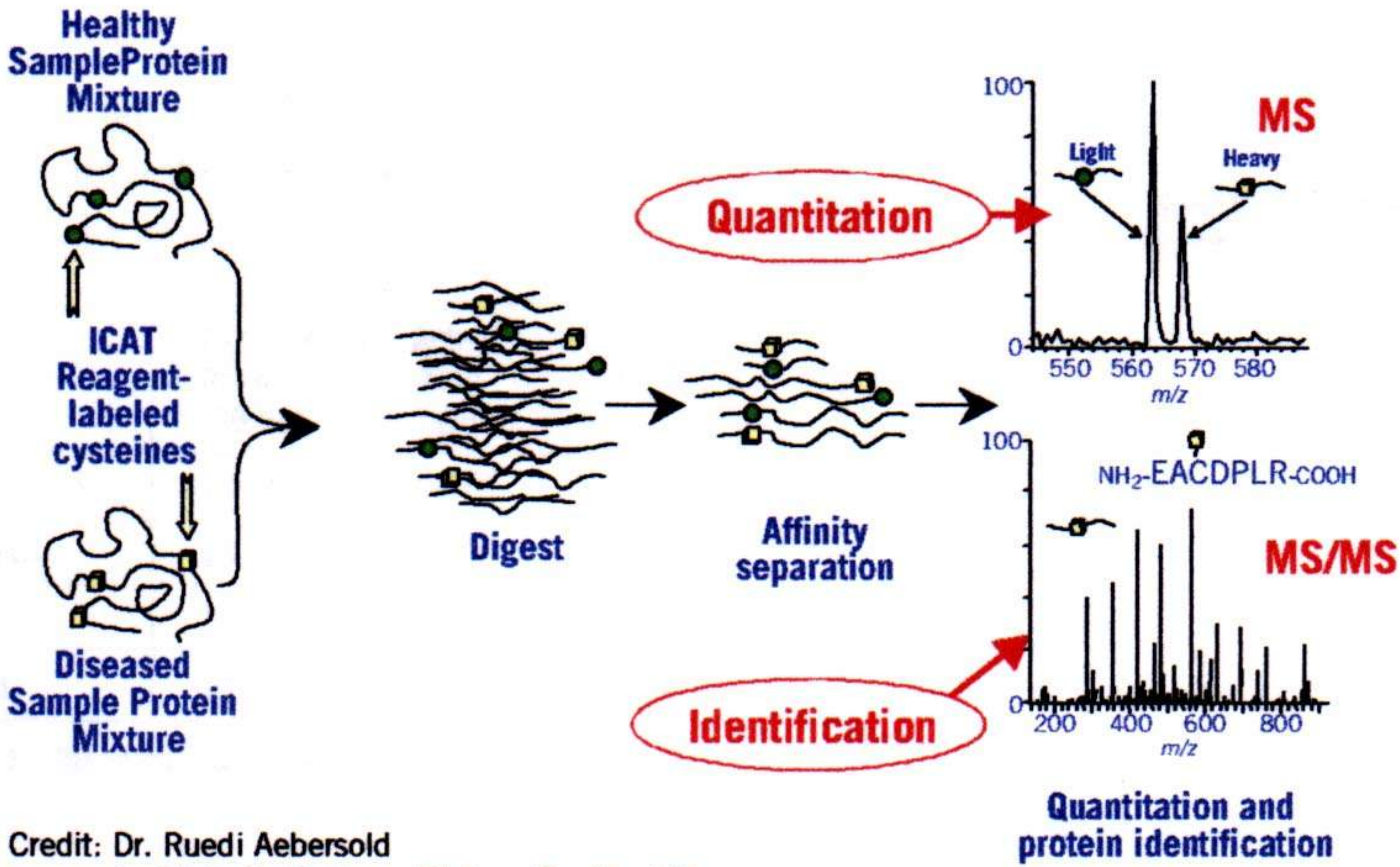
# Quantitation Tools

## Introduction to some differential proteomics analysis

# Hacia una "Proteómica de Segunda Generación" (II): ICAT (*isotope-coded affinity tags*) (Aebersold y cols.)

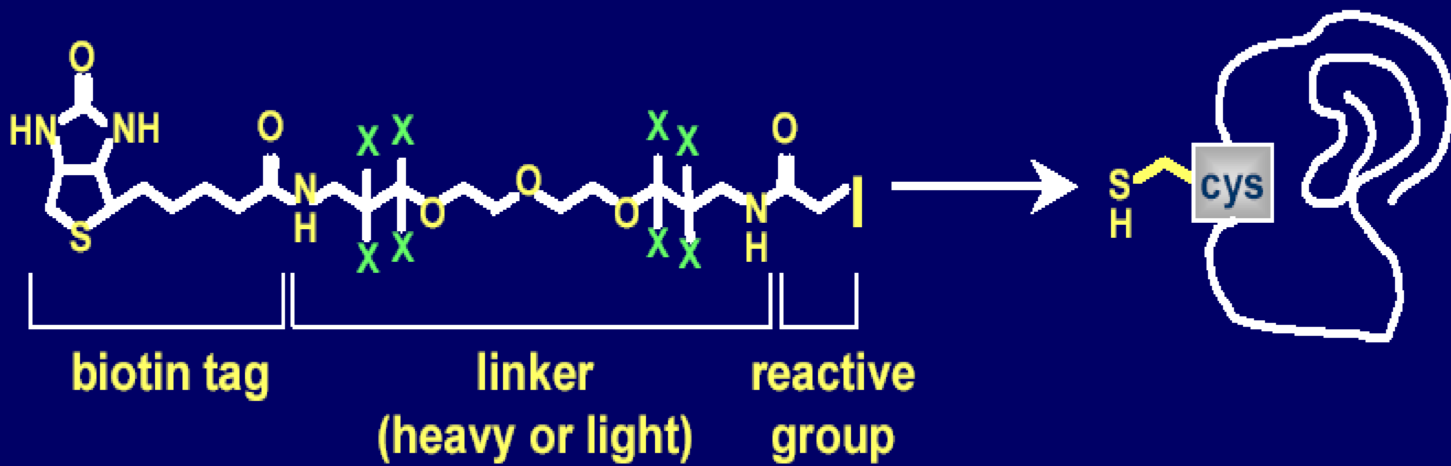



Credit: Dr. Ruedi Aebersold
Institute for Systems Biology, Seattle, WA

# ICAT

## Quantitation - ICAT™ technology

heavy reagent: d8-ICAT (X=deuterium)
light reagent: d0-ICAT (X=hydrogen)




Gygi, Rist et al. 1999, *Nature Biotechnol.* **17**: 994

# ICAT

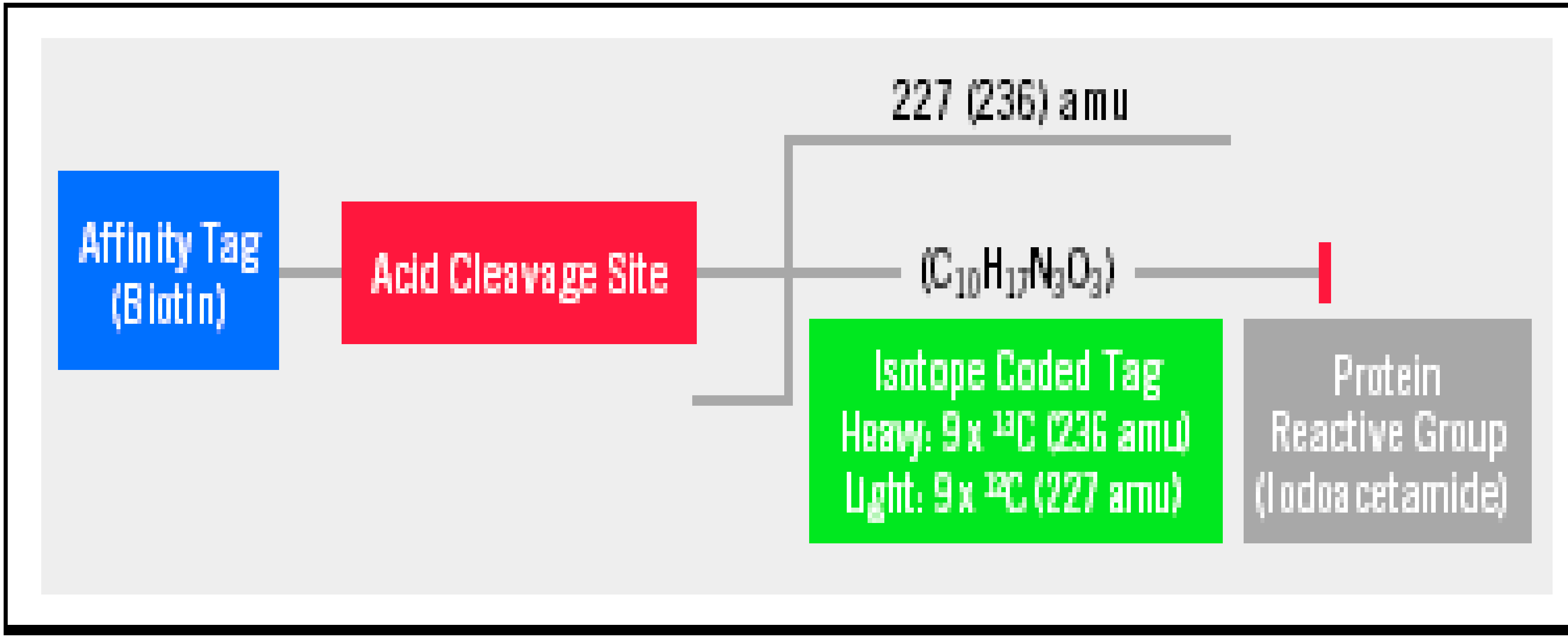


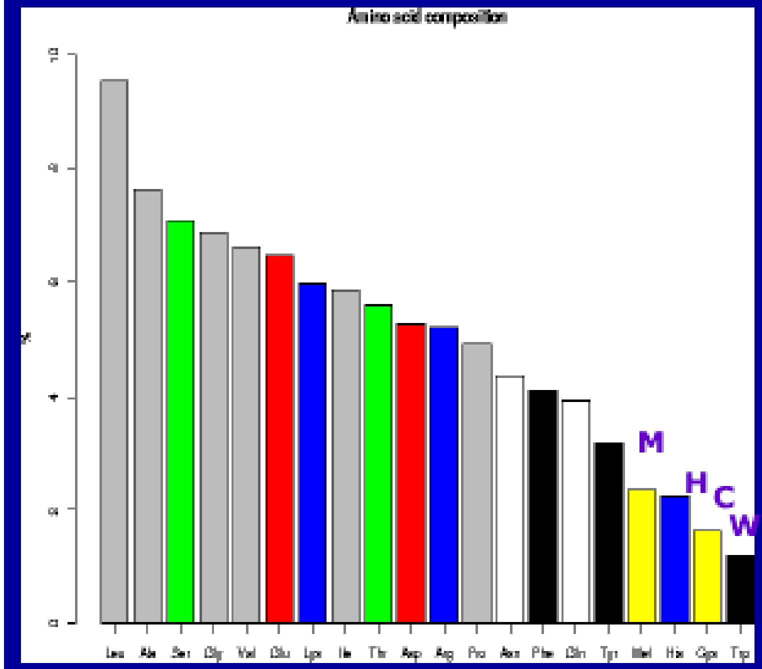

Figure 1. Cleavable ICAT™ Reagent Structure.

## ICAT Assumptions

- All/most proteins have at least 1 cys residue.
- Pairs of ICAT peptides tagged with heavy and light ICAT reagent are chemically identical and serve as mutual internal standards for quantification.
- The ICAT light and heavy labels contribute no difference in elution times of the tagged Cys peptides.
- The entire sample must be analyzed everytime to identify proteins which have undergone quantitative changes.
- Pair-wise comparison of samples.

# ICAT

## Frequency of Amino Acids In Different Species




| | *E.Coli* | Human | Yeast |
|---|---|---|---|
| % of Proteins lacking Met | 0.33 | 2.02 | 0.9 |
| % of Proteins lacking Cys | 14.56 | 4.42 | 8.69 |
| % of Proteins lacking His | 4.12 | 3.8 | 2.55 |
| % of Protein lacking Trp | 11.34 | 8.74 | 10.32 |

Classification of the amino acids by their frequency

Leu, Ala, Ser, Gly, Val, Glu, Lys, Ile, Thr, Asp,

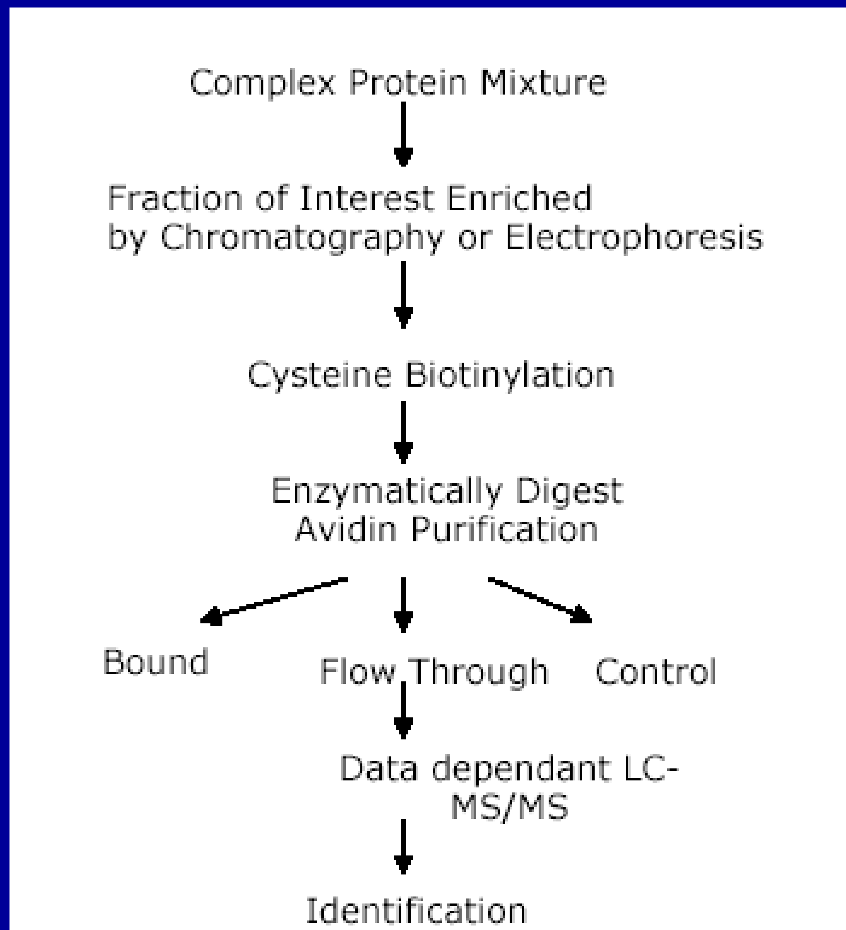
Arg, Pro, Asn, Phe, Gln, Tyr, Met, His, Cys, Trp

J. Vandekerckhove et al.
in Proteomic Forum 2001

Source: http://www.expasy.ch/sprot/relnotes/relstat.html

# ICAT

## Reducing Complexity In Discovery Proteomics Analyzing Cys Peptides in A Mixture




Spahr CS, Susin SA, Bures EJ, Robinson JH, Davis MT, McGinley MD, Kroemer G, Patterson SD. Simplification of complex peptide mixtures for proteomic analysis: reversible biotinylation of cysteinyl peptides.

## ICAT Reagent Technology Benefits

- *Identifies and quantifies important membrane and low-abundance proteins.*
- *Allows relative protein quantitation as well as identification using a mass spectrometer.*
- *Reduces sample complexity by selecting for cysteine-containing peptides, leading to a deeper analysis of a proteome.*

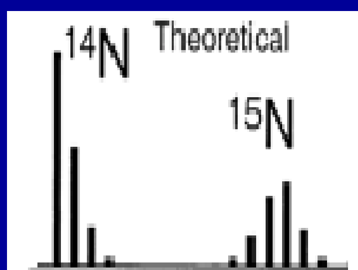
# Stable Isotope Dilution Method

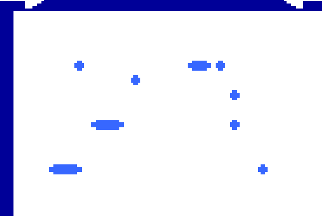
Cell State A → Standard Media (light)

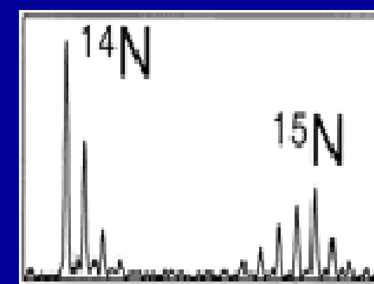
Cell State B → $^{15}$N-enriched Media (heavy)

Harvest/Sample Prep

Combine then 2DE






Excision/Digestion

MALDI-MS of each spot

Identification
Quantitation by Measuring Peak Ratios.

Oda Y, Huang K, Cross FR, Cowburn D, Chait BT. Accurate quantitation of protein expression and site-specific phosphorylation.
Proc Natl Acad Sci U S A. 1999 Jun 8;96(12):6591-6.

# SILAC

## Stable Isotope Dilution Method with Cys Affinity Tags and Metabolic Labeling

Cell State A → Standard Media (light)

Cell State B → $^{15}$N-enriched Media (heavy)

Combine then Biotinylate Cys residues.

↓

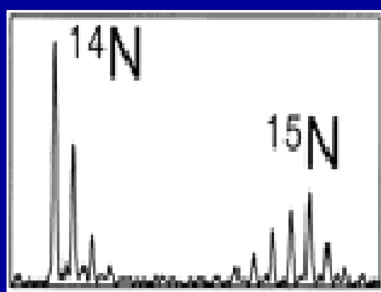
LC-MS of Cys Peptides with FTICR

↓

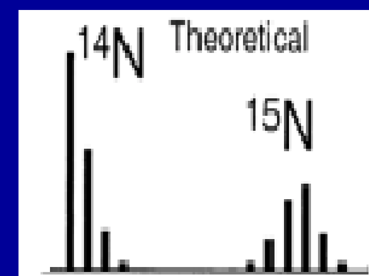
Identify Isotopic Pairs

↓

Quantitate and Identify with High Mass Accuracy
MS/MS performed if needed



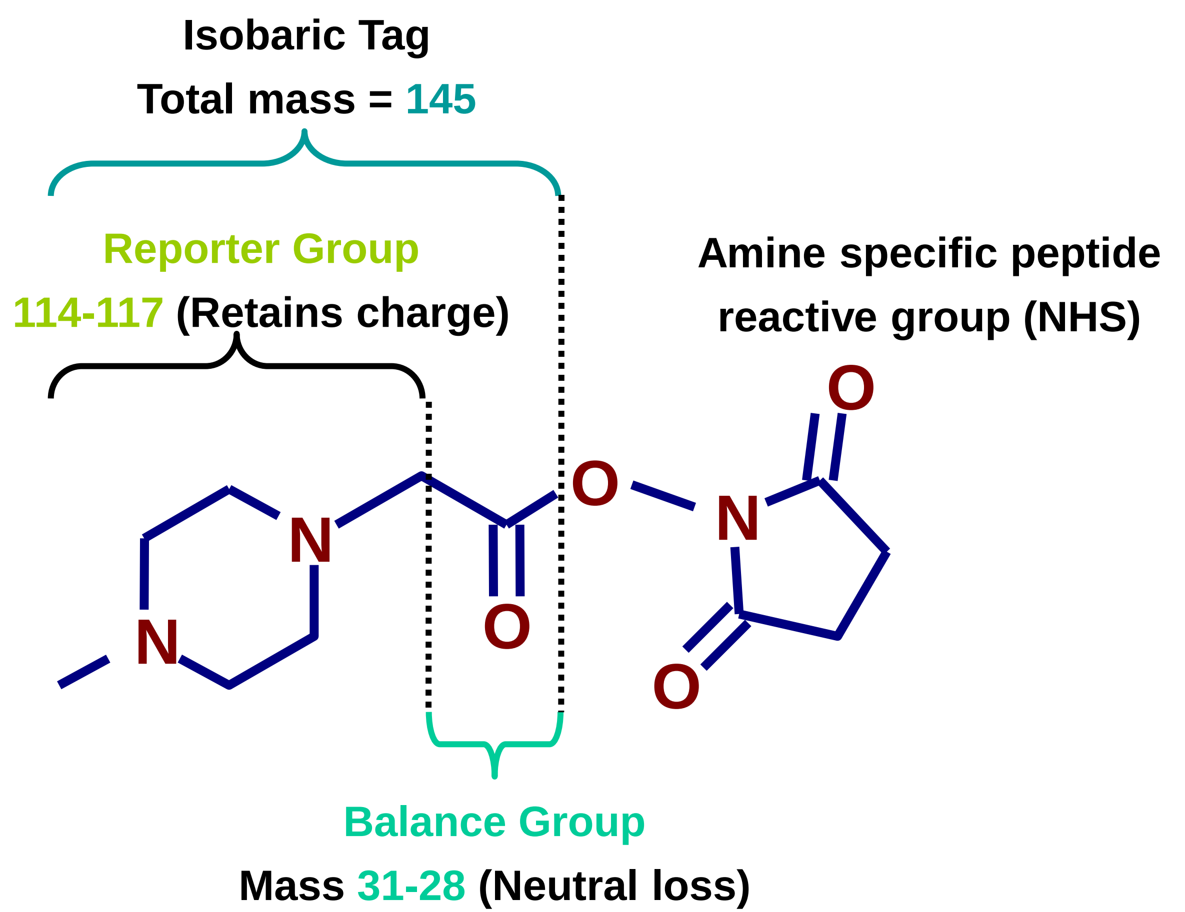

Mass Difference Varies with no. of N atoms



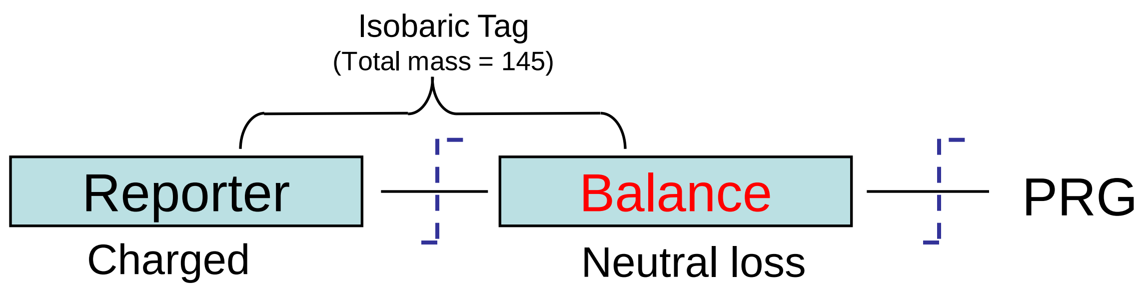

Advantage: Coeluting Peptide Pairs


Conrads TP, Alving K, Veenstra TD, Belov ME, Anderson GA, Anderson DJ, Lipton MS, Pasa-Tolic L, Udseth HR, Chrisler WB, Thrall BD, Smith RD. Quantitative analysis of bacterial and mammalian proteomes using a combination of cysteine affinity tags and 15N-metabolic labeling.
Anal Chem. 2001 May 1;73(9):2132-9.

# iTRAQ$^{TM}$ reagents



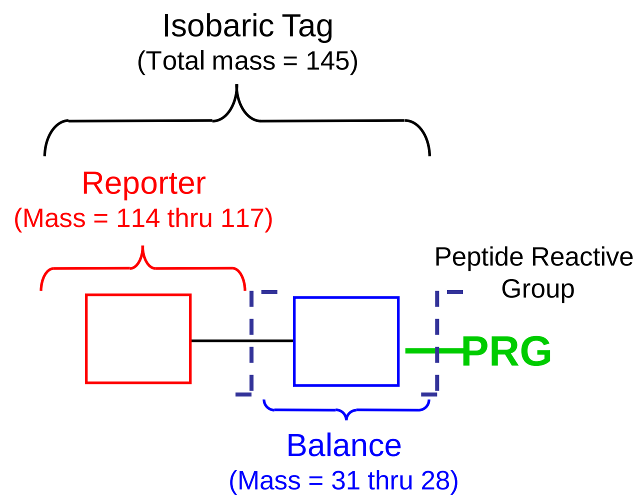
# iTRAQ™ Reagent Design




- ✓ Gives characteristic signature ion in MS/MS
- ✓ Gives good b- and y-ion series
- ✓ Maintains charge state
- ✓ Maintains ionization efficiency of peptide
- ✓ Signature ion masses lie in quiet region

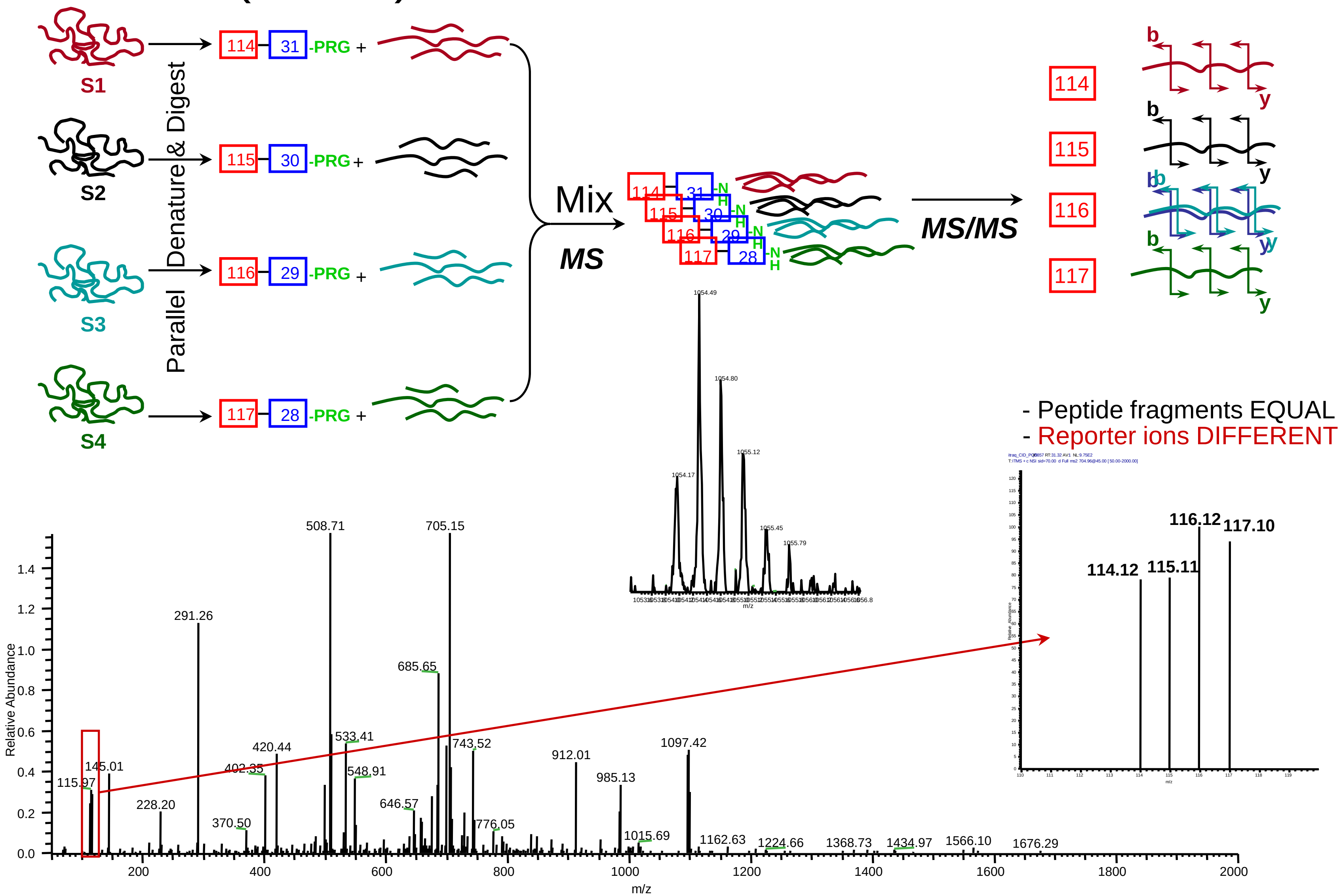
- ✓ Balance changes in concert with reporter mass to maintain total mass of 145
- ✓ Neutral loss in MS/MS

- ✓ Amine specific

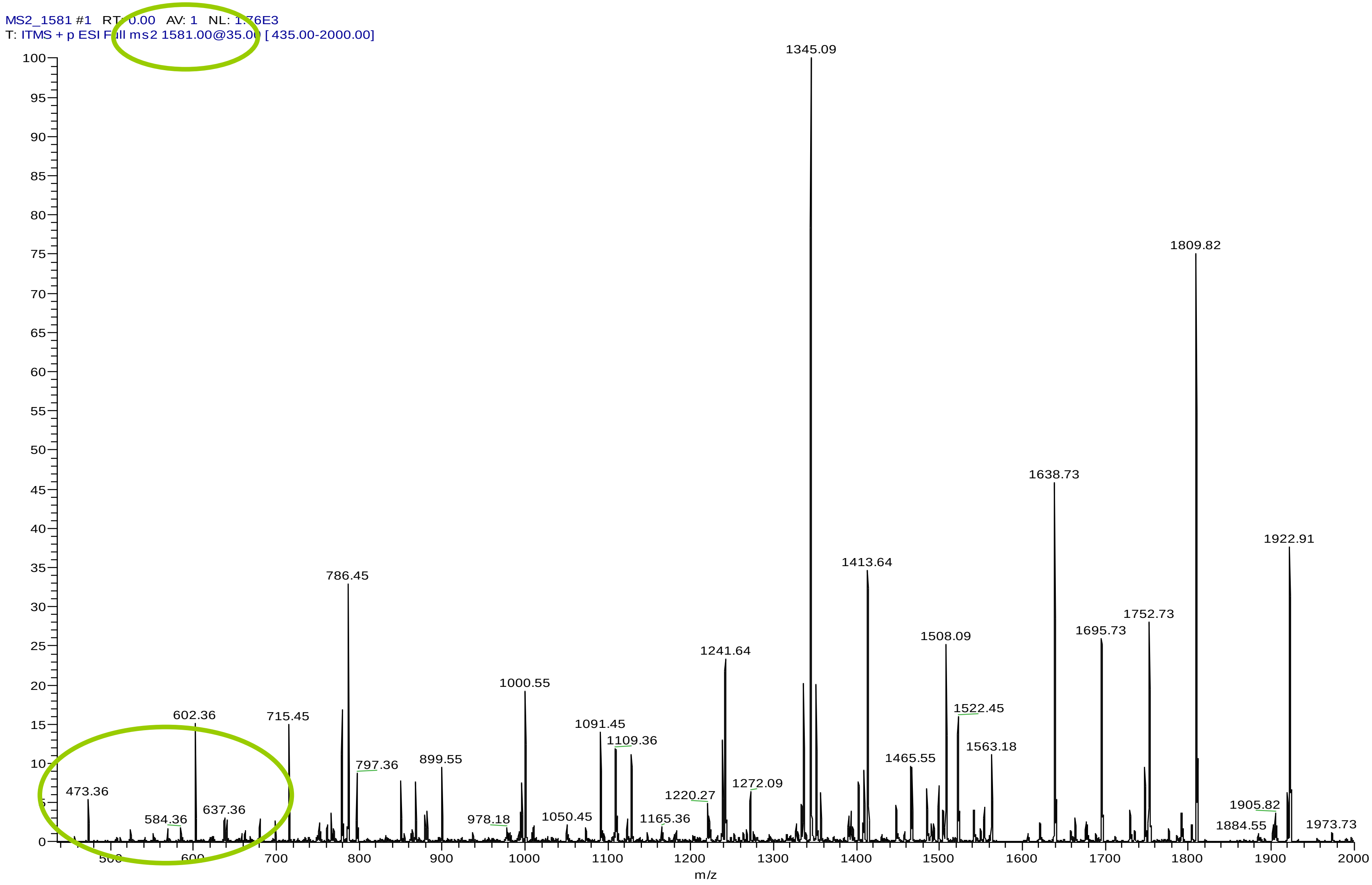



Multiplexed protein quantitation in saccharomyces cerevisiae using amine-reactive isobaric tagging reagents Ross, PL., et al, Mol Cell Proteomics, 2004. **3**(12): p. 1154-69

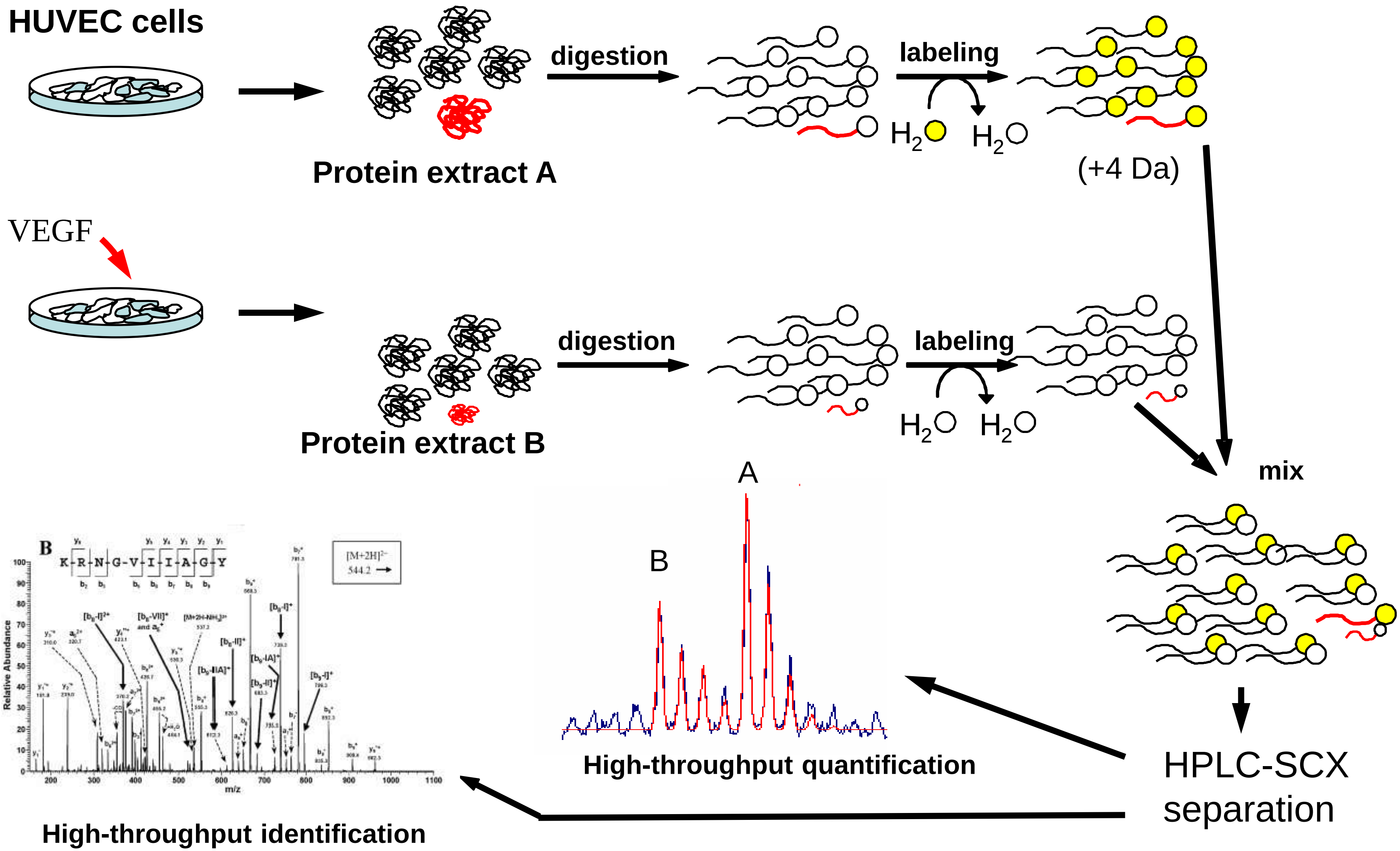
# Isobaric Tagging - General Method (4-Plex)



# Mass cutoff typically observed with ion traps for $MS^2$ spectra (1/3rd rule)

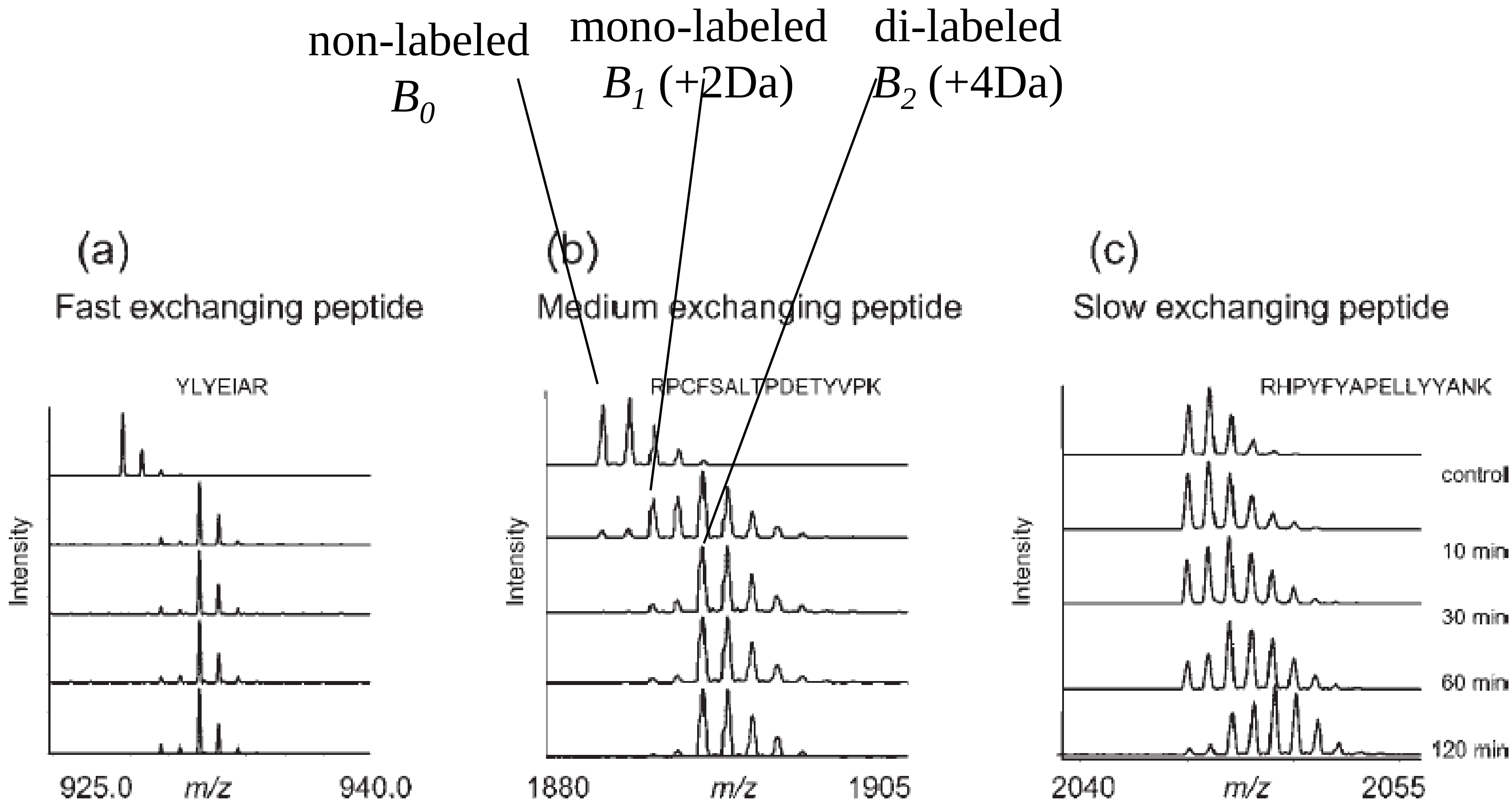


# Differential Proteomics study using $^{18}O$ stable isotope labeling




*Ramos, López-Ferrer and Vázquez Proteomics 2006; Molecular and Cellular Proteomics (2007)*

# Enzymatic post-digestion incorporation of $^{18}O$ in peptides




*Bantscheff et al. Rapid Comm. Mass Spectrom. 2004*

$^{18}O$

# Towards a kinetic correction algorithm: the rationale:

$$UU \xrightarrow{k} UL \xrightarrow{\alpha k} LL \qquad (1)$$

The kinetic model

$$\frac{dUU}{dt} = -k\,UU \qquad (2)$$

$$\frac{dLL}{dt} = \alpha k UL = \alpha k(1 - UU - LL) \qquad (3)$$

The kinetic equations

Note that this eq. does not depend on *k* nor *t*!

$$\frac{dLL}{dUU} = \frac{\alpha(UU + LL - 1)}{UU} \qquad (4)$$

An universal diff. equation!

$$LL = -2\sqrt{UU} + UU + 1 \qquad (6)$$

Solution of eq.6 for trypsin exchange ($\alpha$=0.5)

$$LL = f^2$$
$$UU = (1 - f)^2 \qquad (7)$$
$$UL = 2f(1 - f)$$

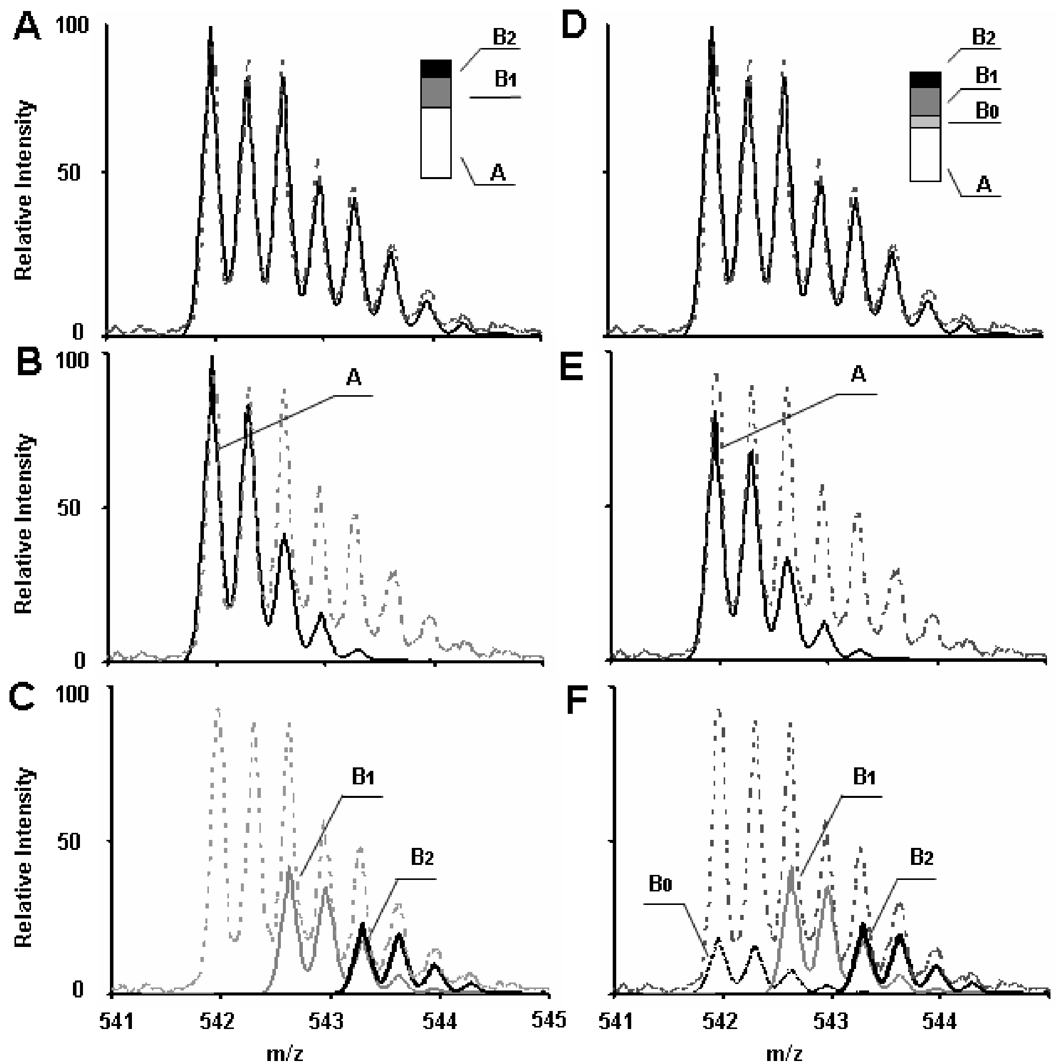
An universal relation!

*Ramos, López-Ferrer and Vázquez, Molecular and Cellular Proteomics (2007)*
*Ramos, López-Ferrer and Vázquez, CSIC, Patent filed 2006*

## $^{18}O$

### The kinetic correction algorithm

| Old algorithm | New algorithm |
| --- | --- |
| Fitting of $A$, $B_1$, $B_2$ | Fitting of $A$, $B$, $f$ |



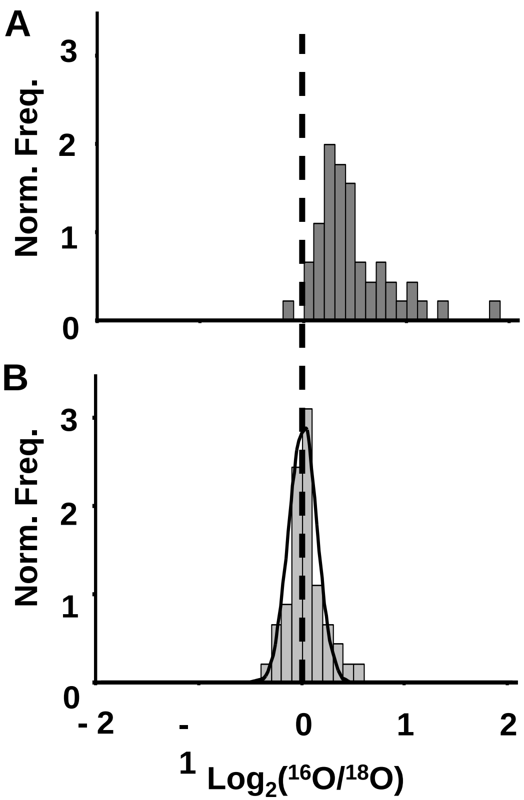

-degrees of freedom of the fitting are maintained

*Ramos, López-Ferrer and Vázquez, Molecular and Cellular Proteomics (2007)*
*Ramos, López-Ferrer and Vázquez, CSIC, Patent filed 2006*

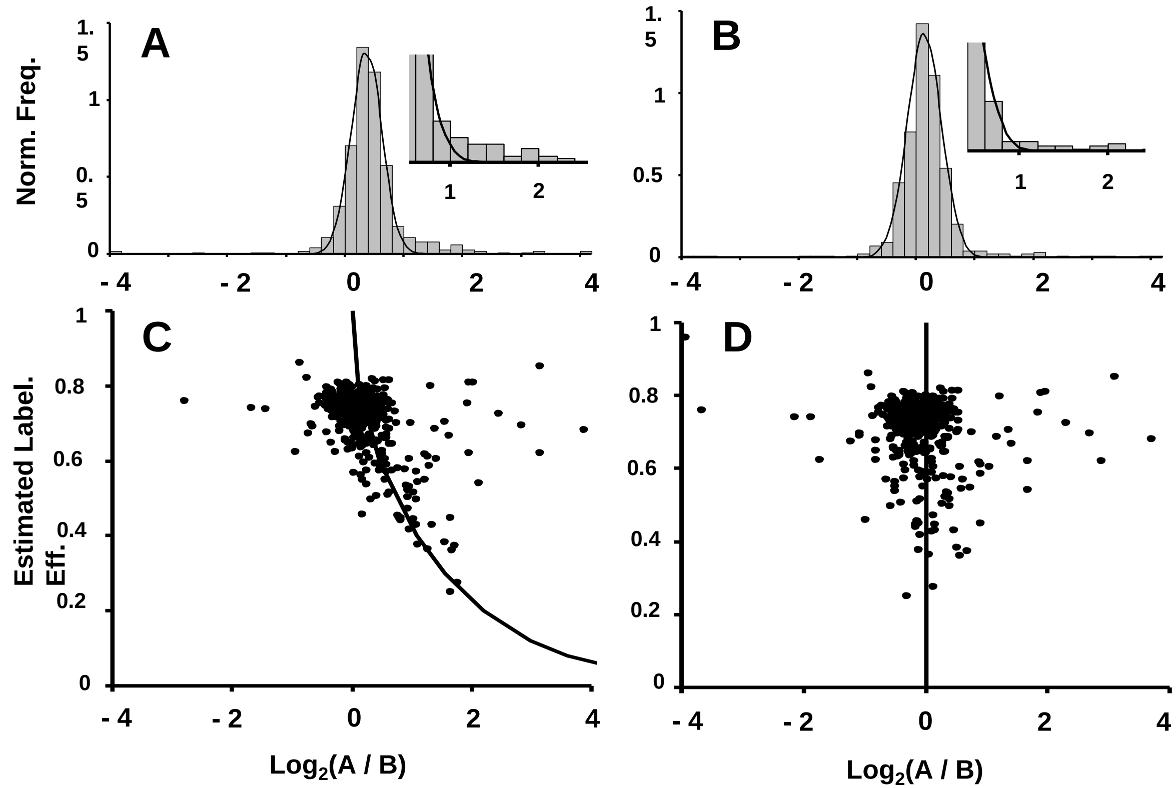
$^{18}O$

# Results before and after correction for incomplete $^{18}O$ labeling

Control experiment:
Proteome vs itself

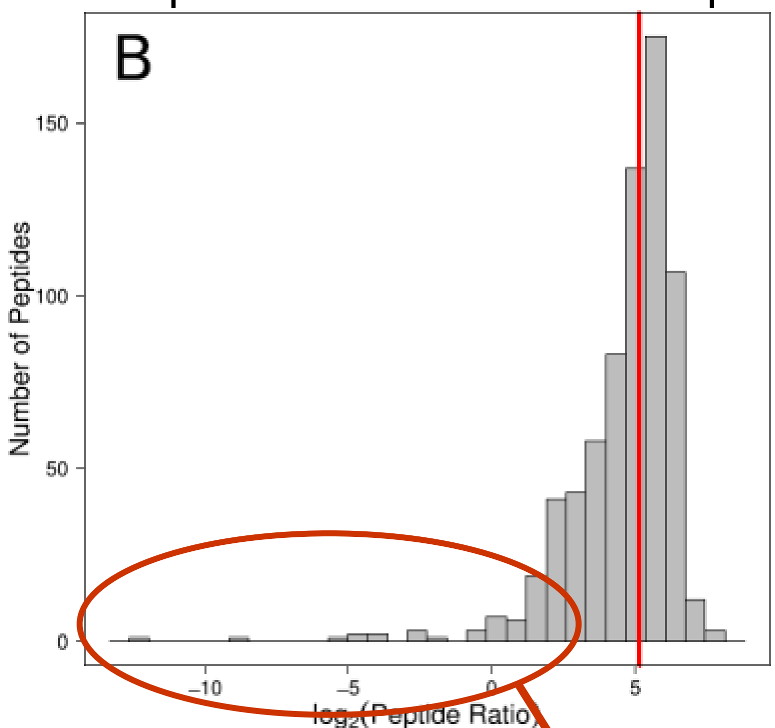



Expression changes after T-cell activation




*Ramos, López-Ferrer and Vázquez, Molecular and Cellular Proteomics (2007)*
*Ramos, López-Ferrer and Vázquez, CSIC, Patent filed 2006*

SILAC labeling eff.:
Aliquot from the labeled sample




Graumann… Mann
MCP (2008)

iTRAQ labeling eff.
Calculated from the pool of data

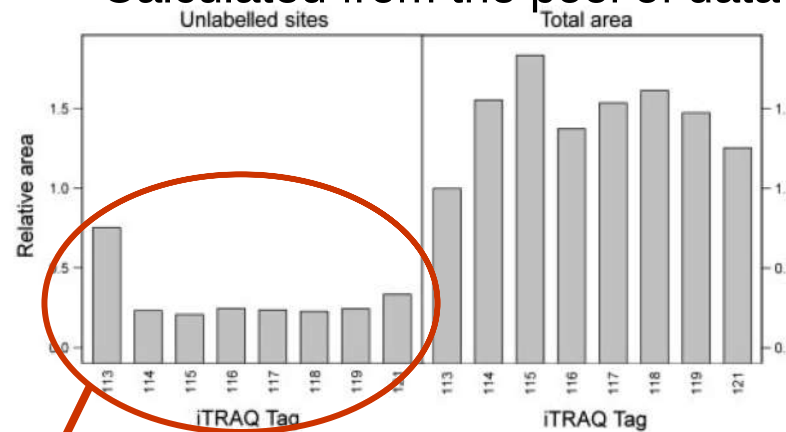



**Figure 6:** Quality control measures applied to shot-gun data to assess labelling efficiency. Unlabelled sites, grouped by tag, are quantified as a function of the relative peak areas of partially and fully labelled peptides. Total area is a measure of total protein identified by isobaric tagging analysis and is quantified as a function of total peak area, grouped by tag.

D'Ascenzo et al.,
BFGenomics &
Proteomics (2008)

Labeling is not complete:
Labeling efficiency must be controlled!!

# A comment on labeling efficiency on SIL experiments

- Both SILAC and iTRAQ are assumed to have a 100% labeling efficiency… This is not true in the practice!
- Labeling efficiency has to be controlled in both cases by analyzing an aliquot of the labeled sample or analyzing the data as a whole, but is not controlled at the individual peptide level
- $^{18}O$ labeling allows a precise (and automated) control of labeling efficiency of each one of the quantified peptides and obviates the need of “external” testing

Müller et al. JPR 2008

EuPA · ProteoRed · SEPROT

**Table 1.** Summary of Software Tools for Isobaric and Isotopic Labeling in Proteomics Experiments[a]

| software | operating systems | tested data types | input format | label | compatible labels | availability | ref |
|---|---|---|---|---|---|---|---|
| | | | | Isobaric Labeling | | | |
| Multi-Q | Windows, Web version | Mascot/Sequest results | mzXML | iTRAQ | specific | http://ms.iis.sinica.edu.tw/Multi-Q | 34 |
| iTracker | Linux, OSX, Windows | via TPP[9] | .mgf, .dat | iTRAQ | specific | http://www.cranfield.ac.uk/health/researchareas/bioinformatics/page3201.jsp | 35 |
| Libra | Linux, OSX, Windows | via TPP[9] | mzXML | iTRAQ | specific | http://tools.proteomecenter.org/wiki/ | 9 |
| ProQuant | Windows | QStar, Qtrap | raw file | iTRAQ | specific | http://www.appliedbiosystems.com | – |
| ProteinPilot | Windows | QStar, Qtrap, Maldi-Tof/Tof | raw file | iTRAQ | specific | http://www.appliedbiosystems.com | – |
| | | | | Isotopic Labeling | | | |
| XPRESS | Linux, OSX, Windows | LTQ, OrbiTrap, Qtof, FT-LTQ | mzXML | ICAT | ICPL, SILAC | http://tools.proteomecenter.org/wiki/ | 46 |
| ASAPRatio | Linux, OSX, Windows | LTQ, OrbiTrap, Qtof, FT-LTQ | mzXML | $^{2}H$ | ICAT, SILAC, ICPL | http://tools.proteomecenter.org/wiki/ | 47 |
| PeakPicker | Windows | Maldi-Tof/Tof | raw file | ICPL | specific | http://www.appliedbiosystems.com | – |
| WARP-LC | Windows | Maldi-Tof/Tof, Qtof | raw file | ICPL | generic | http://www.bdal.com/ | – |
| ZoomQuant | Linux, OSX, Windows | LTQ | raw file | $^{18}O$ | specific | http://proteomics.mcw.edu/zoomquant/ | 53 |
| STEM | Windows | Mascot results | .pkl file | $^{18}O$ | generic | http://www.sci.metro-u.ac.jp/proteomicslab/STEMDLP-0.html | 54 |
| MSQuant | Windows | QStar, Qtof, FT-LTQ | raw file | SILAC | ICAT, ICPL | http://msquant.sourceforge.net/ | 55 |
| **QuiXoT** | **Windows** | **Thermo ion traps** | **raw file** | **18O** | **iTRAQ??** | **http://www.cbm.uam.es/jvazquez/bioinformatics.htm** | |