TECHNICAL MANUAL

AccuMAP™ Low pH Protein Digestion Kits

Instructions for Use of Products
VA1040 and VA1050

Revised 7/18
TM504

AccuMAP™ Low pH Protein Digestion Kits

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

The AccuMap™ Low pH Protein Digestion Kit is designed for accurate, reproducible characterization of biotherapeutic proteins by peptide mapping using LC/MS and/or UV HPLC. The entire sample preparation procedure is performed at low (mildly acidic) pH to suppress artificial deamidation and disulfide bond scrambling. The kit also contains an optional agent for suppression of protein oxidation during sample preparation.

Features

- Suppression of sample preparation-induced artificial deamidation, disulfide bond scrambling and oxidation over the course of sample preparation. Pre-existing nonenzymatic modifications remain intact.
- Efficient reduction, alkylation and digestion at low pH.
- Complete sample preparation in 4.5–5 hours.
- High reproducibility.
- Digestion generates tryptic or Lys-C peptides. The kit can be used to digest reduced and nonreduced proteins.
- Two-step digestion with the first step conducted under strong denaturing conditions to ensure efficient proteolysis of tightly-folded proteins or protein domains.
- Low baseline noise.
- Procedural flexibility: Options to use alternative reducing and alkylating agents and to perform desalting step with size exclusion chromatography prior to digestion; reaction steps are amenable to optimization.

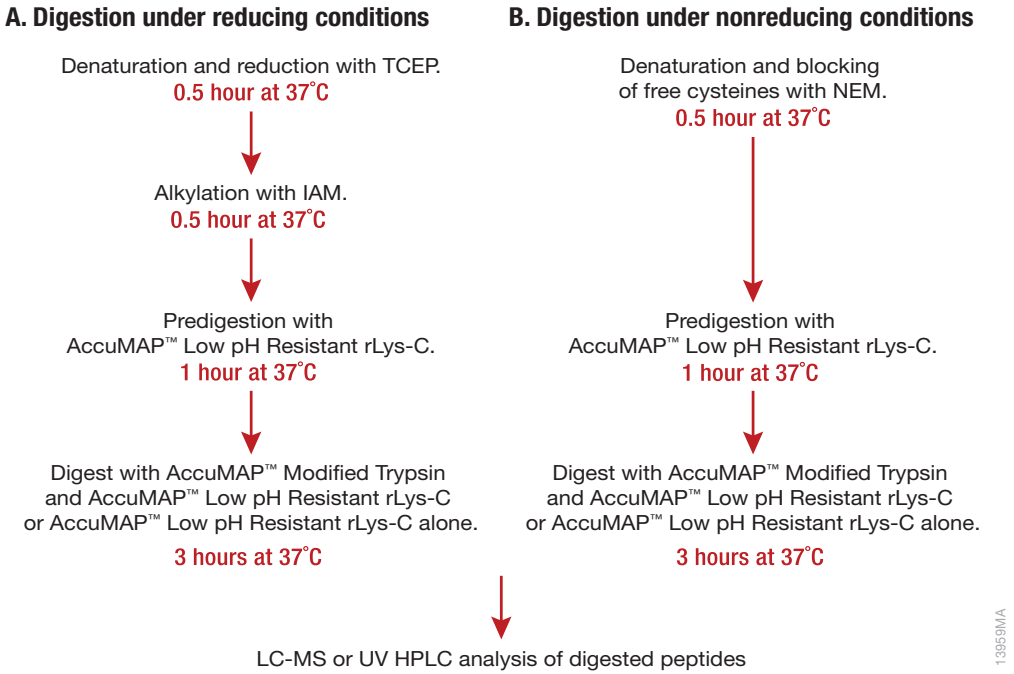

Figure 1. Dilution-based protein sample preparation with AccuMAP™ Low pH Protein Digestion Kit.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
AccuMAP™ Low pH Protein Digestion Mini Kit	**1 each**	**VA1040**

Contains sufficient reagents to prepare and digest 500µg of protein. Includes:

- 1ml AccuMAP™ Denaturing Solution (contains GuHCl)
- 1ml AccuMAP™ 10X Low pH Reaction Buffer
- 3 vials TCEP (Tris(2-carboxyethyl)phosphine), solid, 15mg/vial
- 2 vials NEM (N-Ethylmaleimide), solid, 15mg/vial
- 3 vials IAM (Iodoacetamide), solid, 15mg/vial
- 1 vial AccuMAP™ 100X Oxidation Suppressant Solution, 50µl/vial
- 2 vials AccuMAP™ Modified Trypsin, frozen solution, 0.5µg/µl, 120µl/vial
- 2 vials AccuMAP™ Low pH Resistant rLys-C, frozen solution, 200µg/ml, 300µl/vial

PRODUCT	SIZE	CAT.#
AccuMAP™ Low pH Protein Digestion Maxi Kit	**1 each**	**VA1050**

Contains sufficient reagents to prepare and digest 5mg of protein. Includes:

- 2 × 5ml AccuMAP™ Denaturing Solution (contains GuHCl)
- 2 × 5ml AccuMAP™ 10X Low pH Reaction Buffer
- 5 vials TCEP (Tris(2-carboxyethyl)phosphine), solid, 15mg/vial
- 4 vials NEM (N-Ethylmaleimide), solid, 15mg/vial
- 5 vials IAM (Iodoacetamide), solid, 15mg/vial
- 2 vials AccuMAP™ 100X Oxidation Suppressant Solution, 250µl/vial
- 3 vials AccuMAP™ Modified Trypsin, frozen solution, 0.5µg/µl, 800µl/vial
- 5 vials AccuMAP™ Low pH Resistant rLys-C, frozen solution, 200µg/ml, 1,200µl/vial

Storage Conditions: Store all kit contents at –65°C or below until ready to use.

Before using either kit, see Section 8.B for information on preparation of reagents and solutions.

3. Protocols for Sample Preparation Under Reducing Conditions

Materials to Be Supplied by the User

- NANOpure® (or equivalent grade) water
- trifluoroacetic acid (TFA)
- urea (for use in Sections 3.B and 4.B)

The protocols in this technical manual do not include a 'capping' step with excess reducing agent following alkylation. A capping step is commonly used to inactivate excess alkylating agent. In contrast, we allow alkylation to continue over the course of the digestion reaction effectively extending the incubation of a reduced protein with IAM to 4.5 hours. This extended alkylation period is required to compensate for decreased IAM activity at low pH. IAM concentration during the digestion period is kept sufficiently low (at ~3mM) to minimize nonspecific alkylation. Digestion should be performed protected from light to ensure IAM stability.

3.A. Sample Preparation with Guanidine HCl

Certain tryptic cleavage sites demonstrate increased proteolytic resistance. The sites located at the tips of peptide termini are particularly resistant. These sites are generated by cleavage of NNNN(K/R)^(K/R)NNNN or NNNN(K/R)^N(K/R)NNNN sequences (the cleavages are indicated by '^'). These sites could not be completely digested. If missed cleavages are observed and need to be minimized, digest protein with AccuMAP™ Low pH Resistant rLys-C alone (choose 'Digestion with AccuMAP™ Low pH Resistant rLys-C' in Step 7 of the protocol below). AccuMAP™ Low pH Resistant rLys-C efficiently digests proteolytically resistant sites. To assure the most complete digestion, allow for overnight incubation with AccuMAP™ Low pH Resistant rLys-C.

Alternatively, use the protocols in Sections 3.B. or 3.C. Trypsin activity increases when guanidine HCl is replaced with urea or removed by size exclusion chromatography. The majority of the missed cleavages mentioned above will be digested using protocols in 3.B or 3.C. Note that these protocols also have disadvantages (see Sections 3.B and 3.C for details).

This protocol is optimized for the digestion of 50µg of protein at a concentration of 10mg/ml. For less concentrated protein samples, please refer to Table 1 in Section 8.A.

Before You Begin

Prepare 100mM TCEP and 300mM IAM as described in Section 8.B.

Reduce and Alkylate

1. Add 5µl of protein solution (50µg of protein) to 20µl of AccuMAP™ Denaturing Solution and mix.
2. Add 6µl of AccuMAP™ 10X Low pH Reaction Buffer and 1µl of 100mM TCEP, mix and incubate for 30 minutes at 37°C.
3. Add 2µl of 300mM IAM, mix and incubate protected from light for 30 minutes at 37°C. Cover a heat block with aluminum foil to protect from light if needed.

Predigest

4. Add 25µl of AccuMAP™ Low pH Resistant rLys-C, mix and incubate protected from light for 1 hour at 37°C.

Digest

5. Add 10µl of AccuMAP™ 10X Low pH Reaction Buffer.
6. Add 2µl of AccuMAP™ 100X Oxidation Suppressant Solution if needed. If suppressant is not needed, add 2µl of distilled water.

   Note: We recommend that you add the suppressant if a protein solution contains polysorbates or other compounds that decompose into oxidizing agents or have inherent protein oxidizing activity. You must clean up the sample prior to UV HPLC or LC/MS analysis if suppressant is used as this reagent can damage reverse phase columns.
7. Complete the digestion using additional AccuMAP™ Low pH Resistant rLys-C or AccuMAP™ Modified Trypsin and AccuMAP™ Low pH Resistant rLys-C as follows:

   Digest with AccuMAP™ Low pH Resistant rLys-C: Add 64µl of NANOpure® water and 25µl of AccuMAP™ Low pH Resistant rLys-C, mix and incubate protected from light for 3 hours at 37°C (the reaction can be continued overnight if desired).

   Digest with AccuMAP™ Modified Trypsin and AccuMAP™ Low pH Resistant rLys-C: Add 44µl of NANOpure® water, 25µl of AccuMAP™ Low pH Resistant rLys-C and 20µl of AccuMAP™ Modified Trypsin. Mix and incubate protected from light for 3 hours at 37°C. (Longer incubation is not recommended as trypsin could generate semi-tryptic peptides that contribute to baseline noise.)

Terminate Reaction

8. Add TFA to a final concentration of 2% (for example, add 17µl of 20% TFA). Check a small amount of sample with pH paper to ensure the reaction is properly acidified.
9. Store digests at –20°C or below until ready for analysis. We recommend that you clean up digested protein with solid phase extraction or a trapping column prior to UV HPLC or LC/MS analysis to remove salts and impurities.

3.B. Sample Preparation with Urea

Urea is an efficient protein denaturing agent. It is also favored by many researchers since trypsin inhibition by urea is minimized by dilution of the urea. However, isocyanic acid, a product of urea decomposition, is commonly present in urea solutions at low levels. This byproduct causes protein modification (carbamylation). Amino groups at peptide N termini are particularly susceptible to carbamylation. To avoid carbamylation, use the procedure based on guanidine HCl protein denaturation (Section 3.A). Guanidine HCl also provides more efficient protein denaturation than urea.

This protocol is optimized for the digestion of 50µg of protein at a concentration of 10mg/ml. For less concentrated protein samples, please refer to Table 2 in Section 8.A.

Before You Begin

Prepare 100mM TCEP, 300mM IAM and 9M urea/1.4X AccuMAP™ Low pH Reaction Buffer mix as described in Section 8.B.

3.B. Sample Preparation with Urea (continued)

Reduce and Alkylate

1. Add 5µl of protein solution (50µg protein quantity) to 42µl of 9M urea/1.4X AccuMAP™ Low pH Reaction Buffer mix.
2. Add 1µl of 100mM TCEP, mix and incubate for 30 minutes at 37°C.
3. Add 2µl of 300mM IAM, mix and incubate protected from light for 30 minutes at 37°C. Cover the heating block with aluminum foil to protect from light if needed.

Predigest

4. Add 25µl of AccuMAP™ Low pH Resistant rLys-C, mix and incubate protected from light for 1 hour at 37°C.

Digest

5. Add 10µl of AccuMAP™ 10X Low pH Reaction Buffer.
6. Add 2µl of AccuMAP™ 100X Oxidation Suppressant if needed. If suppressant is not needed, add 2µl of distilled water. We recommend adding the suppressant if a protein solution contains polysorbates or other compounds that decompose into oxidizing agents or have inherent protein oxidizing activity. You must clean up the sample prior to UV HPLC or LC/MS analysis if suppressant is used as this reagent can damage reverse phase columns.
7. Complete the digestion using additional AccuMAP™ Low pH Resistant rLys-C or AccuMAP™ Modified Trypsin and AccuMAP™ Low pH Resistant rLys-C as follows:

   Digest with AccuMAP™ Low pH Resistant rLys-C: Add 48µl of NANOpure® water and 25µl of AccuMAP™ Low pH Resistant rLys-C. Mix and incubate protected from light for 3 hours at 37°C (the reaction can be continued overnight if desired).

   Digest with AccuMAP™ Modified Trypsin and AccuMAP™ Low pH Resistant rLys-C: Add 28µl of NANOpure® water, 25µl of AccuMAP™ Low pH Resistant rLys-C and 20µl of AccuMAP™ Modified Trypsin. Mix and incubate protected from light for 3 hours at 37°C (longer incubation is not recommended as trypsin could generate semi-tryptic peptides that contribute to baseline noise).

Terminate Reaction

8. Add TFA to a final concentration of 2% (for example, add 17µl of 20% TFA). Check a small amount of sample with pH paper to ensure the reaction is properly acidified.
9. Store digests at –20°C or below until ready for analysis. We recommend that you clean up digested protein with solid phase extraction or a trapping column prior to UV HPLC or LC/MS analysis to remove salts and impurities.

3.C. Sample Preparation with Desalting by Size Exclusion Chromatography

Some sample preparation protocols make use of a desalting step following denaturing, reduction and alkylation in order to remove the denaturants and other components that inhibit proteolytic activity. While these desalting procedures can be effective in that regard, they also have significant drawbacks that stem from loss of protein and protein refolding. If possible, use the protocol described in Section 3.A, which is optimized for high reproducibility and digestion efficiency.

This protocol is optimized for sample preparation of 50µg of protein at a concentration of 10mg/ml. For less concentrated protein samples, please refer to Table 3 in Section 8.A. This procedure was tested with BioSpin® 6 (BioRad) and Zeba® Spin (Thermo Fisher) desalting columns.

Before You Begin

Prepare 100mM TCEP and 300mM IAM as described in Section 8.B.

Reduce and Alkylate

1. Add 5µl of protein solution (50µg of protein) to 20µl of AccuMAP™ Denaturing Solution.
2. Add 6µl of AccuMAP™ 10X Low pH Reaction Buffer and 1µl of 100mM TCEP, mix and incubate for 30 minutes at 37°C.
3. Add 2µl of 300mM IAM, mix and incubate protected from light for 30 minutes at 37°C. Cover a heat block with aluminum foil to protect from light if needed.
4. Desalt according to manufacturer's procedure.

Digest

The protocol below uses 1µg/µl protein solution. We recommend that you determine the protein concentration after desalting to ensure that most of the protein was recovered. Adjust volumes of protein solution and water accordingly if the protein concentration is not 1µg/µl. The reaction is designed to use 20µg of protein in a 100µl reaction volume.

5. Transfer 20µl of 1µg/µl desalted protein solution (20µg of protein) to a new vial.
6. Add 1µl of 300mM IAM prepared as described in Section 8.B.
7. Digest using AccuMAP™ Low pH Resistant rLys-C or AccuMAP™ Modified Trypsin and AccuMAP™ Low pH Resistant rLys-C Mix as follows:

   Digest with AccuMAP™ Low pH Resistant rLys-C: Add 10µl of AccuMAP™ 10X Low pH Reaction Buffer, 10µl of AccuMAP™ Low pH Resistant rLys-C and 59µl of NANOpure® water. Mix and incubate protected from light for 4 hours at 37°C (the reaction can be continued overnight if desired).

   Digest with AccuMAP™ Modified Trypsin and AccuMAP™ Low pH Resistant rLys-C: Add 10µl of AccuMAP™ 10X Low pH Reaction Buffer, 10µl of AccuMAP™ Low pH Resistant rLys-C, 4µl of AccuMAP™ Modified Trypsin and 55µl of NANOpure® water. Mix and incubate protected from light for 4 hours at 37°C.

   If protein digestion is incomplete, digest with AccuMAP™ Low pH Resistant rLys-C overnight instead of 4 hours. To generate tryptic peptides, add 4µl of AccuMAP™ Modified Trypsin in the morning and continue digestion for an additional 4 hours at 37°C.

Terminate Reaction

8. Add TFA to a final concentration of 2% (for example, add 11µl of 20% TFA). Check a small amount of sample with pH paper to ensure the reaction is properly acidified.
9. Store digests at –20°C or below until ready for analysis. We recommend that you clean up digested protein with solid phase extraction or a trapping column prior to UV HPLC or LC/MS analysis to remove salts and impurities.

3.D. Sample Preparation of Proteolytically Resistant Proteins

This protocol is optimized for the digestion of 50µg of protein at a concentration of 10mg/ml. For less concentrated protein samples, please refer to Table 4 in Section 8.A.

Before You Begin

Prepare 100mM TCEP and 300mM IAM as described in Section 8.B.

Reduce and Alkylate

1. Add 5µl of protein solution (50µg of protein) to 20µl of AccuMAP™ Denaturing Solution.
2. Add 6µl of AccuMAP™ 10X Low pH Reaction Buffer and 1µl of 100mM TCEP, mix and incubate for 30 minutes at 37°C.
3. Add 2µl of 300mM IAM, mix and incubate protected from light for 30 minutes at 37°C. Cover a heat block with aluminum foil to protect from light if needed.

Predigest

4. Add 25µl of AccuMAP™ Low pH Resistant rLys-C, mix and incubate protected from light for 4 hours at 37°C.

Digest

5. Add 30µl of AccuMAP™ 10X Low pH Reaction Buffer.
6. Add 2µl of AccuMAP™ 100X Oxidation Suppressant if needed. If suppressant is not needed, add 2µl of distilled water.

   Note: We recommend the addition of suppressant if a protein solution contains polysorbates or other compounds that decompose into oxidizing agents or have inherent protein oxidizing activity. You must clean up the sample prior to UV HPLC or LC/MS analysis if suppressant is used as this reagent can damage reverse phase columns.

7. Complete the digestion using AccuMAP™ Low pH Resistant rLys-C or AccuMAP™ Modified Trypsin and AccuMAP™ Low pH Resistant rLys-C as follows:

   Digest with AccuMAP™ Low pH Resistant rLys-C: Add 204µl of NANOpure® water and 25µl of AccuMAP™ Low pH Resistant rLys-C. Mix and incubate protected from light overnight at 37°C.

   Digest with AccuMAP™ Modified Trypsin and AccuMAP™ Low pH Resistant rLys-C: Add 184µl of NANOpure® water and 25µl of AccuMAP™ Low pH Resistant rLys-C. Mix and incubate protected from light overnight at 37°C. In the morning, add 20µl of AccuMAP™ Modified Trypsin. Mix and incubate for 3 hours at 37°C (longer incubation with trypsin is not recommended as trypsin could generate semi-tryptic peptides, which contribute to baseline noise).

Terminate Reaction

8. Add TFA to a final concentration of 2% (for example, add 35µl of 20% TFA). Check a small amount of sample with pH paper to ensure the reaction is properly acidified.
9. Store digests at –20°C or below until ready for analysis. We recommend that you clean up digested protein with solid phase extraction or a trapping column prior to UV HPLC or LC/MS analysis to remove salts and impurities.

4. Protocols for Sample Preparation Under Nonreducing Conditions

The protocols below include blocking of free cysteines with N-Ethylmaleimide (NEM) prior to digestion. Free cysteines are commonly present in proteins at low levels and could induce disulfide bond scrambling during sample preparation. NEM is used as an alkylating agent in this protocol as it retains high alkylating activity at low pH and quickly blocks free cysteines. NEM is not recommended for alkylation in the protocols for sample preparation under reducing conditions due to side effects (see Section 5.B).

4.A. Sample Preparation with Guanidine HCl

The protocol below is optimized for digestion of 50µg of protein at a concentration of 10mg/ml. For less concentrated protein samples, please refer to Table 5 in Section 8.A.

Before you Begin

Prepare 200mM NEM as described in Section 8.B.

Block Free Cysteines

1. Add 5µl of protein solution (50µg of protein) to 20µl of AccuMAP™ Denaturing Solution.
2. Add 6µl of AccuMAP™ 10X Low pH Reaction Buffer and 2µl of 200mM NEM. Mix and incubate for 30 minutes at 37°C.

Predigest

3. Add 25µl of AccuMAP™ Low pH Resistant rLys-C, mix and incubate for 1 hour at 37°C.

4.A. Sample Preparation with Guanidine HCl (continued)

Digest

4. Add 10µl of AccuMAP™ 10X Low pH Reaction Buffer.
5. Complete the digestion using AccuMAP™ Low pH Resistant rLys-C or AccuMAP™ Modified Trypsin and AccuMAP™ Low pH Resistant rLys-C as follows:

   Digest with AccuMAP™ Low pH Resistant rLys-C: Add 67µl of NANOpure® water and 25µl of AccuMAP™ Low pH Resistant rLys-C. Mix and incubate for 3 hours at 37°C (the reaction can be continued overnight if desired).

   Digest with AccuMAP™ Modified Trypsin and AccuMAP™ Low pH Resistant rLys-C: Add 47µl of NANOpure® water, 25µl of AccuMAP™ Low pH Resistant rLys-C and 20µl of AccuMAP™ Modified Trypsin. Mix and incubate for 3 hours at 37°C (longer incubation is not recommended as trypsin could generate semi-tryptic peptides that contribute to baseline noise).

Terminate Reaction

6. Add TFA to a final concentration of 2% (for example, add 17µl of 20% TFA). Check a small amount of sample with pH paper to ensure the reaction is properly acidified.
7. Store digests at –20°C or below until ready for analysis. We recommend that you clean up digested protein with solid phase extraction or trapping column prior to UV HPLC or LC/MS analysis to remove salts and impurities.

4.B. Sample Preparation with Urea

Urea is an efficient protein denaturing agent. It is also favored by many researchers as trypsin inhibition by urea is eliminated by dilution of urea solution. However, isocyanic acid, a product of urea decomposition, is commonly present in urea solutions at low levels and can cause protein modification (carbamylation). Amino groups at peptide N termini are particularly susceptible to carbamylation. To avoid carbamylation, use the procedure based on guanidine HCl protein denaturation (Section 4.A). Guanidine HCl also provides more efficient protein denaturation than urea.

The protocol below is optimized for digestion of 50µg of protein at a concentration of 10mg/ml. For less concentrated protein samples, please refer to Table 6 in Section 8.A.

Before You Begin

Prepare 200mM NEM and 9M urea/1.4X AccuMAP™ Low pH Reaction Buffer as described in Section 8.B.

Block Free Cysteines

1. Add 5µl of protein solution (50µg of protein) to 42µl of 9M urea/1.4X AccuMAP™ Low pH Reaction Buffer.
2. Add 2µl of 200mM NEM, mix and incubate for 30 minutes at 37°C.

Predigest

3. Add 25µl of AccuMAP™ Low pH Resistant rLys-C, mix and incubate for 1 hour at 37°C.

Digest

4. Add 10µl of AccuMAP™ 10X Low pH Reaction Buffer.

5. Complete the digestion using AccuMAP™ Low pH Resistant rLys-C or AccuMAP™ Modified Trypsin and AccuMAP™ Low pH Resistant rLys-C as follows:

   Digest with AccuMAP™ Low pH Resistant rLys-C: Add 51µl of NANOpure® water and 25µl of AccuMAP™ Low pH Resistant rLys-C. Mix and incubate for 3 hours at 37°C (the reaction can be continued overnight if desired).

   Digest with AccuMAP™ Modified Trypsin and AccuMAP™ Low pH Resistant rLys-C: Add 31µl of NANOpure® water, 25µl of AccuMAP™ Low pH Resistant rLys-C and 20µl of AccuMAP™ Modified Trypsin. Mix and incubate for 3 hours at 37°C (longer incubation is not recommended as trypsin could generate semi-tryptic peptides that contribute to baseline noise).

Terminate Reaction

6. Add TFA to a final concentration of 2% (for example, add 17µl of 20% TFA). Check a small amount of sample with pH paper to ensure the reaction is properly acidified.

7. Store digests at –20°C or below until ready for analysis. We recommend that you clean up the digested protein with solid phase extraction or a trapping column prior to UV HPLC or LC/MS analysis to remove salts and impurities.

4.C. Sample Preparation with Desalting by Size Exclusion Chromatography

Some sample preparation protocols make use of a desalting step following denaturing, reduction and alkylation in order to remove the denaturants and other components that inhibit proteolytic activity. While these desalting procedures can be effective in that regard, they also have significant drawbacks that stem from loss of protein and protein refolding. If possible, use the protocol described in Section 4.A. It is optimized for high reproducibility and digestion efficiency.

The protocol below is optimized for preparation of 50µg of protein at a concentration of 10mg/ml. For less concentrated protein samples, please refer to Table 7 in Section 8.A.

Before You Begin

Prepare 200mM NEM as described in Section 8.B.

4.C. Sample Preparation with Desalting by Size Exclusion Chromatography (continued)

Block Free Cysteines

1. Add 5µl of protein solution (50µg of protein) to 20µl of AccuMAP™ Denaturing Solution.
2. Add 6µl of AccuMAP™ 10X Low pH Reaction Buffer and 2µl of 200mM NEM. Mix and incubate for 30 minutes at 37°C.
3. Perform a desalting procedure according to the manufacturer's protocol.

Digest

This protocol uses 1µg/µl protein solution. We recommend that you determine the protein concentration after desalting to ensure that most of the protein was recovered. Adjust volumes of protein solution and water accordingly if the protein concentration is higher or lower. The reaction is designed to use 20µg of protein in 100µl of reaction volume.

4. Transfer 20µl of 1µg/µl desalted protein solution (20µg of protein) to a new vial.
5. Digest using AccuMAP™ Low pH Resistant rLys-C or AccuMAP™ Modified Trypsin and AccuMAP™ Low pH Resistant rLys-C as follows:

   Digest with AccuMAP™ Low pH Resistant rLys-C: Add 10µl of AccuMAP™ 10X Low pH Reaction Buffer, 10µl of AccuMAP™ Low pH Resistant rLys-C and 60µl of NANOpure® water. Mix and incubate for 4 hours at 37°C (the reaction can be continued overnight if desired).

   Digest with AccuMAP™ Modified Trypsin and AccuMAP™ Low pH Resistant rLys-C: Add 10µl of AccuMAP™ 10X Low pH Reaction Buffer, 10µl of AccuMAP™ Low pH Resistant rLys-C, 4µl of AccuMAP™ Modified Trypsin and 56µl of NANOpure® water. Mix and incubate for 4 hours at 37°C.

   If protein digestion is not complete, digest overnight instead of for 4 hours using AccuMAP™ Low pH Resistant rLys-C. To generate tryptic peptides, add 4µl of AccuMAP™ Modified Trypsin in the morning and continue digestion for an additional 4 hours at 37°C.

Terminate Reaction

6. Add TFA to a final concentration of 2% (for example, add 11µl of 20% TFA). Check a small amount of sample with pH paper to ensure the reaction is properly acidified.
7. Store digests at –20°C or below until ready for analysis. We recommend cleaning up the digested protein with solid phase extraction or a trapping column prior to UV HPLC or LC/MS analysis to remove salts and impurities.

4.D. Sample Preparation of Proteolytically Resistant Proteins

This protocol is optimized for digestion of 50µg of protein at a concentration of 10mg/ml. For less concentrated protein samples, please refer to Table 8 in Section 8.A.

Before You Begin

Prepare 200mM NEM as described in Section 8.B.

Block Free Cysteines

1. Add 5µl of protein solution (50µg of protein) to 20µl of AccuMAP™ Denaturing Solution.
2. Add 6µl of AccuMAP™ 10X Low pH Reaction Buffer and 2µl of 200mM NEM. Mix and incubate for 30 minutes at 37°C.

Predigest

3. Add 25µl of AccuMAP™ Low pH Resistant rLys-C, mix and incubate for 4 hours at 37°C.

Digest

4. Add 30µl of AccuMAP™ 10X Low pH Reaction Buffer.
5. Complete the digestion using AccuMAP™ Low pH Resistant rLys-C or AccuMAP™ Modified Trypsin and AccuMAP™ Low pH Resistant rLys-C as follows:

   Digest with AccuMAP™ Low pH Resistant rLys-C: Add 207µl of NANOpure® water and 25µl of AccuMAP™ Low pH Resistant rLys-C. Mix and incubate overnight at 37°C.

   Digest with AccuMAP™ Modified Trypsin and AccuMAP™ Low pH Resistant rLys-C:
   Add 187µl of NANOpure® water and 25µl of AccuMAP™ Low pH Resistant rLys-C. Mix and incubate overnight at 37°C. In the morning, add 20µl of AccuMAP™ Modified Trypsin, mix and incubate for 3 hours at 37°C (longer incubation with trypsin is not recommended as trypsin could generate semi-tryptic peptides, which contribute to baseline noise).

Terminate Reaction

6. Add TFA to a final concentration of 2% (for example, add 35µl of 20% TFA). Check a small amount of sample with pH paper to ensure the reaction is properly acidified.
7. Store digests at –20°C or below until ready for analysis. We recommend cleaning up digested protein with solid phase extraction or a trapping column prior to UV HPLC or LC/MS analysis to remove salts and impurities.

5. Background Information

Nonenzymatic posttranslational modifications (PTMs) spontaneously occur in biotherapeutic proteins during manufacturing and storage. The major nonenzymatic PTMs are deamidation, disulfide bond scrambling and oxidation. These modifications can affect efficacy and stability of biotherapeutic proteins and are the subject of careful monitoring. Unfortunately, these nonenzymatic PTMs are also induced during protein preparation for peptide mapping. This compromises analysis of nonenzymatic PTMs. Major causes of nonenzymatic PTMs induced during sample preparation include alkaline pH as well as excipients and impurities with protein oxidizing activity (1–3). With the AccuMap™ Low pH Protein Digestion Kit, nonenzymatic PTMs are suppressed because sample preparation is performed at low pH and in the presence of an oxidation suppressing agent.

5.A. Reduction and Alkylation at Low pH

Common reducing and alkylating agents favor alkaline pH. Because alkaline pH induces deamidation and disulfide bond scrambling, we modified the reduction and alkylation procedure to be compatible with low pH. To ensure efficient reduction at low pH we use TCEP (Tris(2-carboxyethyl)phosphine), which maintains high reducing activity at low pH. Alkylation is performed with IAM (iodoacetamide). Activity of this reagent is decreased at low pH. To compensate for decreased IAM activity at low pH, alkylation is allowed to proceed through the digestion reaction. Accordingly, our procedure lacks a post-alkylation 'capping' step with reducing agent, which is intended to inactivate excess alkylating agent prior to digestion. Alkylating efficiency in this procedure is typically >99%.

5.B. Blocking of Free Cysteines at Low pH Prior to Digestion under Nonreducing Conditions

Free cysteines are commonly present in proteins at low levels and may induce disulfide bond scrambling. Disulfide bond scrambling is prevented by blocking free cysteines with NEM (N-Ethylmaleimide) prior to digestion in our procedure. NEM retains high activity at low pH and promptly blocks cysteines at these conditions.

Note: NEM is not recommended for alkylation in sample preparation procedure under reducing conditions. NEM generates stereoisomers for each alkylated cysteine (4), and as a result, NEM-alkylated peptides produce double peaks in LC/MS and UV HPLC. In addition, NEM forms dominant peaks in the early part of the RP-HPLC gradient, which may interfere with analysis of hydrophilic peptides.

5.C. Protein Digestion at Low pH

Trypsin and other proteases commonly used in peptide mapping sample preparation favor alkaline pH in order to efficiently digest proteins. To avoid artificial nonenzymatic PTMs induced at these conditions, we developed protocols for trypsin digestion at low pH. Our studies show that low pH primarily inhibits tryptic cleavages at lysine sites whereas arginine cleavage sites are still efficiently digested (Figure 2). To restore trypsin cleavage efficiency at lysine sites we supplemented trypsin with a special, low pH resistant recombinant Lys-C (rLys-C) protease. By supplementing trypsin with low pH resistant rLys-C we achieved efficient tryptic digestion at low pH (Figure 3). Under the conditions established in this protocol, artificial deamidation and disulfide bond scrambling were completely suppressed (Figure 4).

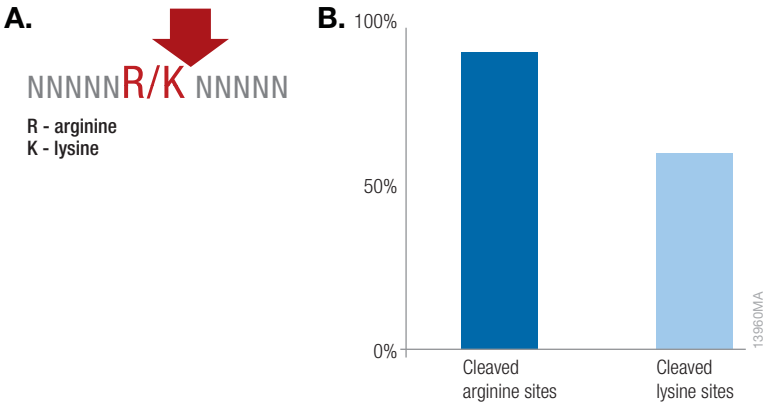

Figure 2. Tryptic cleavages at low pH. Yeast total protein extract was digested overnight at 37°C at low pH and analyzed by LC-MS/MS with a Q Exactive™ (Thermo Fisher). **Panel A** shows tryptic cleavage sites. **Panel B** shows cleavages in yeast total protein extract digested with trypsin. The data indicate that low pH primarily inhibits tryptic cleavages at lysine sites.

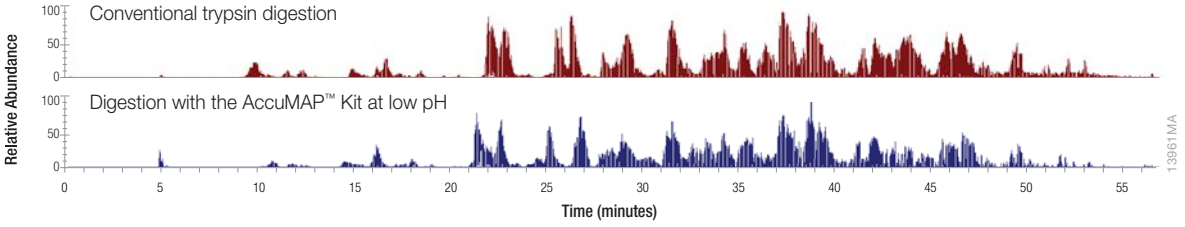

Figure 3. Efficient digestion with AccuMAP™ Low pH Protein Digestion Kit. Rituximab was digested according to a conventional protocol (at pH 8) or with the AccuMAP™ Low pH Protein Digestion Kit and analyzed with LC/MS. The data show that digestion at low pH was comparable to digestion at conventional conditions.

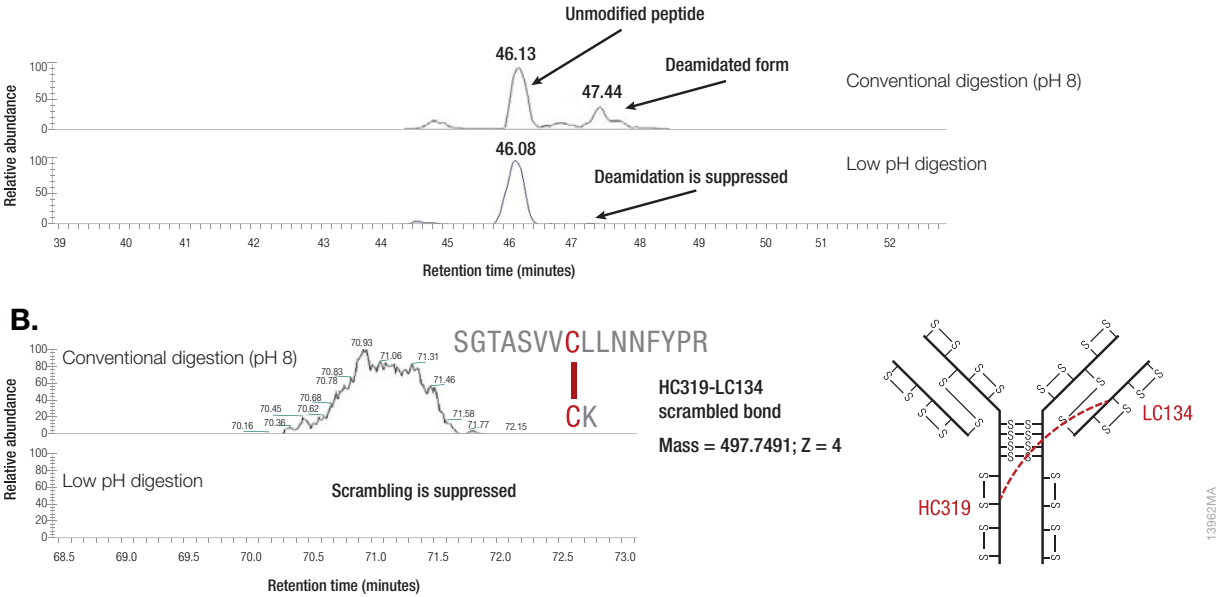

Figure 4. Suppression of deamidation and disulfide bond scrambling in IgG digested with AccuMAP™ Low pH Protein Digestion Kit. Panel A shows an extracted ion chromatogram of a GLEWIGAIYPGNGDTSYNQK peptide from Rituximab digested at conventional conditions (pH 8) and at low pH with AccuMAP™ Low pH Protein Digestion Kit. The data show that asparagine (highlighted in red) was deamidated in this peptide at pH 8. In contrast, deamidation was fully suppressed at low pH. **Panel B** shows an extracted ion chromatogram of a peptide with a scrambled disulfide bond from Panitumumab antibody digested under conventional conditions (pH 8) and at low pH with the AccuMAP™ Low pH Protein Digestion Kit. Disulfide bond scrambling is evident at pH 8 while it is fully suppressed at low pH. Both digests were analyzed with a Q Exactive™ (Thermo Fisher).

5.C. Protein Digestion at Low pH (continued)

Low pH Digestion with AccuMAP™ Low pH Resistant rLys-C

Trypsin is the protease of choice due to the optimal distribution of trypsin cleavage sites in proteins. However, digestion with rLys-C has advantages. In contrast to trypsin, AccuMAP™ Low pH Resistant rLys-C is not prone to generation of semi-specific peptides (see Section 5.D for details). Thus use of AccuMAP™ Low pH Resistant rLys-C eliminates the need for special precautions required to maintain low baseline noise during digest analysis. Our kit offers the option to digest proteins at low pH with rLys-C. rLys-C-generated peptides are just 1.4-fold longer than tryptic peptides, on average. Furthermore, in many proteins including IgGs, the majority of tryptic cleavage sites are lysines. For example, in IgGs, the lysine:arginine ratio is typically 3:1. In fact, Rituximab has 41 lysine and 12 arginine residues, and another therapeutic antibody, Panitumumab, has 35 and 12, respectively. Digestion of these proteins with Lys-C protease generates a peptide pattern very similar to the peptide pattern generated by trypsin.

Predigestion Step

Often, certain protein areas are resistant to proteolysis even after reducing disulfide bonds. This resistance is caused by a tightly folded conformation of these areas. Tight folding prevents protease access to internal cleavage sites. A common approach to overcome proteolytic resistance of tightly folded proteins or protein domains is predigestion with Lys-C protease under denaturing conditions (5). Digestion under denaturing conditions is possible due to the unique ability of Lys-C proteases to tolerate denaturing conditions. We used this property to ensure efficient digestion of proteolytically resistant domains. As with all other steps in this protocol, predigestion is performed at low pH. Subsequently a denaturing agent is diluted and digestion is completed with trypsin and rLys-C or optionally, rLys-C alone.

5.D. Baseline Noise in Tryptic Digest

Baseline noise is a common problem in tryptic digests. The major cause of this problem is trypsin overdigestion, which results in the generation of semi-tryptic peptides. Semi-tryptic peptides are the products of specific cleavage at lysine or arginine residue at one terminus and nonspecific cleavage (primarily at an aromatic residue) at the other terminus. Semi-tryptic peptides were evident in overnight trypsin digestions at 1:20 or lower trypsin:protein ratio in our study.

We offer two potential solutions to this problem. One solution is to remove trypsin from a reaction and digest a protein with the AccuMAP™ Low pH Resistant rLys-C alone. In contrast to trypsin, AccuMAP™ Low pH Resistant rLys-C does not generate semi-specific peptides.

An alternative solution is based on optimization of the trypsin:protein ratio and digestion time. In our experience, optimal digestion was achieved at a 1:5 trypsin:protein ratio and 3-hour incubation. Baseline noise was minimal and digestion was efficient at these conditions (Figure 5).

Autoproteolysis is another common source of baseline noise. AccuMAP™ Modified Trypsin and AccuMAP™ Low pH Resistant rLys-C protease included in the AccuMAP™ Low pH Protein Digestion Kit remain stable over the course of the digestion reaction.

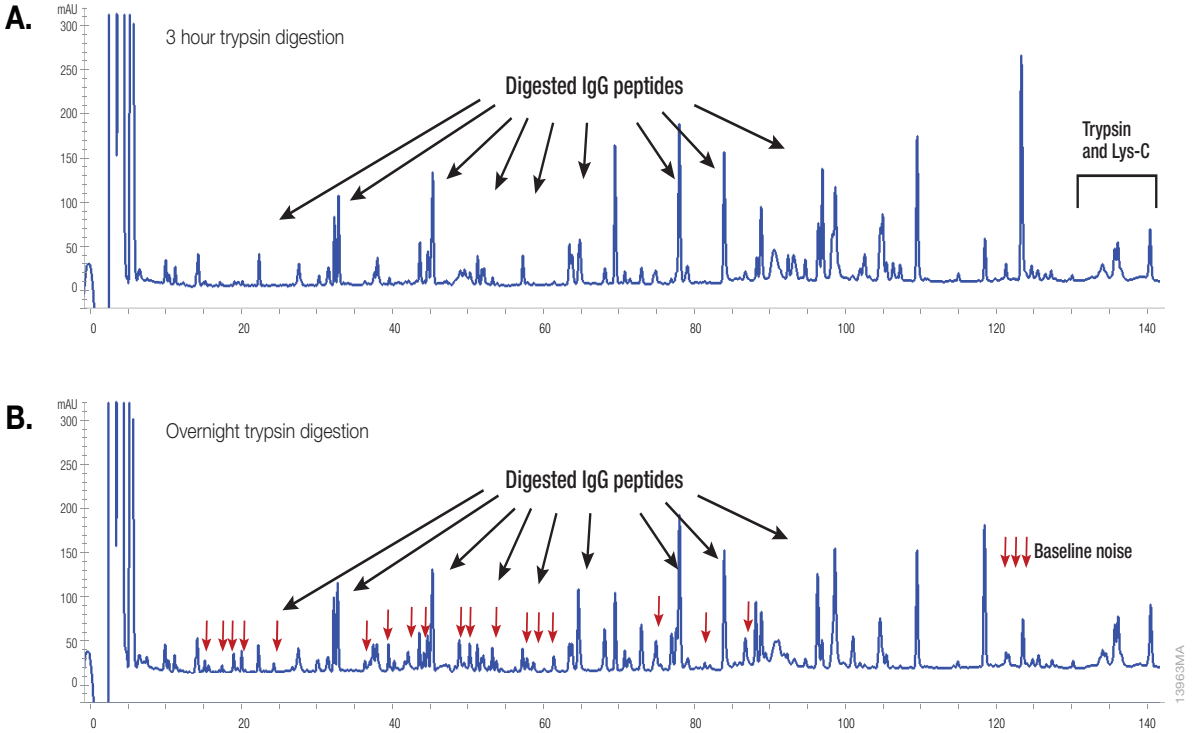

Figure 5. UV-HPLC chromatograms of Panitumumab digests. Panitumumab was predigested with AccuMAP™ Low pH Resistant rLys-C, then the reaction was diluted and digestion was completed by incubation with AccuMAP™ Modified Trypsin for 3 hours (**Panel A**) or overnight (**Panel B**). In this experiment we used a 1:5 trypsin:protein ratio. Note the accumulation of baseline noise for the overnight digest shown in Panel B.

5.E. Oxidation Suppression

Certain excipients and impurities have protein oxidation activity. For example, polysorbates, which are popular surfactants used to improve solubility of biotherapeutic proteins, decompose upon storage into hydrogen peroxide, which oxidizes proteins (3). Protein oxidation increases during proteolytic digestion due to the increased temperature. To suppress oxidation during digestion we recommend adding the optional AccuMAP™ Oxidation Suppressant (included in the kit).

You must clean up your sample prior to UV HPLC or LC/MS analysis if suppressant is used as this reagent can damage reverse phase columns. If you have to avoid a clean-up step, we recommend replacing the oxidation suppressant with methionine. In our experiments methionine suppressed protein oxidation activity, although the suppression efficiency was lower than the efficiency of the oxidation suppressant. We recommend adding methionine to a final concentration of 15mM in a digestion reaction.

5.F. Flexibility in Choice of Reagents and Procedural Steps

Reduction and Alkylation

We recommend TCEP and IAM for reduction and alkylation steps at low pH in sample preparation under reducing conditions. However, we have observed that DTT and IAA (iodoacetic acid) can provide similar modification efficiency at low pH in this procedure.

Proteolysis Optimization

Predigestion (with AccuMAP™ Low pH Resistant rLys-C protease) and digestion (with AccuMAP™ Low pH Resistant rLys-C and AccuMAP™ Modified Trypsin) steps should be performed for 1 hour and 3 hours, respectively. However, either step can be extended if required. For example, longer digestion is recommended if a protein is incompletely digested. Certain precautions should be taken in this case to avoid baseline noise. Generally, digestion with AccuMAP™ Low pH Resistant rLys-C can be extended for up to 20 hours with no signs of baseline noise. If trypsin digestion is required, first digest a protein with AccuMAP™ Low pH Resistant rLys-C for the desired amount of time, then add trypsin and digest for an additional 3–4 hours (see Sections 3.D and 4.D).

We generally do not recommend sample desalting prior to digestion. Desalting can compromise analytical reproducibility due to poor control over protein recovery and incomplete removal of denaturing agent. However, if desalting is desired, it can be incorporated into the protocol. Desalting-based procedures are described in Sections 3.C and 4.C.

6. Frequently Asked Questions (FAQs)

The following questions (Q) and answers (A) were developed by our researchers to aid your successful use of the AccuMAP™ Low pH Protein Digestion Kit.

Q: What pH is used by the kit?

A: Reduction and alkylation steps are performed at pH 5.6–5.8. pH is shifted to 5.2–5.4 at the digestion step.

Q: What denaturing agent is used in the AccuMAP™ Denaturing Solution?

A: Guanidine HCl (GuHCl).

Q: Reduction is commonly performed at 50°C or higher temperature. Why is reduction performed at 37°C in your procedure?

A: High temperature is used to unfold a protein and provide access of a reducing agent to disulfide bonds. We achieve the same goal using a denaturing agent. High temperature is avoided in our procedure because it can induce nonenzymatic PTMs and protein precipitation.

Q: Can I use DTT instead of TCEP?

A: Yes. We have found that DTT enables efficient reduction in our procedure.

Q: Can I use IAA (iodoacetic acid) instead of IAM in sample preparation procedure under reducing conditions?

A: Yes. Alkylation with IAA is typically >99% efficient at low pH in our procedure.

Q: Does the kit use Tris or phosphate buffer?

A: No.

Q: Is the reaction buffer compatible with mass spectrometry and reverse phase chromatography?

A: Yes. It is fully compatible with MS and RP HPLC. However, we recommend cleaning up a protein digest with solid phase extraction prior to analysis to remove salts coming from protein preparation steps.

Q: Can I use the kit to digest proteins at conventional conditions (pH 7.5–8.5)?

A: AccuMAP™ Low pH Resistant rLys-C and AccuMAP™ Modified Trypsin in this kit are designed to work at low pH. We do not guarantee digestion efficiency and stability of these proteases at conventional reaction conditions.

Q: Can I desalt protein prior to digestion?

A: Yes. See Sections 3.C and 4.C for protocol information.

7. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**.

Symptoms	Causes and Comments
Observed prominent peaks in the end of RP HPLC gradient.	The peaks could be AccuMAP™ Modified Trypsin and AccuMAP™ Low pH Resistant rLys-C. These proteases remain intact after digestion and elute in the end of the gradient (see Figure 5 for an UV HPLC chromatogram). We suggest conducting a control digestion reaction with a no substrate protein and analyzing by UV HPLC to verify which peaks are due to AccuMAP™ Modified Trypsin and AccuMAP™ Low pH Resistant rLys-C.
	If peaks still appear late in the gradient and do not match AccuMAP™ Modified Trypsin and AccuMAP™ Low pH Resistant rLys-C peaks, the cause may be incomplete digestion. In this case, refer to Sections 3.D or 4.D to improve digestion. Incomplete digestion could also occur if you desalt a protein prior to digestion. Desalting removes denaturing agent and certain protein areas may refold. Protein refolding can lead to proteolytic resistance due to obstruction of protease cleavage sites, which results in incomplete digestion. Try to digest the protein according to the above procedure first and desalt it after rather than before digestion.
Very few peaks appear in the gradient.	Digestion was severely inhibited. To improve digestion, be sure that you properly stored AccuMAP™ Modified Trypsin and AccuMAP™ Low pH Resistant rLys-C (at –65°C or below). These proteases will lose activity if stored at temperatures of –20°C or above. If proteases were stored at –20°C or above, please order a new kit.
	Protease inhibitors may be present in a protein sample. Perform dialysis or another clean up method prior to sample preparation.
Observed posttranslational modifications (PTMs).	If the kit was used as described in this Technical Manual, artificial nonenzymatic PTMs induced during sample preparation should be fully suppressed. If you still observe nonenzymatic PTMs, they are likely artifacts of manufacturing and/or storage.

7. Troubleshooting (continued)

Symptoms	Causes and Comments
Observed deterioration of reverse phase column performance including peptide peak broadening	Sample clean-up is recommended, particularly, if you use oxidation suppressant. This reagent can damage reverse phase columns and must be removed from the sample prior to analysis.

8. Appendix

8.A. Sample Preparation Protocol Tables

Table 1. Sample Preparation with Guanidine HCl Under Reducing Conditions.

		Concentration of Test Protein (mg/ml)		
		2	5	10
Denature and Reduce (30 minutes)	**Reagent**	**Volume to Add (µl)**		
	50µg Protein	25	10	5
	AccuMAP™ Denaturing Solution	40	24	20
	AccuMAP™ 10X Low pH Reaction Buffer	7	7	6
	100mM TCEP	2	1	1
Alkylate (30 minutes)	300mM IAM	4	2	2
Predigest (1 hour)	Water	12	0	0
	AccuMAP™ 10X Low pH Reaction Buffer	5	0	0
	AccuMAP™ Low pH Resistant rLys-C Solution	25	25	25
Dilute and Digest (3 hours)	AccuMAP™ 10X Low pH Reaction Buffer	20	12	10
	Water	132	62	44
	AccuMAP™ 100X Oxidation Suppressant	3	2	2
	AccuMAP™ Low pH Resistant rLys-C Solution	25	25	25
	AccuMAP™ Modified Trypsin Solution (replace with water if AccuMAP™ Low pH Resistant rLys-C only is used)	20	20	20
	Total Volume (µl)	320	190	160

8.A. Sample Preparation Protocol Tables (continued)

Table 2. Sample Preparation with Urea Under Reducing Conditions.

		Concentration of Test Protein (mg/ml)		
		2	5	10
Denature and Reduce	**Reagent**	**Volume to Add (µl)**		
	50µg Protein	25	10	5
	9M urea/1.4X AccuMAP™ Low pH Reaction Buffer	85	52	42
	100mM TCEP	2	1	1
Alkylate (30 minutes)	300mM IAM	4	2	2
Predigest (1 hour)	AccuMAP™ Low pH Resistant rLys-C Solution	25	25	25
Dilute and Digest (3 hours)	AccuMAP™ 10X Low pH Reaction Buffer	20	12	10
	Water	121	51	28
	AccuMAP™ 100X Oxidation Suppressant	3	2	2
	AccuMAP™ Low pH Resistant rLys-C Solution	25	25	25
	AccuMAP™ Modified Trypsin Solution (replace with water if rLys-C only is used)	20	20	20
	Total Volume (µl)	330	200	160

Table 3. Sample Preparation with Desalting by Size Exclusion Chromatography Under Reducing Conditions.

		Concentration of Test Protein (mg/ml)		
		2	5	10
	Reagent	**Volume to Add (µl)**		
Denature and Reduce (30 minutes)	50µg Protein	25	10	5
	AccuMAP™ Denaturing Solution	40	24	20
	AccuMAP™ 10X Low pH Reaction Buffer	7	7	6
	100mM TCEP	2	1	1
Alkylate (30 minutes)	300mM IAM	4	2	2
Desalt according to manufacturer's protocol and digest as described in Section 3.C.				

Table 4. Sample Preparation of Proteolytically Resistant Proteins Under Reducing Conditions.

		Concentration of Test Protein (mg/ml)		
		2	5	10
	Reagent	**Volume to Add (µl)**		
Denature and Reduce (30 minutes)	50µg Protein	25	10	5
	AccuMAP™ Denaturing Solution	40	24	20
	AccuMAP™ 10X Low pH Reaction Buffer	7	7	6
	100mM TCEP	2	1	1
Alkylate (30 minutes)	300mM IAM	5	3	2
Predigest (4 hours)	Water	12	0	0
	AccuMAP™ 10X Low pH Reaction Buffer	5	0	0
	AccuMAP™ Low pH Resistant rLys-C Solution	25	25	25
Dilute and Digest (overnight)	AccuMAP™ 10X Low pH Reaction Buffer	52	32	30
	Water	417	240	184
	AccuMAP™ 100X Oxidation Suppressant	5	3	2
	AccuMAP™ Low pH Resistant rLys-C Solution	25	25	25
Add Trypsin and Digest an Additional 3 Hours	AccuMAP™ Modified Trypsin Solution (replace with water if rLys-C only is used)	20	20	20
	Total Volume (µl)	640	390	320

8.A. Sample Preparation Protocol Tables (continued)

Table 5. Sample Preparation with Guanidine HCl Under Nonreducing Conditions.

		Concentration of Test Protein (mg/ml)		
		2	5	10
Denature and Block Free Cysteines (30 minutes)	**Reagent**	**Volume to Add (µl)**		
	50µg Protein	25	10	5
	AccuMAP™ Denaturing Solution	40	24	20
	AccuMAP™ 10X Low pH Reaction Buffer	7	7	6
	200mM NEM	3	2	2
Predigest (1 hour)	Water	15	0	0
	AccuMAP™ 10X Low pH Reaction Buffer	5	0	0
	AccuMAP™ Low pH Resistant rLys-C Solution	25	25	25
Dilute and Digest (3 hours)	AccuMAP™ 10X Low pH Reaction Buffer	20	12	10
	Water	135	65	47
	AccuMAP™ Low pH Resistant rLys-C Solution	25	25	25
	AccuMAP™ Modified Trypsin Solution (replace with water if rLys-C only is used)	20	20	20
	Total Volume (µl)	320	190	160

8.A. Sample Preparation Protocol Tables (continued)

Table 6. Sample Preparation with Urea Under Nonreducing Conditions.

		Concentration of Test Protein (mg/ml)		
		2	5	10
Denature and Reduce	**Reagent**	**Volume to Add (µl)**		
	50µg Protein	25	10	5
	9M urea/1.4X AccuMAP™ Low pH Reaction Buffer	85	52	42
	200mM NEM	3	2	2
Predigest (1 hour)	AccuMAP™ Low pH Resistant rLys-C Solution	25	25	25
Dilute and Digest (3 hours)	AccuMAP™ 10X Low pH Reaction Buffer	20	12	10
	Water	127	54	31
	AccuMAP™ Low pH Resistant rLys-C Solution	25	25	25
	AccuMAP™ Modified Trypsin Solution (replace with water if rLys-C only is used)	20	20	20
	Total Volume (µl)	330	200	160

Table 7. Sample Preparation with Desalting by Size-Exclusion Chromatography Under Nonreducing Conditions.

		Concentration of Test Protein (mg/ml)		
		2	5	10
Denature and Block Free Cysteines (30 minutes)	**Reagent**	**Volume to Add (µl)**		
	50µg Protein	25	10	5
	AccuMAP™ Denaturing Solution	40	24	20
	AccuMAP™ 10X Low pH Reaction Buffer	7	7	6
	200mM NEM	3	2	2
Desalt according to manufacturer's protocol and digest as described in Section 4.C.				

Table 8. Sample Preparation of Proteolytically Resistant Proteins Under Nonreducing Conditions.

		Concentration of Test Protein (mg/ml)		
		2	5	10
Denature and Block Free Cysteines (30 minutes)	**Reagent**	**Volume to Add (µl)**		
	50µg Protein	25	10	5
	AccuMAP™ Denaturing Solution	40	24	20
	AccuMAP™ 10X Low pH Reaction Buffer	7	7	6
	200mM NEM	3	2	2
Predigest (4 hours)	Water	15	0	0
	AccuMAP™ 10X Low pH Reaction Buffer	5	0	0
	AccuMAP™ Low pH Resistant rLys-C Solution	25	25	25
Dilute and Digest (overnight)	AccuMAP™ 10X Low pH Reaction Buffer	52	32	30
	Water	423	245	187
	AccuMAP™ Low pH Resistant rLys-C Solution	25	25	25
Add Trypsin and Digest an Additional 3 Hours	AccuMAP™ Modified Trypsin Solution (replace with water if AccuMAP™ Low pH Resistant rLys-C only is used)	20	20	20
	Total Volume (µl)	640	390	320

8.B. Preparation of Reagents and Solutions

AccuMAP™ Denaturing Solution

Completely thaw the frozen solution at room temperature or thaw in hand. Thoroughly mix with a vortex until completely dissolved. After thawing, store AccuMAP™ Denaturing Solution at room temperature for up to 6 months.

AccuMAP™ 10X Low pH Reaction Buffer

Completely thaw the frozen solution at room temperature or thaw in hand. Mix the solution with a vortex. After thawing, store AccuMAP™ 10X Low pH Reaction Buffer at 4°C–8°C for up to 6 months.

AccuMAP™ 100X Oxidation Suppressant Solution

Thaw a vial with the frozen solution at room temperature or thaw in hand, then mix the solution with a vortex. Place the vial into ice until ready to use. Take a required aliquot, snap-freeze the remaining solution in dry ice or in liquid nitrogen and return to a freezer (–65°C or below). To retain maximum activity, do not freeze and thaw more than three times.

AccuMAP™ Modified Trypsin Solution

Thaw a vial of the frozen solution by hand and gently mix with a vortex or by tapping the vial. Place the vial into ice until ready to use. Take a required aliquot, snap-freeze the remaining solution in dry ice or in liquid nitrogen and return to a freezer (–65°C or below). To retain maximum activity, do not freeze and thaw more than three times.

AccuMAP™ Low pH Resistant rLys-C Solution

Thaw a vial of the frozen solution by hand and gently mix with a vortex or by tapping the vial. Place the vial into ice until ready to use. Take a required aliquot, snap-freeze the remaining solution on dry ice or in liquid nitrogen and return to a freezer (–65°C or below). To retain maximum activity, do not freeze and thaw more than three times.

100mM TCEP (M.W. 286.65)

Briefly centrifuge vial to collect the powder. To 15mg of powder, add 520µl of NANOpure® water. Vortex to dissolve. Store at room temperature. Use within a few hours of preparation. Discard any unused TCEP.

300mM IAM (M.W. 184.96)

Briefly centrifuge vial to collect the powder. To 15mg of powder, add 270µl of NANOpure® water. Vortex to dissolve. Store at room temperature, protected from light. Use within a few hours of preparation. Discard any unused IAM.

9M urea (M.W. 60.06)/1.4X AccuMAP™ Low pH Reaction Buffer Mix

Weigh 540mg of urea in a 1.5ml vial. Add 140µl of AccuMAP™ 10X Low pH Reaction Buffer and water to 1ml. Vortex to dissolve. Warming the vial by hand or in a 37°C water bath may be needed to completely dissolve the urea.

200mM NEM (M.W. 125.13)

Briefly centrifuge vial to collect the powder. To 15mg of powder, add 600µl of NANOpure® water. Vortex to dissolve. Store at room temperature. Use within a few hours of preparation. Discard any unused NEM.

8.C. References

1. Hao, P. *et al*. (2011) Detection, evaluation and minimization of nonenzymatic deamidation in proteomic sample preparation. *Mol. Cell Prot*. **10(10)**:0111.009381. DOI: 10.1074/mcp.0111.009381.
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5. McDonald, W.H. *et al*. (2002) Comparison of three directly coupled HPLC MS/MS strategies for identification of proteins from complex mixtures: Single dimension LC-MS/MS, 2-phase MudPIT and 3-phase MudPIT. I*nt. J. Mass Spectrom*. **219,** 245–51.

8.D. Related Products

The following AccuMAP™ Low pH Protein Digestion Kit components are available separately:

Product	Size	Cat.#
AccuMAP™ Denaturing Solution	1ml	VA1000
AccuMAP™ 10X Low pH Reaction Buffer	1ml	VA1010
AccuMAP™ 100X Oxidation Suppressant	50µl	VA1020
AccuMAP™ Modified Trypsin	120µl	V5285
AccuMAP™ Low pH Resistant rLys-C Solution	300µl	VA1030
TCEP	15mg	VB1000
Iodoacetamide	15mg	VB1010

Additional Mass Spec Reagents and Tools

Product	Size	Cat.#
Trypsin/Lys-C Mix, Mass Spec Grade	20µg	V5071
	100µg	V5072
	100µg (5 × 20µg)	V5073
IdeS Protease	5,000 units	V7511
	25,000 units	V7515
IdeS Protease, Frozen	2,000 units	V7512
PNGase F	500 units	V4831
6 × 5 LC-MS/MS Peptide Reference Mix	50µl	V7491
	200pmol	V7495

8.E. Summary of Changes

The following changes were made to the 7/2018 revision of TM504:

The Predigest protocol step in Sections 3.A, 3.D, 4.A and 4.D was revised.