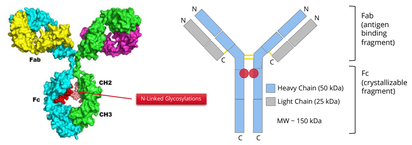
Peptide Mapping for mAb Characterization

Introduction to mAB Peptide Mapping

LC-MS/MS Conditions

Immune system IgG-like protein that recognizes specific targets (antigens). Contains ~ 1320 amino acids in 4 polypeptide chains:

- Two identical heavy chains (long chains)
- Two identical light chains (short chains)



Critical Quality Attributes

Critical Quality Attribute: "physical, chemical, biological or microbiological property or characteristics that should be within an appropriate limit, range, or distribution to ensure the desired product quality." ICH guidance Q8(R2)

Attribute Category	What can be measured?
Product- specific variants	• MW • Sequence variants • Charge distribution • PTMs (oxidation, deamidation/isomerization, N/C-terminal processing, glycosylations) • Aggregation (higher order structure variants)
Process-related impurities	• Host cell proteins • DNA • Raw materials (cell culture media, buffers, protein A) • Leachables from product contact materials
Obligatory (drug product attributes)	• pH • Excipients • Concentration • Osmolality • Adventitious agents (viruses, mycoplasma, endotoxins). • Appearance

Risk Assessment of pCQAs (example)

Biologicals 44 (2016) 291e305.
http://dx.doi.org/10.1016/j.biologicals.2016.06.005

Sample Prep Steps

Protein Digestion Steps

- **Protein Denaturation** - Proteins can be denatured using high temperatures or by addition of chaotropic agents such as urea, guanidinium hydrochloride, and acetonitrile.
- **Reduction of disulfide bonds** - Dithiothreitol (DTT) reduces the disulfide bonds between cysteine residues, allowing the protein to become fully unfolded.
- **Alkylation** - An alkylating agent such as iodoacetic acid (IAA) is added to alkylate all of the cysteine residues, preventing the formation of disulfide bonds.
- **Dilution** - Salts and other reagents that may denature the enzyme must be removed or diluted to ensure successful digestion.
- **Digestion** – Enzyme is added to the protein solution.

Denaturants

Denaturation	Limitations
8 M Urea	• Heat breaks urea down to cyanate which can carbamylate lysine and arginine residues • Needs dilution to <2M urea for trypsin activity
6M GnHCl	• Lower trypsin digestion efficiency, even at 0.9 M concentration. • Needs extensive dilution or buffer exchange
SDS	• Not MS compatible (ion suppressing) • Must be removed prior to MS
MS-friendly surfactants	• Acid-labile surfactants (PPS Silent® Surfactant, Rapigest™, ProteaseMax) • Surfactants yielding insoluble precipitates under low pH (e.g., DOC) • Invitrosol™ does not interfere with your chromatography separations and does not contaminate LC/MS instrumentation

Removal of Denaturant

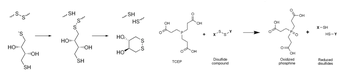
Cleanup type	Strengths	Weaknesses
Dialysis	• Minimal sample loss	• Long clean up times
Desalting column	• Rapid desalting/Cleanup	• Final sample concentration lower
Molecular weight cut-off filters (MWCO) FASP In-Stage Tip SP3	• Integrated protein digestion, denaturant and detergent removal. • Can be easily automated	• Susceptible to sample loss • Final sample concentration higher

SP3 - Single-Pot Solid-Phase-enhanced Sample Preparation
FASP - Filter-aided sample preparation

Reducing reagents

Dithiothreitol (DTT)
- Needs pH values above 7
- Can promote Met oxidation (metal-catalyzed reaction)

Tris(2-carboxyethyl)phosphine (TCEP)
- Odorless and more resistant to oxidation
- Can reduce natively oxidized Met

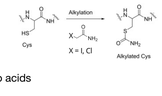


Beta-mercaptoethanol (BME)

Alkylating Reagents

Iodoacetic acid (IAA):
- Slower reacting
- Carboxymethyl (+58 Da) is added to reduced cysteines

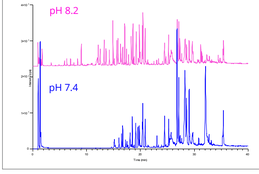
Iodoacetamide (IAM):
- Most common (fastest reacting),
- Carbamidomethyl (+57 Da) added to reduced cysteines
- Quenching with DTT can help with non-specific alkylation of other amino acids



Chloroacetamide:
- Less reactive and more stable in solution than iodoacetamide
- Less non-specific alkylation of other amino acids and side reactions
- Light sensitive reagents

Trypsin

- Serine protease considered the gold standard for protein digestion.
- Well-defined specificity for C-terminal K and R.
- Potential missed cleavages when K and R are followed at N-term by D/Q and C-term by P.
- Optimal pH 8–9. Generally buffered with Tris or ammonium bicarbonate.
- Tolerates: 0.1 % SDS, 1 M urea, and 10 % acetonitrile.



N – ala – ser – pro – lys + gly –gly – arg + trp – asp – glu – lys + cys - **C**

N – asp – lys – glu – lys – glu –arg – trp – gly – arg – pro – cys - **C**

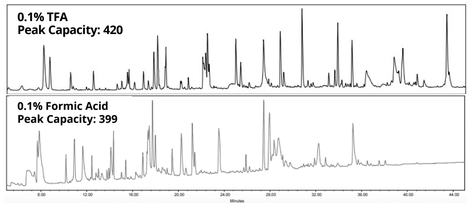
Column: Biozen 2.6 µm XB-C18
Dimensions: 150 x 2.1 mm
Part No.: 00F-4768-AN
Mobile Phase: A: 0.1 % Formic Acid in H2O
B: 0.1 % Formic Acid in ACN
Gradient: 1-50 % B in 50 minutes
Flow Rate: 0.3 mL/min
Temperature: 40 °C
Detection: Q-TOF
Sample: Trastuzumab

Selectivity Drivers

Mobile Phase and Acidic Modifiers

Commonly used mobile phase contains:
- Water
- Acetonitrile, MeOH, IPA ➡ IPA: Better recovery of hydrophobic peptides
- Acidic modifier: Formic acid (FA)
  Trifluoroacetic acid (TFA) ➡ ✓ Better peptide unfolding ✓ Less free-silanol groups ✓ Better retention with TFA (IP)
  Acetic acid

Effect of TFA and Formic Acid

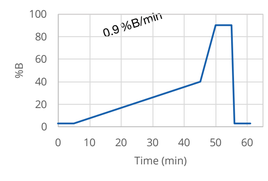


Column: Biozen 1.6 µm PS-C18
Dimensions: 150 x 2.1 mm
Mobile Phase: A: 0.1 % Acidic Modifier in H20
B: 0.1 % Acidic Modifier in ACN
Gradient: 1-50 % B in 50 minutes
Flow Rate: 0.3 mL/min
Temperature: 40 °C
Detection: UV @ 214 nm
Sample: Tryptic Digest, NIST mAb

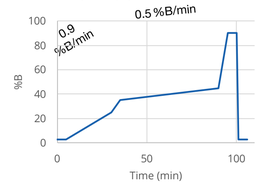
Comprehensive Evaluation of Reversed-Phase Columns for Peptide Mapping
Gain insights from a detailed comparison of 13 reversed-phase columns tested with complex peptide mixtures.
The study assesses performance across acidic, basic, and long-chain peptides, revealing how column chemistry and hardware influence mapping quality.
Findings support data-driven column selection for improved resolution, efficiency, and reproducibility in biopharma applications.
- Review the study to strengthen your peptide mapping strategy.

Type of Gradients

Shallow gradients:
- Slope 0.5 – 1 %B/min
- Provide the best separation.
- Could cause a switch in peak order.



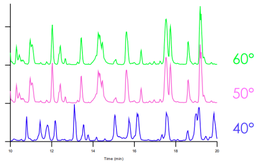
Step gradients:
- Quickly skip time windows where peptides don't elute and increase overall peak capacity.
- Focus on specific peptides



Temperature

- ✓ Increase in efficiency with temperature. More significant for fully porous particles e.g. bioZen peptide PS-C18
- ✓ Higher temperatures improve peak shape of peptides from proline-rich proteins (cis-trans isomerization rate)
- ✗ On-column thermal peptide cleavage in presence of 0.1 % formic acid (Asp-X and X-Asp)
- ✗ On-column thermal promotion of artificial modifications: oxidation of Met, Pyro-Glu (N-term Q/E), deamidation of Asn to Asp, and isomerization of Asp.

Column: Biozen 1.6 µm Peptide PS-C18
Dimensions: 150 x 2.1 mm
Mobile Phase: A: 0.1 % Formic Acid in Water
B: 0.1 % Formic Acid in Acetonitrile
Gradient: 1-50 % B in 50 minutes
Flow Rate: 0.3 mL/min
Temperature: 40 °C
Detection: Q-TOF
Sample: Trastuzumab Digest, 1 µg



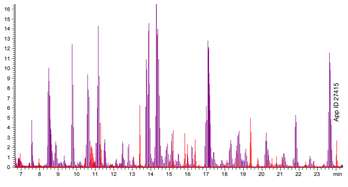
Temp (°C)	Peak Capacity
60	457
50	513
40	343

Effect of Column Dimensions

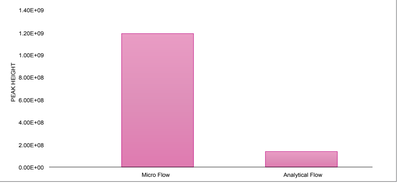
What can we modify?
- Column Length
- Column ID

Column Length effect:
Efficiency is proportional to column length.
- Longer columns provide higher resolution, but longer run times
- Very complex samples could warrant the use of a longer column (250 mm)
- In general, ID of 2.1 mm are the common choice for LC-MS/MS
- For tryptic peptides pore sizes of ~100 Å is ideal. Larger pore sizes can be considered for larger peptides (middle-out/down approach).

Overlap of TIC Traces for Analytical Flow (Red) and Micro Flow (Purple) of AAV9 Viral Proteins Tryptic Digest.

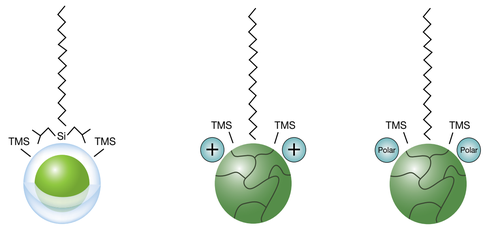


Increase in Ion Intensity by Using Micro Flow.



Selection of Stationary Phases

Biozen™ by Phenomenex

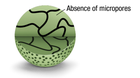


	Biozen Peptide XB-C18	Biozen Peptide PS-C18	Biozen Peptide Polar
Recommended Use	Overall retention of both acidic and basic peptides for both peptide mapping and peptide quantitation applications	Excellent retention of peptides for both peptide mapping and peptide quantitation applications	Enhanced selectivity and retention for polar peptides without diminishing non-polar retention mechanism.
Particle Size (µm)	1.7, 2.6, 5	1.6, 3	1.6, 3
Pore Size (Å)	100	100	100
Surface Area (m^2/g)	200	260	260
Carbon Load	10	9	9
pH Stability	1.5-9	1.5-8.5	1.5-8.5

Core-Shell Technology

Using sol-gel nano-structuring, a durable and uniform porous shell is formed over a solid silica core. Combined with advanced packing technology, this process delivers highly reproducible columns with excellent efficiency and sensitivity

Thermally Modified Fully Porous

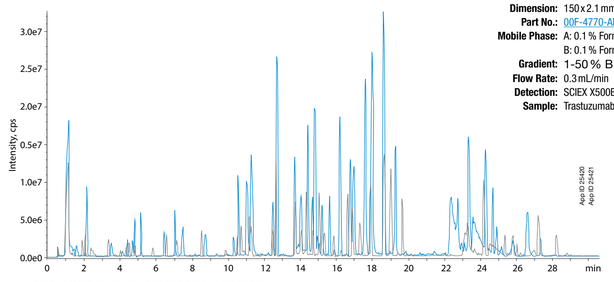


Through a proprietary series of thermal processing steps, we eliminate micropores and further improve consistency, leading to higher column efficiency and reproducibility.

Biozen provides improved sequence coverage and sensitivty compared to equivalent alternatives

Biozen 1.6 µm Peptide PS-C18, 137 Unique Peptides
Waters® ACQUITY® 1.7 µm Peptide CSH, 103 Unique Peptides

Method Conditions:
Column: Biozen 1.6 µm Peptide PS-C18
Waters ACQUITY 1.7 µm Peptide CSH
Dimension: 150 x 2.1 mm
Part No.: 00F-4770-AN (PS-C18)
Mobile Phase: A: 0.1 % Formic Acid in Water
B: 0.1 % Formic Acid in Acetonitrile
Gradient: 1-50 % B in 50 minutes
Flow Rate: 0.3 mL/min
Detection: SCIEX X500B Q-TOF
Sample: Trastuzumab Digest, 1 µg



Comparative separations may not be representative of all applications.

Comprehensive Evaluation of Reversed-Phase Columns for Peptide Mapping

- Compare 13 reversed-phase columns with complex peptide mixtures.
- See performance across acidic, basic, and long-chain peptides.
- Discover how column chemistry and hardware shape mapping quality.
- Choose columns confidently for better resolution, efficiency, and reproducibility in biopharma

Review the study to strengthen your peptide mapping strategy.

GET THE INSIGHTS

Have questions or want more details? We would love to help!
*Visit **www.phenomenex.com/Chat** to get in touch with one of our Technical Specialists*

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