



# 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



# Outline



## Introduction - Ariel

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

## Improve confident peptide identification - Ariel

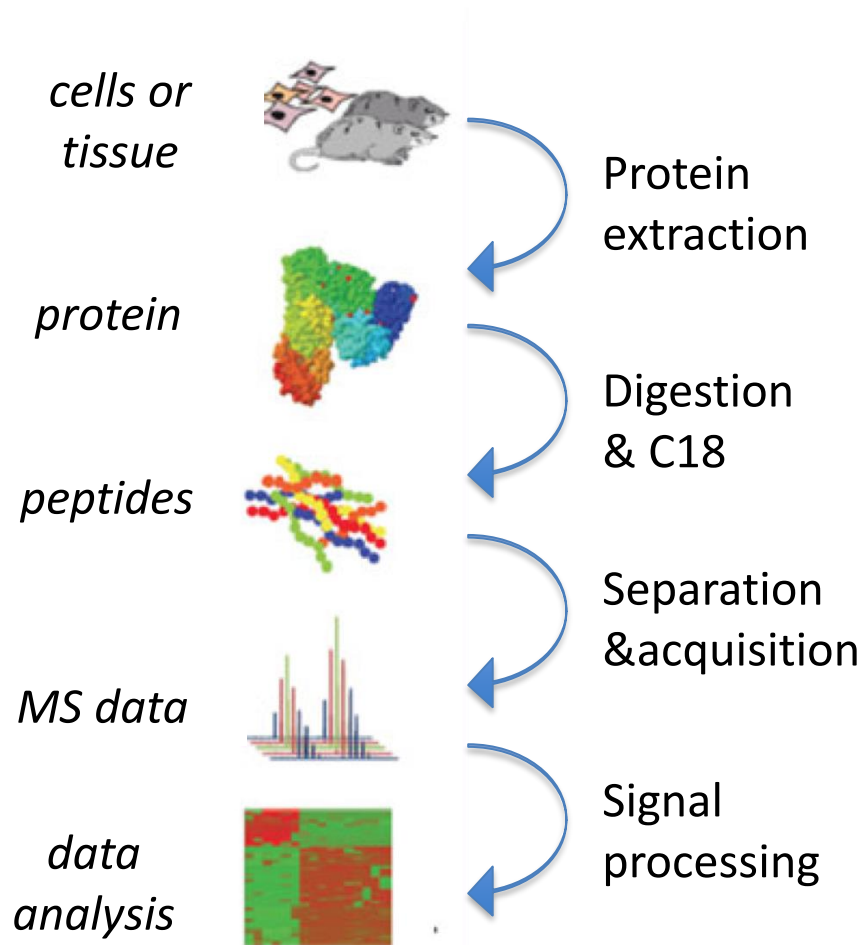
- Generating a reference for identification
- Using a reference for peak selection
- Using a reference for optimal quantification

## 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

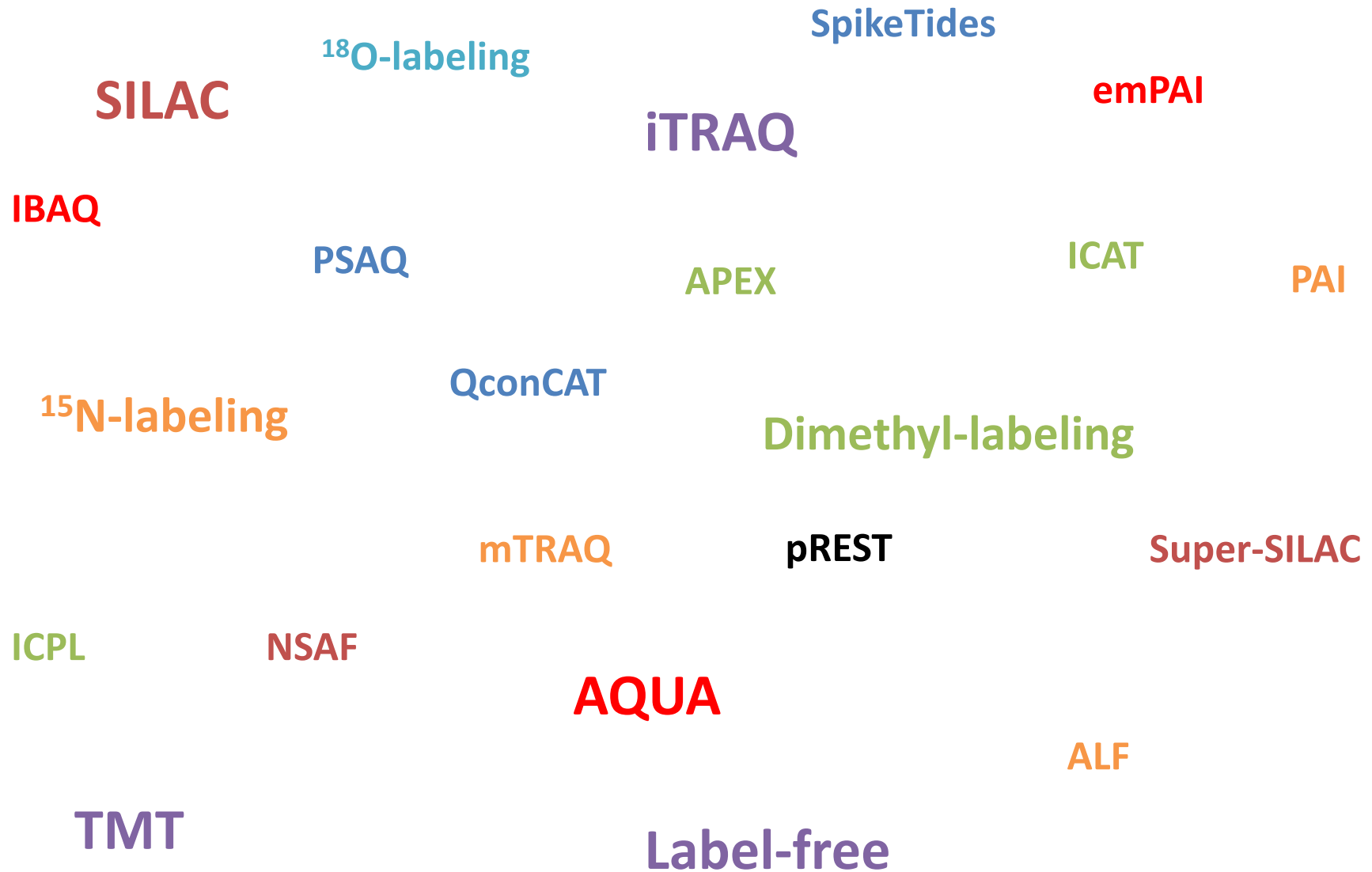
# Motivation – why use isotope labeled standards ?

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



The **earlier** an isotope-labeled standard is added into the samples during sample preparation, the **more precise and accurate** the results!

# The multitude of quantitative MS-applications



# The multitude of quantitative MS-applications



# Label-free versus label-based quantification



label-free



label-based

APEX IBAQ ALF  
emPAI **Label-free**  
NSAF

SpikeTides QconCAT  
ICAT pREST TMT **SILAC**  
Dimethyl-labeling ICPL Super-SILAC  
<sup>15</sup>N-labeling PSAQ <sup>18</sup>O-labeling  
mTRAQ **AQUA** iTRAQ

# Label-free versus label-based quantification



label-free



## Major advantages label-free:

- **Cost-** and **work-effective** (at least in terms of sample preparation)
- applicable to all identified/quantified proteins – **proteome-wide**
- no increase in sample complexity - **high sensitivity**



label-based



## Major advantages label-based:

- Can account for **preparative and analytical variabilities**
- Improved quantitative **precision and accuracy** compared to label-free quant.
- Improved **confidence** in peak identification

# Label-free versus label-based quantification



label-free



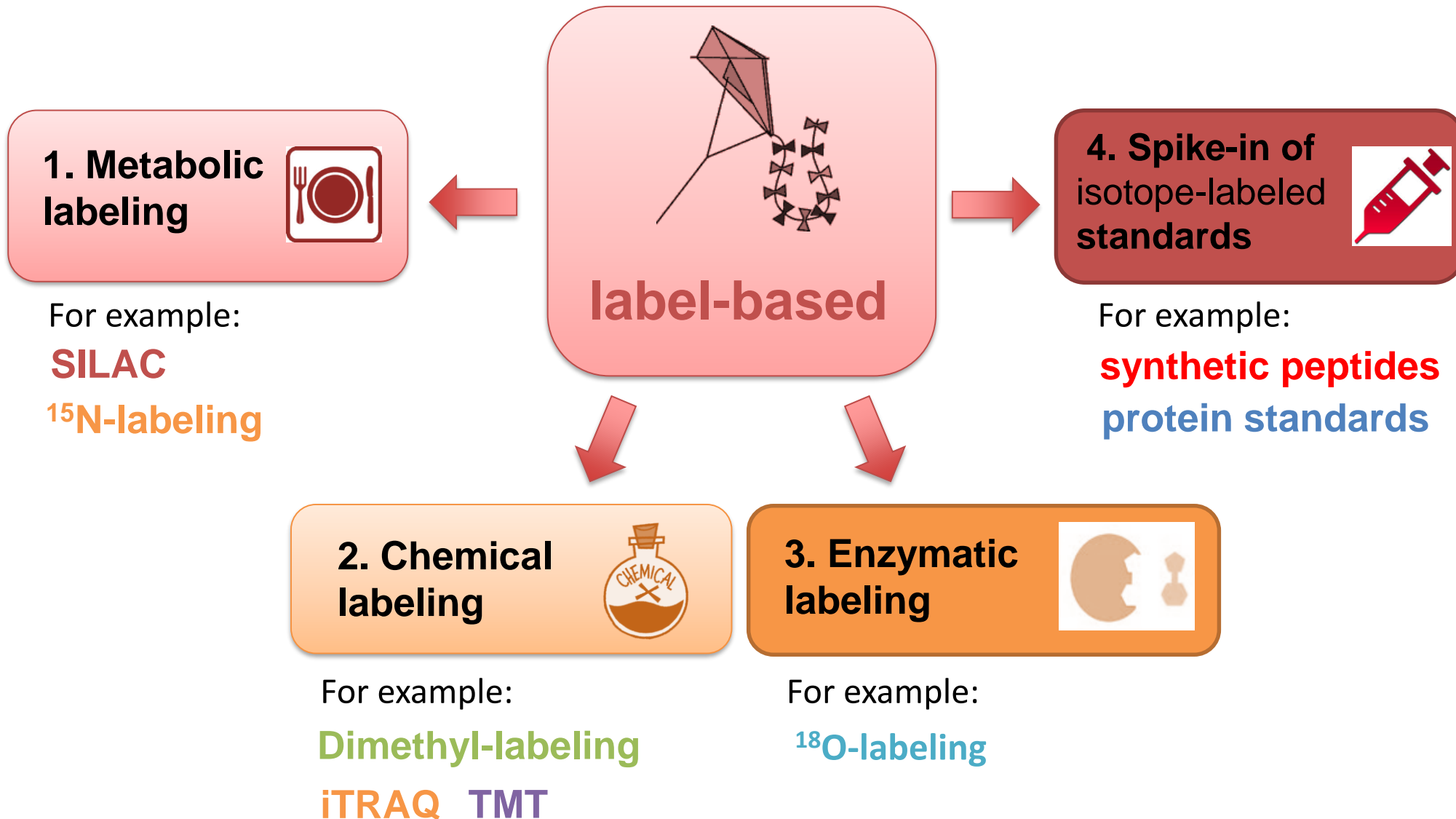
label-based

APEX IBAQ ALF  
emPAI Label-free  
NSAF

SpikeTides QconCAT  
ICAT pREST TMT SILAC  
Dimethyl-labeling ICPL Super-SILAC  
<sup>15</sup>N-labeling PSAQ <sup>18</sup>O-labeling  
mTRAQ AQUA iTRAQ



# Strategies for incorporating stable-isotopes



# Pros and Cons of stable-isotope labeling strategies

## 1. Metabolic labeling



### Strengths

- + eliminates best preparative and analytical variabilities (combination of light and heavy very early, on cell level)

### Weaknesses

- compatible with growing cells only
- significantly increased sample complexity

label-based

## 4. Spike-in of isotope-labeled standards



### Strengths

- + Compatible with any sample type
- + Maximal confidence in correct peak identification
- + Minimal increase in sample complexity

### Weaknesses

- expensive
- applicable to limited number of proteins

## 2. Chemical labeling



### Strengths

- + Compatible with any protein source
- + Multiplexing possible

### Weaknesses

- Potential for side reactions
- incomplete reactions
- Typically carried out on the peptide level

## 3. Enzymatic labeling



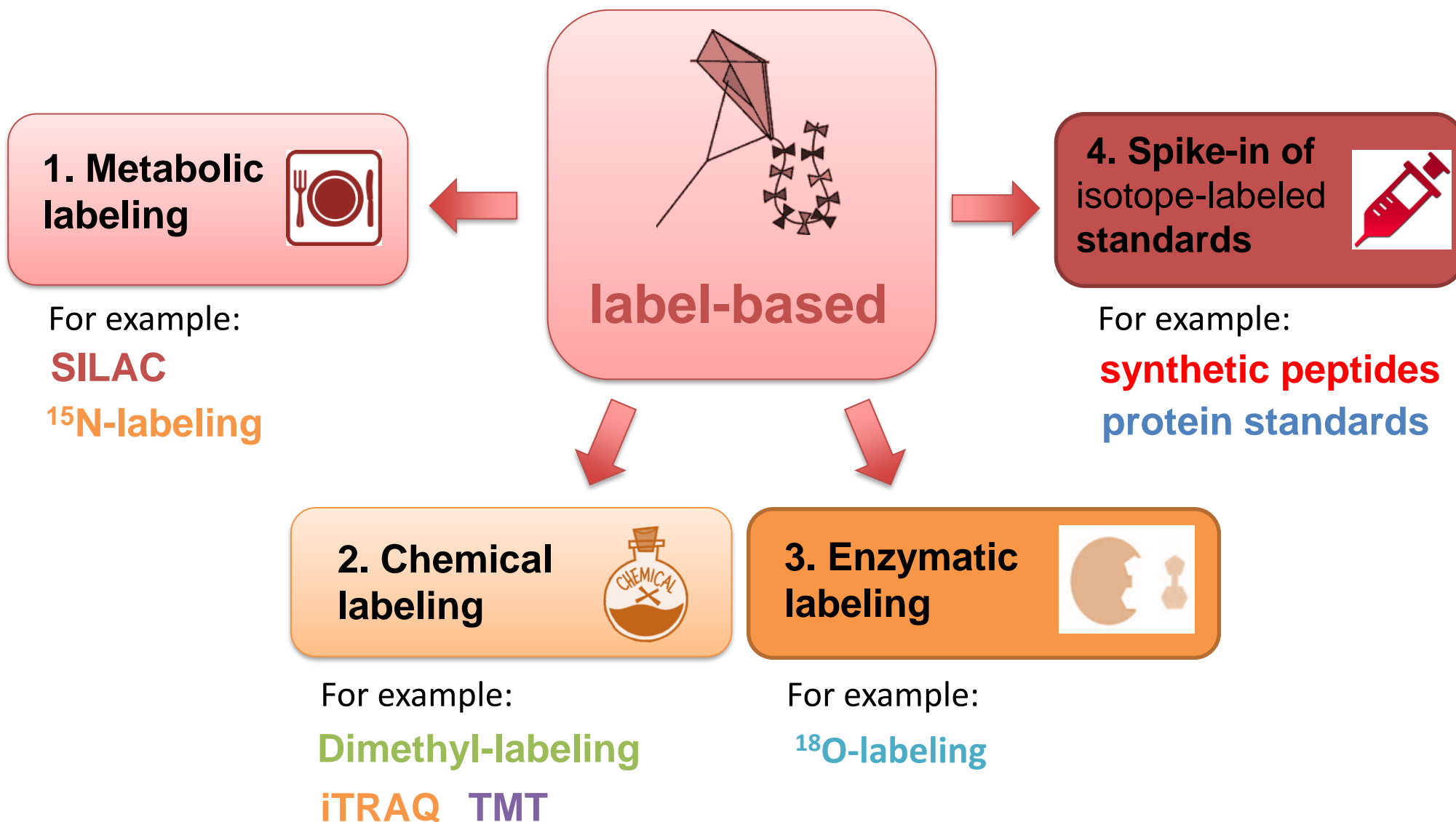
### Strengths

- + Compatible with any protein source
- + protein digestion and labeling one step

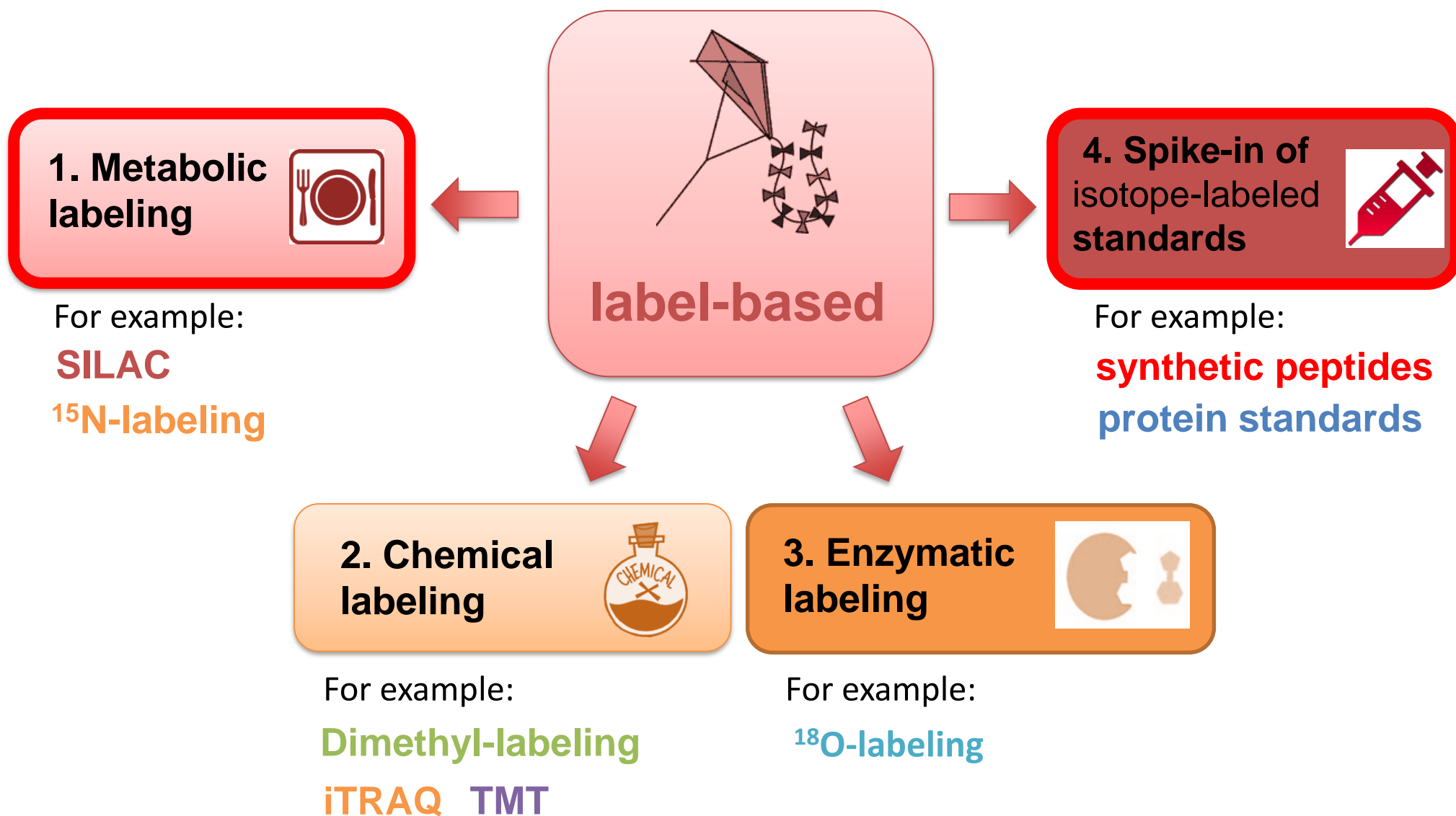
### Weaknesses

- variable incorporation of  $^{18}\text{O}$  atoms
- Small mass shifts of 2 to 4 Da

# Strategies for incorporating stable-isotopes



# Strategies for incorporating stable-isotopes



# Skyline supports metabolic labeling

## 1. Metabolic labeling

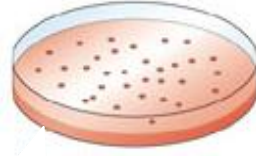


### SILAC



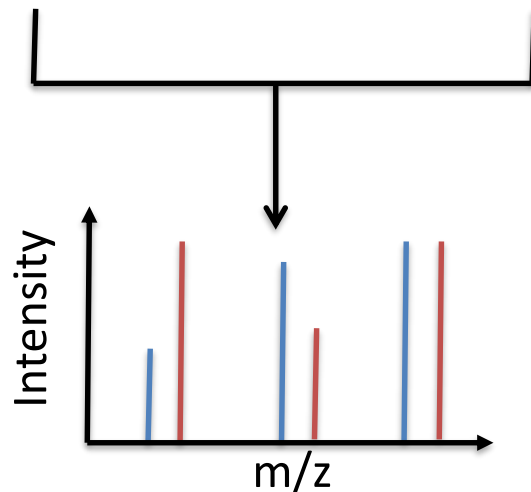
Condition A  
light ( $^{12}\text{C}$   $^{14}\text{N}$ )

arginine and lysine



Condition B  
heavy ( $^{13}\text{C}$   $^{15}\text{N}$ )

arginine and lysine



→ **constant mass shift**  
8 Da (lysine) or 10 Da (arginine)

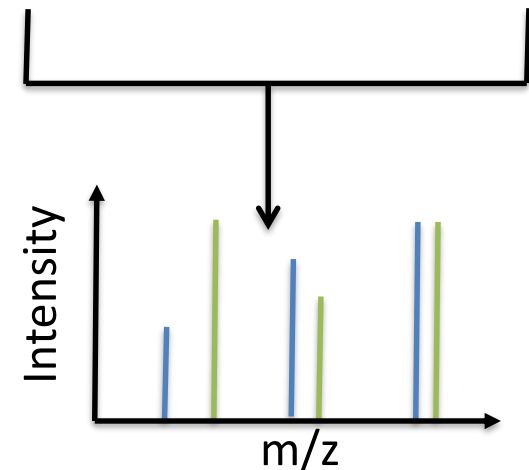
### $^{15}\text{N}$ labeling



Condition A  
light  $^{14}\text{N}$



Condition B  
heavy  $^{15}\text{N}$   
( $^{15}\text{N}$ -labeled  $(\text{NH}_4)_2\text{SO}_4$ )



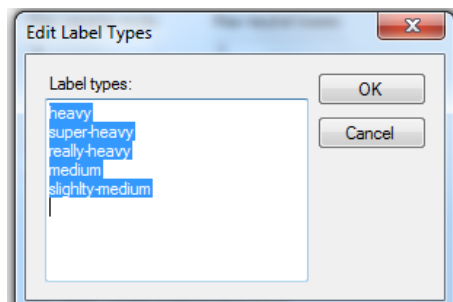
→ **variable mass shift** depending on  
the number of N-atoms in the  
peptide/fragment

# Skyline supports metabolic labeling

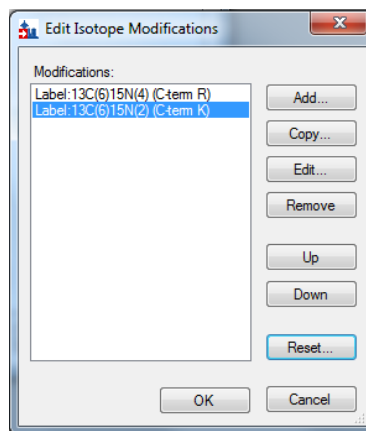
## 1. Metabolic labeling



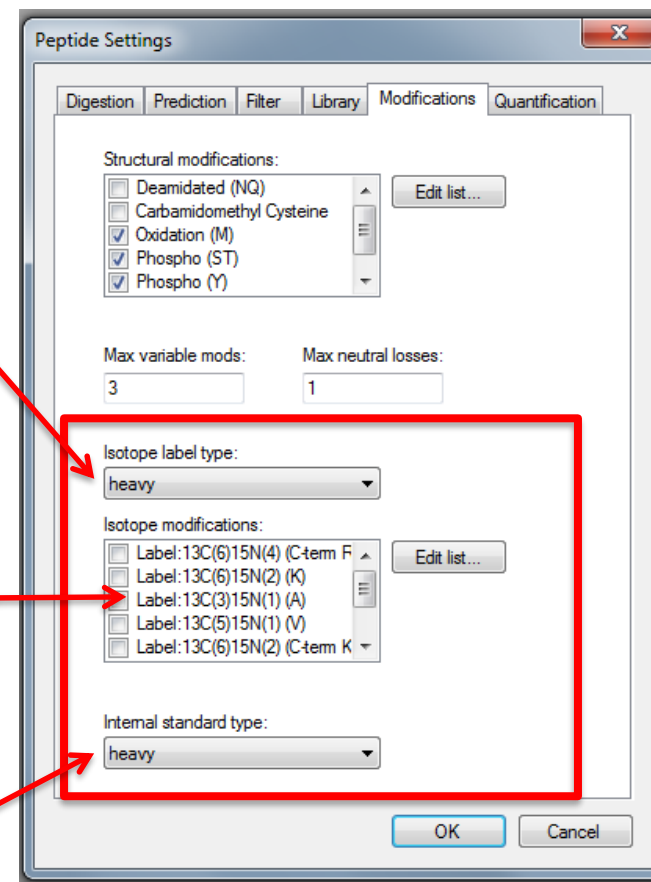
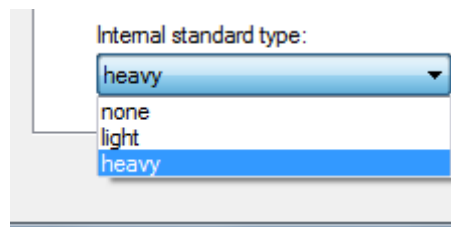
*You can define and name labels ; select those relevant for your experiment.*



*You can select a set of possible isotope modifications; enable those relevant for the label.*



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



# Skyline supports metabolic labeling

## 1. Metabolic labeling



### 1. Define isotope modifications

→ Peptide Settings → Modifications → Isotope Modification

#### SILAC

#### Edit Isotope

Name:  
Label: 13C(6)15N(4) (R)

Amino acid: R Terminus:

☐ Chemical formula

☒ 13C ☒ 15N ☐

Monoisotopic mass: 10.008269 Average mass: 9.92

Relative retention time: Matching

#### Edit Isotope Modification

Name:  
Label: 13C(6)15N(2) (K)

Amino acid: K Terminus:

☐ Chemical formula

☒ 13C ☒ 15N ☐ 18O ☐ 2H

Monoisotopic mass: 8.014199 Average mass: 7.941847

Relative retention time: Matching

OK  
Cancel

#### <sup>15</sup>N labeling

#### Edit Isotope Modification

Name:  
Label: 15N

Amino acid: Terminus:

☐ Chemical formula

☐ 13C ☒ 15N ☐ 18O ☐ 2H

Monoisotopic mass: Average mass:

Relative retention time: Matching

OK  
Cancel

# Skyline supports metabolic labeling

## 1. Metabolic labeling



## 2. Create additional label type

Peptide Settings

Digestion Prediction Filter Library Modifications Quantification

Structural modifications:

☒ Carbamidomethyl (C) Edit list...

Max variable mods: 3 Max neutral losses: 1

Isotope label type: <Edit list...>

Isotope modifications:

☐ Label:  $^{13}\text{C}(6)^{15}\text{N}(4)$  (R) Edit list...

☐ Label:  $^{13}\text{C}(6)^{15}\text{N}(2)$  (K)

Internal standard type: heavy

OK Cancel

SILAC



$^{15}\text{N}$  labeling

Edit Label Types

Label types:

SILAC

OK Cancel

Edit Label Types

Label types:

$^{15}\text{N}$

OK Cancel



# Skyline supports metabolic labeling

## 1. Metabolic labeling



### 3. Define Label Types

#### SILAC

Peptide Settings

Digestion Prediction Filter Library Modifications Quantification

Structural modifications:

☒ Carbamidomethyl (C) Edit list...

Max variable mods: 3 Max neutral losses: 1

Isotope label type: SILAC

Isotope modifications:

☒ Label:13C(6)15N(2) (K) Edit list...

☒ Label:13C(6)15N(4) (R)

Internal standard type: SILAC

none

light

SILAC

OK Cancel

#### $^{15}\text{N}$ labeling

Peptide Settings

Digestion Prediction Filter Library Modifications Quantification

Structural modifications:

☒ Carbamidomethyl (C) Edit list...

Max variable mods: 3 Max neutral losses: 1

Isotope label type:  $^{15}\text{N}$

Isotope modifications:

☒ Label:15N Edit list...

Internal standard type:  $^{15}\text{N}$

none

light

$^{15}\text{N}$

OK Cancel

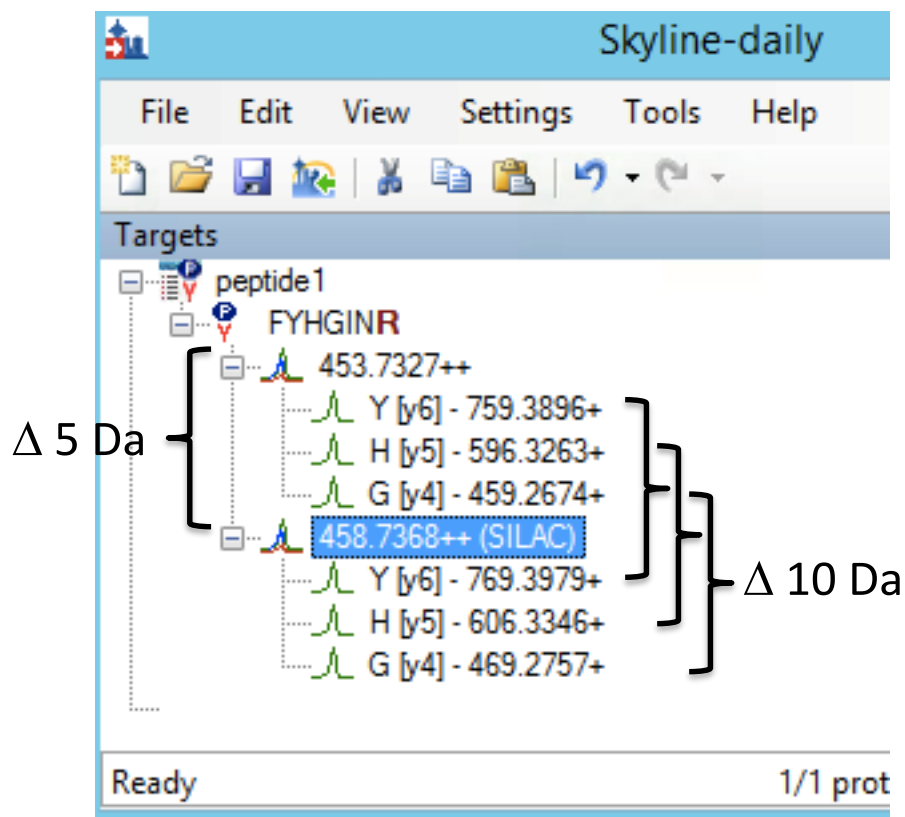
# Skyline supports metabolic labeling

## 1. Metabolic labeling

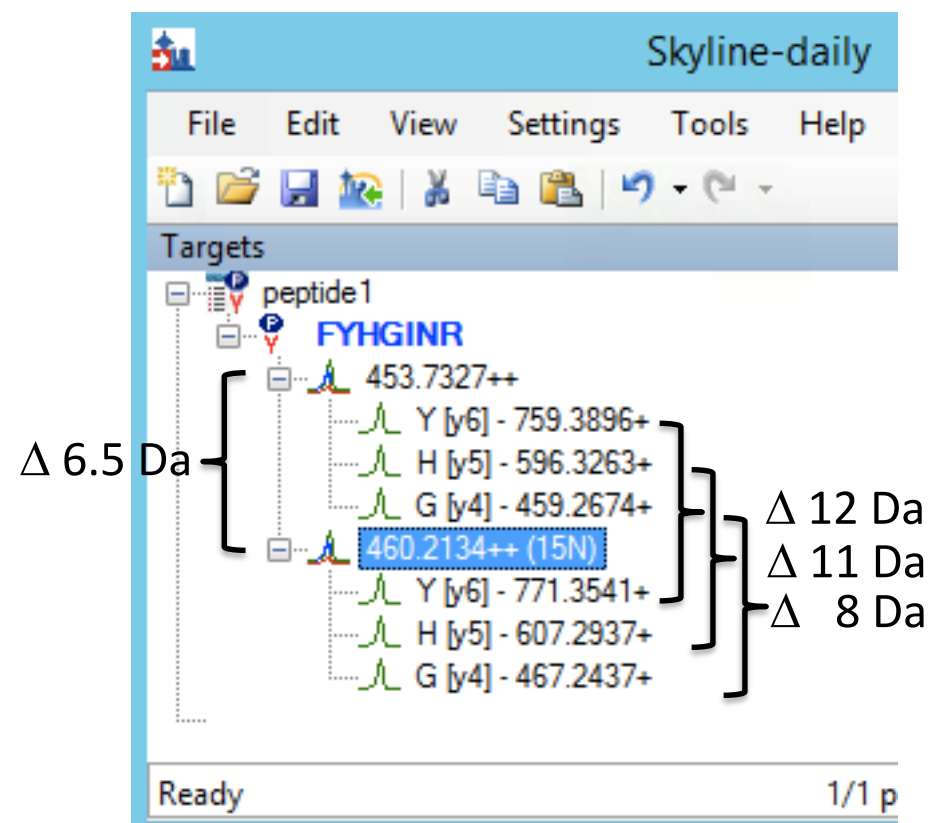


### 4. Isotope labeled variants will appear in peptide list

#### SILAC



#### $^{15}\text{N}$ labeling



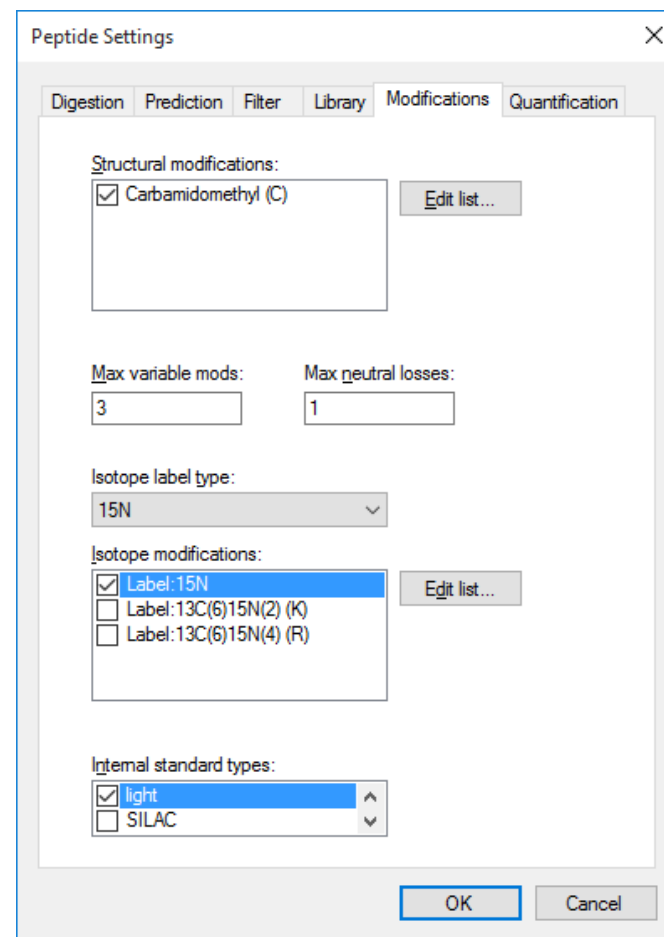
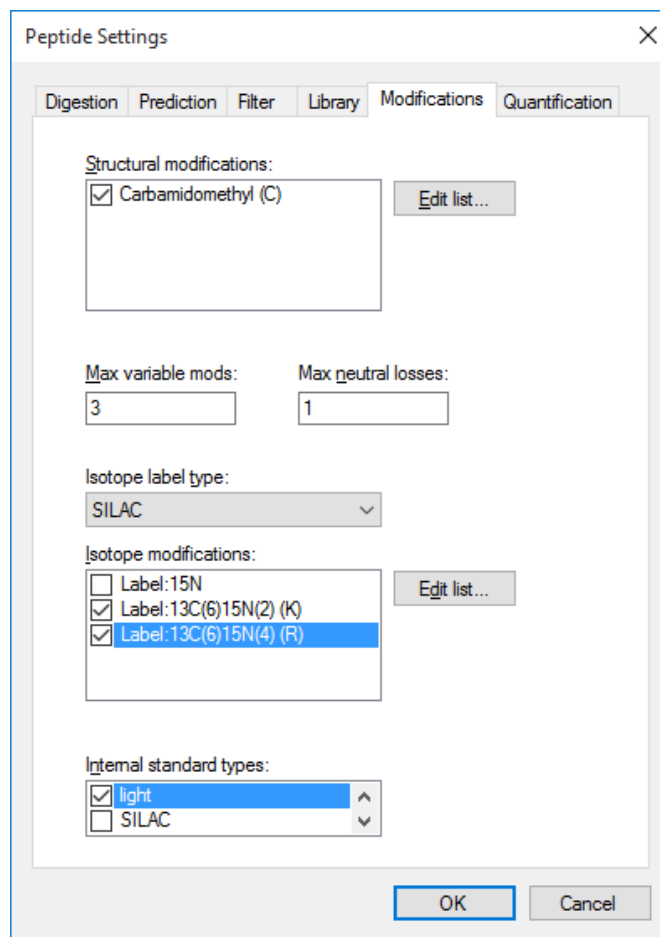
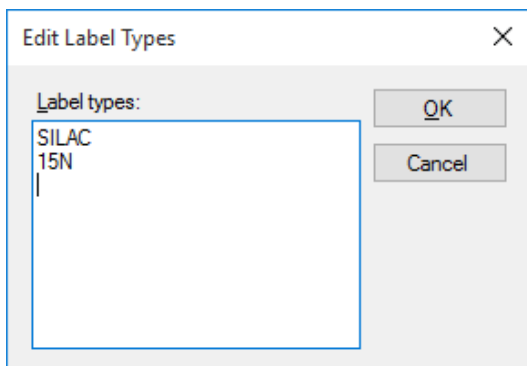
# Skyline supports metabolic labeling

## 1. Metabolic labeling



Combination of several isotope modification types in a single Skyline document is possible!!!!

light AND SILAC and  $^{15}\text{N}$  labeling

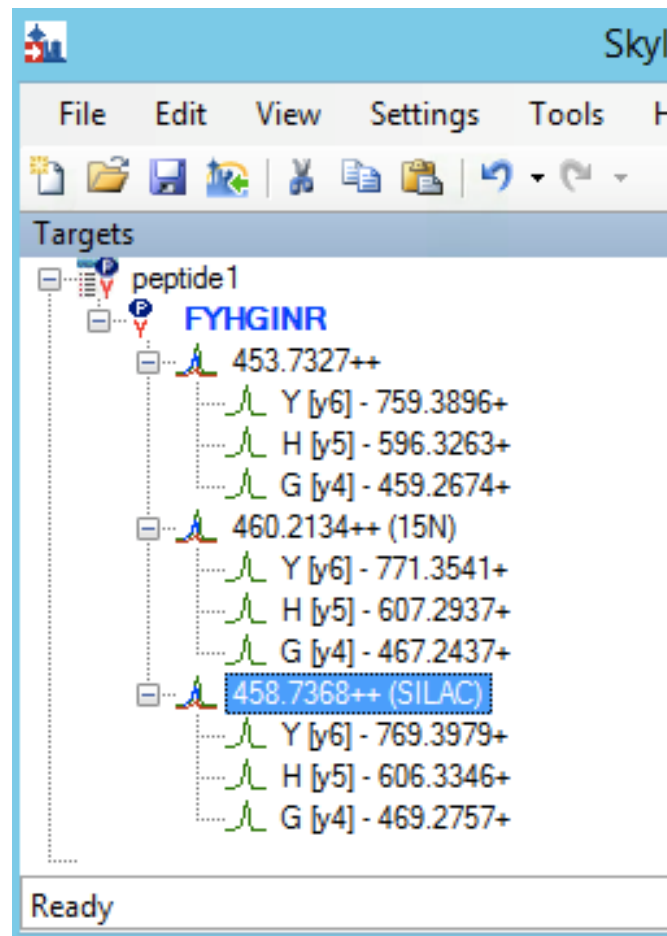


# Skyline supports metabolic labeling

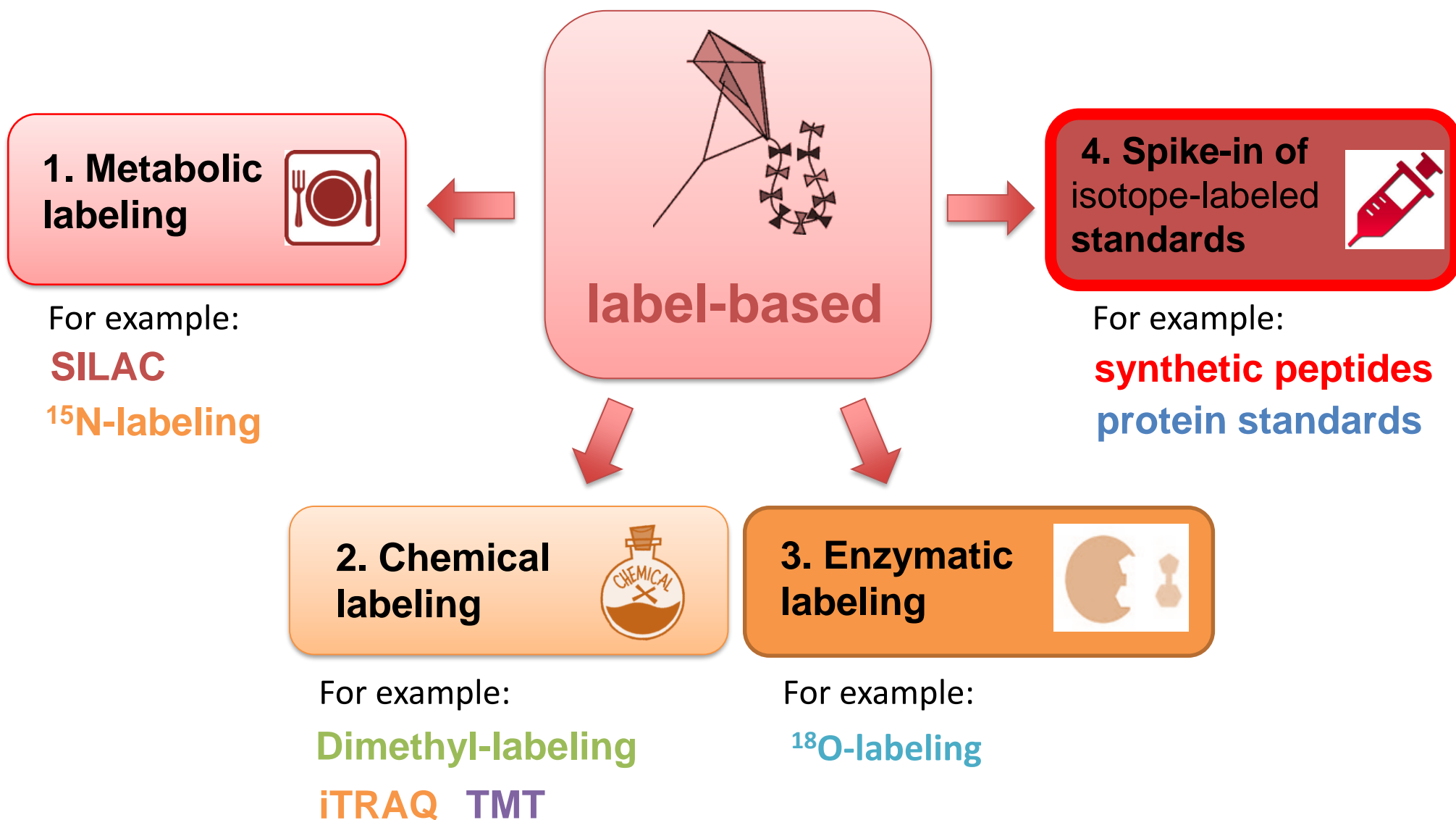
## 1. Metabolic labeling



**Combination of several isotope modification types in a single Skyline document is possible!!!!**



# Strategies for incorporating stable-isotopes





### synthetic peptides

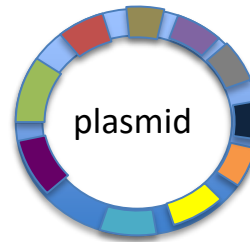


$^{13}\text{C}$ -  $^{15}\text{N}$ -labeled C-terminal amino acid (lysine/arginine)

Different purity forms available from various companies:

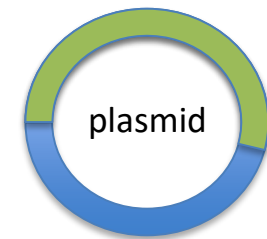
- **Crude peptides** = cheap  
→ Library generation and relative quant.
- **Highly purified peptides (AQUA/SIS)** = expensive  
→ Absolute quant.

### concatenated peptides



- **cloning** of concatenated peptides (possibly from different proteins) **QconCAT**
- **overexpression** in labeled medium
- **purification** and concentration determination
- **co-digestion** with endogenous proteins

### full-length recombinant proteins



- **cloning** of the complete protein
- **overexpression** of the target protein in labeled medium
- **purification** and concentration determination
- **co-digestion** with endogenous protein



### synthetic peptides



$^{13}\text{C}$ -  $^{15}\text{N}$ -labeled C-terminal amino acid (lysine/arginine)

Different purity forms available from various companies:

- **Crude peptides** = cheap  
→ Library generation and relative quant
- **Highly purified peptides (AQUA/SIS)** = expensive  
→ Absolute quant.

## 1. Define isotope modifications

Edit Isotope Modification

Name: Label:  $^{13}\text{C}(6)^{15}\text{N}(2)$  (C-term K)

Amino acid: K Terminus: C

☐ Chemical formula

☒  $^{13}\text{C}$  ☒  $^{15}\text{N}$  ☐  $^{18}\text{O}$  ☐  $2\text{H}$

Monoisotopic mass: 8.014199 Average mass: 7.941847

Relative retention time: Matching

OK Cancel

Different to SILAC modification!!!

Edit isotope Modification

Name: Label:  $^{13}\text{C}(6)^{15}\text{N}(4)$  (C-term R)

Amino acid: R Terminus: C

☐ Chemical formula

☒  $^{13}\text{C}$  ☒  $^{15}\text{N}$  ☐  $^{18}\text{O}$  ☐  $2\text{H}$

Monoisotopic mass: 10.008269 Average mass: 9.928665

Relative retention time: Matching

OK Cancel



### synthetic peptides



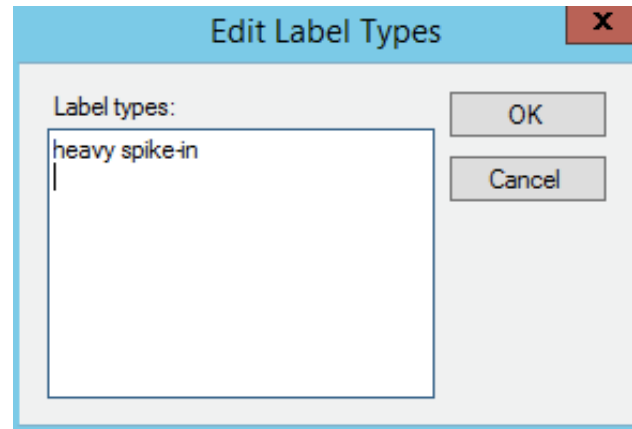
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Different purity forms available from various companies:

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## 1. Define isotope modifications

## 2. Create additional label type







### synthetic peptides



$^{13}\text{C}$ -  $^{15}\text{N}$ -labeled C-terminal amino acid (lysine/arginine)

Different purity forms available from various companies:

- **Crude peptides** = cheap  
→ Library generation and relative quant
- **Highly purified peptides (AQUA/SIS)** = expensive  
→ Absolute quant.

## 1. Define isotope modifications

## 2. Create additional label type

## 3. Define Label Types

Isotope label type:

heavy spike-in

Isotope modifications:

- ☒ Label:  $^{13}\text{C}(6)^{15}\text{N}(2)$  (C-term K)
- ☒ Label:  $^{13}\text{C}(6)^{15}\text{N}(4)$  (C-term R)



### synthetic peptides

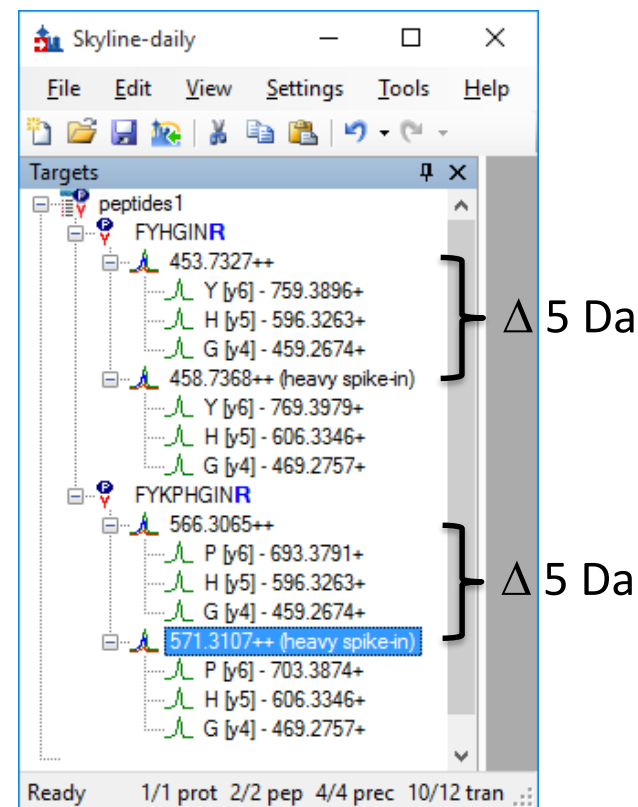


$^{13}\text{C}$ -  $^{15}\text{N}$ -labeled C-terminal amino acid (lysine/arginine)

Different purity forms available from various companies:

- **Crude peptides** = cheap  
→ Library generation and relative quant
- **Highly purified peptides (AQUA/SIS)** = expensive  
→ Absolute quant.

1. Define isotope modifications
2. Create additional label type
3. Define Label Types
4. Isotope labeled variants will appear in peptide list



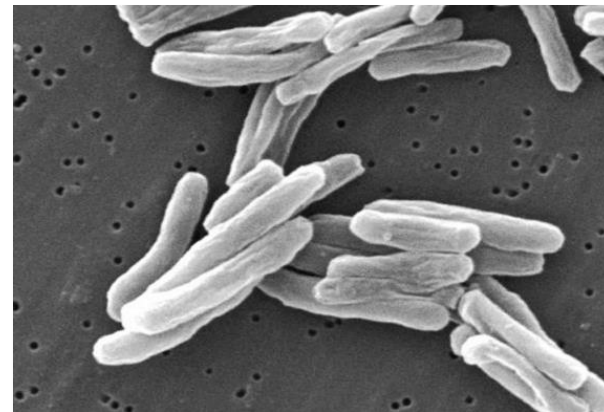
# Tutorial-6 “Manual SRM data analysis in Skyline” Zürich course

<http://targetedproteomics.ethz.ch/downloads.html>

[http://targetedproteomics.ethz.ch/tutorials2014/Tutorial-6\\_ManualAnalysis.pdf](http://targetedproteomics.ethz.ch/tutorials2014/Tutorial-6_ManualAnalysis.pdf)

*Mycobacterium tuberculosis* (Mtb) total cell extracts

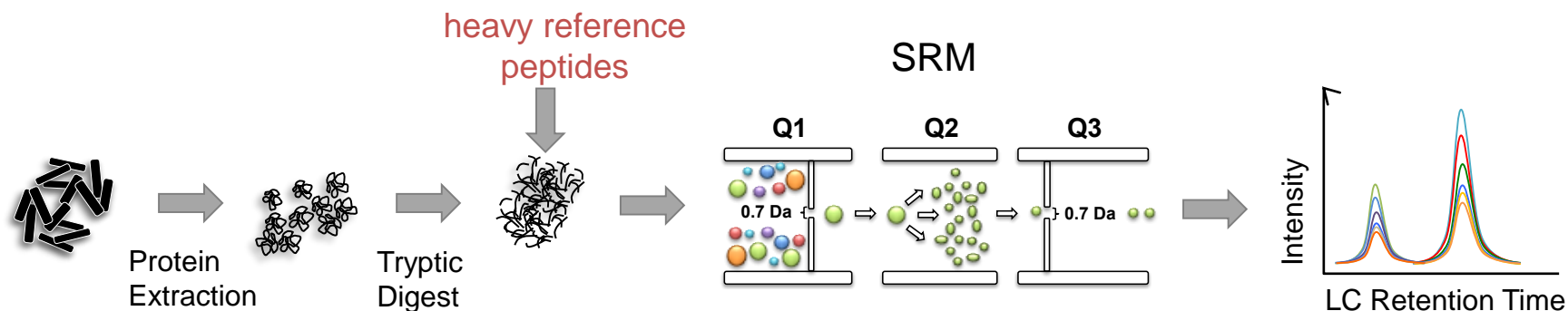
- **Three** different **time points** of hypoxia: **0h, 6h, and 48h**
- **10 target proteins** represented by 3 peptides each  
→ total of 30 peptides
- **30** isotopically labelled **crude synthetic reference peptides** available and spiked into each sample at the same concentration



## Task:

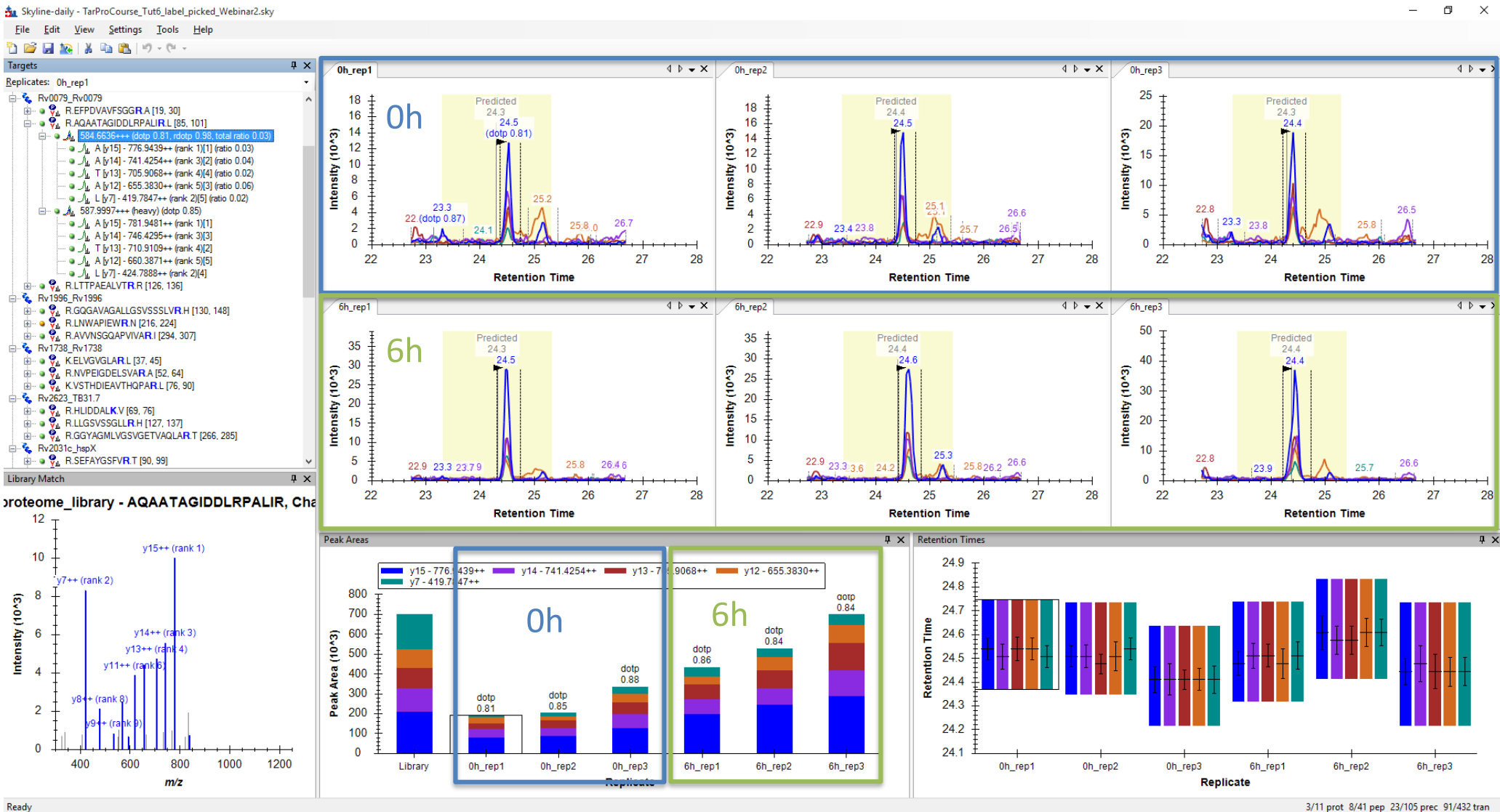
Analyze the data twice: **Once label-free, once label-based!**

Investigate the quantitative differences.

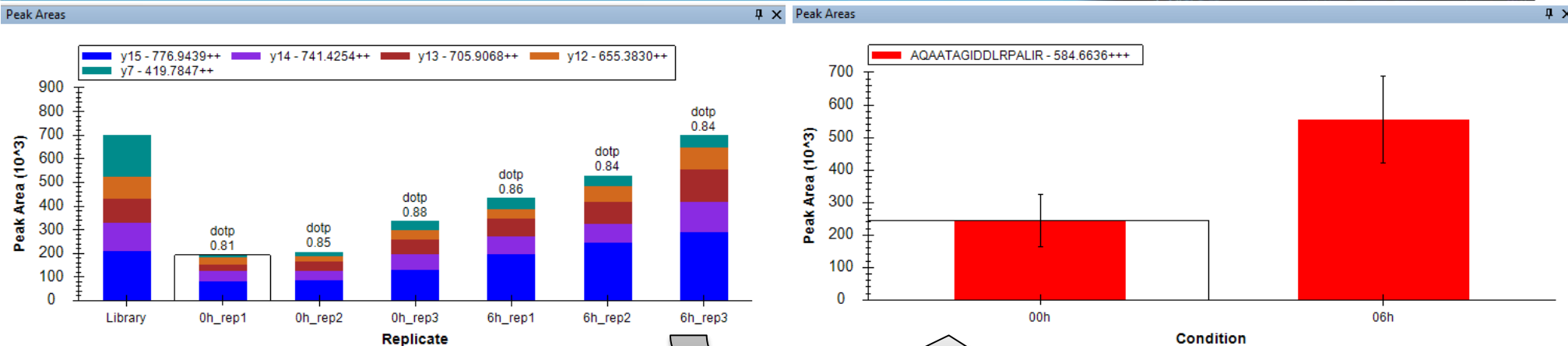


# Tutorial-6 “Manual SRM data analysis in Skyline” Zürich course

[http://targetedproteomics.ethz.ch/tutorials2014/Tutorial-6\\_ManualAnalysis.pdf](http://targetedproteomics.ethz.ch/tutorials2014/Tutorial-6_ManualAnalysis.pdf)



# “Peak Area” viewing options in Skyline



## 1. Settings → Document Settings

Document Settings

Annotations | Group Comparisons | Reports

Annotations are extra pieces of data which you can attach to elements in a Skyline document. Use this dialog to control which annotations are available in this document, as well as to define new annotations.

☒ Condition ☒ BioReplicate

Add... Edit List...

OK Cancel

## 2. View → Results Grid

Replicate	Sample Type	Analyte Concentration	Condition	BioReplicate
0h_rep1	Unknown		00h	1
0h_rep2	Unknown		00h	2
0h_rep3	Unknown		00h	3
6h_rep1	Unknown		06h	1
6h_rep2	Unknown		06h	2
6h_rep3	Unknown		06h	3

## 3. right mouse click on „Peak Areas“ window

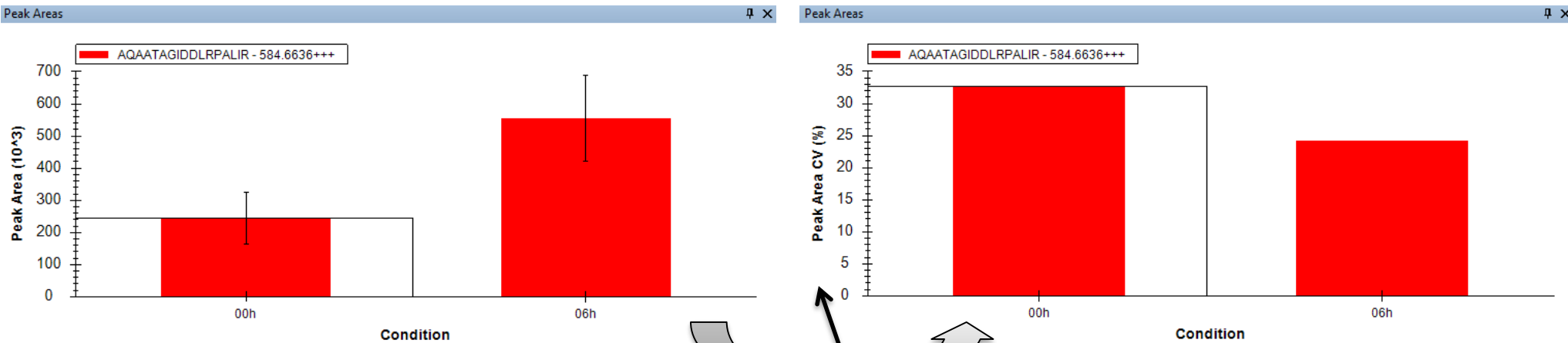
**Group By**

- Replicate
- ☒ Condition
- BioReplicate

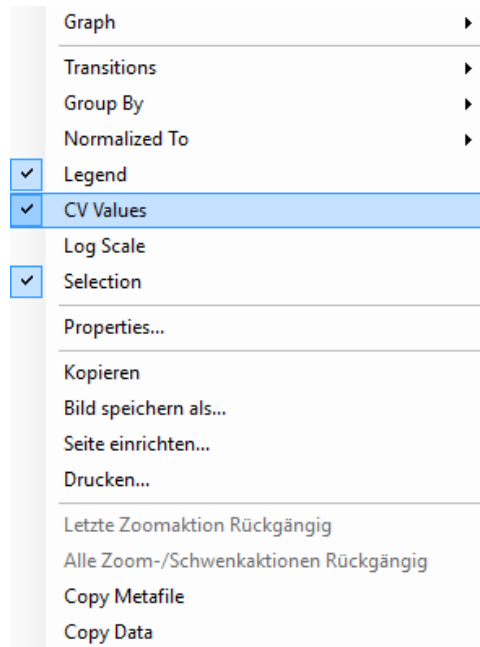
**Transitions**

- All
- Single
- ☒ Total
- Split Graph

# “Peak Area” viewing options in Skyline



right mouse click on „Peak Areas“ window



**Coefficient of variation in %**

Is the detected fold-change of peptide AQAATAGIDDLRPALIR between condition 0h and 6h **statistical significant**?



# Simple group comparison within Skyline

Tutorial „Processing Grouped Study data“: [https://skyline.gs.washington.edu/labkey/tutorial\\_grouped.url](https://skyline.gs.washington.edu/labkey/tutorial_grouped.url)

View Settings Tools Help

- Targets ▶
- Text Zoom ▶
- Spectral Libraries
- Arrange Graphs ▶
- Library Match Alt+1
- Ion Types ▶
- Charges ▶
- ✓ Ranks
- Chromatograms ▶
- Transitions ▶
- Transform ▶
- Auto-Zoom ▶
- Retention Times ▶
- Peak Areas ▶
- Calibration Curve
- Group Comparisons ▶
  - Add...
  - Edit List...
- Results Grid Alt+2
- Document Grid
- ✓ Tool Bar
- ✓ Status Bar

Label-free 6h - 0h:Settings

Control group annotation:  
Condition

Control group value:  
00h

Value to compare against:  
06h

Identity annotation (for technical replicates):  
BioReplicate

Normalization method:  
None

Confidence level:  
99 %

Scope  
☒ Peptide ☐ Protein

Label-free 6h - 0h:Grid

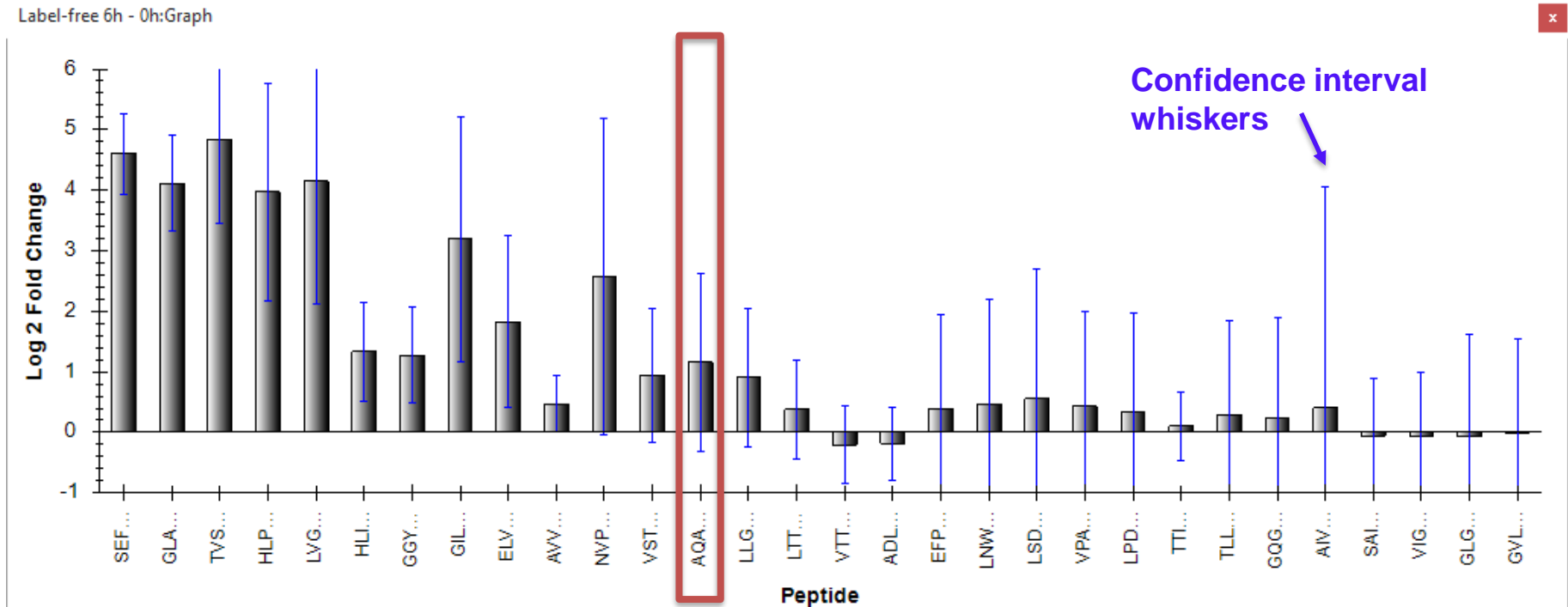
Show Graph Change Settings

Views 1 von 30 Export... Find:

	Protein	Peptide	Fold Change Result	Adjusted P-Value
▶	Rv2031c_hspX	SEFAYGSFVR	24.25 (99% CI:15.24 to 38.58)	0.0002
	Rv2626c_hrp1	GLAAGLDPNATAGELAR	17.35 (99% CI:10.06 to 29.92)	0.0003
	Rv2031c_hspX	TVSLPVGADEDIIK	28.53 (99% CI:10.85 to 75.05)	0.0009
	Rv2626c_hrp1	HLPEHAIVQFVK	15.67 (99% CI:4.5 to 54.57)	0.0040
	Rv2626c_hrp1	LVGIVTEADIAR	17.74 (99% CI:4.36 to 72.11)	0.0042
	Rv2623_TB31.7	HLIDDALK	2.53 (99% CI:1.44 to 4.45)	0.0071
	Rv2623_TB31.7	GGYAGMLVGSVGETVA...	2.42 (99% CI:1.39 to 4.23)	0.0071
	Rv2031c_hspX	GILTVSVAVSEGKPTKE	9.17 (99% CI:2.26 to 37.31)	0.0071
	Rv1738_Rv1738	ELVGVGILAR	3.56 (99% CI:1.33 to 9.52)	0.0135
	Rv1996_Rv1996	AVVNSGQAPVIVAR	1.38 (99% CI:1 to 1.91)	0.0286
	Rv1738_Rv1738	NVPEIGDELSVAR	5.94 (99% CI:0.98 to 36.14)	0.0286
	Rv1738_Rv1738	VSTHDIEAVTHQPAR	1.91 (99% CI:0.88 to 4.14)	0.0449
	Rv0079_Rv0079	AQAATAGIDDLRPALIR	2.22 (99% CI:0.8 to 6.17)	0.0484
	Rv2623_TB31.7	LLGSVSSGLLR	1.88 (99% CI:0.85 to 4.16)	0.0484
	Rv0079_Rv0079	LTTPAEALVTR	1.3 (99% CI:0.74 to 2.29)	0.2044
	Rv1812c_Rv1812c	VTTSTGASYSYDR	0.87 (99% CI:0.55 to 1.36)	0.4144
	Rv1812c_Rv1812c	ADLLAAAPR	0.88 (99% CI:0.58 to 1.34)	0.4196
	Rv0079_Rv0079	FFPDVAVFSGGR	1.32 (99% CI:0.45 to 3.84)	0.4319
	Rv1996_Rv1996	LNWAPIEWR	1.38 (99% CI:0.42 to 4.62)	0.4319
	Rv3132c_devS	LSDVVDLDQDVQIEIR	1.47 (99% CI:0.33 to 6.44)	0.4319
	Rv3133c_devR	VPAARPDVAVLVDR	1.35 (99% CI:0.46 to 3.96)	0.4319
	Rv3133c_devR	LPDNGIELCR	1.26 (99% CI:0.41 to 3.89)	0.5324
	Rv3132c_devS	TTIYDLHGASQGIR	1.07 (99% CI:0.72 to 1.59)	0.5733
	Rv3133c_devR	TLLGLLSEGLTNK	1.22 (99% CI:0.41 to 3.62)	0.5733
	Rv1996_Rv1996	GQGAVAGALLGSVSSS...	1.18 (99% CI:0.37 to 3.75)	0.6608
	Rv2027c_dosI	AIVHTAAELVDAR	1.32 (99% CI:0.11 to 16.56)	0.7374
	Rv2027c_dosI	SAIFDLHAGPSR	0.96 (99% CI:0.5 to 1.86)	0.8822
	Rv1812c_Rv1812c	VIGVPAMFAAGDVAAAR	0.96 (99% CI:0.46 to 2)	0.8911
	Rv3132c_devS	GLGVIGLLIEDPKPLR	0.96 (99% CI:0.3 to 3.09)	0.9047
	Rv2027c_dosI	GVLGALIEEPKPIR	1 (99% CI:0.34 to 2.92)	0.9884

# Simple group comparison within Skyline

Tutorial „Processing Grouped Study data“: [https://skyline.gs.washington.edu/labkey/tutorial\\_grouped.url](https://skyline.gs.washington.edu/labkey/tutorial_grouped.url)

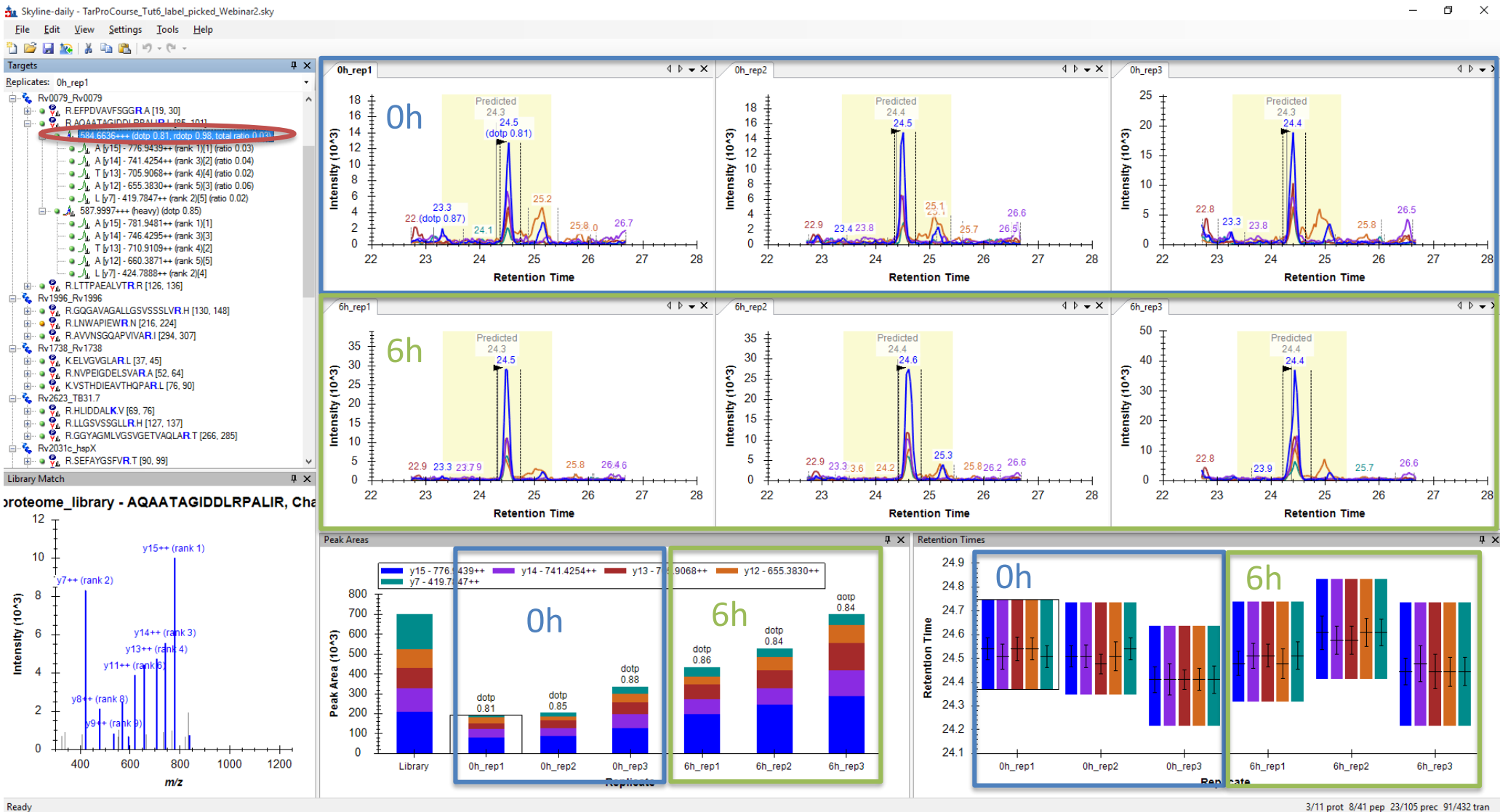


With a confidence of 99% (adj. p-value < 0.01) peptide AQAATAGIDDLRPALIR does **not change significant between 0h and 6h!**



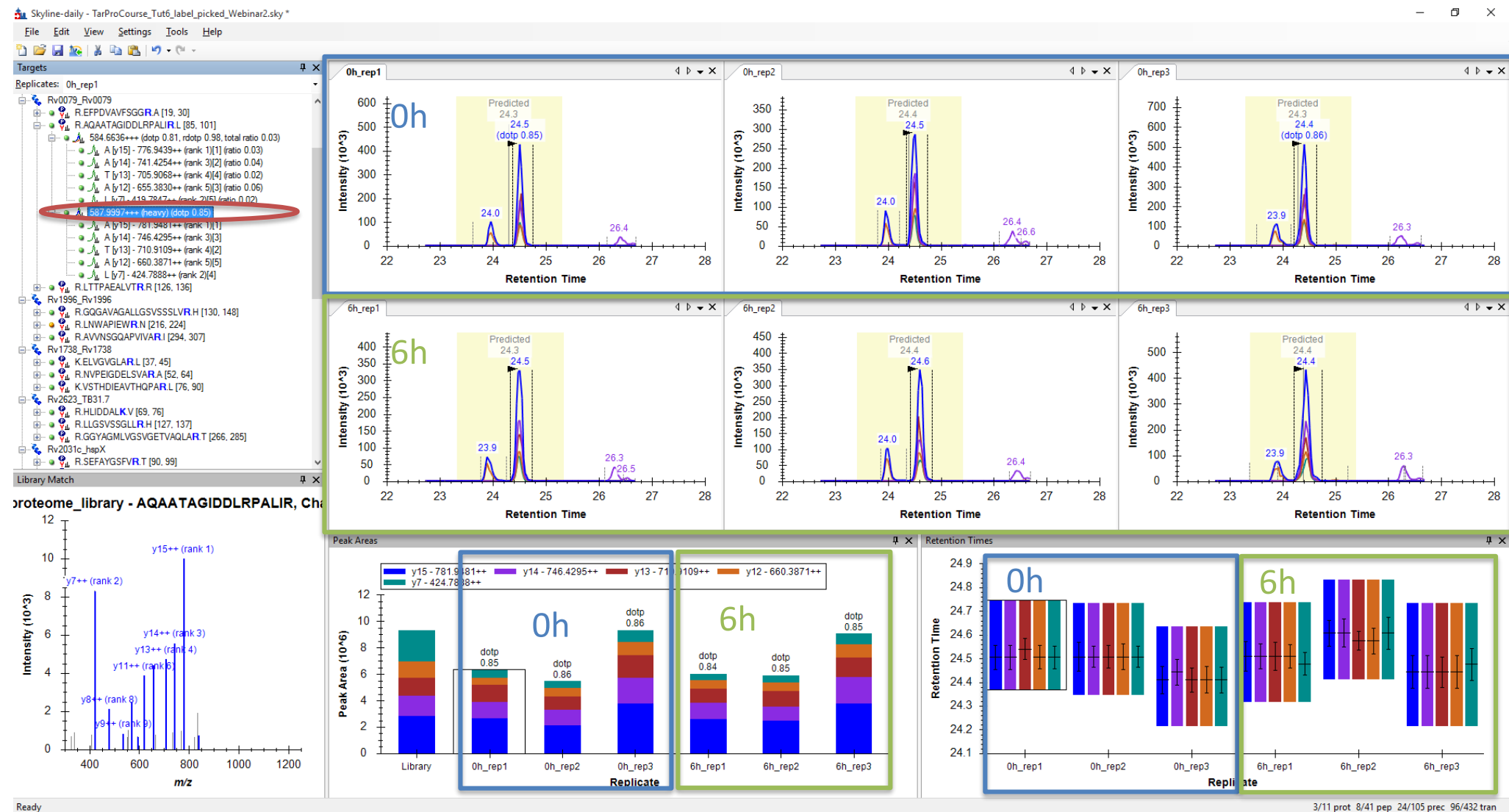
# Tutorial-6 “Manual SRM data analysis in Skyline”

[http://targetedproteomics.ethz.ch/tutorials2014/Tutorial-6\\_ManualAnalysis.pdf](http://targetedproteomics.ethz.ch/tutorials2014/Tutorial-6_ManualAnalysis.pdf)



# Tutorial-6 “Manual SRM data analysis in Skyline”

[http://targetedproteomics.ethz.ch/tutorials2014/Tutorial-6\\_ManualAnalysis.pdf](http://targetedproteomics.ethz.ch/tutorials2014/Tutorial-6_ManualAnalysis.pdf)



# Tutorial-6 “Manual SRM data analysis in Skyline”

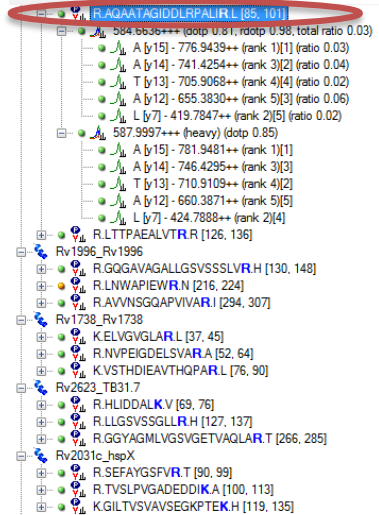
[http://targetedproteomics.ethz.ch/tutorials2014/Tutorial-6\\_ManualAnalysis.pdf](http://targetedproteomics.ethz.ch/tutorials2014/Tutorial-6_ManualAnalysis.pdf)

Skyline-daily - TarProCourse\_Tut6\_label\_picked\_Webinar2.sky

File Edit View Settings Tools Help

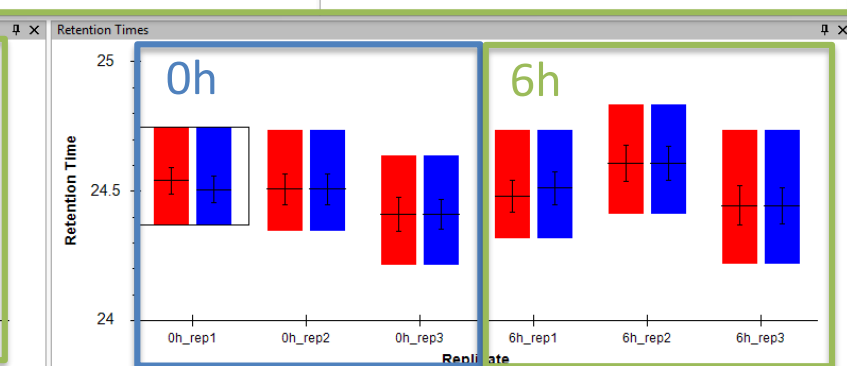
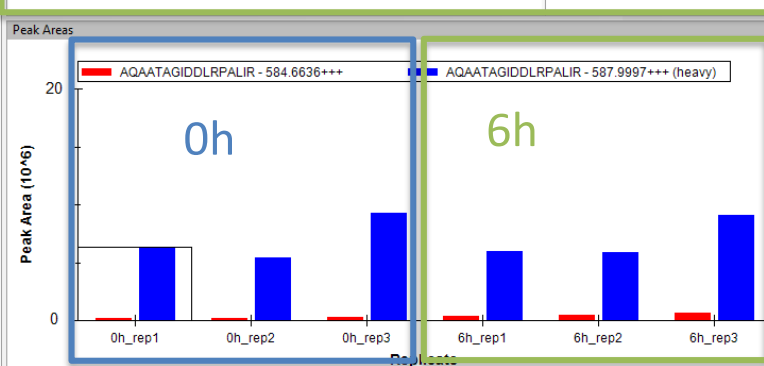
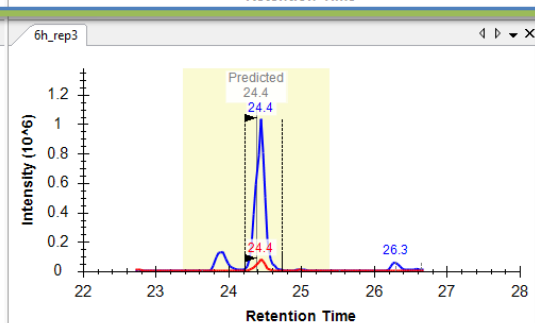
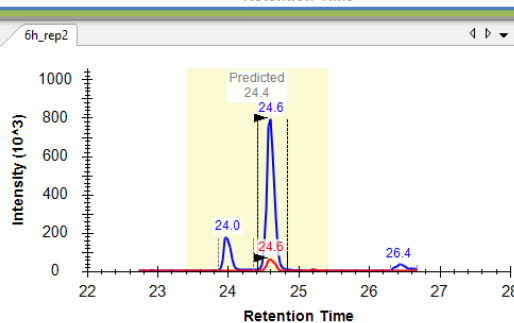
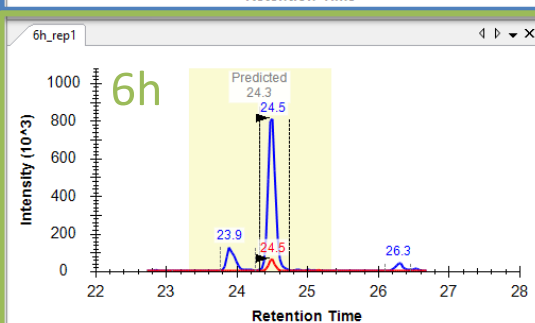
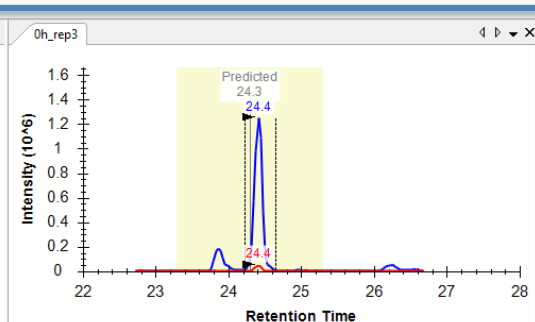
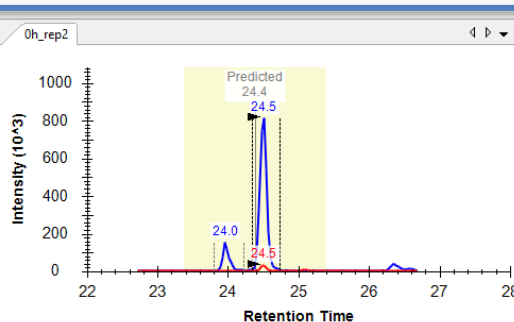
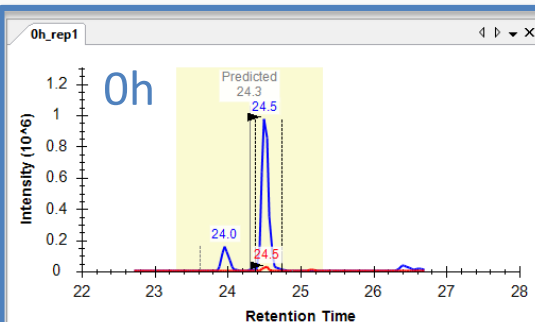
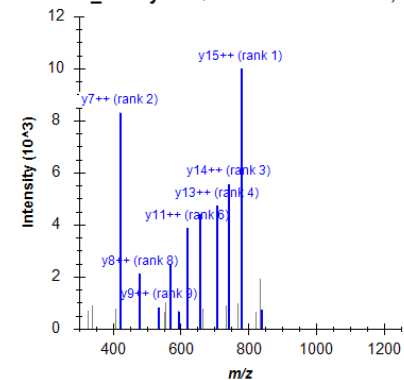
Targets

Replicates: 0h\_rep1

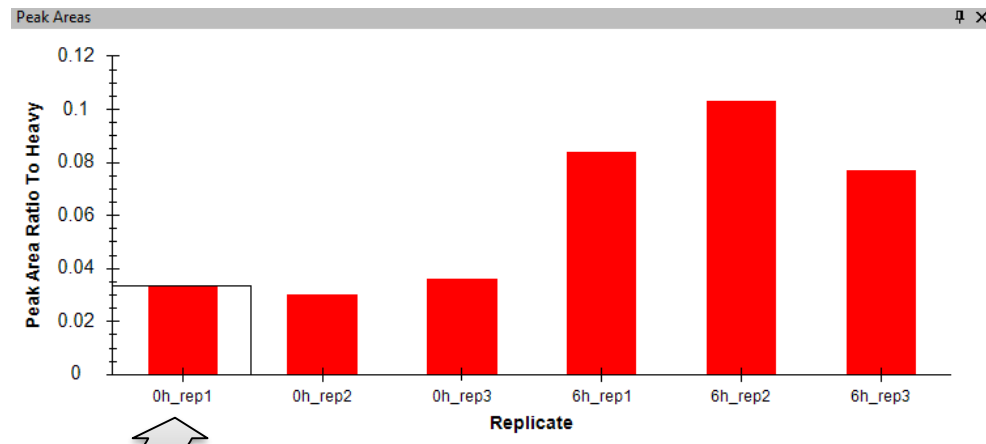
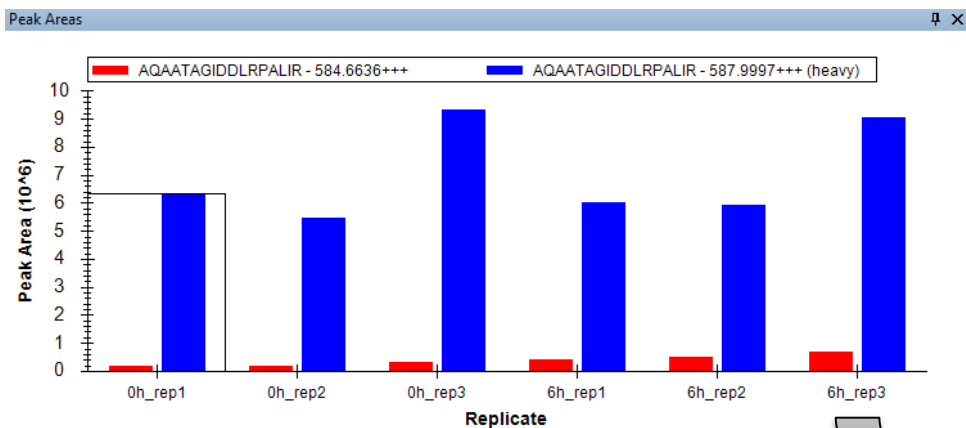


Library Match

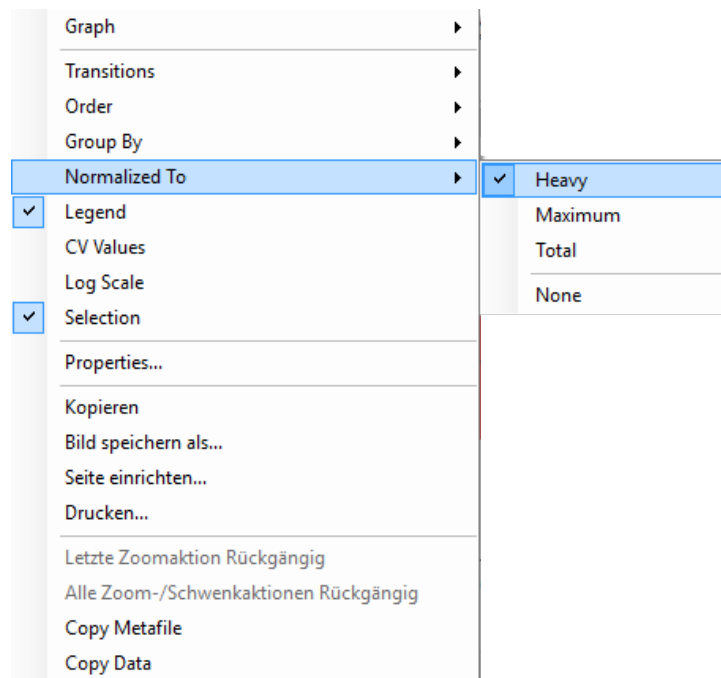
proteome\_library - AQAATAGIDDLRPALIR, Ch



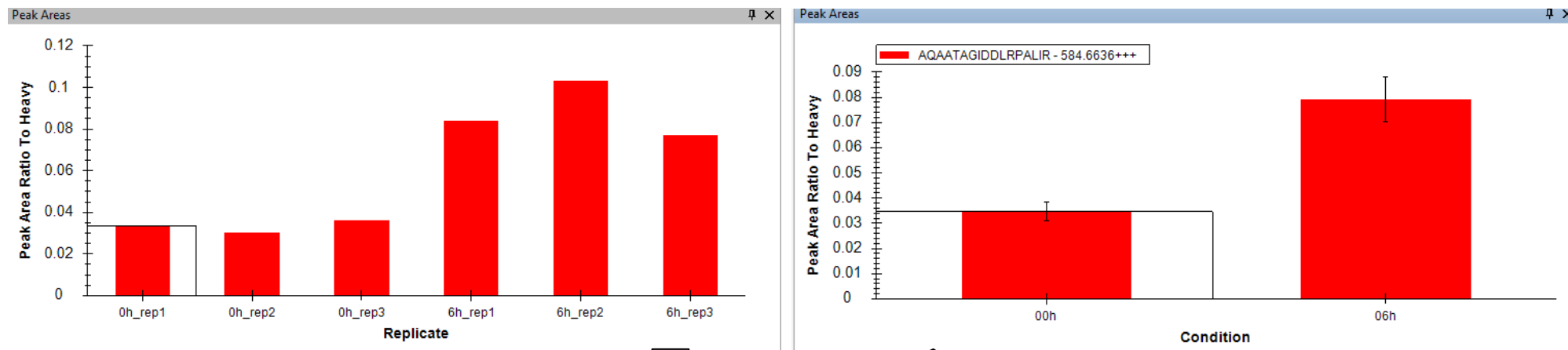
# “Peak Area” viewing options in Skyline



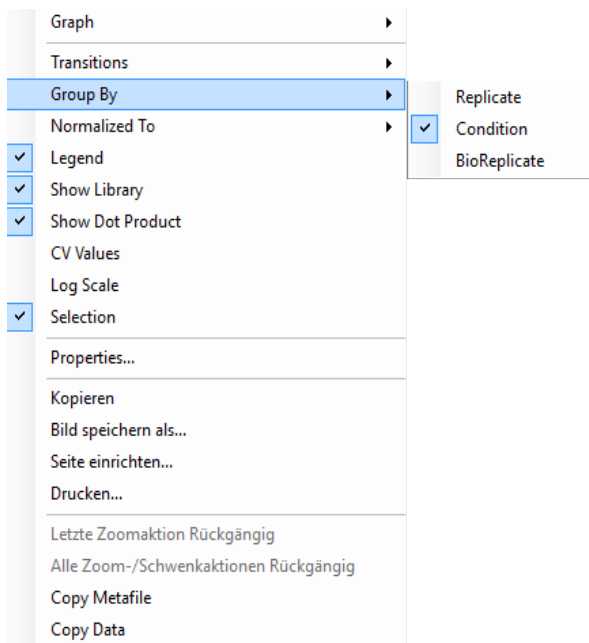
right mouse click on „Peak Areas“ window



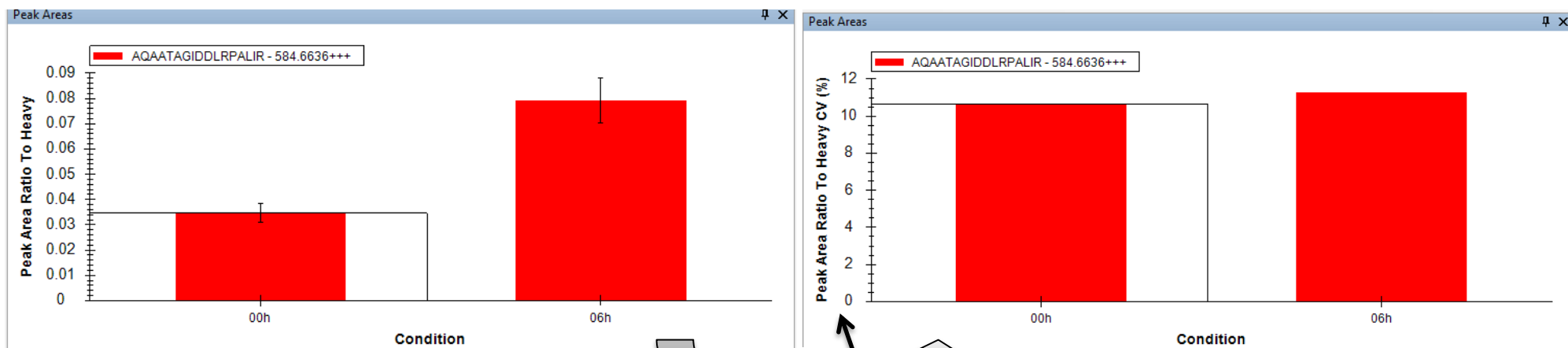
# “Peak Area” viewing options in Skyline



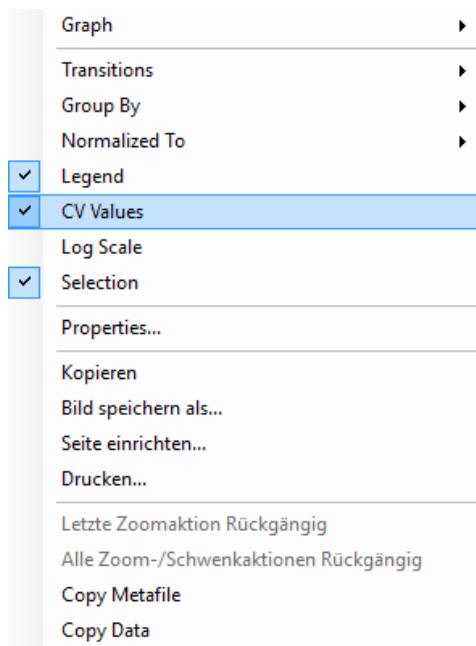
right mouse click on „Peak Areas“ window



# “Peak Area” viewing options in Skyline



right mouse click on „Peak Areas“ window



**Coefficient of variation in %**



# Simple group comparison within Skyline

Tutorial „Processing Grouped Study data“: [https://skyline.gs.washington.edu/labkey/tutorial\\_grouped.url](https://skyline.gs.washington.edu/labkey/tutorial_grouped.url)

View Settings Tools Help

- Targets
- Text Zoom
- Spectral Libraries
- Arrange Graphs
- Library Match Alt+1
- Ion Types
- Charges
- ☒ Ranks
- Chromatograms
- Transitions
- Transform
- Auto-Zoom
- Retention Times
- Peak Areas
- Calibration Curve
- Group Comparisons
  - Add...
  - Edit List...
- Results Grid Alt+2
- Document Grid
- ☒ Tool Bar
- ☒ Status Bar

Label-based 6h - 0h:Settings

Control group annotation:  
Condition

Control group value:  
00h

Value to compare against:  
06h

Identity annotation (for technical replicates):  
BioReplicate

Normalization method:  
Ratio to Heavy

Confidence level:  
99 %

Scope  
☒ Peptide ☐ Protein

Label-based 6h - 0h:Grid

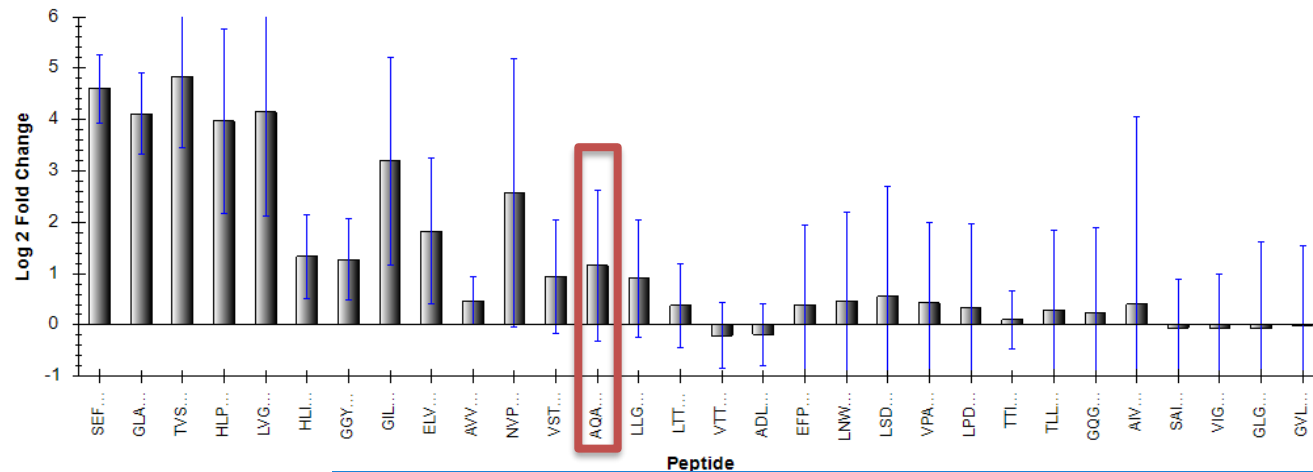
Show Graph Change Settings

Views 1 von 30 Export... Find:

	Protein	Peptide	Fold Change Result	Adjusted P-Value
▶	Rv2623_TB31.7	GGYAGMLVGSVGETVAQLAR	2.32 (99% CI:1.97 to 2.72)	0.0002
	Rv2031c_hspX	SEFAYGSFVR	25.13 (99% CI:12.66 to 49.87)	0.0002
	Rv2031c_hspX	TVSLPVGADEDDIK	31.03 (99% CI:15.68 to 61.4)	0.0002
	Rv2626c_hrp1	GLAAGLDPTATAGELAR	17.29 (99% CI:9.72 to 30.75)	0.0002
	Rv2031c_hspX	GILTVSVAVSEGKPTK	8.79 (99% CI:4.26 to 18.15)	0.0010
	Rv2626c_hrp1	HLPEHAIVQFVK	17.13 (99% CI:6.33 to 46.41)	0.0010
	Rv2626c_hrp1	LVGIVTEADIAR	18.2 (99% CI:5.45 to 60.8)	0.0016
	Rv2623_TB31.7	HLIDDALK	2.62 (99% CI:1.65 to 4.17)	0.0025
	Rv0079_Rv0079	AQAATAGIDDLRPALIR	2.29 (99% CI:1.52 to 3.47)	0.0026
	Rv1738_Rv1738	ELVGVLAR	3.77 (99% CI:1.52 to 9.35)	0.0074
	Rv1738_Rv1738	NVPEIGDELSVAR	6.11 (99% CI:1.73 to 21.56)	0.0074
	Rv1996_Rv1996	AVVNSGQAPVIVAR	1.41 (99% CI:1.08 to 1.84)	0.0096
	Rv1738_Rv1738	VSTHDIEAVTHQPAR	2.1 (99% CI:0.97 to 4.53)	0.0243
	Rv2623_TB31.7	LLGSVSSGLLR	1.95 (99% CI:0.98 to 3.88)	0.0243
	Rv0079_Rv0079	LTTPAEALVTR	1.37 (99% CI:0.88 to 2.12)	0.0623
	Rv0079_Rv0079	EFPDVAVFSGGR	1.36 (99% CI:0.69 to 2.66)	0.1938
	Rv3133c_devR	VPAARPDVAVLDVR	1.3 (99% CI:0.68 to 2.49)	0.2360
	Rv3133c_devR	LPDGNGIELCR	1.39 (99% CI:0.49 to 3.92)	0.3600
	Rv1812c_Rv1812c	ADLLAAAPR	0.91 (99% CI:0.67 to 1.24)	0.3713
	Rv3132c_devS	TTIYDLHGASQGITR	1.09 (99% CI:0.82 to 1.44)	0.3713
	Rv1812c_Rv1812c	VTTSTGASYSYDR	0.9 (99% CI:0.6 to 1.35)	0.4045
	Rv1996_Rv1996	LNWAPIEWR	1.39 (99% CI:0.38 to 5.08)	0.4045
	Rv3133c_devR	TLGLLSEGLTNK	1.18 (99% CI:0.62 to 2.22)	0.4045
	Rv1812c_Rv1812c	VIGVPAMFAAGDVAAAR	0.91 (99% CI:0.58 to 1.43)	0.4958
	Rv1996_Rv1996	GQGAVAGALLGSVSSSLVR	1.22 (99% CI:0.45 to 3.33)	0.4970
	Rv2027c_dosT	AIVHTAAELVDAR	1.33 (99% CI:0.27 to 6.5)	0.5271
	Rv2027c_dosT	GVLGALIEPKPIR	1.05 (99% CI:0.73 to 1.51)	0.6450
	Rv2027c_dosT	SAIFDLHAGPSR	0.96 (99% CI:0.64 to 1.43)	0.6945
	Rv3132c_devS	LSDVDDLQDVQIEIR	1.05 (99% CI:0.56 to 1.99)	0.7483
	Rv3132c_devS	GLGVGLIEDPKPLR	0.98 (99% CI:0.46 to 2.12)	0.9204

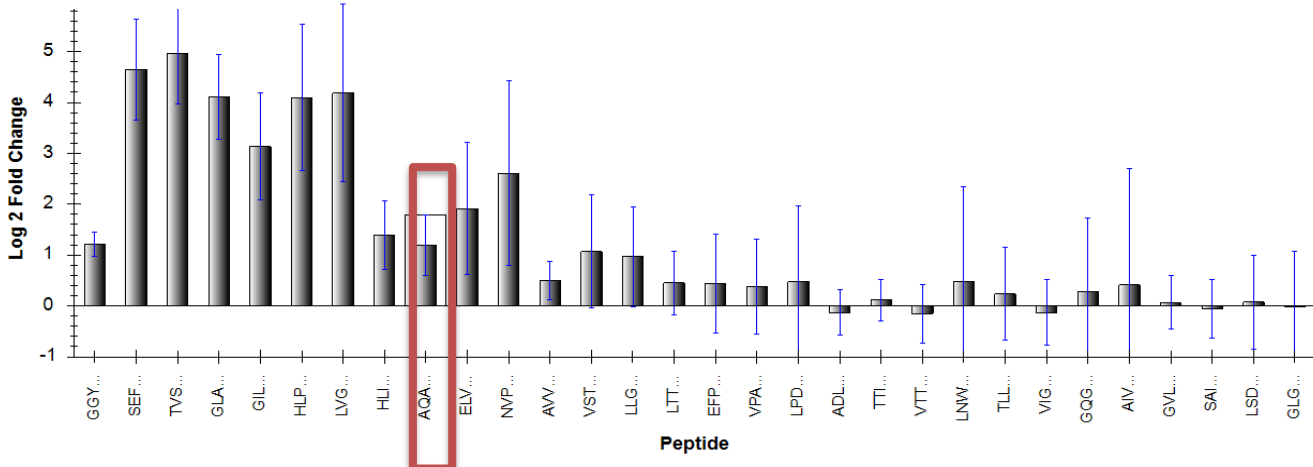
# Simple group comparison within Skyline

## Label-free



**Conclusion:** Spike-in of isotope labeled peptides helps to **account for sample preparation variabilities** from the spike-in moment on (no protein extraction or digestion variabilities!!!).

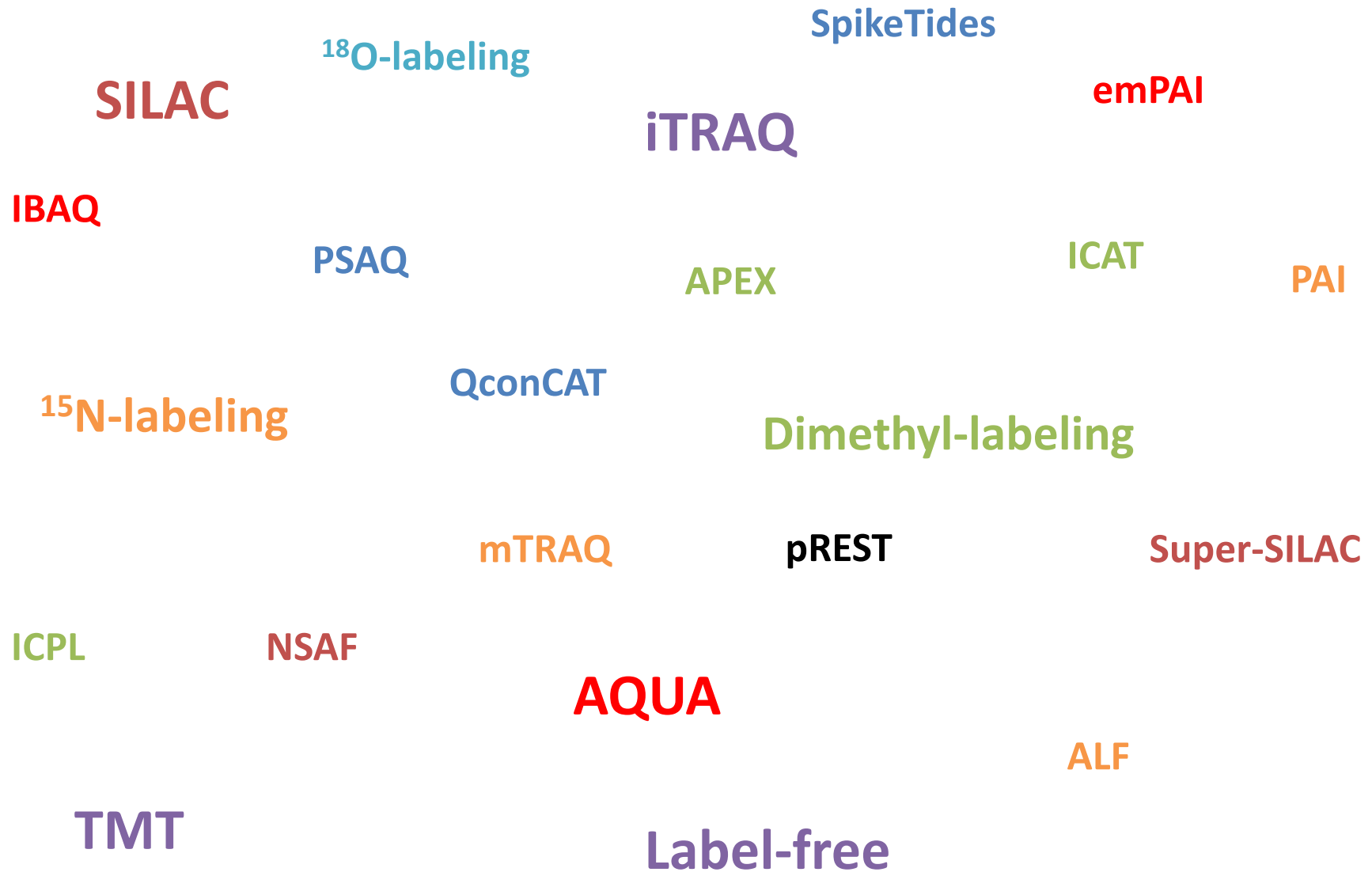
## Label-based



Thereby **quantitative precision and accuracy** get improved!

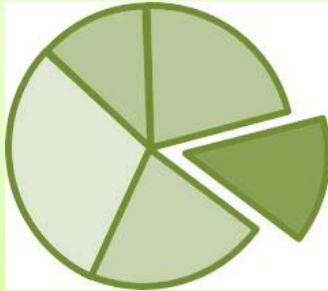


# The multitude of quantitative MS-applications

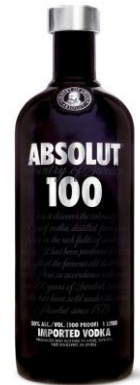


# Relative versus absolute protein quantification

relative



absolute



<sup>15</sup>N-labeling    <sup>18</sup>O-labeling  
IBAQ    mTRAQ    APEX  
SILAC  
ICPL    emPAI    SpikeTides  
iTRAQ    ICAT    pREST  
TMT  
QconCAT    Dimethyl-labeling  
ALF    PSAQ    AQUA    Label-free

PSAQ    NSAF  
QconCAT    APEX  
ALF    AQUA  
IBAQ    emPAI

# Definition of absolute versus relative protein quantification

## Relative quantification

## Absolute quantification

samples

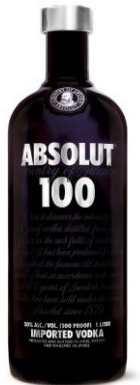
$\geq 2$  samples

output

protein ratio



copies/cell  
fmol/ $\mu$ g extract  
ng/ml body fluid

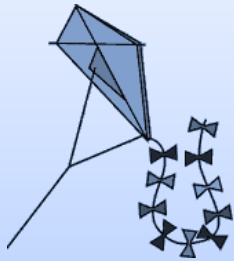


relative comparison of  
same protein between samples

comparison of same protein  
between samples +  
comparison of different proteins  
within the same sample



# MS-based absolute protein quantification strategies



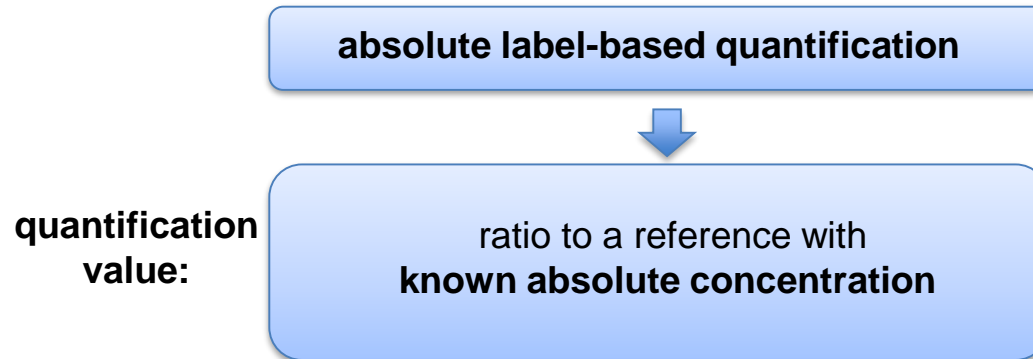
**absolute  
label-based**

**gold-standard -  
more precise !!!!**

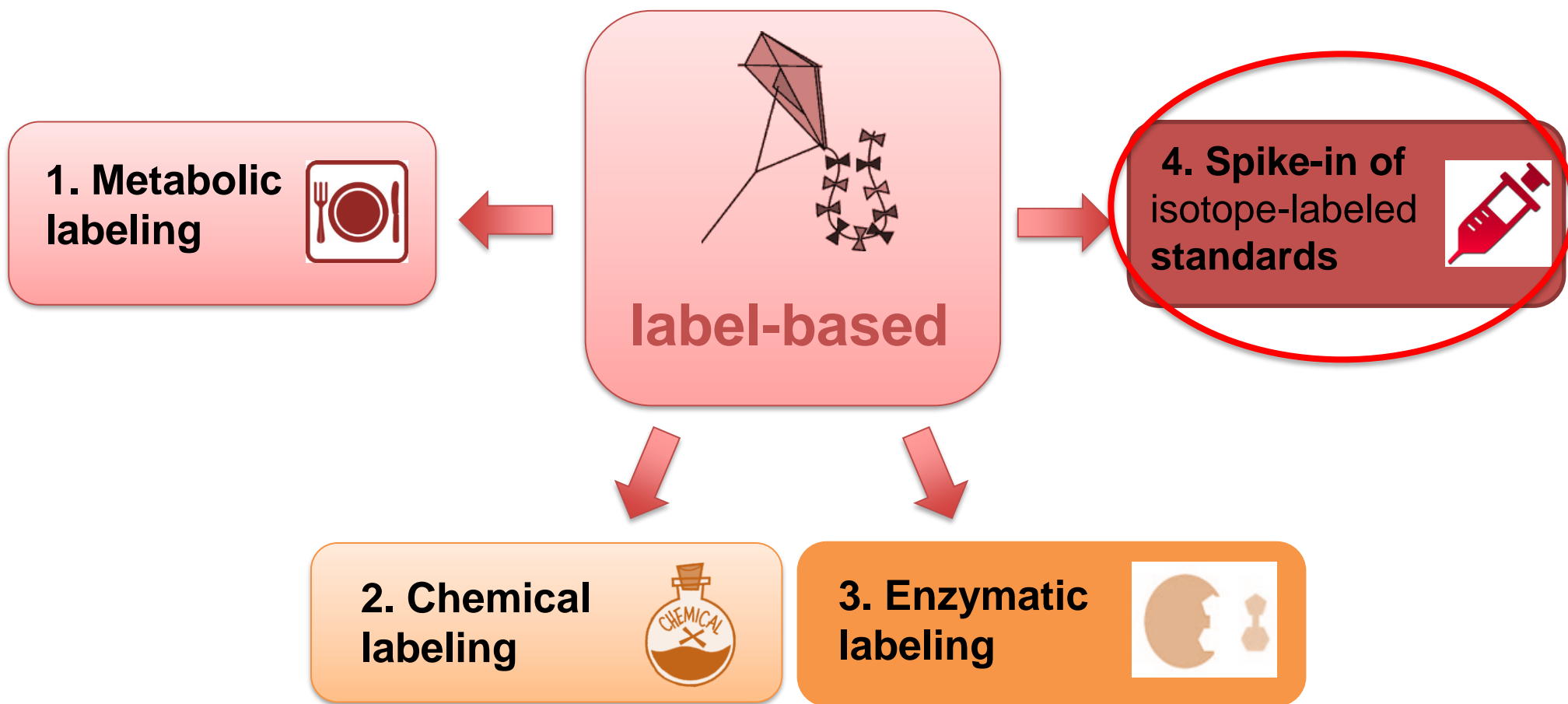


**absolute  
label-free**

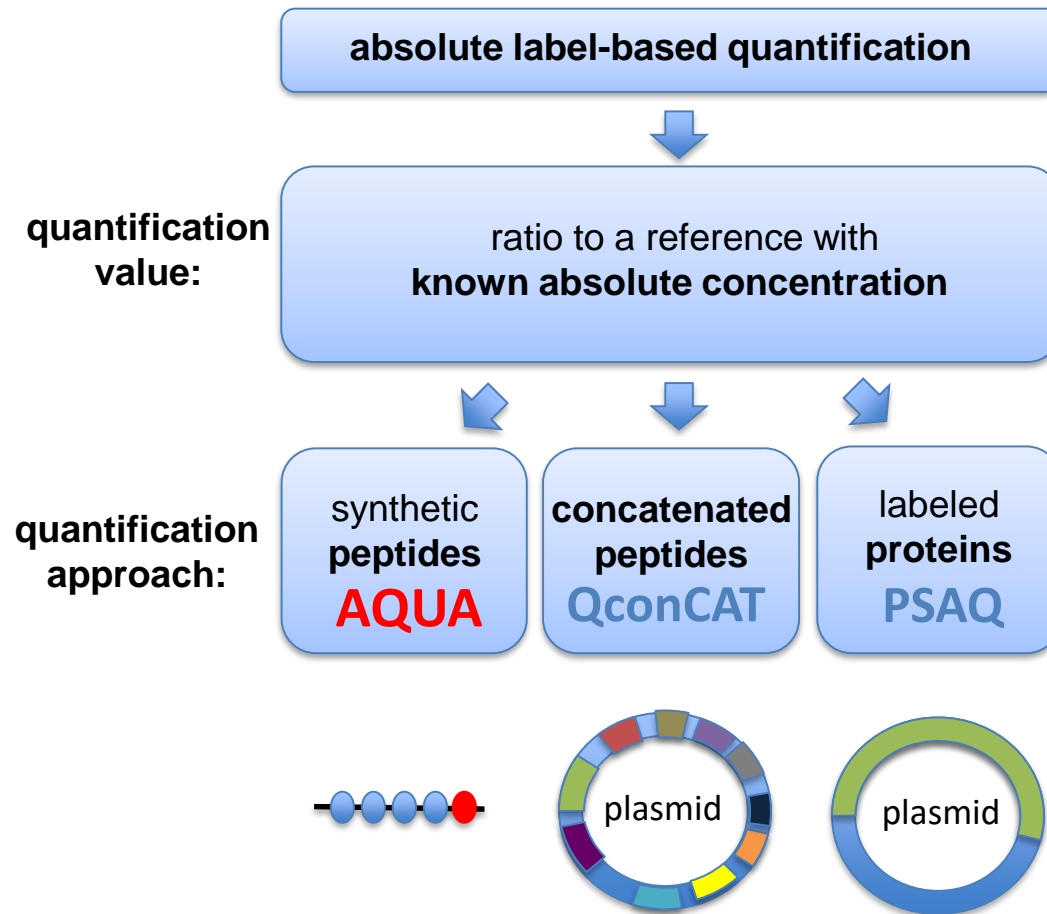
# Label-based absolute protein quantification



# Strategies for incorporating stable-isotopes

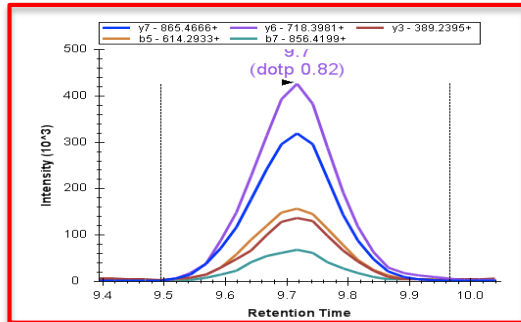


# Label-based absolute protein quantification

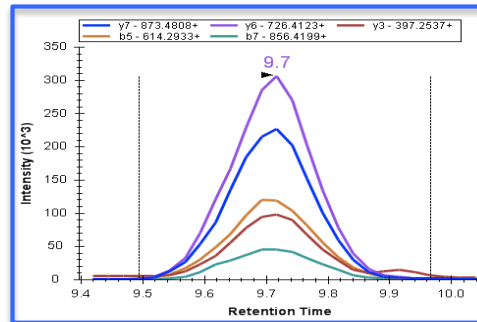


# Absolute quantification – internal single point calibration

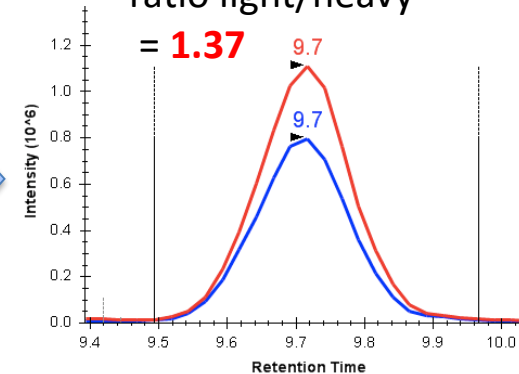
endogenous peptide (light)



reference peptide (heavy)



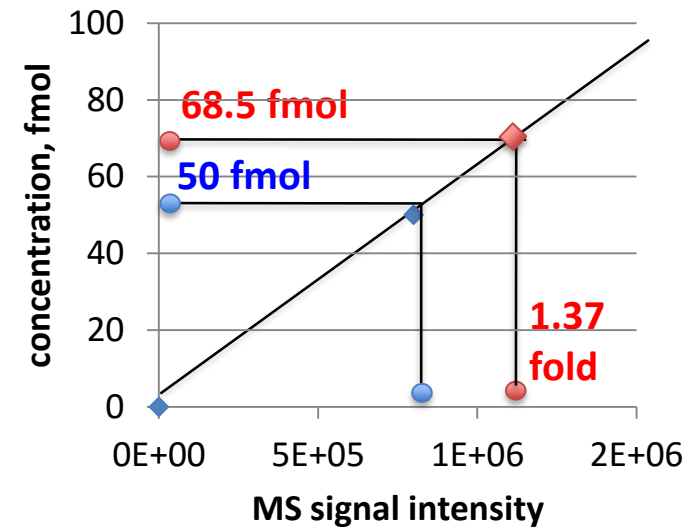
ratio light/heavy



**Absolute quantification** requires comparison to a **reference** molecule at a **known concentration**.

**spike-in concentration**  
**50 fmol**

Single point calibration



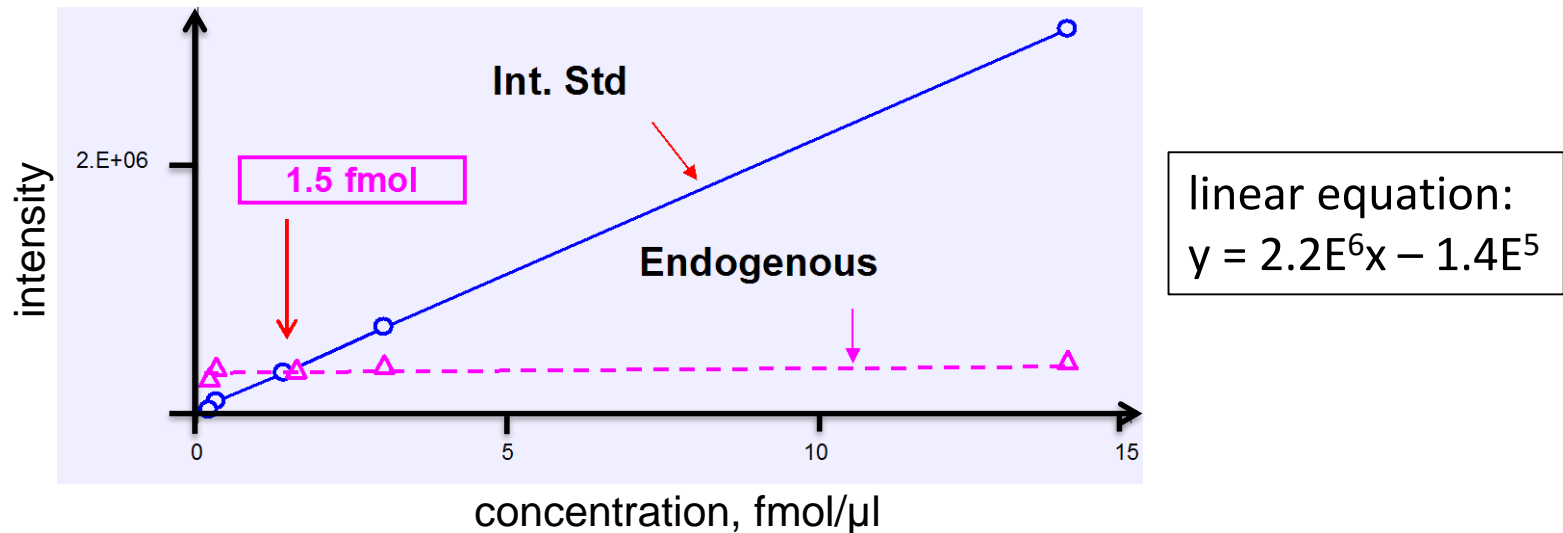
## Assumptions of internal single point calibration:

- Measurements are carried out **within the linear dynamic range** of the mass spectrometer
- Linear correlation between MS signal intensity and absolute concentration with a **slope of 1** and an **axis intercept of 0**.



# Absolute quantification – multiple point calibration curve

Work with dilution series of isotope-labeled reference peptides/proteins



## Advantages of multiple point calibration:

- You can **account** for linear peptide responses which **do NOT** have **slope = 1** and **axis intercept = 0**

## Limitations of multiple point calibration:

- Requires **multiple injections** (time and sample investment)

**Best** → Generate an **external calibration curve once** and use the obtained linear equation for absolute quantification

# Isotope-labeled absolute quantification supported within Skyline

Current Skyline Tutorial  
online available:


Home

Skyline

Start Page > Tutorials > Absolute Quantification

Get hands-on experience using Skyline with Excel to estimate the absolute mol

[download]



\* - written on Skyline v1.1, updated for v1.4

Also, see our paper in Nature Methods  
Rapid empirical discovery of optimal peptides for targeted proteomics  
[abstract]

DISCUSSION ▶

[https://skyline.gs.washington.edu/labkey/tutorial\\_absolute\\_quant.url](https://skyline.gs.washington.edu/labkey/tutorial_absolute_quant.url)

New implementations for **internal single point** and **multiple point calibration curves** are already available in **Skyline-daily** and are **coming soon in Skyline public**....

# Internal single-point absolute quantification supported within Skyline

## Published data set:

Schubert O. T. & Ludwig C., *et al. Cell host & microbe* **18**, 96-108, doi:10.1016/j.chom.2015.06.001 (2015).

**Panorama link:** [https://panoramaweb.org/labkey/Mtb\\_anchors.url](https://panoramaweb.org/labkey/Mtb_anchors.url)

## Motivation of the data set:

Absolutely quantify **30 proteins** in *Mycobacterium Tuberculosis* **over 24 samples** using **1-2 AQUA peptides per protein**.

## Points to consider regarding sample preparation:

- Spike-in **AQUA peptides** into all samples.
- Keep the concentration for a **given AQUA peptide** over all samples **constant**
- Try to select a spike-in AQUA concentration close to **1:1 light : heavy**
  - If a peptide is strongly regulated over the samples of interest a 1:1 ratio is of course not possible, try to select a spike-in concentration between highest and lowest sample

# Internal single-point absolute quantification supported within Skyline

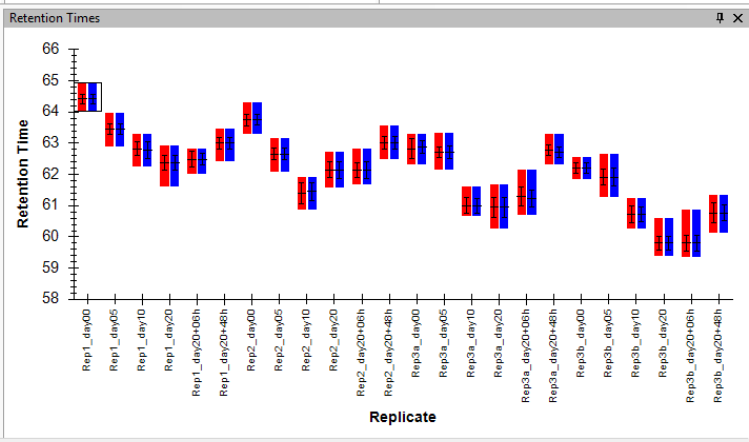
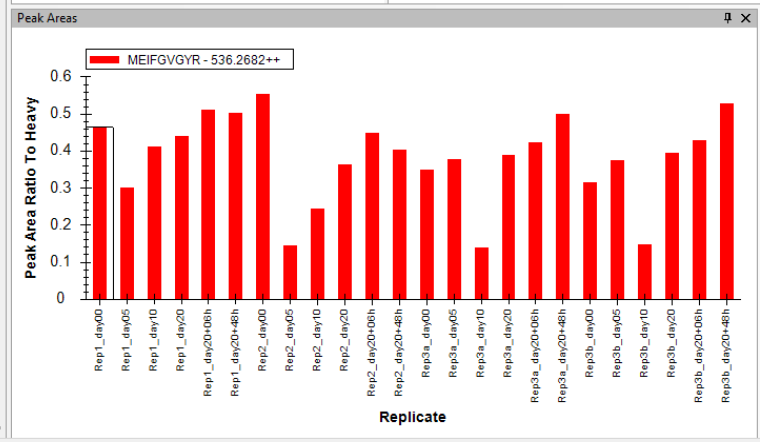
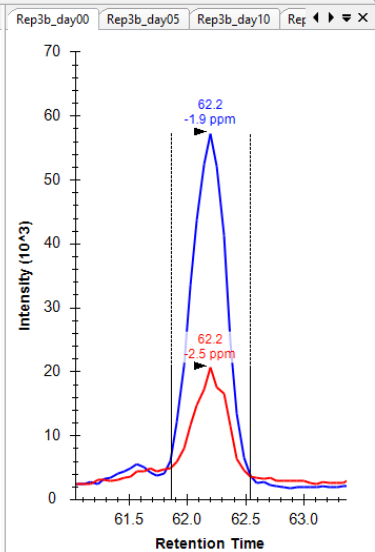
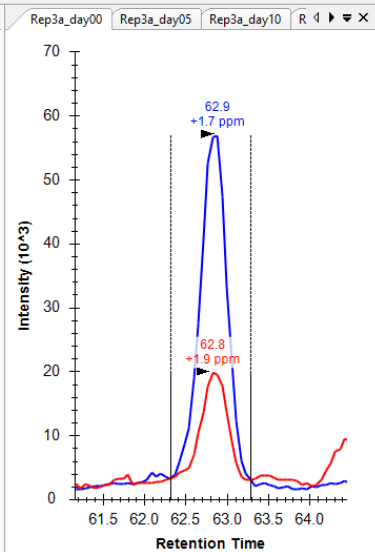
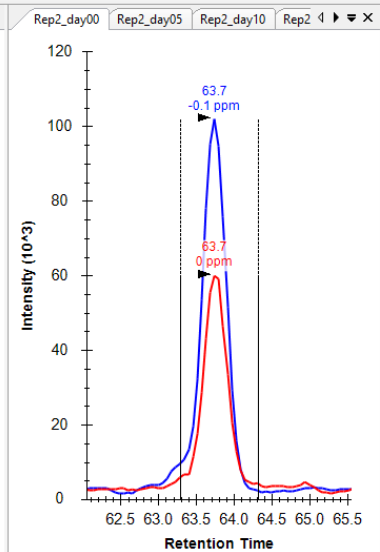
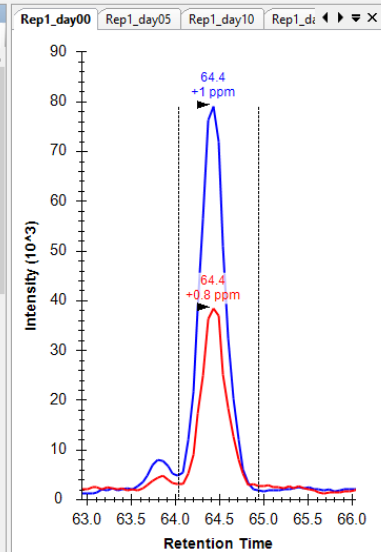
Skyline-daily - Anchor\_proteins\_absolute\_quantification\_Panorama\_14\_01\_2015\_test\_for\_Brendan.sky

File Edit View Settings Tools Help



Targets  
Replicates: Rep1\_day00

- Rv0041leuS
  - R.TINGVSEDFEALR.N [823, 835]
    - 689.3722++ (dotp 0.93, rdotp 0.97, total ratio 0.41)
    - 694.3764++ (heavy) (dotp 0.96)
  - R.VVVAADTEETLKA [923, 935]
    - 695.3590++ (dotp 0.98, rdotp 1, total ratio 0.96)
    - 699.3661++ (heavy) (dotp 0.97)
- Rv0044cRv0044c
  - R.IGVQLGPQHAPHYR.A [12, 25]
    - 548.6303+++ (dotp 0.97, rdotp 1, total ratio 0.56)
    - 551.9664+++ (heavy) (dotp 0.98)
- Rv0234cigabD1
  - R.FAAPALMAGNVLLK.H [196, 210]
    - 736.9183+ (dotp 0.95, rdotp 1, total ratio 0.85)
    - 740.9254++ (heavy) (dotp 0.95)
  - R.ELSAHGIRIE [492, 499]
    - 441.7432+ (dotp 0.9, rdotp 0.99, total ratio 0.98)
    - 446.7474++ (heavy) (dotp 0.93)
- Rv0440lproEL2
  - K.EIELEDPEYK.I [57, 66]
    - 632.8008++ (dotp 0.99, rdotp 1, total ratio 2.05)
    - 636.8079++ (heavy) (dotp 0.98)
  - R.NVAAGANPLGLK.R [104, 115]
    - 562.8248++ (dotp 0.94, rdotp 0.98, total ratio 2.64)
    - 566.8319++ (heavy) (dotp 0.96)
- Rv0657pso8
  - K.EASQTLLENLFFKE [283, 295]
    - 770.4063++ (dotp 0.97, rdotp 1, total ratio 0.77)
    - 774.4134++ (heavy) (dotp 0.97)
  - 513.9400+++ (dotp 0.96, rdotp 1, total ratio 0.68)
  - 516.6114+++ (heavy) (dotp 0.95)
  - K.GENIPEPIGESFK.V [1102, 1115]
    - 757.3803+ (dotp 0.99, rdotp 1, total ratio 0.69)
    - 761.3874++ (heavy) (dotp 0.98)
- Rv0719lplf
  - K.GTLGLTVAEPIK.V [30, 41]
    - 599.8557++ (dotp 0.97, rdotp 1, total ratio 0.7)
    - 603.8628++ (heavy) (dotp 0.98)
  - K.MEIFGVGYR.V [87, 95]
    - 536.2682++ (dotp 0.99, rdotp 1, total ratio 0.47)
    - 541.2724++ (heavy) (dotp 0.97)
- Rv0927cRv0927c
  - K.VAVITGGGR.G [11, 19]
    - 415.2482++ (dotp 0.97, rdotp 1, total ratio 0.59)
    - 420.2523++ (heavy) (dotp 0.97)
  - R.TSSELDVAEGLR.A [42, 54]
    - 709.8597++ (dotp 0.96, rdotp 1, total ratio 0.35)
    - 714.8639++ (heavy) (dotp 0.99)
- Rv1133cimetE
  - R.SWLAFGAKE.V [347, 355]
    - 504.7611++ (dotp 0.97, rdotp 1, total ratio 1.36)
    - 508.7682++ (heavy) (dotp 0.97)
  - R.DGHDVADEIASSR.A [368, 381]
    - 721.8290++ (dotp 0.99, rdotp 1, total ratio 0.47)
    - 726.8331++ (heavy) (dotp 0.99)
  - 481.5551+++ (dotp 0.98, rdotp 1, total ratio 0.5)
  - 484.8811+++ (dotp 0.99, rdotp 1, total ratio 0.6)



Ready

6/31 prot 11/62 pep 23/131 prec 95/522 tran

# Internal single-point absolute quantification supported within Skyline

Peptide Settings → Quantification

Peptide Settings

Digestion Prediction Filter Library Modifications **Quantification**

Regression Fit:  
None

Normalization Method:  
Ratio to Heavy

Regression Weighting:  
None

MS Level  
All

Units  
fmol/ug

OK Cancel

View → Document Grid → Views → Peptide Quantification

Document Grid: Peptide Quantification

Views 1 von 62 Export... Find:

Peptide	Protein	Peptide Modified Sequence	Standard Type	Internal Standard Concentration	Concentration Multiplier	Calibration Curve	Peptide Note
TIVGVSEDFAAALB	Rv0041IeuS	TIVGVSEDFAAALR		10.4		Slope: 9.6154E-2	
VVVAADTDEET...	Rv0041IeuS	VVVAADTDEETLK		3.4		Slope: 2.9412E-1	
IGVQLQPQHAP...	Rv0044cRv004...	IGVQLQPQHAP...		19.8		Slope: 5.0505E-2	
FAAPALMAGNV...	Rv0234cIgabD1	FAAPALMAGNV...		15.4		Slope: 6.4935E-2	
ELSAHGIR	Rv0234cIgabD1	ELSAHGIR		10.4		Slope: 9.6154E-2	
EIELEDPYEK	Rv0440IgroEL2	EIELEDPYEK		413.9		Slope: 2.4160E-3	
NVAAGANPLGLK	Rv0440IgroEL2	NVAAGANPLGLK		304.2		Slope: 3.2873E-3	
ESQATLLENLFFK	Rv0667IpoB	ESQATLLENLFFK		63.6		Slope: 1.5723E-2	
GENIPEGPICES...	Rv0667IpoB	GENIPEGPICESFK		41.4		Slope: 2.4155E-2	
GTGLTVAEPIK	Rv0719IplF	GTGLTVAEPIK		41.4		Slope: 2.4155E-2	
MEIFGVGYR	Rv0719IplF	MEIFGVGYR		39.6		Slope: 2.5253E-2	
VAVITGGGR	Rv0927cRv092...	VAVITGGGR		10.4		Slope: 9.6154E-2	
TSSELDVAEQIR	Rv0927cRv092...	TSSELDVAEQIR		20.6		Slope: 4.8544E-2	
SWLAFGAEK	Rv1133cImetE	SWLAFGAEK		55.2		Slope: 1.8116E-2	
DGHDAVADEIA...	Rv1133cImetE	DGHDAVADEIA...		67.5		Slope: 1.4815E-2	
IIENSAEDLAAR	Rv1294IthrA	IIENSAEDLAAR		10.6		Slope: 9.4340E-2	
VTADDVYR	Rv1294IthrA	VTADDVYR		10.4		Slope: 9.6154E-2	
VTGPVVDVEFPR	Rv1310IatoD	VTGPVVDVEFPR		82.8		Slope: 1.2077E-2	
KPPAFEELEPR	Rv1310IatoD	KPPAFEELEPR		72.3		Slope: 1.3831E-2	
SAIDTGSDDTT...	Rv1329cIdinG	SAIDTGSDDTTA...		0.4		Slope: 2.5000E+0	
ESAPVDVTDR	Rv1331Rv1331	ESAPVDVTDR		5.5		Slope: 1.8182E-1	
DAESDEVLGK	Rv1388ImlhF	DAESDEVLGK		342		Slope: 2.9240E-3	
VSALLEALPK	Rv1388ImlhF	VSALLEALPK		331.1		Slope: 3.0202E-3	
IDTPGEADYYR	Rv1475cIacn	IDTPGEADYYR		20.7		Slope: 4.8309E-2	
NGGILQYVLR	Rv1475cIacn	NGGILQYVLR		21.7		Slope: 4.6083E-2	
TVADVDLSLR	Rv1605IhisF	TVADVDLSLR		10.4		Slope: 9.6154E-2	
AGFDLALLR	Rv1605IhisF	AGFDLALLR		5.9		Slope: 1.6949E-1	
YFNDGDIVEGTI...	Rv1630IpsA	YFNDGDIVEGTI...		82.8		Slope: 1.2077E-2	
VEEGIEGLVHIS...	Rv1630IpsA	VEEGIEGLVHIS...		61.6		Slope: 1.6234E-2	
AAALDEIAAEP...	Rv1773cRv177...	AAALDEIAAEP...		1.1		Slope: 9.0909E-1	
ALGDNVIAISVP...	Rv1773cRv177...	ALGDNVIAISVP...		2		Slope: 5.0000E-1	
VPDIHVALME...	Rv1837cIalcB	VPDIHVALME...		51.3		Slope: 1.9493E-2	
HGVITSADVR	Rv1837cIalcB	HGVITSADVR		41.4		Slope: 2.4155E-2	
ADLPFAELLAR	Rv2129cRv212...	ADLPFAELLAR		5.5		Slope: 1.8182E-1	
IPDEDLAGLR	Rv2244IacpM	IPDEDLAGLR		266.8		Slope: 3.7481E-3	

Copy paste spike-in concentrations of each AQUA peptide in fmol/ug

# Internal single-point absolute quantification supported within Skyline

View → Document Grid → Views → Peptide Ratio Results

Document Grid: Peptide Ratio Results

Views 1 von 1488 Export... Find:

	Peptide Sequence	Protein Name	Replicate Name	Peptide Peak Found Ratio	Peptide Retention Time	Ratio To Standard	Quantification
▶	TIVGVSEDFAAALR	Rv0041IleuS	Rep1_day00	1	68.44	0.4123	<a href="#">4.2877 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep1_day05	1	67.73	0.3701	<a href="#">3.8491 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep1_day10	1	67.24	0.4582	<a href="#">4.7653 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep1_day20	1	67.18	0.4168	<a href="#">4.3344 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep1_day20+06h	1	66.49	0.4924	<a href="#">5.1208 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep1_day20+48h	1	67.52	0.5674	<a href="#">5.9006 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep2_day00	1	67.89	0.4466	<a href="#">4.6444 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep2_day05	1	66.95	0.4311	<a href="#">4.4834 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep2_day10	1	66.07	0.4592	<a href="#">4.7756 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep2_day20	1	66.72	0.4649	<a href="#">4.8351 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep2_day20+06h	1	66.69	0.5054	<a href="#">5.2558 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep2_day20+48h	1	67.06	0.4717	<a href="#">4.9058 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep3a_day00	1	67.06	0.4931	<a href="#">5.1286 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep3a_day05	1	67.18	0.4665	<a href="#">4.8521 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep3a_day10	1	65.89	0.4034	<a href="#">4.1953 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep3a_day20	1	65.52	0.4622	<a href="#">4.8066 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep3a_day20+06h	1	65.58	0.4521	<a href="#">4.7015 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep3a_day20+48h	1	66.95	0.5389	<a href="#">5.6048 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep3b_day00	1	66.38	0.524	<a href="#">5.45 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep3b_day05	1	66.52	0.5711	<a href="#">5.9392 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep3b_day10	1	65.78	0.4972	<a href="#">5.1709 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep3b_day20	1	64.89	0.3764	<a href="#">3.9148 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep3b_day20+06h	1	65.04	0.3677	<a href="#">3.8239 fmol/ug</a>
	TIVGVSEDFAAALR	Rv0041IleuS	Rep3b_day20+48h	1	65.52	0.518	<a href="#">5.387 fmol/ug</a>
	VVAADTDEETLK	Rv0041IleuS	Rep1_day00	1	36.09	0.9584	<a href="#">3.2587 fmol/ug</a>
	VVAADTDEETLK	Rv0041IleuS	Rep1_day05	1	34.61	1.0584	<a href="#">3.5984 fmol/ug</a>
	VVAADTDEETLK	Rv0041IleuS	Rep1_day10	1	35.31	0.9909	<a href="#">3.3692 fmol/ug</a>
	VVAADTDEETLK	Rv0041IleuS	Rep1_day20	1	35.26	0.783	<a href="#">2.6622 fmol/ug</a>
	VVAADTDEETLK	Rv0041IleuS	Rep1_day20+06h	1	34.97	0.9748	<a href="#">3.3144 fmol/ug</a>
	VVAADTDEETLK	Rv0041IleuS	Rep1_day20+48h	1	35.09	1.2843	<a href="#">4.3665 fmol/ug</a>
	VVAADTDEETLK	Rv0041IleuS	Rep2_day00	1	35.6	1.0328	<a href="#">3.5115 fmol/ug</a>
	VVAADTDEETLK	Rv0041IleuS	Rep2_day05	1	35.26	1.0548	<a href="#">3.5863 fmol/ug</a>
	VVAADTDEETLK	Rv0041IleuS	Rep2_day10	1	33.74	0.9577	<a href="#">3.2561 fmol/ug</a>
	VVAADTDEETLK	Rv0041IleuS	Rep2_day20	1	34.54	0.7541	<a href="#">2.564 fmol/ug</a>
	VVAADTDEETLK	Rv0041IleuS	Rep2_day20+06h	1	33.66	0.8693	<a href="#">2.9556 fmol/ug</a>
	VVAADTDEETLK	Rv0041IleuS	Rep2_day20+48h	1	34.57	1.0229	<a href="#">3.4778 fmol/ug</a>

# Multiple-point absolute quantification supported within Skyline

Peptide Settings → Quantification

Peptide Settings

Digestion Prediction Filter Library Modifications **Quantification**

Regression Fit:  
Linear

Normalization Method:  
None

Regression Weighting:  
None

MS Level  
All

Units  
fmol/ug

OK Cancel

View → Document Grid → Views → Replicates

Document Grid: Replicates

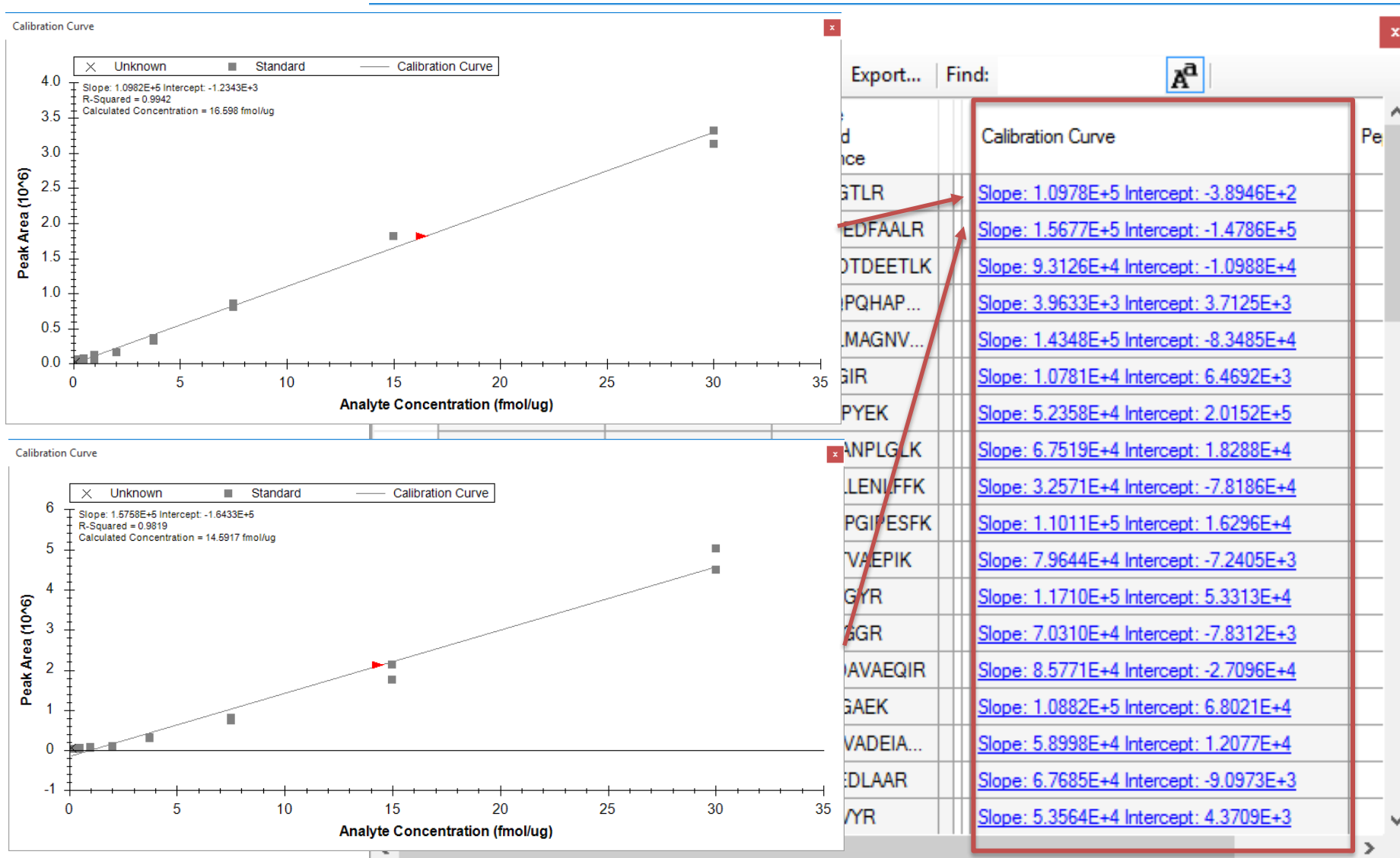
Views 1 von 18

	Replicate	Sample Type	Analyte Concentration
▶	<a href="#">30.0fmol/ug_A</a>	Standard	30
	<a href="#">15.0fmol/ug_A</a>	Unknown	15
	<a href="#">07.5fmol/ug_A</a>	Standard	7.5
	<a href="#">03.75fmol/ug_A</a>	Quality Control	3.75
	<a href="#">02.0fmol/ug_A</a>	Solvent	2
	<a href="#">01.0fmol/ug_A</a>	Blank	1
	<a href="#">00.5fmol/ug_A</a>	Double Blank	0.5
	<a href="#">00.25fmol/ug_A</a>	Standard	0.25
	<a href="#">00.1fmol/ug_A</a>	Standard	0.1
	<a href="#">30.0fmol/ug_B</a>	Standard	30
	<a href="#">15.0fmol/ug_B</a>	Standard	15
	<a href="#">07.5fmol/ug_B</a>	Standard	7.5
	<a href="#">03.75fmol/ug_B</a>	Standard	3.75
	<a href="#">02.0fmol/ug_B</a>	Standard	2
	<a href="#">01.0fmol/ug_B</a>	Standard	1
	<a href="#">00.5fmol/ug_B</a>	Standard	0.5
	<a href="#">00.25fmol/ug_B</a>	Standard	0.25
	<a href="#">00.1fmol/ug_B</a>	Standard	0.1



# Multiple-point absolute quantification supported within Skyline

View → Document Grid → Views → Peptide Quantification

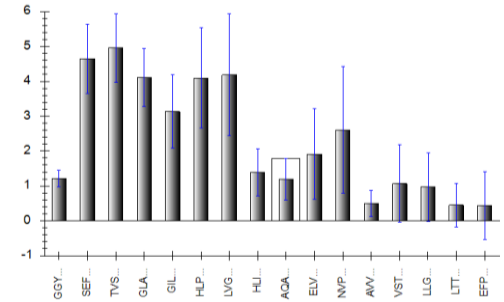




# Take-home messages - Quantification

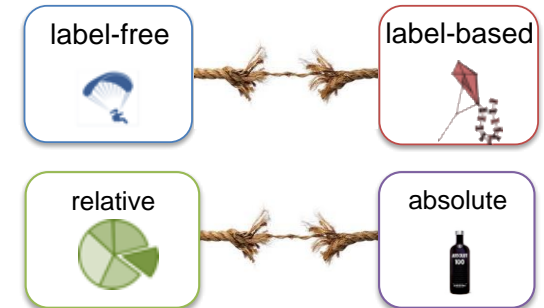


- Isotope-labeling helps to account for **various types of variabilities** (during sample preparation and LC-MS) and thereby to **improve quantitative precision and accuracy!**
- For every research project the **optimal quantitative approach** must be selected accordingly (label-free or label-based, relative or absolute)



→ **Skyline** supports **label-free** as well as all **4 different subtypes of label-based** quantification approaches (metabolic, enzymatic, chemical, spike-in)

→ **Skyline** supports **internal single-point** as well as **multiple point** absolute quantification



# Acknowledgements

## Targeted Proteomics Course Zürich – Team:

- **Prof. Ruedi Aebersold (ETH Zürich)**
- **Brendan MacLean (University of Washington)**
- **Prof. Mike MacCoss (University of Washington)**
- **The whole Skyline Team**
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- Prof. Bruno Domon (Luxembourg Institute of Health)
- **Olga Schubert (UCLA)**
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- Betty Friedrich (ETH Zürich)
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- Lukas Reiter (Biognosys)
- Claudio Escher (Biognosys)
- Oliver Rinner (Biognosys)
- Eduard Sabido (CRG Barcelona)
- Dario Amodei (Google)
- Jarrett Egertson (University of Washington)
- Ralph Schiess (ProteoMedix)
- Paola Picotti (ETH Zürich)

**ETH** Zürich



Technische Universität München



# Learn More

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- ▶ Webinar #13: in January
- ▶ Weeklong Course at IIT-Bombay
  - ▶ December 10-14 - Full
- ▶ Weeklong Courses 2016
  - ▶ ETH, Zurich – February 8-12 - Full
  - ▶ Buck Institute, San Francisco – March 7-11 – Register now!
  - ▶ Northeastern University, Boston – May 2-6
  - ▶ University of Washington, Seattle – July 18-22
  - ▶ PRBB, Barcelona – November 13-18
- ▶ Workshops 2016
  - ▶ US HUPO, Boston – March 13



# Questions?

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- ▶ Ask any questions you have on modifications in Skyline at the following form:

<http://tinyurl.com/QA4Skyline>

- ▶ Take the post-webinar survey:

<http://tinyurl.com/Survey4Webinar>





# Skyline Tutorial Webinar #12

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This ends this Skyline Tutorial Webinar.

Please give us feedback on the webinar at the following survey:

<http://tinyurl.com/Survey4Webinar>

A recording of today's meeting will be available shortly at the Skyline website.

We look forward to seeing you at a future Skyline Tutorial Webinar.





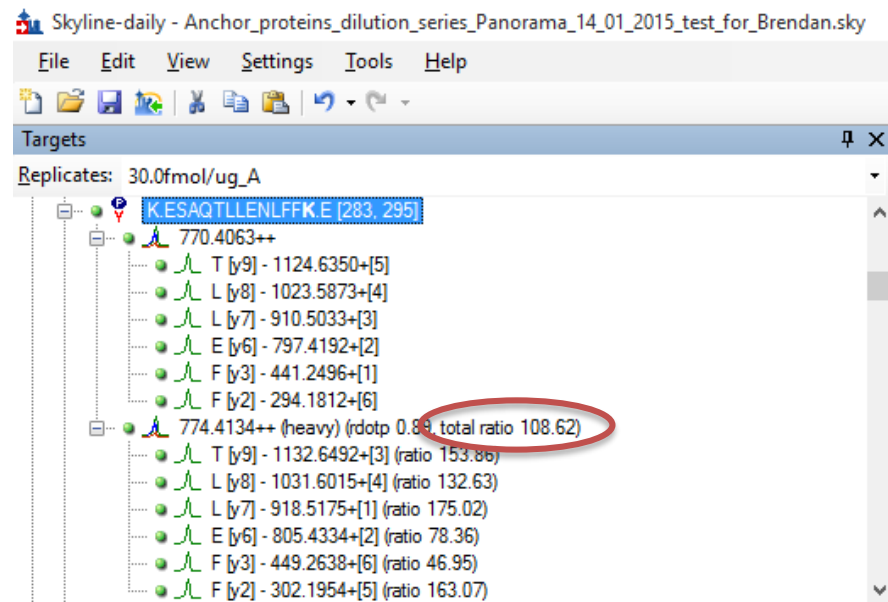
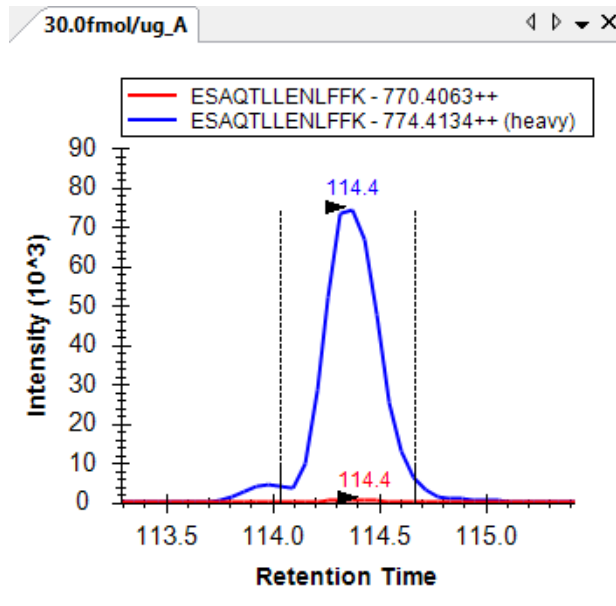
**Additional slide material Christina Ludwig**

# Tips and tricks: Isotope impurity!



Isotope-labeled synthetic peptides can be “**polluted**” with the light form.

→ Make sure what you are measuring in the light channel is not a “pollution” coming from the synthetic peptide (ratios close to 1:1 help!)

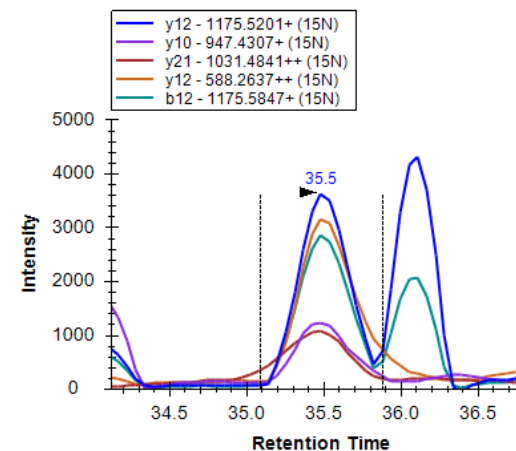
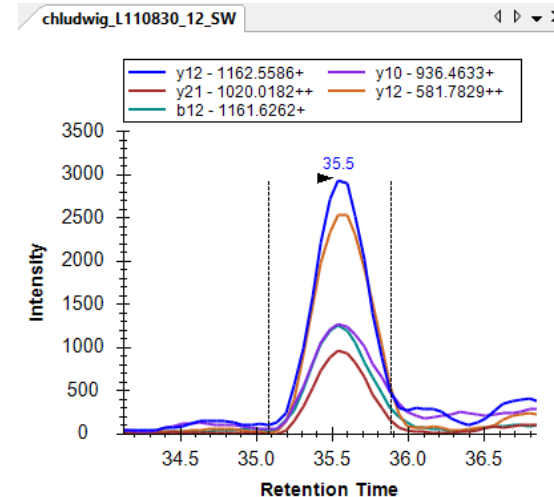
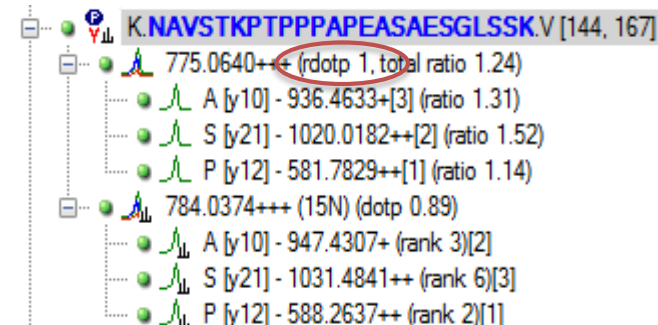
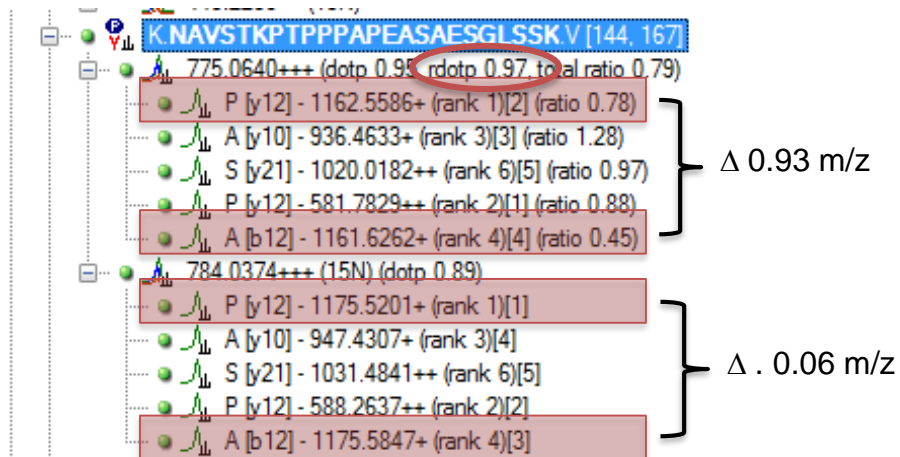


# Tips and tricks: Isobaric transition interferences



**Isobaric transition interferences** between **heavy** and **light** form of a peptide can make exclusions of such transitions necessary!

→ Investigate your data!





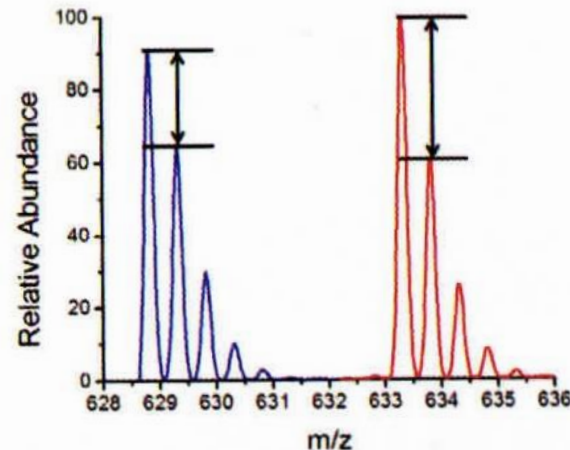
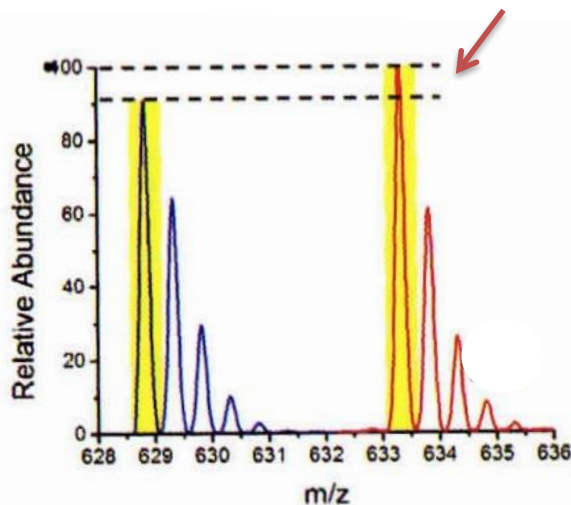
## Tips and tricks: Different isotopic envelopes between light and heavy



The **isotopic pattern** of the light and heavy peptide form is **not perfectly equal!**

→ Hence, **high resolution accurate mass data (PRM, DIA)**, where quantification gets based on the **monoisotopic peak** (as performed in Skyline!), **must get corrected** for the different theoretical isotopic pattern.

7% increase for 75 C-atom peptide



Standard peptide  
(15 aa 75 C-atoms)

1.1%  $^{13}\text{C}$  in  
75 C-atoms

1.1%  $^{13}\text{C}$  in  
69 (75-6) C-atoms

Figure adapted from Mike MacCoss

Mass Spectrometry Signal Calibration for Protein Quantitation, technical note, Cambridge Isotope Laboratories

## Tips and tricks: Handling AQUA peptides



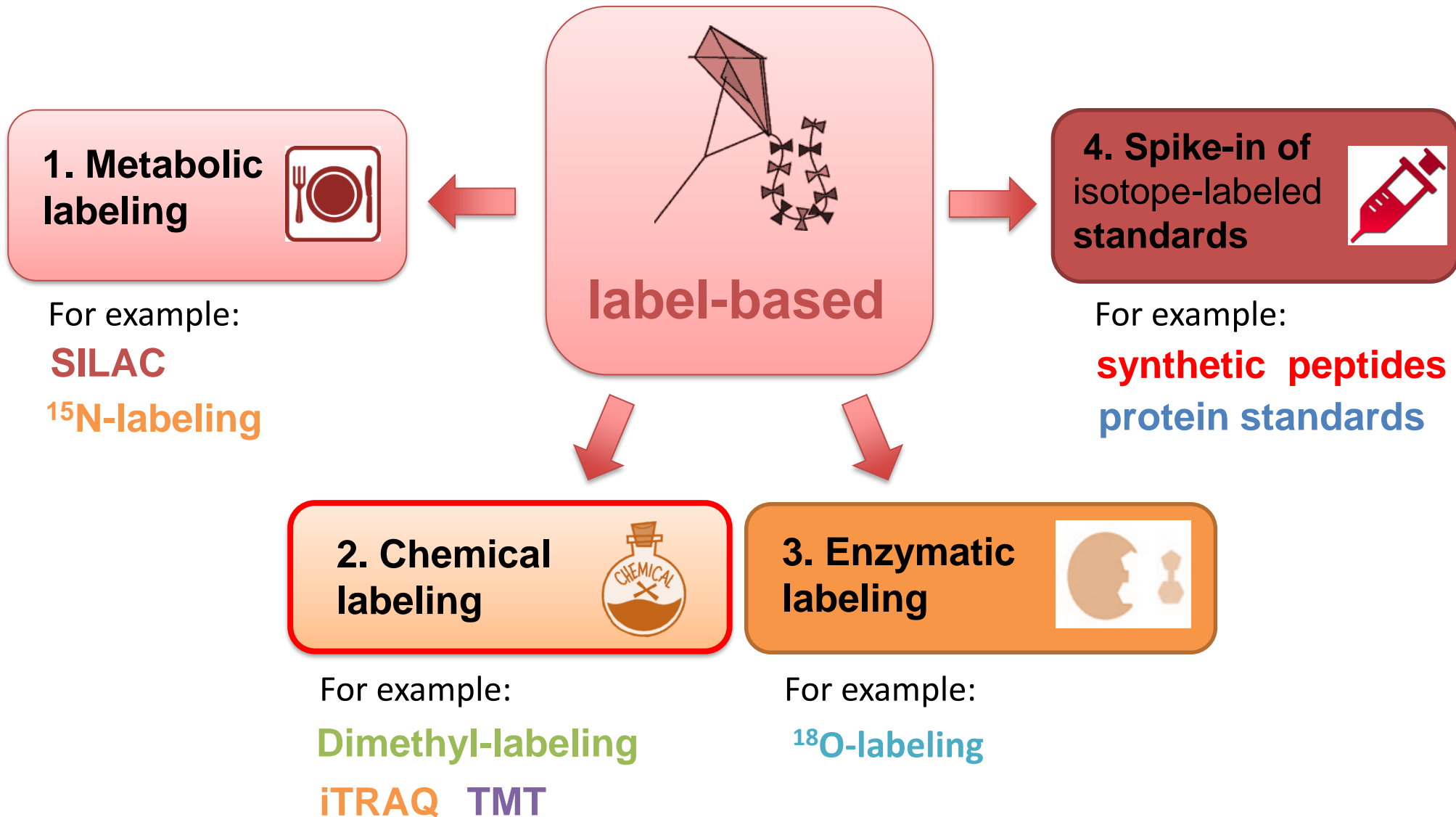
**Issue** when **handling AQUA peptides** (highly purified peptide solutions with precisely determined concentration):

**Significant Losses of AQUA** peptides due to **attachment to surfaces, degradation, freezing-thawing cycles**, etc.

### Tips:

- work with **low-binding vessels** and **low-binding pipetting tips**
- whenever possible **use glass ware**
- whenever possible use a complex sample background or a BSA solution as **blocking reagent**
- **aliquote** your standard stock solution upon arrival to **avoid freezing-thawing cycles**
- only purchase standards with known concentration **in solution** (not lyophilized to avoid any resuspension step)

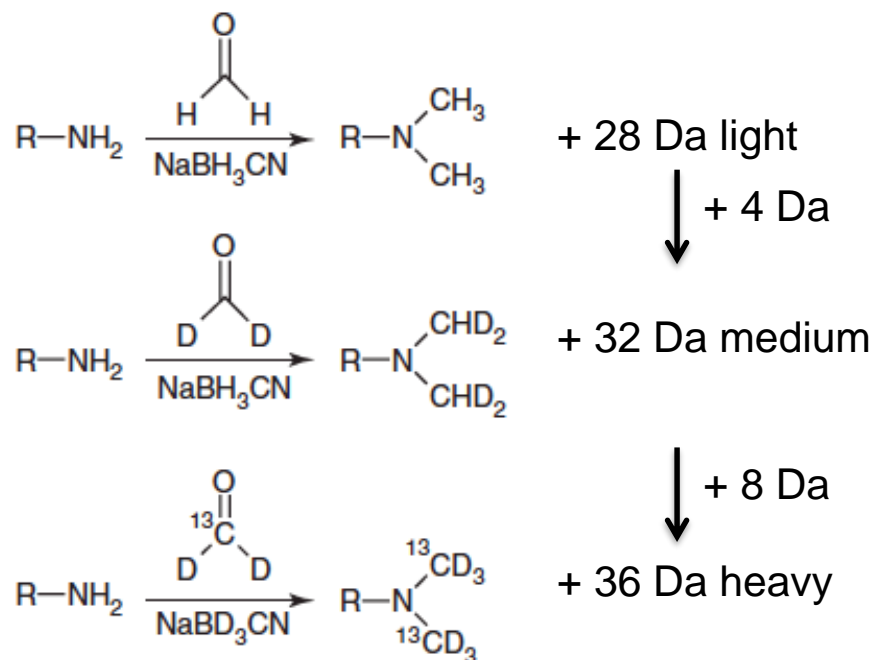
# Strategies for incorporating stable-isotopes





### Dimethyl labeling

→ reactivity for primary amines  
(N-terminus and Lysine residues)



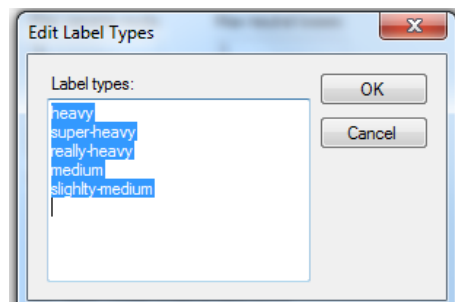
Boersema, P. J. et al., Nature Protocols, 4, 484-494 (2009).

# Skyline supports chemical labeling

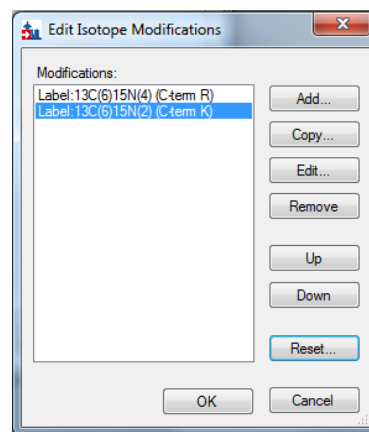
## 2. Chemical labeling



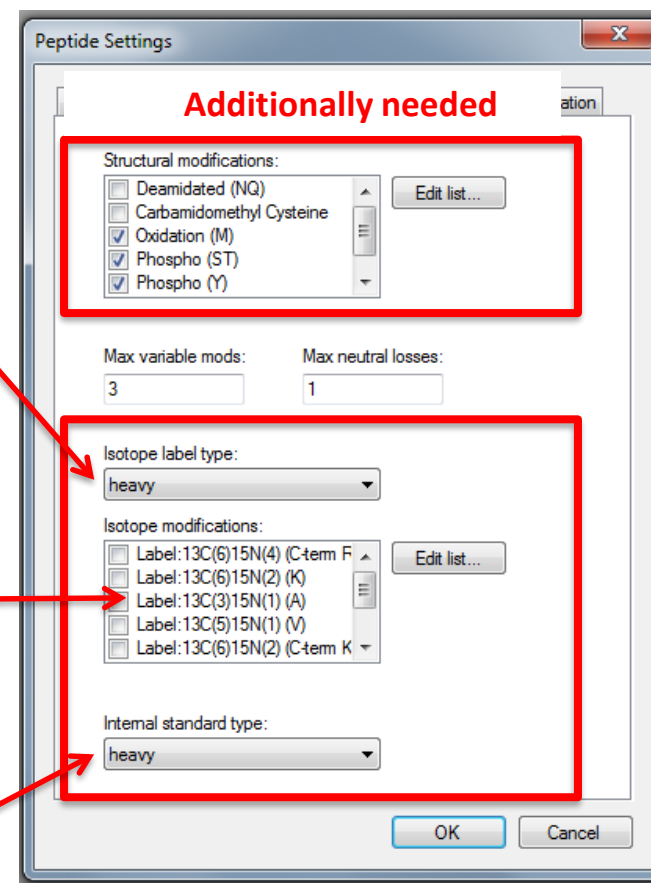
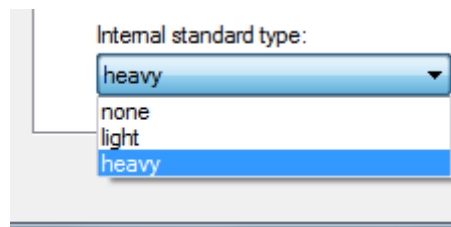
*You can define and name labels ; select those relevant for your experiment.*



*You can select a set of possible isotope modifications; enable those relevant for the label.*



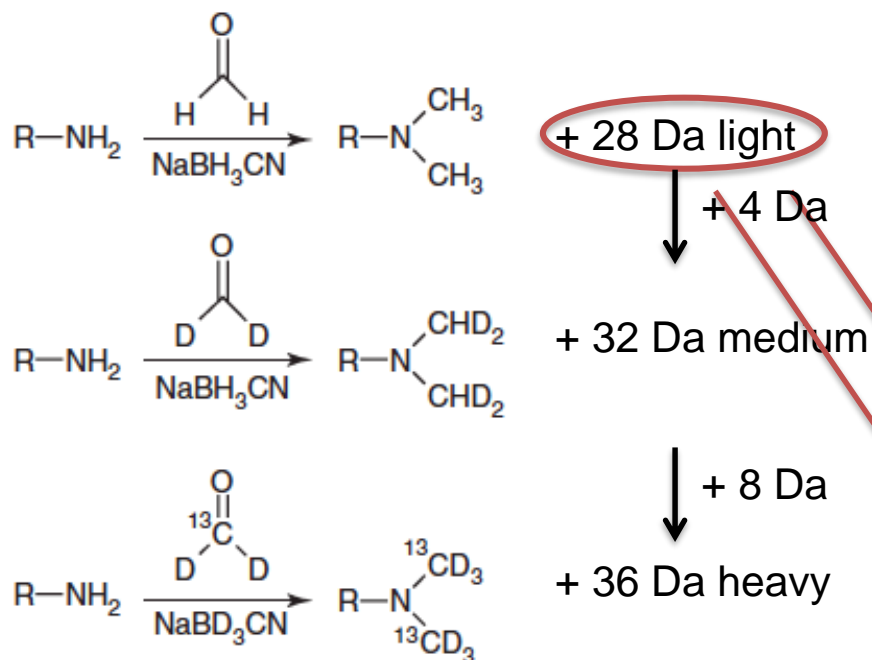
*You can select which (if any) label is the internal standard*





### Dimethyl labeling

→ reactivity for primary amines  
(N-terminus and Lysine residues)



Boersema, P. J. et al., Nature Protocols, 4, 484-494 (2009).

### 1. Define structural modification

Two screenshots of the 'Edit Structural Modification' dialog box in Skyline software, illustrating the configuration for dimethyl labeling.

**Top Screenshot:**

- Name: Dimethyl (K)
- Amino acid: K
- Terminus: (empty)
- Variable: ☐
- Chemical formula: C2H4
- Monoisotopic mass: 28.0313

**Bottom Screenshot:**

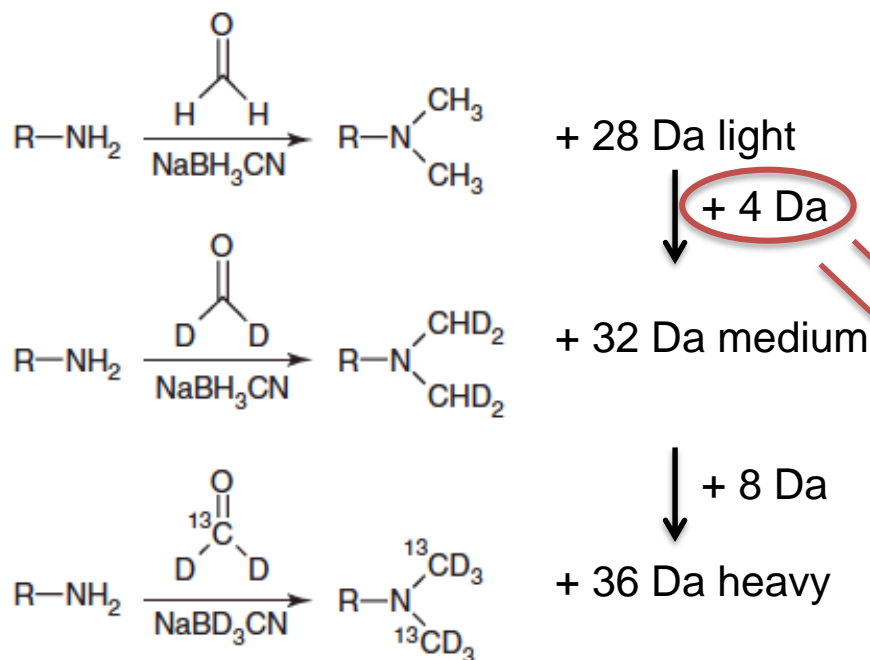
- Name: Dimethyl (N-term)
- Amino acid: (empty)
- Terminus: N
- Variable: ☐
- Chemical formula: C2H4
- Monoisotopic mass: 28.0313
- Average mass: 28.05346
- Loss >>

Red circles and arrows highlight the 'Name' field, 'Amino acid' field, 'Terminus' field, 'Variable' checkbox, 'Chemical formula' field, and 'Monoisotopic mass' field in both dialog boxes.



### Dimethyl labeling

→ reactivity for primary amines  
(N-terminus and Lysine residues)



Boersema, P. J. et al., Nature Protocols, 4, 484-494 (2009).

### 2. Define isotope modification

Two screenshots of the 'Edit Isotope Modification' dialog box in Skyline software.

**Top Screenshot:**

- Name: Dimethyl:2H(4) (N-term)
- Amino acid: [dropdown]
- Terminus: N
- Chemical formula: ☒ H<sup>4</sup> - H<sub>4</sub>
- Monoisotopic mass: 4.025107
- Relative retention time: Matching

**Bottom Screenshot:**

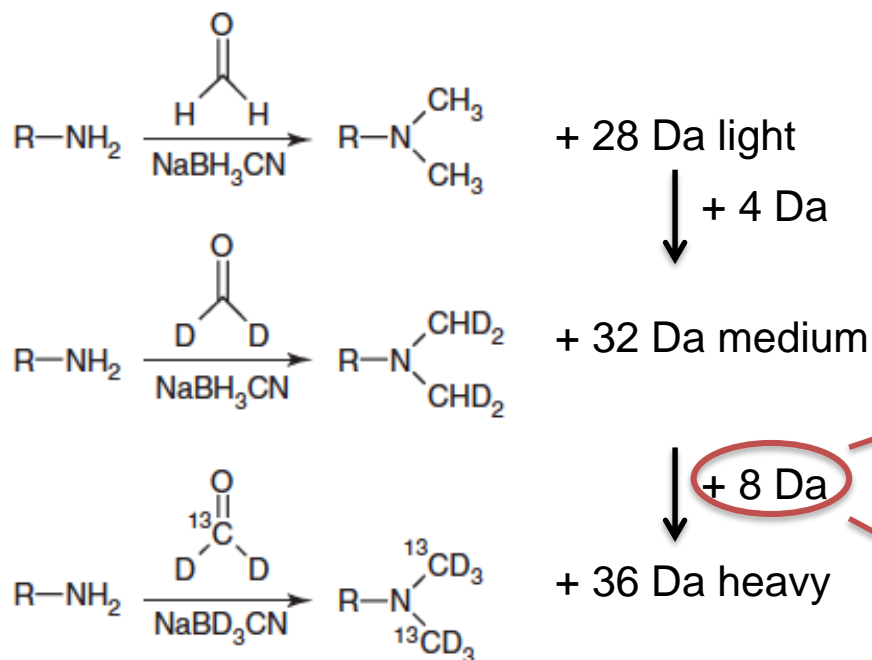
- Name: Dimethyl: 2H(4) (K)
- Amino acid: K
- Terminus: [dropdown]
- Chemical formula: ☒ H<sup>4</sup> - H<sub>4</sub>
- Monoisotopic mass: 4.025107
- Average mass: 4.024647
- Relative retention time: Matching

Red circles and arrows highlight the 'Name' field and the 'Monoisotopic mass' field in both dialog boxes.



### Dimethyl labeling

→ reactivity for primary amines  
(N-terminus and Lysine residues)



### 2. Define isotope modification

**Edit Isotope Modification**

Name: Dimethyl:2H(6)13C(2) (K)

Amino acid: K Terminus:

☒ Chemical formula

Chemical formula: C\*2H\*6 - C2H6

Monoisotopic mass: 8.04437

Relative retention time: Matching

**Edit Isotope Modification**

Name: Dimethyl:2H(6)13C(2) (N-term)

Amino acid: N Terminus: N

☒ Chemical formula

Chemical formula: C\*2H\*6 - C2H6

Monoisotopic mass: 8.04437 Average mass: 8.02198

Relative retention time: Matching

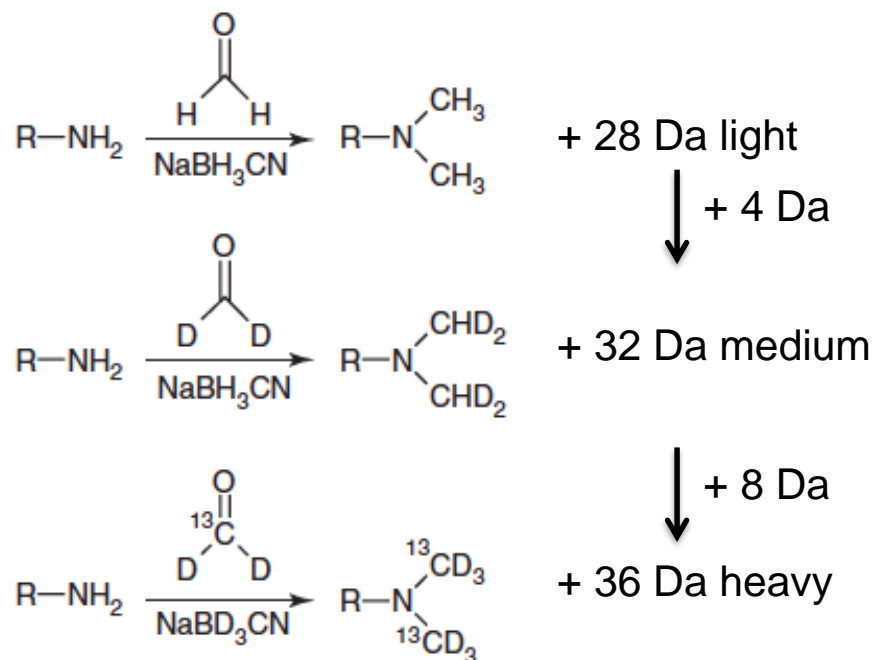
Boersema, P. J. et al., Nature Protocols, 4, 484-494 (2009).





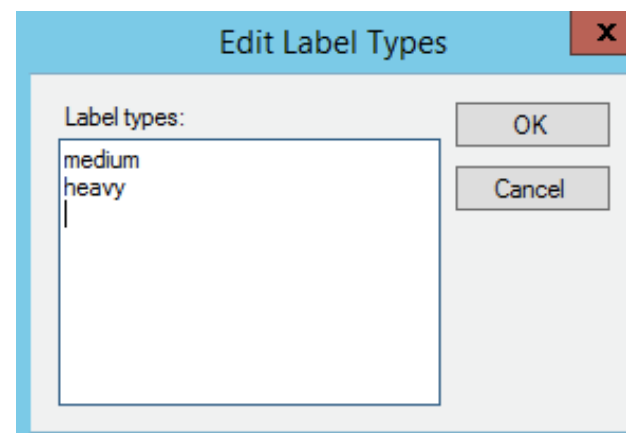
### Dimethyl labeling

→ reactivity for primary amines  
(N-terminus and Lysine residues)



Boersema, P. J. et al., Nature Protocols, 4, 484-494 (2009).

### 3. Create additional label type



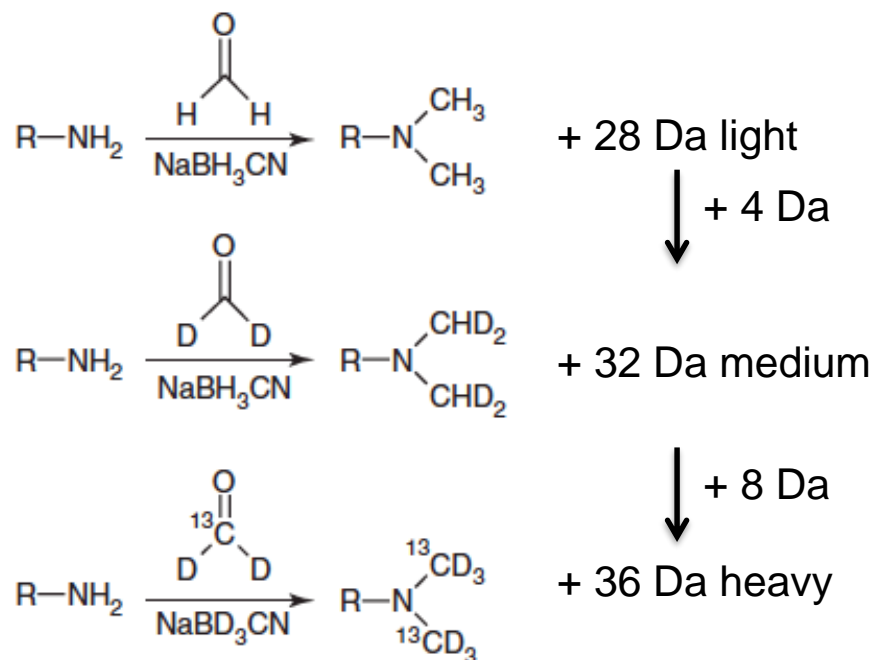
### 4. Define Label Types

Isotope label type:	Isotope label type:
medium	heavy
Isotope modifications:	Isotope modifications:
<input checked="" type="checkbox"/> Dimethyl: 2H(4) (K)	<input type="checkbox"/> Dimethyl: 2H(4) (K)
<input checked="" type="checkbox"/> Dimethyl: 2H(4) (N-term)	<input type="checkbox"/> Dimethyl: 2H(4) (N-term)
<input type="checkbox"/> Dimethyl: 2H(6)13C(2) (K)	<input checked="" type="checkbox"/> Dimethyl: 2H(6)13C(2) (K)
<input type="checkbox"/> Dimethyl: 2H(6)13C(2) (N-term)	<input checked="" type="checkbox"/> Dimethyl: 2H(6)13C(2) (N-term)



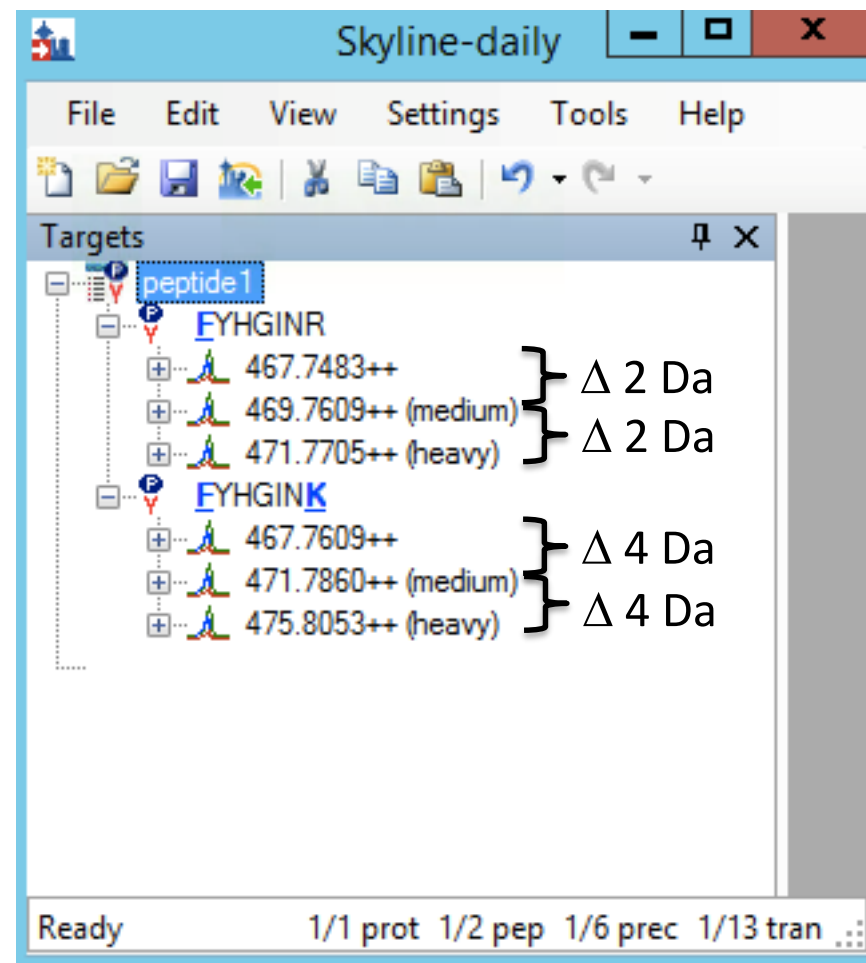
### Dimethyl labeling

→ reactivity for primary amines  
(N-terminus and Lysine residues)



Boersema, P. J. et al., Nature Protocols, 4, 484-494 (2009).

### 5. Isotope labeled variants will appear in peptide list

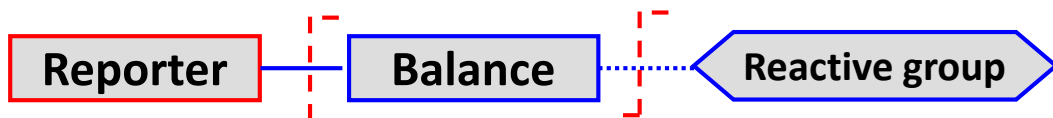




### iTRAQ/TMT

(isobaric tags for relative and absolute quantification / tandem mass tags)

→ reactivity for primary amines  
(N-terminus and Lysine residues)



Amine specific

114	31
115	30
116	29
117	28

*Mix all samples  
for one  
MS/MS analysis*

$$M_{Reporter} + M_{Balance} = \text{Constant}$$

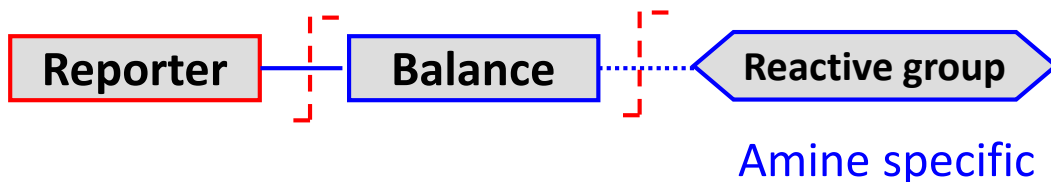
### 1. Define structural modification



### iTRAQ/TMT

(isobaric tags for relative and absolute quantification / tandem mass tags)

→ reactivity for primary amines  
(N-terminus and Lysine residues)



114	31
115	30
116	29
117	28

*Mix all samples for one MS/MS analysis*

$$M_{Reporter} + M_{Balance} = \text{Constant}$$

## 2. Define reporter ions

# Skyline supports chemical labeling

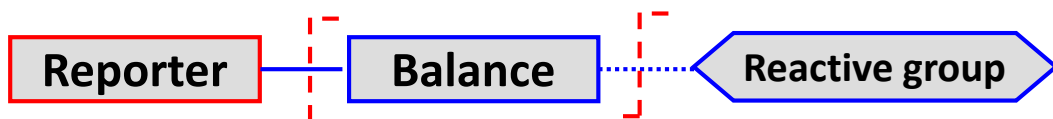
## 2. Chemical labeling



### iTRAQ/TMT

(isobaric tags for relative and absolute quantification / tandem mass tags)

→ reactivity for primary amines  
(N-terminus and Lysine residues)



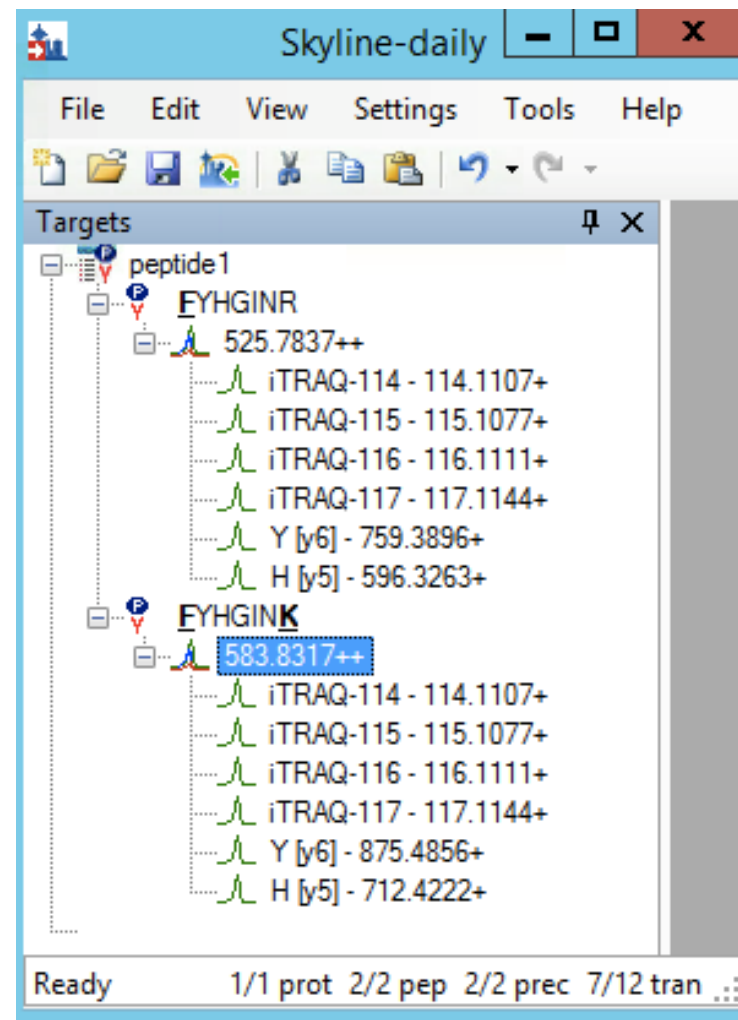
Amine specific

114	31
115	30
116	29
117	28

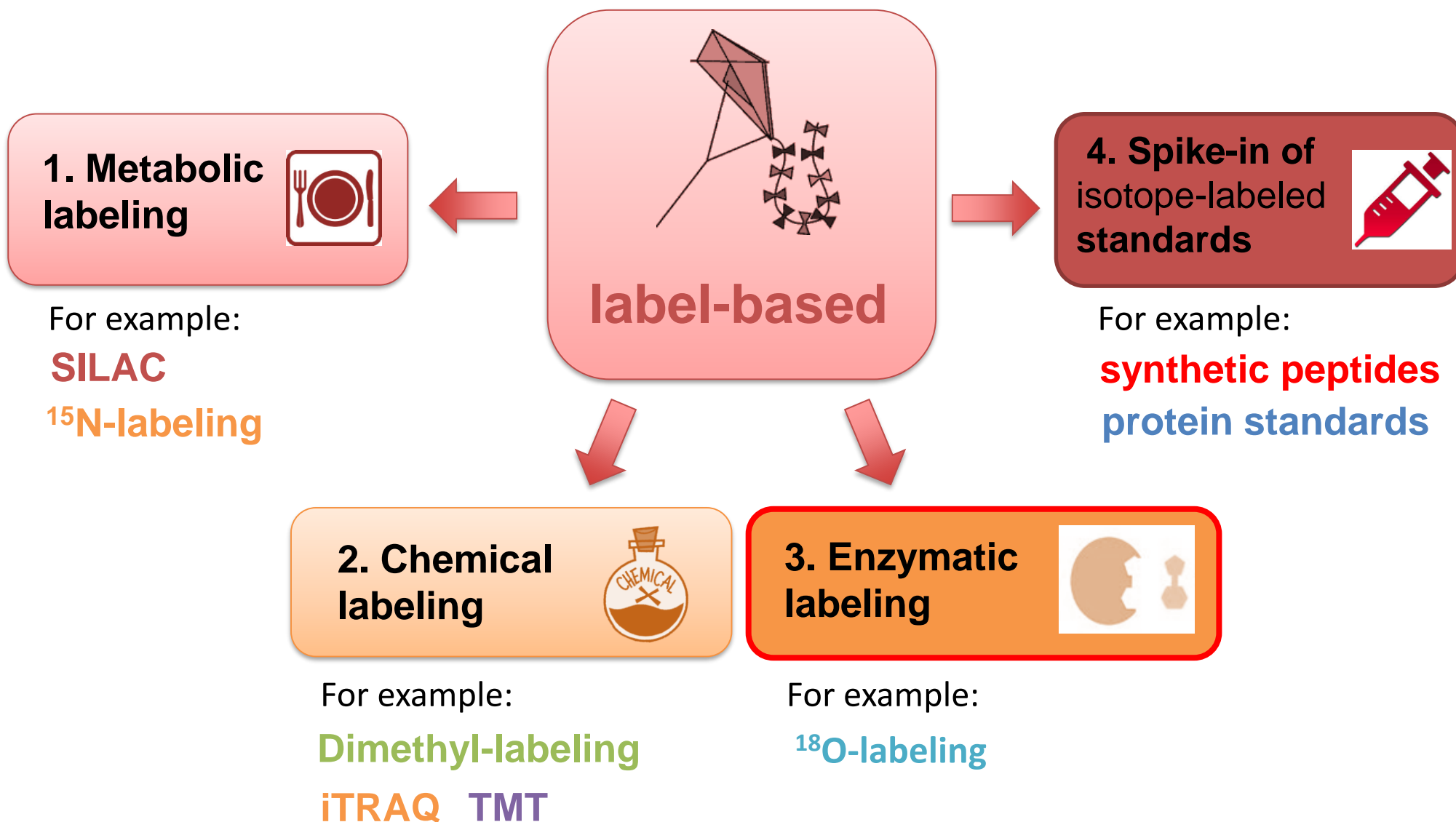
*Mix all samples  
for one  
MS/MS analysis*

$$M_{Reporter} + M_{Balance} = \text{Constant}$$

### 3. Labeled variants will appear in peptide list



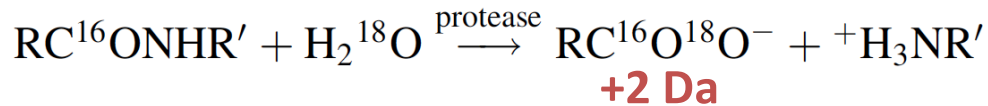
# Strategies for incorporating stable-isotopes



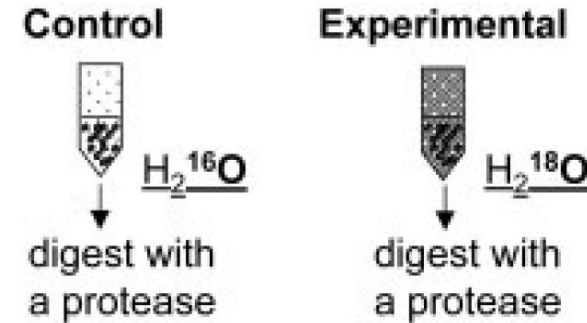
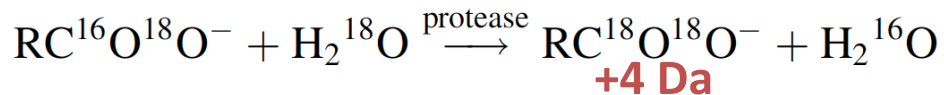


### Proteolytic $^{18}\text{O}$ labeling

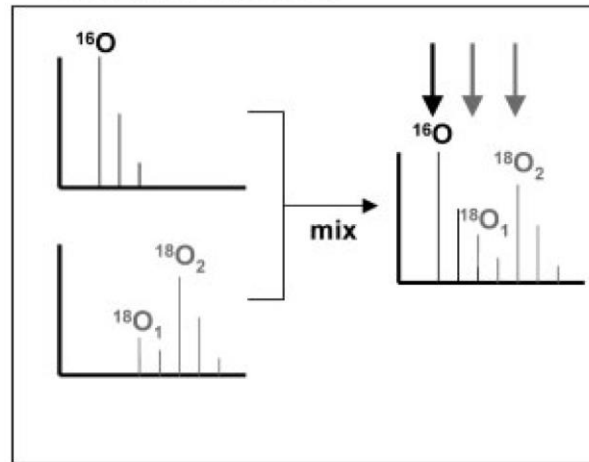
**Reaction 1 (peptide bond cleavage):**



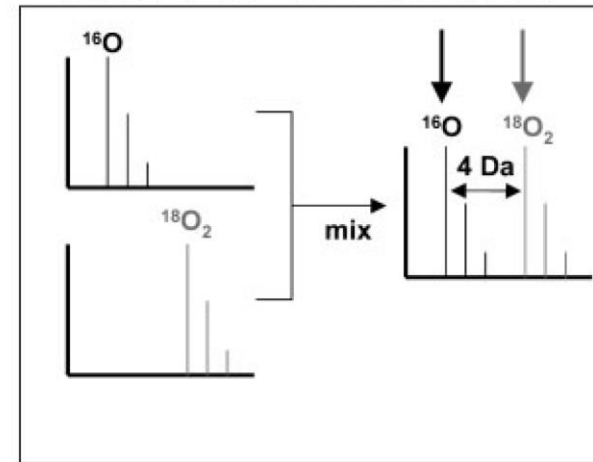
**Reaction 2 (carboxyl oxygen exchange):**



**A** Variable Incorporation



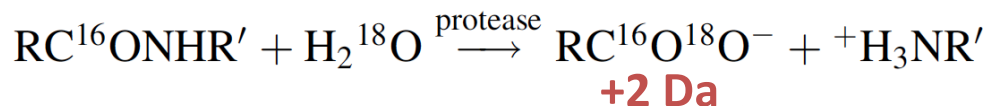
**B** Complete Two  $^{18}\text{O}$  Atoms Incorporation



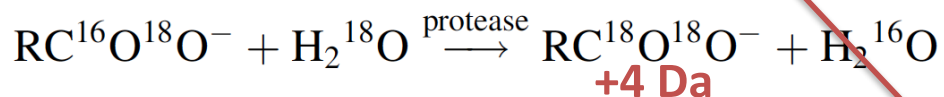


### Proteolytic $^{18}\text{O}$ labeling

Reaction 1 (peptide bond cleavage):



Reaction 2 (carboxyl oxygen exchange):



### 1. Define isotope modifications

**Edit Isotope Modification**

Name: Label:18O(1) (C-term)

Amino acid:  Terminus:

☒ Chemical formula

Chemical formula:

Monoisotopic mass: 2.004246

Relative retention time:

**Edit Isotope Modification**

Name: Label:18O(2) (C-term)

Amino acid:  Terminus:

☒ Chemical formula

Chemical formula:

Monoisotopic mass: 4.008492 Average mass:

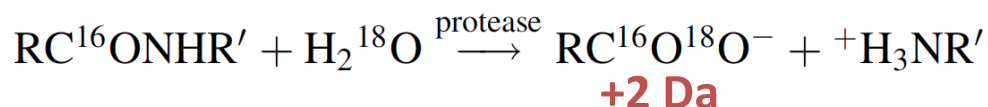
Relative retention time:



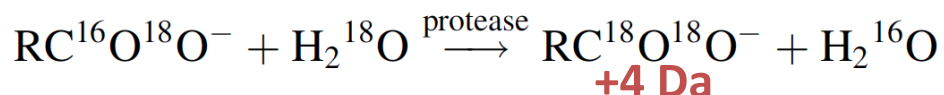


### Proteolytic $^{18}\text{O}$ labeling

Reaction 1 (peptide bond cleavage):

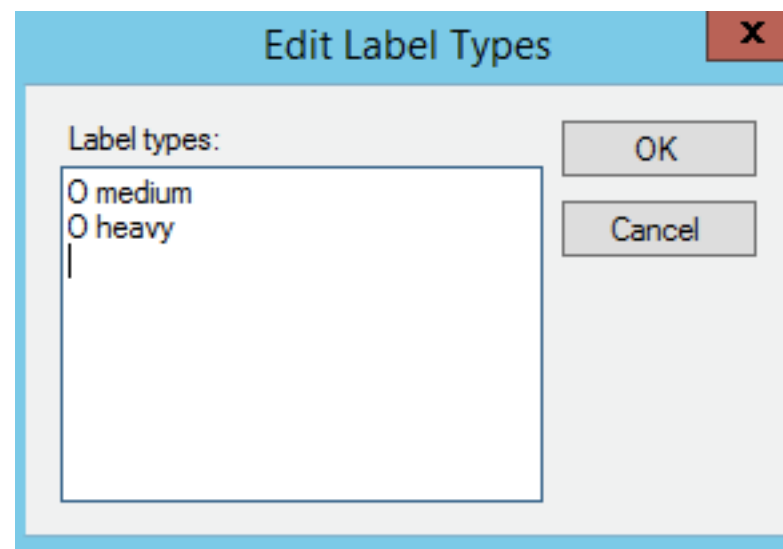


Reaction 2 (carboxyl oxygen exchange):



1. Define isotope modifications

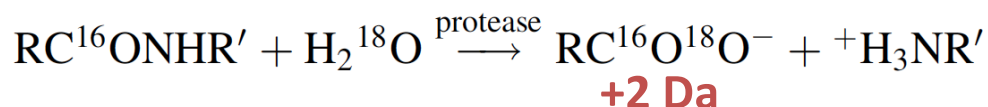
2. Create additional label type



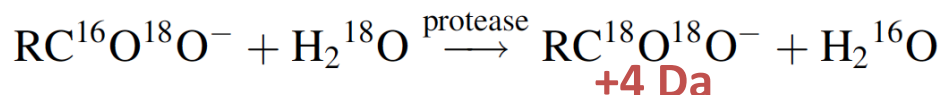


### Proteolytic $^{18}\text{O}$ labeling

Reaction 1 (peptide bond cleavage):



Reaction 2 (carboxyl oxygen exchange):



1. Define isotope modifications

2. Create additional label type

3. Define Label Types

Isotope label type:

O medium

Isotope modifications:

- ☒ Label:18O(1) (C-term)
- ☐ Label:18O(2) (C-term)

Isotope label type:

O heavy

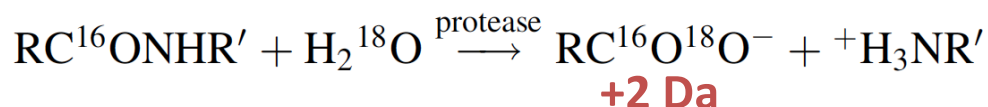
Isotope modifications:

- ☐ Label:18O(1) (C-term)
- ☒ Label:18O(2) (C-term)

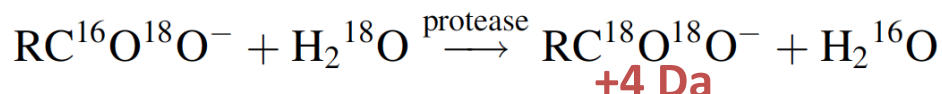


### Proteolytic $^{18}\text{O}$ labeling

Reaction 1 (peptide bond cleavage):



Reaction 2 (carboxyl oxygen exchange):

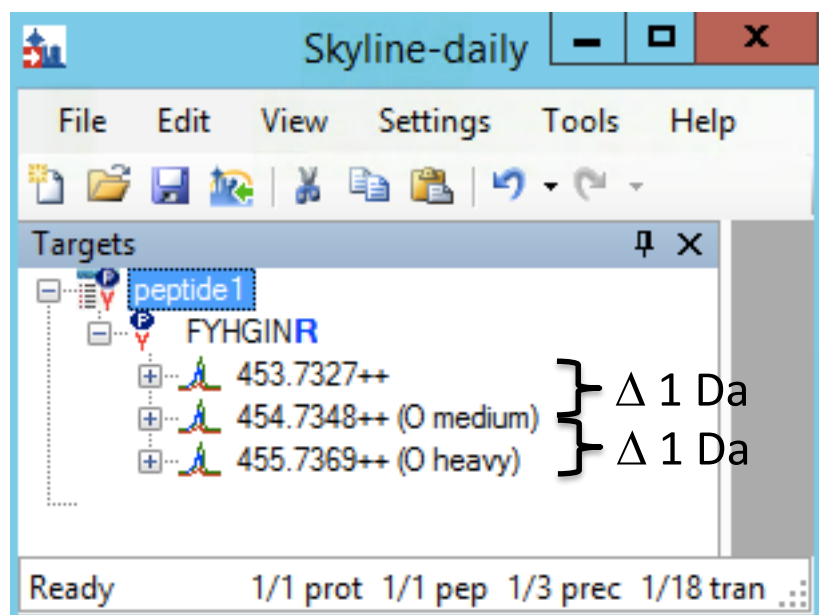


1. Define isotope modifications

2. Create additional label type

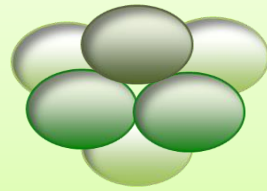
3. Define Label Types

4. Isotope labeled variants will appear in peptide list



**For which projects  
does absolute  
protein quantification  
really matter?**

**protein complex  
stoichiometries**



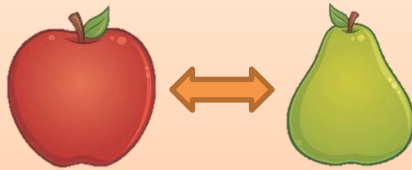
3 : 2 : 1

**mathematical  
modeling**



- rate constants
- kinetic fluxes
- energy budget
- etc.

**data cross-comparison**



- inter-experimental
- inter-laboratory
- Inter-MS platform
- inter-organism

**absolute  
protein  
concentration**

**pharmaceutical and  
biomedical industry**



- quality control
- therapeutic analysis
- food safety
- sports drug testing
- etc.

**protein biomarker  
development**



# The time-point of heavy-light sample combination matters!

The **more early** an isotope-labeled standard is added into the samples during sample preparation, **more accurate** the results!

