# BMG 744 Proteomics-Mass Spectrometry

# Quantitative analysis of the proteome

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## Proteomics Data Standards

- 2005 MCP - Paris guidelines
- 2008 HUPO – MIAPE (Minimum Information About a Proteomics Experiment) and mzML
- 2008 NCI - Amsterdam principles
  - (1) timing, (2) comprehensiveness, (3) format, (4) deposition to repositories, (5) quality metrics, and (6) responsibility for proteomics data release.
- 2011 NCI – Sydney
  - For users of public data
  - Reviewers of journals
  - Multi-site projects with unpublished data
- 2013 HUPO Proteomics Standards Initiative
  - http://www.psidev.info/

Kissinger et al MCP 10:1-9, 2011

# Proteomics Data Standards

- Common descriptive terms
- Sufficient experimental description
- Data format
- Data quality
  - Mass accuracy (evidence of calibration)
  - Repeatability (technical and biological replicates)
  - False discovery rate (MRM and pseudoMRM)
  - Degeneracy of MRM
  - # of peptides to make a match
- Reference materials

Kissinger et al MCP 10:1-9, 2011

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# Quantitative proteomics

Use of isotopes

- ICAT ($d_o/d_8$) and ICAT $^{13}C_0/^{13}C_8$
- $d_0/d_{10}$ propionic anhydride (N-terminal labeling)
- $^{15}N/^{14}N$ (whole cell labeling)
- $^{18}O/^{16}O$ (trypsin)
- iTRAQ labeling

- Non-isotope methods
  - Peptide coverage
  - Classical triple quadrupole methods (MRM)

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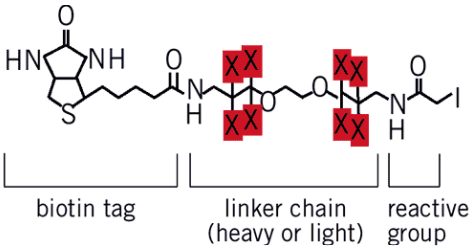
# Isotope-coded affinity technology

**Isotope-Coded Affinity Tags**

heavy reagent: D8-ICAT Reagent (X=deuterium)
light reagent: D0-ICAT Reagent (X=hydrogen)




**This reagent reacts with cysteine-containing proteins (80-85% of proteome)**

**Labeling can be replacement of hydrogens (X) with deuterium, or better to exchange $^{12}C$ with $^{13}C$ in the linker region (this avoids chromatography issues)**

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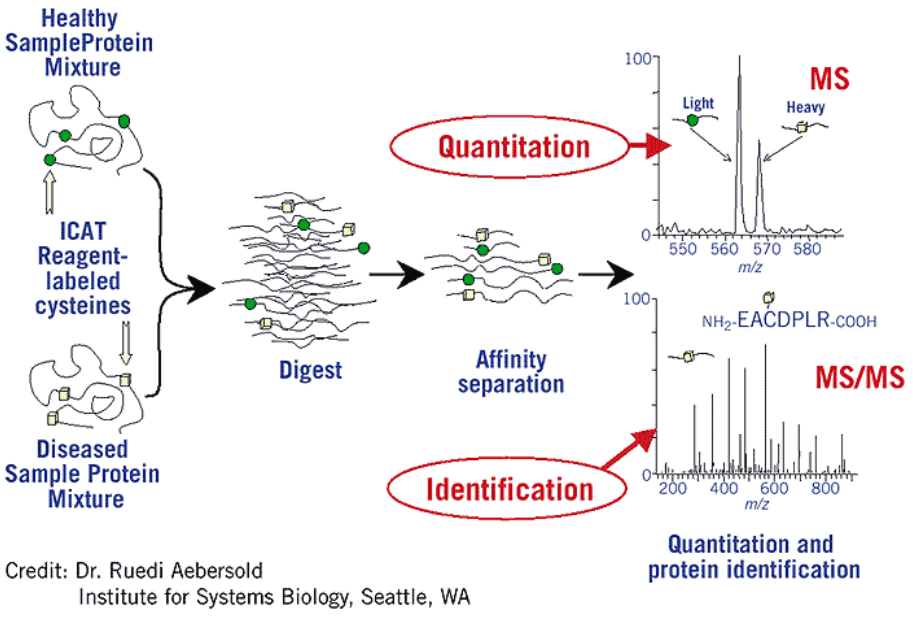


Credit: Dr. Ruedi Aebersold
Institute for Systems Biology, Seattle, WA

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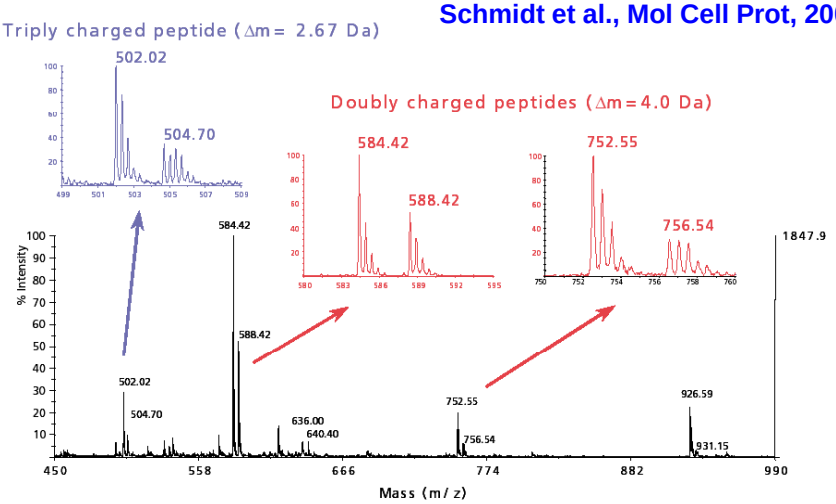
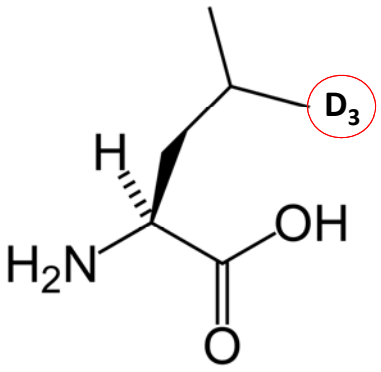
# Quantification from ESI-mass spectrum

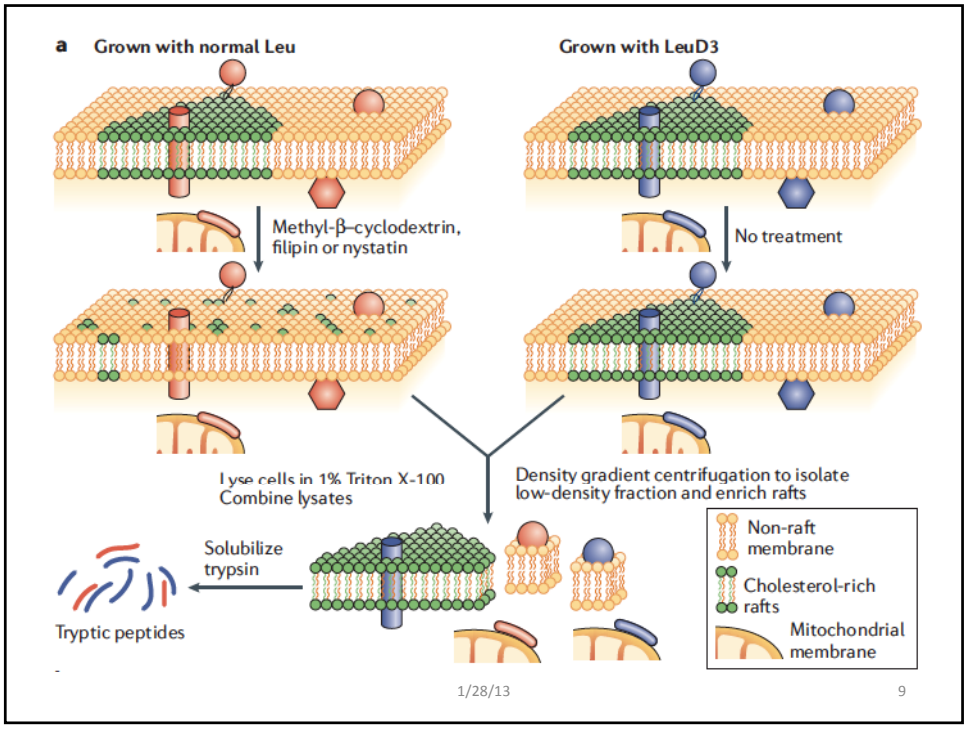
Schmidt et al., Mol Cell Prot, 2003






# Quantification with isotopically labeled amino acids




a
Grown with normal Leu
Grown with LeuD3
Methyl-β–cyclodextrin, filipin or nystatin
No treatment
Lyse cells in 1% Triton X-100
Combine lysates
Density gradient centrifugation to isolate low-density fraction and enrich rafts
Solubilize trypsin
Tryptic peptides
Non-raft membrane
Cholesterol-rich rafts
Mitochondrial membrane
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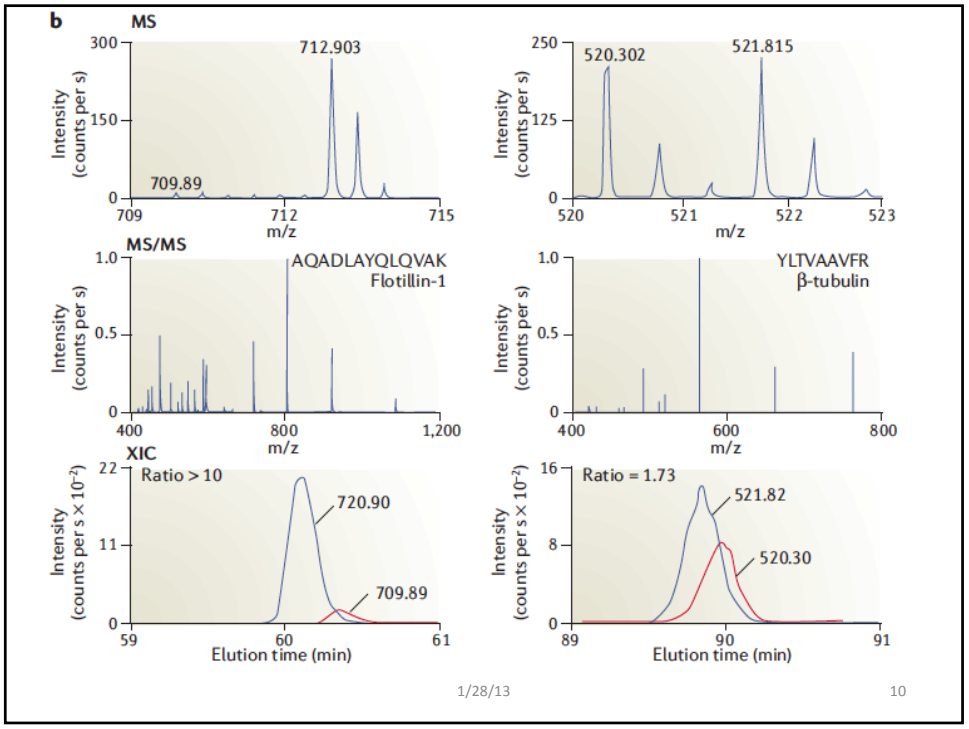
b
MS
712.903
709.89
520.302
521.815
Intensity (counts per s)
m/z
MS/MS
AQADLAYQLQVAK
Flotillin-1
YLTVAAVFR
β-tubulin
XIC
Ratio > 10
720.90
709.89
Ratio = 1.73
521.82
520.30
Intensity (counts per s × 10⁻²)
Elution time (min)
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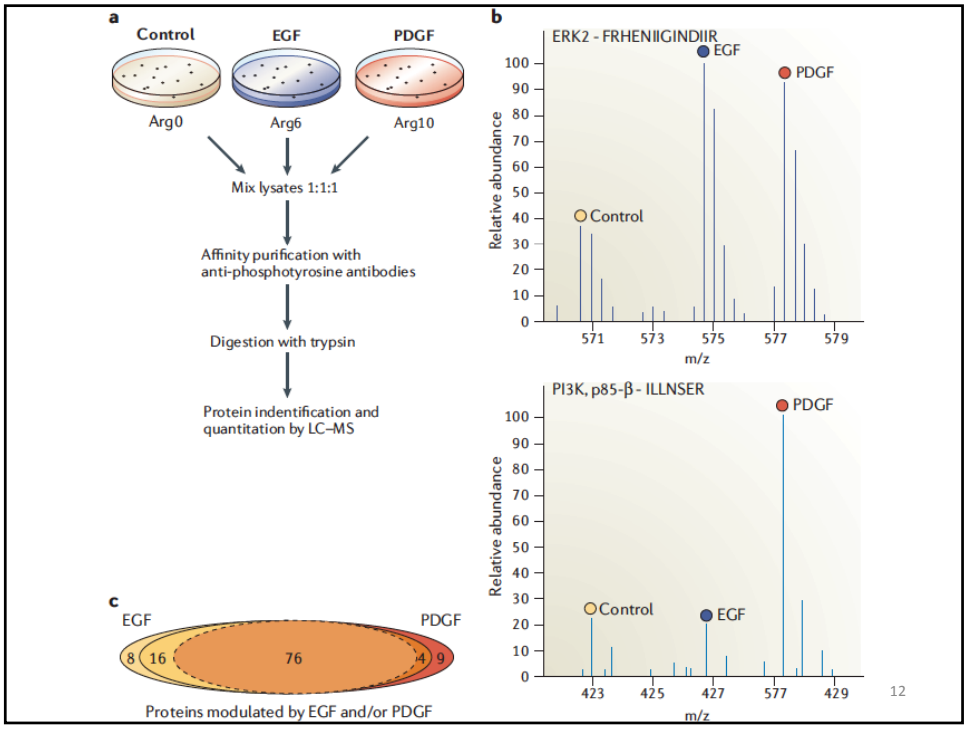
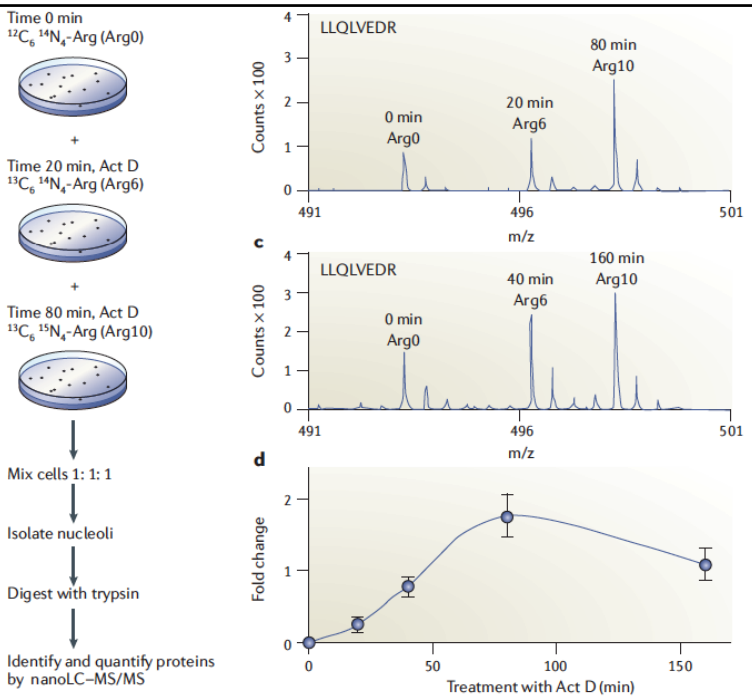
# Arginine-$^{13}C/^{15}N$ isotopic labeling





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# Isotope labeling with $^{13}C^{15}N$-lysine

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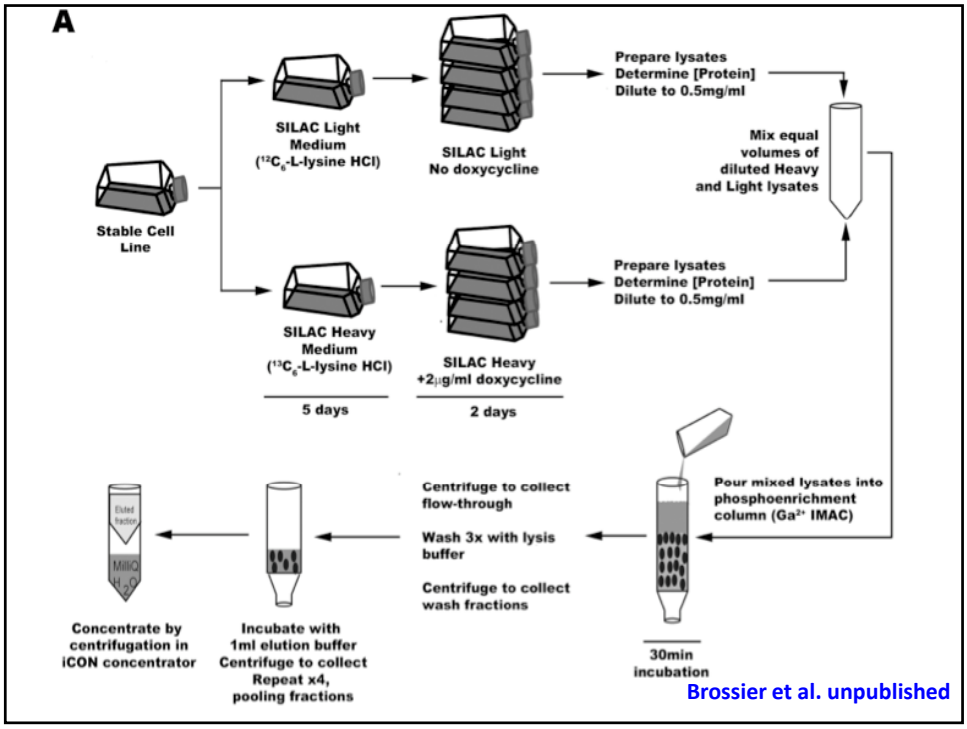
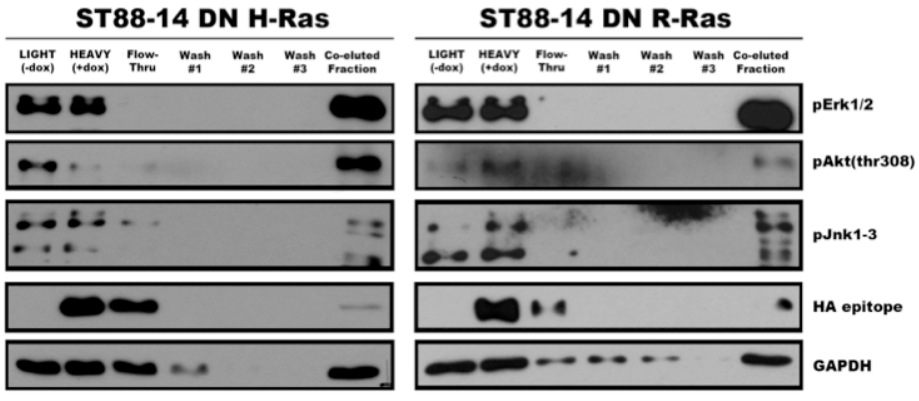
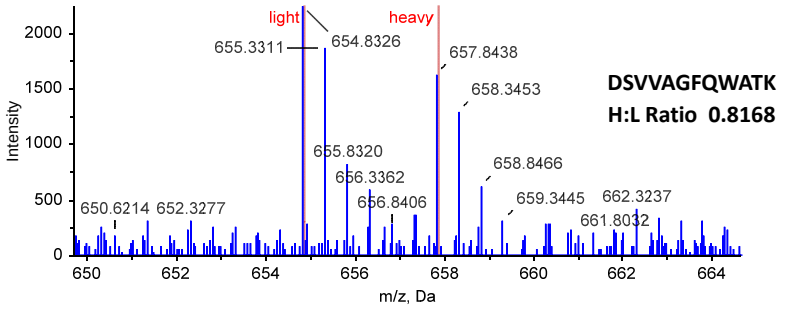


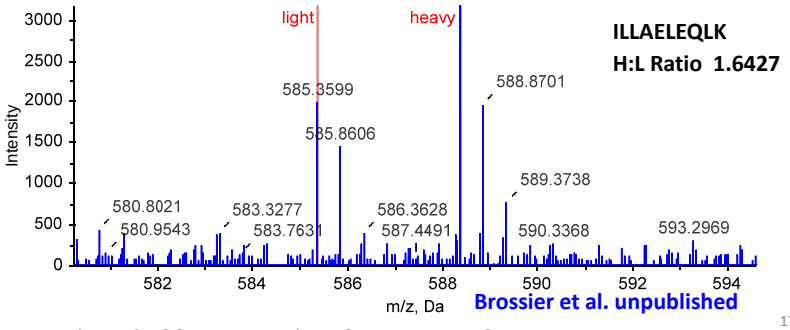

# Verifying absorption of phosphoproteins onto IMAC




Brossier et al. unpublished




Elongation factor 2 OS=Homo sapiens GN=EEF2 PE=1 SV=4




Vimentin OS=Homo sapiens GN=VIM PE=1 SV=4

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# $^{18}$O-labeling

- Trypsin catalyzes the transfer of $^{18}$O in $^{18}$O-enriched water to both the carboxylate oxygens of the C-terminus of tryptic peptides

  $$R\text{-}COOH \longrightarrow R\text{-}C^{18}O_2H$$

- The peptides have an increase in mass of 4 Da
- Generally not considered a large enough mass difference

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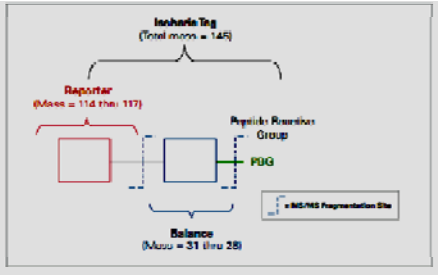
# Tandem mass tag reagents

- TMT reagents are isobaric, i.e., they have the same molecular weight and are chemically the same, but their "parts" have different masses
- Some reagents have four parts:
  - A mass reporter (different for each reagent)
  - A cleavable region
  - An isotopic balancing region
  - A lysine-$NH_2$ reacting reagent

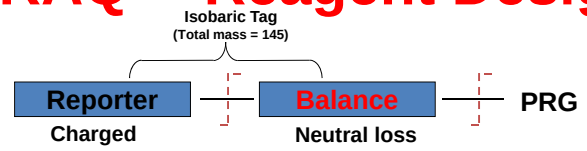


# iTRAQ quantification

- The iTRAQ™ reagents
  - React with Lys amino groups and each one adds 145 Da to the molecular weight of the peptide
  - Fragmentation produces reporter ions from *m/z* 114, 115, 116 and 117
  - Current iTRAQ kit contains 8 forms with reporter fragment ions of *m/z* 114, 115, 116, 117, 118, 119 and 121



# iTRAQ™ Reagent Design

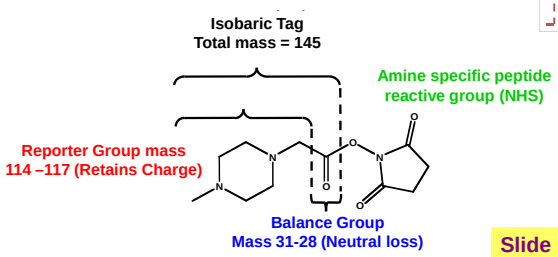



- Gives strong signature ion in MS/MS
- Gives good b- and y-ion series
- Maintains charge state
- Maintains ionization efficiency of peptide
- Signature ion masses lie in quiet region

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

Amine specific

= MS/MS Fragmentation Site




Slide provided by Applied Biosystems

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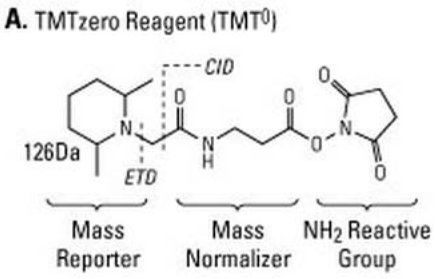
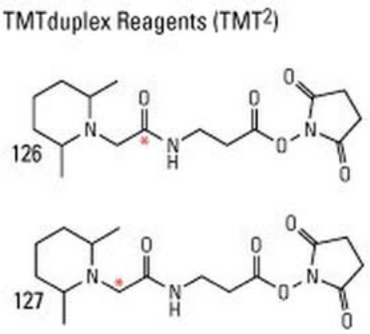
# TMT reagent from Pierce

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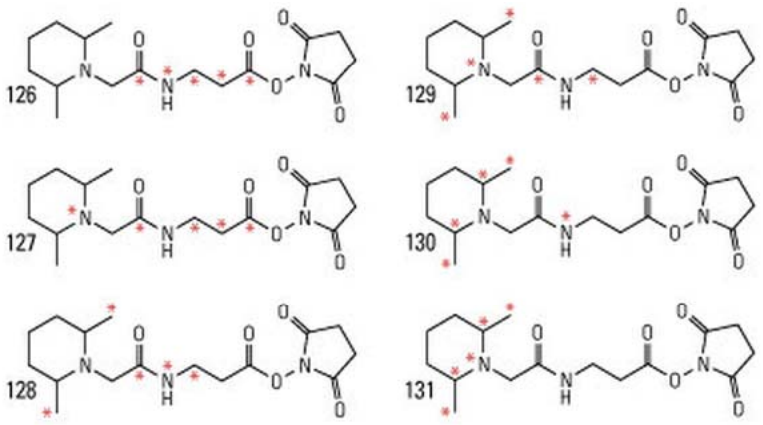
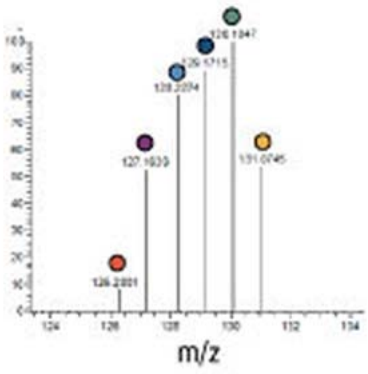
# A 6-plex TMT reagent






# MS/MS spectrum of TMT tags




The mass of the tryptic peptide when reacted with anyone of the TMT reagents is the same.

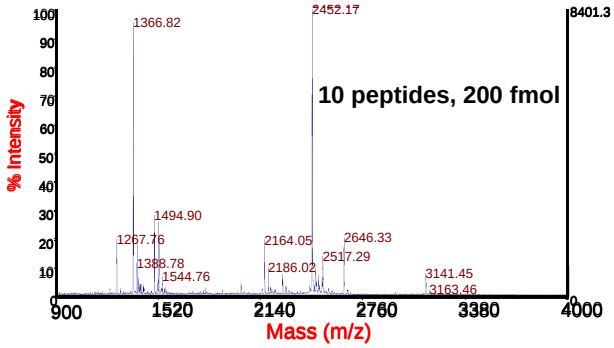
However, each reagent gives a separate reporter mass (m/z 126, 127, 128, 129, 130 and 131.

Therefore, samples from different experimental conditions can be combined and analyzed in a single run.

# Other non-isotopic quantitative methods in proteomics

**The coverage (the number of peptides observed for a protein) is sensitive to the amount of the protein**

- **This can be used to calculate whether a treatment affects the abundance of a protein where fold-change > 2**
- **Applies to LC-MS (MUDPIT methods)**






# Method of the Year 2012

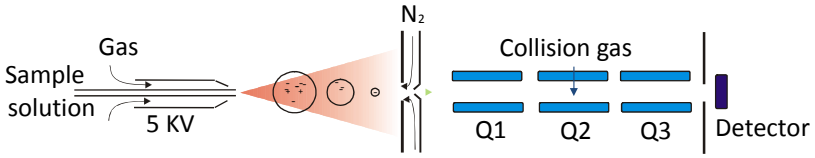
New method and tool developments are helping to bring targeted proteome analysis technologies to a broader array of biologists.

**This quote comes from the January 2013 issue of Nature Methods. It noted there are several methods for measuring proteins (antibodies, immunofluorescence, protein arrays)**

> But there is another way. Mass spectrometry, perhaps most familiar for its use in discovery-based proteomics, can also be applied to specifically analyze target proteins of interest. In the most mature technology for targeted analysis, known as selected (or multiple) reaction monitoring, a mass spectrometer called a triple quadrupole is programmed to detect specific peptides that uniquely represent proteins of interest, allowing researchers to quantitatively monitor these proteins with high sensitivity and reproducibility.

# Triple quad MRM analysis

**Peptides of interest can be analyzed like small molecules**

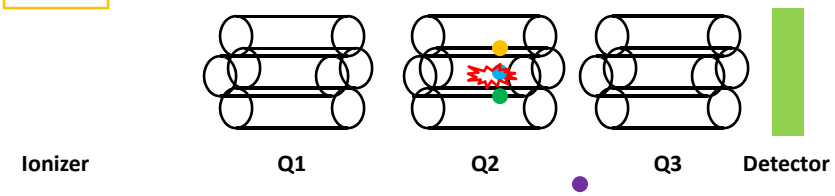



- **Multiple reaction ion scanning**
  - **First filter the $[M+nH]^{n+}$ precursor ion of the analyte (Q1)**
  - **Fragment the precursor ion with $N_2$ gas (Q2)**
  - **Select a specific (and unique) product ion (Q3)**
  - **Measure ion current reaching the detector for 20-50 msec**
  - **Repeat with a precursor/product ion combination for another peptide, etc.**



# Multiple reaction ion monitoring

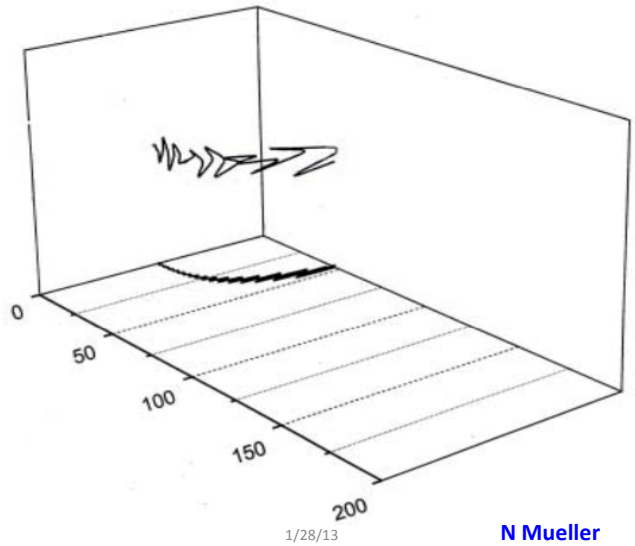

**Quantitative analysis of peptides in a complex mixture carried out using a triple quadrupole instrument**




**Based on precursor ion/product ion pair(s)**

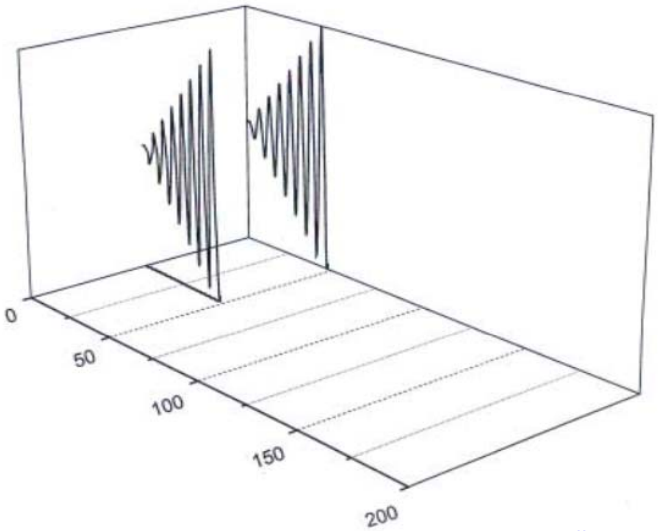
 *Courtesy, John Cutts* 

## Flight path of ions through the quadrupole
## ion mass is higher than the set mass

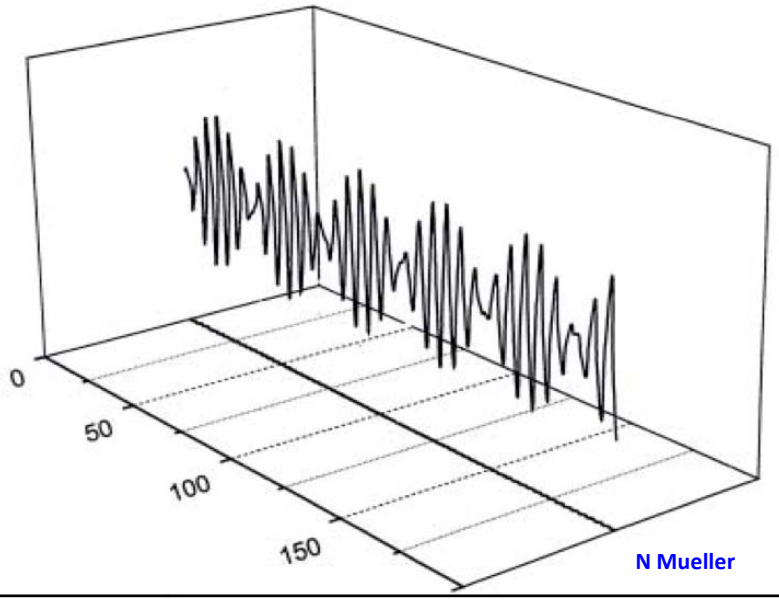





## Flight path of ions through the quadrupole
## ion mass is lower than the set mass

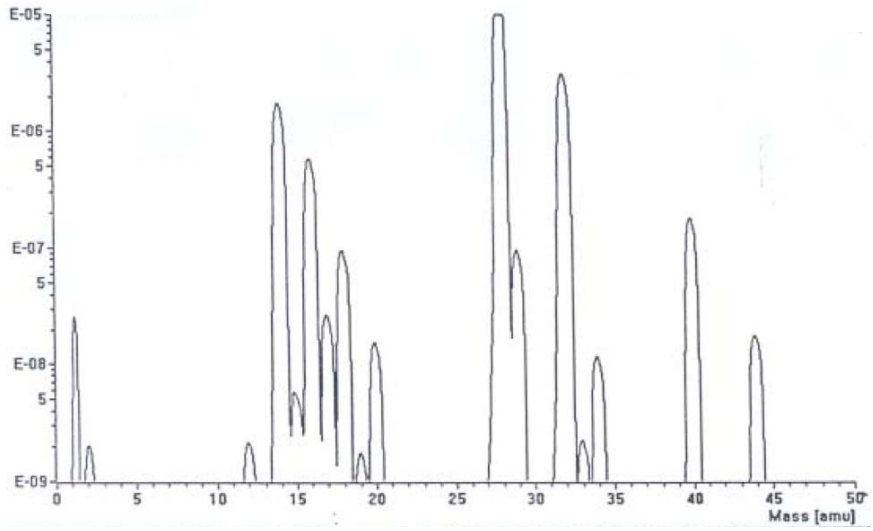


## Flight path of ions through the quadrupole
## ion mass is the same as the set mass




N Mueller

## The real quadrupole ions
## 0.7 m/z wide

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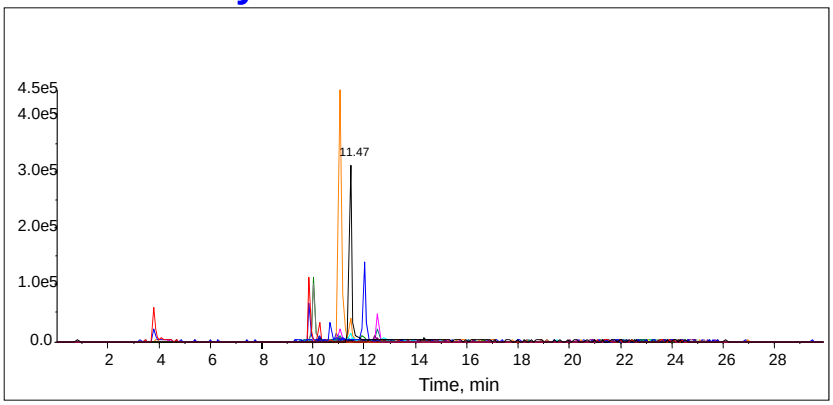
# Quantitation experiment for biotinylated cytochrome c

## MRM analysis monitored in 50 channels

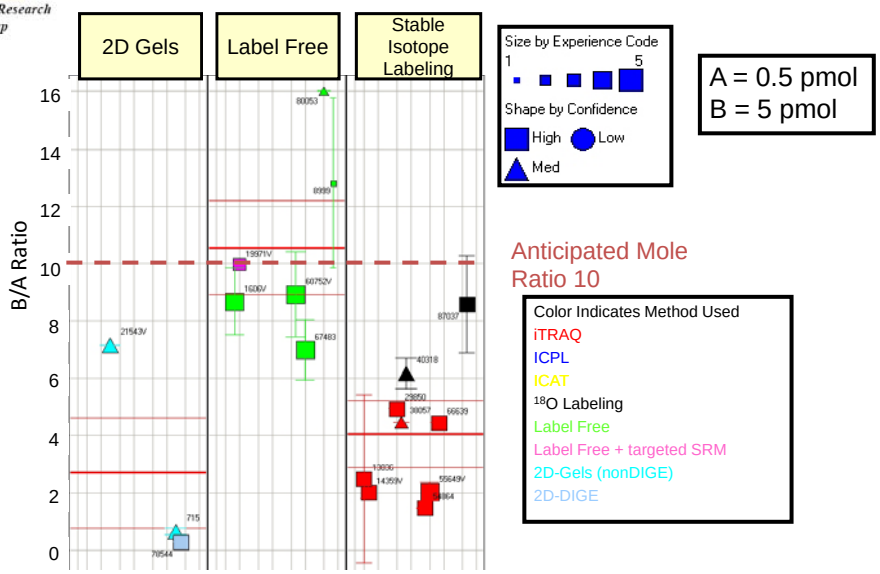



**Each colored peak represents a different biotinylated peptide**

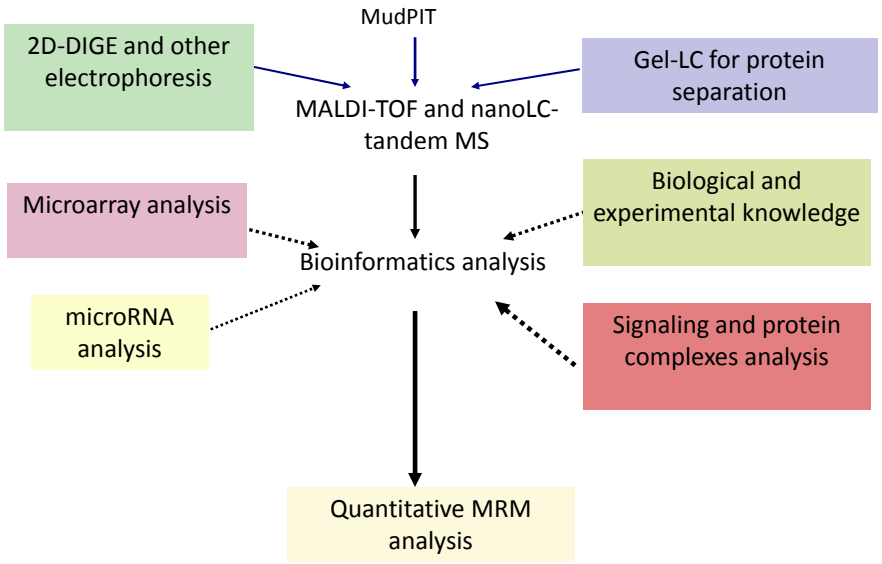


# Quantitative Accuracy: Myoglobin

Proteomics Research Group



## Workflow for generation of proteomics data

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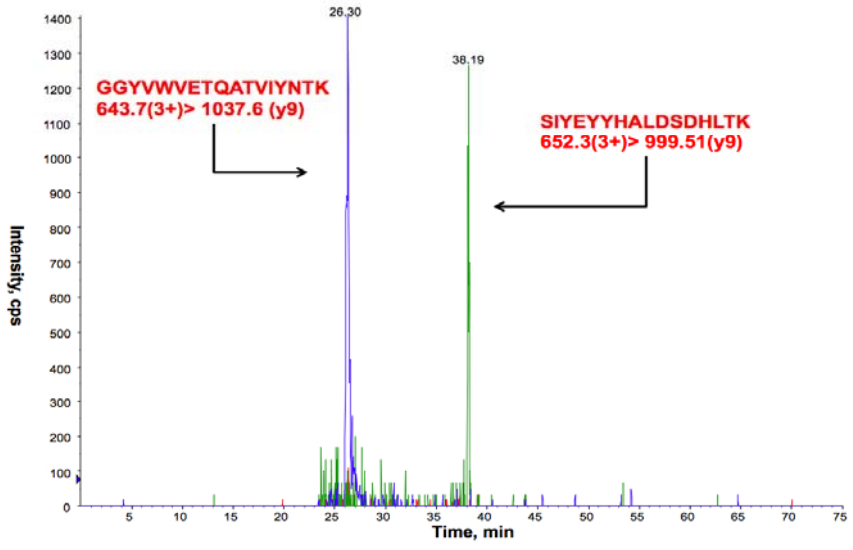
## HIF-1α in kidney cytosol by LC-MRM-MS

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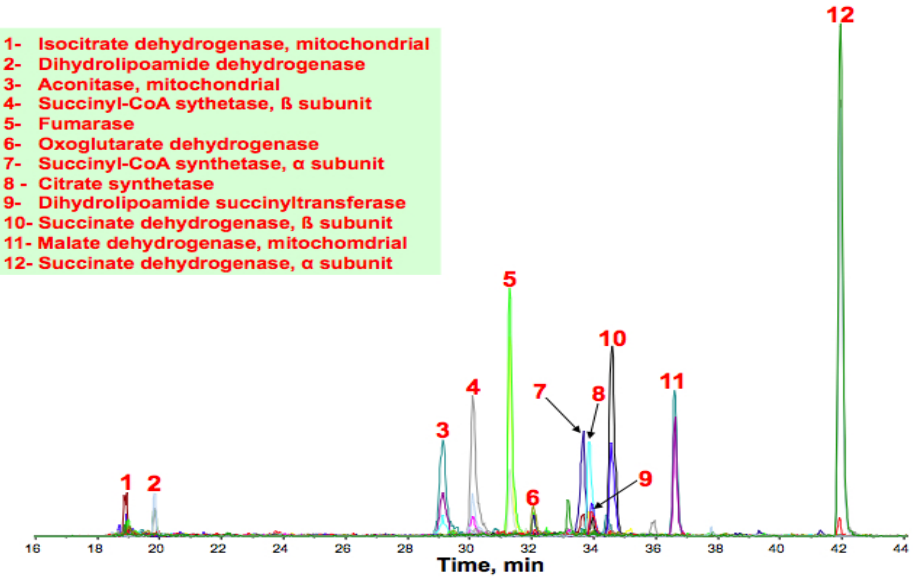
## Multiple reaction ion monitoring of Krebs cycle enzymes

1- Isocitrate dehydrogenase, mitochondrial
2- Dihydrolipoamide dehydrogenase
3- Aconitase, mitochondrial
4- Succinyl-CoA sythetase, ß subunit
5- Fumarase
6- Oxoglutarate dehydrogenase
7- Succinyl-CoA synthetase, α subunit
8 - Citrate synthetase
9- Dihydrolipoamide succinyltransferase
10- Succinate dehydrogenase, ß subunit
11- Malate dehydrogenase, mitochomdrial
12- Succinate dehydrogenase, α subunit

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## Splicing generates a new sequence at the junction between exon 4 and exon 6

MDFWLWPLYFLP

VSGALRILPEVKVEGELGGSVTIKCPLPEMHVRIYLCREMAGSGTCGTVVSTTNFI

KAEYKGRVTLKQYPRKNLFLVEVTQLTESDSGVYACGAGMNTDRGKTQKVTLNVHS

EYEPSWEEQPMPETPKWFHLPYLFQMPAYASSSKFVTR

VTTPAQRGKVPPVHHSSPTTQITHRPRVSRASSVAGDK

PRTFLPSTTASKISALEGLLKPQTPSYNHHTRLHRQR Exon 4

ALDYGSQSGREGQGFHILIPTILGLFLLALLGLVVKRAVERRK

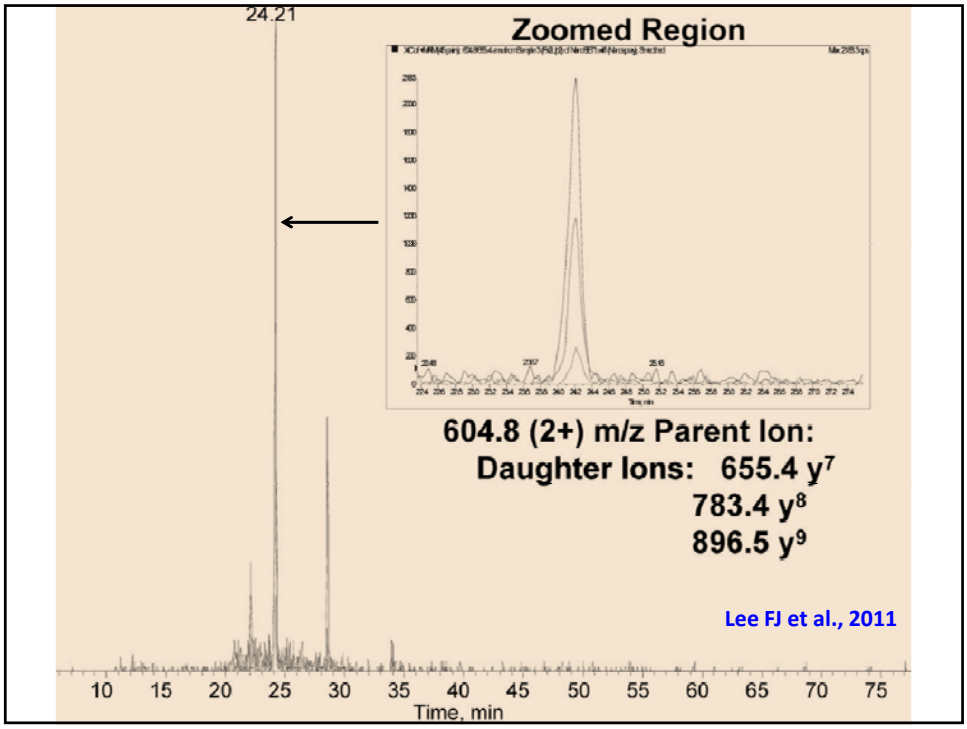
ALSRRARRLAVRMRALESSQRPRGSPRPRSQNNIYSACPRRARGADAA

P·QAGPPTGREDA·PG·LPEA··VAATALPKQHLQRLPAA·SWSGRCR Exon 6

GTGEAPVPGPGAPLPPAPLQ

HR·GPRSRPRS·VA·RPAAG

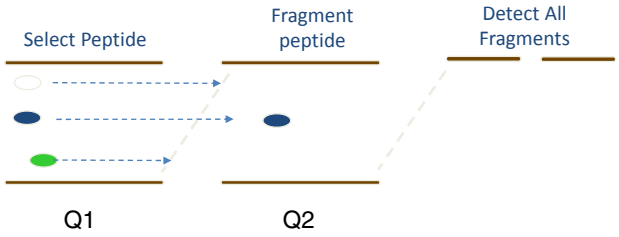
VSESPWLHAPSLKTSCEYVSLYHQPAAMMEDSDSDDYINVPA




# Limitations of MRM-MS

- A single precursor/product ion combination is not sufficiently specific (see class on MRMPath)
  - Need 3-4 product ions to provide specificity
  - This decreases the number of <u>different</u> peptides that can be monitored per second
- The quadrupole analyzer has a low mass accuracy
  - Typically the peak is passed through a 0.7 *m/z* filter
- In an ideal world, we need an MS instrument that can collect high mass accuracy (2-3 ppm) MSMS spectra in 20-50 msec

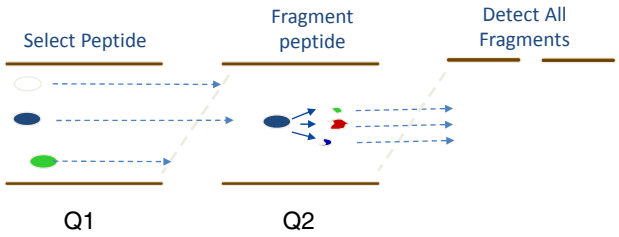
# Pseudo MRM Analysis




- **High resolution TOF Analyzer for detection of fragment ions**

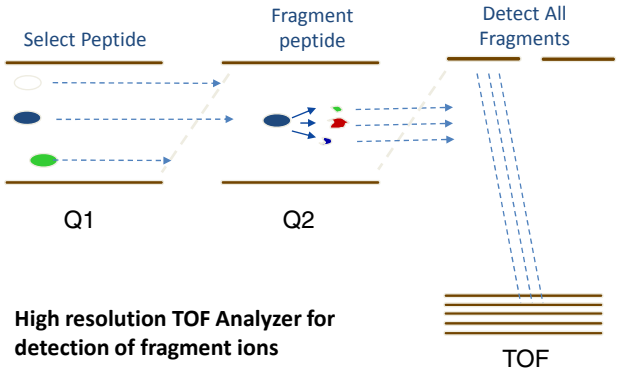



# Pseudo MRM Analysis




- **High resolution TOF Analyzer for detection of fragment ions**

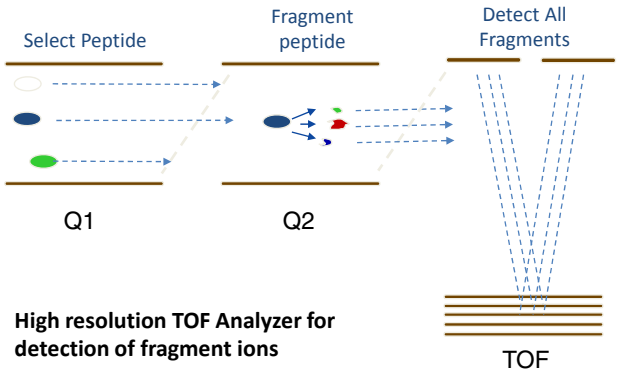
# Pseudo MRM Analysis




- **High resolution TOF Analyzer for detection of fragment ions**

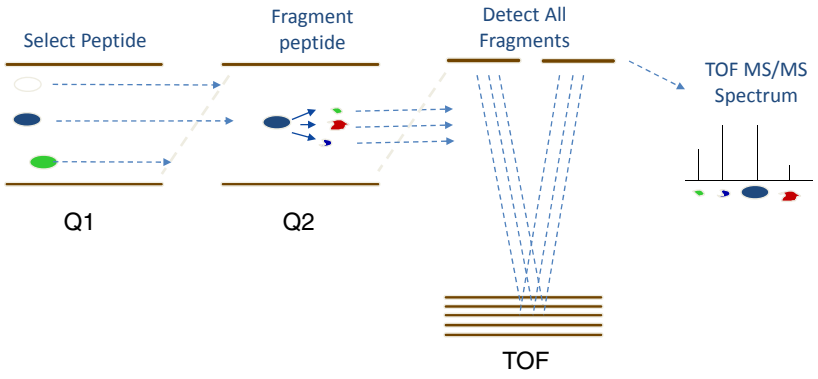


# Pseudo MRM Analysis




- **High resolution TOF Analyzer for detection of fragment ions**

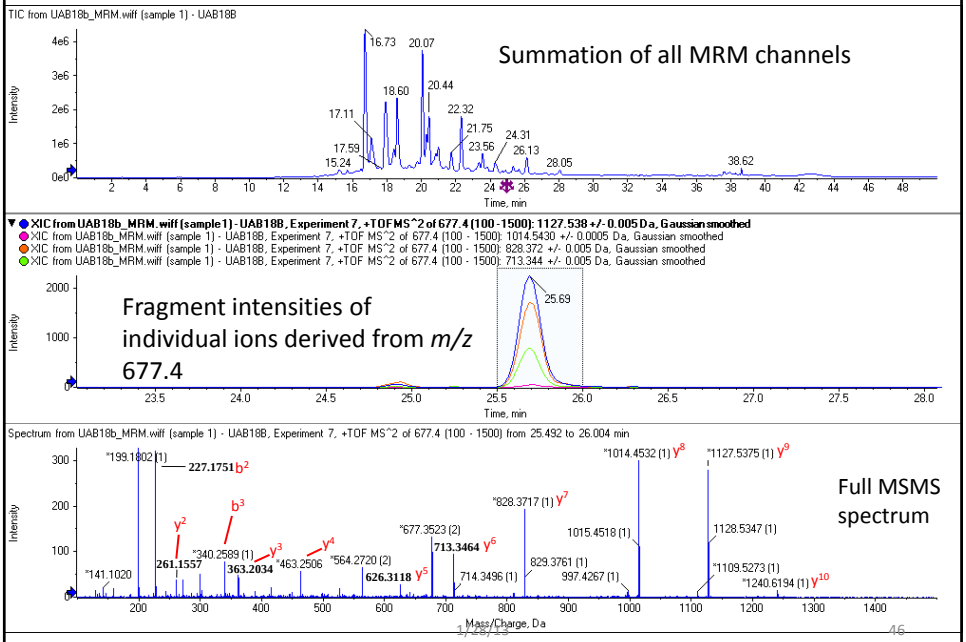
# Pseudo MRM Analysis




- **The key difference between the TripleTOF and the triple quad is that the entire MSMS spectrum is collected by the TripleTOF in a single 50 sec (or shorter) data acquisition – the selection of product ion to follow is made post-data acquisition**



MS/MS Fragmentation of **LIIWDSYTTNK**
Found in **gi|11055998**, guanine nucleotide-binding protein, beta-4 subunit [Homo sapiens]



# Verifying and quantifying C-truncation

- αA crystallin is supposedly processed to a 173aa form from the 196aa translated product. Interestingly, what we see is the removal of an interior 23aa peptide, so it must be differential splicing, not posttranslational processing.
- Processed rat αA crystallin has a chymotrypsin cleavage site at $^{141}$Phe
- This peptide can be observed as a triply charged peptide
  - FSGPKVQSGLDAGHSERAIPVSREEKPSSAPSS
- The C-truncations observed by mass spectrometry imaging are the following:
  - SGPKVQSGLD (truncation at 151)
  - SGPKVQSGLDAGHSE (truncation at 156)
  - SGPKVQSGLDAGHSER (truncation at 157)
  - SGPKVQSGLDAGHSERAIPVSR (truncation at 163)
  - SGPKVQSGLDAGHSERAIPVSREEKPS (truncation at 168)

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# Fragmentation of a chymotryptic peptide

$NH_2$-SGPKVQSGLD-COOH

$[M+2H]^{2+}$ = 494.55

| b-ions | y-ions |
|---|---|
| $b_1$ = - | $y_1$ = (*134*) |
| $b_2$ = 145 | $y_2$ = (*247*) |
| $b_3$ = 242 | $y_3$ = (*304*) |
| $b_4$ = 370 | $y_4$ = (*391*) |
| $b_5$ = 469 | $y_5$ = (*519*) |
| $b_6$ = 597 | $y_6$ = (*618*) |
| $b_7$ = 684 | $y_7$ = 746 |
| $b_8$ = 741 | $y_8$ = 843 |
| $b_9$ = 854 | $y_9$ = 900 |
| $b_{10}$ = 969 | $y_{10}$ = 987 |

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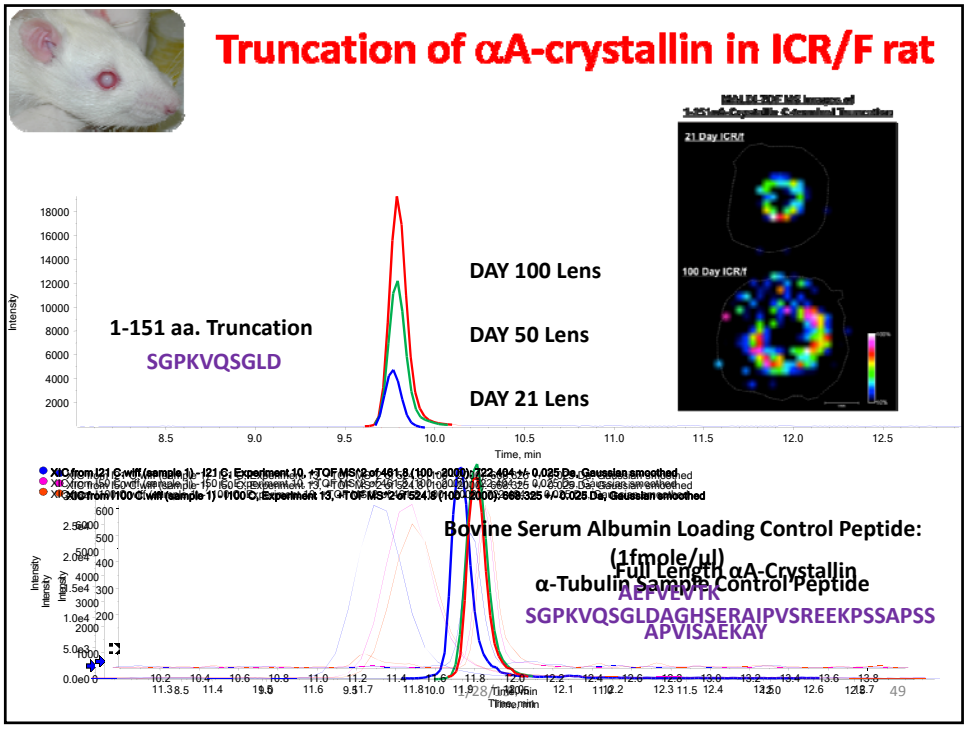
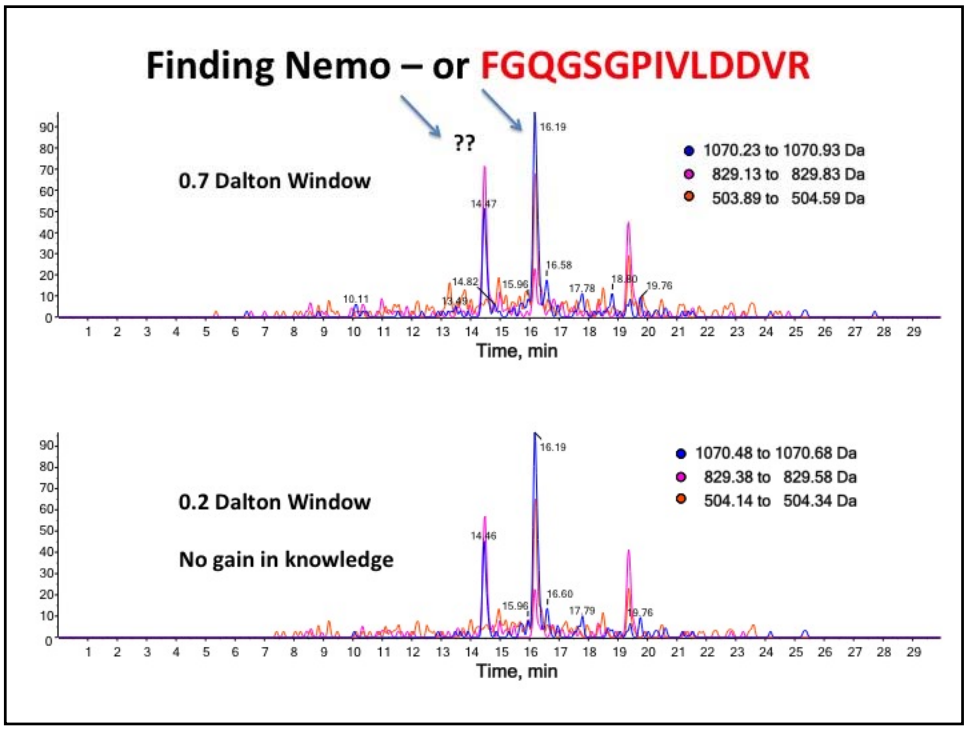
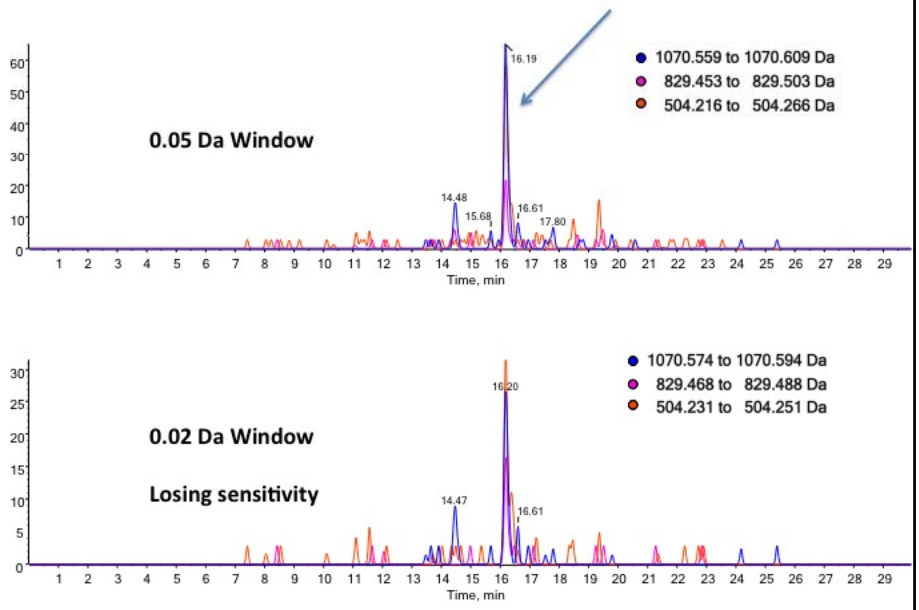
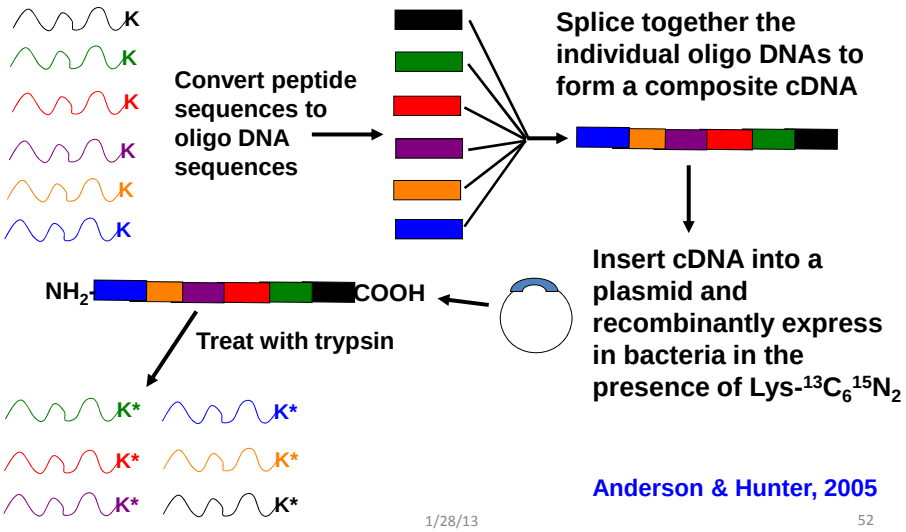

Truncation of αA-crystallin in ICR/F rat
1-151 aa. Truncation
SGPKVQSGLD
DAY 100 Lens
DAY 50 Lens
DAY 21 Lens
21 Day ICR/f
100 Day ICR/f
Intensity
Time, min
Bovine Serum Albumin Loading Control Peptide:
(1fmole/ul)
Full Length αA-Crystallin
α-Tubulin Sample Control Peptide
AEFVEVTK
SGPKVQSGLDAGHSERAIPVSREEKPSSAPSS
APVISAEKAY
128/11
49


Finding Nemo – or FGQGSGPIVLDDVR
??
0.7 Dalton Window
1070.23 to 1070.93 Da
829.13 to 829.83 Da
503.89 to 504.59 Da
16.19
14.47
16.58
14.82
15.96
17.78
18.80
19.76
10.11
13.99
Time, min
0.2 Dalton Window
No gain in knowledge
1070.48 to 1070.68 Da
829.38 to 829.58 Da
504.14 to 504.34 Da
16.19
14.46
16.60
15.96
17.79
19.76
Time, min

## Higher resolution identifies FGQGSGPIVLDDVR




## Concatenation - making $^{13}C$-labeled peptide internal standards




Anderson & Hunter, 2005

## Quantitative peptide MRM-MS

- The albumin-depleted plasma proteome is mixed with the composite $^{13}C$,$^{15}N$-labeled protein internal standard and then treated with trypsin
- The molecular ions (doubly charged) and the specific y ions for each peptide and its labeled form are entered into the MRM script one channel at a time
- A single run may consist of 30 peptides in 60 channels
- Sensitivity is compromised by "sharing out" measurement time, but can be compensated for by carrying out nanoLC



## The future – SWATH MS

- http://www.youtube.com/watch?v=VZAZtA_qEbg
- **Data independent analysis with a mass spectrometer that has a fast enough analyzer (TOF) to allow comprehensive quantitative analysis of ALL peptides that elute from a LC column**

# References for these talks (1)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