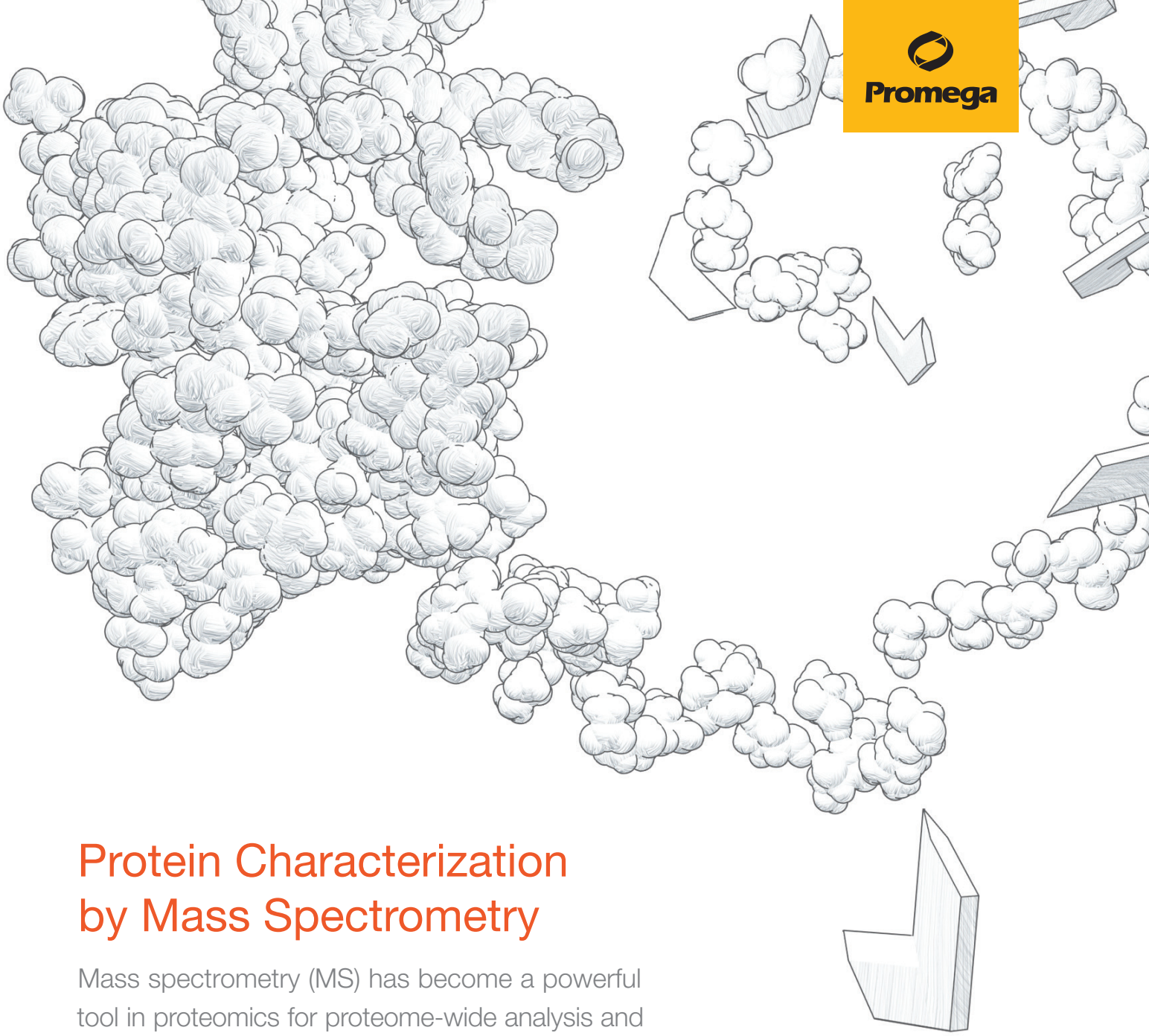
7 Protein Characterization by Mass Spectrometry



Protein Characterization by Mass Spectrometry

Mass spectrometry (MS) has become a powerful tool in proteomics for proteome-wide analysis and characterization of proteins from a variety of organisms and cell types. Recent advances in mass spectrometry provide tools for protein identification, protein characterization, relative and absolute quantitation, and the study of post-translational modifications and protein:protein interactions. Proteins are generally digested with proteases to generate peptides for MS analysis followed by sequencing (tandem MS). Promega provides high-quality proteases and other reagents that are critical to sample preparation for mass spectrometry.

7.1 Trypsin: Mass Spectrometry Sample Preparation

OVERVIEW

Trypsin is the most widely used protease in mass spectrometry sample preparation. It is a highly-specific serine protease, which cleaves at the carboxylic side of lysine and arginine residues. Protein digestion with trypsin generates peptides of optimal sizes for mass spec analysis. Tryptic peptides have a strong C-terminal charge, and therefore they can be efficiently ionized.

Trypsin is used for in-gel or in-solution digestion of proteins. Post digestion, the resulting peptides are introduced into the mass spectrometer and identified by *peptide mass fingerprinting or tandem mass spectrometry* (MS/MS). This approach is called "bottom-up" proteomics and uses identification at the peptide level to detect and characterize proteins.

The stringent specificity of trypsin is essential for characterizing proteins using mass spectrometry. Promega's high quality trypsin is derived from porcine trypsin and modified to give the highest proteolytic activity and cleavage specificity **(Figure 7.1)**.

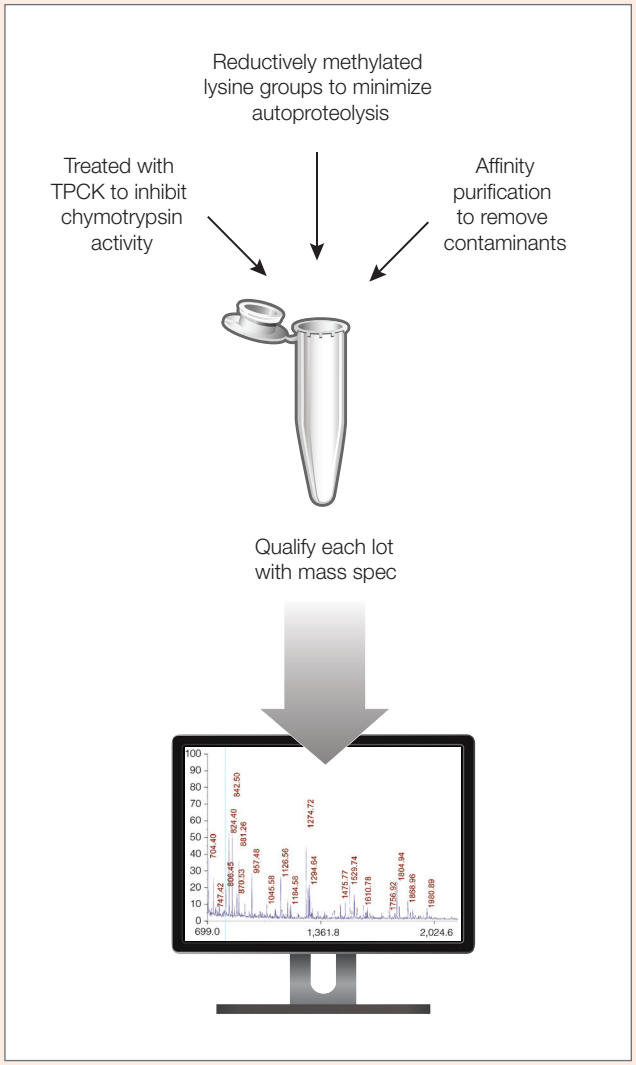


Figure 7.1. The high quality of Promega trypsin is achieved by chemical modification, affinity purification and strict lot-release criteria. Trypsin Gold, Mass Spectrometry Grade (Cat.# V5280) is mass spectrometry-qualified.

Trypsin Gold, Mass Spectrometry Grade

Digestion of proteins into peptides via in-gel or in-solution techniques, followed by mass spec. Typical applications include protein identification, protein quantitation and post-translation modifications via liquid chromatography-mass spectrometry/ mass spectrometry analysis.

Description

Trypsin Gold, Mass Spectrometry Grade, is manufactured to provide maximum specificity. Lysine residues in porcine trypsin are modified by reductive methylation, yielding a highly active and stable molecule that is extremely resistant to autolytic digestion. The specificity of the purified trypsin is further improved by TPCK treatment, which inactivates chymotrypsin. Additionally, the high performance of Trypsin Gold is assured by selecting affinity fractions with the highest proteolytic activity.

Principle

For in-gel digestion, protein samples are run on SDS-PAGE. Protein bands are stained and the bands of interest are excised. A buffer containing resuspended trypsin is added and the reaction is incubated overnight at 37°C. The resulting peptides are then extracted from the gel and analyzed by mass spec.

For in-solution digestion, protein samples are denatured, reduced and alkylated. Trypsin is added and the solution is incubated for 3-12 hours at 37°C. The resulting peptides are desalted and analyzed by mass spectrometry.

Features and Benefits

- **Application Qualified:** Each lot is qualified by mass spectrometry.
- **Pure:** Trypsin Gold is prepared by TPCK treatment followed by affinity purification.
- **Good Value:** Stable for up to 5 freeze-thaw cycles, thus minimizing reagent waste.
- **Referenced in Thousands of Papers:** Reliable and customer proven.

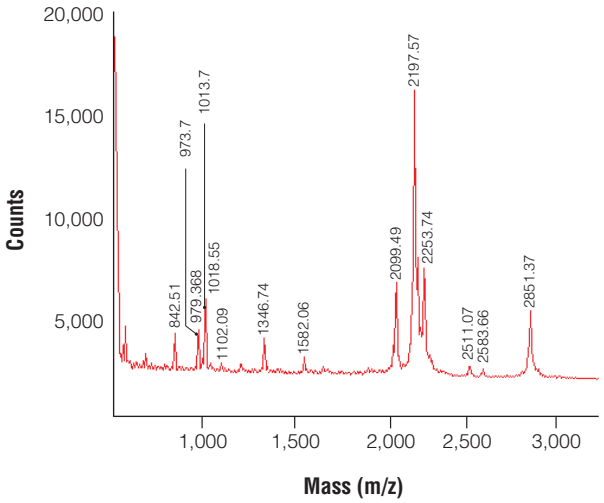


Figure 7.2. Spectrogram of bovine carbonic anhydrase II digested by Trypsin Gold, Mass Spectrometry Grade. A 500ng sample of carbonic anhydrase II was separated by gel electrophoresis and digested with 500ng of Trypsin Gold, Mass Spectrometry Grade, overnight at 37°C.*

Ordering Information

Trypsin Gold, Mass Spectrometry Grade (Cat.# V5280)

Trypsin/Lys-C Mix, Mass Spectrometry Grade

Enhanced in-solution protein digestion. Applications include: protein identification, protein quantitation and characterization of post-translation modifications via LC-MS/MS analysis.

Description

Trypsin/Lys-C Mix, Mass Spectrometry Grade, is a mixture of Trypsin Gold, Mass Spectrometry Grade, and rLys-C, Mass Spec Grade. The Trypsin/Lys-C Mix is designed to improve digestion of proteins or protein mixtures in solution. Replacing trypsin with Trypsin/Lys-C Mix has multiple benefits for protein analysis, including more accurate mass spectrometry-based protein quantitation and improved analytical reproducibility. Trypsin/Lys-C Mix also provides greater tolerance to trypsin-inhibiting agents, assuring efficient digestion of proteins for which purification is limited or not feasible.

Principle

Typical trypsin reactions do not digest proteins to completion, missing 10–30% of cleavage sites **(Figure 7.3)**. Incomplete digestion affects protein identification, reproducibility of mass spectrometry analysis and accuracy of protein quantitation. The number of missed cleavage sites may be even higher if the protein is not properly purified or contains protease-inhibiting contaminants. Using the conventional trypsin digestion protocol (i.e., overnight incubation under nondenaturing conditions), Trypsin/Lys-C Mix improves protein digestion by eliminating the majority of missed lysine cleavages. The mixture is stable since trypsin is modified and therefore resistant to digestion by Lys-C.

References

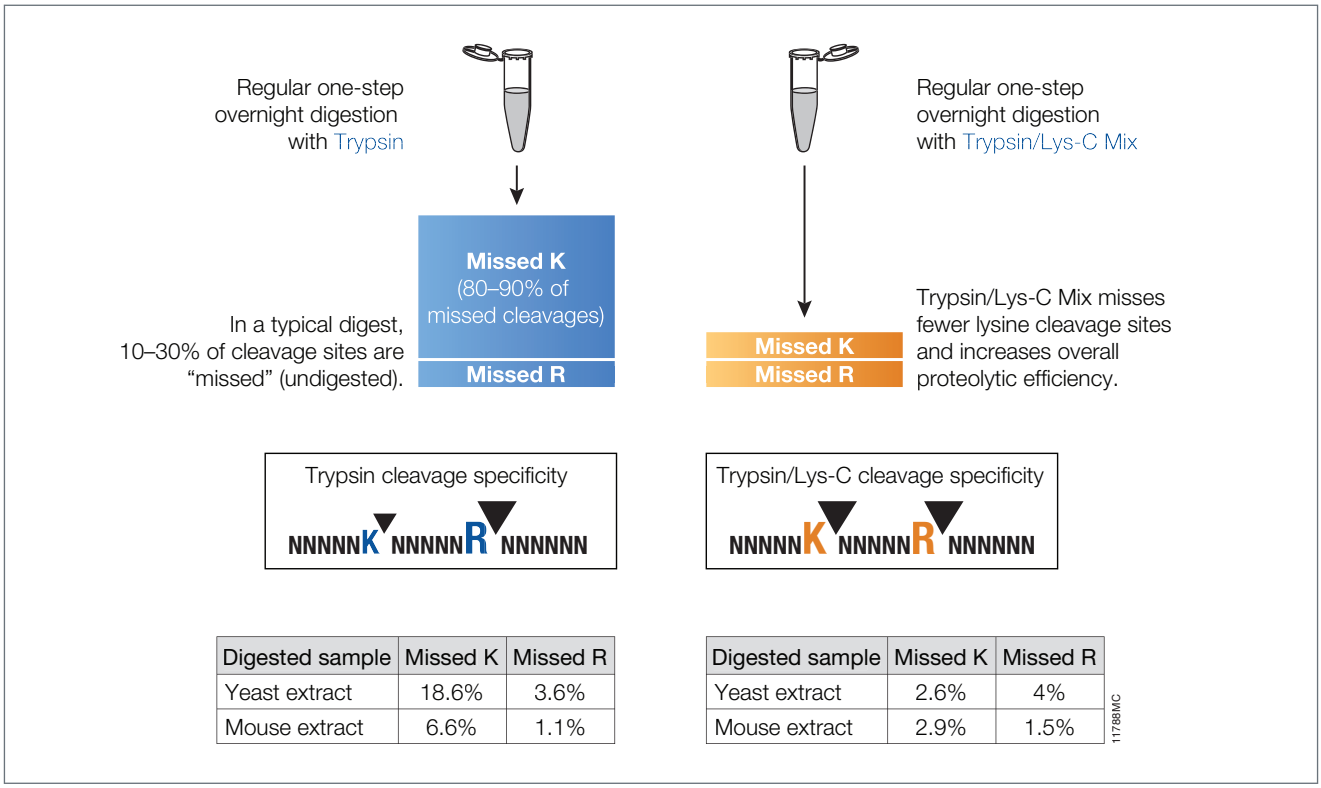
Saveliev, S. *et al.* (2013) Trypsin/Lys-C protease mix for enhanced protein mass spectrometry analysis. *Nature Methods* 10, Published online 30 November 2013. http://www.nature.com/app_notes/nmeth/2013/131211/pdf/nmeth.f.371.pdf

Features and Benefits

- **Simple to Use:** Use standard overnight digestion with non-denaturing conditions.
- **Enhanced Proteolysis:** Increase peptide recovery by enhancing cleavage efficiency at lysine residues and eliminating the majority of missed cleavages **(Figures 7.3 and 7.4)**.
- **Tolerant to Trypsin-Inhibiting Contaminants:** Generate mass spectrometry data from low-quality sample material.

Ordering Information

Trypsin/Lys-C Mix, Mass Spec Grade
(Cat.# V5071, V5072, V5073)



Digested sample	Missed K	Missed R
Yeast extract	18.6%	3.6%
Mouse extract	6.6%	1.1%

Digested sample	Missed K	Missed R
Yeast extract	2.6%	4%
Mouse extract	2.9%	1.5%

Figure 7.3. Side-by-side comparison of cleavage sites missed by trypsin or the Trypsin/Lys-C Mix using a standard digestion protocol.

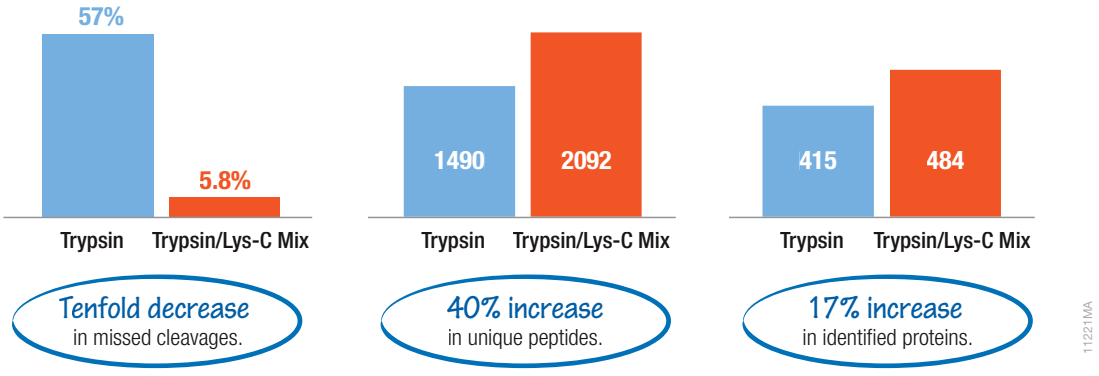


Figure 7.4. Improved mass spec analysis of a protein extract containing residual amounts of methanol and chloroform, which inhibit trypsin but have no effect on Trypsin/Lys-C Mix performance.

Sequencing Grade Modified Trypsin

Digestion of proteins into peptides via in-gel or in-solution techniques, followed by mass spec analysis. Typical applications include protein identification, protein quantitation and analysis of post-translation modifications via LC-MS/MS analysis.

Description

Trypsin is a serine protease that specifically cleaves at the carboxylic side of lysine and arginine residues. The stringent specificity of trypsin is essential for protein identification. Native trypsin is subject to autoproteolysis, generating pseudo-trypsin, which exhibits a broadened specificity including a chymotrypsin-like activity. Such autoproteolysis products present in a trypsin preparation could result in additional peptide fragments that may interfere with database analysis of the mass of fragments detected by mass spectrometry. Sequencing Grade Modified Trypsin is chemically modified to prevent auto-proteolysis and assure high proteolytic activity over the course of digestion reaction.

Principle

For in-gel digestion, protein samples are run on SDS-PAGE. Protein bands are stained and bands of interest are excised. A buffer containing resuspended trypsin is added and incubated overnight at 37°C. The resulting peptides are then extracted from the gel and analyzed by mass spec.

For in-solution digestion, protein samples are denatured, reduced and alkylated. Trypsin is then added and the solution is incubated for 3-12 hours at 37°C. The resulting peptides are desalted and analyzed by mass spec.

Features and Benefits

- **Maximum Specificity and Purity:** Trypsin is prepared by TPCK treatment followed by affinity purification.
- **High Stability:** Due to reductive methylation of lysine residues.
- **Reliable and Customer-Proven:** Referenced in more than 2,000 publications.
- **Additional Value:** Stability is ensured for up to 5 freeze-thaw cycles, minimizing leftover reagents.
- **Variety of Packaging:** We offer flexibility in packaging depending on experimental design and scope.

Ordering Information

Sequencing Grade Modified Trypsin (Cat.# V5111, V5117)

Sequencing Grade Modified Trypsin, Frozen (Cat.# V5113)

References

Trask. S. *et al.* (2013) Mutations in the rotavirus spike protein VP4 reduce trypsin sensitivity but not viral spread. *J. Gen, Vir.* **94**, 1296-300.

Trautner. C. *et al.* (2013) The sll1951 gene encodes the surface layer protein of Synechocystis sp. strain PCC 6803. *J. Bact.* **195**(23), 5370-80.

Lord, M. *et al.* (2013) Sulfation of the bikunin chondroitin sulfate chain determines heavy chain·hyaluronan complex formation. *J. Biol. Chem.* **288**(22), 22930-41.

Gordon,S. *et al.* (2013) Multi-dimensional co-separation analysis reveals protein-protein interactions defining plasma lipoprotein subspecies. *Mol. Cell, Prot.* **12**(11), 3123-34.

Jiang,W. *et al.* (2013) Mass spectrometry method to identify aging pathways of Sp- and Rp-tabun adducts on human butyrylcholinesterase based on the acid labile P-N bond. *Toxicol. Sci.* **132**(2), 390-98.

Burkhart J. *et al.* (2012) Systematic and quantitative comparison of digest efficiency and specificity reveals the impact of trypsin quality on MS-based proteomics. *J. Proteomics* **75**(4), 1454-1462.

Immobilized Trypsin

Protein digestion of simple or complex protein mixtures over a wide range of concentrations.

Description

Trypsin in solution is the most widely used protease for digestion of proteins. However, there are a few limitations in using free trypsin. The limitations include:

1) The digestion requires a long time (three hours to overnight).
2) High concentrations of trypsin cannot be used because of the generation of dominant trypsin autolytic fragments in the digested samples.

To overcome these limitations we offer Immobilized Trypsin. It provides a fast and convenient method for protein digestion.

Principle

Immobilized Trypsin reduces digestion time and allows easy removal of trypsin from the digestion reaction **(Figure 7.5)**. Immobilized Trypsin provides the ability to digest 20–500µl of protein in solution simply by adjusting the amount of resin used in the reaction. This flexibility facilitates digestion while decreasing potential trypsin interference in downstream sample analysis. The percentage of digestion and sequence coverage is comparable to overnight digestion with free trypsin. Immobilized Trypsin has been used to study protein expression profiling in serum, microwave-assisted digestion of proteins, phoshopeptide analysis, analysis of membrane proteins and for $^{18}O/^{16}O$ labeling of peptides.

Features and Benefits

- **Fast:** Digestion can be accomplished in as little as 30 minutes.
- **Scalable:** Easily adjustable protocol to accommodate various protein concentrations.
- **Easy Setup:** Minimal centrifugation steps.

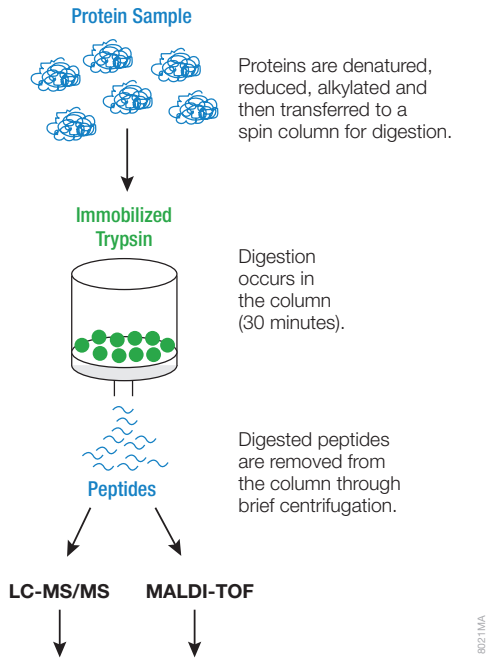


Figure 7.5. Overview of the Immobilized Trypsin digestion protocol.

Ordering Information

Immobilized Trypsin (Cat.# V9012, V9013)

7.2 Alternative Proteases for Mass Spectrometry

OVERVIEW

Trypsin is the most widely used endoprotease. It cleaves proteins in a highly specific manner and generates peptides 7–20 amino acids in length, with a strong C-terminal charge, making trypsin ideal for mass spectrometry analysis.

However, there are certain proteins and protein mixtures where trypsin digestion alone is not efficient enough. Examples include digestion of membrane proteins and analysis of histone post-translational modifications (PTMs). Furthermore, certain tryptic peptides are too small or too large for mass spec analysis. In these situations alternative proteases provide a viable solution, either alone or in combination with trypsin.

Table 7.1. Alternative Proteases and Corresponding Cleavage Sites.

Protease	Cleavage Site	Optimal pH	Example of Use
Lys-C Specific Protease	NNNN**K**⌄NNN (**K** is lysine)	7.0–9.0	Digests membrane and other proteolytically resistant proteins; generates larger peptides than trypsin, an advantage for certain mass spec methods (for example, ETD).
Arg-C Specific Protease	NNNN**R**⌄NNN (**R** is arginine) Arg-C can, at a lesser degree, cleave at lysine also.	7.6–7.9	Facilitates analysis of histone post-translational modifications; used in proteome-wide analysis.
Glu-C Specific Protease	NNNN**E**⌄NNN (**E** is glutamate) Glu-C can, at a lesser degree, cleave at aspartate residue also.	4.0–9.0	Used as alternative to trypsin if trypsin produces too short or too long peptides or if tryptic cleavage sites are not accessible.
Asp-N Specific Protease	NNNN⌄**D**NNN (**D** is aspartate)	4.0–9.0	Similiar to Glu-C.
Chymotrypsin Low Specific Protease	NNNN(**F**/**Y**/**W**)⌄NNN (**F**,**Y** and **W**) are aromatic residues phenylalanine, tyrosine and tryptophan)	7.0–9.0	Digests hydrophobic proteins (for example, membrane proteins).
Pepsin Nonspecific Protease	Nonspecific Protease (advantage: most active at low pH)	1.0–3.0	Used in structural protein studies and antibody analysis; digests proteolytically resistant, tightly folded proteins.
Thermolysin Nonspecific Protease	Nonspecific Protease (advantage: remains active at high temperature)	5.0–8.5	Digests proteolytically resistant, tightly folded proteins; used in structural protein studies.
Elastase Nonspecific Protease	Nonspecific Protease	9.0	Used to increase protein coverage.

Digestion with Alternative Proteases Increase Protein Coverage

Digestion with an alternative protease, individually or in combination with trypsin, creates a unique peptide map that may include sequences not seen with trypsin digestion alone. Overlaying peptides obtained with alternative proteases and trypsin, increases protein coverage and overall confidence in protein identification **(Figure 7.6)**.

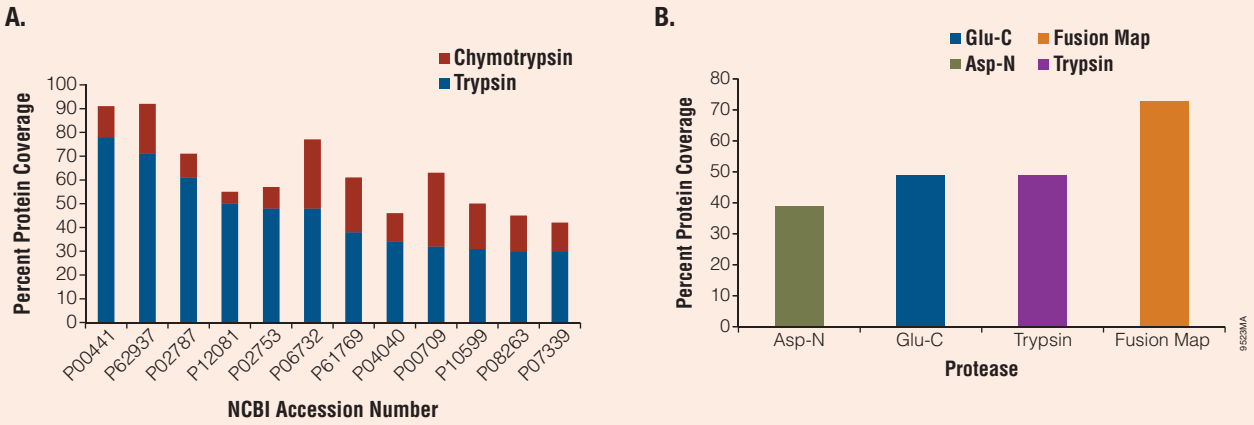


Figure 7.6. Increased protein coverage using **(Panel A)** both trypsin and chymotrypin; **(Panel B)** multiple proteases in separate digestion reactions. Note that the fusion map is the combination of the results obtained by digestion with each protease alone.

Alternative Proteases Improve Analysis of Post-Translational Modifications (PTMs)

In certain cases, trypsin is not suitable for PTM analysis. For example, histone PTMs are complex and some, such as acetylation and methylation, prevent trypsin digestion. As shown in **Figure 7.7**, trypsin digestion of histone H4 identified several PTMs; however, certain PTMs were missing. By digesting histone H4 with Arg-C, additional PTMs were identified, including mono- and dimethylated, and acetylated lysine and arginine residues.

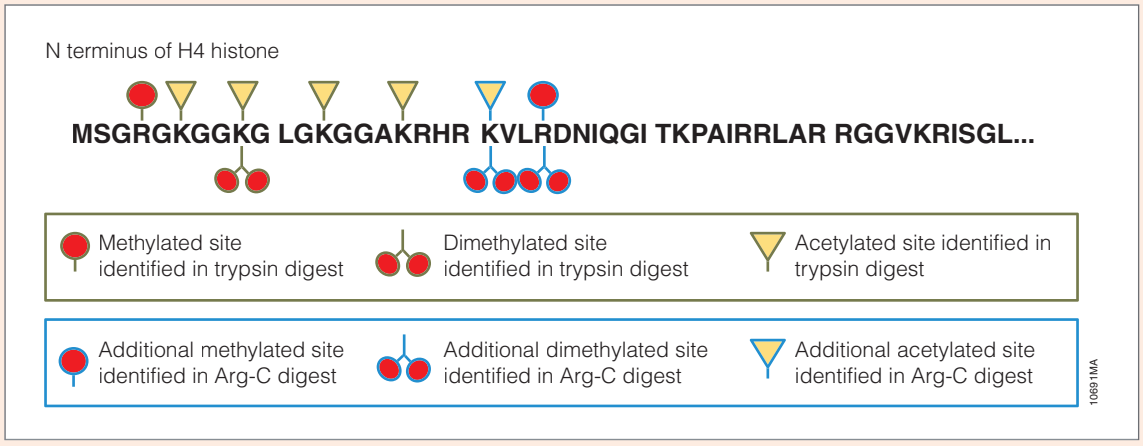


Figure 7.7. Histone H4 post-translational modifications identified in trypsin and Arg-C digests.

Alternative Proteases

Improve protein sequence coverage, enhance digestion of tightly-folded proteins, improve the identification of post-translation modifications (PTMs).

Specific Proteases

Arg-C (Clostripain), Sequencing Grade is an endopeptidase that cleaves at the C-terminus of arginine residues, including the sites next to proline. Arg-C activity is optimal in the pH range of 7.6–7.9.

Asp-N, Sequencing Grade is an endoproteinase that hydrolyzes peptide bonds on the N-terminal side of aspartic acid residues. Asp-N activity is optimal at pH 4.0–9.0.

Glu-C, Sequencing Grade is a serine protease that specifically cleaves at the C-terminus of glutamic acid residues. In ammonium bicarbonate and ammonium acetate the enzyme specificity is higher at the glutamic residues. In phosphate buffers cleavage occurs at the aspartic and glutamic residues. Glu-C activity is optimal at pH 4.0–9.0.

Endoproteinase Lys-C, Sequencing Grade is a serine protease isolated from *Lysobacter enzymogenes* as a highly purified protease, that hydrolyzes specifically at the C-terminus of Lys residues. Lys-C activity is optimal at pH 7.0–9.0.

rLys-C, Mass Spec Grade is a recombinant Lys-C expressed in *E. coli*. The sequence origin of rLys-C is Protease IV from *Pseudomonas aeruginosa*. Similarly to a native Lys-C, rLys-C cleaves at the C-terminus of lysine residues with exceptional specificity. rLys-C retains proteolytic activity under protein denaturing conditions such as 8M Urea, used to improve digestion of proteolytically resistant proteins. rLys-C activity is optimal at pH 8.0–9.0.

Low-specific Proteases

Chymotrypsin, Sequencing Grade is a highly purified serine endopeptidase derived from bovine pancreas that preferentially hydrolyzes at the carboxyl side of aromatic amino acids: tyrosine, phenylalanine and tryptophan. Chymotrypsin activity is optimal at pH 7.0–9.0.

Nonspecific Proteases

Elastase is a serine protease that preferentially cleaves at the C-terminus of alanine, valine, serine, glycine, leucine or isoleucine. Elastase activity is optimal at pH 9.0.

Pepsin preferentially cleaves at the C-terminus of phenylalanine, leucine, tyrosine and tryptophan. Pepsin activity is optimal at pH 1.0–3.0.

Thermolysin is a thermostable metalloproteinase. Thermolysin preferentially cleaves at the N-terminus of the hydrophobic residues leucine, phenylalanine, valine, isoleucine, alanine and methionine. The optimal digestion temperature range is 65–85°C. Thermolysin activity is optimal at pH 5.0–8.5.

Poster

Enhancing Trypsin Digestion with Lys-C and Arg-C Proteases. Promega Scientific Poster: **www.promega.com/resources/scientific_posters/posters/enhancing-trypsin-digestion-with-lysc-and-argc-proteases-scientific-poster/**

Alternative Proteases for Mass Spectrometry

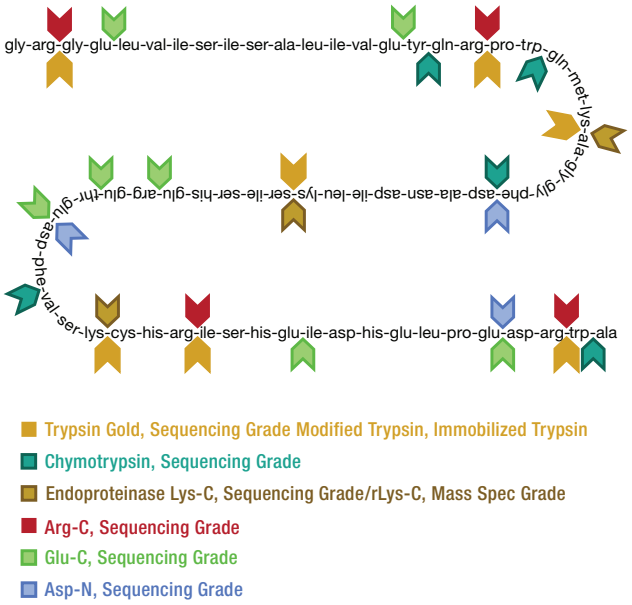


Figure 7.8. Cleavage sites of proteases frequently used in mass spectrometry sample preparation.

Ordering Information

Specific Proteases:

Arg-C (Clostripain), Sequencing Grade (Cat.# **V1881**)

Asp-N, Sequencing Grade (Cat.# **V1621**)

Glu-C, Sequencing Grade (S. aureus V8; Cat.# **V1651**)

Endoproteinase Lys-C, Sequencing Grade (Cat.# **V1071**)

rLys-C, Mass Spec Grade (Cat.# **V1671**)

Low-specific Proteases:

Chymotrypsin, Sequencing Grade (Cat.# **V1061, V1062**)

Nonspecific Proteases:

Elastase (Cat.# **V1891**)

Pepsin (Cat.# **V1959**)

Thermolysin (Cat.# **V4001**)

7.3 Glycosidases

OVERVIEW

Glycosylation is the most abundant post-translational modification (PTM) in eukaryotic proteins. Glycosylation structures on proteins have diverse functions and can serve as disease biomarkers, such as for the progression of cancer. Therefore, tools that facilitate glycoprotein characterization are extremely important.

Endoglycosidases and exoglycosidases are tools for structural and functional analysis of glycoproteins. Endoglycosidases like PNGase F **(see Figure 7.9)** and Endo H are routinely used to remove carbohydrates from N-glycosylated proteins. For the analysis of O-glycosylated proteins, a mixture of endoglycosidases and exoglycosidases is recommended.

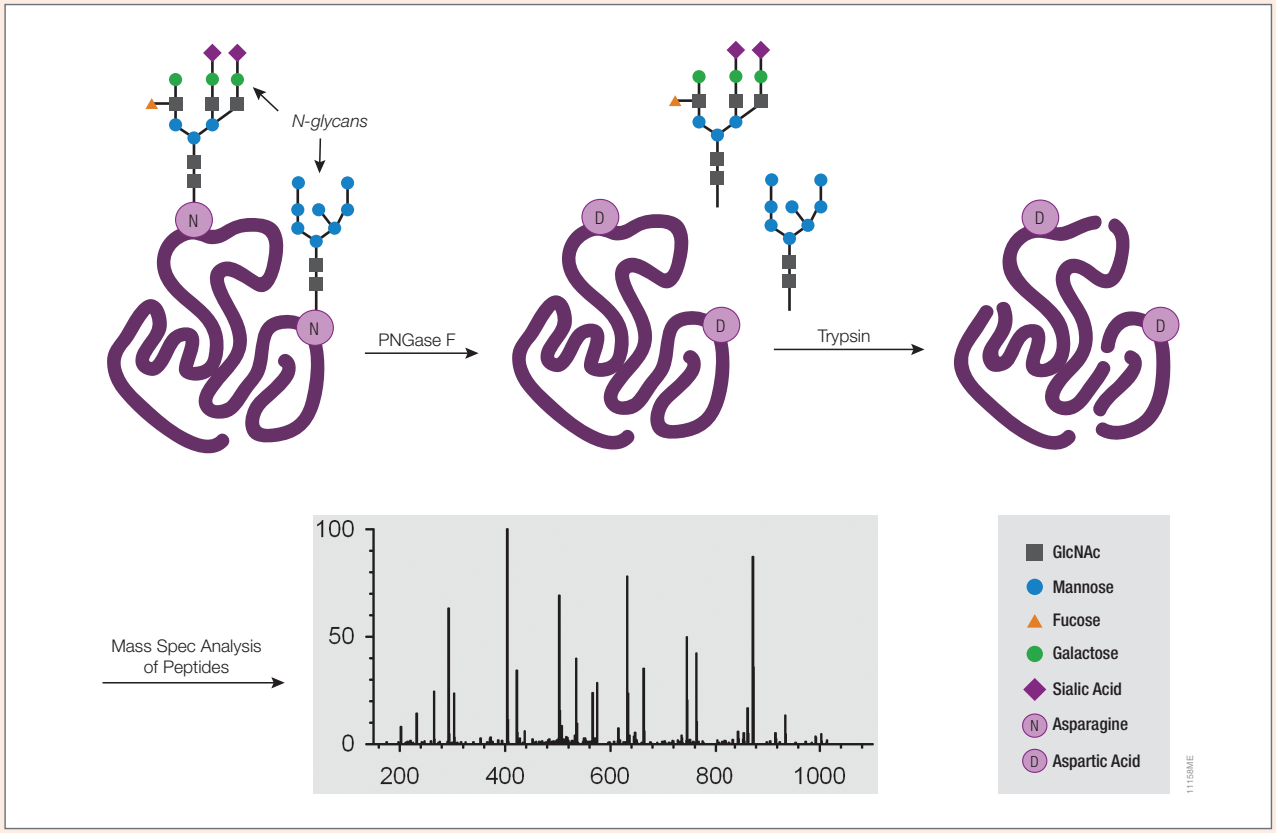


Figure 7.9. Identification of N-glycosylated sites in proteins. Asn-linked glycans can be cleaved enzymatically by PNGase F, yielding intact oligosaccharides and a protein in which Asn residues at the site of N-glycosylation are converted to Asp.

Glycosidases

Identification of glycosylation sites by LC-MS/MS; characterization of glycoproteins and glycan structures; monitor protein trafficking (endoplasmic reticulum, golgi); correlation of structural and functional data.

Description

PNGase F is a recombinant glycosidase cloned from *Elizabethkingia miricola* and overexpressed in *E. coli.* PNGase F has a molecular weight of 36kDa. PNGase F catalyzes the cleavage of N-linked oligosaccharides between the innermost N-Acetylglucosamine (GlcNAc) and asparagine residues (Asn) of high mannose, hybrid, and complex oligosaccharides from N-linked glycoproteins **(Figure 7.10)**. Due to the enzymatic reaction, intact oligosaccharides are released and Asn residues are converted to aspartic acids (Asp) at the former sites of N-glycosylation. The conversion (deamination) of Asn to Asp leads to a monoisotopic mass shift of 0.9840 Da that is used in tandem mass spectrometry (MS/MS) for the identification of N-glycosylated sites in proteins. PNGase F will not remove oligosaccharides containing α-(1,3)-linked core fucose commonly found on plant glycoproteins.

Endo H is a recombinant glycosidase cloned from *Streptomyces plicatus* and overexpressed in *E. coli.* Endo H cleaves within the chitobiose core of high mannose and some hybrid oligosaccharides from N-linked glycoproteins. In contrast to PNGase F, Endo H does not cleave N-glycosylated proteins of the complex type. The enzymatic activity of EndoH leaves one GlcNac residues at the N-glycosylated protein site leading to a shift in molecular weight (M.W.). This M.W. shift is used in mass spectrometry for the identification of N-glycosylated sites.

Protein Deglycosylation Mix is a mixture of five protein deglycosidases (PNGase F, O-Glycosidase, Neuraminidase, β1-4 Galactosidase, β-N- Acetylglucosaminidase) capable of removing glycans from both O-linked and N-linked glycosylation sites. Fetuin is provided as a deglycosylation substrate control.

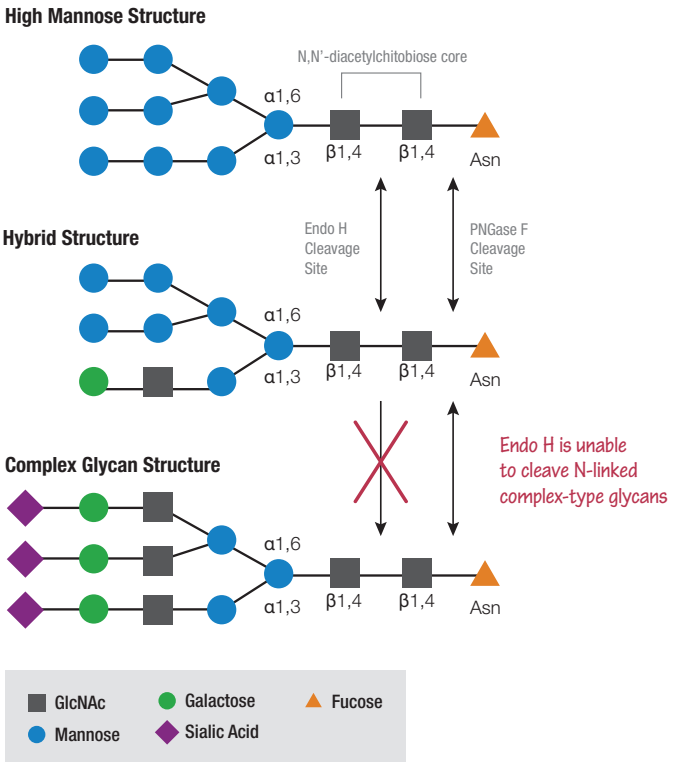


Figure 7.10. Cleavage specificity of PNGase F and Endo H on N-glycans.

Glycosidases (continued)

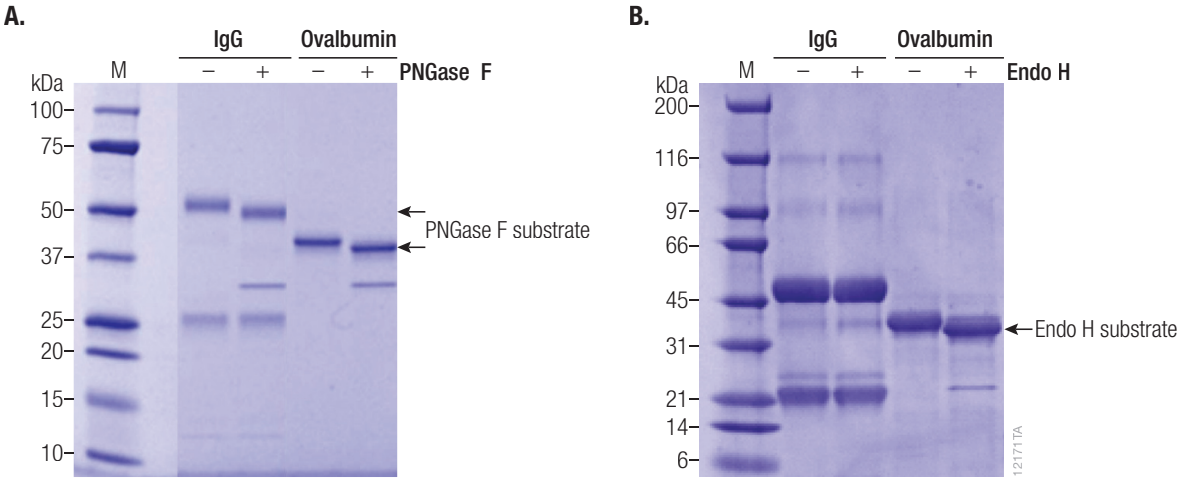


Figure 7.11. Detection of N-glycosylated proteins in SDS-PAGE. After glycan cleavage, the respective proteins show a higher mobility in SDS-PAGE. The application of both PNGase F and Endo H is informative for the presence of complex glycan structures, as shown here for Immunoglobulin G (IgG).

Ordering Information

PNGase F (Cat.# **V4831**)

Endo H (Cat.# **V4871, V4875**)

Protein Deglycosylation Mix (Cat.# **V4931**)

Fetuin (Cat.# **V4961**)

7.4 ProteaseMAX™ Surfactant

OVERVIEW

Detergents are routinely used for protein extraction, solubilization and/or denaturation. However, detergents are known to interfere with mass spectrometry analysis. ProteaseMAX™ Surfactant is a mass spec-compatible detergent that can be used for improved protein extraction from cells and tissues, for protein solubilization/denaturation as well as for improved in-gel and in-solution protein digestion. Unlike other detergents/denaturants such as SDS and Urea, ProteaseMAX™ Surfactant is compatible with MS analysis. It degrades over the course of digestion reaction. Samples can then be easily analyzed with mass spectrometry.

A comparison of different detergents in cell-lysis buffers (Urea, SDC, ProteaseMAX™ Surfactant) revealed that ProteaseMAX™ Surfactant is optimal for efficient cell lysis/protein extraction for mass spectrometry sample preparation. The results of Pirmoradian *et al.* suggest that the addition of ProteaseMAX™ Surfactant to the cell-lysis buffer/extraction buffer provides the highest number of peptide/protein identifications **(Table 7.2)**.

References

Pirmoradian, M. *et al.* (2013) Rapid and deep human proteome analysis by single-dimension shotgun proteomics. *Mol. Cell. Prot.* **12**, 3330–8.

Table 7.2. Comparison of Cell-lysis/Protein Extraction Methods

Cell-lysis/ Extraction Buffer	Number of Peptides	Number of Proteins
Urea	17,024 ± 148	3,326 ± 20
SDC*	22,171 ± 403	3,698 ± 18
ProteaseMAX™ Surfactant	29,884 ± 228	4,465 ± 100
ProteaseMAX™ Surfactant and Cell Debris**	33,098 ± 283	4,655 ± 51

*SDC: Sodium desoxycholate.

** Cell debris was included in the trypsin digestion step.

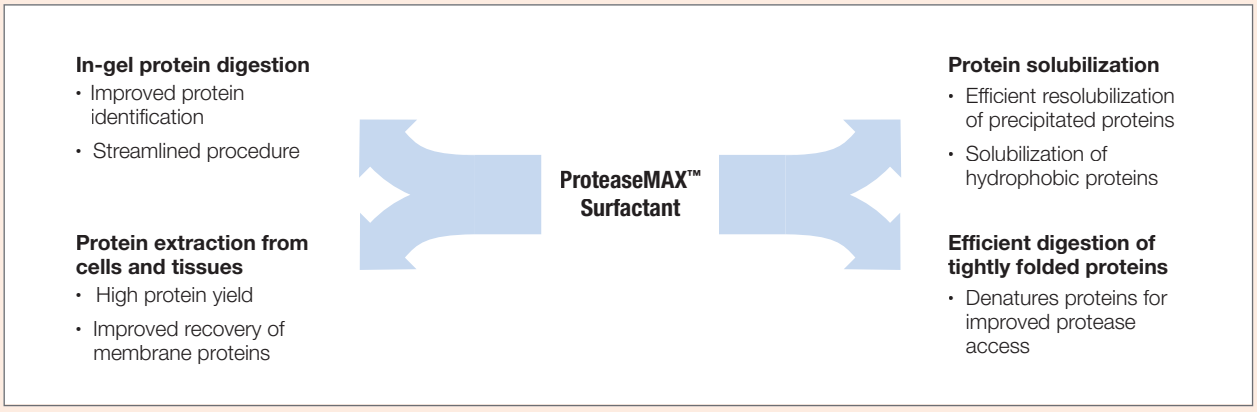


Figure 7.12. Multiple applications of ProteaseMAX™ Surfactant for improved protein mass spectrometry sample preparation.

ProteaseMAX™ Surfactant, Trypsin Enhancer

Enhanced in-gel and in-solution protein digestions; protein solubilization/ denaturation; protein extraction from cells and tissues.

Description

ProteaseMAX™ Surfactant, Trypsin Enhancer is designed to improve in-gel and in-solution protein digestions. It ensures fast and efficient protein digestion with proteases such as Trypsin, Chymotrypsin and Lys-C. ProteaseMAX™ Surfactant degrades over the course of a digestion reaction **(Figure 7.13)**, resulting in products that are compatible with mass spectrometry (MS) and liquid chromatography (LC). No long-term negative effect of the residual surfactant on the ion optics and capillary of mass spectrometry instruments has been observed. ProteaseMAX™ Surfactant can be used with existing in-gel or in-solution digestion protocols.

Principle

In-gel protein digestion is the major application for ProteaseMAX™ Surfactant. Benefits for in-gel digestion include increased peptide recovery from gels, minimized peptide absorption to plasticware and a streamlined digestion procedure. Trypsin and ProteaseMAX™ Surfactant (final concentration 0.01%) are added to a gel slice containing the protein band of interest. The digestion is incubated at 50°C for 1 hour. ProteaseMAX™ Surfactant also improves recovery of longer peptides that are retained in the gel under a standard extraction protocol.

ProteaseMAX™ Surfactant solubilizes proteins, including difficult proteins (i.e., membrane proteins) and enhances in-solution protein digestion by providing a denaturing environment. For cytoplasmic proteins, add ProteaseMAX™ Surfactant to a final concentration of 0.03%; for membrane proteins add to a final concentration 0.05%.

Features and Benefits

- **No Interference with Mass Spec Analysis:** Avoid the negative effects of common solubilizing agents (such as SDS).
- **Improved Peptide Recovery from Gels:** Protein sequence coverage and identification increased.
- **Enhanced Protein Solubilization :** Avoid high temperature.
- **Enhanced proteolysis rate:** Better access to cleavage sites.
- **Degrades Over Course of Digestion Reaction:** No need for post-digestion inactivation.

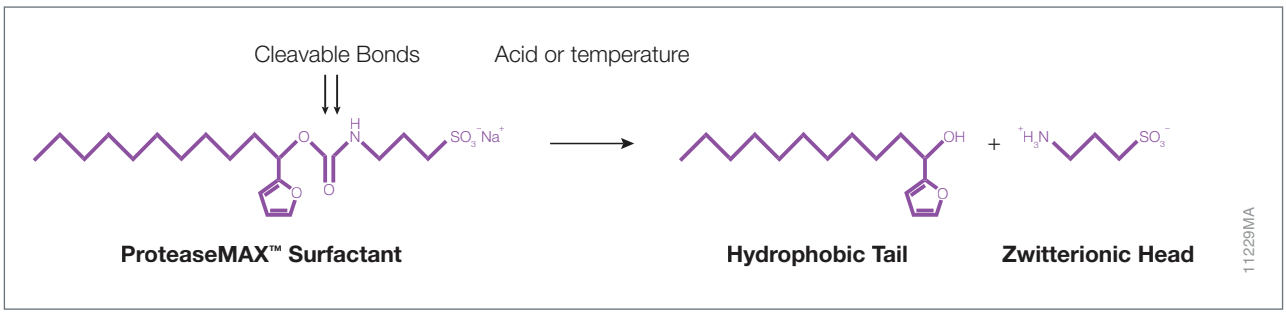


Figure 7.13. Chemical Structure of ProteaseMAX™ Surfactant and its decomposition pathway.

ProteaseMAX™ Surfactant

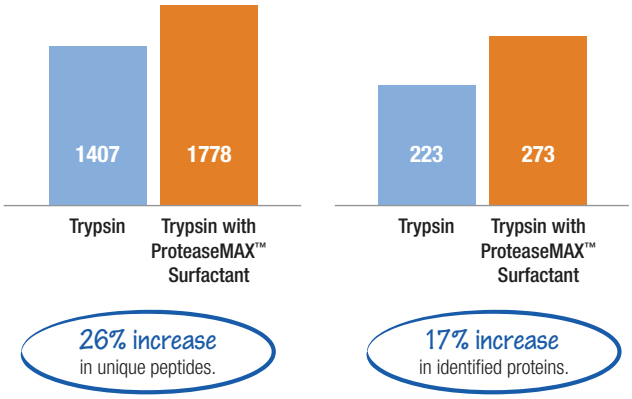


Figure 7.14. Example of improved peptide/protein identifications when using ProteaseMAX™ Surfactant for in-gel digestion of a complex protein sample.

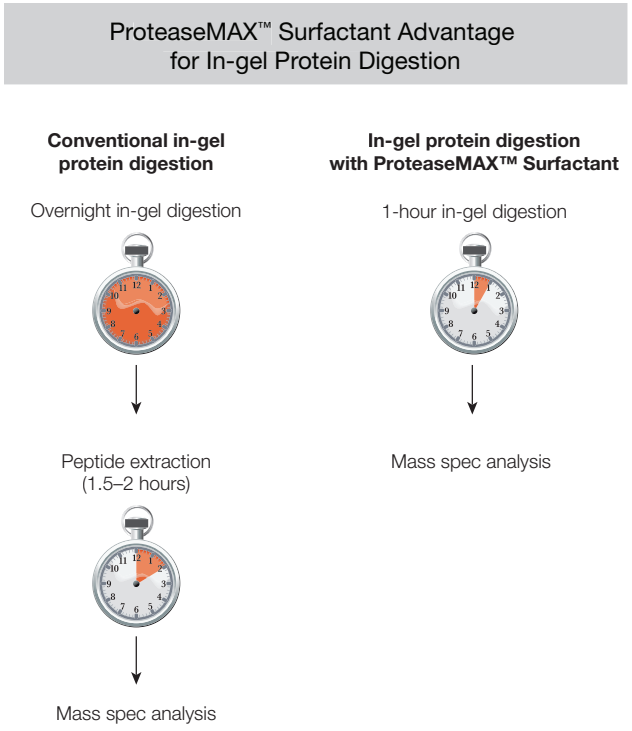


Figure 7.15. Comparison of ProteaseMAX ™ Surfactant-assisted versus standard in-gel digestion procedure ProteaseMAX™ Surfactant offers time and labor savings.

References

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Kalashnikova, A. *et al.* (2013) Linker histone H1.0 interacts with an extensive network of proteins found in the nucleolus. *Nucl. Acids. Res.* **41**(7), 4026-35.

Silva, C. *et al.* (2013) Interaction of Mycobacterium leprae with human airway epithelial cells: adherence, entry, survival, and identification of potential adhesins by surface proteome analysis. *Infect. Immun.* **81**(7), 2645-59.

Pearson, R. *et al.* (2013) Regulation of H_2O_2 stress-responsive genes through a novel transcription factor in the protozoan pathogen Entamoeba histolytica. *J. Biol. Chem.* **288**(6), 4462-74.

Ordering Information

ProteaseMAX™ Surfactant, Trypsin Enhancer (Cat.# **V2071, V2072**)

7.5 Protein Extracts for LC/MS Instrument Performance Monitoring

OVERVIEW

Adequate monitoring of instrument performance for proteomic studies requires complex protein reference materials. Whole-cell protein extracts provide the needed complexity. We offer mass spec-compatible whole-cell reference protein extracts from yeast and human cells, in a convenient, pre-digested lyophilized form. Consistency is monitored by various protein and peptide qualitative and quantitative methods including amino acid analysis and LC/MS analysis (Table 7.3).

For routine reporting on MS instrument sensitivity and dynamic range as well various LC parameters, we recommend use of the 6 x 5 LC-MS/MS Peptide Reference Mix. The product is a mixture of 30 peptides: 6 sets of 5 isotopologues of the same peptide sequence. The isotopologues differ only by the number of stable, heavy-labeled amino acids incorporated into the sequence. The labels consist of uniform 13C and 15N atoms. Chromatographically, each of the isotopologues is indistinguishable; however, since they differ in mass, they are clearly resolved by mass spectrometry. The isotopologues of each peptide are present in a series of tenfold dilutions. This format allows assessment of instrument dynamic range and sensitivity from a single run.

Table 7.3. Promega Human Extract has Enhanced Quality Control Specifications and Higher Performance than Other Commercial Extracts.

	Promega (MS Compatible Human Protein Extract, Digest, Cat.# V6951)	Vendor X (HeLa Protein Digest Standard)
Nonbiological post-translational modifications	Deamidation spectra: <12%	Not tested; no specification
	Oxidation spectra: <5%	Oxidation spectra: <10%
	Carbamylation spectra: <5%	Carbamylation spectra: <10%
Missed clevages	<10%	<10%
Peptide quality	Measured by Amino Acid Analysis (AAA) to quantitate the amount of each amino acid in the sample	A_{280}
	Protein fragmentation: 1% or less	Not tested; no specification
	Matching spectra: >65%	Not tested; no specification
Lot-to-lot consistency	≥12,462 unique peptides	LC-MS chromatogram conforms to reference
	Total proteins: ≥1805	4.8%
	Protein identification >85% of 1,194 human core peptides	Ratio of peptide area to reference = 0.75–1.125
	Protein quantitation reproducibility is monitored by relative abundance of ten reference proteins	Not tested

Promega

Protein Extracts for LC/MS Instrument Performance Monitoring

MS Compatible Human Protein Extract, Digest

Ready-to-use predigested human extracts for Liquid Chromatography/Mass Spectrometry (LC/MS) instrument performance monitoring and method development.

Description

MS Compatible Human Protein Extract, Digest is a lyophilized tryptic peptide mixture from K562 human cells (myelogenous leukemia cell line) designed for monitoring of LC/MS instrument performance. The digest is also suitable for MS method development such as isobaric mass tag labeling. Stringent quality control over the manufacturing process assures reproducibility of this reference material. MS Compatible Human Protein Extract, Digest is provided in ready-to-use format eliminating the need for sample preparation prior to MS analysis.

Preparation

MS Compatible Human Protein Extract, Digest, is prepared by digestion of K562 whole-cell protein extract with the Trypsin/Lys-C Mix. The digestion procedure is optimized to assure the most efficient proteolysis with less than 8% tryptic missed cleavages. The digest is SPE (C_{18}) cleaned-up to remove nonpeptide material and lyophilized. The digest can be reconstituted in formic acid or TFA and used for instrument performance monitoring. In general, 1µg of digest per injection is recommended.

Features and Benefits

- **Ready-to-Use:** No clean-up or digestion required.
- **High Material Complexity:** Complex proteome with a large dynamic range.
- **Lot-to-Lot Consistency:** Stringent QC over the production process.
- **Validated Reference Material:** Tested with all the major LC/MS platforms.

Ordering Information

MS Compatible Human Protein Extract, Digest (Cat.# V6951)

MS Compatible Human Protein Extract, Intact (Cat.# V6941)

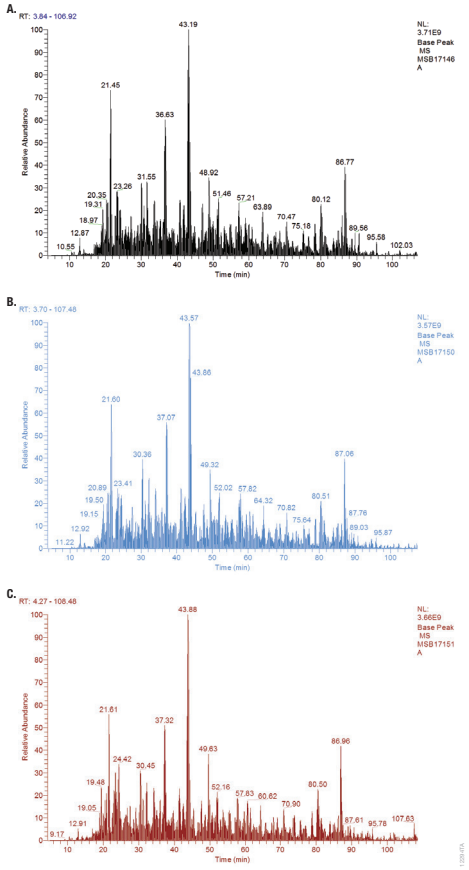

Figure 7.16. Lot-to-lot reproducibility. Base peak chromatograms of three different human protein extract batches analyzed by LC/MS. Each run used 1µg of human pre-digested protein extract injected into the instrument (Waters NanoAquity HPLC system interfaced to a ThermoFisher Q Exactive). Peptides were resolved with 2h gradient.

Promega

Protein Extracts for LC/MS Instrument Performance Monitoring

MS Compatible Yeast Protein Extract, Digest

Ready-to-use predigested yeast extracts for Liquid Chromatography/Mass Spectrometry LC/MS instrument performance monitoring and method development.

Description

MS Compatible Yeast Protein Extract, Digest from *Saccharomyces cerevisiae* is a lyophilized tryptic peptide mixture designed for monitoring LC/MS instrument performance. The digest is also suitable for MS method development such as isobaric mass tag labeling. Stringent quality control over the manufacturing process assures reproducibility of this reference material.

Preparation

MS Compatible Yeast Protein Extract, Digest, is prepared by digestion of yeast whole-cell protein extract with the Trypsin/Lys-C Mix. The digestion procedure is optimized to assure the most efficient proteolysis with less than 8% tryptic missed cleavages. The digest is SPE (C_{18}) cleaned-up to remove nonpeptide material and lyophilized. The digest can be reconstituted in formic acid or TFA and used for instrument performance monitoring. In general, 1µg of digest per injection is recommended.

Features and Benefits

- **Ready-to-Use:** No clean-up or digestion required.
- **Well-Annotated Proteome:** The small yeast proteome is a convenient reference material.
- **Lot-to-Lot Consistency:** Stringent QC throughout the production process.
- **Validated Reference Material:** Tested with all the major LC/MS platforms.

Ordering Information

MS Compatible Yeast Protein Extract, Digest (Cat.# V7461)

MS Compatible Yeast Protein Extract, Intact (Cat.# V7341)

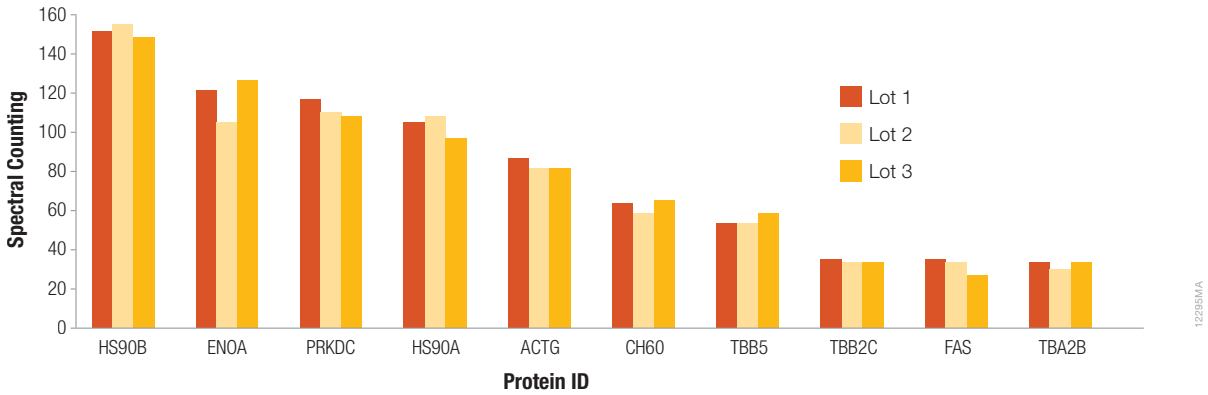

Figure 7.17. Lot-to-lot quantitative reproducibility of yeast extract. Relative protein quantitative levels were determined by spectral counting. The data showed high protein quantitative reproducibility between the different lots.

Promega

Peptide Mix for LC/MS Instrument Performance Monitoring

6 × 5 LC-MS/MS Peptide Reference Mix

Unique peptide mix/software designed to monitor, analyze and record a variety of LC-MS/MS parameters.

Description

The 6 × 5 LC-MS/MS Peptide Reference Mix is a unique reagent designed to monitor liquid chromatography (LC) and mass spectrometry (MS) instrument performance and to assist in method development/optimization. The product is a mixture of 30 peptides: 6 sets of 5 isotopologues of the same peptide sequence. The isotopologues differ only by the number of stable, heavy-labeled amino acids incorporated into the sequence. The labels consist of uniform 13C and 15N atoms. Chromatographically, each of the isotopologues is indistinguishable; however, since they differ in mass, they are clearly resolved by mass spectrometry. The isotopologues of each peptide are present in a series of tenfold dilutions. This format allows assessment of instrument dynamic range and sensitivity from a single run (Figure 7.19). Peptides with a wide range of hydrophobicities were chosen to enable reporting of LC column performance. In addition there is a mass separation of at least 4 daltons between the isotopologues, so that even low resolution instruments can distinguish the masses.

PReMiS™ Software Tool

The 6 × 5 LC-MS/MS Peptide Reference Mix is designed for use with the complementary PReMiS™ Software tool (available by download) that reports on key liquid chromatography and mass spec parameters. The parameter reports can be exported to CSV or saved as ".pdf" files. In addition to the general reporting feature, performance parameters can be tracked over time, allowing a clear assessment of trends to pinpoint poor performance and maintenance needs. For those laboratories that have multiple instruments, the ability to compare parameters across instruments will also be available. Thermo (.raw) and ABSCIEX (.wiff) are available for direct importing. Other vendor formats can be imported after conversion to ".mzml."

Features and Benefits

Unique peptide formulation

- Save time by assessing LC and MS parameters (e.g., dynamic range and sensitivity) in one run with a single reagent
- Optimize and streamline method development

Complementary software provides routine analysis and historical monitoring

- Eliminate time-consuming manual calculations
- Ensure consistent instrument performance over time
- Compare performance of multiple instruments over time

Peptides are AAA qualified

- Accurate reporting of instrument sensitivity and dynamic range

Multiple applications

- Compatible with neat or complex mixture analysis

Ordering Information

6 x 5 LC-MS/MS Peptide Reference Mix
(Cat.# V7491, V7495)

Peptide Mix for LC/MS Instrument Performance Monitoring (continued)

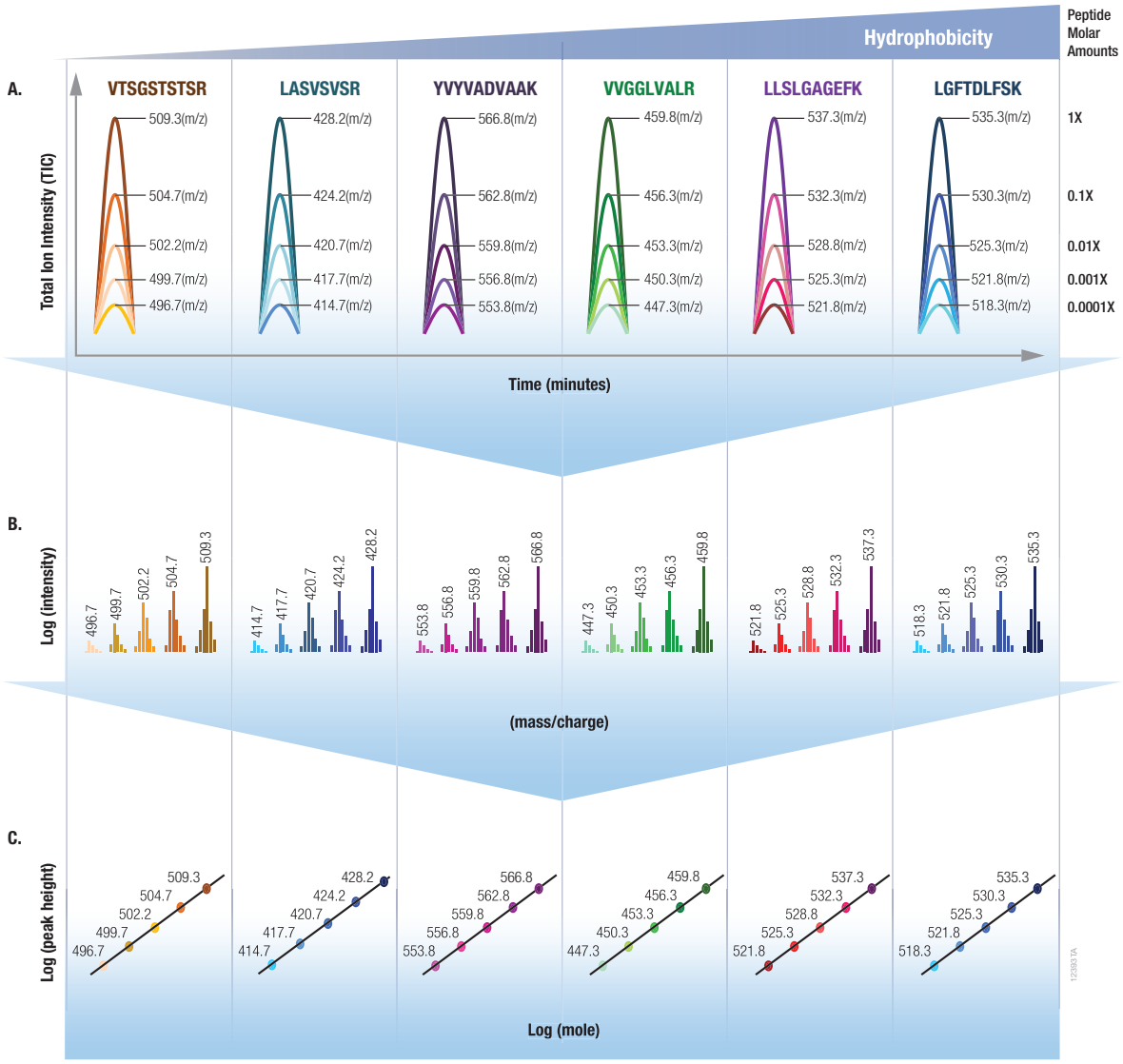

Figure 7.18. Schematic illustrating standard workflow and correlating features of the 6 × 5 LC-MS/MS Peptide Reference Mix. A mixture of 6 peptide sets is actually 30 peptides, in total. Each of the peptides sets is a mixture of 5 isotopologues that are distinguished only by mass through the incorporation of stable, heavy-labeled amino acids. **Panel A.** The isotopologues are chemically and chromatographically identical and therefore appear as 6 peaks. **Panel B.** Each of the isotopologues is mixed with a 10-fold molar increase (lightest to heaviest). **Panel C.** A plot of the log of the peak height versus the log (molar amount) on column has a slope of 1. This analysis enables confirmation of detection limits and can also be used to confirm linearity of up to 5 logs of dynamic range.

7.6 Antibody Characterization/Fragmentation

OVERVIEW

Therapeutic monoclonal antibodies are large, complex molecules that undergo numerous post-translational modifications (PTMs). Numerous varieties of PTMs are known to exist on antibodies. Because these PTMs can affect the efficacy and safety profile of the drug, the structural variants must be assessed and quantified. In-depth characterization of antibody PTMs remains a significant hurdle because their large size (~150kDa) makes mass spectrometry analysis extremely challenging.

IdeS Protease is highly specific and cleaves human (and some other species) IgG specifically at one site in the lower hinge region. Because of the exquisite specificity of the enzyme, it produces highly homogeneous Fc and Fab fragments which are then readily analyzed using techniques such as mass spectrometry (Figure 1) or X-ray crystallography.

IdeS Protease

Recombinant protease engineered to simplify antibody fragmentation and characterization.

Description

The Immunoglobulin-degrading enzyme from *Streptococcus pyogenes* (IdeS) is a highly specific protease that cleaves Immunoglobulin G (IgG) at a single site below the hinge region, yielding F(ab')^2 and Fc fragments. The IdeS Protease can be used to characterize therapeutic antibody candidates using LC/MS. Promega IdeS Protease is a recombinant, engineered version of the enzyme from *S. pyogenes.*

Features and Benefits

- **Fast and Easy:** Digestion in 30 minutes with no optimization.
- **Highly Specific and Reproducible:** Cleaves exclusively at a single site below the hinge to produce F(ab')^2 and Fc fragments.
- **High Performance:** Essentially 100% complete digestion.
- **Versatile:** Effectively cleaves human IgG1–IgG4, monkey, sheep, rabbit, humanized and chimeric IgGs as well as Fc-fusion proteins.

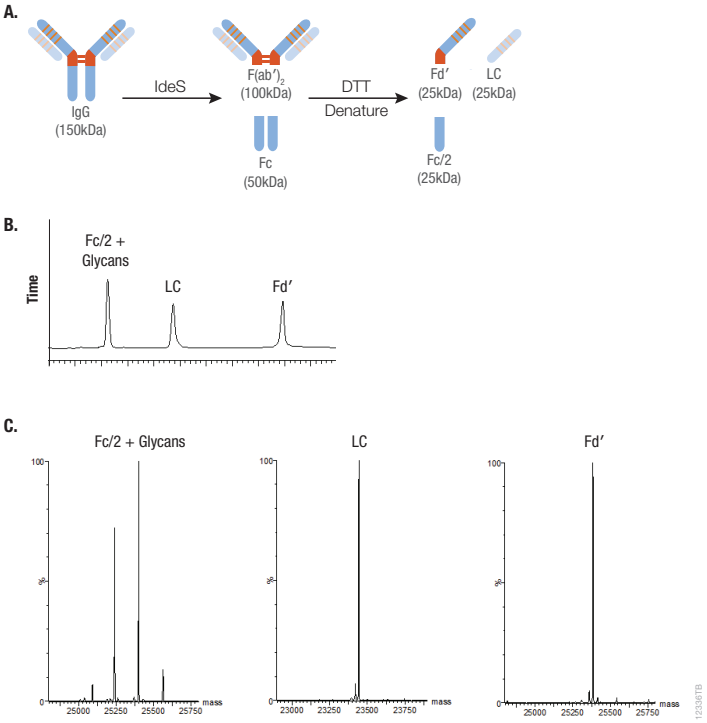

Figure 7.19. Schematic showing cleavage specificity of IdeS Protease. The orange cysteines (C) indicate where the interchain disulfide bonds are formed to the opposite heavy chain. The orange glycines (G) indicate the IdeS cleavage site.

Ordering Information

IdeS Protease (Cat.# V7511, V7515)

Product may not be available in all countries. Please contact your local Promega representative for information.

7.7 Proteases for Mass Spectrometry Sample Preparation

ADDITIONAL INFORMATION

Table 7.4. Characteristics and Reaction Conditions for Trypsin Proteases.

	Trypsin Gold, Sequencing Grade Modified Trypsin, Sequencing Grade Modified Trypsin, Frozen	Immobilized Trypsin	Trypsin/Lys-C Mix
Cat.#	V5280; V5111; V5117; V5113	V9012; V9013	V5071; V5072; V5073
Source and Size	Porcine Pancreas (23.8kDa)	Porcine Pancreas (23.8kDa)	Mix of Tryspin-Gold & rLys-C
Cleavage Sites	C-terminal of Lys, Arg. Does not cleave if Lys and Arg are followed by Pro. Asp and Glu at C-terminal side of Lys and Arg inhibit cleavage.	C-terminal of Lys, Arg. Does not cleave if Lys and Arg are followed by Pro. Asp and Glu at C-terminal side of Lys and Arg inhibit cleavage.	C-terminal of Lys, Arg. Does not cleave if Lys and Arg are followed by Pro. In contrast to trypsin, Trypsin/Lys-C efficiently tolerates presence of Glu and Asp at C-terminal side of Lys and Arg.
Protease Protein Ratio (w/w)	1:20 to 1:100	see Technical Manual	1:25 to 1:50
pH Range for Digestion	pH 7–9	pH 5–9	pH 8
Reaction Conditions	50–100mM Tris-HCl (pH 8) or 50–100mM NH_4HCO_3(pH 7.8). Digest overnight hours at 37°C.	50mM NH_4HCO_3 (pH 7.8). Digest for 30 minutes at room temperature.	50–100mM Tris-HCl (pH 8) or 50mM NH_4HCO_3 (pH7.8). Digest overnight at 37°C.
Buffer Compatibility	Tris-HCl, NH_4HCO_3	Tris-HCl, NH_4HCO_3	Tris-HCl, NH_4HCO_3
In-Gel Digestion Compatibility	Yes	No	Yes
ProteaseMAX™ Compatibility	Yes	Yes	Not tested
Notes	Most widely used protease in mass spectrometry. All Promega trypsin is treated with TPCK to inactivate chymotrypsin contamination and chemically modified (methylated) to minimize autoproteolysis. All Promega trypsin is resistant to mild denaturing conditions (1–2M urea and 0.1% SDS). They retain 48% activity in 2M Guanidine Chloride.	Used if rapid digestion is required. Urea ≤ 4M; guanidine HCl ≤ 3M; methanol < 60%; DTT ≤ 50mM; TCEP ≤ 5mM; pH 5–9; Iodoacetamide ≤ 300mM.	Trypsin/Lys-C mix is the most robust trypsin preparation. It is designed to improve general protein digestion. Trypsin/Lys-C mix uses the same regular overnight digestion at 37°C under non- or mildly denaturing conditions as trypsin alone.

Table 7.5. Characteristics and Reaction Conditions for Specific Alternative Proteases.

	rLys-C	Endoproteinase Lys-C	Arg-C	Asp-N	Glu-C
Cat.#	V1671	V1071	V1881	V1621	V1651
Source and Size	*Pseudomonas aeruginosa.* Expressed in *E.coli* (27.7kDa)	*Lysobacter enzymogenes* (30kDa)	*Clostridium histolyticum* (Subunits: 45kDa and 12kDa)	*Pseudomonas fragi* (24.5kDa)	*Staphylococcus aureus V8* (27kDa)
Cleavage Sites	C-terminal of Lys. Does not cleave if Lys is followed by Pro. Asp and Glu at C -terminal side of Lys inhibit cleavage.	C-terminal of Lys. Does not cleave if Lys is followed by Pro. Asp and Glu at C -terminal side of Lys inhibit cleavage.	C-terminal of Arg. Also cleaves at Lys albeit at lower efficiency.	N-terminal of Asp.	C-terminal of Glu. Low level cleavages might occur at Asp residues too albeit at 100–300 fold lower efficiency.
Protease Protein Ratio (w/w)	1:20 to 1:50	1:20 to 1:100	1:20 to 1:350	1:20 to 1:200	1:20 to 1:200
pH Range for Digestion	pH 8–9	pH 7–9	pH 7.6–7.9	pH 4–9	pH 4–9
Reaction Conditions	50-100mM Tris-HCl (pH 8) or 50mM NH_4HCO_3 (pH 7.8). Digestion 2–18 hours at 37°C.	50-100mM Tris-HCl (pH 8) or 50mM NH_4HCO_3 (pH 7.8) Digestion 2–18 hours at 37°C.	50mM Tris-HCl (pH 7.6-7.9), 5mM $CaCl_2$, 2mM EDTA, >2mM DTT. Digestion 2–18 hours at 37°C.	50mM Tris-HCl (pH 8).Digestion 2–18 hours at 37°C.	100mM NH_4HCO_3 (pH 7.8), 50-100 mM HCL (pH8). Digestion 2–18 hours at 37°C.
Buffer Compatibility	Tris-HCl, NH_4HCO_3	Tris-HCl, NH_4HCO_3	Tris-HCl, NH_4HCO_3	Tris-HCl, NH_4HCO_3	NH_4HCO_3, Ammonium acetate
In-Gel Digestion Compatibility	Yes	Yes	Yes	Yes	Yes
ProteaseMAX™ Compatibility	Yes	Yes	Yes	Yes	Yes
Notes	Inexpensive alternative to a native Lys-C protease. Similarly to a native protease, rLys-C tolerates high denaturing conditions such as 8M urea. Used to digest tightly folded proteolytically resistant proteins. Also used as a trypsin alternative if larger peptides are preferable for the analysis. If urea is used in protein sample preparation, avoid high temperature. High temperature induces protein carbamylation in the presence of urea.	Tolerates high denaturing conditions such as 8M urea. Used to digest tightly folded proteolytically resistant proteins. Also used as a trypsin alternative if larger peptides are preferable for analysis. If urea is used in protein sample preparation, avoid high temperature. High temperature induces protein carbamylation in the presence of urea.	Used in analysis of histone modifications. Requires DTT, cysteine or other reducing agent and $CaCl_2$ for activity.	Can be used as a trypsin alternative to achieve better distribution of cleavage sites.100% activity retained in the presence of urea (up to 3.5 M), guanidine HCL (1M), SDS (up to 0,028%), ProteaseMax™ Surfactant (up to 0,026%), acetonitrile (up to 60%), EDTA (up to 2 mM); DTT or ß-mercaptoethanol	Can be used as a trypsin alternative to achieve better distribution of cleavage sites. Glu-C activity and cleavage specificity is affected by buffer conditions. In ammonium biocarbonate and other non-phosphate buffers, Glu-C cleaves at C-term of Glu. Glu-C cleaves at C-term of Glu and Asp in phosphate buffer.

Table 7.6. Characteristics and Reaction Conditions for Low Specific Alternative Proteases.

	Chymotrypsin
Cat.#	V1061; V1062
Source and Size	Bovine pancreas (25kDa)
Cleavage Sites	Preferentially C-terminal of Trp, Tyr and Phe but it also cleaves at other residues albeit at a lower efficiency.
Protease Protein Ratio (w/w)	1:20 to 1:200
pH Range for Digestion	pH 7–9
Reaction Conditions	100mM Tris HCl (pH 8), 10mM $CaCl_2$. Digestion 2–18 hours at 25°C.
Buffer Compatibility	Tris-HCl, NH_4HCO_3
In-Gel Digestion Compatibility	Yes
ProteaseMax™ Compatibility	Yes
Notes	Often used to digest hydrophobic proteins including membrane proteins. 80% activity retained in the presence of urea (up to 1M) or 1M guanidine HCl (up to 1M). No reduction in activity in the presence of ProteaseMAX™ Surfactant (up to 0,025%).

Table 7.7. Characteristics and Reaction Conditions for Nonspecific Proteases.

	Elastase	Pepsin	Thermolysin
Cat.#	V1891	V1959	V4001
Source and Size	Porcine pancreas (25.9kDa)	Porcine stomach (34.6kDa)	*Bacillus thermoproteolyticus rokko* (36.2kDa)
Cleavage Sites	Preferentially C-terminal of Ala, Val, Ser, Gly, Leu and Ile.	Preferentially C-terminal of Phe, Leu, Tyr and Trp	Preferentially N-terminal of Leu, Phe, Val, Ile and Met.
Protease Protein Ratio (w/w)	1:20 to 1:100	1:20 to 1:100	1:20 to 1:50
pH Range for Digestion	pH 9	pH 1–3	pH 5.0–8.5
Reaction Conditions	50-100mM Tris-HCl (pH 8.5-9.5), digestion 2–18 hours at 37°C.	Adjust protein solution to pH 1-3 with 1N HCl prior to digestion. Digestion 1–18 hours at 37°C.	50mM Tris-HCl (pH 8), 0.5mM $CaCl_2$. Digestion 0.5-6 hours at 70–95°C.
Buffer Compatibility	Tris-HCl, NH_4HCO_3	(—)	Tris-HCl
In-Gel Digestion Compatibility	Yes	Yes	Not tested
ProteaseMAX™ Compatibility	Yes	Yes	Yes
Notes	Used as a trypsin alternative to increase protein coverage.	Used in structural protein studies (HDX exchange based) and antibody analysis; used to digest proteolytically resistant, tightly folded proteins.	Used to digest proteolytically resistant, tightly folded proteins and in structural studies.