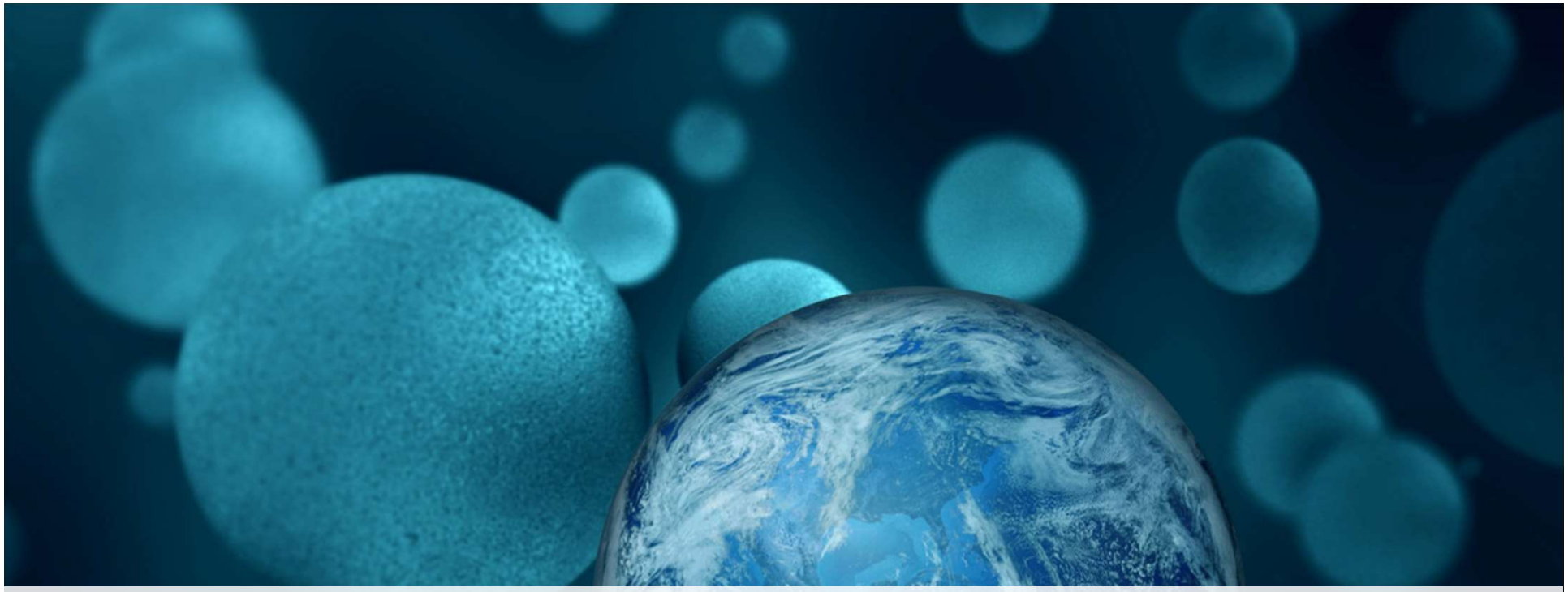


ThermoFisher SCIENTIFIC

Multiple studies with a single experiment:

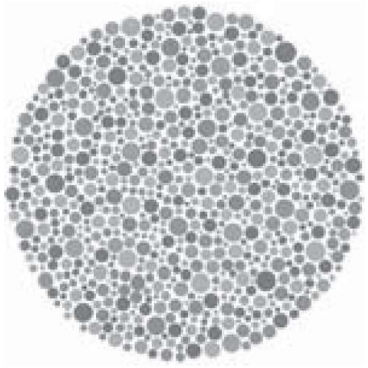
The Power of Quantitative Multiplexing

Jitnapa Voranitikul
LCMS Product Specialist, SciSpec Co., Ltd.

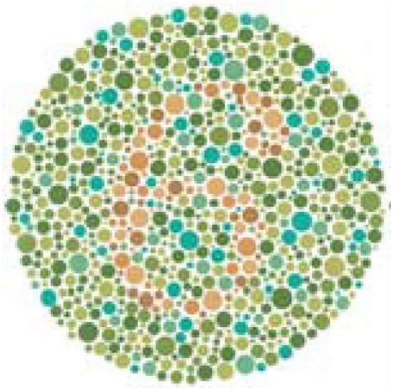
Moving Beyond Qualitative Proteomics

Problem: Quantitative information about expression level of a protein is essential to understanding its biological role in response to change or disease.

Qualitative

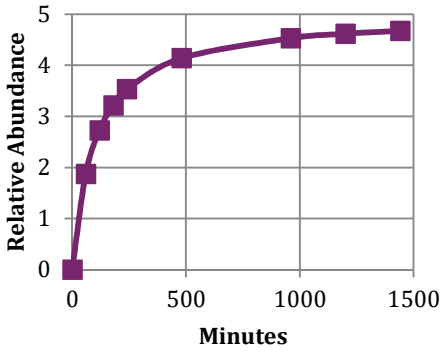


Quantitative

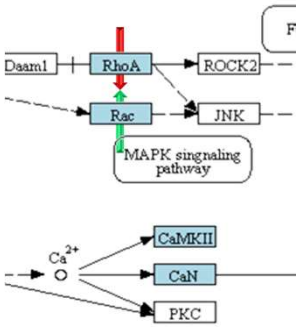


Add another dimension to any experiment by determining the relative abundance of each identified protein

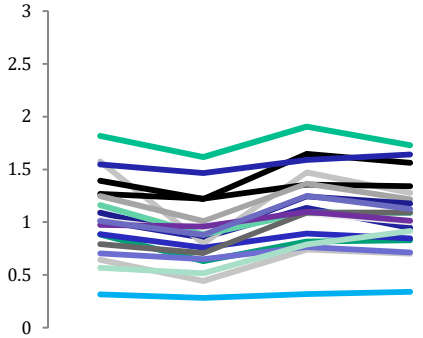
Alterations in expression can reveal a meaningful biological pattern not apparent in a pure identification experiment, which provides only a list of detected proteins



Changes with time



Changes with treatment



Changes with cell line

ThermoFisher SCIENTIFIC

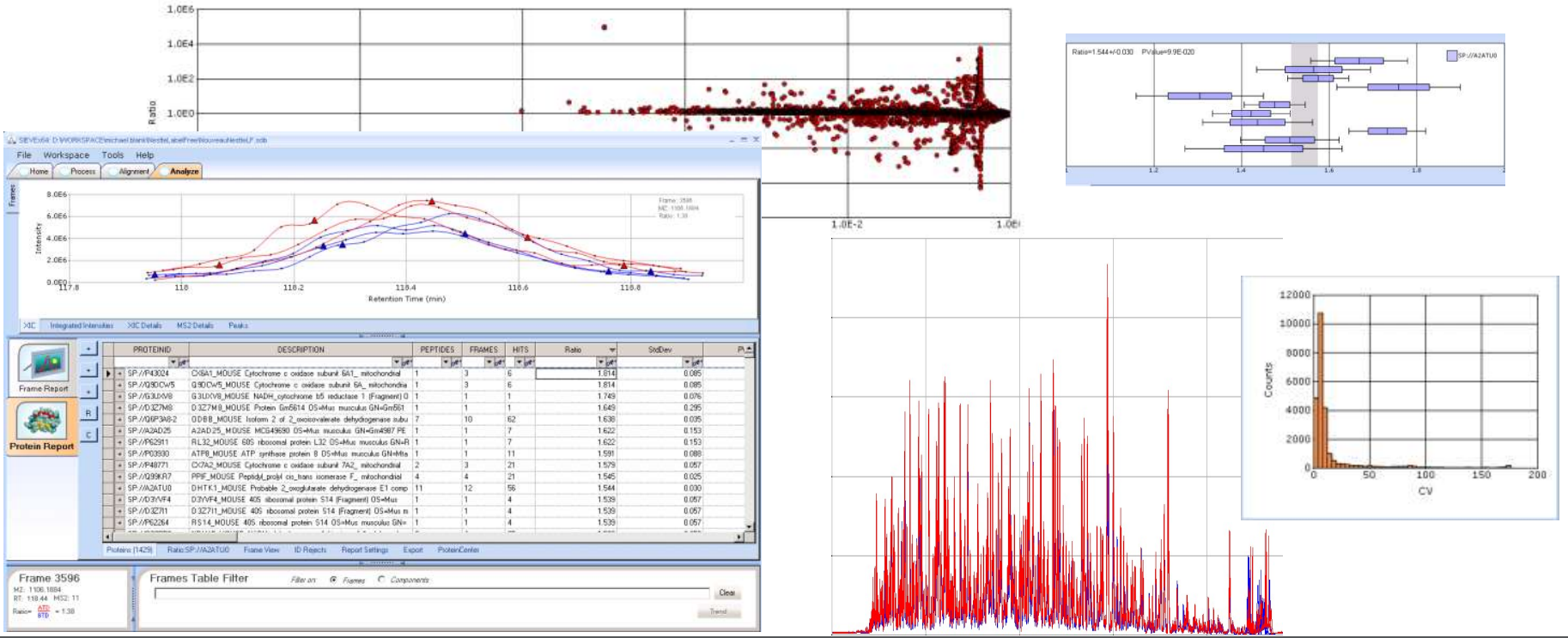
Label Free Quantitation

Several well established pipelines for the quantitation of label-free data from a data dependent (or DDA informed DIA experiment) exist. Among these:

Label Free

- Multiple LC/MS Runs
- Compare a few conditions
- Requires replicate sample material

SIEVE 2.2



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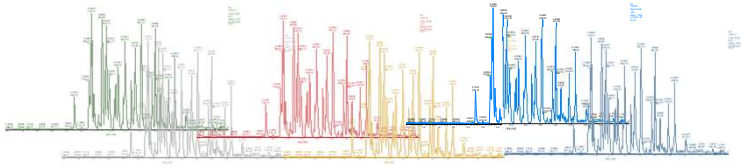
Label Free Quantitation

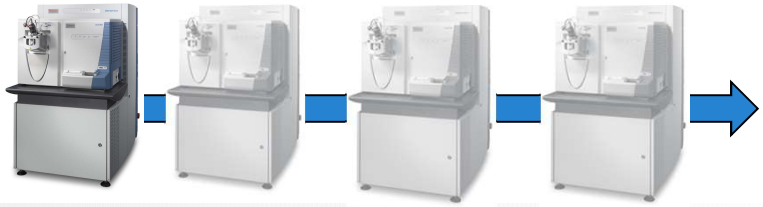
Problem: Requires multiple LC/MS analyses and is thus sample intensive

A differential analysis of 2 biological conditions with 3 technical replicates each would require **six** LC/MS injections and analyses:



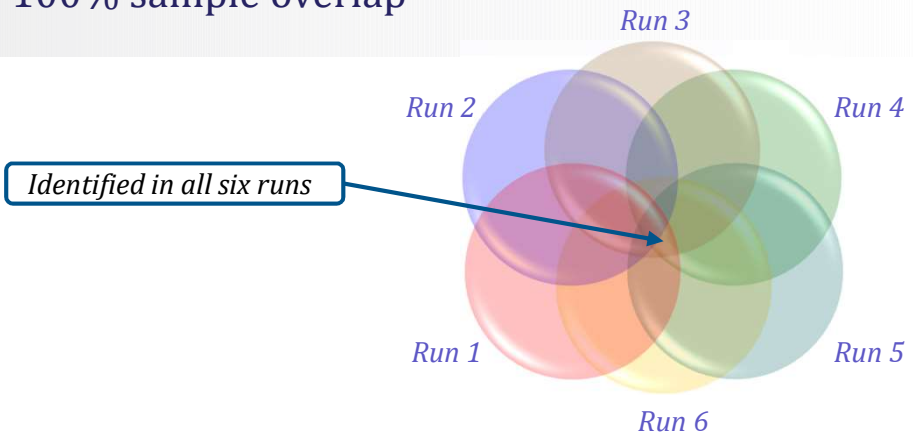
Problem: Substantial instrument time to compare only a few conditions simultaneously

Comparing just two conditions with a two hour gradient would take more than 14 hours of instrument time



Problem: Irreproducibility due to less than 100% sample overlap

Even with 85% overlap run to run
AND
4000 proteins identified in each run

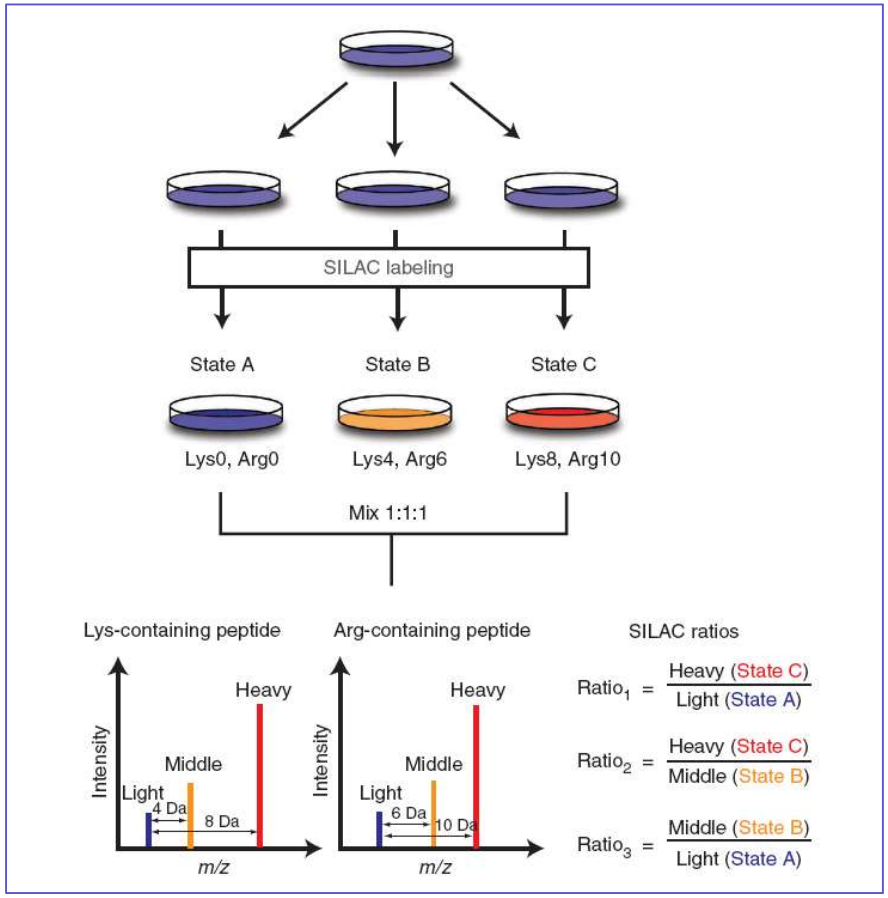


...less than 2500 common proteins

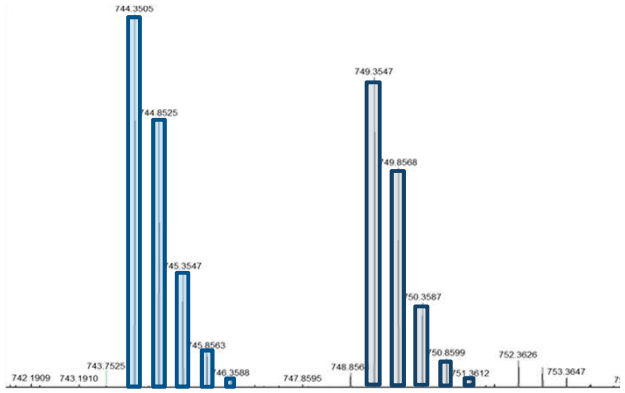
ThermoFisher SCIENTIFIC

Improving Quantitation Throughput: SILAC

SILAC Workflow



SILAC MS1 Quantitation



Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC)

- Low variation between samples
- Requires Hi-Res Mass Spectrometry
- Compare up to 3 conditions
- Applicable to cell culture
- Peptide ID not required

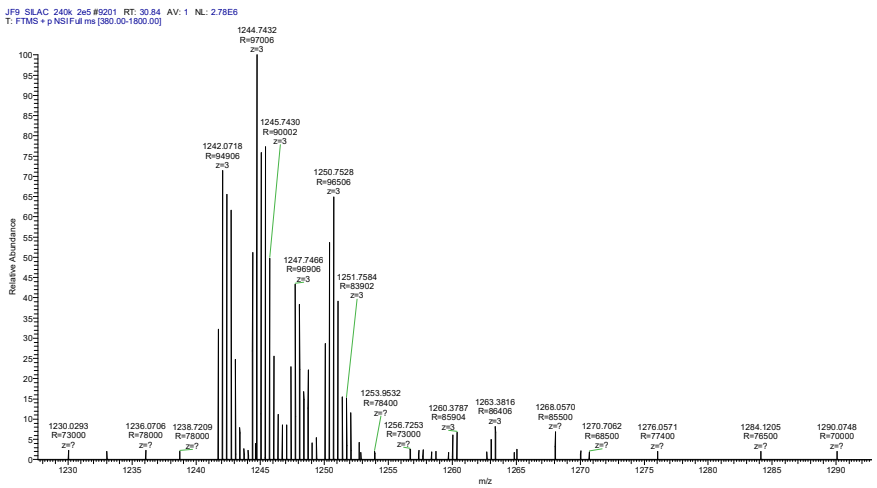
Geiger T., *et al*, Nature protocols(2011):147-157

ThermoFisher SCIENTIFIC

SILAC Quantitation

Problem: Increases MS1 Spectral Complexity

High resolution and intelligent precursor selection (i.e. selection of only one SILAC labeled peptide per pair or triad) is required for best quantitative results



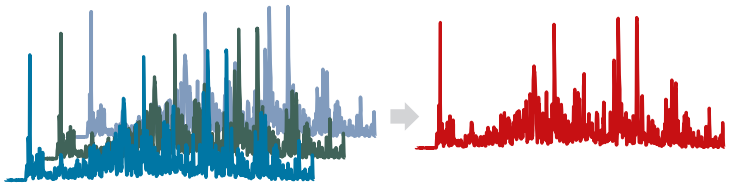
Problem: Requires cell labeling in culture

Proteins must be able to be metabolically labelled and thus is not suitable for all organisms/conditions

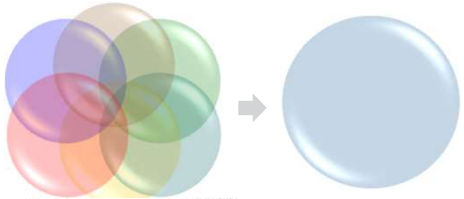
With SILAC began a trend towards increased multiplexing…

A Better Multiplexing Method– Isobaric Mass Tagging

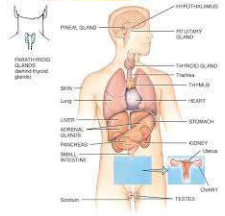
- Less MS1 Complexity
- Increased Throughput
  - Concurrent MS analysis of multiple samples
  - Less consumed samples and less instrument time



- Fewer Missing Values
  - Identification and quantification achieved in a single run
  - No worries about irreproducibility

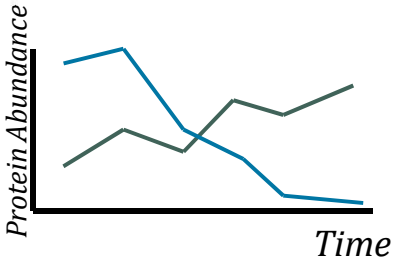


- Sample Origin Flexibility
  - Samples can be derived from cells, tissues or biological fluids



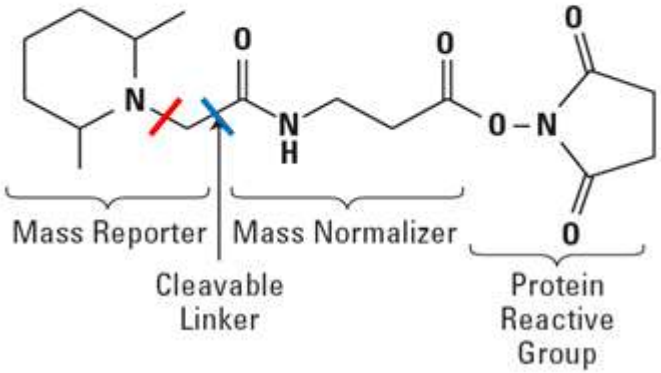
- Increased Multiplexing
  - Compare more than 3 conditions

- Multiple Comparisons and Improved Statistics
  - Incorporate replicates with multiple conditions: dose-response, time-course, multiple tissues, subcellular fractions, etc



Thermo Scientific Tandem Mass Tag (TMT) Isobaric Tag Family

TMT⁰
Method Development & SRM

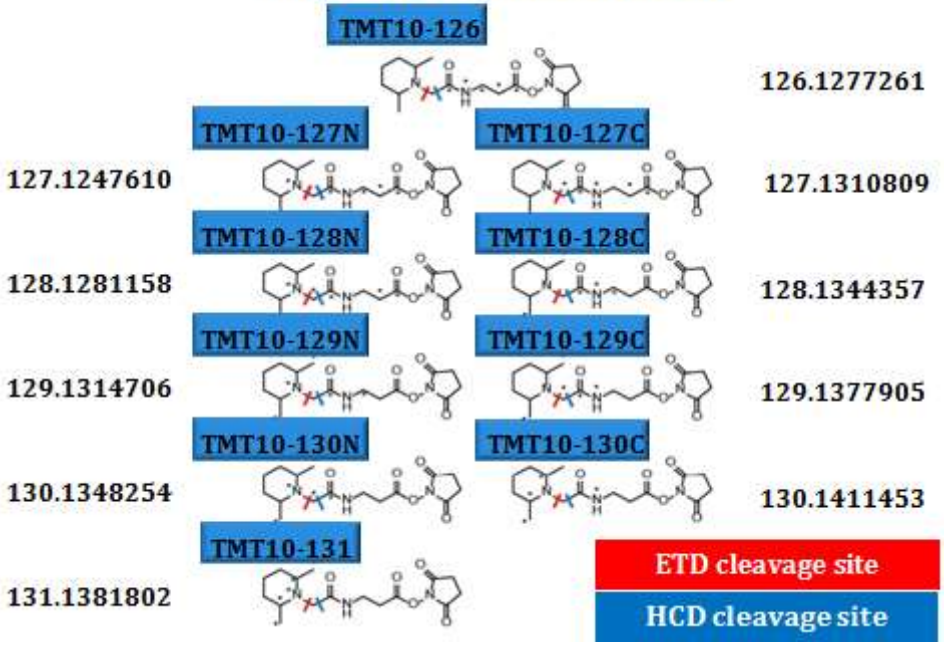


- 13C and 15N labeled reporter
- Isotopes balanced between linker region and reporter region keeping all tags exactly isobaric
- Fragments by ETD or HCD
- Up to 10 different tags
- Other reactive tags :Iodo TMT and Aminoxy TMT

TMT
Duplex Quantitation

TMT
Six Plex Quantitation

TMT
10plex Quantitation



ThermoFisher SCIENTIFIC

The Multiplexing Revolution –Not Only Consumables…

SILAC

Compare 3 Conditions

Ong SE, Blagoev B, et al.
Mol Cell Proteomics. 2002 May;1(5):376-86

TMT6plex

Compare 6 Conditions in MS^2 with amine reactive tags

Andrew Thompson , Juergen Schaefer , Karsten Kuhn, et al. *Anal. Chem.*, **2006**, *78* (12), pp 4235–4235

iTRAQ8plex

Label and compare 8 Conditions

Choe, L., D'Ascenzo, M., Relkin, et al. (2007),. Proteomics, 7: 3651–3660

TMT8 and TMT10

Concurrently quantify up to 10 sample conditions

McAlister, G., Huttlin, E.L.; Haas, W.; et. al.
Anal Chem. 2012. 84, 7469-7478.

TMT11

Concurrently quantify up to 11 sample conditions

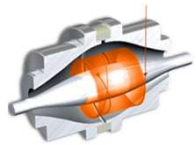
Tabiwang, N., Rosa, V.; Ryan, D.B.; et. al.
Thermo Fisher Scientific GmbH 2017. Poster Presentation.

2002
2005
2006
2007
2009
2011
2013
2017
2019

Orbitrap Classic

High Resolution Orbitrap Mass Analyzer

Hu, Q., Noll, R. J., Li, H., Makarov, A., et al. (2005), J. Mass Spectrom., 40: 430–443



Orbitrap Velos

New Axial Field HCD Cell for Improved MS^2

Olsen, JV; Schwartz, JC, et al.
Mol Cell Proteomics. 2009 December; 8: 2759-2769

Orbitrap Elite

Hybrid; Single Notch MS^3; PTR

Wenger CD, Lee MV, Hebert AS, McAlister GC, Phanstiel DH, Westphall MS, Coon JJ.
Nat Methods. 2011 Oct 2;8(11):933-5

Orbitrap Fusion

Tribrid, Parallelized Analysis, Multinotch

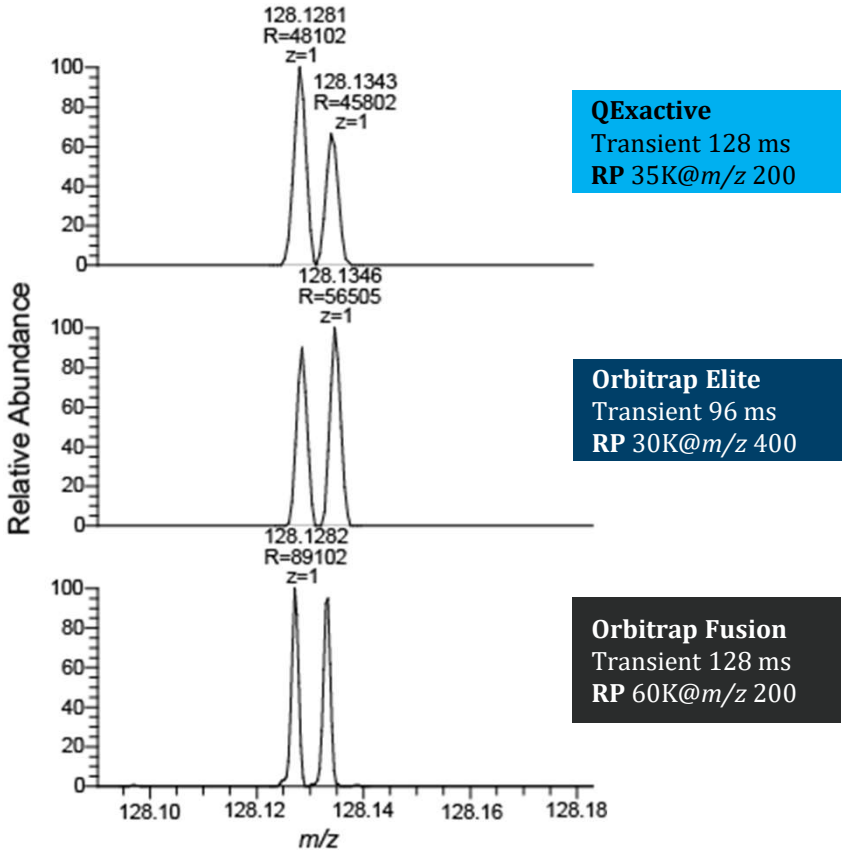
Erickson BK, Jedrychowski MP, McAlister GC, Everley RA, Kunz R, Gygi SP.
Anal Chem 2015 Jan 20;87(2):1241-9

Orbitrap Eclipse

Newest Tribrid, highest sensitivity and selectivity

ThermoFisher SCIENTIFIC

High Performance Depends Upon High Resolution Instruments



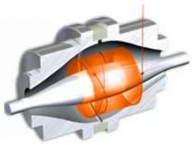
QExactive
Transient 128 ms
RP 35K@*m/z* 200

Orbitrap Elite
Transient 96 ms
RP 30K@*m/z* 400

Orbitrap Fusion
Transient 128 ms
RP 60K@*m/z* 200

HIGH RESOLVING POWER IS ESSENTIAL FOR ACCURATE QUANTIFICATION OF THE TMT10PLEX REAGENTS

Result: Get accurate quantitation using the high resolution of **Orbitrap Mass Analyzer**



ThermoFisher SCIENTIFIC

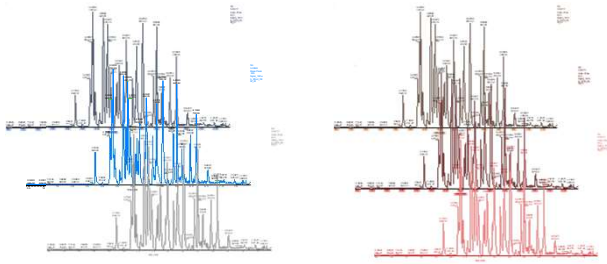
A Real Example

Sample: Mouse mitochondrial extract untreated or treated with phosphatase inhibitor

Orbitrap Elite

- *75 um x 50 cm PepMap C18*
- *210 min gradient: 250 min run*
- *1 ug of sample on column*

LABEL FREE



Untreated
- Three Analyses
- 750 minutes
- 3 ug

Treated
- Three Analyses
- 750 minutes
- 3 ug

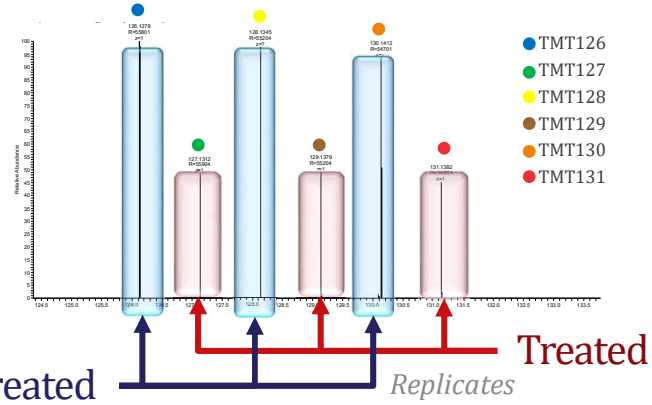
Quantified

1423 protein groups

in **1.04 days**

using **6 ug** material

TMT6



Single Analysis | 250 minutes | 1 ug

Quantified

1310 protein groups

in **4.16 hours**

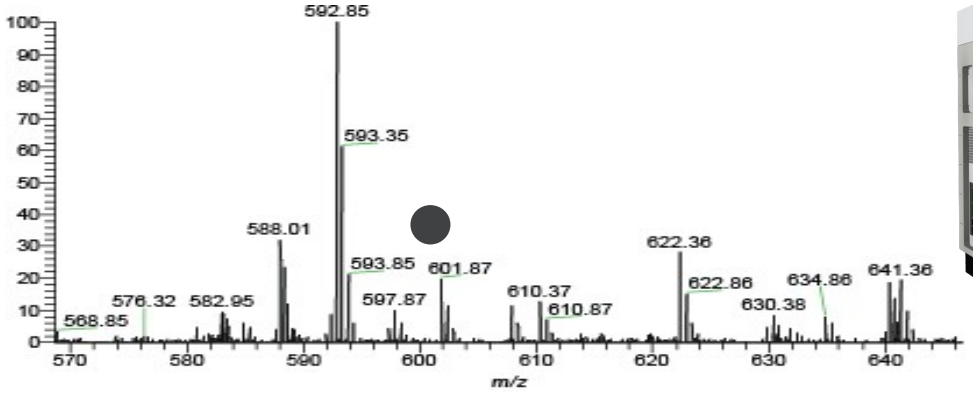
using **1 ug** material

Thermo Poster Note : Liver Mitochondria Proteomics Employing High –Resolution MS Technology; J.Ho. et al

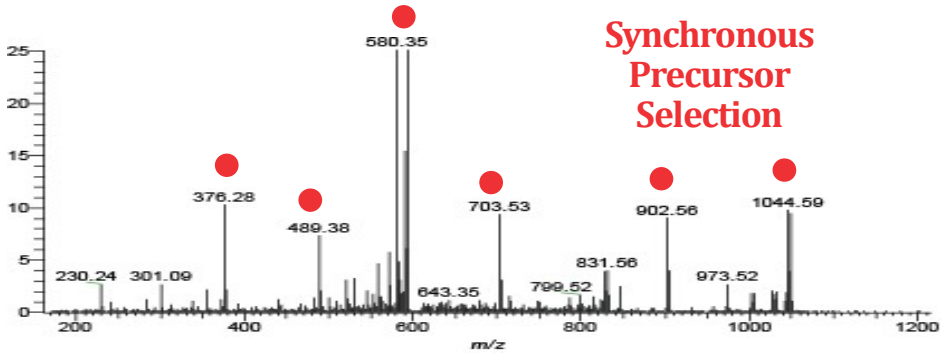
ThermoFisher SCIENTIFIC

Synchronous Precursor Selection (SPS) for Accurate Quantification

Precursor Ion

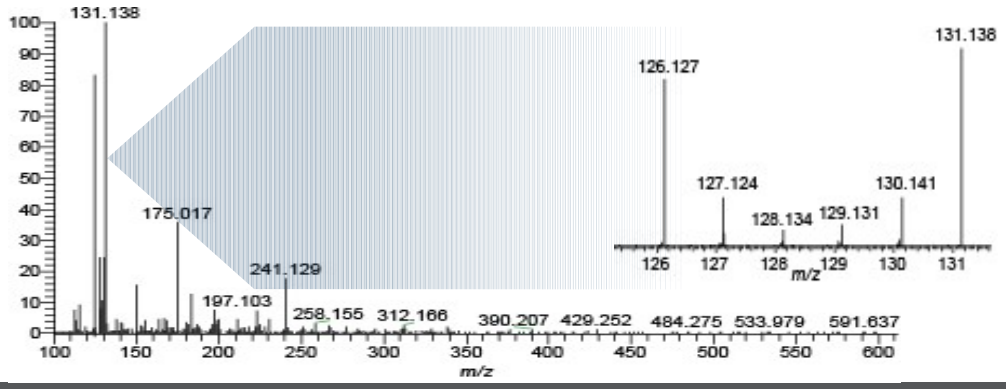


CID MS^2, Ion Trap



Synchronous Precursor Selection

HCD MS^3, OT



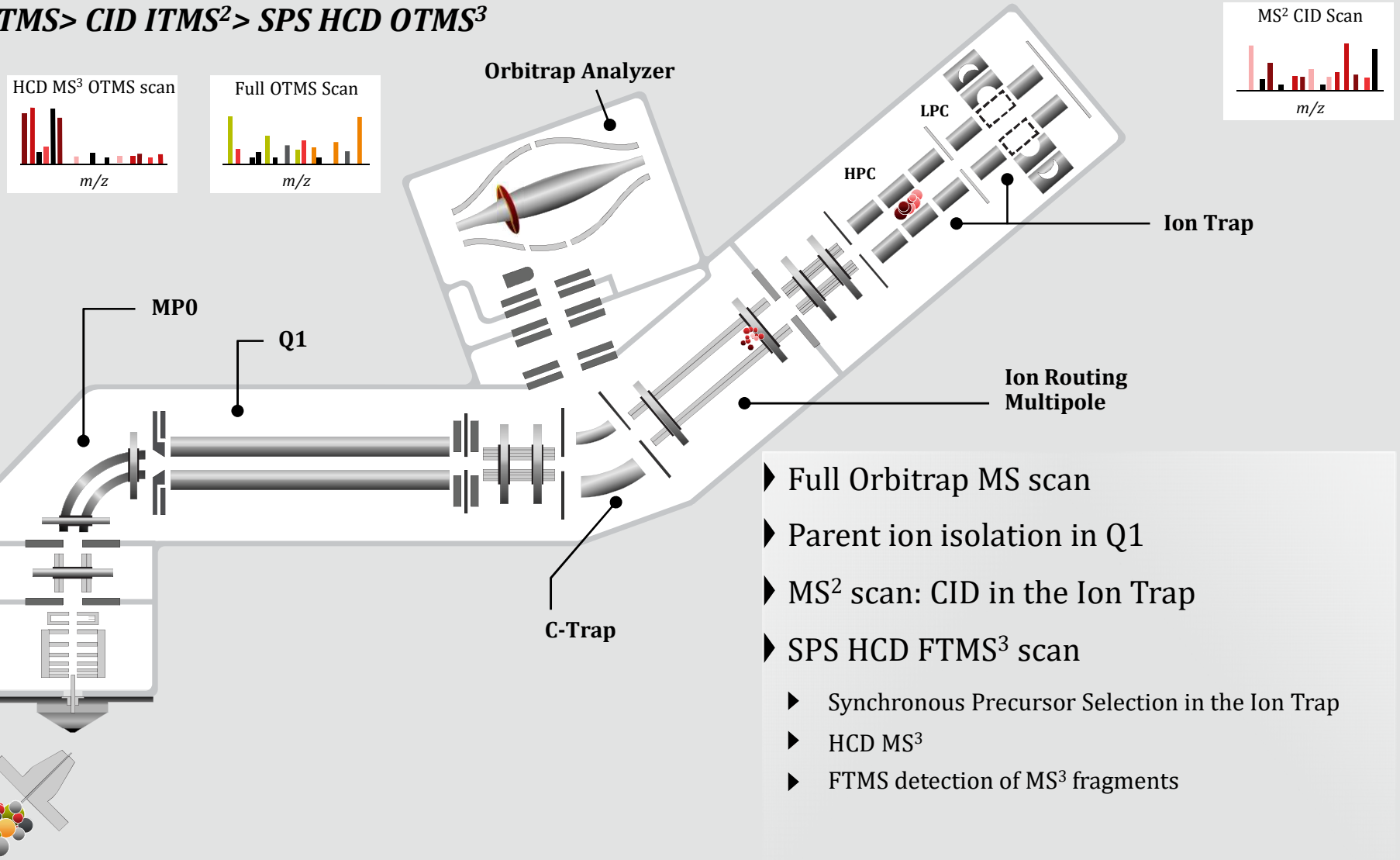
2013

Orbitrap Fusion Tribrid Mass Spectrometer

ThermoFisher SCIENTIFIC

TMT^3 Experiment, Powered by SPS

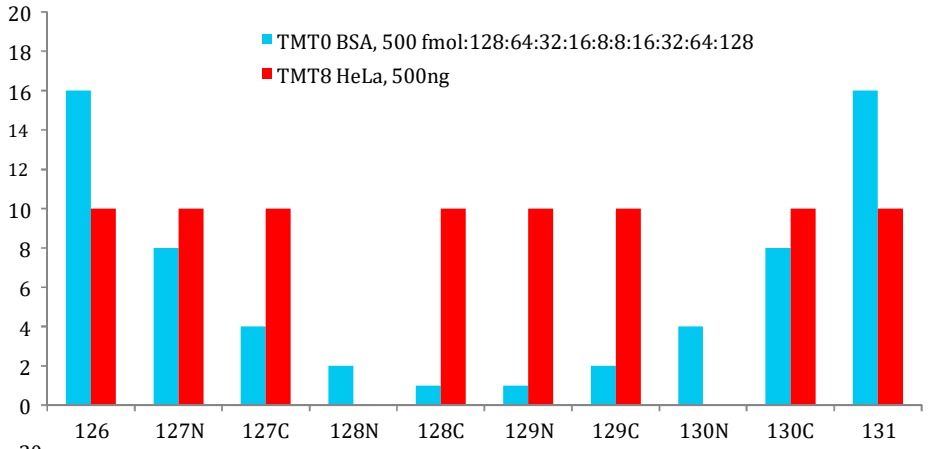
OTMS> CID ITMS^2> SPS HCD OTMS^3



- Full Orbitrap MS scan
- Parent ion isolation in Q1
- MS^2 scan: CID in the Ion Trap
- SPS HCD FTMS^3 scan
  - Synchronous Precursor Selection in the Ion Trap
  - HCD MS^3
  - FTMS detection of MS^3 fragments

Co-isolation of Interfering Ions Affects Accuracy

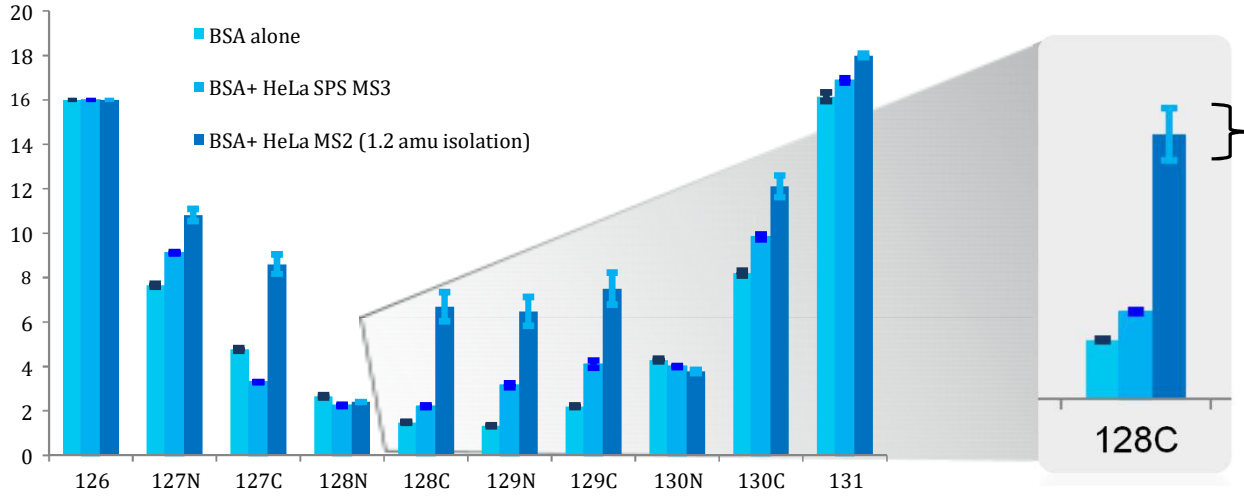
Expected



- 500fmol of BSA in 500ng HeLa digest
- Narrow precursor isolation with 4 hour gradient

Constant background

Observed



Less precision

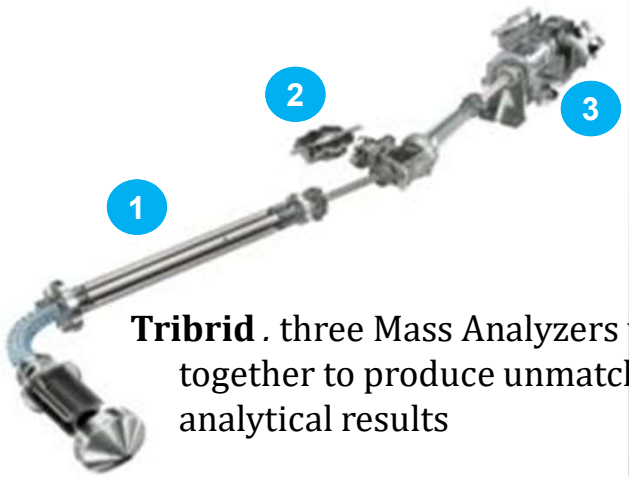
Less accuracy (ratio distortion)

Results: Best possible accuracy and precision by reducing co-isolated interfering ions.

ThermoFisher SCIENTIFIC

Orbitrap Fusion Lumos Tribrid Mass Spectrometer

2015



Tribrid . three Mass Analyzers working together to produce unmatched analytical results

Unmatched Analytical Performance

Revolutionary performance

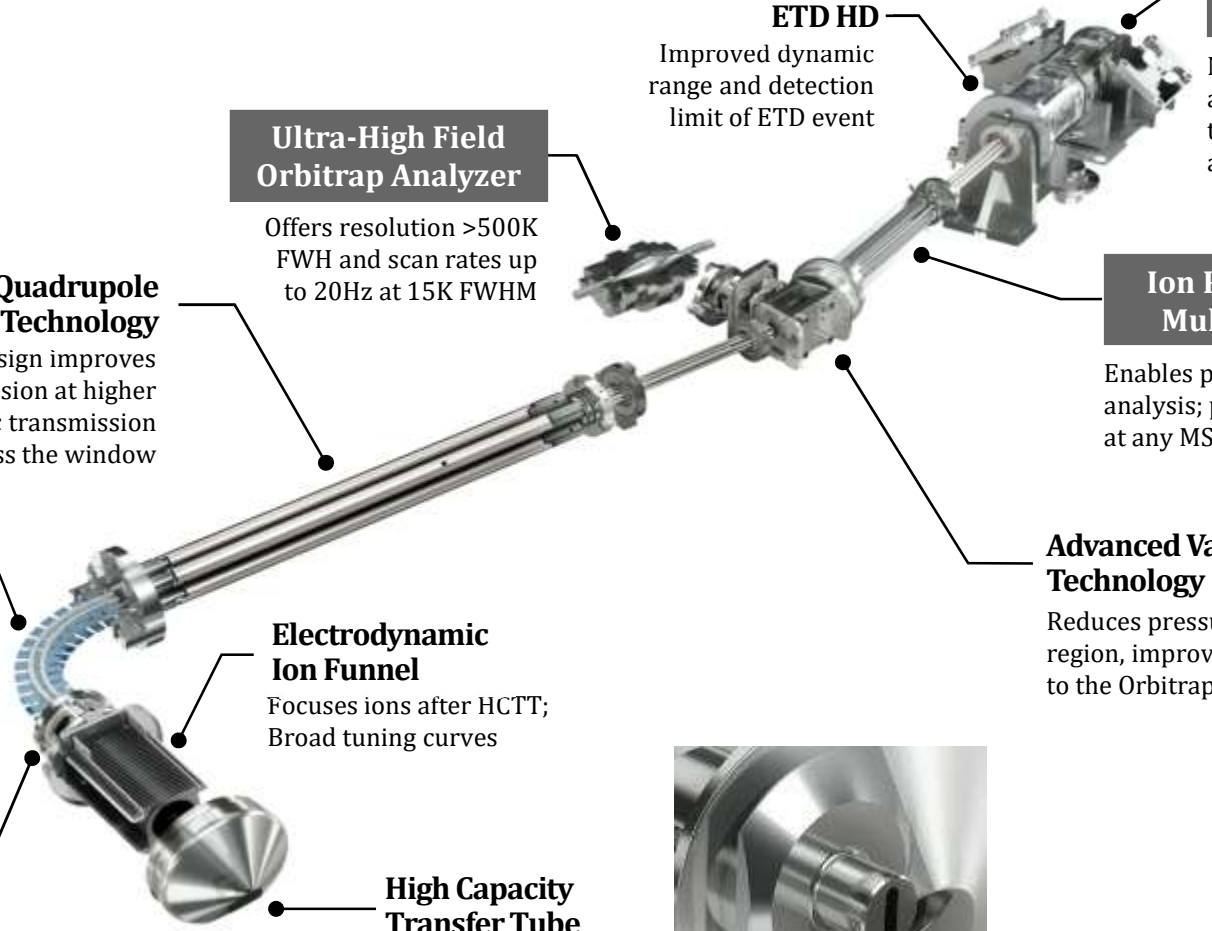
Exceptional versatility

Unprecedented usability

Highest sensitivity

ThermoFisher SCIENTIFIC

Orbitrap Fusion Lumos Tribrid Mass Spectrometer



Dual-Pressure Linear Ion Trap

MS^n and sensitive mass analysis of four fragmentation types: CID, HCD, ETD and EThcD

ETD HD

Improved dynamic range and detection limit of ETD event

Ultra-High Field Orbitrap Analyzer

Offers resolution >500K FWH and scan rates up to 20Hz at 15K FWHM

Ion Routing Multipole

Enables parallel analysis; performs HCD at any MS^n stage

Advanced Quadrupole Technology

Segmented design improves transmission at higher resolution; symmetric transmission across the window

Advanced Vacuum Technology

Reduces pressure in UHV region, improving transmission to the Orbitrap analyzer

Advanced Active Ion Beam Guide

Prevents neutrals and high velocity clusters from entering mass resolving quadrupole

Electrodynamic Ion Funnel

Focuses ions after HCTT; Broad tuning curves

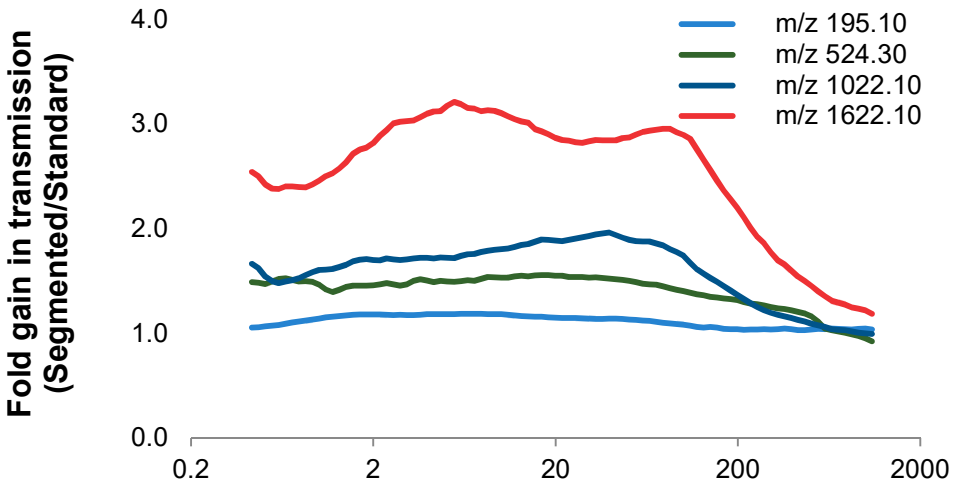
EASY ETD Source

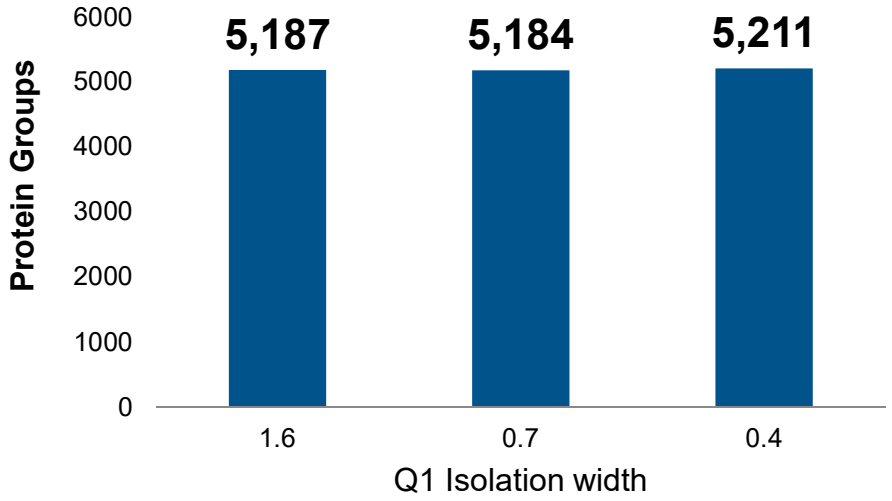
Based on Townsend discharge; reliable and easy to use

High Capacity Transfer Tube

Increases ion flux into the mass spectrometer

Better Ion Transmission With Segmented Quadrupole

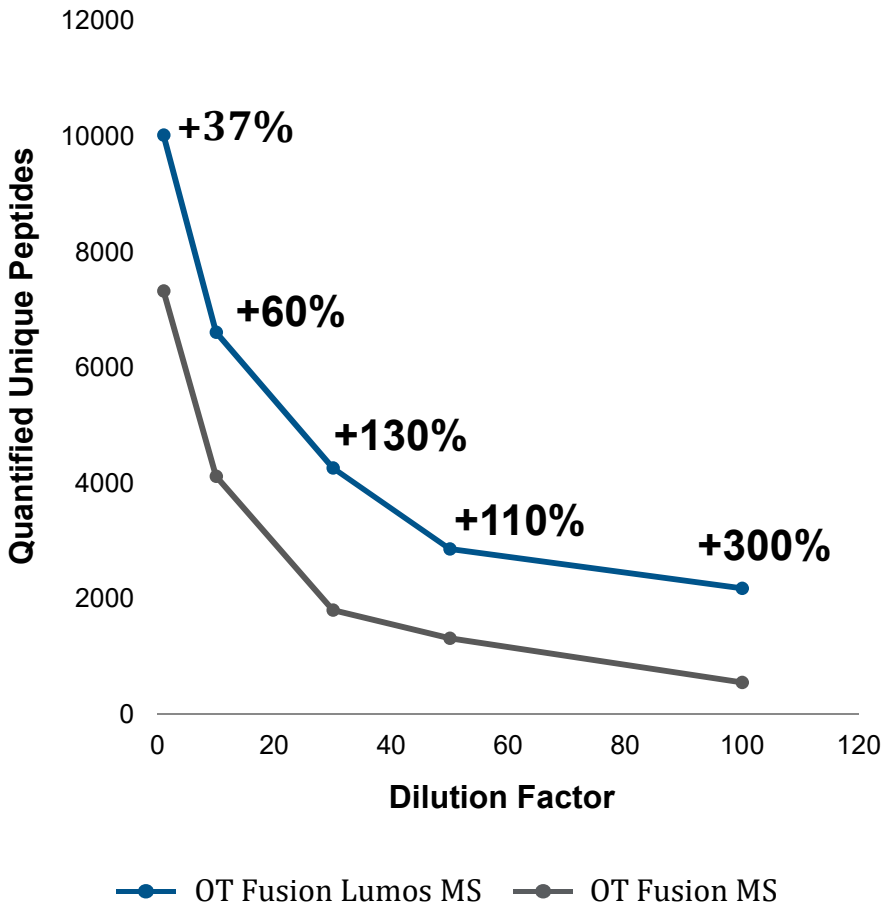




Segmented Quadrupole

- Improved transmission across m/z range and for narrow windows
- Brighter Source and Segmented Quad allows the use of a 0.4 amu isolation without loss of IDs (here for 1 ug HeLa, DD OT IT CID, 2 h runs, n=2)
- Improved performance for TMT quantitation
- Improved performance for PRM and DIA
- Improved performance for top down

Improved TMT SPS MS^3 Performance



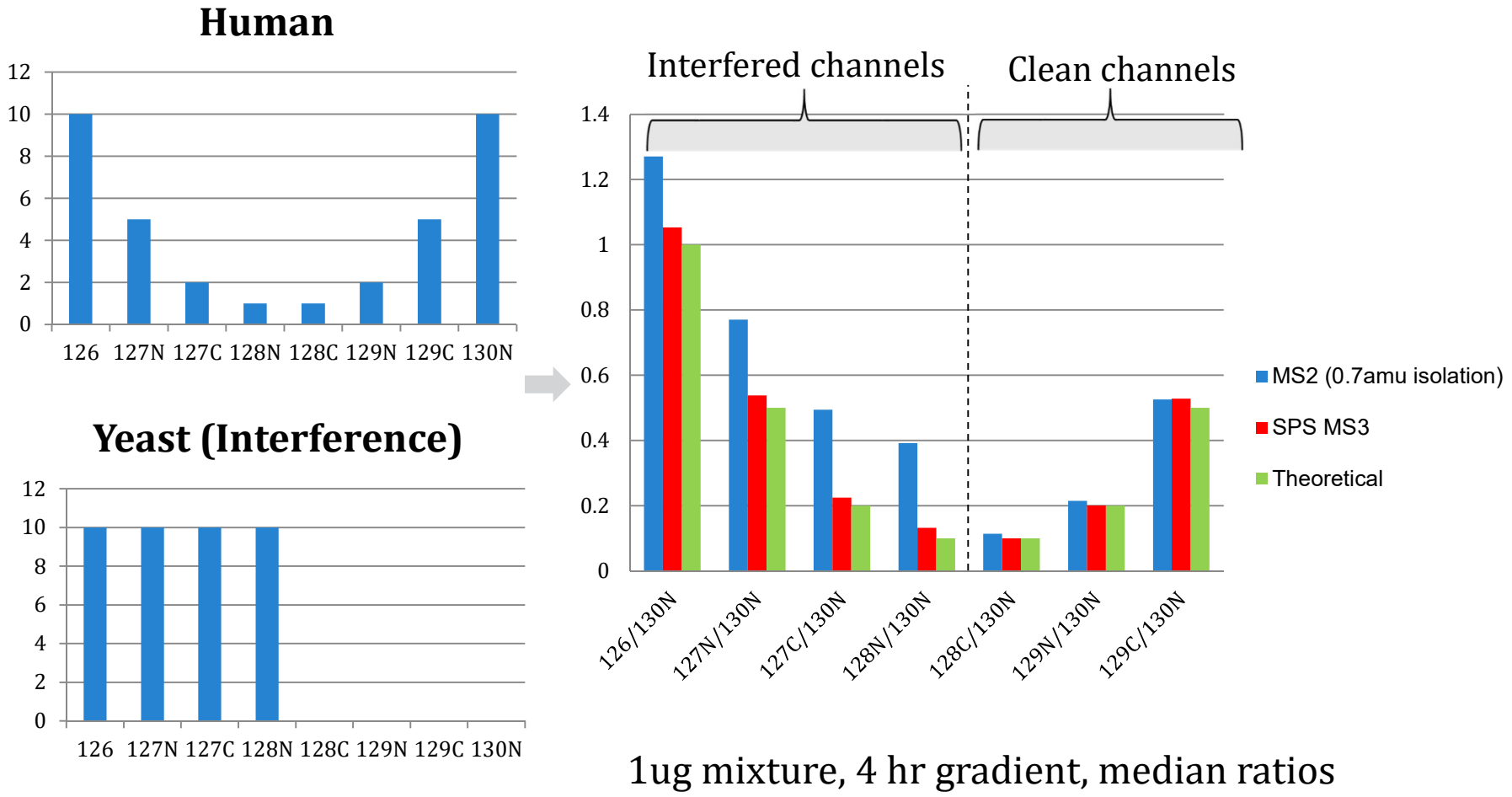
Chris Rose, Gygi's lab, Harvard Medical School

TMT Dilution

- Standard HeLa digest, labeled with TMT0 analyzed with an 85 min gradient using SPS-MS^3
- Sample diluted 1:1, 1:10, 1:30, 1:50, 1:100
- The number of MS^3 acquisitions was similar in both analyses
- The number of unique peptides quantified was systematically higher with the Orbitrap Fusion Lumos MS

SPS MS^3 Quantification on Orbitrap Fusion Lumos MS

Results: Best possible accuracy by reducing co-isolated interferences.



1ug mixture, 4 hr gradient, median ratios

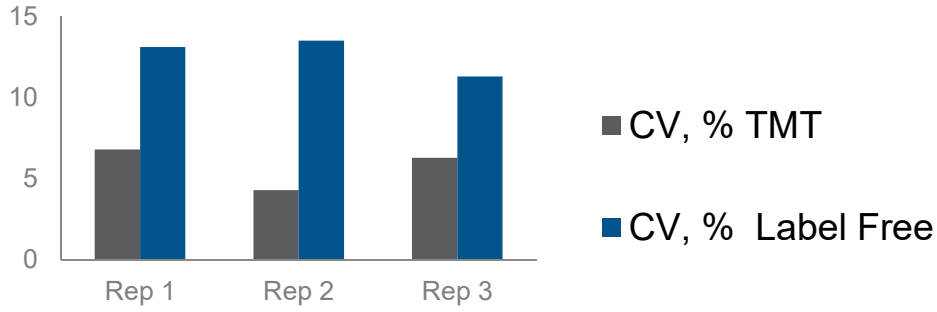
ThermoFisher SCIENTIFIC

TMT Technology is More Precise than Label Free Quan

Roman Zubarev
Karolinska Institute

"We compared the average and median CVs (calculated for the whole dataset containing ca. 4000 proteins quantified with ≥2 peptides) between the three biological replicates of the same treatment. Ignoring the fact that the cell lines were different, the results are clearly in favor of TMT.

In other words, TMT produced two times lower CVs than our label-free quantification, which we thought was pretty good. *I am stunned…*"



Additional Key Customers Include:

Clinical Proteomic Tumor Analysis Consortium
OFFICE OF CANCER CLINICAL PROTEOMICS RESEARCH
Washington University in St. Louis
JOHNS HOPKINS UNIVERSITY
Stanford University
Pacific Northwest NATIONAL LABORATORY
WISCONSIN UNIVERSITY OF WISCONSIN-MADISON
BROAD INSTITUTE
TCMP The ThermoFisher CENTER for MULTIPLEXED PROTEOMICS
DEPARTMENT OF CELL BIOLOGY • HARVARD MEDICAL SCHOOL

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TMT Used for Protein Research in…

Stem Cells

nature COMMUNICATIONS

Received 28 Aug 2014 | Accepted 21 Oct 2014 | Published 10 Dec 2014 DOI: 10.1038/ncomms6613

Proteome adaptation in cell reprogramming proceeds via distinct transcriptional networks

Marco Benevento[1,2], Peter D. Tonge[3], Mira C. Puri[3,4], Samer M.I. Hussein[3], Nicole Cloonan[5], David L. Wood[5], Sean M. Grimmond[5], Andras Nagy[3,6,7], Javier Munoz[1,2,†] & Albert J.R. Heck[1,2]

Viromics

Quantitative Temporal Viromics: An Approach to Investigate Host-Pathogen Interaction

Cell

Cell *157*, 1460–1472, June 5, 2014

Michael P. Weekes,[1,3,4,*] Peter Tomasec,[2,4] Edward L. Huttlin,[1] Ceri A. Fielding,[2] David Nusinow,[1] Richard J. Stanton,[2] Eddie C.Y. Wang,[2] Rebecca Aicheler,[2] Isa Murrell,[2] Gavin W.G. Wilkinson,[2] Paul J. Lehner,[3] and Steven P. Gygi[1,*]

Drug Discovery

Tracking cancer drugs in living cells by thermal profiling of the proteome

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Cancer

Quantification of Pancreatic Cancer Proteome and Phosphorylome: Indicates Molecular Events Likely Contributing to Cancer and Activity of Drug Targets

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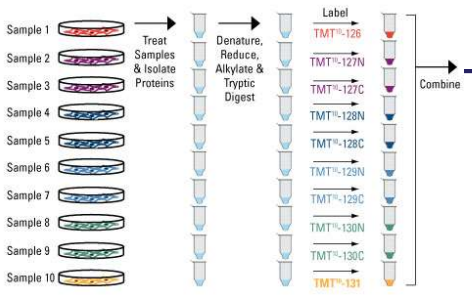
Straightforward Workflow

Sample Preparation

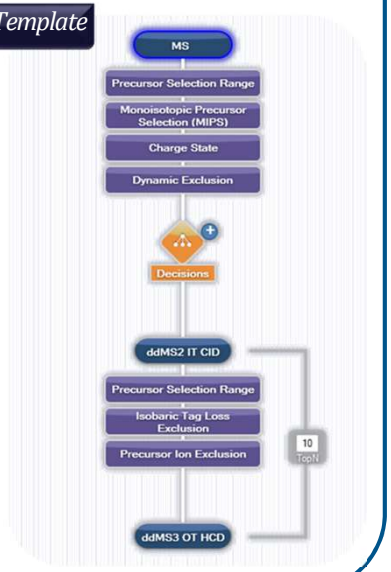


TMT10plex

Mass Spectrometry

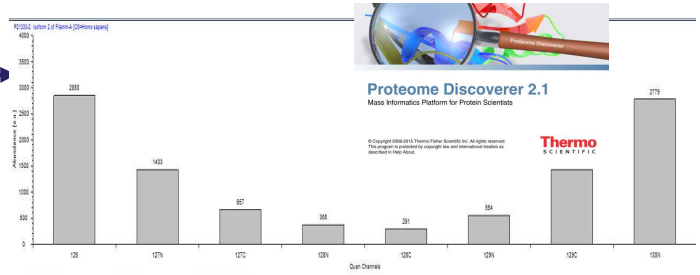
OT Fusion Lumos

Method Template

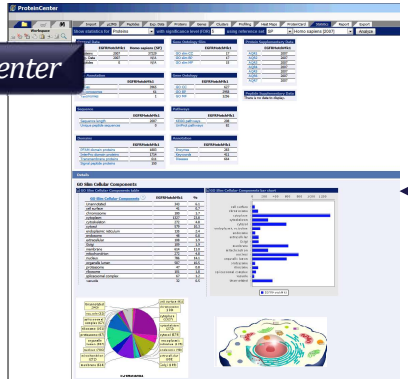


Data Analysis and Interpretation

Proteome Discoverer 2.1



Protein Center



Result: Complete software and method development suite from reagents to data analysis

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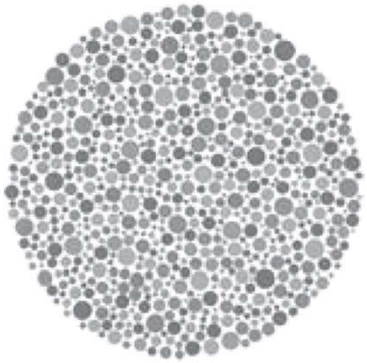
Competitive Advantages

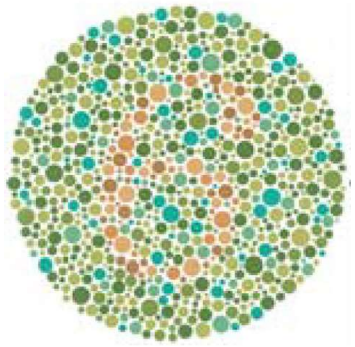
Trust your quantitation!

- **Multinotch MS^3 quantification is more accurate than other MS^2 Methods**
- **The accuracy of Multinotch MS^3 quantification means not missing important expression level changes due to co-isolated interference**
- **Multinotch MS^3 quantification is only available on the Orbitrap Fusion and Orbitrap Fusion Lumos**
- **Orbitrap Fusion Lumos provides highest sensitivity, highest selectivity and lowest detection limit for best quantification**

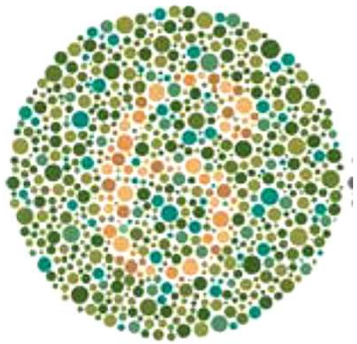
No Quan



MS^2



Multinotch



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Additional Resources

- **Online Resources**
  - http://planetorbitrap.com/ (Published Articles, Posters, Brochures, Product Support Bulletins, Technical Guides, Webinars, Protocols, Application Workflows.)

- **Some More Publications**

Relative Quantitation of TMT-Labeled Proteomes - Focus on Sensitivity and Precision
Viner R, Scigelova M, Zeller M, Oppermann M, Moehring T, Zabrouskov V.
Application Note 566

Increasing the multiplexing capacity of TMTs using reporter ion isotopologues with isobaric masses
McAlister GC, Huttlin EL, Haas W, Ting L, Jedrychowski MP, Rogers JC, Kuhn K, Pike I, Grothe R, Blethrow JD, Gygi SP.
Anal Chem. 2012 Sep 4;84(17):7469-78.

MS3 eliminates ratio distortion in isobaric multiplexed quantitative proteomics
Ting L, Rad R, Gygi SP, Haas W.
Nat Methods. 2011 Oct 2;8(11):937-40.

Evaluating multiplexed quantitative phosphopeptide analysis on a hybrid quadrupole mass filter/linear ion trap/orbitrap mass spectrometer
Erickson BK , Jedrychowski MP, McAlister GC, Everley RA, KUNZ R, Gygi SP
Anal Chem.2015 Jan 20;87(2):1241-9.

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  - Zezong Gu, University of Missouri-Columbia
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