COMPUTATIONAL PROTEOMICS AND METABOLOMICS

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6. Quantification III: SRM/MRM, SWATH

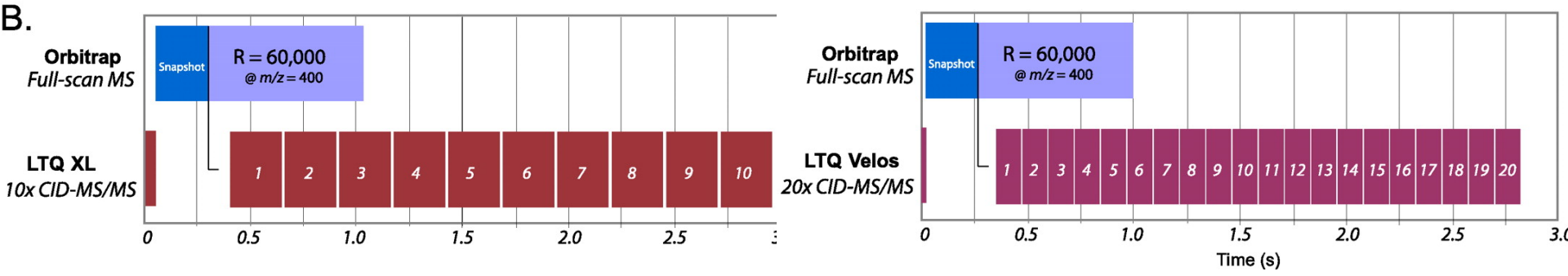
Overview

- iTRAQ quantification
  - Labeling
  - Data analysis
- Targeted proteomics: SRM/MRM
  - Definition of targeted proteomics
  - Data analysis
  - Human Proteome Project
- Other quantification methods
  - Spectral counting
- Comparison of quantification methods

LEARNING UNIT 6A
ISOBARIC LABELING

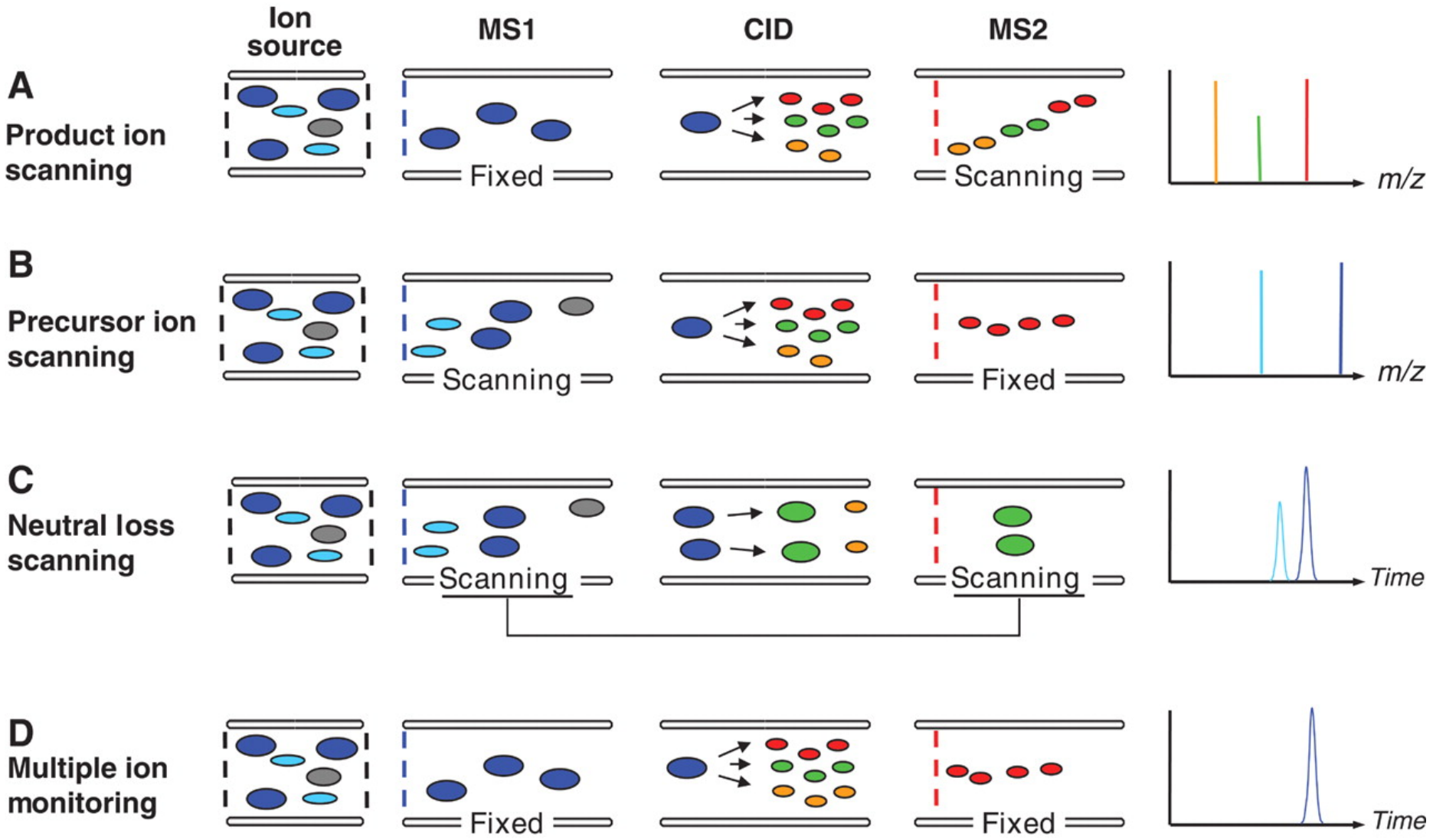
LC-MS/MS

- **Data-Driven Acquisition**: MS spectrum (survey spectrum) controls the selection of peptide ions for CID fragmentation
- **Peptide ion intensity** determines fragmentation order (most intense first)
- 'TOP10' means that the 10 most intense peptide peaks from each survey spectrum will be chosen for fragmentation before a new survey spectrum is acquired
- Direct re-fragmentation of the same mass is prevented by (dynamic) exclusion lists



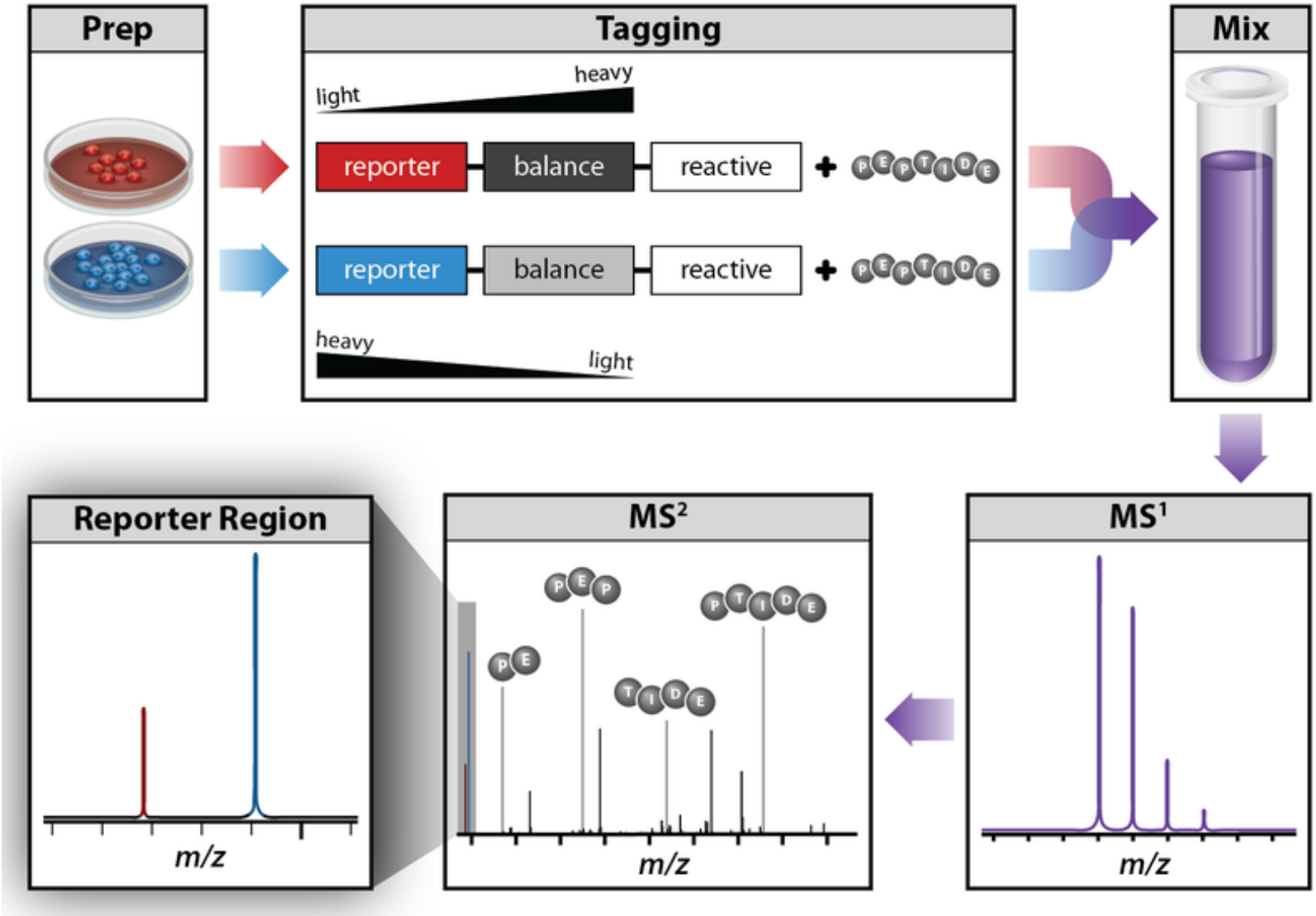
Olsen J V et al. Mol Cell Proteomics 2009;8:2759-2769 B Domon, R Aebersold Science 2006;312:212-217

MS/MS Techniques



B Domon, R Aebersold Science 2006;312:212-217

Isobaric Labeling



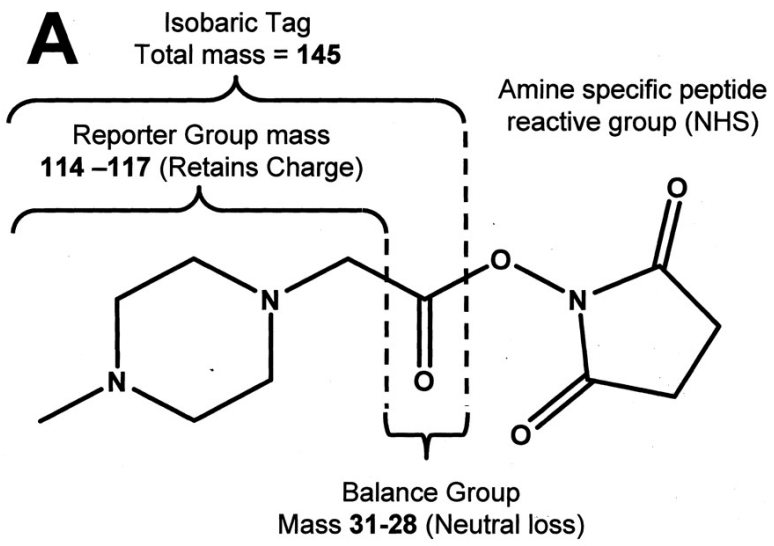
http://en.wikipedia.org/wiki/File:Isobaric_labeling.png [accessed 19.11.11, 19:48 CET]

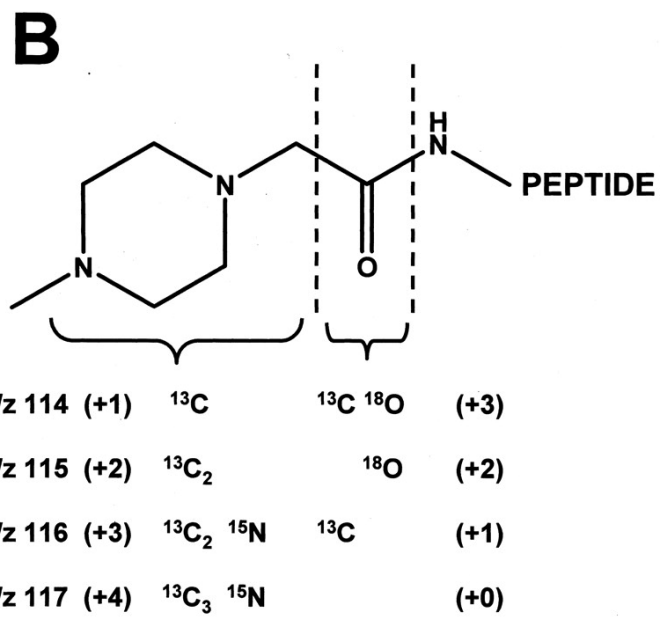
Isobaric Labeling

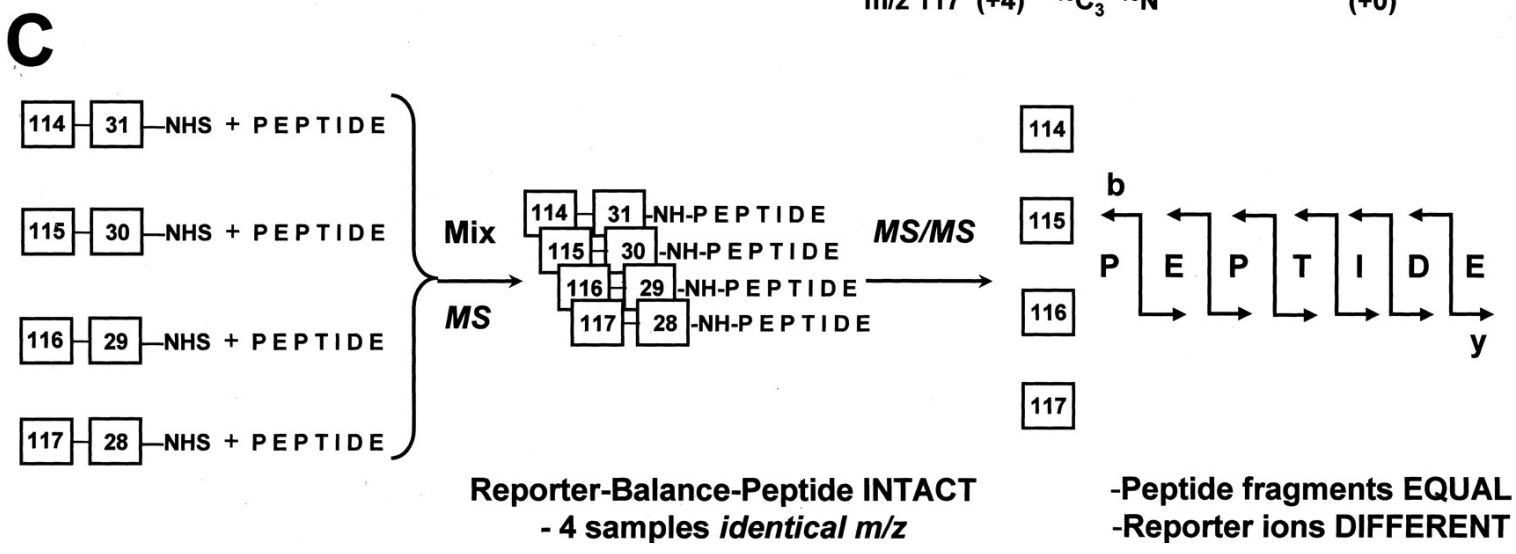
- **Idea**
  - Label the different samples with labels of the **same mass (isobaric)**
  - Design the label in a way the fragmentation pattern allows to distinguish them upon collision-induced dissociation
  - MS^2 spectra will then contain **reporter ions**
  - Quantification and identification are then both based on tandem spectra only

- Key method: **iTRAQ – isobaric tags for relative and absolute quantification**
  - Based on covalent modification of N-terminus of peptides
  - Labeling performed after digestion (also applicable to clinical samples)
  - Kits available for 4 or 8 distinct labels (‘quadruplex’, ‘octuplex’)

iTRAQ

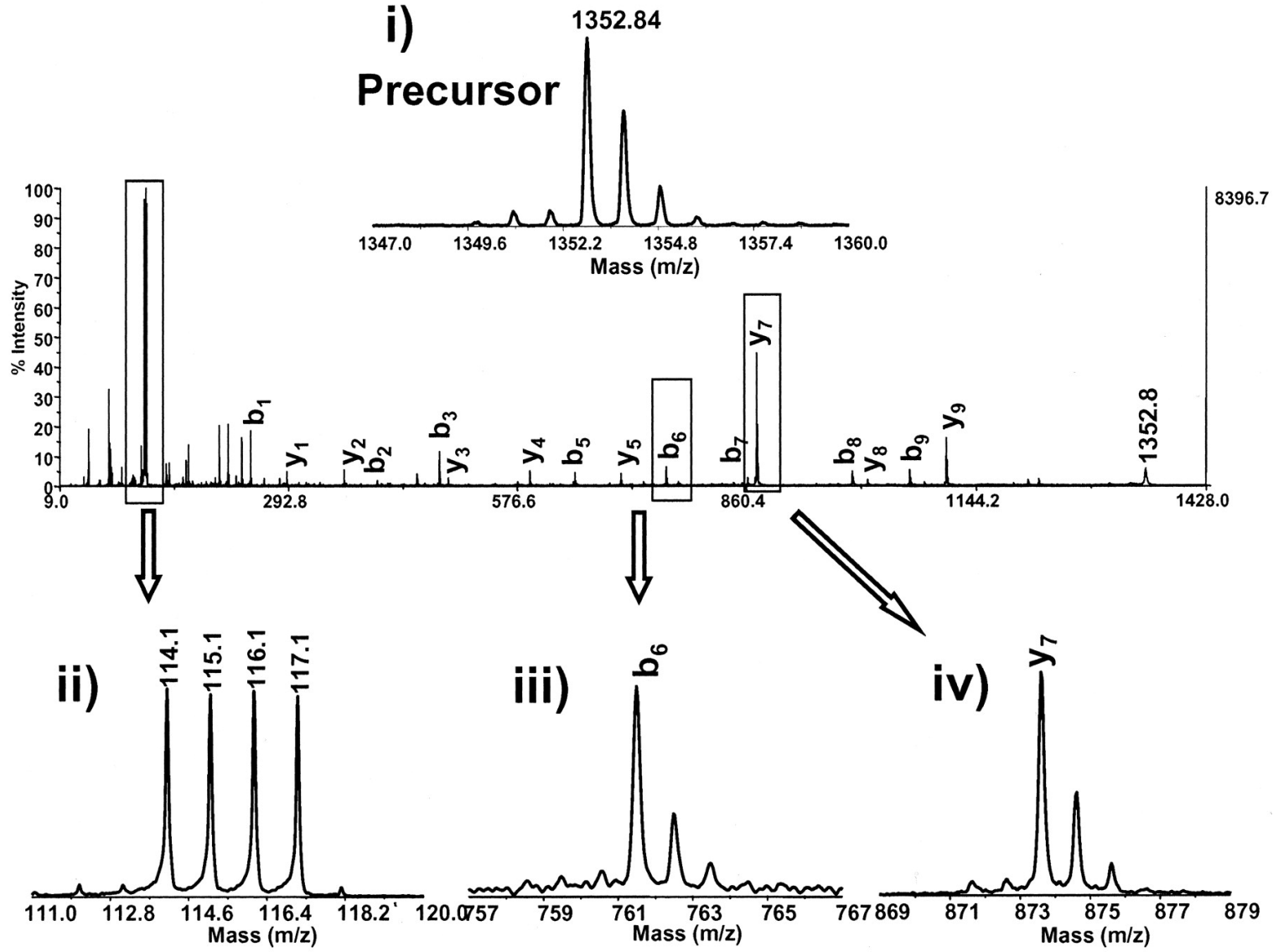






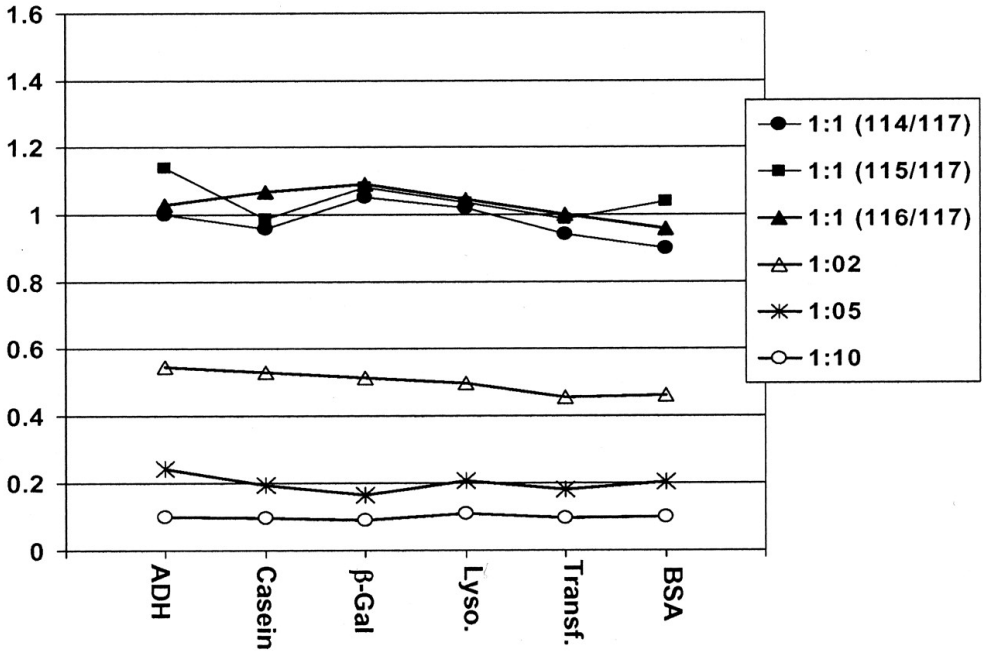
Ross et al., Mol Cell Prot (2004), 3, 1154-1169.

iTRAQ



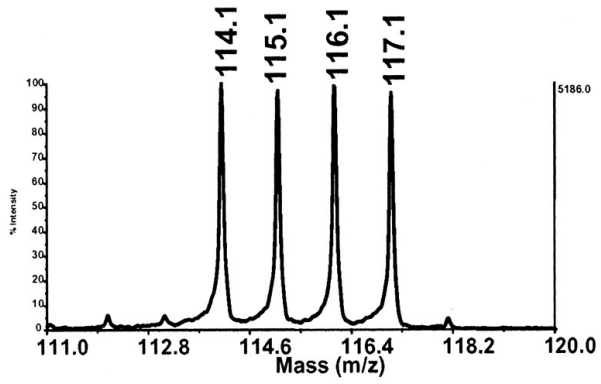
Ross et al., Mol Cell Prot (2004), 3, 1154-1169.

iTRAQ

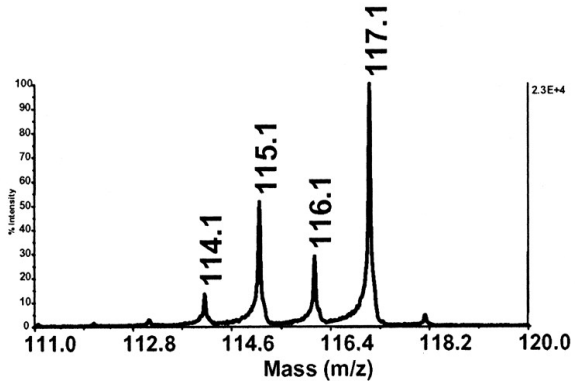


Ratio	Mean	SD
1:1	1.03	0.16
1:2	0.514	0.12
1:5	0.204	.045
1:10	0.097	0.023

1:1:1:1 Mixture



1:5:2:10 Mixture



Ross et al., Mol Cell Prot (2004), 3, 1154-1169.

iTRAQ

- iTRAQ reagents contain **isotopic impurities**
- The intensity of each reporter ion peak will thus influence the intensities (areas) of adjacent peaks (+/- 2 nominal masses)
- **Correction factors** can be determined for each of the reporter ions (by mass spectrometry of the individual reagents)
- Observed peak intensities and real (corrected) channel intensities can thus be related by a **system of linear equations**
- This system of linear equations can be solved

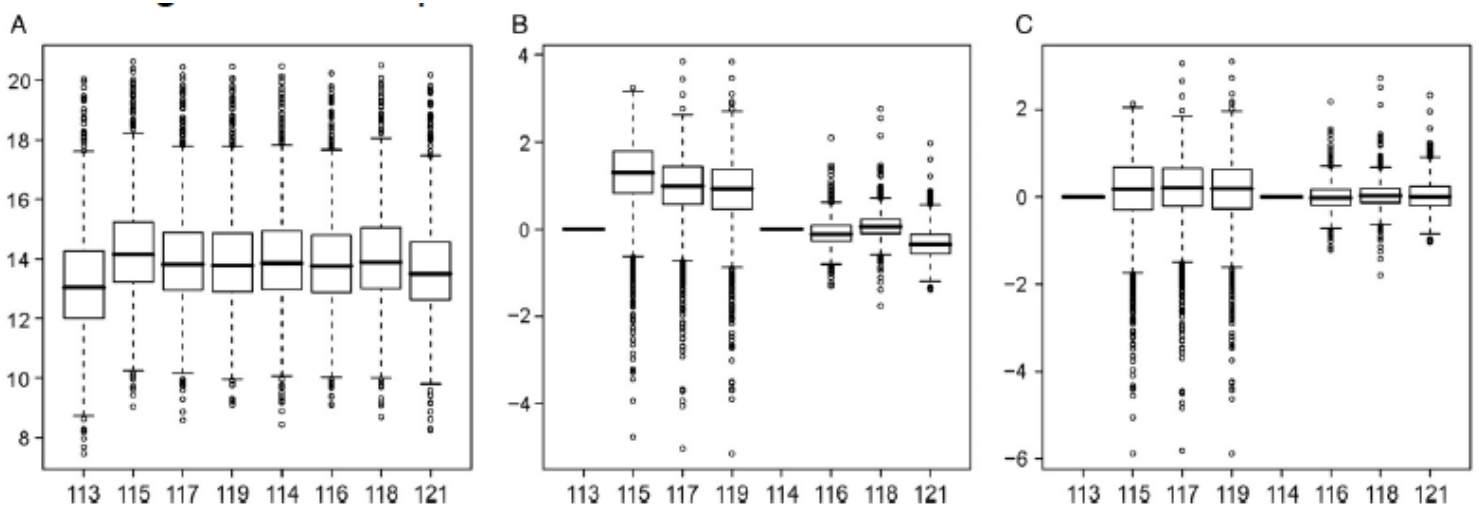
Tag	-2	-1	+1	+2
113	0	2.5	3	0.1
114	0	1	5.9	0.2
115	0	2	5.6	0.1
116	0	3	4.5	0.1
117	0.1	4	3.5	0.1
118	0.1	2	3	0.1
119	0.1	2	4	0.1
121	0.1	2	3	0.1

Isotopic impurities of iTRAQ 8-plex tags for nominal mass shifts -2...+2 in percent (remainder to 100%: intensity of reporter ion).

D'Ascenzo et al., Brief Funct Genomic Proteomic. 2008 Mar; 7'(2):127-35

Correction and Normalization

- Isotopic correction and normalization relative to a specific channel to improve quantification results
- Example below:
  - 8-plex containing two time series (t=0 on channel 113 and 114 respectively)
  - Left: unnormalized raw peak intensities
  - Middle: log fold changes for both time series relative to their respective t=0
  - Right: after isotopic correction and media normalization

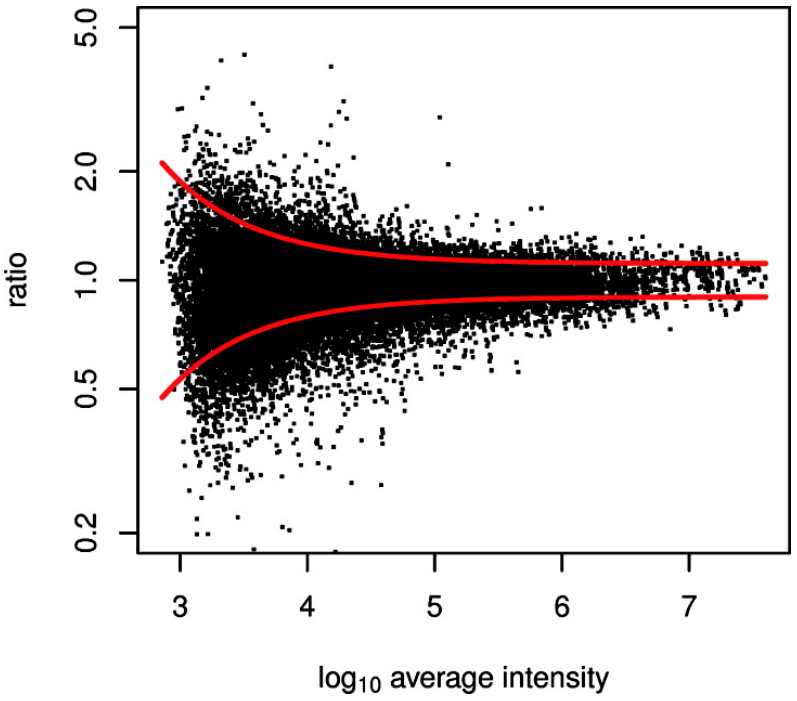


D'Ascenzo et al., Brief Funct Genomic Proteomic. 2008 Mar; 7'(2):127-35

iTRAQ

- **Noise model**
  - Reliability of the signal intensity reduces with the intensity
  - Low-intensity peaks thus have a higher error than high-intensity peaks
  - This behavior is known in statistics as **heteroscedasticity**: different subpopulations of the samples have different variance
  - The noise in iTRAQ (and most other quantification methods) is thus **heteroscedastic noise**

Noise from an iTRAQ experiment as determined from a 1:1:1:1 experiment. All iTRAQ channels should show the same intensities. For low intensities, ratios spread out further.



Breitwieser et al., J. Proteome Res., 2011, 10 (6), pp 2758-2766

iTRAQ

- Peptide quantification => protein quantification
- Different isoforms make the translation from peptide to protein quantities non-trivial
- Peptides can only be mapped to so-called protein groups, a set of proteins containing this peptide
- For iTRAQ: some peptides can not be used to distinguish between protein isoforms
- Regression methods are used to unravel some of this information
- See protein inference problem

Breitwieser et al., J. Proteome Res., 2011, 10 (6), pp 2758–2766

iTRAQ Analysis

- **isobar** is an R package for iTRAQ analysis that
  - Reads the MS data (spectra and identifications)
  - Corrects for isotopic impurities
  - Implements a heteroscedastic noise model
  - Quantifies full proteins based on its peptides
- Output: a full report on differentially quantified proteins

A

Significantly regulated proteins

	ch1	ch2	protein	group	peptides	spectra	ratio
1	Control	Treatment	Serpina1e: Q00898	1/1	7	1	0.22
2	Control	Treatment	Acaca: $Q5SWU9_{1,2}$	2/2	5	4	0.40
3	Control	Treatment	Atp5j: P97450	1/1	4	19	0.49
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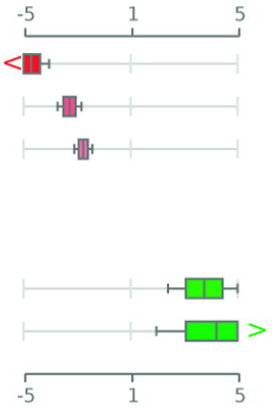
.		.		.	.		.
131	Control	Treatment	Postn: $Q62009_{1-5}$	5/5	1	3	3.05
132	Control	Treatment	Myh7: Q91Z83	1/1	128	62	3.66

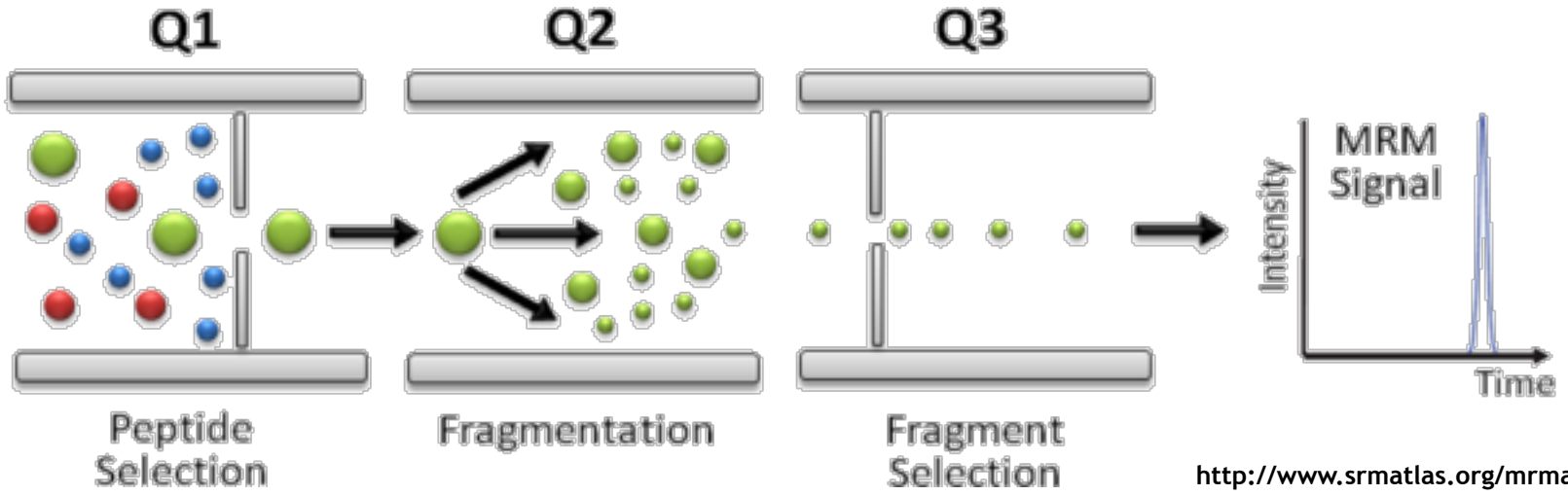


Breitwieser et al., J. Proteome Res., 2011, 10 (6), pp 2758-2766

LEARNING UNIT 6B
SRM/MRM

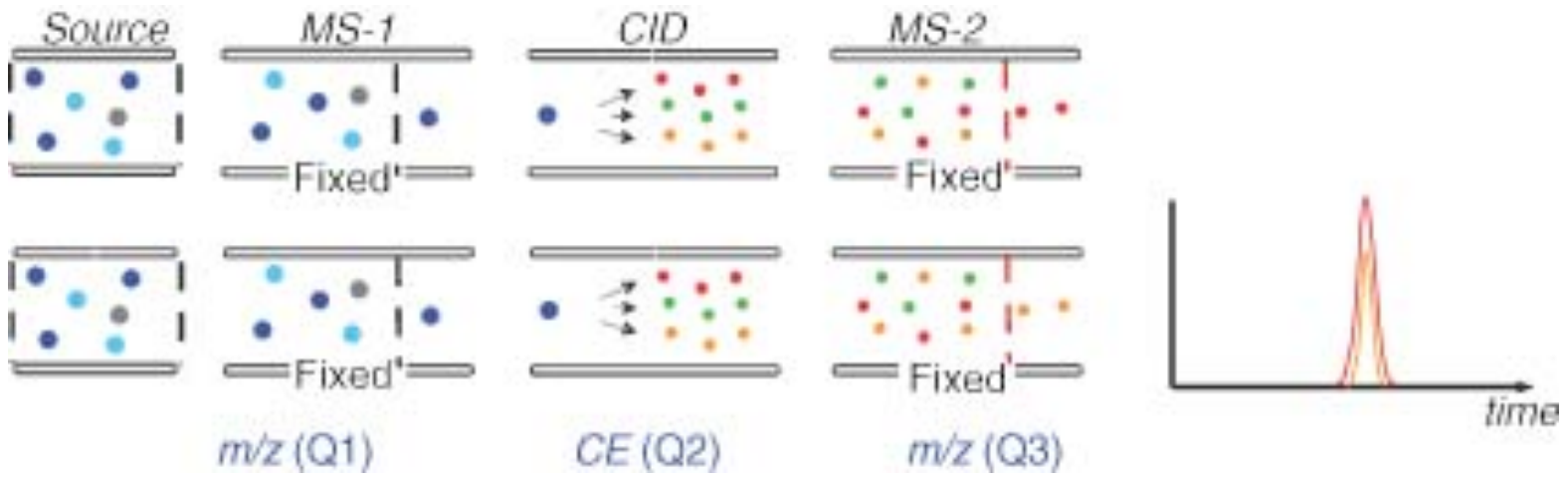
SRM/MRM

- **Selected Reaction Monitoring** (SRM) **and Multiple Reaction Monitoring** (MRM) use the signal of selected MS^2 fragment ions for quantification
- It is typically performed on **triple-quadrupole instruments**: Q_1 selects a peptide ion, Q_2 fragments the peptide, and Q_3 selects a specific fragment ion for the detector
- Double mass selection reduces possible interferences between ions, quantification through MRM signal area



http://www.srmatlas.org/mrmassays.php

SRM vs. MRM



- **SRM**: monitor a single fixed mass window only
- **MRM**: scan rapidly over multiple (very narrow) mass windows and thus acquire traces of multiple fragment ion masses in parallel

Gallien et al., J. Mass Spectrom, 2011, 46(3), 298-312

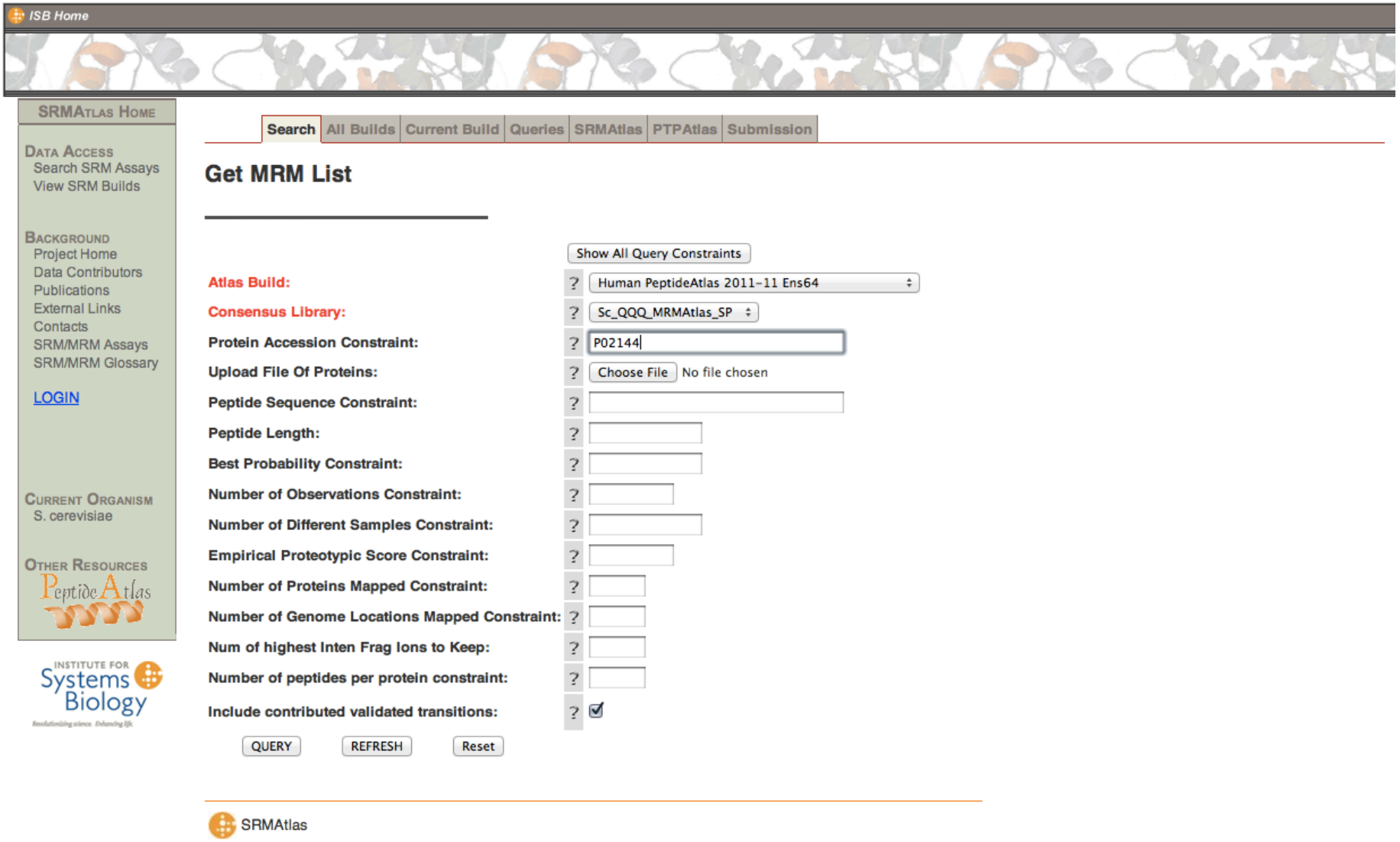
Targeted Assays

- Targeted proteomics/metabolomics is based on a list of known analytes (proteins, metabolites)
- Targeted methods are in contrast to so-called discovery mode or shotgun proteomics, where proteins/metabolites are identified and quantified as comprehensively, as possible
- MRM Assay:
  - Consists of a transition list
  - For each SRM transition, the expected retention time, precursor ion m/z, and fragment ion m/z need to be specified
  - Transition list is uploaded to the instrument prior to the analysis and controls
- Advantages of SRM/MRM
  - Minimal fractionation only (second separation in the MS)
  - Better sensitivity
  - Better linear range (4-5 orders of magnitude)

Computational Challenges

- Assay construction
  - Given a list of proteins, determine a transition list
  - Based on either experimentally determined tandem spectra (to identify the most intense fragment ions) or on predicted spectra
  - Assays need to be optimized (avoid interferences, optimize instrument settings for each transition)
- Automated assay analysis
  - Given an assay, automatically quantify a sample

SRM Atlas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SRMAtlas Home
Data Access
Search SRM Assays
View SRM Builds
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Project Home
Data Contributors
Publications
External Links
Contacts
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Sc_QQQ_MRMAtlas_SP
Protein Accession Constraint:
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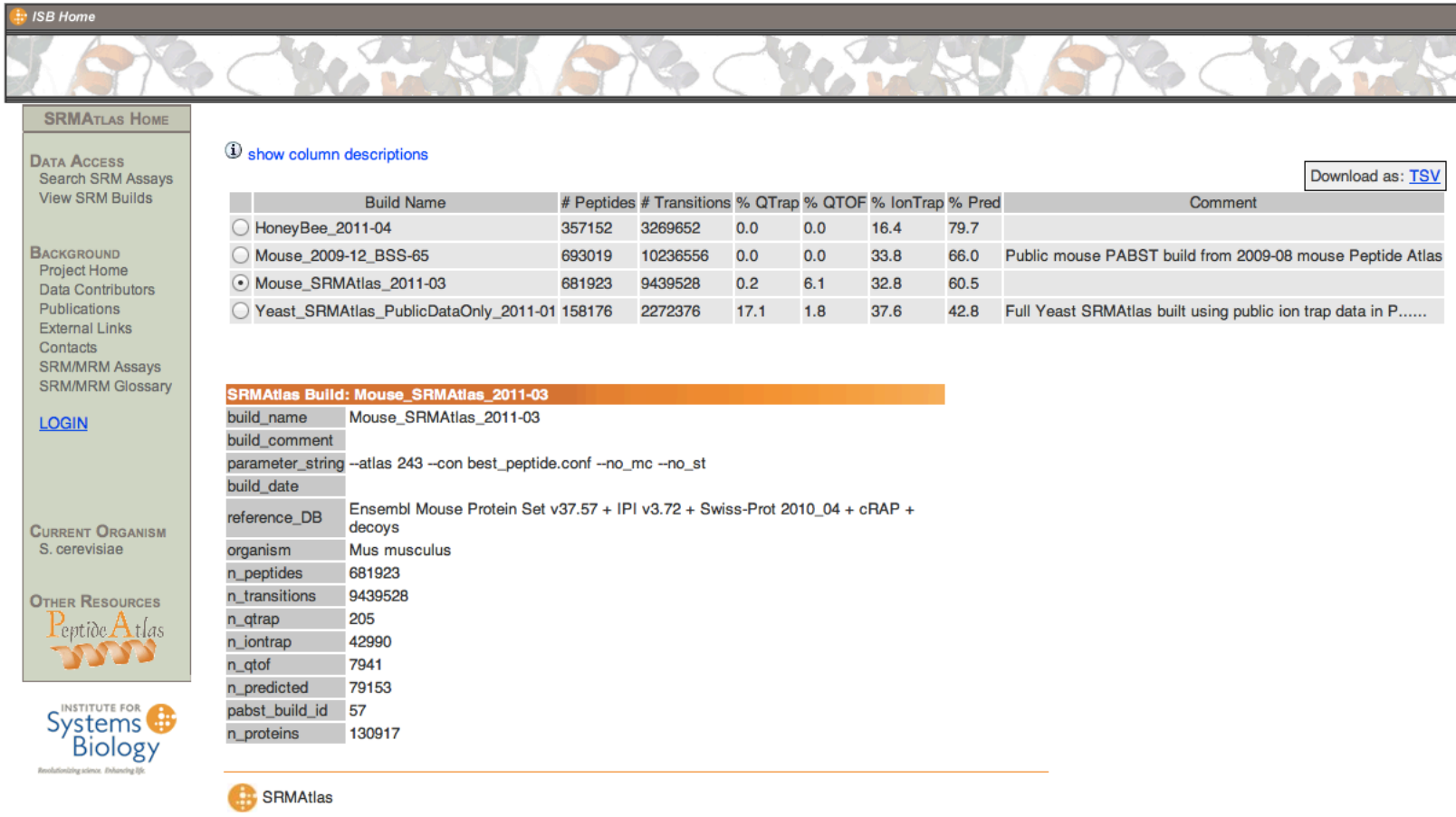
Upload File Of Proteins:
Choose File
No file chosen
Peptide Sequence Constraint:
Peptide Length:
Best Probability Constraint:
Number of Observations Constraint:
Number of Different Samples Constraint:
Empirical Proteotypic Score Constraint:
Number of Proteins Mapped Constraint:
Number of Genome Locations Mapped Constraint:
Num of highest Inten Frag Ions to Keep:
Number of peptides per protein constraint:
Include contributed validated transitions:
QUERY
REFRESH
Reset
SRMAtlas

SRM Atlas

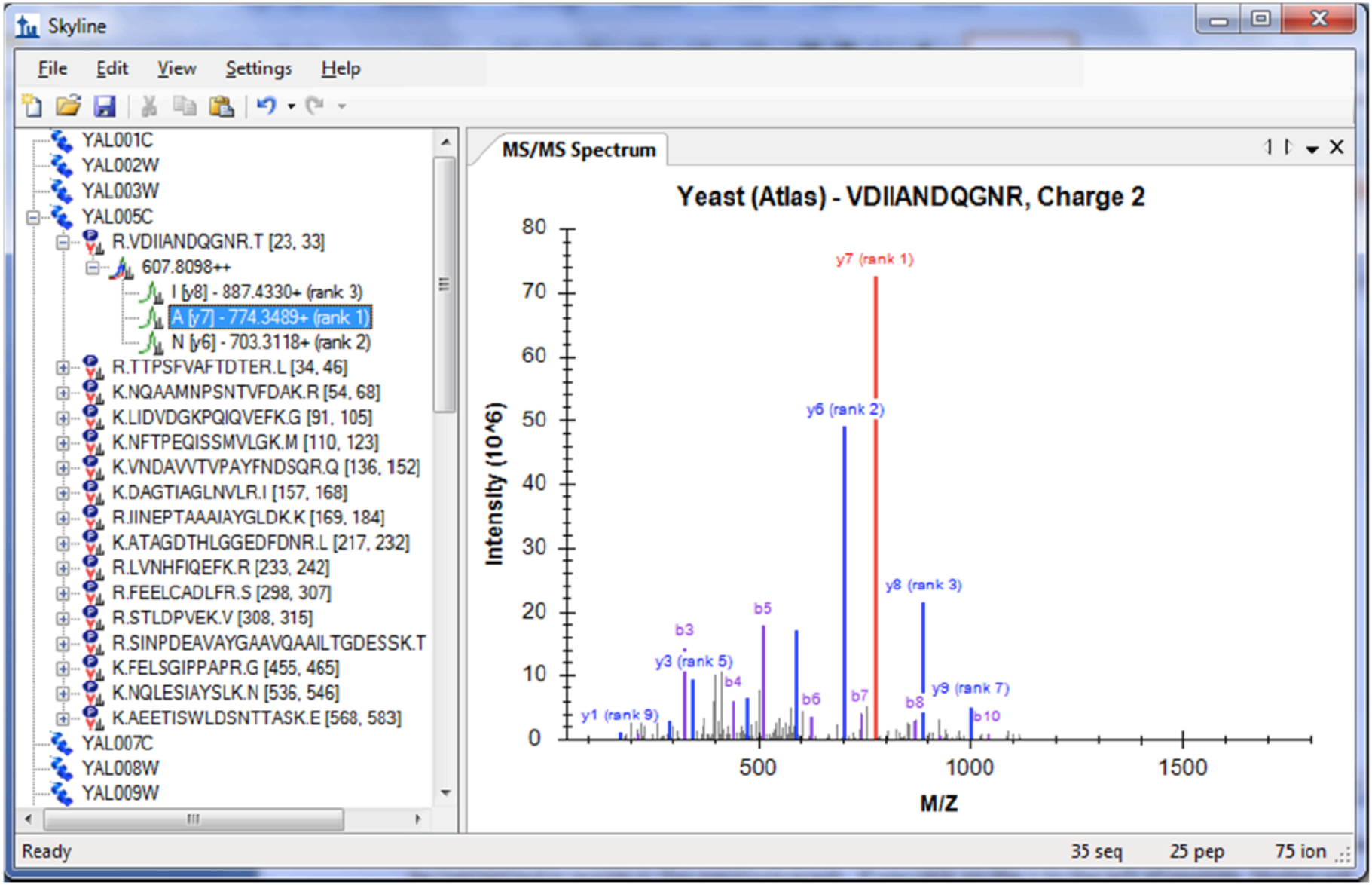


- Apart from the search interface, SRM Atlas also offers downloadable transition lists for several organisms (based on experimentally validated and predicted transitions)

Skyline

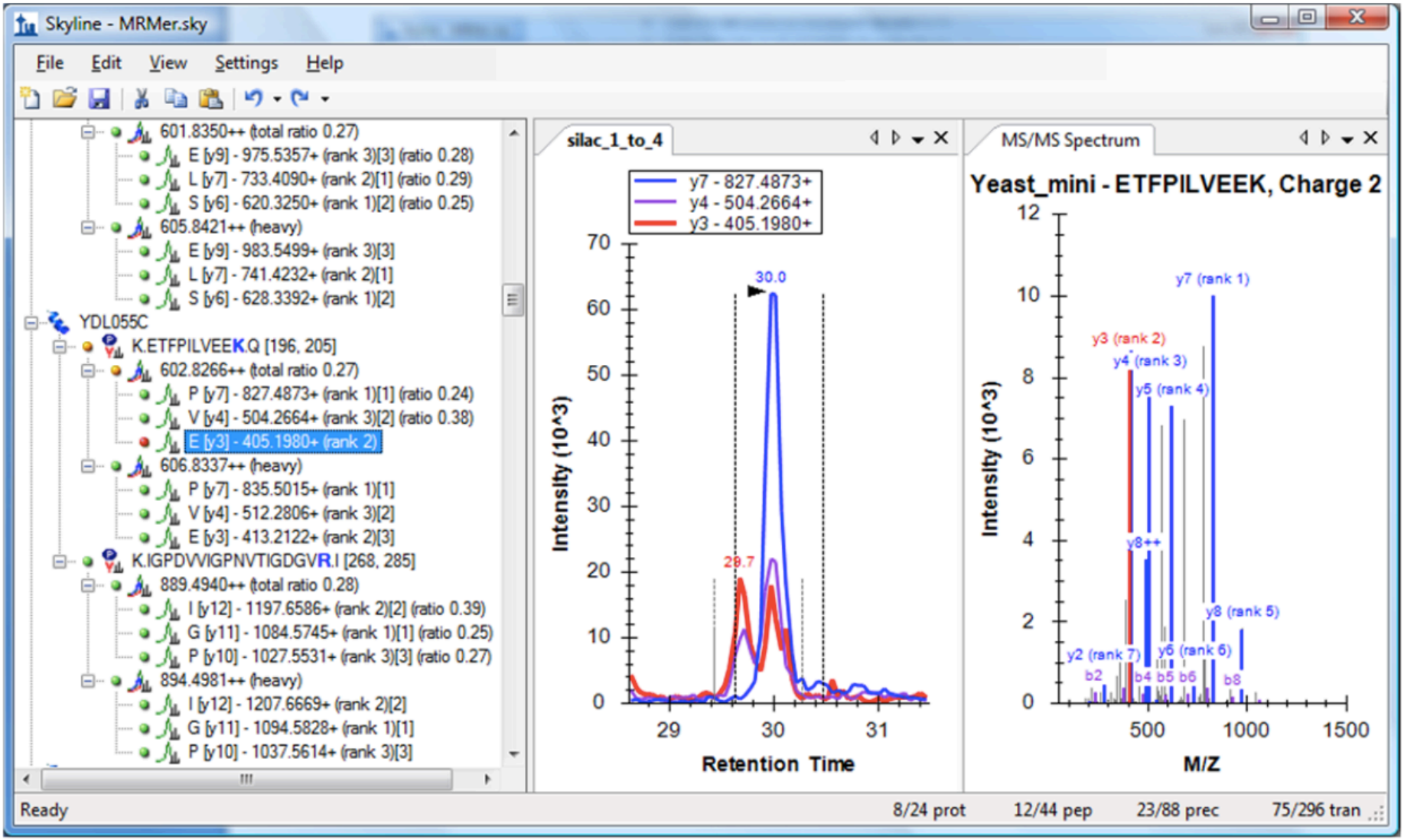
- Skyline is a software package (Windows only) for the construction and analysis of MRM assays
- Skyline permits the construction and optimization of MRM assays based on experimental data
- The graphical user interface also permits the analysis of the resulting datasets and (semi-)automatic processing of larger datasets

Skyline – Assay Construction



https://skyline.gs.washington.edu/tutorials/MethodEdit-1_1.pdf

Skyline

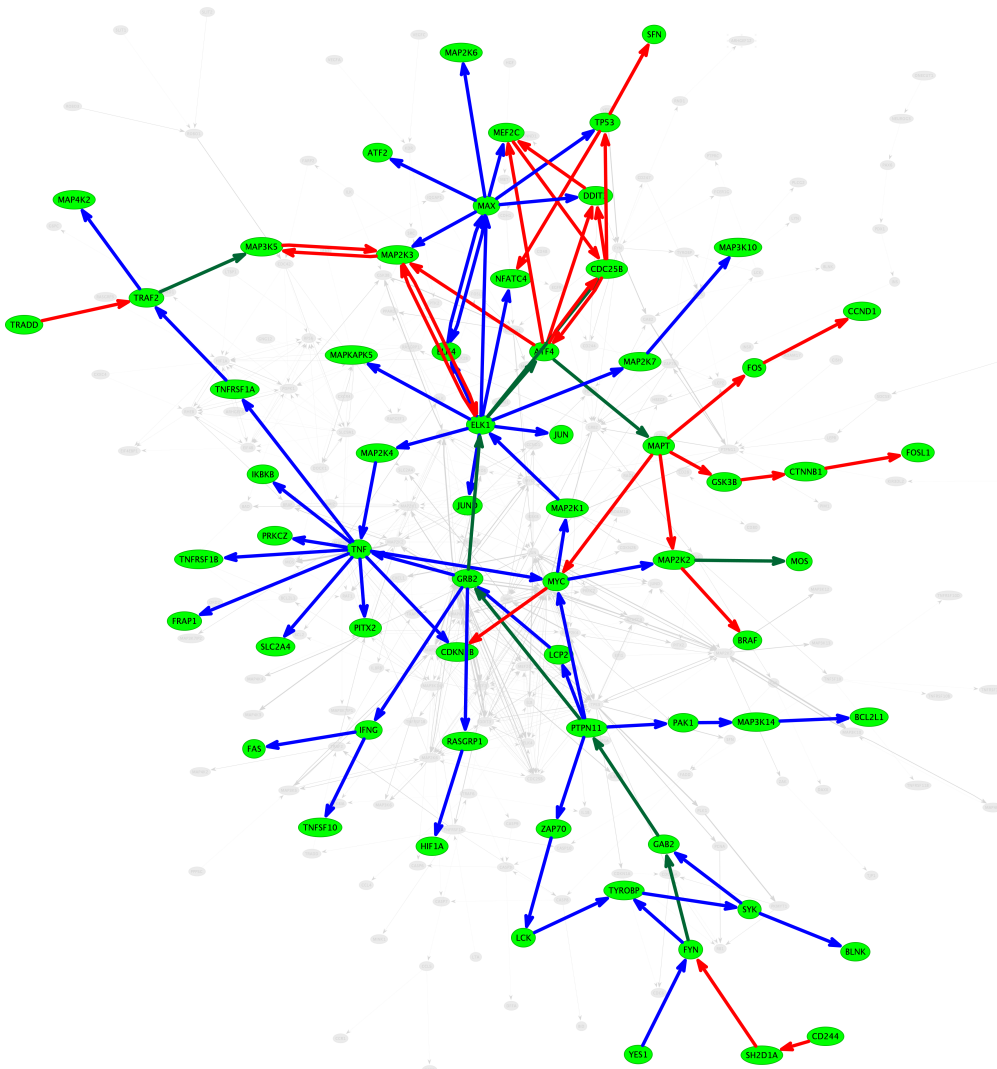


https://skyline.gs.washington.edu/tutorials/ExistingQuant-1_1.pdf

MRM Transition Scheduling

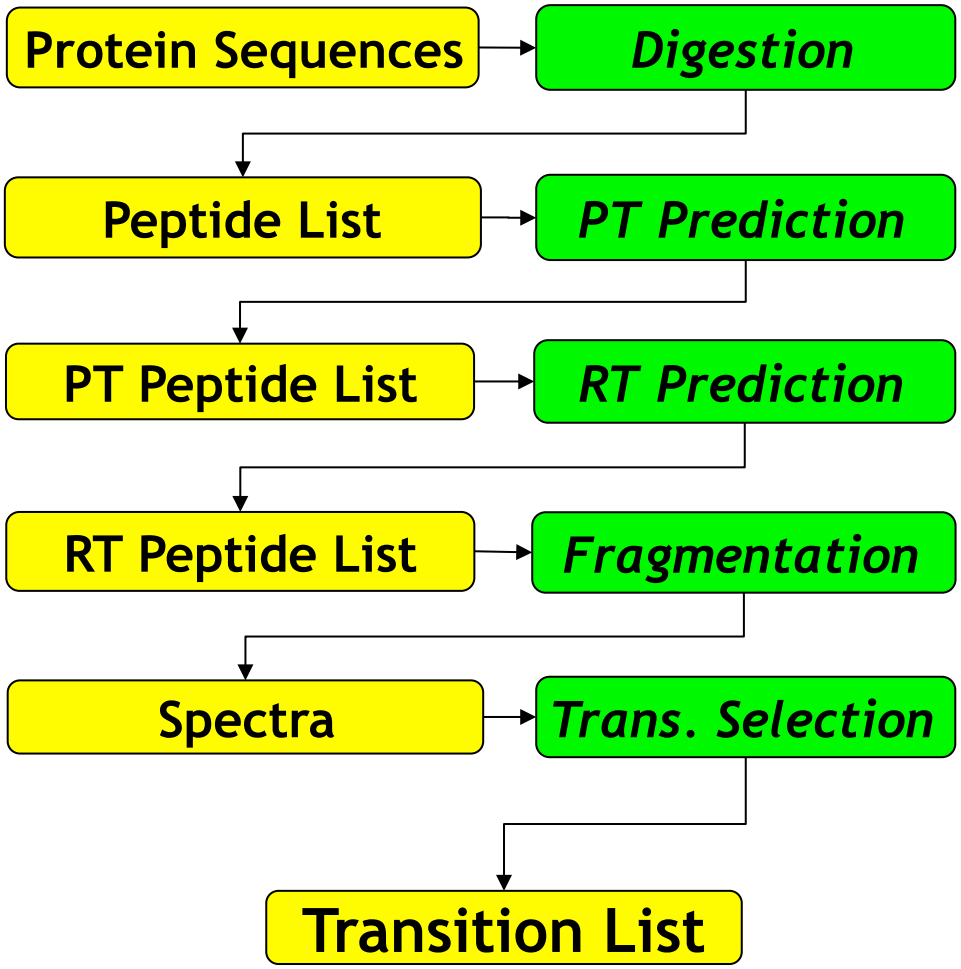
Idea

- Identify proteins of interest (e.g., from a pathway)
- Predict MRM transitions to cover all proteins
- Predict optimal scheduling of transitions
- Formulate as a combinatorial optimization problem (ILP)



Targeted MRM Scheduling

- For a set of given **protein sequences**
  - *in silico* digest
  - Predict **proteotypicity**
  - Predict **retention time**
  - Predict **tandem spectrum**
  - Select **transitions** from the predicted values

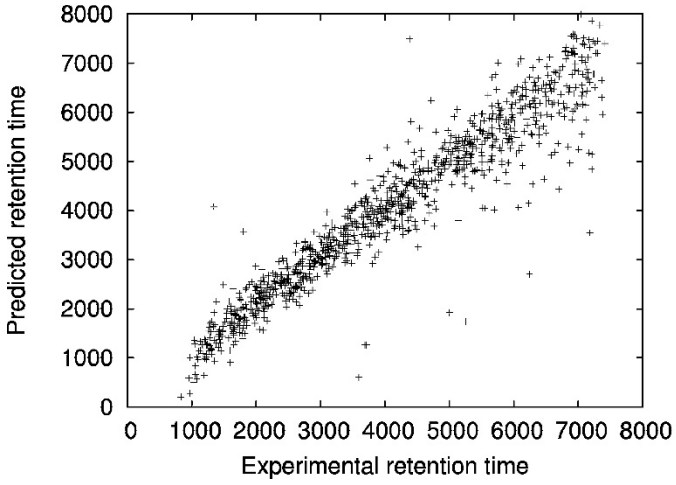


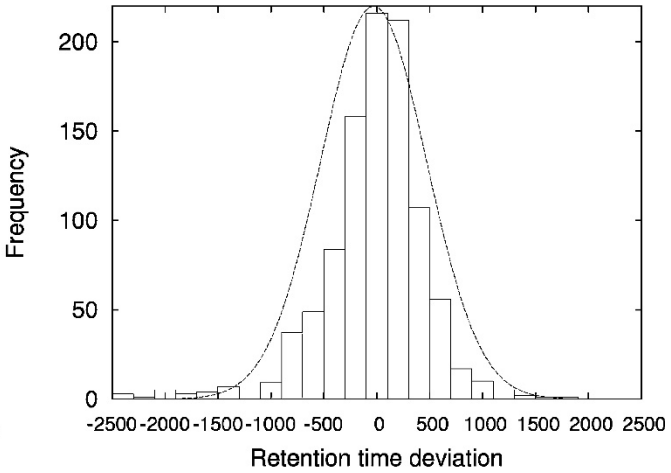
Prediction Methods

- **Proteotypicity prediction**
  - Predict whether the peptide is a so-called proteotypic peptide
  - Proteotypic peptides are peptides that are typically observed for a given peptide and allow unique protein mapping
  - This corresponds to predicting the response factors: proteotypic peptides have high response factors, ionize well, yield strong signals and are thus observed whenever the protein is present
- **Retention time prediction**
  - Predict at what time the peptide will elute
  - Depends on the separation system
  - For a given separation system, the retention time will depend on the peptide
- Both properties, proteotypicity and retention time, depend on the sequence of the peptide) **sequence-based machine learning can solve both problems**

Retention Time Prediction

- Retention time (as well as proteotypicity) can be predicted using support vector regression (SVR)
- All that is needed is a sufficiently large training set (100+ peptides) and their retention time
- The predictor can then predict retention times of arbitrary peptides given their sequence alone
- Accuracy is excellent (r^2 = 0.94)



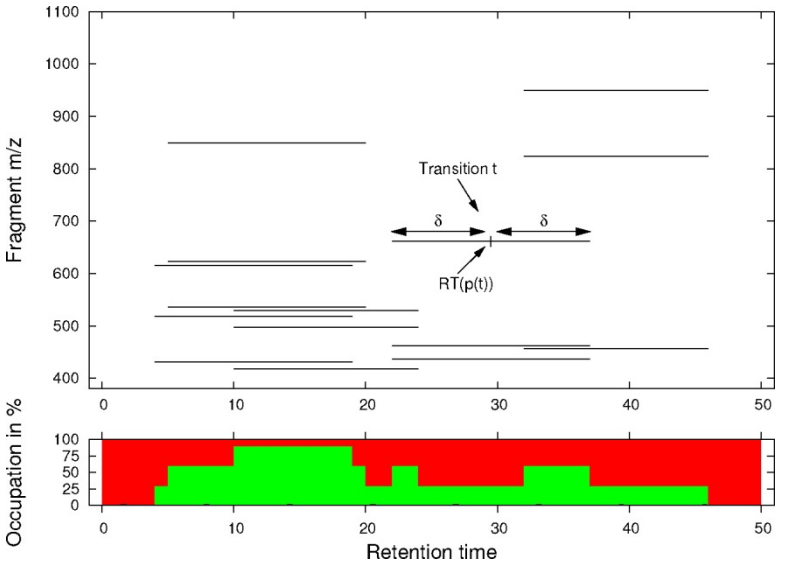


Bertsch et al., J. Proteome Res. (2010), 2010, 9(5):2696-704

Optimization Problem

- **Given**
  - A set of possible transitions
- **Objective**
  - Pack as many of the transitions into a list as possible
  - Maximize coverage of the proteins
- Combinatorial optimization problem, can be solved **using integer linear programming (ILP)**

- The number of transitions at any time is limited
- Each transition has to be scheduled for a certain retention time window
- Given the choice between multiple transitions, those should be preferred that stem from peptides/ proteins not yet measured



Bertsch et al., J. Proteome Res. (2010), 2010, 9(5):2696-704

Optimization Problem

Definitions

- A set of protein sequences $S = \{s_1,\ldots,s_k\}$
- S can be digested in silico into a set of tryptic peptides $P=\{p_1,\ldots,p_n\}$
- The sequence of a peptide is mapped to a predicted
  - Retention time RT(p),
  - Proteotypicity PT(p), and
  - A set of predicted fragment ion intensities FI(p)
- A set of possible transitions $T=\{t_1,\ldots,t_l\}$, where each transition t is defined by its peptide parent mass p(t) and fragment ion m/z m(t)
- δ denotes the length in RT of a scheduled transition (based on the std. deviation of the retention time prediction)

Optimization Problem

ILP Formulation

$$\text{maximize} \sum_{t \in T} x_t d_t - \omega^p \sum_{p \in P} y_p - \omega^s \sum_{s \in S} \sum_{0 \leq j < \rho} z_s^j (\rho - j)^2$$

subject to

Binary decision variables x_t: x_t= 1 if the transition in T is choosen, 0 otherwise

Weight d_t describes the detectability (log value of combined proteotypicity and fragment ion probability)

Binary decision variables y_p: y_p= 1 if peptide $p \in P$ is NOT covered by at least τ transitions.

Binary decision variables z_s^j: z_s^j= 1 if protein sequence s is NOT represented by at least j peptides. ρ Is the given minimum number of peptides.

ω^s, ω^p are constants appropriately chosen

Bertsch et al., J. Proteome Res. (2010), 2010, 9(5):2696-704

Optimization Problem

subject to

$$\tau y_p + \sum_{i \in T_p} x_i \geq \tau, \forall\ p \in P$$

$$(j+1)z_s^j + \sum_{p \in P_s} tcov(p) \geq j+1, \forall\ s \in S, \forall\ 0 \leq j < \rho$$

with

$$tcov(p) = \begin{cases} 1, & \sum_i x \geq \tau \\ 0, & \text{otherwise} \end{cases}$$

and

$$\sum_{j \in TS_i} x_j \leq C, \forall\ 1 \leq i \leq N$$

τ

First equation ensures coverage by

The second equation ensures protein *s* is covered by at least *j* peptides

The last constraint given restricts the number of transitions in parallel to at most *C*, the maximal number of transitions that can be measured in parallel.

Bertsch et al., J. Proteome Res. (2010), 2010, 9(5):2696-704

Optimization Problem

ILP Formulation (complete)

$$\text{maximize} \sum_{t \in T} x_t d_t - \omega^p \sum_{p \in P} y_p - \omega^s \sum_{s \in S} \sum_{0 \le j < \rho} z_s^j (\rho - j)^2$$

subject to

$$\tau y_p + \sum_{i \in T_p} x_i \ge \tau, \forall\ p \in P$$

$$(j+1) z_s^j + \sum_{p \in P_s} tcov(p) \ge j + 1, \forall\ s \in S, \forall\ 0 \le j < \rho$$

with

$$tcov(p) = \begin{cases} 1, & \sum_i x \ge \tau \\ 0, & \text{otherwise} \end{cases}$$

and

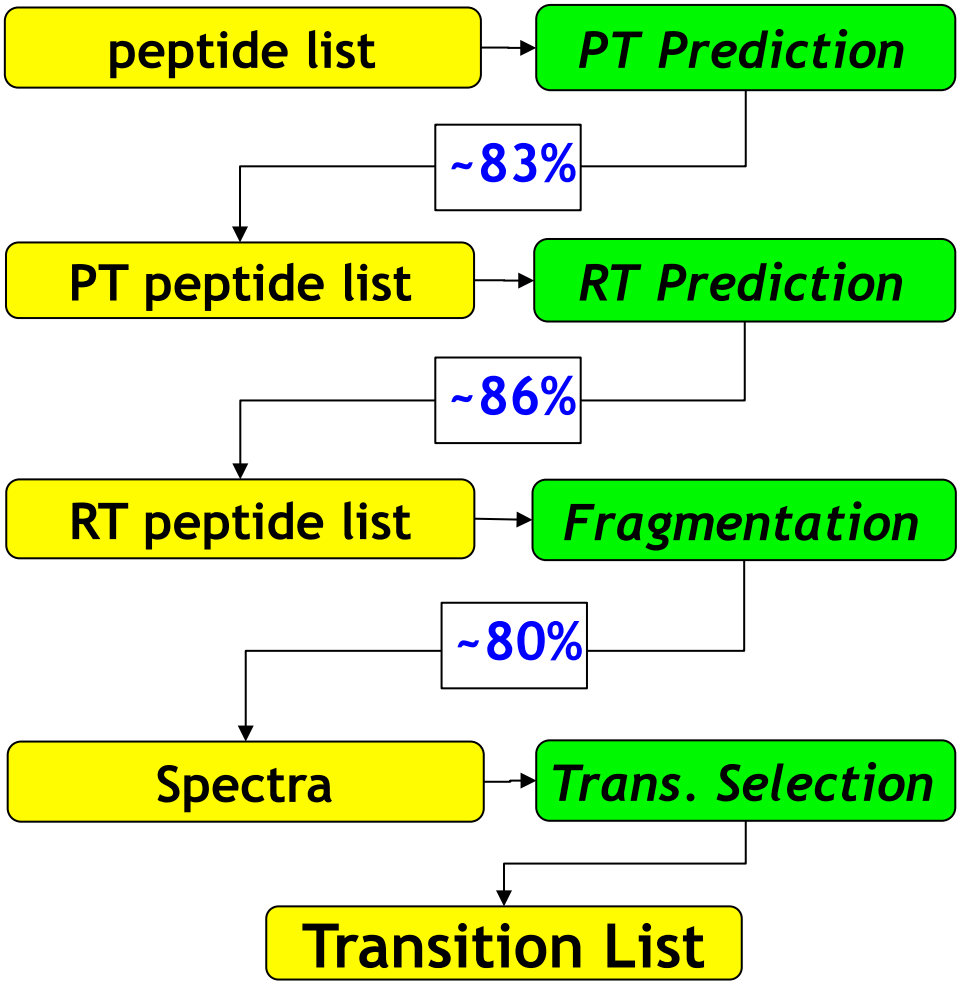
$$\sum_{j \in TS_i} x_j \le C, \forall\ 1 \le i \le N$$

Bertsch et al., J. Proteome Res. (2010), 2010, 9(5):2696-704

Training Performance

- Predictions are between 80% and 86% correct
- Expected accuracy of an individual predicted transition is thus

  0.83 x 0.86 x 0.8 = 0.57

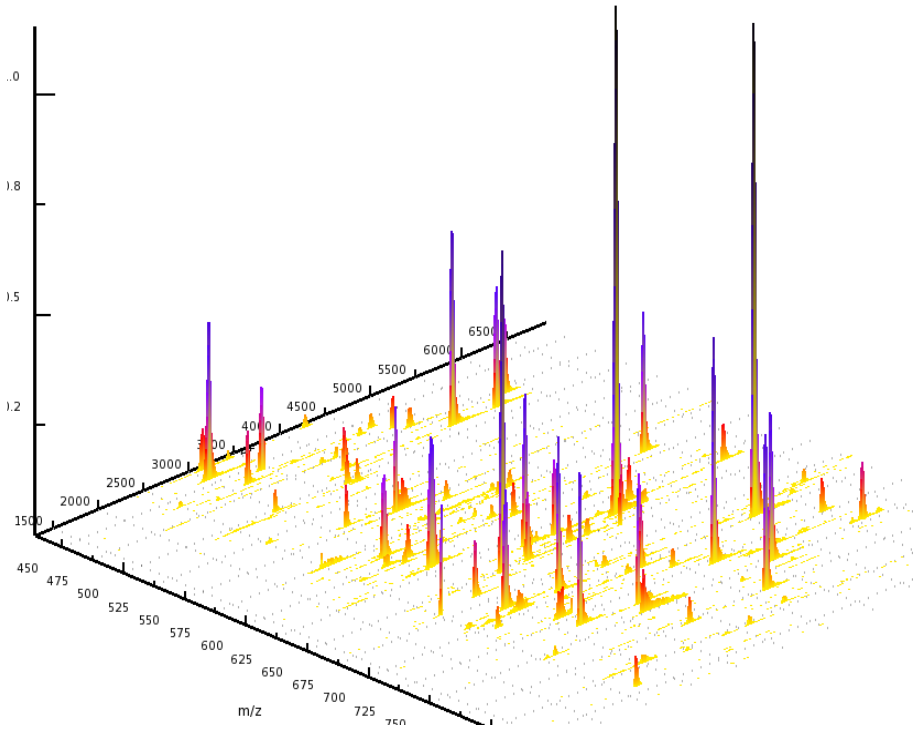


Expectation:

about 57% success rate

Proof of Principle

- Test: 48 protein mix
- 154 out of the 306 generated transitions showed signals (50%)
- As expected, about half of the transitions were successful
- Most showed clean signals and good intensities



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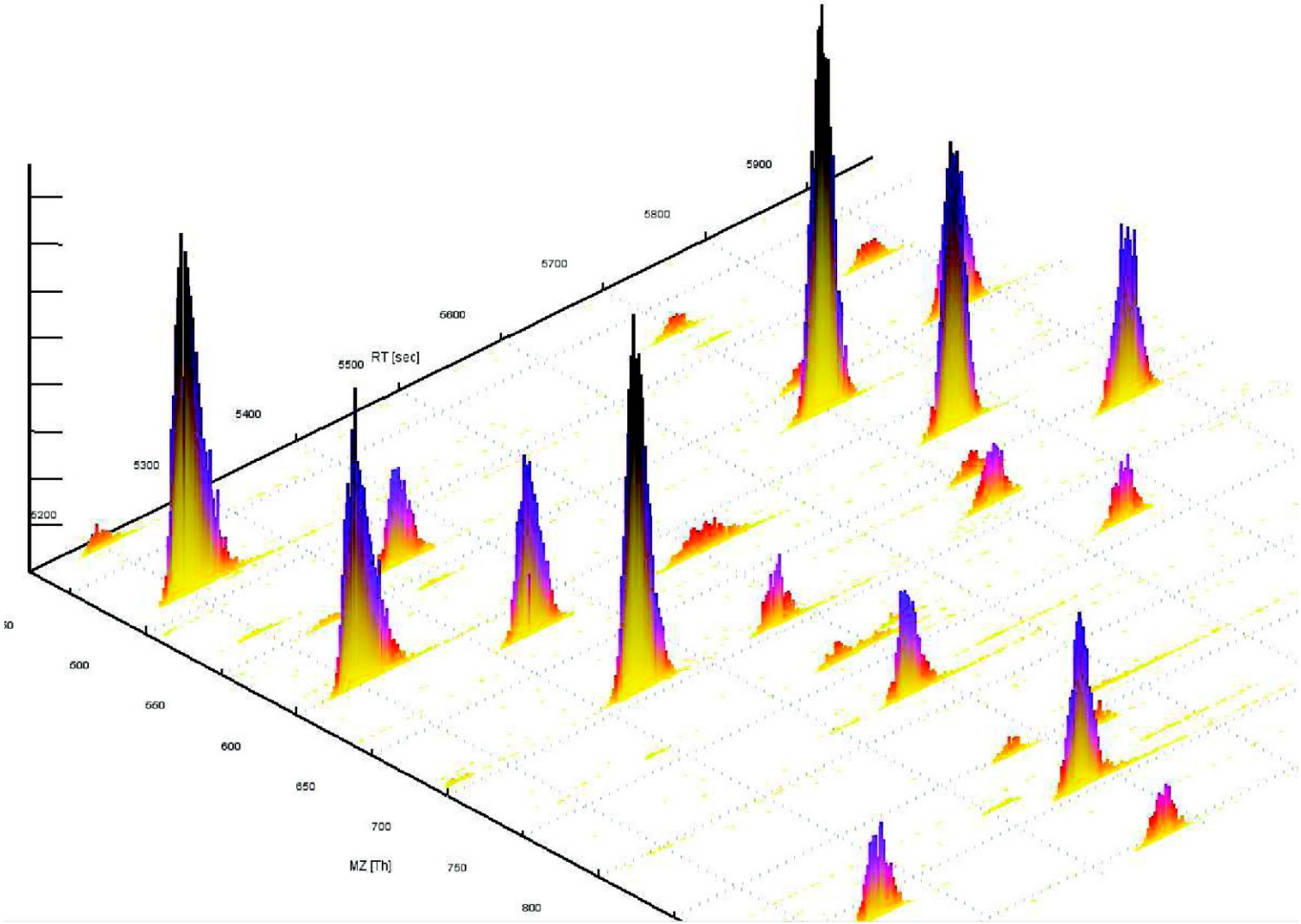
SRM Maps



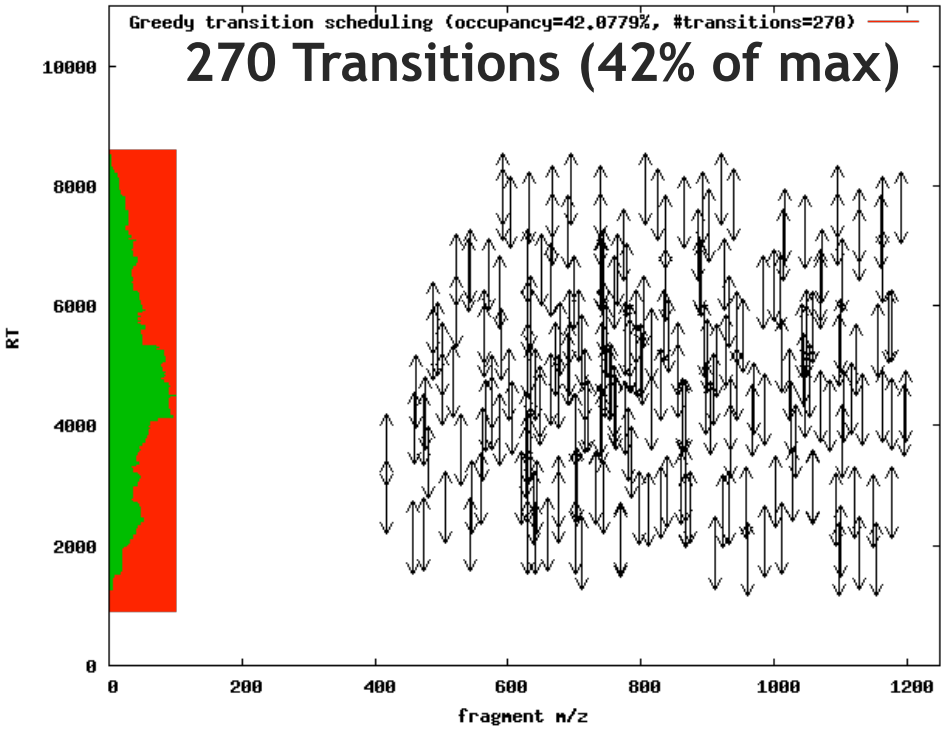
Small region of the whole pseudo 2D HPLC−MS map of the UPS1 protein mixture sMRM experiment in 3D view. The *m/z* axis shows the product ion *m/z* values of the transitions.

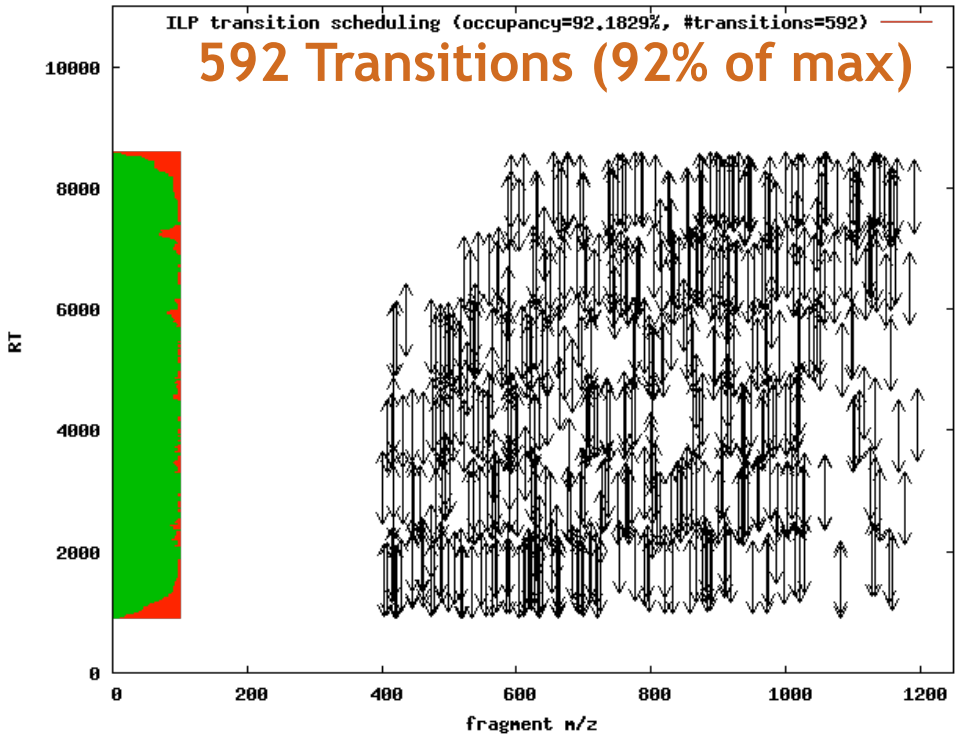
Bertsch et al., J. Proteome Res. (2010), 2010, 9(5):2696-704

Optimal Scheduling

Combinatorial optimization problem

- Ensure minimum number of transitions per protein
- Maximize the number of transitions measured to increase accuracy and coverage
- Make optimal use of the instrument's acquisition time





Bertsch et al., J. Proteome Res. (2010), 2010, 9(5):2696-704

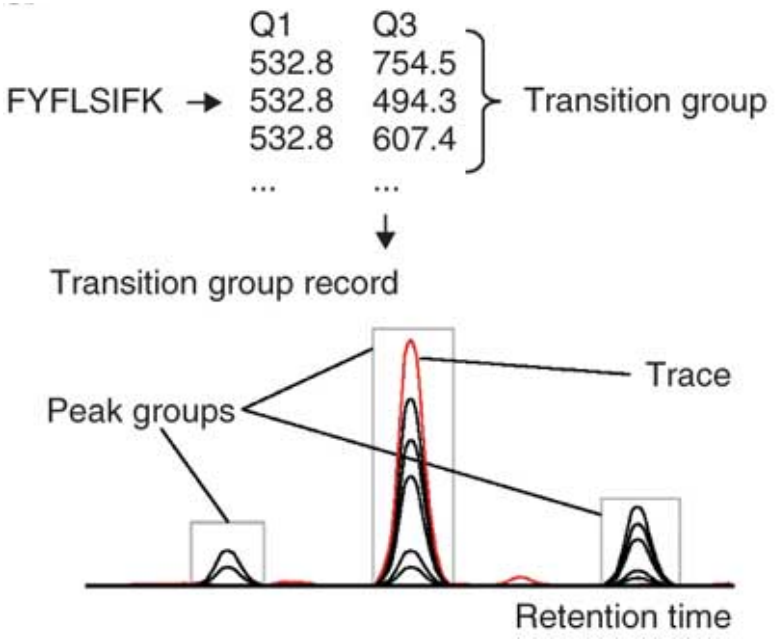
SRM Analysis – mQuest/mProphet

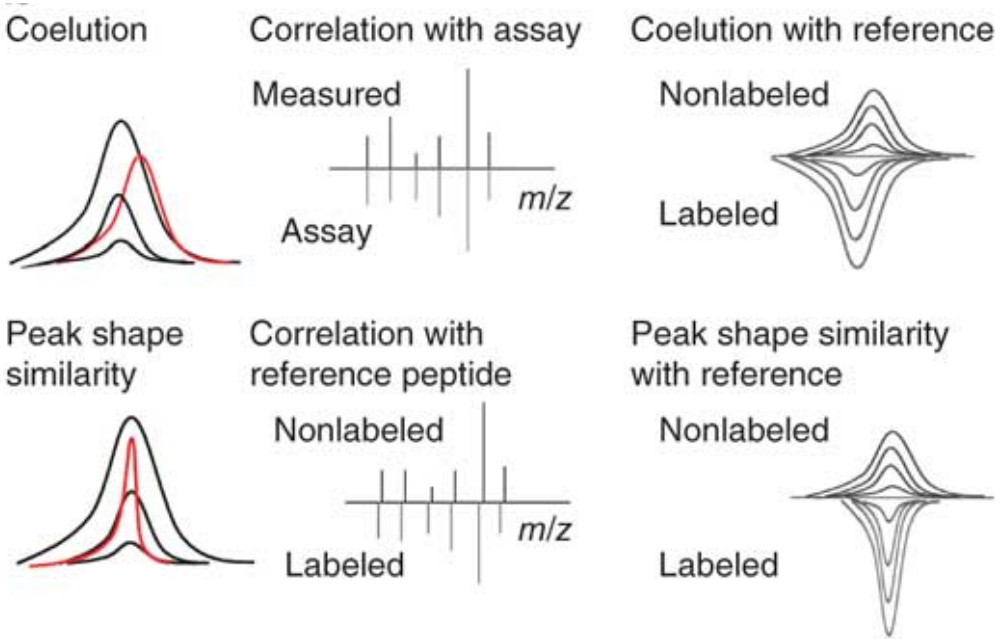
- mQuest/mProphet is a suite of (Perl and R) tools for the analysis of MRM data sets
- **Input**
  - Acquired SRM dataset (mzXML)
  - Transition list (Excel spreadsheet)
- **Output**
  - Quantified proteins
- mQuest maps the transition list onto the acquired data
- mProphet performs the statistical analysis

Reiter et al., . Nat Methods. 2011 May;8(5):430-5.

SRM Analysis – mQuest/mProphet

- Peak shape scoring
  - Transitions caused by the same peptide should have the same peak shape ("coelution")
  - Interferences would most likely show a different shape
  - Coeluting peaks in different traces form a peak group





Reiter et al., . Nat Methods. 2011 May;8(5):430-5.

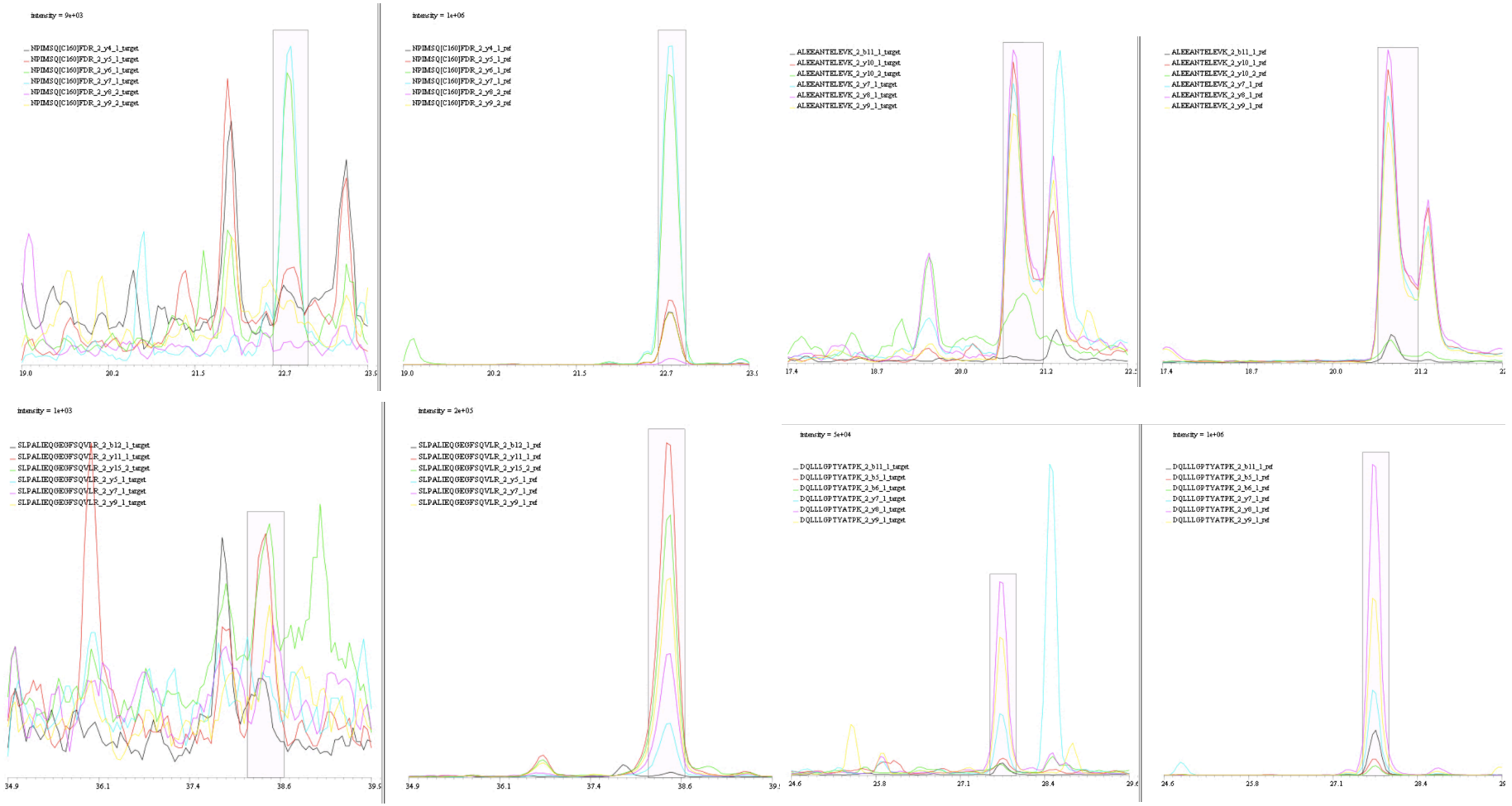
Scoring

- Scoring consists of various subscores
  - **Coelution** – how well do the peaks elute at the same time (and at the same time as the assay reference)
  - **Peak shape** – how well do the peak shapes agree within a peak group
  - **Peak intensities** – how well do relative intensities of the different transitions agree with the reference (the tandem spectrum of the peptide)
- Subscores are combined linearly into a complete score and then converted into p-values
- Coefficients of the linear combination are adjusted automatically based on the analysis of artificial decoy transitions

Scoring

- **Coelution**
  - Compute cross-correlation of each peak of the peak group with each other peak
  - Peak RT shift is determined as the maximum of the pair-wise cross-correlation (more robust than difference of peak maxima in noisy signals)
  - Mean of these shifts is reported as a score
- **Peak shape**
  - Based on the maximum correlation of two peaks as well
- **Peak intensities**
  - Pearson correlation coefficient between peak intensities of the peak group and intensities of the corresponding intensities in the reference peptide

Reality



Real-world MRM traces. Left: measurement from a complex sample, right: reference transition.

Reiter et al., . Nat Methods. 2011 May;8(5):430-5.

LEARNING UNIT 6C
COMPARISON OF METHODS

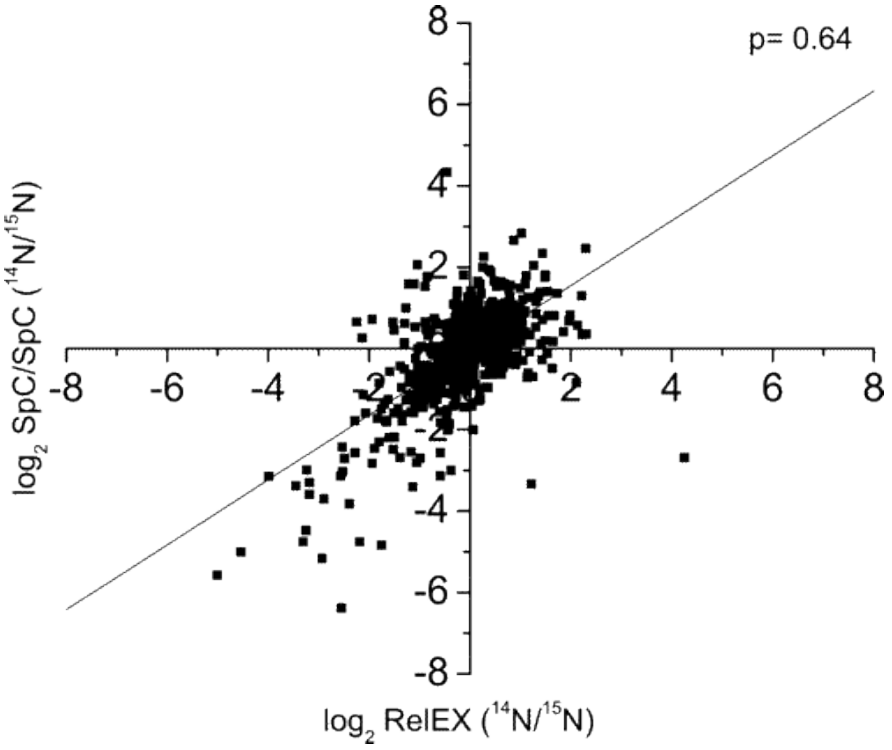
Spectral Counting

- Spectral counting is a trivial quantitation method based on counting tandem MS spectra matching the same peptide
- **Idea**
  - If the peptide is more abundant, then it will trigger a tandem spectrum more often
- **Advantages**
  - Trivial to implement
- **Disadvantages**
  - Depends on instrument settings (dynamic exclusion time)
  - No physical basis for the quantification
  - Rather inaccurate

Spectral Counting vs. Labeling

- Zybailov et al. compared spectral counting and metabolic labeling ($^{14}N/^{15}N$ labeling) in yeast
- Both methods – according to the authors – yield good quantification results

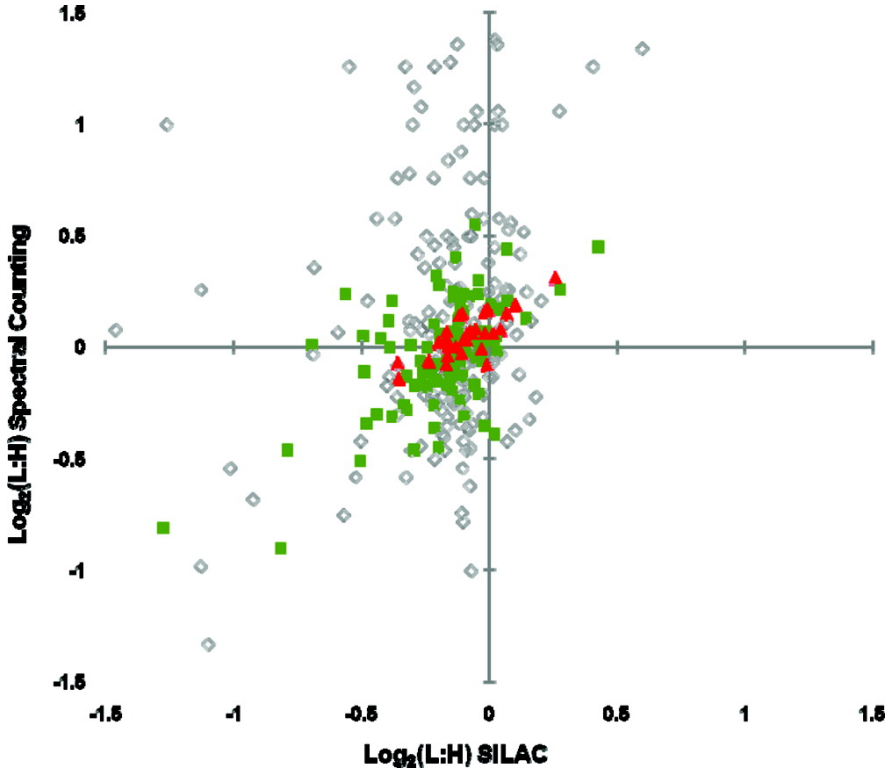
"We demonstrate that spectrum counting and mass spectrometry derived ion chromatograms strongly correlate for determining quantitative changes in protein expression. Spectrum counting proved more reproducible and has a wider dynamic range contributing to the deviation of the two quantitative approaches from a perfect positive correlation."



Spectral Counting vs. SILAC

- Collier et al. compared SILAC to spectral counting on human embryonic stem cells

"With respect to protein quantification, spectral counting was inherently able to quantify more proteins (885) than SILAC (450), although less accurately unless a 5 spectral count limit was established for protein quantification, reducing the number of proteins quantified by spectral counting to 340. In a normal experimental setting, a label-free strategy allows for double the total protein amount to be analyzed using spectral counting compared to SILAC."

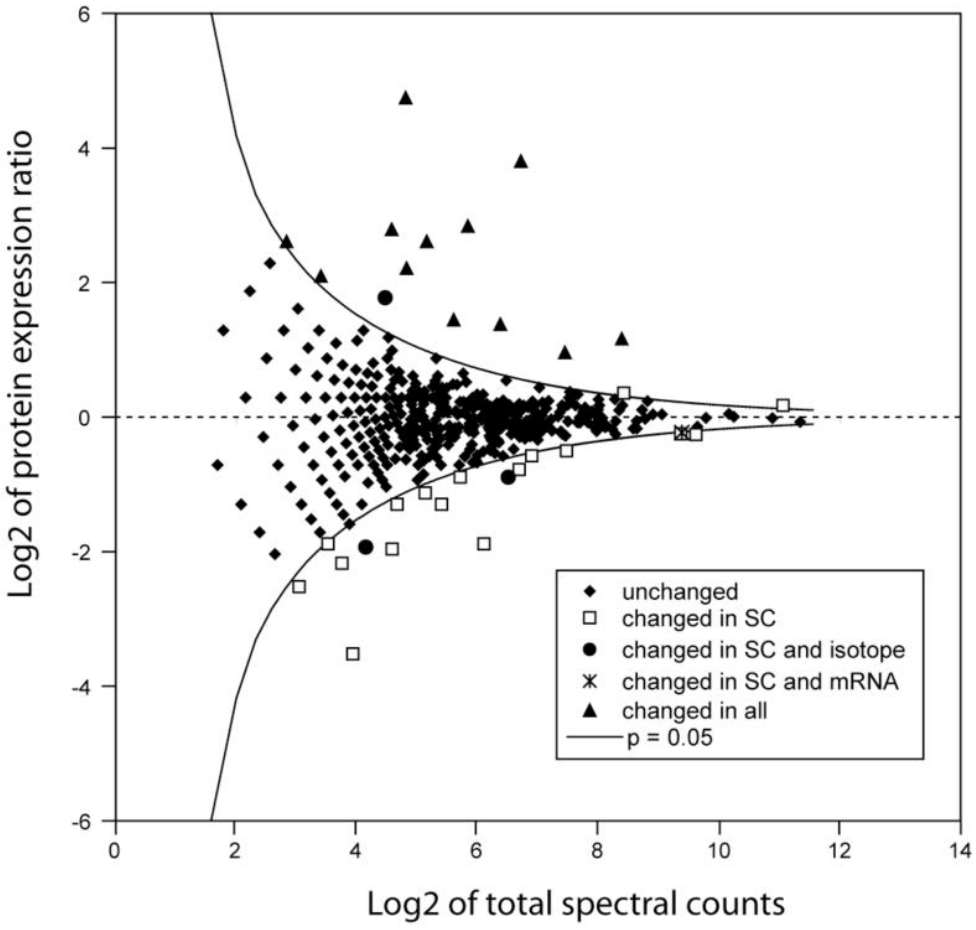


Collier et al., Anal Chem. 2010 Oct 15;82(20):8696-702.

Spectral Counting vs. SILAC

- Hendickson et al. compared metabolic labeling to spectral count on microbial proteomes (wt vs. mutant)

"Spectral counting showed lower overall sensitivity defined in terms of detecting a two-fold change in protein expression, and in order to achieve the same level of quantitative proteome coverage as the stable isotope method, it would have required approximately doubling the number of mass spectra collected."



Hendickson et al., Analyst. 2006 Dec;131(12):1335-41.

iTRAQ vs. Label-Free

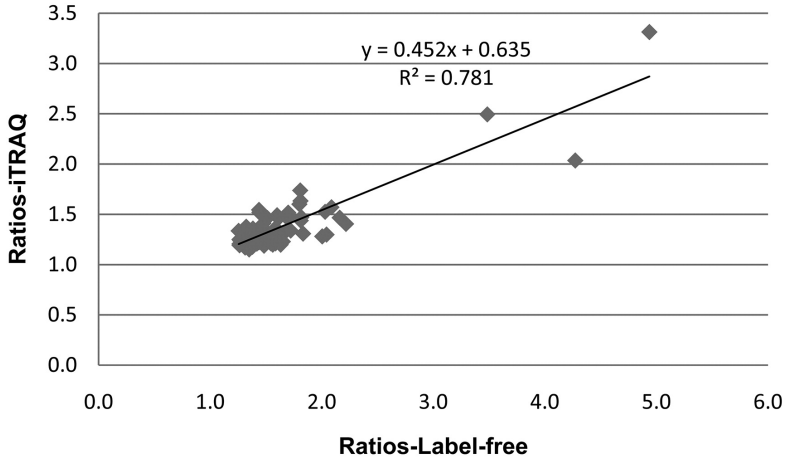
- Wang et al. compared iTRAQ labeling and label-free quantification
- *Chlamydomonas* proteome samples were analyzed and four proteins added in various concentrations as internal standards
- Samples were analyzed in technical and biological replicate on an Thermo Orbitrap Velos
- iTRAQ quantification was performed using MASCOT distiller
- Label-free quantification was performed using Progenesis LC-MS

Wang et al., J Proteome Res. 2011 Dec 1. [Epub ahead of print, PMID: 22059437]

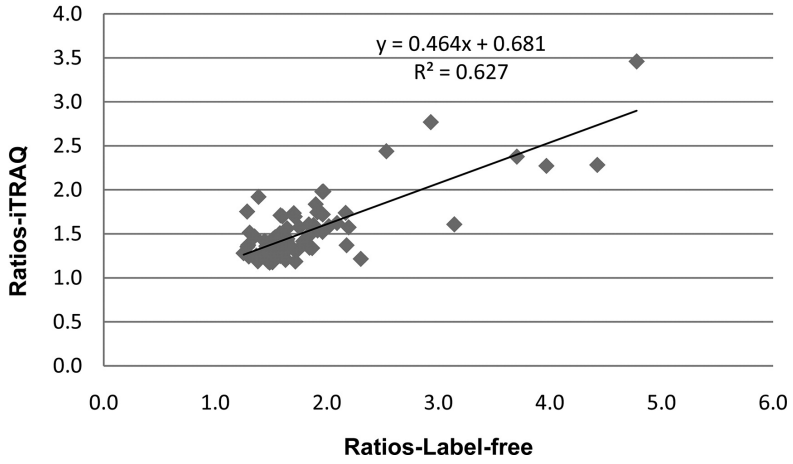
iTRAQ vs. Label-Free

"The comparison between both methods indicates that the label-free method provided better quantitation accuracy for high fold change ratios; however, quantitation precision is better when using iTRAQ. […] The results from both approaches have a good correlation of protein ratios for the commonly quantified proteins; […] iTRAQ, with its higher quantitation accuracy when ratios are close to 1, would allow the identification of smaller changes often times responsible for important biological changes "

A – Technical replicates

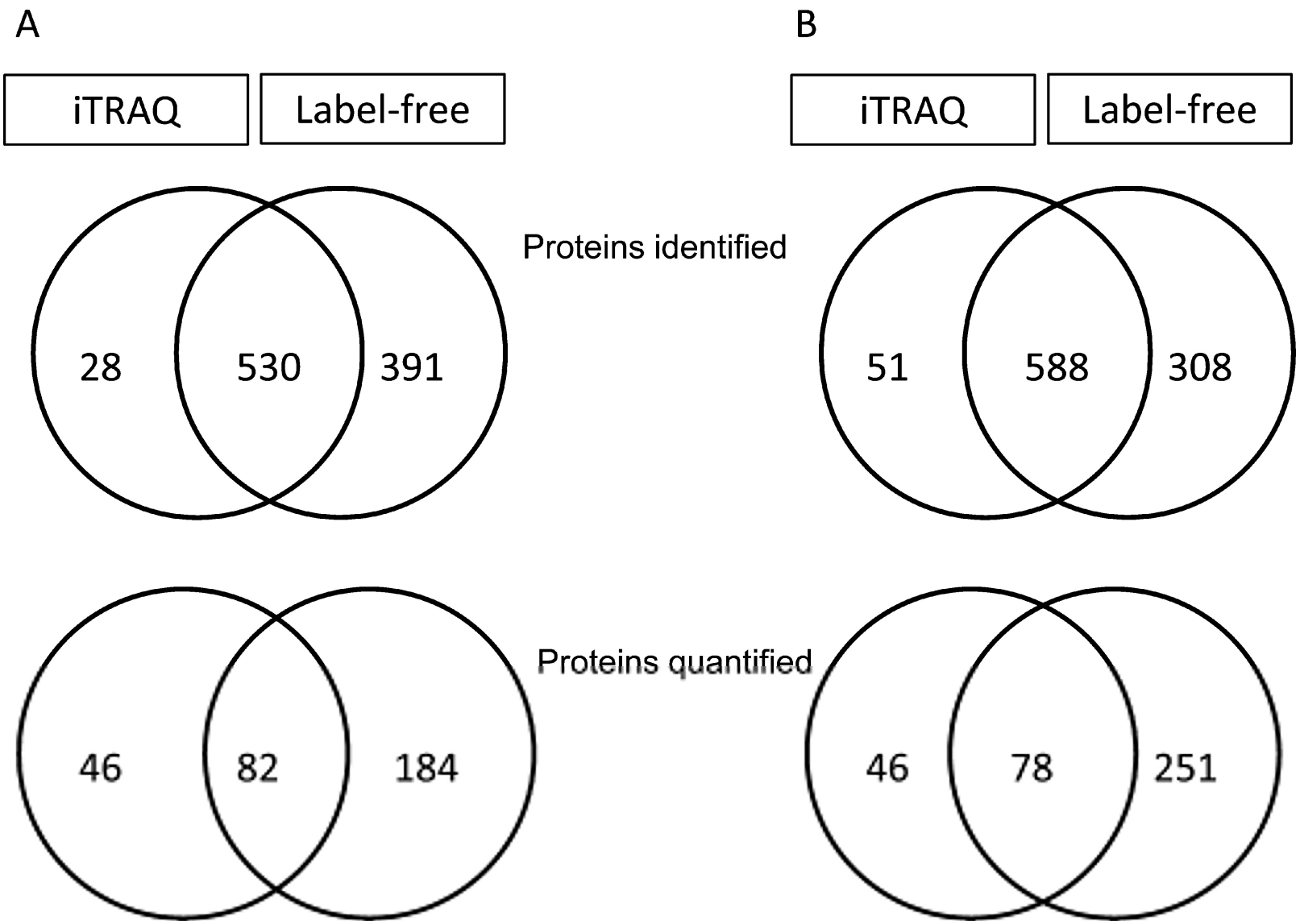


B – Biological replicates



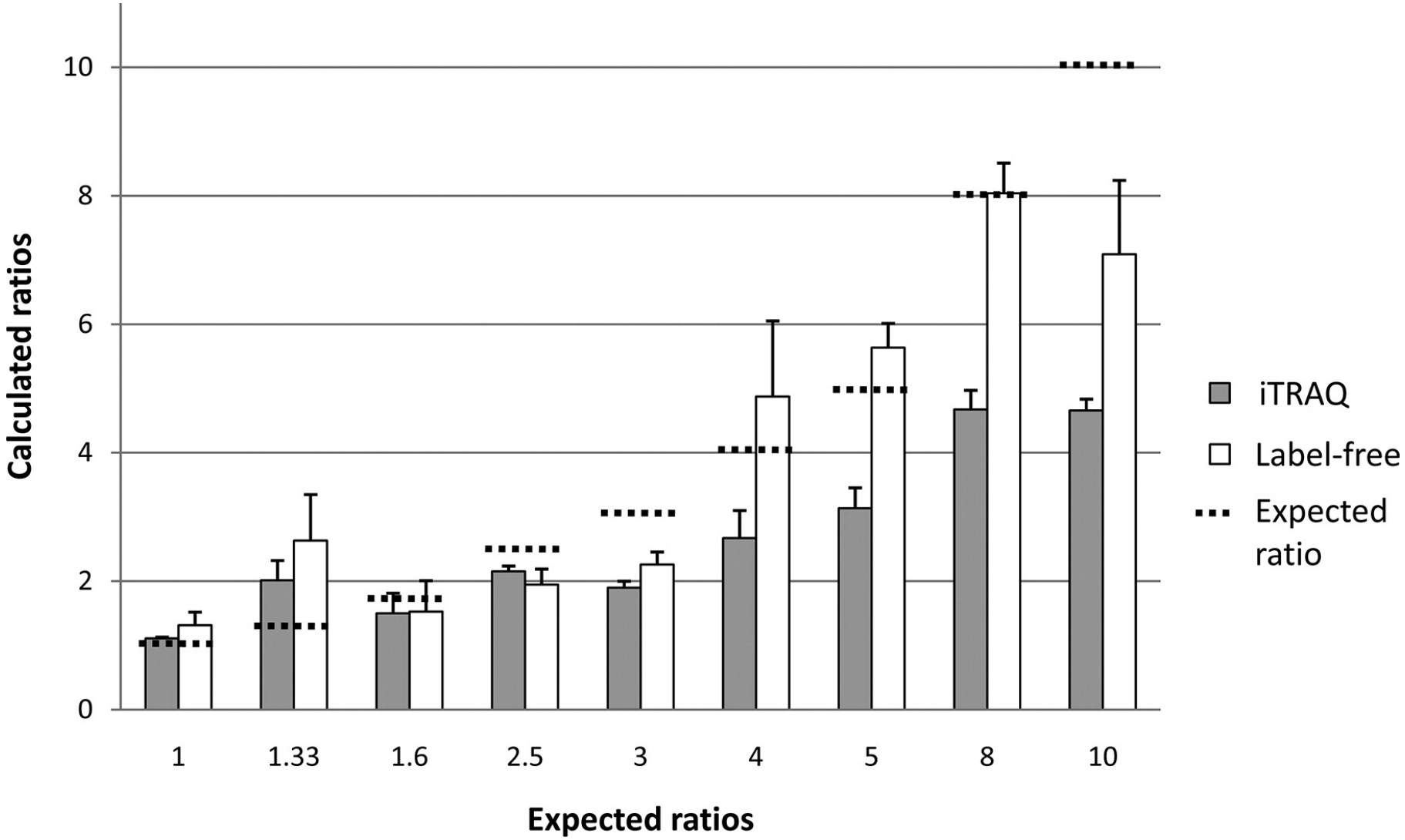
Wang et al., J Proteome Res. 2011 Dec 1. [Epub ahead of print, PMID: 22059437]

iTRAQ vs. Label-Free



Wang et al., J Proteome Res. 2011 Dec 1. [Epub ahead of print, PMID: 22059437]

iTRAQ vs. Label-Free



Wang et al., J Proteome Res. 2011 Dec 1. [Epub ahead of print, PMID: 22059437]

Comparison of Methods

- Comparison of quantification methods is difficult: few studies really benchmark multiple methods
- Most experimental labs have established one or two methods at best
- Quantitative proteomics is still pretty much an open field
- No standard method has been firmly established yet
- Currently, SILAC, and label-free are probably the most 'popular' methods, followed by MRM
- Choice of the quantification method depends on the application, the available instrument and the available bioinformatics expertise

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- **mProphet**
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- **Quantification in general**
  - Bantscheff *et al.*, Quantitative mass spectrometry in proteomics: a critical review, Anal Bioanal Chem (2005), 389, 1017-1031 [PMID: 17668192]

Materials

- Online Materials
  - Learning Unit 6A, B, C