KEYS FOR ENABLING OPTIMUM PEPTIDE CHARACTERIZATIONS:

A Peptide Mapping "How to" Guide

The Measure of Confidence

Agilent Technologies

INTRODUCTION

Peptide mapping – an invaluable tool for biopharmaceuticals – is a very powerful method and the most widely used identity test for proteins, particularly those produced by recombinant means. It most commonly involves enzymatic digestion (usually using trypsin) of a protein to produce peptide fragments, followed by separation and identification of the fragments in a reproducible manner, allowing the detection and monitoring of single amino acid changes, oxidation, deamidation, and other degradation products. It also enables the direct detection of common monoclonal antibody variants such as N-terminal cyclization, C-terminal lysine processing, and N-glycosylation, as well as other post-translational modifications.

A peptide map is a fingerprint of a protein and the end product of several processes that provide a comprehensive understanding of the protein being analyzed. It involves four major steps: isolation and purification of the protein; selective cleavage of the peptide bonds; chromatographic separation of the peptides; and validated analysis of the peptides.

Peptide mapping is considered a comparative procedure that confirms the primary structure of the protein and detects alterations in structure. Additionally, it demonstrates process consistency and genetic stability. A peptide map should include positive identification of the protein, maximize coverage of the complete peptide sequence, and provide additional information and sequence identification beyond that obtained at the non-digested protein level.

The selection of a chromatographic technique to separate peptides and generate peptide maps depends on the protein, experimental objectives, and anticipated outcome. However, the excellent resolving power of reversed-phase chromatography (RPC) makes this technique the predominant HPLC technique for peptide mapping separations. It is also ideal for both analytical and preparative separations because of the availability of volatile mobile phase eluents. It is important to note that the preferred columns for peptide mapping separations are similar to those used for small molecules, but because most peptide mapping separations are performed at low pH and elevated temperature, columns with excellent pH stability and minimal silanol effects are routinely used.

Careful inspection of the complete characterization strategy is required to generate successful peptide maps. A profile may consist of over 100 peaks representing individual peptides and their derivatives, so it requires knowledge of sample preparation methods, powerful separation techniques and validated protocols. Having the skill and information to develop a successful peptide map will help you achieve the best possible separation of your proteolytic digests and deliver a successful and reliable peptide characterization outcome.

The objective of this peptide mapping "how-to" guide is to highlight the areas which are important to generating peptide maps by reversed-phase chromatography, share some of the fundamental techniques used for peptide mapping procedures and emphasize considerations for optimizing your peptide mapping separations to achieve the best possible results.
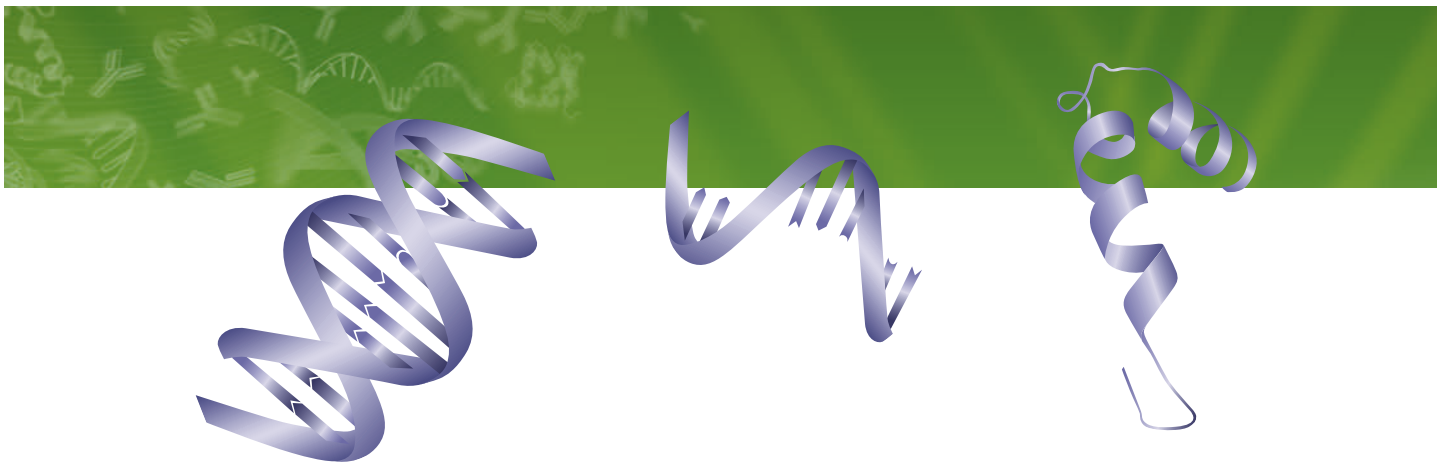
Protein Digestion:
Preparing Your Protein to Enhance
the Peptide Mapping Separation

A good understanding of the steps for digesting a protein prior to analysis will help to ensure a complete, successful digestion and provide a high degree of confidence in your chosen strategy. Often the digestion method requires its own set of development protocols to provide an adequate and stable sample for LC injection. Although there are many options to consider for optimizing the digestion, a number of common approaches should be followed. The five steps used for protein digestion, summarized in Table 1, are (1) sample preparation (2) selection of cleavage agents (3) alkylation/reduction (4) digestion process (5) enrichment/cleanup.

Table 1

Five steps for protein digestion		
Procedure	**Intended Effect**	**General Experiment**
1. Sample Preparation	Preparing sample for digestion	Depletion, enrichment, dialysis, desalting
2. Selection of Cleavage Agent	Specific cleavage requirement	None
3. Reduction and Alkylation	Reduction reduces disulphide bonds Alkylation caps SH groups	Reduction: DTT, 45 min, 60 °C Alkylation: IAM, 1 hr, in the dark
4. Digestion Process	Cleavage of proteins	Digestion: pH 8, 37 °C, overnight Quenching: TFA addition
5. Enrichment/Cleanup	Preparing sample for LC or LC/MS analysis	C18 tips, concentrating, dialysis, affinity columns

Learn more about Agilent columns for peptide mapping at **agilent.com/chem/advancebio**

Step 1: **Sample Preparation**

Depending on the size or the configuration of the protein, there are different approaches for pretreatment of your sample. Under certain conditions, it might be necessary to enrich the sample or to separate the protein from added substances and stabilizers used in formulation of the product, especially if these interfere with the mapping procedure. There are many methods for performing these procedures and each protein has its own set of cleanup measures or processes. However, some of the more common approaches used for sample cleanup prior to digestion include depletion/enrichment dialysis and desalting by gel filtration.

Depletion and enrichment strategies have been developed to remove high abundance proteins or isolate target proteins in the sample, respectively. Depletion is more often used in proteomics applications to reduce the complexity of biological samples such as serum, which contain high concentrations of albumin and immunoglobulins. The Agilent Multiple Affinity Removal System (MARS) HPLC columns and spin cartridges enable the identification and characterization of high-value, low abundance proteins and biomarkers found in serum, plasma, and other biological fluids. Through depletion of the 14 high-abundance proteins with MARS, ~94% of the total protein mass is removed. The depletion process is robust, easily automated, and highly efficient.

Depletion strategies utilize immunoaffinity techniques (e.g., immunoprecipitation, co-immunoprecipitation and immunoaffinity chromatography). Alternatively, enrichment techniques isolate subclasses of cellular proteins based on unique biochemical activity, post-translational modifications (PTMs) or spatial localization within a cell. Post-translational modifications – such as phosphorylation and glycosylation – can be enriched using affinity ligands such as ion-metal affinity chromatography (IMAC) or immobilized lectins, respectively. To introduce unique protein chemistries, other techniques entail metabolic or enzymatic incorporation of modified amino acids or PTMs.

Whether simple or complex, samples often need dialysis or desalting to ensure they are compatible and optimized for digestion. For example, because mass spectrometry (MS) measures charged ions, salts – especially sodium and phosphate salts – should be removed prior to MS to minimize their interference with detection. Dialysis and desalting products allow buffer exchange, desalting, or small molecule removal to prevent interference with downstream processes.

Dialysis is an established procedure for reducing the salt concentration in samples. It requires filling a dialysis bag (membrane casing of defined porosity), tying the bag off, and placing the bag in a bath of water or buffer where the concentration of salt will equilibrate through diffusion. Large molecules that can't diffuse through the bag remain in the bag. If the bath is water, the concentration of the small molecules in the bag will decrease slowly until the concentration inside and outside is the same. Once equilibration is complete, the bag is ruptured and the solution poured off into a collection vessel. Dialysis can be used for volumes up to a few liters, but it is not practical for large sample volumes because it can take several days for complete salt removal.

To desalt samples prior to digestion, Gel Filtration (GF) is the most practical laboratory procedure. This method is a non-adsorptive chromatography technique that separates molecules on the basis of molecular size. Desalting is used to completely remove or lower the concentration of salt or other low molecular weight components in the sample, while buffer exchange replaces the sample buffer with a new buffer.

MARS is available in a variety of LC column dimensions and in spin cartridge formats. Proteins depleted include Albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, alpha2-macroglobulin, alpha1-acid glycoprotein, IgM, apolipoprotein AI, apolipoprotein AII, complement C3, and transthyretin.

Agilent Bio SEC columns can effectively classify (by size) and desalt protein mixtures prior to peptide mapping applications.

Gel filtration is one of the easiest chromatography methods to perform because samples are processed using an isocratic elution. In its analytical form, gel filtration (also known as size exclusion chromatography) can distinguish between molecules (e.g. proteins) with a molecular weight difference of less than a factor of 2 times. In these applications, the size difference between the substances being separated is very large (i.e. proteins vs. salts). A gel filtration media is chosen that completely excludes the larger molecules while allowing the smaller molecules to freely diffuse into all of the pore spaces. The column is equilibrated with a buffer, which may be the same or different from that of the sample. Following application of the sample to the column, more of the column buffer (eluting buffer) is added to carry the sample molecules down the column. The larger molecules – which can't enter the pores of the media – elute first from the column, followed by the smaller molecules that diffuse into the pores, slowing them down relative to the larger molecules. If the eluting buffer is different from the sample that was applied, the larger molecules will be displaced from the original salts and elute in this new buffer, completely separated from the original sample buffer.

Captiva Low Protein Binding Filters

Regardless of what sample prep you are performing, it's a good idea to filter your sample with a low protein binding filter.

Agilent PES filters provide superior and consistent low protein binding for protein-related filtration. The PES filter membranes are a better option than PVDF membranes for most LC analyses. Agilent PES has similar compatibility to PVDF filters for common LC solvents and is superior in terms of protein binding and cleanliness. Learn more at **agilent.com/chem/filtration**

Captiva PES Filters

Diameter (mm)	Pore Size (µm)	Certification	Housing	Part Number
15	0.2	LC/MS	Polypropylene	5190-5096
4	0.45	LC	Polypropylene	5190-5095
4	0.2	LC/MS	Polypropylene	5190-5094
15	0.45	LC	Polypropylene	5190-5097
25	0.2	LC/MS	Polypropylene	5190-5098
25	0.45	LC	Polypropylene	5190-5099

Learn more about Agilent columns for peptide mapping at **agilent.com/chem/advancebio**

Step 2: **Selection of cleavage agents**

There are two methods employed for the cleavage of peptide bonds, chemical and enzymatic. Chemical cleavage involves the use of nucleophilic non-enzymatic reagents such as cyanogen bromide (CNBr) to chemically cleave the peptide bond at a specific region while proteolytic enzymes, such as trypsin, have been proven highly useful for a variety of site specific cleavage locations. The cleavage method and agent will depend on the protein under test and the specific outcome expectations of the analysis. Additionally, the selection process involves careful examination of the entire peptide mapping process and considerations for related characterizations. The most common cleavage agent used for peptide mapping is trypsin due to its well defined specificity. Trypsin hydrolyzes only the peptide bonds in which the carbonyl group is followed either by an arginine (Arg) or lysine (Lys). Several common cleavage agents and their specificity are shown in **Table 2**.

Table 2

Cleavage Type		
Cleavage Type	**Cleavage Agent**	**Specificity**
Enzymatic	Trypsin	C-terminal side of Arg & Lys
	Pepsin	Non-specific
	Chymotrypsin	C-terminal side of hydrophobic residues
	Glutamyl endopeptidase	C-terminal side of Glu and Asp
Chemical	Cyanogen bromide	C-terminal side of Met
	Dilute acid	Asp and Pro
	BNPS-skatole	Trp

Step 3: **Denaturation, reduction, and alkylation**

For the proteolytic enzyme to efficiently cleave the peptide chains, most samples need to be denatured, reduced, and alkylated, using various reagents. Denaturation and reduction can often be carried out simultaneously by a combination of heat and a reagent, like 1,4-dithiothreitol (DTT), mercaptoethanol, or tris(2-carboxyethyl)phosphine. Most used is DTT, which is a strong reducing agent that reduces the disulfide bonds and prevents inter- and intra-molecular disulfide formation between cysteines in the protein. By combining denaturation and reduction, renaturation – a problem when using heat solely as the denaturation agent – due to reduction of the disulfide bonds can be avoided. Following protein denaturation and reduction, alkylation of cysteine is necessary to further reduce the potential renaturation. The most commonly used agents for alkylation of protein samples prior to digestion are iodoacetamide (IAM) and iodoacetic acid (IAA).

Figure 1 provides a good example of a reversed-phase chromatographic separation method used to evaluate the reduction and alkylation completeness of a monoclonal antibody prior to digestion.

Figure 1 – **Reduction/alkylation profile by reversed-phase chromatography**
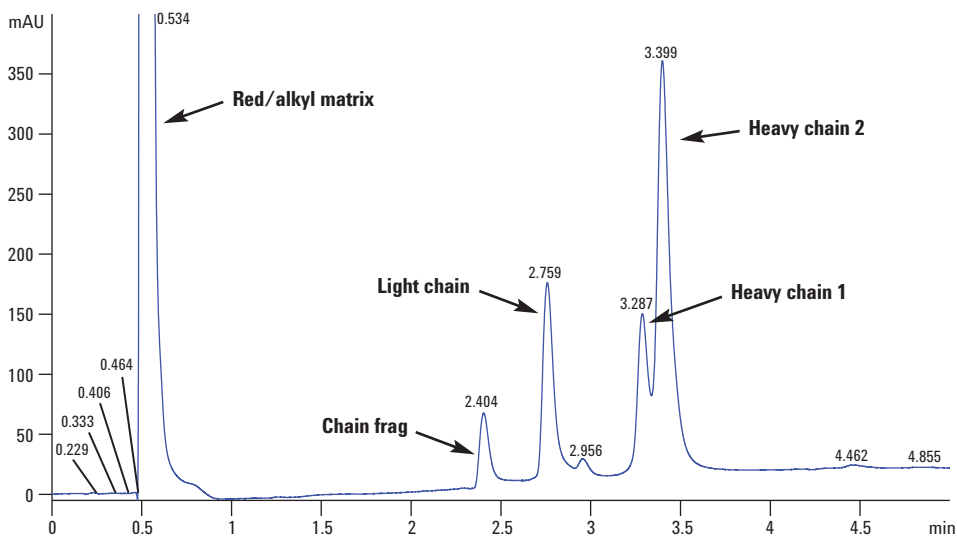

Figure 1 – Reversed-phase separation of a reduced and alkylated monoclonal antibody prior to digestion protocol using an Agilent Rapid Resolution High Definition (RRHD) 300SB-C8, 2.1 x 50 mm column (Agilent p/n 857750-906). The separation was performed at 0.5 mL/min, 75 °C using water (0.1% TFA)/ACN (0.08%) multi-segmented conditions on an Agilent 1290 Infinity LC.

Step 4: **Digestion**

As already mentioned, trypsin is the most commonly used protease for digestion due to its well defined specificity. Since trypsin is a protein, it may digest itself in a process called autolysis. However, Ca++, naturally present in most samples, binds at the Ca++ binding loop in trypsin and prevents autolysis. With the modified trypsin presently used in most laboratories, autolysis is additionally reduced and not typically a large concern.

Tryptic digestion is performed at an optimal pH in the range 7.5-8.5, and commonly at 37 °C. To provide an optimal pH for the enzymatic cleavage, a buffer is added (usually 50 mM triethyl ammonium bicarbonate (tABC) or 12.5 mM ammonium bicarbonate (ABC) prior to the addition of trypsin. A 2-amino-2-hydroxymethyl propane-1,3-diol (Tris) buffer may also be used for this purpose, but it should be taken into consideration that the Tris buffer is incompatible with MS analysis, such as MALDI and ESI-MS, and needs to be depleted through solid phase extraction (SPE) or ZipTips. To ensure a sufficient – but not too high – amount of enzyme to perform the digestion, it is crucial to have the right enzyme-to-protein ratio.

Proteins may act differently in different environments and when model proteins were digested in a mixture vs. separately, less effective digestions have been observed. One reason could be increased competition for the trypsin cleavage sites, when more proteins are digested together. Additionally, there can be many factors and conditional parameters that could affect the completeness and effectiveness of digestion of proteins, causing a variety of anticipated outcomes. If these factors are more carefully understood or controlled, the digestion results can be greatly improved. The pH of the reaction, digestion time and temperature and the amount of cleavage agent used are all critical to the effectiveness of the digestion.

- **Digestion pH**. In general, the pH of the digestion mixture is empirically determined to ensure the optimization of the performance of a given cleavage agent. For example, when using cyanogen bromide as a cleavage agent, a highly acidic environment (e.g. pH 2, formic acid) is necessary; however, when using trypsin as a cleavage agent, a slightly alkaline environment (pH 8) is optimal. As a general rule, the pH of the reaction milieu must not alter the chemical integrity of the protein during the digestion or the course of the fragmentation reaction.

- **Digestion Time & Temperature**. Time and temperature play an important role for optimum digestion. To minimize chemical reactions, a temperature between 25 °C and 37 °C is adequate – and recommended – for most protein digestions (e.g., trypsin digestions are commonly run at 37 °C). However, the type and size of protein will ultimately determine the temperature of the reaction due to protein denaturation as the temperature of the reaction increases. Reaction time is also a factor for consideration in optimizing the digestion protocol. If sufficient sample is available, an experimental study should be considered in order to determine the optimum time to obtain a reproducible map while avoiding incomplete digestion. Time of digestion varies from 2 h to 30 h depending on sample size and type, while the reaction is stopped by the addition of an acid, which does not interfere in the map or by freezing.

- **Concentration of cleaving enzyme**. The concentration of the cleaving agent should be minimized to avoid its contribution to the map patterns. An excessive amount of cleavage agent is commonly used to accomplish a reasonably rapid digestion time (i.e. 6 to 20 hours); however, careful consideration should be given to these increased amounts. A protein-to-protease ratio between 10:1 and 200:1 is generally used and it is recommended that the cleavage agent be added in two or more stages to optimize cleavage. In many standard trypsin digestion procedures, the trypsin is added in this manner. Nonetheless, the final reaction volume remains small enough to facilitate separation – the next step in peptide mapping. To sort out digestion artifacts that might interfere with the subsequent analysis, a blank determination is performed using a digestion control with all the reagents, except the test protein.

The Trypsin digestion method described below and summarized in **Figure 2 and 3** is a common procedure routinely used for the reduction, alkylation, in-solution digestion, and cleanup of protein (0.5 mg). This procedure is scalable for smaller amounts of proteins and additionally provides a useful list of Agilent reagents and part numbers.

Figure 2 – **Trypsin digestion procedure (Parts I-V)**

0.5 mg protein
25 µL ammonium bicarbonate stock
25 µL TFE
1.0 µL DTT stock

Add 4.0 µL IAM stock

Add 1.0 µL DTT stock

1. Re-suspend, denature, and reduce protein.

  (Vortex; heat 1 hr at 60 °C or 20 min at 90 °C)

2. Alkylate.

  Perform in the dark.
  (1 hr at room temp.)

3. Quench excess IAM.

  Perform in the dark.
  (1 hr at room temp.)

Add
300 µL water +
100 µL ammonium bicarbonate stock

Add trypsin stock

Add 1 µL formic acid or TFA

4. Dilute and adjust pH.
  (pH 7-9)

5. Digest.
  (37 °C for 4-18 hours)

6. Reduce pH to <4.

Figure 3 – **Expected timeline for digestion procedure**

Allow 3.5 continuous hours for these steps

Resuspend, denature, reduce
60 min

Alkylate
60 min

Quench
60 min

Dilute
Adjust pH
10 min
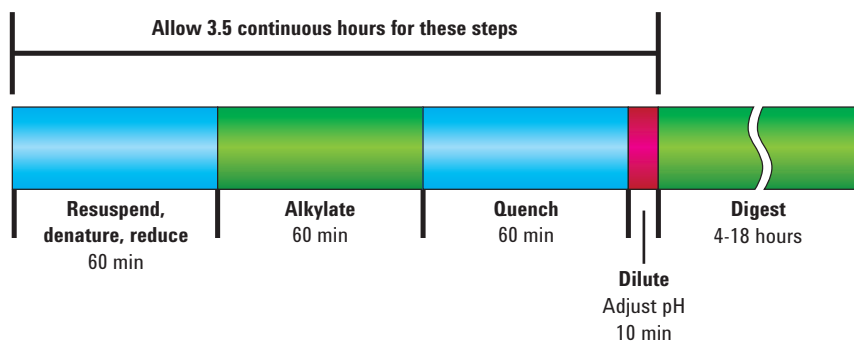
Digest
4-18 hours

Reduction, alkylation, digestion solution preparation: Summary

100 mM ammonium bicarbonate: Add 100 mL water to 0.7906 g ammonium bicarbonate. Store in refrigerator at 4 °C for up to 2 months.

Trypsin stock: Modified trypsin can be purchased: Agilent Proteomics Grade Trypsin (P/N 204310), see next page "Reagents and equipment"). It is lyophilized and may be stored in this form at -20 °C for more than one year without significant loss in activity. When required, prepare trypsin stock solution by hydrating the lyophilized trypsin in 100 μL of 50 mM acetic acid, to a final concentration of 1 μg/mL. To minimize freeze-thaw cycles and to increase storage stability, divide the hydrated trypsin into ten separate tubes of ~10 μL each. Store each aliquot at -20 °C in a non-frost-free freezer. This 1 μg/μL solution is used to prepare the trypsin intermediate solution as needed (see below). Note that the Agilent Proteomics Grade Trypsin comes with technical literature that provides an alternate protocol for tryptic digestion. We have used the method below and find it to be straightfoward and reliable.

200 mM DTT: Add 1 mL water to 0.031 g DTT in a 1.5 mL Eppendorf tube. Vortex. Divide the DTT solution into convenient (e.g., 100 μL) aliquots in microcentrifuge tubes. Store each aliquot at -20 °C for up to one month in a non-frost-free freezer. Do not thaw and re-freeze.

200 mM IAM (prepare just before use): Add 1 mL water to 0.037 g IAM in a 1.5 mL Eppendorf tube. Vortex.

Trypsin digestion protocol

Resuspension, denaturing, and reduction of protein

1. Add 0.5 mg total protein to 0.5 mL Eppendorf tube.
2. Add 25 μL ammonium bicarbonate stock solution.
3. Add 25 μL TFE denaturation agent.
4. Add 1.0 μL DTT stock solution.
5. Vortex to mix.
6. Heat under one of the following sets of conditions to denature:
   - 60 °C for 45 minutes to 1 hour
   - 90 °C for 20 minutes (hydrophilic proteins) to 1 hour (hydrophobic proteins)
7. Cool to room temperature.

Akylation

1. Add 4.0 μL IAM stock solution.
2. Vortex briefly.
3. Incubate sample in the dark (foil-covered rack) at room temperature for 1 hour.

Quenching of excess IAM

1. Add 1.0 μL DTT stock solution to destroy excess IAM.
2. Allow to stand for 1 hour in the dark (foil-covered rack) at room temperature.

Dilution and pH adjustment

1. Add 300 μL water to dilute denaturant.
2. Add 100 μL ammonium bicarbonate stock solution to raise pH.
3. Optionally check pH by placing 0.5 to 1 μL on a strip of pH indicator paper. Typical value is 7.5 to 8.0. It is more important to check pH when the pH of the starting sample is unknown.
4. Add more base (ammonium bicarbonate) if pH is not in the 7 to 9 range.

Digestion

1. Make fresh stock solution of trypsin in trypsin storage solution. Allow 15 min for complete re-suspension.
2. If you plan to digest less than 20 μg total protein, prepare trypsin intermediate solution by diluting stock 10-fold by adding 45 μL ultrapure water. This 100 ng/μL solution may be stored at -20 °C for 2 months without significant loss of activity.
   CAUTION: If IAM is not destroyed, it will slowly alkylate lysines.
3. Add trypsin stock solution at 1:20 to 1:50 by mass of enzyme:substrate. For example, for 500 μg protein, add between 10 and 25 μg trypsin (10 to 25 μL trypsin stock).
4. Vortex briefly.
5. Place tube in heater and incubate at 37 °C for 4 to 18 hours.
6. Cool solution.

Lowering of pH to halt trypsin activity

1. Add 1 μL neat formic acid or TFA to lower the pH and stop trypsin activity.

   If you are planning to desalt, use TFA because it aids in the peptide binding to the resin during cleanup.
2. Vortex briefly.
3. If you are concerned about the pH of the original sample, check pH (3.0 to 3.3 typically). Add more acid if pH is greater than 4.

Digestion Cleanup

1. Depending on sample origin, it may be necessary to desalt prior to MS analysis.
2. If desalting is not necessary, but the sample appears opaque, filter the sample prior to MS. Use Agilent spin filters, P/N 5185-5990.

   The opacity may be caused by cellular debris in the sample.
3. Dilute an aliquot of sample as necessary for analysis.

   If protein has a molecular weight of 50 kDa, and if digestion went to completion, solution is about 20 pmol/μL.

   If you have a less complex sample, dilute to achieve a 50 fmol/μL solution.

Step 5: **Cleanup and enrichment of digests**

Prior to peptide mapping, cleanup and/or enrichment is usually required for the successful analysis of peptide maps. There are many types of methods to accomplish cleanup and enrichment dependent on sample type and targeted objective. For example, enrichment for specific PTMs (e.g., phosphorylation, ubiquitination and glycosylation) is performed by affinity purification using PTM-specific antibodies or ligands, while phosphopeptides can be enriched by IP using anti-phospho-specific antibodies or by pull-down using TiO2, which selectively binds phosphorylated serine, tyrosine or threonine.

After peptide enrichment, salts and buffers can be removed using either graphite or C-18 tips or columns, and detergents can be removed using affinity columns or detergent-precipitating reagents. Dilute samples can also be concentrated using concentrators of varying molecular weight cutoff (MWCO) ranges. Once purified, peptide samples are then ready for the final preparation for MS analysis, which varies based on the type of analysis. For LC/MS or LC-MS/MS analysis, the proper choice of mobile phases and ion-pairing reagents is required to achieve good LC resolution and analytical results. MALDI-MS requires combining the peptide sample with specific matrices (crystalline energy absorbing dye molecules), which are then dried on MALDI plates prior to analysis.

Reagents and equipment

Item needed	Example
Ammonium bicarbonate, reagent grade	Sigma catalog #A-6141
Dithiothreitol (DTT), >99+%	Sigma catalog #D-5545
Iodoacetamide (IAM), 97%	Sigma-Aldrich catalog #I-670-9
Trifluoroethanol (TFE), 99+%	Sigma-Aldrich catalog #T63002-100G
Trypsin, modified	Agilent Proteomics Grade Trypsin (P/N 204310)
Water, 18 megohm or equivalent	Agilent P/N 8500-2236
Formic acid, analytical grade or trifluoroacetic acid, sequencing grade	Agilent P/N G2453-85060
Eppendorf Safe-Lock microcentrifuge tubes, natural, not siliconized	Eppendorf P/N 022363611 (0.5 mL, box of 500), or P/N 022363204 (1.5 mL, box of 500)
Micropipettors and tips: 1-1000 µL range	
Tube heater/shaler	Eppendorf Thermomixer
pH indicator strips, pH ranges 2.5-4.5 and 7.0-9.0	EM Science ColorpHast strips, catalog #700181-2
Analytical balance	
Bond Elut OMIX Tips, 10 µL (elution volume 2-10 µL)	1x96 tips (Agilent P/N A5700310); 6x96 tips (Agilent P/N A5700310K)
Bond Elut OMIX Tips, 100 µL (elution volume 10-100 µL)	1x96 tips (Agilent P/N A57003100); 6x96 tips (Agilent P/N A57003100K)

For small volumes of peptides for cleanup:
Bond Elut OMIX tips

Bond Elut OMIX (10 μL volume) method for peptide digest cleanup	
Sample Pretreatment	Adjust sample to a 0.5%-1.0% trifluoroacetic acid (TFA) concentration using a 2.5% TFA solution
Conditioning and Equilibration	Aspirate 10 μL of 50% acetonitrile (ACN):water and discard solvent. Repeat. Aspirate 10 μL of 1.0% TFA solution and discard solvent. Repeat.
Sample Application	Aspirate up to 10 μL of pre-treated sample into OMIX Tip. Dispense and aspirate sample 3-5 cycles for maximum efficiency. Up to 10 cycles may be used for improved binding.
Rinsing	Aspirate 10 μL of 0.1% TFA buffer and discard solvent. Repeat.
Elution	LC/MS or LC/MS/MS Analysis: Aspirate 2-10 μL of 0.1% Formic Acid or 0.1% Acetic Acid in either a 50-75% acetonitrile or 50-75% methanol solution and dispense into an autosampler vial or well plate.

For best results, set the pipettor to match the tip volume – 10 μL – for equilibration, sample application, and rinsing steps. For elution, aliquot the exact volume of elution solution into a separate container and maintain your pipettor at the maximum volume setting to match the tip volume, 10 μL.

For high-throughput peptide applications:
Automated sample prep solutions for peptide mapping

"Using the combination of extremely consistent, parallelized digestion with automated reversed-phase cleanup via AssayMAP... has enabled us to contemplate collaborative studies of previously unheard of scales and throughputs."
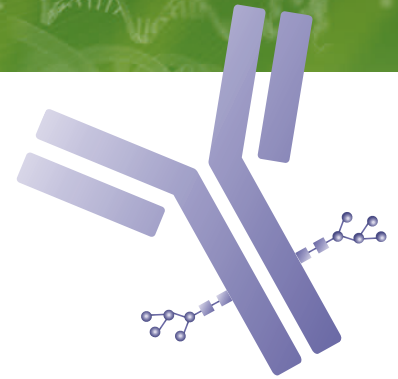
Jacob D. Jaffe, Ph.D.
Assistant Director – Proteomics Platform

See more information about automated sample prep for peptide mapping on **page 22**.

Learn more about Agilent columns for peptide mapping at **agilent.com/chem/advancebio**

Reversed-Phase Chromatography – The Superior Choice for Peptide Mapping

The selection of a column and method to generate peptide maps ultimately depends on the protein being mapped and the goals of the workflow. The most widely used peptide mapping column method, especially among the biopharmaceutical industry, is reversed-phase chromatography (RPC). Excellent resolving power and the use of volatile mobile phases (compatible with mass spectrometry) has resulted in this technique becoming the predominant HPLC method for most peptide separations. It is superior to other modes of HPLC separations with respect to both speed and efficiency. **Figure 4** displays a well resolved peptide separation using bovine serum albumin and demonstrates the multitude of peptide peak fragments that can be resolved by employing RPC for peptide mapping.

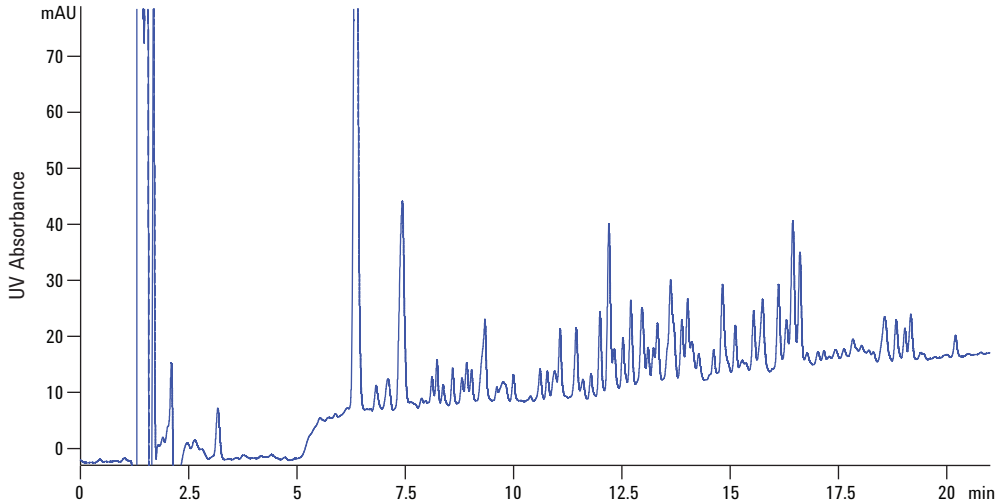## Figure 4 – **Reversed-phase peptide mapping separation**

Figure 4 – Reversed-phase separation of BSA using an Agilent Polaris C18-A, 2.0 x 150 mm column (Agilent P/N A2001150X020).

Requirements for a Successful Peptide Mapping Separation

The general approach in developing a practical RPC method for peptide mapping requires a good understanding of peptide specific column requirements and chromatographic method development. Although many of the same chromatographic principles apply to the separation of peptides compared to small molecule separations, there are a number of condition specific variables for optimizing the peptide method and achieving a reproducible and robust separation. Column selection, column quality, mobile phase selection and detection requirements are all important components to peptide mapping separations that can vastly improve the quality of your peptide maps.

Column selection

The most important aspect for achieving a reliable, well-resolved peptide mapping separation is the selection of a suitable column. The column pore size, particle type and size, bonded phase chemistry and stability (chemical and packed bed) all play a significant role in facilitating the peptide mapping separation, optimization strategy and spectrometric analysis. For peptide separations, the preferred column pore sizes range from 100Å to 120Å, while the optimum phase selection is typically C18. Although some commercial columns offer pore sizes for peptides down to 60Å, these are typically related to smaller peptide fragment separations or standards analysis. Likewise, there are smaller bonded phase carbon chain lengths used, but these have relationships to specific methods and have limited practicality for achieving retention across a broad spectrum of peptide hydrophobicity.

Separations of peptides deliver smaller plate numbers due to their higher diffusion coefficients, and have favored the use of smaller diameter totally porous column materials at slower flows. This has spawned an increase in sub-2 µm packings for achieving more efficient peptide maps. However, more recently, superficially porous columns have become increasingly popular for biological separations — especially among the biopharmaceutical industry — because they address the limitations of protein and peptide mass diffusion. These columns offer a shorter diffusion path allowing the separations of larger molecules at high linear velocities without the system backpressure increases associated with the smaller particles. **Figure 5** provides an example of a rapid high resolution peptide map achieved using a superficially porous column.

Learn more about Agilent columns for peptide mapping at **agilent.com/chem/advancebio**

Figure 5 – **Fast and efficient high resolution peptide mapping sequence of bovine serum albumin (BSA)**
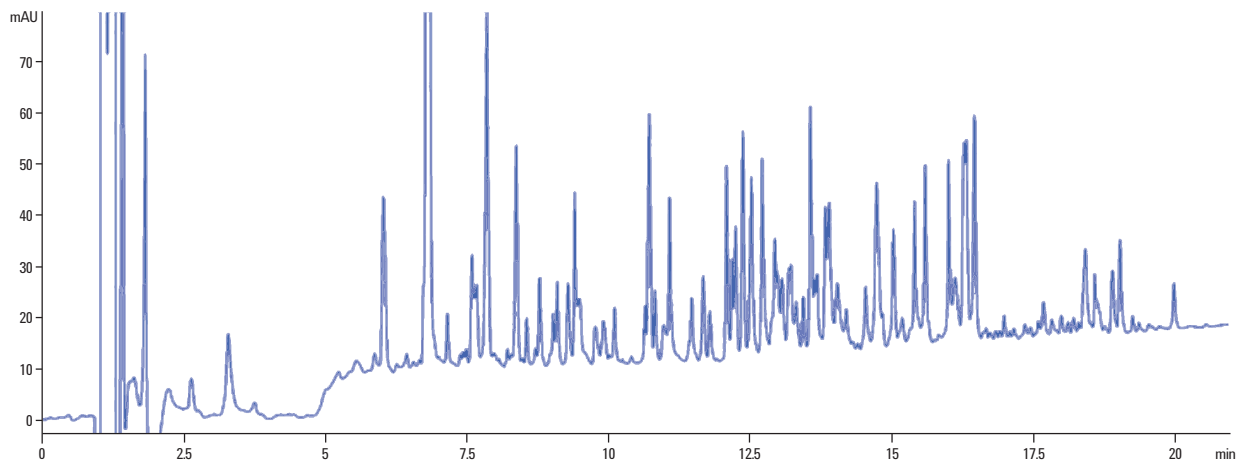

Figure 5 – Reversed-phase separation of BSA using an Agilent AdvanceBio Peptide Mapping 2.1 x 150 mm column (Agilent P/N 653750-902). The peptide mapping separation was performed at 0.3 mL/min, 40 °C using water (0.1% TFA)/ACN (0.08%) linear gradient.

Column quality – run-to-run reproducibilty and stability – is a critical, and sometimes overlooked, requirement for maintaining reproducible and robust peptide mapping separations. Reversed-phase separations of peptides are commonly carried out at low pH (pH<3) and elevated temperatures (>40 °C). Peptide maps rely on repeatable operation of the column for delivering precise mapping fingerprints and repeated validation protocols. When choosing a column for peptide mapping, column quality should be at the forefront of the decision making process. **Figure 6** provides an excellent example of a reproducible peptide map of a monoclonal antibody tryptic digest separated under low pH and elevated temperature conditions during an LC/MS analysis.

Figure 6 – **Peptide mapping reproducibility during LC/MS analysis**
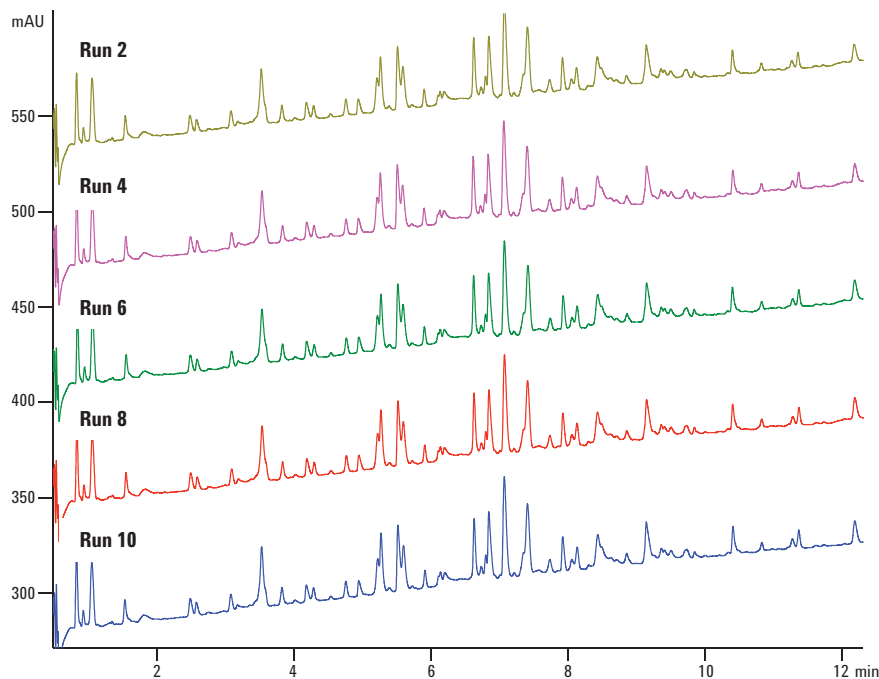

Figure 6 – Five replicate injections of a monoclonal antibody tryptic digest using a 3.0 x 150 mm Agilent AdvanceBio Peptide Mapping column (Agilent P/N 653950-302) on an Agilent 1200 LC system coupled to a 6520 Q-TOF. Separation was performed at 0.3 mL/min, 40 °C using water (0.1% FA)/ACN (0.1% FA) gradient.

Mobile phase selection

The most commonly used solvent in peptide mapping is water with acetonitrile as the organic modifier to which not more than 0.1% of ion pairing agent is recommended. Under certain circumstances, propyl alcohol or isopropyl alcohol can be added to solubilize the digest components, provided that the addition does not unduly increase the viscosity of the components. Buffered mobile phases containing phosphate are used to provide some flexibility in the selection of pH conditions, since shifts of pH in the 3.0-5.0 range enhance the separation of peptides containing acidic residues (e.g. glutamic and aspartic acids). Sodium or potassium phosphates, ammonium acetate, phosphoric acid at a pH between 2 and 7 (or higher for polymer-based supports) have also been used with acetonitrile gradients. Acetonitrile containing trifluoroacetic acid is used quite often.

Mobile phases used in RPC for the analysis of proteins and peptides contain an additive which works as an ion-pairing agent. This component increases the hydrophobicity of peptides by forming ionic pairs with their charged groups. As a consequence, interaction of the peptides with the hydrophobic stationary phase is possible and, therefore, so is their improved separation through increased retention. More common additives such as trifluoroacetic acid (TFA), formic acid (FA), and acetic acid (AcOH) can yield very low pHs and promote protein unfolding and denaturation. Thus, molecules such as peptides, elute in sharper and more symmetrical bands. The ion-pairing agent most widely used for the separation of proteins and peptides is TFA for both its compatibility (high volatility) with mass spectrometry and affinity for the charged peptide.

Detection

Detection for peptides is usually 210 nm to 220 nm and/or 280 nm (**Figure 7**). Detection at 280 nm is often performed in parallel with detection at 210 nm in peptide mapping. Tryptophan, tyrosine, and phenylalanine are sensitive at 280 nm while 210 nm detection is relatively unselective for a host of other biologicals in the sample matrix. However, sensitivity at 210 nm and 220 nm is two to four fold higher than 280 nm. Additionally, of some importance to the detection profile for peptide maps is the blending of 0.1% TFA in water (A-solvent) and 0.08% TFA (B-solvent) in ACN which is used to minimize baseline drift caused by changes in absorbance over the course of the elution gradient. **Figure 7** provides an example comparison of a peptide mapping separation as wavelength is varied between 220 nm and 280 nm and details the differences in absorbance sensitivity and UV peak profiles.

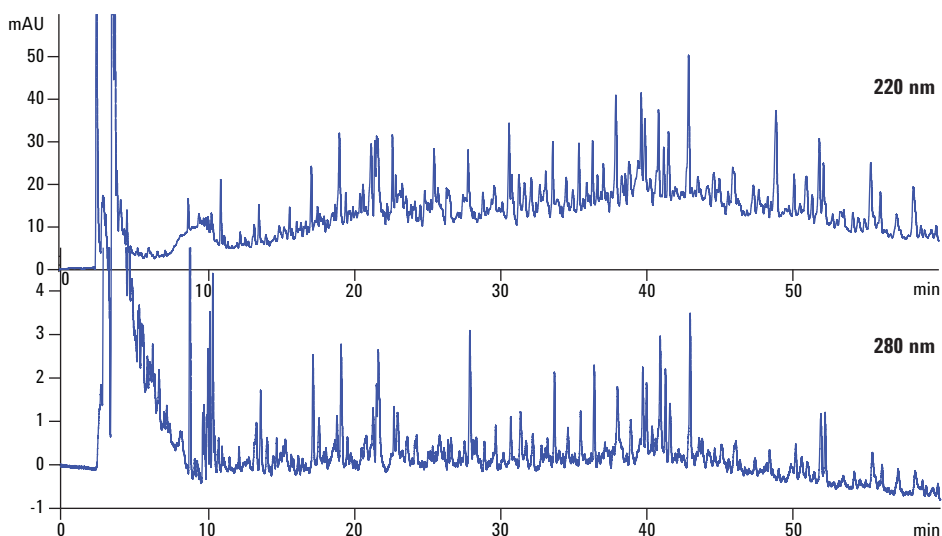## Figure 7 – **Peptide mapping at different wavelengths**

Figure 7 – AdvanceBio Peptide Mapping column (Agilent P/N 651750-902), 2.1 x 250 mm, profiling e.coli digest at 220 nm (top) and 280 nm (bottom) on an Agilent 1290 Infinity LC.

Learn more about Agilent columns for peptide mapping at **agilent.com/chem/advancebio**

Developing an Efficient Peptide Mapping Method

The general approach to developing an RPC method for a peptide mapping separation is the same employed by typical RP method development practices, however, there are special requirements unique to peptide mapping development. This section will provide a recommended basic approach for preparing a well resolved peptide map via (1) optimization of gradient conditions for retention, (2) variables for changing selectivity and (3) further optimizing column conditions to improve the compromise between run time and resolution. At each step of this method development process careful attention should always be given to sample type and the intended purpose of your peptide mapping experiment.

(1) Optimizing the gradient conditions

A low pH ACN buffer gradient is always highly recommended for the separation of peptides, because it:

• Facilitates the separation of a wide range of peptide types and structures.

• Suppresses ionization of silanols, which can have undesirable interactions with basic amino side chains in the molecule, resulting in poor peak shapes.

• Helps to denature the peptide fragment improving retention and resolution.

• Allows for low UV detection (<210 nm) for maximizing detection sensitivity.

• Provides narrower bands due to the lower viscosity of the mobile phase.

• Increases retention of small poorly retained peptides by ion-pairing with the free amino terminus and basic amino acids (when TFA is used in buffer).

Propanol or iso-propanol (IPA) can be substituted for ACN as the organic modifier to provide better recovery of hydrophobic peptides. However, they are more viscous, resulting in higher column backpressure and somewhat broader bands in some cases. These solvents also require a higher wavelength for detection (>220 nm) and have a loss in detection sensitivity.

Most peptides are eluted with less than 60% ACN, but occasionally a higher ACN concentration is required. A good starting point for an initial peptide mapping development run is 0 to 60% in 45 minutes (2%/min). However a flatter gradient often is necessary in the final method to obtain the desired resolution. Gradient steepness, or the %B/min, determines the average retention (k′) of a sample band during its migration through a column. The value of k′ depends on the column dimensions, flow, sample weight and gradient steepness.

(2) Variables for changing selectivity (α) of the peptide map

Chromatographers working with biological samples generally postpone a change of column conditions (N) until band spacing (α) has been improved. Changes in temperature and gradient steepness are convenient to perform (no change in mobile phase or column) and should be explored first to improve band spacing (α) for optimizing a peptide mapping separation.

A change in temperature is a powerful means of changing selectivity and could result in retention switching for particular peptide residues. Elevating the temperature of a peptide mapping separation produces narrower bands, lowers system backpressure and changes selectivity. An initial temperature of 30-50 °C is recommended; however, the optimum temperature for a particular mapping separation will depend on many factors based on digestion type and composition. Some very hydrophobic peptides require a temperature of 60-80 °C for maximum recovery, while selectivity for a given sample will often be best for a particular temperature in the range of 30-60 °C.

Figure 8 details a comparison between two identical gradient regions when temperature was increased from 30 °C (top chromatogram) to 60 °C (bottom chromatogram) for a myoglobin tryptic digest. At an elevated temperature of 60 °C, the separation profile details changes in band shape and peak position highlighted by the peaks 1-7. Clearly some of the notable changes in this region of the chromatogram are the improved separation between peaks 1, 2 and 3 and the band positioning differences (selectivity) between peaks 4 and 5.

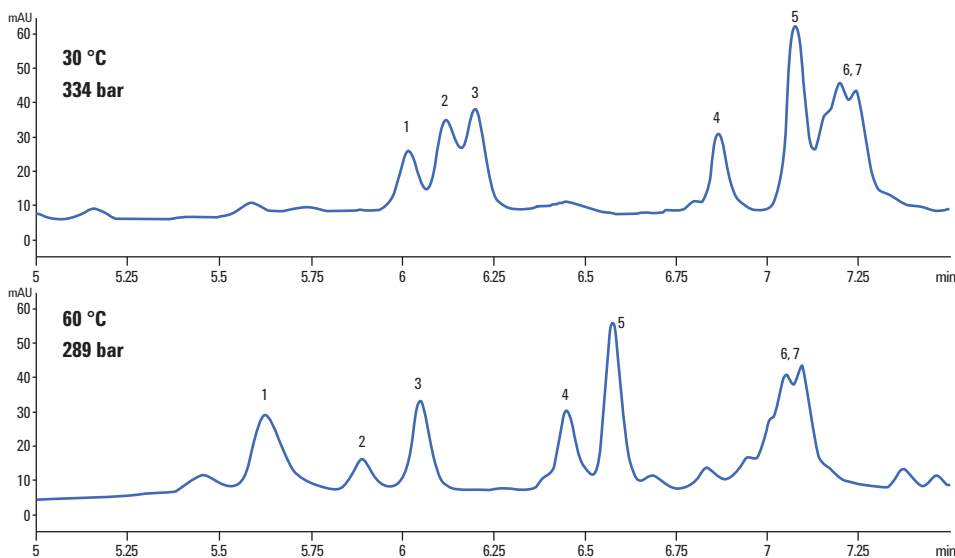## Figure 8 – Effect of temperature on selectivity for a myoglobin tryptic digest

Figure 8 – Myoglobin tryptic digest gradient separation at 5.0-8.0 min of a 20 min gradient with a 2.1 x 150 mm AdvanceBio Peptide Mapping column (Agilent P/N 653950-302). Both separations were completed with a water (1.0% TFA)/ACN (0.08% TFA) linear gradient, 0.3 mL/min at 215 nm on an Agilent 1260 Infinity Bio-inert Quaternary LC system. The top chromatogram was separated at a temperature of 30 °C and the bottom chromatogram was completed at a temperature of 60 °C.

Learn more about Agilent columns for peptide mapping at **agilent.com/chem/advancebio**

Changes in gradient steepness can also dramatically improve band spacing and change selectivity of the peptide mapping separation. Gradient steepness can be varied in two ways by either keeping the flow rate constant and changing the elution time to shorter (Increasing steepness) or longer (decreasing steepness) run times or by keeping run time constant and changing the flow rate.

Figure 9 demonstrates selectivity changes resulting from varying gradient steepness. Using a myoglobin tryptic peptide digest, a steep gradient run time of 15 minutes (top chromatogram) was compared to longer gradient run time of 40 minutes (bottom chromatogram), while both separations were maintained at a flow of 0.6 mL/min at 50 °C. A comparison on the chromatograms – and identifying the same peaks (asterisks) in each separation – shows numerous changes to band spacing, peak counts and peak shape.

Figure 9 – **Effect of gradient slope during a myoglobin tryptic digest separation**

15 minute gradient
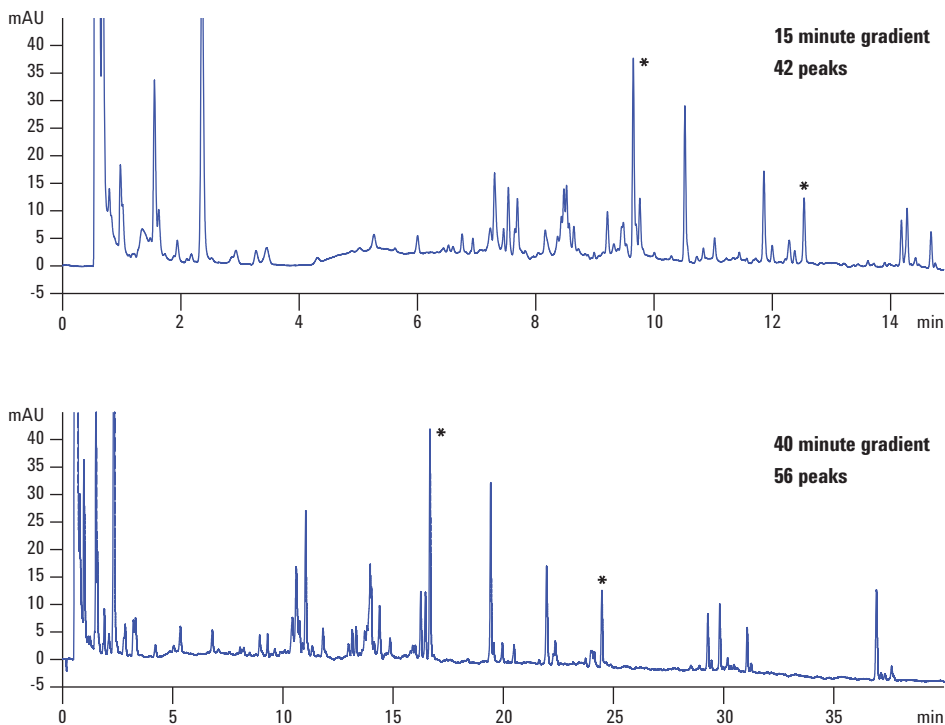**42 peaks**

40 minute gradient
56 peaks

Figure 9 – Myoglobin tryptic digest gradient separations with a 2.1 x 150 mm AdvanceBio Peptide Mapping column (Agilent P/N 653950-302) on an Agilent 1260 Infinity Bio-inert Quaternary LC system using water (1.0%TFA)/ACN (0.08% TFA) linear gradient, 0.6 mL/min at 50 °C. The top chromatogram was completed in 15 minutes while the bottom chromatogram was completed in 40 minutes. Asterisks in each chromatogram represent same peaks.

(3) Adjusting column conditions for further optimization

Once the gradient has been optimized in terms of retention (k') and selectivity (α), further improvements in separation are possible by varying column length and flow rate. The choice of which column condition to vary in gradient elution is essentially the same as for an isocratic separation. In both cases, larger values of efficiency (N) can be obtained at the expense of longer run times. For minor improvements in resolution, where an increase in the run time is less important, it is convenient to reduce flow rate. However, when a larger increase in resolution is needed, an increase in column length is usually preferred. If resolution is greater than required after optimizing selectivity, this excess resolution can be traded for a shorter run time by increasing flow rate and/or reducing column length. **Figure 10** provides an example of improved peptide mapping resolution for a myoglobin tryptic digest when column length was increased from 150 mm to 250 mm. In this comparison, conditions and gradient time were held constant while column length was increased from 150 mm to 250 mm. A red box was added to the same areas of the separations to highlight the increased resolution enabled by the 250 mm length and to emphasize the gains in peak capacity per unit time.

The gradient elution, subsequent variables associated in optimizing selectivity and the column condition optimizations discussed in (1), (2) & (3) above are proven basic strategies for improving any separation strategy including peptide mapping. The methods described above can be best outlined in the steps below:

Peptide mapping method development steps

1. **Select the initial gradient conditions: column length, mobile-phase composition, flow rate, temperature, and detection.** The initial separation should be optimized for retention (k'). This requires a gradient that is not too steep.

2. **Adjust the gradient range.** This is used to minimize run time by eliminating wasted space at the beginning and end of the chromatogram.

3. **Vary selectivity.** If overlapping bands are observed or run time is too long, options discussed for selectivity adjustments can be tried.

4. **Consider gradient shape.** Additional band spacing may be achieved with the use of a non-linear gradient shape as an option to further improve the separation.

5. **Adjust column conditions.** When band spacing and selectivity are optimized, consider varying run time and/or column length to improve resolution and/or analysis speed.

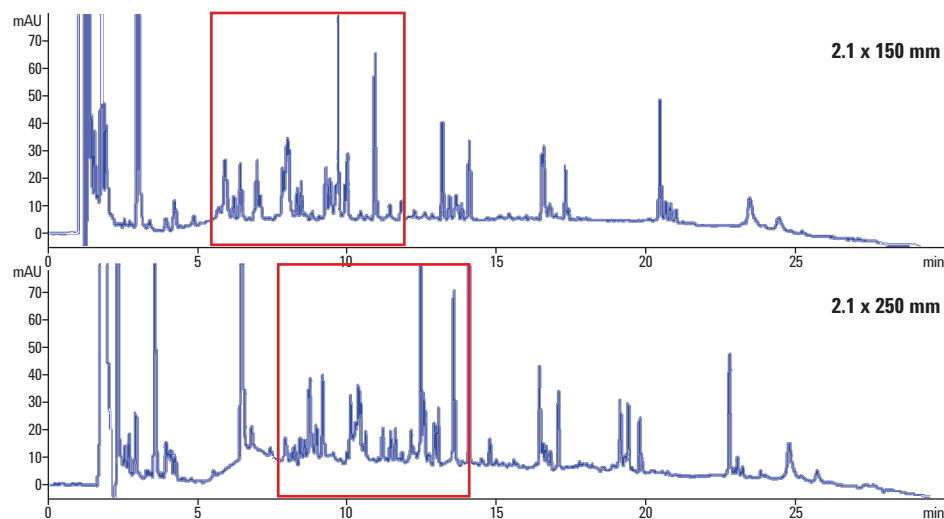## Figure 10 – **Effect of column length on resolution**

Figure 10 – Effect of column length on resolution, a peptide mapping comparison using a myoglobin tryptic digest (Agilent P/N 651750-902). Areas highlighted in red indicate equivalent areas of separation to emphasize resolution and peak shape. The separations were performed using an Agilent AdvanceBio Peptide Mapping Column, 2.1 x 150 mm (Agilent P/N 651750-902), on an Agilent 1260 Infinity Bio-inert Quaternary LC system using a water (1.0% TFA)/ACN (0.08% TFA) linear gradient, 10-60% B in 30 minutes, 0.3 mL/min, 45 °C.

Learn more about Agilent columns for peptide mapping at **agilent.com/chem/advancebio**

Peptide Mapping Characterizations by Mass Spectrometry

The use of RPC with mass spectrometry has made this combined technique the method of choice for characterizing peptides and peptide maps. For example, in the biopharmaceutical industry, establishing and monitoring the sequence identity of a therapeutic target is critical, and the stability of a protein biologic is an important aspect of therapeutic development for monitoring modifications such as oxidation, reduction, glycosylation, and truncation. MS can be used as a non-regulatory purity test for establishing the genetic stability of a product throughout its lifecycle.

Peptides are analyzed by mass spectrometry by direct infusion of the isolated peptides – or by the use of on-line LC/MS for structure analysis – and then correlated to the protein amino acid sequence. The identified peptides thus confirm the specific amino acid sequences covered by the peptide map, as well as the identity of the protein. Mass spectrometric peptide mapping is applied to:

- Confirm the identity of a specific protein.

- Get detailed characterization of the protein, such as confirmation of N-terminal and C-terminal peptides, high sequence coverage peptide maps, amino acid substitutions, etc.

- Screen and identify post translational modifications. (e.g. glycosylations, disulfide bonds, N-terminal pyroglutamic acid, methionine and tryptophan oxidation, etc.)

In general, types of MS analysis include electrospray and MALDI-TOF-MS, as well as fast-atom bombardment (FAB). Tandem MS has also been used to sequence a modified protein and to determine the type of amino acid modification that has occurred. Using electrospray ionization (ESI) or MALDI-MS, proteolytic peptides can be ionized intact into the gas phase and their masses accurately measured. Most peptide separations are performed on electrospray ionization (ESI) LC/MS instruments due to the convenience of LC coupling and better quality of tandem mass spectra for confident protein identification. For example, a quadrupole time-of-flight (QTOF) MS instrument often gives more structural information, especially for larger peptides, due to its high resolving power and mass accuracy.

Based on MS information, proteins can readily be identified in which measured masses are compared to the predicted values derived from the intact protein or protein database to elucidate mass and sequence coverage information. The goal of a characterization of a protein through peptide mapping is to reconcile and account for at least 95% sequence coverage of the theoretical composition of the protein structure. **Figure 11** is an example of a highly optimized peptide map of erythropoietin protein (EPO) digest using ESI-MS. The optimized chromatographic conditions and MS parameters have enabled 100% sequence coverage and highlight a well characterized peptide mapping separation.

Use Agilent Peptide Mapping standards to ensure your system is operating at peak performance for the application.
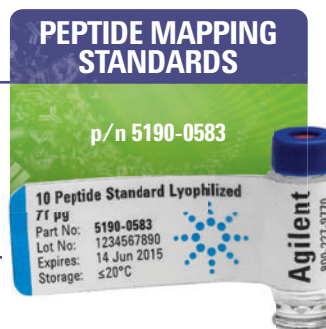
PEPTIDE MAPPING STANDARDS

p/n 5190-0583

10 Peptide Standard Lyophilized
7f µg
Part No: 5190-0583
Lot No: 1234567890
Expires: 14 Jun 2015
Storage: ≤20°C

Agilent
800-227-9770

Figure 11 – **Optimized peptide map of EPO protein providing 100% sequence coverage**

Cpd 8270: 32.698: +ESI ECC Scan Frag=170.0V EPOdigest1d0010.d
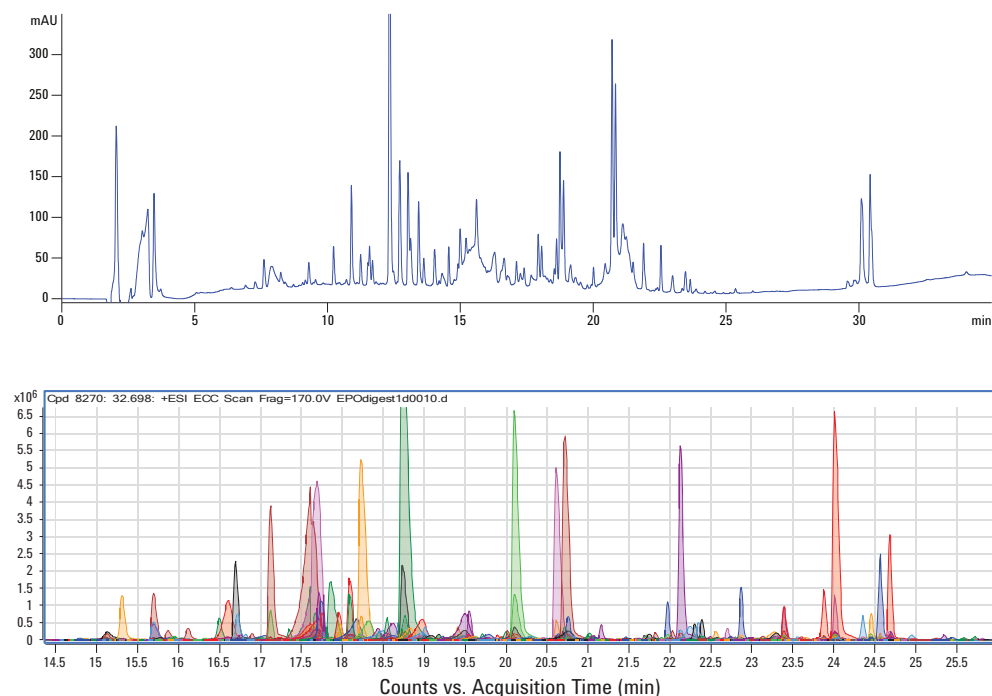
Counts vs. Acquisition Time (min)

Figure 11 – The top chromatogram displays a fully optimized EPO digest peptide mapping separation performed on a 2.1 x 150 mm AdvanceBio Peptide Mapping column. The bottom chromatogram shows the qualitative analysis (using a molecular feature extractor) for sequence coverage generated by on an Agilent Q-TOF.

Ordering Information

For peptide mapping, Agilent recommends:

AdvanceBio Peptide Mapping –
the first choice for most applications

Description	Part Number	Fast Guard Part Number
4.6 x 150 mm, 2.7 µm	653950-902	850750-911
3.0 x 150 mm, 2.7 µm	653950-302	853750-911
2.1 x 250 mm, 2.7 µm	651750-902	851725-911
2.1 x 150 mm, 2.7 µm	653750-902	
2.1 x 100 mm, 2.7 µm	655750-902	

*Fast Guards extend column lifetime without slowing down the separation or affecting resolution.

ZORBAX RRHD 300-C18 for incomplete digest or samples containing a hydrophobic core

Description	Part Number
2.1 x 50 mm, 1.8 µm	857750-902
2.1 x 100 mm, 1.8 µm	858750-902

ZORBAX RRHD 300-HILIC
for additional data on hydrophilic and glycopeptides

Description	Part Number
2.1 x 50 mm, 1.8 µm	857750-901
2.1 x 100 mm, 1.8 µm	858750-901

Peptide Quality Control Standard

Use Agilent's 10-Peptide Quality Control Standard, the same standard Agilent uses to QC its columns, to evaluate your column performance over its lifetime. It can be used for HPLC or LC/MS. Approximately 20 injections per vial.

Description	Part Number
Peptide quality control standard, 71 µg in 2 mL vial	5190-0583

Learn more about Agilent columns for peptide mapping at **agilent.com/chem/advancebio**

Peptide sample preparation for mass spec analysis, intelligently automated

Manual sample preparation of peptides is a time-consuming process. If you are doing peptide mapping applications on MS, you are likely looking for increased throughput. And you are going to be reliant on a highly reproducible end-to-end workflow to ensure your results are consistent.

AssayMAP transforms digestion, cleanup, and fractionation workflows to enable previously unachievable precision and throughput:

- Improved reproducibility, due to decreased human error – <5% CVs

- Increased throughput – up to 384 samples each day

- Significantly reduces hands-on time – freeing up scientists to do analytical work

- Faster method development – the automated platform enables you to quickly optimize methods

AssayMAP Peptide Sample Prep Solution is based on the powerful combination of miniaturized, packed bed chromatography, the state-of-the-art Bravo Liquid Handling Platform and a simple, applications-based user interface that creates an open access environment for both novices and experienced users and simplifies the most challenging sample preparation workflows.

AssayMAP Peptide Sample Prep Solution
For Mass Spec Analysis

Digest → **Cleanup** → **Fractionate**
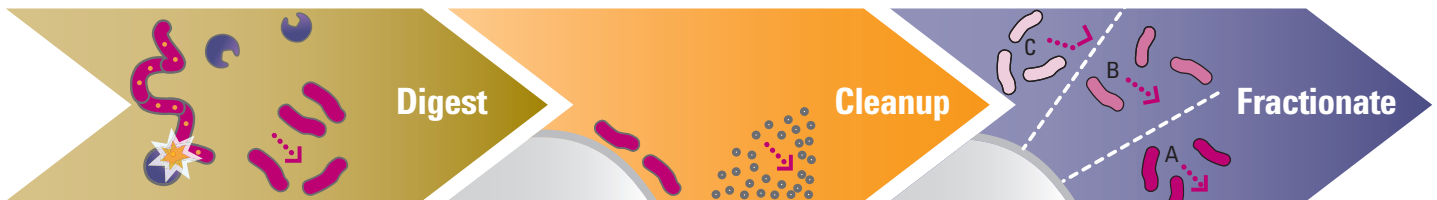
Digestion:

- In-solution digestion with user-supplied reagents

- Parallel process up to 4x96-well plates

- 1 manual pipetting step

Benefits:
- Reduce user variability

- Improve throughput and reproducibility

Cleanup:

- Quantitative separation method using reversed-phase cartridges

- Parallel process 1x96-well plate

Benefits:
- 10 µL elution equals short dry down times or "dilute and shoot" method

- Process control – every sample is treated identically

Fractionation:

- Strong cation exchange (SCX) cartridges generate up to 6 fractions to simplify the sample using step-wise elution with pH or salt

- Parallel process 1x96-well plate

Benefits:
- Increases LC/MS throughput by taking fractionation offline, reducing long LC gradient times

- Powerful enrichment tool for simplifying samples and isolating target peptides prior to analysis

Total workflow benefit:

- User interfaces for workflows are standardized for ease-of-use and linked for workflow integration.

- AssayMAP reduces the need for sample replicates and requires fewer repeated samples.

Achieve total workflow reproducibility with Agilent AssayMAP solution for sample prep before mass spec analysis

The AssayMAP Peptide Sample Prep Solution was used to digest 64 replicates each of two sample types: BSA in urea and guanidine HCL. The samples were cleaned using AssayMAP reversed-phase cartridges and analyzed using an Agilent AdvanceBio Peptide Mapping column, Agilent 1290 Infinity LC, and an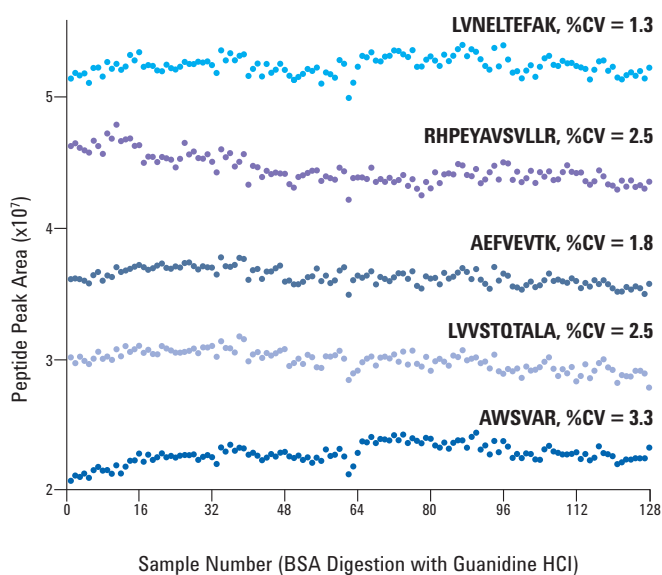 Agilent 6550 iFunnel Q-TOF mass spectrometer. The experiment was repeated on day two to examine reproducibility. %CV was determined for 25 peptides within each sample as shown in **Table 1**. The different %CV bins are shown. Illustrating the contributions of the total average %CV. To further showcase the reproducibility, peak area for representative peptides are shown in **Figure 12**.

Figure 12 – Scatter plots showing peak area of 4 peptides over 2 days.

25 Peptides	Urea (n=64, 62)		Guanidine HCl (n=64, 64)	
	Day 1	Day 2	Day 1	Day 2
Average Peak Area %CV	3.3	3.7	2.3	2.6
Peptides with %CV<5	**23**	**21**	**25**	**23**
Peptides with 5>%CV<10	2	3		1
Peptides with %CV>10		1		1

Table 1 – %CV by day with different %CV bins.

The AssayMAP sample prep took about four hours per day, with only two hand-on hours per day. Manual sample prep for the same workflow would take about eight hours per day, with four hands-on hours each day.

Total workflow CVs were <4%. The full workflow included AssayMAP Peptide Sample Prep system, an Agilent AdvanceBio Peptide Mapping Column, the 1290 Infinity LC System, and an Agilent 6550 iFunnel Q-TOF mass spectrometer.

For more details about this application, see Agilent publication 4991-2474EN.

Learn more about Agilent columns for peptide mapping at **agilent.com/chem/advancebio**

Partnering with you to get great results

Increasing challenges require better answers. Our solutions enable biopharmaceutical scientists to innovate in disease research, accelerate drug discovery, and have greater confidence throughout development and manufacturing. A broad range of Agilent solutions in genomics research, automation, separation, and detection technologies – along with workflow-driven software solutions – helps deliver the answers required to bring effective therapeutics to market.

Learn about the Agilent solutions for biopharma
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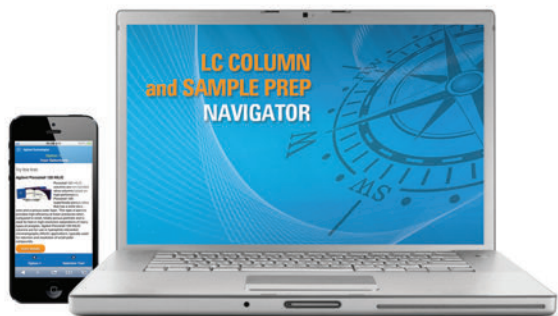
Navigate your way to a successful result

agilent.com/chem/navigator

With a wealth of biocolumns and small columns available, Agilent has introduced the LC columns and Sample Prep NAVIGATOR to help you choose the right column for your application.

The NAVIGATOR presents four easy search options:

- By part number – cross-reference for LC columns and sample prep products to find the best Agilent replacement
- By column – recommendations based on method
- By compound – drop down list
- By USP method

In addition, the tool offers column support to optimize chromatography, sample prep product recommendations, and quick access to technical support resources and other tools.

Agilent Technologies