

TECHNICAL MANUAL

Trypsin/Lys-C Mix, Mass Spec Grade

Instructions for Use of Products
V5071, V5072 and V5073



Trypsin/Lys-C Mix, Mass Spec Grade

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1. Description

Trypsin/Lys-C Mix, Mass Spec Grade, is a mixture of Trypsin Gold, Mass Spectrometry Grade, and rLys-C, Mass Spec Grade. Proteolysis with Trypsin/Lys-C Mix, Mass Spec Grade, is specific and generates tryptic peptides (i.e., peptides with C-terminal arginine and lysine residues). Trypsin/Lys-C Mix, Mass Spec Grade, addresses several known limitations to trypsin digestion. Typical trypsin reactions do not digest proteins to completion, missing 15–30% of cleavage sites (Figure 1). 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 poorly purified or contains protease-inhibiting contaminants. For example, guanidine chloride, a common agent for solubilizing and denaturing proteins, inhibits trypsin (1). While both trypsin and Trypsin/Lys-C Mix are inhibited by guanidine chloride to some extent, the Trypsin/Lys-C Mix is more tolerant of guanidine chloride than trypsin alone. Tightly folded proteins represent another challenge for trypsin. Theoretically, proteolytically resistant proteins can be digested under strong denaturing conditions, for example using urea; however, denaturing conditions inhibit trypsin. Using the Trypsin/Lys-C Mix instead of trypsin alone helps overcome these limitations.

1. Description (continued)

Analysis of a typical trypsin digest shows that trypsin does not cleave arginine and lysine cleavage sites with equal efficiency. While trypsin misses few arginine cleavage sites, it leaves a high number of undigested lysine sites. Figure 1 shows an example of the distribution of missed trypsin cleavages for a complex protein mixture. Yeast protein extract was digested with trypsin overnight at 37°C. Mass spectrometry analysis showed 22.2% of cleavage sites remained undigested. The majority (84%) of the missed sites occurred at lysine residues. Digestion efficiency improved when the Trypsin/Lys-C Mix was used. The Trypsin/Lys-C Mix digested the missed lysine residues with high efficiency, and the percent of missed lysines decreased. The remaining uncleaved lysines were, for the most part, found at N termini or at K/(D/E) sites. These sites are known to be particularly difficult to digest (2).

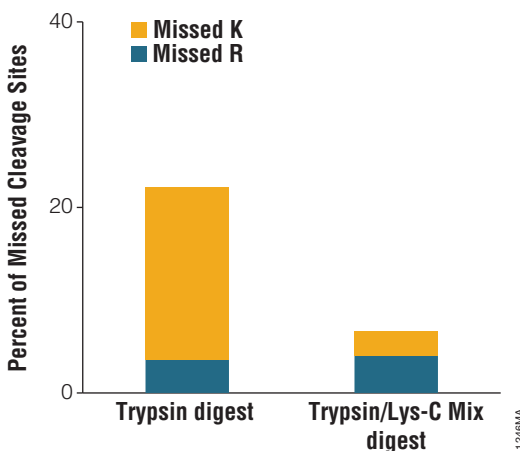
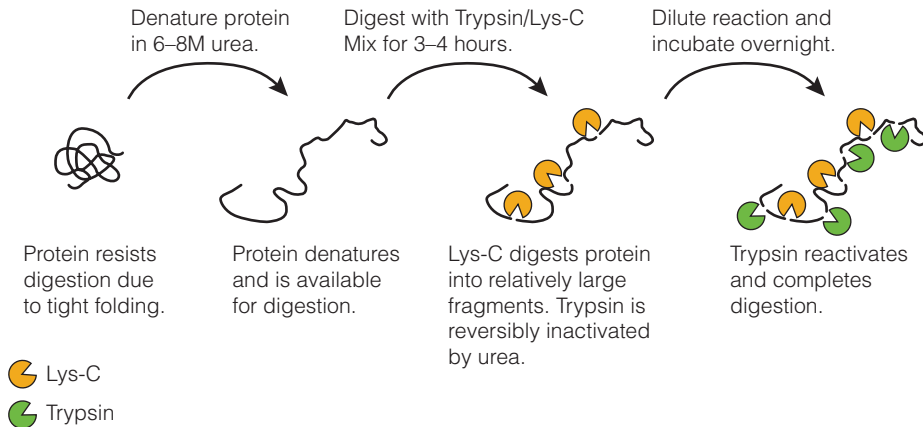


Figure 1. Improved digestion of a complex protein mixture with the Trypsin/Lys-C Mix. Yeast total protein extract was reduced and alkylated, then digested with trypsin or Trypsin/Lys-C Mix overnight at 37°C in 50mM Tris-HCl (pH 8). The digests were analyzed using an Orbitrap® Velos mass spectrometer (Thermo Fisher Scientific).

The Trypsin/Lys-C Mix also can efficiently cleave difficult-to-digest proteins due to the ability of Lys-C to remain active under denaturing conditions. Proteolysis is performed in two steps (Figure 2). The first step uses a strong protein denaturing agent such as urea, which allows Lys-C to cleave previously inaccessible sites. Lys-C digests proteins into relatively large fragments, while trypsin activity is inhibited. In the second step, the digestion mixture is diluted to reduce urea concentration. This reactivates trypsin and allows complete proteolysis. This two-step digestion procedure is based on a sequential digestion protocol developed by Yates's group (3). Figure 3 shows an example using the Trypsin/Lys-C Mix to digest a protein resistant to proteolysis. Digestion with trypsin left horse myoglobin largely undigested, while digestion with Lys-C alone generated peptides ~5kDa in size. However, the Trypsin/Lys-C Mix completely digested myoglobin.

Each lot of Trypsin/Lys-C Mix is qualified for use under non-denaturing and denaturing conditions and validated for mass spectrometry analysis using a single protein digest. HPLC chromatograms of QC results may be obtained by contacting Promega Technical Services at: techserv@promega.com



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Figure 2. Two-step digestion of proteolytically resistant proteins with Trypsin/Lys-C Mix.

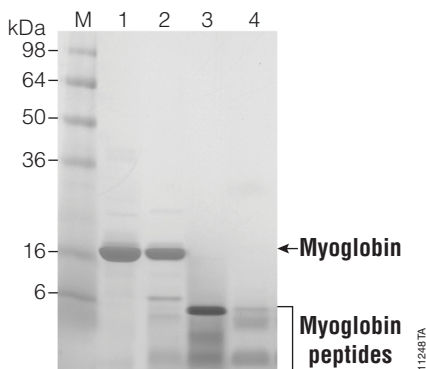


Figure 3. Digestion of a protein resistant to proteolysis with Trypsin/Lys-C Mix. Horse myoglobin was digested using the indicated protease in 25mM Tris-HCl (pH 8) with or without urea at 37°C. The reactions were analyzed by 4–20% SDS-PAGE. Lane M, SeeBlue® Plus2 Pre-Stained Standard (Life Technologies); lane 1, no-protease digestion control; lane 2, trypsin-only digestion under nondenaturing conditions; lane 3, digestion with Lys-C in 8M urea; lane 4, two-step digestion with Trypsin/Lys-C Mix as described in Section 4.B.



2. Product Components and Storage Conditions

Product	Size	Cat.#
Trypsin/Lys-C Mix, Mass Spec Grade	20µg	V5071

Includes:

- 20µg Trypsin/Lys-C Mix, Mass Spec Grade, lyophilized
- 500µl Resuspension Buffer

Product	Size	Cat.#
Trypsin/Lys-C Mix, Mass Spec Grade	100µg	V5072

Includes:

- 100µg Trypsin/Lys-C Mix, Mass Spec Grade, lyophilized
- 500µl Resuspension Buffer

Product	Size	Cat.#
Trypsin/Lys-C Mix, Mass Spec Grade	5 × 20µg	V5073

Includes:

- 5 × 20µg Trypsin/Lys-C Mix, Mass Spec Grade, lyophilized
- 500µl Resuspension Buffer

Storage Conditions: Store lyophilized Trypsin/Lys-C Mix at -20°C . Prior to use, reconstitute the lyophilized Trypsin/Lys-C Mix with Resuspension Buffer. Store on ice for a few hours or at -70°C for up to 30 days. Immediately after thawing, place Trypsin/Lys-C Mix on ice. To retain maximum activity, do not freeze and thaw more than once.

Note: The Resuspension Buffer is 50mM acetic acid ($\text{pH} < 3$). Low pH reversibly inhibits the proteases, minimizing proteolysis during storage.

3. Recommendations for Protein Preparation Prior to Digestion

Typically, disulfide bonds are reduced to ensure efficient proteolysis; however, proteins often precipitate as a result of the reducing step. We recommend using urea to prevent protein precipitation. Urea also denatures the protein, allowing the reducing agent access to disulfide bonds without applying heat. This prevents protein carbamylation, which occurs in urea solutions at high temperatures as reduction occurs at relatively low temperature (37°C). The data showing lack of urea-induced carbamylation under the recommended conditions may be obtained by contacting Promega Technical Services at: techserv@promega.com

Note: Guanidine chloride inhibits trypsin even at relatively low concentrations. We have observed that more than half of tryptic peptides were underdigested after overnight incubation with trypsin under the conditions described by Saveliev *et al.* (4). Trypsin/Lys-C addresses this inhibition by reducing the number of missed tryptic cleavages to more conventional levels. To minimize protease inhibition due to guanidine chloride, we recommend diluting guanidine chloride to 0.5M or less prior to digestion, replacing trypsin with Trypsin/Lys-C Mix and following the standard overnight digestion protocol (Section 4.A or 4.B).

Materials to Be Supplied by the User

- NANOpure® (or equivalent grade) water
- 6–8M urea
- 50mM Tris-HCl (pH 8)
- 1M dithiothreitol (DTT)
- 1M iodoacetamide (IAA)

Reduction and Alkylation Protocol

1. Solubilize a protein or protein extract in 6–8M urea/50mM Tris-HCl (pH 8).
2. Add DTT to a final concentration of 5mM, mix and incubate for 30 minutes at 37°C.
3. Add iodoacetamide to a final concentration of 15mM, mix and incubate in the dark at room temperature for 30 minutes.

If the sample is not used immediately, snap-freeze the reduced and alkylated protein solution on dry ice and store at –20°C for up to 1 month.

4. Digestion Protocols

The Trypsin/Lys-C Mix was developed to improve digestion of proteins or protein mixtures (e.g., cellular protein extracts) in solution. The mix is also suitable for in-gel protein digestion, although improvements in digestion may be minor when compared to in-gel digestion with trypsin. An in-gel digestion protocol can be found in Section 6.B.

Trypsin/Lys-C Mix is recommended for use in two applications: standard in-solution digestion and in-solution digestion of proteolytically resistant proteins. The first application is intended for users who would like to improve proteolytic efficiency using a standard digestion protocol (i.e., overnight incubation under non-denaturing or mildly denaturing conditions). Digestion of proteolytically resistant proteins uses a two-step procedure, with the first step occurring under strong denaturing conditions.

Note: Guanidine chloride inhibits trypsin even at relatively low concentrations. We have observed that more than half of tryptic peptides were underdigested after overnight incubation with trypsin under the conditions described by Saveliev *et al.* (4). Trypsin/Lys-C addresses this inhibition by reducing the number of missed tryptic cleavages to more conventional levels. To minimize protease inhibition due to guanidine chloride, we recommend diluting guanidine chloride to 0.5M or less prior to digestion, replacing trypsin with Trypsin/Lys-C Mix and following the standard overnight digestion protocol (Section 4.A or 4.B).

Materials to Be Supplied by the User

- NANOpure® (or equivalent grade) water
- 50mM Tris-HCl (pH 8)
- trifluoroacetic acid (TFA)
- urea



4.A. Standard In-Solution Protein Digestion

Note: If the reduction and alkylation protocol provided above was used, dilute the reduced and alkylated protein solution sixfold or greater with 50mM Tris-HCl (pH 8) before digestion to reduce the urea concentration to 1M or less. Urea concentrations higher than 1M inhibit trypsin.

1. Add Trypsin/Lys-C Mix to a protein or protein mixture at a 25:1 protein:protease ratio (w/w), mix and incubate overnight at 37°C.
We recommend using 50mM Tris-HCl (pH 8) as the digestion buffer.
2. Terminate digestion by adding trifluoroacetic acid to a final concentration of 0.5–1%. Remove particulate material by centrifuging at 14,000–16,000 × *g* for 10 minutes.
3. Analyze the supernatant.

4.B. Two-Step In-Solution Digestion of Proteolytically Resistant Proteins

Note: If our reduction and alkylation protocol was used, proceed to Step 2.

1. Add urea and Tris-HCl (pH 8) to a protein or protein mixture to a final concentration of 6–8M and 50mM, respectively, before the digestion.
2. Add Trypsin/Lys-C Mix to protein at a 25:1 protein:protease ratio (w/w), mix and incubate for 3–4 hours at 37°C.
3. Dilute the reaction sixfold or greater with 50mM Tris-HCl (pH 8) to reduce urea concentration to 1M or below, and continue incubating overnight at 37°C.
4. Terminate digestion by adding trifluoroacetic acid to a final concentration of 0.5–1%, and remove particulate material by centrifuging at 14,000–16,000 × *g* for 10 minutes.
5. Analyze the supernatant.

5. 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

Inefficient digestion (e.g., high number of missed cleavages or few identified proteins and peptides)

Causes and Comments

Protein solution contains protease inhibitor. Purify the protein, and digest again.

pH of the digestion reaction is low. Using large volumes of Trypsin/Lys-C Mix can decrease the pH of the digestion reaction due to the low pH of Trypsin/Lys-C Resuspension Buffer (pH<3). Neutralize the pH to make it more compatible with the digestion reaction.

Trypsin/Lys-C Mix has lost activity.

- The provided Resuspension Buffer is the recommended reconstitution buffer. Reconstituting the Trypsin/Lys-C Mix in other buffers will inactivate the proteases.
- The Trypsin/Lys-C Mix was stored at -20°C rather than the recommended -70°C .

Protein is proteolytically resistant. Use the Two-Step In-Solution Digestion of Proteolytically Resistant Proteins protocol in Section 4.B.

Low peptide yield

Protein was poorly solubilized or may have precipitated, resulting in only a small fraction of protein being digested. Solubilize and reduce the protein in the presence of high urea concentrations (see Recommendations for Protein Preparation Prior to Digestion, Section 3).

6. Appendix

6.A. References

1. Trinidad, J.C. *et al.* (2006) Comprehensive identification of phosphorylation sites in postsynaptic density preparations. *Mol. Cell. Proteomics* **5**, 914–22.
2. Yen, C.Y. *et al.* (2006) Improving sensitivity in shotgun proteomics using a peptide-centric database with reduced complexity: Protease cleavage and SCX elution rules from data mining of MS/MS spectra. *Anal. Chem.* **78**, 1071–84.
3. 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. *Int. J. Mass Spectrom.* **219**, 245–51.
4. Saveliev, S. *et al.* (2013) Trypsin/Lys-C protease mix for enhanced protein mass spectrometry analysis. *Nature Methods* **10**, i–ii.

6.B. In-Gel Protein Digestion

Washing

1. Resolve proteins by gel electrophoresis, and stain the gel. Wash with an appropriate destaining solution to remove nonspecifically bound stain.
2. Excise the protein band or spot of interest. Cut the gel slice into 1mm³ pieces, and transfer to a 1.5ml microcentrifuge tube.
3. Add 200µl of 25mM ammonium bicarbonate, and incubate at room temperature for 15 minutes. Remove the supernatant, and discard.
4. Add 200µl of acetonitrile, and incubate at room temperature for 15 minutes. Remove the supernatant, and discard.
5. Repeat Steps 3 and 4 three times for a total of four washes.

Reduction/Alkylation

6. Add 200µl of 10mM DTT in 25mM ammonium bicarbonate, and allow the gel to swell.
7. Incubate for 30 minutes at 65°C. Remove the supernatant, and discard.
8. Add 200µl of 25mM ammonium bicarbonate, and incubate at room temperature for 15 minutes. Remove the supernatant, and discard.
9. Add 200µl of acetonitrile, and incubate at room temperature for 15 minutes. Remove the supernatant, and discard.
10. Repeat Steps 8 and 9 three times for a total of four washes.
11. Add 200µl of freshly prepared 10mM iodoacetamide in 25mM ammonium bicarbonate, and incubate the gel in the dark for 45 minutes at 37°C. Remove the supernatant, and discard.
12. Add 200µl of 25mM ammonium bicarbonate, and incubate at room temperature for 15 minutes. Remove the supernatant, and discard.

13. Add 200µl of acetonitrile, and incubate at room temperature for 15 minutes. Remove the supernatant, and discard.
14. Repeat Steps 12 and 13 three times for a total of four washes.

Digestion

15. Rehydrate Trypsin/Lys-C Mix in Reconstitution Buffer to 1µg/µl. Dilute an aliquot of the 1µg/µl Trypsin/Lys-C Mix in 25mM ammonium bicarbonate to a final concentration of 20ng/µl.
16. Rehydrate the gel pieces in a minimal volume (10–20µl) of 20ng/µl Trypsin/Lys-C solution for 10 minutes. Cover the gel with 60µl of 25mM ammonium bicarbonate.
17. Incubate overnight at 37°C.
18. Add 10µl of 1% formic acid, remove the supernatant and analyze the sample.

Optional: Clean up the peptides with ZipTip® pipette tips (Millipore Corporation) following the manufacturer's directions.

6.C. Related Products

Product	Size	Cat. #
Trypsin Gold, Mass Spectrometry Grade	100µg	V5280
Sequencing Grade Modified Trypsin	100µg	V5111
Sequencing Grade Modified Trypsin, Frozen	100µg	V5113
rLys-C, Mass Spec Grade	15µg	V1671
Arg-C, Sequencing Grade	10µg	V1881
Asp-N, Sequencing Grade	2µg	V1621
Chymotrypsin, Sequencing Grade	25µg	V1061
	100µg	V1062
Glu-C, Sequencing Grade	50µg	V1651
Endoproteinase Lys-C, Sequencing Grade	5µg	V1071
Elastase	5mg	V1891
Thermolysin	25mg	V4001

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