Tandem Mass Tag (TMT) Labeling Workflow

IDeA National Resource for Proteomics Workshop for Core Directors and Staff
Renny Lan
4/4/2017
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
Tandem Mass Tags for multiplexed Proteomics

Precursor ion (MS1 scan) → Peptide Sequencing (MS2 scan) → Reporter ion quantification (MS3 scan)
Six reporter ions quantify the same peptide from six samples in one spectrum.
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

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

Quantified
- 1423 protein groups in 1.04 days using 6 ug material
- 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
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
“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*...”
Procedure schematic for using the Thermo Scientific TMT10plex Label Reagents

1. Treat Samples & Isolate Proteins
2. Denature, Reduce, Alkylate & Tryptic Digest
3. Label: TMT<sup>10</sup> - 126
   - TMT<sup>10</sup> - 127N
   - TMT<sup>10</sup> - 127C
   - TMT<sup>10</sup> - 128N
   - TMT<sup>10</sup> - 128C
   - TMT<sup>10</sup> - 129N
   - TMT<sup>10</sup> - 129C
   - TMT<sup>10</sup> - 130N
   - TMT<sup>10</sup> - 130C
   - TMT<sup>10</sup> - 131
4. Combine
5. Fractionate, Perform, LC-MS/MS (SPS MS<sup>3</sup>) Analysis
6. Data Analysis & Quantitation using Proteome Discoverer Software
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
- CHES
- 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 μl (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

Clean up

UHPLC

Combine Samples

Ratio Check(MS^3)

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.** 88328
- **Description**
  - 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
- **Chemistry:** Indirect Cu-reduction and chelation
- **Time:** 30 mins
- **Measurement:** Abs 480nm
- **Linearity:** 15-1000 µg/mL
- **Sensitivity:** 15 µg/mL
- **Minimum sample:** 0.3 µg
- **Not recommended for:** Single peptides

#### Fluorimetric Peptide Quantitation (FPQ) assay
- **Chemistry:** Direct N-terminal labeling induced fluorescence
- **Time:** 5 mins
- **Measurement:** Ex. 390nm/Em. 475nm
- **Linearity:** 5-1000 µg/mL
- **Sensitivity:** 5 µg/mL
- **Minimum sample:** 0.05 µg
- **Not recommended for:** TMT Reagent-labeled samples

*ThermoFisher SCIENTIFIC*
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.
iFASP: Combining Isobaric Mass Tagging with Filter-Aided Sample Preparation

Gary S. McDowell,†,‡ Aleksandr Gaun,† and Hanno Steen*†,‡
More than 10 samples (with replicates)

Each block includes all experimental and control groups.

www.random.org
### Mouse Tissue Study (TMT 10-plex for more than 10 samples)

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Mouse #</th>
<th>TMT block</th>
<th>TMT labels</th>
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<tbody>
<tr>
<td>Group A</td>
<td>1</td>
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<td>1</td>
<td>127C</td>
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<tr>
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<td>128C</td>
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<tr>
<td></td>
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<td>2</td>
<td>131</td>
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</tbody>
</table>

Mouse lung tissue in RIPA buffer

Homogenization and Incubation

Digestion: Load 50 ug protein in FASP including pool from 18 samples

SPE C18 clean up and dry

Liquid-liquid extraction

TEAB buffer and TMT labeling

5% of mixture for test run

Ratio check and volume correction before mixing

Normalization

SPE C18 clean up

High pH fractionation
Quantitative Phospho-Proteomics Workflow

Protein samples (1 mg per sample) → Peptides → TMT Labeling

5% → LCMS/MS Analysis

Protein Profiling
Changes in Protein expression

95% → Phospho-Peptide enrichment → LCMS/MS Analysis

Phospho-Peptide Profiling
Changes in Phosphorylation site

Distinguish
site occupancy vs protein expression changes
# iTRAQ vs. TMT

<table>
<thead>
<tr>
<th></th>
<th>TMT</th>
<th>iTRAQ</th>
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<tbody>
<tr>
<td>Maximum plex level</td>
<td>10</td>
<td>8</td>
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<tr>
<td>Vendor</td>
<td>Thermo Scientific</td>
<td>AB Sciex</td>
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<tr>
<td>Reporter ion mass</td>
<td>126-131</td>
<td>113-119, 121&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>Balance group mass</td>
<td>103-98</td>
<td>192-184</td>
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<tr>
<td>Total mass</td>
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<td>305</td>
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<tr>
<td>Required resolution</td>
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<td>Price/kit</td>
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<td>$750</td>
</tr>
</tbody>
</table>

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

<sup>b</sup>The resolution to resolve the isotopic patterns of the C- and N-ion series
Summary

- Isobaric (same nominal mass) chemical tags: reporter + normalizer $\rightarrow$ 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