ (heavy) (dotp 0.85)
R.LTTPAEALVTR.R [126, 136]
586.3377++ (dotp 0.95, rdotp 0.99, total ratio 0.27)
591.3418++ (heavy) (dotp 0.97)
Rv1996_Rv1996
Library Match
Spectrum: Mtb_proteome_library
b_proteome_library - ADLLAAAAPR, Charg
Intensity (10^3)
y5 = 485.3
precursor++ = 484.8
485.3
(rank 4)
943.2
944.2
y8 (rank 5)
782.5
782.5
m/z
ADLLAAAAPR - 484.7798++
ADLLAAAAPR - 489.7840++ (heavy)
Predicted
19.2
19.4
Intensity (10^3)
Apply Peak to All
Apply Peak to Subsequent
Remove Peak
Legend
Peak Boundaries
Retention Times
Retention Time Prediction
Peptide ID Times
Transitions
Transform
Auto-zoom X-axis
Auto-scale Y-axis
Synchronize Zooming
Show iRT_SRMcourse Score
Properties...
Copy
Copy Metafile
Copy Data
Save Image As...
Page Setup...
Print...
All
Single
Total
Split Graph

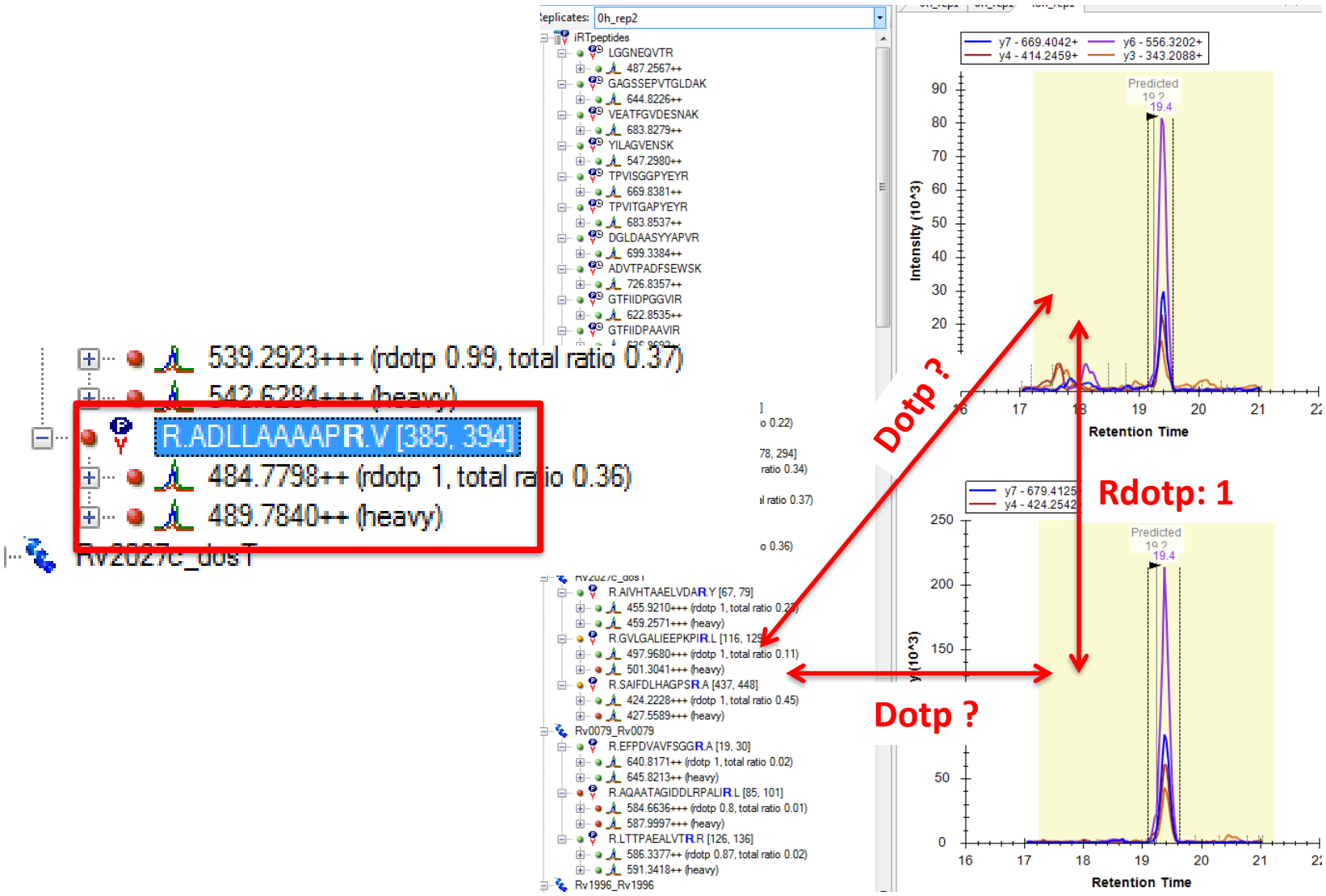

y7 - 669.4042+
y6 - 556.3202+
y4 - 414.2459+
y3 - 343.2088+
Predicted
19.2
19.4
Intensity (10^3)
Retention Time
y7 - 679.4125+ (heavy)
y6 - 566.3284+ (heavy)
y4 - 424.2542+ (heavy)
y3 - 353.2171+ (heavy)
Predicted
19.2
19.4
Intensity (10^3)
Retention Time

Dotp, rdotp with library

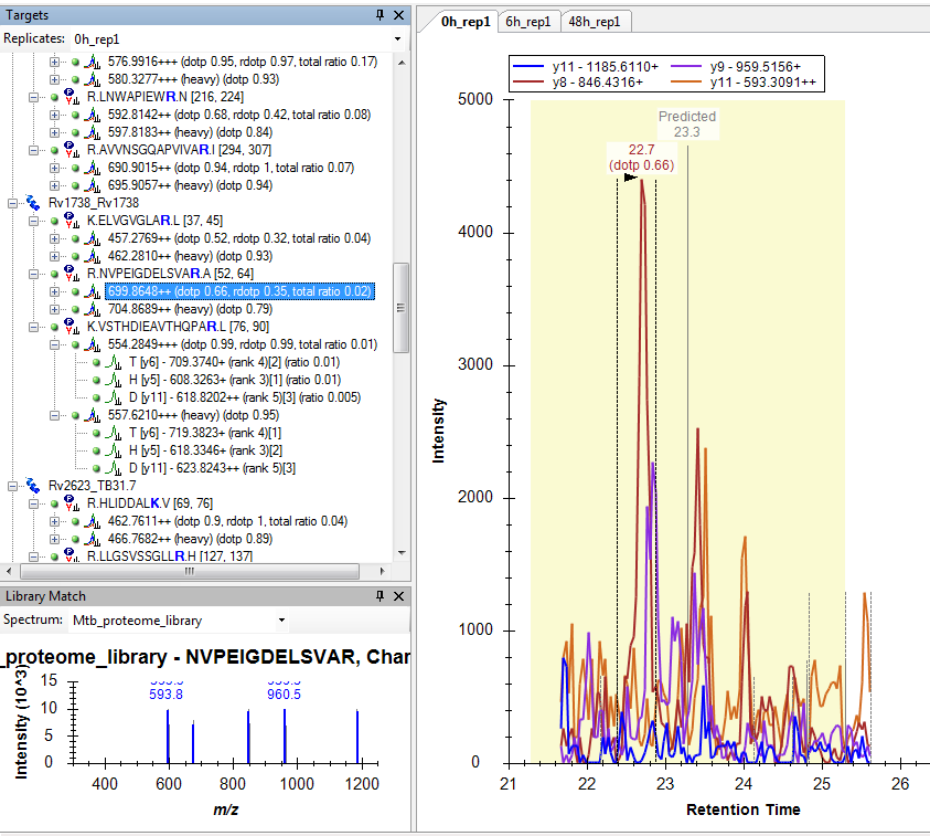




Dotp, rdotp without library

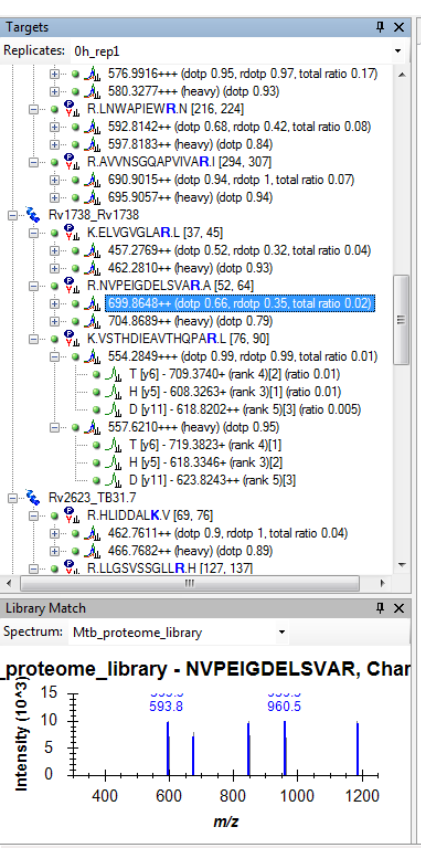


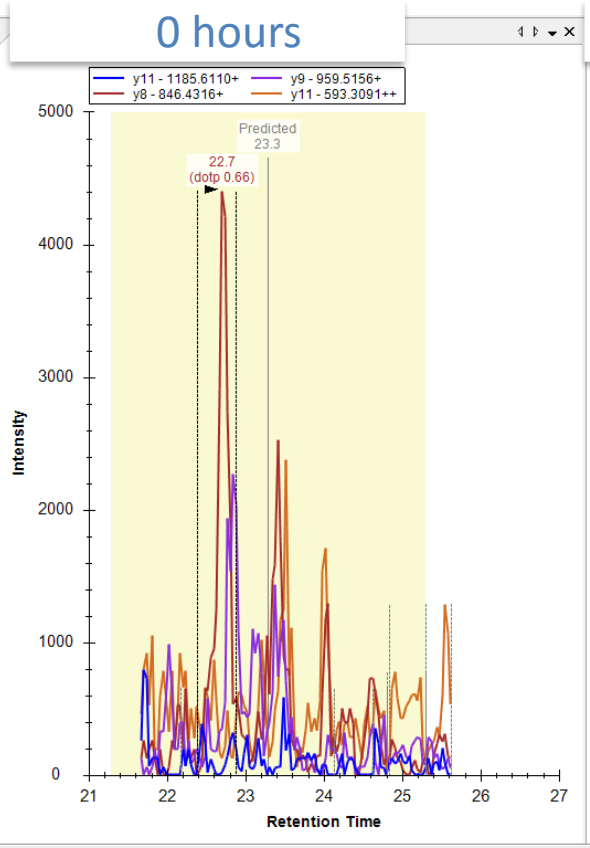
Using reference standards for peak selection

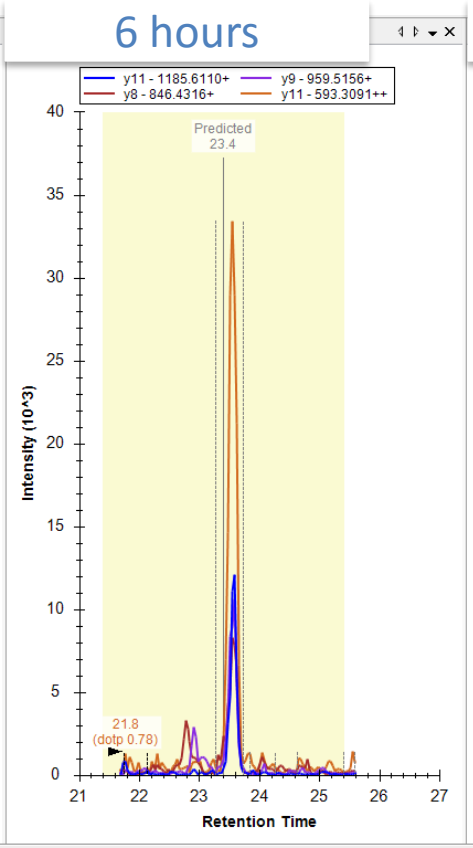


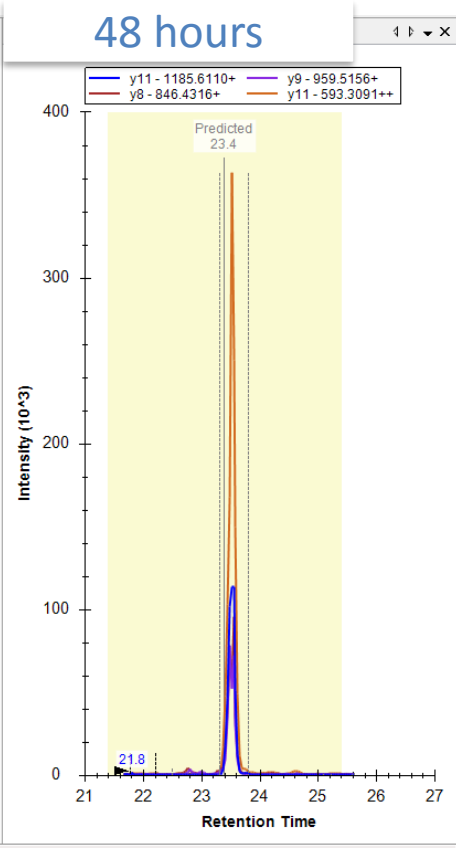
No clear peak, A low dotp
Which should we select ?

Using reference standards for peak selection

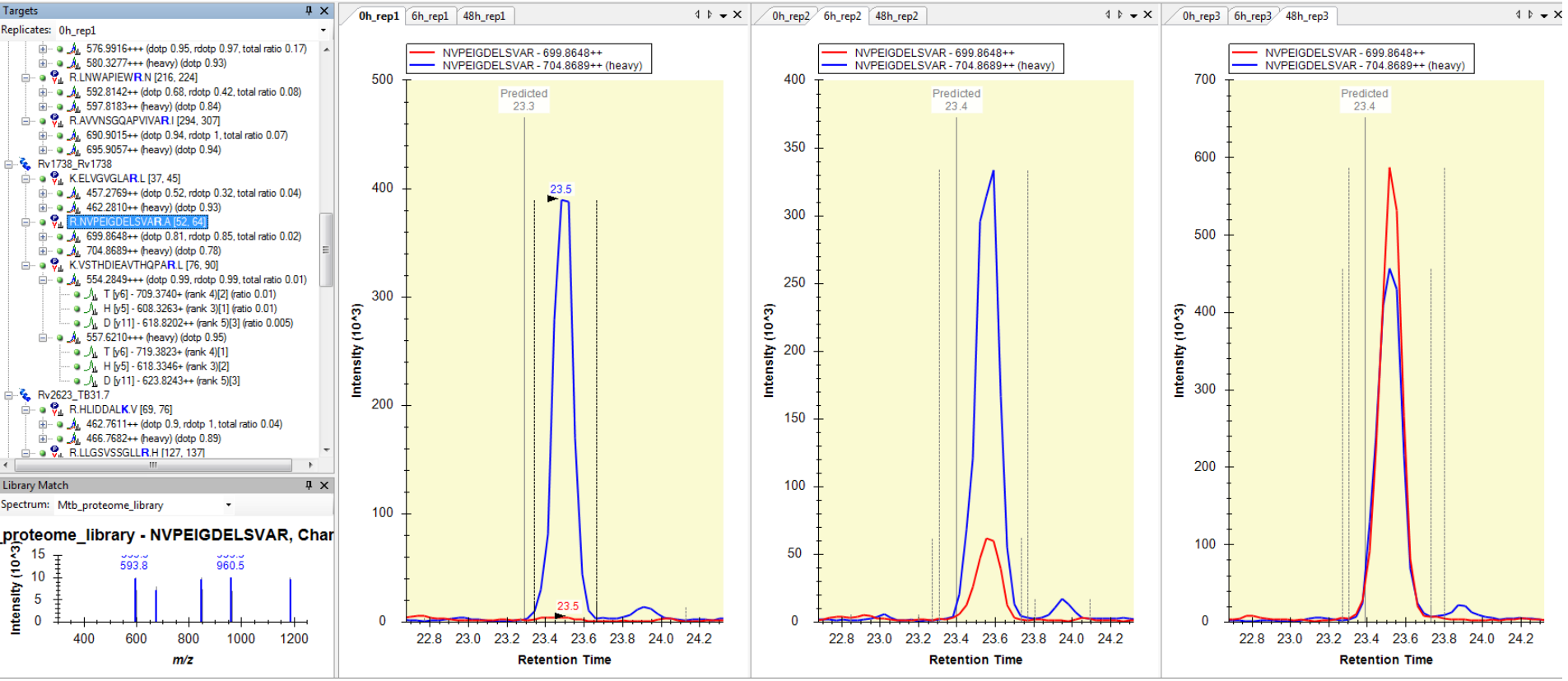






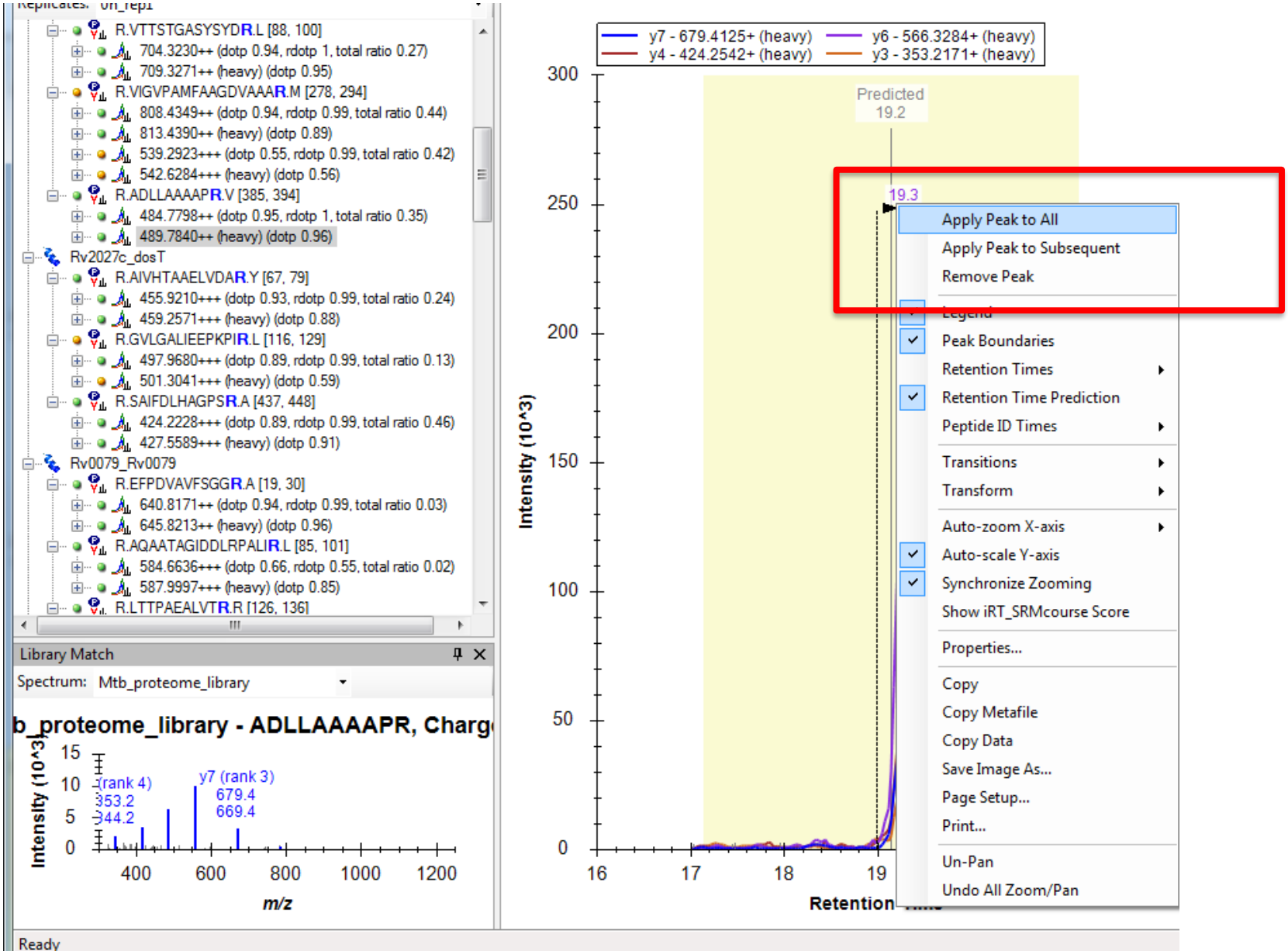


Using reference standards for peak selection

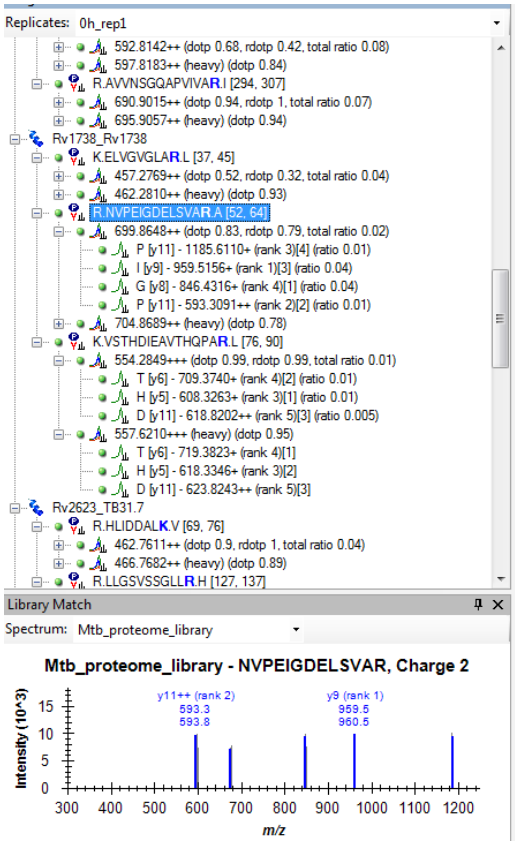


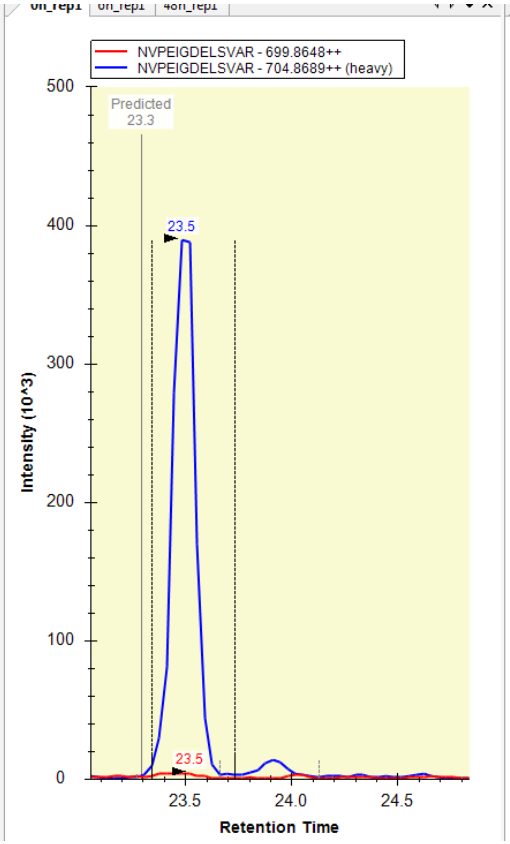
We can confidently state about absence :
the endogenous peptide is below detectability

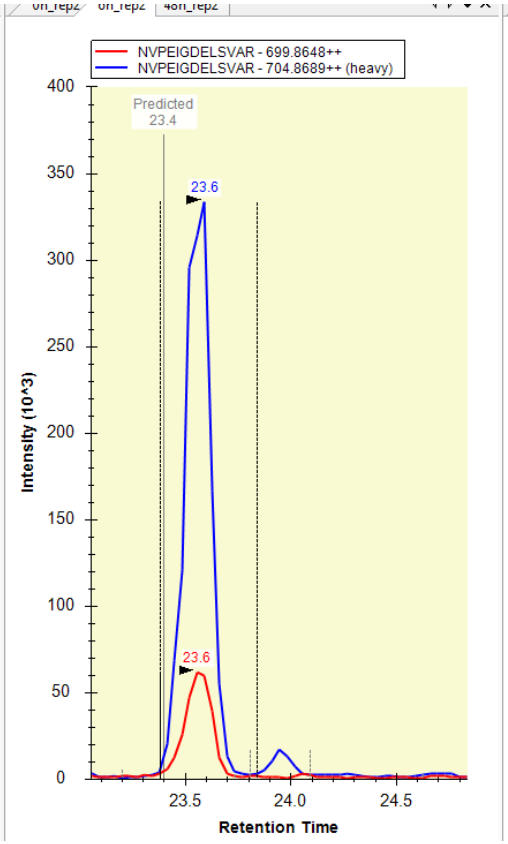
Using reference standards for peak selection

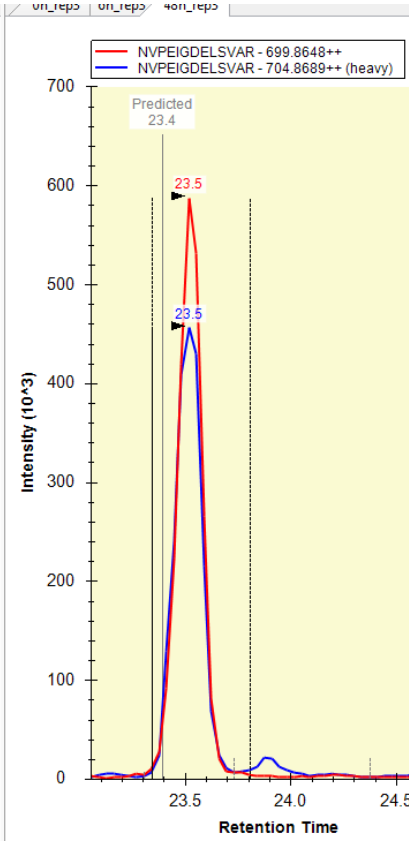


Using reference standards for peak selection

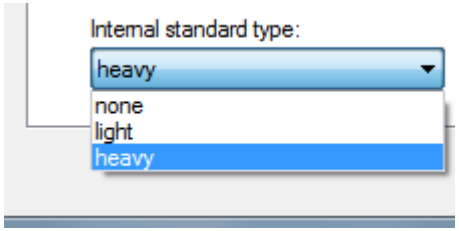






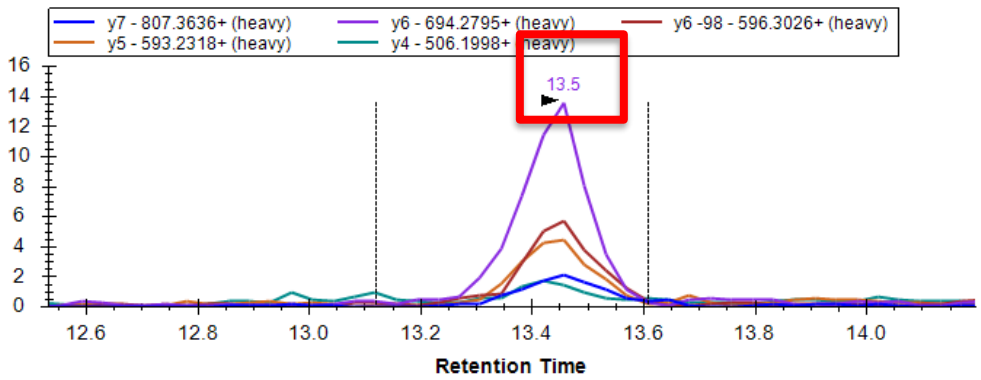


If the standard is set to heavy, it will be used in peak picking



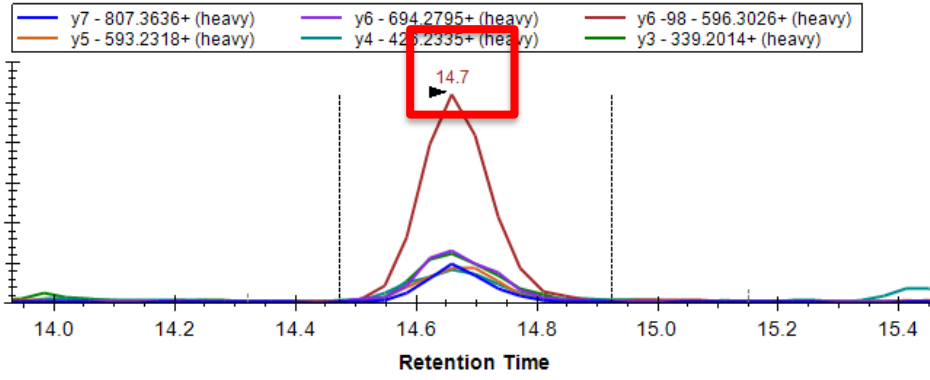
Using reference standards for peak selection

- Modified peptides & localization: we can use synthetic standards to generate a reference for the identification and quantification of the correct modification site (an example with phosphorylation):



reference

TDALTS**S[Phos]**PG**R**

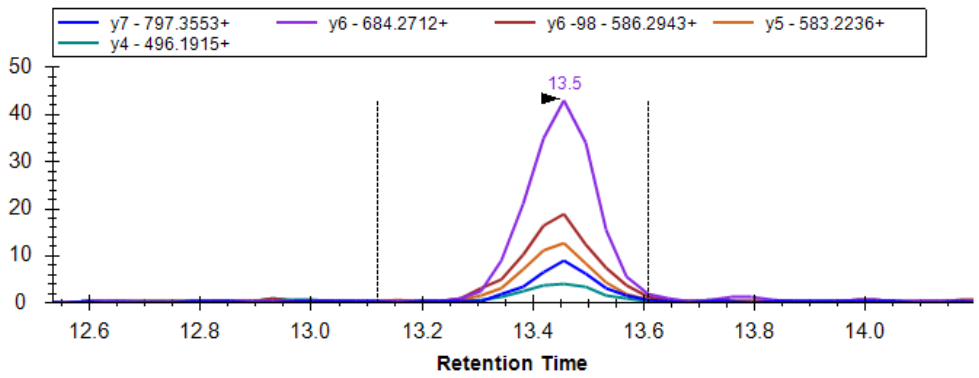


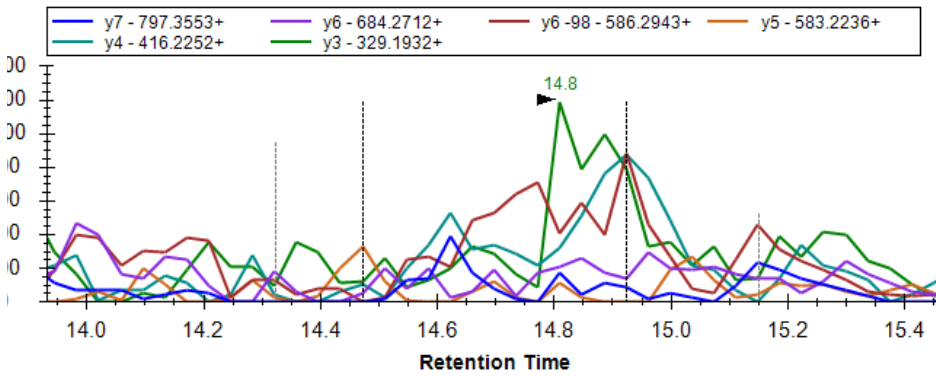
reference

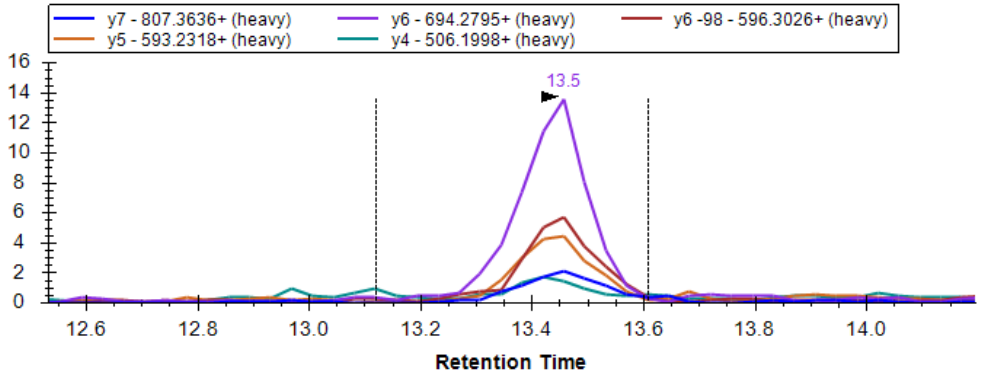
TDALT**S[Phos]**SPG**R**

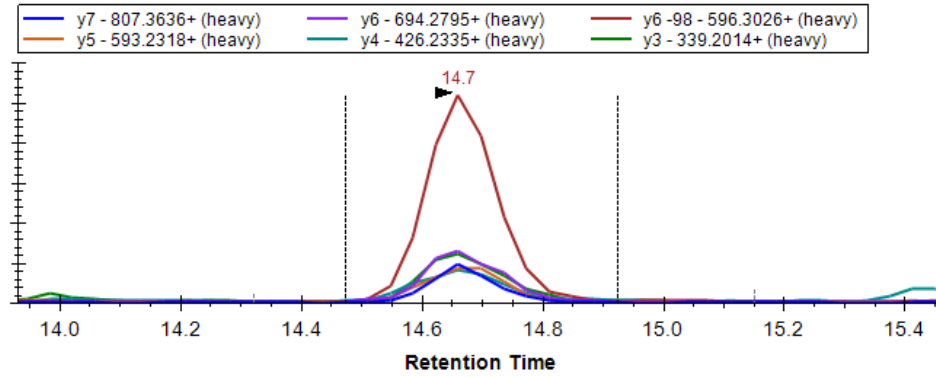
http://targetedproteomics.ethz.ch/videos.html

Using reference standards for peak selection









reference

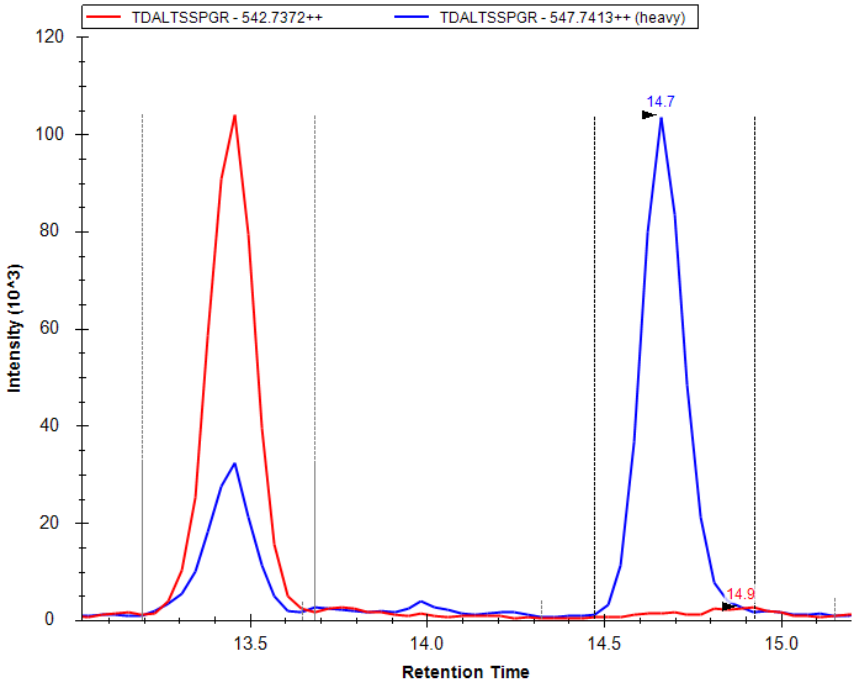
TDALTS**S[Phos]**PG**R**

reference

TDALT**S[Phos]**SPG**R**

Using reference standards for peak selection

- Modified peptides & localization: we can use synthetic standards to generate a reference for the identification and quantification of the correct modification site :



reference *reference*

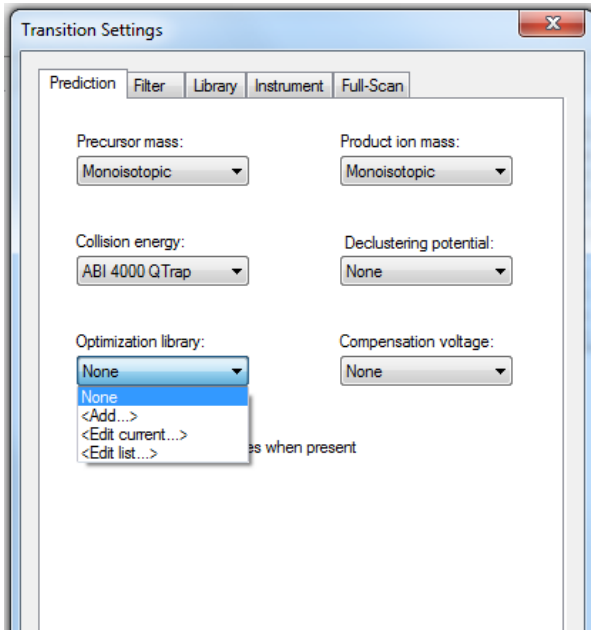
TDALTS**S[Phos]**PG**R** TDALT**S[Phos]**SPG**R**

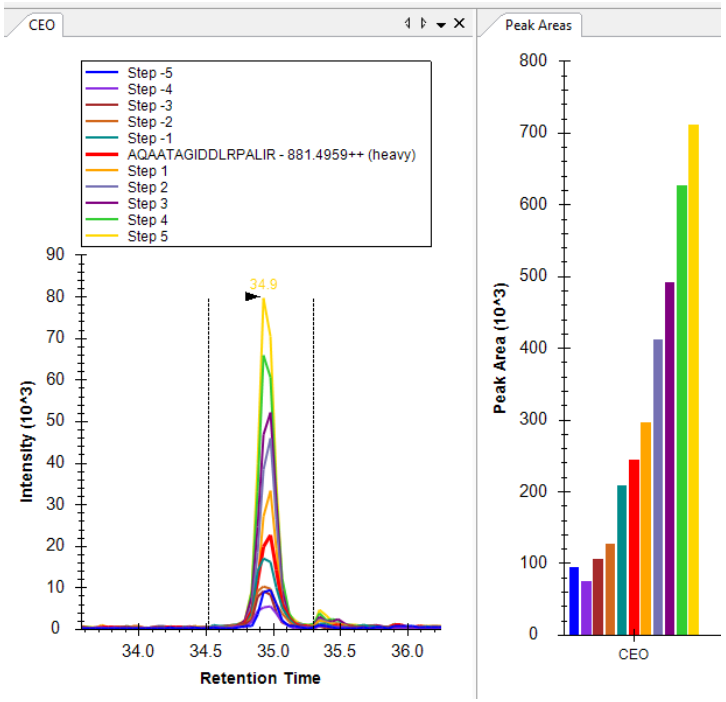
Generating a reference for quantification

- We can use a synthetic standard to generate a reference for the best quantitative assay :

PRM data
SRM data
→ Library building → Reference assay for maximal sensitivity

for example collision energy optimization





https://skyline.gs.washington.edu/labkey/wiki/home/software/Skyline/page.view?name=tutorial_optimize_ce
http://targetedproteomics.ethz.ch/downloads.html

Using standards for peptide identification - summary

- Generating a reference for identification
- Using a reference for peak selection
- Using a reference for optimal quantification

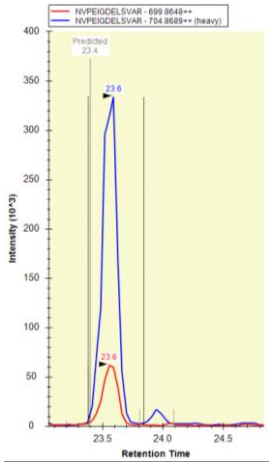
Can be stored in Skyline, Panorama

- Reference for retention time
- Reference spectrum in library
- Reference chromatogram In library
- Reference for parameters (CE)

Per se, does not require isotope label standards

Peptide identification In targeted proteomics

Reference chromatogram Peak selection: RT, rdotp, transition selection



Using isotope label standards

Improve confident peptide identification

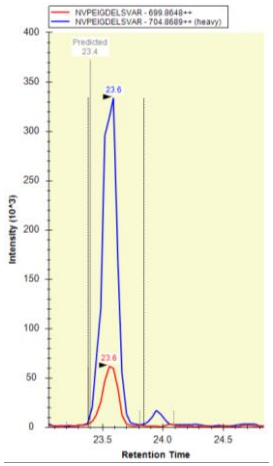
- Generating a reference for identification
- Using a reference for peak selection
- Using a reference for optimal quantification

Peptide identification In targeted proteomics

Reference chromatogram Peak selection: RT, rdotp, transition selection

Tina:

Improve quantitative precision and accuracy



Using isotope label standards