Lecture:
Isotope Labeled Standards in Skyline

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ETH Zürich
Switzerland

Technische Universität München

ETH Zürich

Outline

Introduction - Ariel

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

Improve confident peptide identification - Ariel

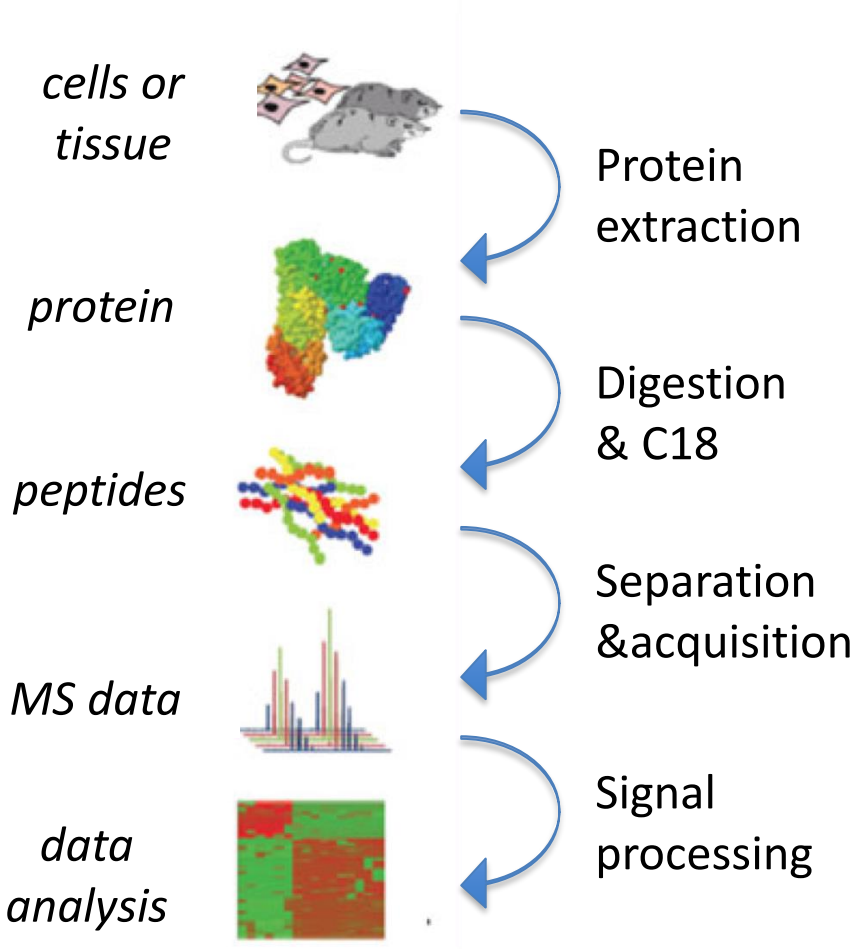
- Generating a reference for identification
- Using a reference for peak selection
- Using a reference for optimal quantification

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!

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

The multitude of quantitative MS-applications

SpikeTides

^{18}O-labeling

SILAC

emPAI

iTRAQ

IBAQ

PSAQ

ICAT

APEX

PAI

QconCAT

^{15}N-labeling

Dimethyl-labeling

mTRAQ

pREST

Super-SILAC

ICPL

NSAF

AQUA

ALF

TMT

Label-free

The multitude of quantitative MS-applications

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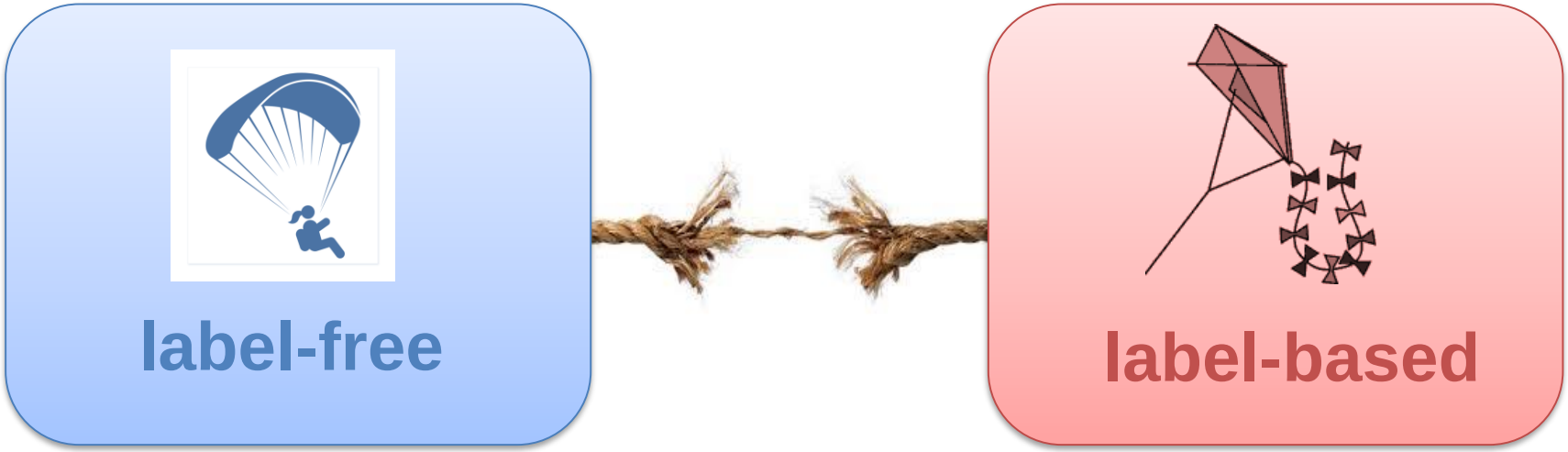
Label-free

Label-free versus label-based quantification



APEX IBAQ ALF
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SpikeTides QconCAT
ICAT pREST TMT SILAC
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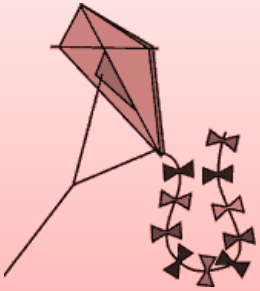
Label-free versus label-based quantification

label-free



label-based

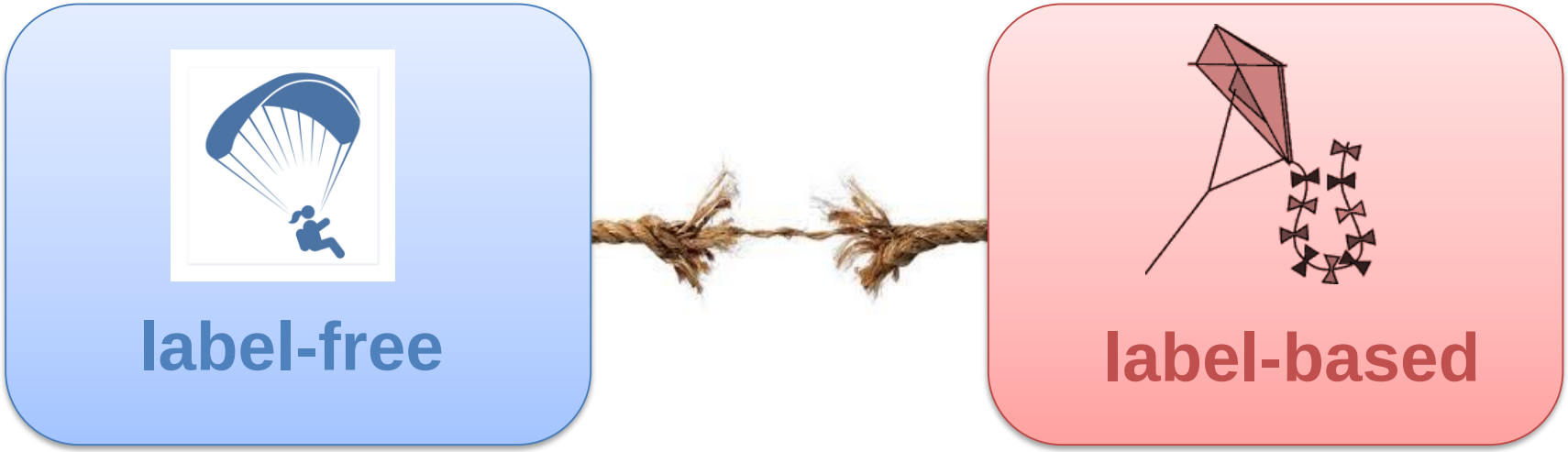
Major advantages label-free:

- **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**

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



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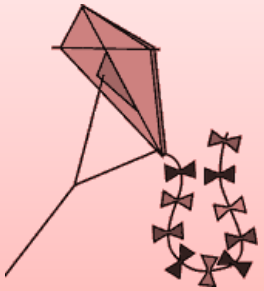
Strategies for incorporating stable-isotopes



label-based

1. Metabolic labeling

For example:

SILAC

^{15}N-labeling

2. Chemical labeling

For example:

Dimethyl-labeling

iTRAQ TMT

3. Enzymatic labeling

For example:

^{18}O-labeling

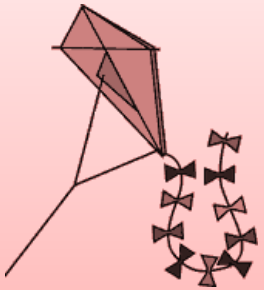
4. Spike-in of isotope-labeled **standards**

For example:

synthetic peptides

protein standards

Pros and Cons of stable-isotope labeling strategies



label-based

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

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

- expansive
- 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

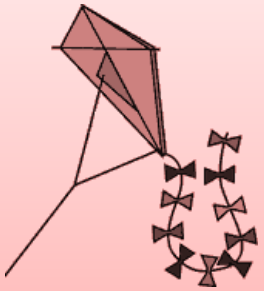
Strengths

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

Weaknesses

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

Strategies for incorporating stable-isotopes



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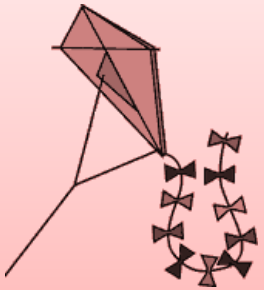

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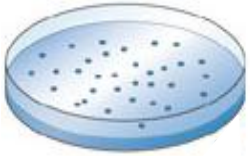
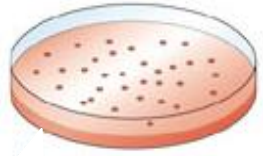
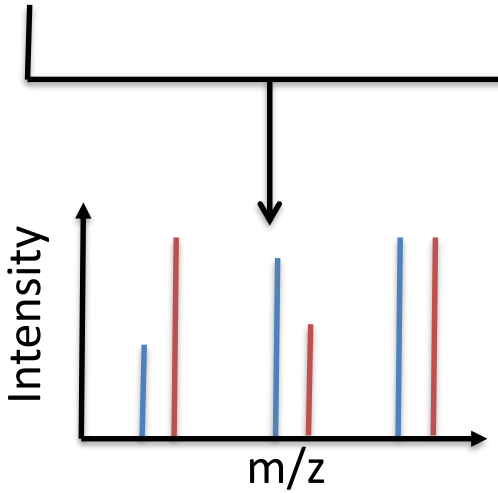


Condition A
light (^{12}C ^{14}N)
arginine and lysine



Condition B
heavy (^{13}C ^{15}N)
arginine and lysine

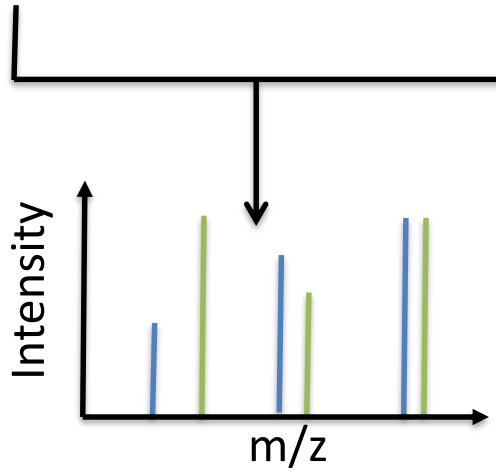


→ constant mass shift
8 Da (lysine) or 10 Da (arginine)

^{15}N labeling

Condition A
light ^{14}N

Condition B
heavy ^{15}N
(^{15}N-labeled $(NH_4)_2SO_4$)

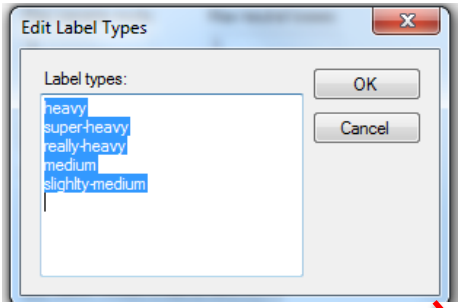


→ variable mass shift depending on the number of N-atoms in the peptide/fragment

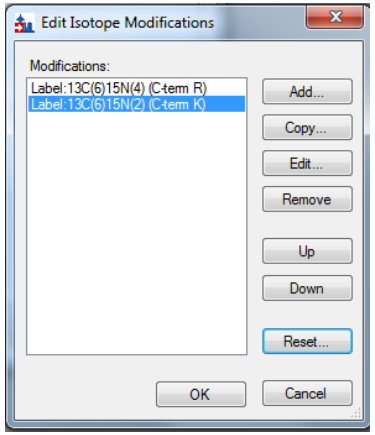
Skyline supports metabolic labeling

1. Metabolic labeling

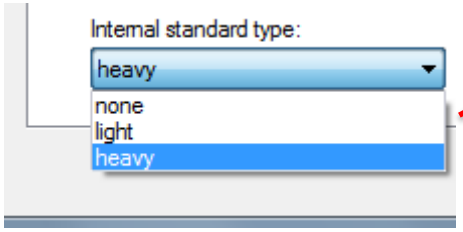
You can define and name labels ; select those relevant for your experiment.

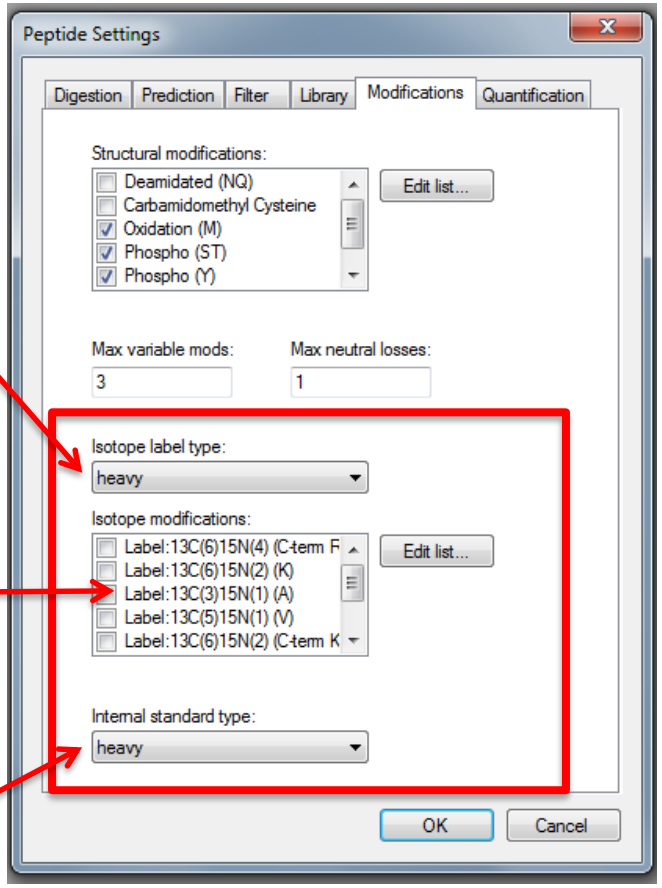


You can select a set of possible isotope modifications; enable those relevant for the label.



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





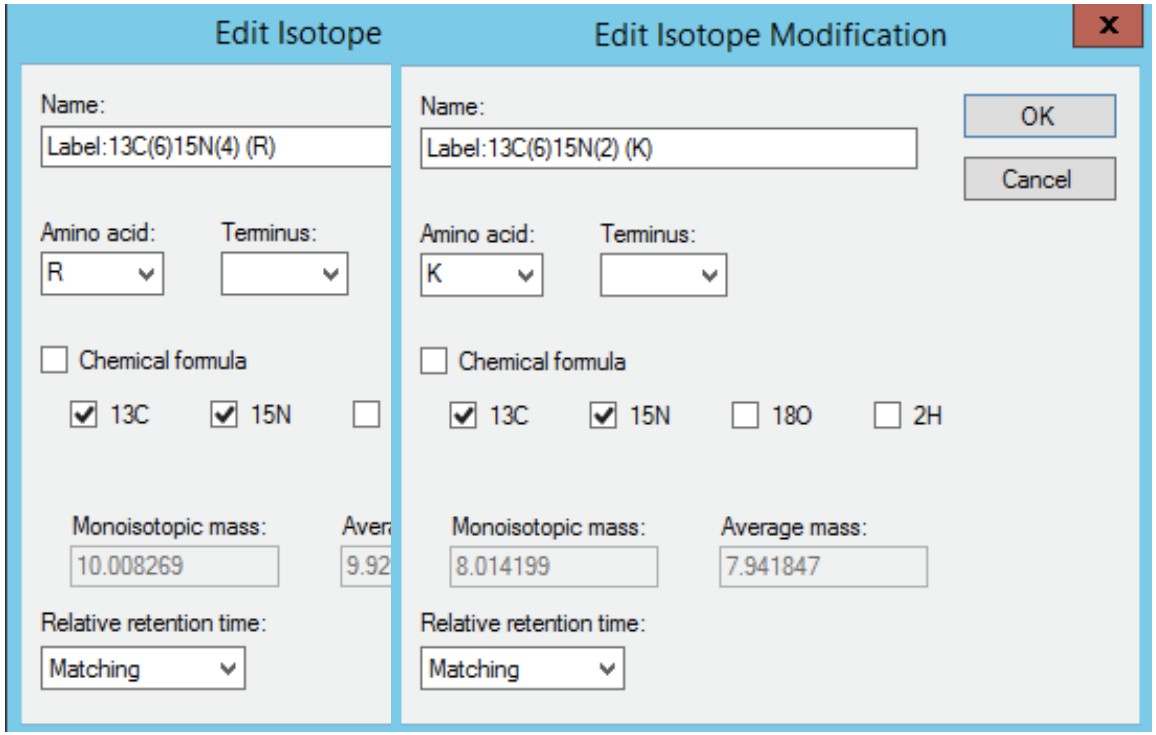
Skyline supports metabolic labeling

1. Metabolic labeling

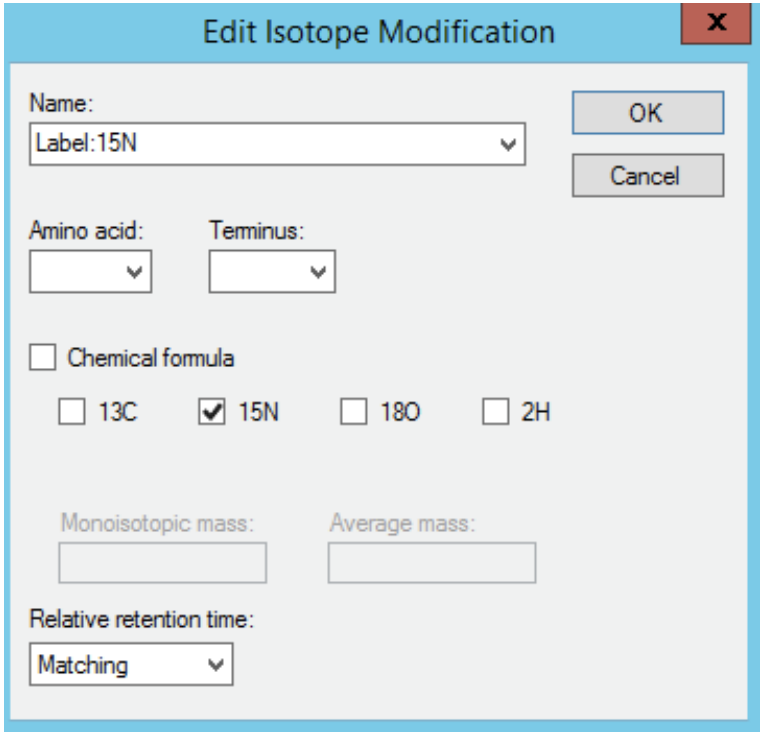
1. Define isotope modifications

→ Peptide Settings → Modifications → Isotope Modification

SILAC



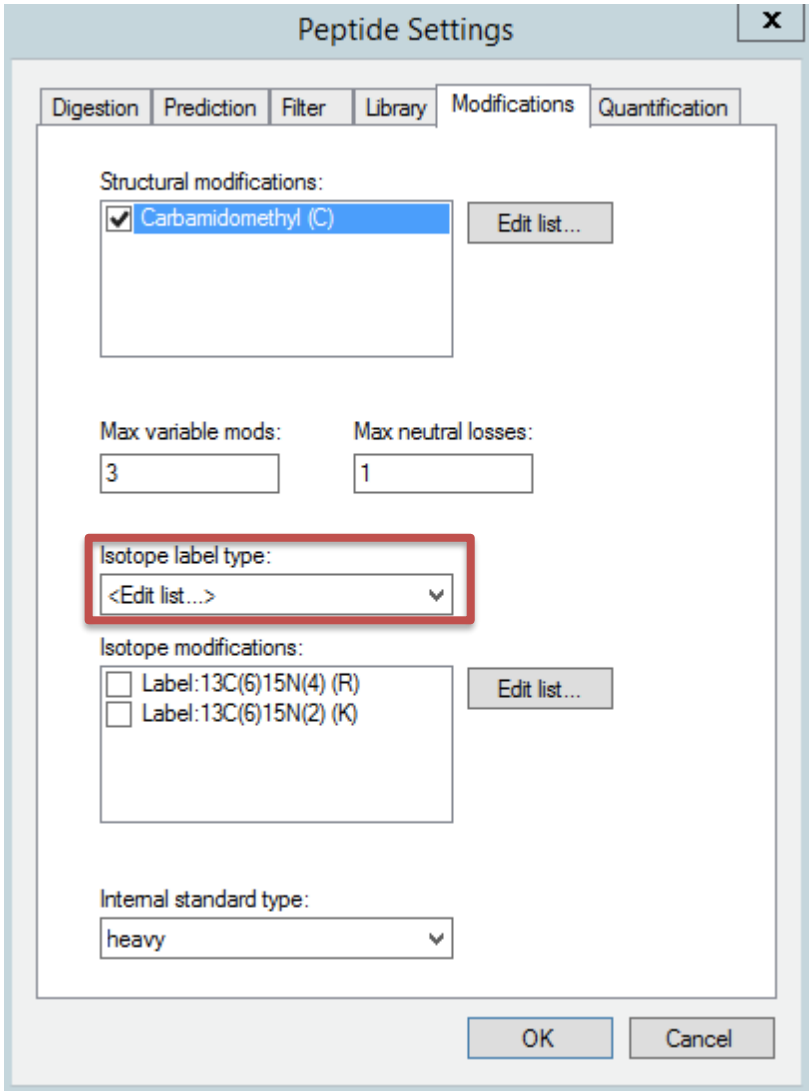
^{15}N labeling



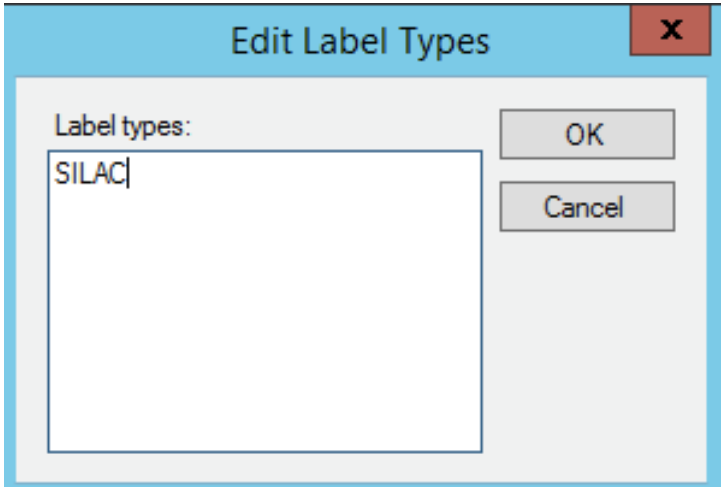
Skyline supports metabolic labeling

1. Metabolic labeling

2. Create additional label type



SILAC



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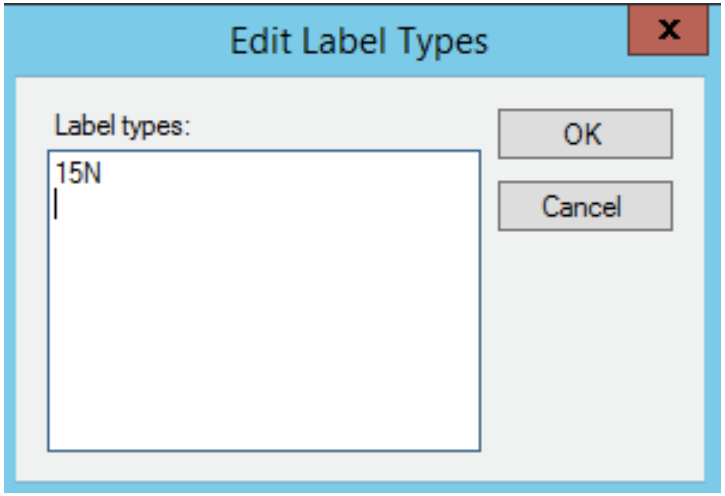
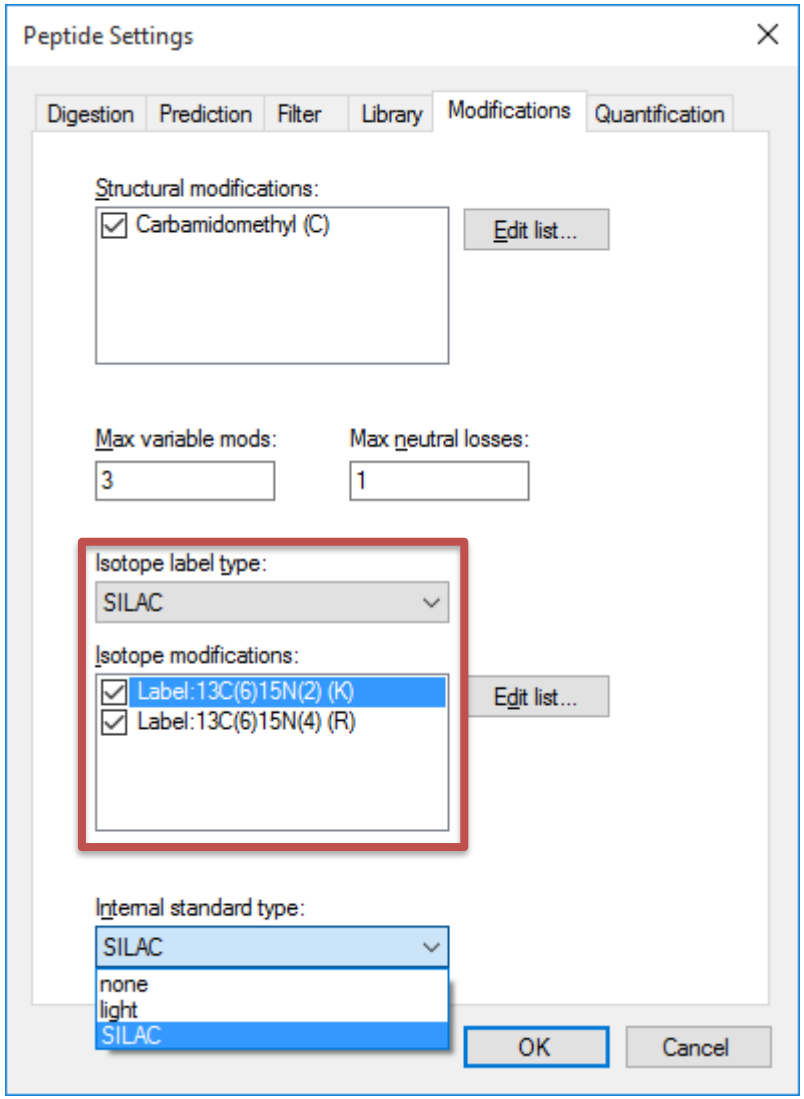


Skyline supports metabolic labeling

1. Metabolic labeling

3. Define Label Types

SILAC



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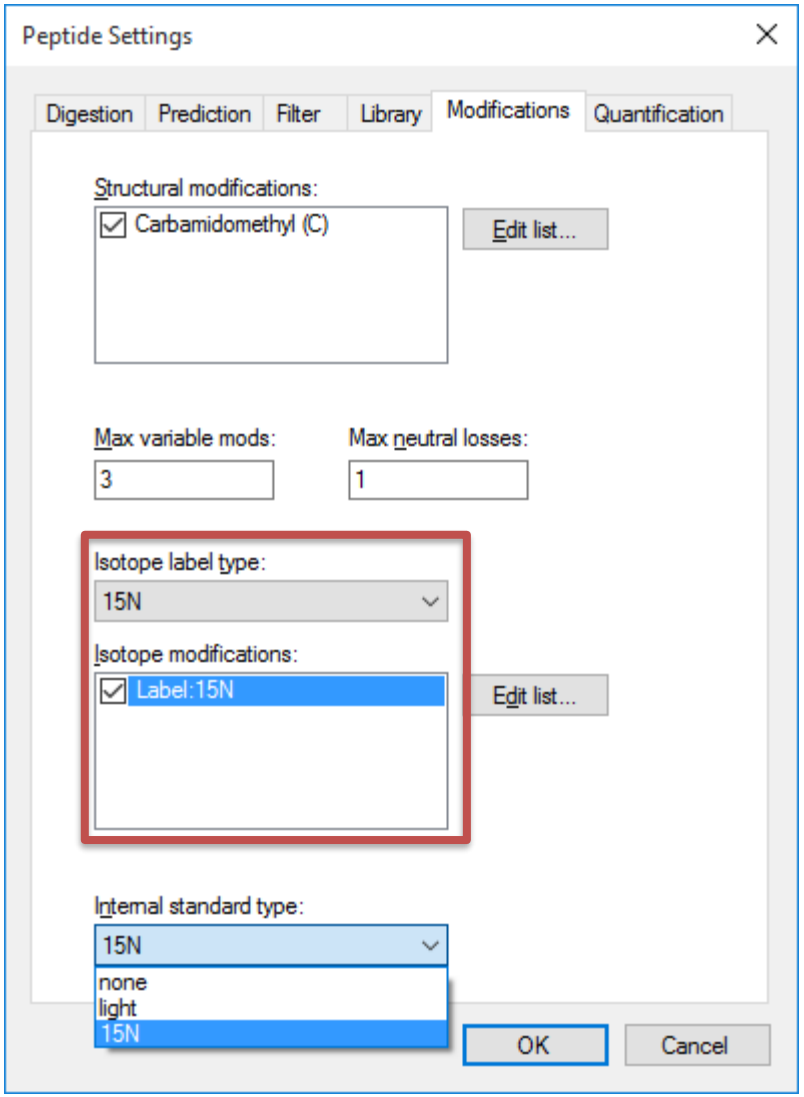
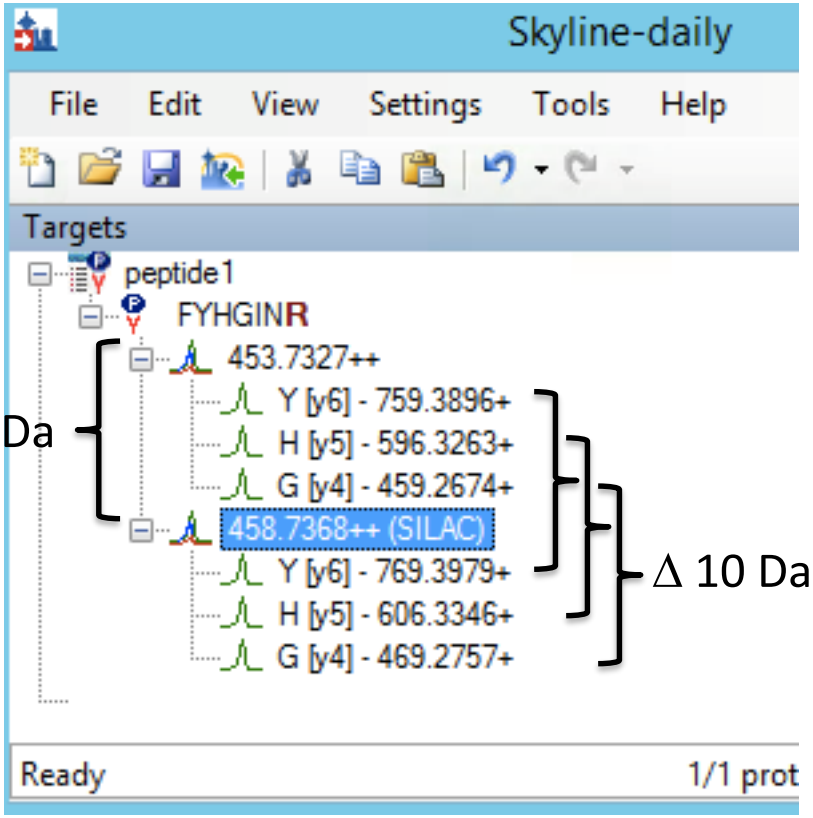


Skyline supports metabolic labeling

1. Metabolic labeling

4. Isotope labeled variants will appear in peptide list

SILAC



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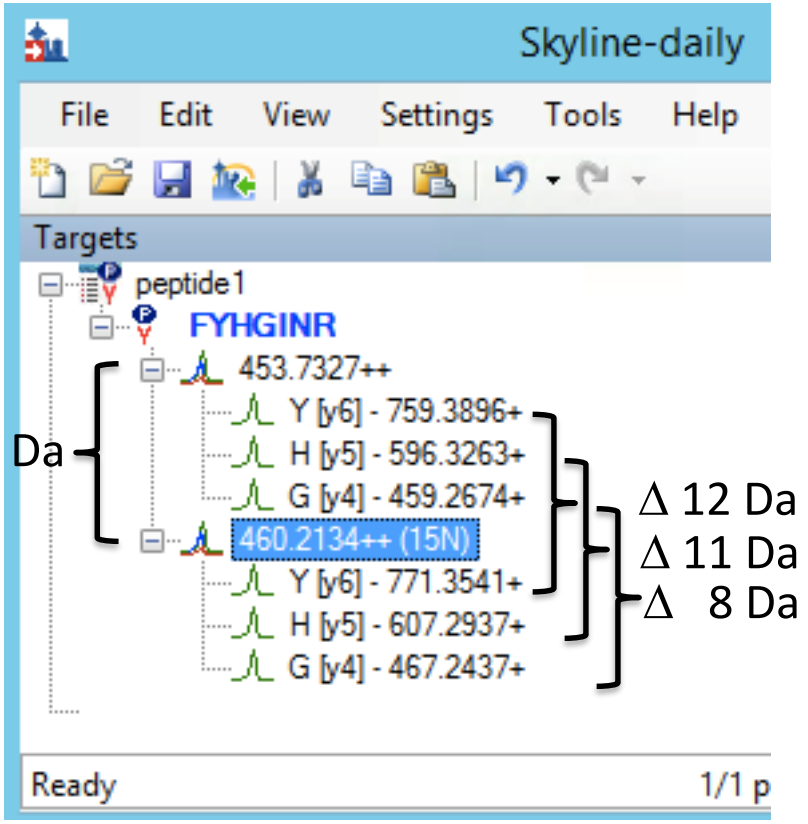
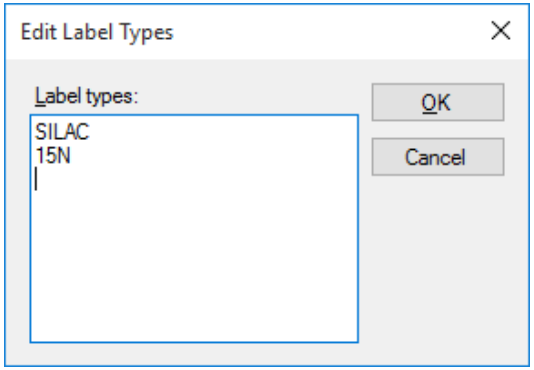


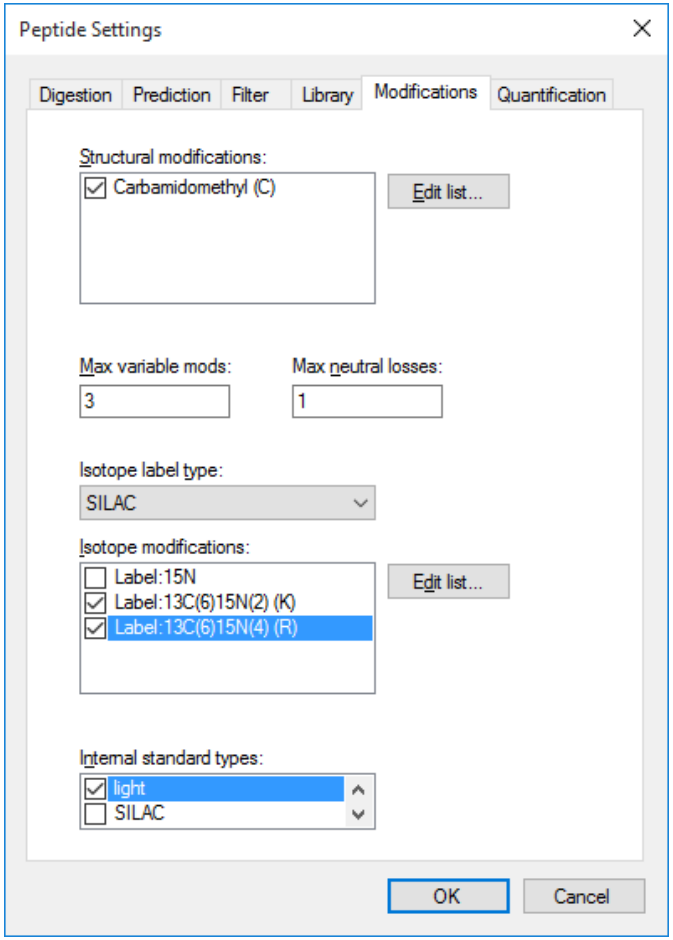
Skyline supports metabolic labeling

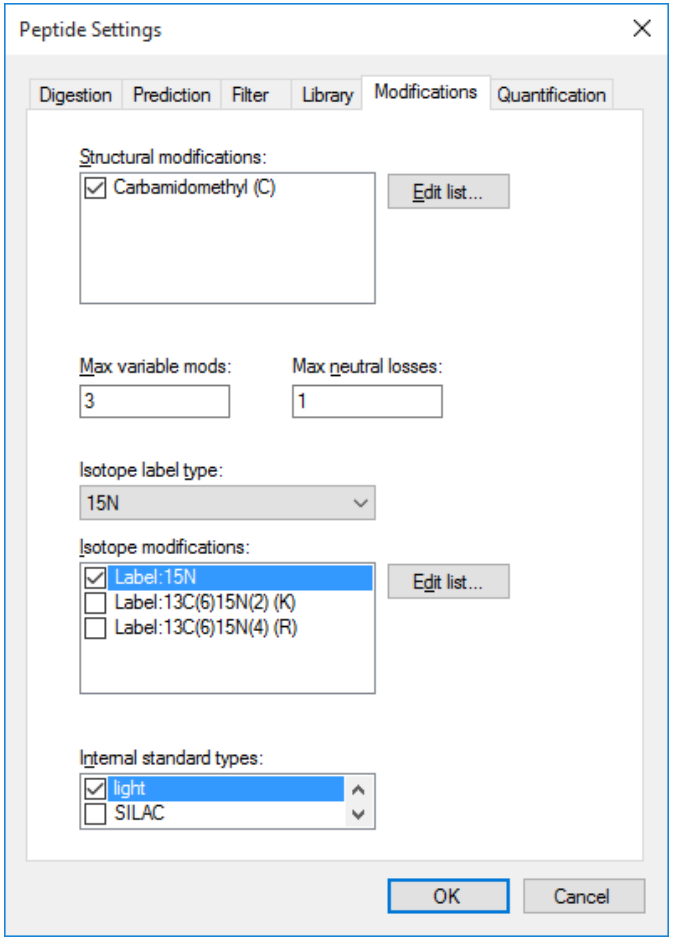
1. Metabolic labeling

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

light AND SILAC and ^{15}N labeling







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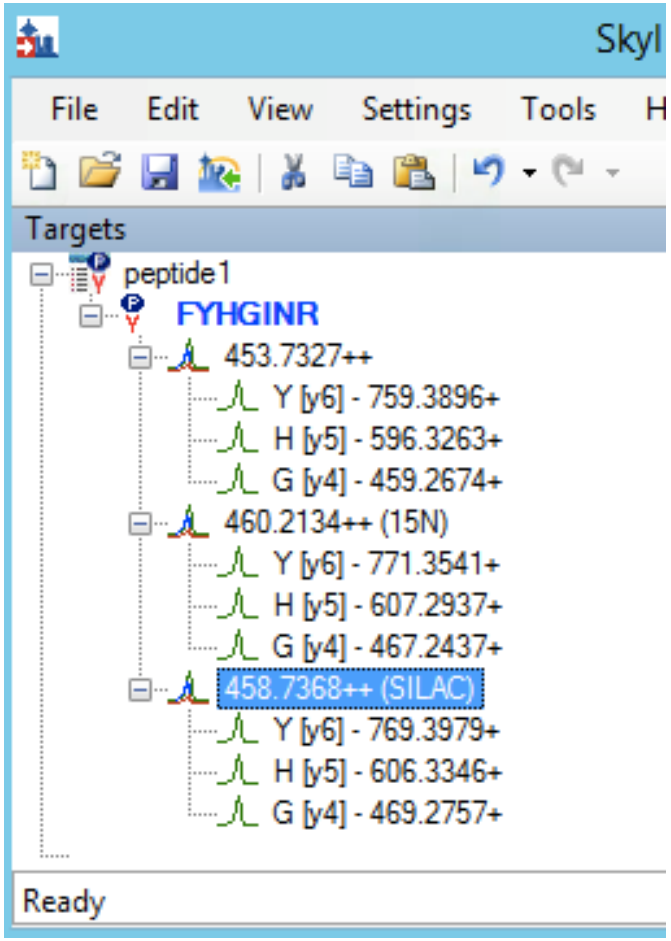
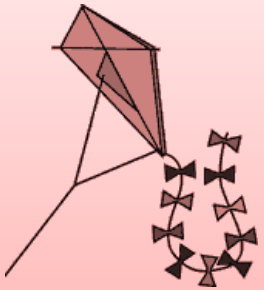


Strategies for incorporating stable-isotopes



label-based

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For example:

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3. Enzymatic labeling

For example:

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

For example:

synthetic peptides

protein standards

Spike-in of isotope-labeled standards

4. Spike-ins

synthetic peptides

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

Different purity forms available from various companies:

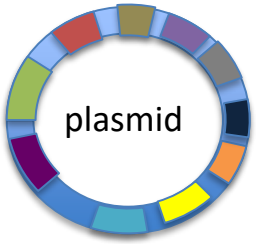
- **Crude peptides** = cheap

→ Library generation and relative quant.

- **Highly purified peptides (AQUA/SIS)** = expansive

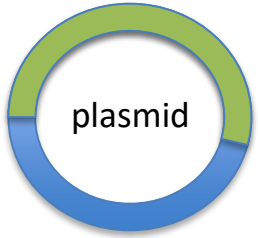
→ Absolute quant.

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

Skyline supports spike-in labeling

4. Spike-in

synthetic peptides

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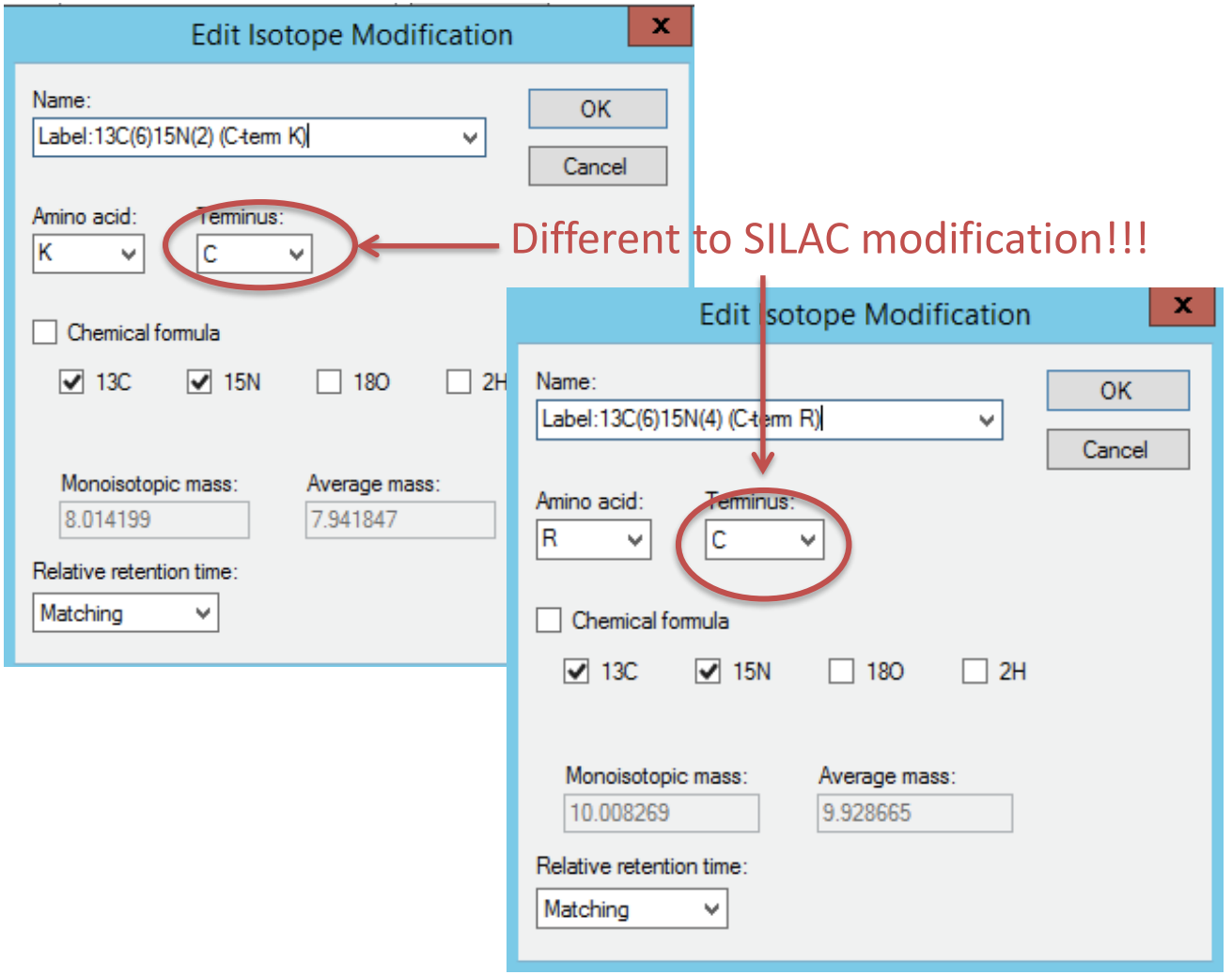

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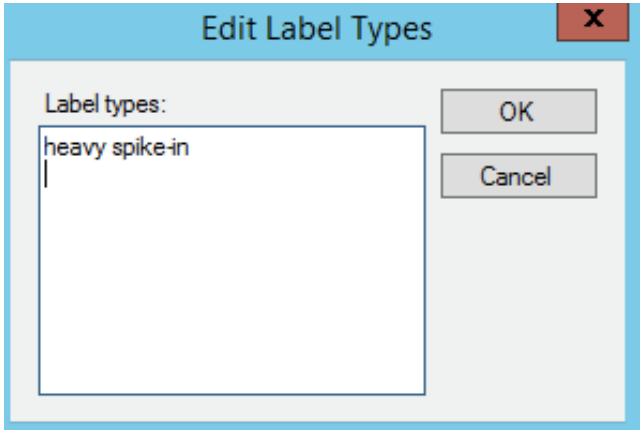

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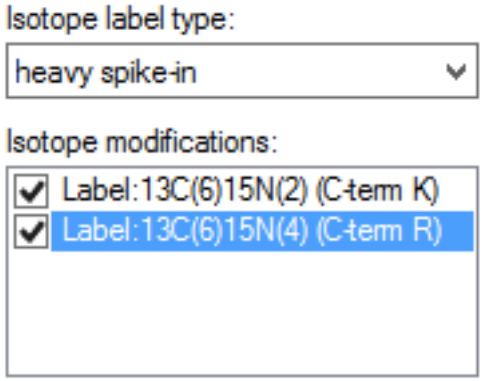
1. **Define isotope modifications**
2. **Create additional label type**
3. **Define Label Types**



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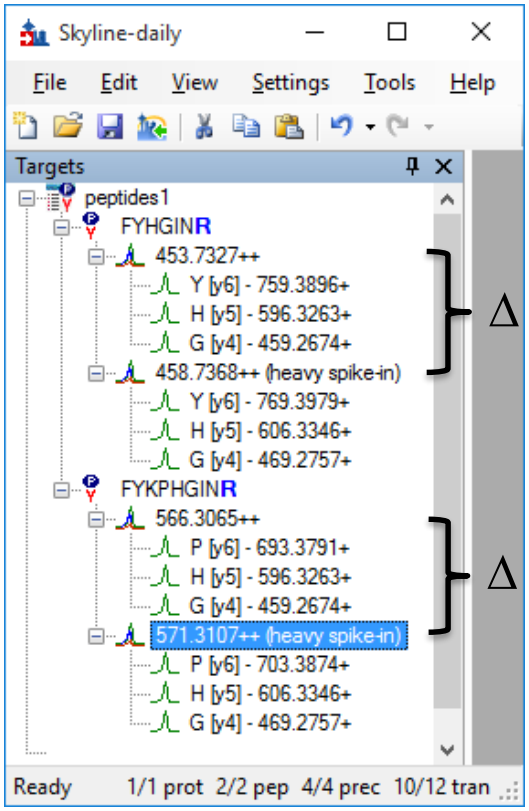


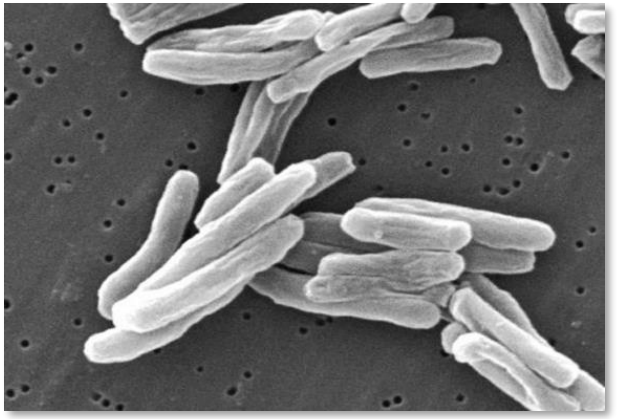
Tutorial-6 “Manual SRM data analysis in Skyline” Zürich course

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

http://targetedproteomics.ethz.ch/tutorials2014/Tutorial-6_ManualAnalysis.pdf

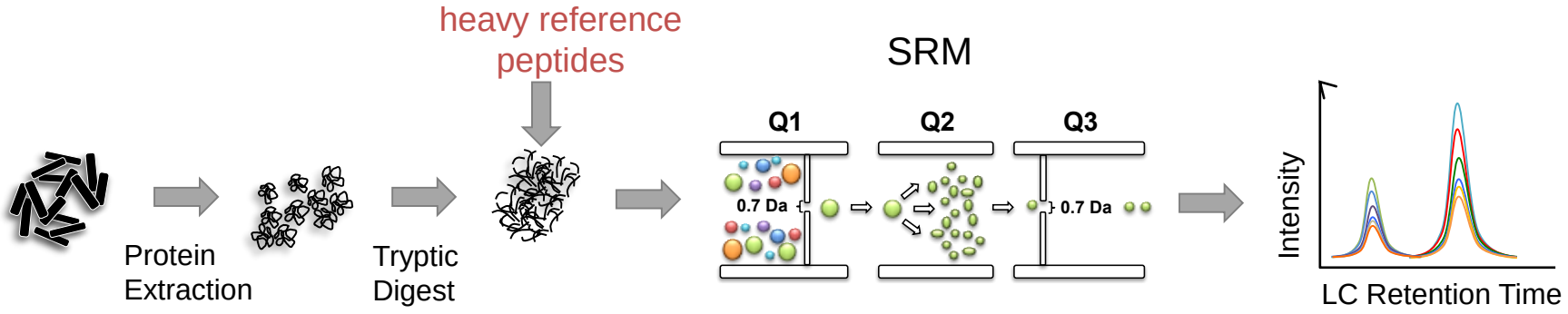
Mycobacterium tuberculosis (Mtb) total cell extracts

- **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.



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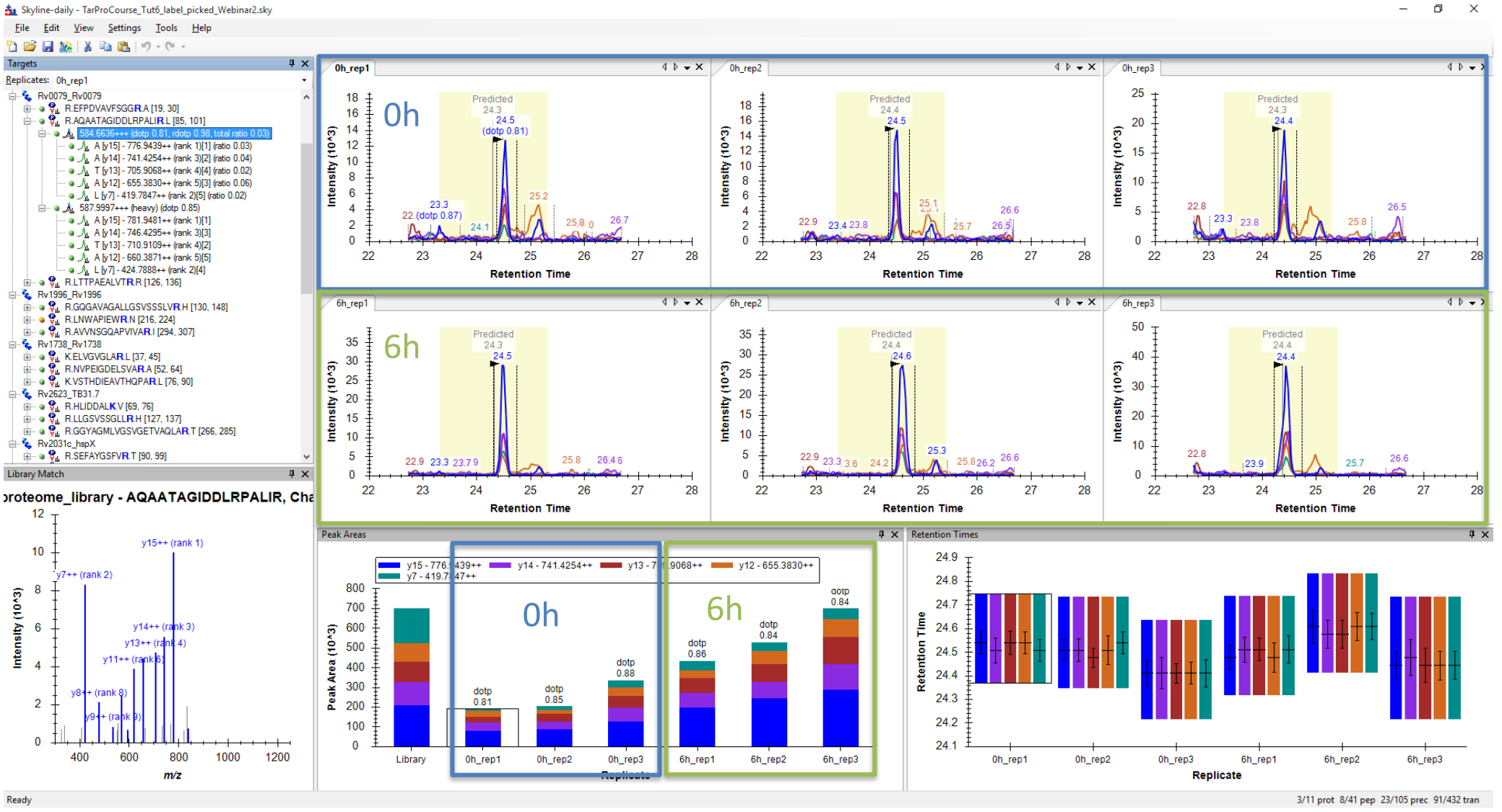
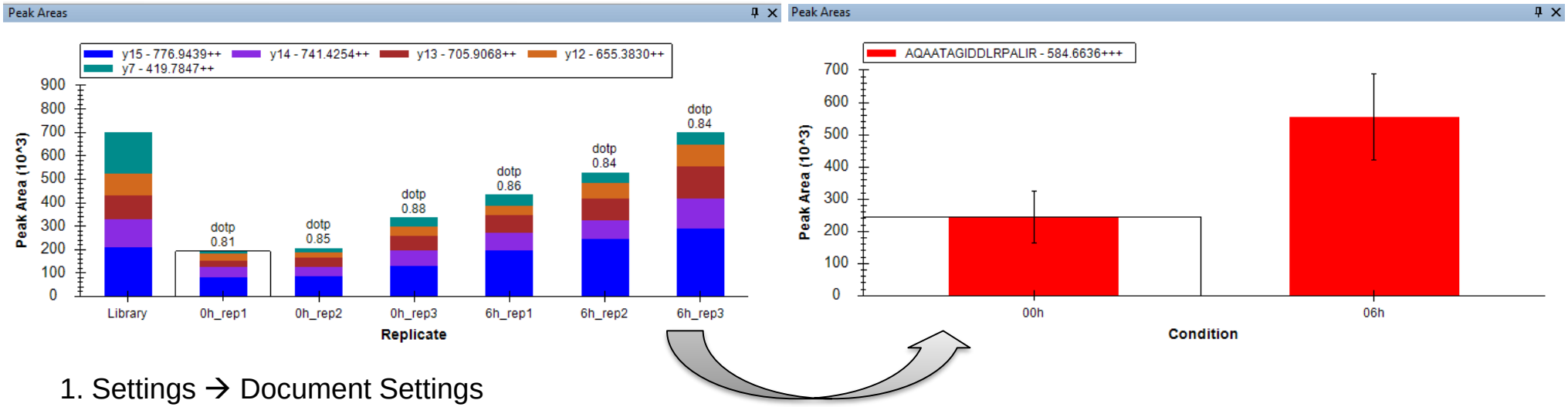
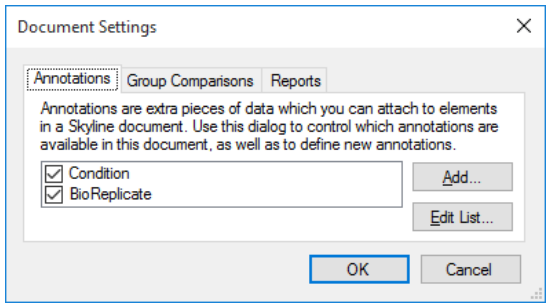


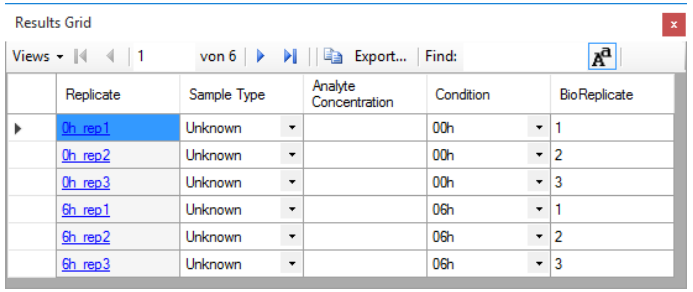
“Peak Area” viewing options in Skyline



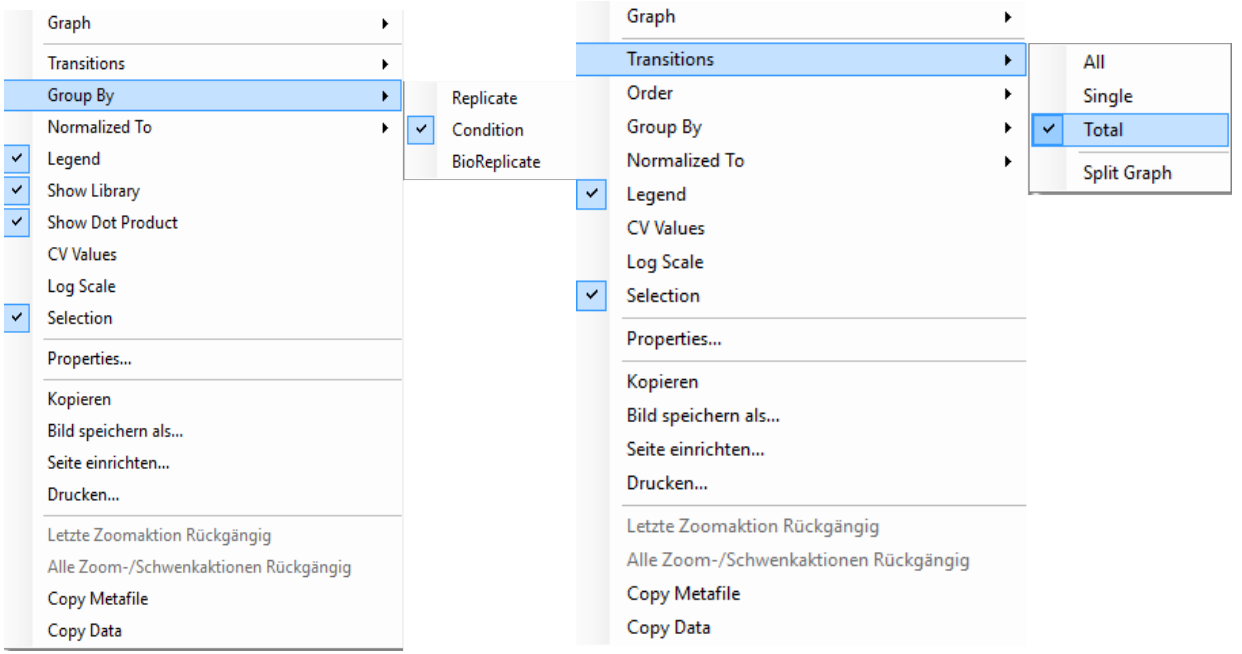
1. Settings → Document Settings



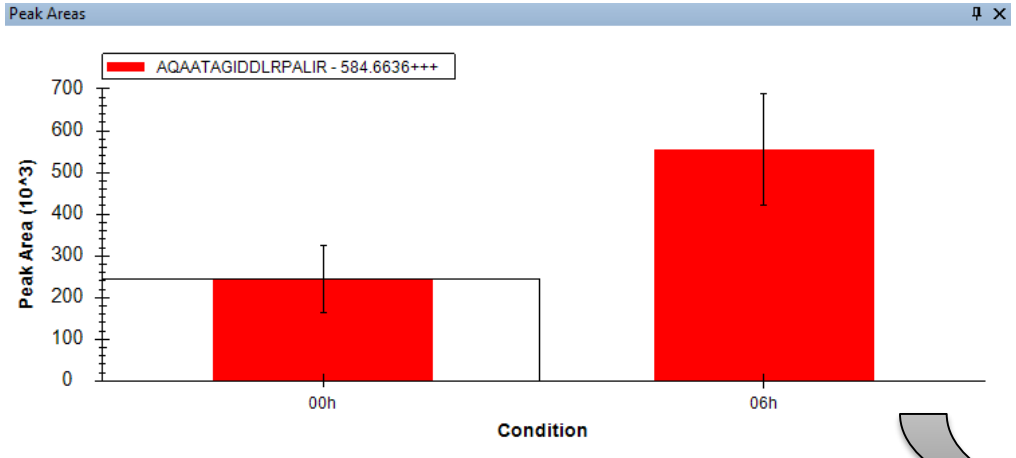
2. View → Results Grid

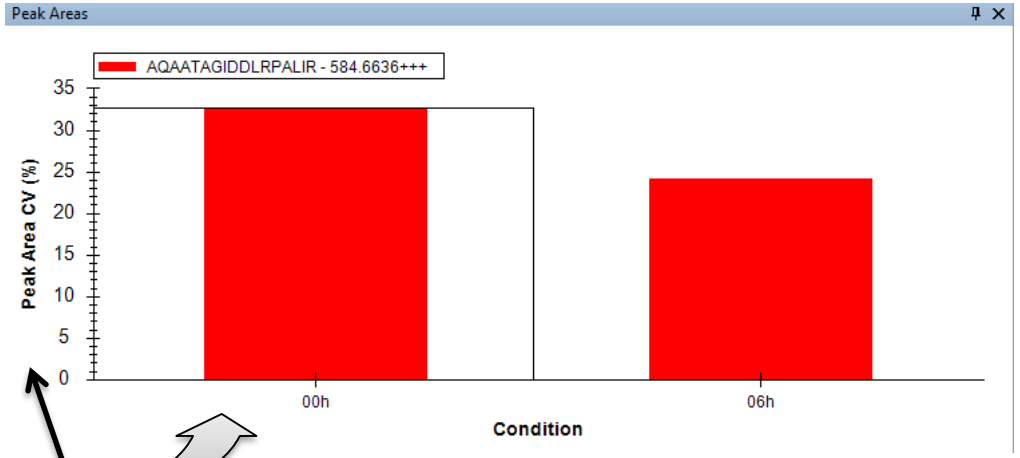


3. right mouse click on „Peak Areas“ window

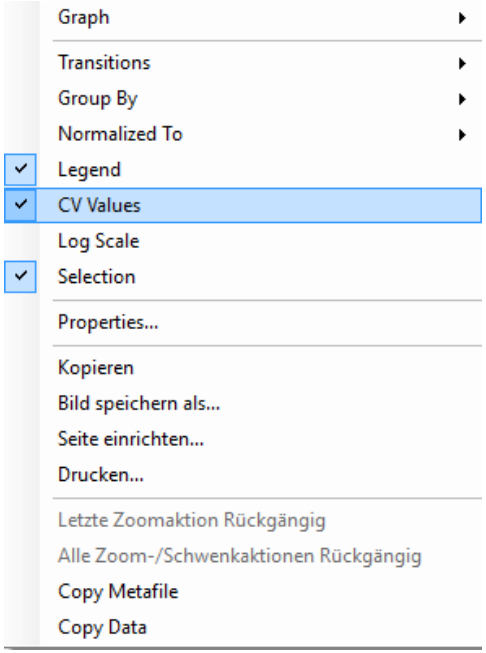


“Peak Area” viewing options in Skyline





right mouse click on „Peak Areas“ window

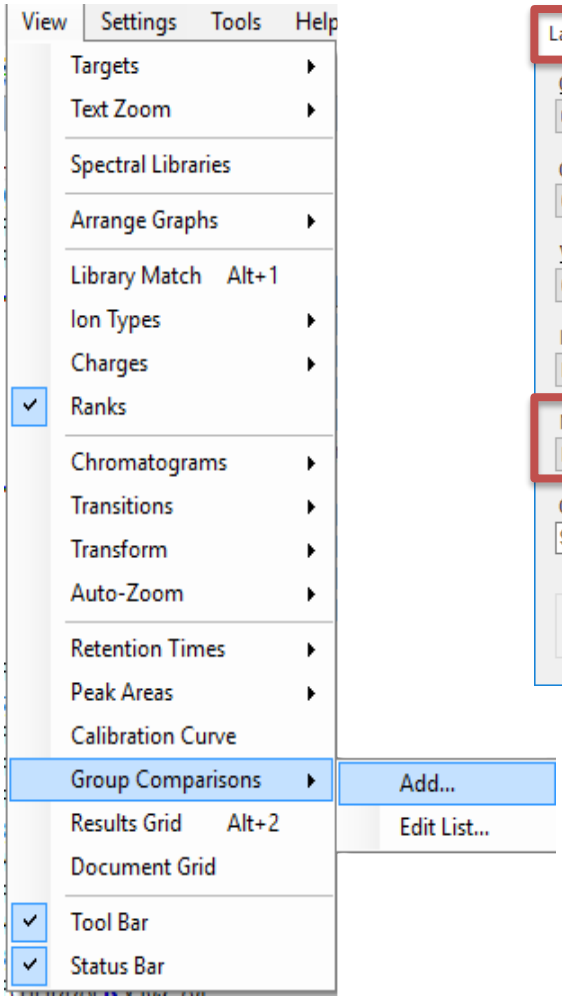


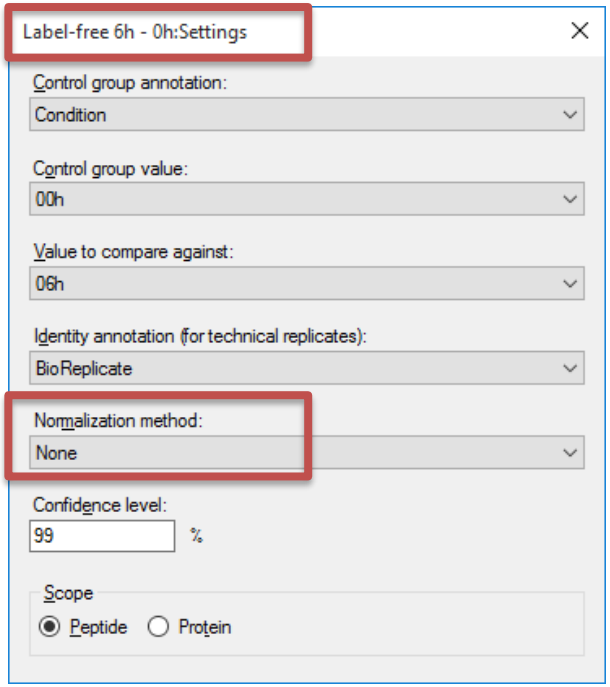
Coefficient of variation in %

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

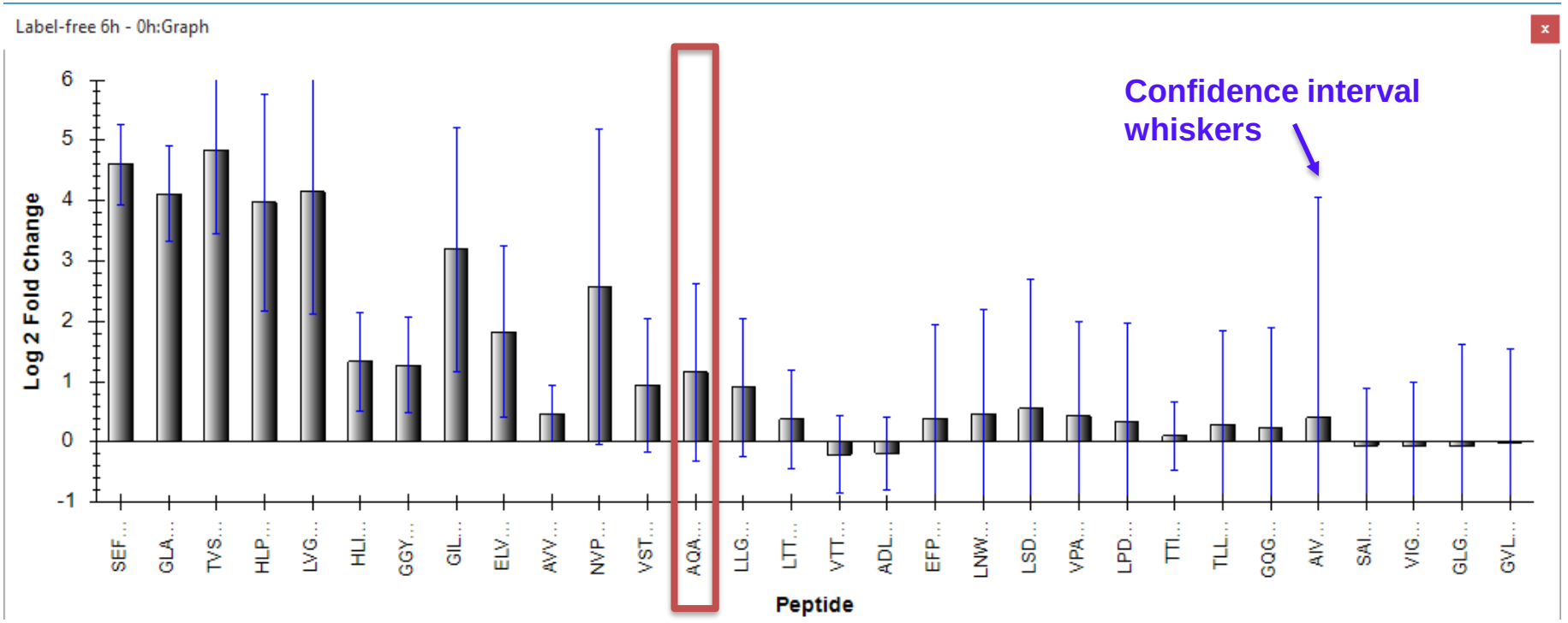




Protein	Peptide	Fold Change Result	Adjusted P-Value
Rv2031c_hspX	SEFAYGSFVR	24.25 (99% CI:15.24 to 38.58)	0.0002
Rv2626c_hrp1	GLAAGLDPNTATAGELAR	17.35 (99% CI:10.06 to 29.92)	0.0003
Rv2031c_hspX	TVSLPVGADEDDIK	28.53 (99% CI:10.85 to 75.05)	0.0009
Rv2626c_hrp1	HLPEHAIVQFVK	15.67 (99% CI:4.5 to 54.57)	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	GILTVSVAVSEGKPTEK	9.17 (99% CI:2.26 to 37.31)	0.0071
Rv1738_Rv1738	ELVGVGLAR	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	ADLLAAAAPR	0.88 (99% CI:0.58 to 1.34)	0.4196
Rv0079_Rv0079	EFPDVAVFSGGR	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	LSDVVDDLQDVIQEIR	1.47 (99% CI:0.33 to 6.44)	0.4319
Rv3133c_devR	VPAARPDVAVLDVR	1.35 (99% CI:0.46 to 3.96)	0.4319
Rv3133c_devR	LPDGNGIELCR	1.26 (99% CI:0.41 to 3.89)	0.5324
Rv3132c_devS	TTIYDLHGASQGITR	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_dosT	AIVHTAAELVDAR	1.32 (99% CI:0.11 to 16.56)	0.7374
Rv2027c_dosT	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_dosT	GVLGALIEEPKPIR	1 (99% CI:0.34 to 2.92)	0.9884

Simple group comparison within Skyline

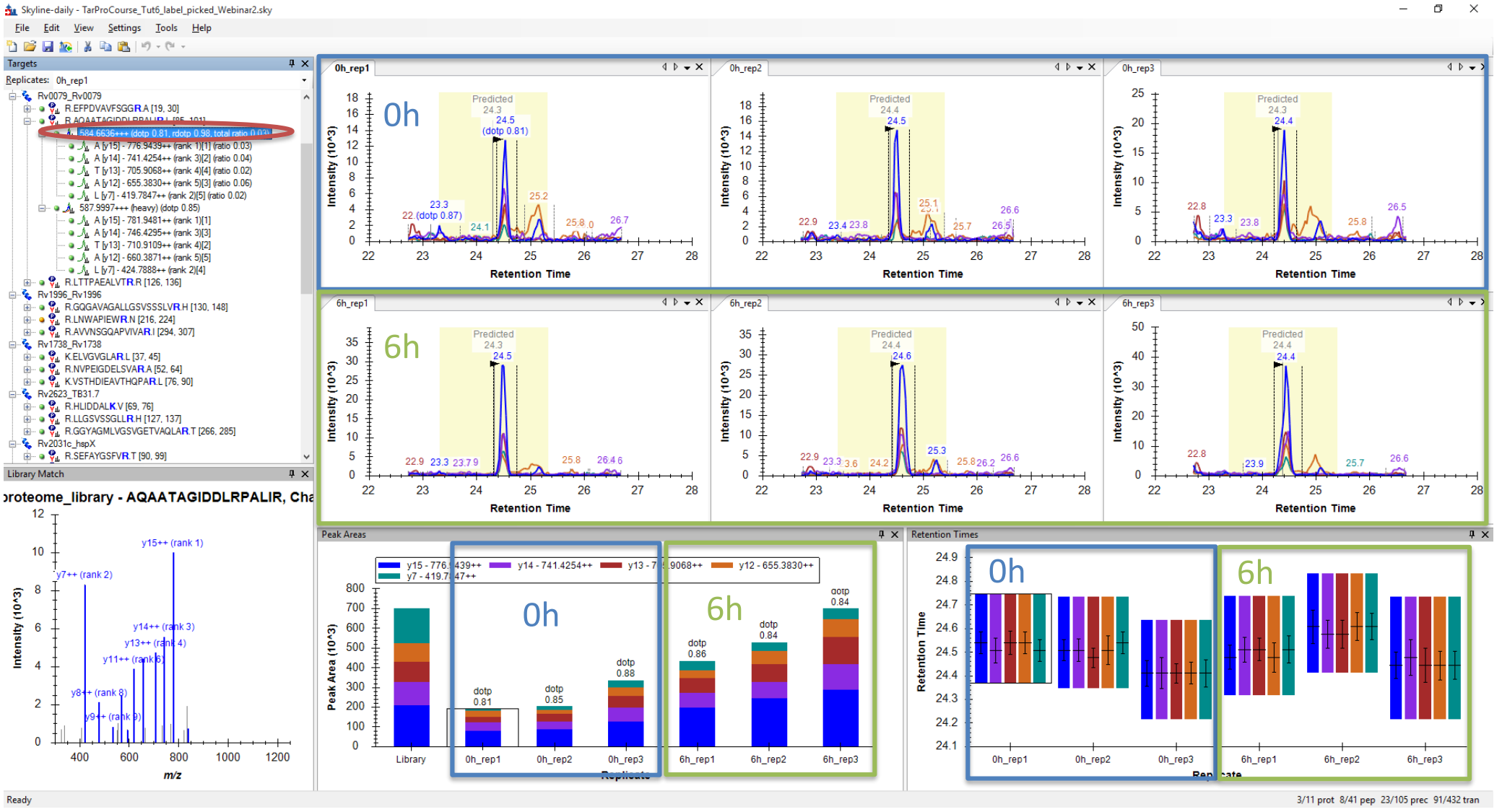
Tutorial „Processing Grouped Study data“: https://skyline.gs.washington.edu/labkey/tutorial_grouped.url



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

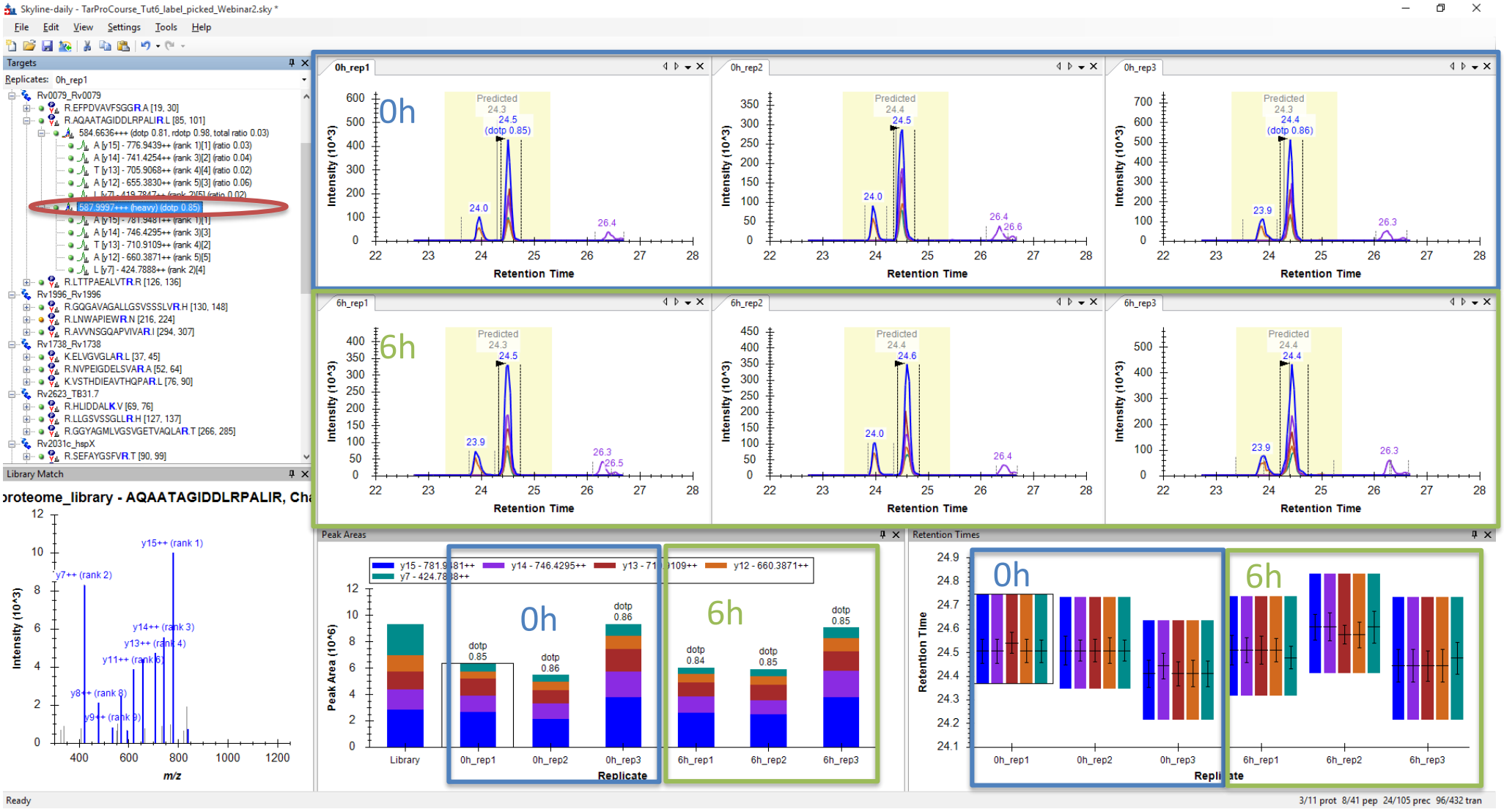
Tutorial-6 “Manual SRM data analysis in Skyline”

http://targetedproteomics.ethz.ch/tutorials2014/Tutorial-6_ManualAnalysis.pdf



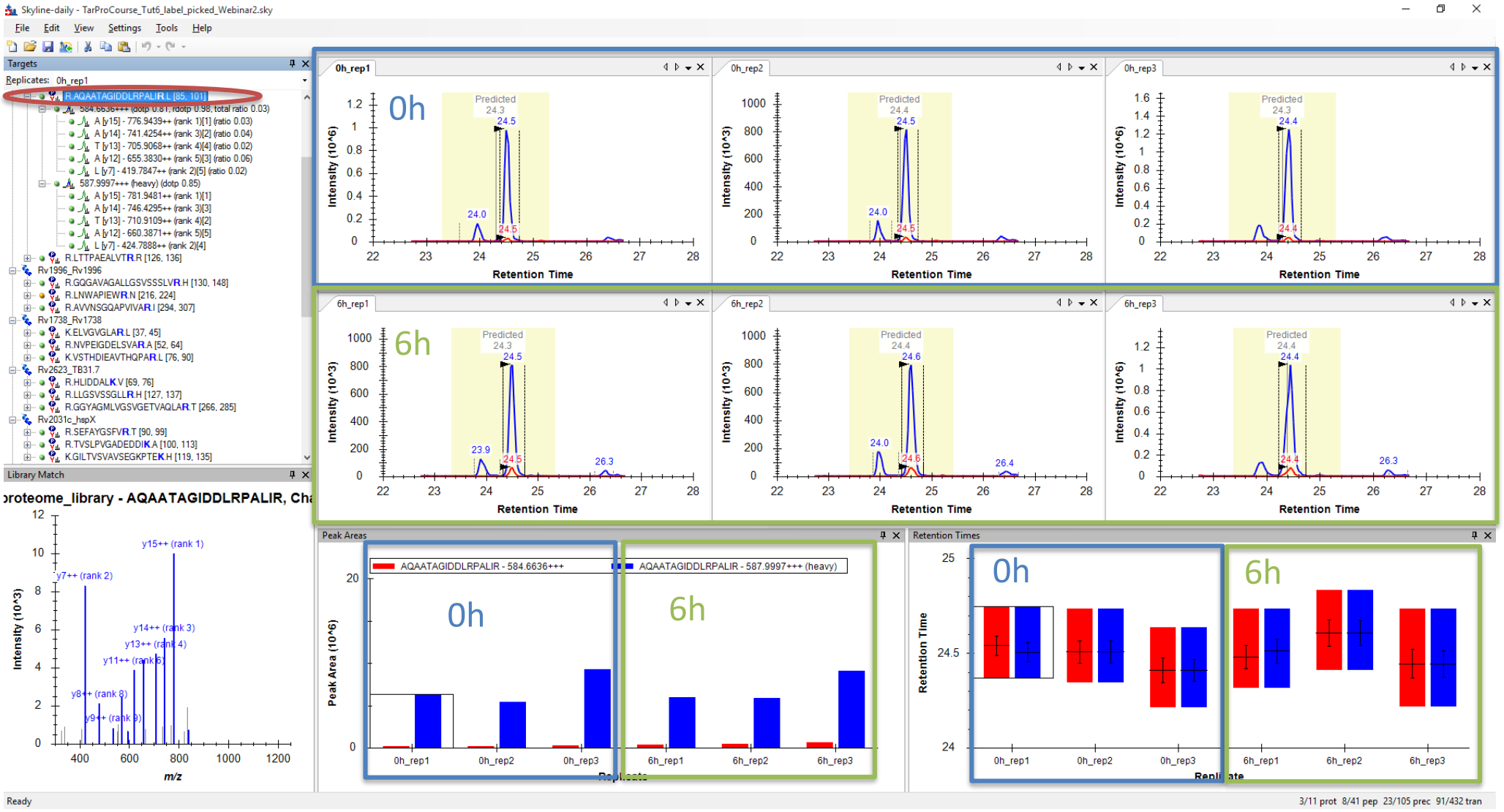
Tutorial-6 “Manual SRM data analysis in Skyline”

http://targetedproteomics.ethz.ch/tutorials2014/Tutorial-6_ManualAnalysis.pdf

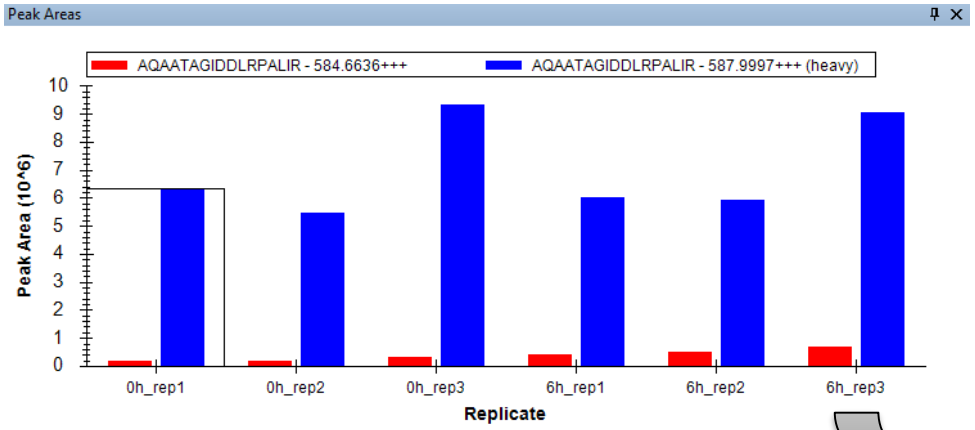


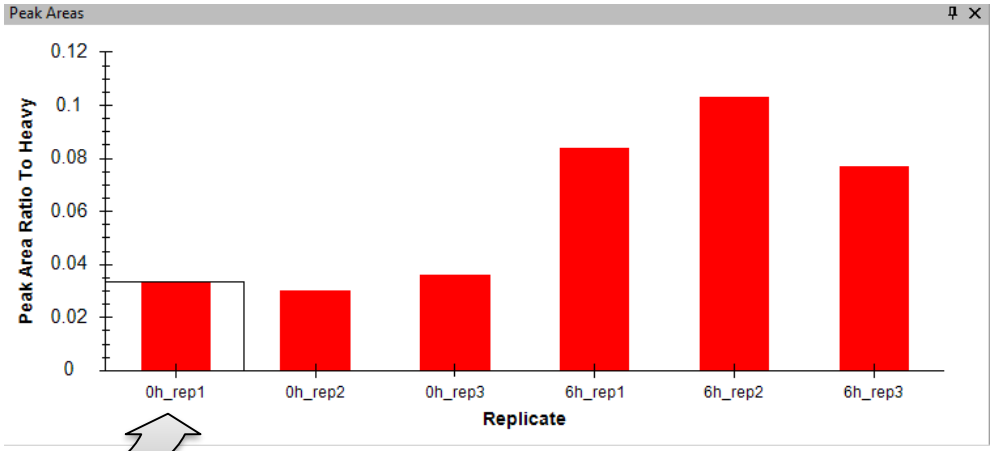
Tutorial-6 “Manual SRM data analysis in Skyline”

http://targetedproteomics.ethz.ch/tutorials2014/Tutorial-6_ManualAnalysis.pdf

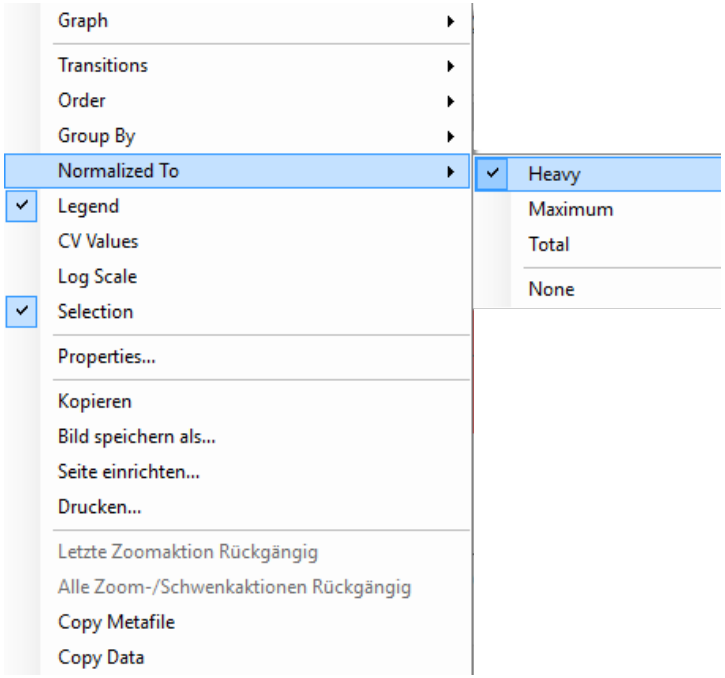


“Peak Area” viewing options in Skyline

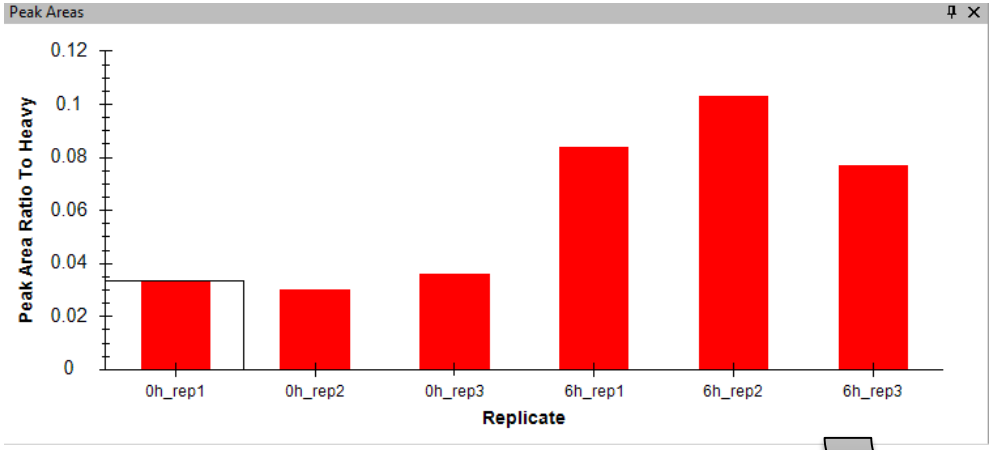


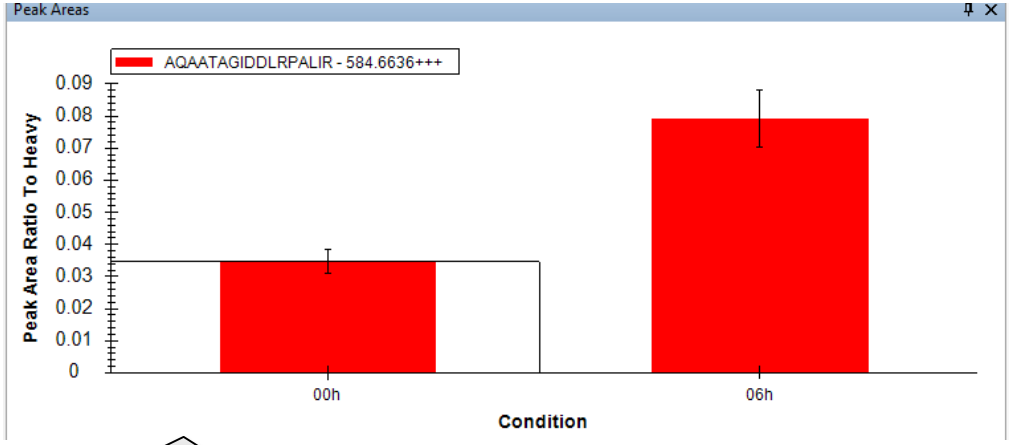


right mouse click on „Peak Areas“ window

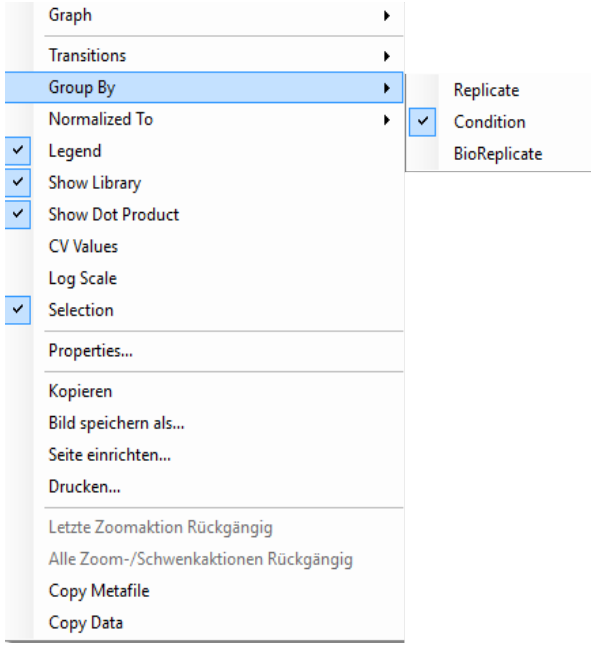


“Peak Area” viewing options in Skyline

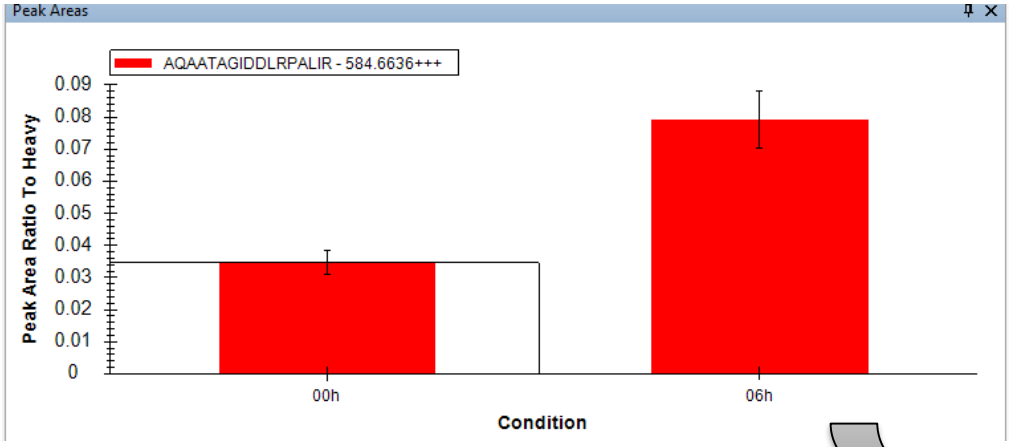


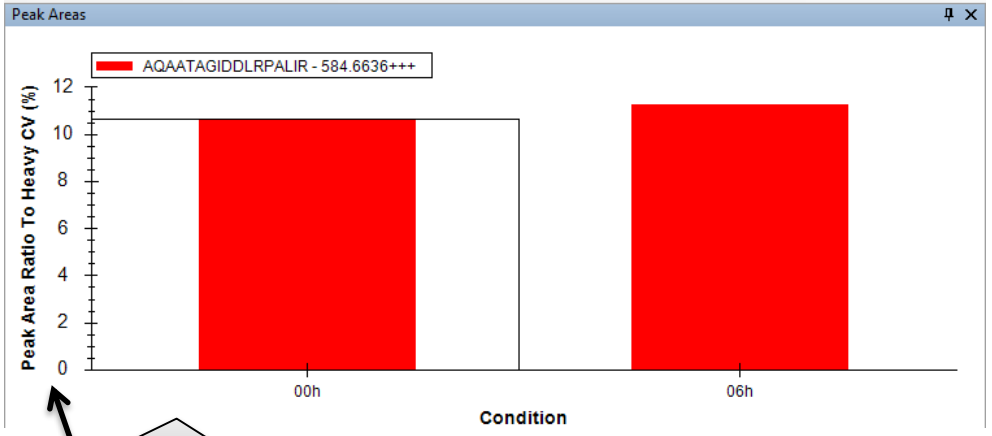


right mouse click on „Peak Areas“ window

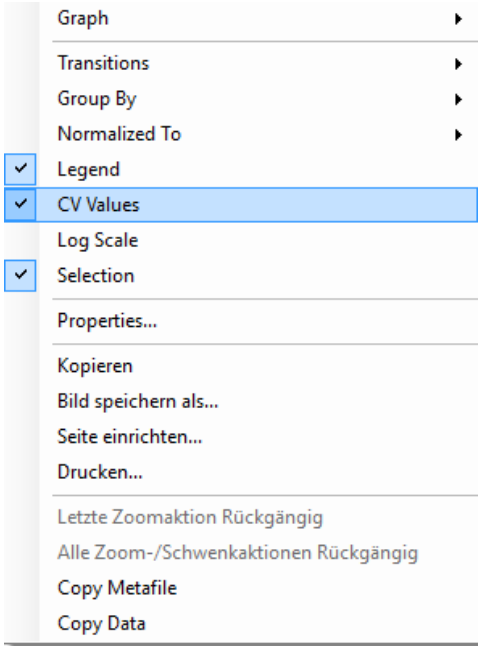


“Peak Area” viewing options in Skyline





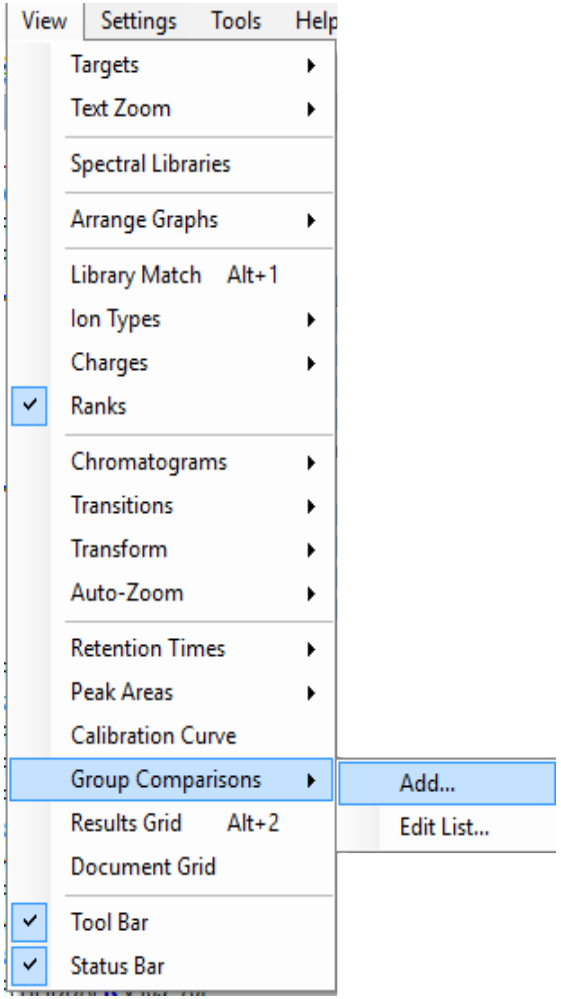
right mouse click on „Peak Areas“ window

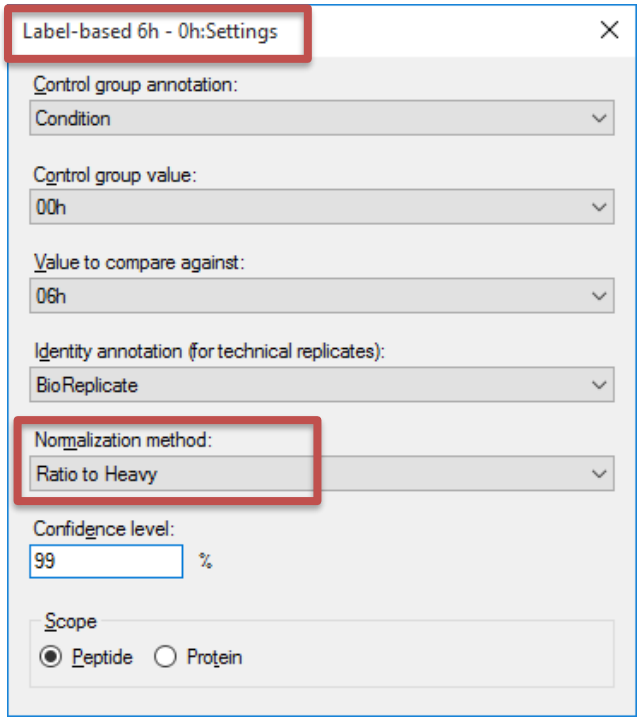


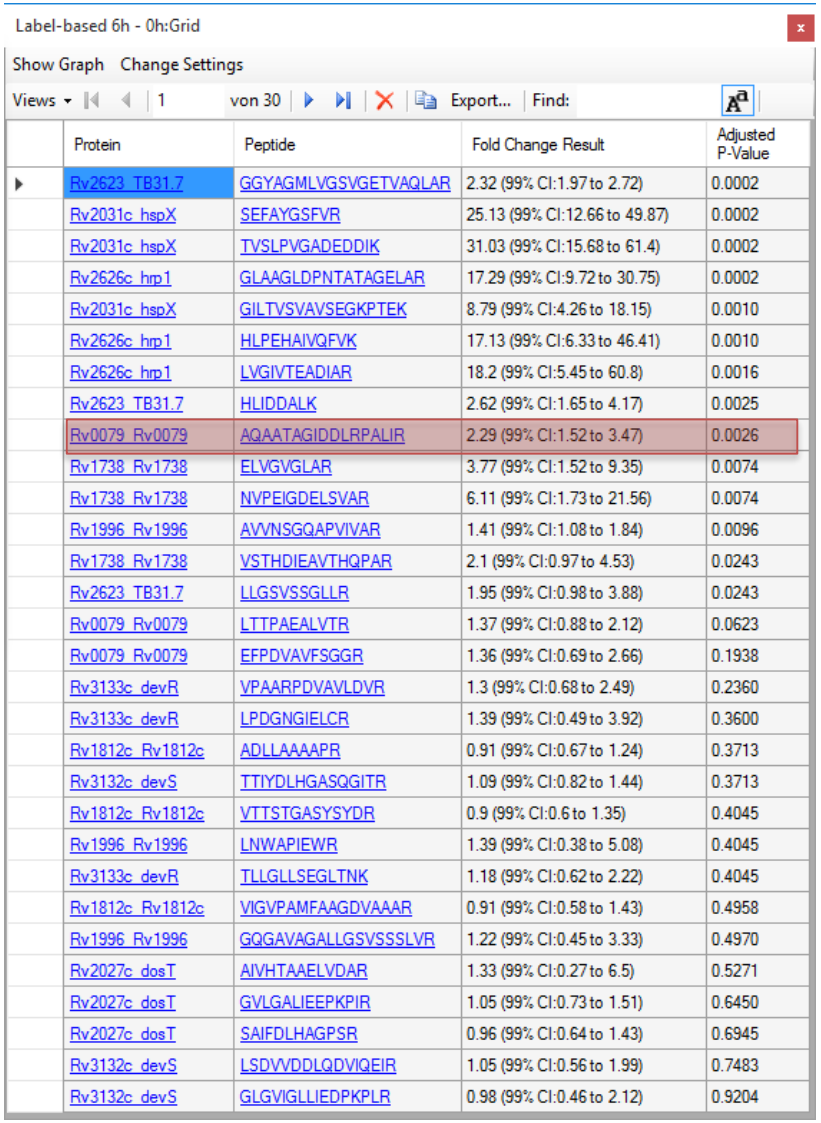
Coefficient of variation in %

Simple group comparison within Skyline

Tutorial „Processing Grouped Study data“: https://skyline.gs.washington.edu/labkey/tutorial_grouped.url



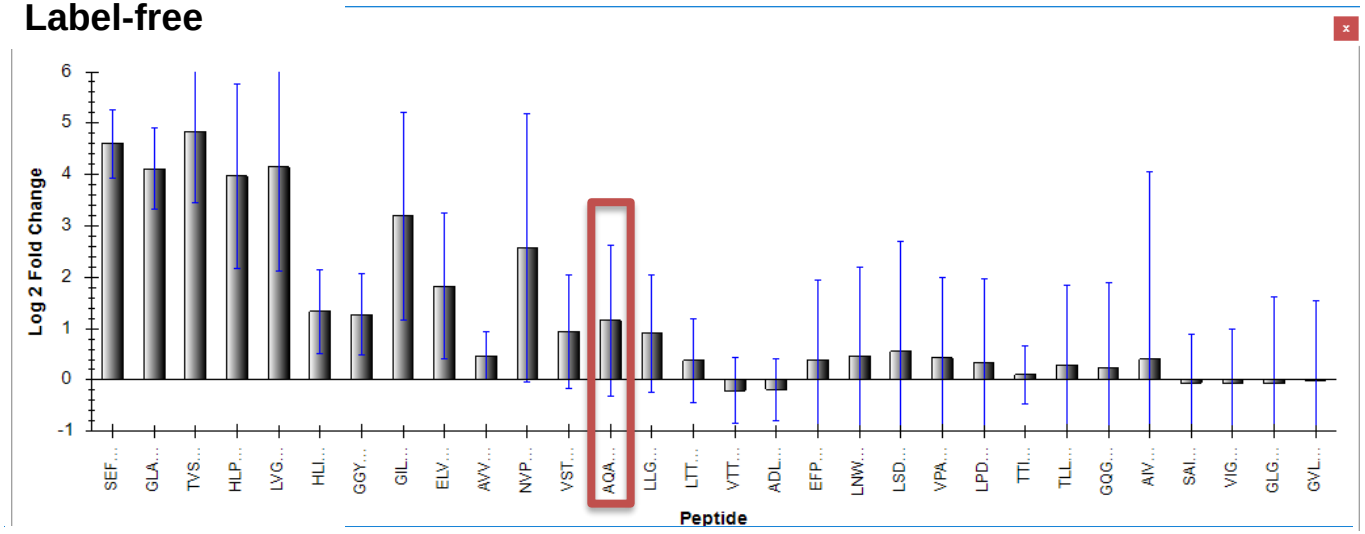




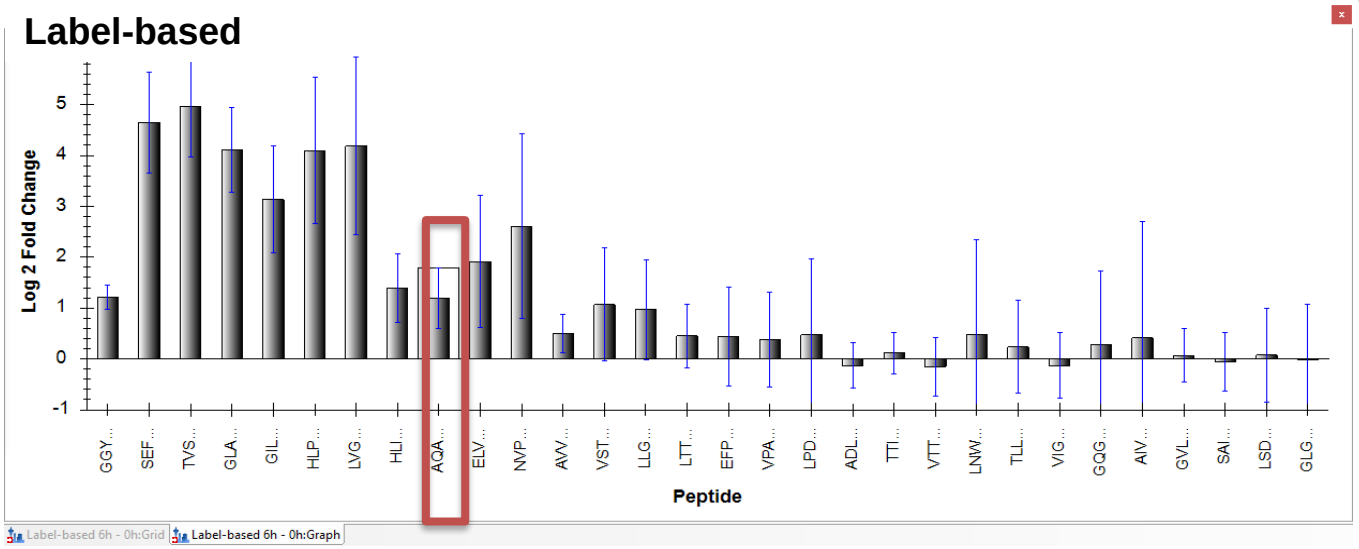
Protein	Peptide	Fold Change Result	Adjusted P-Value
Rv2623_TB31.7	GGYAGMLVGSVGETVAQLAR	2.32 (99% CI:1.97 to 2.72)	0.0002
Rv2031c_hspX	SEFAYGSFVR	25.13 (99% CI:12.66 to 49.87)	0.0002
Rv2031c_hspX	TVSLPVGADEDDIK	31.03 (99% CI:15.68 to 61.4)	0.0002
Rv2626c_hrp1	GLAAGLDPNTATAGELAR	17.29 (99% CI:9.72 to 30.75)	0.0002
Rv2031c_hspX	GILTVSVAVSEGKPTEK	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	ELVGVGLAR	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	ADLLAAAAPR	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	TLLGLLSEGLTNK	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	GVLGALIEEPKPIR	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	LSDVVDDLQDVIQEIR	1.05 (99% CI:0.56 to 1.99)	0.7483
Rv3132c_devS	GLGVIGLLIEDPKPLR	0.98 (99% CI:0.46 to 2.12)	0.9204

Simple group comparison within Skyline

Label-free



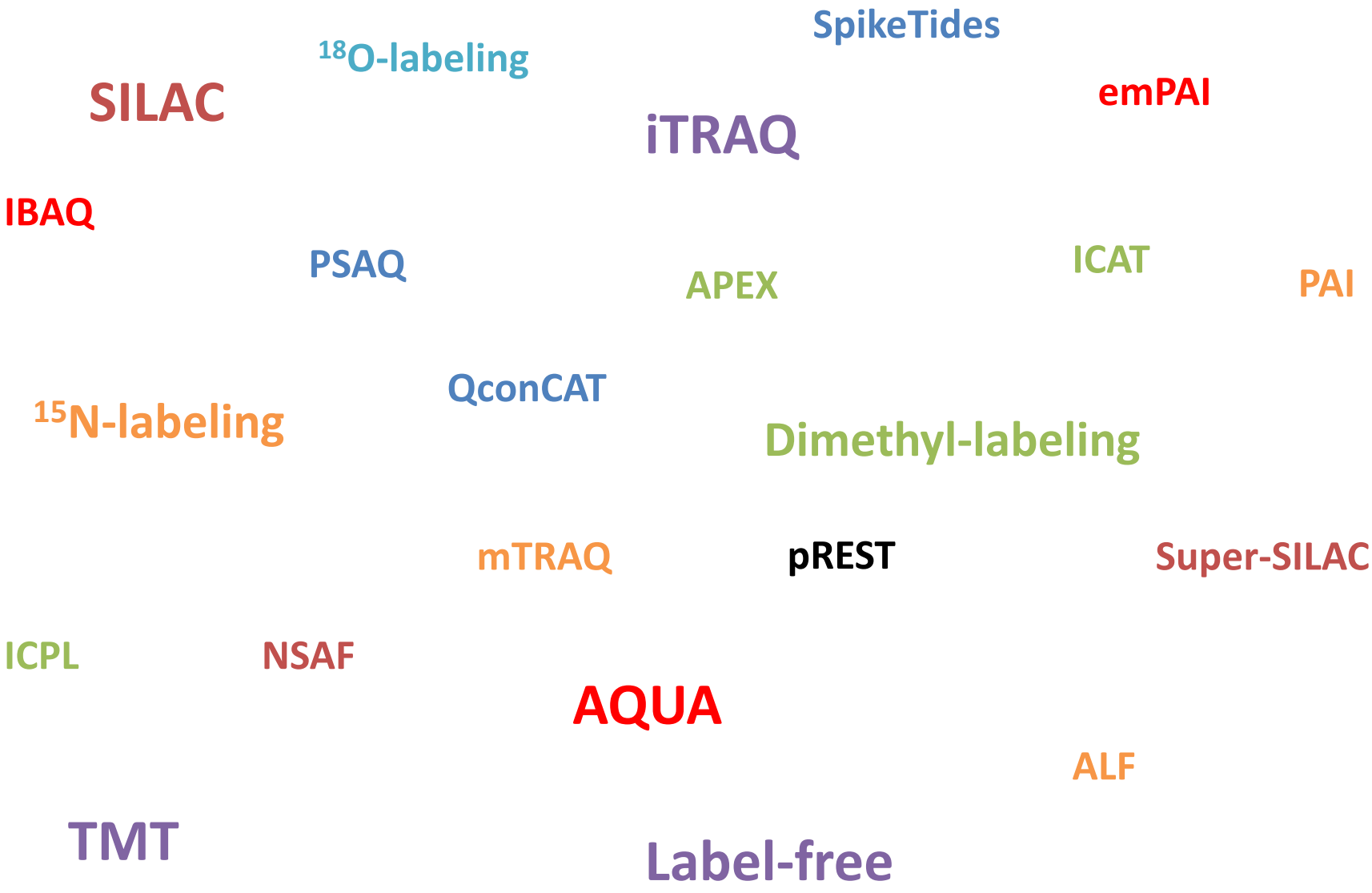
Label-based



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

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

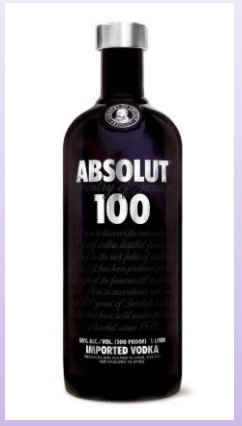
The multitude of quantitative MS-applications



Relative versus absolute protein quantification

relative

absolute

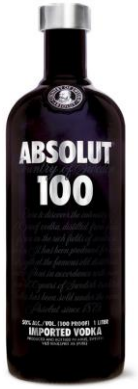


^{15}N-labeling
^{18}O-labeling
IBAQ
mTRAQ
APEX
SILAC
emPAI
SpikeTides
ICPL
iTRAQ
TMT
ICAT
pREST
Dimethyl-labeling
QconCAT
PSAQ
AQUA
Label-free
ALF

PSAQ
NSAF
QconCAT
APEX
AQUA
ALF
emPAI
IBAQ

Definition of absolute versus relative protein quantification

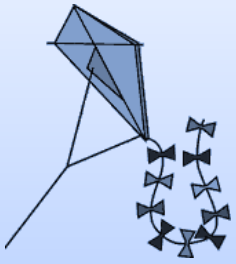
	Relative quantification	Absolute quantification
samples	≥ 2 samples	
output	protein ratio	copies/cell fmol/µg extract ng/ml body fluid



relative comp… same protein between samples

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

MS-based absolute protein quantification strategies

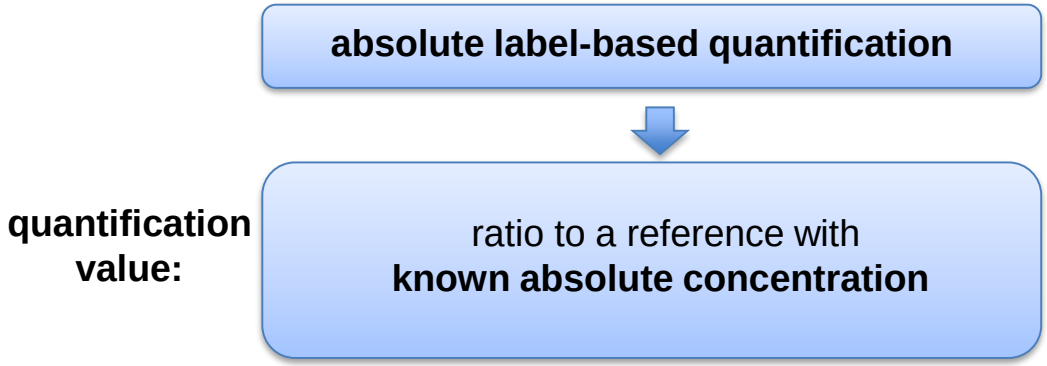


absolute label-based

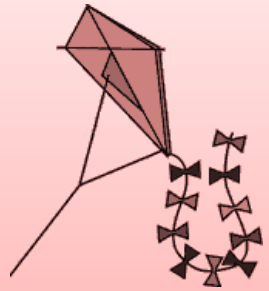
absolute label-free

gold-standard - more precise !!!!

Label-based absolute protein quantification



Strategies for incorporating stable-isotopes



label-based

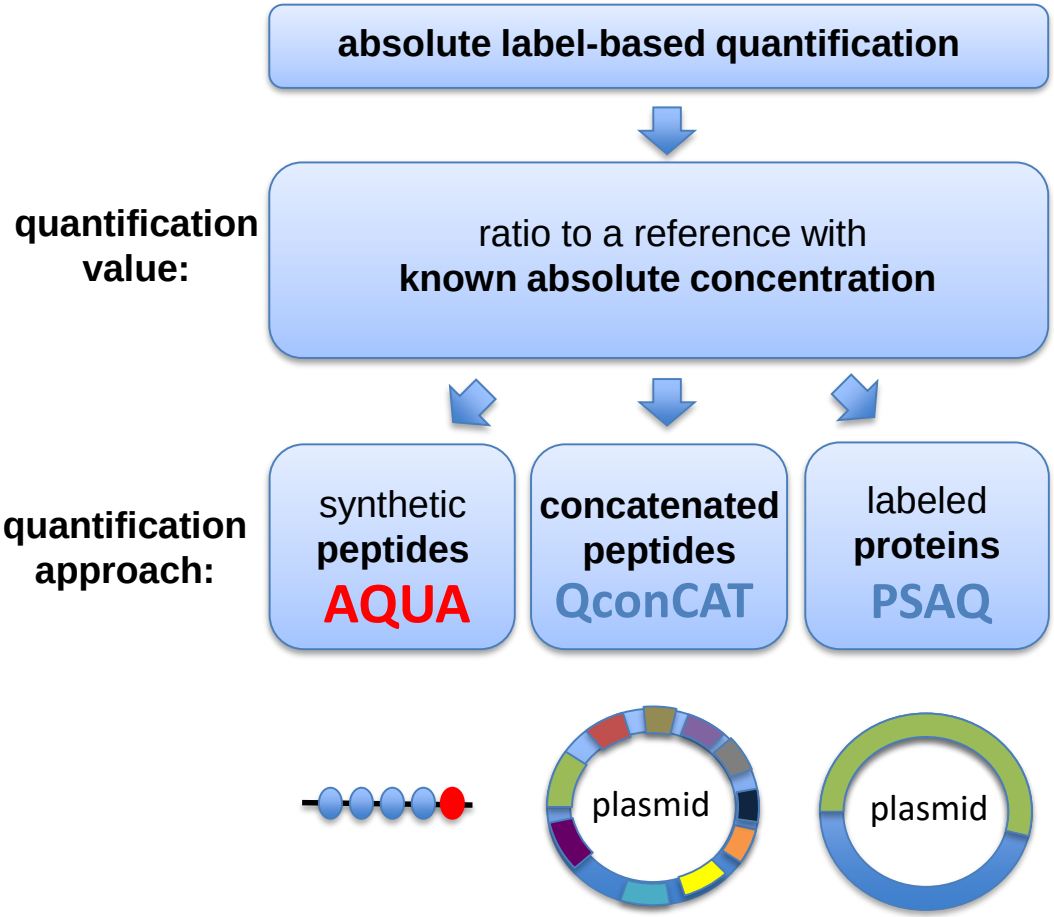
1. Metabolic labeling

2. Chemical labeling

3. Enzymatic labeling

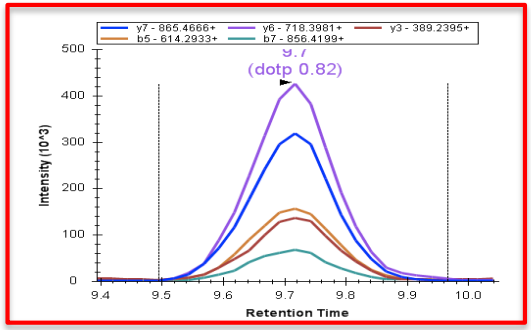
4. Spike-in of isotope-labeled **standards**

Label-based absolute protein quantification

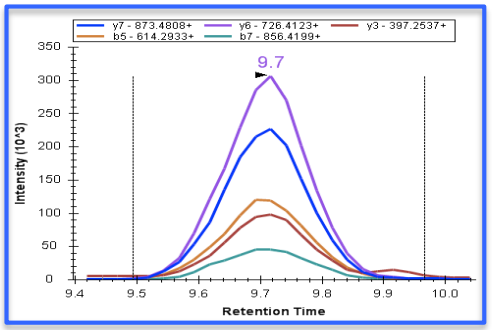


Absolute quantification – internal single point calibration

endogenous peptide **(light)**

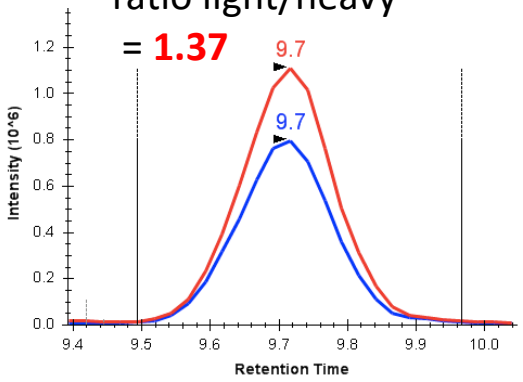


reference peptide **(heavy)**



ratio light/heavy

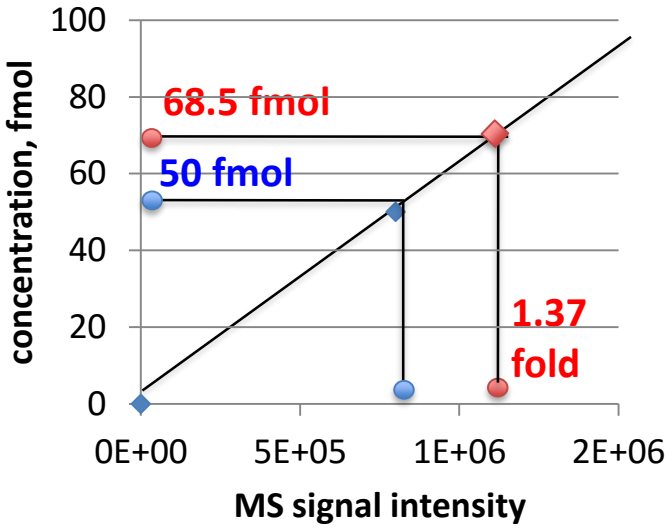
= 1.37



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

spike-in concentration 50 fmol

Single point calibration

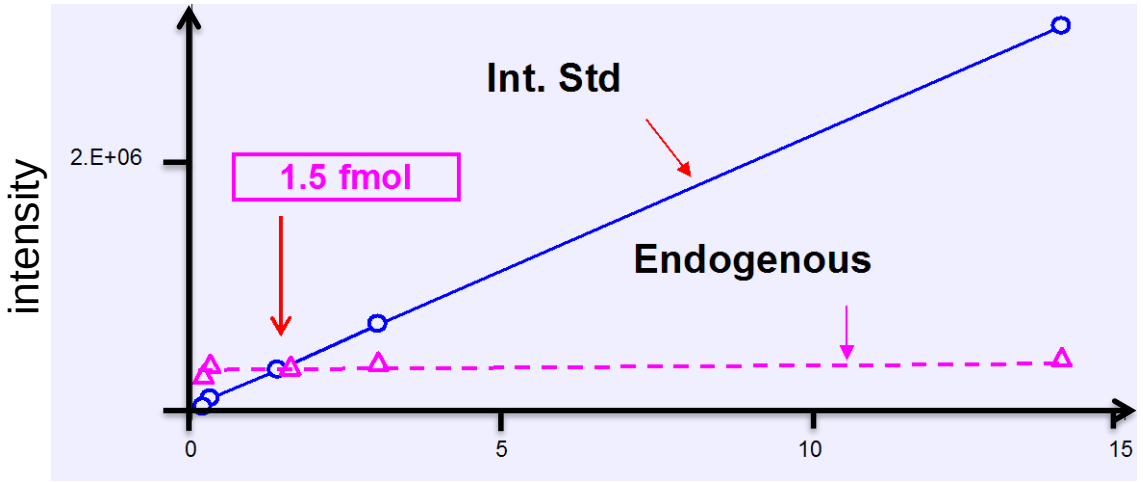


Assumptions of internal single point calibration:

- 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



linear equation:
$y = 2.2E^6x - 1.4E^5$

Advantages of multiple point calibration:

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

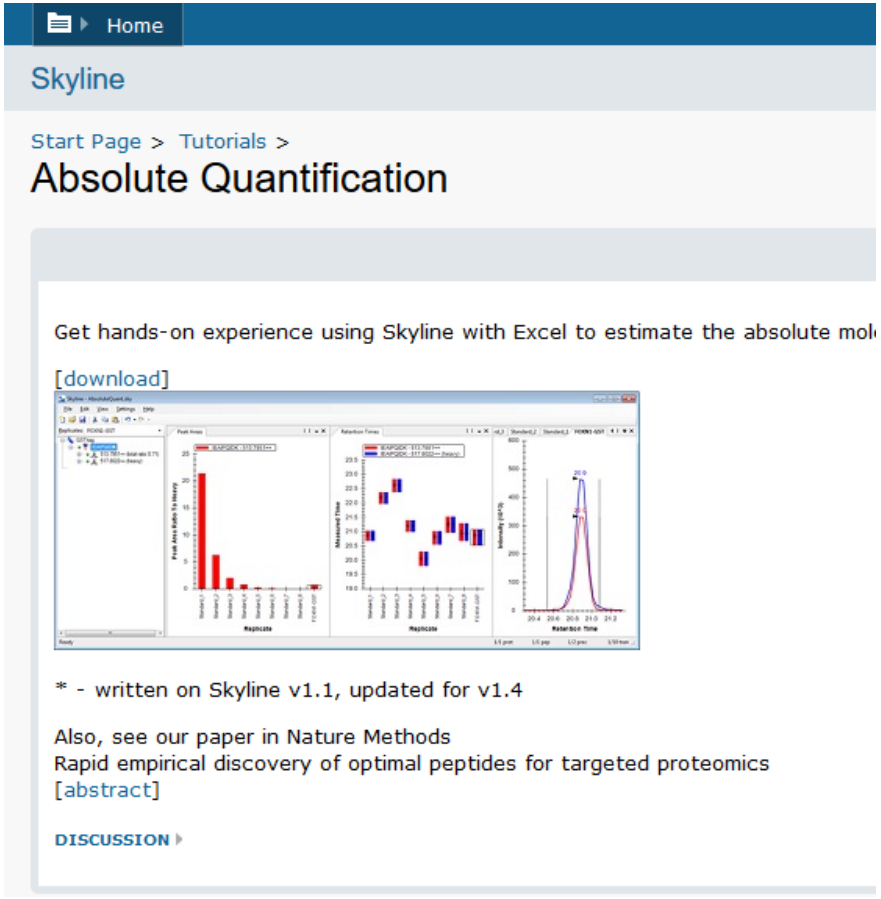
Limitations of multiple point calibration:

- 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:



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

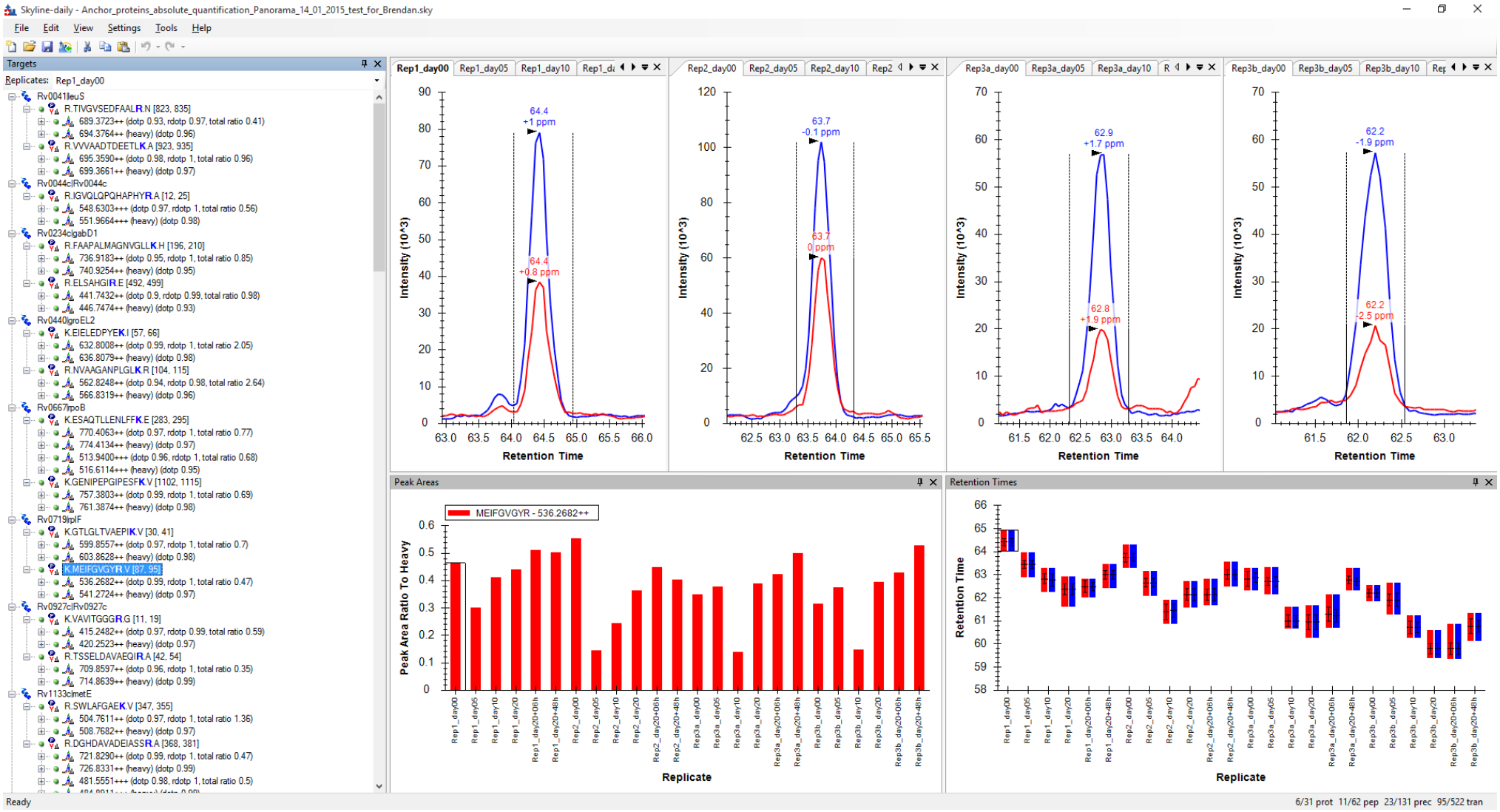
Motivation of the data set:

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

Points to consider regarding sample preparation:

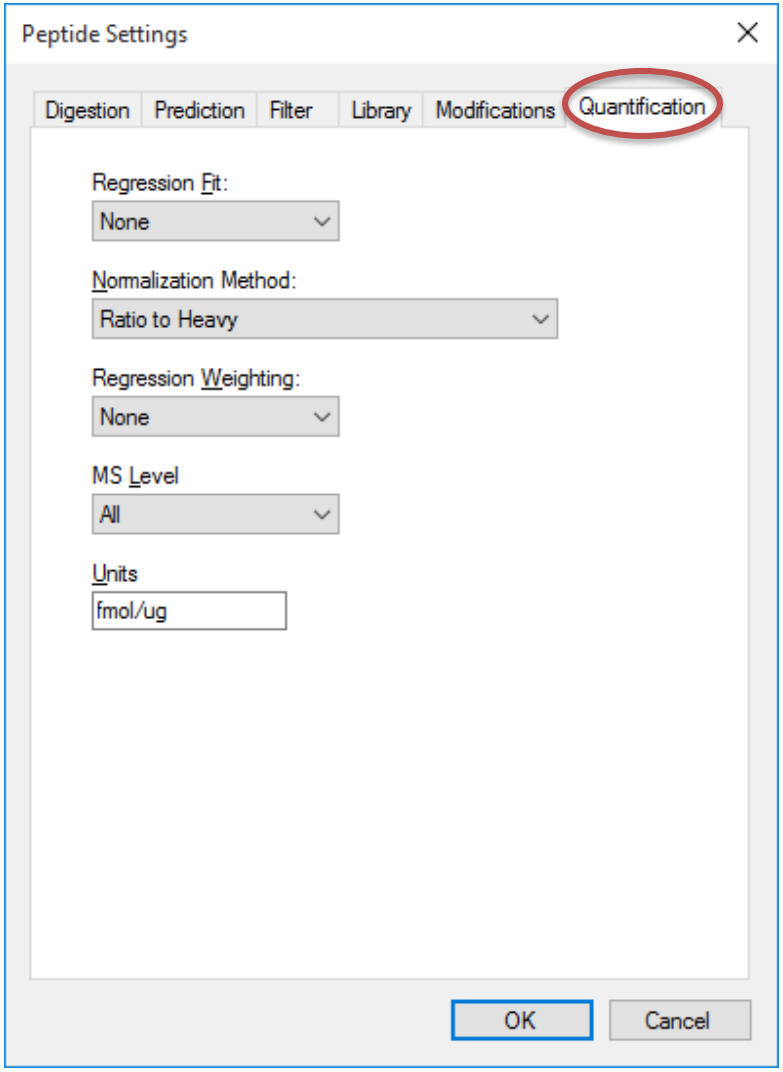
- Spike-in **AQUA peptides** into all samples.
- Keep the concentration for a **given AQUA peptide** over all samples **constant**
- Try to select a spike-in AQUA concentration close to **1:1 light : heavy**
  → If a peptide is strongly regulated over the samples of interest a 1:1 ratio is of course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

Internal single-point absolute quantification supported within Skyline

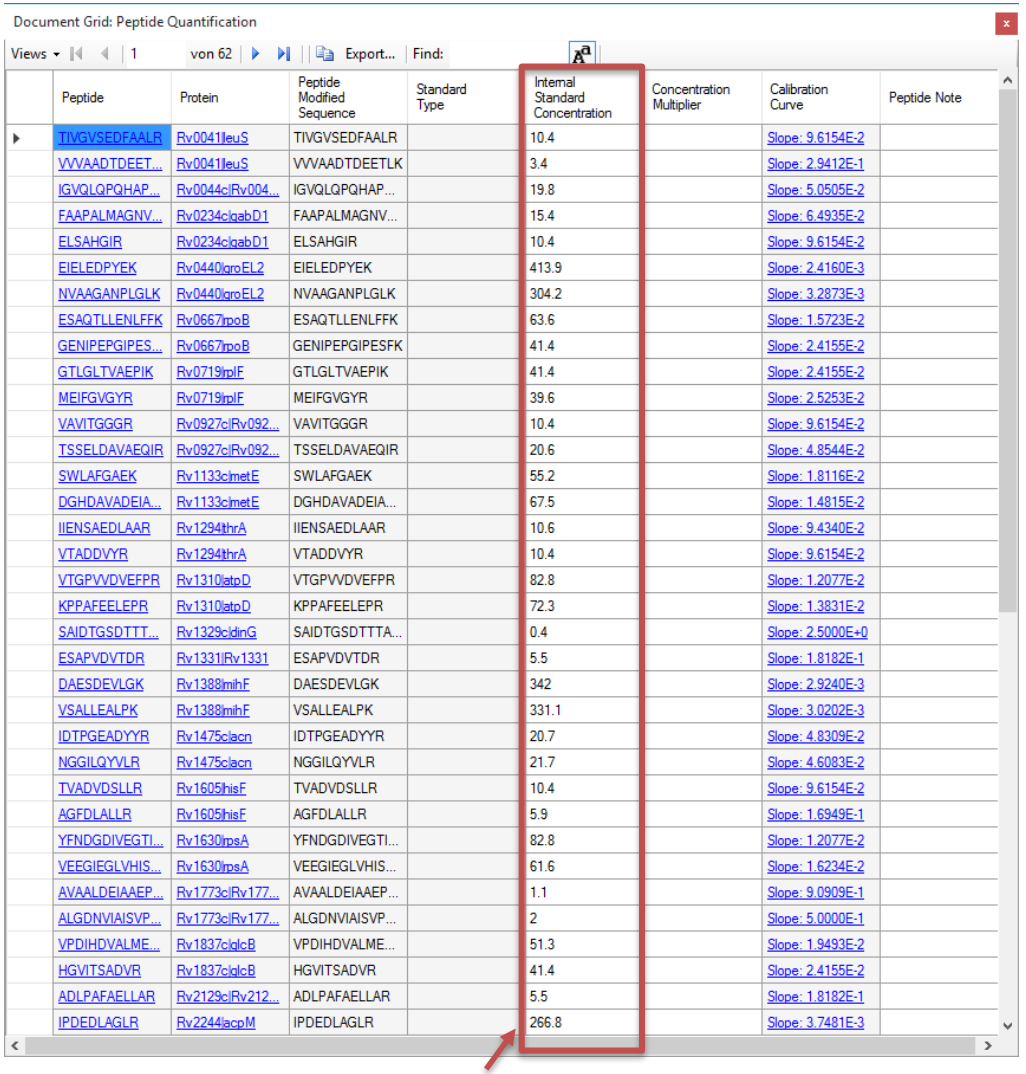


Internal single-point absolute quantification supported within Skyline

Peptide Settings → Quantification



View → Document Grid → Views → Peptide Quantification



Peptide	Protein	Peptide Modified Sequence	Standard Type	Internal Standard Concentration	Concentration Multiplier	Calibration Curve	Peptide Note
TIVGVSEDFAALR	Rv0041|leuS	TIVGVSEDFAALR		10.4		Slope: 9.6154E-2	
VVVAADTDEET...	Rv0041|leuS	VVVAADTDEETLK		3.4		Slope: 2.9412E-1	
IGVQLQPQHAP...	Rv0044c|Rv004...	IGVQLQPQHAP...		19.8		Slope: 5.0505E-2	
FAAPALMAGNV...	Rv0234c|gabD1	FAAPALMAGNV...		15.4		Slope: 6.4935E-2	
ELSAHGIR	Rv0234c|gabD1	ELSAHGIR		10.4		Slope: 9.6154E-2	
EIELEDPYEK	Rv0440|groEL2	EIELEDPYEK		413.9		Slope: 2.4160E-3	
NVAAGANPLGLK	Rv0440|groEL2	NVAAGANPLGLK		304.2		Slope: 3.2873E-3	
ESAQTLLENLFFK	Rv0667|rpoB	ESAQTLLENLFFK		63.6		Slope: 1.5723E-2	
GENIPEPGIPES...	Rv0667|rpoB	GENIPEPGIPESFK		41.4		Slope: 2.4155E-2	
GTLGLTVAEPIK	Rv0719|rplF	GTLGLTVAEPIK		41.4		Slope: 2.4155E-2	
MEIFGVGYR	Rv0719|rplF	MEIFGVGYR		39.6		Slope: 2.5253E-2	
VAVITGGGR	Rv0927c|Rv092...	VAVITGGGR		10.4		Slope: 9.6154E-2	
TSSELDAVAEQIR	Rv0927c|Rv092...	TSSELDAVAEQIR		20.6		Slope: 4.8544E-2	
SWLAFGAEK	Rv1133c|metE	SWLAFGAEK		55.2		Slope: 1.8116E-2	
DGHDAVADEIA...	Rv1133c|metE	DGHDAVADEIA...		67.5		Slope: 1.4815E-2	
IIENSAEDLAAR	Rv1294|thrA	IIENSAEDLAAR		10.6		Slope: 9.4340E-2	
VTADDVYR	Rv1294|thrA	VTADDVYR		10.4		Slope: 9.6154E-2	
VTGPVVDVEFPR	Rv1310|atpD	VTGPVVDVEFPR		82.8		Slope: 1.2077E-2	
KPPAFEELEPR	Rv1310|atpD	KPPAFEELEPR		72.3		Slope: 1.3831E-2	
SAIDTGSDTTT...	Rv1329c|dinG	SAIDTGSDTTTA...		0.4		Slope: 2.5000E+0	
ESAPVDVTDR	Rv1331|Rv1331	ESAPVDVTDR		5.5		Slope: 1.8182E-1	
DAESDEVLGK	Rv1388|mihF	DAESDEVLGK		342		Slope: 2.9240E-3	
VSALLEALPK	Rv1388|mihF	VSALLEALPK		331.1		Slope: 3.0202E-3	
IDTPGEADYYR	Rv1475c|acn	IDTPGEADYYR		20.7		Slope: 4.8309E-2	
NGGILQYVLR	Rv1475c|acn	NGGILQYVLR		21.7		Slope: 4.6083E-2	
TVADVDSLLR	Rv1605|hisF	TVADVDSLLR		10.4		Slope: 9.6154E-2	
AGFDLALLR	Rv1605|hisF	AGFDLALLR		5.9		Slope: 1.6949E-1	
YFNDGDIVEGTI...	Rv1630|rpsA	YFNDGDIVEGTI...		82.8		Slope: 1.2077E-2	
VEEGIEGLVHIS...	Rv1630|rpsA	VEEGIEGLVHIS...		61.6		Slope: 1.6234E-2	
AVAALDEIAAEP...	Rv1773c|Rv177...	AVAALDEIAAEP...		1.1		Slope: 9.0909E-1	
ALGDNVIAISVP...	Rv1773c|Rv177...	ALGDNVIAISVP...		2		Slope: 5.0000E-1	
VPDIHDVALME...	Rv1837c|glcB	VPDIHDVALME...		51.3		Slope: 1.9493E-2	
HGVITSADVR	Rv1837c|glcB	HGVITSADVR		41.4		Slope: 2.4155E-2	
ADLPAFAELLAR	Rv2129c|Rv212...	ADLPAFAELLAR		5.5		Slope: 1.8182E-1	
IPDEDLAGLR	Rv2244|acpM	IPDEDLAGLR		266.8		Slope: 3.7481E-3	

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

Internal single-point absolute quantification supported within Skyline

View → Document Grid → Views → Peptide Ratio Results

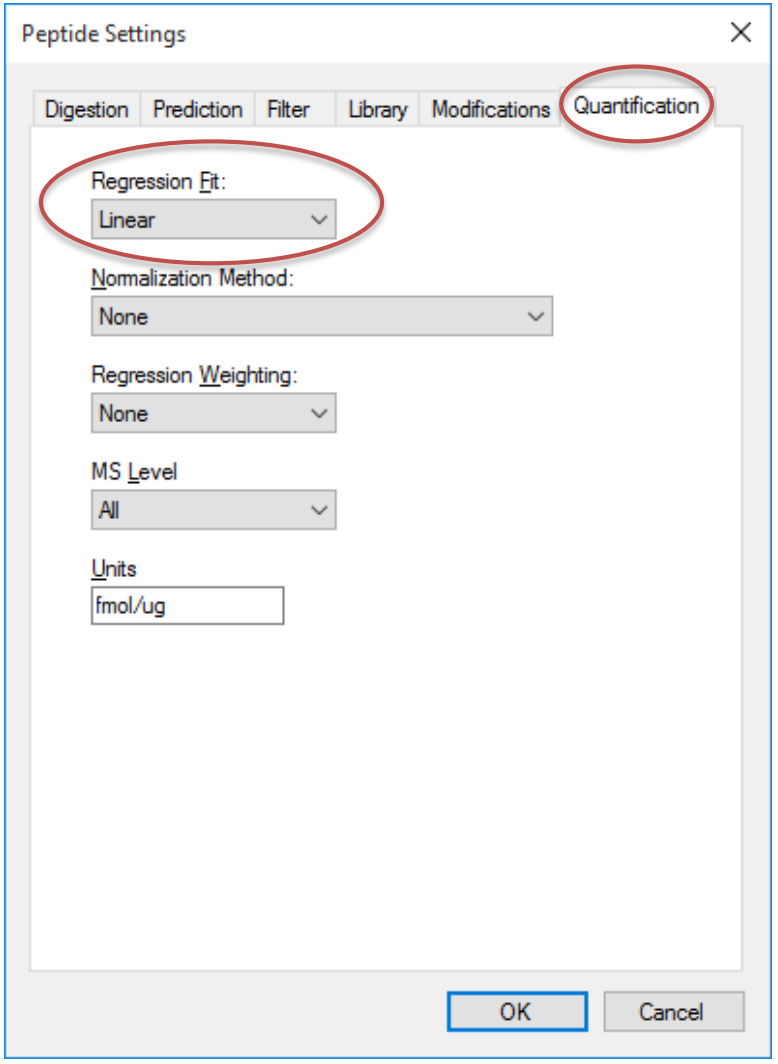
Document Grid: Peptide Ratio Results

Views | 1 | von 1488 | Export... | Find:

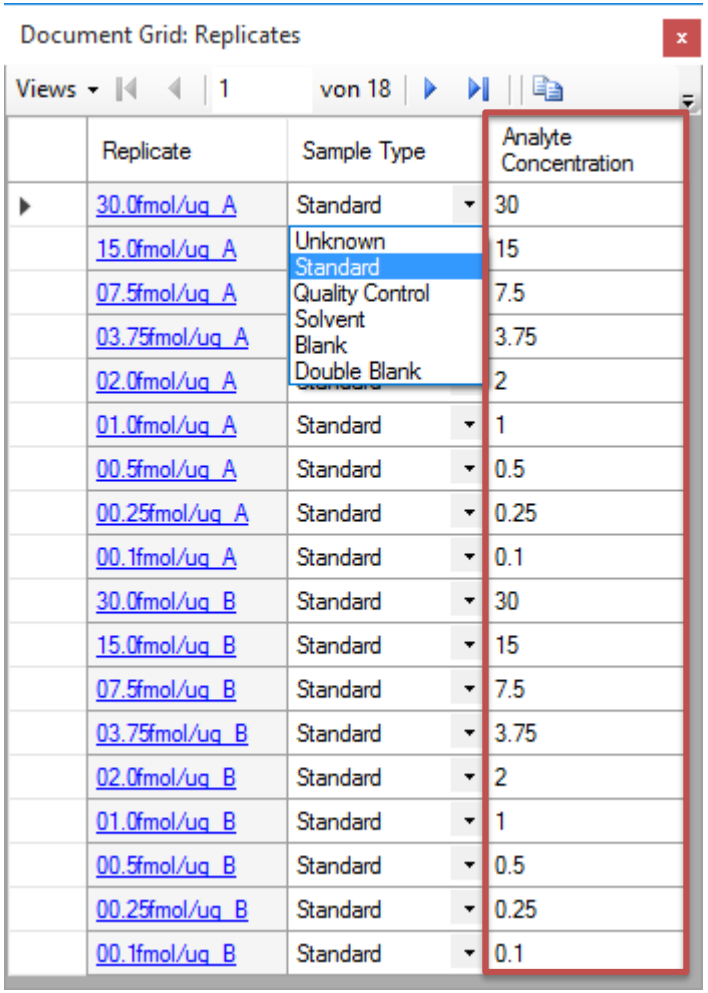
Peptide Sequence	Protein Name	Replicate Name	Peptide Peak Found Ratio	Peptide Retention Time	Ratio To Standard	Quantification
TIVGVSEDFAALR	Rv0041IleuS	Rep1_day00	1	68.44	0.4123	4.2877 fmol/ug
TIVGVSEDFAALR	Rv0041IleuS	Rep1_day05	1	67.73	0.3701	3.8491 fmol/ug
TIVGVSEDFAALR	Rv0041IleuS	Rep1_day10	1	67.24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

Multiple-point absolute quantification supported within Skyline

Peptide Settings → Quantification

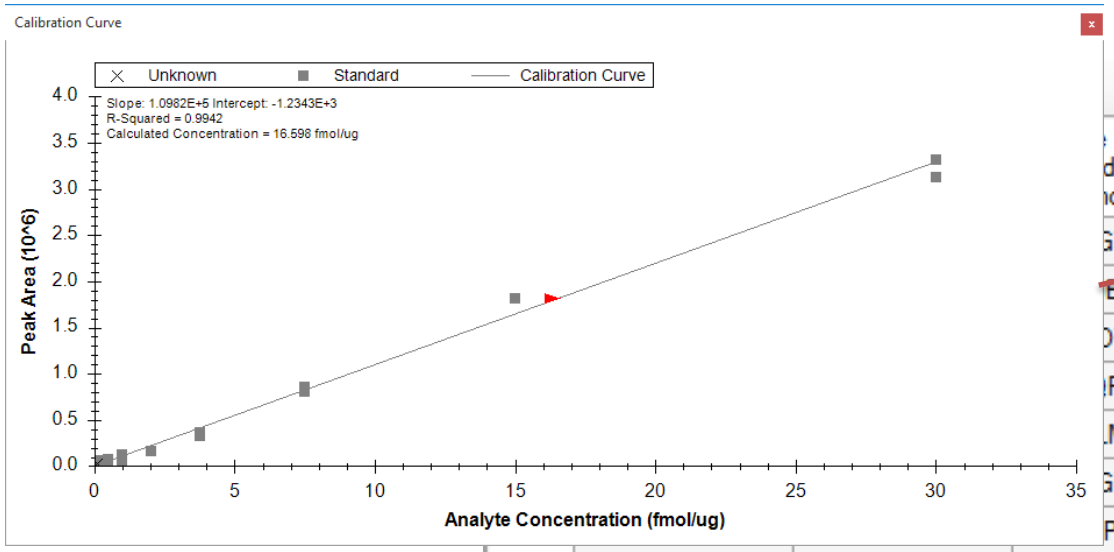


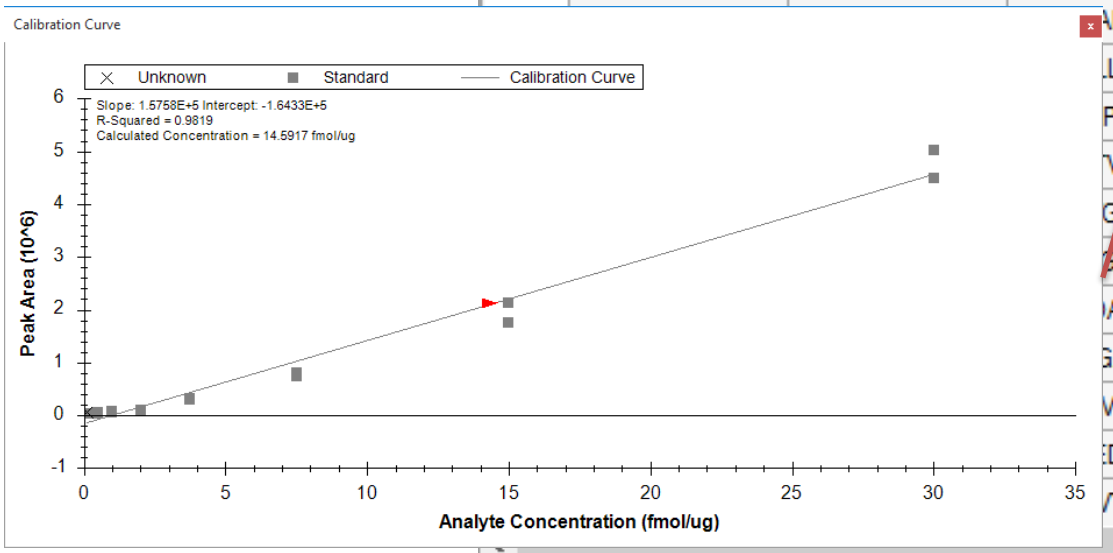
View → Document Grid → Views → Replicates



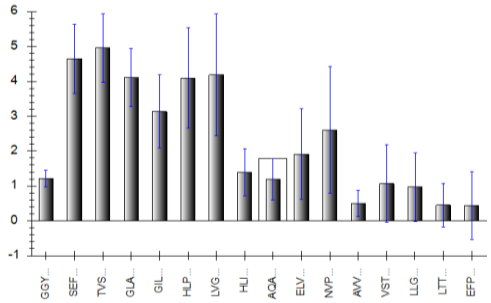
Multiple-point absolute quantification supported within Skyline

View → Document Grid → Views → Peptide Quantification



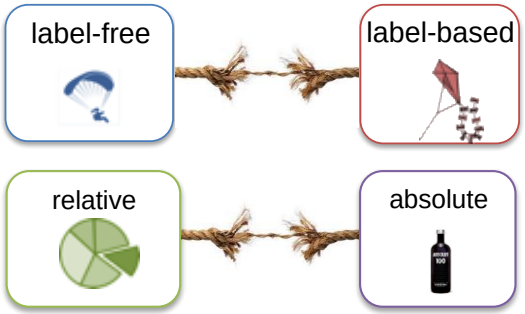


Take-home messages - Quantification



- 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)**
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- **Prof. Mike MacCoss (University of Washington)**
- **The whole Skyline Team**
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- Prof. Bruno Domon (Luxembourg Institute of Health)
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- Betty Friedrich (ETH Zürich)
- Ludovic Gillet (ETH Zürich)
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- 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

Skyline

Learn More

- 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?

- 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

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.

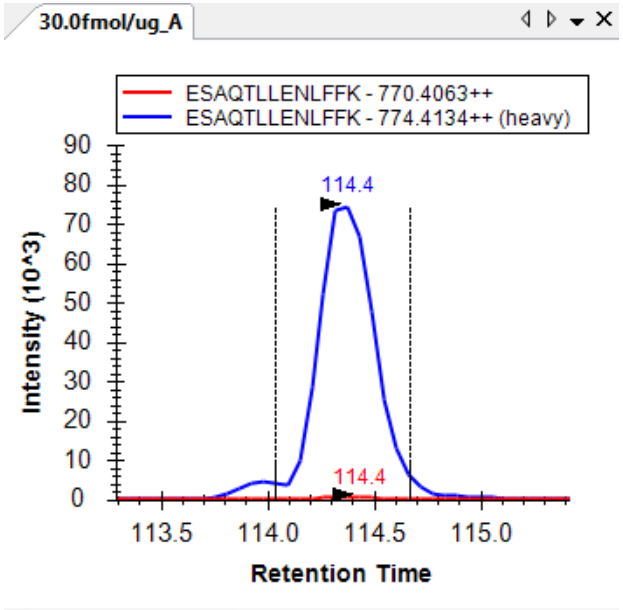
We look forward to seeing you at a future Skyline Tutorial Webinar.

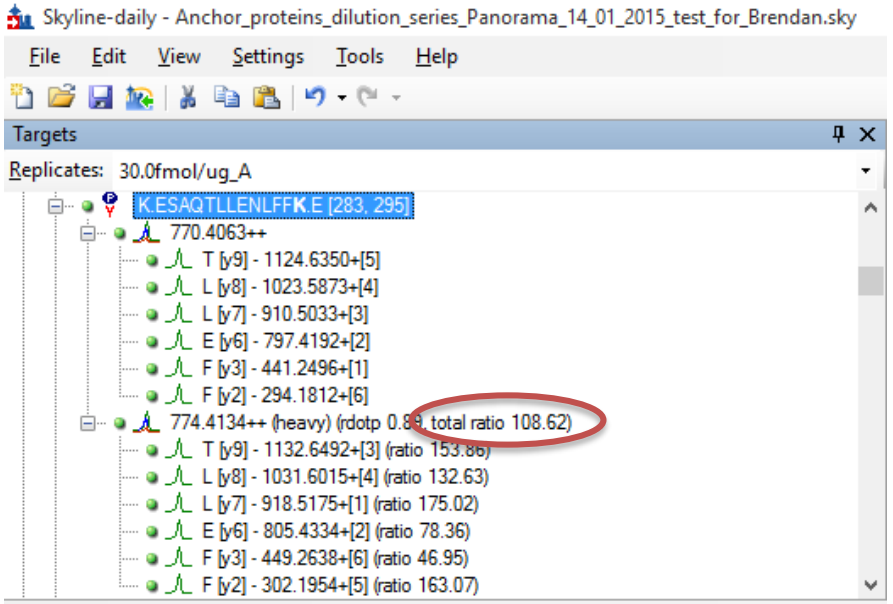
Additional slide material Christina Ludwig

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 form the synthetic peptide (ratios close to 1:1 help!)

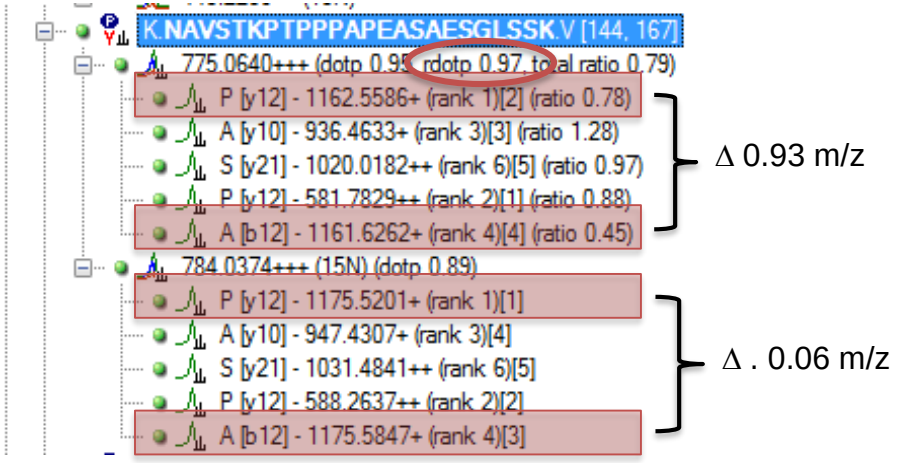


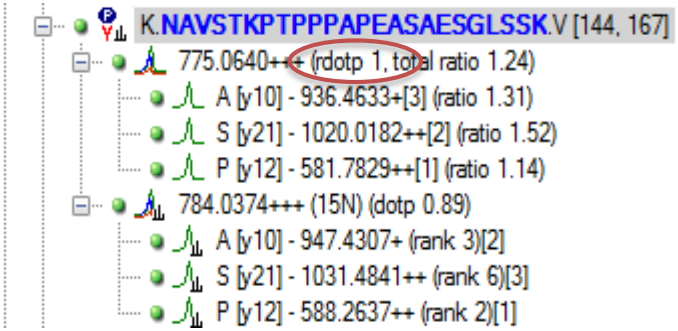


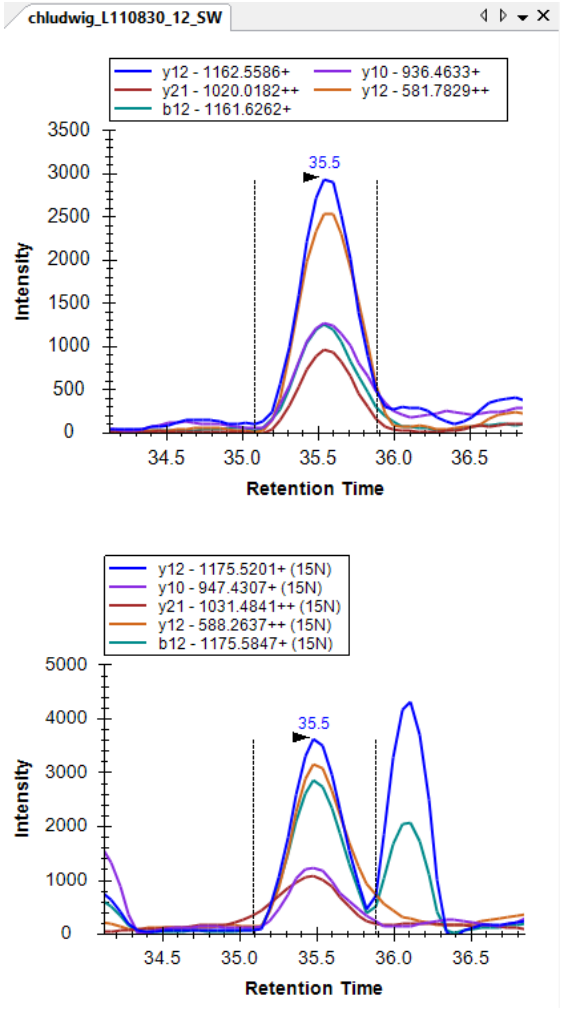
Tips and tricks: Isobaric transition interferences

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

→ Investigate your data!





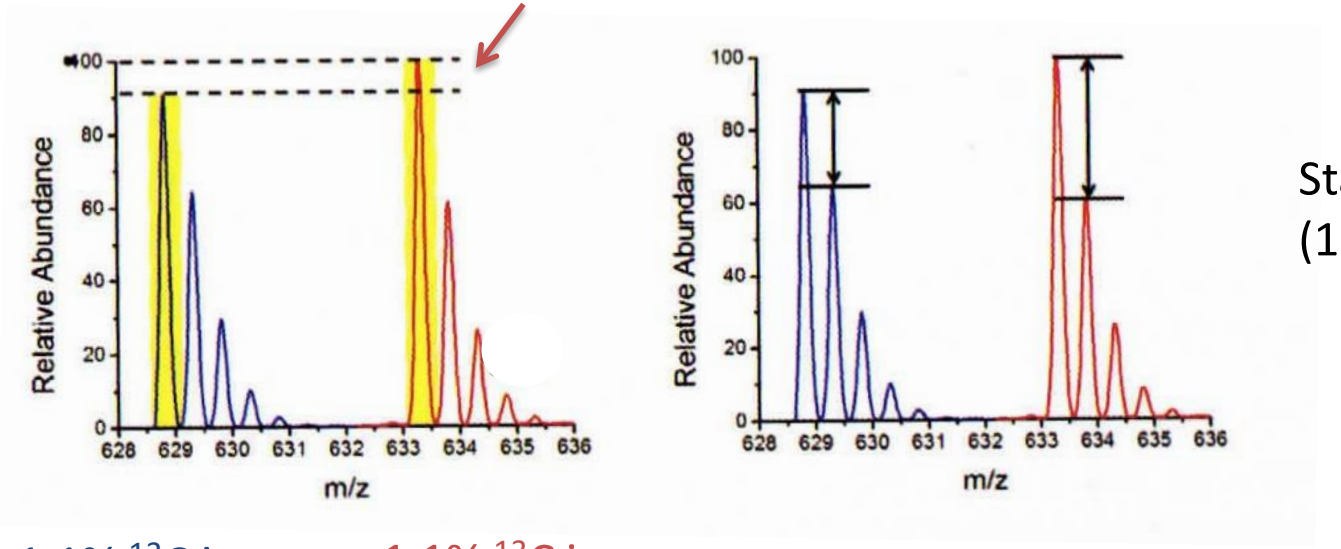


Tips and tricks: Different isotopic envelopes between light and heavy

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}C in
75 C-atoms

1.1% ^{13}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

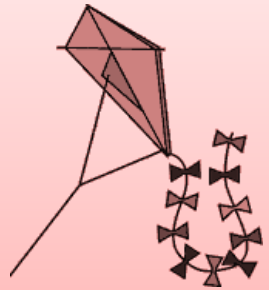
Issue when **handling AQUA peptides** (highly purified peptide solutions with precisely determined concentration):

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



label-based

1. Metabolic labeling

For example:

SILAC

^{15}N-labeling

2. Chemical labeling

For example:

Dimethyl-labeling

iTRAQ TMT

3. Enzymatic labeling

For example:

^{18}O-labeling

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

For example:

synthetic peptides

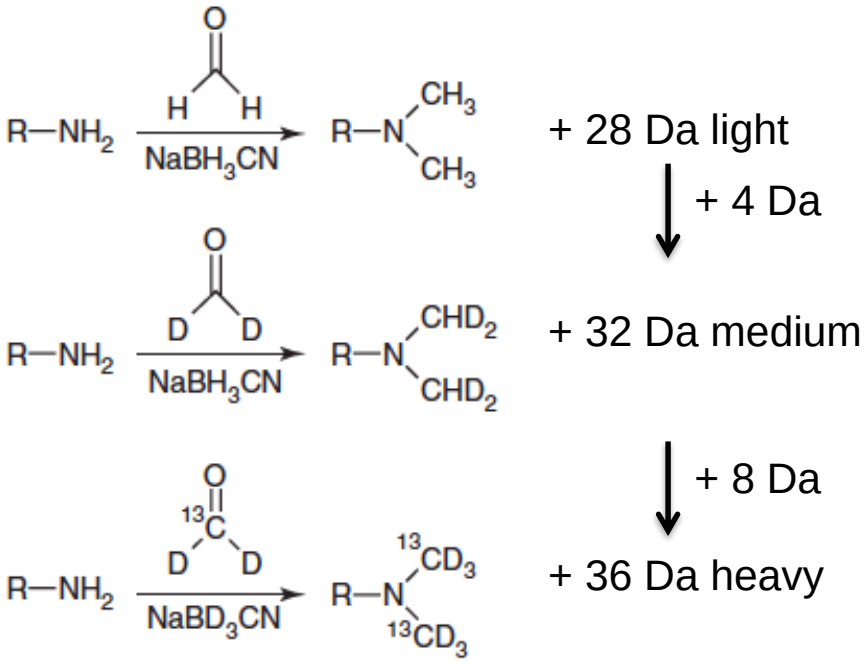
protein standards

Skyline supports chemical labeling

2. Chemical labeling

Dimethyl labeling

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

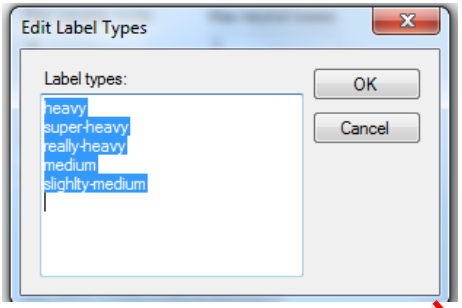


Boersema, P. J. et al., Nature Protocols, 4, 484-494 (2009).

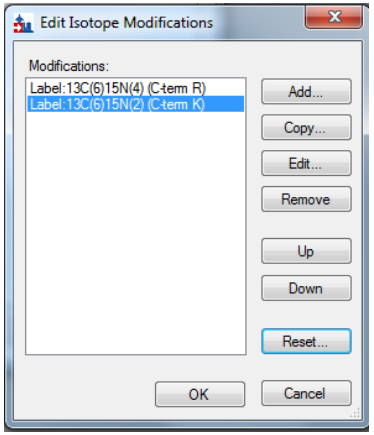
Skyline supports chemical labeling

2. Chemical labeling

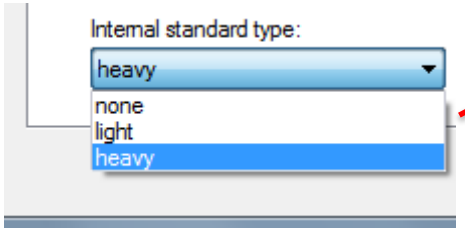
You can define and name labels ; select those relevant for your experiment.

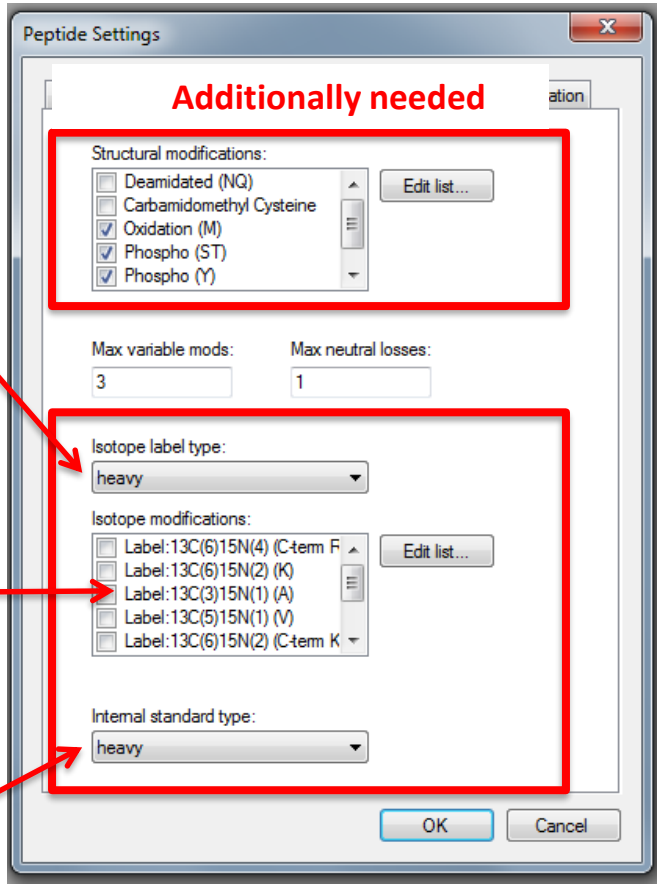


You can select a set of possible isotope modifications; enable those relevant for the label.



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





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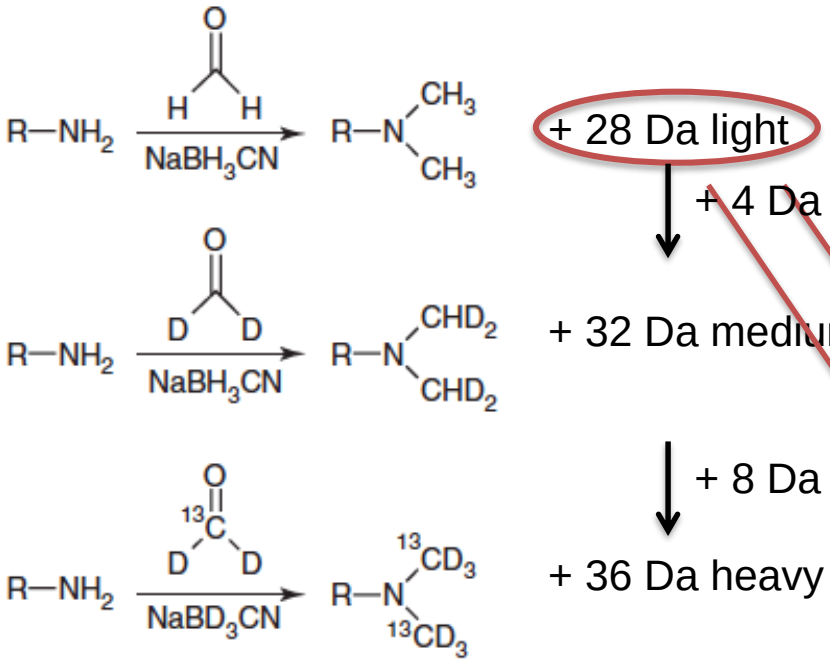
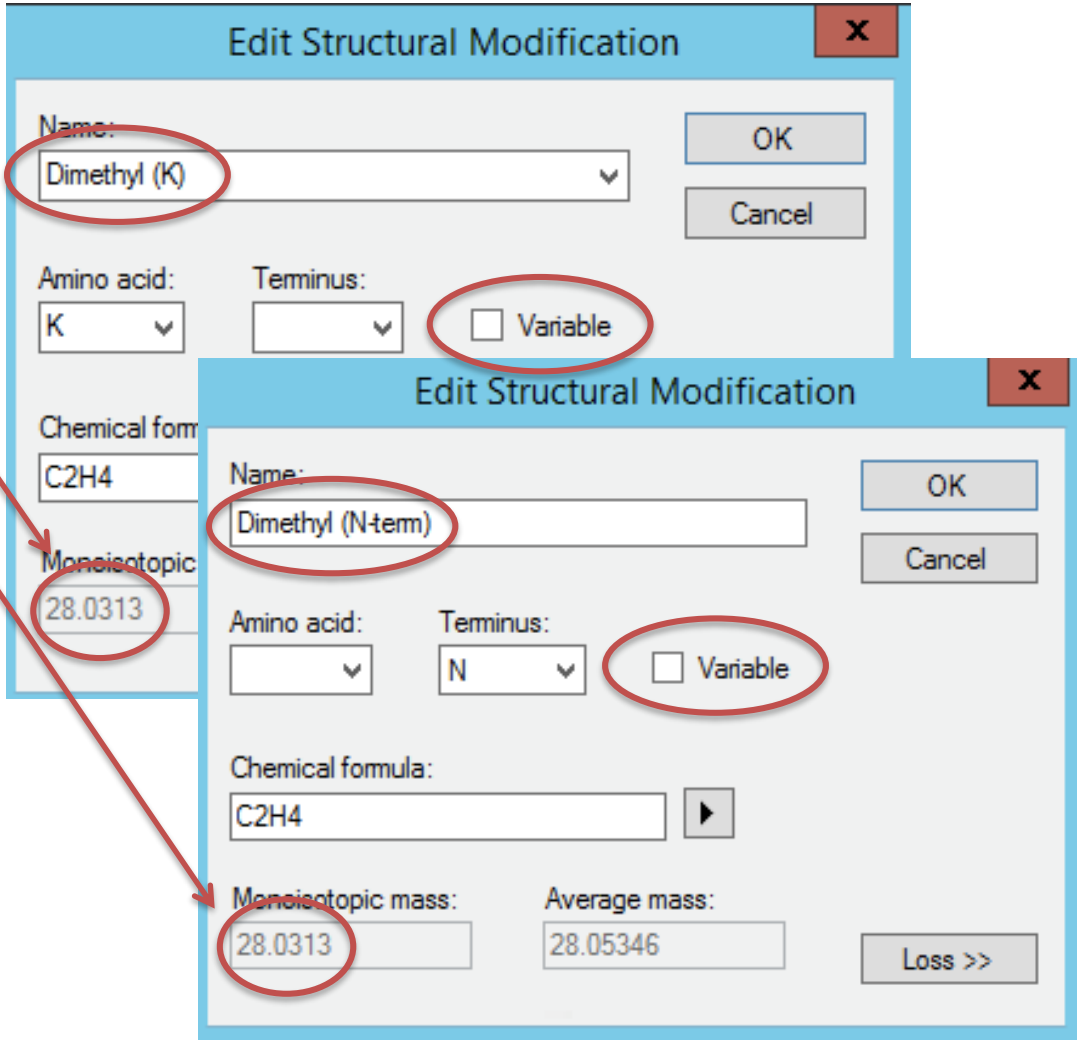


Boersema, P. J. et al., Nature Protocols, 4, 484-494 (2009).

1. Define structural modification



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Dimethyl labeling

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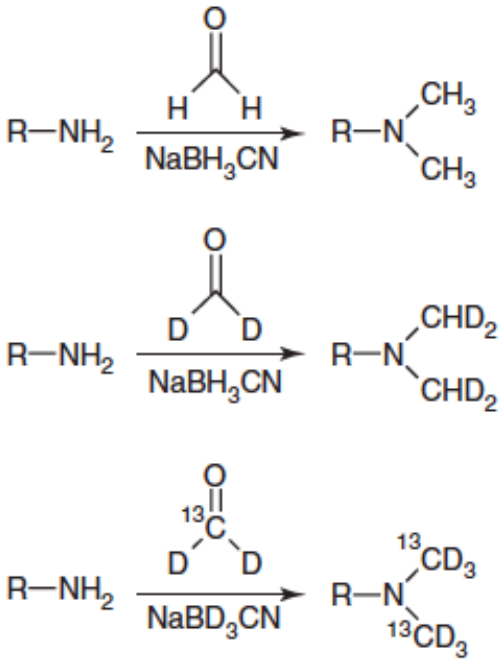


+ 28 Da light

+ 4 Da

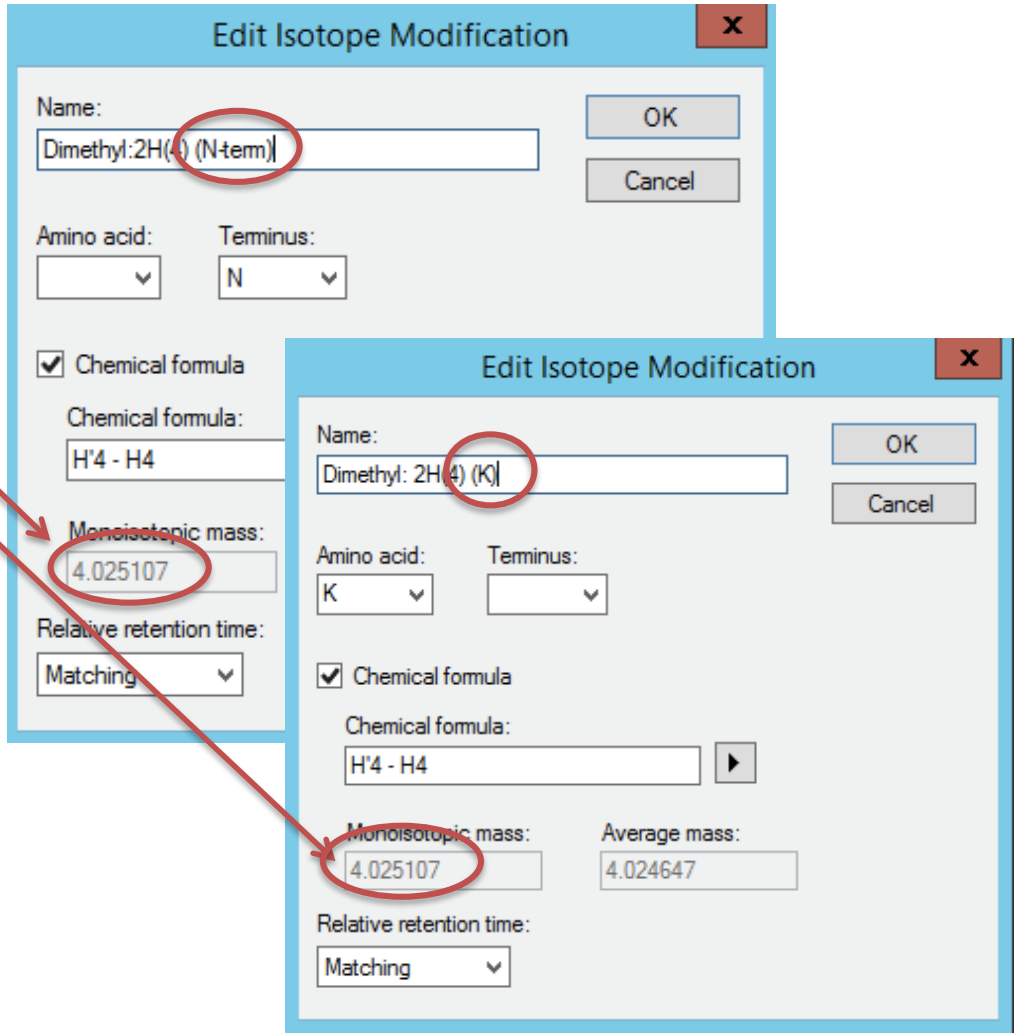
+ 32 Da medium

+ 8 Da

+ 36 Da heavy

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

2. Define isotope modification



Skyline supports chemical labeling

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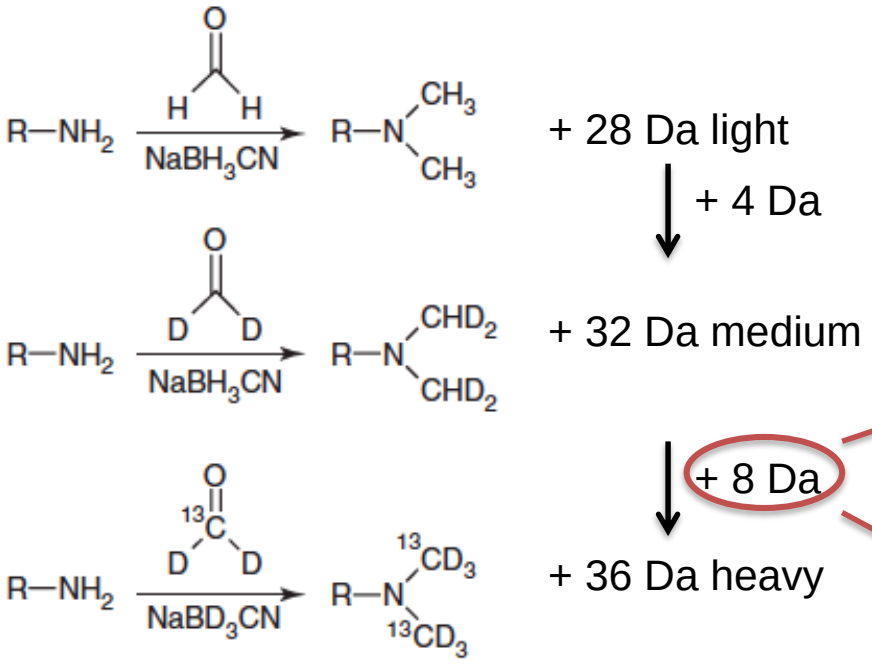

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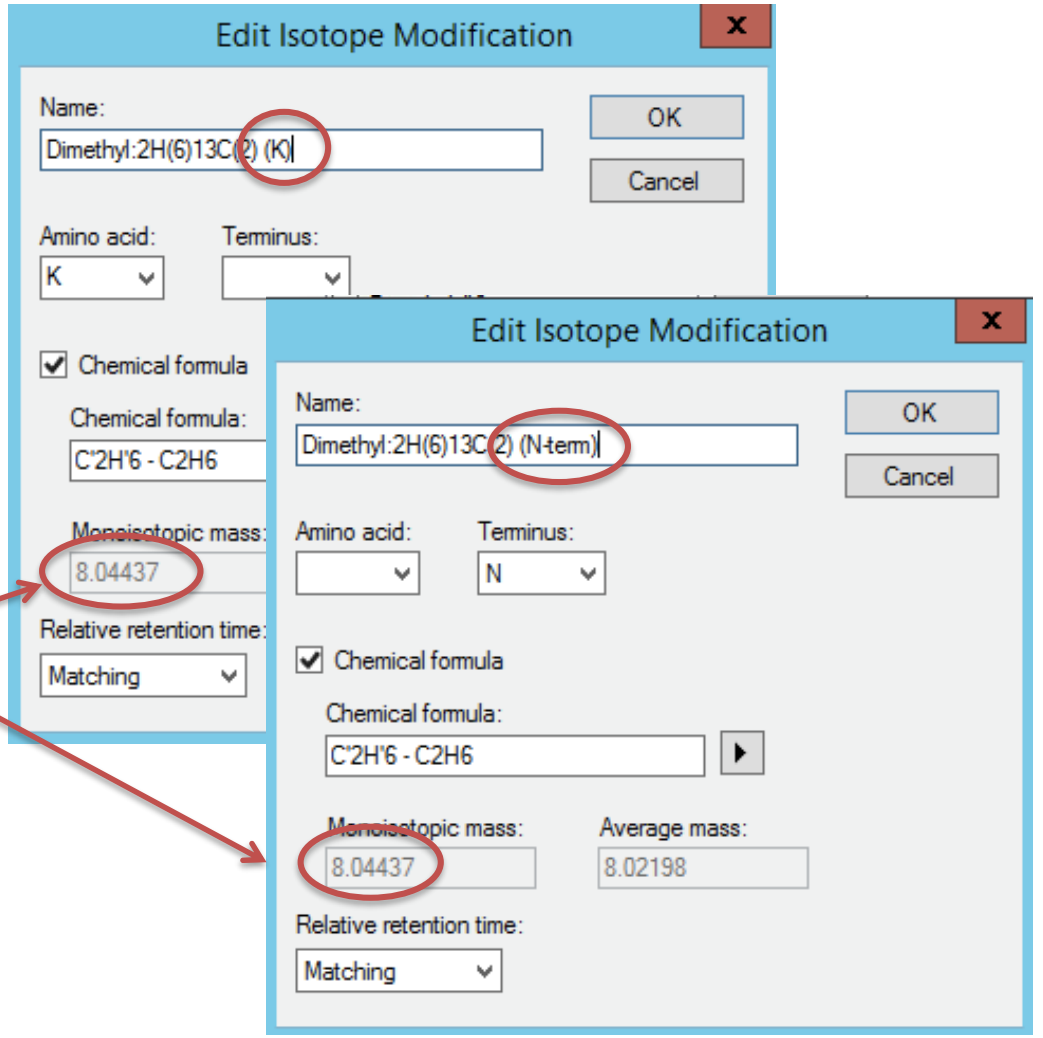

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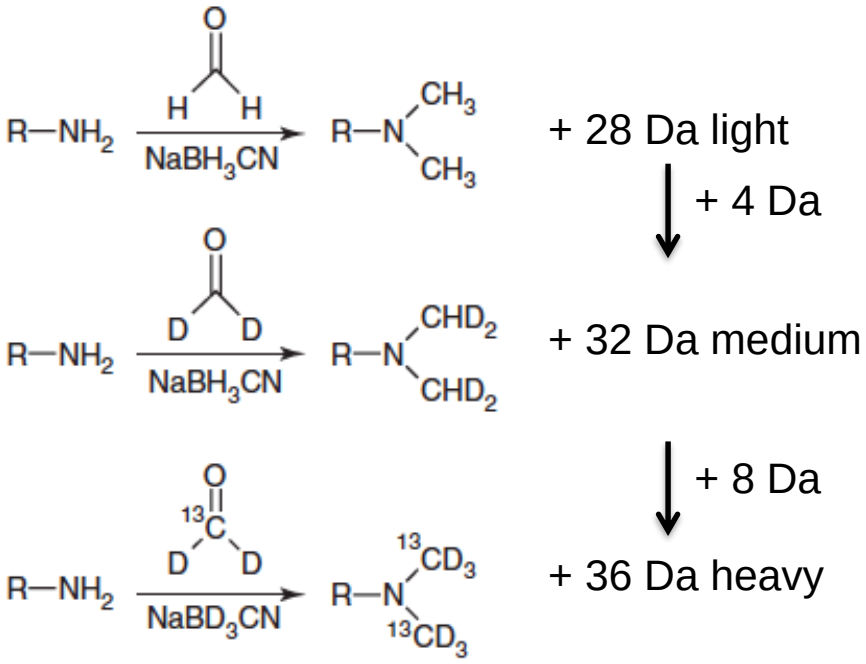

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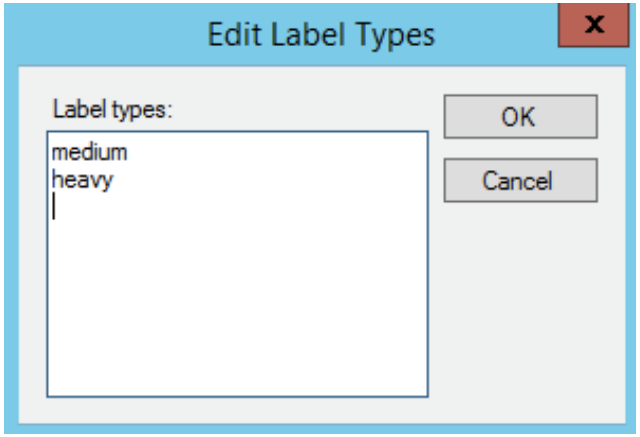
3. Create additional label type



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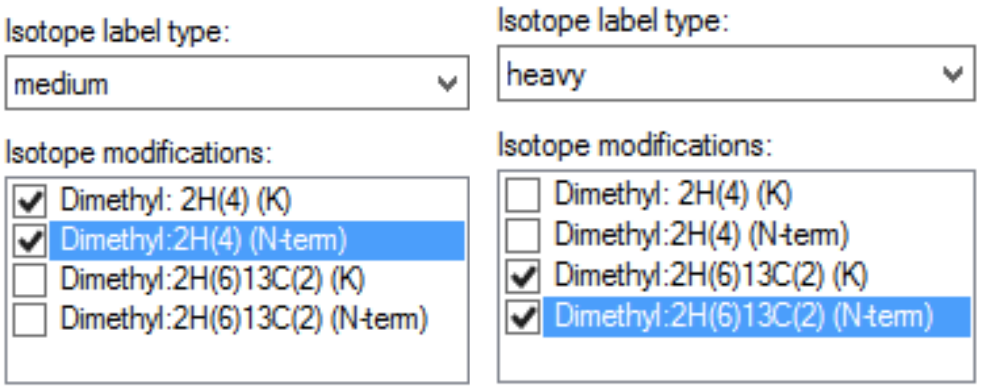

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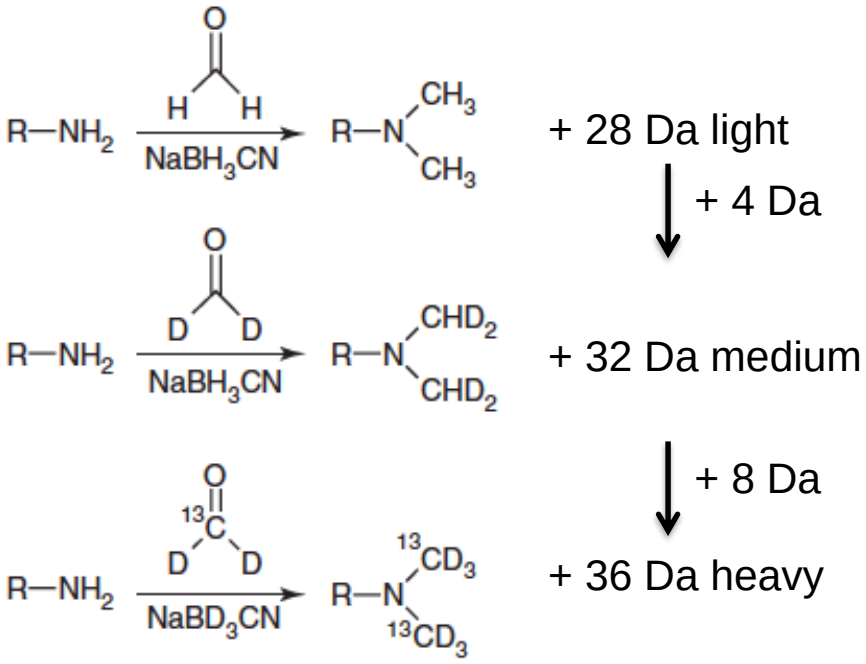

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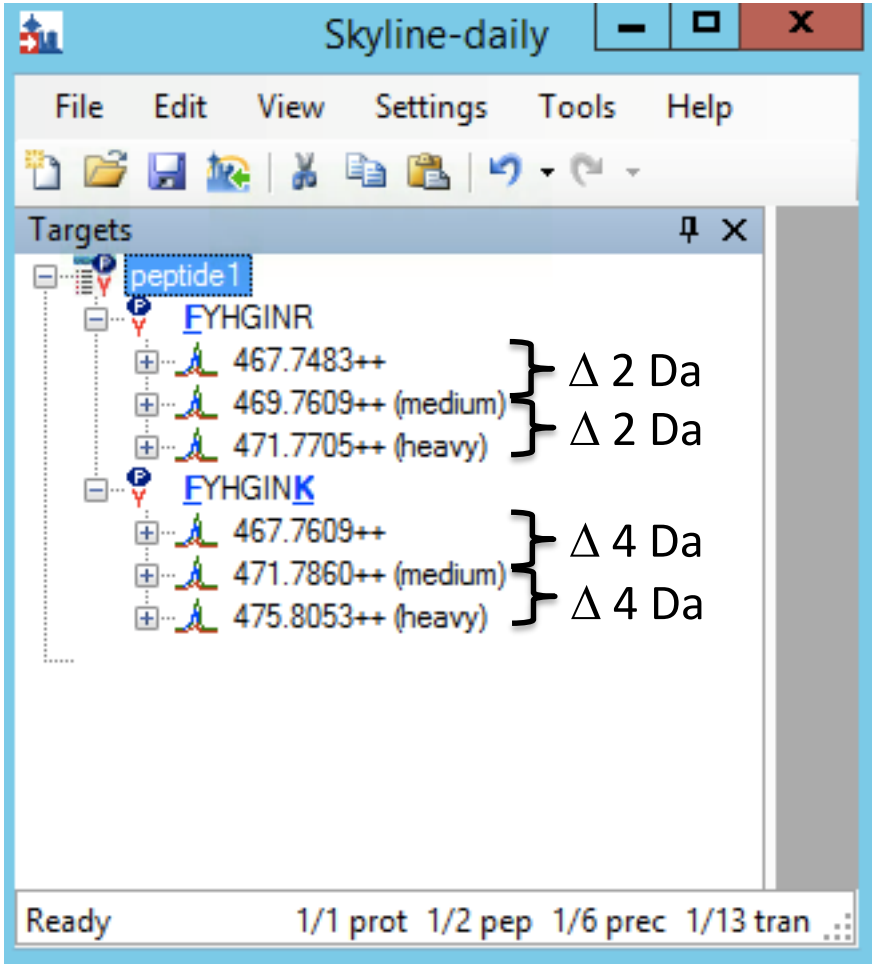
5. Isotope labeled variants will appear in peptide list



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

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

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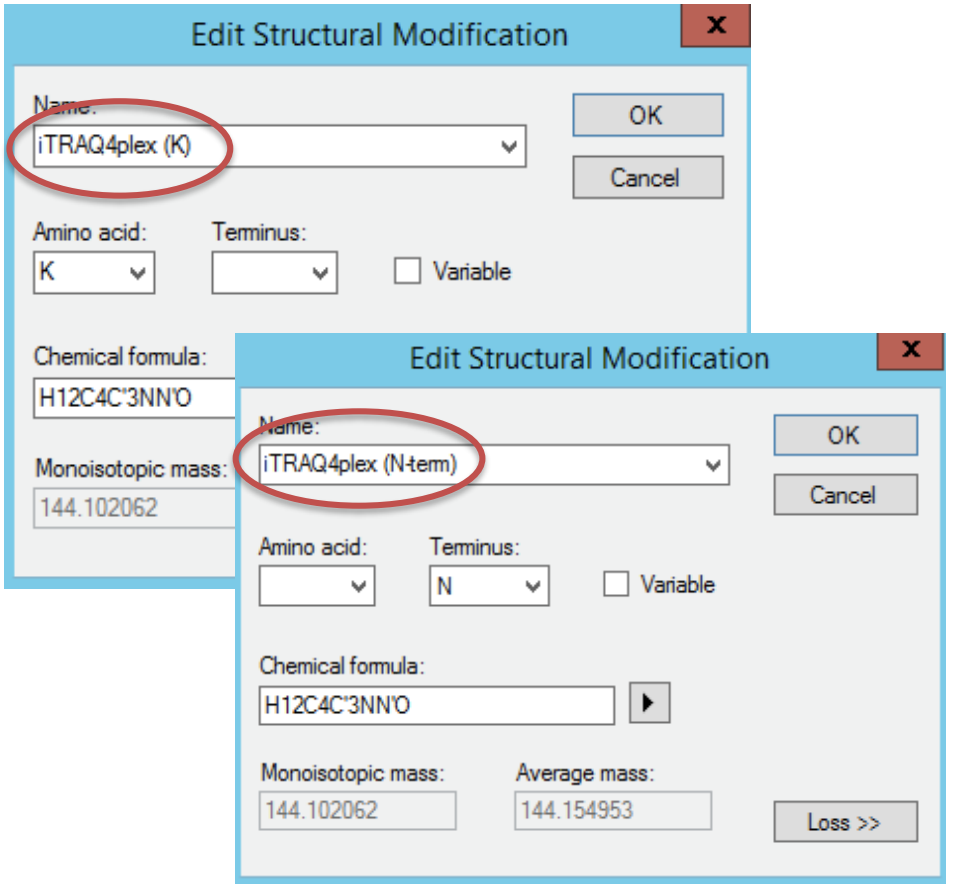

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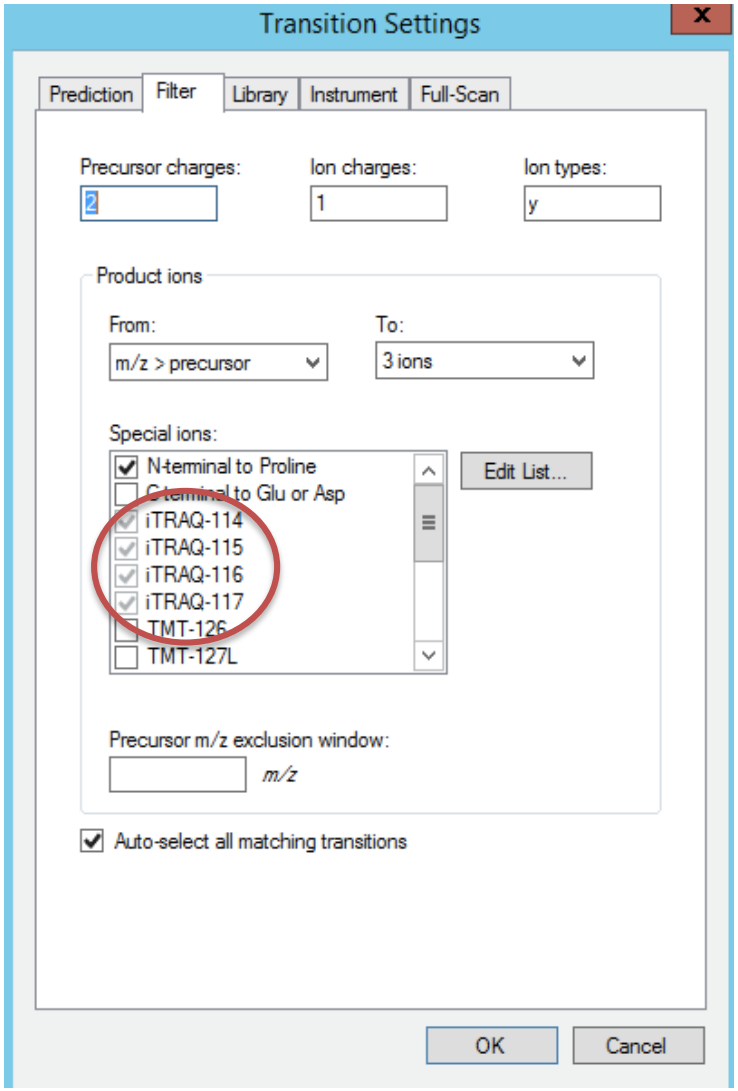

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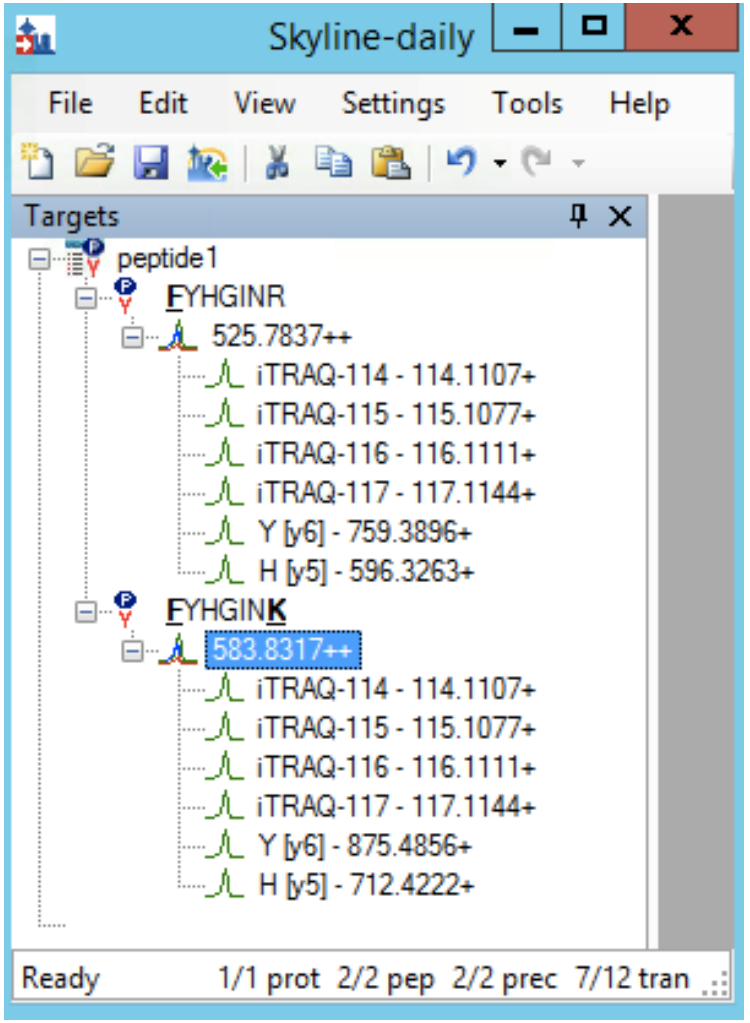
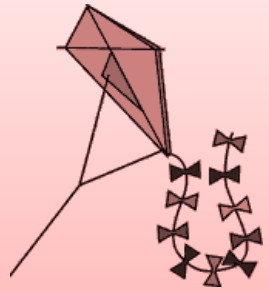


Strategies for incorporating stable-isotopes



label-based

1. Metabolic labeling

For example:

SILAC

^{15}N-labeling

2. Chemical labeling

For example:

Dimethyl-labeling

iTRAQ TMT

3. Enzymatic labeling

For example:

^{18}O-labeling

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

For example:

synthetic peptides

protein standards

Skyline supports enzymatic labeling

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Proteolytic ^{18}O labeling

Reaction 1 (peptide bond cleavage):

$$RC^{16}ONHR' + H_2{}^{18}O \xrightarrow{\text{protease}} RC^{16}O^{18}O^- + {}^+H_3NR'$$

+2 Da

Reaction 2 (carboxyl oxygen exchange):

$$RC^{16}O^{18}O^- + H_2{}^{18}O \xrightarrow{\text{protease}} RC^{18}O^{18}O^- + H_2{}^{16}O$$

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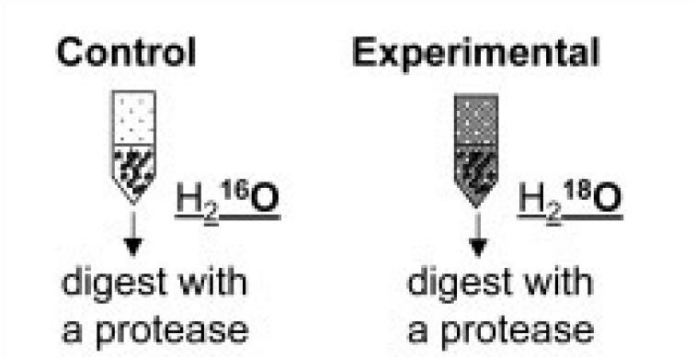

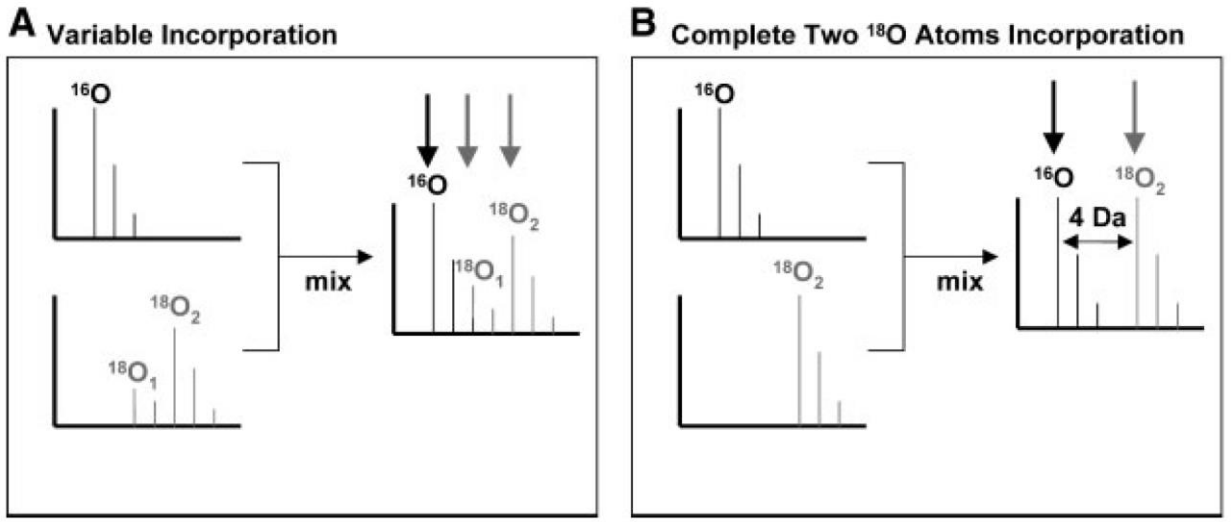

Miyagi, M. & Rao, K. C. S., Mass Spectrom Rev 26, 121–136 (2007).

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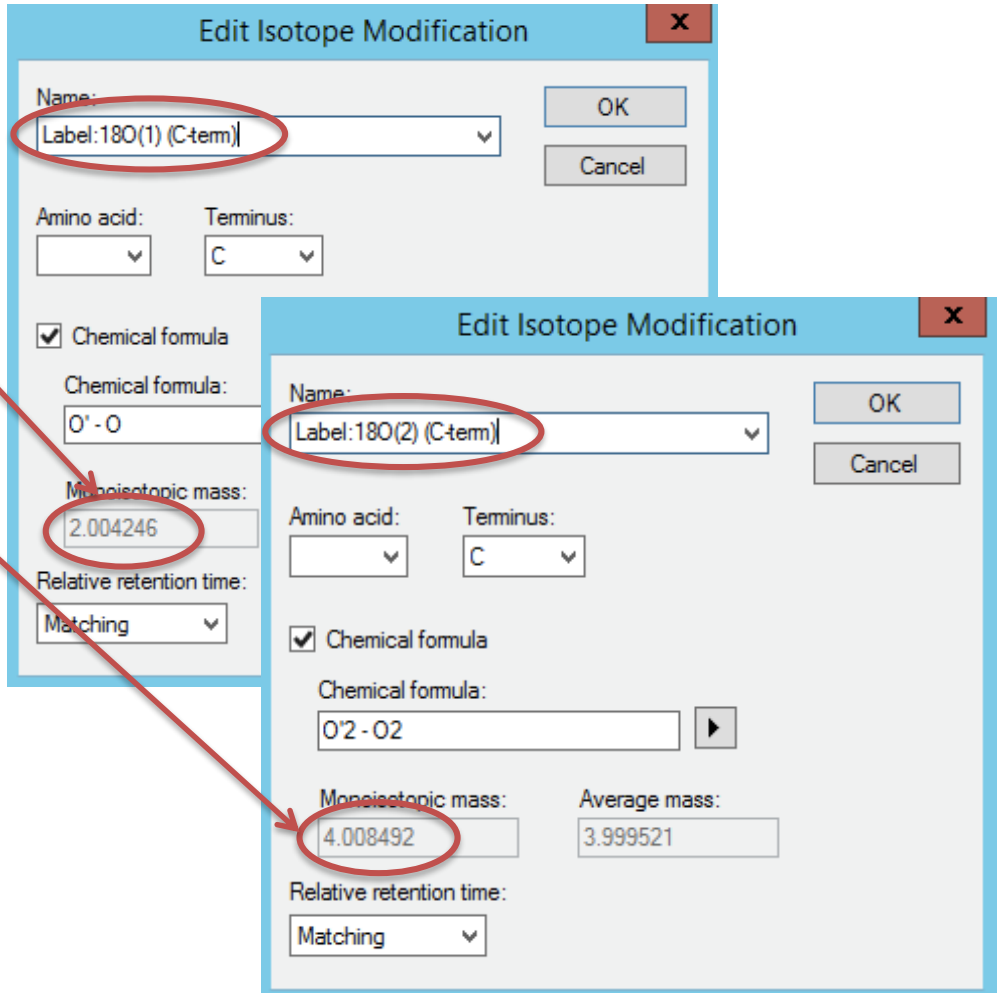
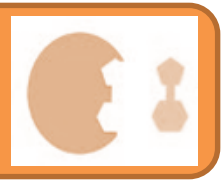


Skyline supports enzymatic labeling

3. Enzymatic labeling



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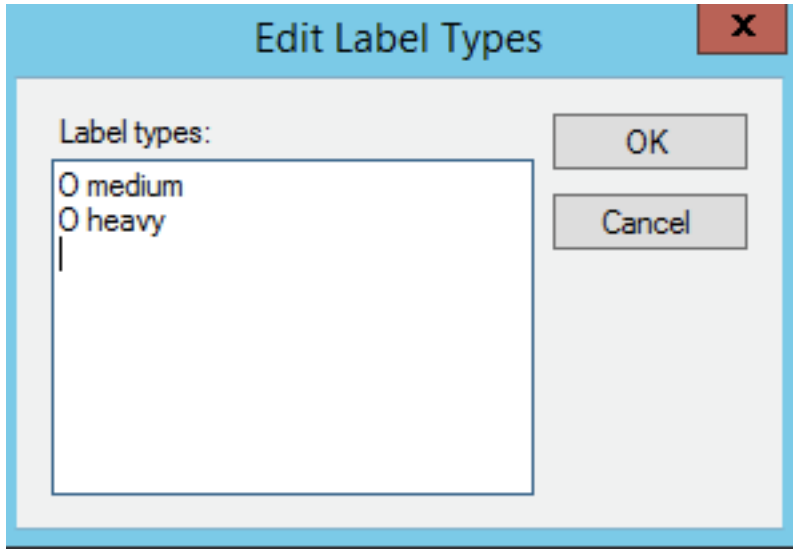

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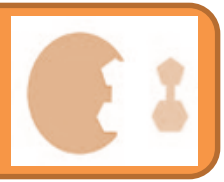

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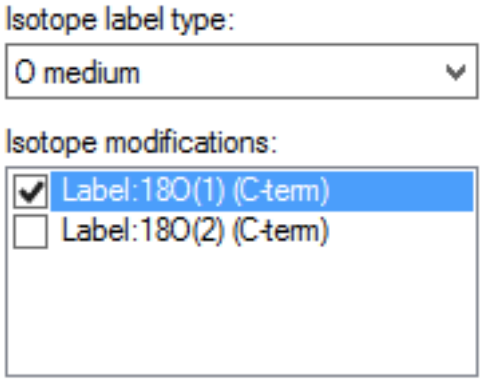
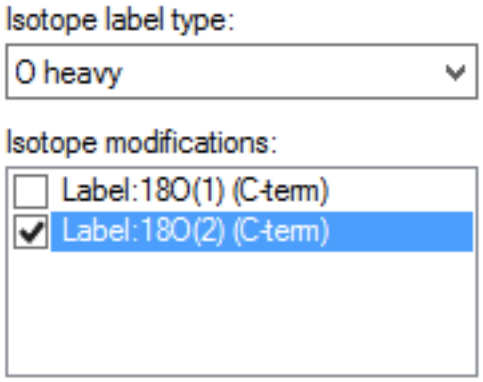
3. Define Label Types





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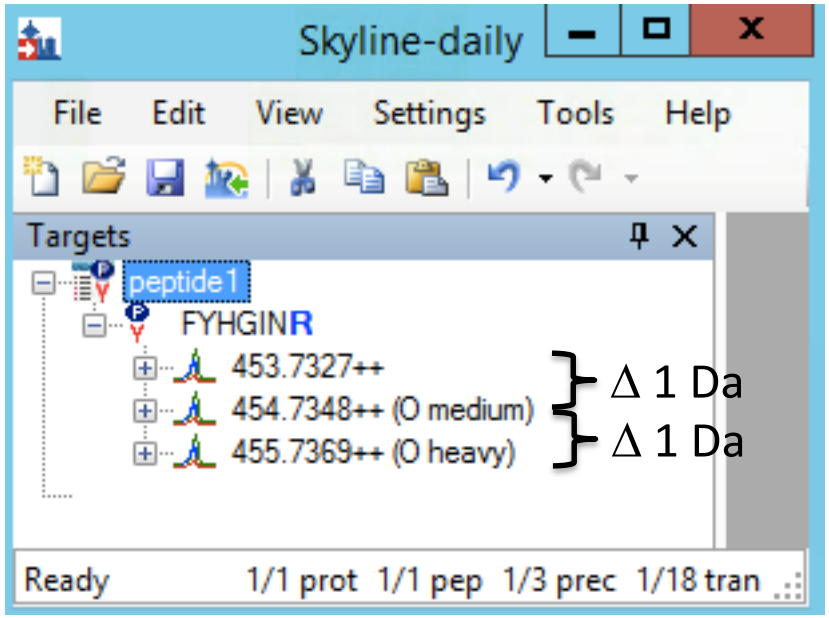
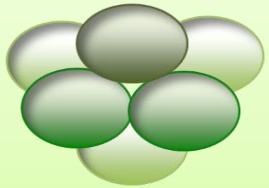


1. Define isotope modifications
2. Create additional label type
3. Define Label Types
4. Isotope labeled variants will appear in peptide list

For which projects does absolute protein quantification really matter?

protein complex stoichiometries

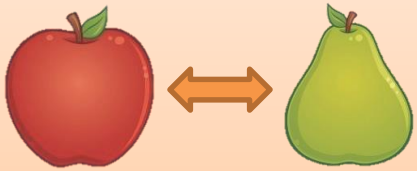


3 : 2 : 1

mathematical modeling

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

data cross-comparison



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

absolute protein concentration

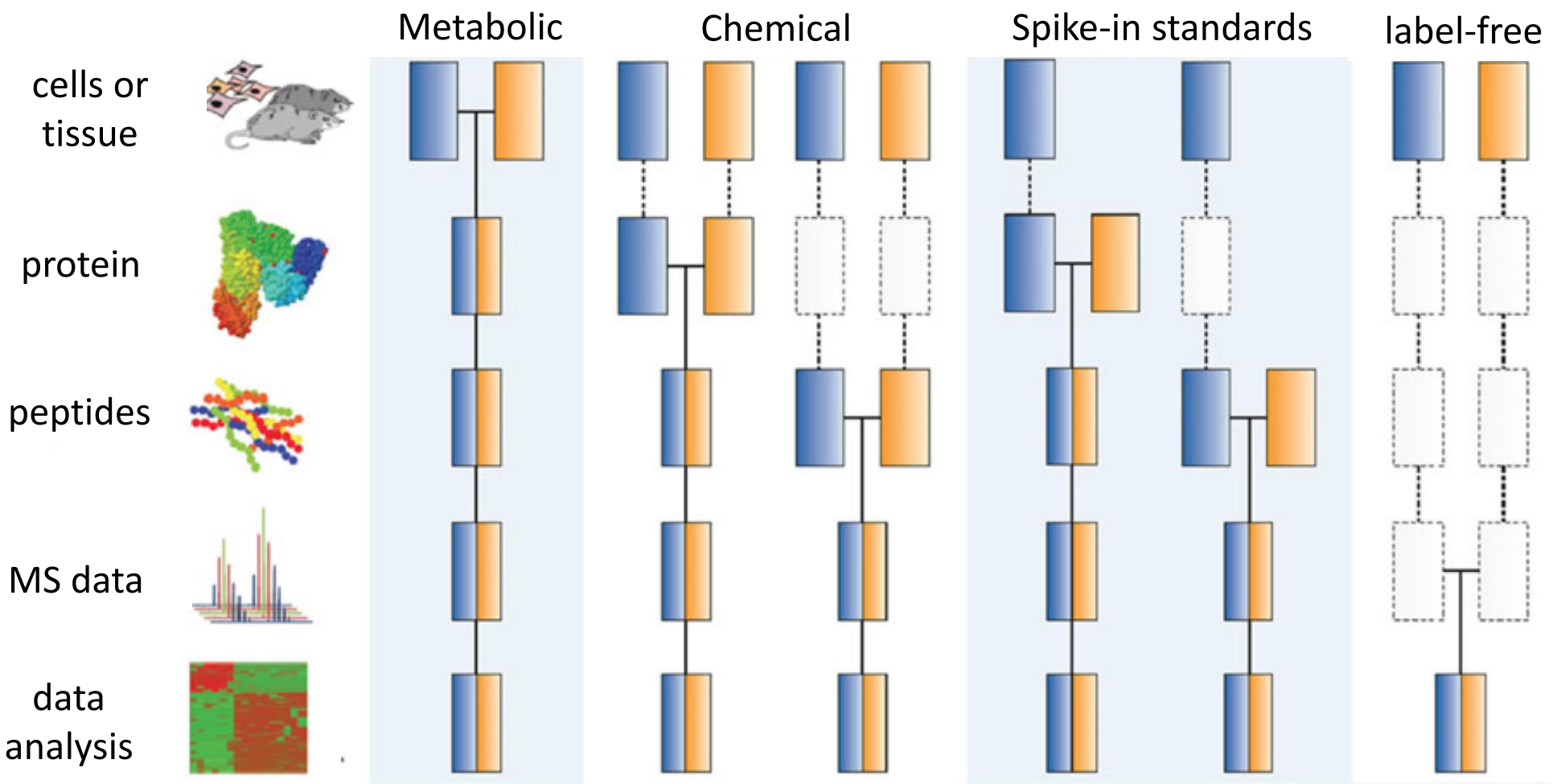
pharmaceutical and biomedical industry

- 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!



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