



# Skyline Tutorial Webinar #12

## Isotope Labeled Internal Standards in Skyline

With

Christina Ludwig (Proteomics Researcher)

Ariel Bensimon (Proteomics Researcher)

# Agenda

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- ▶ 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

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Germany

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ETH Zürich  
Switzerland

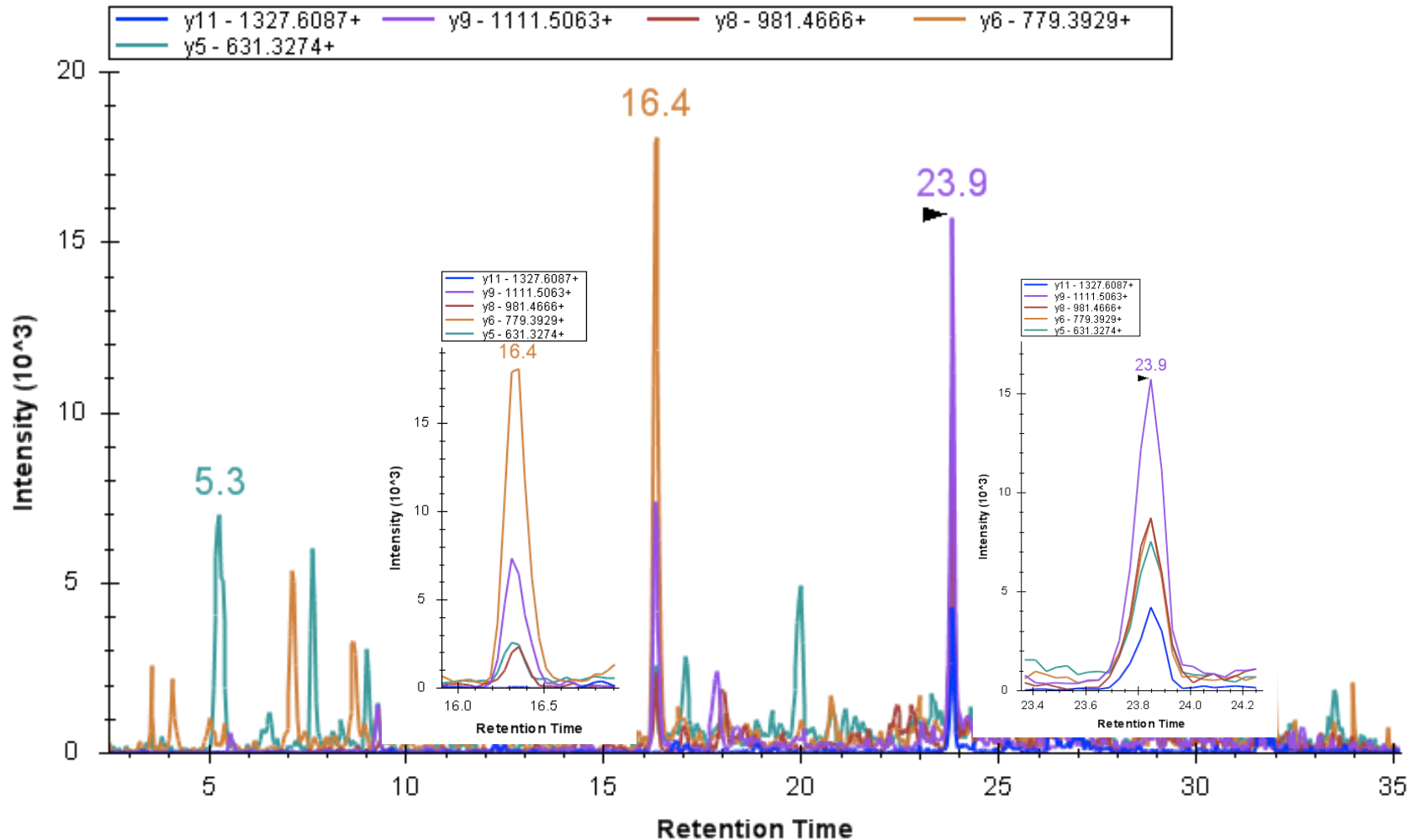


Technische Universität München



# Motivation – why use isotope labeled standards ?

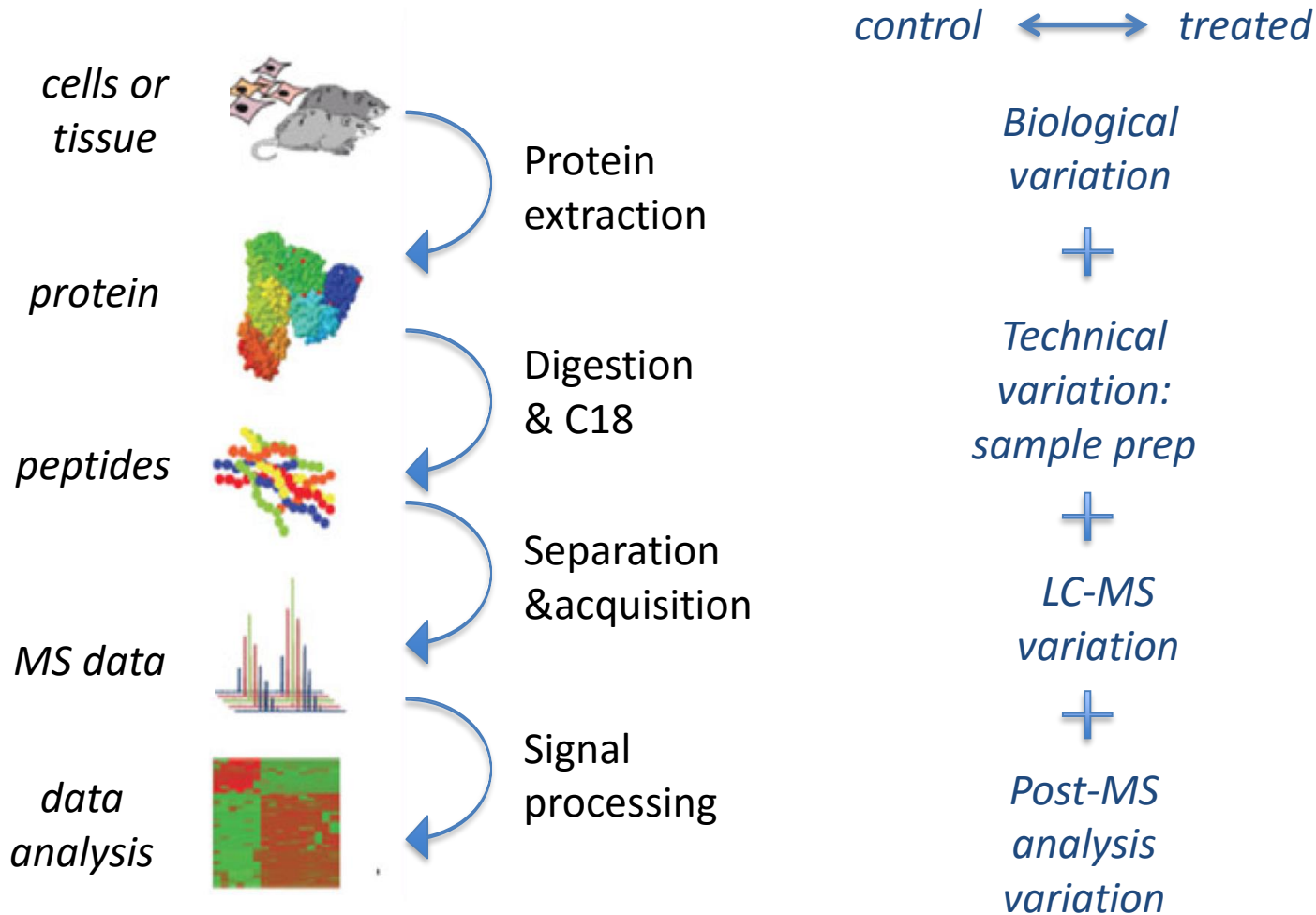
For the correct identification of a peptide: selecting the correct peak



# 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



# 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

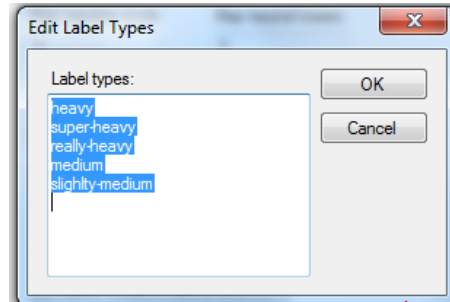
# Stable-isotope labeling

- **Heavy** reference standards are generated such that they carry one or several isotope-labeled atoms.
  - typically used isotope-labeled atoms:  $^{13}\text{C}$ ,  $^{15}\text{N}$ ,  $^{18}\text{O}$
  - most common amino acids: K, R but also A, L, I, F, P, V.
  - use of  $^2\text{H}$  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

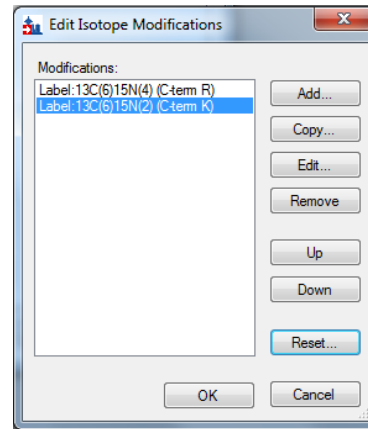
# Modifications tab

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

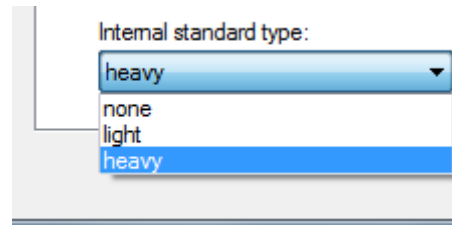
*You can define and name labels ; select those relevant for your experiment. Some appear in default*



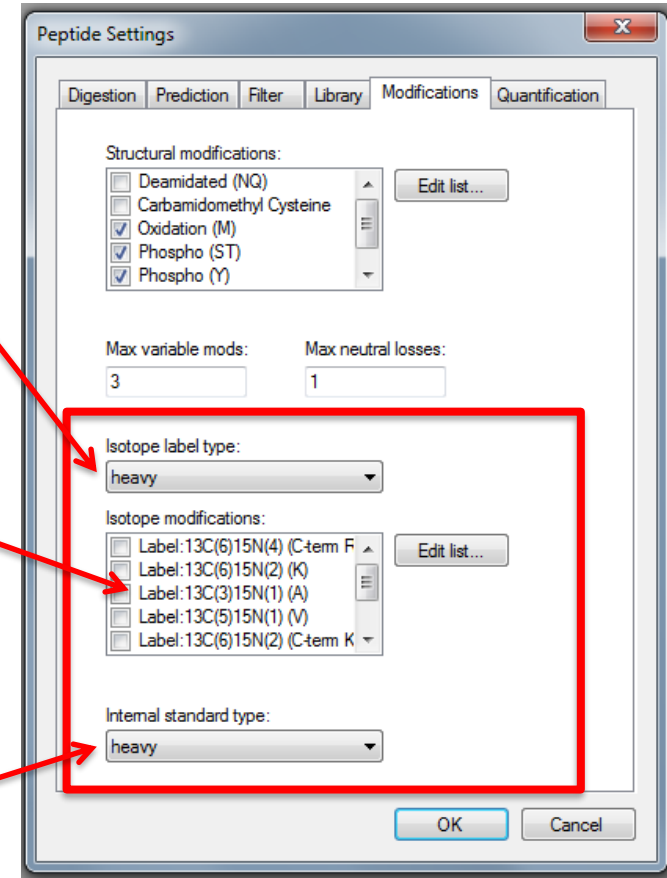
*You edit a set of possible isotope modifications; select those relevant for each of the labels*



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



*You assume the standard is present; Reverse is possible*

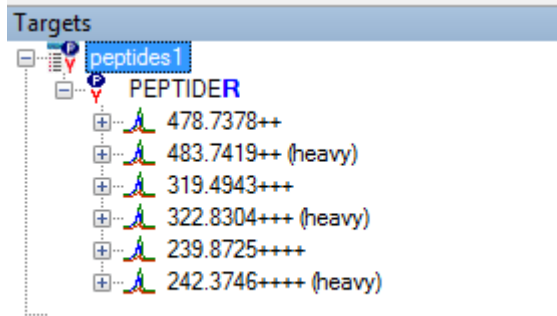




# Insert peptides

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

- Insert a light peptide sequence



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

Peptide Sequence	Protein Name	Protein Description
PEPTIDESR[Label:13C(6)15N(4) (C-term R)]		
THEPEPTIDESR[+10]		
MANYPEPTIDESR{+10}		

# Select precursors & transitions

File Edit View Settings Tools Help

Targets

peptides1

- PEPTIDER
  - 478.7378++
    - P [y6] - 730.3730+
    - T [y5] - 633.3202+
    - I [y4] - 532.2726+
  - 483.7419++ (heavy)
    - P [y6] - 740.3813+
    - T [y5] - 643.3285+
    - I [y4] - 542.2808+
- THEPEPTIDER
  - 662.3124++ ✓
  - 667.3165++ (heavy) ✗
  - 441.8774+++ ✗
  - 445.2135+++
  - 331.6598+++
  - 334.1619+++

**THEPEPTIDER Precursors**

  - 1323.6175+
  - 1333.6258+ (heavy)
  - 662.3124++
  - 667.3165++ (heavy)
  - 441.8774+++
  - 445.2135+++ (heavy)
  - 331.6598+++
  - 334.1619+++ (heavy)
  - 265.5293, +5
  - 267.5310, +5 (heavy)
  - 221.4423, +6
  - 223.1104, +6 (heavy)

PEPTIDER

- 478.7378++
  - P [y6] - 730.3730+
  - T [y5] - 633.3202+
  - I [y4] - 532.2726+
- 483.7419++ (heavy)
  - P [y6] - 740.3813+
  - T [y5] - 643.3285+
  - I [y4] - 542.2808+

THEPEPTIDER

- 662.3124++
- 441.8774+++
- 331.6598+++

**Transitions**

- precursor [M-1] - 483.2415++ (rank 4)
- precursor - 483.7419++ (rank 1)
- precursor [M+1] - 484.2435++ (rank 2)
- precursor [M+2] - 484.7447++ (rank 3)
- precursor [M+3] - 485.2460++ (rank 5)
- E [y7] - 869.4239+
- P [y6] - 740.3813+
- T [y5] - 643.3285+
- I [y4] - 542.2808+
- D [y3] - 429.1968+
- E [y2] - 314.1698+
- R [y1] - 185.1272+

Synchronize isotope label types

# Modify peptides

File Edit View Settings Tools Help

Targets

- peptides1
  - PEPTIDER
    - 478.7378++
    - 483.7419++ (heavy)
    - 319.4943+++
    - 322.8304+++ (heavy)
    - 239.8725++++
    - 242.3746++++ (heavy)
    - THEPEPTIDER**

Context menu options:

- Cut
- Copy
- Paste
- Delete
- Pick Children
- Set Standard Type
- Modify...**
- Edit Note
- Replicates

File Edit View Settings Tools Help

targets

- peptides1
  - PEPTIDER
    - 478.7378++
    - 483.7419++ (heavy)
    - 319.4943+++
    - 322.8304+++ (heavy)
    - 239.8725++++
    - 242.3746++++ (heavy)
  - THEPEPTIDER
    - 662.3124++
    - 441.8774+++
    - 331.6598++++

Edit Modifications dialog:

Structural:		Isotope heavy:
	T	
	H	
	E	
	P	
	E	
	P	
	T	
	I	
	D	
	E	
	R	Label:13C(6)15N(4) (C-term R)

Buttons: OK, Cancel, Create copy, Reset

Dropdown menu options:  
Label:13C(6)15N(4) (C-term R)  
Label:13C(6)15N(4) (R)  
<Add...>  
<Edit list...>

*Make sure these are first set in Modifications tab*

# Add label

The screenshot shows a software application's menu system. The 'Edit' menu is open, and the 'Refine' option is highlighted. A sub-menu is visible, listing various actions such as '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...', and 'Advanced...'. The 'Advanced...' option is highlighted in blue.

- File
- Edit
  - 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
- View
- Settings
- Tools
- Help

- 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...**

The screenshot shows the 'Refine' dialog box. It contains several settings for document refinement. The 'Add label type' dropdown is set to 'heavy', and the 'Add' checkbox is checked. The 'Auto-select all' section has three unchecked checkboxes: 'Peptides', 'Precursors', and 'Transitions'. The 'Min peptides per protein' and 'Min transitions per precursor' fields are empty. The 'Remove repeated peptides' and 'Remove duplicate peptides' checkboxes are also unchecked. The 'OK' and 'Cancel' buttons are at the bottom right.

Document

Min peptides per protein:

Remove repeated peptides  Remove duplicate peptides

Min transitions per precursor:

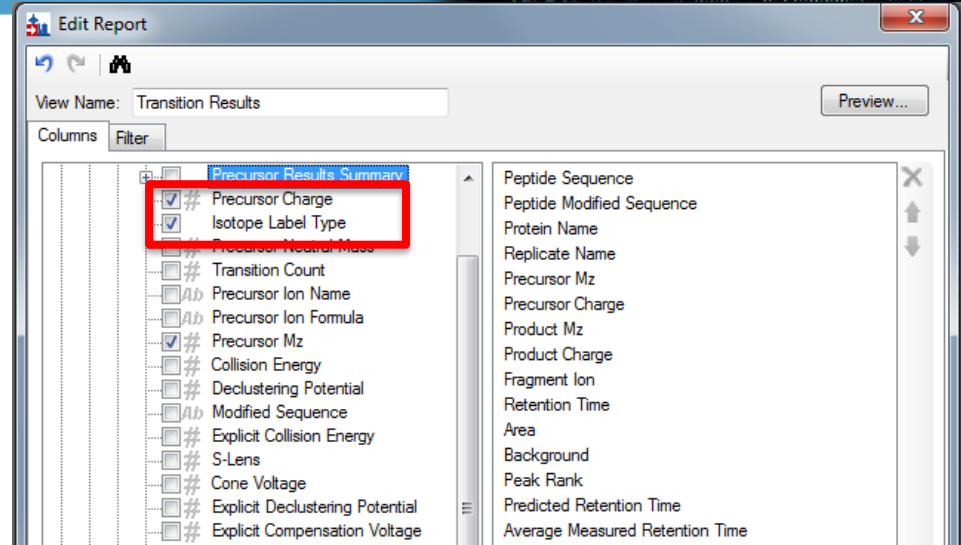
Add label type:  
heavy  Add  
light  
heavy

Auto-select all:  
 Peptides  
 Precursors  
 Transitions

OK Cancel

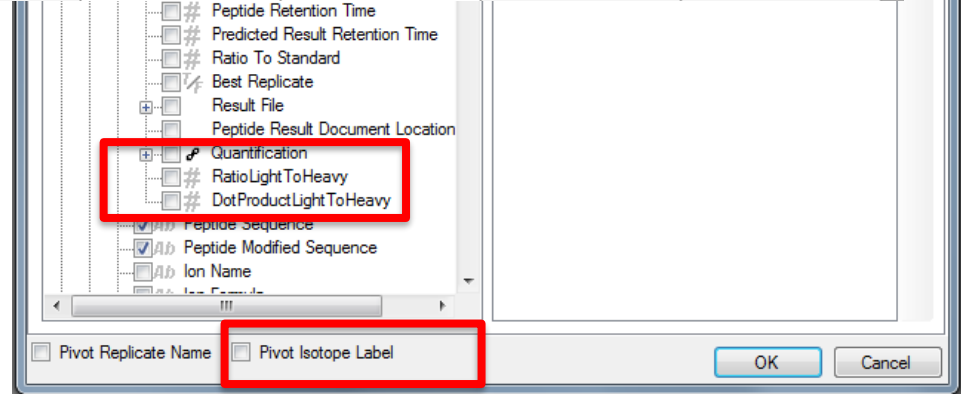
# Export & Results

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



Views 3 of 9 Export... Find:

Replicate	Peptide Peak Found Ratio	Peptide Retention Time	Ratio To Standard	BioReplicate	Run	RatioLight To Heavy	DotProductLight To
<a href="#">0h_rep1</a>	1	31.12	0.1705	0H	1	0.1705	0.9669
<a href="#">6h_rep1</a>	1	31.06	0.171	6H	1	0.171	0.9927



Results tutorial :

[https://skyline.gs.washington.edu/labkey/\\_webdav/home/software/Skyline/@files/tutorials/CustomReports-1\\_2.pdf](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 ?

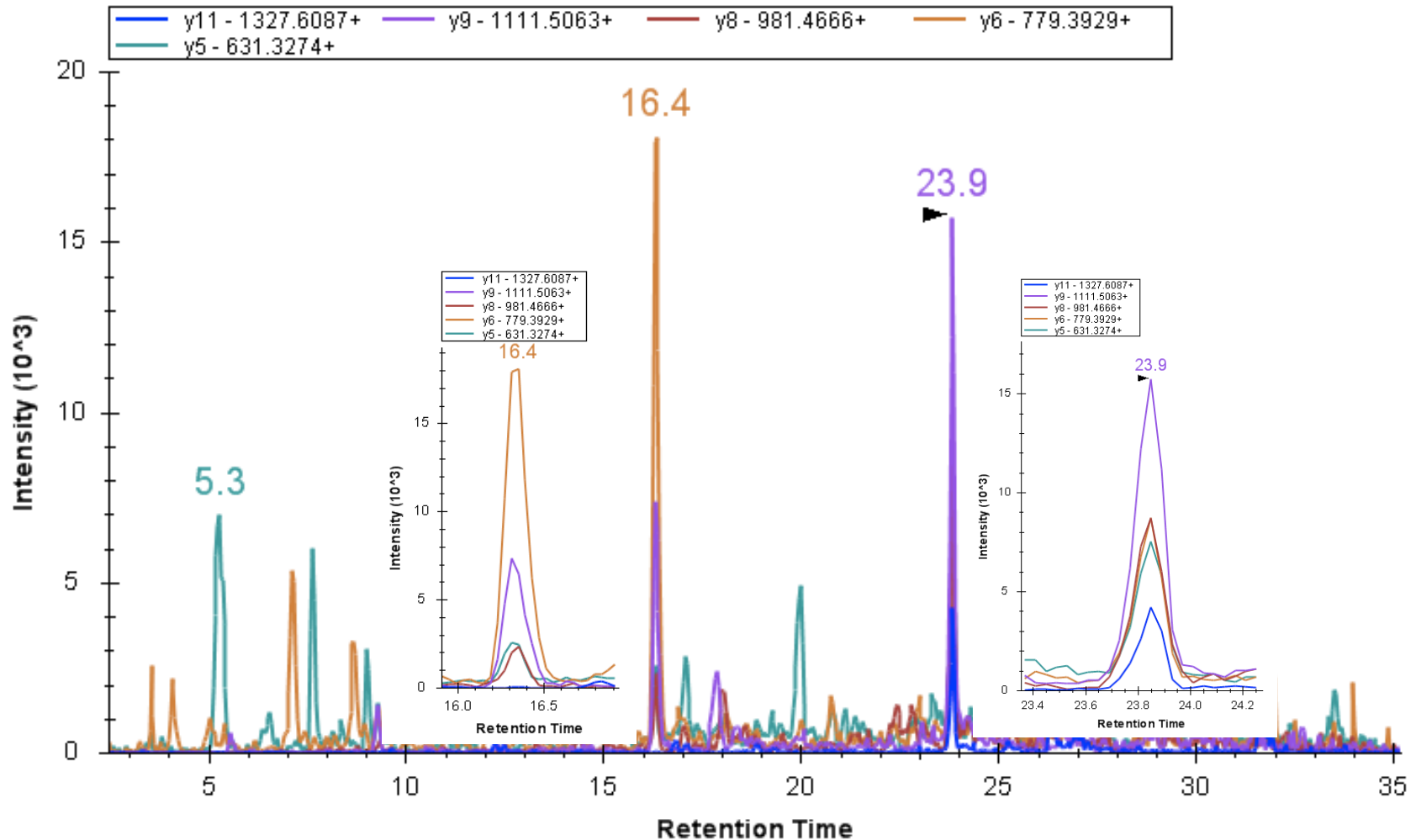
## Improve confident peptide identification - Ariel

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



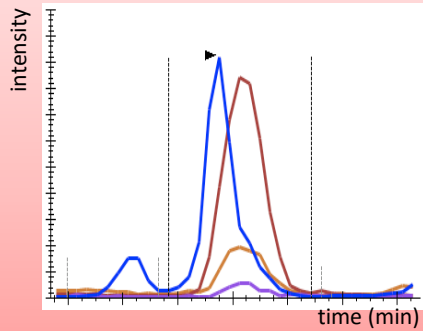
# Motivation – why use isotope labeled standards ?

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

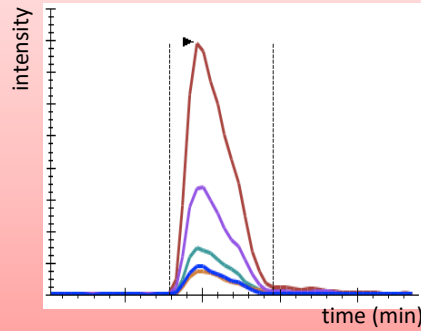


# Criteria for reliable peak identification

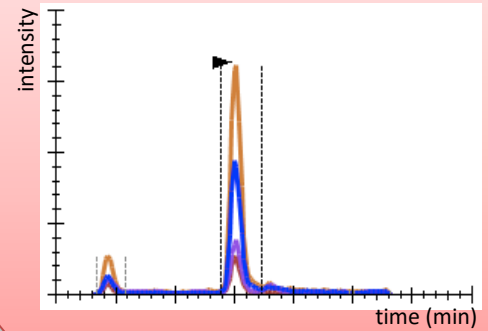
## 1. Co-elution



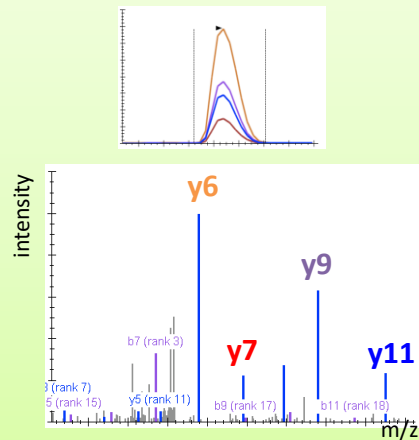
## 2. Peak shape



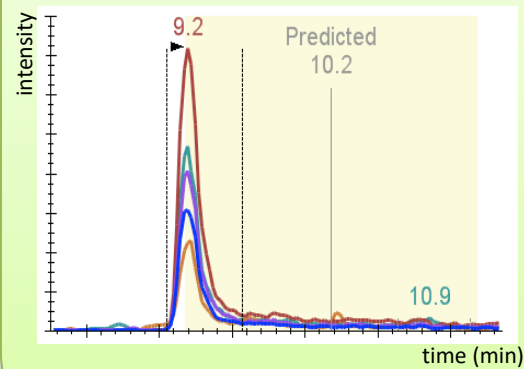
## 3. Signal intensity



## 4. Correlation peak intensities to library spectrum



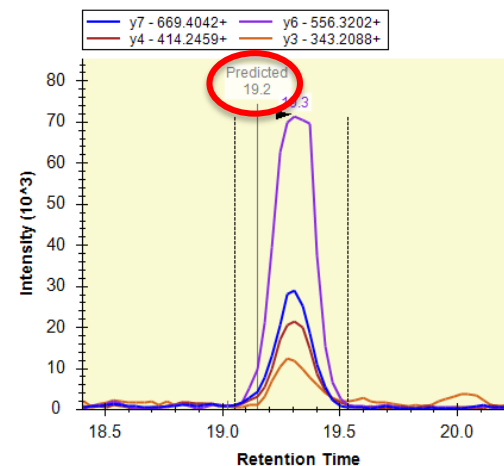
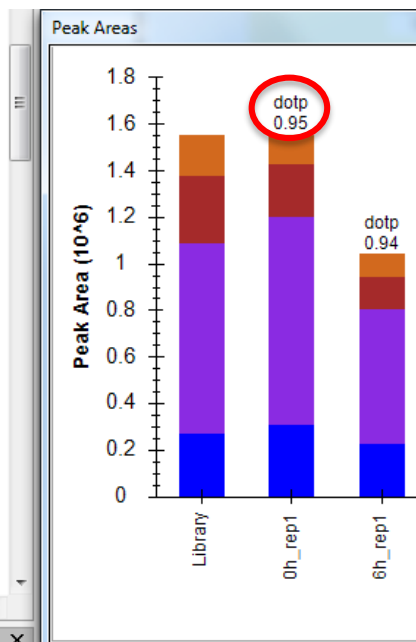
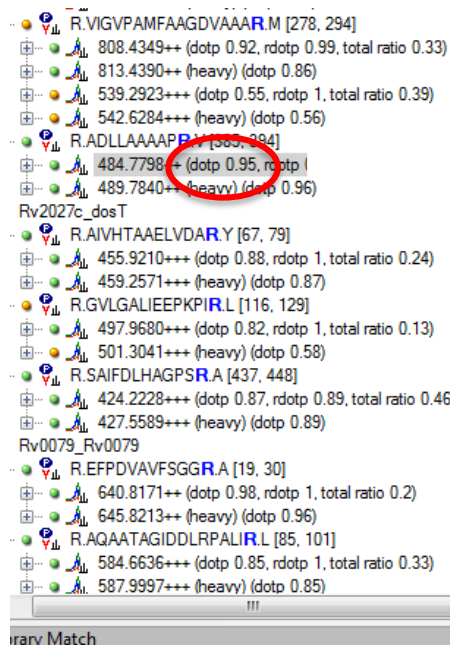
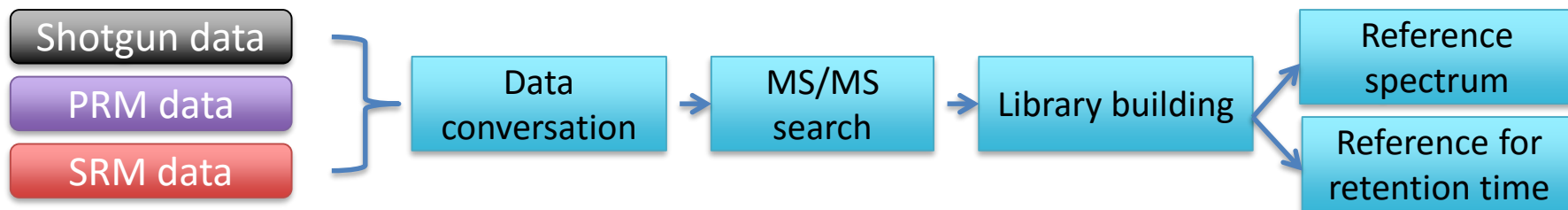
## 5. Correlation retention time (empirical)





# Generating a reference for identification

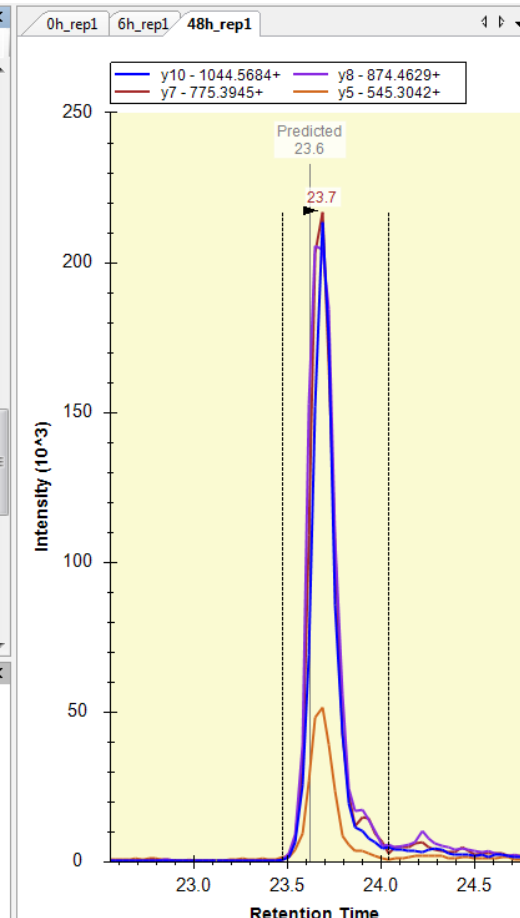
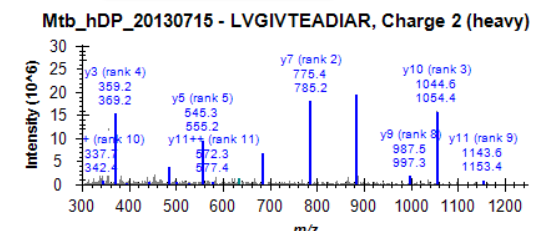
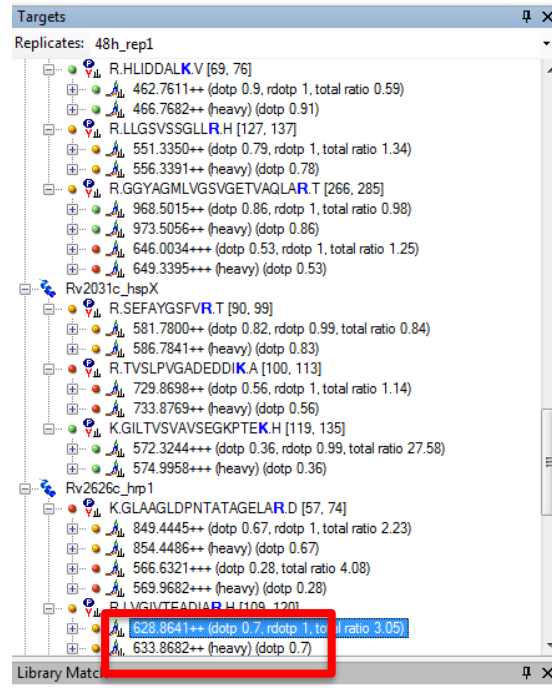
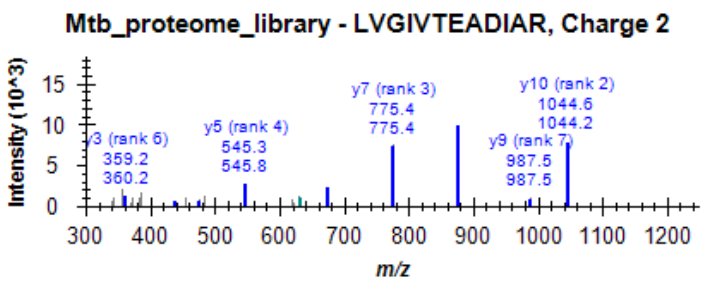
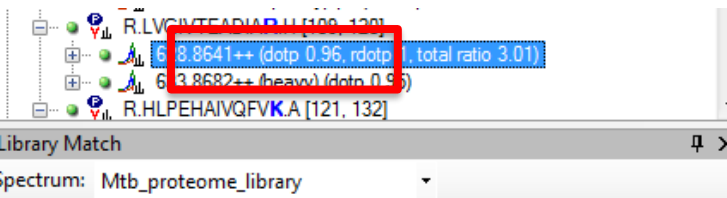
- We can use a synthetic standard to generate :
  - A reference spectrum for the spectral library (see also webinar 4, tutorials)
  - A reference retention time value , for the RT library (see webinar 7, tutorials)



# Generating a reference for identification

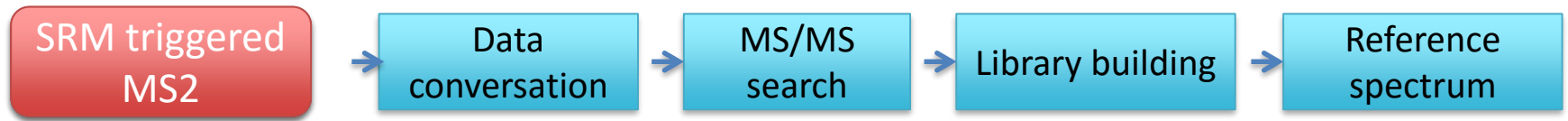
- We can use a synthetic standard to generate a reference:
  - peptide or protein standard.
  - heavy or light standard (chemically identical).
  - Skyline transfers information, if you activate the heavy label in Modifications.

- 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.

# 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)

Transition Settings

Prediction Filter Library Instrument **Full-Scan**

MS1 filtering

Isotope peaks included: Count  
Precursor mass analyzer: QIT

Peaks: 1  
Resolution: 0.7 m/z

Isotope labeling enrichment:

MS/MS filtering

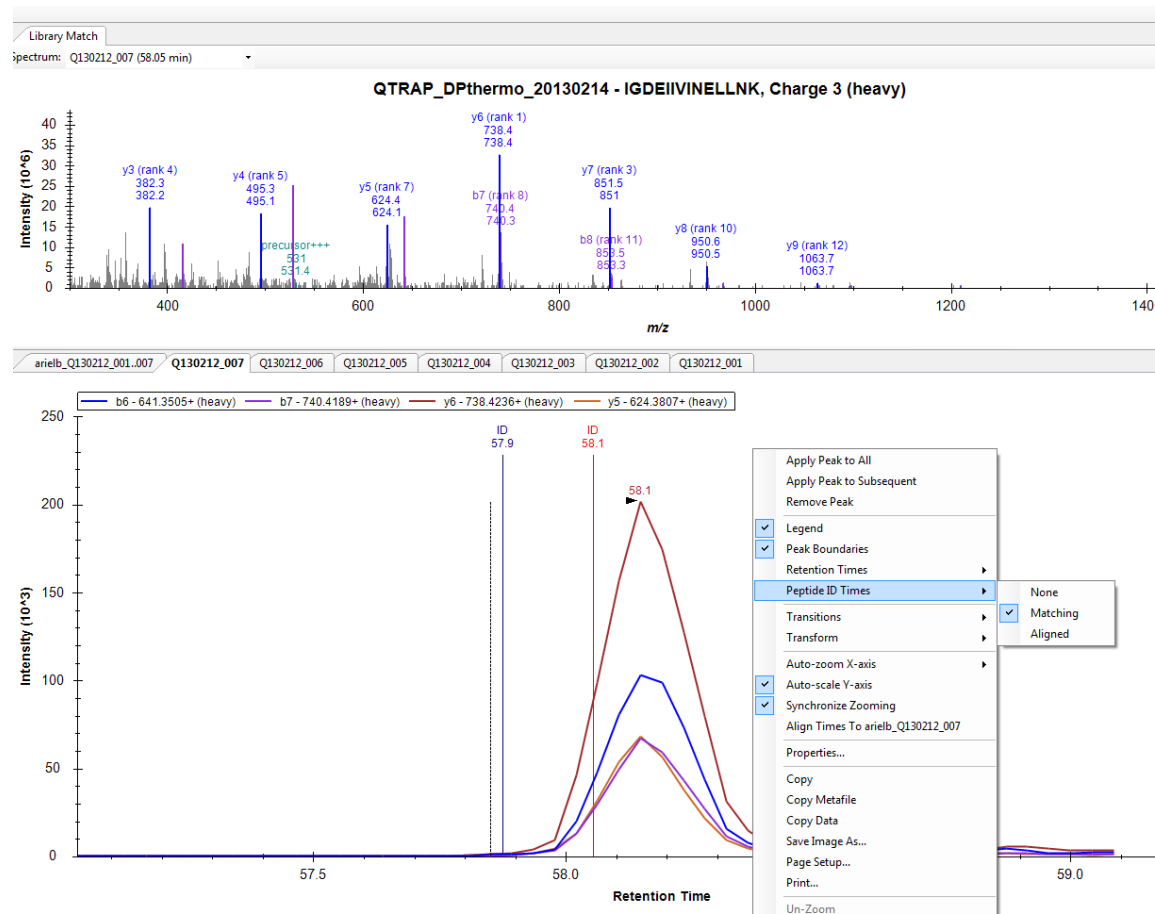
Acquisition method: None  
Product mass analyzer:

Isolation scheme:  
Resolution: m/z

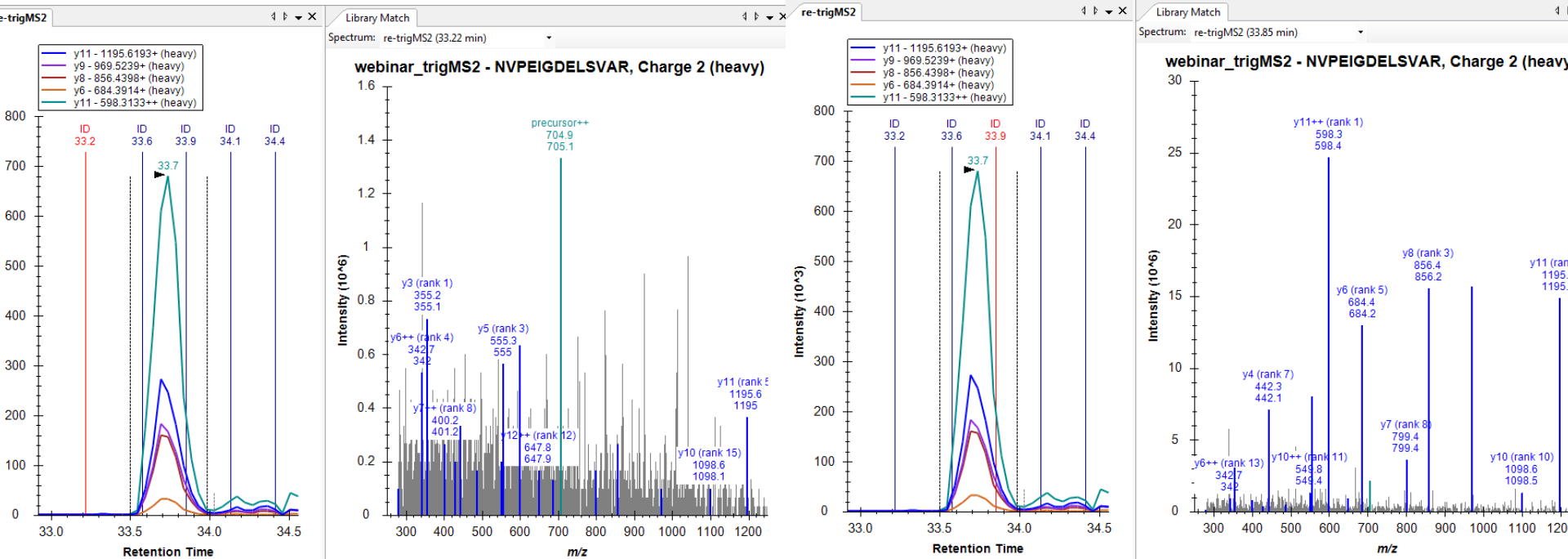
Retention time filtering

Use only scans within 5 minutes of MS/MS IDs  
 Use only scans within 5 minutes of predicted RT  
 Include all matching scans

OK Cancel



# Targeted MS2 in Skyline

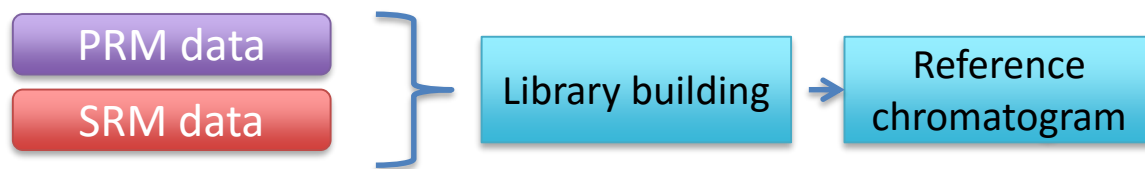


- When working with with synthetic standards for assay generation : ensure the quality of your spectra

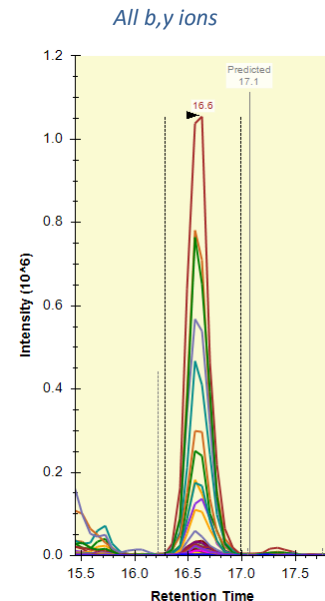
[https://skyline.gs.washington.edu/labkey/tutorial\\_library\\_explorer.url](https://skyline.gs.washington.edu/labkey/tutorial_library_explorer.url)

# Generating a reference for identification

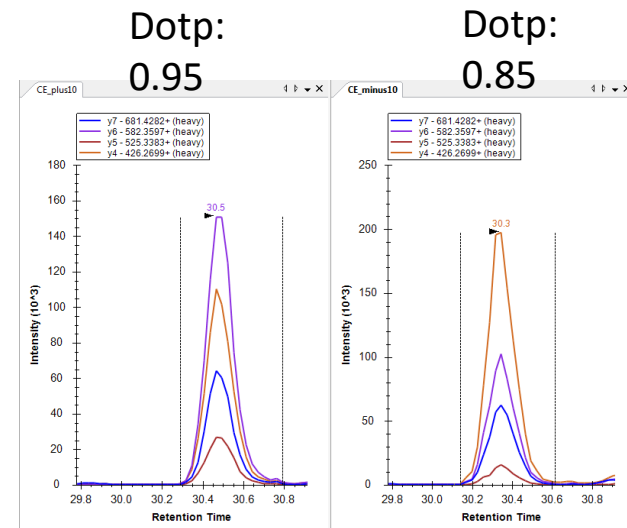
- We can use a synthetic standard to generate a reference for the chromatogram library: heavy or light standard; peptide or protein standard.



Webinar 11; Tutorial  
(chromatogram  
libraries)



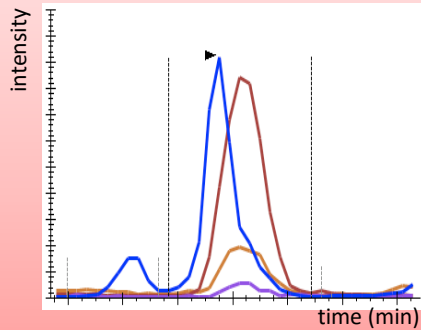
- Notes:
  - In SRM: you would need to measure all the desired transitions.
  - You will still get a dotp from a chromatogram library.
  - Ensure similarity in MS setup (CE etc).



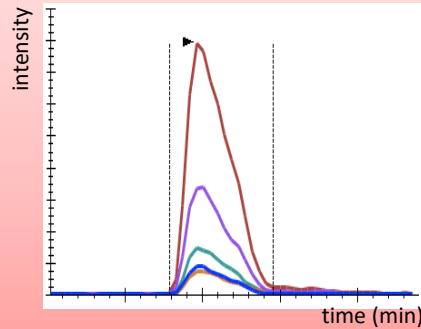
Same peptide with different CEs

# Criteria for reliable peak identification

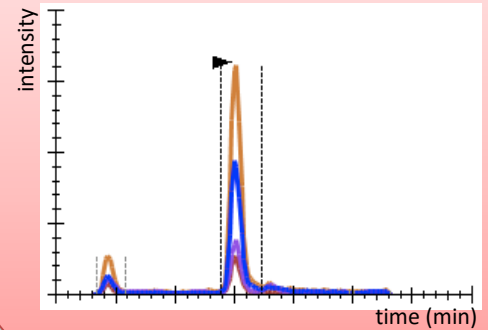
## 1. Co-elution



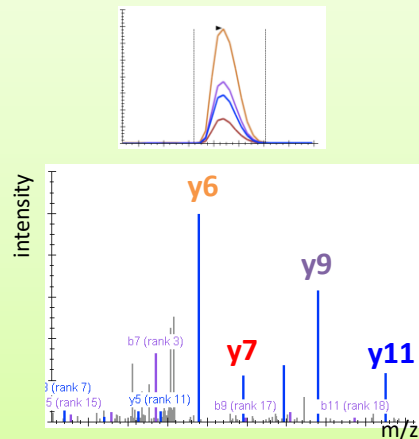
## 2. Peak shape



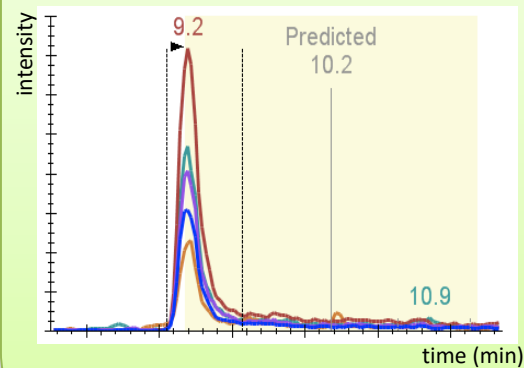
## 3. Signal intensity



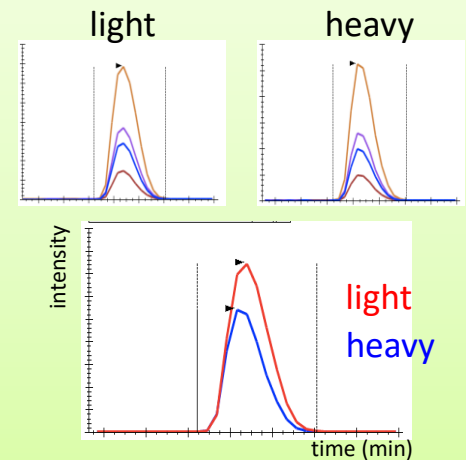
## 4. Correlation peak intensities to library spectrum



## 5. Correlation retention time (empiric or predicted\*)



## 6. Correlation with heavy-labeled standard

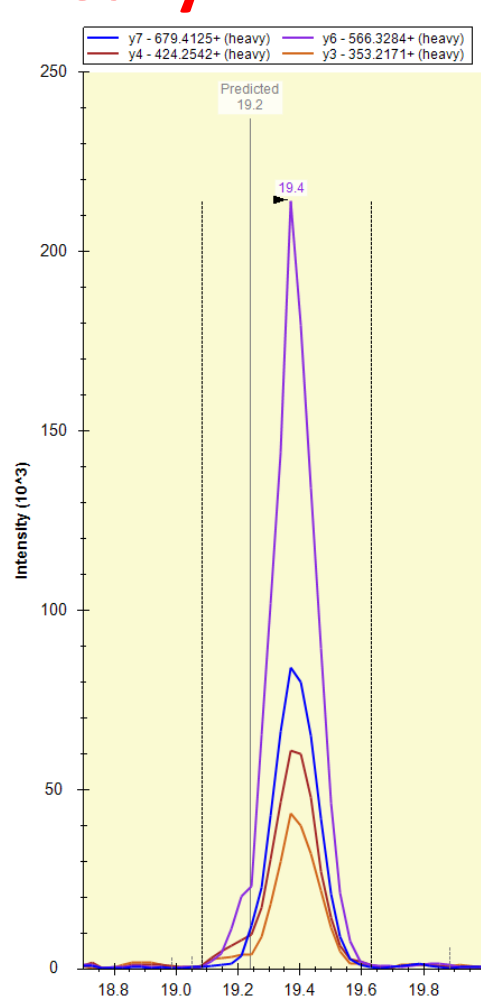
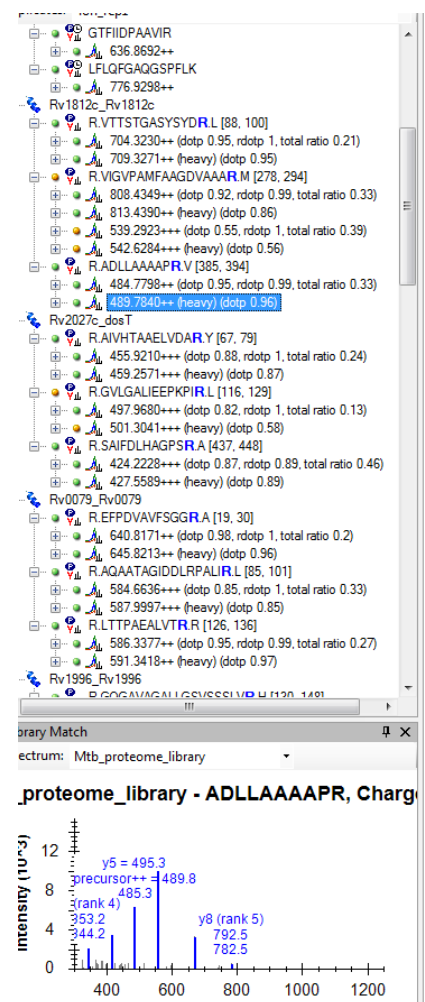
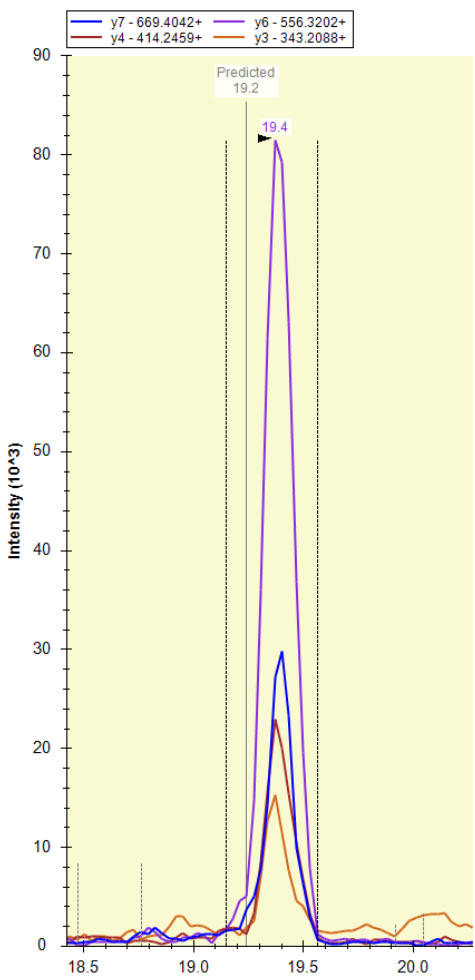
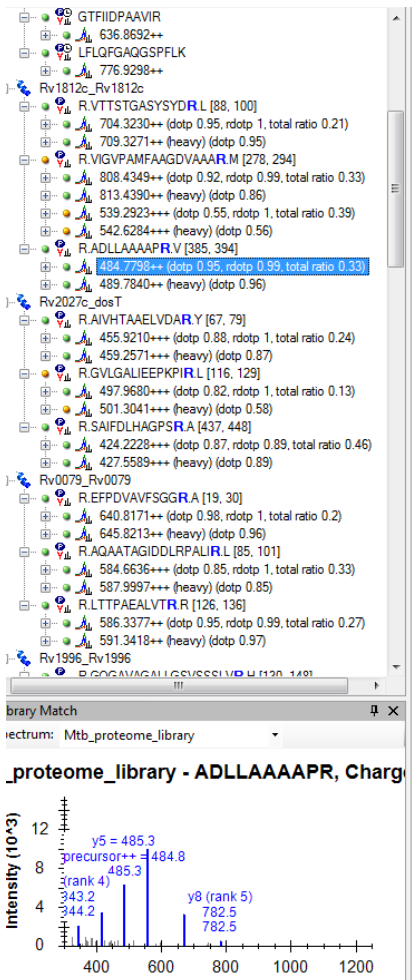




# View individual precursors

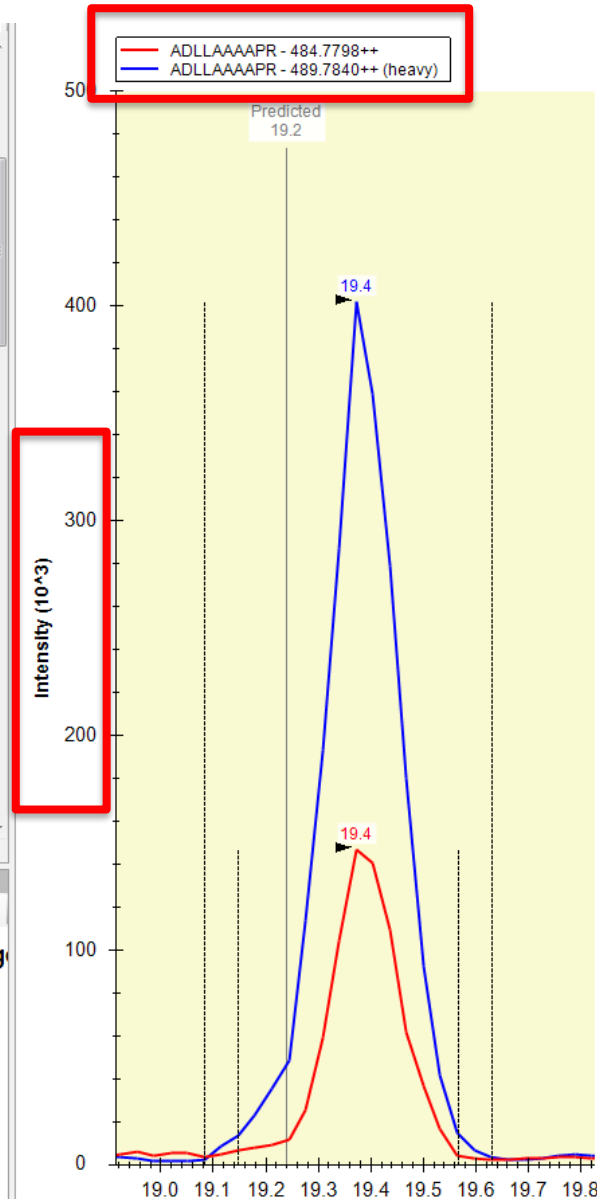
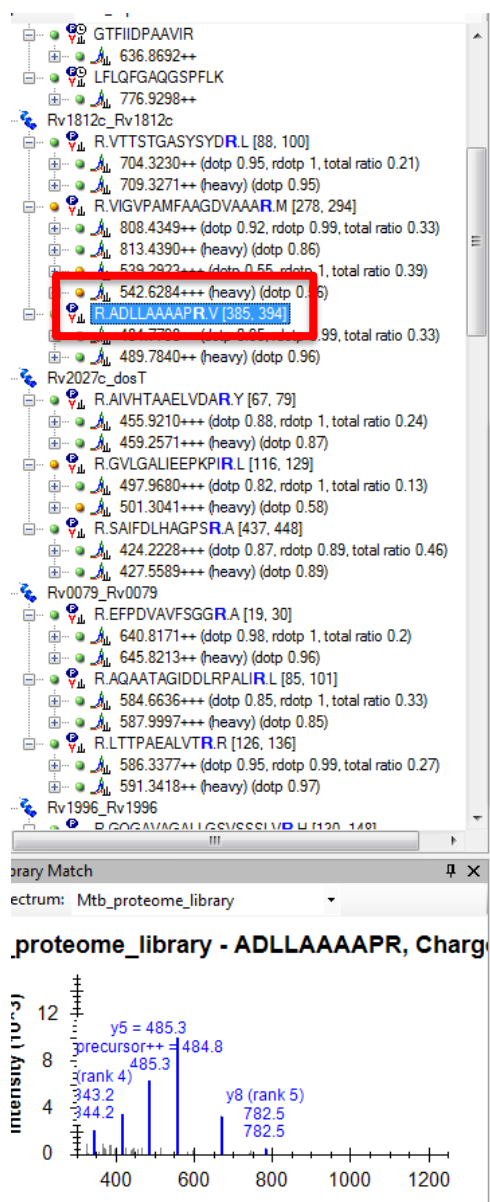
light

heavy





# View pairs



View:

Total sum, no transition info  
Same boundaries

These **heavy** references are **chemically identical** to the endogenous (**light**):  
Co-elution  
Same peak boundaries

# Split graph

Replicates: 48h\_rep1

- GTFFIDPAAVIR
- 636.8692++
- LFLQFGAQGSPFLK
- 776.9298++
- Rv1812c\_Rv1812c
- R.VTTSTGASYSYDRL [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.ADLLAAAAPRV [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.GVLGALIEEPKIR L [116, 129]
  - 497.9680+++ (dotp 0.82, rdotp 1, total ratio 0.13)
  - 501.3041+++ (heavy) (dotp 0.58)
- R.SAIFDLHAGPSRA [437, 448]
  - 424.2228+++ (dotp 0.87, rdotp 0.89, total ratio 0.46)
  - 427.5589+++ (heavy) (dotp 0.89)
- Rv0079\_Rv0079
- R.EFPDVAVFSGGRI A [19, 30]
  - 640.8171++ (dotp 0.98, rdotp 1, total ratio 0.2)
  - 645.8213++ (heavy) (dotp 0.96)
- R.AQAATAGIDLRPALIR L [85, 101]
  - 584.6636+++ (dotp 0.85, rdotp 1, total ratio 0.33)
  - 587.9997+++ (heavy) (dotp 0.85)
- R.LITTPAEALVTR R [126, 136]
  - 586.3377++ (dotp 0.95, rdotp 0.99, total ratio 0.27)
  - 591.3418++ (heavy) (dotp 0.97)
- Rv1996\_Rv1996
- R.GGAVAGALGSVSSIV R [120, 149]

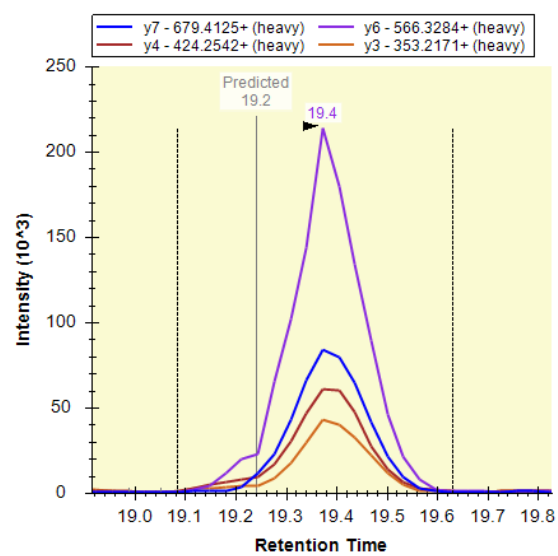
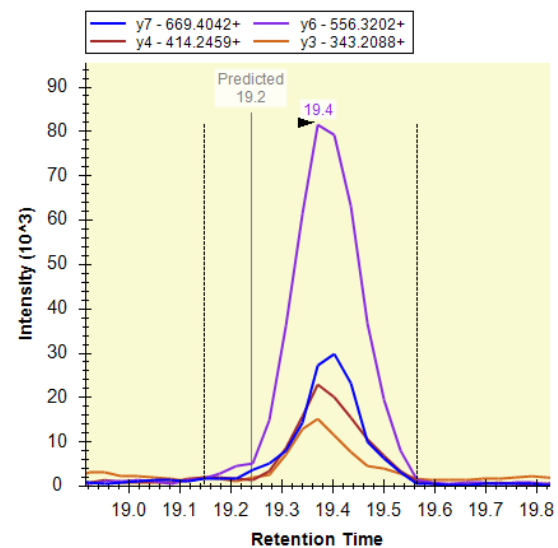
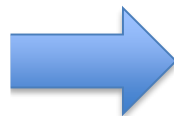
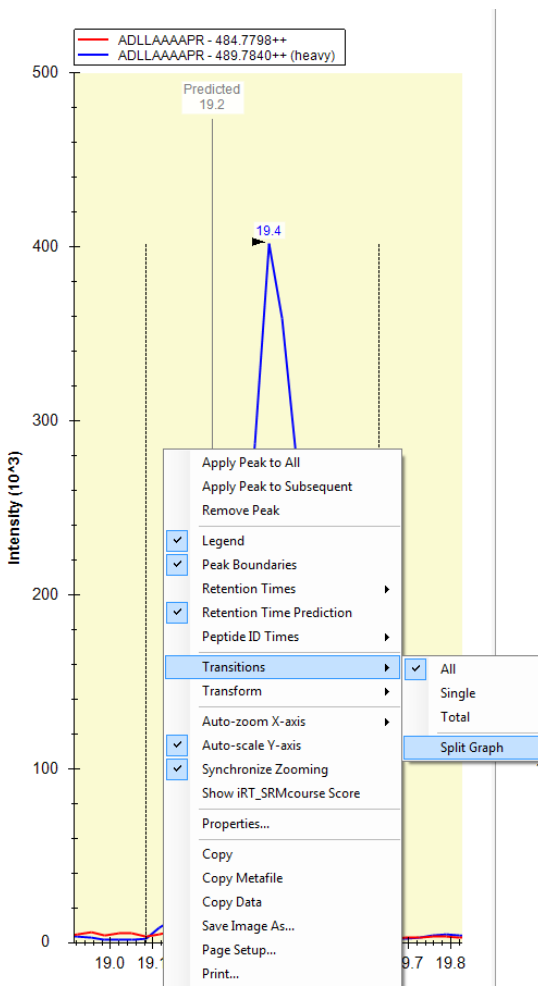
Library Match

Spectrum: Mtb\_proteome\_library

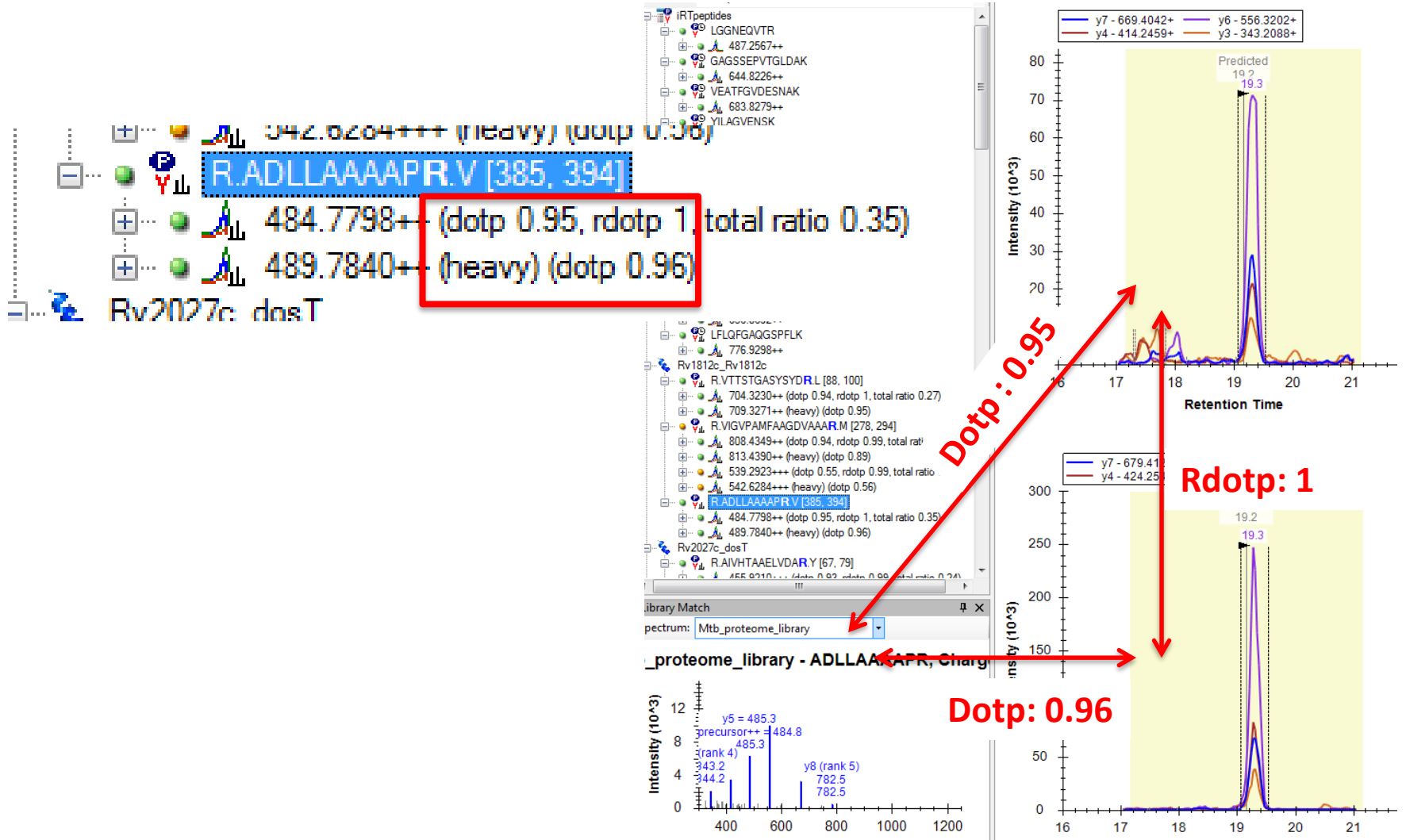
**b\_proteome\_library - ADLLAAAAPR, Charg**

Intensity (10<sup>3</sup>)

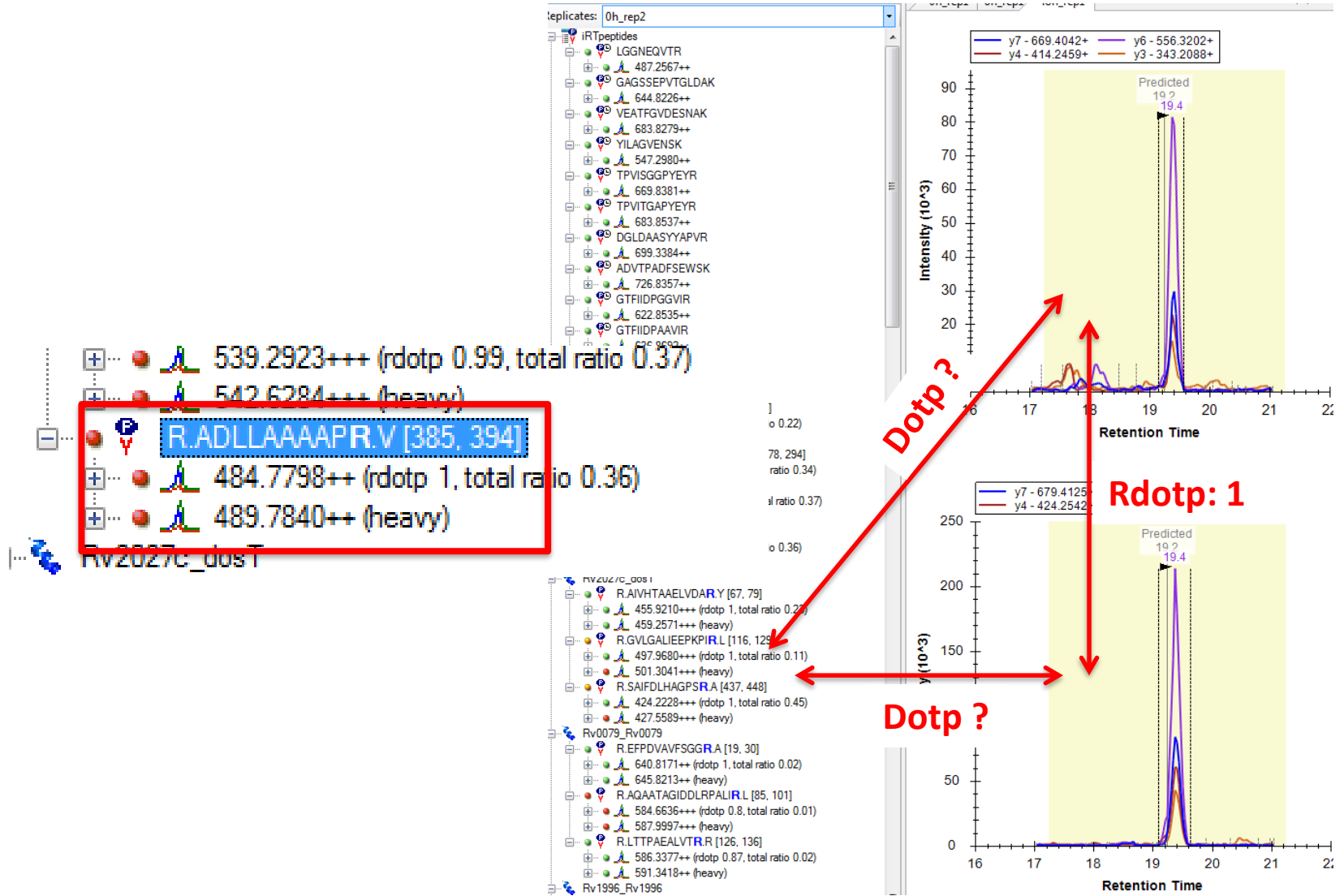
y5 = 485.3  
precursor++ = 484.8  
485.3  
343.2 (rank 4)  
344.2  
y8 (rank 5)  
782.5  
782.5



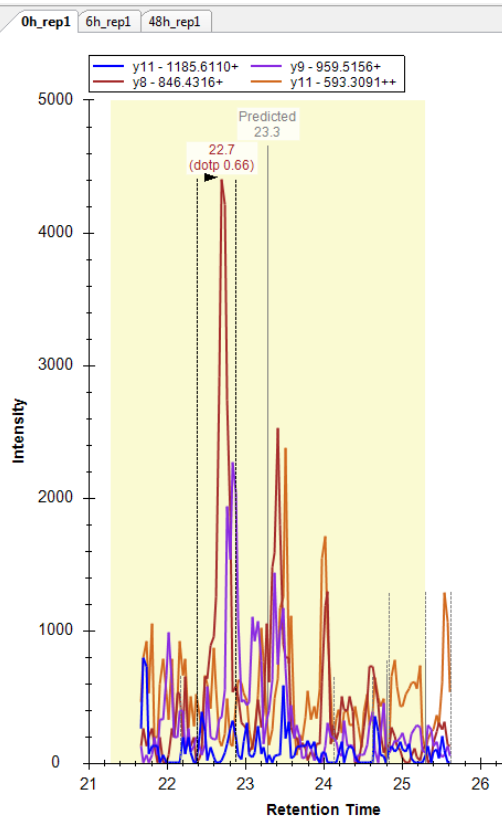
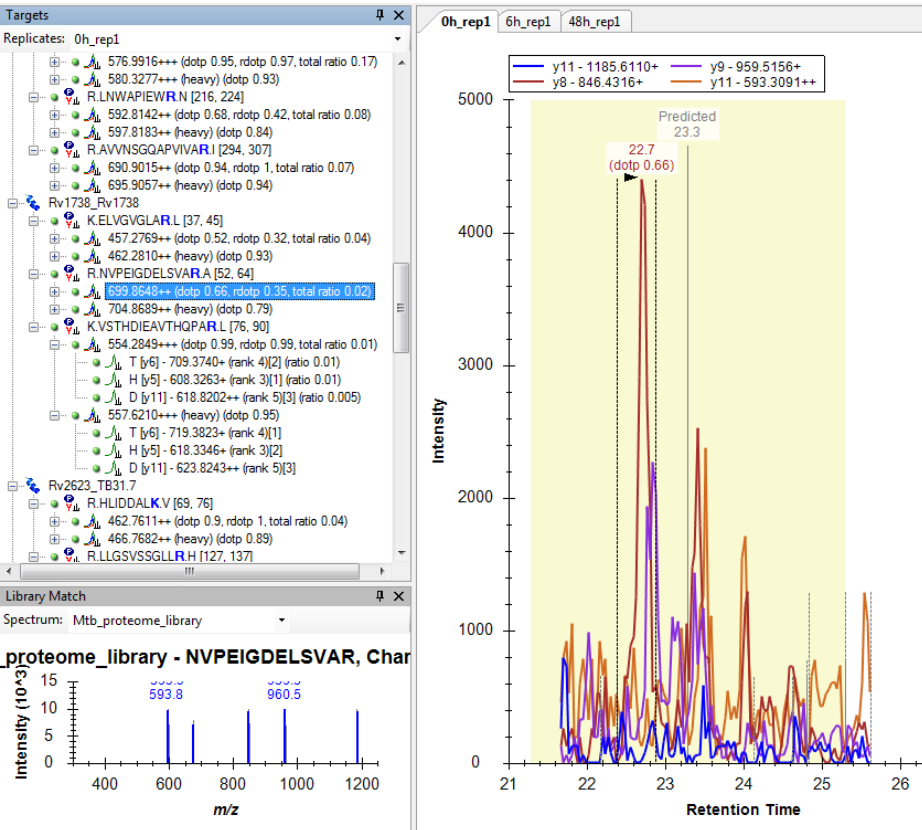
# Dotp, rdotp with library



# 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

Targets

Replicates: 0h\_rep1

- 576.9916+++ (dotp 0.95, rdotp 0.97, total ratio 0.17)
- 580.3277+++ (heavy) (dotp 0.93)
- R.LNWAPIEW.R.N [216, 224]
- 592.8142++ (dotp 0.68, rdotp 0.42, total ratio 0.08)
- 597.8183++ (heavy) (dotp 0.84)
- R.AVNSGAPVIVAR.I [294, 307]
- 690.9015++ (dotp 0.94, rdotp 1, total ratio 0.07)
- 695.9057++ (heavy) (dotp 0.94)
- Rv1738\_Rv1738
- KELVGVGLAR.L [37, 45]
- 457.2769++ (dotp 0.52, rdotp 0.32, total ratio 0.04)
- 462.2810++ (heavy) (dotp 0.93)
- R.NVPEIGDELSVAR.A [52, 64]
- 699.8648++ (dotp 0.66, rdotp 0.35, total ratio 0.02)
- 704.8689++ (heavy) (dotp 0.79)
- K.VSTHDIEAVTHQPAR.L [76, 90]
- 554.2849+++ (dotp 0.99, rdotp 0.99, total ratio 0.01)
- T [y6] - 709.3740+ (rank 4)[2] (ratio 0.01)
- H [y5] - 608.3263+ (rank 3)[1] (ratio 0.01)
- D [y11] - 618.8202++ (rank 5)[3] (ratio 0.005)
- 557.6210+++ (heavy) (dotp 0.95)
- T [y6] - 719.3823+ (rank 4)[1]
- H [y5] - 618.3346+ (rank 3)[2]
- D [y11] - 623.8243++ (rank 5)[3]
- Rv2623\_TB31.7
- R.HLIDDALK.V [69, 76]
- 462.7611++ (dotp 0.9, rdotp 1, total ratio 0.04)
- 466.7682++ (heavy) (dotp 0.89)
- R.LLGSVSSGLLR.H [127, 137]

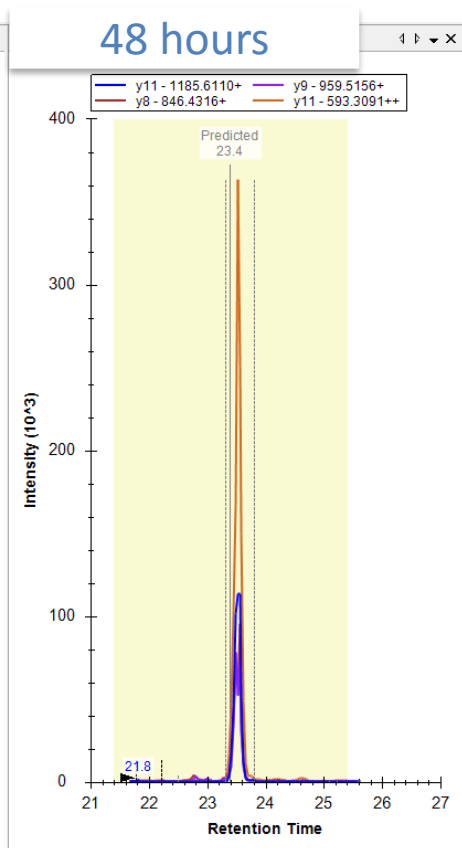
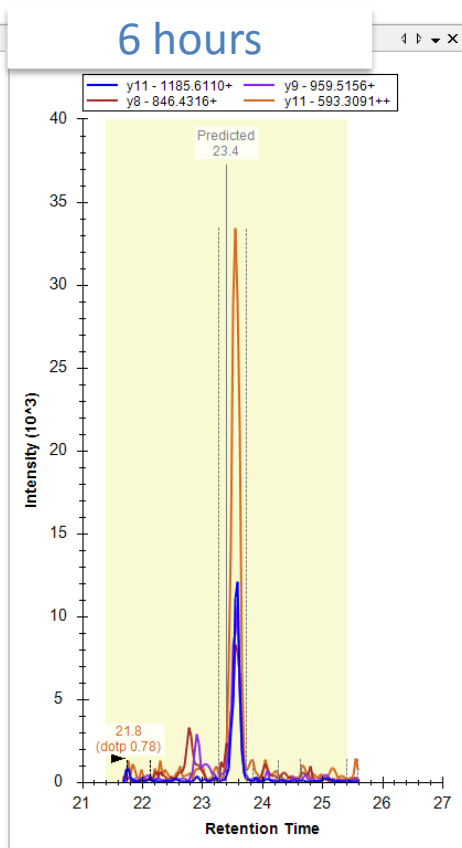
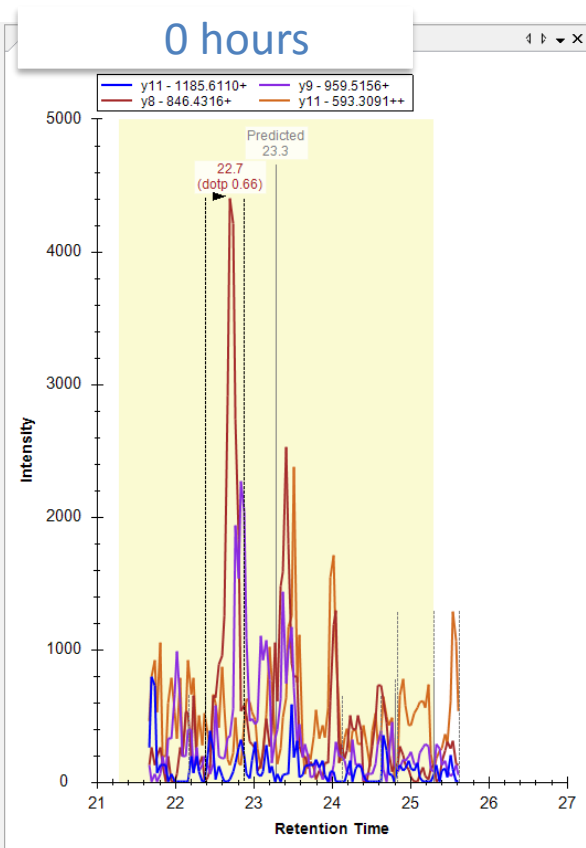
Library Match

Spectrum: Mtb\_proteome\_library

proteome\_library - NVPEIGDELSVAR, Char

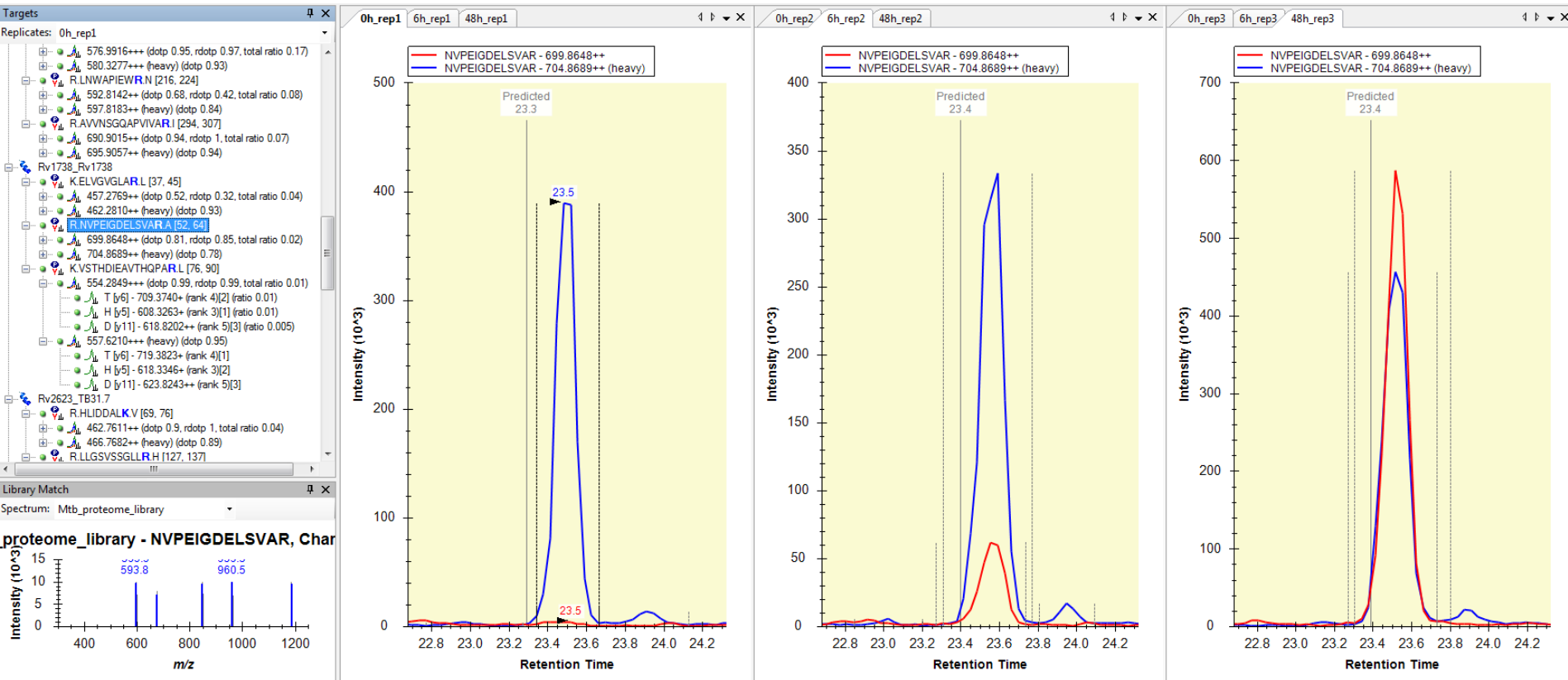
Intensity (10<sup>3</sup>)

m/z



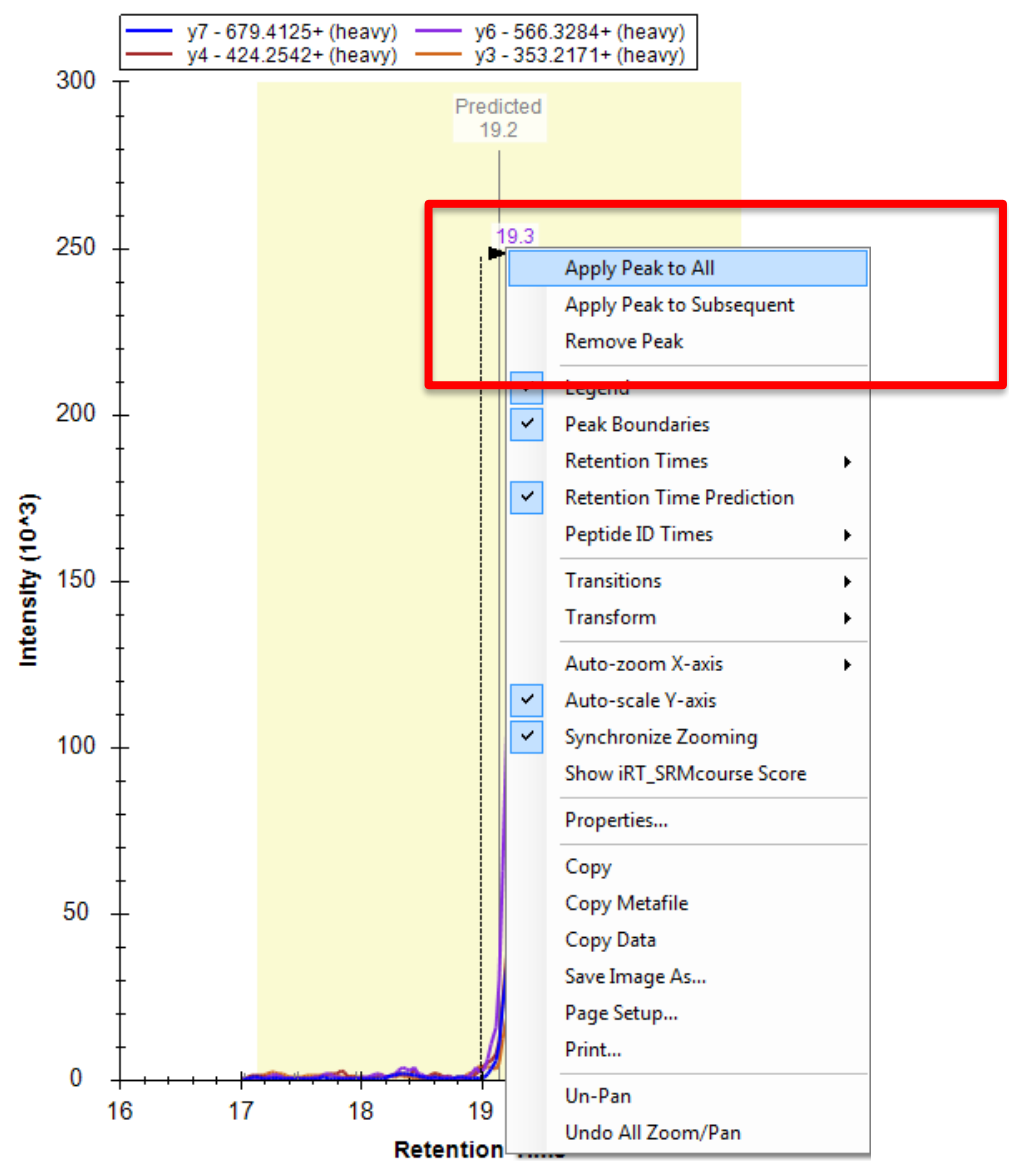
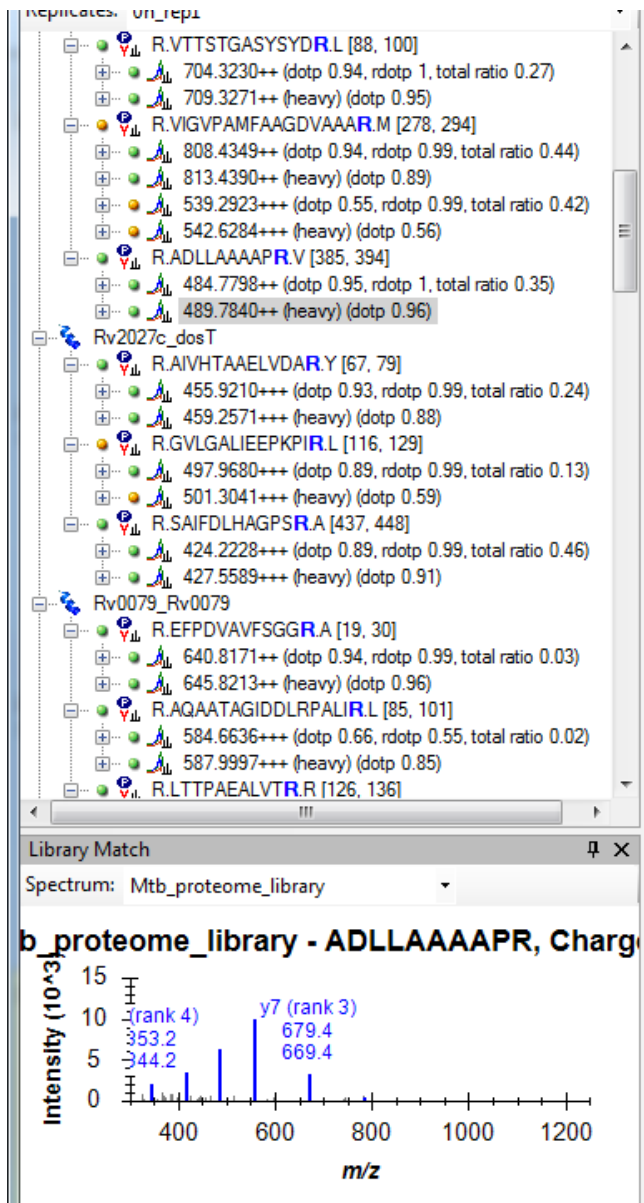


# Using reference standards for peak selection



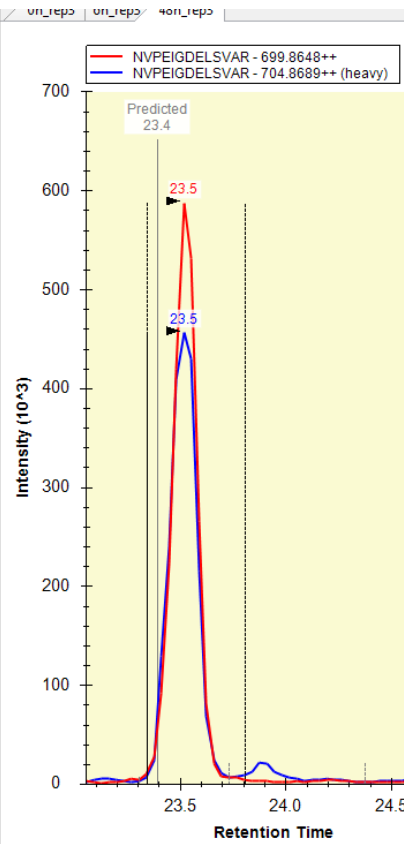
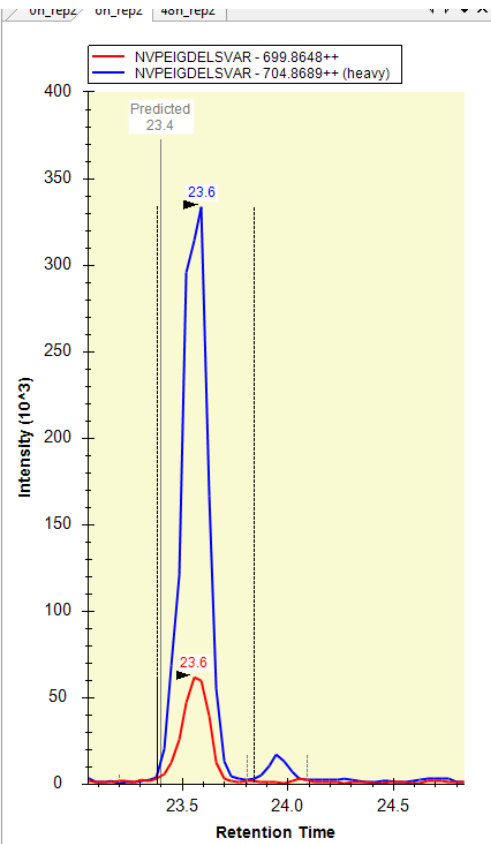
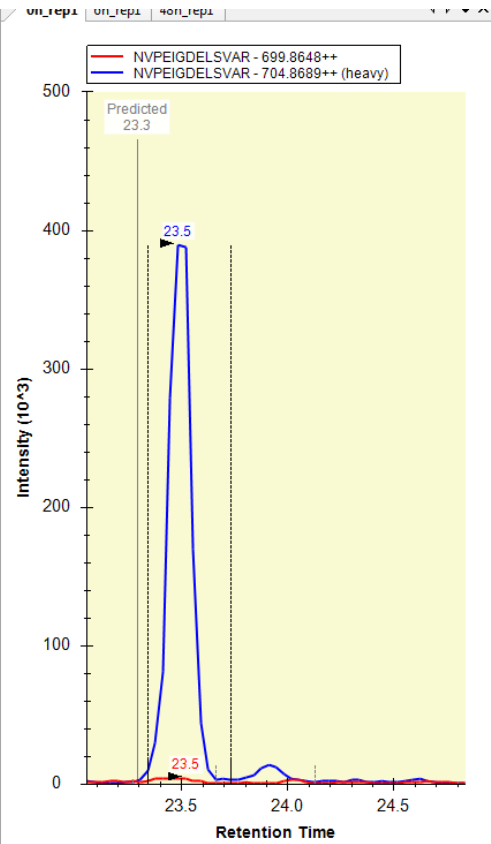
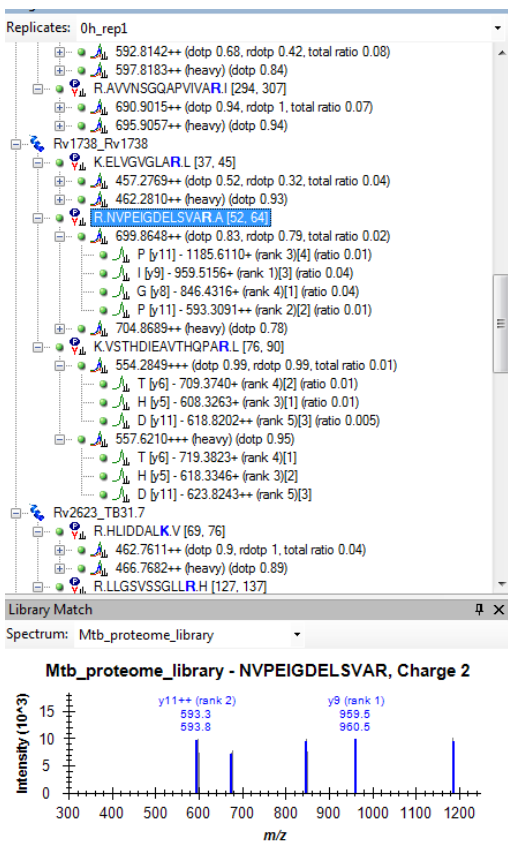
We can confidently state about absence :  
the endogenous peptide is below detectability

# Using reference standards for peak selection





# Using reference standards for peak selection



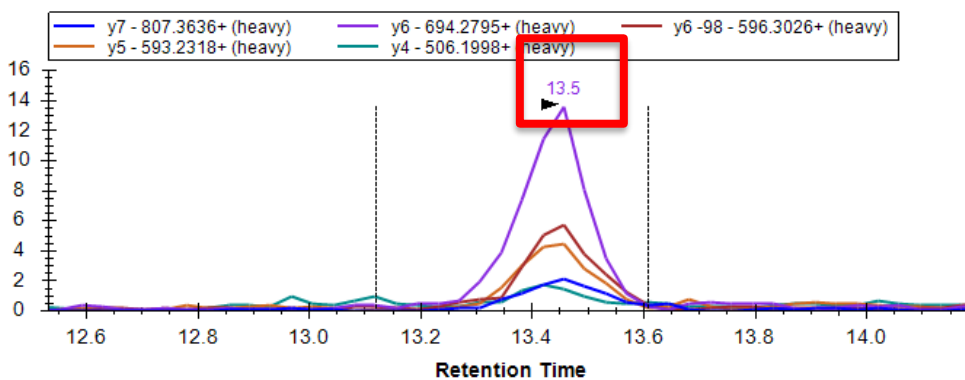
If the standard is set to heavy, it will be used in peak picking

Internal standard type:

- heavy
- none
- light
- heavy

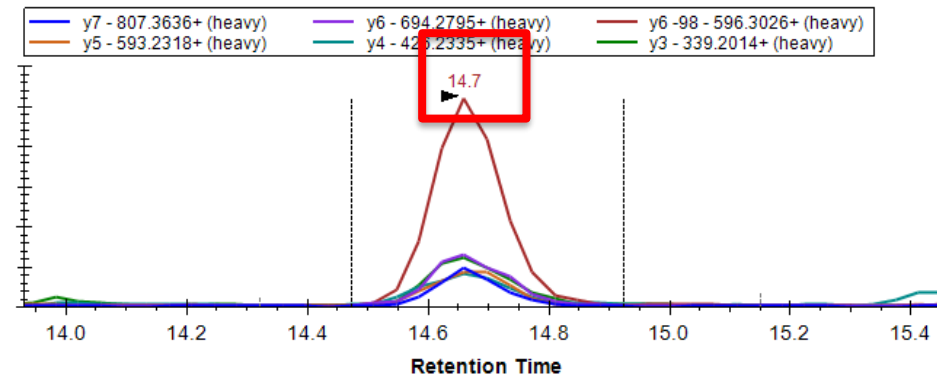
# Using reference standards for peak selection

- 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*

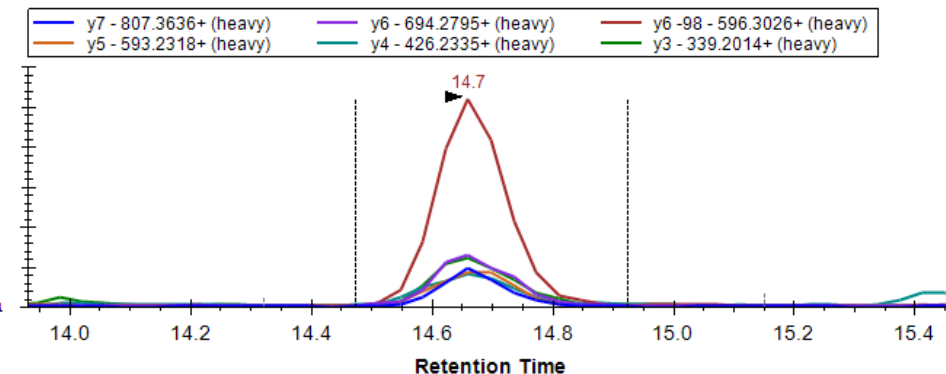
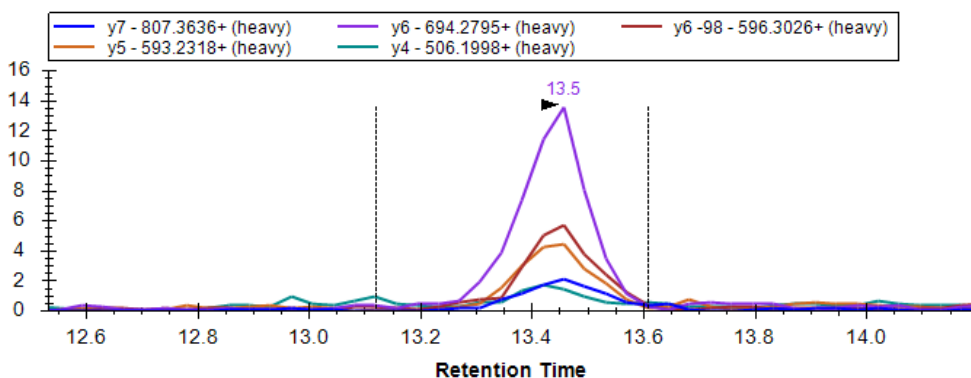
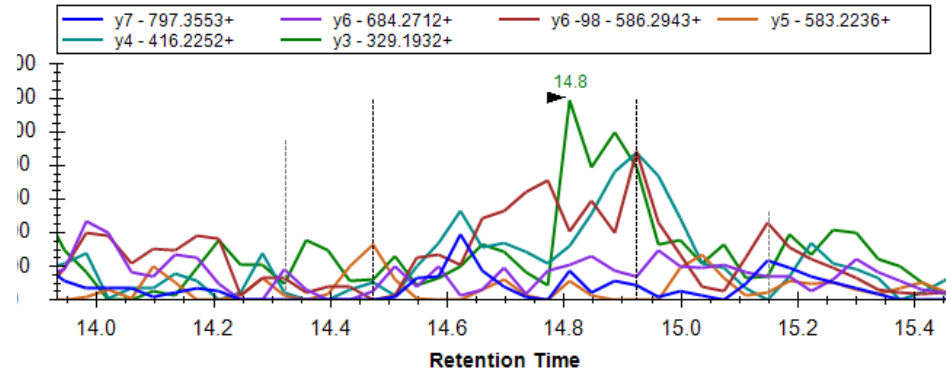
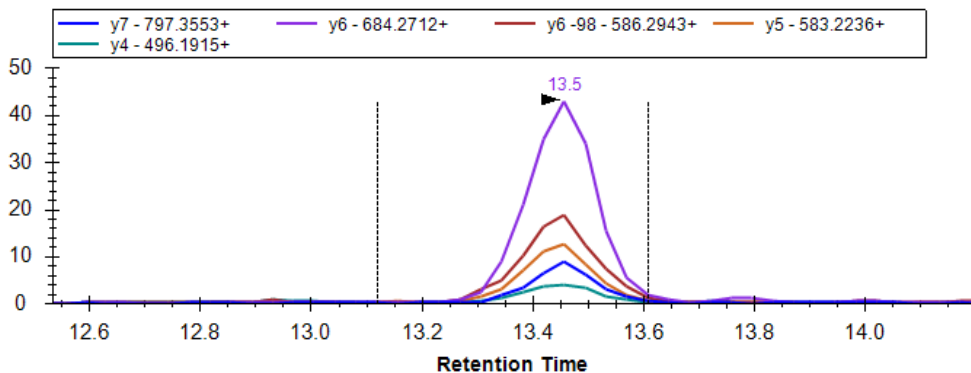
TDALTSS[Phos]PGR



*reference*

TDALTS[Phos]SPGR

# Using reference standards for peak selection



*reference*

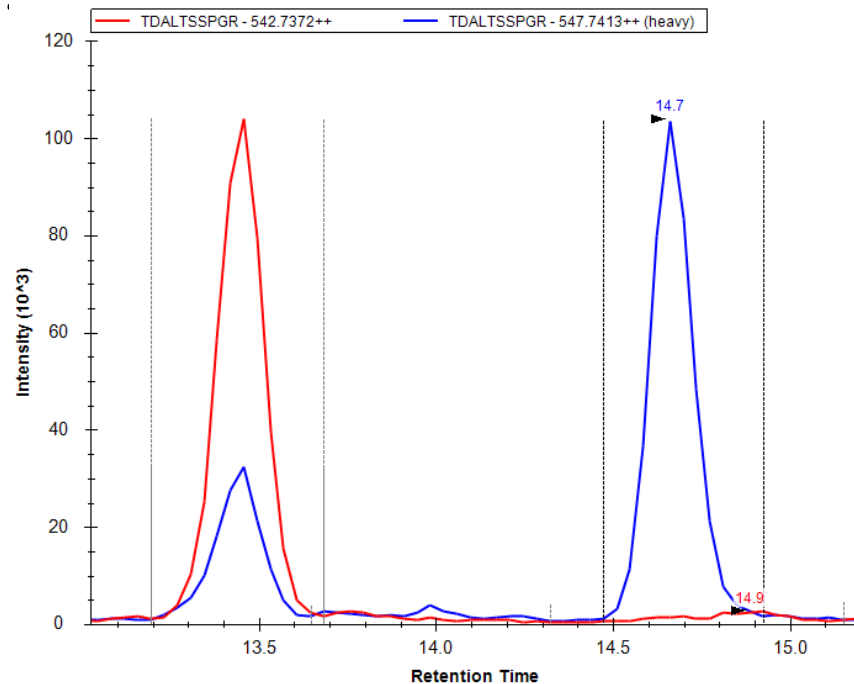
TDALTSS[Phos]PGR

*reference*

TDALTS[Phos]SPGR

# 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*

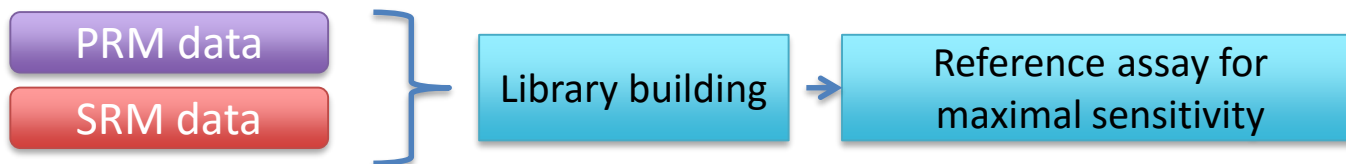
TDALTSS[Phos]PGR

*reference*

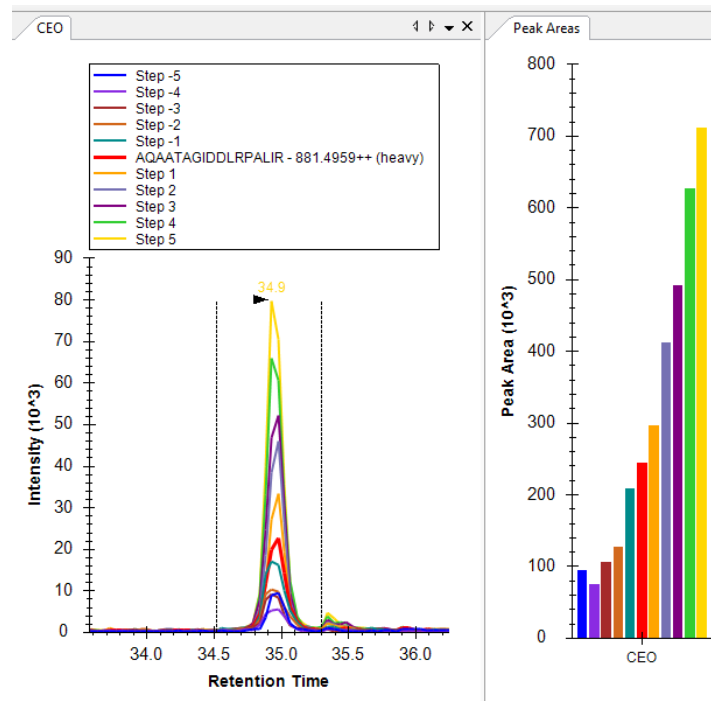
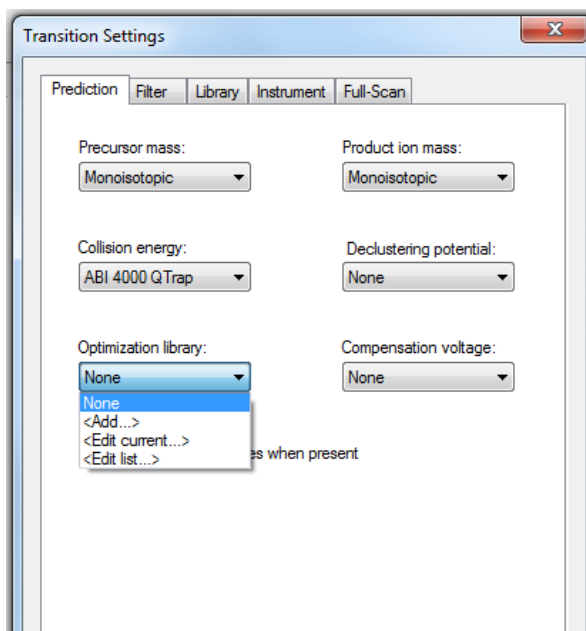
TDALTS[Phos]SPGR

# Generating a reference for quantification

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



for example collision energy optimization



# 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

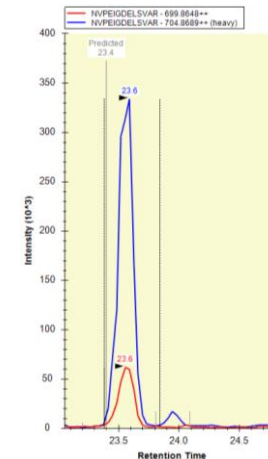
Reference for parameters (CE)

Peptide identification  
In targeted proteomics

Reference chromatogram  
Peak selection: RT, rdotp, transition selection



*Per se, does not require isotope label standards*



*Using isotope label standards*

# Improve confident peptide identification

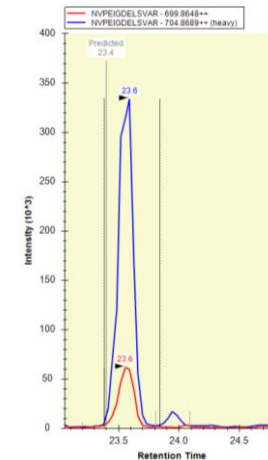
- Generating a reference for identification
- Using a reference for peak selection
- Using a reference for optimal quantification

Peptide  
identification  
In targeted  
proteomics

Reference  
chromatogram  
Peak selection: RT,  
rdotp, transition  
selection

**Tina:**

Improve  
quantitative  
precision and  
accuracy



*Using isotope  
label standards*