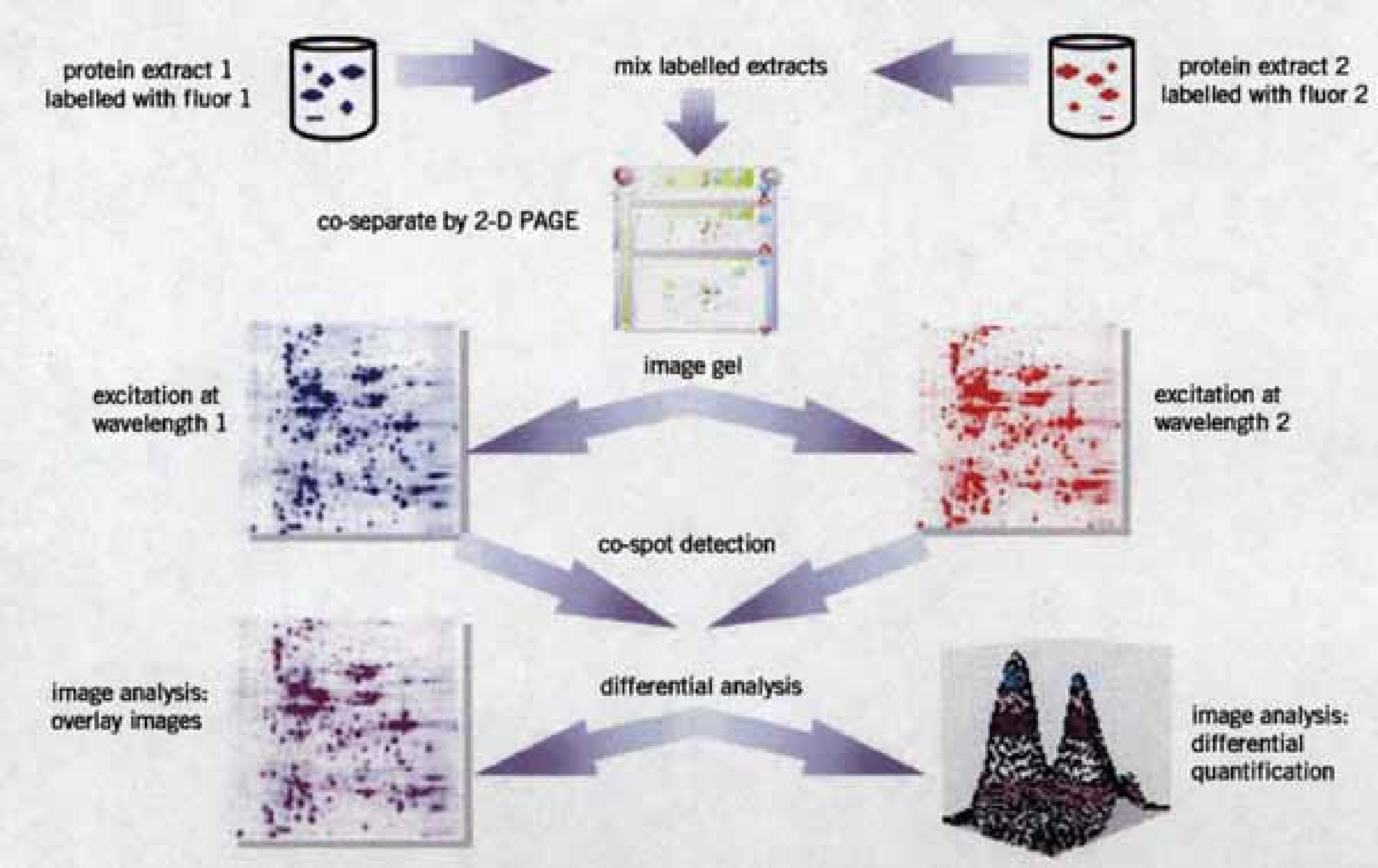
DIGE (difference gel electrophoresis)

- Built upon the classical gel approach to protein quantification (gel densitometry)
- Separate Samples are treated with unique fluorophore tags (binding covalently with lysine ε-amino groups)
- Samples are combined and run on the same 2D gel (ΔMW of proteins is negligible)
- Quantitative Analysis is based on relative intensities of fluorescing labels at specific spots (relative quantitation) or to labeled standard (absolute quantitation).

Practical Approach to DIGE

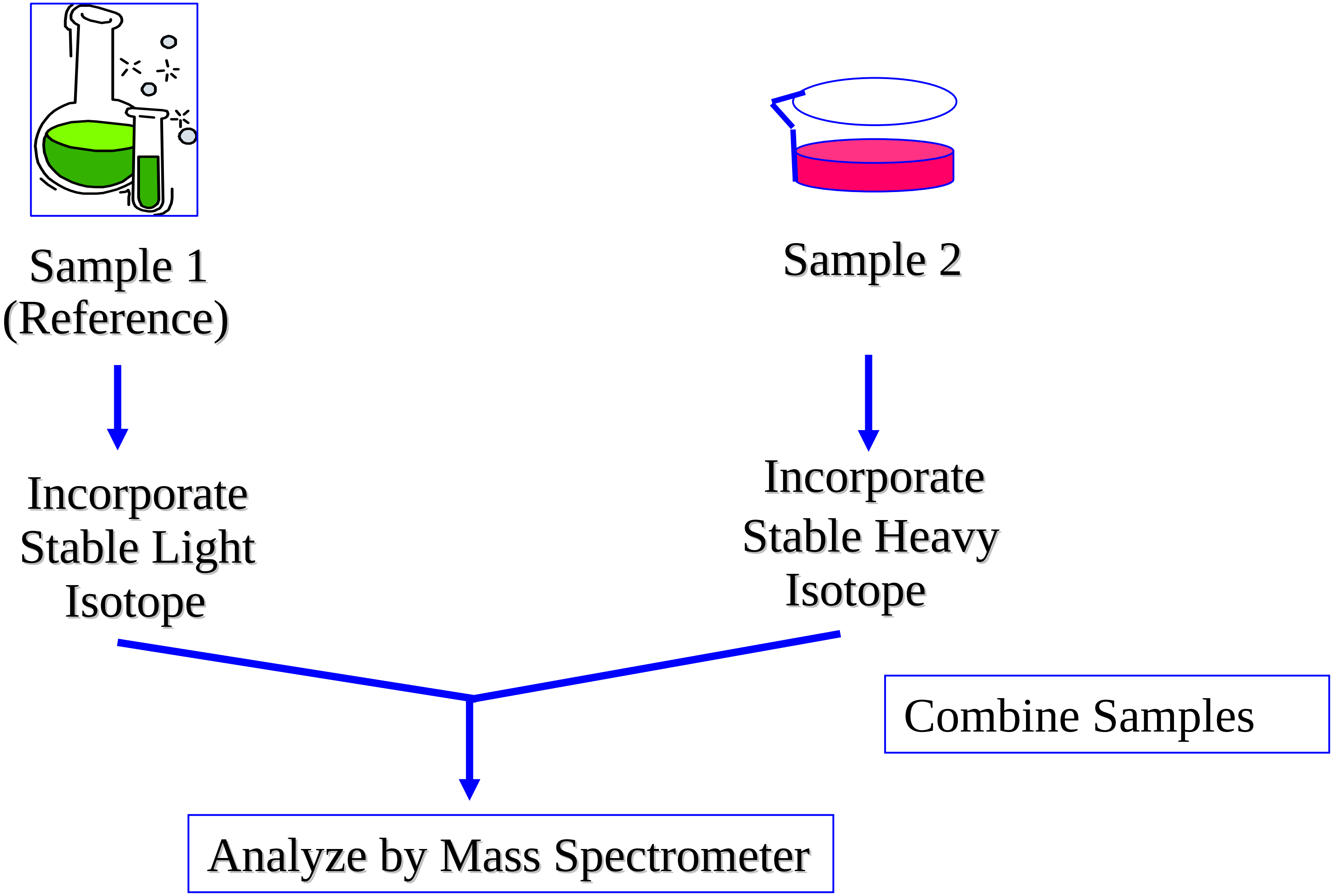


Amersham Pharmacia Biotech, Life Science News, 7, 2001

Available Methodology

1) Isotope-Coded Affinity Tag (ICAT) technology
2) iTRAQ tagging technology
3) Absolute QUAntitation of proteins (AQUA)
4) Stable Isotope Labeling with Amino acids in Cell culture (SILAC)

Accurate Quantitation Using Isotope Dilution



Sample 1
(Reference)

Incorporate
Stable Light
Isotope

Sample 2

Incorporate
Stable Heavy
Isotope

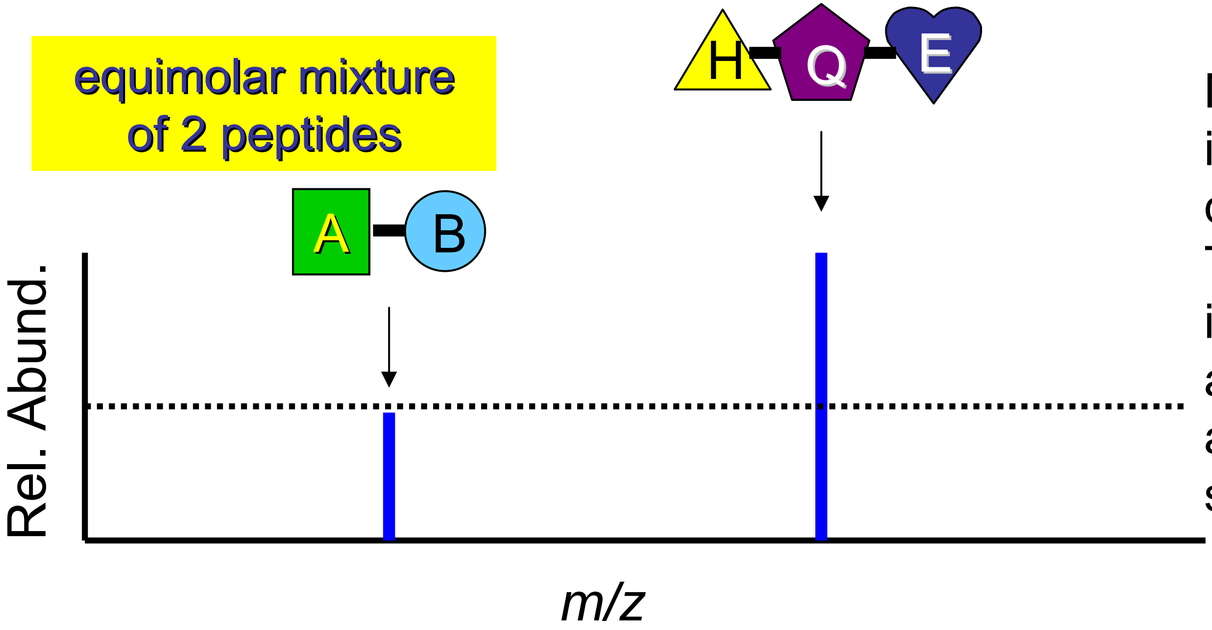
Combine Samples

Analyze by Mass Spectrometer

- heavy/light analytes are chemically identical ⇒ identical specific signal in MS
    - Ratio of heavy/light signals indicates ratio of analytes

Mass Spectrometry and Quantitative Measurements

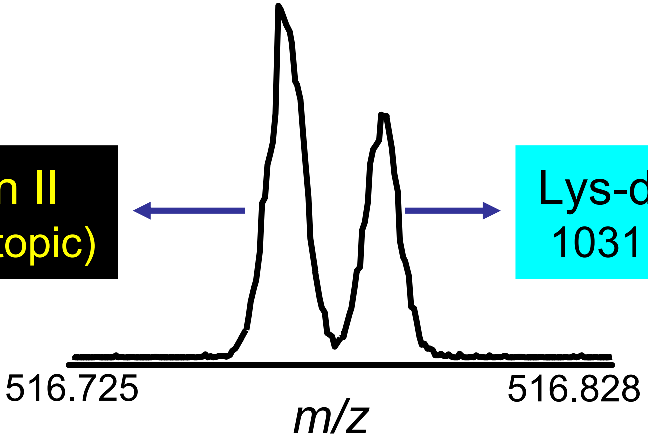
equimolar mixture
of 2 peptides



Mass spectrometry is inherently not a quantitative technique. The intensity of a peptide ion signal does not accurately reflect the amount of peptide in the sample.

equimolar mixture
of 2 peptides

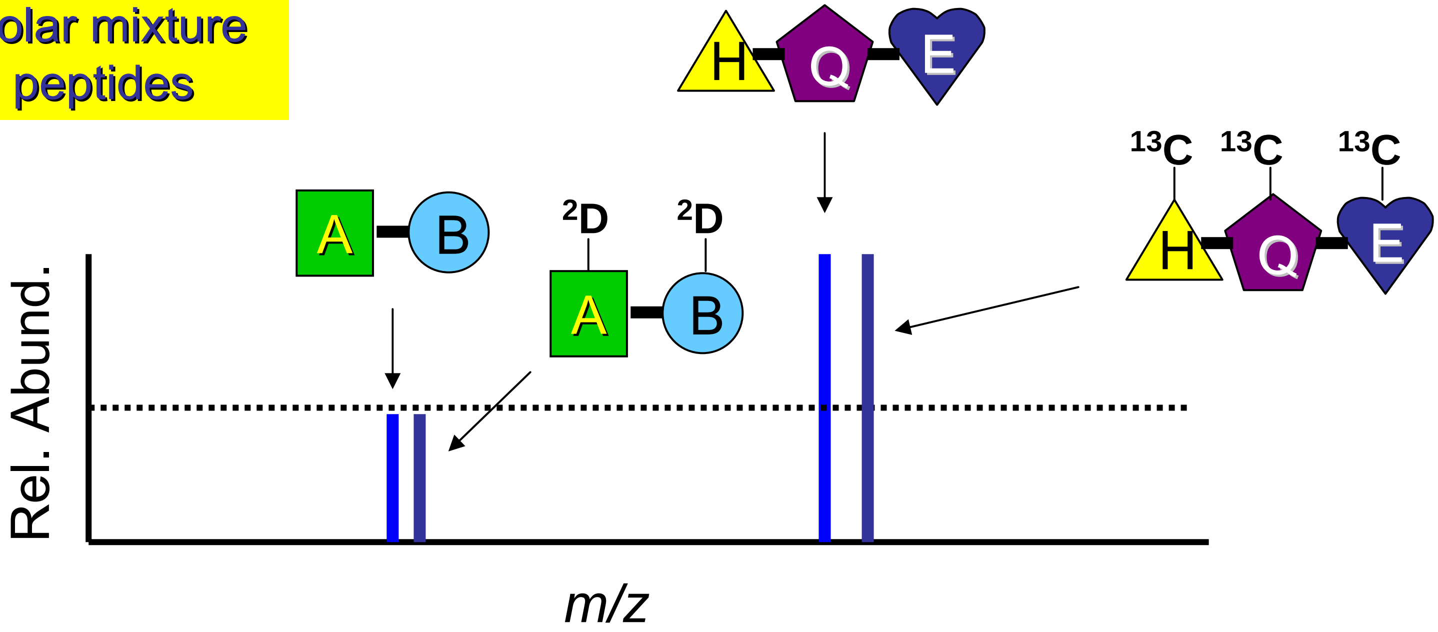
[Val^5]-Angiotensin II
1031.5188 (monoisotopic)



Lys-des-Arg^9-Bradykinin
1031.5552 (monoisotopic)

Mass Spectrometry and Quantitative Measurements

equimolar mixture of 2 peptides



Two peptides of identical chemical structure that differ in mass because they differ in isotopic composition are expected to generate identical specific signals in a mass spectrometer.

Methods coupling mass spectrometry and stable isotope tagging have been developed for quantitative proteomics.

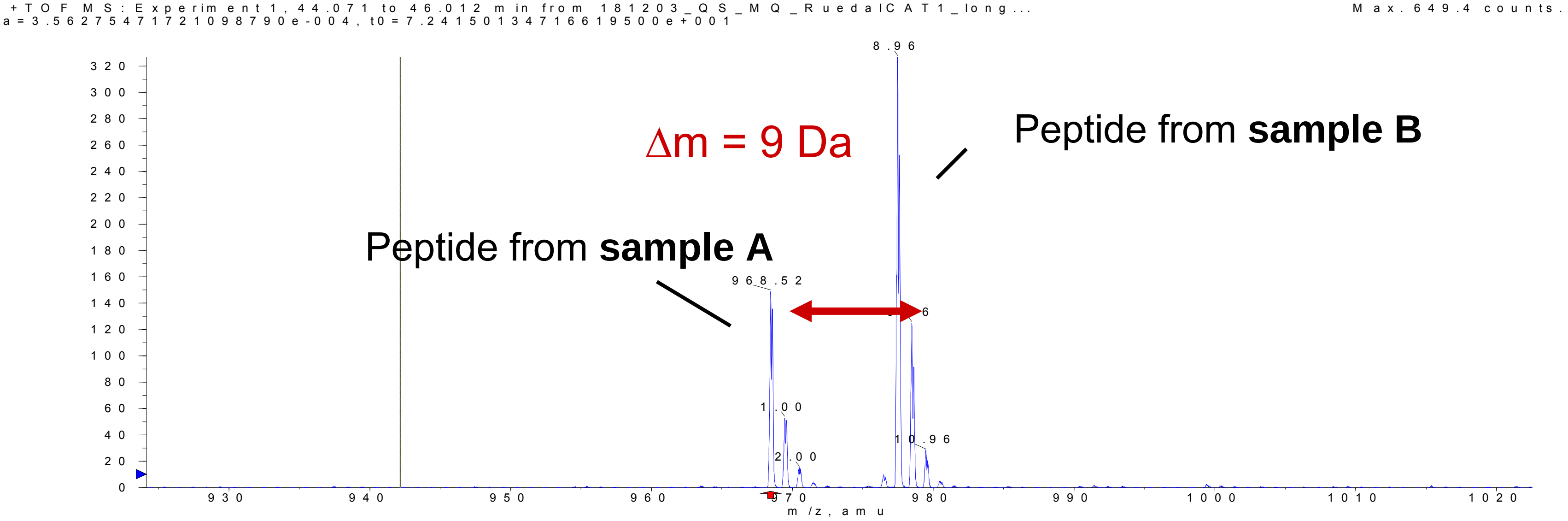
Relative quantitation : stable isotope labelling is very fashionable!

Sample A : light isotope

Sample B : heavy isotope

mix, digest

Quantitate and identify (MS)



Δm = 9 Da

Peptide from **sample B**

Peptide from **sample A**

How to label ?

-chemically, post protein synthesis

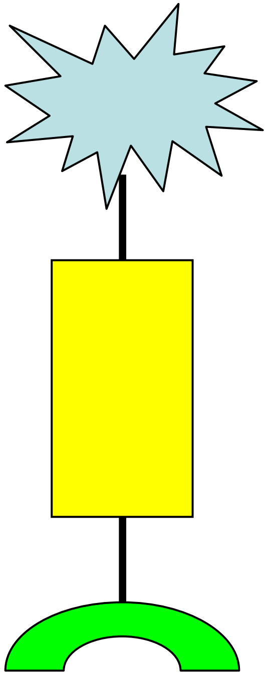
- **→ “specific” chemical modification of AA side chain**
  - **(+)** ***any sample can be done***
  - **(-)** ***side reactions***

-metabolically, during protein synthesis

- **→Incorporation of one or more labelled amino acid**
  - ***(+) “native” proteins***
  - ***(-) need cultivable organism***

ISOTOPE-CODED AFFINITY TAG (ICAT): a quantitative method

- Label protein samples with heavy and light reagent
- Reagent contains affinity tag and heavy or light isotopes



Chemically reactive group: forms a covalent bond to the protein or peptide

Isotope-labeled linker: heavy or light, depending on which isotope is used

Affinity tag: enables the protein or peptide bearing an ICAT to be isolated by affinity chromatography in a single step

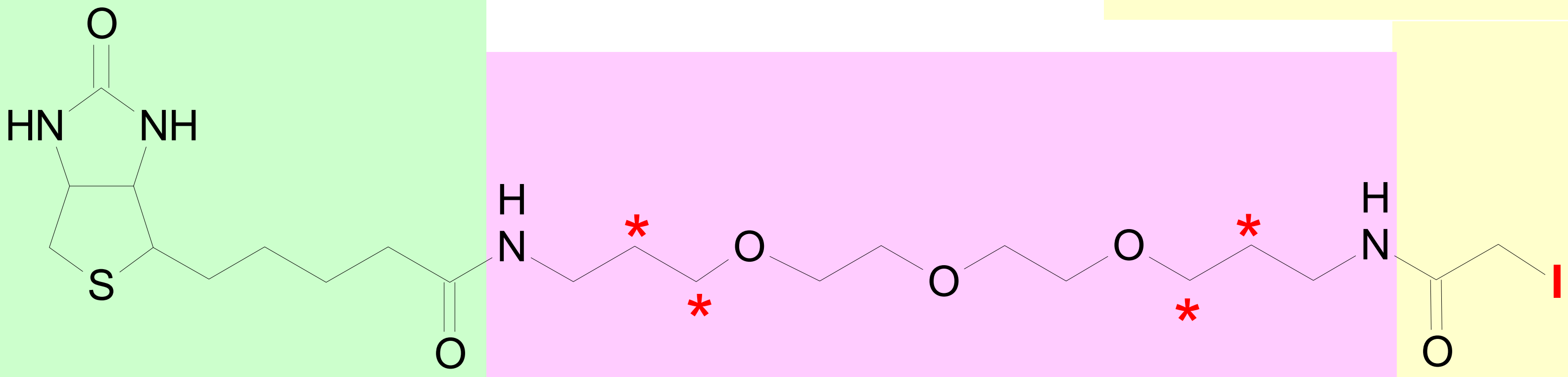
Example of an ICAT Reagent

Biotin affinity tag

binds tightly to streptavidin-agarose resin

Thiol-reactive group

note similarity to iodoacetamide

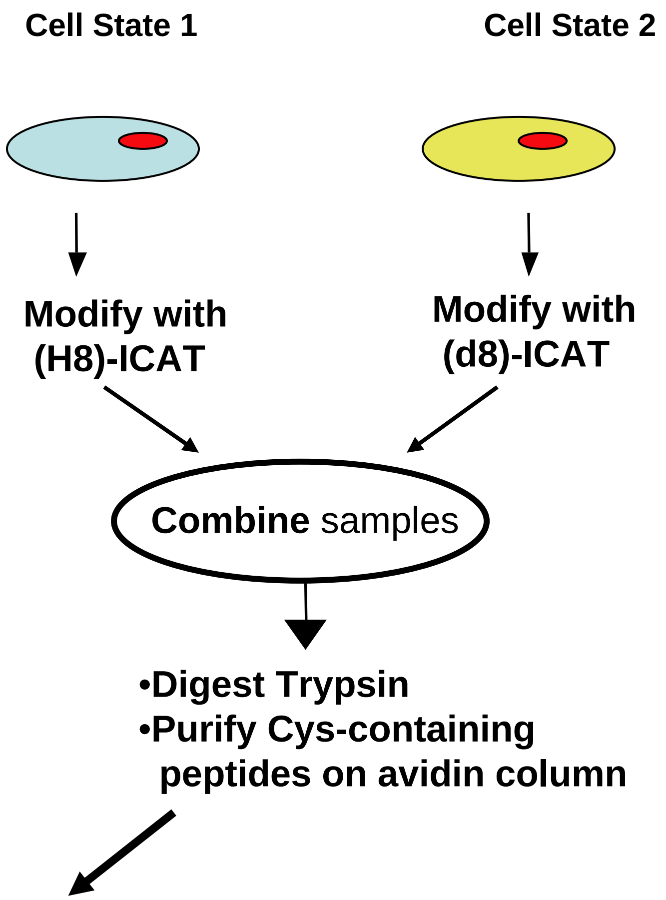


Linker

Heavy version will have deuteriums at *

Light version will have hydrogens at *

New Methods : ICAT:quantitation and identification



Cell State 1

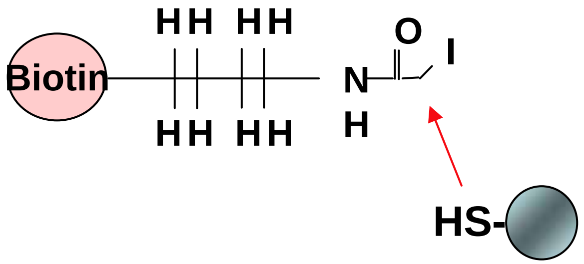
Cell State 2

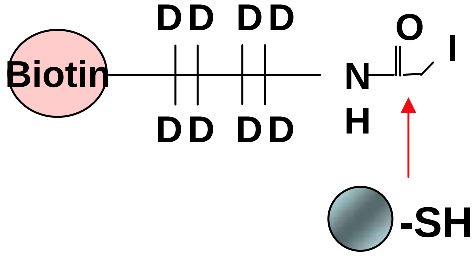
Modify with (H8)-ICAT

Modify with (d8)-ICAT

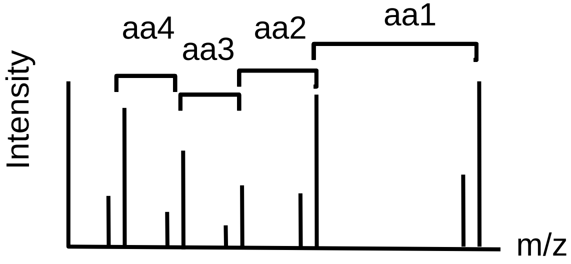
Combine samples

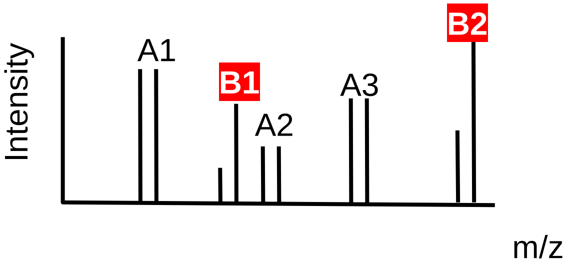
- **Digest Trypsin**
- **Purify Cys-containing peptides on avidin column**





Identify proteins by MS/MS





Quantitate protein levels
by H8 / D8 peak heigth ratios

ICAT (+) and (-)

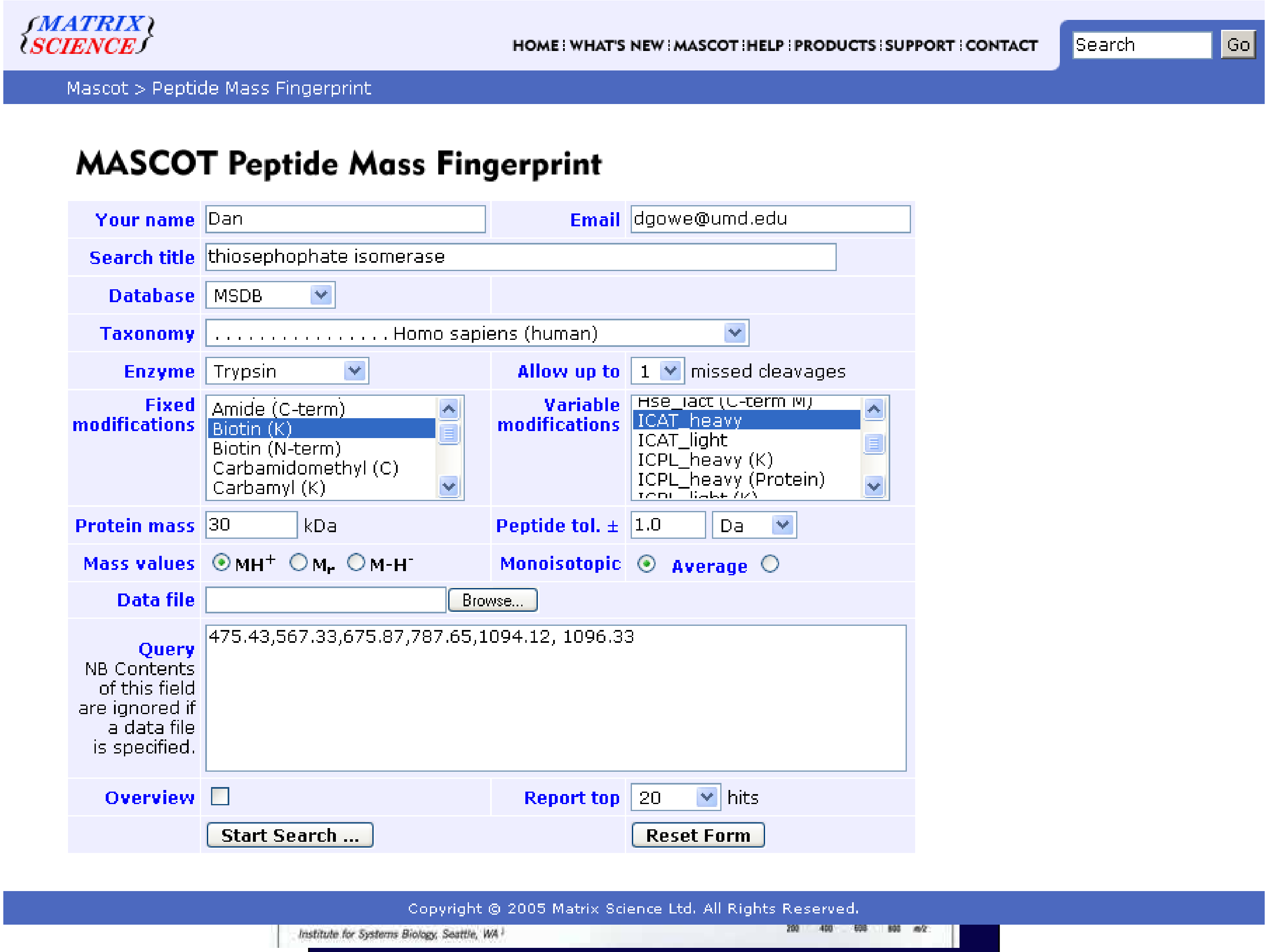
+

- relative protein quantification by MS
- simplification of complex mixtures by selecting a subset of peptides after digestion
- eliminate analytical variability by mixing samples

-

- protein quantification unreliable for weak signals
- affinity purification (avidin) : losses for low amounts
- multiple side reactions possible

~15 different isotope labelling methods developed in the last 5 years !!



MASCOT Peptide Mass Fingerprint

Your name	Dan	**Email**	dgowe@umd.edu
Search title	thiosephophate isomerase		
Database	MSDB		
Taxonomy	. . . . Homo sapiens (human)		
Enzyme	Trypsin	**Allow up to**	1 missed cleavages
Fixed modifications	Amide (C-term) Biotin (K) Biotin (N-term) Carbamidomethyl (C) Carbamyl (K)	**Variable modifications**	Hse_lact (C-term M) ICAT_heavy ICAT_light ICPL_heavy (K) ICPL_heavy (Protein) ICPL_light (K)
Protein mass	30 kDa	**Peptide tol. ±**	1.0 Da
Mass values	● MH^+ ○ M_r ○ $M\text{-}H^-$	**Monoisotopic** ● **Average** ○	
Data file	Browse...		
Query NB Contents of this field are ignored if a data file is specified.	475.43,567.33,675.87,787.65,1094.12, 1096.33		
Overview	☐	**Report top**	20 hits
	Start Search ...		Reset Form

Institute for Systems Biology, Seattle, WA

http://dir.niehs.nih.gov/proteomics/emerg2.htm

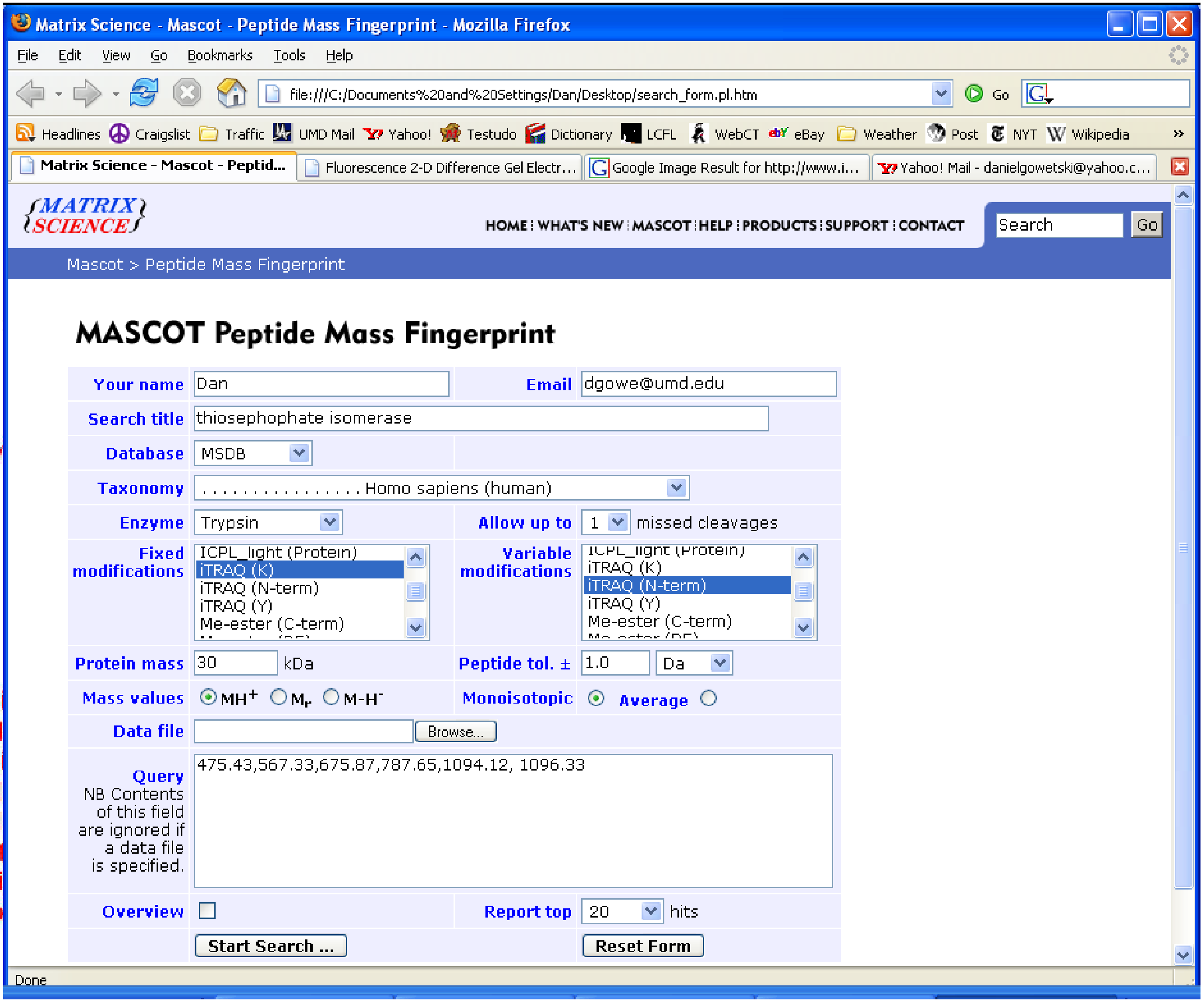
iTRAQ (isobaric tags for relative and absolute quantification)

- Uses up to 4 tag reagents that bind covalently to the N-terminus of the peptide and any Lysine side chains at the anime group (global tagging).
- Each sample set is digested separately and then mixed with the specific iTRAQ tag

A	B	C	D
DIGEST	DIGEST	DIGEST	DIGEST
Label A	**Label B**	**Label C**	**Label D**

iTRAQ (isobaric tags for relative and absolute quantification)

- Fragmentation of the precursor ion (MS/MS) will reliably separate the tag fragment from the peptide
- The fragmented tag ions will show up from 114 to 117 m/z on the spectrum
- Quantitative analysis can be made by comparing the relative intensities of each of the tagged peaks

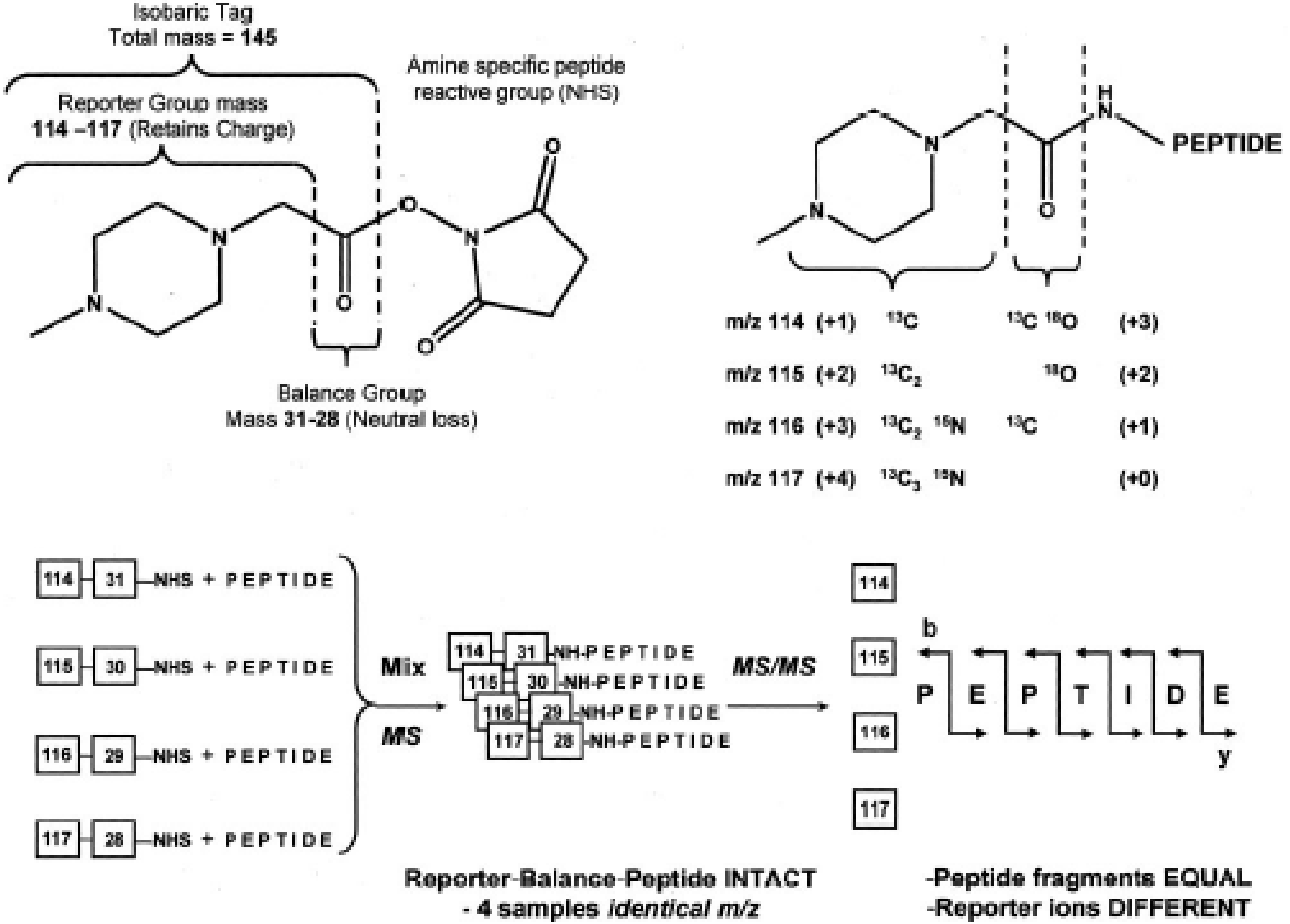


MASCOT Peptide Mass Fingerprint

Your name	Dan	**Email**	dgowe@umd.edu
Search title	thiosephophate isomerase		
Database	MSDB		
Taxonomy	. . . . Homo sapiens (human)		
Enzyme	Trypsin	**Allow up to**	1 missed cleavages
Fixed modifications	ICPL_light (Protein) iTRAQ (K) iTRAQ (N-term) iTRAQ (Y) Me-ester (C-term)	**Variable modifications**	ICPL_light (Protein) iTRAQ (K) iTRAQ (N-term) iTRAQ (Y) Me-ester (C-term)
Protein mass	30 kDa	**Peptide tol. ±**	1.0 Da
Mass values	● MH^+ ○ M_r ○ $M-H^-$	**Monoisotopic**	● **Average** ○
Data file			
Query NB Contents of this field are ignored if a data file is specified.	475.43,567.33,675.87,787.65,1094.12, 1096.33		
Overview	☐	**Report top**	20 hits

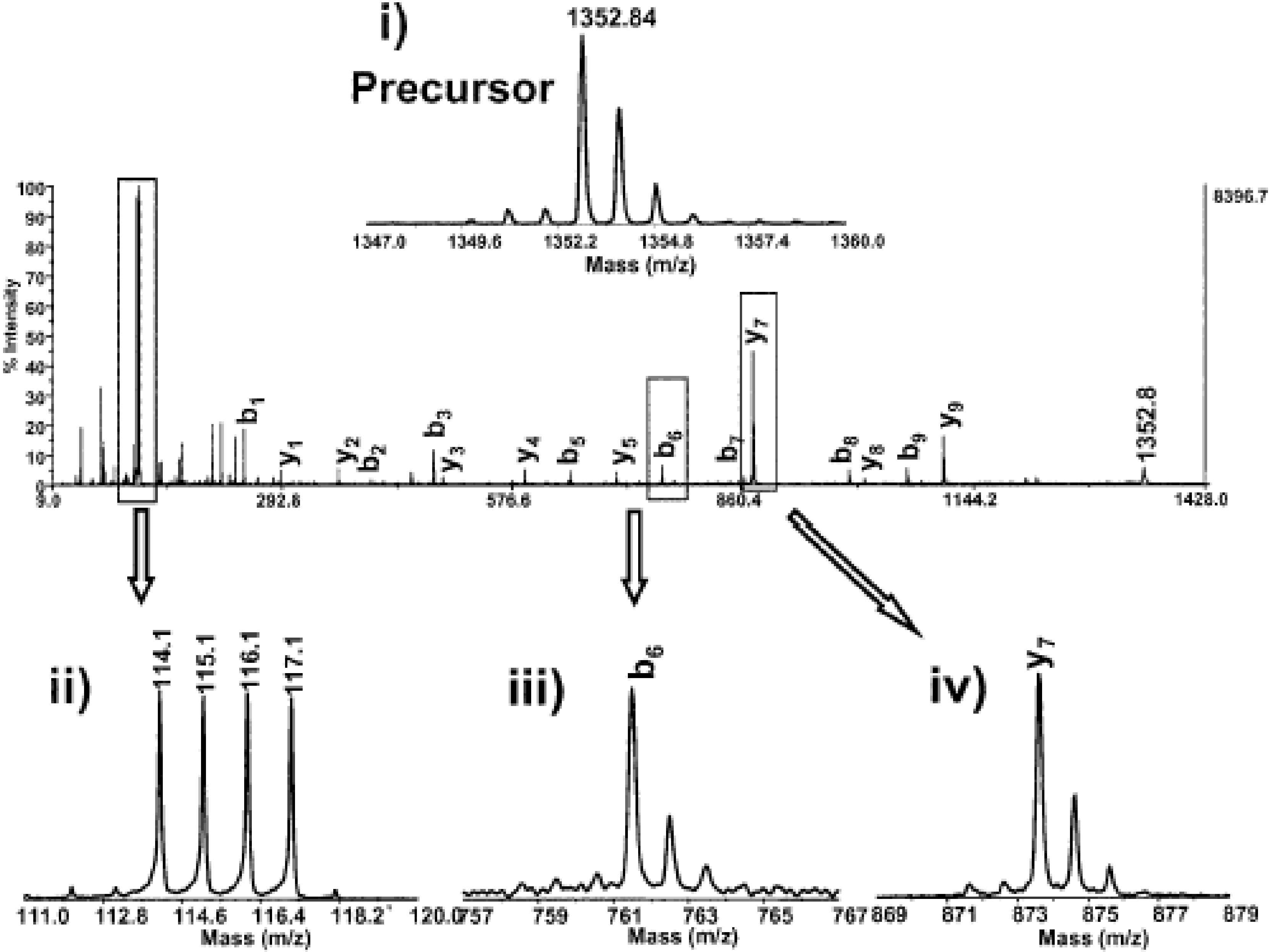
✓ Gi
M
✓ Gi
✓ M
✓ M
o
✓ Si
lo

iTRAQ



Ross, P. L. et al. (2004) Mol Cell Proteomics **3**(12): 1154-69

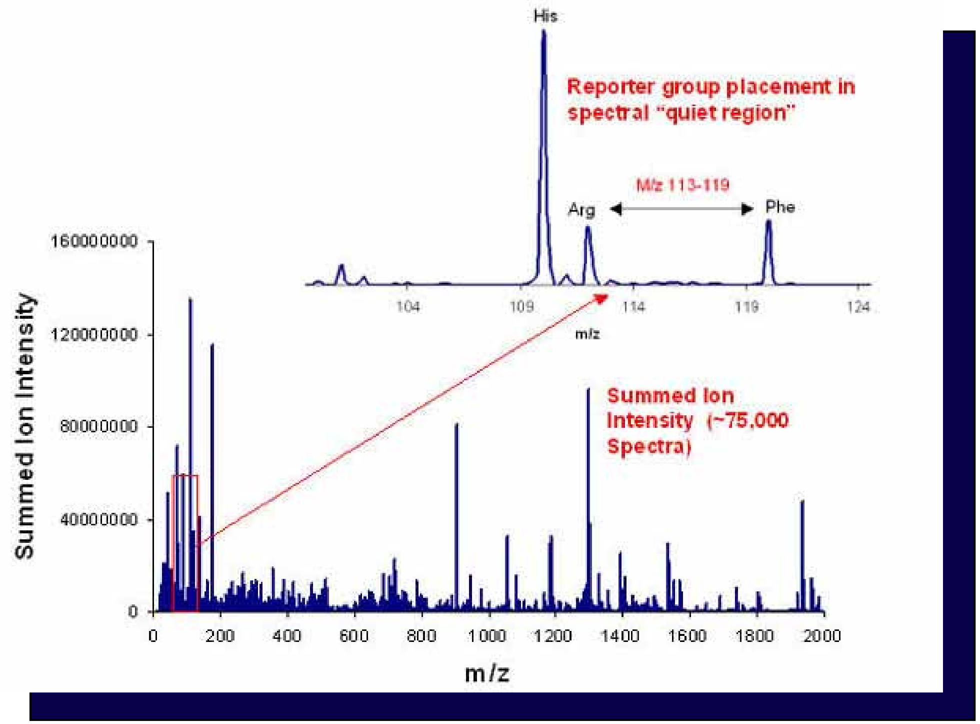
iTRAQ



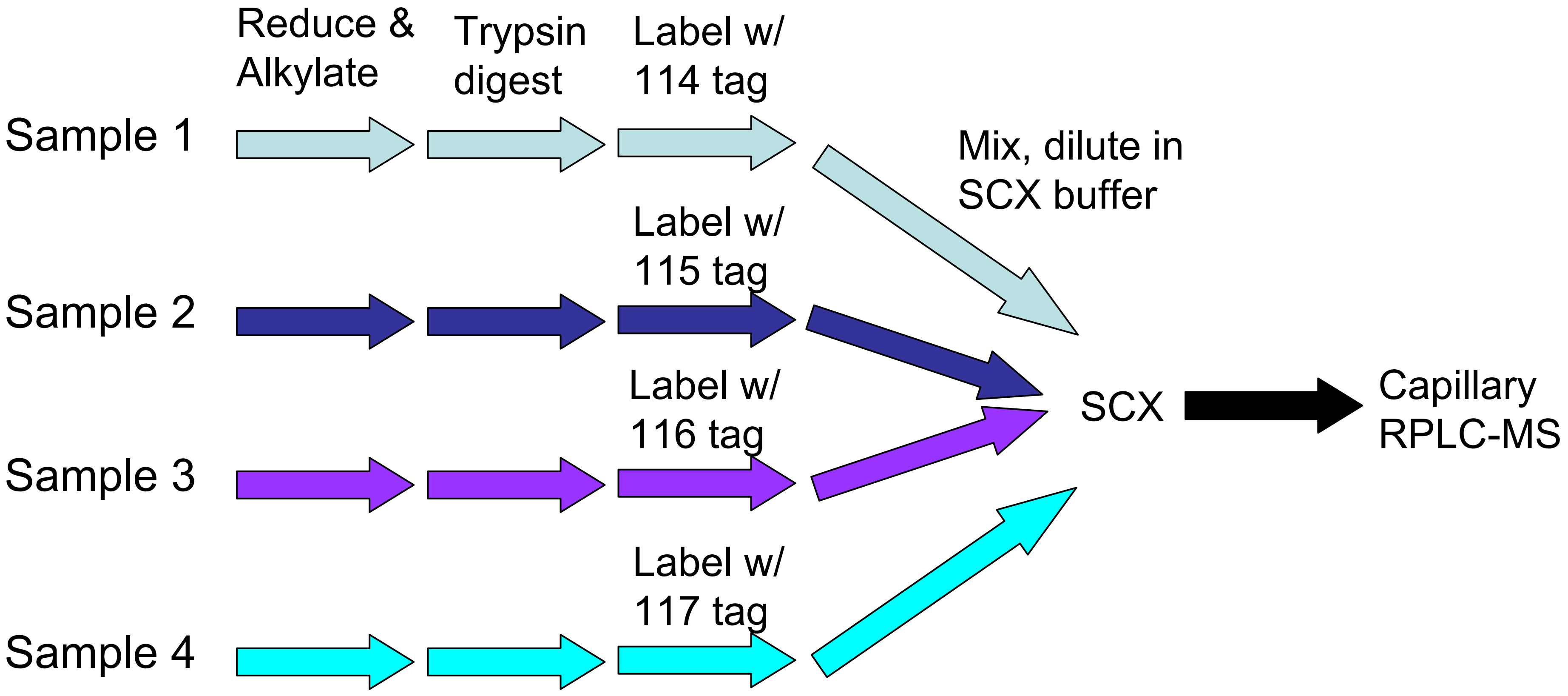
Ross, P. L. et al. (2004) Mol Cell Proteomics **3**(12): 1154-69

iTRAQ (isobaric tags for relative and absolute quantification)

- Tags were designed to produce fragments in a “quiet” spectral region



iTRAQ



Reduce & Alkylate

Trypsin digest

Label w/ 114 tag

Mix, dilute in SCX buffer

Sample 1

Label w/ 115 tag

Sample 2

Label w/ 116 tag

SCX

Capillary RPLC-MS

Sample 3

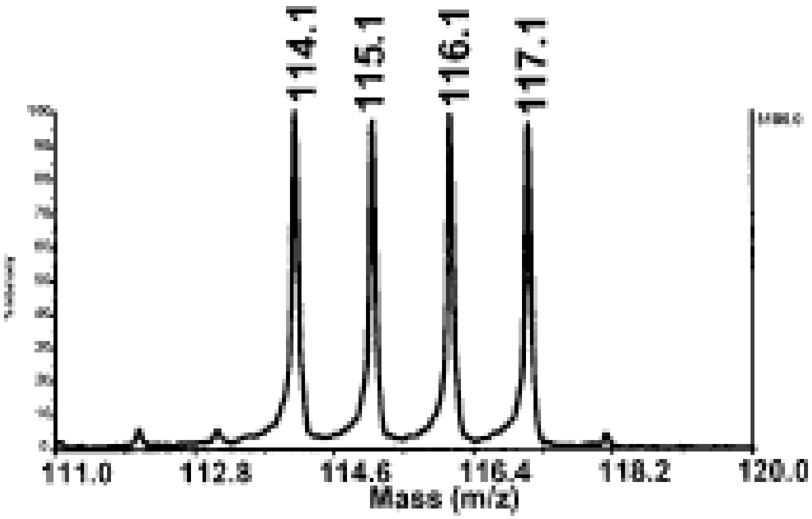
Label w/ 117 tag

Sample 4

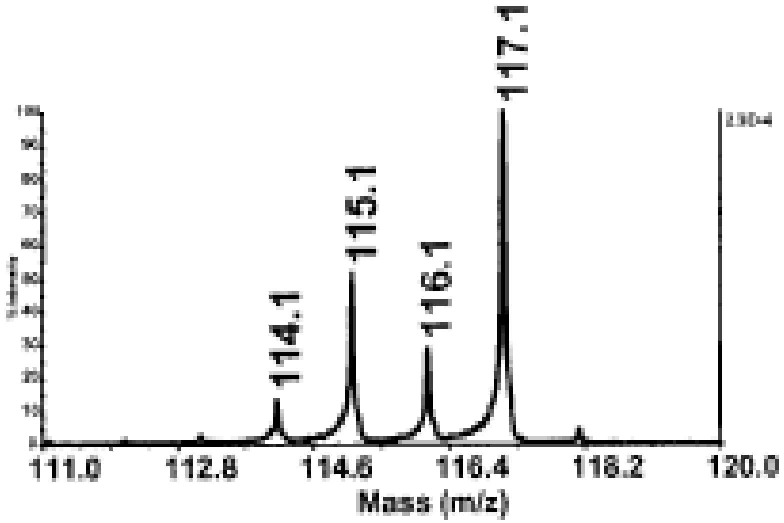
Ross, P. L. et al. (2004) Mol Cell Proteomics **3**(12): 1154-69

iTRAQ

1:1:1:1 Mixture



1:5:2:10 Mixture



- Peptides have the same mass from each of the samples
- MS/MS of selected mass yields
    - Fragmentation spectra for the identification of peptide
    - Reporter group gives relative abundance information

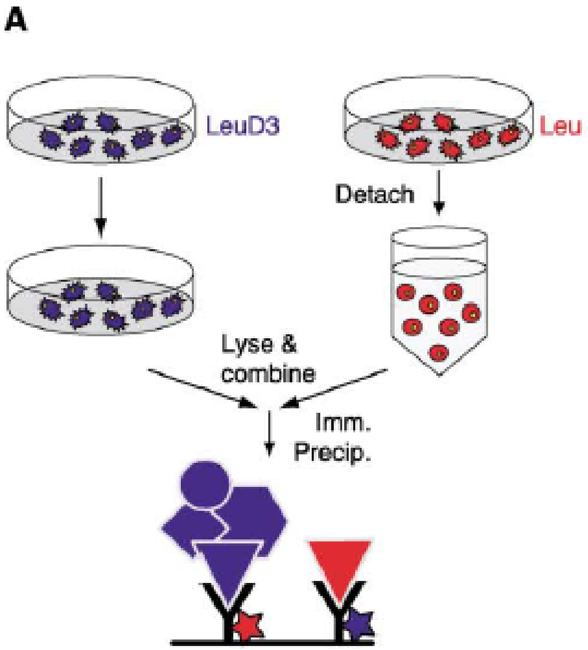
Ross, P. L. et al. (2004) Mol Cell Proteomics **3**(12): 1154-69

SILAC

Ong SE, Blagoev B, Kratchmarova I, Kristensen DB, Steen H, Pandey A, Mann M.

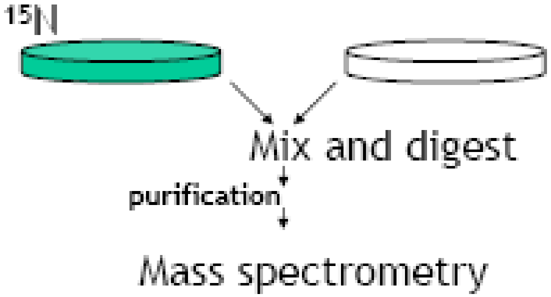
Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics.

Mol Cell Proteomics. 2002 May;1(5):376-86.



- Label light / heavy cultures (Leu d0 / d3)
- Stimulate heavy cells
- Mix cells or lysates
- Purify fraction of interest
- Analyse by LC-MS/MS (->ID)
- Quantify signals of ion pairs

Stable Isotope Labeling with Amino acids in Cell culture (SILAC)



^{15}N

Mix and digest

purification

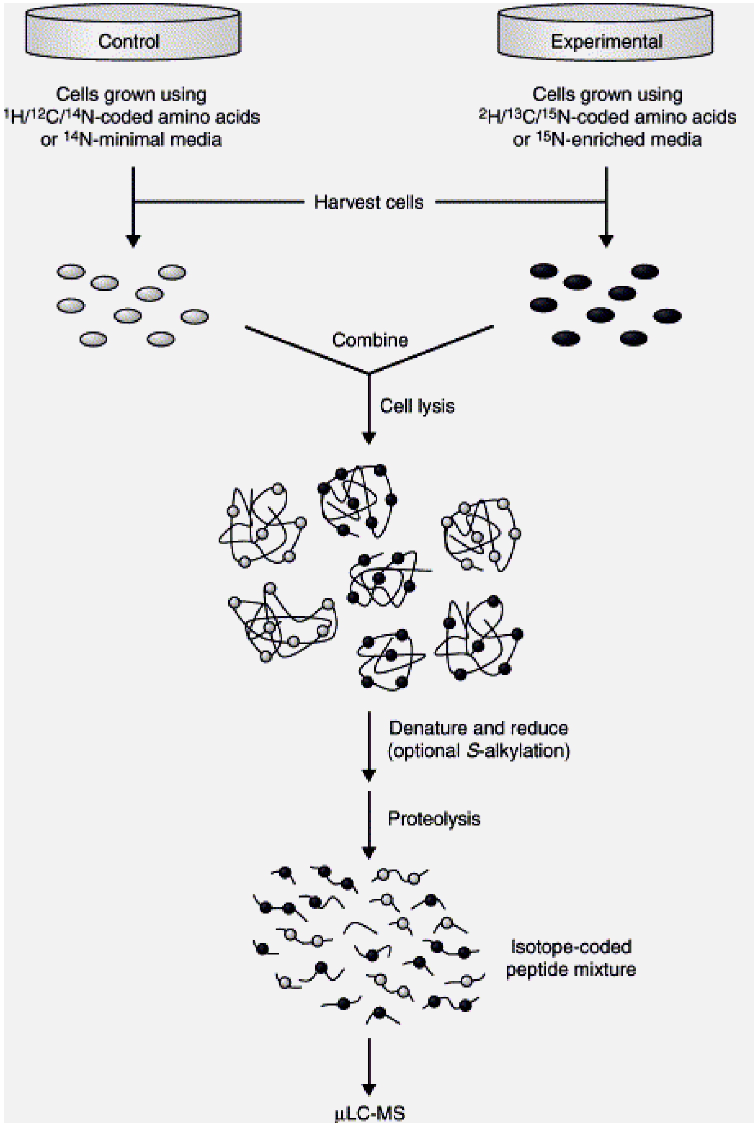
Mass spectrometry

- One of the most accurate procedures for quantitation of proteins

- Each peptide produced by the cell and cleaved by enzymatic digestion will have an isotopically identical internal standard

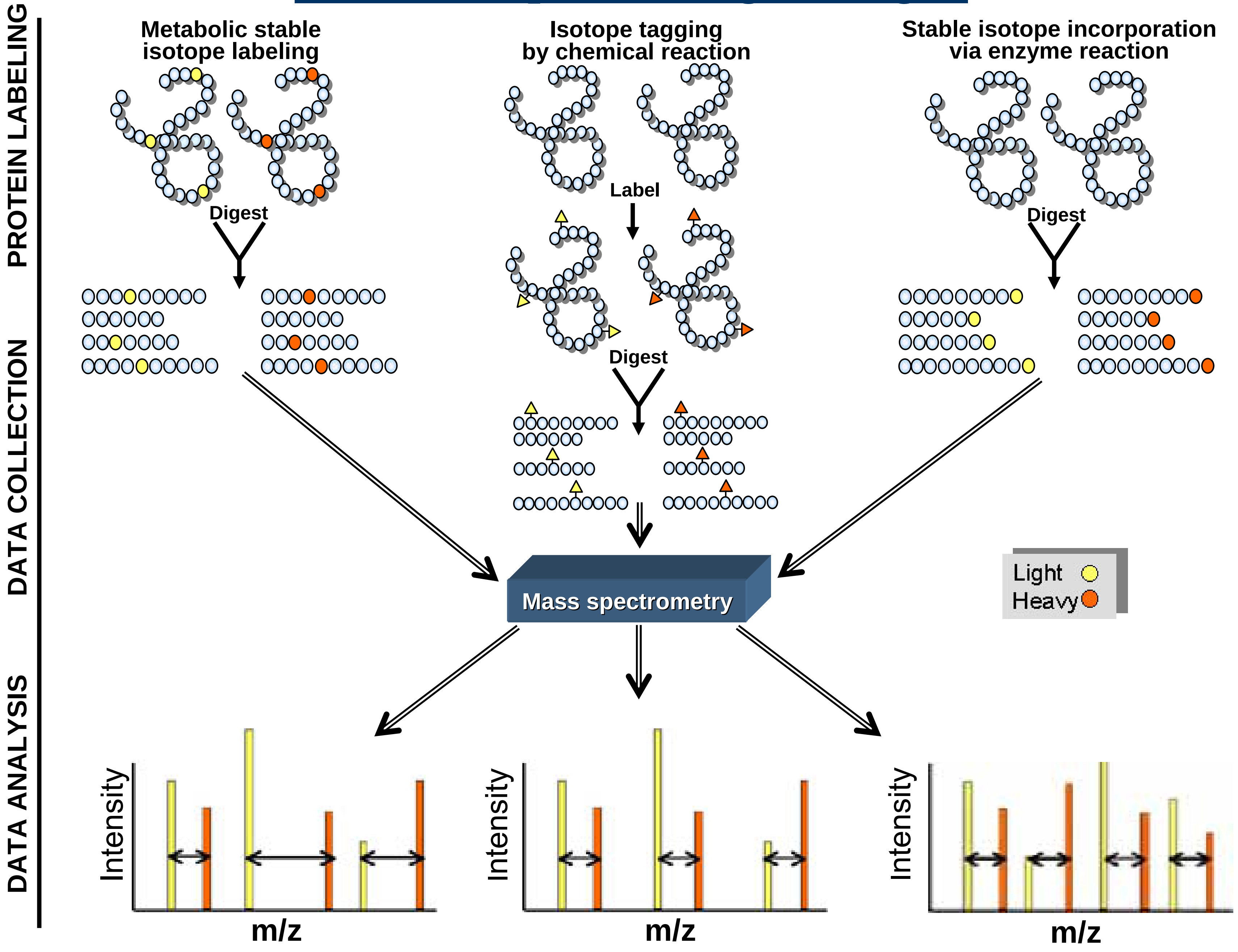
- Works best with cell lines only

Stable Isotope Amino Acid or ^{15}N- *in vivo* Labeling



- Metabolic stable isotope coding of proteomes
- An equivalent number of cells from 2 distinct cultures are grown on media supplemented with either normal amino acids or ^{14}N-minimal media, or stable isotope amino acids (^{2}D/^{13}C/^{15}N) or ^{15}N-enriched media.
- These mass tags are incorporated into proteins during translation.

Stable Isotope Labeling Strategies



PROTEIN LABELING

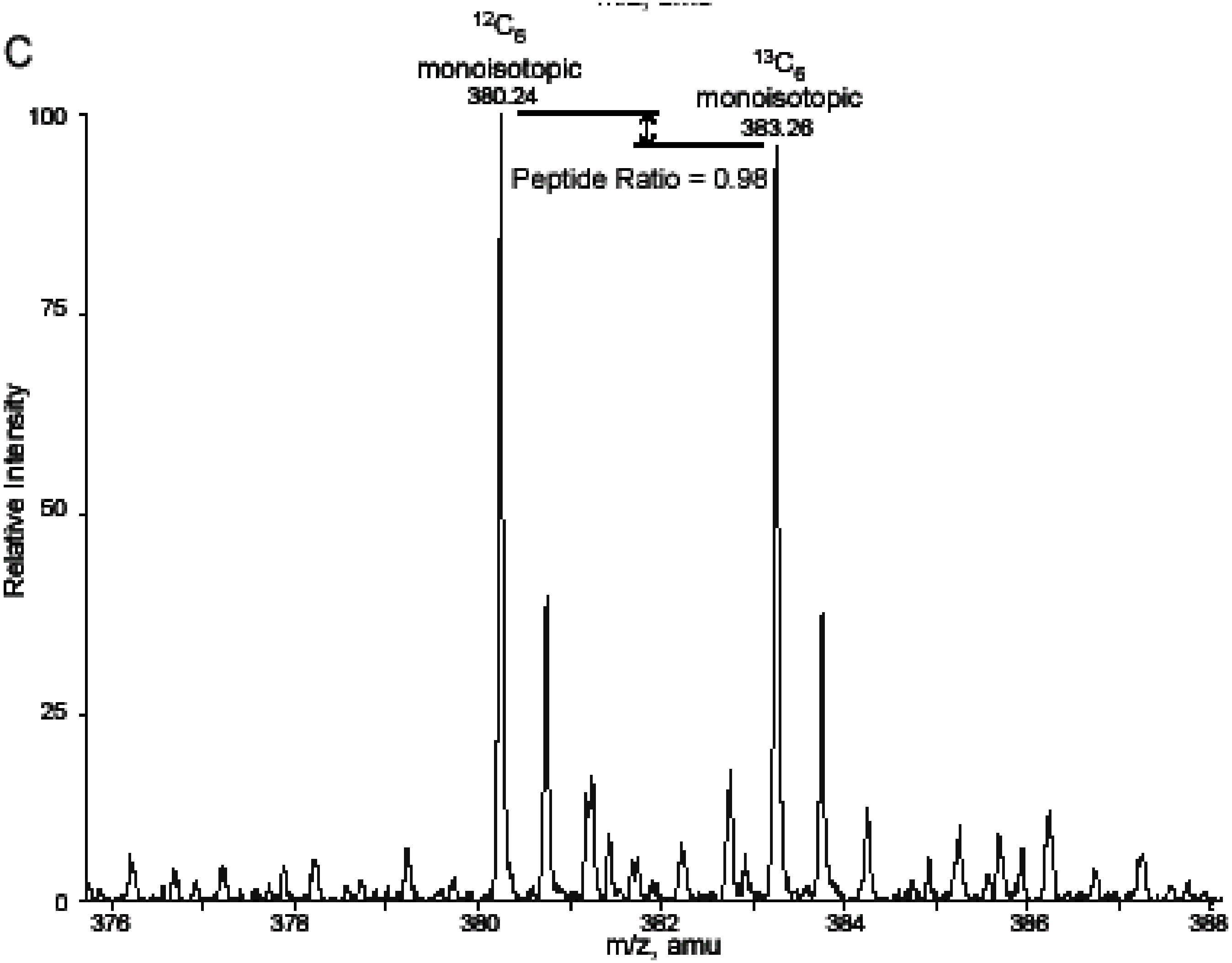
DATA COLLECTION

DATA ANALYSIS

Metabolic stable isotope labeling

Isotope tagging by chemical reaction

Stable isotope incorporation via enzyme reaction


C
$^{12}C_6$
monoisotopic
380.24
$^{13}C_6$
monoisotopic
383.26
Peptide Ratio = 0.98
Relative Intensity
100
75
50
25
0
376
378
380
382
384
386
388
m/z, amu

SILAC (+) and (-)

+

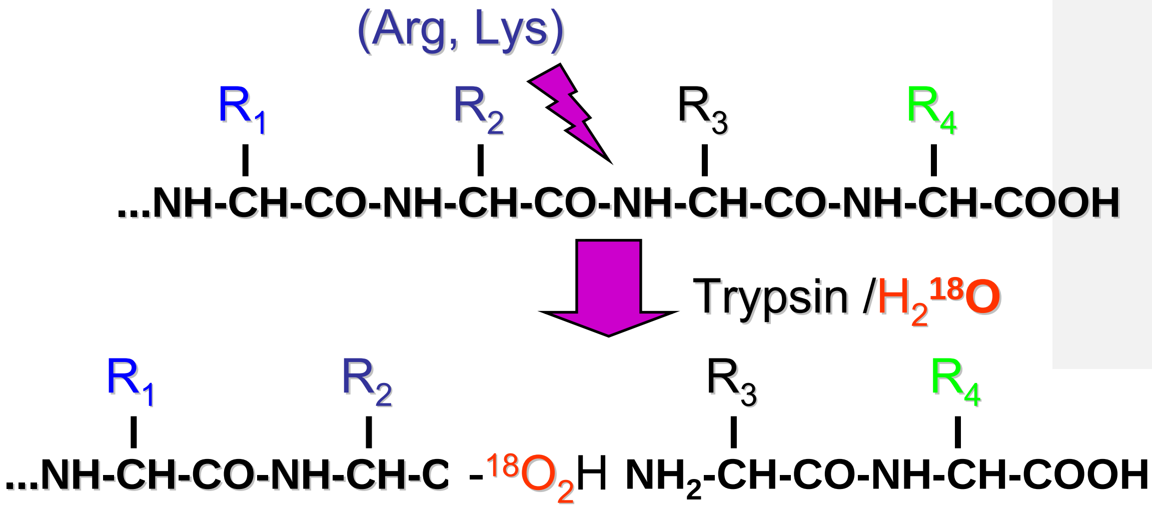
- relative protein quantification by MS
- eliminate praparative variability by mixing samples immediately after culture
- eliminate analytical variability
- peptides in native state (no side reactions)

-

- protein quantification unreliable for very weak signals
- mass shift variable (dependent on number of residues)
- only feasible with organisms in culture

Enzymatic Stable Isotope Coding of Proteomes

- Enzymatic digestion in the presence of ^{18}O-water incorporates ^{18}O at the carboxy-terminus of peptides
- Proteins from 2 different samples are enzymatically digested in normal water or $H_2{}^{18}O$.



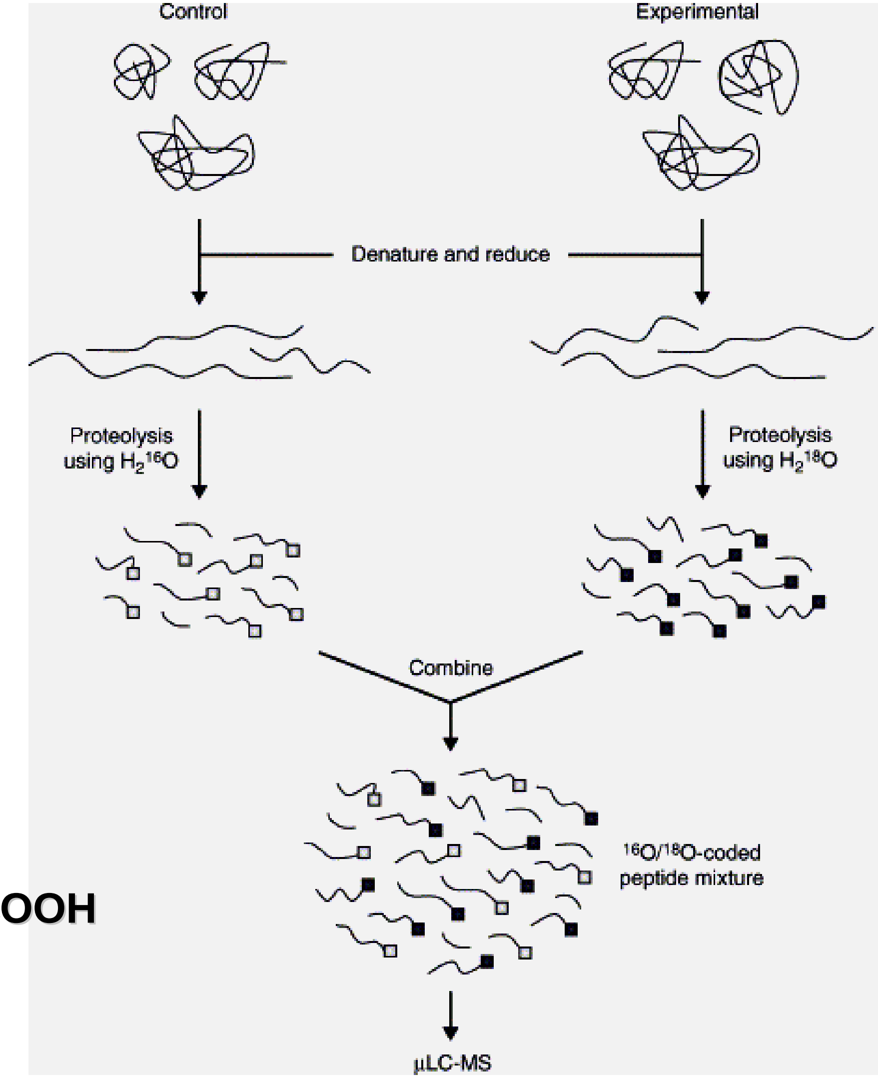
(Arg, Lys)

$$\cdots\mathrm{NH\text{-}\overset{R_1}{CH}\text{-}CO\text{-}NH\text{-}\overset{R_2}{CH}\text{-}CO\text{-}NH\text{-}\overset{R_3}{CH}\text{-}CO\text{-}NH\text{-}\overset{R_4}{CH}\text{-}COOH}$$

Trypsin /$H_2{}^{18}O$

$$\cdots\mathrm{NH\text{-}\overset{R_1}{CH}\text{-}CO\text{-}NH\text{-}\overset{R_2}{CH}\text{-}C}\ \text{-}{}^{18}\mathrm{O_2H}\ \ \mathrm{NH_2\text{-}\overset{R_3}{CH}\text{-}CO\text{-}NH\text{-}\overset{R_4}{CH}\text{-}COOH}$$

C-terminal peptide



Advantages vs. Disadvantages

- Estimates relative protein levels between samples with a reasonable level of accuracy (within 10%)
- Can be used on complex mixtures of proteins
- Cys-specific label reduces sample complexity
- Peptides can be sequenced directly if tandem MS-MS is used

- Yield and non specificity
- Slight chromatography differences
- Expensive
- Tag fragmentation
- Meaning of relative quantification information
- No presence of cysteine residues or not accessible by ICAT reagent