Put the PRO in Protein Characterization

Aaron Boice
LC/Q-TOF Product Manager
5-June-2017
Agenda

The Right Tools for the Job

Tackling Intact Proteins

Break it Down – Peptides and PTMs

Sweet Success – Released Glycan Profiling

Conclusion
Agenda

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Conclusion
Protein Characterization Poses Many Questions

Intact Mass?
Relative Glycoforms?
ADC DAR Calculation?
Peptide Sequence Mapping?
Post Translational Modifications?
Disulfide Bonds?
Host Cell Proteins?
Glycan Profile?
...

For Research Use Only. Not for use in diagnostic procedures.
Multiple Experiments – One Set of Tools

Sample Preparation

Data Processing

Separation

Detection
Agilent BioPharma Workflow Platform for LC/MS
Step 1
Sample Prep Separation Detection Analysis

Agilent AssayMAP Bravo

• Bravo Liquid Handling Robot

• Graphical Software (no programming language needed!)

• Micro-chromatography tips
Step 2
Sample Prep → Separation → Detection → Analysis

Agilent 7100 CE
Agilent 1290 Infinity II UHPLC
2D–LC
Agilent AdvanceBio LC Columns

LC/MS compatible columns designed for:

- Intact Proteins, Heavy/Light Chains, Fab/Fc Regions
- Peptide Mapping, PTM separations
- Glycan Profiling
Sample Prep > Separation > Detection > Analysis

**Intact Protein**
Industry leading data quality and sensitivity at the intact level

**Peptide Mapping and PTMs**
Ready for routine confirmation or digging deeper with IterativeMS/MS

**Glycan Profiling**
Full released glycan workflow with BioConfirm B.09

**New**
6545XT AdvanceBio LC/Q-TOF
Step 4
Sample Prep ➔ Separation ➔ Detection ➔ Analysis

MassHunter BioConfirm – One Stop Protein Analysis Software

Input

Protein Sequence

Experimental Conditions

Output

• Deconvolved mass confirmation
• Glycoform results
• Peptide Sequence Coverage
• Glycan Profile
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Intact Protein Characterization Workflow

Sample Prep > Separation > Detection > Analysis

- Sample Prep:
  - Enrich

- Separation:
  - No Enzyme
  - Deglycosylate
  - Met Enzyme

- Detection:
  - Intact mAb
  - Deglycosylated mAb

- Analysis:
  - ProZyme InstantPC glycan tag or 2-AB tag
  - Fc
  - F(ab')2
  - Fd' & LC

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Intact Protein Characterization

Sample Prep  Separation  Detection  Analysis

Agilent 1290 Infinity II  Agilent AdvanceBio LC Columns

PLRP-S 1000 Å
AdvanceBio RP mAb
Zorbax SB 300 RRHD
Poroshell 300

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Intact Protein Characterization

**Sample Prep**
- Intact protein spectral clarity (10E-8 vacuum)
- Large molecule SWARM autotune
- 30k m/z mass range
- Vent-free capillary removal
- Quick start methods with PLRP-S column
Intact ADC Drug Antibody Ratio (DAR) Calculation

DAR peak list shows all components and % area for each species

calculated DAR

For Research Use Only. Not for use in diagnostic procedures.
Reduced ADC DAR Calculation

Total DAR from both HC and LC

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Intact Protein Spectral Quality

Sample:
0.5 μg NISTmAb

Chromatography:
Agilent PLRP-S 1000 Å, 2.1 × 50 mm, 5 μm, PL1912-1502

5 min H₂O/ACN gradient w/0.1% FA @500 μL/min
In a previously published multi-lab study\(^1\) characterizing the NISTmAb, 18 proteoforms are described by three labs using different HRAM systems. Each lab missed some of the proteoforms, while injecting 5 µg or performing an infusion.

<table>
<thead>
<tr>
<th>Proteoform</th>
<th>Theoretical Mass (Da)</th>
<th>Observed Mass (Da)</th>
<th>Lab 1</th>
<th>Lab 2</th>
<th>Lab 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. G0F/Glycosylated</td>
<td>146591.8</td>
<td>ND</td>
<td>146595.3 (t)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>2. G1F/Glycosylated</td>
<td>146754.0</td>
<td>ND</td>
<td>146756.0 (t)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>3. G2F/Glycosylated</td>
<td>146916.1</td>
<td>ND</td>
<td>146915.8 (t)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>4. G0F/G0F – 2GlcNAc</td>
<td>147630.8</td>
<td>147633.1 (t)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5. G0F/G0F – GlcNAc</td>
<td>147834.0</td>
<td>147837.9 (m)</td>
<td>147837.4 (m)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>6. G0F/G1F – GlcNAc</td>
<td>147996.1</td>
<td>147993.2 (t)</td>
<td>148000.6 (m)</td>
<td>148000.0 (m)</td>
<td></td>
</tr>
<tr>
<td>7. G0F/G0F</td>
<td>148037.2</td>
<td>148040.5 (m)</td>
<td>148039.3 (m)</td>
<td>148045.0 (m)</td>
<td></td>
</tr>
<tr>
<td>8. G0F/G0F + K</td>
<td>148165.3</td>
<td>148154.6 (t)</td>
<td>148164.9 (m)</td>
<td>148163.0 (m)</td>
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</tr>
<tr>
<td>9. G0F/G1F</td>
<td>148199.3</td>
<td>148201.9 (m)</td>
<td>148201.7 (m)</td>
<td>148206.5 (m)</td>
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</tr>
<tr>
<td>10. G0F/G1F + K</td>
<td>148327.5</td>
<td>148325.5 (t)</td>
<td>148328.1 (m)</td>
<td>148302.0 (m)</td>
<td></td>
</tr>
</tbody>
</table>
Know More at the Intact Level

- The 6545XT AdvanceBio LC/Q-TOF detects every proteoform described, while injecting less material, and with the best mass accuracy for 13 of the 18 peaks

### Missed 2 Labs
<table>
<thead>
<tr>
<th>Proteoform</th>
<th>Theoretical Mass (Da)</th>
<th>Agilent 6545XT</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. G0F/Agycosylated</td>
<td>146591.8</td>
<td>146592.5</td>
</tr>
<tr>
<td>2. G1F/Agycosylated</td>
<td>146754.0</td>
<td>146755.8</td>
</tr>
<tr>
<td>3. G2F/Agycosylated</td>
<td>146916.1</td>
<td>146918.5</td>
</tr>
<tr>
<td>4. G0F/G0F – 2GlcNAc</td>
<td>147630.8</td>
<td>147632.5</td>
</tr>
<tr>
<td>5. G0F/G0F – GlcNAc</td>
<td>147834.0</td>
<td>147836.0</td>
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<tr>
<td>6. G0F/G1F – GlcNAc</td>
<td>147996.1</td>
<td>147998.5</td>
</tr>
<tr>
<td>7. G0F/G0F</td>
<td>148037.2</td>
<td>148038.6</td>
</tr>
<tr>
<td>8. G0F/G0F + K</td>
<td>148165.3</td>
<td>148161.9</td>
</tr>
<tr>
<td>9. G0F/G1F</td>
<td>148199.3</td>
<td>148200.9</td>
</tr>
<tr>
<td>10. G0F/G1F + K</td>
<td>148327.5</td>
<td>148324.7</td>
</tr>
</tbody>
</table>

### missed 1 Lab

### Missed 2 Labs

<table>
<thead>
<tr>
<th>Proteoform</th>
<th>Theoretical Mass (Da)</th>
<th>Agilent 6545XT</th>
</tr>
</thead>
<tbody>
<tr>
<td>11. G1F/G1F</td>
<td>148361.4</td>
<td>148363.1</td>
</tr>
<tr>
<td>12. G1F/G1F + K</td>
<td>148489.6</td>
<td>148488.1</td>
</tr>
<tr>
<td>13. G1F/G2F</td>
<td>148523.6</td>
<td>148525.2</td>
</tr>
<tr>
<td>14. G1F/G1F + 2K</td>
<td>148617.8</td>
<td>148623.6</td>
</tr>
<tr>
<td>15. G1F/G2F + K</td>
<td>148651.8</td>
<td>148652.9</td>
</tr>
<tr>
<td>16. G2F/G2F</td>
<td>148685.7</td>
<td>148686.6</td>
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<tr>
<td>17. G2F/G2F + Hex</td>
<td>148847.7</td>
<td>148846.7</td>
</tr>
<tr>
<td>18. G2F/G2F + 2Hex</td>
<td>149010.0</td>
<td>149003.5</td>
</tr>
</tbody>
</table>

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Improved Spectral Clarity from Improved TOF Vacuum

1 ug trastuzumab

Grey – 6545 LC/Q-TOF

Green – 6545XT AdvanceBio LC/Q-TOF

Narrower peaks

Deeper valley between glycoforms

Counts (%) vs. Deconvoluted Mass (amu)
Optimizing for Proteins with Large Molecule SWARM Autotune

Unprocessed mAb spectra zoomed to show charge states

Same AdvanceBio LC/Q-TOF

Blue – Large Molecule SWARM Autotune
Red – Standard Mass Range SWARM Autotune

Counts vs. Mass-to-Charge (m/z)

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Intact Protein Analysis: Denatured vs. Native MS of NIST mAb

**Denaturing Conditions**
- 0.1% Formic Acid in H₂O
- 0.1% Formic Acid in Acetonitrile

**Native MS**
- 100 mM Ammonium Acetate, pH 7
NativeMS Posters at ASMS

Thursday 10:30am – 1:00pm
ThP 603
Higher Order Structure of Intact Proteins by Capillary Electrophoresis Native Ion Mobility Mass Spectrometry

CE/MS of proteins under native conditions

Thursday 12:00pm – 2:30pm
ThP 616 Enhanced Detection of Non-Covalently Bound Enzyme Complexes Using a Dedicated Large Molecule Autotune on a Q-TOF Mass Spectrometer

800 kDa GroEL complex under native conditions
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Sweet Success – Released Glycan Profiling

Conclusion
Peptide Sequence Mapping and PTM Identification

Sample Prep  ➔  Separation  ➔  Detection  ➔  Analysis

- Preconfigured protocols that required no programming language
- Get reproducible digestion
- Run parallel digestions for method development or multiple enzyme preparations

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Peptide Sequence Mapping and PTM Identification

Sample Prep  Separation  Detection  Analysis

Agilent 1290 Infinity II

Agilent AdvanceBio LC Columns

AdvanceBio Peptide Mapping

Tried and true for routine peptide mapping applications.

NEW – AdvanceBio Peptide Plus

The latest AdvanceBio column specifically designed for LC-MS peptide applications. The charged surface provides sharp symmetrical peaks with just formic acid and provide alternate separation for critical PTMs at higher mass loads.
Peptide Sequence Mapping and PTM Identification

- Quick-start peptide mapping method w/columns
- Identification confidence (<1 ppm mass error, 50k resolution)
- One-click optimization with SWARM autotune
- IterativeMS/MS mode
- Agilent Jet Stream ion source

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MassHunter BioConfirm

1. Find Peptides
2. Identify Peptides
3. Assign Possible PTMs
4. Confirm Sequence

AUTOMATICALLY
Sample:
250 ng NISTmAb digested with Trypsin and Lys-C on AssayMAP Bravo

Chromatography:
Agilent AdvanceBio Peptide Mapping, 2.1 x 150 mm, 2.7 μm (p/n 653750-902)

20 min H₂O/ACN gradient w/0.1% FA @400 μL/min

99.4% Sequence Coverage single injection, 250 ng protein
Methionine Oxidation PTM

Relative % oxidation = 1.6%

Oxidized peptide

Non-oxidized peptide
Methionine Oxidation Localization by MS/MS

<table>
<thead>
<tr>
<th></th>
<th>y3</th>
<th>y4</th>
<th>y5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-oxidized</td>
<td>375.2340</td>
<td>506.2769</td>
<td>619.3600</td>
</tr>
<tr>
<td>Oxidized</td>
<td>375.2351</td>
<td>522.2694</td>
<td>635.3547</td>
</tr>
</tbody>
</table>

same +16 +16

AdvanceBio Peptide Plus Column
AdvanceBio LC/Q-TOF
See more details in App Note: 5991-8046EN
Enhanced Analysis of Host Cell Proteins from CHO Cell Cultured mAb Using a Newly Developed Q-TOF Instrument, Linfeng Wu

**Introduction**

High-throughput LC/MS/MS is a powerful method for the identification of peptides and proteins. However, the high sensitivity and specificity of MS/MS can fail to detect proteins with low abundance or those that are difficult to digest. Iterative MS/MS is a strategy that allows for the detection and identification of additional proteins.

**Experimental**

**Results and Discussion**

**Wednesday 12:00 – 2:30pm**

**WP-694**

Enhanced Analysis of Host Cell Proteins from CHO Cell Cultured mAb Using a Newly Developed Q-TOF Instrument, Linfeng Wu

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AssayMAP Glycan Profiling Preparation

Sample Prep ➔ Separation ➔ Detection ➔ Analysis

- **Sample Prep**:
  - Enrichment

- **Separation**:
  - No Enzyme ➔ Elute ➔ Reduce
  - Deglycosylate ➔ Elute ➔ Reduce
  - IdoS Digest ➔ Flowthrough

- **Detection**:
  - Intact mAb ➔ HC & LC
  - Deglycosylated mAb ➔ Deglycosylated HC & LC
  - ProZyme InstantPC glycan tag or 2-AB glycan tag

- **Analysis**:
  - Fc
  - F(ab)’2 ➔ Fd’ & LC
AdvanceBio Glycan Mapping Column

**AdvanceBio Glycan Mapping column, 1.8 μm**

**Non-Agilent sub-2 μm glycan column**

<table>
<thead>
<tr>
<th>Time</th>
<th>%A</th>
<th>%B</th>
<th>Flow rate mL/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20</td>
<td>80</td>
<td>0.5</td>
</tr>
<tr>
<td>25</td>
<td>40</td>
<td>60</td>
<td>0.5</td>
</tr>
<tr>
<td>26</td>
<td>100</td>
<td>0</td>
<td>0.5</td>
</tr>
<tr>
<td>27</td>
<td>40</td>
<td>80</td>
<td>0.5</td>
</tr>
</tbody>
</table>

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AdvanceBio LC/Q-TOF

Adjusts instrument conditions to prevent unwanted fragmentation of labile molecules like glycans
B.09 Preview of Glycan Profiling

Use AM database for formula match

Optional: choose glycan tag

BioConfirm runs Find By Formula algorithm to find glycan features

Found glycans are reported in the Biomolecule Table
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Conclusion
• Tools to address protein characterization at multiple levels
• Efficiency
• Results you can trust
Collaborate - Ask and answer questions

Connect - Interact with other Agilent users

Discover - Find relevant discussions, documents, and videos

Share - Contribute your insights

• Enter our $50 Amazon gift card contest:
  – Register for the Agilent Community
  – Take our 3 question survey:
    • community.agilent.com/asms