Technical Manual

Immobilized Trypsin

INSTRUCTIONS FOR USE OF PRODUCTS V9012 AND V9013.

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1. Description

Immobilized Trypsin provides a fast and convenient method for digesting purified or complex proteins over a wide range of concentrations. With the spin column format, digested peptides are easily separated from the Immobilized Trypsin, reducing enzyme interference during analysis.

Trypsin is a proteolytic enzyme that cleaves at the carboxyl side of positively charged Lysine (Lys) and Arginine (Arg) residues. When these amino acids are followed by the nonpolar Proline (Pro), the digestion of the site is not efficient. When Lys and Arg are followed by Aspartic Acid (Asp) and Glutamic Acid (Glu), the digestion is also not as efficient.

Native trypsin is subject to autolysis, generating pseudotrypsin, which exhibits a decreased specificity including a chymotrypsin-like activity (1). Such autolysis products, if present in a trypsin preparation, would result in additional peptide fragments.Immobilized Trypsin has been treated with N-*p*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK) to block chymotrypsin activity, and formaldehyde to block amine groups on the trypsin to decrease autolysis. These treatments reduce the presence of trypsin autolytic peptides that may interfere with sample analysis and other downstream applications (2).

The trypsin is conjugated to a cellulose resin and provided in a 1:1 v/v slurry in 50mM acetic acid, 1mM $CaCl_2$, 0.02% sodium azide.

Advantages of Immobilized Trypsin:

- **Complete your digestion in as little as 30 minutes.** The high trypsin concentration on the resin allows a higher protease:protein ratio than in-solution digests, resulting in faster digestion.
- **Adjust resin volume to accommodate varying protein concentrations.** Optimal range is 10–500µg of protein.
- **Perform fewer steps.** No shaking or water baths are required.

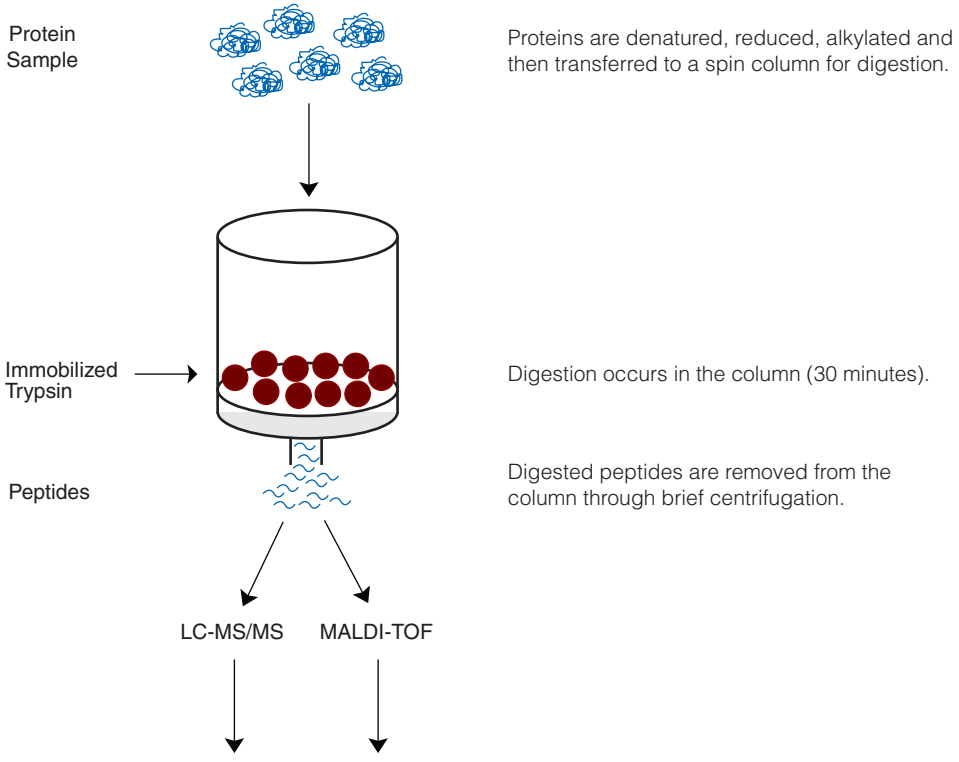

Figure 1. Overview of protein digestion using Immobilized Trypsin.

2. Product Components and Storage Conditions

Product	Size	Cat.#
Immobilized Trypsin	2ml	V9012

This system contains sufficient reagents for 10 reactions. Includes:

2ml	Immobilized Trypsin
10 each	Spin Columns with caps

Product	Size	Cat.#
Immobilized Trypsin	4ml (2 × 2ml)	V9013

This system contains sufficient reagents for 20 reactions. Includes:

4ml	Immobilized Trypsin
20 each	Spin Columns with caps

Storage Conditions: Store Immobilized Trypsin resin at 4°C.

3. Digestion

Materials to be Supplied by User

(See Section 5.D for the composition of buffers and solutions.)

- 0.5ml centrifuge tubes
- 1.5ml centrifuge tubes
- pipettes and tips
- 50mM ammonium bicarbonate (pH 7.5)
- acetonitrile
- microcentrifuge
- trifluoroacetic acid (TFA) [formic acid (FA) can be substituted for TFA]
- speed vacuum

Note: Always use gloves while handling tubes, preparing buffers and performing the experiments to avoid any possible keratin contamination. Working in a sterile hood also helps to prevent contamination.

3.A. Protein Preparation: Denaturation, Reduction and Alkylation

Denature, reduce and alkylate proteins using your preferred protocol. For best results, protocols for denaturing need to be optimized for individual sample sources or proteins. A basic protocol for denaturation, reduction and alkylation is given in Section 5.A.

Note: We recommend starting with higher concentrations (>20mg/ml) of denatured protein. Otherwise the protein in the digestion sample will be diluted when buffers are added to reduce the concentration of denaturants. We recommend diluting the denaturant to a concentration that will not interfere with digestion efficiency. See Table 5 for information on common denaturatants and immobilized trypsin compatibility.

3.B. Immobilized Trypsin Digestion Protocol

1. Determine the volume of digestion mixture to use for the amount of protein being digested based on the information provided in Table 1.

Table 1. Reaction Volumes Required for Digestion.

Amount of Protein to Be Digested	Volume of Digestion Mixture
20–49µg	20µl
50–149µg	40µl
150–299µg	80µl
300–500µg	120µl

2. Make protein digestion mixture:
   a. Add protein to a 1.5ml tube.
   b. Add acetonitrile to a 40% final concentration.
   c. Bring the digestion to the final reaction volume specified in Table 1 using 50mM ammonium bicarbonate buffer (pH 7.5).
   d. Vortex to mix and set aside.

Example: Protein Digestion Mixture for 100µg of Protein.	
100µg of protein at (20mg/ml)	5µl
acetonitrile (40%)	16µl
ammonium bicarbonate	19µl
Final Volume	**40µl**

3. Label each spin column. Remove the plug at the base of the column by twisting.
4. Place the columns into 1.5ml centrifuge tubes.
5. Determine the amount of resuspended resin volume to use for digestion based on the information in Table 2.

Table 2. Amount of Resuspended Resin Volume to Use for Digestion.

Amount of Protein to Be Digested	Resuspended Resin Volume
20–49µg	100µl
50–149µg	200µl
150–299µg	400µl
300–500µg	600µl

6. Resuspend the resin by gentle rocking, shaking or tapping.
7. Using a 1ml pipette, dispense the resuspended resin and place it into the spin column.
8. Centrifuge for 5 seconds to remove liquid from the resin.
9. Wash resin with ammonium bicarbonate in the volumes indicated (Table 3). Centrifuge for 5 seconds.

Table 3. Recommended 50mM Ammonium Bicarbonate Wash Volumes.

Amount of Resuspended Resin	50mM Ammonium Bicarbonate Wash Volume
100µl	100µl
200µl	200µl
400µl	400µl
600µl	400µ1

10. Remove liquid from the tube.
11. Repeat Steps 9 and 10 two more times for a total of three washes.
12. Centrifuge for 5 seconds to remove any excess liquid.

    Note: When using >400µl of resuspended resin, two additional washes will be necessary to keep the wash volume below the top of the column.
13. Remove the spin columns, and place them into clean, labeled 1.5ml tubes.
14. Add the protein digestion solution from Step 2 directly to the resin in the spin basket. If a droplet remains on the pipette tip, gently lower it to the resin. Avoid placing the pipette tip into the resin.
15. Cap the tube loosely.
16. Incubate at room temperature for 30 minutes on the bench top.

3.C. Peptide Recovery

1. Prepare Peptide Recovery Buffer as described in Section 5.D.
2. Add appropriate volume of Peptide Recovery Buffer to the spin column, as indicated in Table 4.

   Note: You can quickly centrifuge without the Peptide Recovery Buffer; however, the recovery amount will be reduced.

Table 4. Recommended Amounts of Peptide Recovery Buffer to Use.

Amount of Resuspended Resin	Peptide Recovery Buffer Volume
100µl	50µl
200µl	100µl
400µl	200µl
600µl	300µl

This table provides the recommended volume of Peptide Recovery Buffer to use for each recovery step.

3. Quickly centrifuge (~5 seconds) to remove the peptide solution from the resin.
4. Leave the peptide solution in the tube.
5. Repeat Steps 1 and 2.
6. Remove the spin column, and save the peptide solution for downstream applications. The recovered peptide solution contains 40% acetonitrile, which may need to be removed by lyophilization before analysis.

   Note: When using resin volumes greater than 400µl, you will need two 1.5ml tubes to collect the peptide recovery solutions and pool them.

4. 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	Possible Causes and Comments
No or insufficient digestion	Inefficient denaturing. Proteins require various denaturing conditions for digestion. Determine the optimal conditions for your sample (i.e., denaturants, reduction and alkylation, amount of immobilized resin required, etc.).
	Proteins are insoluble. Test various denaturants or detergents to increase solubilization.
	Loss of immobilized trypsin activity because of inappropriate storage conditions.
Poor peptide detection	Loss of peptides in downstream processing.
	Check whether or not the final concentration of acetonitrile has been adjusted (if the samples were not concentrated by evaporation). The peptide elution buffer contains 40% acetonitrile, which will interfere with downstream analysis using C18 tips or columns.
Keratin contamination	Keratin is a contaminant from skin and hair that is commonly introduced during sample handling. Wear gloves. Consider working in a sterile hood. Use good laboratory practices when using this product.
Peaks other than peptides in HPLC analysis	Extraneous peaks may be caused by detergents or denaturants. If you have used detergents or surfactants, follow the manufacturer's protocols for removal of these components before sample analysis.
Poor peak intensities in MALDI-TOF analysis	If you use C18 resins or ZipTips® for sample preparation, the final concentration of acetonitrile should be adjusted below 10% (if the samples were not concentrated by evaporation).

5. Appendix

5.A. Protein Denaturation, Reduction and Alkylation Protocol

Preparation of Protein: In general, proteins require efficient solubilization, denaturation and disulphide bond reduction (with modification) for optimal digestion and more complete sequence coverage. The following optional steps are provided as a guideline to facilitate protease digestion with this product.

Solubilization/Denaturation: Dissolve protein in 50mM ammonium bicarbonate (pH 7.5). Proteins that are difficult to dissolve or require denaturation for efficient digestion can be solubilized in a denaturant such as 6–8M urea or 6M guanidine HCl at room temperature to 37°C for up to 1 hour. For some proteins it may be beneficial to further heat the sample to 60°C over this time period (95°C for 15–20 minutes can be used in extreme cases).

Disulphide reduction: To the dissolved protein, add DTT for a final concentration of 5mM or TCEP (≤5mM); heat this sample at 50–60°C for 20 minutes. (See Section 5.D.)

Alkylation: Add iodoacetamide to the reduced protein solution at room temperature to a final concentration of 15mM, and incubate in the dark for 15 minutes at room temperature. (See Section 5.D.)

5.B. Compatibility Chart

Table 5. Compatibility of Denaturants, Reductants and Detergents with Immobilized Trypsin.

Urea	≤4M
Guanidine HCl	≤3M
Methanol	<60%
DTT	≤50mM
TCEP	≤5mM
pH	5–9
Iodoacetamide	≤300mM
ProteaseMAX™ Surfactant	Use as described in ProteaseMAX™ denaturation protocol (Technical Bulletin #TB373)

5.C. References

1. Keil-Dlouha, V. *et al*. (1971) Proteolytic activity of pseudotrypsin. *FEBS LETT*. **16**, 291–5.

2. Rice, R.H., Means, G.E. and Brown, W.D. (1977) Stabilization of bovine trypsin by reductive methylation. *Biochem. Biophys. Acta* **492**, 316–21.

5.D. Composition of Buffers and Solutions

50mM ammonium bicarbonate buffer (pH ~7.8)

Dissolve 98.8mg of NH_4HCO_3 in 25ml of NANOpure® water. Prepare the buffer immediately before use, and store at room temperature until use.

8M urea

Dissolve 480.5mg of mass spectrometry grade urea crystals in a final volume of 1ml of NANOpure® water.

1M dithiothreitol (DTT)

Dissolve 154.25mg of DTT in a final volume of 1ml of NANOpure® water. Store on ice.

80mM dithiothreitol (DTT) solution

Pipet 80µl of 1M DTT and add 920µl of NANOpure® water. Freeze in aliquots at –20°C.

0.55M iodoacetamide (IAA)

Dissolve 40.7mg of IAA in 400µl of 50mM NH_4HCO_3. Prepare immediately before use, and protect from light.

100mM iodoaetamide (IAA) solution

Dispense 181µl of the 0.55mM IAA and add 819µl of 50mM NH_4HCO_3 immediately before use. Protect from light.

10% trifluoroacetic acid (TFA)

900µl	NANOpure® water
100µl	trifluoracetic acid

peptide recovery buffer, 1ml

400µl	acetonitrile
20µl	10% TFA
580µl	50mM ammonium bicarbonate

5.E. Related Products

Product	Size	Cat.#
Trypsin Gold, Mass Spectrometry Grade	100µg	V5280
ProteaseMAX™ Surfactant, Trypsin Enhancer	1mg	V2071
	5 × 1mg	V2072
Sequencing Grade Modified Trypsin*	100µg	V5111

*For Laboratory Use.