

# In-Gel Tryptic Digestion Kit

89871

1468.2

Number	Description
89871	<b>In-Gel Tryptic Digestion Kit</b> , sufficient reagents for approximately 150 in-gel digestions <b>Kit Contents:</b> <b>Trypsin, Modified</b> , 20 µg <b>Trypsin Storage Solution</b> , 40 µl <b>Acetonitrile</b> , 3 × 24 ml <b>Ammonium Bicarbonate</b> , 300 mg <b>TCEP (Tris[2-carboxyethyl]phosphine)</b> , 500 µl <b>Iodoacetamide (IAA)</b> , 500 mg <b>Storage:</b> Upon receipt store Trypsin, Modified at -20°C in a nonfrost-free freezer. Store all other components at 4°C. Product shipped on gel pack. See Important Product Information for additional storage and handling information for each component upon receipt.

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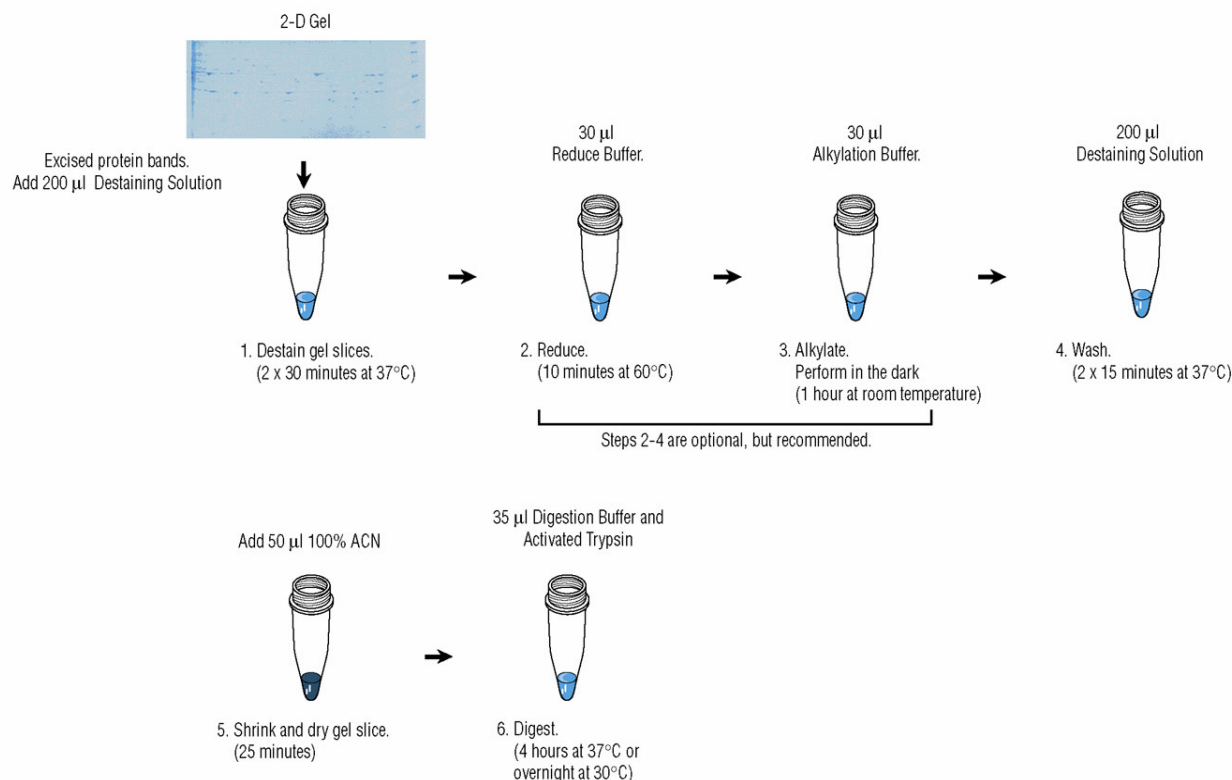
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## Introduction

In-gel digestion coupled with mass spectrometric analysis is a powerful tool for the identification and characterization of proteins.<sup>1,2</sup> The In-Gel Tryptic Digestion Kit provides a complete set of reagents to perform ~150 digestions on colloidal coomassie or fluorescent dye-stained protein bands. The kit includes modified trypsin, destaining buffers, digestion buffers, reduction reagents and alkylation reagents. The methodology of this kit has been designed to function with a wide range of protein band concentrations producing complete and accurate digest for dependable mass spectrometric (MS) analysis.

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## Procedure Summary



## Important Product Information

- Trypsin is a serine protease that specifically cleaves peptide bonds at the carboxyl side of lysine and arginine residues. However, cleavage can be blocked or slowed by a proximal acidic, aromatic or proline residue; proline having the most significant effect. Peptide fragments with one missed cut are common and should be taken into consideration during mass analysis.
- The Modified Trypsin provided in this kit displays only limited autolytic activity that should not interfere with mass spectral analysis. A trypsin fragment of mass 842.51 ( $m/z$ ,  $M + H$ ) will be the most common using standard conditions and can be used as an internal standard.
- The Trypsin In-Gel Digestion Kit is designed for colloidal coomassie or fluorescent dye-stained acrylamide gel slices. For protein bands stained with mass spectrometry-compatible silver stains or reversible zinc staining (Product No. 24582), alternative destaining procedures will be required.<sup>3-4</sup>
- For SDS-PAGE separations, use polyacrylamide gels of 1 mm thickness. Gels of other thicknesses may result in reduced peptide recovery yield.<sup>5</sup>
- Reduction and alkylation of cystine residues using TCEP and IAA, respectively, improves the recovery of cystine-containing peptides from in-gel digests and minimizes the appearance of unknown masses in MS analysis from disulfide bond formation and side chain modification. Alkylation is optional, but highly recommended.<sup>6</sup> A reliable and optimized method for reduction and alkylation, as part of the in-gel digestion protocol, is provided below. Nevertheless, alkylation can be preformed in a variety of ways dependent on the application,<sup>7-9</sup> and no one method is optimal for all applications.

**Note:** Alkylation with iodoacetamide increases the mass of a peptide by 57.02 for each cystine present. Acrylamide modification of cystine results in a peptide mass increase of 71.04.

**Note:** When separating and examining proteins by 2-D gel electrophoresis using alkaline conditions (i.e., pH >8), alkylate the sample before isoelectric focusing (IEF). The use of an alternative reducing agent (e.g., hydroxyethyl disulfide) may help to avoid spurious banding in the alkaline regions caused by disulfide bond formation.<sup>7,10</sup> Alkylation of sample before 2-D electrophoresis is not required for proteins with a pI <8.0.

## Additional Materials Required

- 600 µl microcentrifuge tubes
- 50 ml capped bottle or equivalent
- 10 ml storage bottle, tube or equivalent
- Ultrapure Water (18 megaohm equivalent)

**Note:** Use ultrapure water in the preparation of all materials.

## Material Preparation

**Note:** Some of the solutions required for the In-Gel Tryptic Digestion Kit require occasional preparation while others need to be prepared just before use as needed; therefore, plan accordingly.

Trypsin Stock:	<p>Modified Trypsin (20 µg) is supplied lyophilized and may be stored in this form at -20°C for &gt;1 year without significant loss in activity.</p> <p>When required, prepare trypsin stock solution by hydrating the lyophilized trypsin with 20 µl of the supplied Trypsin Storage Solution. This solution contains components that inactivate and protect the enzyme from autodigestion.</p> <p>To minimize freeze-thaw cycles and to increase storage stability, divide the hydrated Trypsin into four separate tubes of ~5 µl each. Store each aliquot at -20°C in a nonfrost-free freezer. This solution is used to form the Trypsin Working Solution as needed (see below).</p>
Trypsin Working Solution:	<p>When required, thaw a Trypsin Stock aliquot on ice. Dilute stock 10-fold by adding 45 µl ultrapure water. This solution may be stored at -20°C for 2 months without significant activity loss.</p>
Destaining Solution:	<p>Mix 80 mg of ammonium bicarbonate with 20 ml of acetonitrile (ACN) and 20 ml of ultrapure water. The Destaining Solution may be stored at 4°C for 2 months. This stock solution is sufficient for 50-100 digestions and can be prepared three times with this kit.</p>
Digestion Buffer:	<p>Mix 10 mg of ammonium bicarbonate with 5 ml of ultrapure water (final concentration ~25 mM). Digestion Buffer may be stored at 4°C for 2 months. This stock solution can be prepared three times with this kit.</p> <p><b>Note:</b> An excess of Digestion Buffer is supplied to minimize the need for long-term storage and weighing minute quantities of ammonium bicarbonate.</p>
Reducing Buffer:	<p>Prepare just before use (Step B.1). Mix 3.3 µl of TCEP with 30 µl of Digestion Buffer for each digest to be performed. Final TCEP concentration is ~50 mM.</p> <p><b>Note: Do not store Reducing Buffer.</b></p>
Alkylation Buffer:	<p>Prepare just before use (Step B.3) in foil-wrapped tubes to avoid exposure to light. To avoid weighing sub-microgram quantities of IAA when a small number of samples are being processed, dissolve 7 mg of IAA in 70 µl water to make a 5X stock (~500 mM final concentration). Dilute 7 µl of the 5X stock solution with 28 µl of Digestion Buffer for each digest being performed to make the final Alkylation Buffer. If greater than 10 samples are being digested simultaneously, increase the volume of stock accordingly. Excess IAA has been supplied with this kit.</p> <p><b>Note: Do not store the Alkylation Buffer or stock solution.</b></p>
Activated Trypsin:	<p>Shortly before use (Step C.3) dilute 1 µl of Trypsin Working Solution with 9 µl of Digestion Buffer for each sample being processed. Final concentration will be ~10 ng/µl. Store Activated Trypsin on ice until use.</p> <p><b>Note: Do not store Activated Trypsin.</b></p> <p><b>Note:</b> The recommended amount of trypsin used per digest is 100 ng (see protocol). This amount of trypsin can be used reliably for a wide variety of protein concentration within an excised gel band. However, if protein band contains significantly less than ~20 ng protein (~300 fmol), 25 ng of trypsin may be used per digest by diluting the Trypsin Working Solution an additional four-fold with Digestion Buffer.</p>

## Protocol for In-gel Digest from 1-D or 2-D Gel Electrophoresis Separated Proteins

### A. Band Preparation and Destaining

**Note:** This procedure is for colloidal coomassie, or fluorescent dye-stained acrylamide gel slices. Alternative destaining procedures are required for silver- or zinc-stained protein bands. See Related Pierce Products section for a listing of compatible protein stains and Additional Information section for alternative destaining procedures.

1. Use a spot picker or scalpel to excise protein band of interest from 1-D or 2-D gel. Cut band into 1x1 to 2x2 mm pieces. Place pieces into a 600 µl receiver tube.

**Note:** Take care to include only stained region of the gel.

2. Add 200 µl Destaining Solution to gel pieces. Incubate sample at 37°C for 30 minutes with shaking.
3. Remove and discard Destaining Solution from the tube.
4. Repeat steps A.2-A.3.
5. Proceed to step B.1 or C.1.

### B. Reduction and Alkylation (Optional)

**Note:** Reduction and alkylation are optional but recommended if high-sequence coverage is desired. If sample is reduced and alkylated before or during electrophoresis, it may be possible to omit these steps without affecting results. However, alkylation is inhibited or slowed by a variety of conditions, such as the presence of thiourea, SDS or a pH <7.0; therefore, alkylation of the sample before electrophoresis may not be complete.

1. Prepare Reducing Buffer as described in the Material Preparation Section. Add 30 µl of Reducing Buffer to the tube containing the sample and incubate at 60°C for 10 minutes.
2. Allow samples to cool; then remove and discard Reducing Buffer from tube.
3. Prepare Alkylation Buffer as described in the Material Preparation Section. Add 30 µl of Alkylation Buffer to the tube. Incubate sample in the dark at room temperature for 1 hour.
4. Remove and discard Alkylation Buffer from tube. Wash the sample by adding 200 µl Destaining Buffer to the tube. Incubate sample at 37°C for 15 minutes with shaking.
5. Remove and discard Destaining Buffer from tube.
6. Repeat steps B.4-B.5.
7. Proceed to step C.1.

### C. Digestion

1. Shrink gel pieces by adding 50 µl of acetonitrile. Incubate sample for 15 minutes at room temperature.
2. Carefully remove acetonitrile and allow gel pieces to air-dry for 5-10 minutes.
3. Prepare Activated Trypsin as described in the Material Preparation Section. Swell gel pieces by adding 10 µl of Activated Trypsin solution to the tube. Incubate sample at room temperature for 15 minutes.  
**Note:** If 10 µl is insufficient to cover and fully swell gel pieces, increase volume accordingly.
4. Add 25 µl Digestion Buffer to the tube. Incubate sample at 37°C for 4 hours or at 30°C overnight with shaking.
5. Remove digestion mixture and place in a clean tube.
6. (Optional) To further extract peptides, add 10 µl 1% trifluoroacetic acid or 1% formic acid solution to gel pieces and incubate for 5 minutes. Remove extraction solution and add to digestion mixture (step 5). This step also serves to inactivate trypsin, stopping additional enzymatic activity. A second extraction generally results in only a minor increase in peptide recovery.
7. Sample is now ready for liquid chromatographic separation and electrospray ionization mass spectrometry (LC-ESI MS) or for additional processing/clean-up as required for matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) or nanospray ionization mass spectrometry (see Product No. 89870).

**Note:** To prevent clogging or column damage, ensure sample is free of any acrylamide pieces before applying to a LC-ESI MC system.

## Troubleshooting

Problem	Cause	Solution
Incomplete digestion	Insufficient enzymatic activity	Increase incubation time
		Ensure gel slice was dry before addition of enzyme to pull trypsin into gel slice and increase hydration volume
	Enzyme is losing activity	Use a new Trypsin Stock aliquot
	Incorrect pH	Ensure gel slice has been completely destained and Trypsin Working solution has been diluted with digestion buffer
	Residual SDS	Ensure gel slice has been completely destained
Poor mass spectrum	Concentration or detection limits of application	Ensure sample is within the detection limit of the specific downstream application; concentrate digest on C-18 sample prep device (Product No. 89870)  <b>Note:</b> Limits vary considerably based on application and instrumentation
	Interfering agents	Clean-up digest with C-18 sample prep device

## Additional Information

Visit the Pierce web site for information related to this product, including the following Tech Tip: Process stained polyacrylamide gel pieces for mass spectrometry.

## Related Pierce Products

<b>24582</b>	<b>E-Zinc® Reversible Stain Kit</b> , sufficient for staining up to 20 SDS-PAGE mini-gels
<b>24590</b>	<b>GelCode® Blue Stain Reagent</b> , 500 ml, sufficient for staining up to 25 SDS-PAGE mini-gels
<b>24600</b>	<b>SilverSNAP® Stain for Mass Spectrometry</b> , sufficient for silver staining 20 gels mini-gels and destaining 500 gel pieces for subsequent in-gel digestion and processing for mass spectrometry
<b>89898</b>	<b>In-Solution Tryptic Digestion and Guandination Kit</b> , sufficient for 90 digests
<b>89853</b>	<b>Phosphopeptide Isolation Kit</b> , 30 spin columns
<b>89870</b>	<b>PepClean™ C-18 Spin Columns</b> , 25/pkg

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