Bringing Precision to Peptide Quantitation Using Agilent Automation and LC/MS Solutions

Ning Tang, Ph.D.

Agilent Technologies
Santa Clara, CA
Outline

- Sample preparation using AssayMAP
  - Affinity purification
  - In solution digestion and peptide cleanup

- Skyline workflow for targeted protein quantitation
  - Skyline based MRM method development
  - MassHunter and Skyline automation workflow
  - All ions DDA and Skyline workflow

- Ultimate sensitivity and robustness with Agilent 6495 QQQ
  - Low attomole LOQ for peptides
  - Six orders of linearity
  - Great robustness
  - Excellent precision at the lowest levels
  - Extended mass range benefits glycopeptides
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AssayMAP Technology Components

Automated workflows designed for analytical chemists

Microchromatography Cartridges
quantitative binding & elution

Protein purification
- PA-W (protein A)
- PG-W (protein G)
- SA-W (streptavidin)

Reversed-phase cleanup:
- C18 (silica)
- RP-S (polymeric)

Fractionation:
- SCX
- RP-S
- C18

Phosphopeptide enrichment:
- TiO$_2$

Simple User Interface
Uses customer language - not automation language
- Affinity (protein) Purification
- In-Solution Digestion
- Peptide Cleanup (desalting)
- Phosphopeptide Enrichment
- Fractionation
- Liquid handling utilities

Positive Displacement Pipetting
Syringes interface directly with cartridges and enable precise, controlled liquid flow through cartridges with no air bubbles to disrupt binding
## General Workflows

<table>
<thead>
<tr>
<th>Workflow</th>
<th>Description</th>
<th>Open</th>
</tr>
</thead>
<tbody>
<tr>
<td>Affinity Purification Workflow</td>
<td>Create custom affinity cartridges and use them to enrich for target molecules. Using AssayMAP Bravo and Cartridges.</td>
<td></td>
</tr>
<tr>
<td>SISCAPA™ Workflow</td>
<td>Addition-only trypsin digest and immunocaptrue of target peptides and internal standards. Using SISCAPA™ antibodies and Bravo 96LT Head.</td>
<td></td>
</tr>
</tbody>
</table>

## Post-Translational Modification Workflows

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<td>N-Glycan Sample Prep, RX Digestion &amp; 2-AB Labeling v1.0</td>
<td>Denature glycoproteins, then release, label and cleanup glycans. Using AssayMAP Bravo and ProZyme GlykoPrep-plus® 2AB Chemistry Kit.</td>
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AssayMAP mAb Quantitation Workflow

Each step in the workflow was performed using the AssayMAP Bravo

Input  Purify  Output

Digest  Cleanup  Output  Separate  Analyze
Antibody Purification Using Protein A Cartridge

\[ \text{CV} = 1.26\% \]
AssayMAP Digestion and Cleanup

Day 1 cleanup = C18
Day 2 cleanup = RP-S

Low %CVs for both intra- and interday digestion and cleanup for samples prepared using urea or guanidine-based denaturation.
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MrM Based LC/MS Protein Quantitation

- Selection of target proteins
- Selection of target peptides that represent the protein list
- Selection of best charge states and MRM transitions for each targeted peptide
- Optimization of collision energy for each MRM transition
- Application of the assays to the detection and quantitation of the proteins
Skyline Targeted Proteomics Environment

- Freely available Windows client application
- Multi-vendor software
- Initially funded with the CPTAC project
- Rapidly evolved using feedback from top proteomics labs
- Widely used and highly regarded
Agilent’s QQQ + Skyline: Collision Energy Optimization
New Automation Link in Skyline

Automation tool automatically

• Creates QQQ methods and acquires data for CE optimization
• reloads and analyzes the results
• exports the final optimized QQQ method (MRM, dMRM, tMRM)
• Quantitates real samples queued up in the worklist
New Agilent Automation Tool For Skyline and the QQQ Acquisition Software

<table>
<thead>
<tr>
<th>Step-A</th>
<th>Step-B</th>
<th>Step-C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Project setting</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Template method: D:\MassHunter\Methods\chris\A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folder: D:\MassHunter\Data\chris</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Name: demo</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Action selections:
- Step-A (Update Retention Times)
- Step-B (Optimize Collision Energy)
- Step-C (Export method, create worklist)
- Execute worklist

Export method name: Demo
- Single method
- One method per protocol
- Multiple method
- Isomeric proteins

Max transitions per sample injection: 200

Optimizing:
- None
- Method type: Standard, Dwell time (ms):
  - Standard: 5

Create Project
Submit to Study Manager
Close

Project is created at: D:\MassHunter\Data\chris\demo.s (3/4/2013 7:57:09 AM)
Total samples: 12

MRM → RT  dMRM CE opt  dMRM/tMRM → Quant
Agilent’s QTOF and Skyline

- Data Dependent Acquisition (DDA): MS1 Filtering
- Data Independent Acquisition (DIA)

**All ions MS/MS**
- No isolation in quadrupole
- 2 or more collision energies applied
- Simplify the MRM method development

**Wide band isolation**
- Isolate 10 Da
- Typically use same formula as DDA
All Ions MS/MS → Skyline → QQQ MRM Workflow

- All Ions acquisition on a Q-TOF
  - Two channels: 0 V and 25 V

- Load All Ions data into Skyline
  - Create new Skyline project and import FASTA
  - Select target peptides for each protein of interest
  - Select charge state for each peptide precursor
  - Select multiple transitions for each peptide (>6)

- Export MRM list or method with CE opt to run on a QQQ
- Quantitation using the optimized method on a QQQ
All Ions MS/MS on Agilent QTOF

mAb Tryptic Digest

CE=0

CE=25 V

y3+ y5+ y7+ y8+ y9+ y10+ y11+
y12++ y14++ y15++
All Ions ⇒ Skyline Workflow

Step 1: Show p & y Ions for Peptide Confirmation

Precursors (p ions) from low energy channel and products (y ions) from high energy channel are automatically extracted and plotted together to easily confirm the identity of the peptides.

Retention time and peptide sequence confirmation
All Ions → Skyline workflow

Step 2: Show only p Ions to Select Best Charge State

Best charge state for each peptide is easily determined by comparing p ions only
All Ions ➔ Skyline workflow

Step 3: Show only p ions to Compare Peptide Abundance

Peptides with the highest signal are easily determined by comparing p ions from the best charge state of all peptides.

Skyline peptide rank refine tool picks top n peptides
All Ions → Skyline workflow

Step 4: Show Only y Ions to Select Top MRMs

Skyline transition rank refine tool picks top n MRMs
All Ions → Skyline workflow

Step 5: CE Optimization

CE optimization process can be automated using new Skyline Automation Tool
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Improved sensitivity (IDL / MDL) – Average 3x in S/N specifications and applications

Improved precision and excellent accuracy at the lowest levels – 4 to 5x in IDL spec

Proven 6 orders of linear dynamic range

Proven robustness in complex matrix – Food matrix and biological matrix (plasma)

Improved mass range, fast scan speed and MRM acquisition rate
LVNEVTEFAK: Excellent Precision and Low Amol IDL

<table>
<thead>
<tr>
<th>Amount measured</th>
<th>Replicates</th>
<th>%RSD</th>
<th>t (99%)</th>
<th>IDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0 amol (LLOQ)</td>
<td>n = 10 injections</td>
<td>14.0</td>
<td>2.821</td>
<td>2.0 amol</td>
</tr>
</tbody>
</table>

\[
\text{MDL} = t \times \left(\frac{\%\text{RSD}}{100}\right) \times \text{Amount} = 2.821 \times \left(\frac{14.0}{100}\right) \times 5.0 \text{ amol} = 2.0 \text{ amol}
\]

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</tr>
<tr>
<td>2</td>
<td>28.7</td>
</tr>
<tr>
<td>3</td>
<td>30.4</td>
</tr>
<tr>
<td>4</td>
<td>33.91</td>
</tr>
<tr>
<td>5</td>
<td>35.1</td>
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<td>6</td>
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</tr>
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</tr>
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<td>8</td>
<td>20.7</td>
</tr>
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<td>26.7</td>
</tr>
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<td>10</td>
<td>26.6</td>
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%RSD 14.0

Counts vs. Acquisition Time (min)

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%RSD 14.0

Counts vs. Acquisition Time (min)
Peptide Quantitation: Outstanding Sensitivity with Standard Flow Chromatography

Instrumentation: 1290 UHPLC + Agilent JetStream + 6495 QQQ
Sample: synthetic peptide standard (LVNEVTEFAK) spiked into enolase tryptic digest
Injection volume: 1 µL
Peptide Quantitation – 6 Orders of Linear Dynamic Range

LVNEVTEFAK
5 amol – 5 pmol on-column
6 orders of linear dynamic range
R2 = 0.998

Calibration Standards (amount on-column; 1 µL injected)

<table>
<thead>
<tr>
<th>LVNEVTEFAK</th>
<th>5.0 amol</th>
<th>7.5 amol</th>
<th>15 amol</th>
<th>30 amol</th>
<th>300 amol</th>
<th>3 fmol</th>
<th>30 fmol</th>
<th>300 fmol</th>
<th>3 pmol</th>
<th>5 pmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>%Accuracy</td>
<td>109.8</td>
<td>108.7</td>
<td>105.0</td>
<td>87.1</td>
<td>85.2</td>
<td>81.4</td>
<td>86.4</td>
<td>87.4</td>
<td>105.6</td>
<td>97.5</td>
</tr>
<tr>
<td>Reproducibility (%RSD, n=10)</td>
<td>14.0</td>
<td>16.0</td>
<td>9.4</td>
<td>9.0</td>
<td>1.6</td>
<td>1.2</td>
<td>0.6</td>
<td>0.7</td>
<td>2.1</td>
<td>1.0</td>
</tr>
<tr>
<td>RT (%RSD, n=100)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.12</td>
<td></td>
</tr>
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</table>
Peptide Quantitation: Ultimate Sensitivity with Nanoflow Chromatography

Instrumentation: 1260 HPLC-Chip/MS + 6495 QQQ
Sample: synthetic peptide standard (LVNEVTEFAK) spiked into enolase tryptic digest
Injection volume: 1 µL
Proven System Robustness in Complex Matrix: Protein Quantitation in Plasma

• Selected peptides from 42 peptides in the QC sample – normalized to Day 1 response
• Peptide QC samples analyzed daily after every ~25 plasma digest injections
• No significant signal degradation observed after 853 injections of 40 µg plasma digest per injection and 3.5 weeks of continuous operation
• Response %RSD: 6 - 15
Increased Mass Range Useful for Peptides

QQQ MRM spectrum for glycopeptide: EEQYN[+1606.6]STYR (G1F)
Increased Mass Range Useful for Peptides

MRM Spectrum

Skyline Overlay of MRM Chromatograms

MRM Spectrum for IgG Peptide: GFYPSDIAVEWESNGQPENNYK
Summary

• The ability to perform reproducible protein sample preparation on a versatile instrument platform which enables scaling of sample preparation makes robust, high-throughput, protein quantification attainable.

• Agilent LCMS coupled with Skyline software enables a simple automated method development workflow for targeted proteomics and Biopharma research.

• The Agilent 6495 Triple Quadrupole LC/MS achieves low attomole sensitivity for peptides. The calibration curves show excellent linearity with six orders of dynamic range with great accuracy and reproducibility.
Acknowledgement

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Jason Russell
Christine Miller
Yanan Yang

THANK YOU!