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Multiple studies with a single experiment:

The Power of Quantitative Multiplexing

Overview

Part 1: Introduction

- An overview of multiplexed isobaric labeling, the basics of Tandem Mass Tags and the SPS MS3 technique

Part 2: Sample Prep

- A summary of the steps involved for a complete TMT workflow including labeling and fractionation

Part 3: Instrument Configuration

- Getting started with building an instrument method on the Orbitrap Tribrids and Benchtops using nanoHPLC

Part 4: Data Analysis

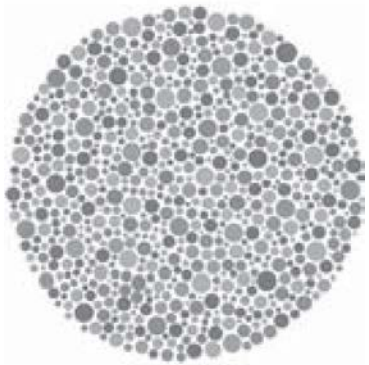
- Data analysis of SPS MS3 data using Proteome Discoverer 2.1 workflow

Introduction

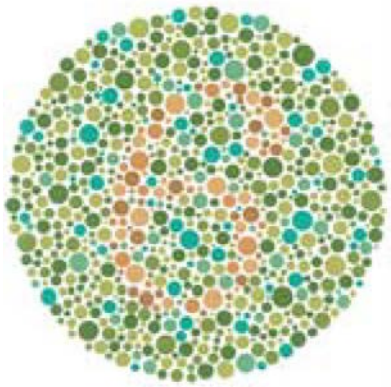
Moving Beyond Qualitative Proteomics

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

Qualitative

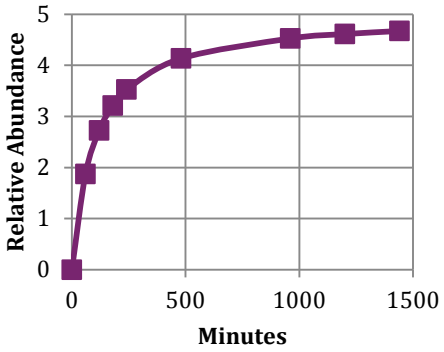


Quantitative

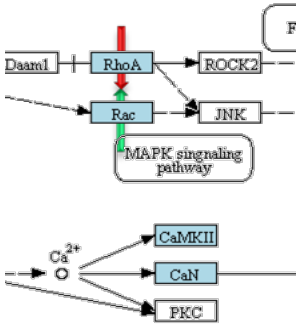


Add another dimension to any experiment by determining the relative abundance of each identified protein

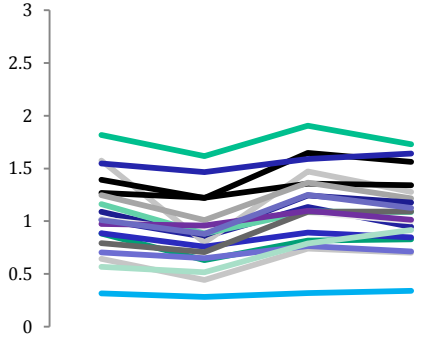
Alterations in expression can reveal a meaningful biological pattern not apparent in a pure identification experiment, which provides only a list of detected proteins



Changes with time



Changes with treatment



Changes with cell line

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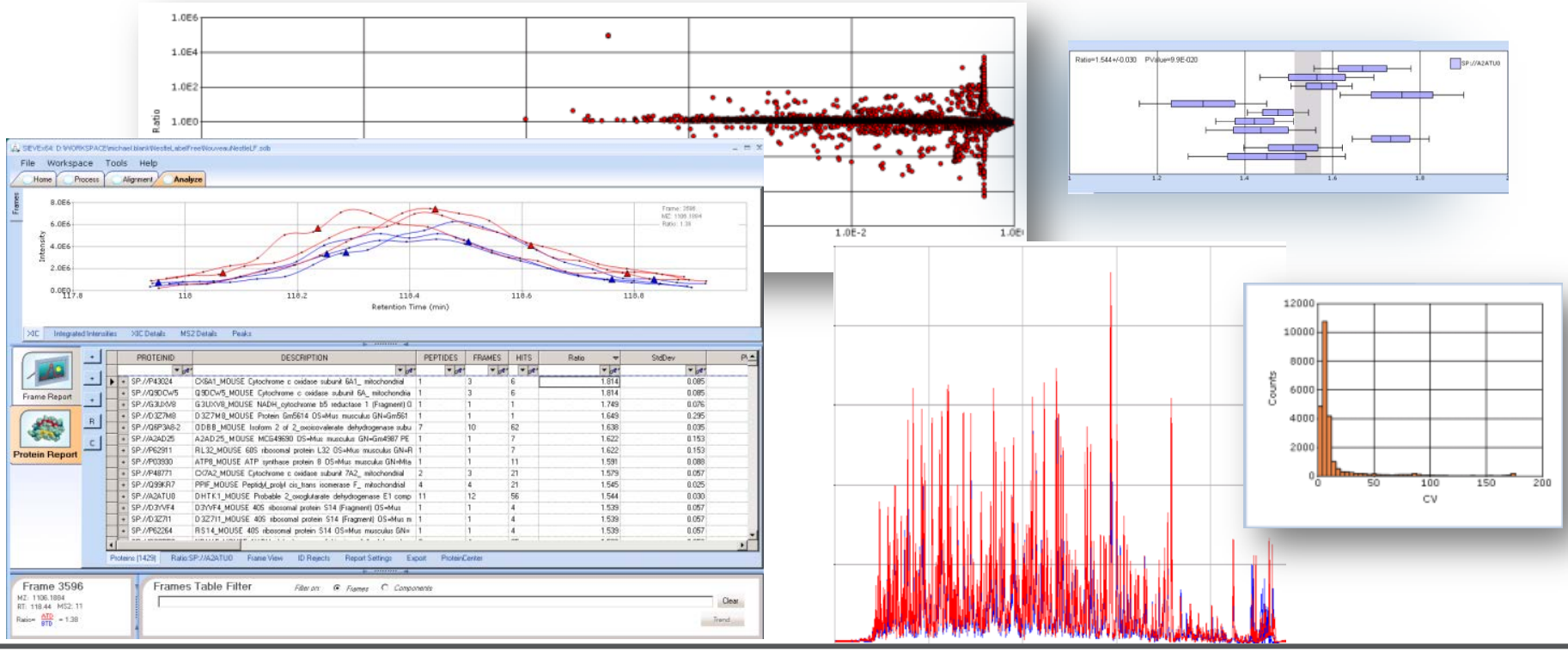
Label Free Quantitation

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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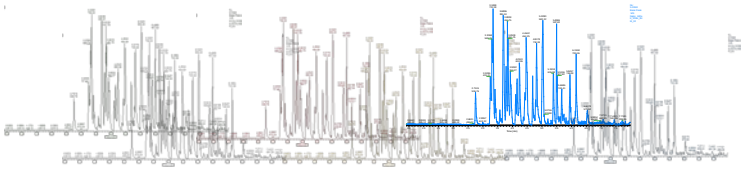
Label Free Quantitation

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:

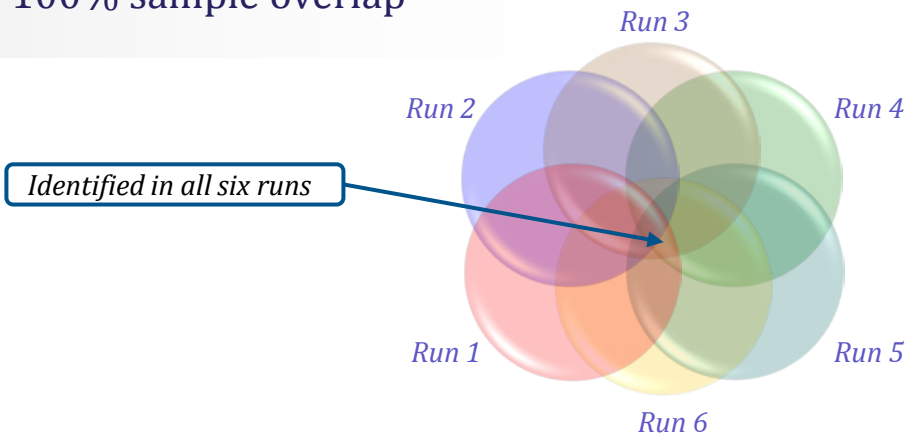


Problem: Substantial instrument time to compare only a few conditions simultaneously

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

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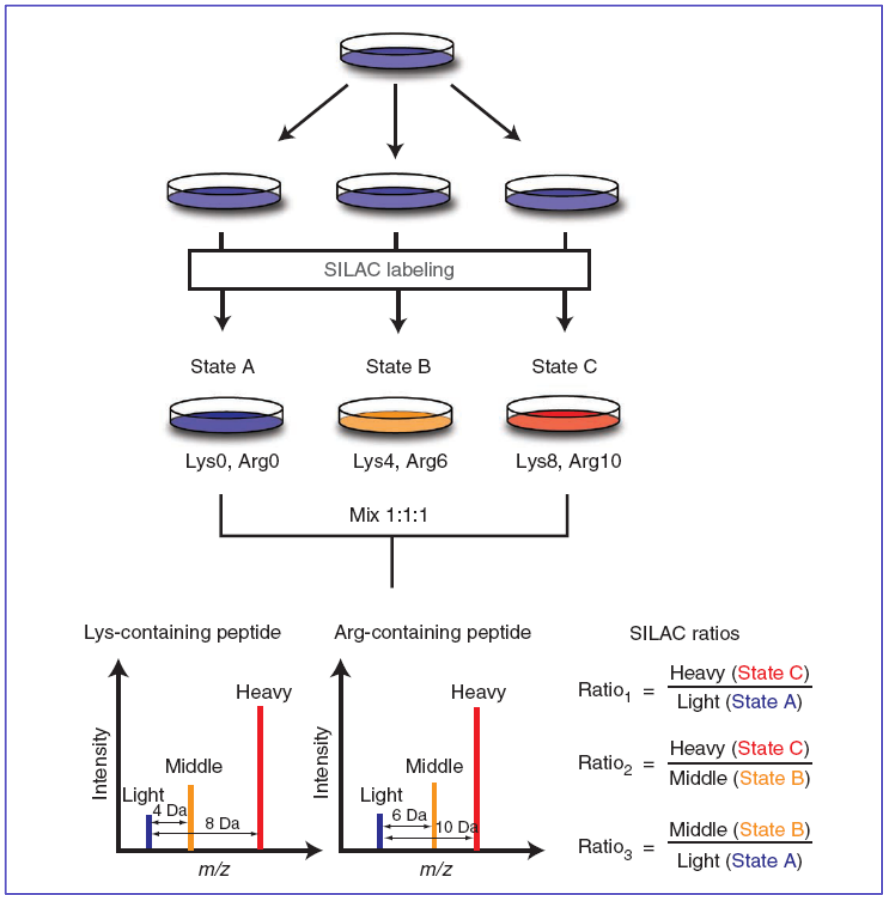
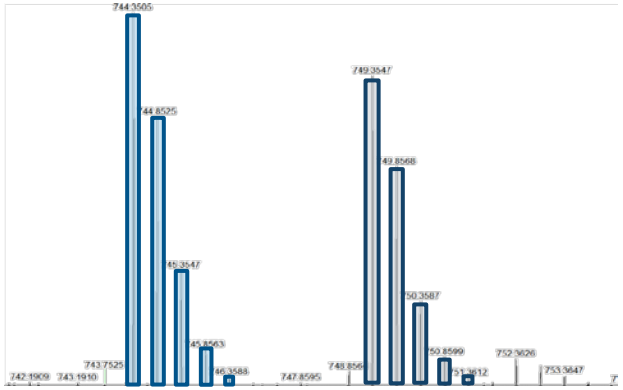
Improving Quantitation Throughput: SILAC

SILAC Workflow



SILAC MS1 Quantitation



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

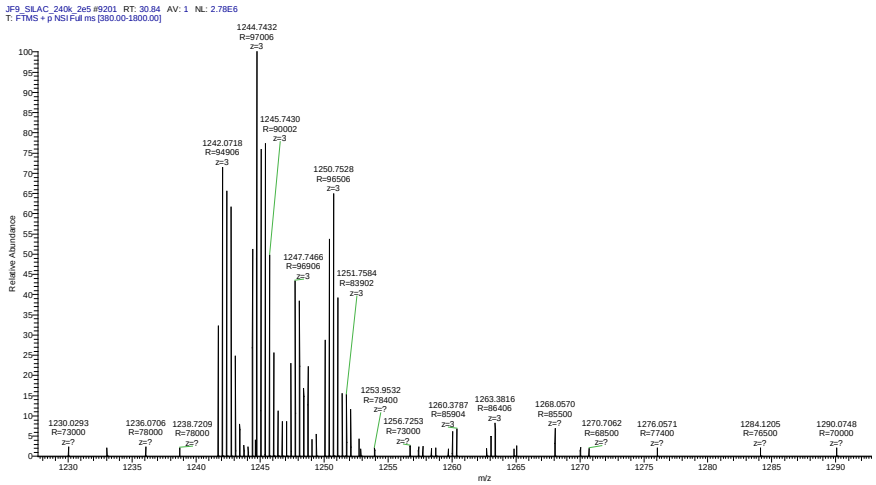
- Low variation between samples
- Requires Hi-Res Mass Spectrometry
- Compare up to 3 conditions
- Applicable to cell culture
- Peptide ID not required

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

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



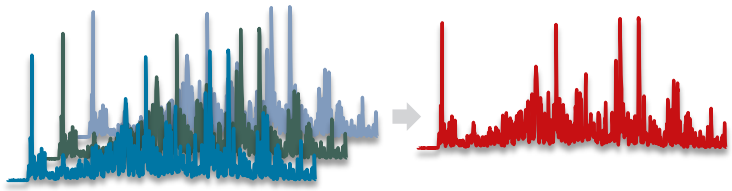
Problem: Requires cell labeling in culture

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

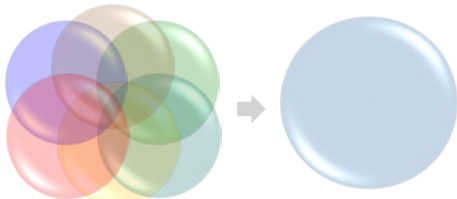
With SILAC began a trend towards increased multiplexing…

A Better Multiplexing Method– Isobaric Mass Tagging

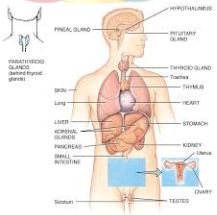
- Less MS1 Complexity
- Increased Throughput
  - Concurrent MS analysis of multiple samples
  - Less consumed samples and less instrument time



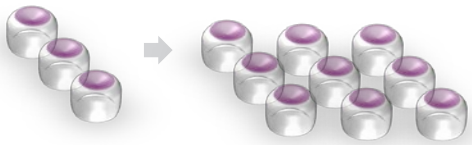
- Fewer Missing Values
  - Identification and quantification achieved in a single run
  - No worries about irreproducibility



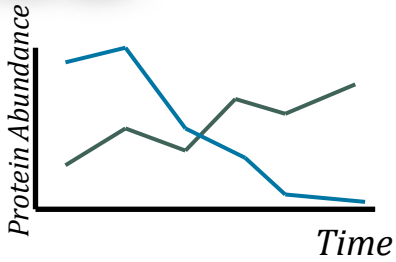
- Sample Origin Flexibility
  - Samples can be derived from cells, tissues or biological fluids



- Increased Multiplexing
  - Compare more than 3 conditions

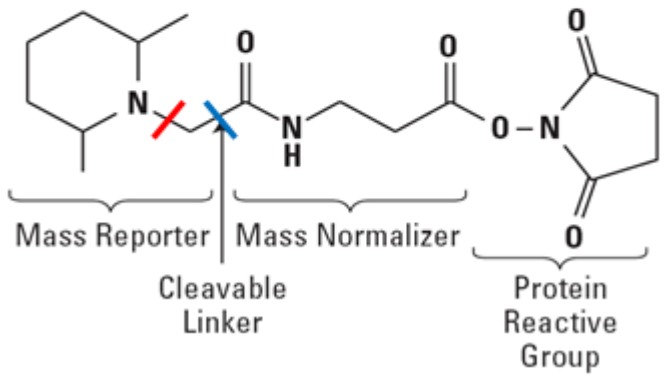


- Multiple Comparisons and Improved Statistics
  - Incorporate replicates with multiple conditions: dose-response, time-course, multiple tissues, subcellular fractions, etc



Thermo Scientific Tandem Mass Tag (TMT) Isobaric Tag Family

TMT^0
Method Development & SRM

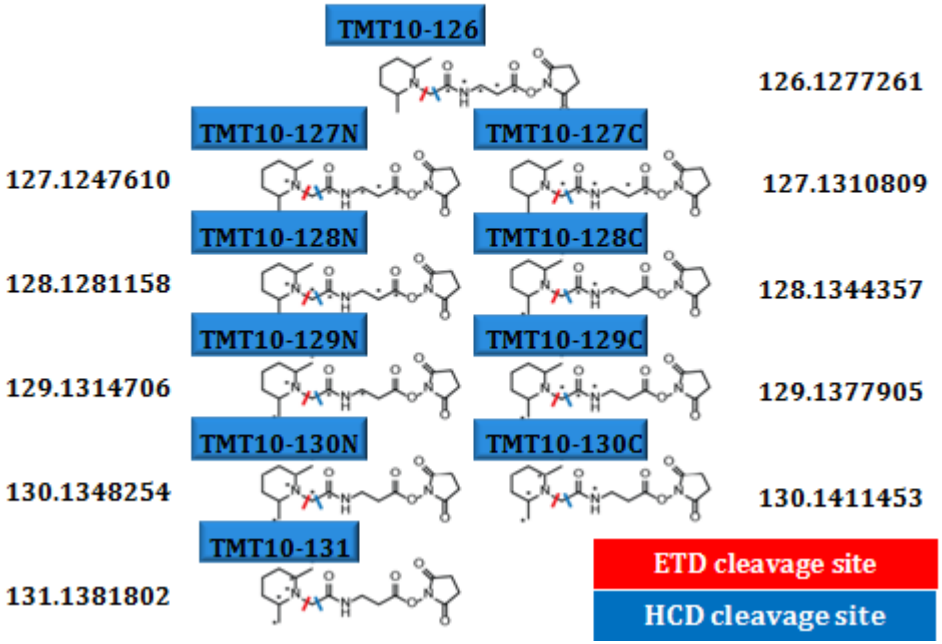


- 13C and 15N labeled reporter
- Fragments by ETD or HCD
- Isotopes balanced between linker region and reporter region keeping all tags exactly isobaric
- Up to 10 different tags
- Other reactive tags :Iodo TMT and Aminoxy TMT

TMT
Duplex Quantitation

TMT
Six Plex Quantitation

TMT
10plex Quantitation



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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 MS²
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

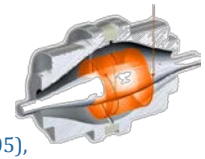
McAlister, G., Huttlin, E.L.; Haas, W.; et. al. Anal Chem. 2012. 84, 7469-7478.

2002
2005
2006
2007
2009
2011
2013
2015

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 MS²

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

Orbitrap Elite

Hybrid; Single Notch MS³; PTR

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

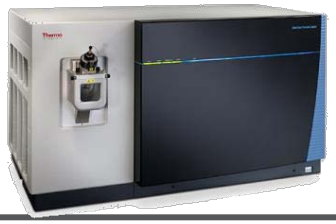
Orbitrap Fusion

Tribrid, Parallelized Analysis, Multinotch

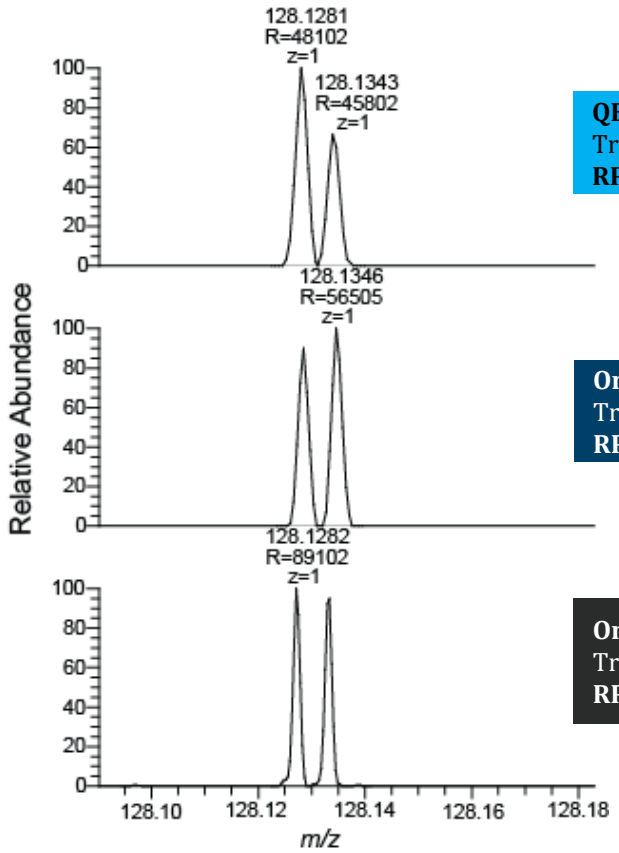
Erickson BK, Jedrychowski MP, McAlister GC, Everley RA, Kunz R, Gygi SP.
Anal Chem 2015 Jan 20;87(2):1241-9

Orbitrap Fusion Lumos

Newest Tribrid, highest sensitivity and selectivity



High Performance Depends Upon High Resolution Instruments



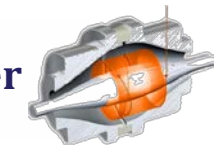
QExactive
Transient 128 ms
RP 35K@*m/z* 200

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 QUANTITATION OF THE TMT10PLEX REAGENTS

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



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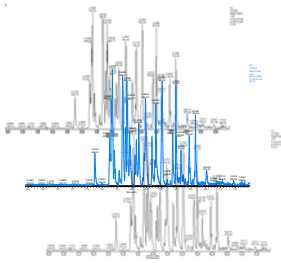
A Real Example

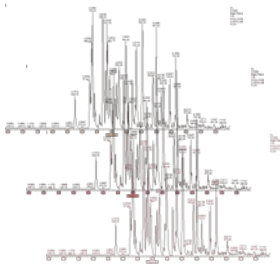
Sample: Mouse mitochondrial extract untreated or treated with phosphatase inhibitor

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

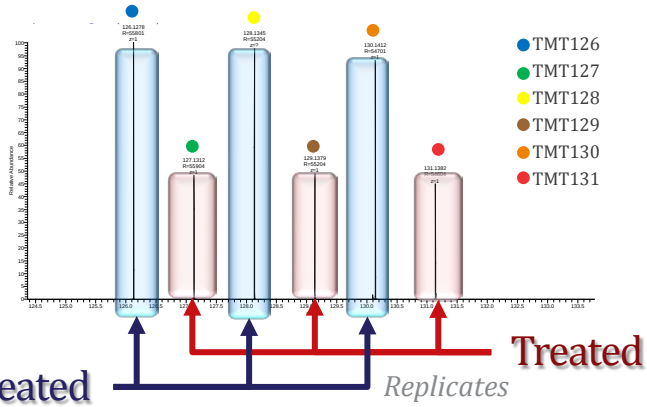
Quantified

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

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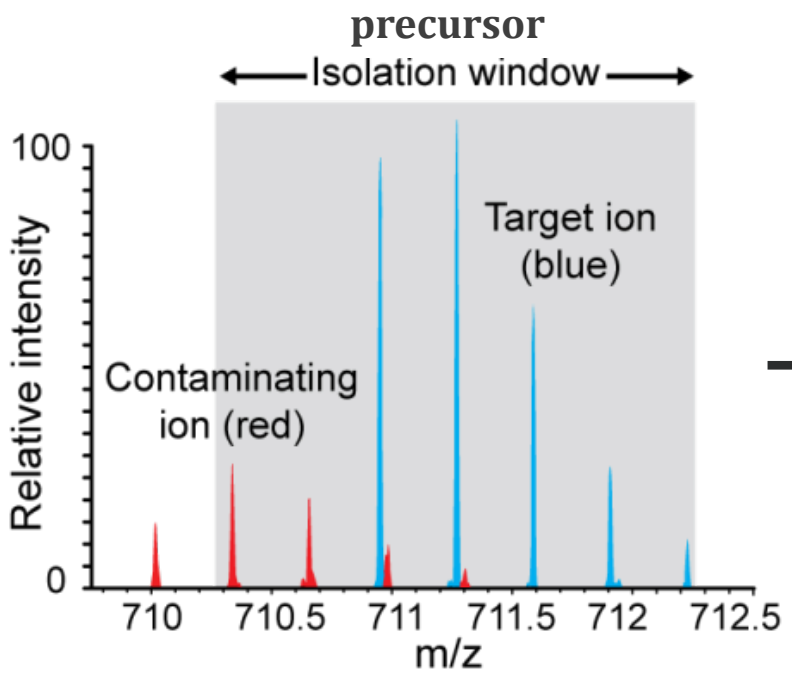
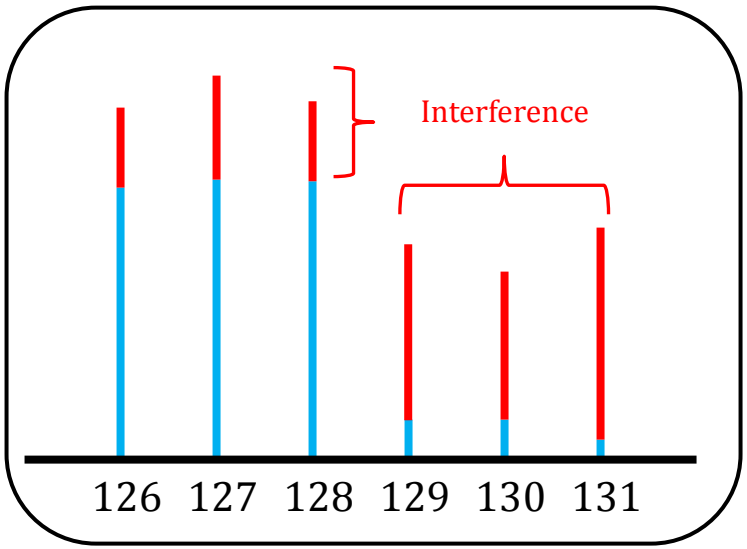
Ratio Distortion with Isobaric Multiplexing

Problem: Quantitation of low-abundance proteins in a complex background is distorted by co-isolated interfering precursor ions



HCD



Ow, S.Y. *et al.* 2009. *JPR* 5347-5355 Ting, L. *et al.* 2011. *Nature Methods* 8: 937-940

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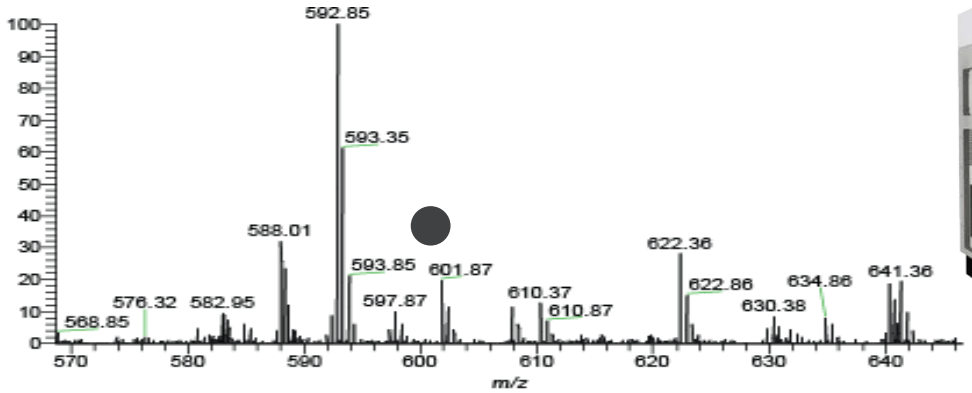
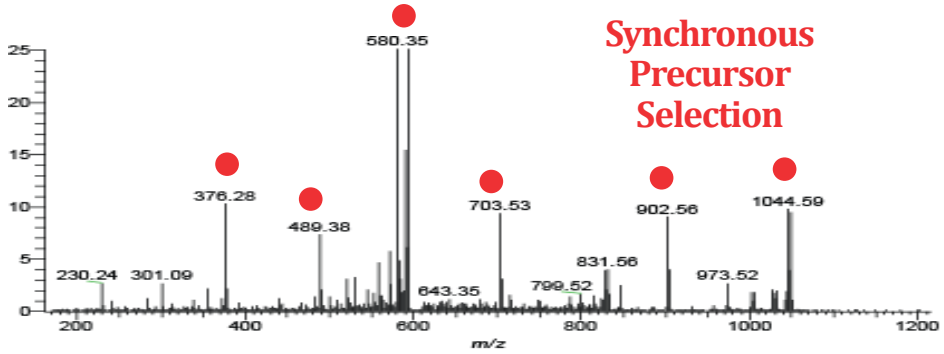
Synchronous Precursor Selection (SPS) for Accurate Quantification

Precursor Ion

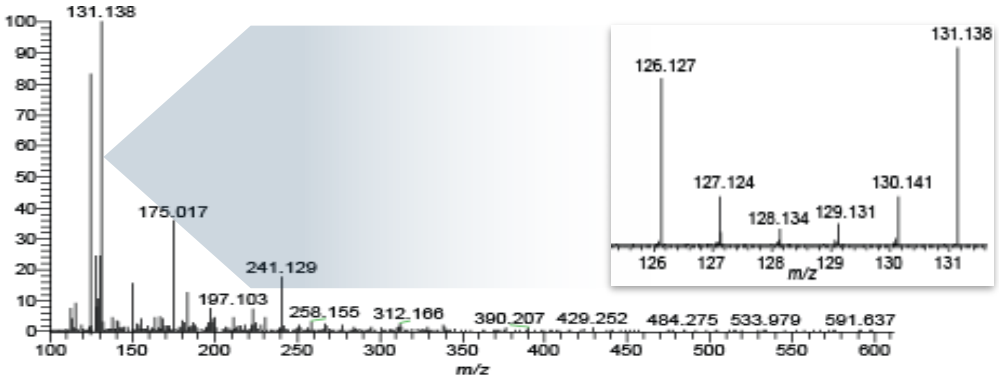


CID MS^2, Ion Trap



Synchronous Precursor Selection

HCD MS^3, OT



2013

Orbitrap Fusion Tribrid Mass Spectrometer

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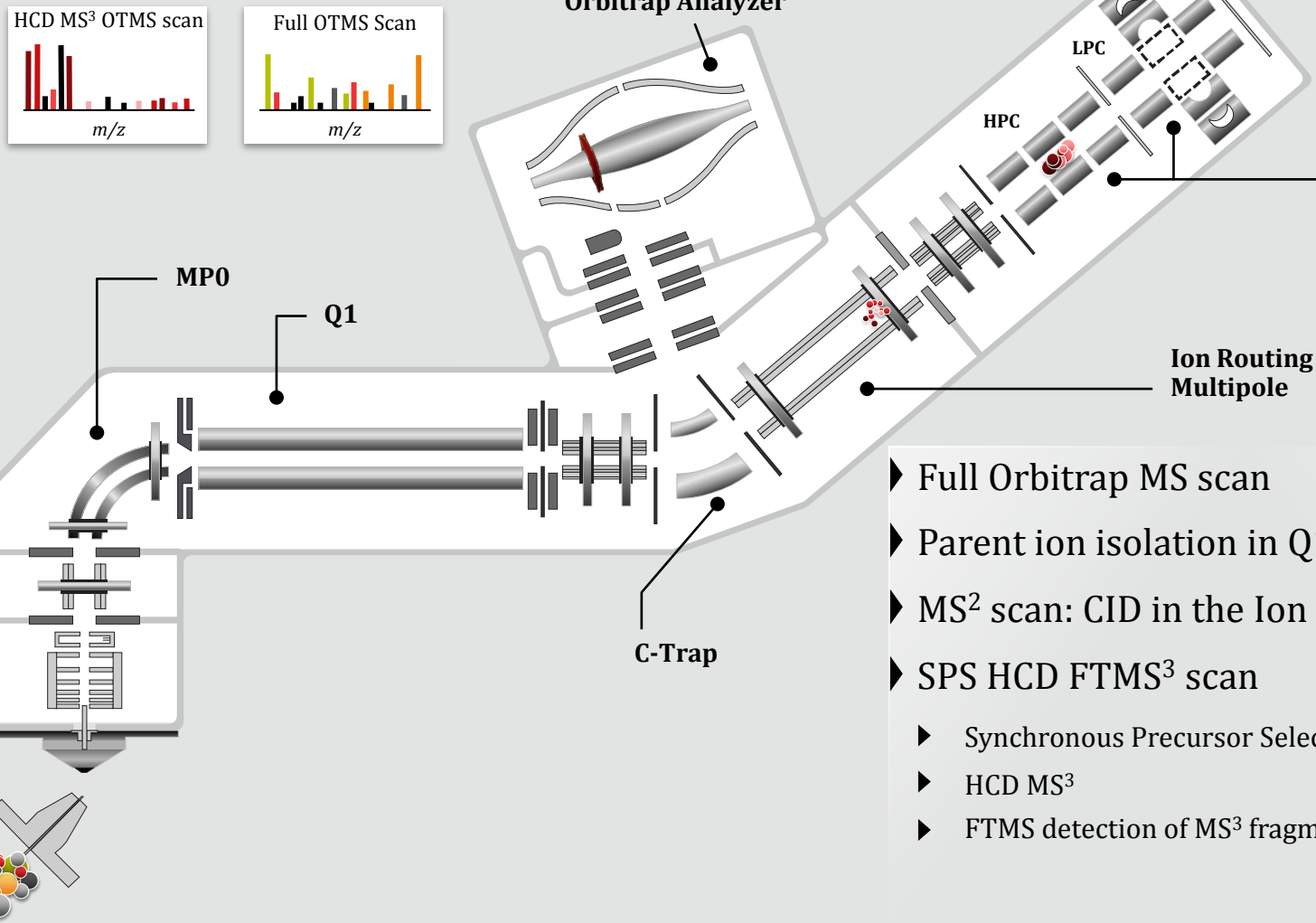
TMT^3 Experiment, Powered by SPS

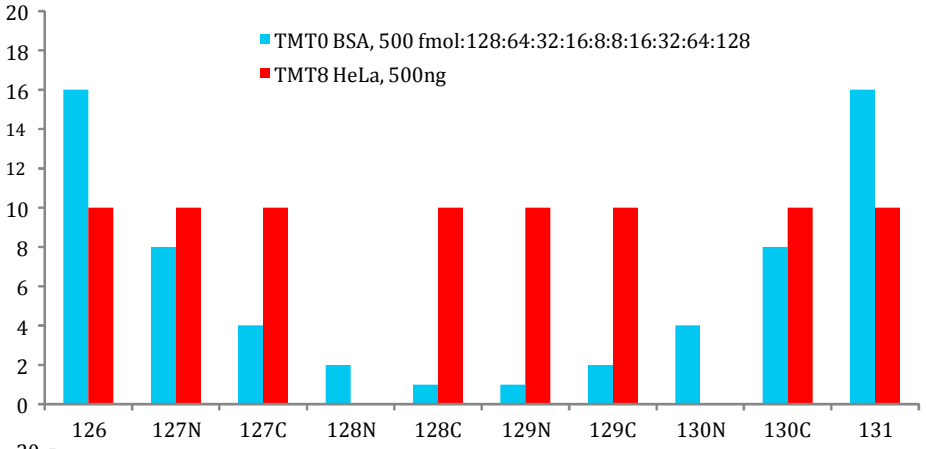
OTMS> CID ITMS^2> SPS HCD OTMS^3



- 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

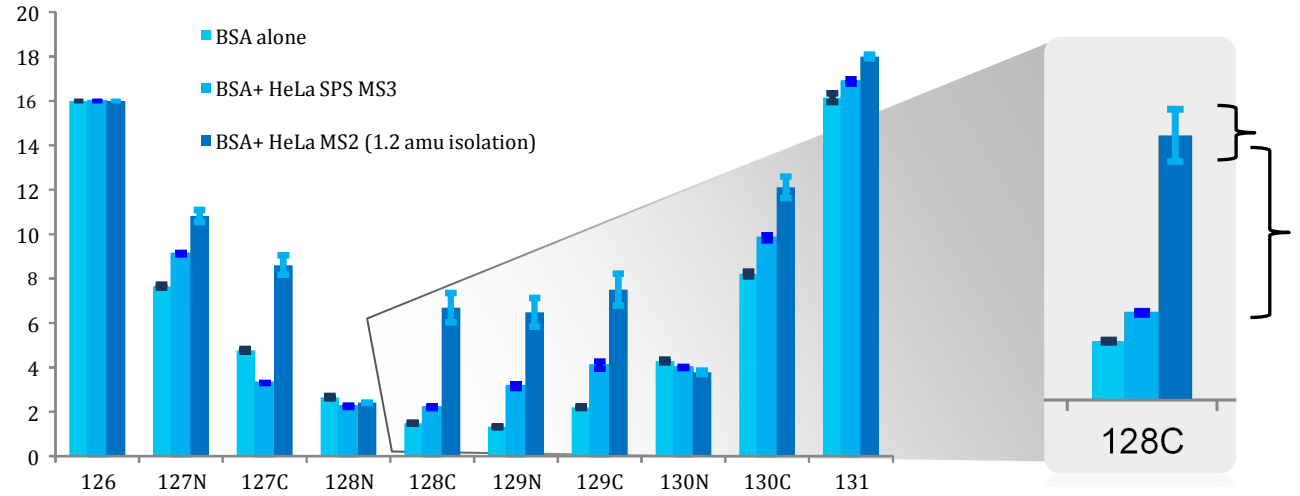
Expected



- 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.

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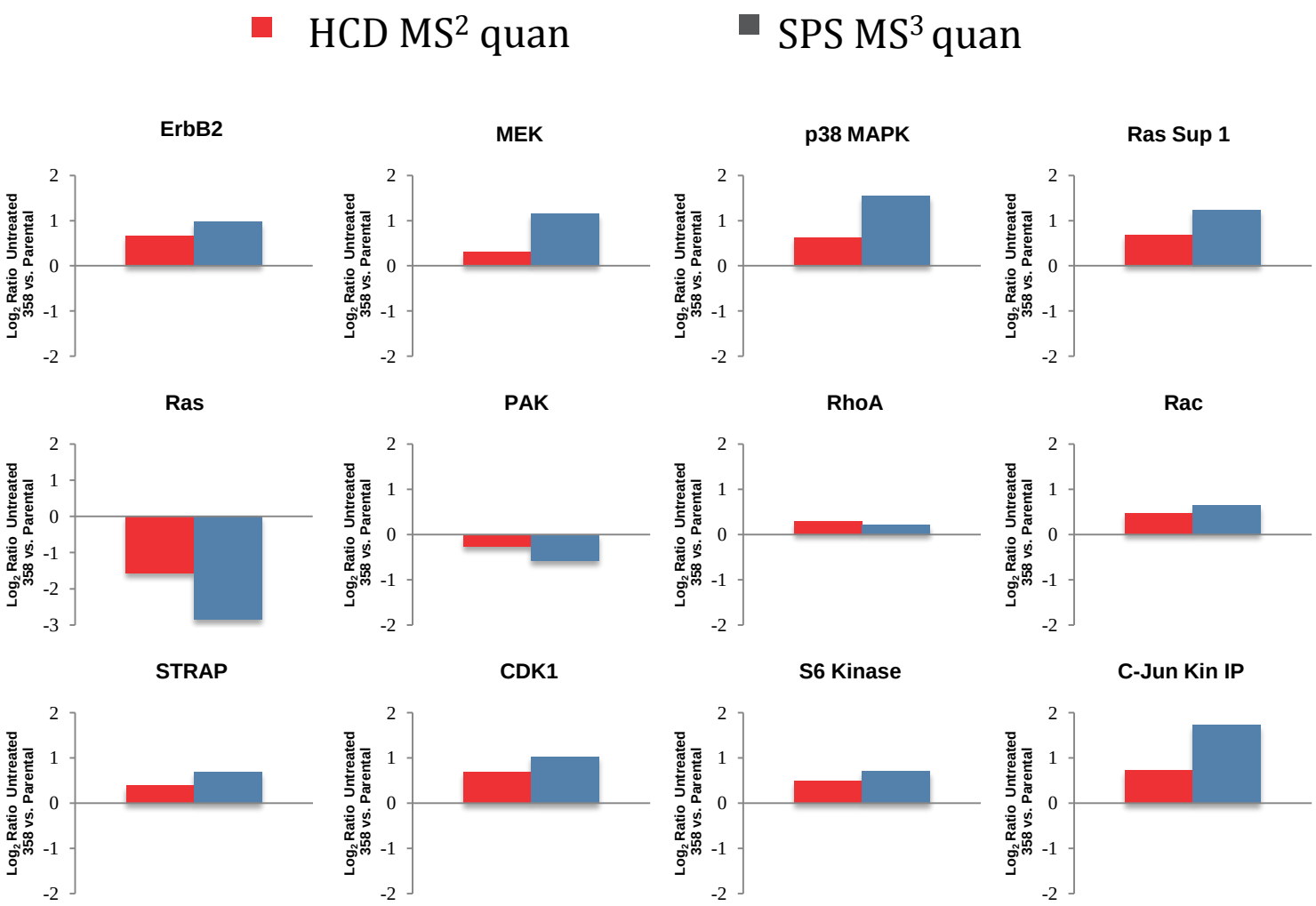
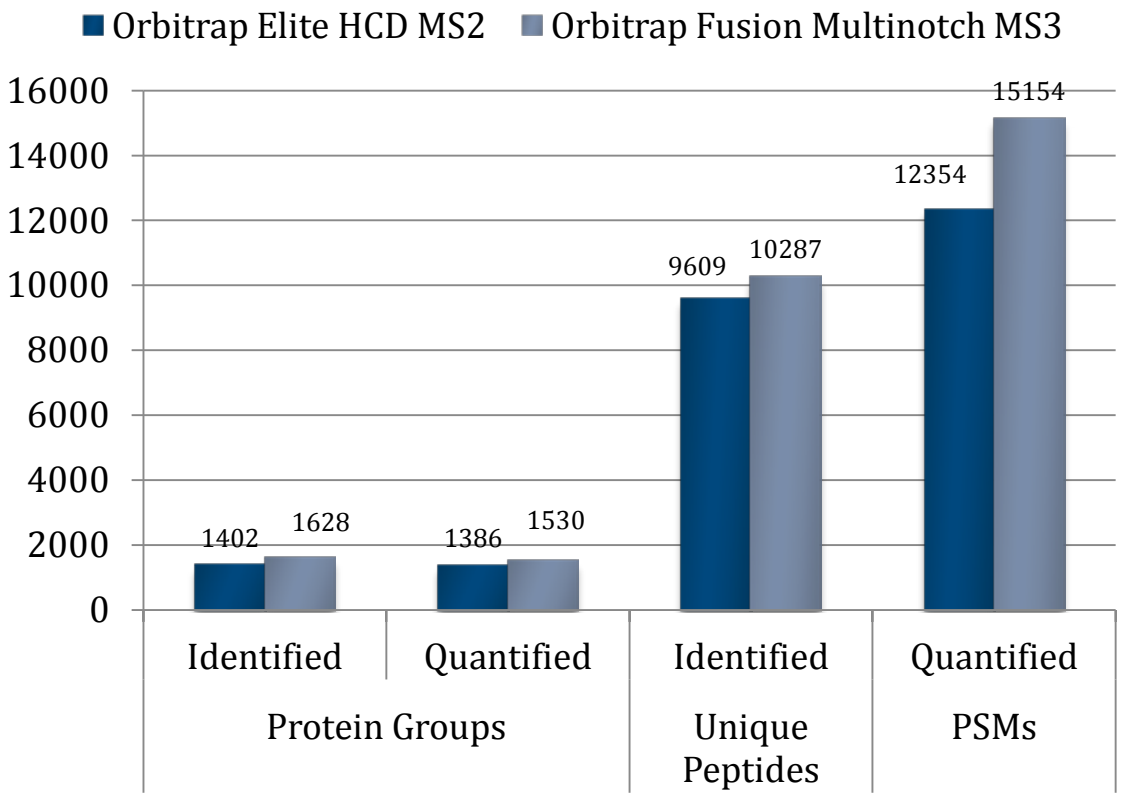
Enhanced Differences Using SPS MS^3 Quantitation



Thermo Poster Note : Towards Mechanism of EGFR Inhibitor Resistance in Non-Small Lung Cancer Cell;M.Blank. et al

…While Still Getting Proteome Coverage

The speed and parallelizable work flow of the Orbitrap Fusion means not choosing between accuracy and coverage…



*The Orbitrap Fusion using multinotch can **Quantify** more proteins than were **Identified** on the previous generation top tier hybrid*

…Don't Settle

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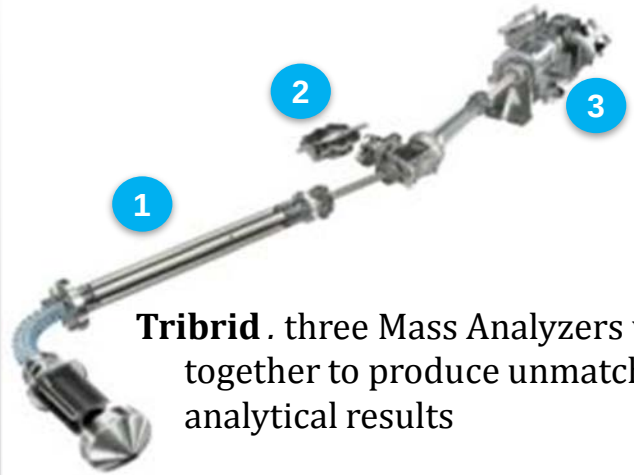
Orbitrap Fusion Lumos Tribrid Mass Spectrometer

2015



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

Unmatched Analytical Performance

Revolutionary performance

Exceptional versatility

Unprecedented usability

Highest sensitivity

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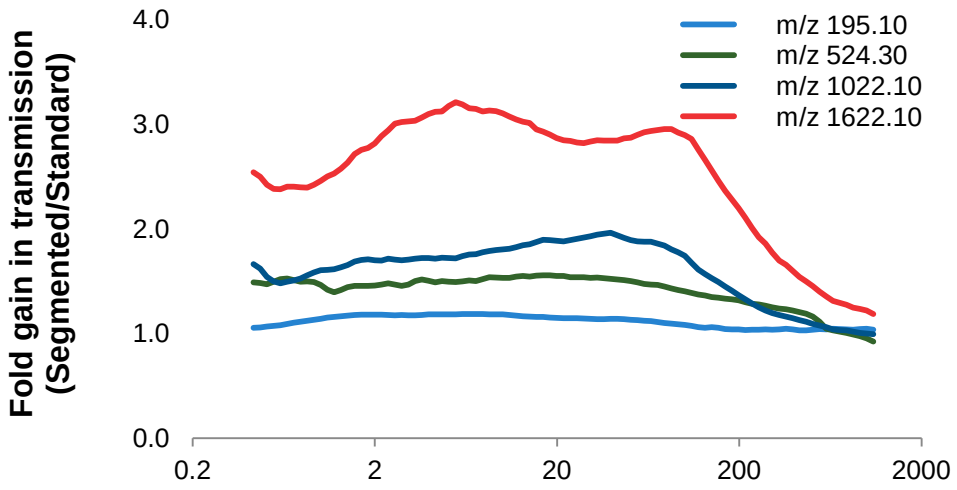
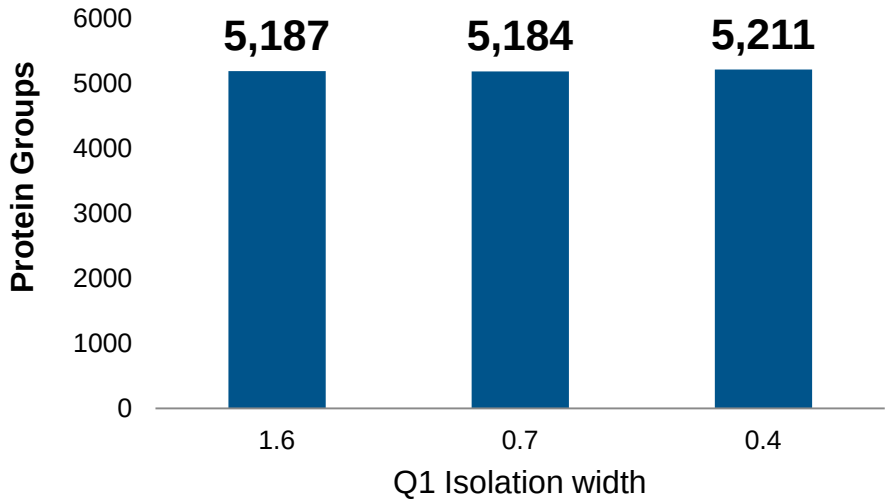
Better Ion Transmission With Segmented Quadrupole





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

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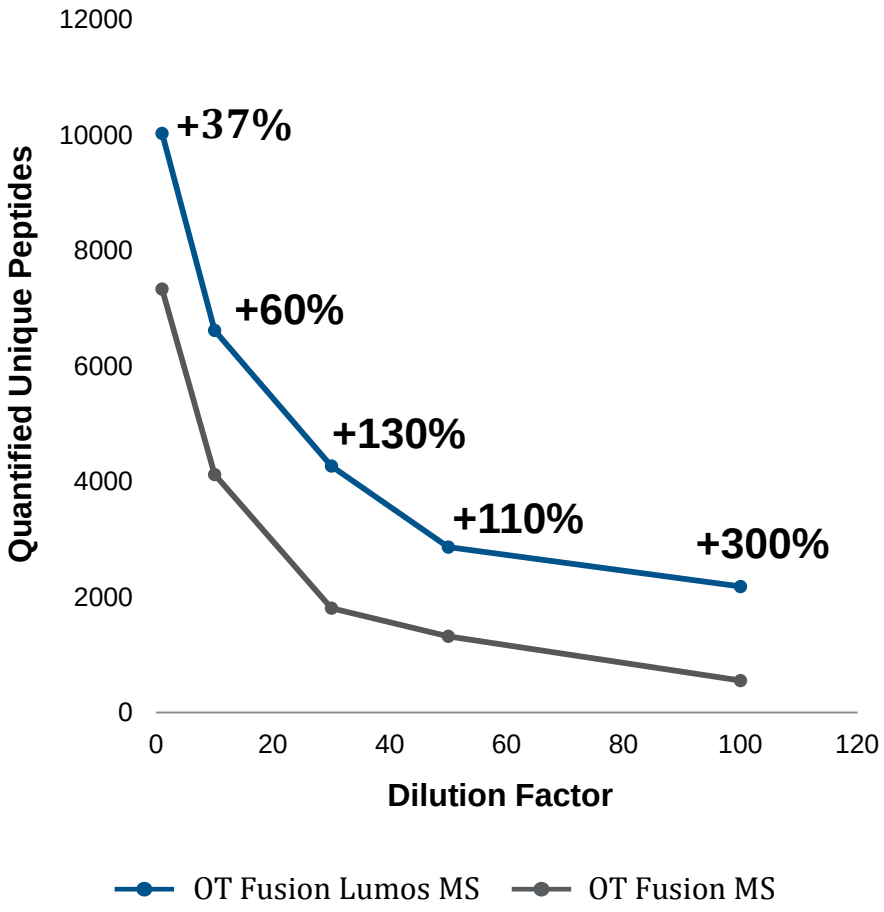
Improved TMT SPS MS^3 Performance

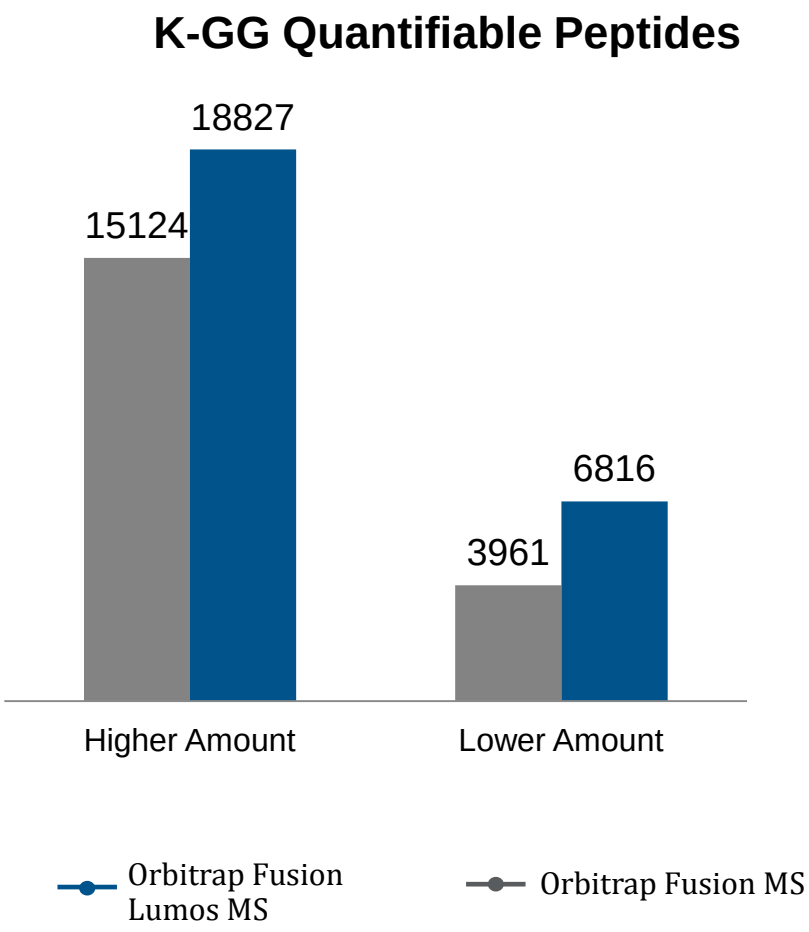


Chris Rose, Gygi's lab, Harvard Medical School

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

Improved Low Level Quan: Ubiquitinated Peptides



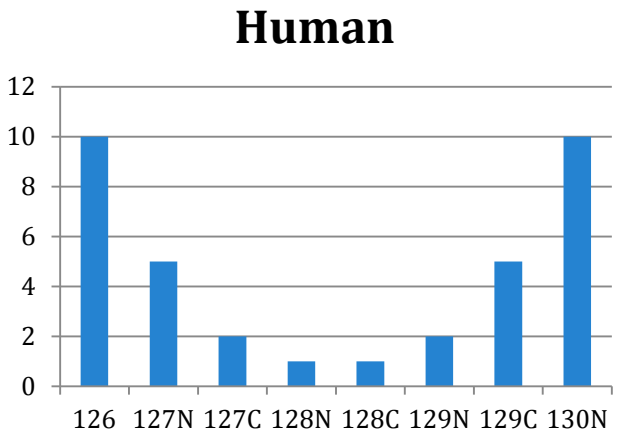
TMT10 Quantitation of Ubiquitinated Peptides

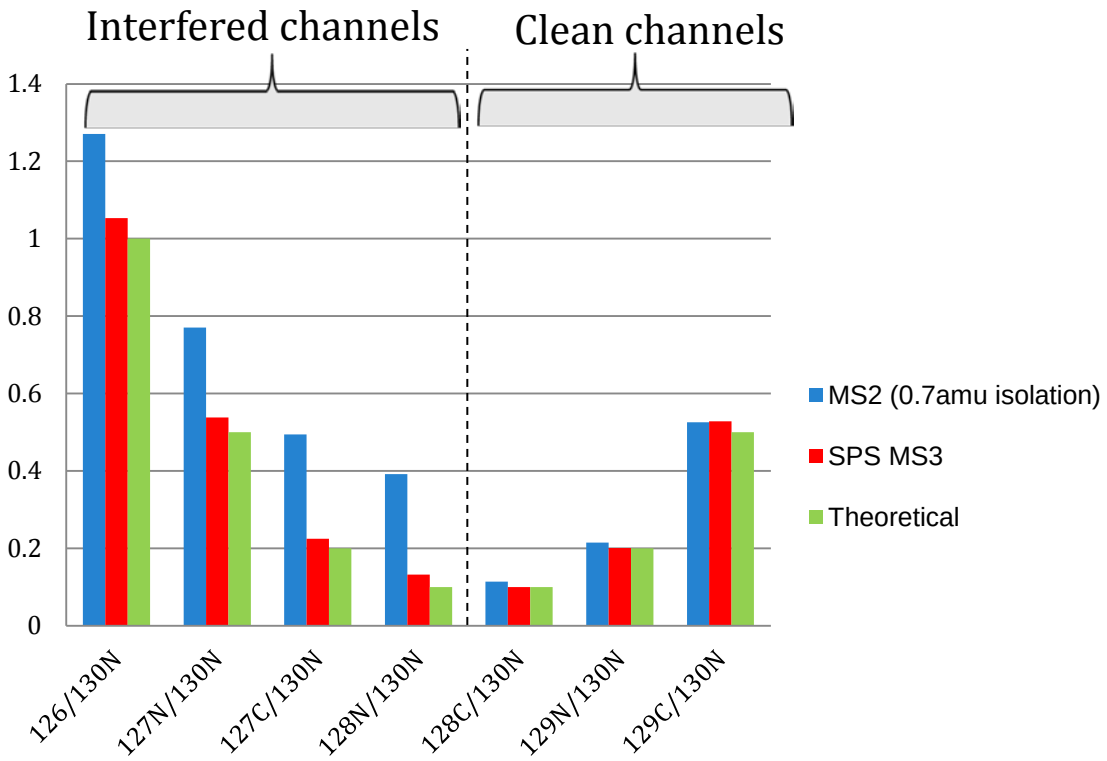
- Human HTC116 cells were treated with a proteasome inhibitor (Bortezomib) for 16 h and analyzed with TMT 10-plex (5 treated vs. 5 untreated)
- Two fractions were prepared
  - With higher amount
  - With lower amount
- 25-73% more quantifiable peptides

ASMS Lecture: Rose et al. Isobaric labeling enables 10-Plex quantitative analysis of ubiquitylated peptides: A diagnostic ion to improve identification and quantification

SPS MS^3 Quantification on Orbitrap Fusion Lumos MS

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





1ug mixture, 4 hr gradient, median ratios

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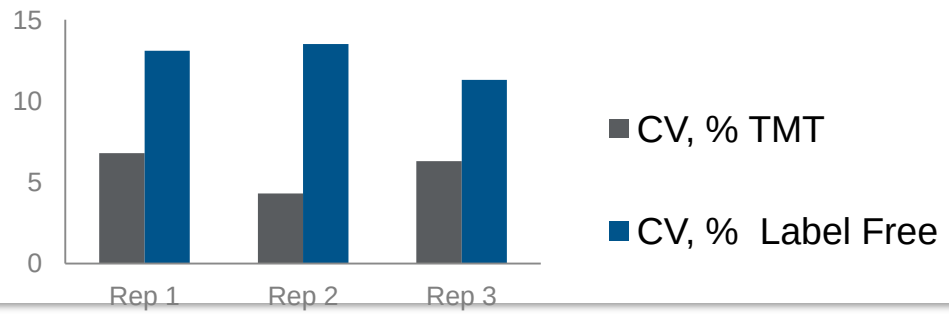
TMT Technology is More Precise than Label Free Quan

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…*"

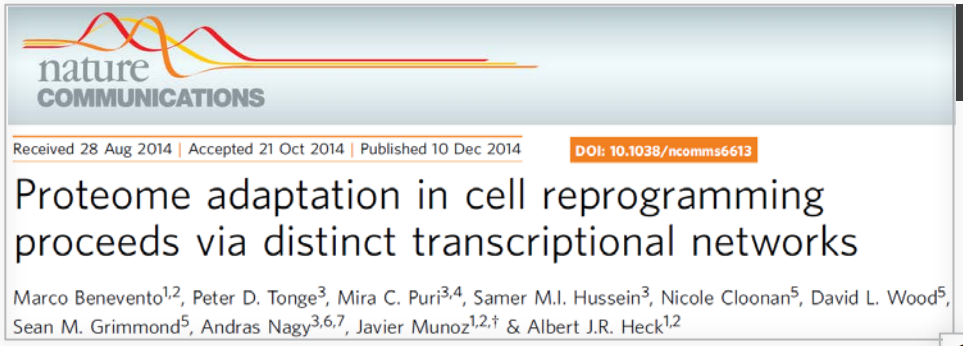


Additional Key Customers Include:

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

ThermoFisher SCIENTIFIC

TMT Used for Protein Research in…



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]

Stem Cells

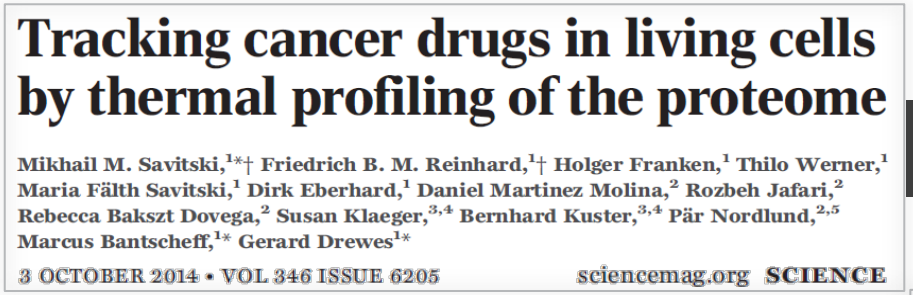
Viromics

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,*]



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

Mikhail M. Savitski,[1*†] Friedrich B. M. Reinhard,[1†] Holger Franken,[1] Thilo Werner,[1] Maria Fälth Savitski,[1] Dirk Eberhard,[1] Daniel Martinez Molina,[2] Rozbeh Jafari,[2] Rebecca Bakszt Dovega,[2] Susan Klaeger,[3,4] Bernhard Kuster,[3,4] Pär Nordlund,[2,5] Marcus Bantscheff,[1*] Gerard Drewes[1*]

3 OCTOBER 2014 • VOL 346 ISSUE 6205 sciencemag.org SCIENCE

Drug Discovery

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

David Britton[1*], Yoh Zen[2], Alberto Quaglia[2], Stefan Selzer[1], Vikram Mitra[1], Christopher Lößner[1], Stephan Jung[1], Gitte Böhm[1], Peter Schmid[1], Petra Prefot[1], Claudia Hoehle[1], Sasa Koncarevic[1], Julia Gee[4], Robert Nicholson[4], Malcolm Ward[1], Leandro Castellano[3], Justin Stebbing[3], Hans Dieter Zucht[1], Debashis Sarker[2], Nigel Heaton[2], Ian Pike[1]

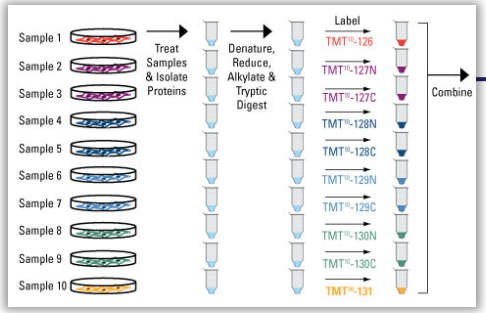
PLOS ONE | www.plosone.org 1 March 2014 | Volume 9 | Issue 3 | e90948

Cancer

ThermoFisher SCIENTIFIC

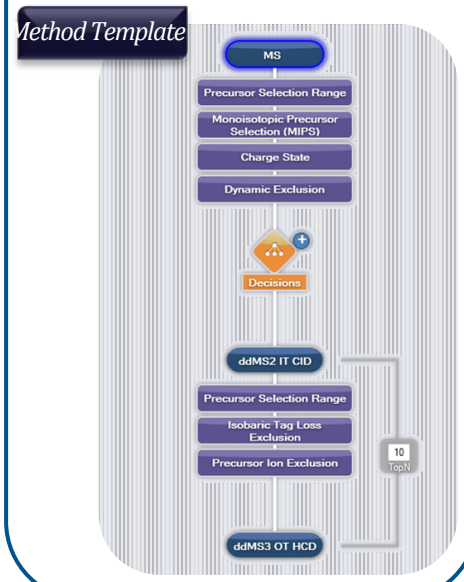
Straightforward Workflow

Sample Preparation

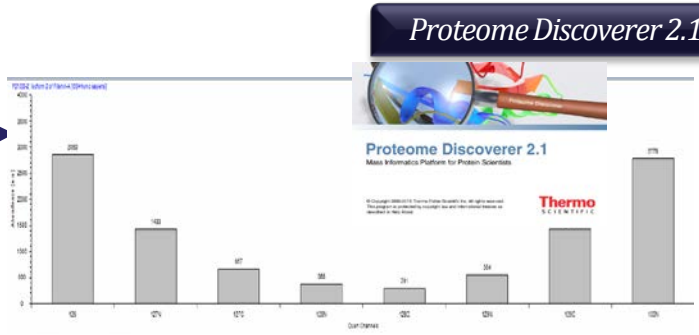


Mass Spectrometry

OT Fusion Lumos



Data Analysis and Interpretation



ProteinCenter
Professional Edition

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

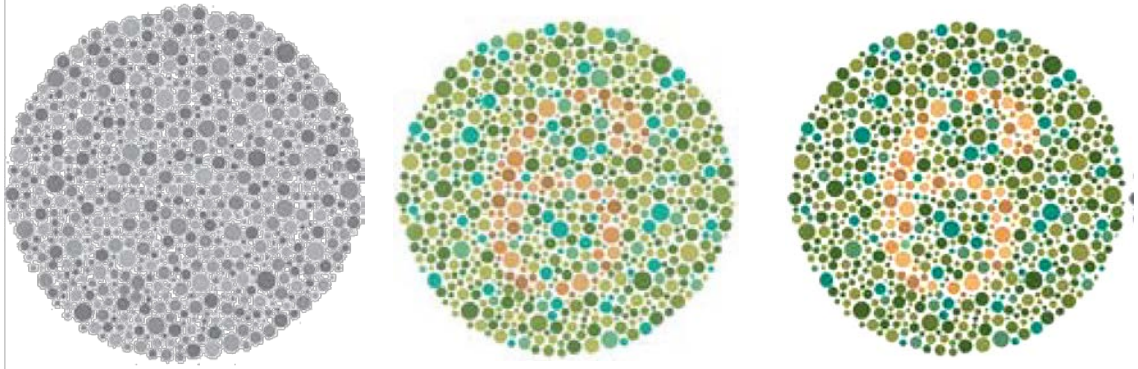
ThermoFisher SCIENTIFIC

Competitive Advantages

Trust your quantitation!

- **Multinotch MS^3 quantitation is more accurate than other MS^2 Methods**
- **The accuracy of Multinotch MS^3 quantitation means not missing important expression level changes due to co-isolated interference**
- **Multinotch MS^3 quantitation 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 quantitation**

No Quan **MS^2** **Multinotch**



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Sample Preparation

Sample Preparation: Materials

Part No.	*Description*
90113	TMT10plex Isobaric Mass Tag Labeling Kit Formulation: Set of ten TMT10 label reagents (3 x 0.8mg each) *Sufficient For: Three 10-plex (3 x 10-way) experiment*

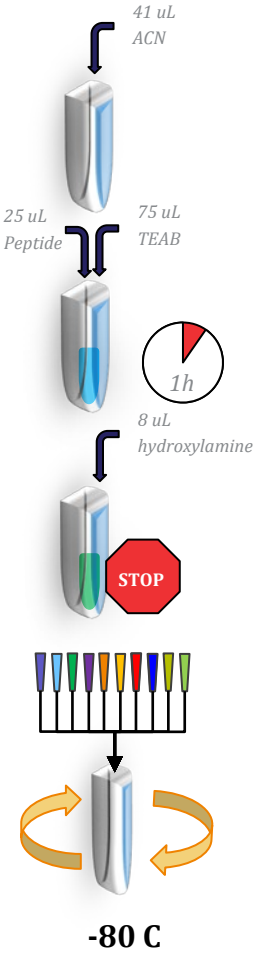
Contents:
TMT10-126 Label Reagent, 3 × 0.8mg
TMT10-127N Label Reagent, 3 × 0.8mg
TMT10-127C Label Reagent, 3 × 0.8mg
TMT10-128N Label Reagent, 3 × 0.8mg
TMT10-128C Label Reagent, 3 × 0.8mg
TMT10-129N Label Reagent, 3 × 0.8mg
TMT10-129C Label Reagent, 3 × 0.8mg
TMT10-130N Label Reagent, 3 × 0.8mg
TMT10-130C Label Reagent, 3 × 0.8mg
TMT10-131 Label Reagent, 3 × 0.8mg
Dissolution Buffer (1M triethyl ammonium bicarbonate), 5mL
Denaturing Reagent (10% SDS), 1mL
Reducing Reagent (0.5M TCEP), 1mL
Iodoacetamide, 12 × 9mg
Quenching Reagent (50% hydroxylamine), 1mL
Pierce Trypsin Protease, MS Grade, 5 × 20µg
Trypsin Storage Solution, 250µL
Albumin, Bovine, 2.5mg

Complete sample preparation kit including alkylation, reduction and digestion

Sample Preparation: Simple Peptide Labeling

Reduced and alkylated trypsin digested proteins
Use Non-Amine Buffer @ pH ~ 8.0 (e.g. TEAB)

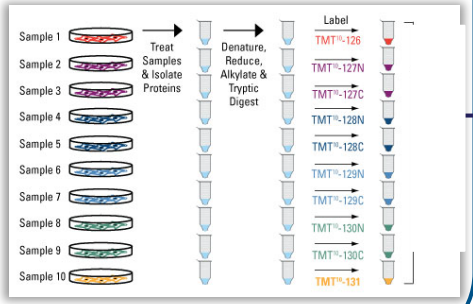
Part No.	*Description*
88328	HeLa Protein Digest Standard Formulation: Lyophilized peptide mixture from a tryptic digest of HeLa S3 cell lysate *Sufficient For: 20 to 100 analyses*



- Add 41µL of anhydrous acetonitrile to each tube. Allow the reagent to dissolve for 5 minutes with occasional vortexing. Briefly centrifuge the tube to gather the solution.
- Transfer 25-100 uL of the reduced and alkylated protein digest (each condition) to the TMT Reagent vial (41 uL). Add sufficient 100 mM TEAB buffer to reach a final volume in vial of 141 uL. Vortex briefly
- Incubate the reaction for 1 hour at room temperature.
- Add 8µL of 5% hydroxylamine to the sample and incubate for 15 minutes to quench the reaction.
- Combine samples in a new microcentrifuge tube at equal amounts and speed vacuum to dryness to remove all TEAB
- Aliquot and Store at -80° C.

A More Complete Workflow For Better Coverage

Sample Preparation

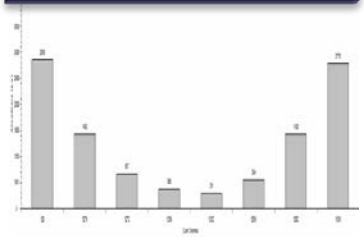


Mass Spectrometry

OT Fusion Lumos

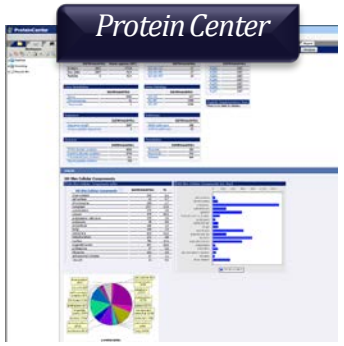
Data Analysis

Proteome Discoverer 2.1

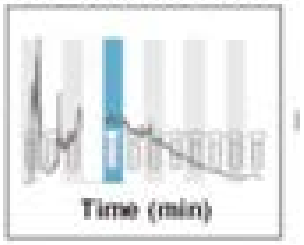


Data Interpretation

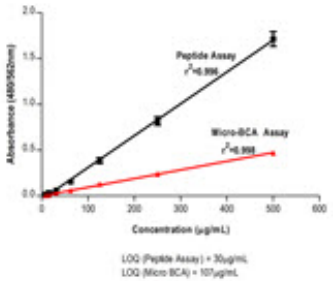
Protein Center



Fractionation



Concentration Measurement



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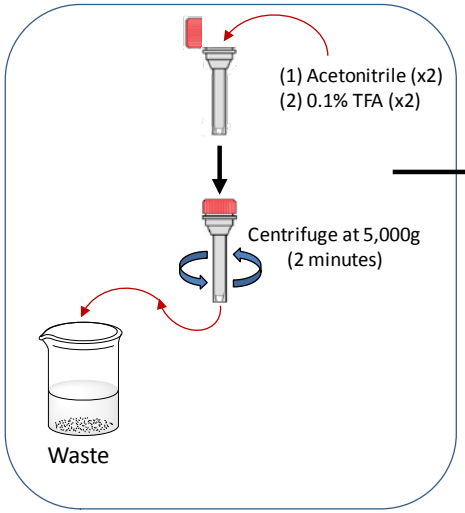
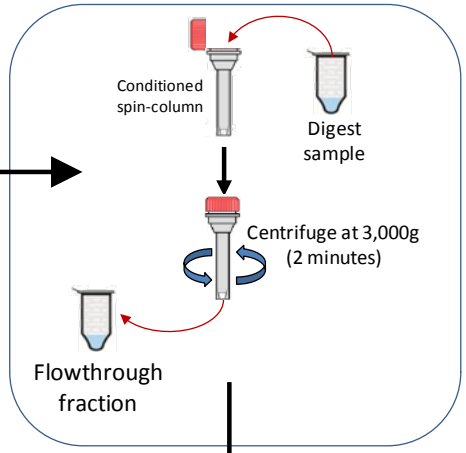
High pH Fractionation Spin Columns

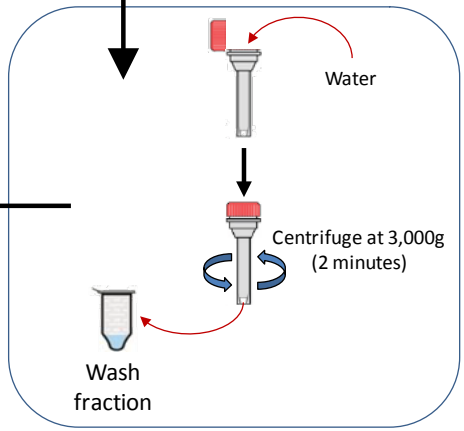
(1) Conditioning



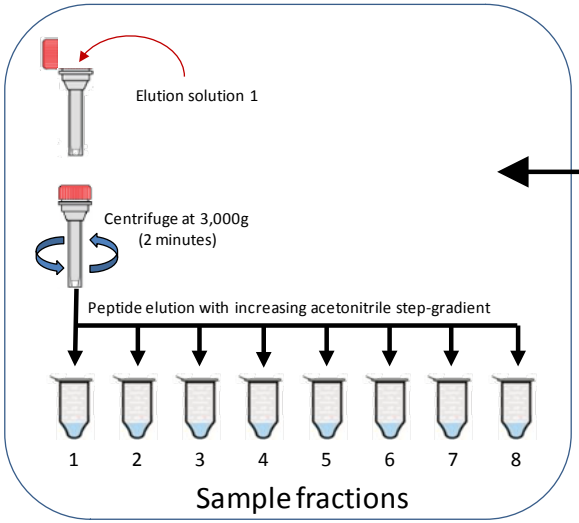
(2) Sample loading



(3) Sample washing (desalting)



(4) Stepwise Fractionation



Thermo Poster Note 64606: High pH Reversed-Phase Peptide Fractionation in a Convenient SpinColumn Format; Snovida S. et al
Thermo Poster Note 64604: Quantitative peptide assay for optimized and reproducible sample preparations for mass spectrometry applications; Jiang X. et al

High pH Reversed Phase vs SCX Fractionation

Strong Cation Exchange (SCX)

Digest

↓

Buffer Exchange

↓

Fractionate

↓

Desalt

↓

LC-MS

High pH Reversed Phase

Digest

↓

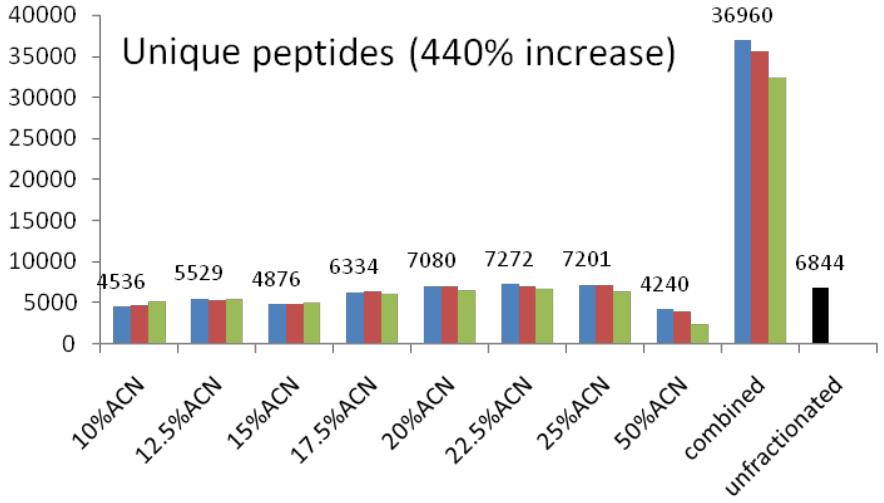
Fractionate

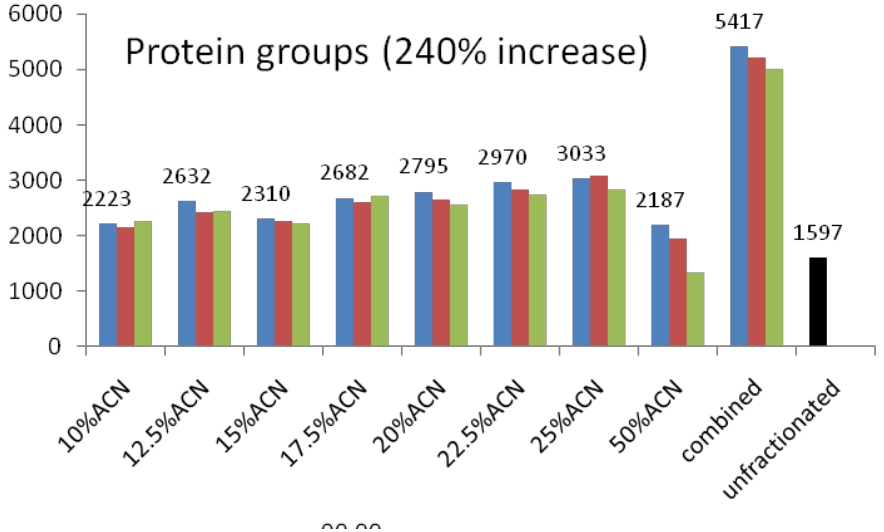
↓

LC-MS

- SCX and high pH reversed phase fractionation are both orthogonal to low pH C18 LC separation
- Strong cation exchange (SCX) requires sample desalting after fractionation

Reproducibly Identify More TMT-labeled Peptides



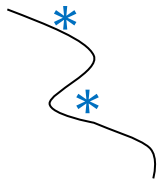


- Significantly increase the number of proteins identified and quantified
- The percentage and number of peptides observed in only 1 fraction are within 10% between runs

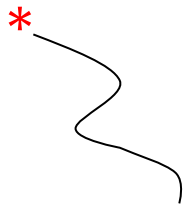
Two New Peptide Quantitation Assays

Colorimetric Peptide Quantitation (CPQ)

TMT compatible



Fluorimetric Peptide Quantitation (FPQ)



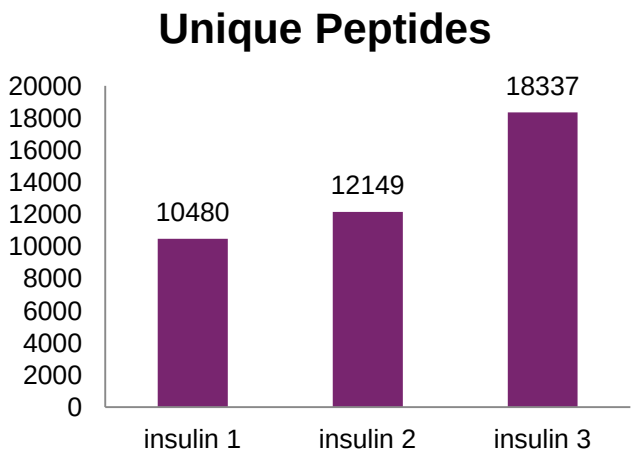
Assay	CPQ assay	FPQ assay
Chemistry	Indirect Cu-reduction and chelation	Direct N-terminal labeling induced fluorescence
Time	30 mins	5 mins
Measurement	Abs 480nm	Ex. 390nm/Em. 475nm
Linearity	15-1000 µg/mL	5-1000 µg/mL
Sensitivity	15 µg/mL	5 µg/mL
Minimum sample	0.3 µg	0.05 µg
Not recommended for	Single peptides	TMT Reagent-labeled samples

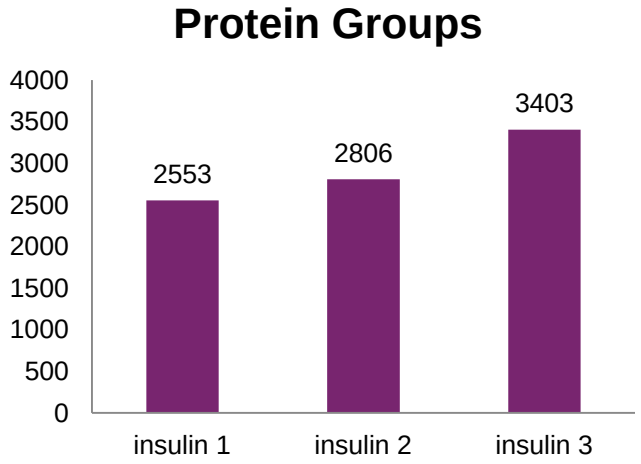
Colorimetric peptide assay is more sensitive than BCA

Thermo Poster Note: Quantitative Peptide Assays for Mass Spectrometry Applications; Haney P. et al

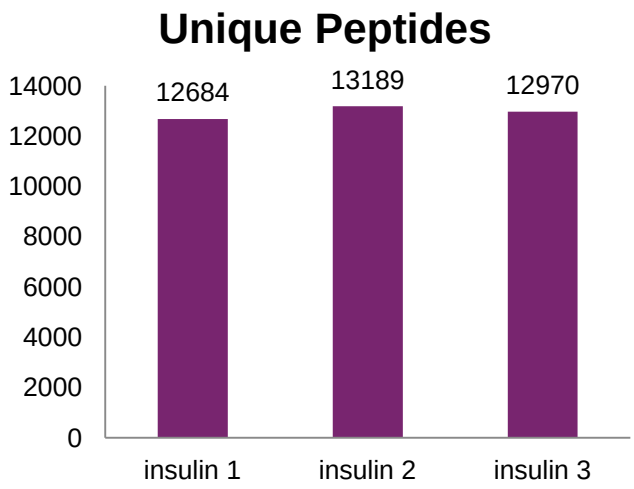
Peptide Quantitation Improves MS Reproducibility

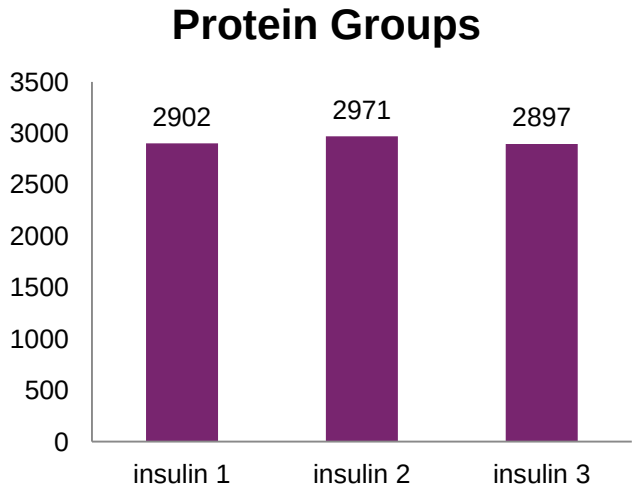
Before Peptide Quantitation





After Peptide Quantitation





Thermo Poster Note 64604: Quantitative peptide assay for optimized and reproducible sample preparations for mass spectrometry applications; Jiang X. et al

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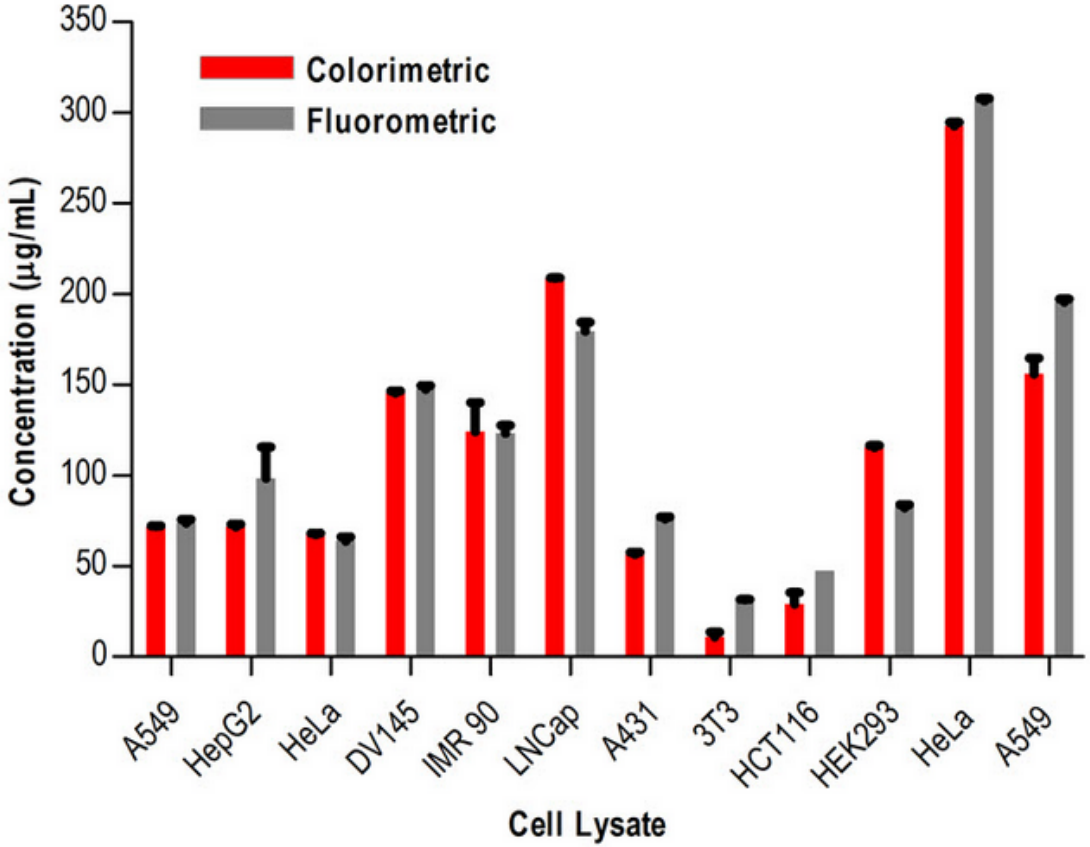
Peptide Assays Provide Consistent Quantification

"We really like those peptide quant kits. We find that peptide mass quantitation is directly relevant for determining the amount of analyte we use for LC-MS/MS analyses, phosphopeptide enrichments, and for TMT labeling.

Josh Coon, UW- Madison



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Instrument Configuration

Introducing A New Powerful Combination

Unlock the performance of your MS with incredibly easy chromatography

[EASY-nLC™ 1200 HPLC System]

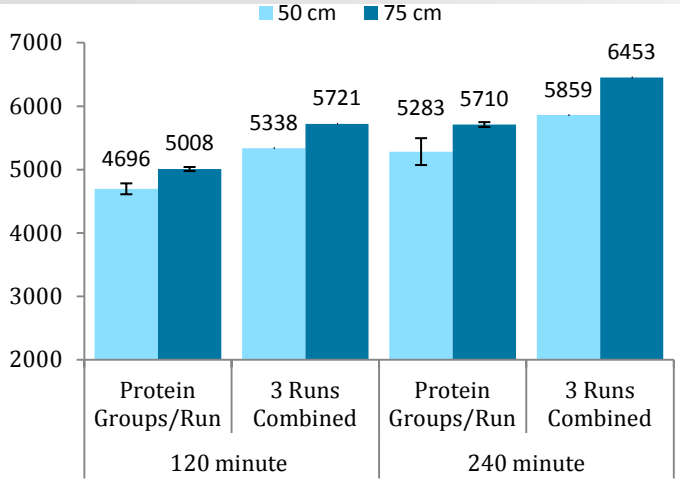
- Industry leading 1200 bar system pressure
- Improved system robustness and easier maintenance
- Temperature control of EASY-Spray columns
- Effortless ultra-high performance for every user, every time

[75cm EASY-Spray™ Column]

- Increased peak capacity
- Even more identifications
- Excellent retention time consistency
- Improved quantitative reproducibility

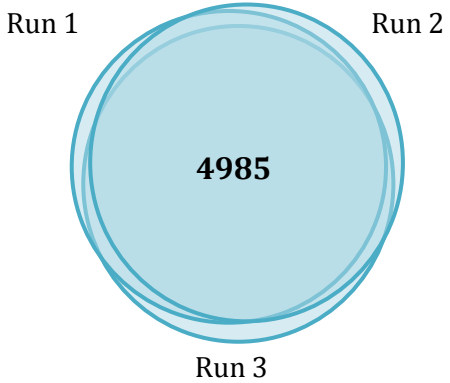
Extend The Performance Of Your MS with 75cm Columns

Improved Protein Identifications



- Peak capacity exceeding 800.
- Deeper proteome coverage with a consistent 5700 protein groups per run.
- 9% increase in protein groups over 3 combined runs compared to 50 cm column.
- Identify more proteins per hour – comparable identifications in 120 minutes to a 50 cm column in 240 minutes.

Increased Quantitative Reproducibility



- Less run-to-run variability 91% identification overlap run to run
- More quantifiable proteins 50% increase in the number of quantifiable proteins with CVs <5%

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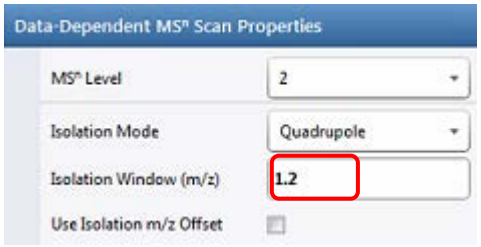
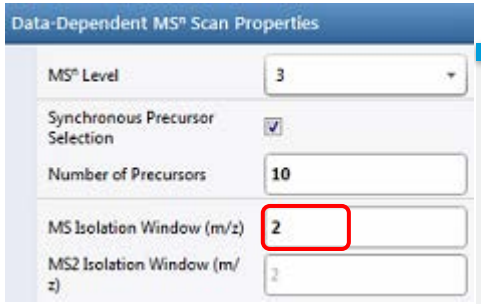
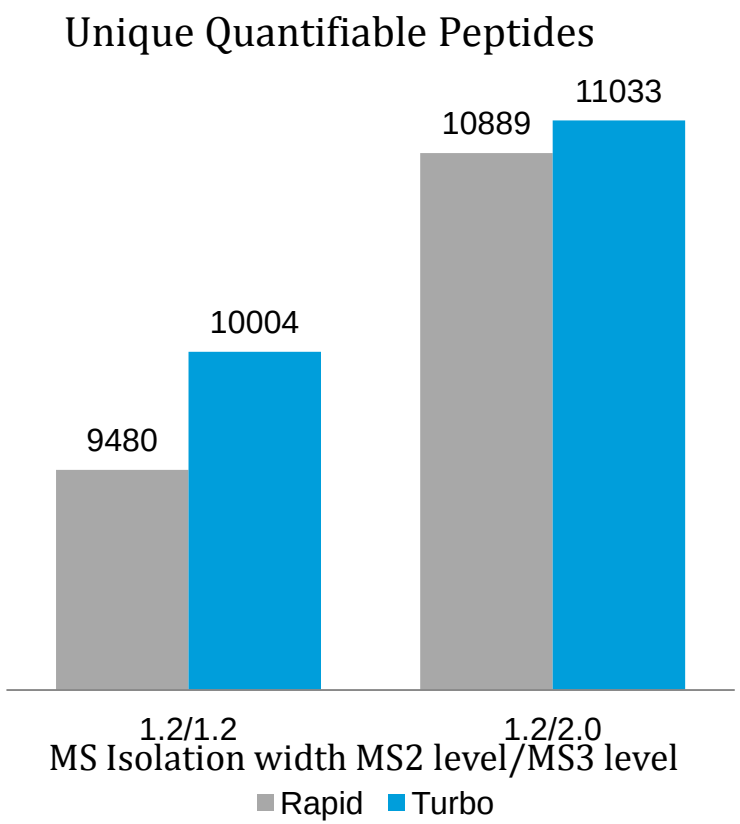
SPS TMT Method Development- What is New on Lumos







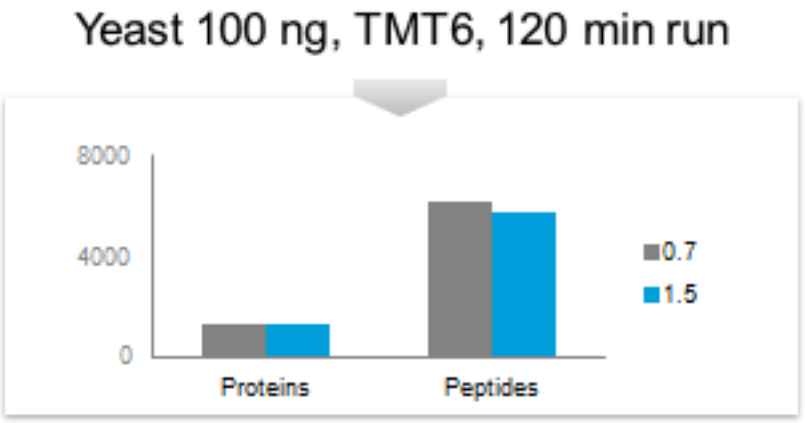
TMT Quantification Improved!

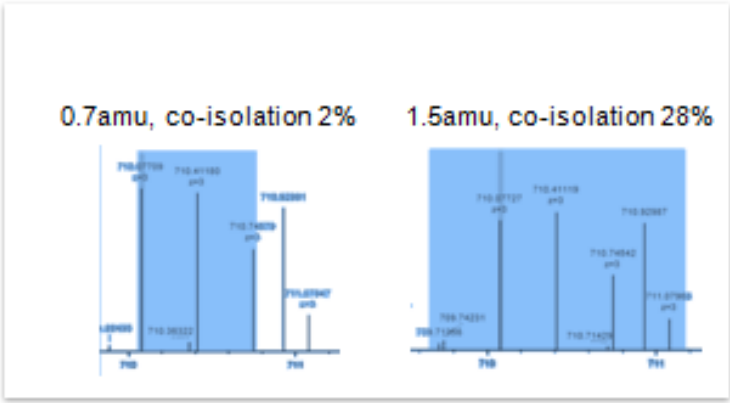
- Turbo Scan MS2
- Single Charge State per Precursor Selection
- Variable Isolation Width MS2/MS3, available in Fusion Lumos and Fusion Tune 2.0

TMT10 HeLa cell lysate (500 ng, 2 hr gradient) was analyzed using different SPS MS3 methods on Fusion with Tune 2.0.
Best results were obtained using all featured settings.

Multiplexing On the Benchtop Orbitrap System (Q Exactive Series)

- Take advantage of segmented quadrupole (QE Plus and HF), for more efficient isolation in narrow windows
- Suited for the analysis of low and medium complexity samples
- Pre-fractionation recommended for high complexity samples to improve quantification accuracy and precision (Pierce Spin Column)





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Data Analysis

Proteome Discoverer 2.1

- New method TMT quantification
  - TMT correction factors (for all TMT reagents) with new user interface
  - Use of “Razor” peptides for protein quantification
  - Modified form of Gygi group’s S/N-based approach to TMT quantification
  - Custom ratio generation
  - New heat map-like coloring of ratios and scaled abundances

Note that almost of all these changes are also applied to isotope-labeled quantification (e.g. SILAC)

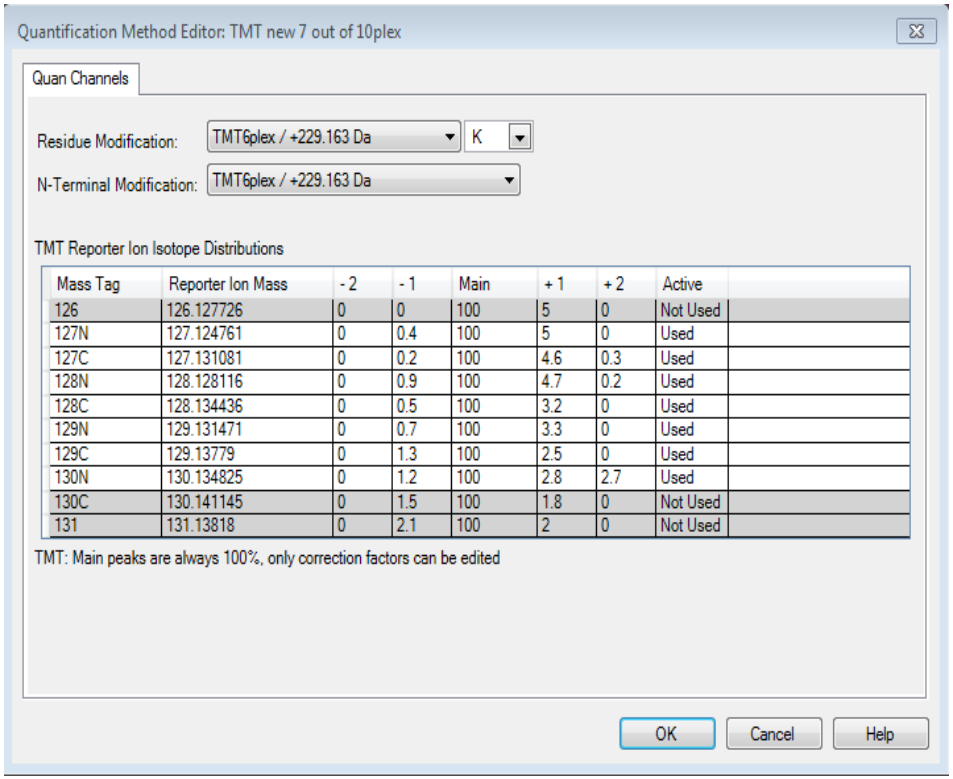
ThermoFisher SCIENTIFIC

TMT Correction Factor Setup

TMT correction factor certificate for each manufacturing lot

Mass Tag	Reporter Ion	-2	-1	Monoisotopic	+1	+2
TMT^{10}-126	126.127726	0.0%	0.0%	100%	5.0% (127C)	0.0% (128N)
TMT^{10}-127N	127.124761	0.0%	0.4%	100%	5.0% (128N)	0.0% (128C)
TMT^{10}-127C	127.131081	0.0%	0.2% (126)	100%	4.6% (128C)	0.3% (129N)
TMT^{10}-128N	128.128116	0.0%	0.9% (127N)	100%	4.7% (129N)	0.2% (129C)
TMT^{10}-128C	128.134436	0.0% (126)	0.5% (127C)	100%	3.2% (129C)	0.0% (130N)
TMT^{10}-129N	129.131471	0.0% (127N)	0.7% (128N)	100%	3.3% (130N)	0.0% (130C)
TMT^{10}-129C	129.137790	0.0% (127C)	1.3% (128C)	100%	2.5% (130C)	0.0% (131)
TMT^{10}-130N	130.134825	0.0% (128N)	1.2% (129N)	100%	2.8% (131)	2.7%
TMT^{10}-130C	130.141145	0.0% (128C)	1.5% (129C)	100%	1.8%	0.0%
TMT^{10}-131	131.138180	0.0% (129N)	2.1% (130N)	100%	2.0%	0.0%

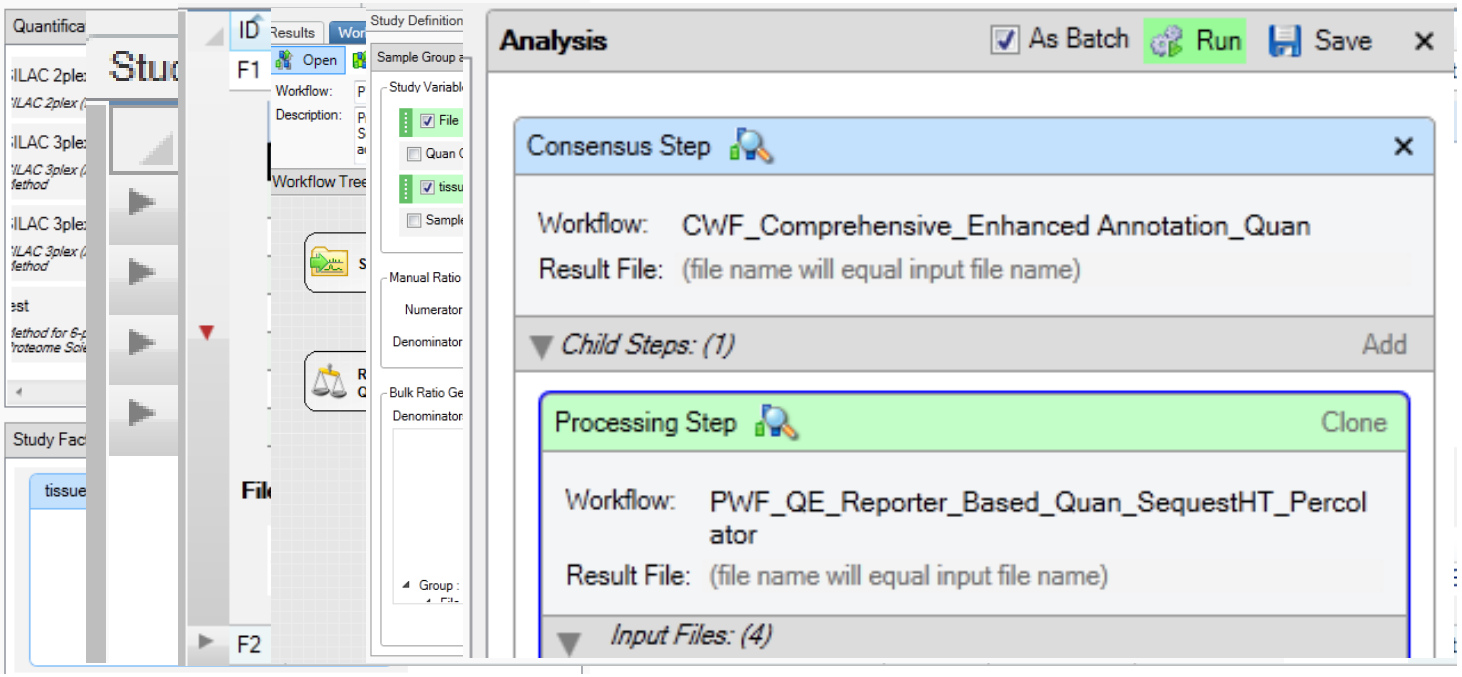
Edit Quantification Method



Certificate of Analysis (CoA) can be found at http://www.thermofisher.com/order/catalog/product/90110?ICID=search-product using the lot number displayed on the reagents packages.

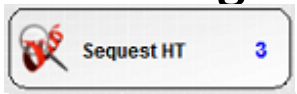
Study Management Setup

- Select quan method and assign study factors
- Input data
- Specify Quan method, match data files and Quan Channels with study factors
- Select processing and consensus workflows and make modifications
- Specify how to group quantification results
- Run

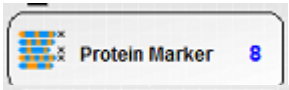


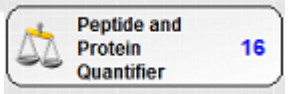
Accommodates the most complex study designs

Select Workflows and Modify Parameters

- **Processing Workflow:**
  - 

:Protein database, enzymes, and modifications
  - 

: MS3 (SPS)
- **Consensus Workflow:**
  - 

: Specify each proteome
  - 

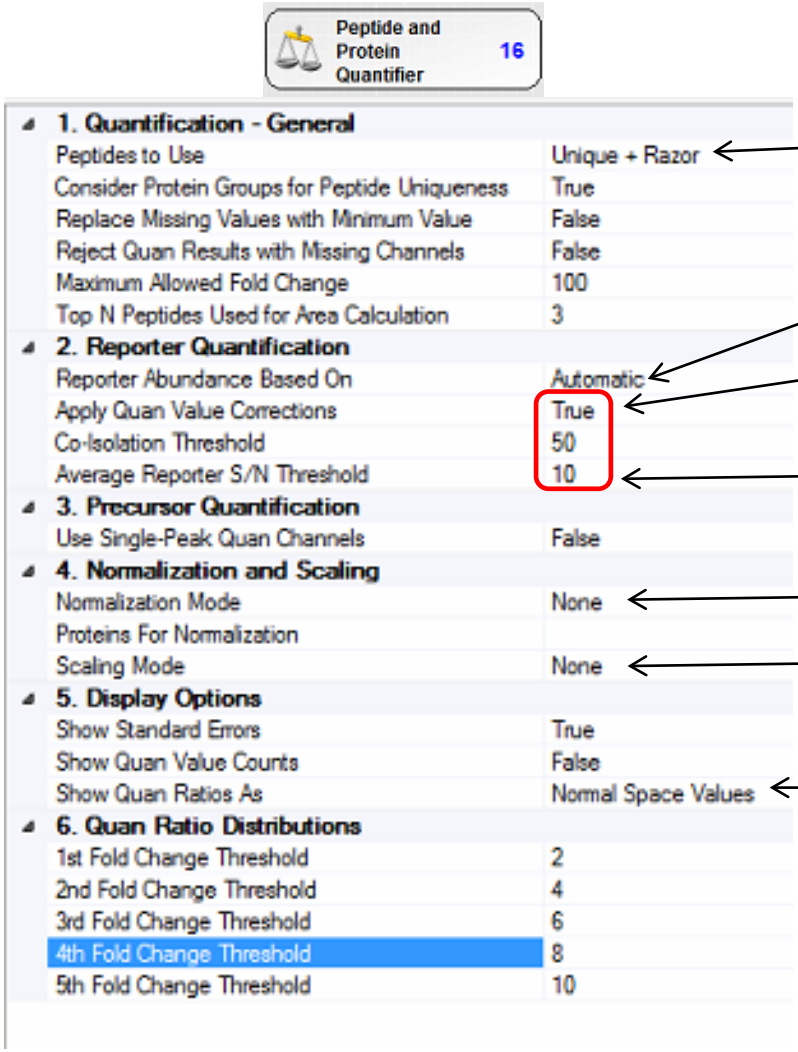
: next slide

2. C-Terminal Modificati	None
3. C-Terminal Modificati	None
6. Dynamic Modifications (protein terminus)	
1. N-Terminal Modificati	Acetyl / +42.011 Da (N-Termi
2. N-Terminal Modificati	None
3. N-Terminal Modificati	None
1. C-Terminal Modificati	None
2. C-Terminal Modificati	None
3. C-Terminal Modificati	None
7. Static Modifications	
Peptide N-Terminus	TMT6plex / +229.163 Da (An
Peptide C-Terminus	None
1. Static Modification	Carbamidomethyl / +57.021 D
2. Static Modification	TMT6plex / +229.163 Da
3. Static Modification	None
4. Static Modification	None
5. Static Modification	None
6. Static Modification	None

1. Peak Integration	
Integration Tolerance	10 ppm
Integration Method	Most Confident Centroid
2. Scan Event Filters	
Mass Analyzer	FTMS
MS Order	MS2
Activation Type	HCD
Min. Collision Energy	0
Max. Collision Energy	1000

1. Contaminant Database	
Protein Database	
2. Additional Marker Database	
Column Name	Ecoli
Protein Database	Escherichia coli K-12 (SwissProt TaxID=83333) (v2015-02-04)
3. Additional Marker Database	
Column Name	Human
Protein Database	Homo sapiens (SwissProt TaxID=9606) (v2015-02-04)

Select Workflows and Modify Parameters (Con'd)



New option for quantification NEW!

Consider S/N first NEW!

Correction factors NEW!

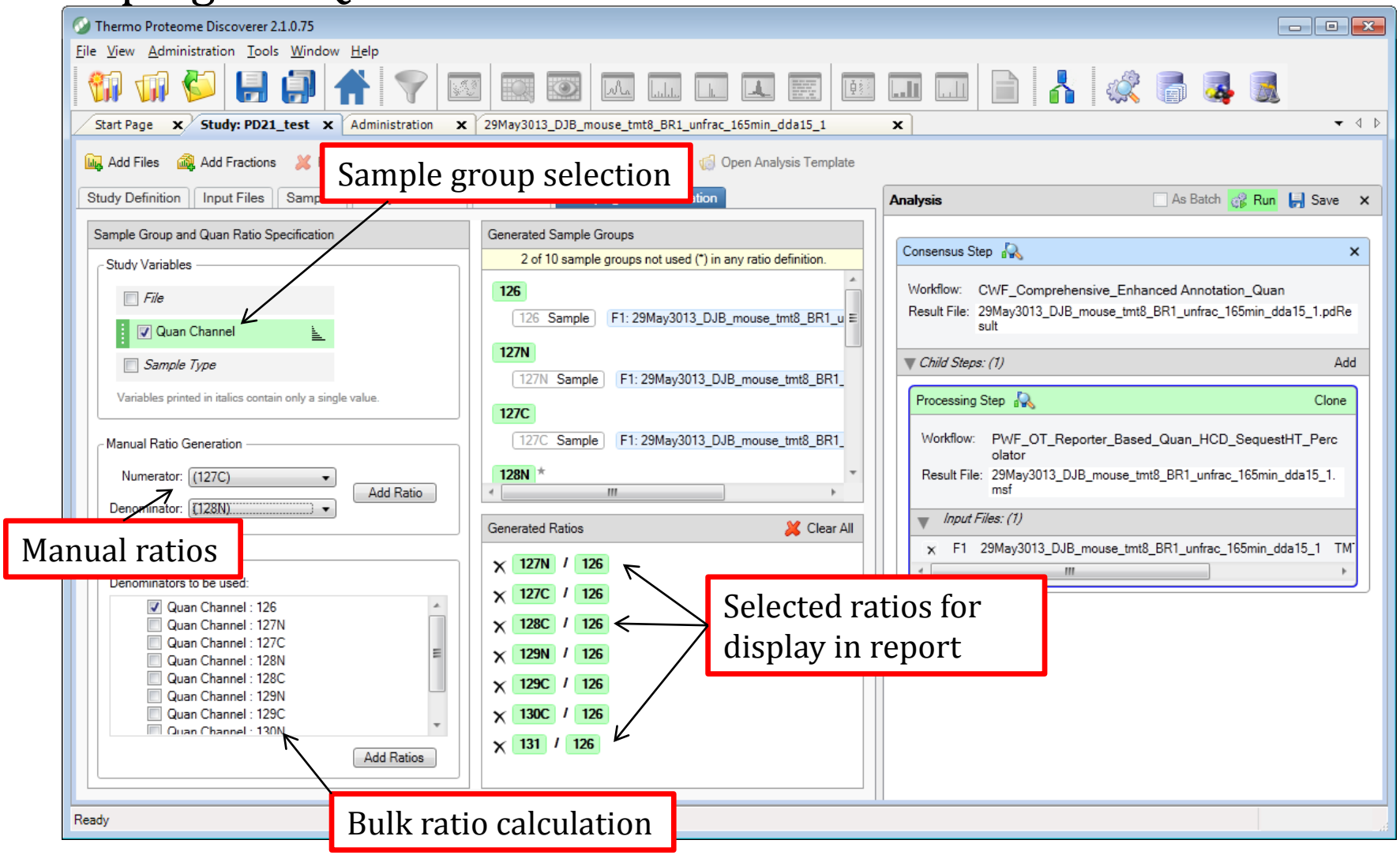
Average S/N per channel NEW!

More options for normalization NEW!

Scale abundances to standard height

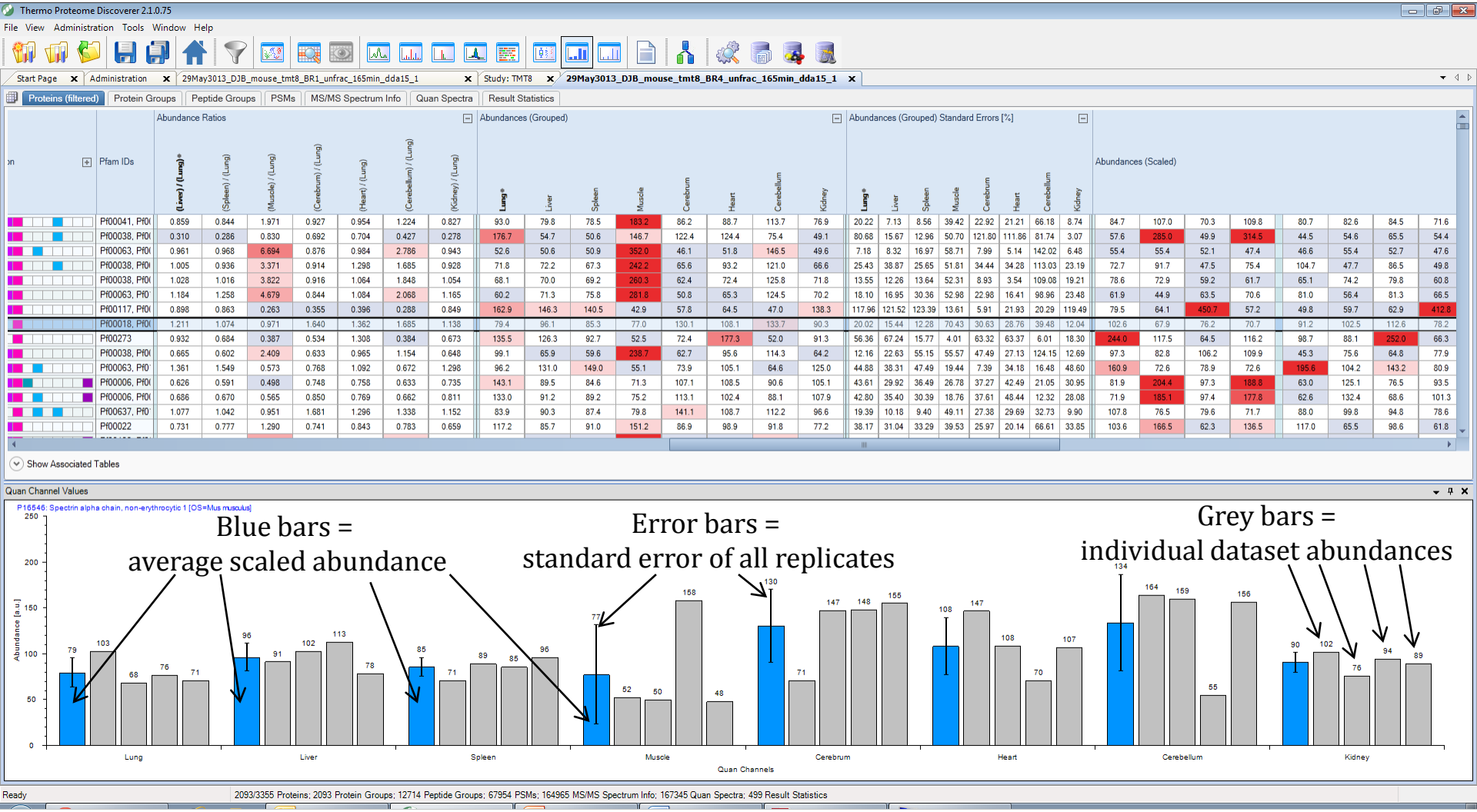
New option for log2 ratios NEW!

Grouping and Quantification



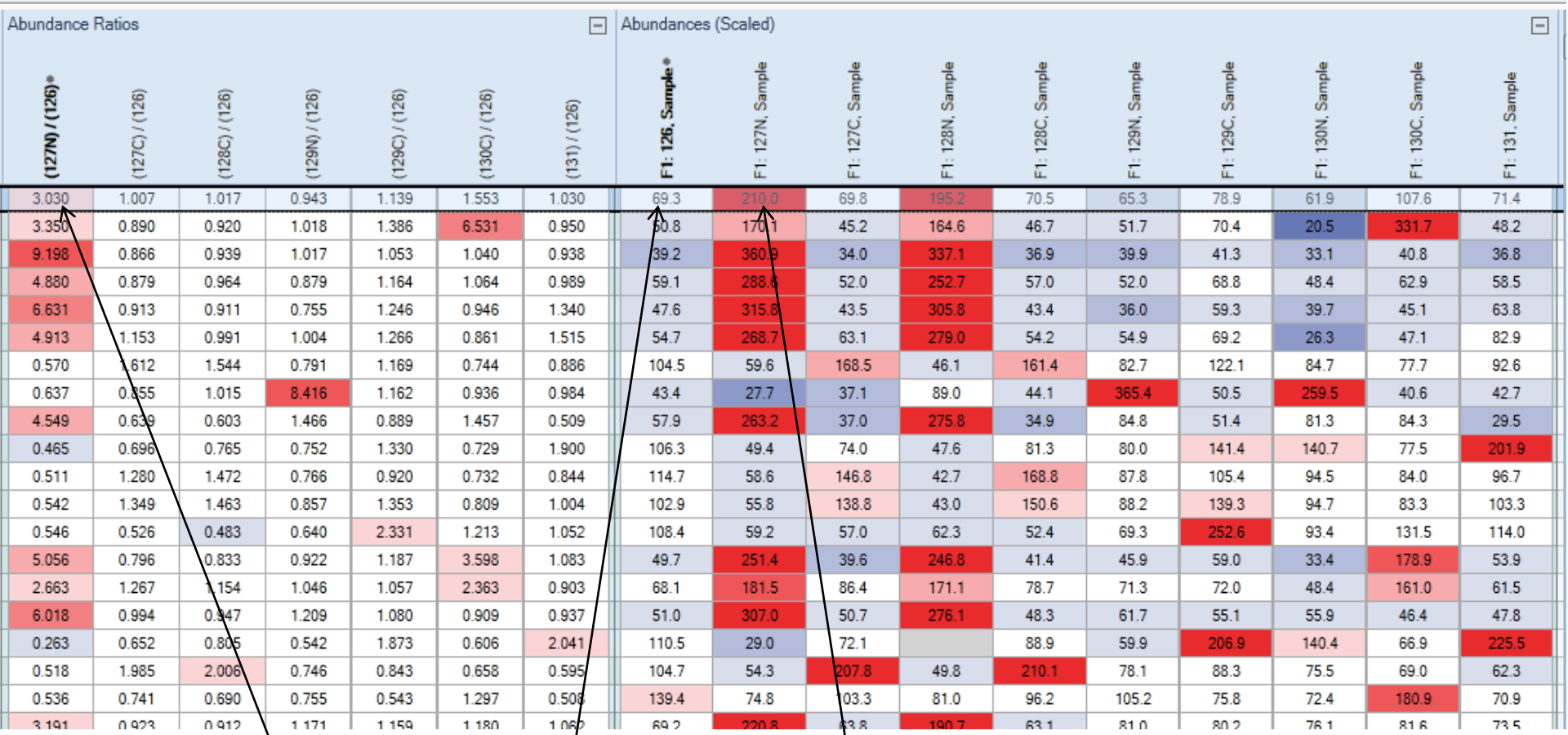
Results From Biological Replicate Search

- Replicates grouped into ratios + standard errors



ThermoFisher SCIENTIFIC

Zoomed Ratios and Scaled Abundances



Abundance Ratios

(127N) / (126)*	(127C) / (126)	(128C) / (126)	(129N) / (126)	(129C) / (126)	(130C) / (126)	(131) / (126)
3.030	1.007	1.017	0.943	1.139	1.553	1.030
3.350	0.890	0.920	1.018	1.386	6.531	0.950
9.198	0.866	0.939	1.017	1.053	1.040	0.938
4.880	0.879	0.964	0.879	1.164	1.064	0.989
6.631	0.913	0.911	0.755	1.246	0.946	1.340
4.913	1.153	0.991	1.004	1.266	0.861	1.515
0.570	1.612	1.544	0.791	1.169	0.744	0.886
0.637	0.855	1.015	8.416	1.162	0.936	0.984
4.549	0.639	0.603	1.466	0.889	1.457	0.509
0.465	0.696	0.765	0.752	1.330	0.729	1.900
0.511	1.280	1.472	0.766	0.920	0.732	0.844
0.542	1.349	1.463	0.857	1.353	0.809	1.004
0.546	0.526	0.483	0.640	2.331	1.213	1.052
5.056	0.796	0.833	0.922	1.187	3.598	1.083
2.663	1.267	1.154	1.046	1.057	2.363	0.903
6.018	0.994	0.947	1.209	1.080	0.909	0.937
0.263	0.652	0.805	0.542	1.873	0.606	2.041
0.518	1.985	2.006	0.746	0.843	0.658	0.595
0.536	0.741	0.690	0.755	0.543	1.297	0.508
3.191	0.923	0.912	1.171	1.159	1.180	1.062

Abundances (Scaled)

F1: 126, Sample*	F1: 127N, Sample	F1: 127C, Sample	F1: 128N, Sample	F1: 128C, Sample	F1: 129N, Sample	F1: 129C, Sample	F1: 130N, Sample	F1: 130C, Sample	F1: 131, Sample
69.3	210.0	69.8	195.2	70.5	65.3	78.9	61.9	107.6	71.4
60.8	170.1	45.2	164.6	46.7	51.7	70.4	20.5	331.7	48.2
39.2	360.9	34.0	337.1	36.9	39.9	41.3	33.1	40.8	36.8
59.1	288.6	52.0	252.7	57.0	52.0	68.8	48.4	62.9	58.5
47.6	315.8	43.5	305.8	43.4	36.0	59.3	39.7	45.1	63.8
54.7	268.7	63.1	279.0	54.2	54.9	69.2	26.3	47.1	82.9
104.5	59.6	168.5	46.1	161.4	82.7	122.1	84.7	77.7	92.6
43.4	27.7	37.1	89.0	44.1	365.4	50.5	259.5	40.6	42.7
57.9	263.2	37.0	275.8	34.9	84.8	51.4	81.3	84.3	29.5
106.3	49.4	74.0	47.6	81.3	80.0	141.4	140.7	77.5	201.9
114.7	58.6	146.8	42.7	168.8	87.8	105.4	94.5	84.0	96.7
102.9	55.8	138.8	43.0	150.6	88.2	139.3	94.7	83.3	103.3
108.4	59.2	57.0	62.3	52.4	69.3	252.6	93.4	131.5	114.0
49.7	251.4	39.6	246.8	41.4	45.9	59.0	33.4	178.9	53.9
68.1	181.5	86.4	171.1	78.7	71.3	72.0	48.4	161.0	61.5
51.0	307.0	50.7	276.1	48.3	61.7	55.1	55.9	46.4	47.8
110.5	29.0	72.1		88.9	59.9	206.9	140.4	66.9	225.5
104.7	54.3	207.8	49.8	210.1	78.1	88.3	75.5	69.0	62.3
139.4	74.8	103.3	81.0	96.2	105.2	75.8	72.4	180.9	70.9
69.2	220.8	63.8	190.7	63.1	81.0	80.2	76.1	81.6	73.5

126 scaled abundance = 69.3, 127N scaled abundance = 210.0

127N/126 = 210.0/69.3 = 3.030

Summary

Comprehend
the fundamentals of isobaric labelling and the dramatically increased throughput enabled by multiplexed quantitation as well as the ease of sample preparation

Configure
an LCMS method for the high accuracy quantitation of TMT labeled samples using the Orbitrap Fusion Lumos with SPS MS3 with the ideal settings

Quantify
peptides labeled with TMT using Proteome Discoverer 2.1 using SequestHT and MS3 quantitation.

Advocate
the complete workflow from sample preparation to data analysis for the multiplexed quantitation of complex samples using TMT and the highly differentiated SPS MS3 on the Orbitrap Fusion Series Instruments

Additional Resources

- **Online Resources**
  - http://portal.thermo-brims.com/ (Software, Manuals, Tutorial Help Videos, Discussion Forum.)
  - 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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