

# Peptide Mapping Using LC/MS

A step-by-step workflow review

#### **Presenters**

Tim Rice – Applications Specialist, Biocolumns

Bob Giuffre – Applications Specialist, LC Instrumentation

Dawn Stickle – Applications Specialist, Mass Spectrometry



## **Getting Started**

- Choose your column carefully for best results
  - Usually, longer columns are better good to start with 150 mm
  - Longer columns enable higher resolution for more complex peptide maps
  - Use 2.1 mm id for mass spec sensitivity
  - Smaller pore sizes— 100Å 150Å are ideal for small protein fragments and peptides

## **Getting Started**

#### Instrument

- 1290 Infinity Binary LC System with autosampler offers significant advantages for peptide mapping
  - Low delay volume (120 μL)
  - Low dead volume
  - Excellent pump mixing performance
  - 1290 Infinity LC Quaternary pump offers even more automated mixing capabilities with BlendAssist
- Agilent 6530 Q-TOF Mass spec
  - State-of-the-art mass spectrometry system
  - BioConfirm software identifies proteins, PTMs, their locations, and protein sequences

### **Getting Started**

#### Mobile Phase

- Acetronitrile and water with a formic acid is ideal
  - Avoid TFA when using MS
- Use a low-pH acetonitrile buffer gradient
  - Facilitates the separation of a wide range of peptides
  - Helps to denature the peptide, improving retention and resolution
  - The formic acid increases retention of small poorly retained peptides

## Agilent Solvent Safety Caps

- Ensure that dangerous fumes don't leach into the lab environment
- Maintain consistency of solvents
- Easy to install and re-use

### Digesting the Protein

#### Reagents to have on hand:

- 100 mM ammonium bicarbonate (ABC)
- Trypsin stock
- 200 mM dithiothreitol (DTT)
- 200 mM iodoacetimide (IAM)

Perform digestion at optimal pH 7.5 – 8.5, 37 °C

#### Protein Digestion Step-by-Step

#### 1) Denature protein

- Start with 0.5 mg total protein in a 0.5 mL Eppendorf tube
- Add 25 μL ammonium bicarbonate (ABC) stock solution
- Add 25 µL tetrafluoroethylene (TFE) denaturation agent
- Add 1.0 μL DTT stock solution, and vortex to mix
- Heat 60 °C for 45 minutes to 1 hour then cool to room temperature

Request a copy of the Peptide Mapping "How to" Guide: visit www.agilent.com/chem/getbioguides

### Protein Digestion Step-by-Step

#### 2) Alkylate

- Add 4.0 μL IAM stock solution
- Vortex briefly
- Incubate sample in a foil-covered rack at room temperature for one hour

#### 3) Quench excess of IAM

- Add 1.0 µL DTT stock solution to destroy excess IAM
- Wrap in foil; stand for one hour at room temperature

#### Protein Digestion Step-by-Step

#### 4) Trypsin Digestion

- Prepare fresh stock of trypsin in 100 μL of 50 mM acetic acid
- Add trypsin stock solution at 1:20 to 1:50 by mass to enzyme: substrate
- Vortex briefly
- Place tube in heater and incubate at 37 °C for 4 18 hours
- Cool solution

#### 5) Lower digestion pH to halt trypsin activity

- Add 1.0 µL neat formic acid
- If desalting, use TFA to aid in peptide binding to the resin
- Vortex briefly
- Check pH and adjust as necessary



### Setting up the Analysis

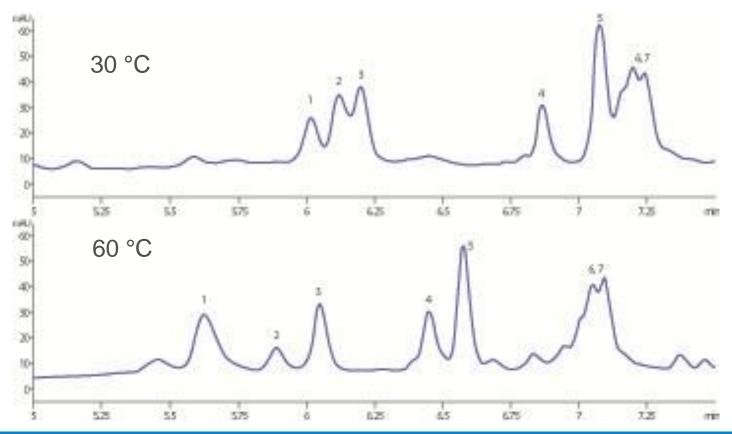
#### Typical starting conditions for peptide mapping:

- 40 °C is ideal starting temperature
- Linear gradient 3 65% of the B bottle
- UV setting: 215 nm and 280 nm
- Run time: 75 min. (starting time may be optimized later) when using AdvanceBio Peptide Mapping column

## Optimizing Peptide Mapping Methods

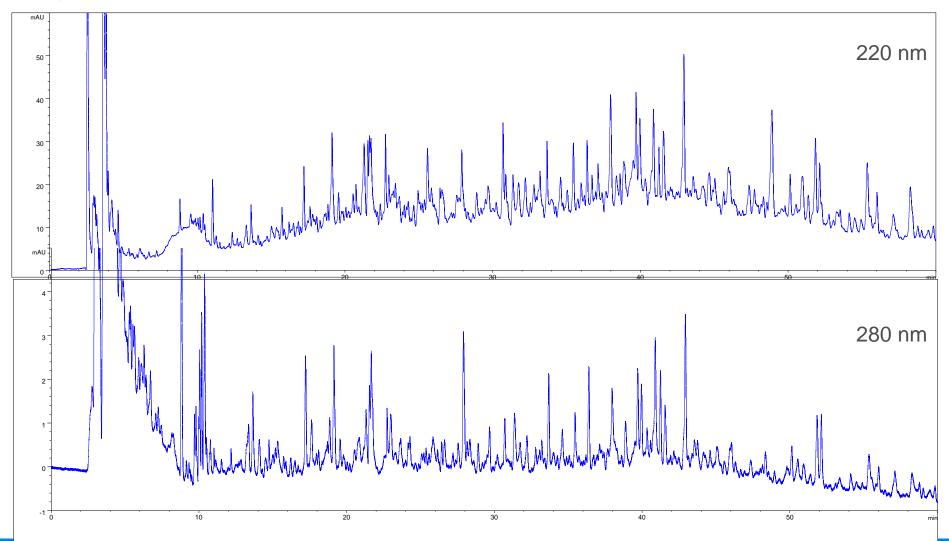
 Elevating temperature produces narrower peaks and may change selectivity

Myoglobin Tryptic Digest

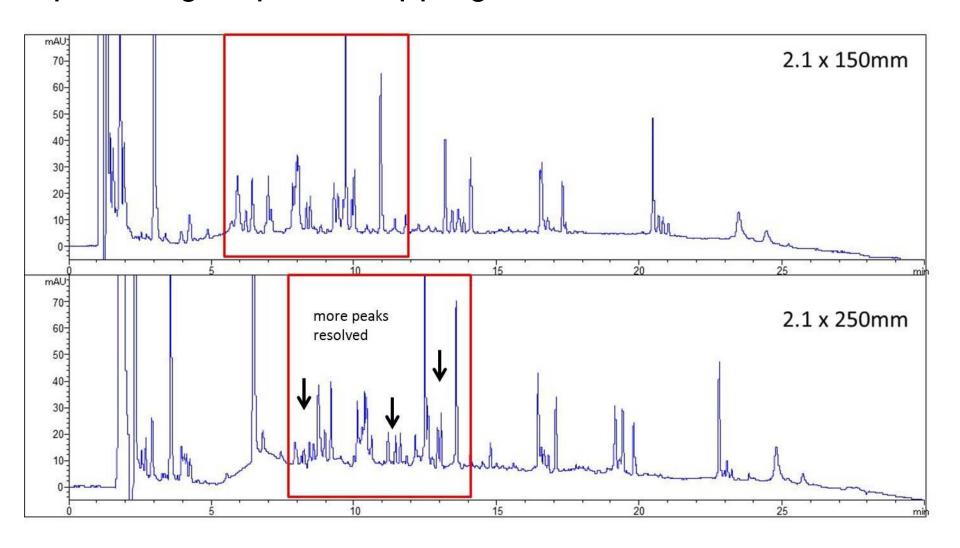


## Optimizing Peptide Mapping Methods

#### Adjustments to detection

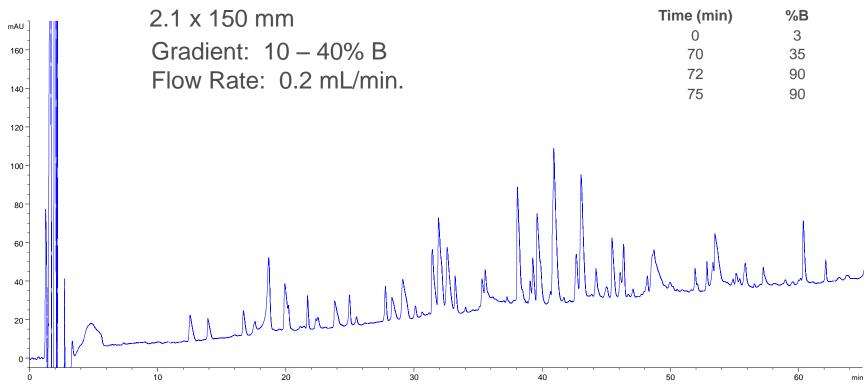


### Optimizing Peptide Mapping Methods



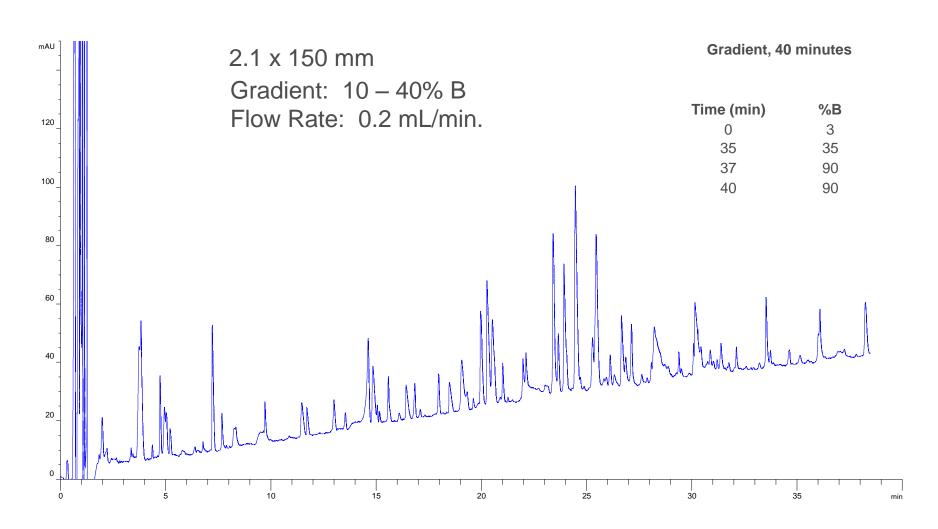
## Optimizing Peptide Mapping Methods: Speed

#### Starting Method: 75 minutes

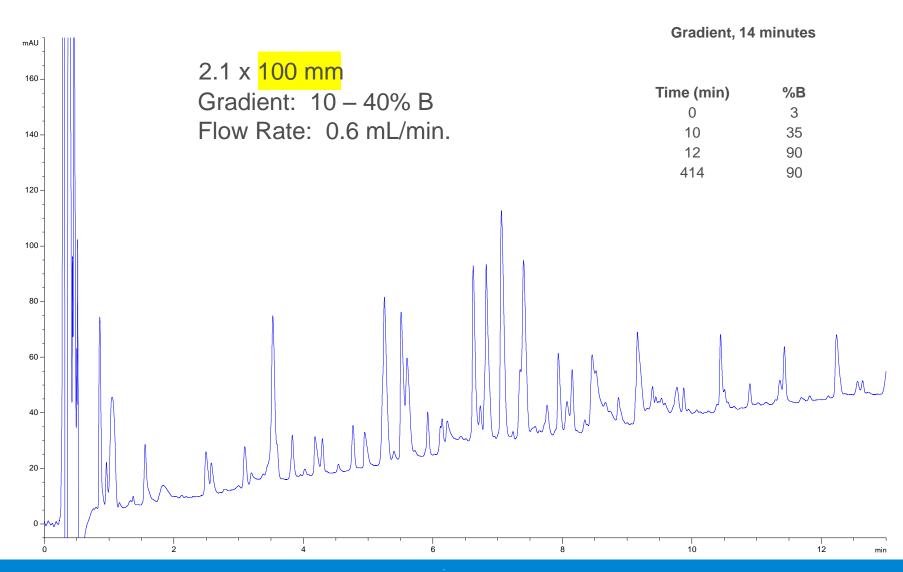


Gradient, 40 minutes

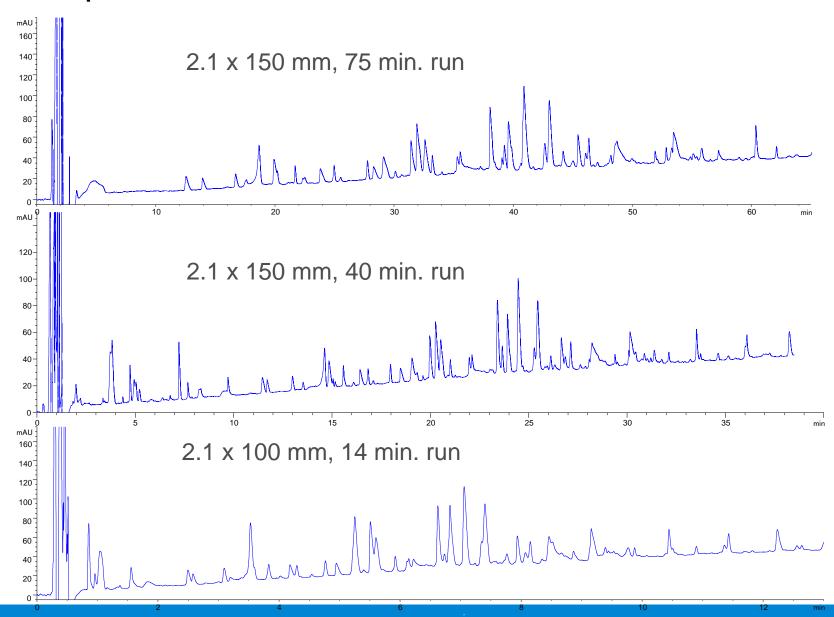
## Optmizing for speed: 40 minute run



#### Going even faster: 14 minute run



#### Comparison of three runs



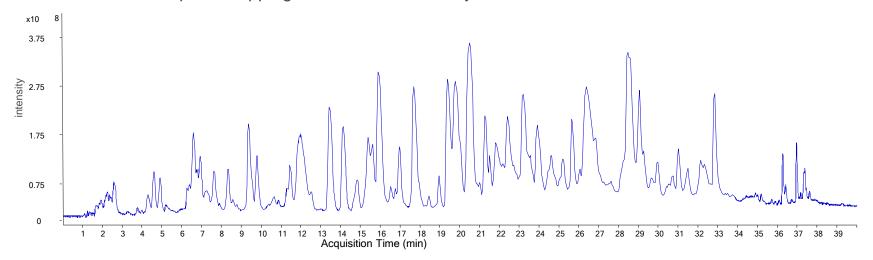
### Verifying Data Accuracy: LC/MS

LC/MS data significantly enhances the information content available from peptide mapping

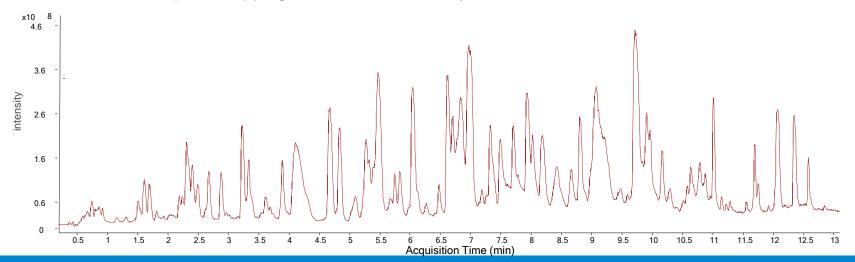
- High sequence coverage is an indicator that an optimal separation has been achieved.

## **Total Ion Chromatograms**

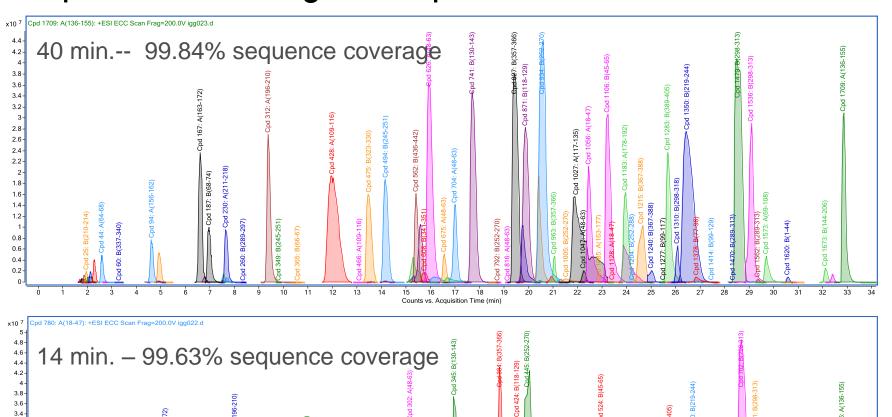
TIC: AdvanceBio Peptide Mapping column, 40 min. Analysis, 0.2mL/min, 140 Bar

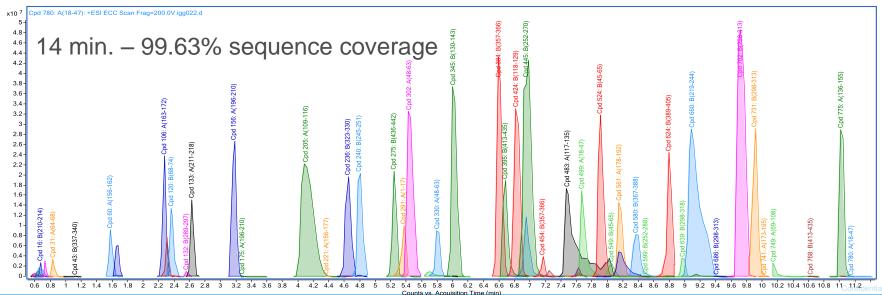


TIC: AdvanceBio Peptide Mapping column, 14 min. Analysis, 0.6mL/min, 433 Bar

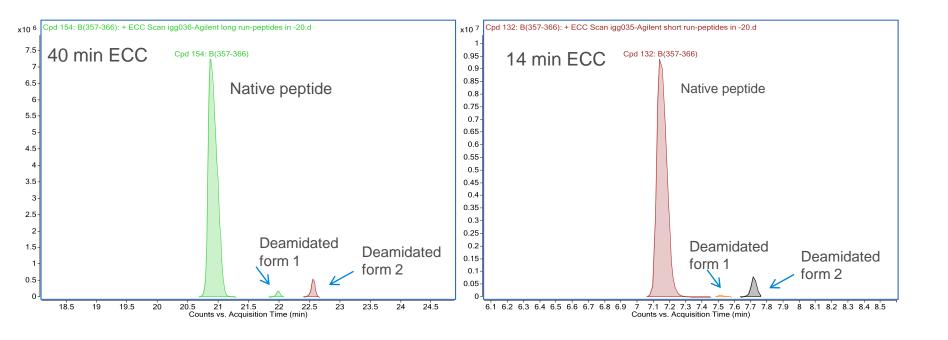


## Sequence Coverage Comparison





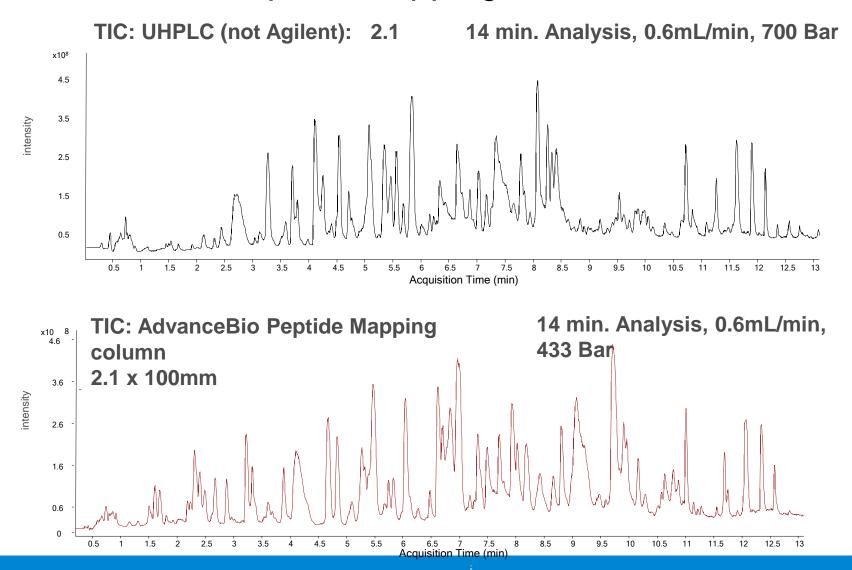
## Rapid PTM Profiling: Deamidation



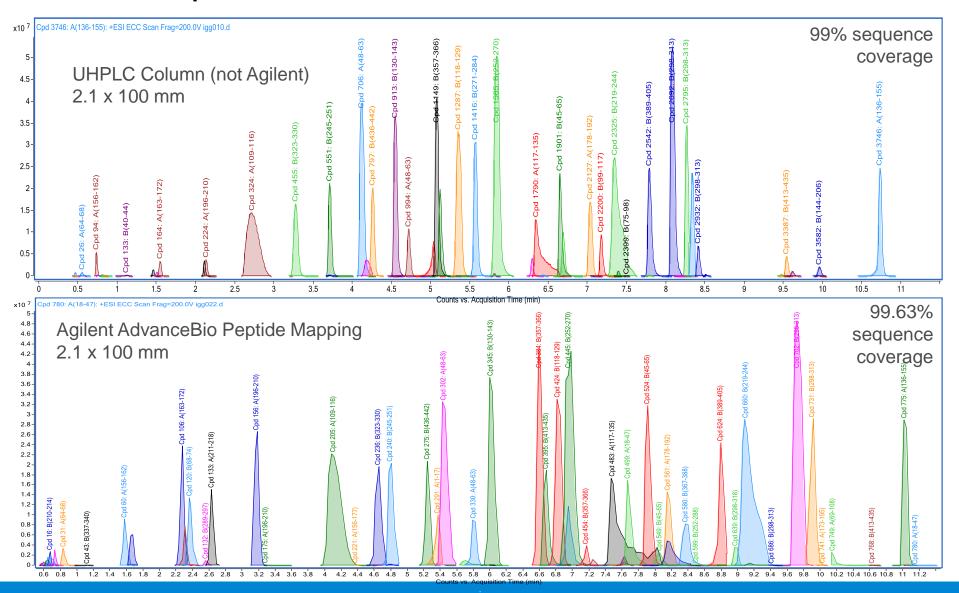
### AdvanceBio Peptide Mapping Columns

Performance (efficiency, resolution) similar to sub-2 µm columns, without the high backpressure

# Comparison of sub-2 µm UHPLC column to AdvanceBio Peptide Mapping columns



#### **ECC** Comparison



## In closing

- Agilent has resources to help you get great peptide mapping results
- Look for biopharma guides, including the Peptide Mapping "How to" guide, at www.agilent.com/chem/getbioguides
- Check out our LC/MS publication warehouse "Inside LCMS" at <u>www.agilent.com/chem/insidelcms</u>

 Look for the Agilent peptide mapping standard to help you ensure optimal system performance – p/n 5190-0583

Thank you!

10 Peptide Standard Lyophilized