Tandem Mass Tag (TMT) Labeling Workflow

IDeA National Resource for Proteomics
Workshop for Core Directors and Staff

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4/4/2017

K-INBRE
IDeA Network of Biomedical Research Excellence

National Institutes of Health IDeA Program
National Resource for Proteomics

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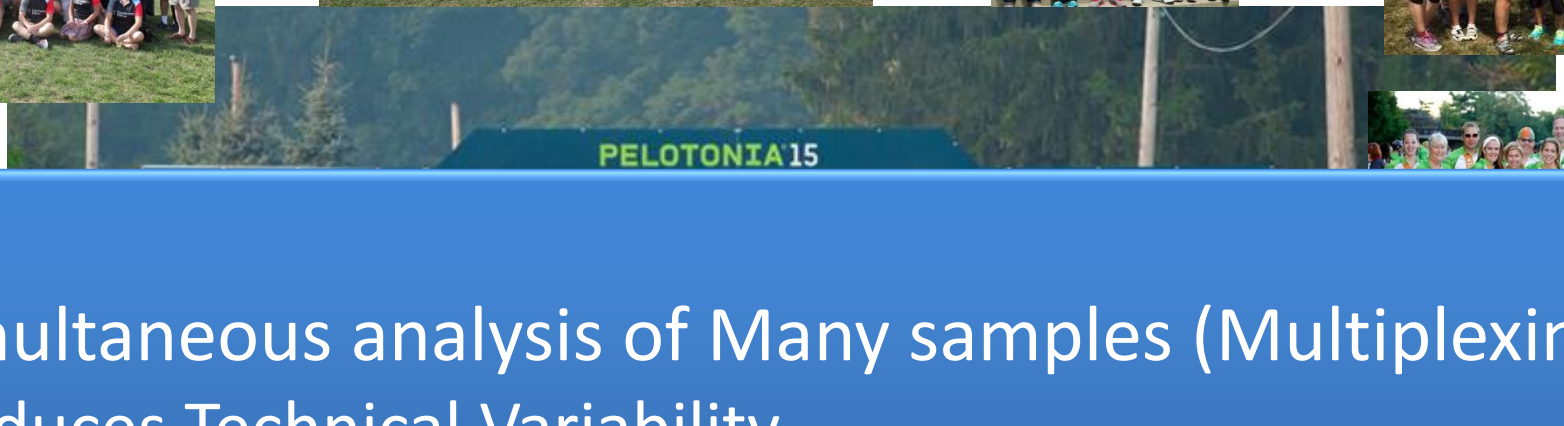


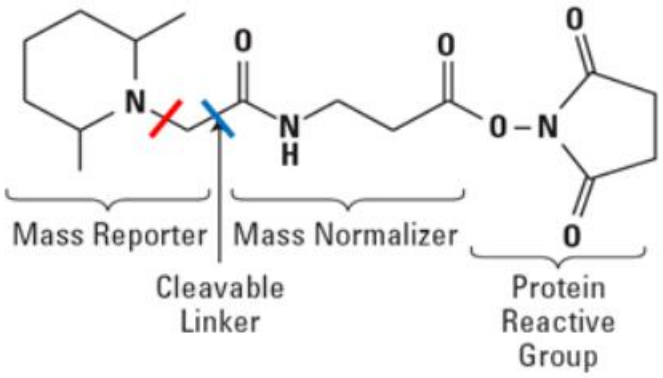
Simultaneous analysis of Many samples (Multiplexing)

- Reduces Technical Variability
- High throughput -> Cuts instrument time (data collection)



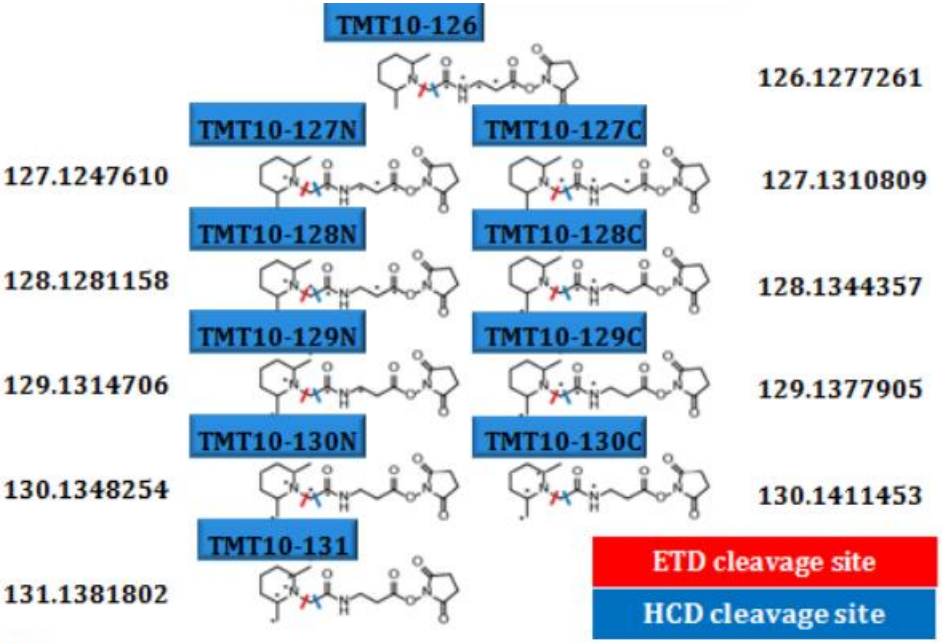

Thermo Scientific Tandem Mass Tag (TMT) Isobaric Tag Family

TMT^0
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
Sixplex Quantitation
TMT
10plex Quantitation



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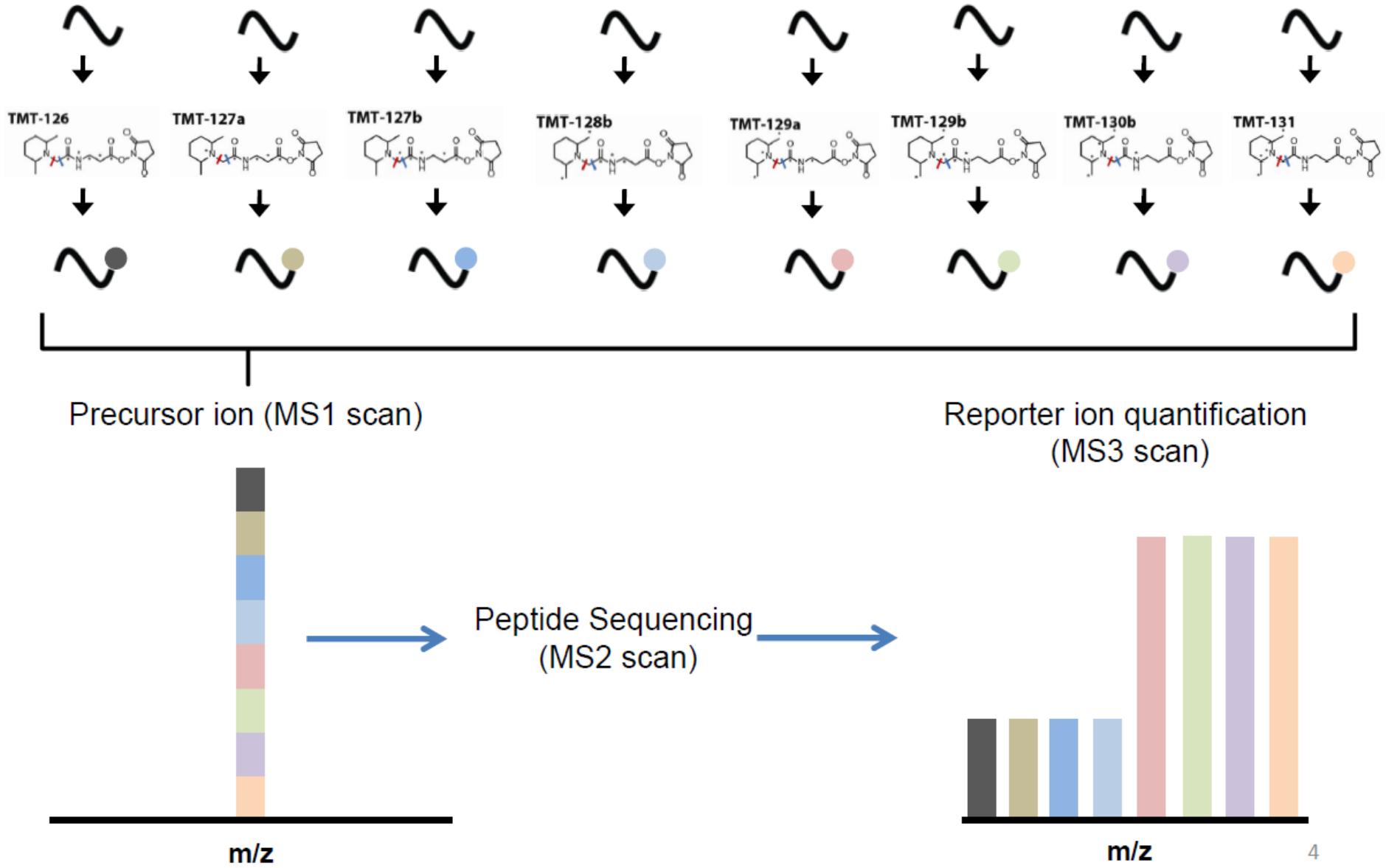
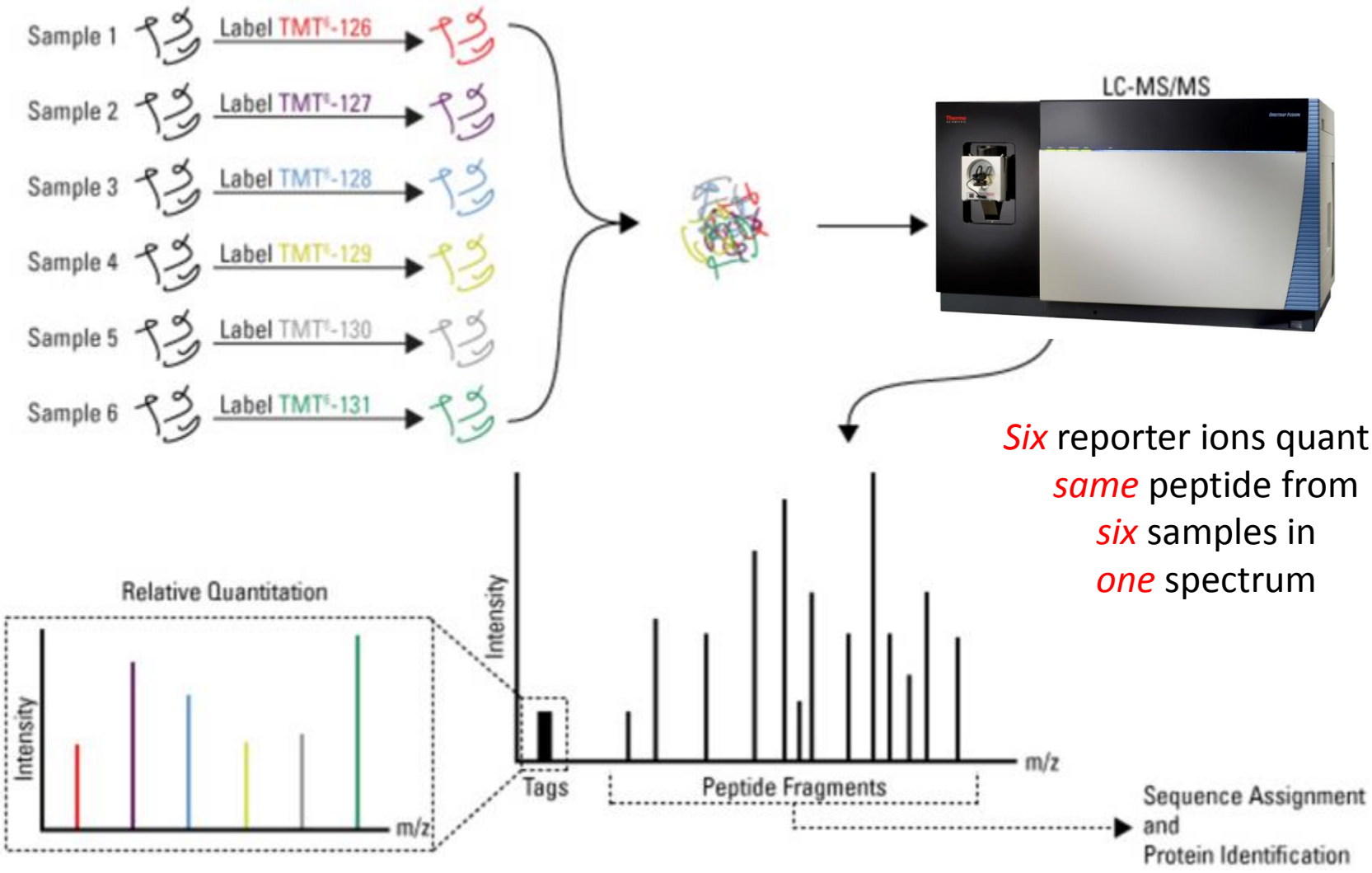
Tandem Mass Tags for multiplexed Proteomics





Six reporter ions quantify *same* peptide from *six* samples in *one* spectrum

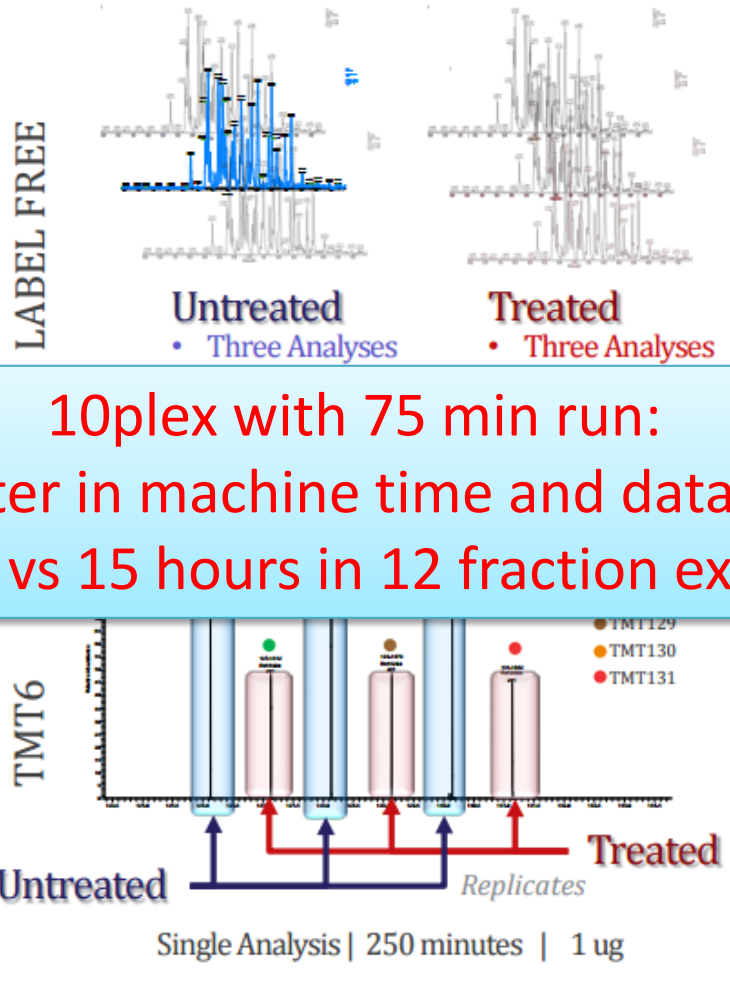
ThermoFisher SCIENTIFIC

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*



Quantified

1423 protein groups

in **1.04 days**

using **6 ug** material

10plex with 75 min run:
10 times faster in machine time and data acquisition
150 hours vs 15 hours in 12 fraction experiment

tified

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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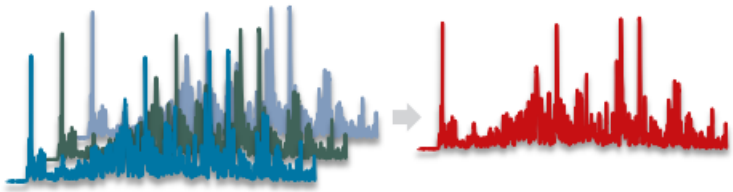
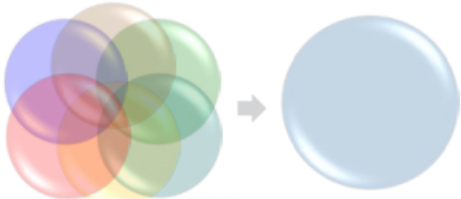
A Better Multiplexing Method– Isobaric Mass Tagging

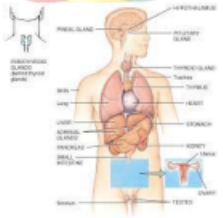
- Less MS1 Complexity
- Increased Throughput
  - Concurrent MS analysis of multiple samples
  - Less consumed samples and less instrument time



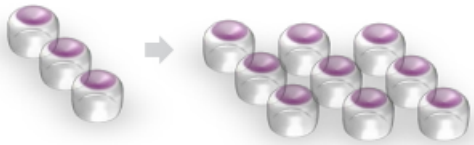
- Fewer Missing Values
  - Identification and quantification achieved in a single run
  - No worries about irreproducibility



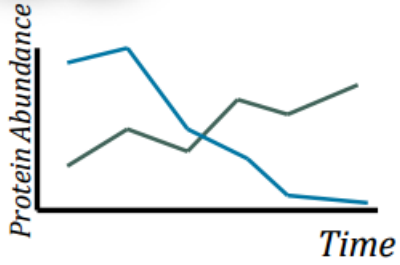
- Sample Origin Flexibility
  - Samples can be derived from cells, tissues or biological fluids



- 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

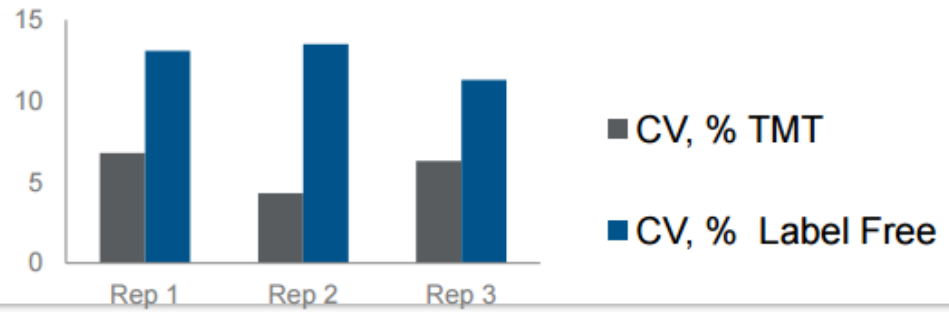


TMT Technology is More Precise than Label Free Quan

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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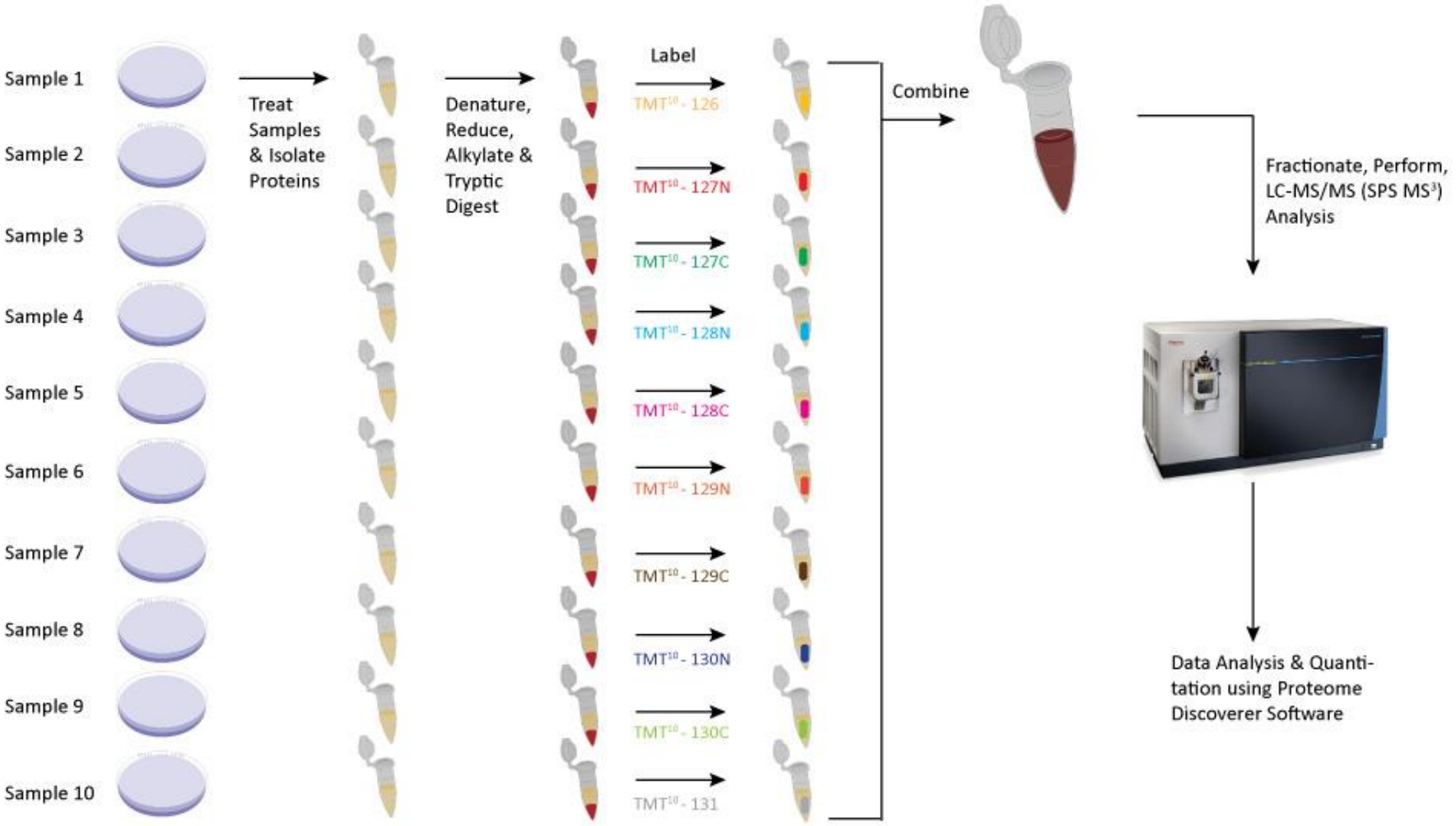
Procedure schematic for using the Thermo Scientific TMT10plex Label Reagents



Sample Preparation (Most Important Step!)

- There is no single protocol for cleaning up the protein samples!
- Reproducibility
  - Reduce Sources of Variability:
    - Technical: “good hands” < “bad hands”
    - Few steps < many steps
    - Biological: cells < tissue < body fluids
    - yeast < nematode < human
- Fractionation (reduce protein amount and complexity, enrich for target proteins)
- Buffer Exchange (remove non-compatible buffer reagents)

Buffer Reagents Compatibility

- Depends on the Proteomic Method
- Three non-compatible categories
  - Detergents
  - Salts
  - Reagents that compete for label
- Buffer exchange to make compatible
  - Filtration (FASP protocol)
  - Precipitation
  - Binding Affinity
- Detecting low abundance proteins requires “Clean samples”, no interfering reagents

Potential interference substances

- Thiols (for example, DTT and mercaptoethanol)
- High amounts of detergents and denaturants
- *Alternative Detergent/Denaturant (Concentration Limit at Trypsin Digestion)*
- SDS (0.05%)
- OG (octyl B-D-glucopyranoside) (0.1%)
- NP®-40 (0.1%)
- Triton® X-100 (0.1%)
- Tween® 20 (0.1%)
- CHAPS (0.1%)
- Urea (<1M)
- Note: When using urea, always use a fresh solution. When reducing a sample containing urea, incubate the tubes at 37 °C for 1 hour

Avoid using any reagents containing primary amines

Amine containing lysis buffer:

- Ammonium acetate
- Ammonium bicarbonate
- Ammonium citrate
- Ammonium tartrate
- AMPD [2-amino-2-methyl-1,3-propanediol]
- Aminoguanidine bicarbonate salt
- AMP [2-amino-2-methyl-1-propanol]
- Ethanolamine
- Gly-gly
- Tris buffers

Alternative Buffer :

- BES
- BICINE
- Boric acid
- CHES
- DIPSO
- EPPS
- HEPBS
- HEPES
- HEPPSO
- MOBS
- MOPS
- Phosphate Buffered
- PIPES
- POPSO

Filter Aided Sample Preparation (FASP) and TMT Labeling

Sample protein amount (2 mg):
- Cell Pellet: 50 ul (15 cm dish)
- Tissue: 25 mg minced; Freeze/thaw; Pestle; Cryo Bead Homogenizer
- Serum: Abundant Protein Depletion
- Protease /phosphatase inhibitor

Lysate Preparation:
- 100 mM DTT
- 2% SDS
- Heat (95°C) 3 min

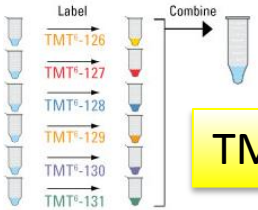
DNA Shredding:
- Sonication
- QIAshredder

Protein Quant (100 µg)
- Bradford or BCA
- Qubit™ 3.0 NGS

FASP processing: 30K filter unit
- Denaturation: 8M Urea
- Alkylation: 0.05M IAA
- Washing: Urea and AMBIC
- Overnight Digestion: Trypsin
- Stop reagent: Formic acid

Digestion clean up:
- Sep-Pak Vac C18 (50 mg)
- High throughput: manifold

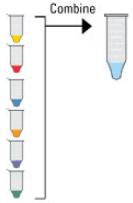
Peptide Quant



TMT Labeling

Orbitrap Fusion

Ratio Check(MS³)



Combine Samples

Clean up

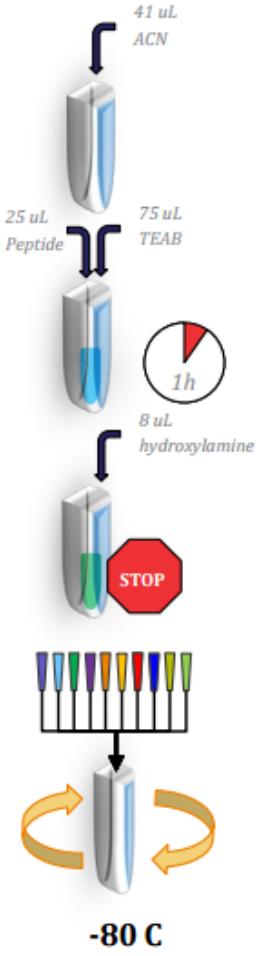
UHPLC

High pH fractionation

Sample Preparation: Simple Peptide Labeling

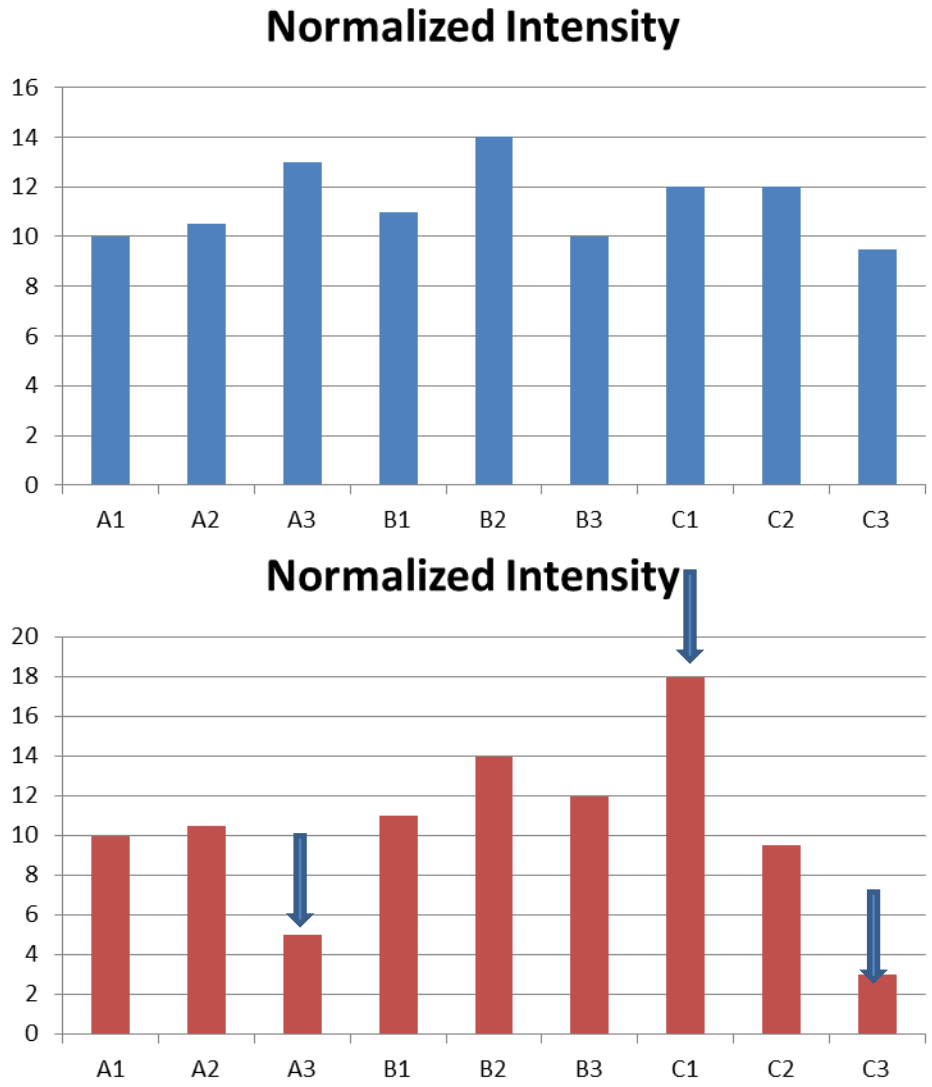
Reduced and alkylated trypsin digested proteins
Use Non-Amine Buffer @ pH ~ 8.0 (e.g. TEAB)

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.

Ratio check



- Small amount of each TMT labelled samples was mixed in equal volume
- Either sum of intensity or median can be used for ratio check

Correction is needed before mixing

Two New Peptide Quantitation Assays

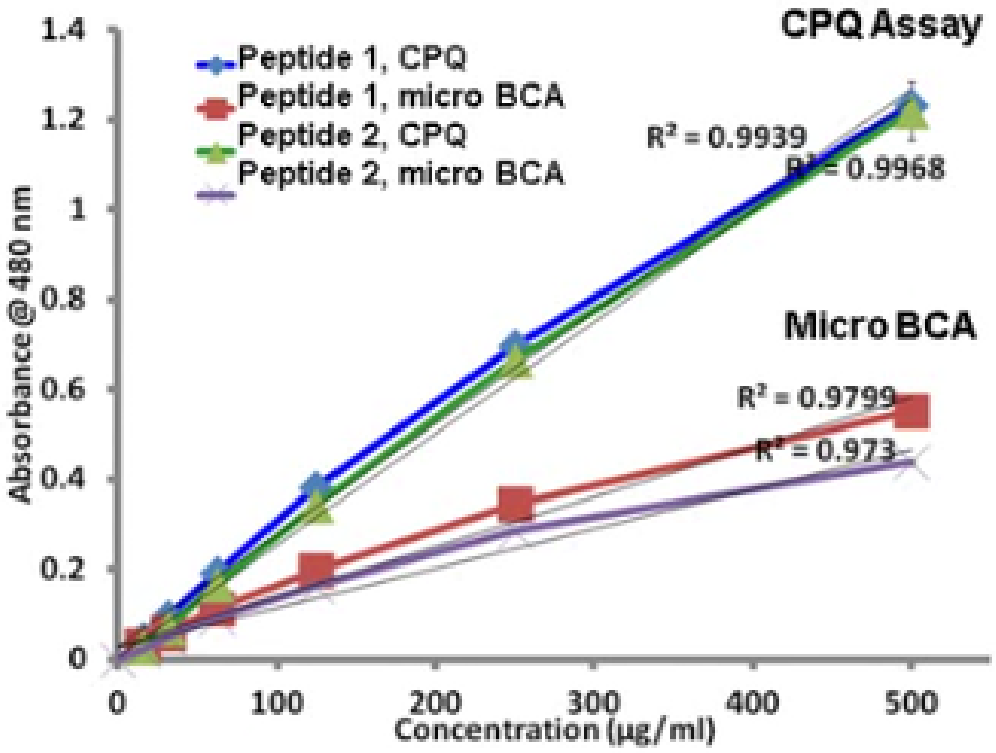
Colorimetric Peptide Quantitation (CPQ) assay

Fluorimetric Peptide Quantitation (FPQ) assay

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 More Sensitive than BCA

- CPQ assay is more linear than micro BCA over a greater dynamic range
- CPQ assay has 3-4 fold increase in S/N and 4 fold increase in sensitivity compared to micro BCA
- Best suited for complex peptide mixtures



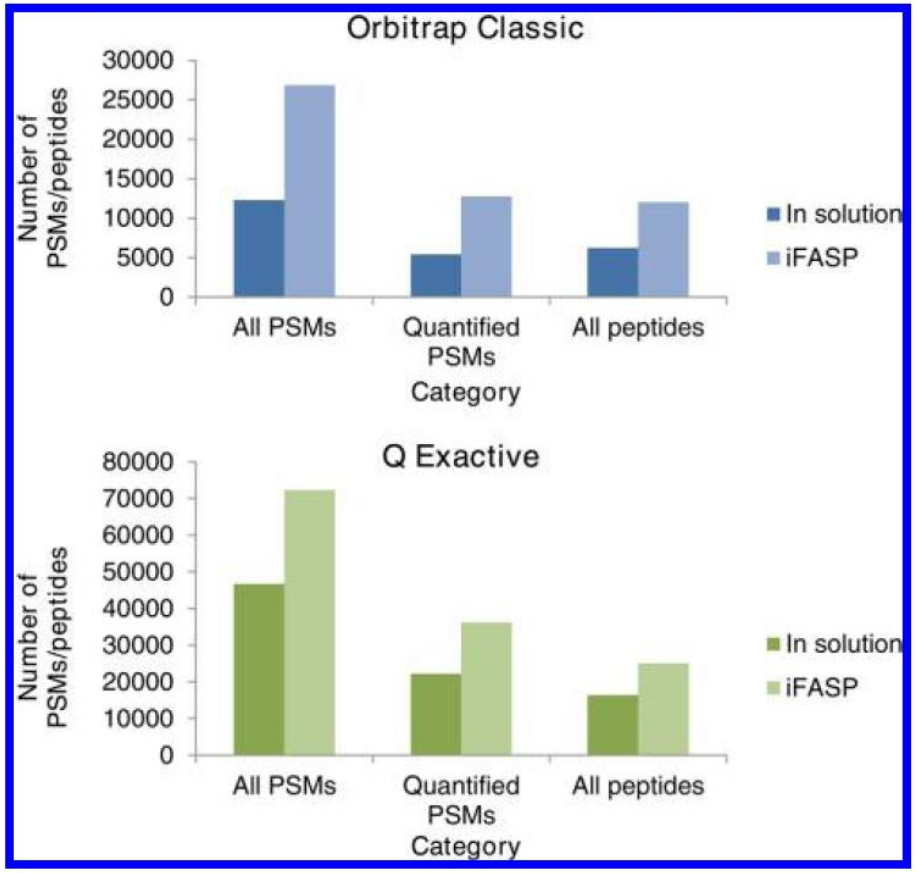
Journal of proteome research

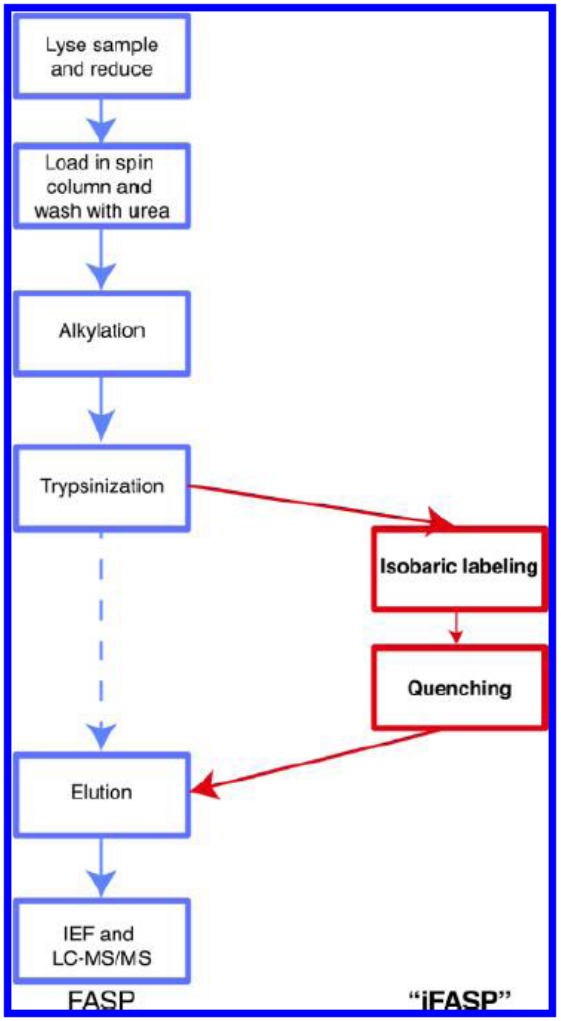
Technical Note

pubs.acs.org/jpr

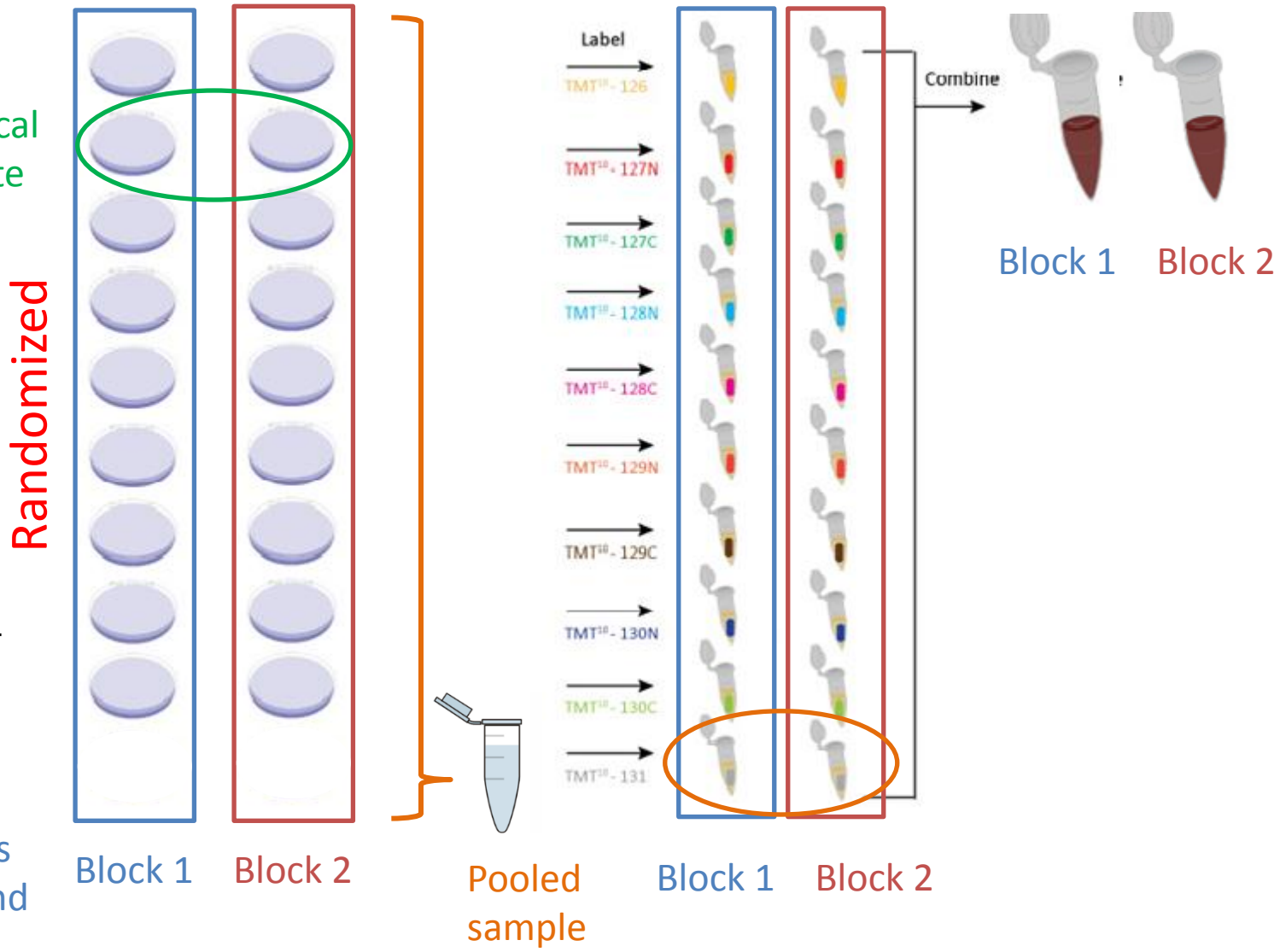
iFASP: Combining Isobaric Mass Tagging with Filter-Aided Sample Preparation

Gary S. McDowell,[†,‡] Aleksandr Gaun,[†] and Hanno Steen*[,†,‡]





More than 10 samples (with replicates)

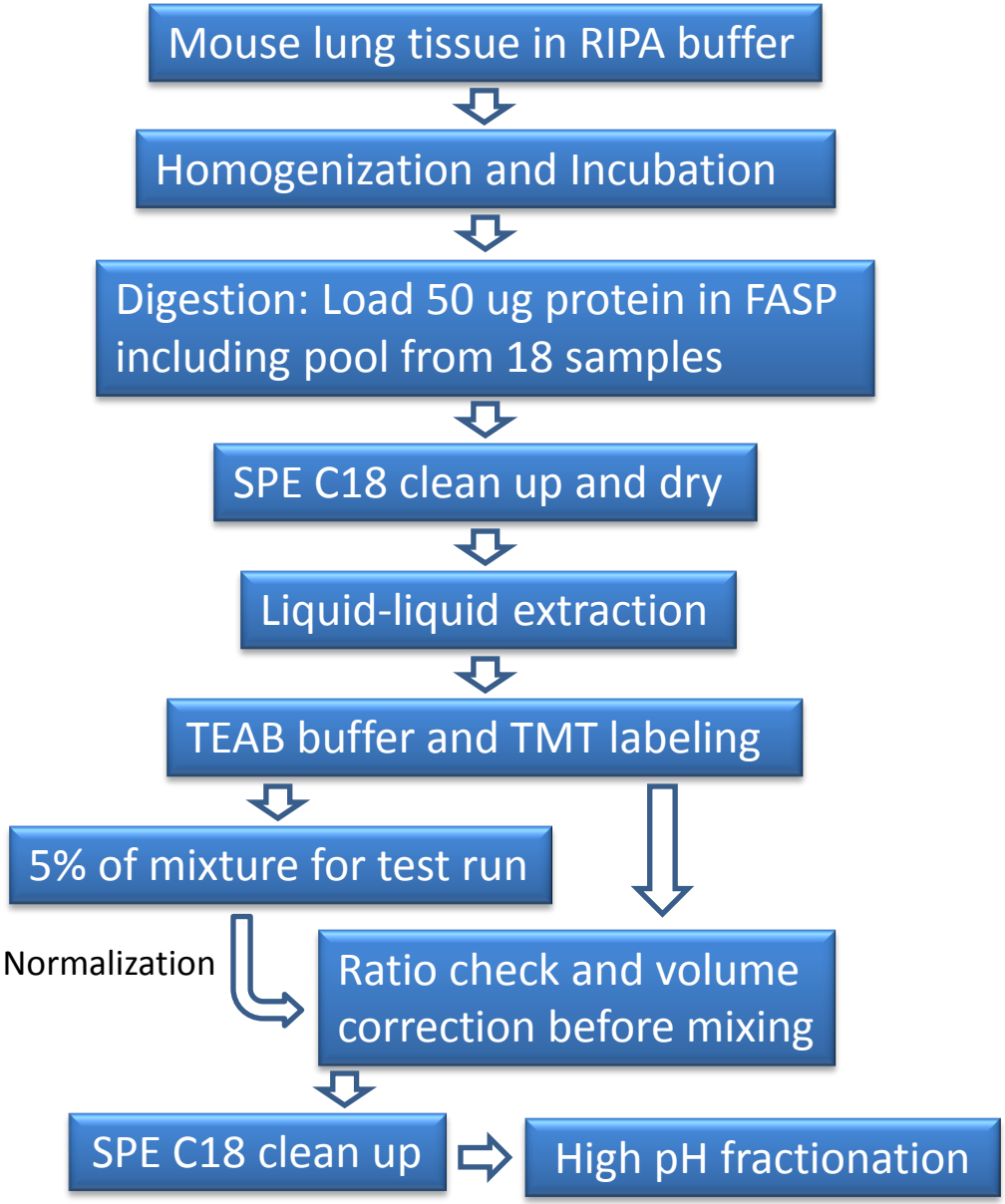


www.random.org

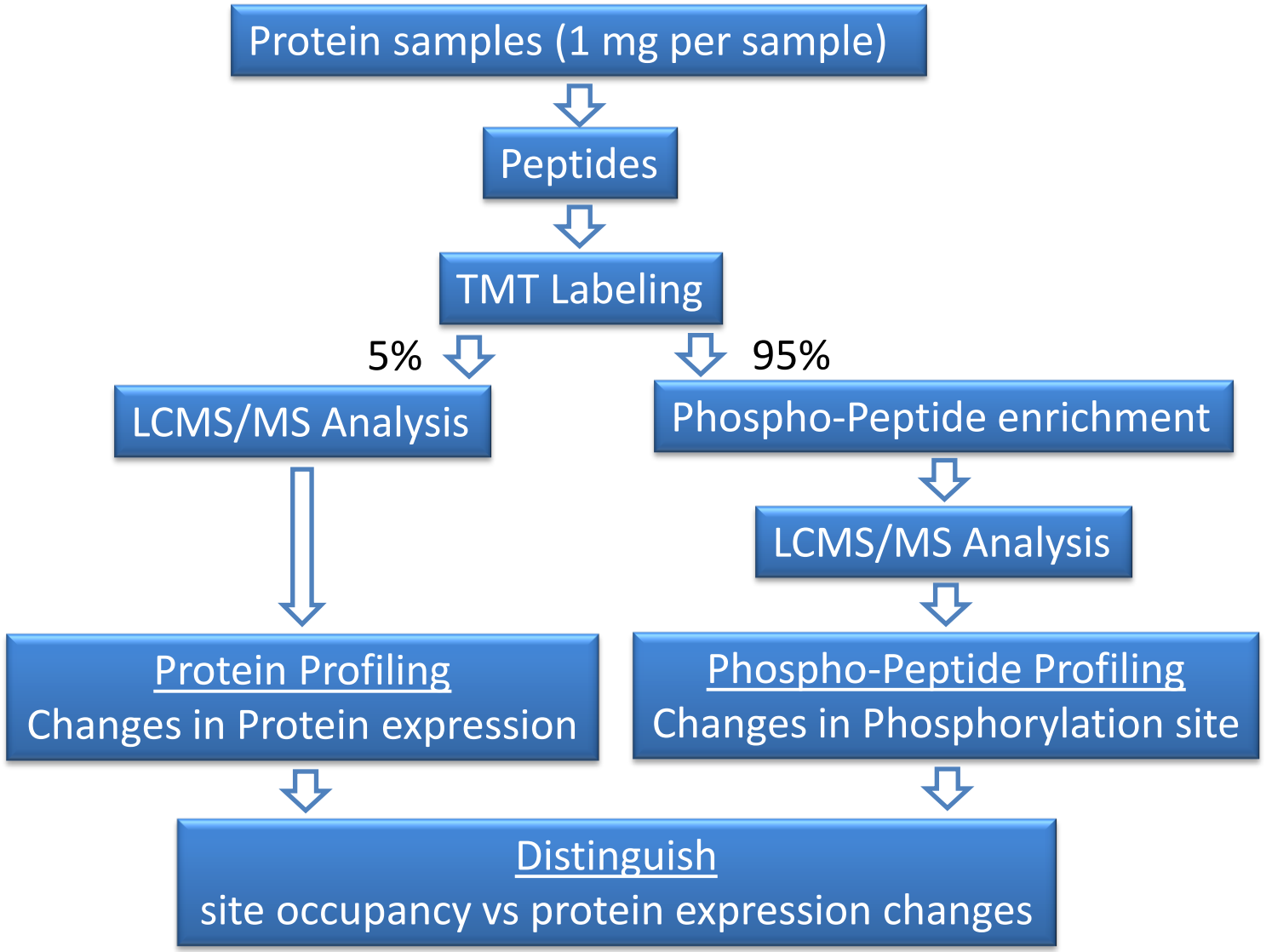
Each block includes all experimental and control groups

Mouse Tissue Study (TMT 10-plex for more than 10 samples)

Treatment group	Mouse #	TMT block	TMT labels
Group A	1	1	126
	2		127N
	3		127C
	11	2	126
	12		127N
	13		127C
Group B	4	1	128N
	5		128C
	6		129N
	14	2	128N
	15		128C
	16		129N
Group C	7	1	130N
	8		130C
	9		130N
	17	2	130N
	18		130C
	19		130N
18 Pooled	10	1	131
	20	2	131



Quantitative Phospho-Proteomics Workflow



iTRAQ vs. TMT

	TMT	iTRAQ
Maximum plex level	10	8
Vendor	Thermo Scientific	AB Sciex
Reporter ion mass	126-131	113-119, 121[a]
Balance group mass	103-98	192-184
Total mass	229	305
Required resolution	>50,000[b]	not specified
Price/kit	$918	$750

[a]Mass 120 is omitted in iTRAQ 8-plex to avoid contamination from phenylalanine immonium ion(m/z 120.08)

[b]The resolution to resolve the isotopic patterns of the C- and N-ion series

Summary

- Isobaric (same nominal mass) chemical tags: reporter + normalizer→ eluted and ionized together
- Reporter group is lost during fragmentation
- Enable relative quantitation and identification simultaneously with multiple conditions
- Quantify thousands of proteins from 10 samples in a single run
- TMT can be used with a variety of samples including cells, tissues, and biological fluids
- Avoid detergent, salt and reagent interfere label
- FASP protocol can be used with TMT labeling
- QC: protein quantification, peptide quantification and ratio check
- Add pooled sample for analysis more than one batch of TMT