TMTpro: Best Practices

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Multiplexed TMT Peptide & Protein Quantitation

**Tandem Mass Tag (TMT) reagents**

- **Reporter**
- **Linker**
- **Amine-reactive region**

*denote $^{13}$C or $^{15}$N stable isotopes

**Precursors (isobaric)**

**Reporter ions (isotopologues)**

**Fractionate, Clean-up, Perform LC-MS/MS Analysis**

**Label**

- TMT<sup>©</sup>-126
- TMT<sup>©</sup>-127N
- TMT<sup>©</sup>-127C
- TMT<sup>©</sup>-128N
- TMT<sup>©</sup>-128C
- TMT<sup>©</sup>-129N
- TMT<sup>©</sup>-129C
- TMT<sup>©</sup>-130N
- TMT<sup>©</sup>-130C
- TMT<sup>©</sup>-131

**Treat Samples & Isolate Proteins**

**Combino**
Higher Multiplexing Requires New, Larger Mass Tag

TMT

TMTpro
Introducing the TMTpro 16plex Reagent Set

- Next generation TMT reagent enabling 16plex sample multiplexing

- New mass tag structure with longer linker supporting 9 stable isotopes

- Identical low mass report ions of TMT reagents with additional channels

- **A44520** TMTpro™ 16plex Label Reagent Set, 1 x 5 mg
- **A44518** TMTpro™ Zero Label Reagent Set, 1 x 5 mg
Reporter Ions Spectra – TMTpro vs TMT reagents

TMT 11plex

TMTpro16plex

No change in m/z!
TMTpro 16plex Samples have Similar Protein/Peptide IDs as TMT 11plex Samples

**QE-HFX**
- Protein overlap:
  - TMT11: 2,165
  - TMTpro16: 2,098
  - 334 overlap
  - 1,831 unique TMT11
  - 267 unique TMTpro16

**Fusion Lumos**
- Protein overlap:
  - TMT11: 2,039
  - TMTpro16: 1,986
  - 387 overlap
  - 1,652 unique TMT11
  - 334 unique TMTpro16

- Peptide overlap:
  - TMT11: 15,466
  - TMTpro16: 14,240
  - 5,079 overlap
  - 10,387 unique TMT11
  - 3,853 unique TMTpro16

  - TMT11: 13,481
  - TMTpro16: 11,948
  - 5,821 overlap
  - 7,660 unique TMT11
  - 4,288 unique TMTpro16

Data courtesy of Joao Paolo & Steven Gygi
Fewer Missing Values among Replicates with TMTpro 16plex vs TMT 11plex

Example: 3 TMT11plex reactions run
In each run, one 131C tag was used as a reference channel for normalization

Example: 2 TMT16plex reactions run
In each run, one channel was used as a reference channel for normalization
Opportunities for normalization in the workflow

Protein level normalization (BCA/Bradford)

Run test for ratio check and correction before mixing
Paulo et al. JPR 2018.

Peptide normalization (Fluorometric/Colorimetric)
Experimental Design of Multiplex Samples

- Include enough bio replicates!
- Block out and randomize sample conditions/replicates across the plex
- Address reporter ion cross talk
  - how much is tolerable to answer your biological question?

<table>
<thead>
<tr>
<th>Conditions</th>
<th>TMT11-plex</th>
<th>Label</th>
<th>TMTpro-16plex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starved</td>
<td>126</td>
<td>126</td>
<td>129N</td>
</tr>
<tr>
<td></td>
<td>129N</td>
<td></td>
<td>131C</td>
</tr>
<tr>
<td>Starved + hEGF</td>
<td>127N</td>
<td>127N</td>
<td>129C</td>
</tr>
<tr>
<td></td>
<td>129C</td>
<td></td>
<td>132N</td>
</tr>
<tr>
<td>Starved + hEGF + Erlotinib</td>
<td>127C</td>
<td>127C</td>
<td>130N</td>
</tr>
<tr>
<td></td>
<td>130N</td>
<td></td>
<td>132C</td>
</tr>
<tr>
<td>Starved + IGF</td>
<td>128N</td>
<td>128N</td>
<td>130C</td>
</tr>
<tr>
<td></td>
<td>130C</td>
<td></td>
<td>133N</td>
</tr>
<tr>
<td>Starved + IGF + Erlotinib</td>
<td>128C</td>
<td>128C</td>
<td>131N</td>
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<tr>
<td></td>
<td>131N</td>
<td></td>
<td>133C</td>
</tr>
<tr>
<td>Pooled</td>
<td>131C</td>
<td></td>
<td>134N</td>
</tr>
</tbody>
</table>

Labeled Peptides
Experimental Design of Multiplex Samples

Assign samples within and betweenplexes to minimize reporter ion interference (RII) and inter batch variability

Non-optimal 10-plex (with RII)

Optimal 10-plex (minimal RII)

2 groups X 5 reps
TMT10-plex

Brenes et al. 2019. MCP.
Experimental Design of Multiplex Samples

3 groups X 3 reps

Optimal 11-plex
(no controls)

TMT11-plex

Optimal 11-plex
(1 control)

Brenes et al. 2019. MCP.
Inter-batch reference control:

- Global pool of all samples across all conditions
- Use 2 reference channels if possible
- Using 126 and 127N channels avoids primary (+1) RII and gives better reproducibility
Analyzing TMTpro Data in Proteome Discoverer

- TMTpro natively supported in PD v2.4
- SEQUEST is preferred/default in workflow
- Other search engines that penalize for unidentified peaks may be problematic (ex. Mascot, MaxQuant)

- For PD v2.2-2.3, TMTpro must be added as a modification
  - See app note and corresponding XML modification and method files
  - Similar to 1st gen TMT, only carbon isotopes corrections supported
TMTpro reporter ion isotopic distributions in PD 2.2/2.3

COA for each lot (as for 1st gen TMT):
- correction factors for natural carbon isotopes (+1, +2) and incomplete stable isotope incorporation (-1, -2)
- COAs available on thermofisher.com; full support in PD 2.4
Assessing Labeling with PMI-Preview in PD

- Fast initial search and quality control check of shotgun dataset
- Use to determine params for full search
- Protein Metrics Preview node option built into Proteome Discoverer
- Open “PMI Preview Template”
- Feed .raw file, protein fasta database, and basic search params
- Returns HTML report (and .pdResult file) with QC metrics on:
  - Labeling efficiency (>98%)
  - Digest efficiency
  - Modifications (over alkylation/labeling)
  - Mass errors
  - Protein/Peptide IDs
Chromatography Considerations

Data courtesy of Jessie Guidry

TMT10/TMTpro labeling

C18 cleanup
(acid wash)

Offline bRPLC fractionation + concatenate

Inject LC-MS/MS
Chromatography Considerations

TMT10/TMTpro labeling

- C18 cleanup
  - (acid wash)
- Offline bRPLC fractionation + concatenate
- Inject LC-MS/MS

Data courtesy of Jessie Guidry
Chromatography Considerations

- TMTpro = more hydrophobic (vs 1st gen TMT)
  - Modify LC gradients with faster/steeper ramping and higher organic strength

- TMTpro unreacted by-products more persistent in online/offline cleanup
  - Modify SPE with low organic washes to eliminate unreacted TMT by product; stronger elution

~10min RT shift

Data courtesy of Jessie Guidry
Assessing Labeling with PMI-Preview in PD

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  - Modify LC gradients with faster/steeper ramping and higher organic strength

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  - Modify SPE with low organic washes to eliminate unreacted TMT by product; stronger elution
Chromatography Considerations

TMT10

Two stage clean-up (C18 RP SPE and bRPLC) with more stringent washes (10% ACN) required to remove unreacted TMT

TMTpro

w/ C18 cleanup
10% ACN wash
70% elution
Chromatography Considerations

Data courtesy of Pandey Lab

- **w/o C_{18} clean-up**

- **w/ C_{18} clean-up**

  - Optimized gradient
  - 5-40% Sol B in 90 min
Chromatography Considerations

Can we get away from two stage clean-up?

- Two stage cleanup (RP SPE and SCX) used in Thompson et al. Analytical Chemistry. 2019. (Proteome Sciences Group)

- If using LC and fraction collector for bRPLC, eliminate the first few fractions

- Other solutions:
  - Use Pierce high pH RP peptide fractionation columns and increase wash to 10% ACN
  - Use EasyPep mixed mode resin clean-up
Other things to try…High pH Fractionation Kit

Pierce High pH RP Peptide Fractionation

Table 1. Preparation of elution solutions for unlabeled, native peptides.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Acetonitrile (%)</th>
<th>Acetonitrile (µL)</th>
<th>Triethylamine (0.1%) (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td></td>
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</tr>
<tr>
<td>1</td>
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<td>950</td>
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<td>2</td>
<td>7.5</td>
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<td>925</td>
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<tr>
<td>3</td>
<td>10.0</td>
<td>100</td>
<td>900</td>
</tr>
<tr>
<td>4</td>
<td>12.5</td>
<td>125</td>
<td>875</td>
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<td>5</td>
<td>15.0</td>
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<td>6</td>
<td>17.5</td>
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<td>7</td>
<td>20.0</td>
<td>200</td>
<td>800</td>
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<tr>
<td>8</td>
<td>50.0</td>
<td>500</td>
<td>500</td>
</tr>
</tbody>
</table>

Table 2. Preparation of elution solutions for Thermo Scientific TMT-labeled peptides.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Acetonitrile (%)</th>
<th>Acetonitrile (µL)</th>
<th>Triethylamine (0.1%) (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wash</td>
<td>5.0</td>
<td>50</td>
<td>950</td>
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<tr>
<td>1</td>
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<td>900</td>
</tr>
<tr>
<td>2</td>
<td>12.5</td>
<td>125</td>
<td>875</td>
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<tr>
<td>3</td>
<td>15.0</td>
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<td>850</td>
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<tr>
<td>4</td>
<td>17.5</td>
<td>175</td>
<td>825</td>
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<tr>
<td>5</td>
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<td>800</td>
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<td>6</td>
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<tr>
<td>8</td>
<td>50.0</td>
<td>500</td>
<td>500</td>
</tr>
</tbody>
</table>

TMT Label

Label Free

↑ to 10% ACN (TMTpro)
Other things to try…EasyPep MS Sample Prep Kit

Stage 1: Chemical & Enzymatic Sample Processing

1. Dry
2. Solubilize proteins (EP lysis solution)
3. Reduction/alkylation
4. Digestion

Depleted Plasma → Dried Depleted Plasma Sample → Solubilized Proteins → Reduced/alkylated proteins plus contaminants* → Peptides plus contaminants*

*contaminants – hydrophilic/hydrophobic buffer salts, reagents, detergent, biomolecules other than proteins

Stage 2: Peptide Clean-Up

1. Flowthrough (a) or Wash A (b)
2. Wash B
3. Elution
4. Drying
5. High pH RP

Peptides plus contaminants → Hydrophilic contaminants (discard) → Hydrophobic contaminants (discard) → Clean Peptide Solution (keep) → Clean peptides

Mixed mode resin clean-up removes unreacted TMT

TMT labeling of protein digest

TMT labeling of clean peptides
Advanced Peak Determination (APD) increases ratio compression for TMT

Evaluation of Advanced Precursor Determination for Tandem Mass Tag (TMT)-Based Quantitative Proteomics across Instrument Platforms

Samuel A. Myers,†‡ Susan Klaeger,† Shankha Satpathy,‡ Rosa Viner,‡ Jae Choi,§ John Rogers,§ Karl Clauser,† Namrata D. Udeshi,† and Steven A. Carr*†

†The Broad Institute of MIT and Harvard, Cambridge, Massachusetts, United States
‡Thermo Fisher Scientific, San Jose, California, United States
§Thermo Fisher Scientific, Rockford, Illinois, United States

Tested with multiple columns loads, gradient lengths, and instrument platforms

• APD designed for label-free
• Increases IDs by sampling overlapping isotope distributions
• This also increases co-isolation and co-isolation interference

Turn APD off for TMT data acquisition!
A Triple Knockout (TKO) Proteomics Standard for Diagnosing Ion Interference in Isobaric Labeling Experiments

Joao A. Paulo, Jeremy D. O’Connell, Steven P. Gygi
Department of Cell Biology, Harvard Medical School, Boston, MA 02115, USA

- True negative control for ion interference
- Assess and monitor instrument performance and readiness
- LC and MS method development
- Test co-isolation interference
- Gygi lab TVT Web based search tool:
  - http://TKOomics.com
Yeast Triple KO TMT Standard for Optimizing Instrumentation

- Thermo App Note 72968
- Optimized instrument params
- Analysis guide with PD
- Pre-built methods in tribrid Tune SW
# Instrument Acquisition Parameters

<table>
<thead>
<tr>
<th>Properties for 120min Acquisition</th>
<th>Fusion SPS-MS3</th>
<th>Fusion MS2</th>
<th>Lumos SPS-MS3</th>
<th>Lumos MS2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MS resolution</strong></td>
<td>120000</td>
<td>120000</td>
<td>120000</td>
<td>120000</td>
</tr>
<tr>
<td><strong>MS AGC target</strong></td>
<td>4e5</td>
<td>4e5</td>
<td>4e5</td>
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</tr>
<tr>
<td><strong>MS max IT</strong></td>
<td>50 ms</td>
<td>50 ms</td>
<td>50 ms</td>
<td>50 ms</td>
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<tr>
<td><strong>Scan range m/z</strong></td>
<td>375-1500</td>
<td>375-1500</td>
<td>375-1500</td>
<td>375-1500</td>
</tr>
<tr>
<td><strong>Top Speed</strong></td>
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<td>3 sec</td>
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<tr>
<td><strong>MS2 Intensity threshold</strong></td>
<td>5e3</td>
<td>5e4</td>
<td>5e3</td>
<td>5e4</td>
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<tr>
<td><strong>MS2 resolution</strong></td>
<td>Turbo</td>
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<td>50000</td>
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<tr>
<td><strong>MS2 AGC target</strong></td>
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<td>1e5</td>
<td>1e4</td>
<td>1e5</td>
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<tr>
<td><strong>MS2 max IT</strong></td>
<td>50 ms</td>
<td>120 ms</td>
<td>50 ms</td>
<td>96 ms</td>
</tr>
<tr>
<td><strong>MS2 Isolation window (charge)</strong></td>
<td>1.2(2), 0.7(3), 0.5(&gt;4)</td>
<td>0.7(2-3), 0.5(&gt;4)</td>
<td>1.2(2), 0.7(3), 0.5(&gt;4)</td>
<td>0.7(2-3), 0.5(&gt;4)</td>
</tr>
<tr>
<td><strong>MS2 NCE %</strong></td>
<td>35</td>
<td>38-40</td>
<td>35</td>
<td>38-40</td>
</tr>
<tr>
<td><strong>Dynamic exclusion</strong></td>
<td>60 s, single charge</td>
<td>60 s, single charge</td>
<td>60 s, single charge</td>
<td>60 s, single charge</td>
</tr>
<tr>
<td><strong>First mass (MS2), mass range (SPS-MS3)</strong></td>
<td>110 – 500 m/z MS3</td>
<td>110 m/z MS2</td>
<td>110 – 500 m/z MS3</td>
<td>110 m/z MS2</td>
</tr>
<tr>
<td><strong>SPS-MS3 Resolution</strong></td>
<td>50000</td>
<td></td>
<td>50000</td>
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<tr>
<td><strong>SPS-MS3 AGC target</strong></td>
<td>1e5</td>
<td>1e5</td>
<td></td>
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<tr>
<td><strong>SPS-MS3 max IT</strong></td>
<td>120 ms</td>
<td></td>
<td>105 ms</td>
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<tr>
<td><strong>SPS-MS3 Isolation window (charge)</strong></td>
<td>1.3(2), 0.7(3), 0.5(&gt;4)</td>
<td>1.3(2), 0.7(3), 0.5(&gt;4)</td>
<td></td>
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<tr>
<td><strong>SPS-MS3 Notches (charge)</strong></td>
<td>5(2), 10(3), 10(&gt;4)</td>
<td>5(2), 10(3), 10(&gt;4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>NCE % SPS-MS3</strong></td>
<td>65</td>
<td>65</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- TMTpro tag fragments easier
  - Drop NCE
  - CID: 32
  - HCD: 45-50

- 16 vs 11 way reporter ion signal
  - Increase ion inject time (IT)
  - Increase sample loading
  - Monitor reporter ion S/N

- IDs vs quantitative accuracy
  - IDs - MS2
  - As with 1st gen TMT, using SPS-MS3 on tribrids will reduce co-isolation interference and ratio compression and give most quantitative accuracy
Summary

• TMTpro 16-plex provides highest level of multiplexing with fewest missing values
• Full analysis support built into PD 2.4
• Design multiplexed experiments to minimize reporter ion interference
• Perform labeling efficiency check
• More hydrophobic => optimize LC gradients
• Unreacted tag => more hydrophobic wash and elution
• Fragments easier => drop NCE
• Monitor reporter ion S/N and adjust IT and sample loading
• Use Yeast triple KO standard to qualify instrument and methods
• Pre-built acquisition methods coming soon for Eclipse and Exploris

Products:

• A44518 TMTpro Zero Label Reagent, 1 x 5mg (August)
• A44519 TMTpro Zero Label Reagent, 5 x 0.5mg (October)
• A44520 TMTpro 16plex Label Reagent Set, 1 x 5mg (August)
• A44521 TMTpro 16plex Label Reagent Set, 1 x 0.5mg (Feb 2020)
• A44522 TMTpro 16plex Label Reagent Set, 6 x 0.5mg (Feb 2020)
Thanks!

- Jessie Guidry (LSU)
- John Rogers
- Ryan Bomgarden
- Jae Choi
- Bhavin Patel
- Penny Jensen
- Leigh Foster
- Amarjeet Flora
- Kratika Singhal
- Kay Opperman
- Rosa Viner
- Aaron Robitaille
- Andreas Huhmer

Questions? Contact your Mass Spec Specialists, BDA TSS, or FAS

Sam Shank (West)

Earl Walker (East)

Tony Herren (North America)
Advantages of Higher Sample Multiplexing

**Increased throughput**
Combine biological samples to reduce fractionation and instrument analysis time.

**Fewer missing values**
Quantified peptides from independent experiments do not overlap completely.

**Internal controls**
An internal reference sample verifies performance and improves comparisons across experiments.

**Multiple comparisons and improved statistics**
Incorporate replicates with multiple conditions: dose-response, time-course, multiple tissues, subcellular fractions, etc.