Today’s lecture

- Quantitative proteomics: \textit{in vitro} labeling
- iTRAQ method
- iTRAQ reagent
- iTRAQ experimental procedure
- Comparison of iTRAQ and ICAT
- Tandem Mass Tag (TMT)
- Comparison of iTRAQ and TMT
Quantitative Proteomics: 
*In vitro* labeling methods

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**In vitro labeling**

- **Amino acid based labeling**
  - ICAT
  - VICAT
  - MCAT
  - QUEST

- **N-terminal peptide labeling**
  - ITRAQ
  - TMT
  - GIST

- **C-terminal peptide labeling**
  - Esterification
  - Proteolysis
  - $^{16}\text{O/}^{18}\text{O}$

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Dass 2006; Robert et al. 2008
Isobaric Tagging for Relative and Absolute Quantification (iTRAQ)

Ross et al. 2004

iTRAQ method

- iTRAQ reagents are set of multiplexed, amine-specific, stable isotope reagents
- It enables simultaneous identification and quantitation, both relative and absolute
- There are two types of iTRAQ Reagents
  - 4-plex - for processing up to 4 samples
  - 8-plex - for processing up to 8 samples
• In iTRAQ all derivatized peptides of a given sequence are isobaric and co-elute
  • derived from control and treatment biological samples
• Upon collision induced dissociation (CID) in MS/MS experiments, it provides reporter ions (signature) that differ in m/z value
  • Reporter ions can be used to monitor the relative quantitation for proteins

iTRAQ Reagent
iTRAQ reagent

• Components of iTRAQ multiplexed isobaric tagging chemistry

(1) Reporter group based on N,N-dimethylpiperazine
(2) Mass balance carbonyl group
(3) A peptide-reactive group (ester of N-hydroxysuccinimide, NHS)

• The m/z value of the reporter group ranges from 114.1 - 117.1
• The balance group mass is 28 - 31 Da
• The overall mass of reporter plus balance components remains constant
  • 145.1 Da for all four reagents
• When reacted with a peptide iTRAQ tag forms an amide linkage to any peptide amine
  • N-terminal or lysine amino group

• Isobaric tag consist of a reporter group, a neutral balance portion, and a peptide reactive group to give an overall mass of 145
iTRAQ reagent

- **Reporter**
  - Provides signature ion in MS/MS
  - Provides good b and y - ion series
  - Maintains the charge state and ionization efficiency of peptide

- **Balancer**
  - Balances mass change of reporter to provide total mass of 145
  - Neutral loss in MS/MS

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iTRAQ reagent: 4-plex

<table>
<thead>
<tr>
<th>Reporter Ions (Da)</th>
<th>Balancer region (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>114</td>
<td>191</td>
</tr>
<tr>
<td>115</td>
<td>190</td>
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<tr>
<td>116</td>
<td>189</td>
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<tr>
<td>117</td>
<td>188</td>
</tr>
</tbody>
</table>

**Peptide**
iTRAQ reagent: 8-plex

<table>
<thead>
<tr>
<th>Reporter Ions (Da)</th>
<th>Balancer region (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>113</td>
<td>192</td>
</tr>
<tr>
<td>114</td>
<td>191</td>
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<td>115</td>
<td>190</td>
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<td>116</td>
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<td>119</td>
<td>186</td>
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<tr>
<td>120</td>
<td>185</td>
</tr>
</tbody>
</table>

iTRAQ Experiment
Sample Preparation: 
Protein reduction and cysteine blocking

- Dissolve protein sample in 0.5 M triethyl ammonium bicarbonate, pH 8.5
- Reduction step by adding a reducing agent
- Incubate samples at 60°C for 1H
- Block cysteine by adding a Cysteine Blocking Reagent

Sample Preparation: 
Protein digestion

- Add trypsin solution
- Incubate overnight at 37°C
- Clean-up samples using ZipTip
Sample Preparation:
Labeling

- Reconstitute the iTRAQ reagent in isopropanol
- Add iTRAQ reagent to digested protein sample

Sample Preparation:
Pooling labeled samples

- Combine labeled samples in one tube
Sample Preparation: Purification

- Pooled samples are purified on a strong cation exchange column to remove excess unbound reagent
- This step facilitates sample clean-up

Overview of protocol

- Control
  - Reduction, Cysteine blocking, Digestion
  - iTRAQ labeling
  - Mix control and treatment samples (iTRAQ labeled)
  - Fractionation and/or clean-up steps
  - LC-MS/MS analysis for protein identification & quantification

- Treatment
  - Reduction, Cysteine blocking, Digestion
  - iTRAQ labeling
  - Mix control and treatment samples (iTRAQ labeled)
  - Fractionation and/or clean-up steps
  - LC-MS/MS analysis for protein identification & quantification
### Overview of protocol

1. Trypsin digestion
2. ITRAQ Labeling
3. Fractionation and/or clean-up steps
4. LC/MS/MS analysis/Quantification

### iTRAQ: MS analysis

- Peptides differentially labeled, mixed together, measured by MS
- Enables simultaneous identification and quantitation
- Labels react with N-terminus
- Reporter group is lost during fragmentation
- Used to determine relative abundance of selected peptide of interest from 4 or 8 samples
iTRAQ: MS analysis

• Samples with 4 independent reagents of same mass (145) give rise to four unique reporter ions (m/z = 114–117) in MS/MS, and subsequently used to quantify different samples

\[
\begin{align*}
114 & \quad \text{NH-GGGGR-COOH} \\
115 & \quad \text{NH-GGGGR-COOH} \\
116 & \quad \text{NH-GGGGR-COOH} \\
117 & \quad \text{NH-GGGGR-COOH}
\end{align*}
\]

MIX

\[
\begin{align*}
114 & \quad \text{NH-GGGGR-COOH} \\
115 & \quad \text{NH-GGGGR-COOH} \\
116 & \quad \text{NH-GGGGR-COOH} \\
117 & \quad \text{NH-GGGGR-COOH}
\end{align*}
\]

MS/MS

iTRAQ: MS analysis

• Quantification occurs at the level of fragment ion spectrum (MS/MS)
• Identification and quantification of peptide are achieved at MS/MS level
iTRAQ: 4-plex MS data

iTRAQ: 8-plex MS data
iTRAQ Technique

Animations

iTRAQ advantages

• Performs relative (or absolute) quantification in up to 4 or 8 samples
• Multiplexing
• Increased analytical precision and accuracy
• Expanded coverage of proteome by tagging tryptic peptides
• Eliminates limitation of ICAT for dependence on cysteine
iTRAQ disadvantages

- Possible errors in quantitation due to
  - differences in efficiency of enzymatic digestion
  - peptide pre-fractionation step
- Variability in initial protein digestion
  - tagging is performed only after individual sample processing is done, which leads to some variations

iTRAQ disadvantages (2)

- Reagents are very costly
- New search algorithms and databases required
• Both ICAT and iTRAQ permits identification and quantifications of proteins
• ICAT amino acid based labeling; iTRAQ on primary amine groups
• ICAT labeling has advantage to reduce sample complexity by eliminating nonlabeled/ noncysteine-containing peptides
• iTRAQ – 4 or 8-plexing; ICAT – only 2 samples

• iTRAQ method provides more complete coverage of original protein sequence than ICAT
• iTRAQ - increased confidence in identification
• iTRAQ - saves MS run time
iTRAQ applications

Animations

Tandem Mass Tag (TMT)

TMT

• TMTs are based on similar principle, with up to 6 possible labels
• TMT isobaric tagging technique can be used to perform absolute quantitation by adding stable isotope labeled internal standard peptides

TMT mass tags

• N-terminal amine and lysine residues labeled through NHS group
• Family of chemical tags based on common structure
  • TMT\(^0\): method development
  • TMT\(^2\): 2-plex profiling and quantitation
  • TMT\(^6\): 6-plex profiling and quantitation
**TMT\(^0\)**

<table>
<thead>
<tr>
<th>Modification</th>
<th>224 Da</th>
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<tbody>
<tr>
<td>MS/MS Reporter</td>
<td>126 Da</td>
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**TMT\(^2\)**

<table>
<thead>
<tr>
<th>Modification</th>
<th>225 Da</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS/MS Reporter</td>
<td>126, 127 Da</td>
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</table>
**TMT**

<table>
<thead>
<tr>
<th>Modification</th>
<th>229 Da</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS/MS Reporter</td>
<td>126, 127, 128, 129, 130, 131 Da</td>
</tr>
</tbody>
</table>

**TMT: MS data**

MS/MS spectrum of TMT-labeled peptide, showing reporter region
**iTRAQ vs. TMT**

**iTRAQ**
- 113-121
- 182-192
- 114-117
- 28-31

**TMT**
- 126-131
- 103-97

**iTRAQ software**
- Protein Pilot
- Mascot

**TMT software**
- Proteome Discoverer
- Mascot

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**Summary**

- iTRAQ technique
- Experimental procedure
- Comparison of iTRAQ with ICAT and TMT


REFERENCES

• Kristin LM Boylan, John D. Andersen, Lorraine B. Anderson, LeeAnn Higgins and Amy PN Skubitz*Quantitative proteomic analysis by iTRAQ® for the identification of candidate biomarkers in ovarian cancer serum. Proteome Science 2010, 8:31


ACKNOWLEDGMENT

• Mr. Sangram Patnaik, ThermoFisher for information related to TMT tags