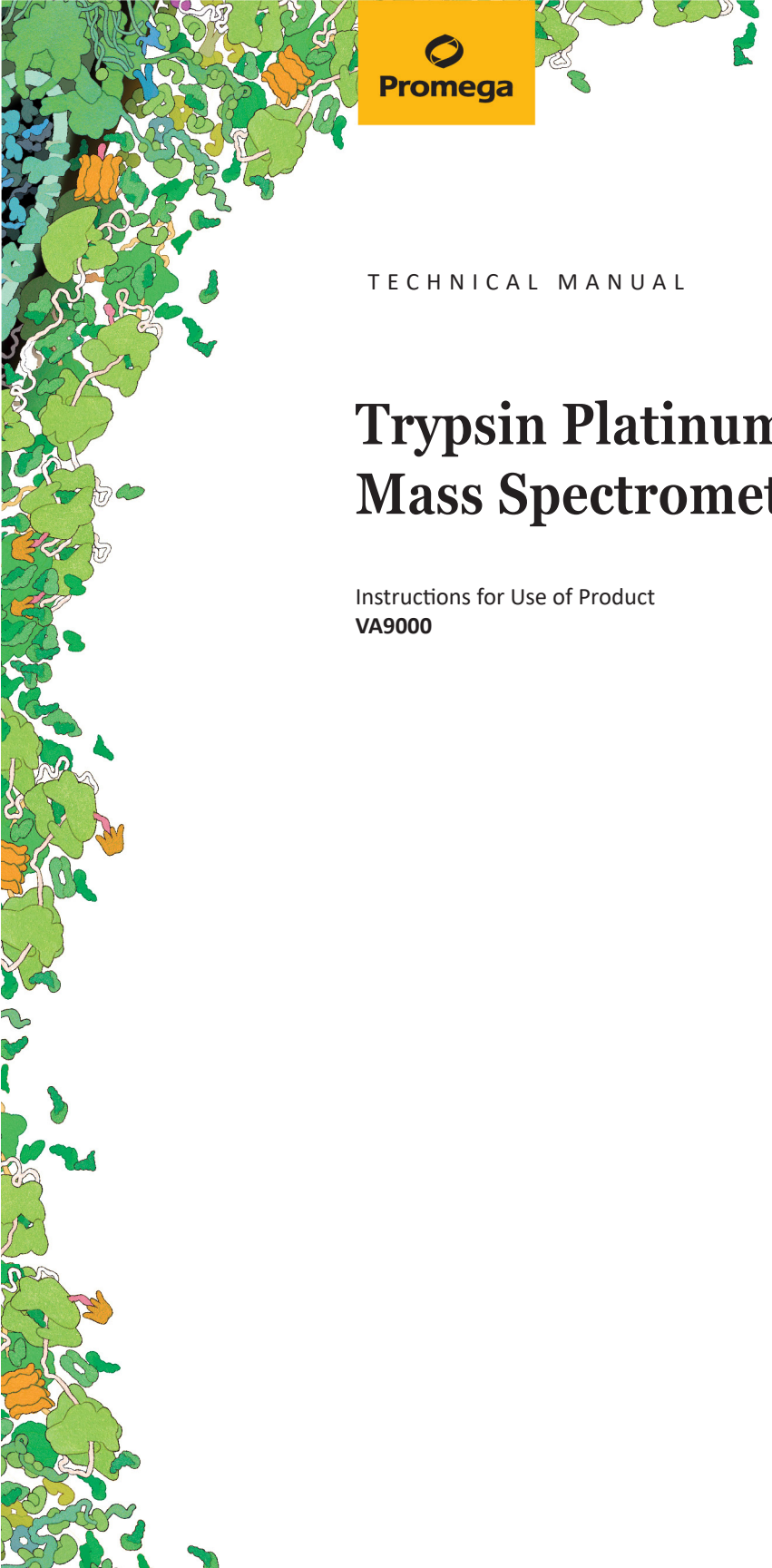

TECHNICAL MANUAL

Trypsin Platinum, Mass Spectrometry Grade

Instructions for Use of Product
VA9000

6/21
TM654

Trypsin Platinum, Mass Spectrometry Grade

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

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1. Description

Trypsin is a serine protease that specifically cleaves at carboxylic termini of arginine and lysine residues. Robust proteolytic efficiency, optimal length and strong C-terminal charge of trypsin-generated peptides makes trypsin the protease of choice in protein mass spectrometry analysis.

Trypsin Platinum, Mass Spectrometry Grade, is a recombinant protease designed for users looking for accurate protein characterization with mass spectrometry and RP-HPLC-UV (reverse phase-high pressure liquid chromatography-UV). It is free of detectable nonspecific proteolytic activity. A novel chemical modification method assures maximal autoproteolytic resistance. Trypsin Platinum has high proteolytic efficiency and is free of contaminating proteins of animal origin.

Commercially available proteomic and mass spectrometry grade trypsins contain nonspecific protease activity at a low but detectable level (1). Close analysis of this activity suggests it is chymotryptic in nature. Trypsin is typically extracted from bovine or porcine pancreas, which, in addition to trypsin, contains chymotrypsin. A certain amount of chymotrypsin is copurified with trypsin during trypsin manufacturing. To suppress chymotryptic activity, trypsin is treated with TPCK, an irreversible chymotrypsin inhibitor. However, trace amounts of chymotrypsin appear to escape this inhibition. The nonspecific, chymotryptic-like cleavage activity becomes evident if large amounts of trypsin are used in a digestion reaction (Figure 1, Panel A). These nonspecific cleavages compromise the quality of protein analysis. Our production procedure assures that Trypsin Platinum is free of any detectable traces of nonspecific cleavage activity (Figure 1, Panel B).

When using Trypsin Platinum, be aware of pre-existing nonspecific cleavages commonly present in proteins. These cleavages are induced by endogenous proteases and mechanical shearing during protein extraction and purification or accumulated during protein storage due to deamidation and other nonenzymatic events (1,2). Nonspecific cleavages are also induced during mass spec analysis as a result of in-source fragmentation (3). The examples of pre-existing and in-source induced, nonspecific cleavages are shown in Figure 2. Typically, these nonspecific cleavages are detected in protein digests as low intensity species and remain at the same low level regardless of the amount of Trypsin Platinum in the reaction or digestion time.

Autoproteolysis is another common negative side effect of trypsin digestion. To suppress trypsin autoproteolysis, trypsins used in protein mass spec sample preparation are chemically modified. Yet, a certain amount of autoproteolysis is still observed (Figure 3, Panel A). The generated tryptic autoproteolytic peptides often compromise protein analysis, particularly if a large amount of trypsin is used in a digestion reaction. Our novel modification method further suppresses Trypsin Platinum autoproteolysis compared to the autoproteolysis observed when using currently available proteomic- and mass spec-grade trypsins (Figure 3, Panel B). This assures that the level of autoproteolytic tryptic peptides remains negligibly low even if large quantities of Trypsin Platinum are used.

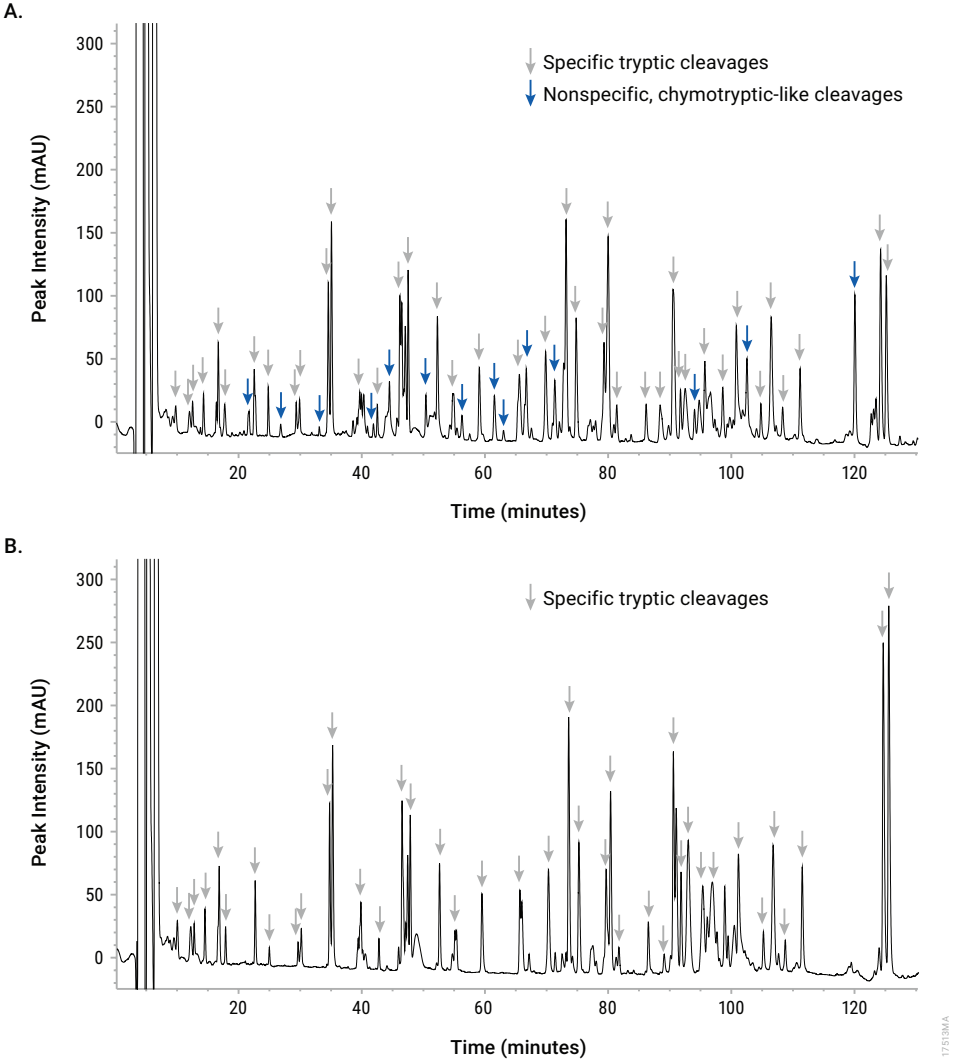

Figure 1. Trypsin Platinum, Mass Spectrometry Grade, cleavage specificity. Panitumumab (Vectibix®) was used as a model protein substrate. The antibody was reduced, alkylated and digested overnight at 37°C as described in the protocol (Section 3). The digestion reactions used 1:10 trypsin:protein ratio. The digested peptides were analyzed with RP-HPLC-UV. The peptide peaks were assigned with LC-MS to differentiate between specific and nonspecific peptides. The analysis showed that commercially available mass spec-grade trypsin (used for comparison) contains prominent nonspecific proteolytic activity (**Panel A**). Close analysis indicated a chymotryptic-like pattern of nonspecific cleavages generated. In contrast, Trypsin Platinum generated specific tryptic cleavages only (**Panel B**).

1. Description (continued)

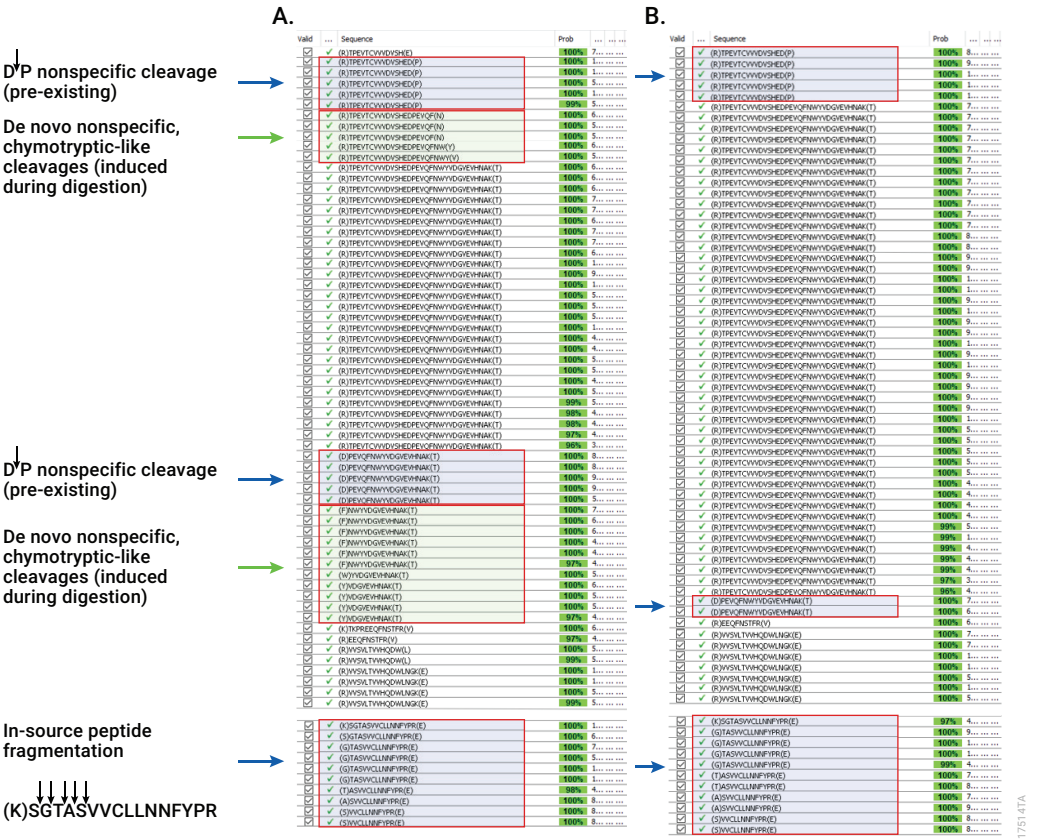

Figure 2. Pre-existing cleavages and in-source peptide fragmentation. Panitumumab digests were prepared using commercially available mass spec-grade trypsin and Trypsin Platinum, Mass Spectrometry Grade, as described in the legend to Figure 1, and analyzed with LC-MS (Q Exactive™ Plus, Thermo Fisher Scientific, Inc.). Here we show a sample fragment of the identified peptide list. Three classes of nonspecific cleavages were identified: pre-existing cleavages, in-source fragmentation and de novo nonspecific cleavages (the cleavages induced during tryptic digestion). The pre-existing cleavage was found at D/P site (highlighted with a gray box in each panel). This is a commonly occurring pre-existing cleavage, induced in proteins at mildly acidic conditions upon long-term storage (3). In-source fragmentation was represented by a typical ladder-like break pattern located at a peptide N-terminus (highlighted with a purple box at the bottom of each panel). As expected, the pre-existing D/P cleavage and in-source fragmentation were detected in both mass spec-grade trypsin and Trypsin Platinum digests. However, de novo nonspecific cleavages, induced during digestion, were detected in mass spec-grade trypsin digest only (**Panel A**). Note that de novo nonspecific cleavages were found at C-termini of aromatic residues. This cleavage pattern is characteristic of chymotryptic proteolytic activity. In contrast, Trypsin Platinum generates only tryptic-specific cleavages (**Panel B**).

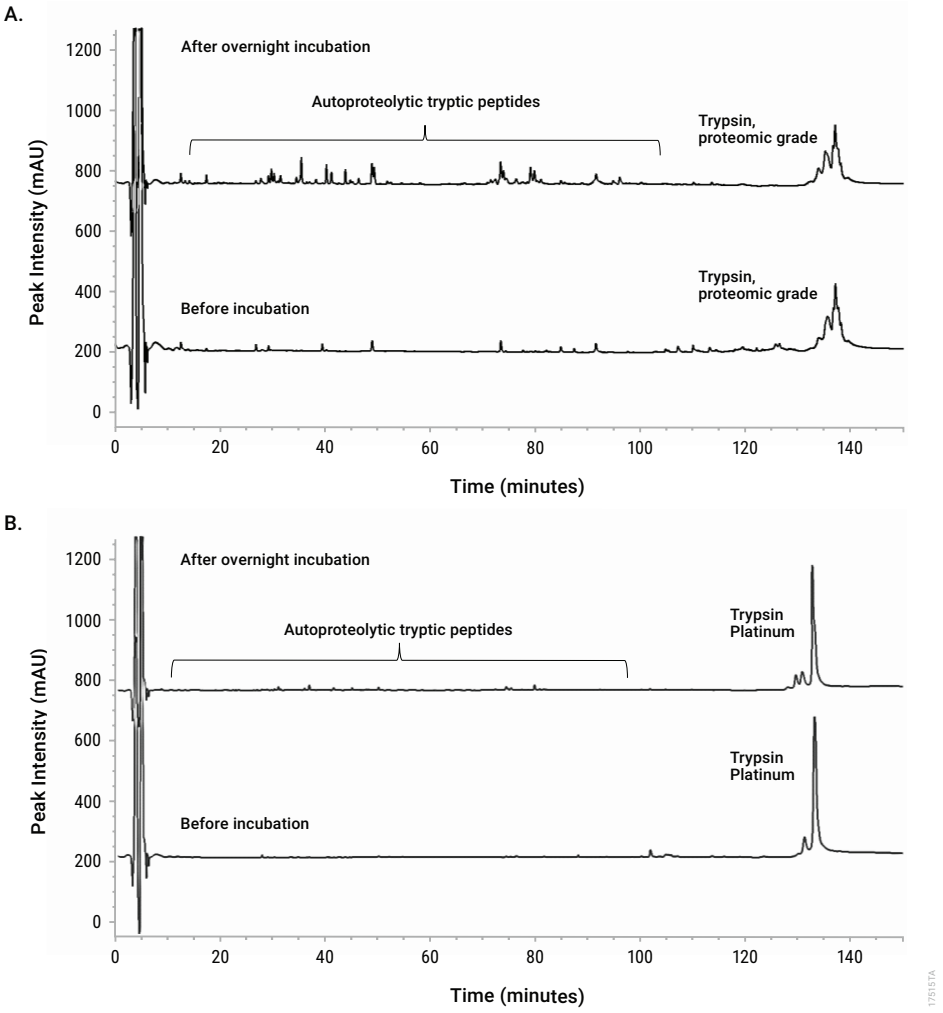

Figure 3. Trypsin Platinum, Mass Spectrometry Grade, autoproteolytic resistance. A commercially available proteomic-grade trypsin (**Panel A**) and Trypsin Platinum, Mass Spectrometry Grade (**Panel B**) were incubated at conventional digestion conditions. Specifically, trypsins were reconstituted in 100mM Tris-HCl (pH 8)/2mM $CaCl_2$ and incubated overnight at 37°C. Autoproteolytic products were then analyzed with RP-HPLC-UV. Fresh, nonincubated aliquots of both trypsins were analyzed as a control. The proteomic-grade trypsin demonstrated prominent autoproteolysis (**Panel A**), whereas Trypsin Platinum showed negligible autoproteolysis (**Panel B**).

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT.#
Trypsin Platinum, Mass Spectrometry Grade	100µg	VA9000

Storage Conditions: Store lyophilized Trypsin Platinum, Mass Spectrometry Grade, at –30°C to –10°C. Reconstitute the enzyme in 50mM acetic acid, dispense into aliquots and store at –30°C to –10°C for up to 1 month. For longer storage, freeze the reconstituted Trypsin Platinum solution at –80°C to –60°C for up to 6 months. Reconstituted Trypsin Platinum solution retains full activity after three freeze-thaw cycles.

3. Protein Digestion Protocol

The following procedure is a sample protocol that has been successfully used in antibody peptide mapping.

Materials to Be Supplied by the User

- NANOpure® (or equivalent grade) water
- guanidinium chloride (GuHCl)
- 300mM Tris-HCl (pH 8)
- 1M $CaCl_2$
- Tris (2-carboxyethyl) phosphine (TCEP)
- iodoacetamide (IAM)
- 50mM acetic acid (for trypsin reconstitution)
- 20% trifluoroacetic acid (TFA)

3.A. Before you Begin

Use NANOpure® water to prepare 8M GuHCl, 300mM Tris-HCl (pH 8)/12mM $CaCl_2$ mix, 100mM TCEP and 300mM IAM. Store IAM solution in the dark until use.

Reconstitute lyophilized Trypsin Platinum, Mass Spectrometry Grade, in 200µl of 50mM acetic acid to create 0.5µg/µl of Trypsin Platinum, Mass Spectrometry Grade, solution. Place on ice.

3.B. Protein Denaturation and Disulfide Bond Reduction

1. Prepare the mix as described in the table below.

Reagent	Volume
20µg/µl protein	30µl
Nanopure® water	30µl
8M GuHCl	240µl
300mM Tris-HCl (pH 8)/12mM $CaCl_2$	72µl
100mM freshly prepared TCEP	12µl
Total	**384µl**

2. Mix and incubate at 37°C for 30 minutes.

3.C. Alkylation

1. Add 24µl of freshly prepared 300mM IAM to the reduced protein, mix and incubate protected from light at 37°C for 30 minutes. The final concentration of the protein after reduction and alkylation will be 1.47µg/µl.
2. After alkylation is complete, transfer 34µl of the reduced/alkylated protein solution to a fresh tube. Store the remaining reduced/alkylated protein solution at –30°C to –10°C for additional analysis.

3.D. Digestion

1. Prepare the mix as described in the table below.
2. Store unused Trypsin Platinum solution at –30°C to –10°C for up to 1 month. The final Trypsin Platinum to protein ratio in the digestion reaction will be 1:10.

Reagent	Volume
Reduced/alkylated protein	34µl
Nanopure® water	106µl
500mM Tris-HCl (pH 8)	10µl
0.5µg/µl Trypsin Platinum	10µl
Total	**160µl**

3. Incubate overnight at 37°C.
4. After digestion is complete, acidify the digestion reaction with 18µl of 20% TFA to terminate proteolysis. The final protein concentration will be 0.28µg/µl.

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4. Troubleshooting

Symptoms	Causes and Comments
Nonspecific cleavages are detected.	Pre-existing cleavages are present in the protein. See information about pre-existing cleavages in Section 1.
	The protein is contaminated with a nonspecific protease. Protein clean-up is required prior to digestion.
Incomplete protein digestion is observed.	Certain excipients present in the protein inhibit trypsin. Protein clean-up is required prior to digestion.
	Agents used to denature proteins (i.e., GuHCl) inhibit trypsin. The extent of the inhibition depends on the agent concentration. Proteolytic efficiency can be increased by diluting or completely removing a denaturing agent from the reaction prior to digestion. Alternatively, the Trypsin Platinum amount can be increased to achieve a 1:3 to 1:5 trypsin:protein ratio to compensate for the inhibitory effect of a denaturing agent. Increasing digestion time beyond the conventional, overnight incubation will afford additional, albeit moderate, improvement in digestion efficiency.
	Some proteins or protein domains are proteolytically resistant due to tight folding. A sequential digestion procedure is recommended to digest these proteins and domains. In this procedure, a protein is first digested with Lys-C protease at strong denaturing conditions, such as 3–3.5M GuHCl. The reaction is next diluted with a reaction buffer to minimize denaturing agent concentration (i.e., to 0.5–1M GuHCl) and the digestion is completed with Trypsin Platinum, Mass Spectrometry Grade.

5. Appendix

5.A. References

1. Fang, P. *et al.* (2015) Controlling nonspecific trypsin cleavages in LC-MS/MS-based shotgun proteomics using optimized experimental conditions. *Analyst* **140(22)**, 7613–21.
2. Vlasak, J. and Ionescu, R. (2011) Fragmentation of monoclonal antibodies. *mAbs* **3(3)**, 253–63.
3. Kim, J-S. *et al.* (2013) In-source fragmentation and the sources of partially tryptic peptides in shotgun proteomics. *J. Proteome Res.* **12**, 910–6.

5.B. Related Products

Product	Size	Cat.#
Trypsin Gold, Mass Spectrometry Grade	100µg	V5280
AccuMAP™ Low pH Protein Digestion Mini Kit	1 each	VA1040
AccuMAP™ Low pH Protein Digestion Maxi Kit	1 each	VA1050
IdeS Protease	5,000 units	V7511
Magne™ Protein A Beads, 20% Slurry	1.0ml	G8781
Magne™ Protein G Beads, 20% Slurry	1.0ml	G7471
rLys-C, Mass Spec Grade	15µg	V1671