Critical Quality Attribute Assessment by Peptide Mapping Using LC/MS with Superficially Porous Columns
Analytical groups are tasked with method development, sample analysis, and method transfer.
Critical Quality Attributes & Testing Methods

Not just used for monoclonal antibodies – same techniques are used for any potentially therapeutic protein.

LC/MS
- Intact mass
- Fragment mass
- Identification
- PTM ID & locations
- Glycan ID

SEC
Aggregation

HILIC
Glycosylation (G0, G1, G2)

IEC
Pyro-Glutamate

IEC
Disulfide Shuffling

IEC
Deamidation/Oxidation

IEC
Fragmentation (Hinge)

IEC
Truncation (Lys 0, 1, 2)

IEC
RP

RP
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Peptide Mapping Workflow Solutions to Accelerate Biomolecule Characterization

Data Acquisition → Peptide mapping chromatogram → Peptide ID by MS/MS → Sequence Matching
Determine Post Translational Modifications

Chromatographic Separation

Sample Preparation

Complete Agilent Workflow Solution

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Sample Preparation for Peptide Mapping

A time consuming, labor-intensive process to do manually, with multiple steps that can be optimized.

- Depletion of matrix proteins, enrichment of target protein, dialysis or desalting
- Reduce disulfide bonds, and block to prevent re-formation
- Trypsin is the most common digestion enzyme, but others may be called for depending on the protein and experiment.
- Sample concentration, enrichment for specific PTMs, or desalting

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AssayMAP Bravo – Sample Prep Automation
General Considerations for Peptide Mapping Column Selection

Instrument capabilities and requirements
- UV vs MS detection or both
- Pressure capabilities

Mobile phase requirements
- High pH required for sample
- TFA vs Formic acid

Sample
- Hydrophilic vs hydrophobic peptides
- Larger polypeptides present

Column dimensions
- Generally prefer longer columns, especially for more complicated maps
  - 50, 150, and 250 mm lengths available
- Use 2.1 mm i.d. for MS-sensitivity
  - 3.0 and 4.6 mm i.d. also available
- Smaller pore sizes ideal, usually 100-150 Å
Detectors for Peptide Mapping

**UV**
- Often used in routine process measurements, QA/QC labs
- No peptide mass or structural information; relies on reproducibility of RT, peak area, and peak area ratios
- Traditionally uses TFA modifier

**MS**
- Often used in discovery/early development stage, R&D labs
- MW and structural information (MS/MS) to assist with co-elution challenges, verify sequence coverage, and to ID unknown peaks when they appear
- Generally prefer formic acid modifier to avoid ion suppression from TFA

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Mobile Phases for Peptide Mapping

Ion pairing agents used for RP LC

- Increase retention of small, poorly retained peptides
- Block exposed silanol sites on silica-based columns
- Acid helps solubilize and ionize peptide and protein samples

TFA works well on “traditional” silica-based columns, but formic acid does not

- Formic acid produces broad, asymmetric peaks
- Poor resolution and poor peak capacity

Need column solution for formic acid modifier

- AdvanceBio Peptide Plus
- Charged surface chemistry solves the problems associated with formic acid

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<table>
<thead>
<tr>
<th>Agilent Bio-LC Column Portfolio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agilent Bio-LC Columns</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Affinity</strong></td>
</tr>
<tr>
<td>Bio-Monolith Protein A</td>
</tr>
<tr>
<td>Bio-Monolith Protein G</td>
</tr>
<tr>
<td>Multiple Affinity Removal System</td>
</tr>
<tr>
<td><strong>Reversed Phase</strong></td>
</tr>
<tr>
<td>AdvanceBio Peptide Plus</td>
</tr>
<tr>
<td>AdvanceBio Peptide Mapping</td>
</tr>
<tr>
<td>AdvanceBio RP mAb</td>
</tr>
<tr>
<td>AdvanceBio Oligonucleotides</td>
</tr>
<tr>
<td>AdvanceBio Desalting-RP</td>
</tr>
<tr>
<td>PLRP-S</td>
</tr>
<tr>
<td>ZORBAX RRHD 300Å, 1.8 μm</td>
</tr>
<tr>
<td><strong>HILIC</strong></td>
</tr>
<tr>
<td>AdvanceBio Glycan Mapping</td>
</tr>
<tr>
<td>ZORBAX RRHD 300-HILIC</td>
</tr>
<tr>
<td><strong>Size Exclusion</strong></td>
</tr>
<tr>
<td>AdvanceBio SEC</td>
</tr>
<tr>
<td>Bio SEC-3</td>
</tr>
<tr>
<td>Bio SEC-5</td>
</tr>
<tr>
<td>ProSEC 300S</td>
</tr>
<tr>
<td>ZORBAX GF-250</td>
</tr>
<tr>
<td>ZORBAX GF-450</td>
</tr>
<tr>
<td><strong>Ion Exchange</strong></td>
</tr>
<tr>
<td>Bio mAb</td>
</tr>
<tr>
<td>Bio IEX (SAX, SCX, WAX, WCX)</td>
</tr>
<tr>
<td>PL SAX</td>
</tr>
<tr>
<td>PL SCX</td>
</tr>
<tr>
<td>Bio-Monolith (QA, DEAE, SO₃)</td>
</tr>
</tbody>
</table>

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## Agilent Bio-LC Column Portfolio

### Agilent Bio-LC Reverse Phase Columns for Protein Analysis

<table>
<thead>
<tr>
<th>Intact Proteins (&amp; Fragments)</th>
<th>Peptides</th>
<th>Amino Acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLRP-S, 5 µm, 1000 Å</td>
<td>AdvanceBio Peptide Plus</td>
<td>AdvanceBio Amino Acid Analysis</td>
</tr>
<tr>
<td>AdvanceBio RP mAb</td>
<td>AdvanceBio Peptide Mapping</td>
<td>ZORBAX Amino Acid Analysis</td>
</tr>
<tr>
<td>ZORBAX RRHD 1.8 µm, 300 Å</td>
<td>ZORBAX Eclipse Plus C18</td>
<td>ZORBAX 300SB*</td>
</tr>
<tr>
<td>ZORBAX 300SB</td>
<td>ZORBAX 300SB*</td>
<td></td>
</tr>
<tr>
<td>Poroshell 300</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AdvanceBio Desalting-RP</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*unusually large pore size for peptide mapping, but some people really like it nonetheless*
AdvanceBio Peptide Plus

**Poroshell particle**
UHPLC performance at lower pressure

**Unique charge hybrid/C18 bonded phase**
Improved peak shape and peak capacity for peptide analysis with formic acid and MS detection

To provide **AdvanceBio Peptide Plus** that gives benefits in **accuracy and reproducibility** of results for characterization of mAb identity, PTMs

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Columns Individually Tested for Efficiency

**LC Column Performance Report**

**SERIAL NUMBER:** USJXB01020  
**PART NUMBER:** 675950-902  
**COLUMN TYPE:** AdvanceBio Peptide Plus 2.1 x 150 mm, 2.7 μm  
**PACKING LOT #:** B17035

**TEST CONDITIONS**  
- MOBILE PHASE = 60% Acetonitrile / 40% Water  
- COLUMN PRESSURE = 328.9 Bar  
- COLUMN FLOW = 0.40 ml/min  
- LINEAR VELOCITY = 0.369 cm/sec  
- TEMPERATURE = AMBIENT (Nominally 23 °C)  
- INJECTION VOLUME = 1 μl

**QUALITY CONTROL PERFORMANCE RESULTS FOR NAPHTHALENE**

<table>
<thead>
<tr>
<th>TEST VALUE</th>
<th>SPECIFICATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>THEORETICAL PLATES = 25583</td>
<td>MIN = 24000</td>
</tr>
<tr>
<td>SELECTIVITY = 1.73</td>
<td>RANGE = 1.68 - 1.78</td>
</tr>
<tr>
<td>USP TAILING FACTOR = 1.20</td>
<td>RANGE = 0.98 - 1.20</td>
</tr>
<tr>
<td>(at 5% Peak Height)</td>
<td></td>
</tr>
</tbody>
</table>

**Sample components with concentrations diluted in mobile phase in the following elution order:**

- Peak # 1: 10 μg/ml Uracil
- Peak # 2: 400 μg/ml Phenol
- Peak # 3: 50 μg/ml 4-Chloro Nitrobenzene
- Peak # 4: 80 μg/ml Naphthalene

---

AdvanceBio Peptide Plus Column

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Column Lots QA Tested Specifically for Peptide Separations

Quality Assurance - Certificate of Analysis for a representative lot using a peptide test sample.

Test Conditions for Peptide Analysis

- Column Dimension: 2.1 x 150mm, 2.7μm
- Test Temperature: 55°C
- Test Standard: HSA 7 peptide mix
- Injection Volume: 1 μl
- Pump Flow Rate: 0.5 ml / minute

Gradient Profile:
- Solvent A: Water 0.1% FA
- Solvent B: Acetonitrile 0.1% FA

<table>
<thead>
<tr>
<th>Time (minutes)</th>
<th>Percent B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>2</td>
</tr>
<tr>
<td>5.00</td>
<td>2</td>
</tr>
<tr>
<td>35.00</td>
<td>35</td>
</tr>
<tr>
<td>36.10</td>
<td>95</td>
</tr>
<tr>
<td>38.00</td>
<td>95</td>
</tr>
<tr>
<td>38.20</td>
<td>2</td>
</tr>
</tbody>
</table>

Peptides Standard (p/n G2455-85001)
LC/MS Instrumentation

- 1290 Infinity II LC system
- 6545XT AdvanceBio LC/Q-TOF
- BioConfirm B.08 for intact protein and peptide mapping data analysis
6545XT Features for Peptide Mapping

- Sensitive peptide detection featuring Agilent Jet Stream
- Quick-start peptide mapping method
- Access low intensity peptides/PTMs with the new IterativeMS/MS mode
- Sub-ppm mass accuracy with 50k resolution from improved beam optics
- Peptide fragmentation performance verification at install
- Ease of maintenance with vent-free capillary removal
## Method Conditions

### 1290 Infinity II LC Column
- **Parameter**: AdvanceBio Peptide Plus 2.1 × 150mm, 2.7µm, 120Å

### Mobile phase
- **A**: 0.1%FA
- **B**: 0.1% FA in ACN

### Gradient
<table>
<thead>
<tr>
<th>Time (min)</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>31</td>
<td>40</td>
</tr>
<tr>
<td>33</td>
<td>95</td>
</tr>
<tr>
<td>34</td>
<td>95</td>
</tr>
<tr>
<td>34.1</td>
<td>3</td>
</tr>
</tbody>
</table>

### Post time
- **10 min**

### Inj vol
- **1 µL**

### Sample thermostat
- **5 °C**

### Column temp
- **55 °C**

### Flow rate
- **0.5 ml/min**

### Sample
- trastuzumab digest (1ug/ul)

### 6545XT AdvanceBio LC/Q-TOF Ion mode
- Positive ion mode, dual AJS ESI (profile)

### Drying gas temp
- **325 °C**

### Drying gas flow
- **13 L/min**

### Sheath gas temp
- **275 °C**

### Sheath gas flow
- **12 L/min**

### Nebulizer
- **35 psi**

### Capillary voltage
- **4000 V**

### Fragmentor voltage
- **175 V**

### Skimmer voltage
- **65 V**

### Oct RF Vpp
- **750 V**

### Acquisition parameters
- Data were acquired at 2 GHz Extended Dynamic Range
- MS mass range 100–1700 m/z
- MS/MS mass range 50 – 1700
- MS scan rate (spectra/second): 8
- MS/MS scan rate (spectra/second): 3

### Ramped collision energy
<table>
<thead>
<tr>
<th>Charge state</th>
<th>Slope</th>
<th>Offset</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>3.1</td>
<td>1</td>
</tr>
<tr>
<td>3 and &gt; 3</td>
<td>3.6</td>
<td>-4.8</td>
</tr>
</tbody>
</table>

### Data analysis
- BioConfirm software B.08.08

---

All results shown are with formic acid mobile phase.

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Effect of Flow Rate on Peak Capacity

- Increasing the flow rate from 0.1 mL/min to 0.5 mL/min leads to an increase of twice the peak capacity.
Effect of Gradient Length on Peak Capacity

- Increasing the gradient time from 0 min to 60 min leads to an increase 2.5 times peak capacity,
- After 30 min, no significant increase in peak capacity.

TIC vs. Acquisition Time (min)

AdvanceBio Peptide Plus Column

Peak capacity vs. Gradient time (min)

1 ug trastuzumab digest
Effect of Gradient Slope on Peak Capacity

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- Higher efficiency → sharper peaks
- Increased sensitivity
- Peak capacity = 402
- Sequence coverage = 96.69%

- Improved resolution
- Peak capacity = 590
- Sequence coverage = 94.59%
Effect of Temperature on Peak Capacity

- Higher temperature produces narrower peaks, improving resolution
- Change in temperature changes selectivity
- ~20% more peaks resolved by increasing the column temperature to 55°C

**AdvanceBio Peptide Plus Column**

**1μg trastuzumab digest**

**Counts vs. Acquisition Time (min)**

**45°C**

**55°C**

**Pc = 504**

**Pc = 600**

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Back Pressure Characterization

Elevated temp produces narrower peak bands and lower pressure

*pressure reading observed (max)
Good Peak Shape & Retention Time Stability with High Mass Load

**Sample**: 3-peptide mixture in 0.1%FA
- Bradykinin
- Angiotensin II
- Venin substrate

Key for transferring methods between LC/MS and LC/UV

All 2.1 x 50 mm columns
Highly Reproducible Separations

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>RT (min)</th>
<th>FWHM (min)</th>
<th>Area (×10^4)</th>
<th>Height (×10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.20</td>
<td>1.13</td>
<td>0.31</td>
<td>1.36</td>
</tr>
<tr>
<td>2</td>
<td>0.06</td>
<td>1.57</td>
<td>5.76</td>
<td>2.54</td>
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<tr>
<td>3</td>
<td>0.06</td>
<td>1.55</td>
<td>4.27</td>
<td>1.39</td>
</tr>
<tr>
<td>4</td>
<td>0.06</td>
<td>1.02</td>
<td>4.60</td>
<td>1.23</td>
</tr>
<tr>
<td>5</td>
<td>0.07</td>
<td>0.91</td>
<td>3.41</td>
<td>1.53</td>
</tr>
<tr>
<td>6</td>
<td>0.05</td>
<td>0.86</td>
<td>2.69</td>
<td>0.74</td>
</tr>
<tr>
<td>7</td>
<td>0.06</td>
<td>3.22</td>
<td>5.79</td>
<td>3.33</td>
</tr>
<tr>
<td>8</td>
<td>0.04</td>
<td>0.99</td>
<td>3.81</td>
<td>1.12</td>
</tr>
<tr>
<td>9</td>
<td>0.02</td>
<td>0.92</td>
<td>2.66</td>
<td>1.31</td>
</tr>
<tr>
<td>10</td>
<td>0.02</td>
<td>0.95</td>
<td>4.22</td>
<td>1.80</td>
</tr>
</tbody>
</table>

- Consistent chromatography
- RSD values demonstrate the robustness of the method and precision of the LC system
- Especially crucial for LC/UV peptide mapping
Lifetime Testing

Column: AdvanceBio Peptide Plus
Sample: 10 peptide standard (5190-0583)
Injection: 3 µL
Flow rate: 0.5 mL/min
Mobile phase: A – 0.1% FA
B – 0.1% FA in ACN

<table>
<thead>
<tr>
<th>Time</th>
<th>% B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>2.01</td>
<td>2</td>
</tr>
<tr>
<td>22</td>
<td>35</td>
</tr>
<tr>
<td>22.01</td>
<td>75</td>
</tr>
<tr>
<td>23.5</td>
<td>75</td>
</tr>
<tr>
<td>23.81</td>
<td>0</td>
</tr>
<tr>
<td>26</td>
<td>0</td>
</tr>
</tbody>
</table>

- The column has subjected to 1000 injections and lost no more than 10% of the initial plate and retention value at the end of the test
- Good chemical stability under formic acid conditions

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Column Options for Alternate Selectivity

10 Peptide Standard (p/n 5190-0583)

1. Bradykinin frag 1-7
2. Bradykinin
3. Angiotensin II (human)
4. Neurotensin
5. Angiotensin I (human)
6. Renin substrate porcine
7. [Ace-F-3,2 H-1] Angiotensinogen (1-14)
8. Ser/Thr Protein Phosphatase (15-31)
9. [F14] Ser/Thr Protein Phosphatase (15-31)
10. Melittin (honey bee venom)

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**mAb Peptide Mapping**

LC/MS of mAb tryptic digest on 2.1 x 150mm AdvanceBio Peptide Plus Column

- **Hydrophilic peptide retention**
- **Narrow Peaks with baseline resolution**
- **Hydrophobic peptide elution**
- **Reduced and fast analysis time**

Critical and desired peptide mapping components to achieve fast, selective, and highly efficient peptide separations across a wide dynamic range.
Tryptic Peptides of Trastuzumab

Light Chain B (1-214)

Heavy Chain A (1-449)

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LC/MS Peptide Map

sequence coverage 99.25%
mass accuracy of 5 ppm

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MassHunter BioConfirm for Peptide Mapping

Sample Prep ▶ Separation ▶ Detection ▶ Data Processing & Report

Acquire data ▶ Extract compounds ▶ Match protein sequences and identify PTMs ▶ Sequence coverage ▶ Report generation
Separation of Oxidized Peptides

**DLTMISR peptide**

Unmodified peptide

Oxidized peptide

Relative % of Oxidation = 1.69%

**AdvanceBio Peptide Plus Column**

**MS/MS spectra**

Increase in mass (16Da)

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Separation of Deamidated Peptides

**NTAYLQMNLSR peptide**
Relative % of Deamidation = 0.21% – 15.9%

**ASQDVNTAVAQYQQKPGK peptide**
Relative % of Deamidation = 9.1%

AdvanceBio Peptide Plus Column

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Critical Quality Attributes & Testing Methods

Not just used for monoclonal antibodies – same techniques are used for any potentially therapeutic protein.

With a Multi-Attribute Method, peptide mapping can monitor many of these CQAs simultaneously.
Multi-Attribute Method (MAM)

Glycans monitored

Deamidation and Oxidation monitored by the MAM (Pep Map)

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Separation of Deamidated Peptides

VVSVLTVLHQDWL\text{ngk} or VVSVLTVLH\text{q}DWLN\text{gk}

m/z 904.9984, doubly charged peptide

- Determination of deamidated peptides is an important goal of peptide mapping. Chromatographic resolution is important. MS/MS confirms which peak corresponds to which deamidation site.
- AdvanceBio Peptide Plus enhances analysis because it has higher selectivity for deamidated forms of peptide over the normal form.
Choosing a Column Chemistry

For LC/MS work, **AdvanceBio Peptide Plus** with formic acid is the first choice.

Best peak shape with formic acid, high peak capacity, high mass load tolerance, excellent PTM resolution (especially deamidation), peak shape and recovery of hydrophobic peptides, unique selectivity

Exception 1: Sample is especially rich in small, hydrophilic peptides.

Poor retention of small, hydrophilic peptides is a feature common to charged surface columns. In this case, try AdvanceBio Peptide Mapping or Eclipse Plus C18 first.

Exception 2: Different selectivity is needed to separate a key pair.

<table>
<thead>
<tr>
<th>Charged surface chemistry</th>
<th>“Regular” silica chemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdvanceBio Peptide Plus</td>
<td>AdvanceBio Peptide Mapping</td>
</tr>
<tr>
<td></td>
<td>Eclipse Plus RRHD</td>
</tr>
<tr>
<td></td>
<td>ZORBAX 300</td>
</tr>
</tbody>
</table>

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Choosing a Column Chemistry

For maximum flexibility to transfer methods across instrument platforms, use AdvanceBio Peptide Plus with formic acid.
Method Development Tips & Reminders

- **Bonded Phase**: C18 is routinely used. Try AdvanceBio Peptide Plus first; for alternate selectivity or highly hydrophilic samples try AdvanceBio Peptide Mapping or a ZORBAX column.

- **Column dimension**: Good start with 2.1mm × 150mm, for higher resolution use longer column

- **Gradient**: Acetonitrile : Water with 0.1% formic acid, other organic solvent substitutions can be used for different selectivity
  Starting gradient time: 0-40% in 30min (1.3%/min)

- **Temperature**: Higher column temperature can dramatically improve both resolution and recovery (60 °C max)

- **Desalt** peptide mixture prior to injection

- **Sample handling** may cause artifacts (peptide modifications)
Troubleshooting – Reasons for < 100% Sequence Coverage

Question to ask: **Looking at the protein sequence, what’s missing?**

- Very small and/or very hydrophilic peptides?
  - If using AdvanceBio Peptide Plus, try AdvanceBio Peptide Mapping for better retention and/or resolution.

- Very large and/or very hydrophobic peptides?
  - Can you go to a higher percent organic mobile phase?
  - Are peptides sticking to sample tubes or otherwise lost during sample preparation? Or on the LC?
  - If using AdvanceBio Peptide Mapping, try AdvanceBio Peptide Plus
    - May even need to consider a C8 column rather than C18

- Very large, or very small peptides of any hydrophobicity?
  - Consider a different digestion enzyme
More Information

How-to Guide

Application Notes:

LC/MS Analysis of Peptide Mapping with Formic Acid Ion-Pairing Agent (5991-7574EN)

Enhancing the Quality of Peptide Mapping Separation for the Analysis of Post-Translational Modifications (5991-7875EN)
Standards & Reagents

10 Peptide Standard (p/n 5190-0583)

HSA peptides Standard (p/n G2455-85001)

Trypsin Digest Methylated BSA Standard (p/n G1990-85000)

Trypsin

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Summary

- High efficiency, high resolution separation of peptides using AdvanceBio Peptide Plus column
- MS-compatible AdvanceBio Peptide Plus column delivers high peak capacity with sharp and narrow peaks using MS-friendly formic acid modifier mobile phase conditions.
- AdvanceBio Peptide Plus and AdvanceBio Peptide Mapping are superficially porous columns that yield low back pressures & STM-like performance, allowing peptide mapping separations to be run on both HPLC and UHPLC systems
- Well-resolved PTM modified peptide peaks enabled reliable mAb peptide maps and development of Multi-Attribute Methods
- High quality separations in ~30 minutes with AdvanceBio Peptide Plus make peptide mapping more time-effective
- Multiple column options for every customer’s unique needs