Oddělení funkční genomiky a proteomiky
Národní centrum pro výzkum biomolekul
Přírodovědecká fakulta MU

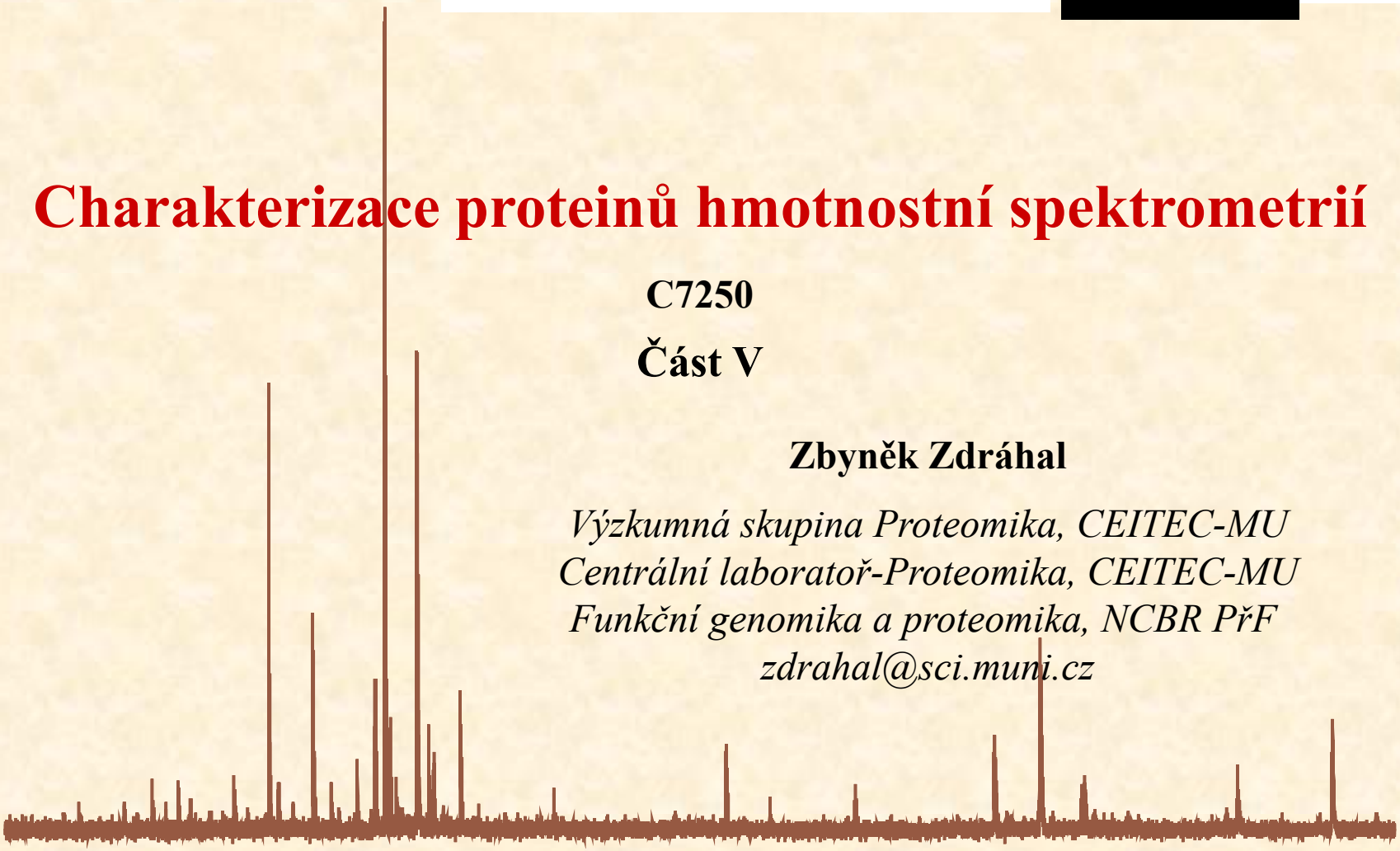
# Charakterizace proteinů hmotnostní spektrometrií

**C7250**

**Část V**

**Zbyněk Zdráhal**

*Výzkumná skupina Proteomika, CEITEC-MU*
*Centrální laboratoř-Proteomika, CEITEC-MU*
*Funkční genomika a proteomika, NCBR PřF*
*zdrahal@sci.muni.cz*



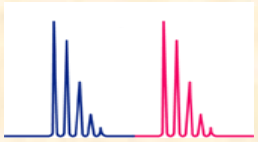
# Kvantifikace proteinů a MS

# Kvantifikace proteinů pomocí MS

**Přístupy:**

- **Absolutní kvantifikace**
  stanoveni koncentrace ve vzorku pomocí standardu o známé koncentraci
- **Relativní kvantifikace**
  poměrné zhodnocení změny koncentrace proteinu ve srovnávaných vzorcích

**Metody:**



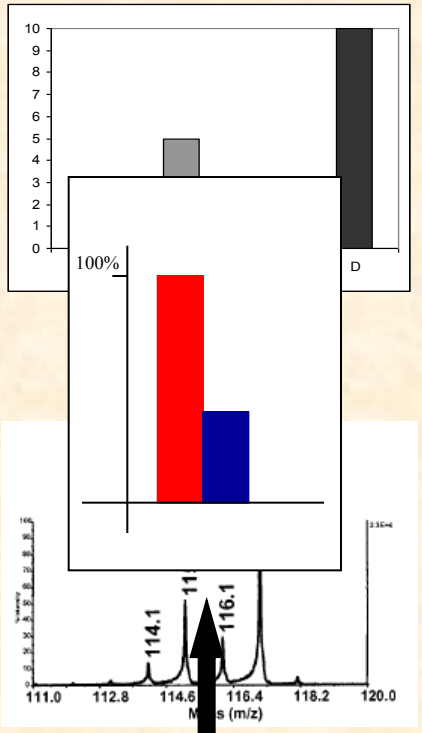
- **s izotopicky odlišnými značkami**
  stejné proteiny/peptidy jsou ve vzorcích odlišeny značkami o různé hmotnosti a takto mohou být měřeny současně

- **bez izotopického značení (label free)**
  metody absolutní a relativní kvantifikace založené na statistickém vyhodnocení MS, resp. MS/MS dat
  výhodou je možnost srovnání neomezeného počtu vzorků a absence derivatizační reakce či izotopicky značených standardů

# Metody relativní kvantifikace

Proteinový vzorek A

Proteinový vzorek B

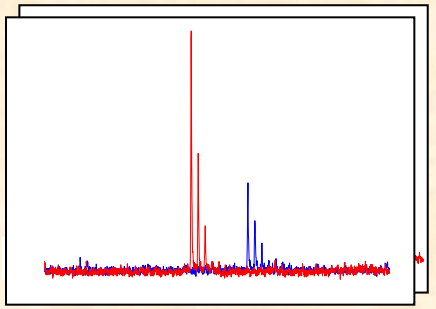



MS/MS

Oddělená digesce vzorků

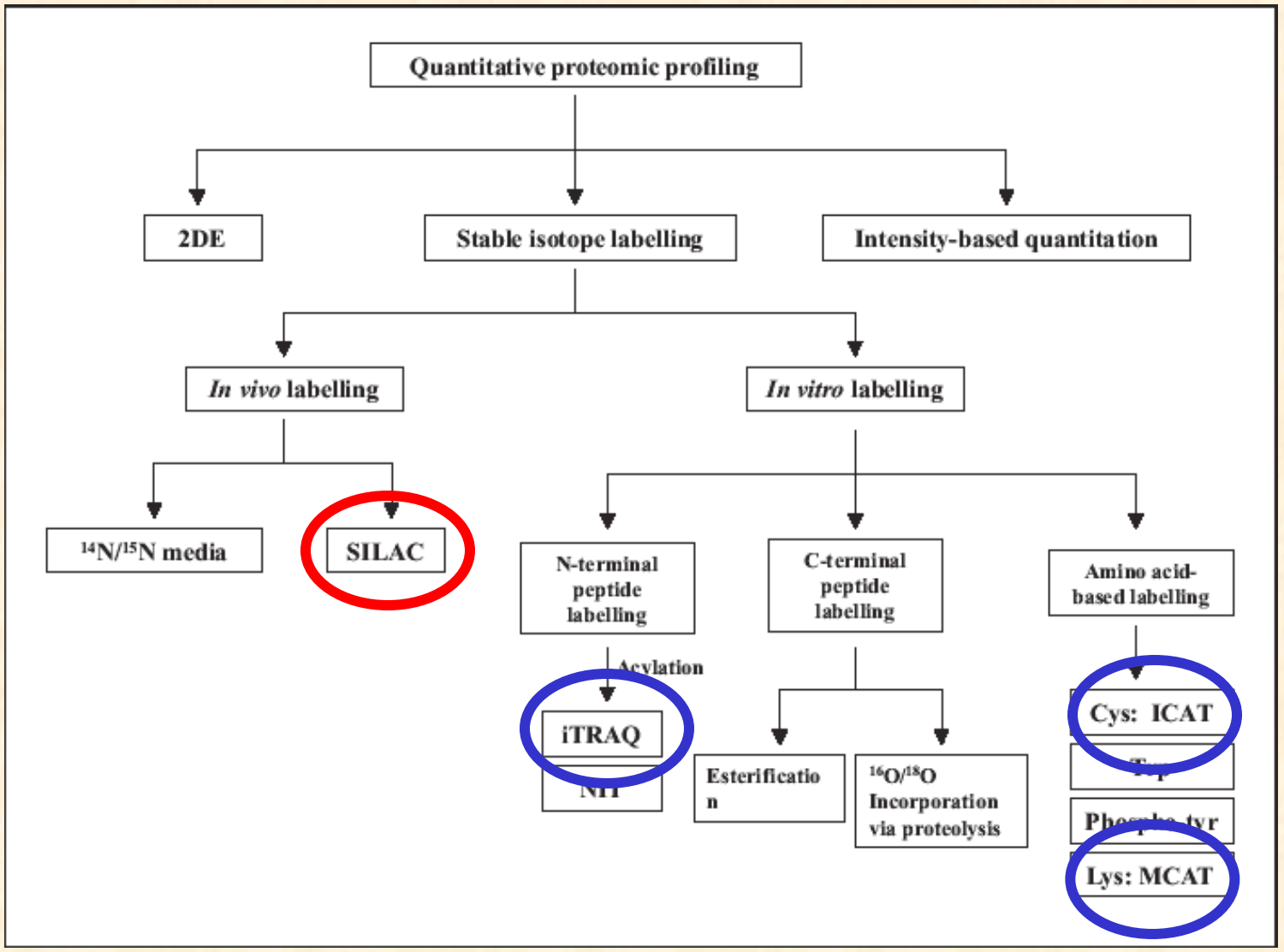
MCAT, alkylace
iTRAQ, TMT

LC-MS
LC-MS/MS



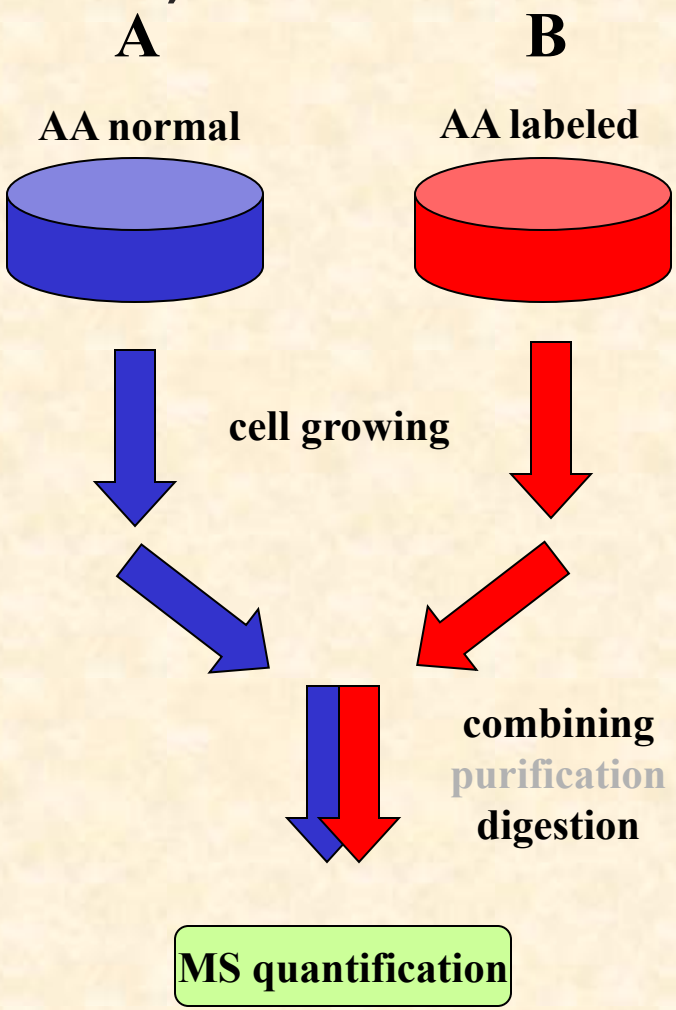
Stejný peptid, odlišen kvantifikační značkou

# Přehled kvantifikačních metod

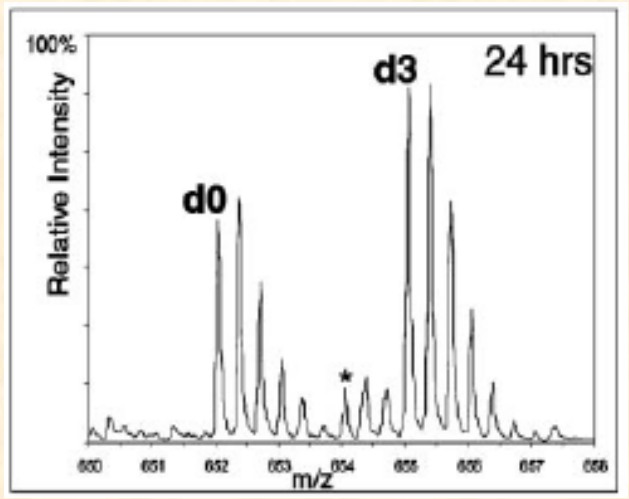



W. Yan, S.S. Chen, *Briefings in functional genomics and proteomics* **4** (1), 1–12, (2005)

# Stable Isotope Labelling with Amino acids in Cell culture (SILAC)




- *in vivo*
- proteins are labeled by growing cells in media containing isotopically labeled amino acids
  (e.g. $^{2}$H-Leu, $^{13}$C-Lys, $^{13}$C-Tyr,$^{13}$C-Arg, $^{13}$C/$^{15}$N-Arg)

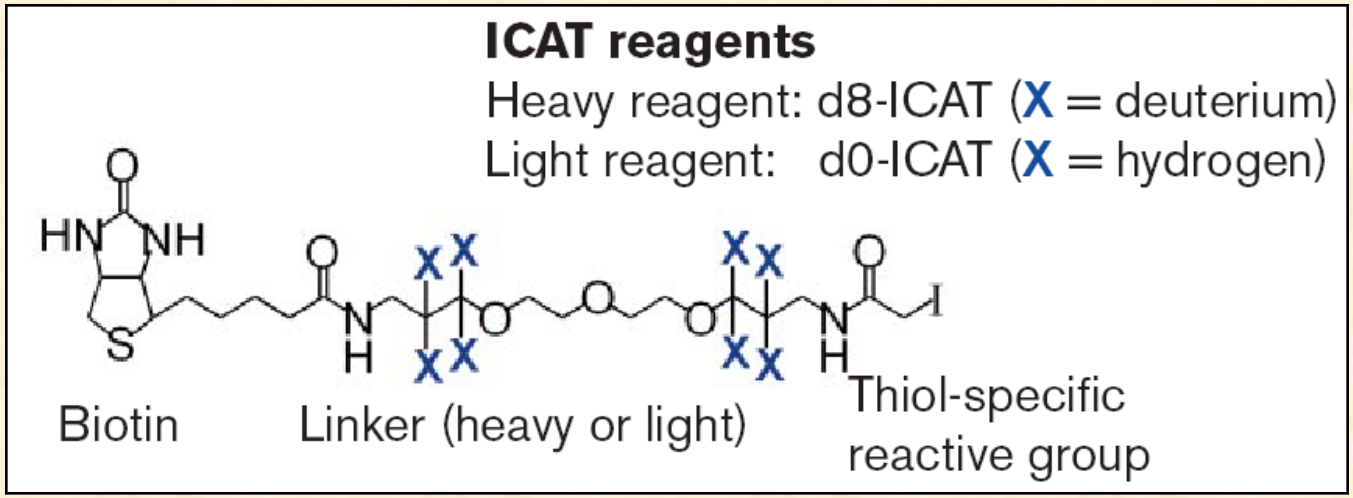



picture from Ong et al.: *MCP* **1**(2002), 376

# ICAT ... Isotope-Coded Affinity Tags

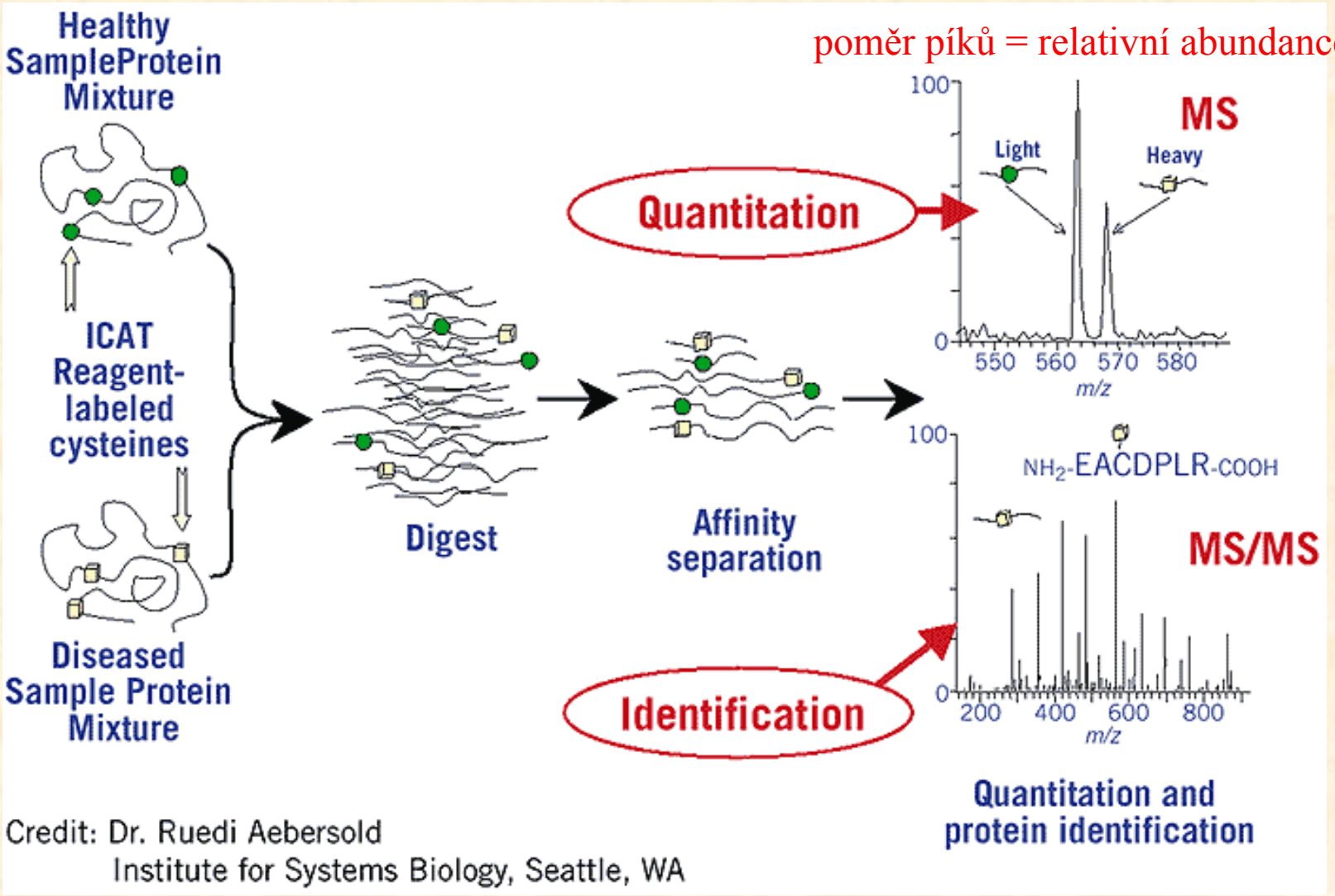
technology for protein expression analysis

- ✓ improved quantitation of a wider range of proteins
- ✓ overcomes limitations of 2-D gel method (e.g. membrane, low abundance proteins)

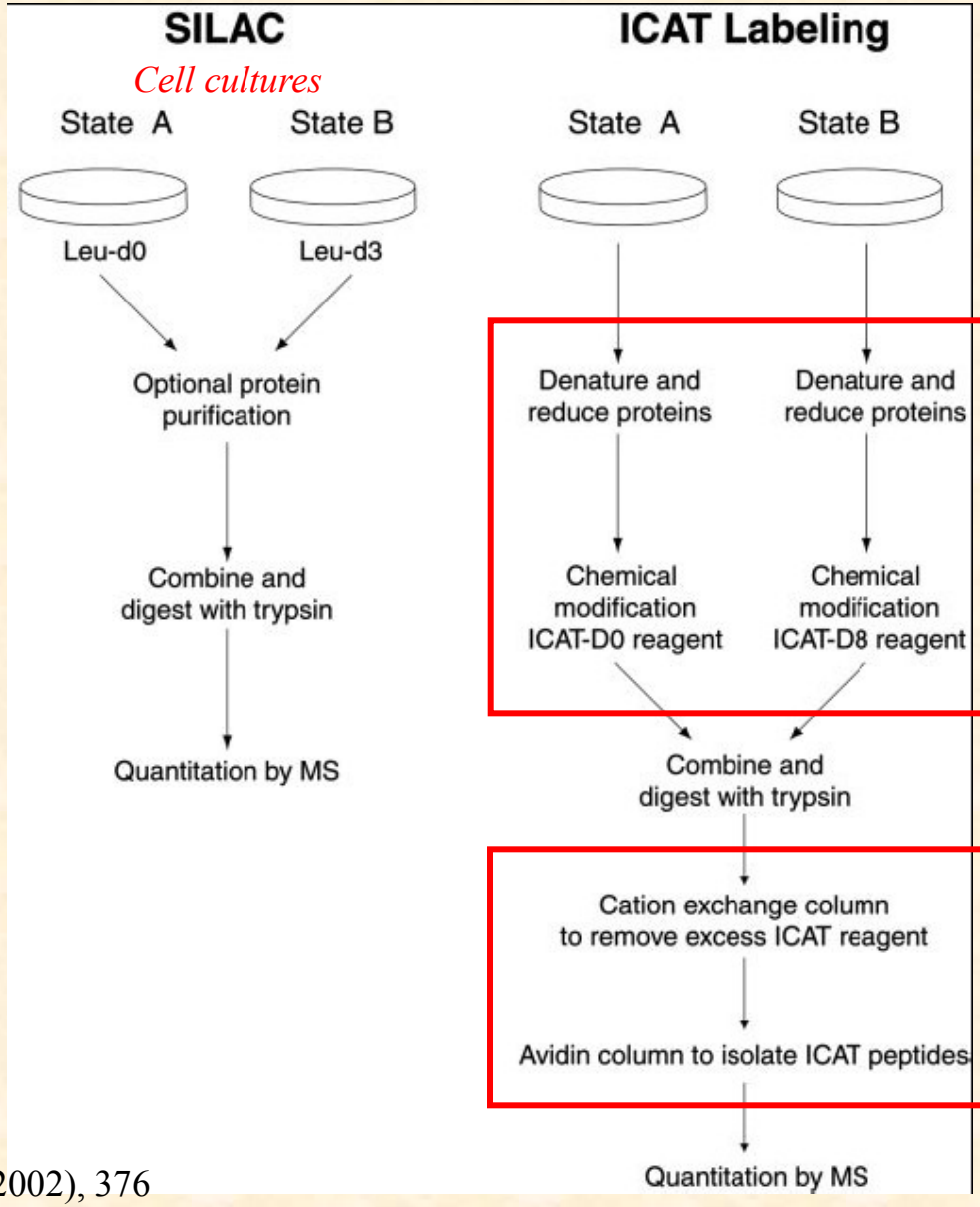



- ➢ tags specific for cysteine-containing peptides (reduction of sample complexity)
- ➢ easy automation of a procedure

# ICAT analysis



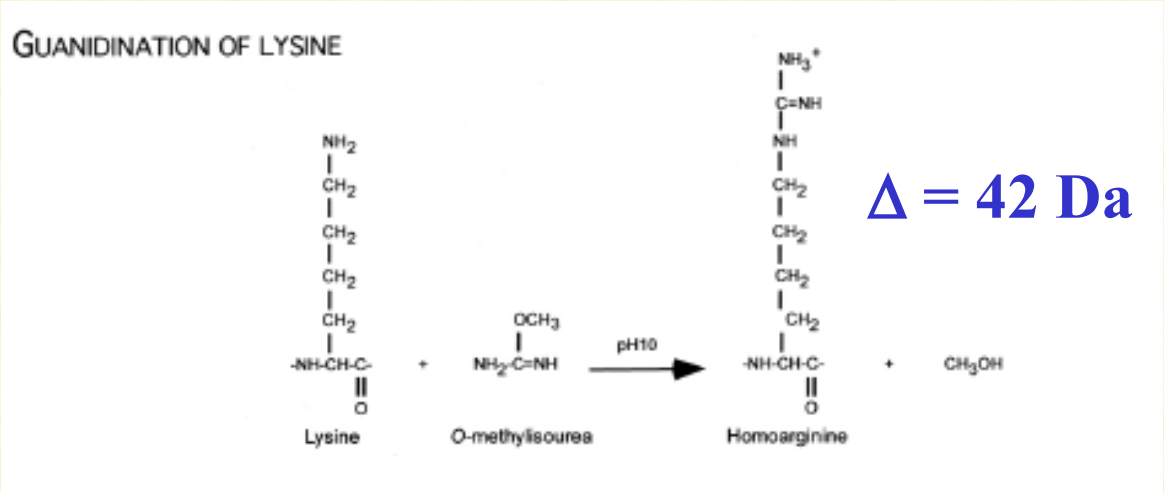
# **Srovnání *in-vivo* a *in vitro* kvantifikačních metod** (SILAC vs ICAT)




from Ong et al.: *MCP* **1**(2002), 376

# Mass Coded Abundance Tagging (MCAT)

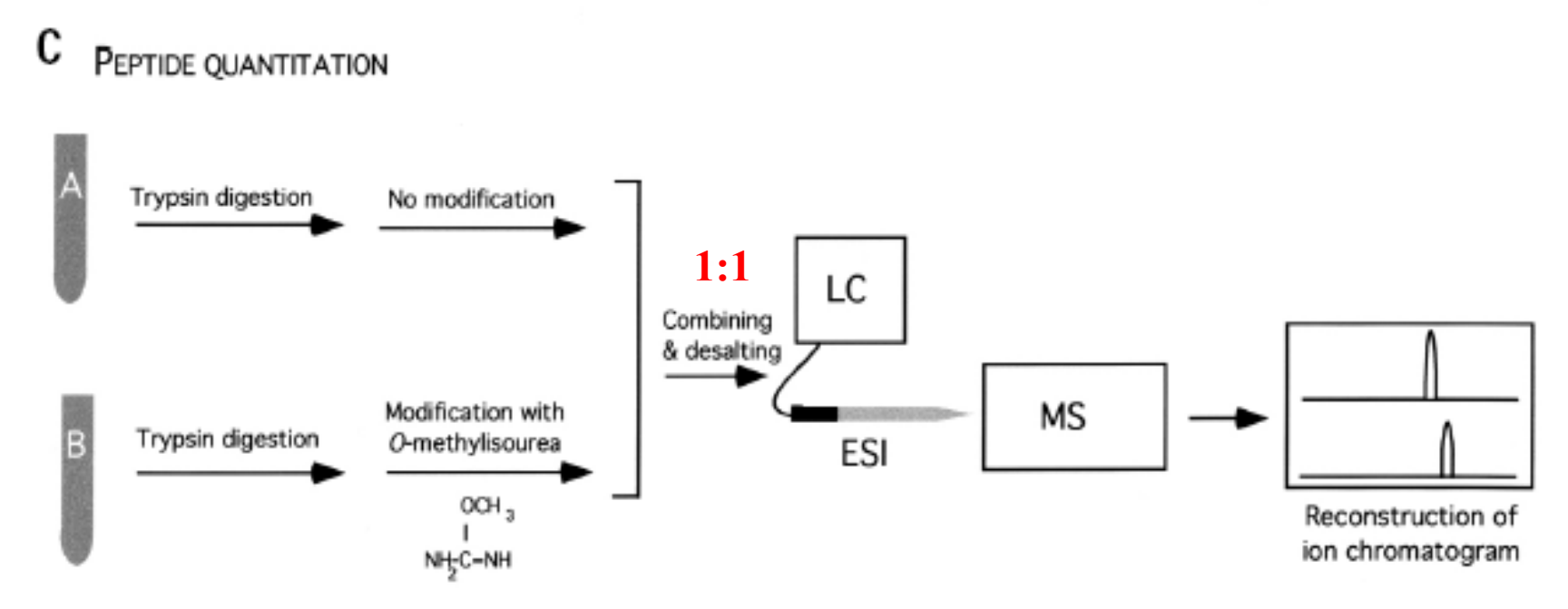
- digesce trypsinem
- modifikace digestu vybraného vzorku (K)




- smíchání **nemodif/modif** v poměru 1:1

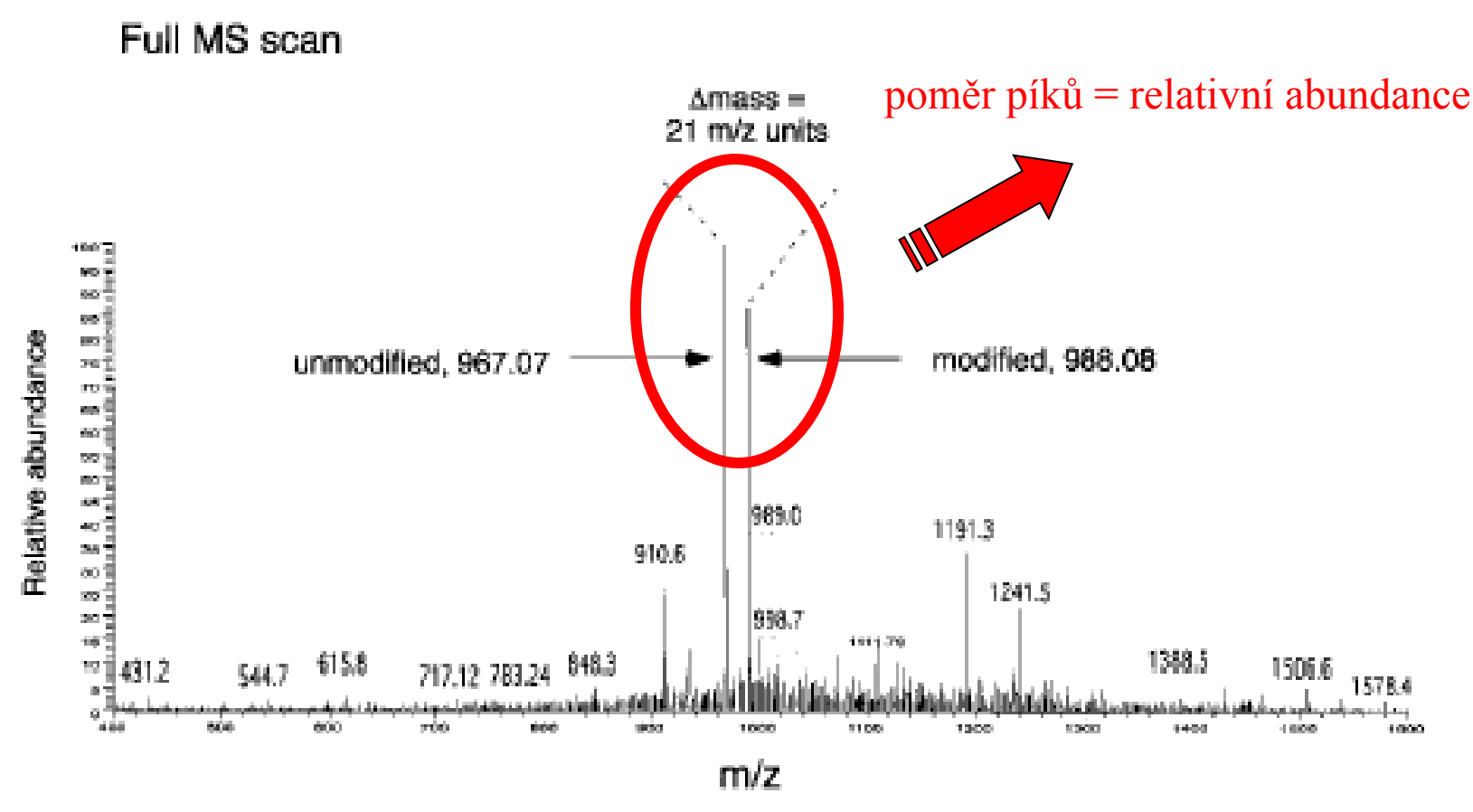
Cagney G., Emili A.: *Nature Biotechnol* **20** (2002), 163-170

# MCAT




Cagney G., Emili A.: *Nature Biotechnol* **20** (2002), 163-170

# MCAT



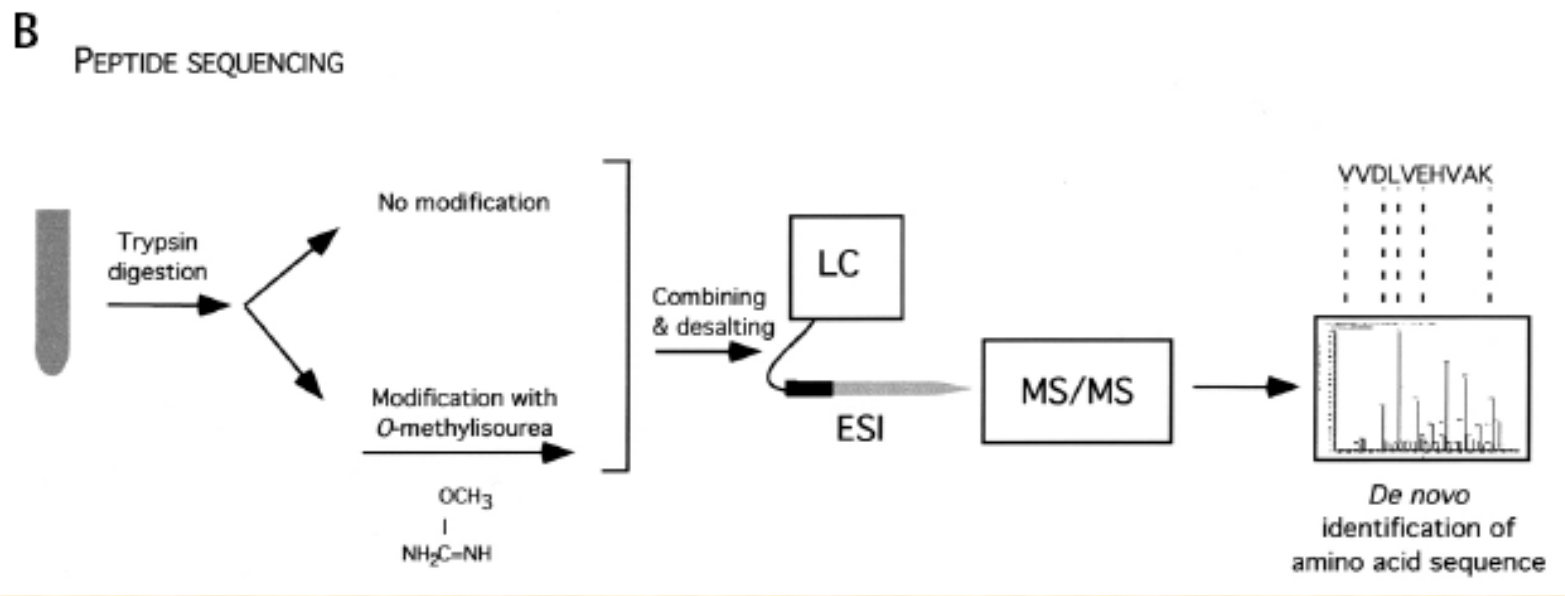

Cagney G., Emili A.: *Nature Biotechnol* **20** (2002), 163-170

# MCAT

derivatizace neni nikdy kompletní, operace se vzorkem
jen ...K peptidy
interní K ????

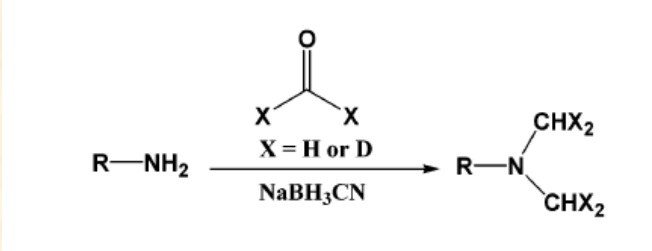
využití derivatizace pro *de novo* sequencing

*b ionty nezměněny, y ionty v dubletech (42 Da)*



# Reduktivní alkylace - dimetylace

- lysin a N-terminus peptidu
- izotopicky značený formaldehyd (D, $^{13}C$)




*Hsu et al., Anal.Chem. 2003*

# Reduktivní alkylace - dimetylace

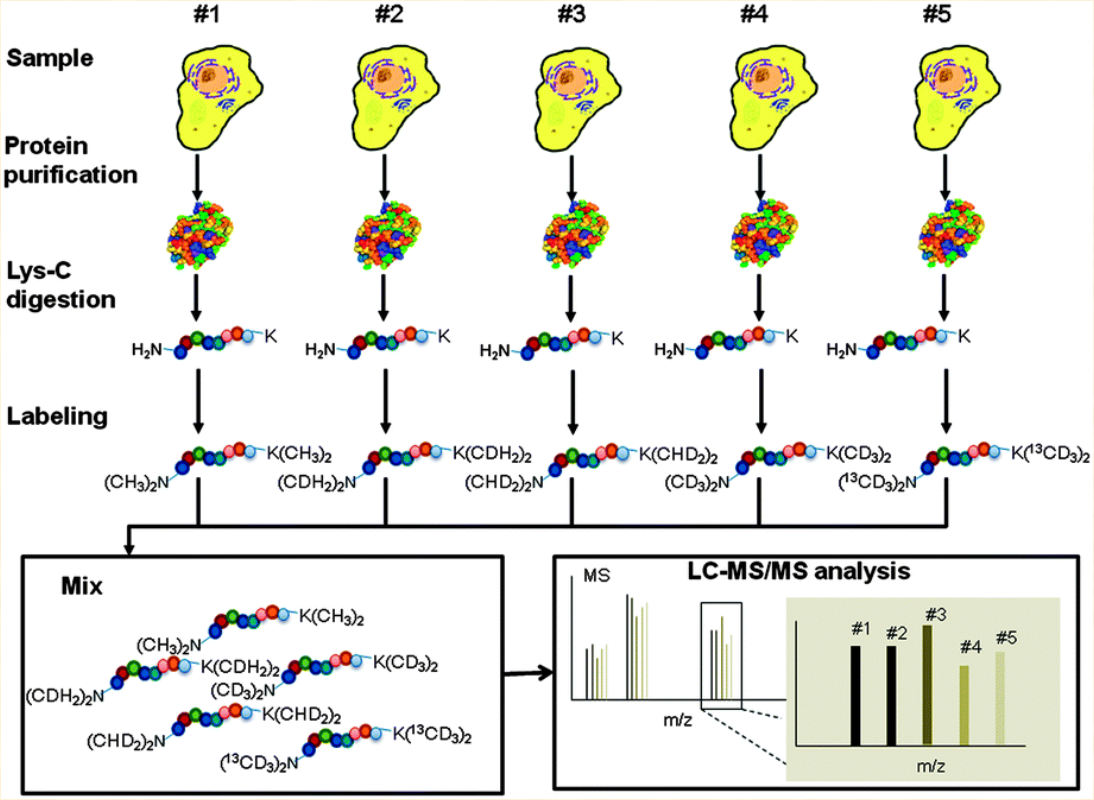



Table 1 The combination of different isotopic reagents in the five-plex isotope dimethyl labeling method

| Label | #1 | #2 | #3 | #4 | #5 |
|---|---|---|---|---|---|
| Formaldehyde isotope | $H_2CO$ | $H_2CO$ | $D_2CO$ | $D_2CO$ | $D_2{}^{13}CO$ |
| Cyanoborohydride isotope | $NaBH_3CN$ | $NaBD_3CN$ | $NaBH_3CN$ | $NaBD_3CN$ | $NaBD_3CN$ |
| ΔMass (Da, one active site) | 28.0313 | 30.0439 | 32.0564 | 34.0690 | 36.0757 |
| ΔMass (Da, two active sites) | 56.0626 | 60.0878 | 64.1128 | 68.1380 | 72.1514 |

*Wu et al., Chem. Commun. 2014*

# Reduktivní alkylace - dimetylace

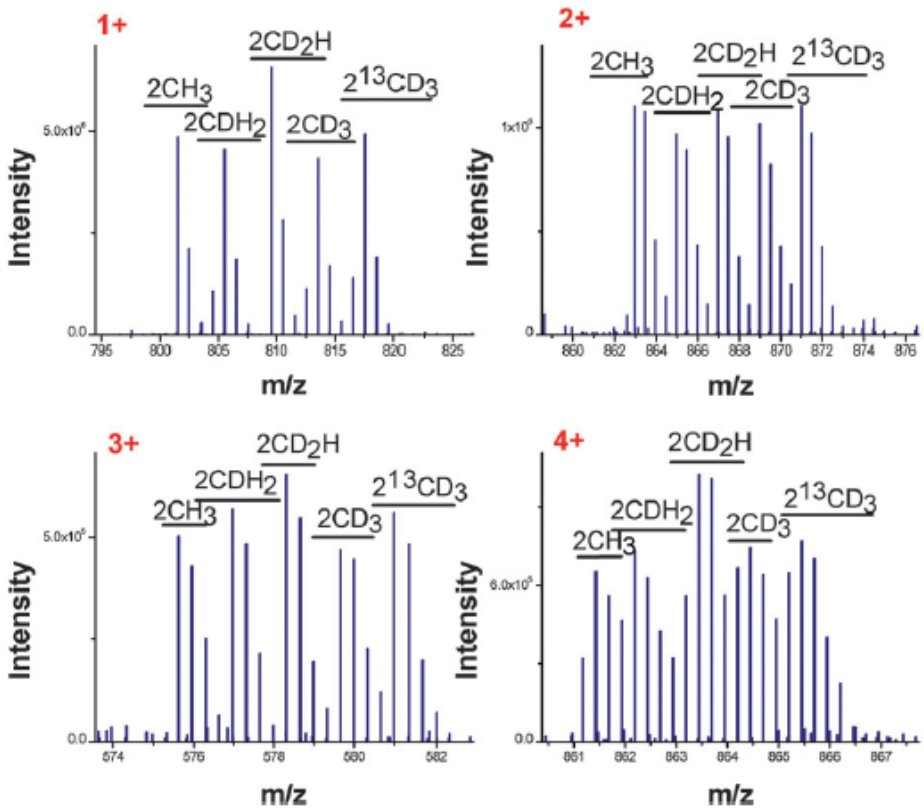
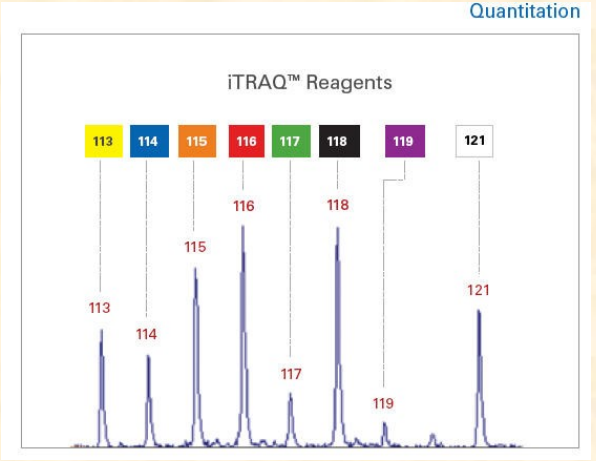


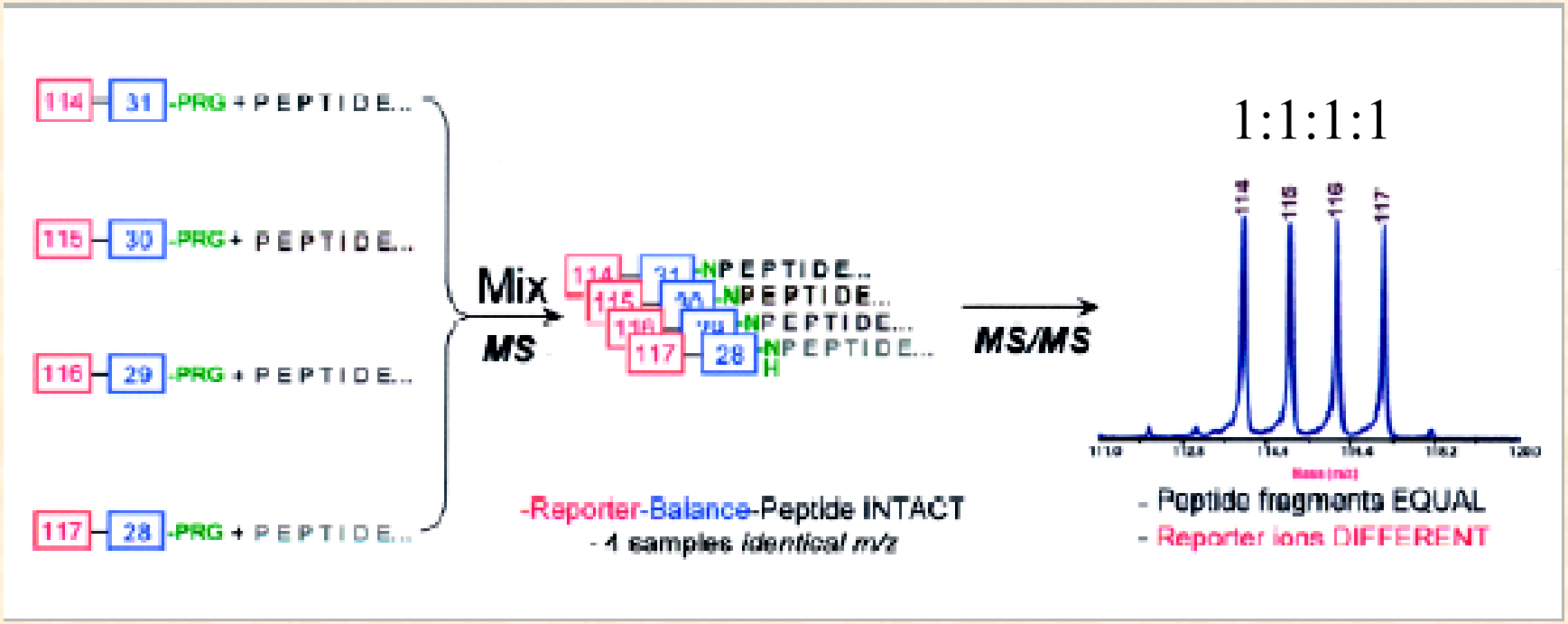

Fig. 2 Mass resolution of five-plex isotopically labeled peptides. MS spectra of Lys-C digested peptides with different charge states (+1, +2, +3 and +4) (1 : 1 : 1 : 1 : 1 ratio).

*Wu et al., Chem. Commun. 2014*

# iTRAQ

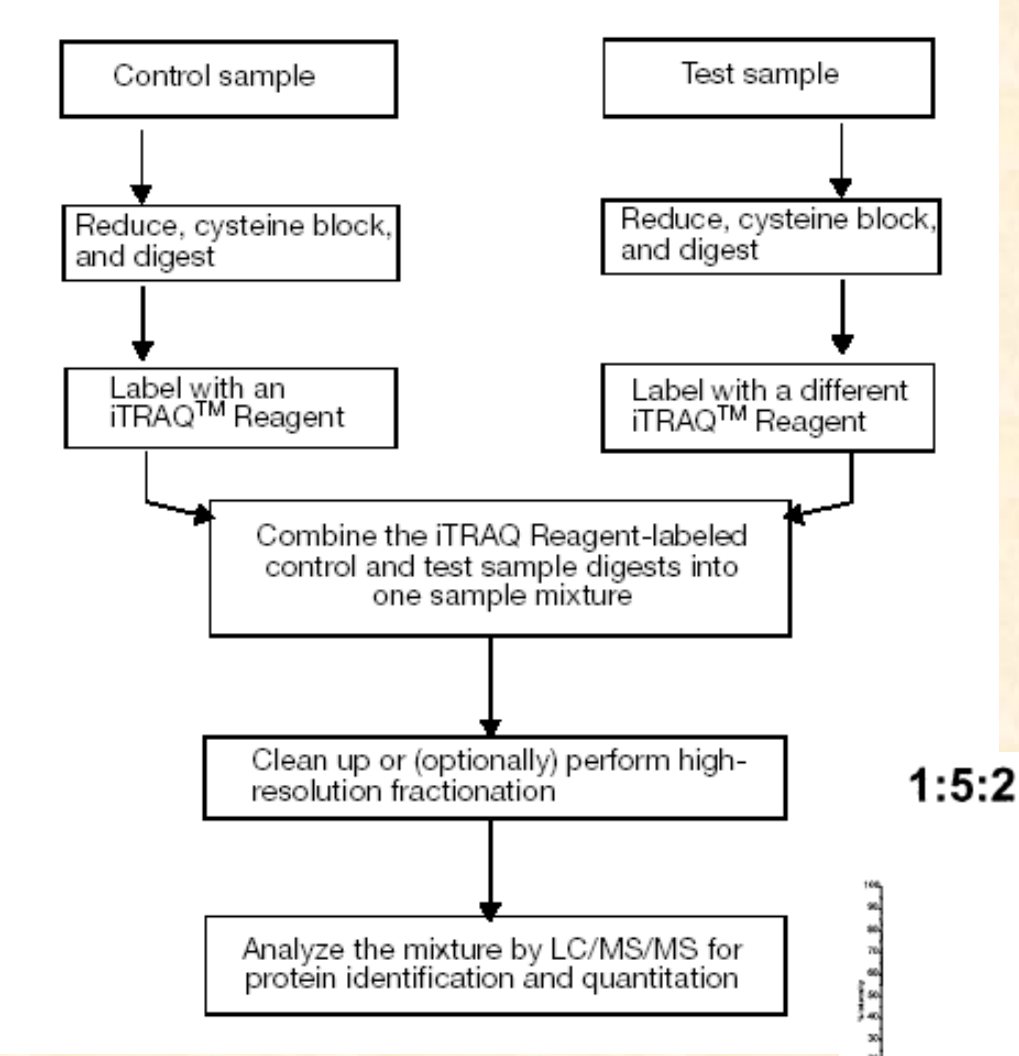


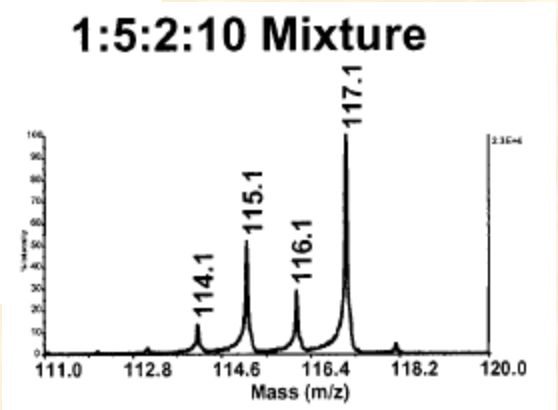

- isobarické značky (4, 8), přednostně na Lys
- označené vzorky při LC separaci a v MS stejné chování
- kvantifikace na základě reporterových iontů po MS/MS




*Applied Biosystems/Sciex*

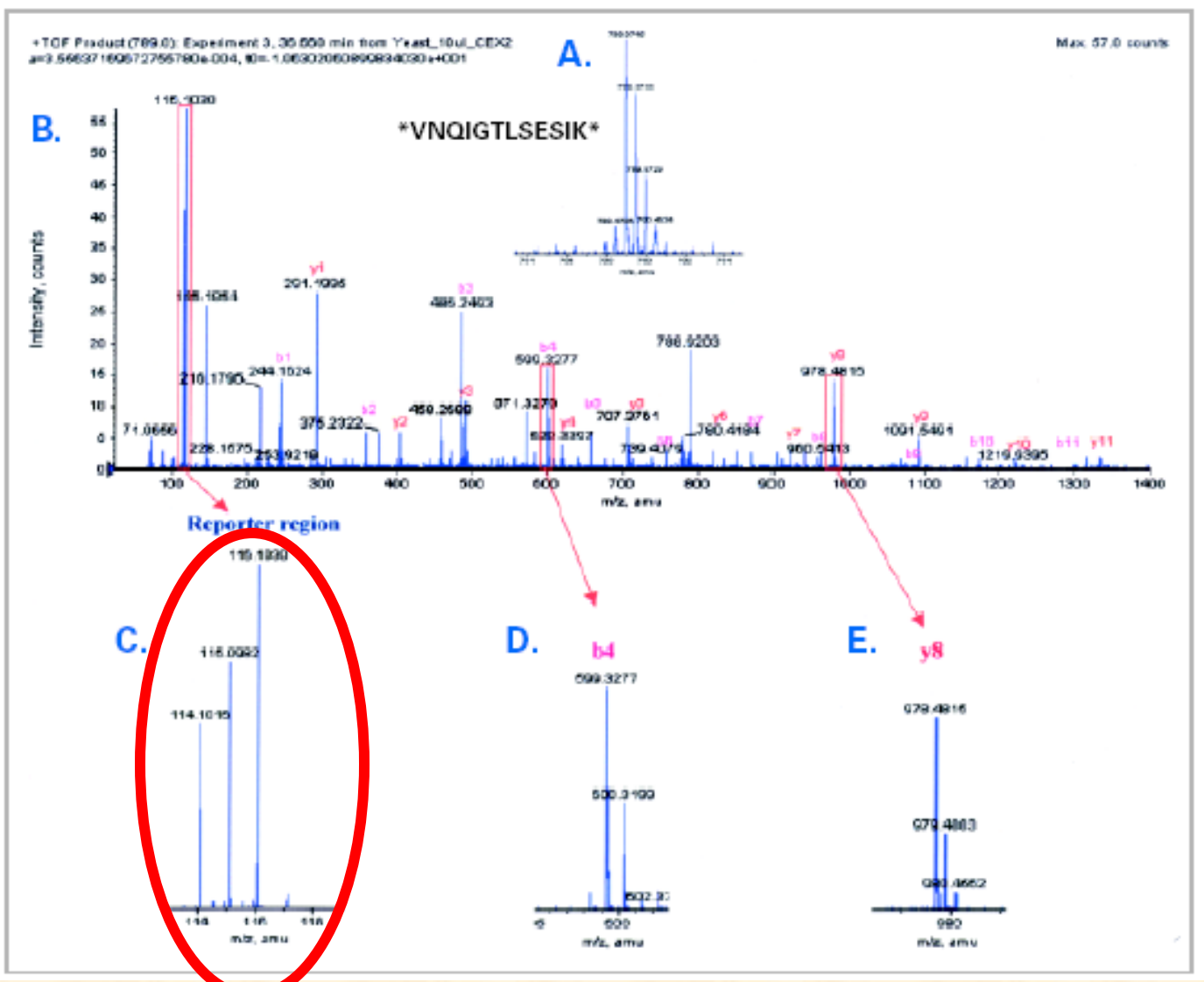
# iTRAQ




*Applied Biosystems/Sciex*

# iTRAQ




*Applied Biosystems/Sciex*

# iTRAQ

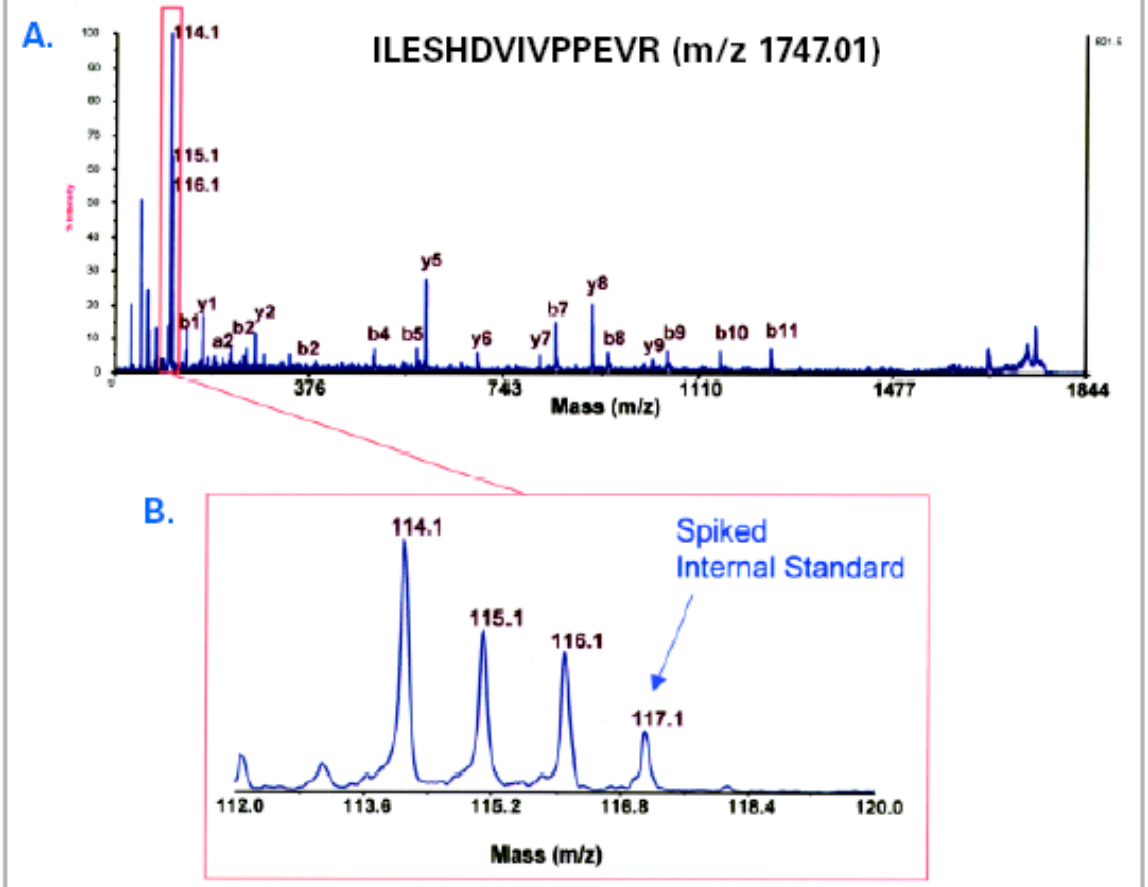
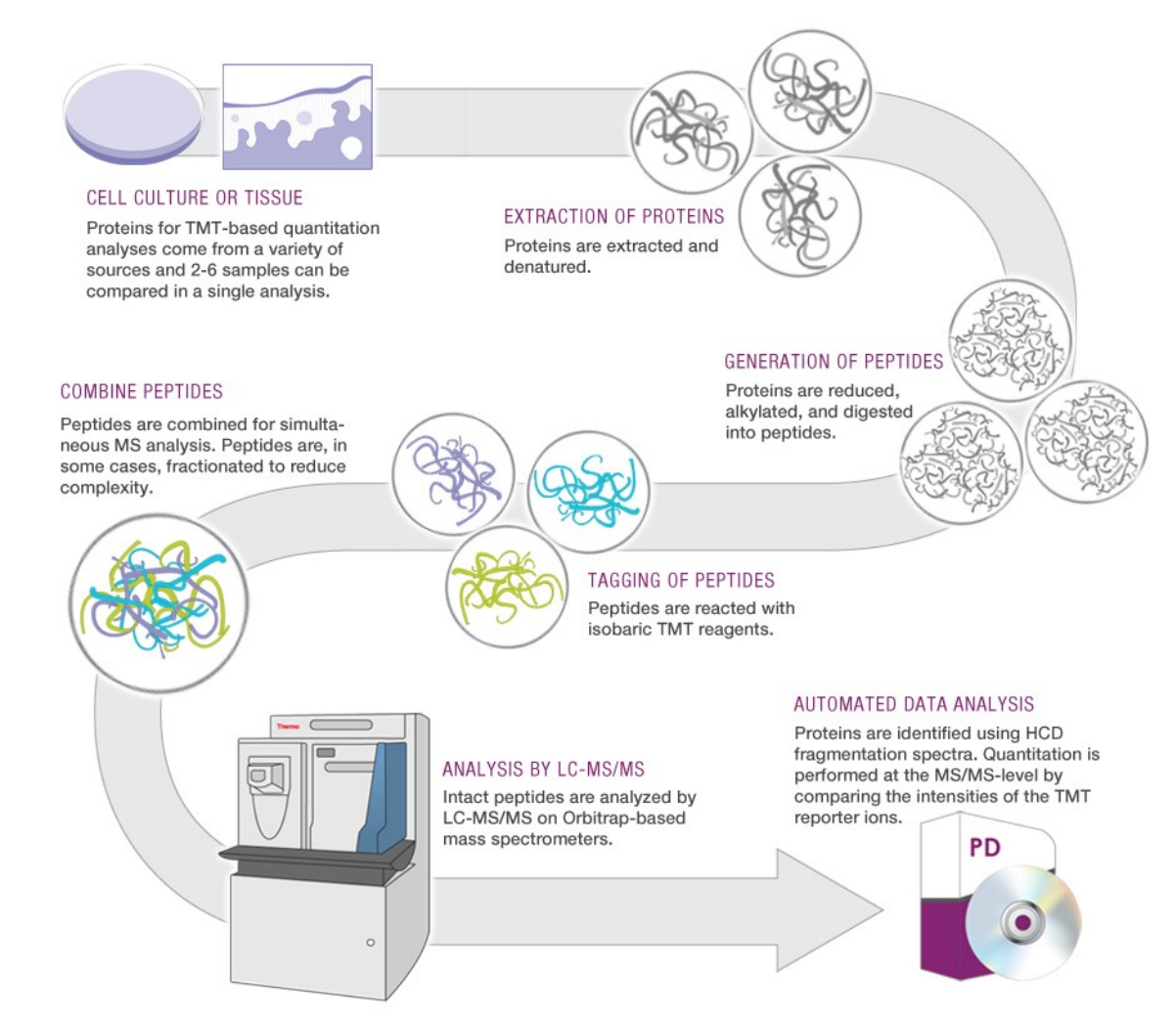



**Figure 8.** Identification and Quantitation of iTRAQ™ Reagent labeled peptide, ILESHDVIVPPEVR, from Carbamoyl-phosphate synthetase, which is up-regulated in both Xrn1Δ and Upf1Δ mutants. Illustrated in panel A is the TOF MS Spectrum and the reporter ion region is expanded in panel B. The diagnostic reporter ions of 114.1, 115.1, and 116.1 are those for the Xrn1Δ, Upf1Δ and wild-type *S. cerevisiae* strains, respectively. The 117.1 peak is from a specific amount of spiked-in synthetic peptide idenpently labeled with the iTRAQ Reagent 117.

*Applied Biosystems/Sciex*

# TMT značky

*Tandem Mass Tags*

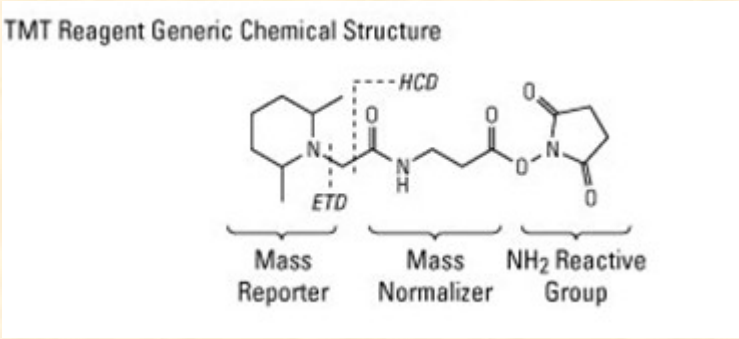



*from http://planetorbitrap.com*

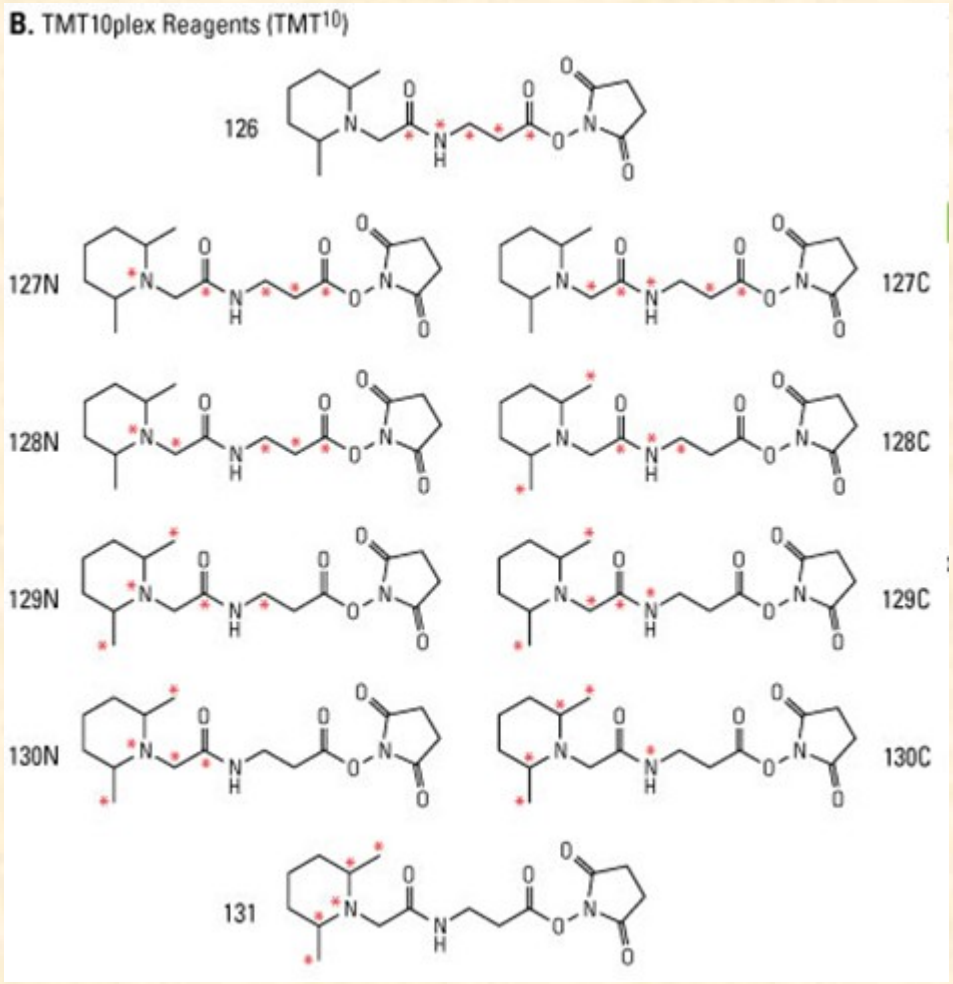
# TMT

C7250

- Isobarické značky (až 16-plex)
- MS/MS




lysine labeling




- Cysteine-reactive mass tags (6-plex)

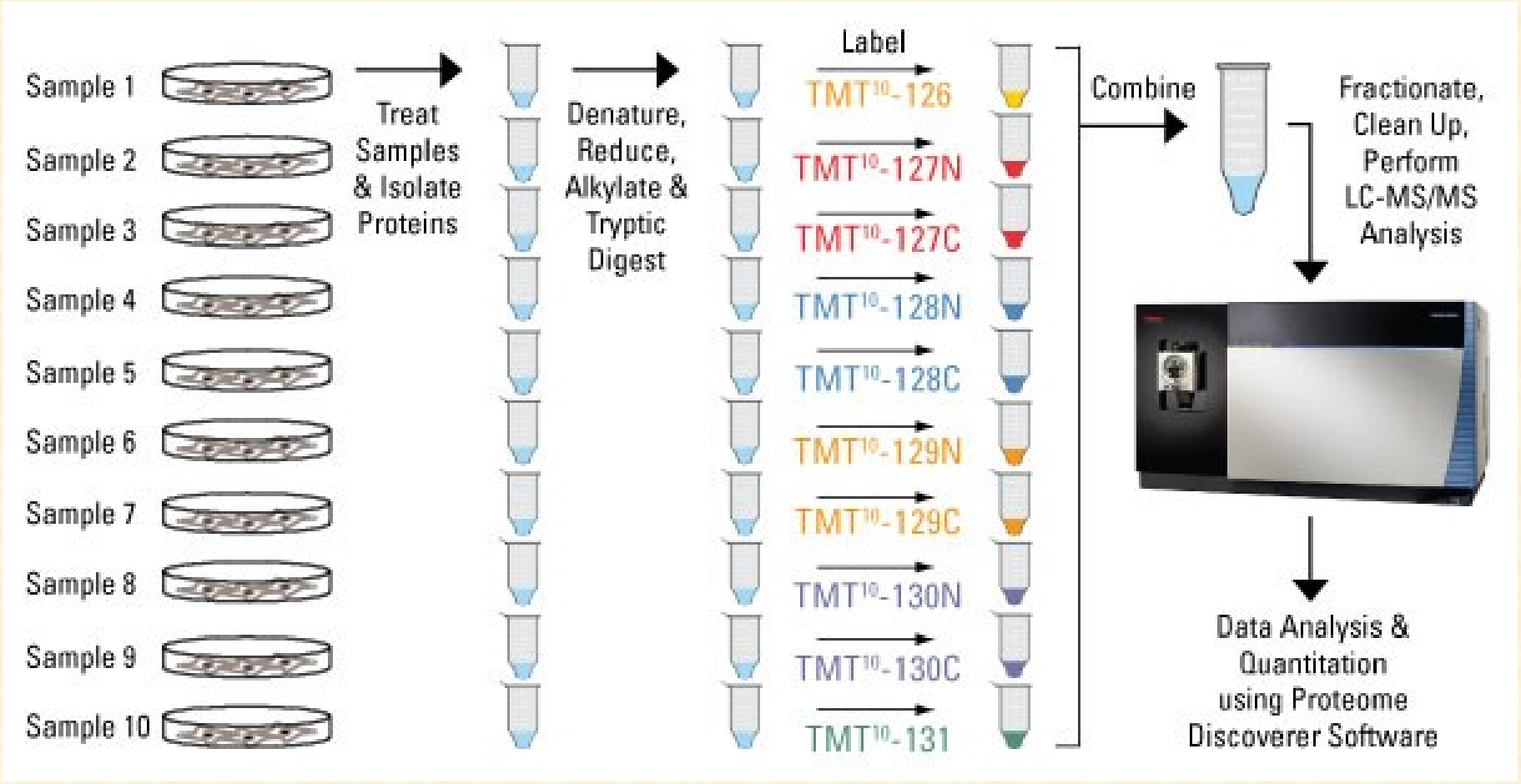
  *quantitation of the relative abundance of cysteine modifications, such as S-nitrosylation, oxidation and disulfide bonds*

- Carbonyl-reactive mass tags (6-plex)

  *glycan, steroids, or oxidized proteins quantification*

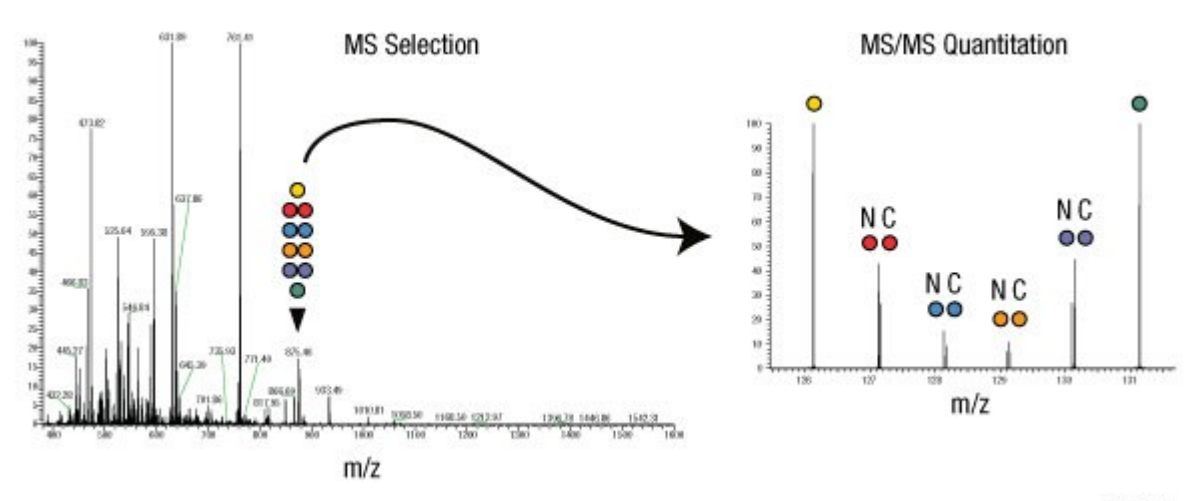
*Thermo Fischer Scientific*

# TMT

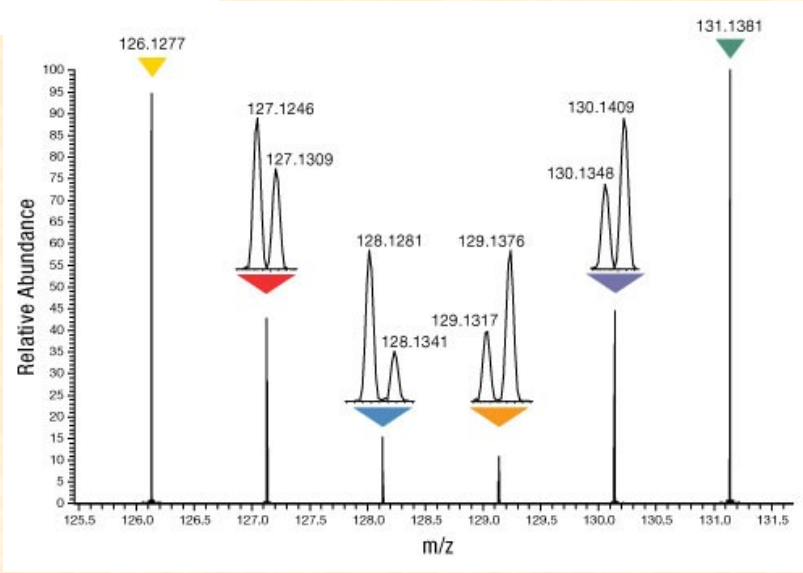



*Thermo Fischer Scientific*

# TMT



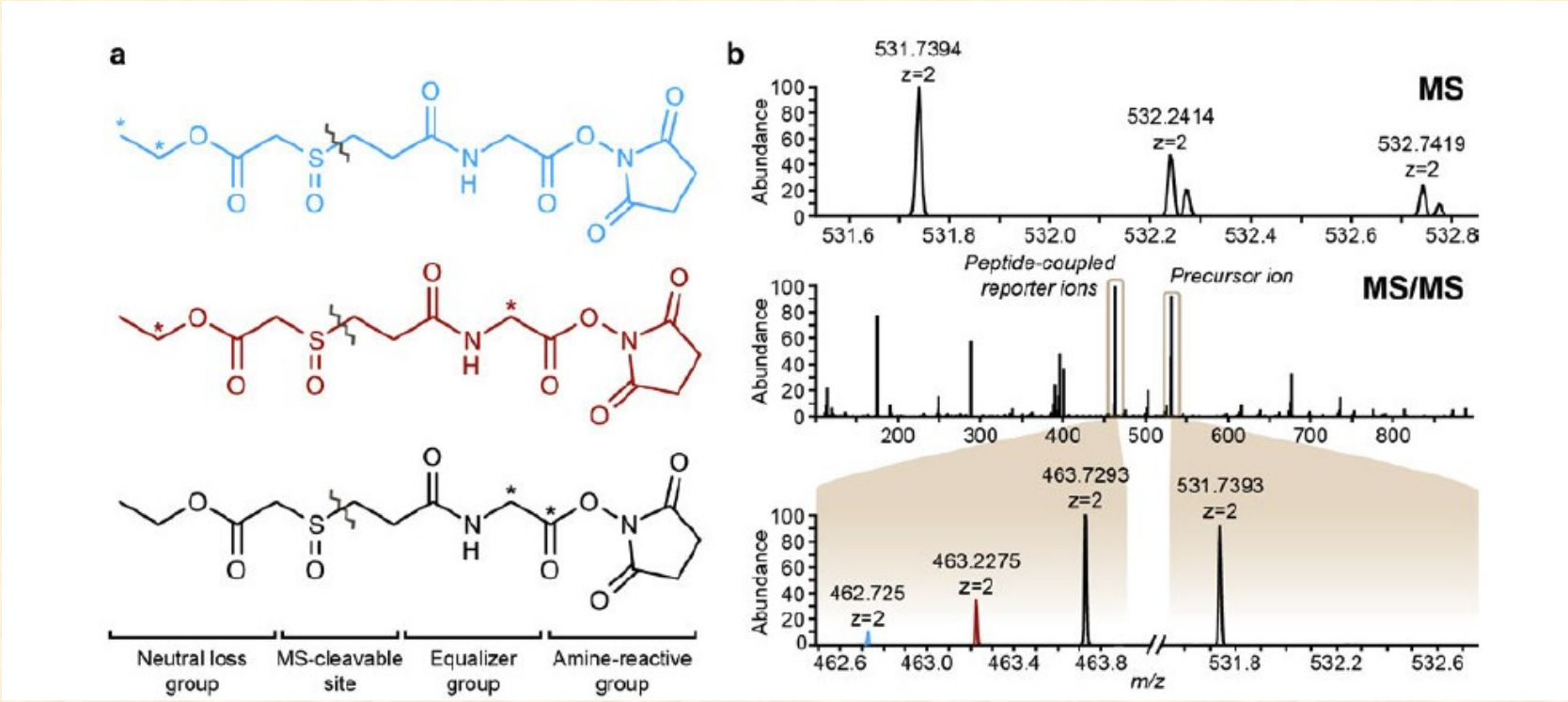

Reagents contain different numbers and combinations of 13C and 15N isotopes in the mass reporter. The different isotopes result in a 10-plex set of tags that have mass differences in the reporter that can be detected using **high resolution** Orbitrap MS instruments.




*Thermo Fischer Scientific*

# EASI-tag

## Easily Abstractable Sulfoxide-based Isobaric tag

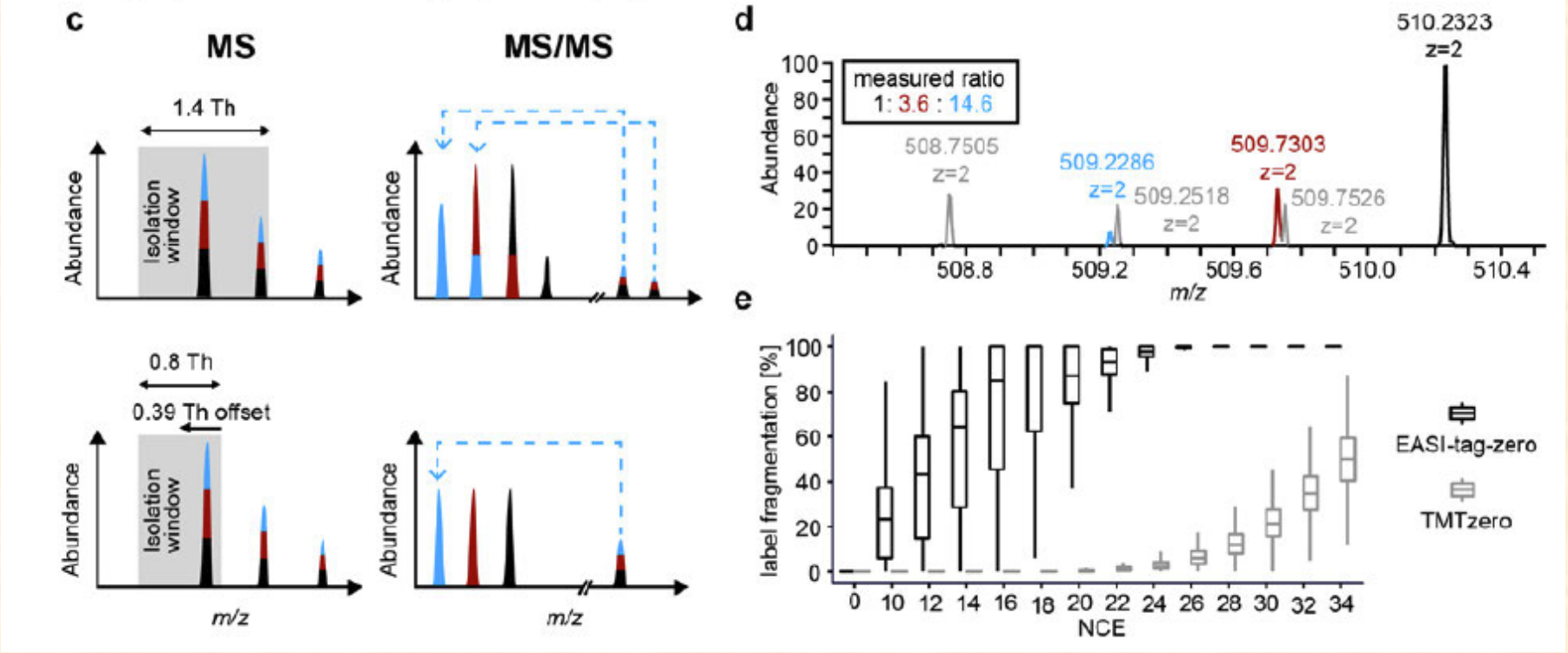



(**a**) Molecular structures of the triplex version of EASI-tag. The isobaric labeling reagents are composed in a modular way of **four functional groups** and feature a central sulfoxide moiety, which introduces an asymmetric, low-energy cleavage site (zig-zag lines indicate fragmentation site). The stable-isotope labeled positions of the neutral loss and equalizer group for multiplexing are indicated by asterisks. Standard labeling protocols can be applied to couple peptides via **the amine-reactive moiety**.
(**b**) Mass spectra of an EASI-tag-labeled yeast peptide mixed in a ratio of 1:3:10. HCD fragmentation of the doubly charged precursor ion abstracts the neutral loss group and yields **the peptide-coupled reporter ion cluster**.

*bioRxiv preprint first posted online Nov. 27, 2017; doi: http://dx.doi.org/10.1101/225649*

# EASI-tag

## Easily Abstractable Sulfoxide-based Isobaric tag




(**c**) Co-isolation of the natural isotope cluster in a standard isolation window centered on the precursor ion (upper panel) convolutes the relative abundance of peptide coupled reporter ions. An asymmetric isolation window (lower panel) that suppresses the signal from adjacent isotope peaks and enables direct quantification of reporter ions.
(**d**) The precursor mass information is retained in the peptide-coupled reporter ions for EASI-tag labeled peptides. Colored peaks indicate the peptide-coupled reporter ions from an identified yeast peptide in a two proteome experiment (mixing ratios: 1:3:10 for yeast & 1:1:1 for human). Grey peaks are peptide-coupled reporter ions from a co-isolated peptide.
(**e**) EASI-tag- and TMT-labeled HeLa peptides were fragmented with normalized collision energies between 10 and 34. (N = 17,565 precursors for EASI-tag & 20,610 for TMT)

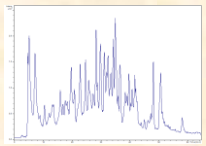
*bioRxiv preprint first posted online Nov. 27, 2017; doi: http://dx.doi.org/10.1101/225649*

# Label – free přístup

- bez značek
- vzorky měřeny jednotlivě
- možnost srovnání „neomezeného“ počtu vzorků

Identifikace na základě MS/MS dat, přiřazení identity jednotlivým signálům

LC-MS/MS



odstranění šumu
detekce píků/údolí
výpočet intenzity/plochy
jednotlivých píků

normalizace
imputace chybějících hodnot

výpočet poměrů
jednotlivých proteinů
mezi vzorky

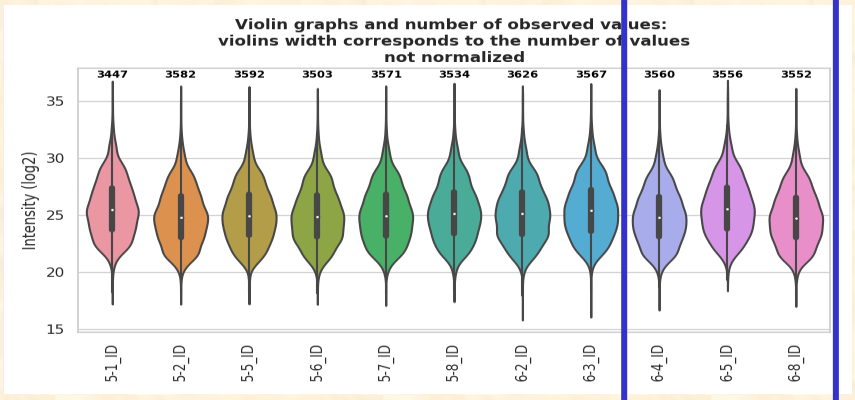
statistické testování

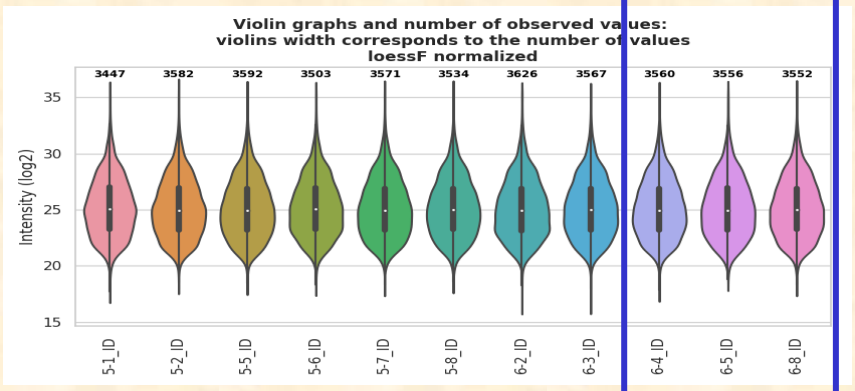
**signifikantní změny**
**v proteomu**

kritické kroky:

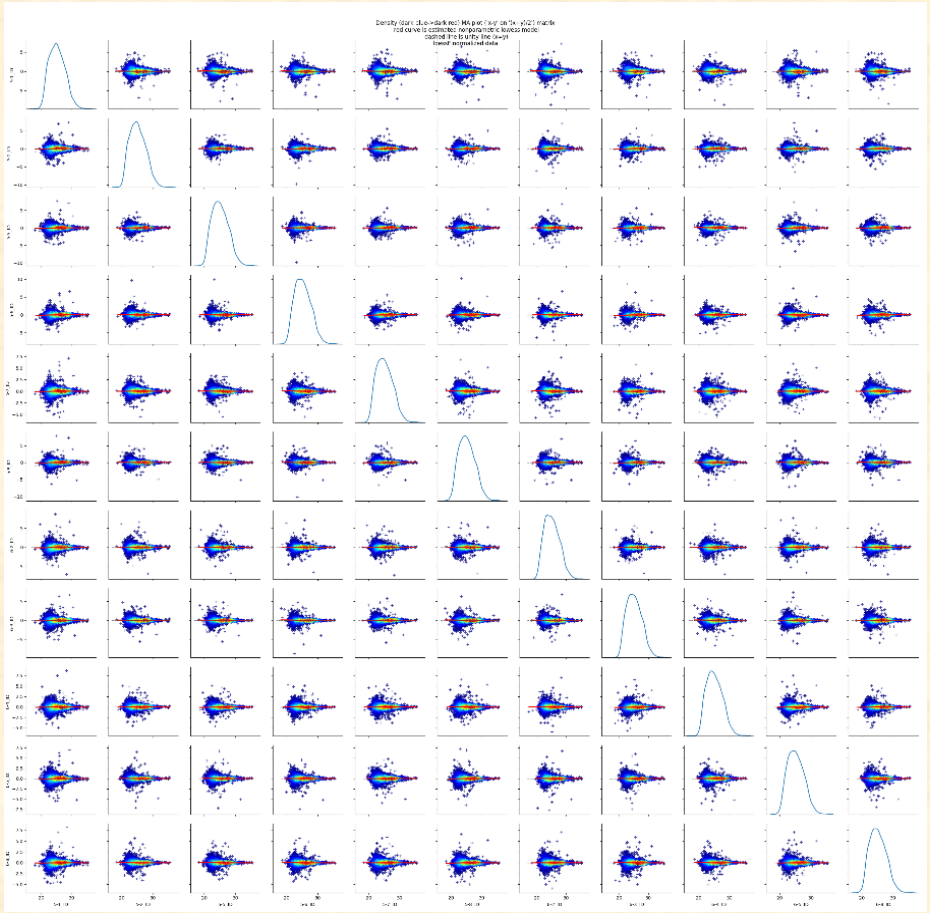
- normalizace dat
- imputace chybějících hodnot (DIA – redukce počtu chybějících hodnot)

# Label – free přístup

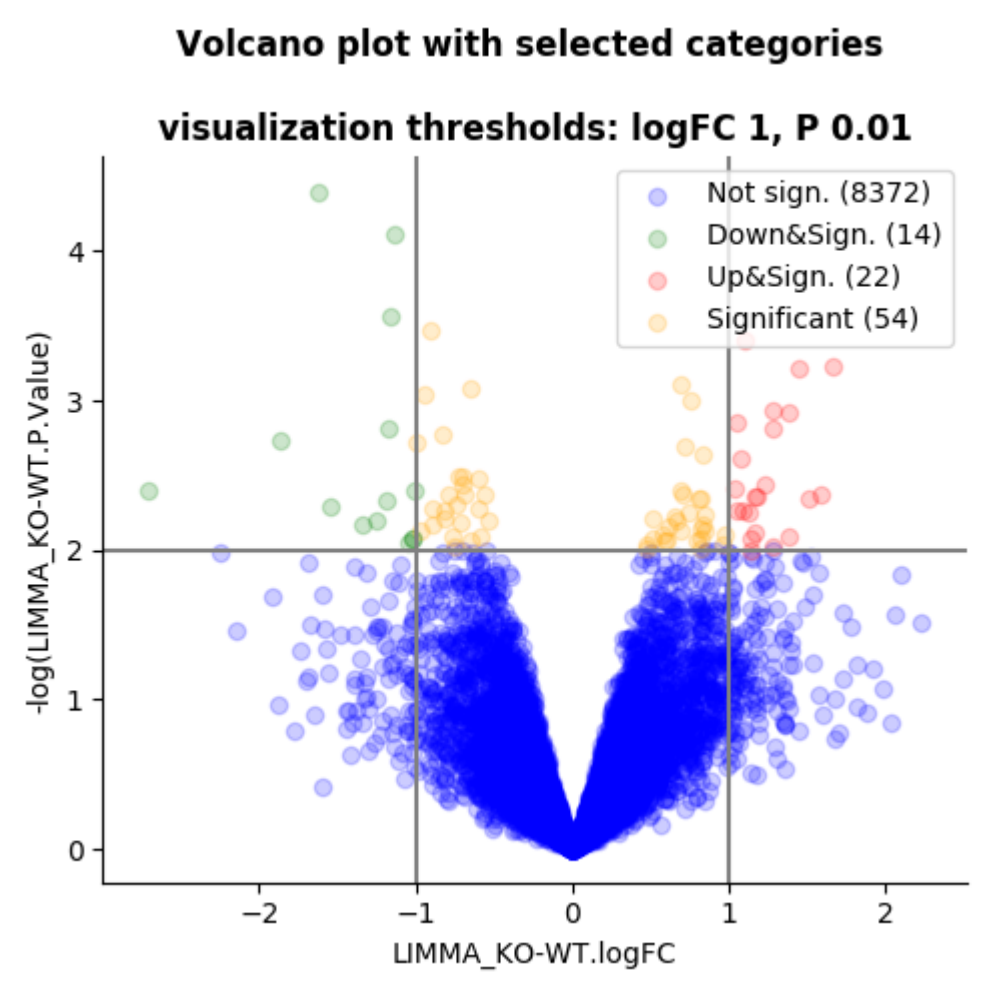


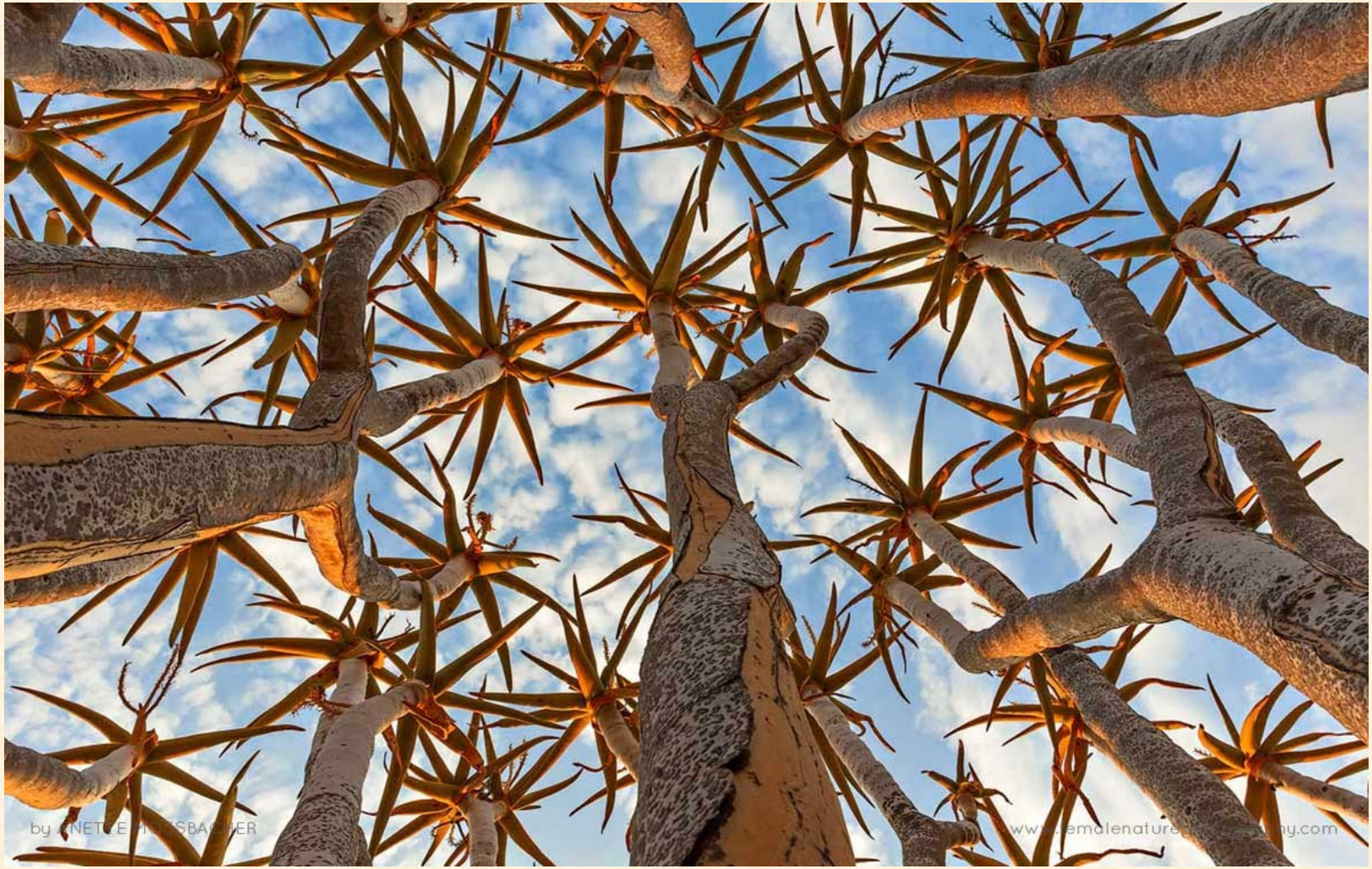




MA plots - dependence of (x-y) on (x+y)/2



# Label – free přístup

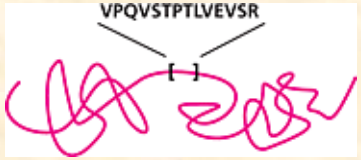




# Absolutní kvantifikace AQUA

- **AQUA Peptide Selection**

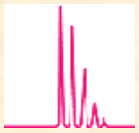
Select an optimal tryptic peptide and stable isotope amino acid from the sequence of your protein of interest




- **Order selected peptide labeled ($^{15}N$, $^{13}C$)**

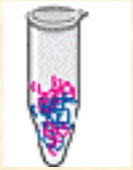
Price !!!

Optimize LC-MS/MS separation protocol for quantitation



- **Adding labeled peptide to protein mix**

- **Digest**



- **Analyze by LC-MS/MS to quantitate protein of interest**



jen pro vybraný protein

# Absolutní kvantifikace

## AQUA peptidy

syntéza
izotopicky označených
proteotypických peptidů

přidání známého množství
do vzorku

digesce

MS analýza

## QconCAT

konstrukce umělého genu
složeného z
proteotypických peptidů až
20 proteinů

exprese v E.coli na
značeném mediu

purifikace umělého proteinu

přidání známého množství
do vzorku

digesce

MS analýza

*Rivers at al., MCP 6, 1416 (2007)*

## PSAQ

exprese
izotopicky označeného
proteinu (s tagem pro
purifikaci)

purifikace proteinu

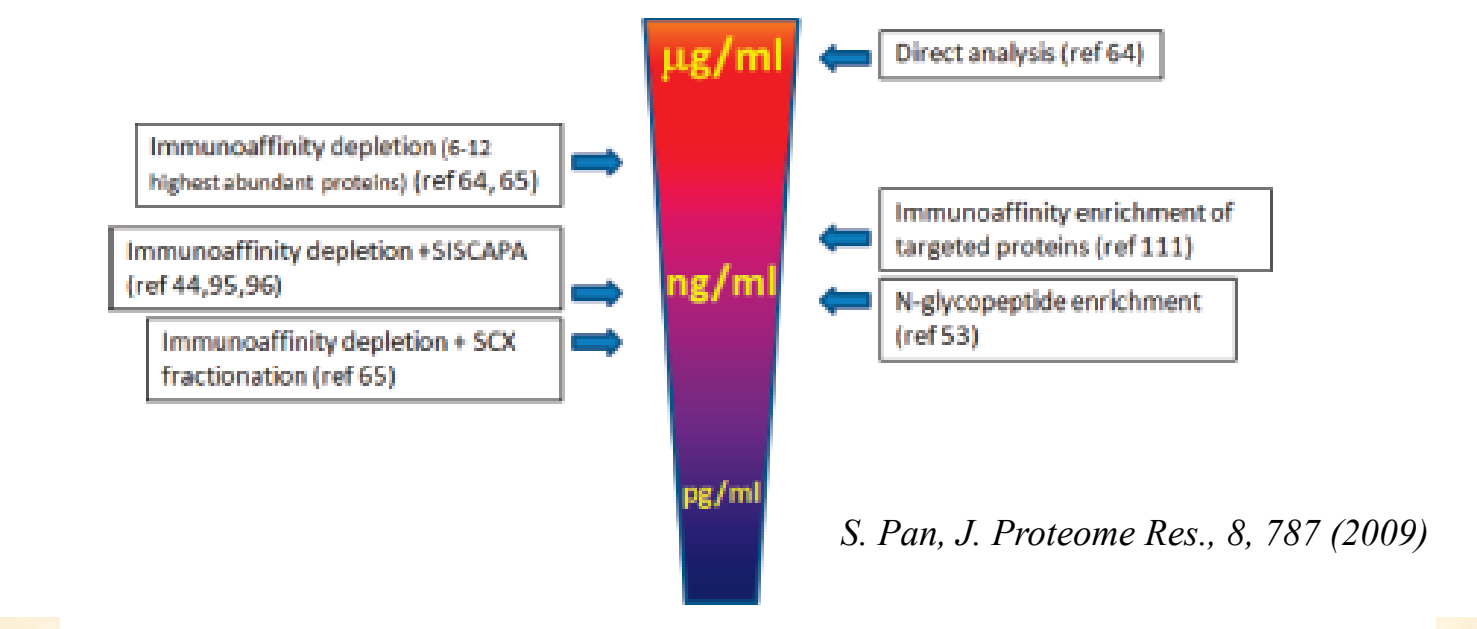
přidání známého množství
do vzorku

digesce

MS analýza

*Brun et al., MCP 6, 2139 (2007)*

# Absolutní kvantifikace
# Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA)




*S. Pan, J. Proteome Res., 8, 787 (2009)*

**Table 1.** Plasma Protein Targets

| protein identity | Swiss-Prot accession no. | protein mass (daltons) | normal range concentration in human serum |
|---|---|---|---|
| interleukin-6 (IL-6) | P05231 | 23 718 | <10 pg/mL |
| hemopexin (hx) | P02790 | 51 676 | 0.5−1.15 mg/mL |
| $\alpha_1$−antichymotrypsin (AAC) | P01011 | 47 650 | 0.3−0.6 mg/mL |
| tumor necrosis factor-α (TNF-α) | P01375 | 25 644 | <10 pg/mL |

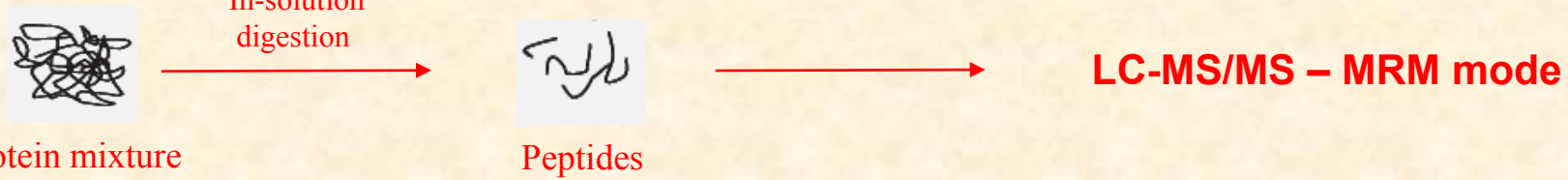
*N.L. Anderson, J. Proteome Res., 3, 235 (2004)*

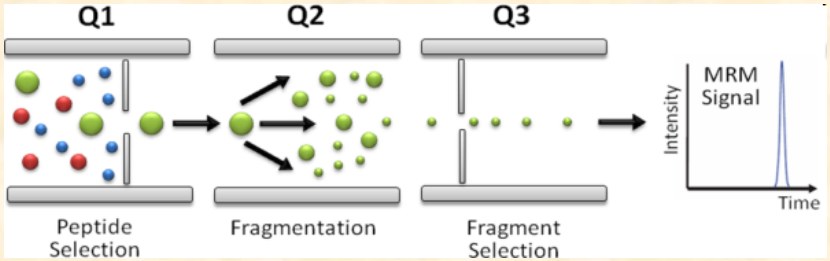
# Cílená MS/MS analýza vybraných proteinů
relativní /absolutní kvantifikace
**multiple reaction monitoring (MRM)**

screening – výběr kandidátního proteinu

příprava metody (výběr MRM přechodů – peptid + vybraný fragment)

vlastní analýza a zpracování dat

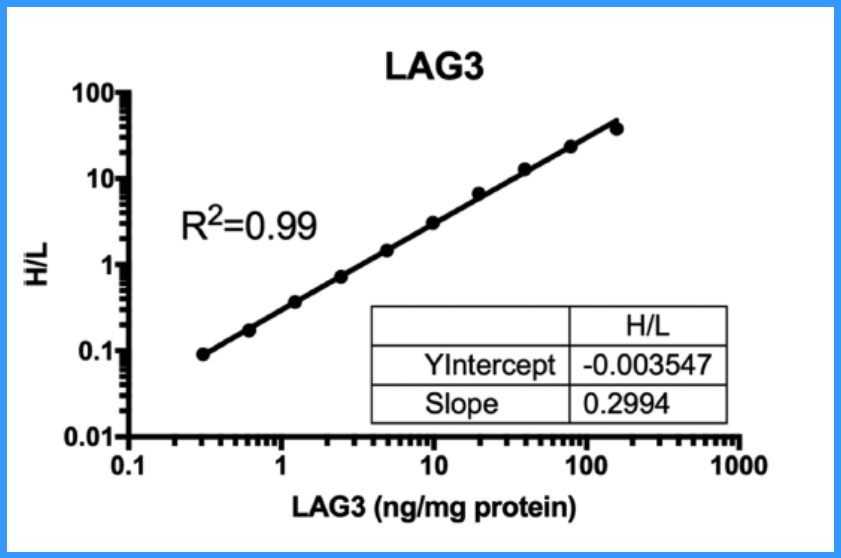



*http://www.srmatlas.org*

# Multiplex Immuno-Liquid Chromatography–Mass Spectrometry–Parallel Reaction Monitoring (LC–MS–PRM)

Quantitation of immune markers CD8A, CD4, LAG3, PD1, PD-L1, and PD-L2 in Frozen Human Tissues



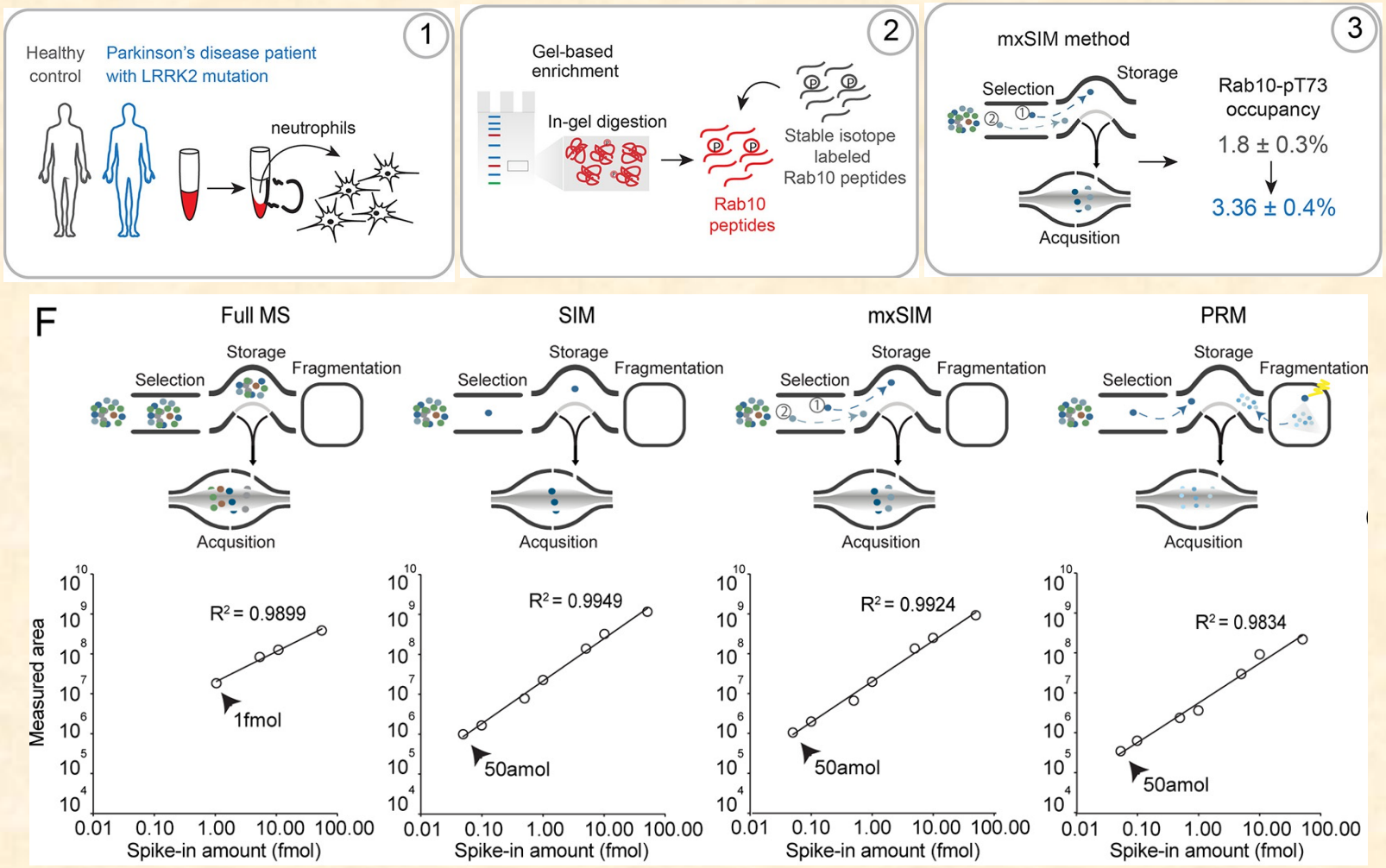

| protein | MW (kDa) | monitored peptide | parent $m/z$ | fragment $m/z$ | HCD energy | LLOQ (ng/mg total protein) |
|---|---|---|---|---|---|---|
| LAG3 | 57.4 | FVWSSLDTPSQR | 711.85 | 990.48 (y9), 588.31 (y5) | 23 | 0.15 |

*Zhang et al., J. Proteome Res. 2018, 17, 3932−3940*

# Accurate MS-based Rab10 Phosphorylation Stoichiometry

*Karayel et al., Mol Cell Proteomics (2020) 19(9) 1546–1560*

assay to measure increased phospho Rab levels using synthetic stable isotope-labeled analogues for both phosphorylated and non-phosphorylated tryptic peptides surrounding Rab10-Thr73




Limit of detection (LOD) of SIL Rab10-pThr73 tryptic peptide (FHpTITTSYYR) with various acquisition methods; full MS, SIM, mxSIM and PRM.

# Absolute Quantification of over 1800 Yeast Proteins

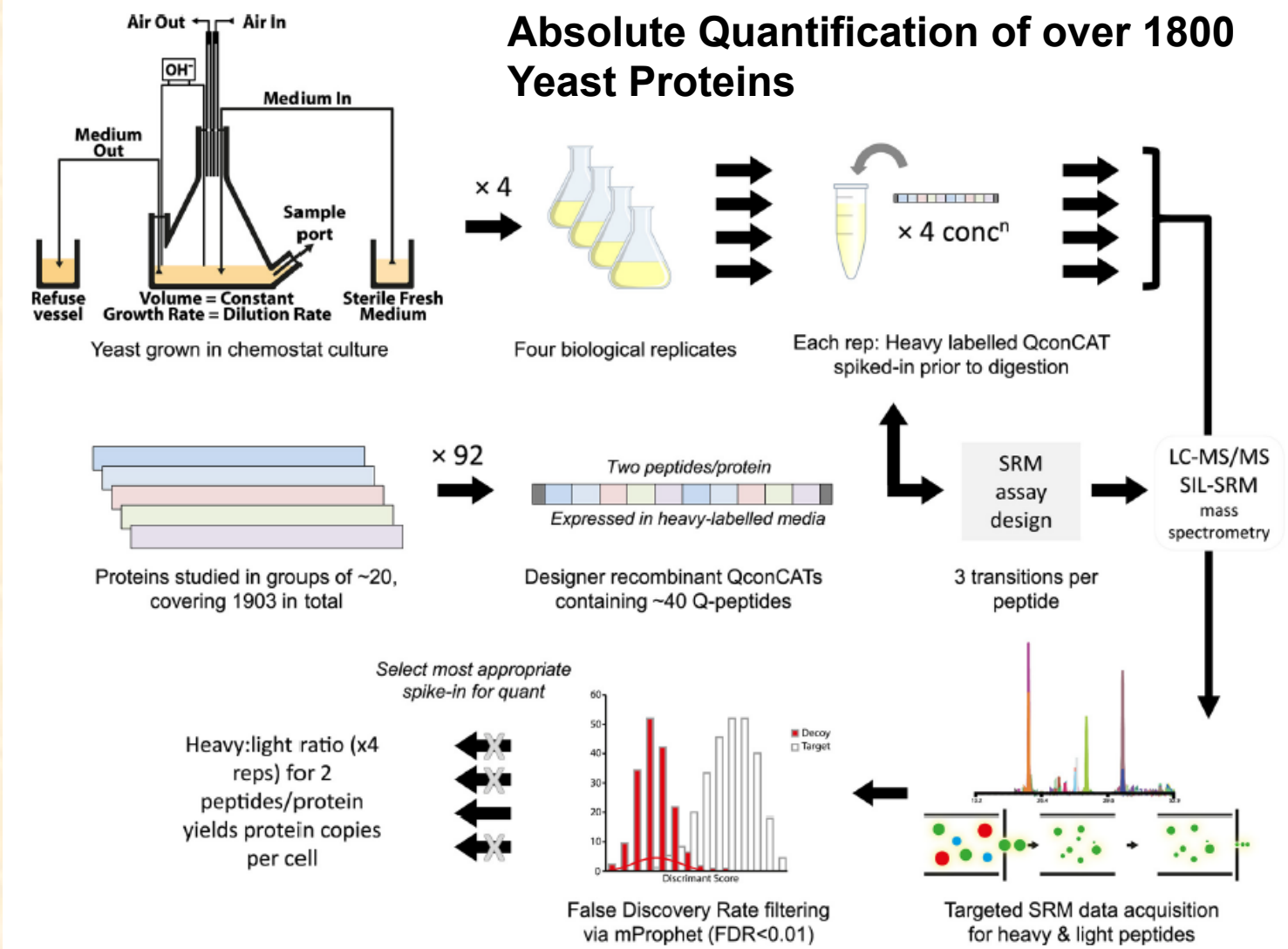



FIG. 1. **Schematic overview of QconCAT-based quantification of the yeast proteome using SIL-SRM methodology.** The experimental workflow is depicted in schematic form, showing how chemostat grown yeast samples are extracted, using four biological replicates, for analysis. These samples were combined with designer QconCAT proteins, containing surrogate quantotypic peptides, expressed in a stable-isotope labeled media. SRM assays, designed using a digest of the expressed QconCATs to generate Q-peptides, were then used to quantify the parent proteins. Mixtures of purified QconCAT and yeast proteins were mixed at four concentrations (one of which contained yeast but no QconCAT) and analyzed by SRM-MS to yield SRM chromatogram peak groups for both light (endogenous yeast) and heavy (Q-) peptides. Subsequent quality control by signal:noise cutoffs and mProphet FDR (estimated from decoy transitions) yielded peptide-level copies per cell values, which were then integrated to the protein level for a final quantification.

*Lawless C., MCP,15, 1309–1322, 2016.*

Konec V. části