Quantitative Proteomics

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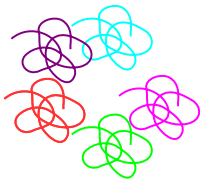
Mass Spectrometry – IOCB AS CR

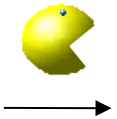
Presentation Outline

- **Bottom-up approach**
- **Before you start**
  - Experimentalal design
- **Quantitation methods**
  - Labelling techniques
    - Metabolic labelling – SILAC
    - Chemical labelling – iTRAQ, dimethyl labelling
  - Label-free techniques
    - Targeted approach – SRM
    - MS/MS^{all} approach –SWATH
- **Summary**

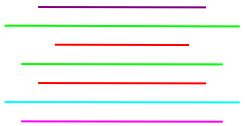
Bottom-up Approach in Proteomics



Complex Protein Mixture

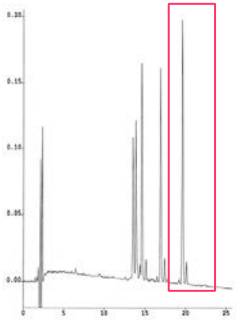


Proteolysis

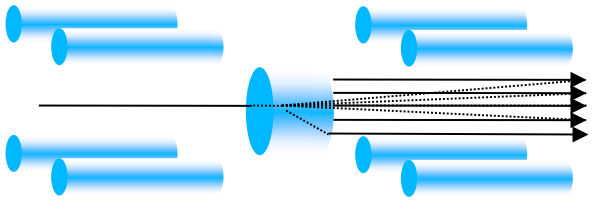


Peptide Digest

Quantitation

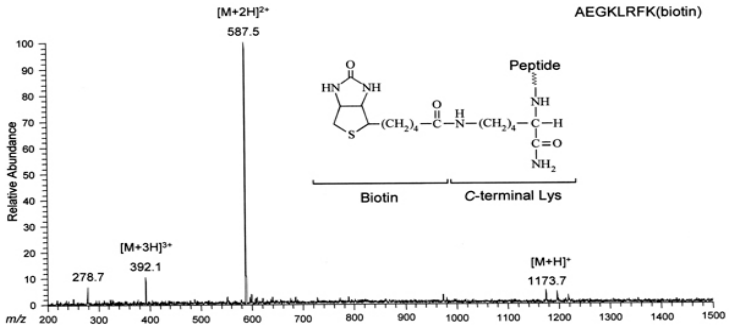


HPLC

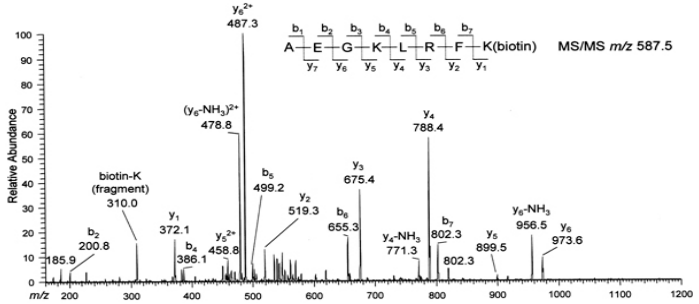


Mass Spectrometry

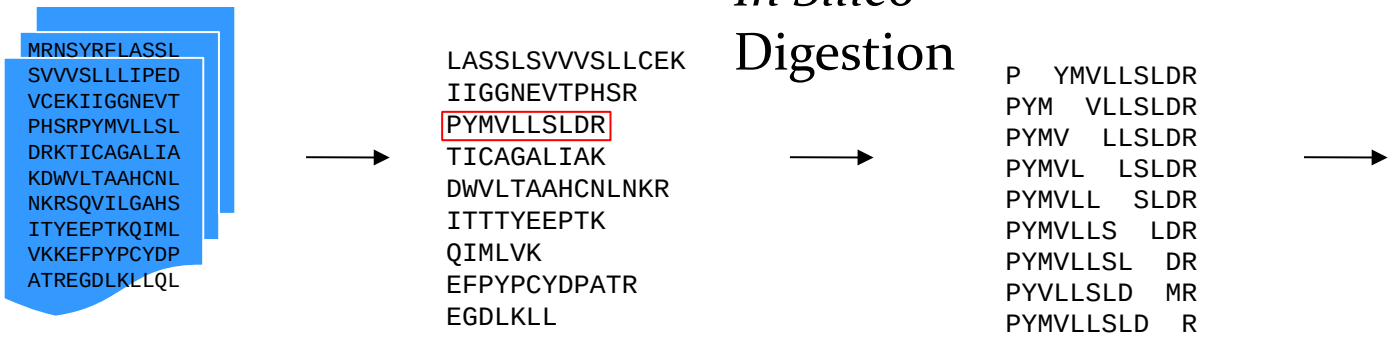
MS



MS/MS



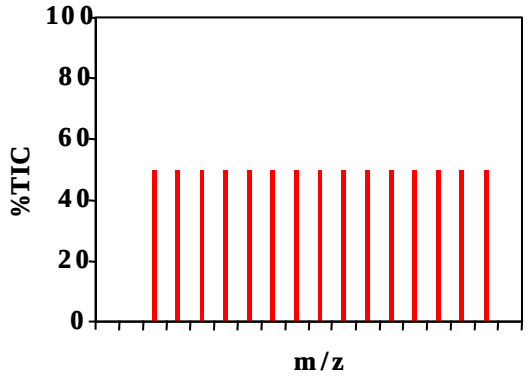
Peptide identification Database Search



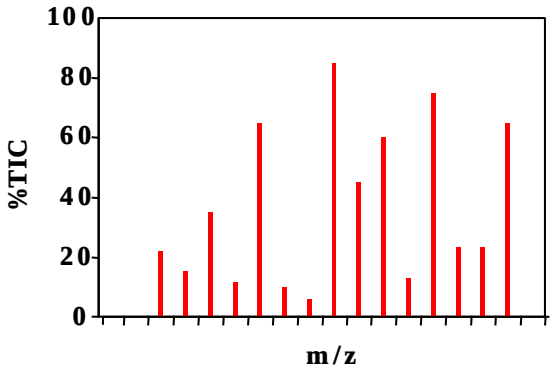
In Silico Digestion

Protein Database

In Silico Fragmentation



Paragon algorithm

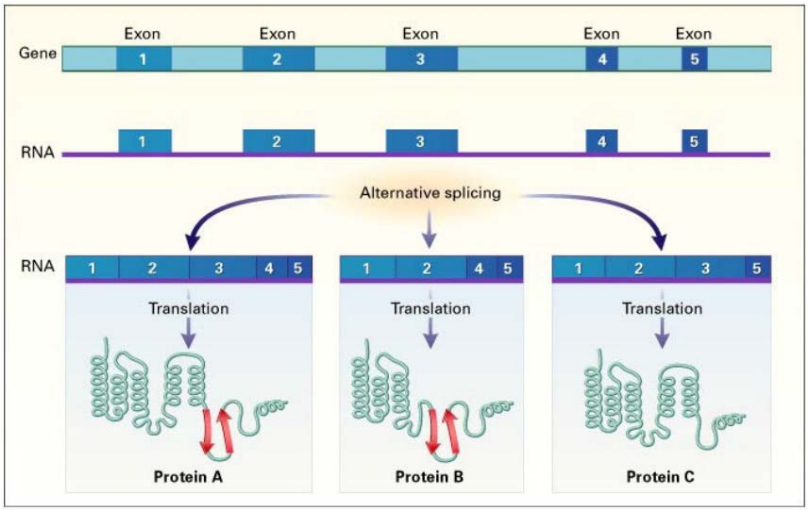


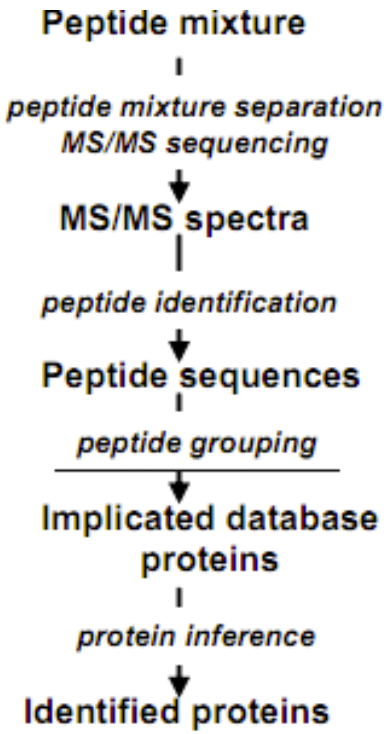
Theoretical Fragmentation Spectrum

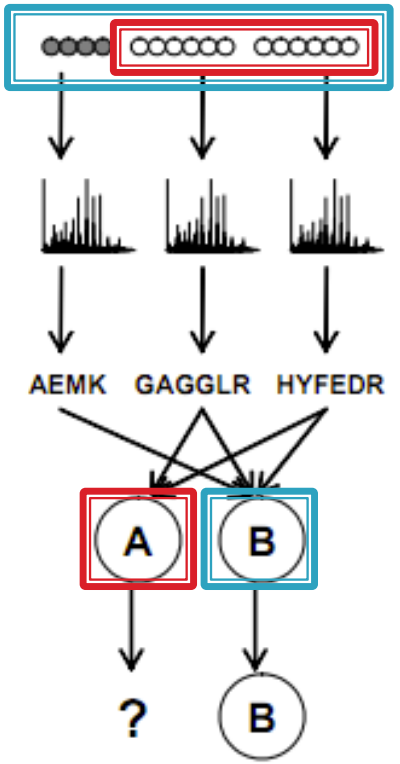
Experimental Fragmentation Spectrum

Limitations of Bottom-up Approach

- Protein level information is inherently lost
  - **Proteins are quantified indirectly**, their ratios are inferred **from peptides** after digestion.
  - **Protein isoforms** impose a problem to protein identification and quantitation. Close *inspection of data on peptide level* is required.







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- Before you start
  - Experimental design
- Quantitation methods
  - Labelling techniques
    - Metabolic labelling – SILAC
    - Chemical labelling – iTRAQ, dimethyl labelling
  - Label-free techniques
    - Targeted approach – SRM
    - MS/MS^{all} approach –SWATH
- Summary

Before you start: Experimental design

What do you want to quantify?

- One protein you know or as many as possible?
- What do you know about the proteins and the sample? Do you expe… …ichment?

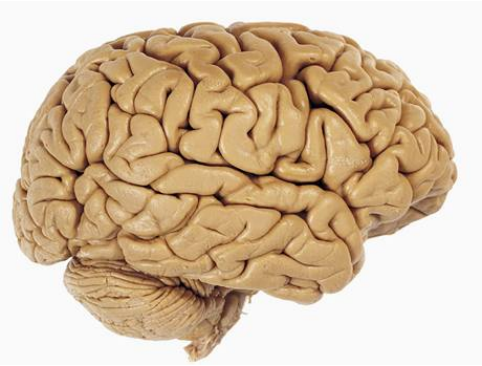
How preci… …esults?

- What is th… …you expect major cha… …ent?
- How man… …Can you validate t… …thod?

Bear in mind: Protein quantitation is a complex task, there is no simple solution. Not all quant. approaches may be suitable in your case.

Consultation is necessary before you begin your experiment!

Experimental design: Choosing your quantitation method



- Sample type
  - Cell culture vs. tissue sample
  - Complexity of the sample and variation in protein abundancy
- Sample preparation prior to LC-MS/MS
  - Clean-up and enrichments strategies
    - sample loss (use of internal STD), but removal of interference
- Shotgun or targeted analysis
  - Large scale screening of up- or down-regulated proteins or biomarker confirmation

→ **Application of label or label-free techniques**

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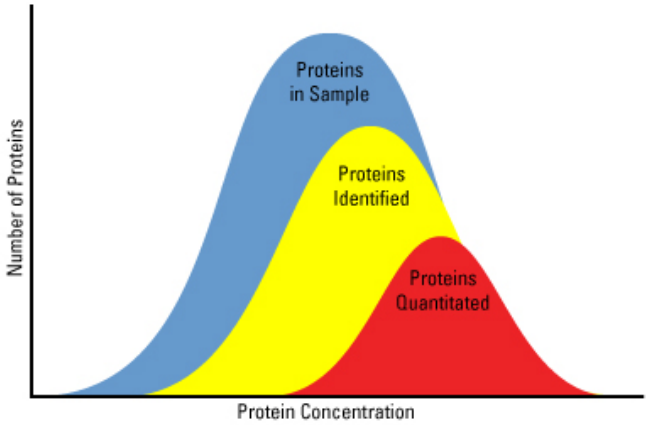
MS quantitation in proteomics

- Often only **relative** determination of quantity
- Based on **peak heights or areas**
- Quantity of a protein can be defined by **peaks** from
  - Precursor peptide(s) m/z (**MS level**)
  - Fragment peptide ion(s) m/z (**MS/MS level**)
- **Labelling techniques**
  - A **mass tag (label)** is introduced into the protein or peptide. Compared **samples are mixed together and analyzed.** The introduced mass shift enables relative quantitation.
- **Label-free techniques**
  - The **mass** of the protein or peptide remains **unchanged**, samples are **analysed separately.**

MS quantitation in proteomics

- **Protein abundance**

and **sample complexity** affect quantitation yield



- **Data dependent acquisition (DDA)** – usually optimized for protein identification not quantitation
  - Multiple injections of the same sample may result in partly different peptide identification lists
  - Only most intense peaks are subjected to MS/MS

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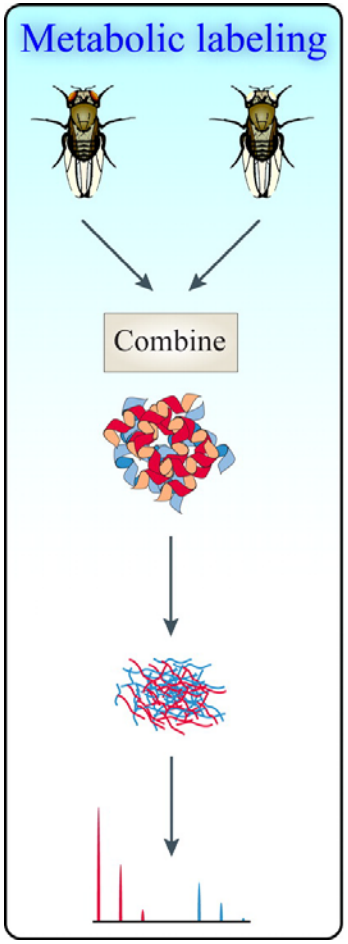
Labelling techniques

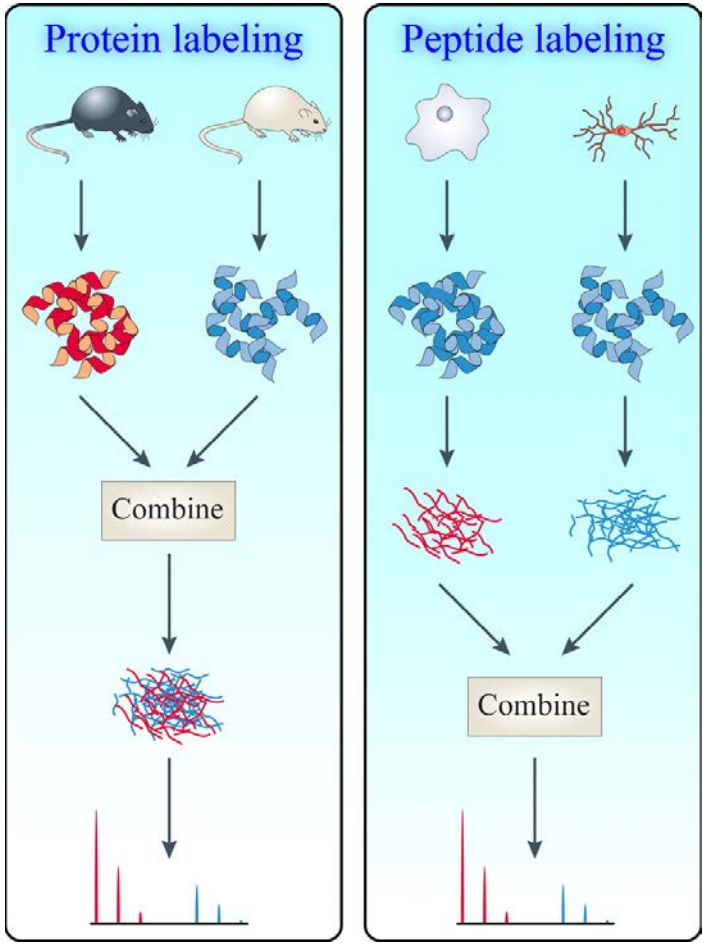
- Stable isotopes
  - Differential mass labels
    Heavy: ^{13}C, ^{15}N, ^{18}O, ^{2}H
    Light: ^{12}C, ^{14}N, ^{16}O, ^{1}H
  - Introduction of single elements
    - Trypsin digestion in $H_2{}^{18}O$
    - ^{15}N labelling of cell cultures
  - Introduction of compounds labelled by multiple heavy isotopes
    - Stable isotope labelling of amino acids in cell culture (SILAC)
    - Isobaric tag for relative and absolute quantitation (iTRAQ)
- Presumptions
  - **Equal** behavior under **chromatographic conditions** – corresponding H/L labeled peptides elute at the same time
  - **Equal MS sampling probability** of the isotopes during their elution window

Labelling techniques

- Metabolical
  - Eg. SILAC



- Chemical
  - Eg. Dimethyl labelling



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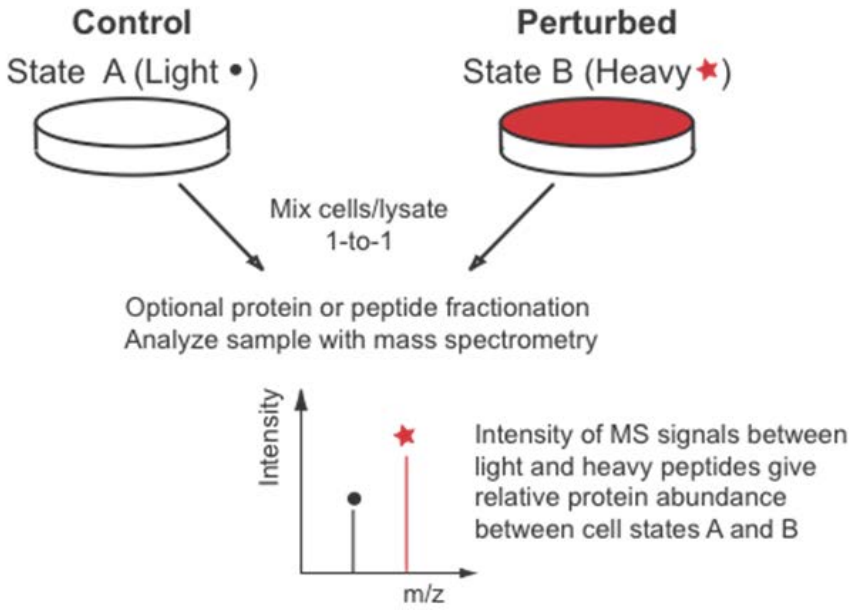
Metabolic Labelling – SILAC

Stable isotope labelling by amino acids in cell culture

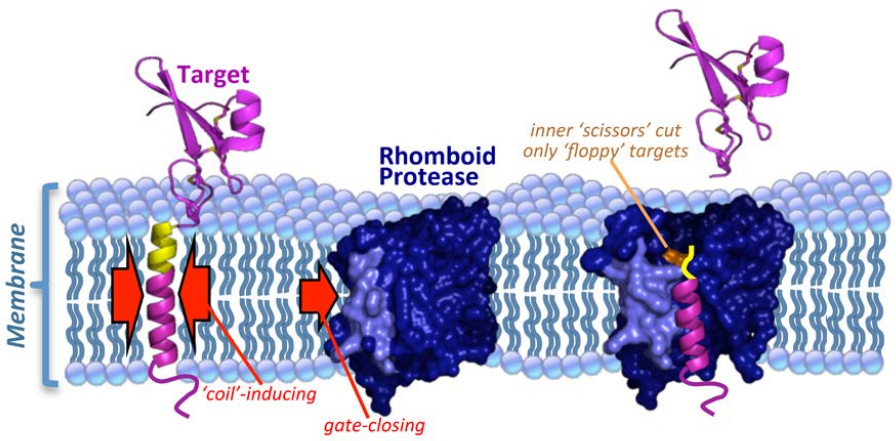
- **Cell culture** is grown on a medium containing either only heavy or light AAs (Arg, Lys) – auxotrophy required
- After at least 5 cell cycles a nearly full incorporation of the heavy AAs is achieved

- Labelled AAs are used as protein building blocks
  - Influence of sample preparation variations on quantitation results is **eliminated**
  - **Label incorporation** needs to be **monitored**

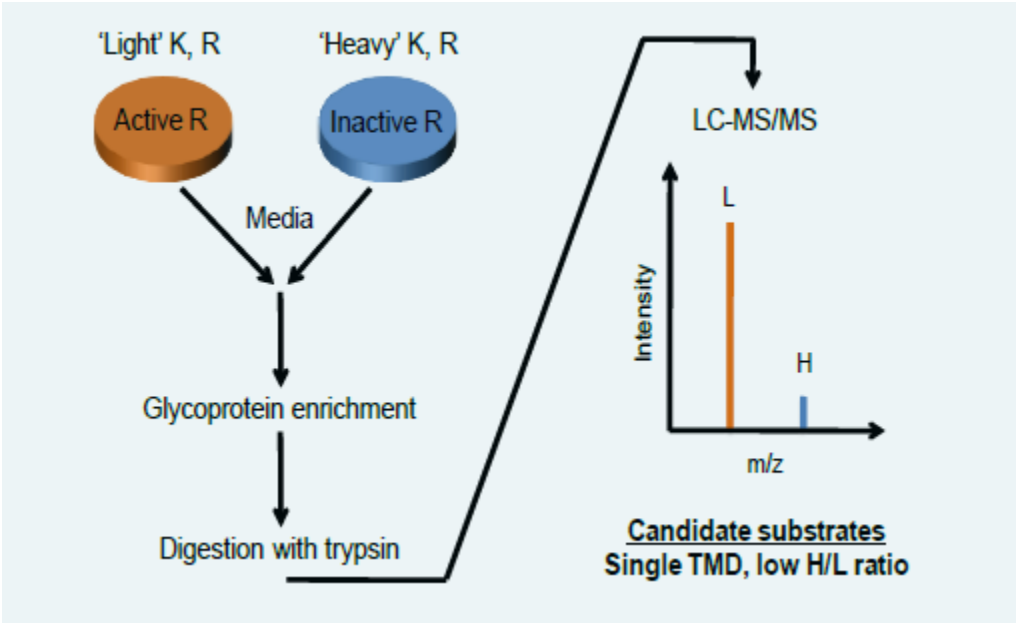


SILAC Application Example

- Rhomboid protease
- Responsible for cleavage of substrates in or near their transmembrane region hereby releasing their N-terminal part into the extracellular space.



- **Quantitative comparison** of secretomes in cell cultures containing active rhomboid and inactive mutant to **identify substrates.**



SILAC Incorporation

- First step: Incorporation level of heavy AAs (^{13}C ^{15}N Arg, Lys) into the cell culture proteins
  - LC-MS/MS quantitation result of a selected protein from a heavy labeled cell culture

Proteins Detected — Results are not bias

N	Unused	Total	% Cov	Accessio...	Name	Species	Peptides(95%)	H:L	Biological Processes	Molecular Functions	PANTHER ID
1	85.47	85.47	82.0	tr|G7RM...	Elongation factor TuOS=Escherichia coli (strain...	ECOC1	76	35.3989			
2	28.15	28.15	25.9	tr|B6HZX...	Beta-galactosidase OS=Escherichia coli (strain...	ECOSE	15	0.0100			

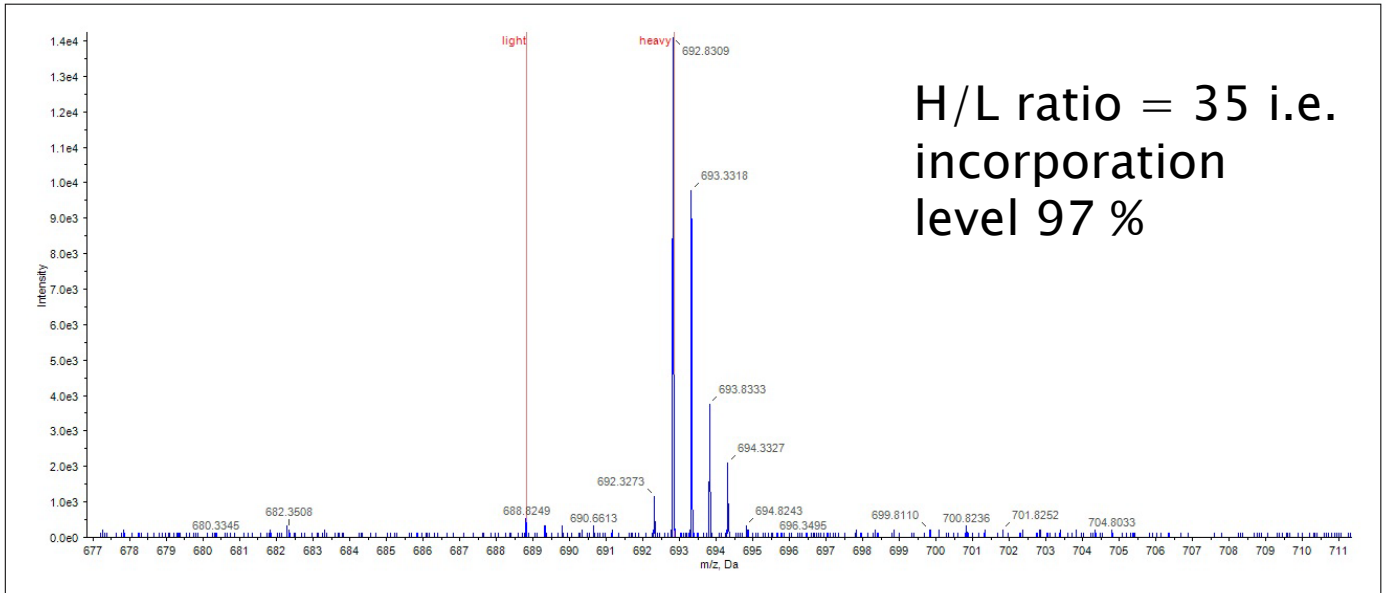
Protein selection

Peptide Quantitation

Used	Annotation	Conf	Sequence	Modifications	Cleavages	ΔMass	Theor m/z	Theor z	Spectrum	H:L
☑	auto	99	AFDQIDNAPEEK			0.0091	688.8201	2	1.1.1.5768.4	29.2134
☑	auto	99	AFDQIDNAPEEK	Label:13C(6)15N(2)(K) @12		0.0070	692.8271	2	1.1.1.5765.4	29.2134
☑	auto	99	AFDQIDNAPEEK	Label:13C(6)15N(2)(K) @12		0.0070	692.8271	2	1.1.1.5766.6	29.2134
☑	auto	99	AFDQIDNAPEEK	Label:13C(6)15N(2)(K) @12		0.0070	692.8271	2	1.1.1.5767.5	29.2134

Peptide selection

Peptide Quantitation Information



H/L ratio = 35 i.e. incorporation level 97 %

SILAC

- Samples are **combined early** in experiment – accounts for any sample losses
- Suited also when **extensive sample preparation** is required
- Both **shotgun** and **targeted** approach possible

Advantages

- **Auxotrophy** for Lys, Arg
- Easily applicable only to **cell cultures**
- Metabolic conversion of Arg to Pro
- **Limited multiplexing**
- **Expensive**

Disadvantages

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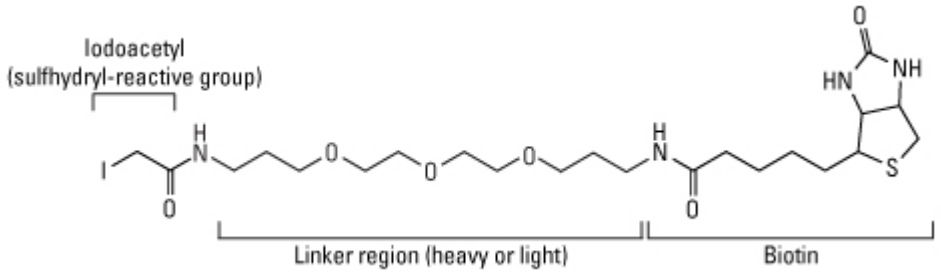
Chemical labelling

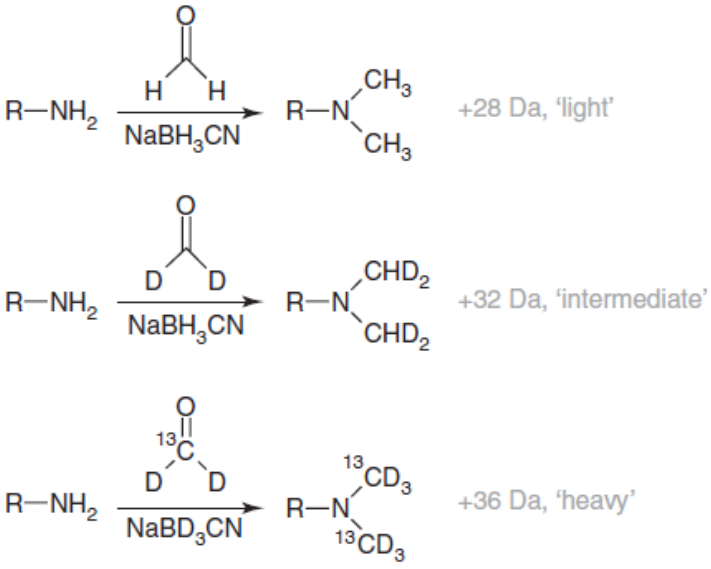
- The label is introduced **in vitro**
- Performed on protein or peptide level
- Cysteine labelling techniques
  - ICAT (Isotope Coded Affinity Tag)

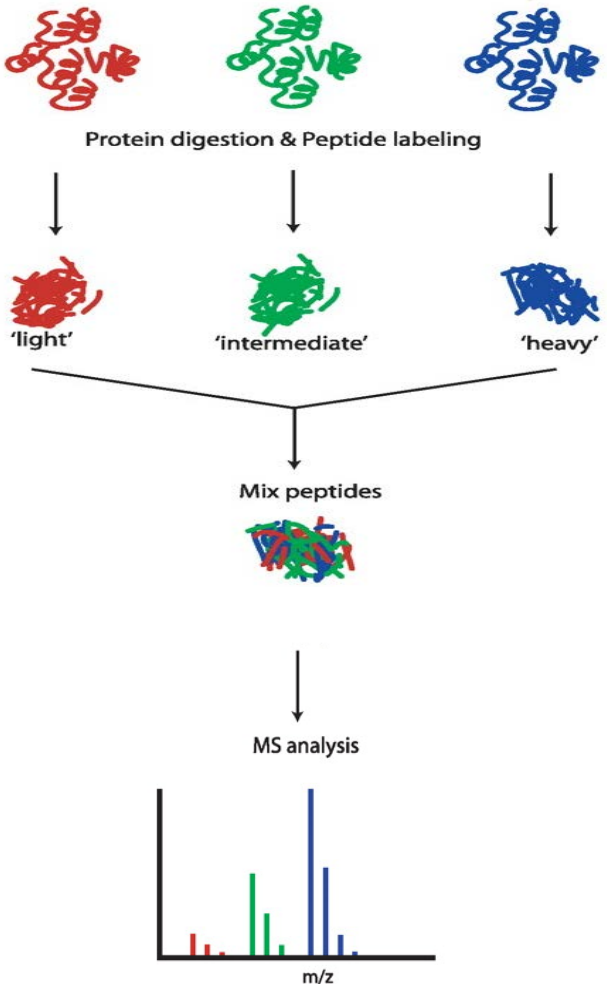


- Primary amine labelling techniques
  - **Dimethyl labelling**
  - **iTRAQ** (AB Sciex), TMT (Thermo)

Dimethyl Labelling

Reaction of N-termini and ϵ-amino group of lysine with formaldehyde followed by a reduction with sodium cyanoborohydride





Boersema, P. J., et al. Triplex protein quantification based on stable isotope labelling by peptide dimethylation applied to cell and tissue lysates. Proteomics. 8, 2008, pp. 4624–4632.

Dimethyl labelling

- **Cheap and easily accessible** reagents
- **Reaction**
  - Fast
  - In solution after digestion

- Other **primary amines** may react with formaldehyde – avoid Tris, Am. Bic, use TEAB
- All steps prior mixing of samples may influence your results – **optimisation required**

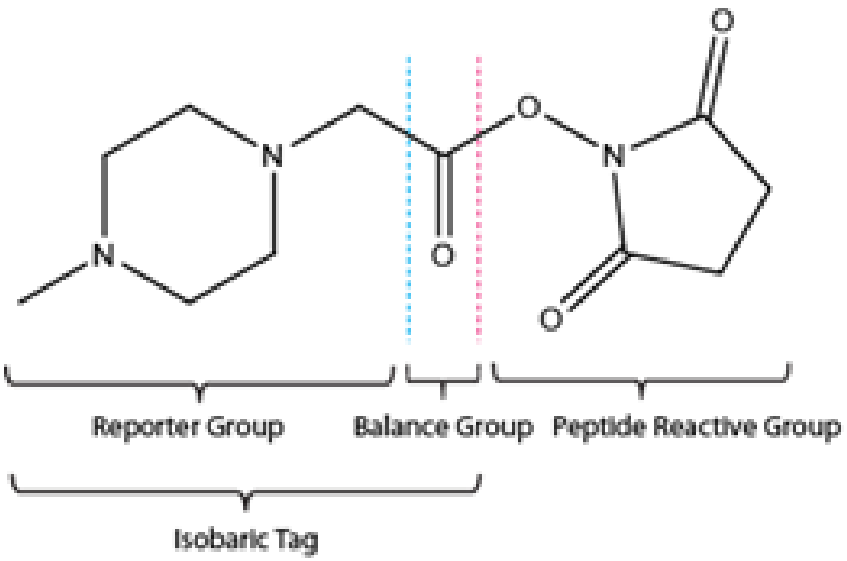
Advantages

Disadvantages

iTRAQ

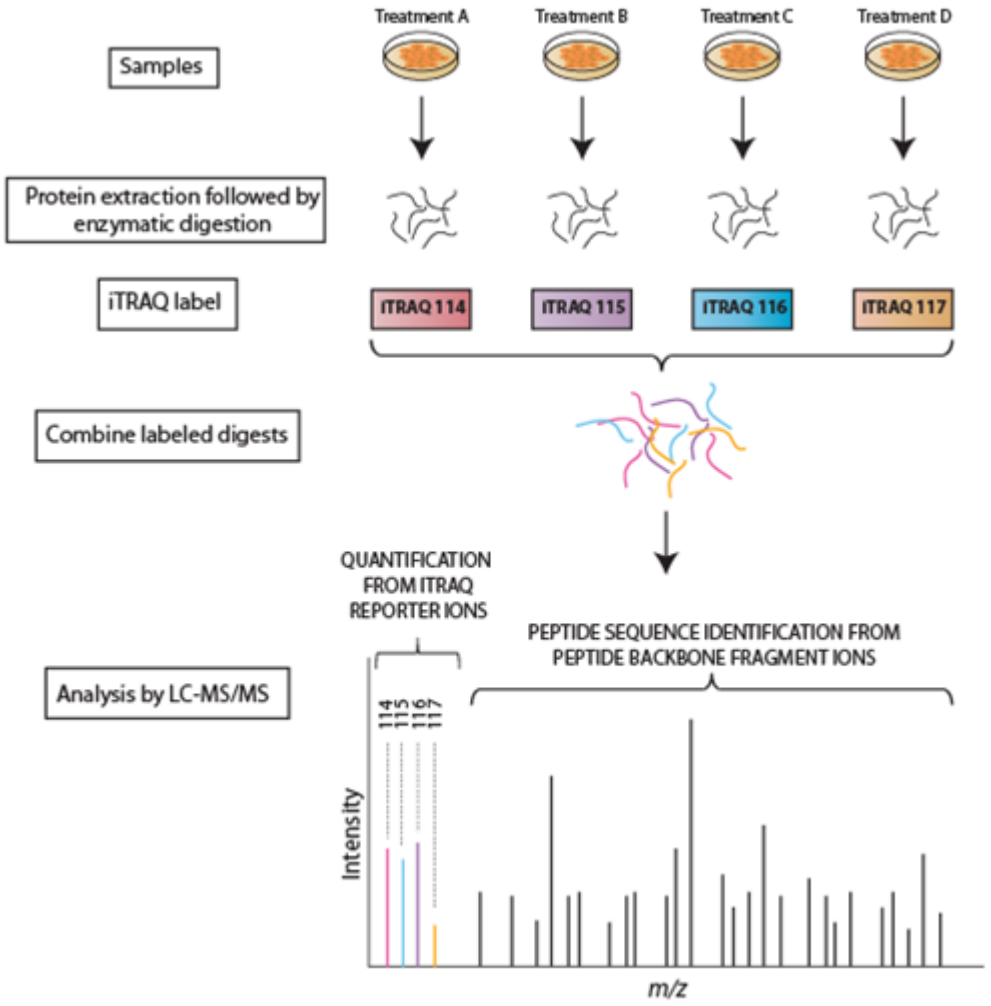
Isobaric tag for relative and absolute quantitation

- Reaction with primary amines
- **Isobaric tag 145 Da** = Reporter group + balance group (both variable heavy/light isotope composition)



iTRAQ

- Tagged peptides are isobaric
- **Ratios of reporter ions** determined after peptide fragmentation
- **Reporter ions** represent peptide levels from diff. exp. conditions



iTRAQ

- **Cheaper than SILAC**
- **Reaction**
  - Fast
  - In solution after digestion
- **Less complex MS spectra**: Isobaric peptides
- Quantitation at MS/MS level – more specific

Advantages

- Other **primary amines** may react with formaldehyde – avoid Tris, Am. Bic, use TEAB
- All steps prior mixing of samples may influence your results – **optimisation required**

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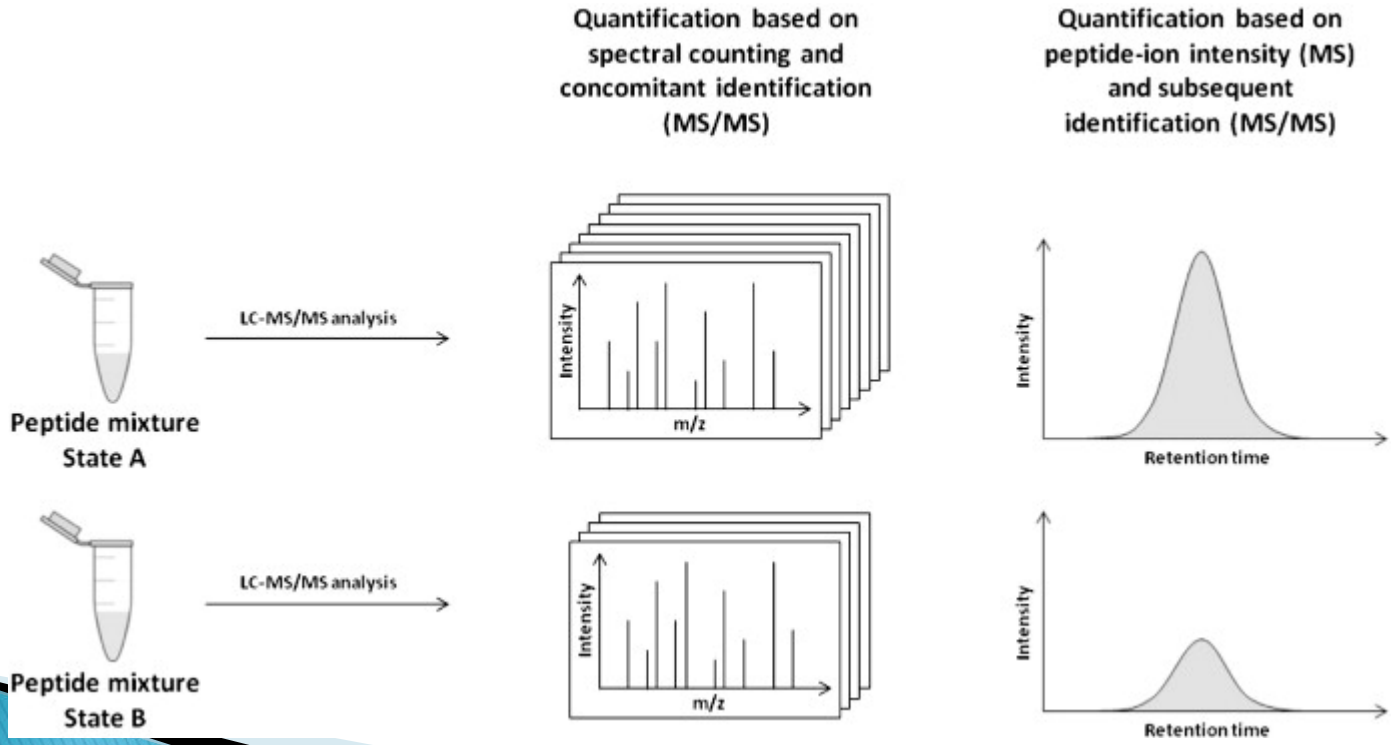
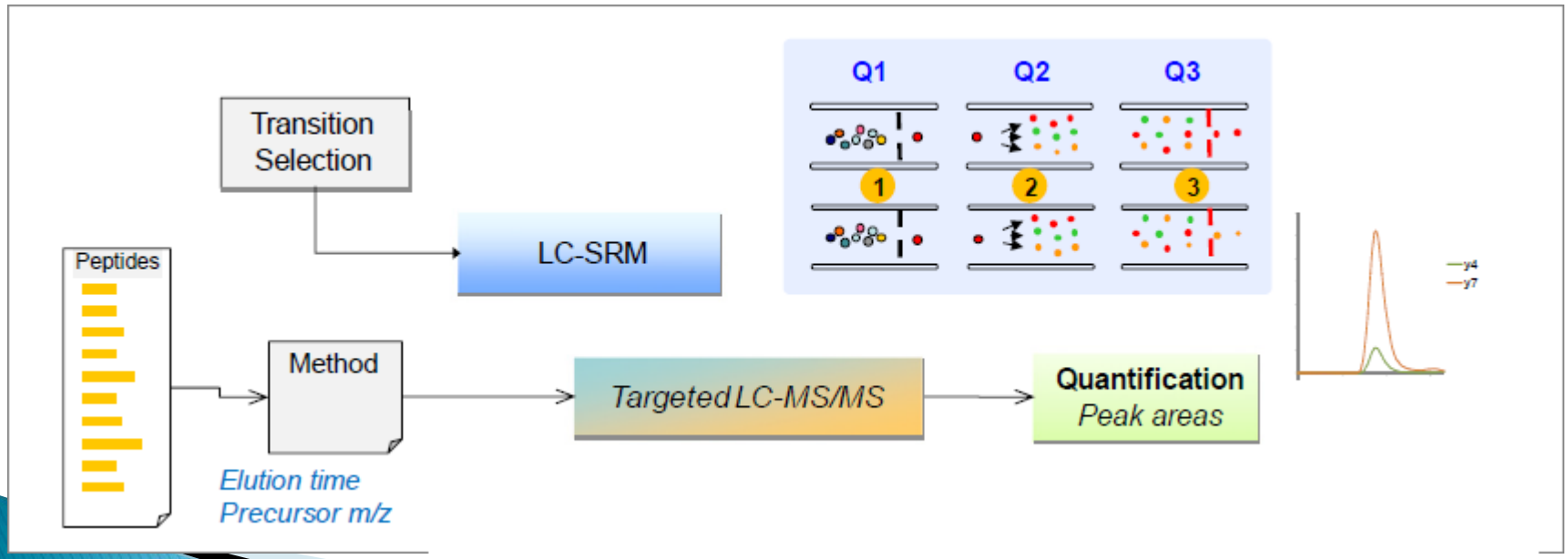
Label-free techniques

- May require special MS instrumentation but no labels
- MS or MS/MS based quantitation possible

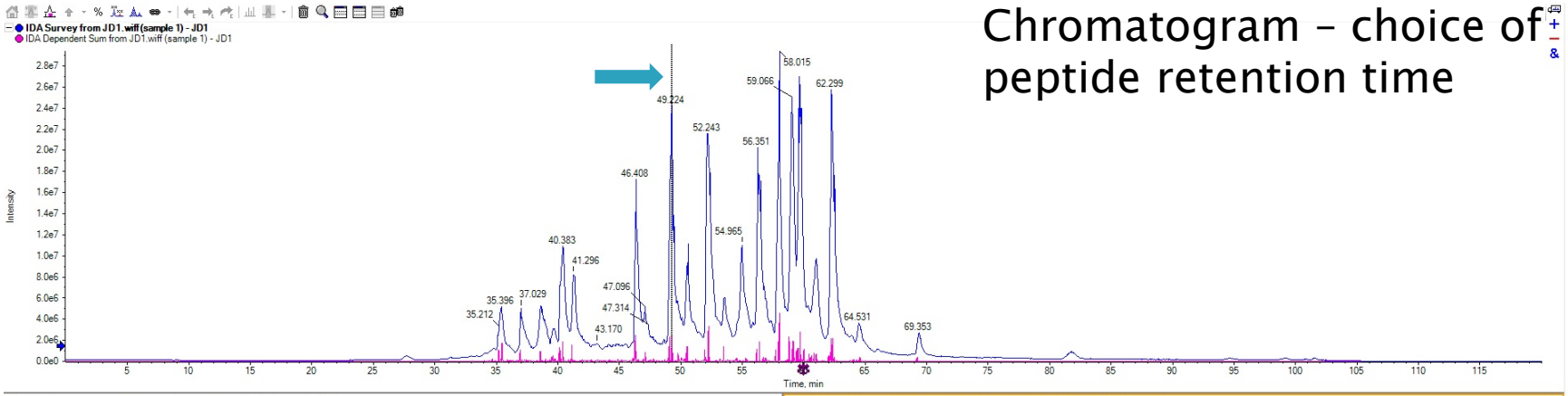


Selected Reaction Monitoring (SRM)

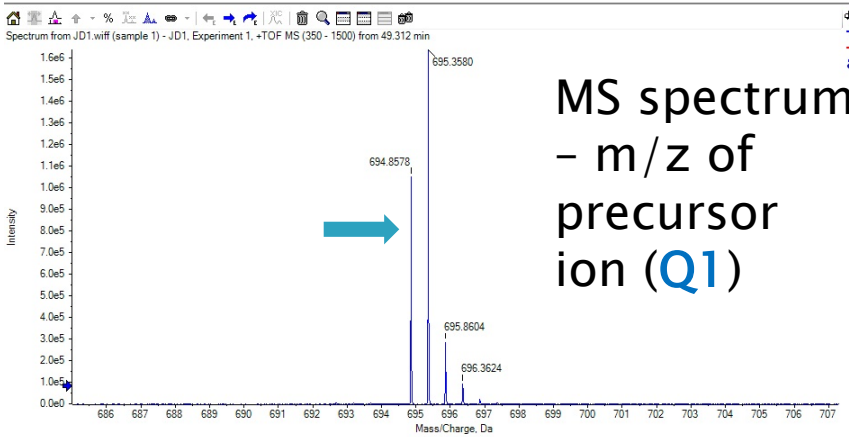
- Targeted approach
  - Precursor selection (Peptide m/z) Q1
  - Fragmentation in the collision cell Q2
  - Fragment ion scan (Peptide fragment m/z) Q3
    - Quantitation



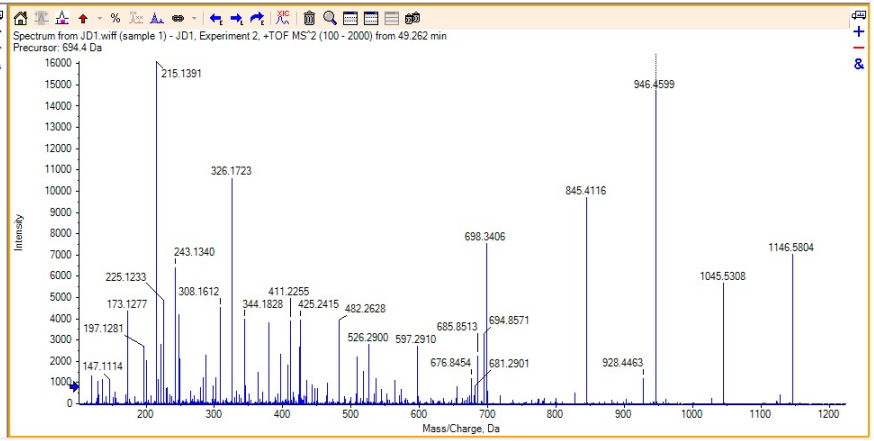
SRM principle



Chromatogram – choice of peptide retention time



MS spectrum – m/z of precursor ion (Q1)



MS/MS fragmentation spectrum (Q3)

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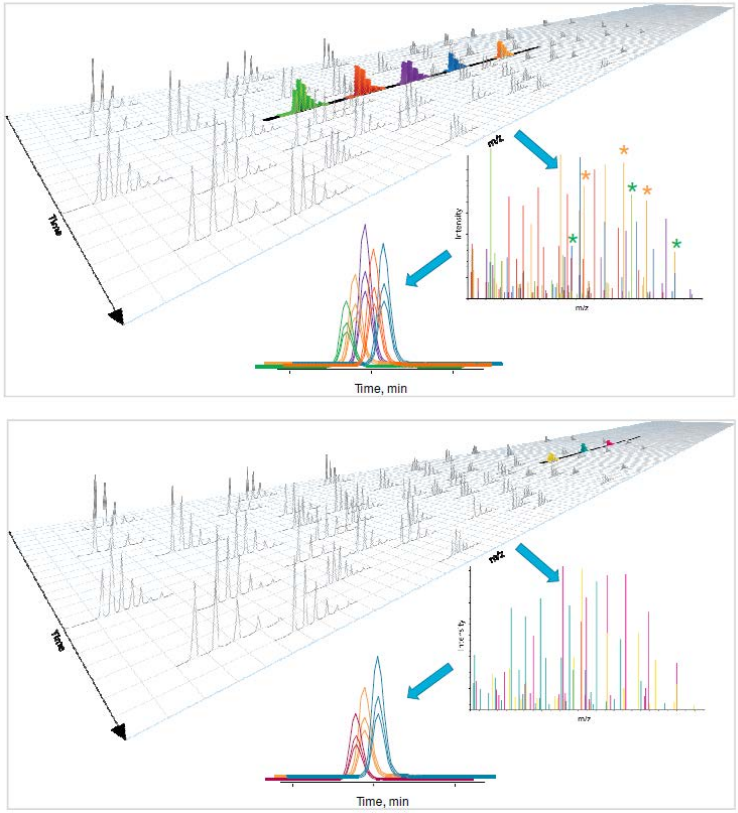
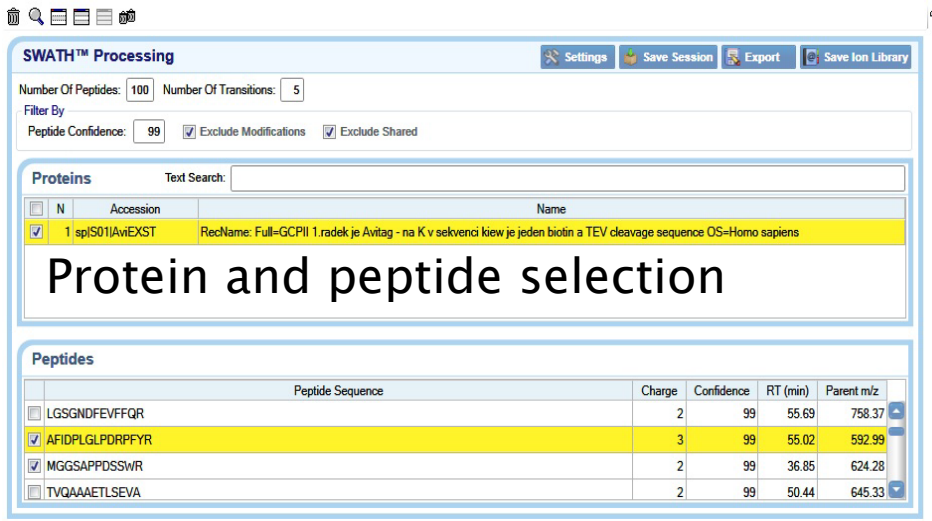
SWATH – MS/MS^{ALL} Acquisition

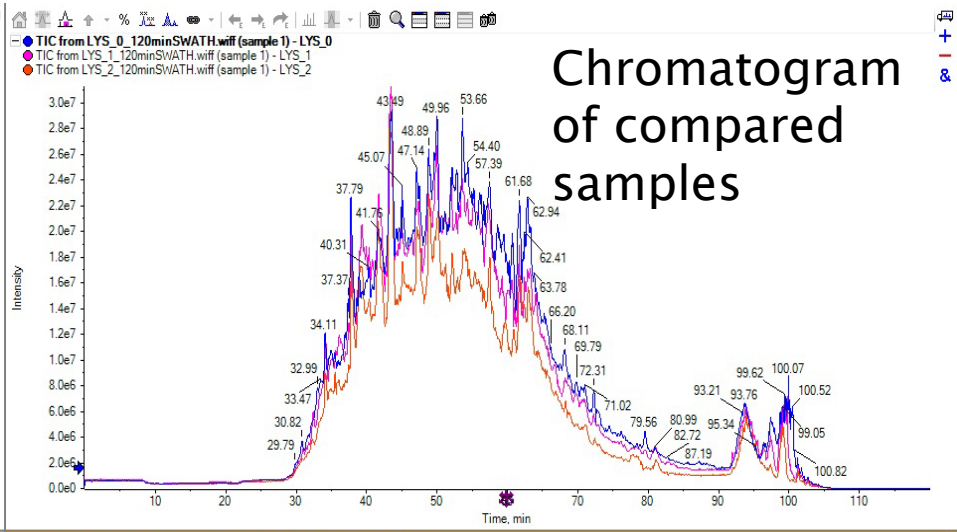


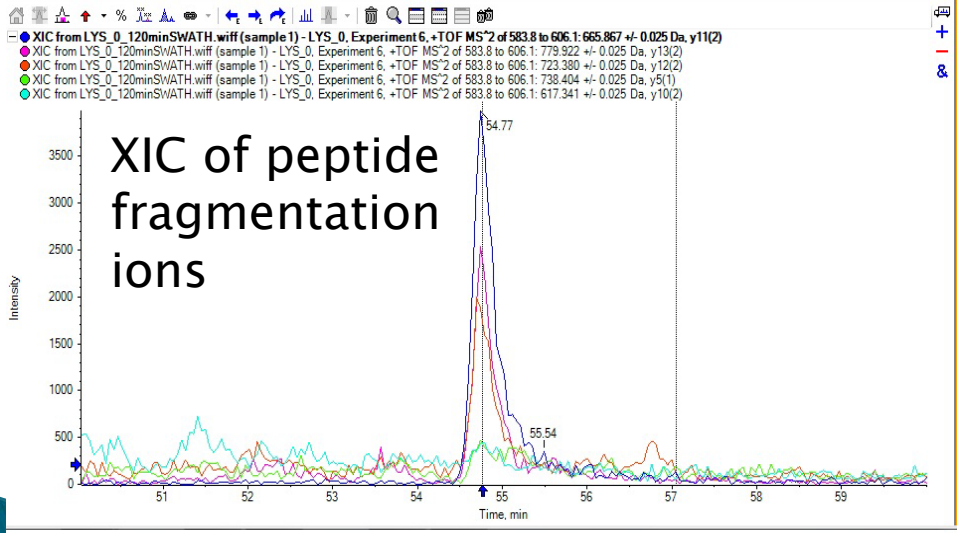
- Precursor selection **window** (in SRM single m/z)
- Fragmentation in the collision cell
- MS/MS scan of fragments originating from **all precursors from the selection window**

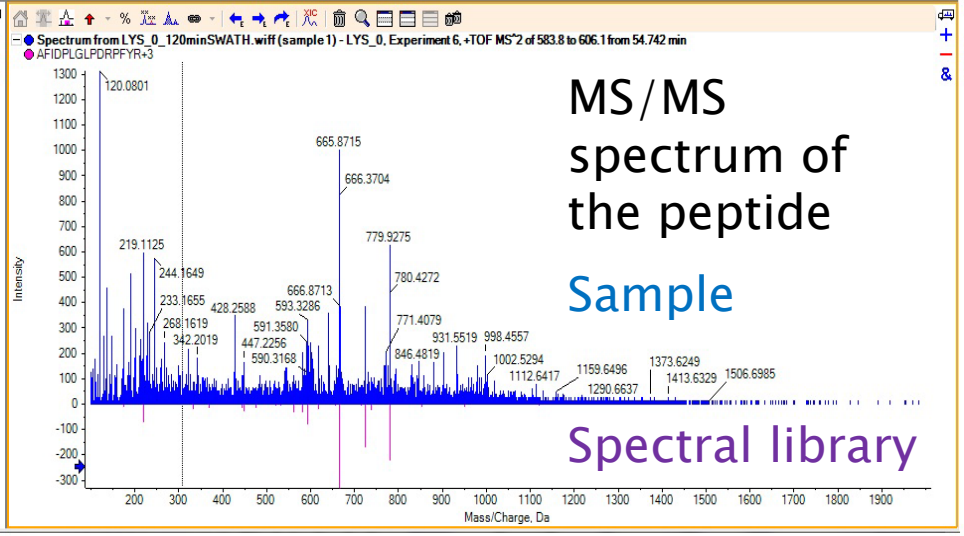
TripleTOF 5600

SWATH Result Example









Data export to a statistical program to perform quantitation

SWATH

- Enables quantititation of previously not considered proteins
- Simplifies SRM method development – choice of precursor ions is less elaborate

- Large and complex data files
- Spectral library needs to be generated in a separate acquisition run
- Internal standards required

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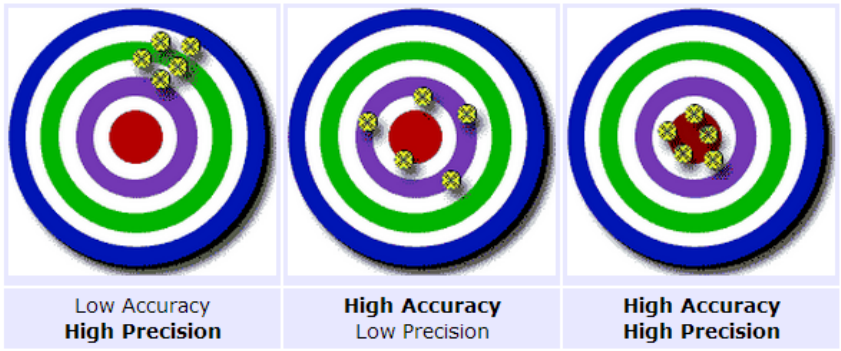

- **Accuracy**
- **Precision/ Reproducibility**

Suggestions

Perform both biological and technical replicates

Randomize your sample preparation to avoid systematic bias

Search engines, quantitation programs are not perfect

Use statistical tests to draw important conclusions

Summary of methods

- More **expensive**
- Combing samples **increases complexity** but **accounts for** any **sample losses**
- Combined samples are treated the same

Label

- **Cheaper**
- Samples analyzed separately – **unlimited multiplexing**, but **increased analysis time**
- Reproducible sample preparation is required
- Extensive **validation** is recommended

Label-free

Thank you
for your attention