



**Promega**

## Technical Bulletin

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# Trypsin Gold, Mass Spectrometry Grade

INSTRUCTIONS FOR USE OF PRODUCT V5280.



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# Trypsin Gold, Mass Spectrometry Grade

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## 1. Description

Trypsin is a serine protease that specifically cleaves at the carboxylic side of lysine and arginine residues. The distribution of Lys and Arg residues in proteins is such that trypsin digestion yields peptides of molecular weights that can be analyzed by mass spectrometry. The pattern of peptides obtained is used to identify the protein.

The stringent specificity of trypsin is essential for protein identification. Native trypsin is subject to autolysis, generating pseudotrypsin, which exhibits a broadened specificity including a chymotrypsin-like activity (1). Such autolysis products, present in a trypsin preparation, would result in additional peptide fragments that could interfere with database analysis of the mass of fragments detected by mass spectrometry.

Trypsin Gold, Mass Spectrometry Grade, has been manufactured to provide maximum specificity. Lysine residues in the porcine trypsin have been modified by reductive methylation, yielding a highly active and stable molecule that is extremely resistant to autolytic digestion (2). The specificity of the purified trypsin is further improved by TPCK treatment, which inactivates chymotrypsin. The treated trypsin is then purified by affinity chromatography and lyophilized to yield Trypsin Gold, Mass Spectrometry Grade.

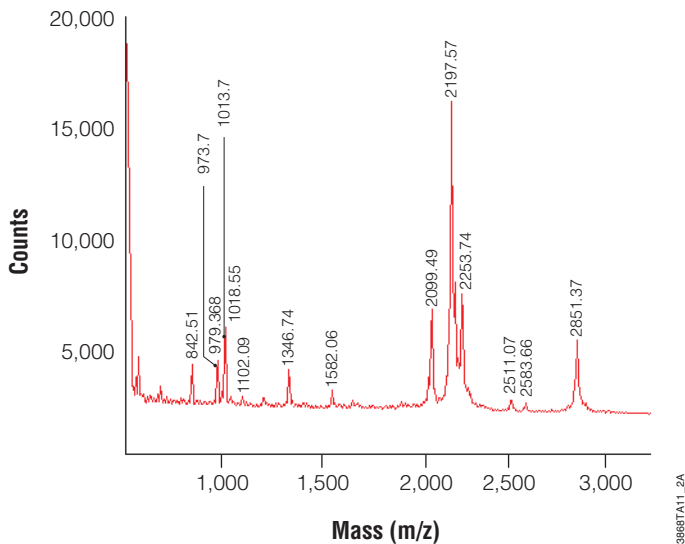
## 1. Description (continued)

Trypsin Gold, Mass Spectrometry Grade, has extremely high specific activity. Modified trypsin is maximally active in the range of pH 7-9 and is reversibly inactivated at pH <4. It is resistant to mild denaturing conditions such as 0.1% SDS, 1M urea or 10% acetonitrile (3) and retains 50% of its activity in 2M guanidine HCl (4). The activity of trypsin is decreased when acidic residues are present on either side of a susceptible bond. If proline is at the carboxylic side of lysine or arginine, the bond is almost completely resistant to cleavage by trypsin.

Trypsin is often used for in-gel digestion. In this procedure, complex protein mixtures such as cell extracts are resolved by gel electrophoresis, and the band or spot of interest is excised from the gel and digested with trypsin. The digestion products are purified and concentrated, then analyzed by mass spectrometry to determine their molecular weights. Database searches can then be performed, using the mass of the peptides to identify the protein(s) resolved on the gel (5).

Figure 1 is a spectrogram generated using this protocol. When the masses observed on this spectrogram were entered into a database, the protein was identified as carbonic anhydrase II.

Each lot of quality-tested Trypsin Gold, Mass Spectrometry Grade, is qualified for use with in-gel digestion and mass spectrometric analysis. A copy of the spectrogram generated with carbonic anhydrase II and any lot of Trypsin Gold, Mass Spectrometry Grade, may be obtained from Promega Technical Services at 1-800-356-9526.



**Figure 1. 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 Trypsin Gold, Mass Spectrometry Grade, overnight at 37°C. The peptides generated were purified as described in Sections 3 and 5 and analyzed using a PerSeptive BioSystems Voyager-DE™ MALDI-TOF system. The peak at 842.51 is due to autolysis of trypsin.

## 2. Product Components and Storage Conditions

Product	Size	Cat.#
Trypsin Gold, Mass Spectrometry Grade	100µg	V5280

**Storage Conditions:** Store the lyophilized powder at -20°C. Reconstitute the powder in 50mM acetic acid and store at -20°C for up to one month. For long-term storage, freeze reconstituted trypsin at -70°C. Thaw the reconstituted trypsin at room temperature, placing on ice immediately after thawing. Remove the amount of trypsin needed, then refreeze the unused portion. To maintain maximum product activity, limit the number of freeze-thaw cycles to 5.

### 3. Trypsin Protocols

#### 3.A. In-Gel Protein Digestion

Numerous protocols for in-gel protein digestion have been described (6–8). The following procedure has been used successfully by Promega scientists.

##### Materials To Be Supplied by the User

- SimplyBlue™ SafeStain (Invitrogen Cat.# LC6060)
- trifluoroacetic acid (TFA)
- acetonitrile (ACN)
- 200mM NH<sub>4</sub>HCO<sub>3</sub> buffer (pH 7.8)
- NANOpure® water
- ZipTip®<sub>SCX</sub> pipette tips (Millipore Cat.# ZTSCXS096)
- α-cyano-4-hydroxycinnamic acid (CHCA)
- MALDI target
- ZipTip®<sub>C18</sub> pipette tips (Millipore Cat.# ZTC18S096)



We recommend wearing gloves throughout the in-gel digestion procedure to avoid contamination of samples by protein present on hands.

1. Separate protein samples by electrophoresis on an SDS-Tris-Glycine gel (see Note 1 at the end of this section).
2. Rinse the gel three times, for 5 minutes each rinse, in NANOpure® water. Stain for 1 hour in SimplyBlue™ SafeStain (Invitrogen Corporation) at room temperature with gentle agitation (see Note 1 at the end of this section). When staining is complete, discard the staining solution.
3. Destain the gel for 1 hour in NANOpure® water at room temperature with gentle agitation. When destaining is complete, discard the solution.
4. Using a clean razor blade, cut the protein bands of interest from the gel, eliminating as much polyacrylamide as possible. Place the gel slices into a 0.5ml microcentrifuge tube that has been prewashed twice with 50% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA).

**Note:** To avoid contamination, razor blades, staining containers and all other equipment that comes into contact with the gel or gel slices should be cleaned with laboratory detergent (e.g., Alconox®) and rinsed well prior to use.

5. Destain the gel slices twice, with 0.2ml of 100mM NH<sub>4</sub>HCO<sub>3</sub>/50% ACN for 45 minutes each treatment, at 37°C to remove the SimplyBlue™ SafeStain.
6. Dehydrate the gel slices for 5 minutes at room temperature in 100µl 100% ACN. At this point, the gel slices will be much smaller than their original size and will be whitish or opaque in appearance.
7. Dry the gel slices in a Speed Vac® for 10–15 minutes at room temperature to remove the ACN.
8. Resuspend the Trypsin Gold at 1µg/µl in 50mM acetic acid, then dilute in 40mM NH<sub>4</sub>HCO<sub>3</sub>/10% ACN to 20µg/ml. Preincubate the gel slices in a minimal volume (10–20µl) of the trypsin solution at room temperature

(do **not** exceed 30°C) for 1 hour. The slices will rehydrate during this time. If the gel slices appear white or opaque after one hour, add an additional 10–20µl of trypsin and incubate for another hour at room temperature.

**Note:** Trypsin specific activities can vary widely, depending on the manufacturer. Procedures describing the use of trypsin by weight may need to be optimized depending on the specific activity of the trypsin being used.

9. Add enough digestion buffer (40mM NH<sub>4</sub>HCO<sub>3</sub>/10% ACN) to completely cover the gel slices. Cap the tubes tightly to avoid evaporation. Incubate overnight at 37°C.
10. Incubate the gel slice digests with 150µl of NANOpure® water for 10 minutes, with frequent vortex mixing. Remove and save the liquid in a new microcentrifuge tube (see Note 2 at the end of this section).
11. Extract the gel slice digests twice, with 50µl of 50% ACN/5% TFA (with mixing) for 60 minutes each time, at room temperature (see Note 2 at the end of the section).
12. Pool all extracts (Steps 10 and 11) and dry in a Speed Vac® at room temperature for 2–4 hours (do **not** exceed 30°C).
13. Purify and concentrate the extracted peptides using ZipTip® pipette tips (Millipore Corporation) following the manufacturer's directions or the ZipTip® protocol described in Section 5.B.
14. The peptides eluted from the ZipTip® tips are now ready for mass spectrometric analysis.

#### **Notes:**

1. Other gel systems and staining reagents can be used for in-gel digestions (6–8) but should be tested to ensure compatibility with the protein of interest and the detection system being used.
2. Alternatively, the buffer solution may be removed from the in-gel digestion immediately following the overnight incubation step, dried with a Speed Vac®, then prepared for mass spectrometry. The effectiveness and utility of the aqueous and organic extractions following in-gel digests depends on the target protein and should be determined by the user. In general, the aqueous and organic extractions are recommended for initial digestions or for previously uncharacterized samples.

### **3.B. Digestion of Proteins in Solution**

In general, proteins require denaturation and disulfide bond cleavage for enzymatic digestion to reach completion (4). If partial digestion of a native protein is desired, begin this protocol at Step 3.

1. Dissolve the target protein in 6M guanidine HCl (or 6–8M urea or 0.1% SDS), 50mM Tris-HCl (pH 8), 2–5mM DTT (or β-mercaptoethanol).

### 3.B. Digestion of Proteins in Solution (continued)

2. Heat at 95°C for 15–20 minutes or at 60°C for 45–60 minutes. Allow the reaction to cool.
3. For denatured proteins, add 50mM NH<sub>4</sub>HCO<sub>3</sub> (pH 7.8) or 50mM Tris-HCl, 1mM CaCl<sub>2</sub> (pH 7.6), until the guanidine HCl or urea concentration is less than 1M. If SDS is used, dilution is not necessary. For digestion of native proteins, dissolve the protein in buffer with a pH between 7 and 9.
4. Add Trypsin Gold to a final protease:protein ratio of 1:100 to 1:20 (w/w). Incubate at 37°C for at least 1 hour. Remove an aliquot and chill the remainder of the reaction on ice or freeze at -20°C.
5. Terminate the protease activity in the aliquot from Step 4 by adding an inhibitor (Section 5.A). Alternatively, precipitate the aliquot by adding TCA to a 10% final concentration. The reaction can also be terminated by freezing at -20°C. Trypsin can also be inactivated by lowering the pH of the reaction below pH 4. Trypsin will regain activity when the pH is raised above pH 4 (4).
6. Determine the extent of digestion by subjecting the aliquot in Steps 4 and 5 to reverse phase HPLC or SDS-PAGE.
7. If no inhibitors were added to the remainder of the reaction and further proteolysis is required, incubate at 37°C until the desired digestion is obtained (9). Reducing the temperature will decrease the digestion rate. Incubations of up to 24 hours may be required, depending on the nature of the protein. With long incubations, take precautions to avoid bacterial contamination.

### 4. Troubleshooting

Symptoms	Causes and Comments
No or insufficient in-gel digestion	Different target proteins may require different digestion conditions. Determine optimum conditions for each target protein. This includes optimizing trypsin:protein ratios and incubation times.
	Peptides not eluting from the ZipTip® tips. Optimize the ZipTip® elution conditions. Follow the manufacturer's directions for use.
	Incompatible reconstitution buffer. Reconstitution of Trypsin Gold in buffers other than 50mM acetic acid is not recommended.
	Loss of trypsin activity due to multiple freeze-thaw cycles. Reconstituted trypsin is stable for up to 5 freeze-thaw cycles.

#### 4. Troubleshooting (continued)

Symptoms	Causes and Comments
The spectrogram contains peaks at 842 and 2211	These peaks are due to autocatalytic trypsin cleavage at arginine residues, which are not protected by reductive methylation. These peaks are often used to calibrate the mass spectrometer.
Apparent chymotrypsin peaks on mass spectrogram	The presence of chymotrypsin is very unlikely, because it is inhibited by TPCK. These peaks are most likely due to pseudotrypsin activity (1). <ul style="list-style-type: none"> <li>• Try using lower trypsin:protein ratios and/or shorter incubation times.</li> <li>• Reconstitution of Trypsin Gold, Mass Spectrometry Grade, in buffers other than 50mM acetic acid is not recommended.</li> <li>• Reconstituted trypsin subjected to more than 5 freeze-thaw cycles may contain products of autolysis.</li> </ul>
Reaction contamination	Keratin is a contaminant from skin and hair commonly introduced during sample handling. Wear gloves. Work in a hood. Thoroughly clean all equipment and use disposable components when possible.

#### 5. Appendix

##### 5.A. Trypsin Inhibitors

The following are general trypsin inhibitors:

Antipain (50µg/ml), antithrombin (1unit/ml), APMSF (0.01–0.04mg/ml), aprotinin (0.06–2µg/ml), leupeptin (0.5µg/ml), PMSF (17–170µg/ml), TLCK (37–50µg/ml), trypsin inhibitors (10–100µg/ml).

##### 5.B. ZipTip® Protocol

Promega scientists have successfully used the following ZipTip® (ZipTip®<sub>SCX</sub> or ZipTip®<sub>C18</sub>) pipette tip protocol for purification and sample concentration. These conditions may not be optimal for all proteins/peptides.

1. Prepare ZipTip® tips by washing with 10µl of 100% ACN, then washing 2–3 times with 10µl of 0.1% TFA.
2. Reconstitute the samples with 10µl of 0.1% TFA.
3. Draw the sample into ZipTip® tips by pipetting fully into and out of tips 4–5 times. Expel liquid.

### 5.B. ZipTip® Protocol (continued)

4. Wash ZipTip® tips 2-3 times with 10µl of 0.1% TFA to remove contaminants.
5. Elute peptides in 2.5µl of 70% ACN/0.1% TFA containing 10mg/ml  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) and spot directly onto MALDI target.

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