INSTRUCTIONS

In-Gel Tryptic Digestion Kit

89871 1468.3

Number	Description
89871	**In-Gel Tryptic Digestion Kit,** sufficient reagents for approximately 150 in-gel digestions
	Kit Contents:
	Pierce Trypsin Protease, MS Grade, 20µg
	Trypsin Storage Solution, 40µL
	Acetonitrile, 3 × 24mL
	Ammonium Bicarbonate, 300mg
	TCEP (Tris[2-carboxyethyl]phosphine), 500µL
	Iodoacetamide (IAA), 500mg
	Storage: Upon receipt store Pierce Trypsin Protease, MS Grade 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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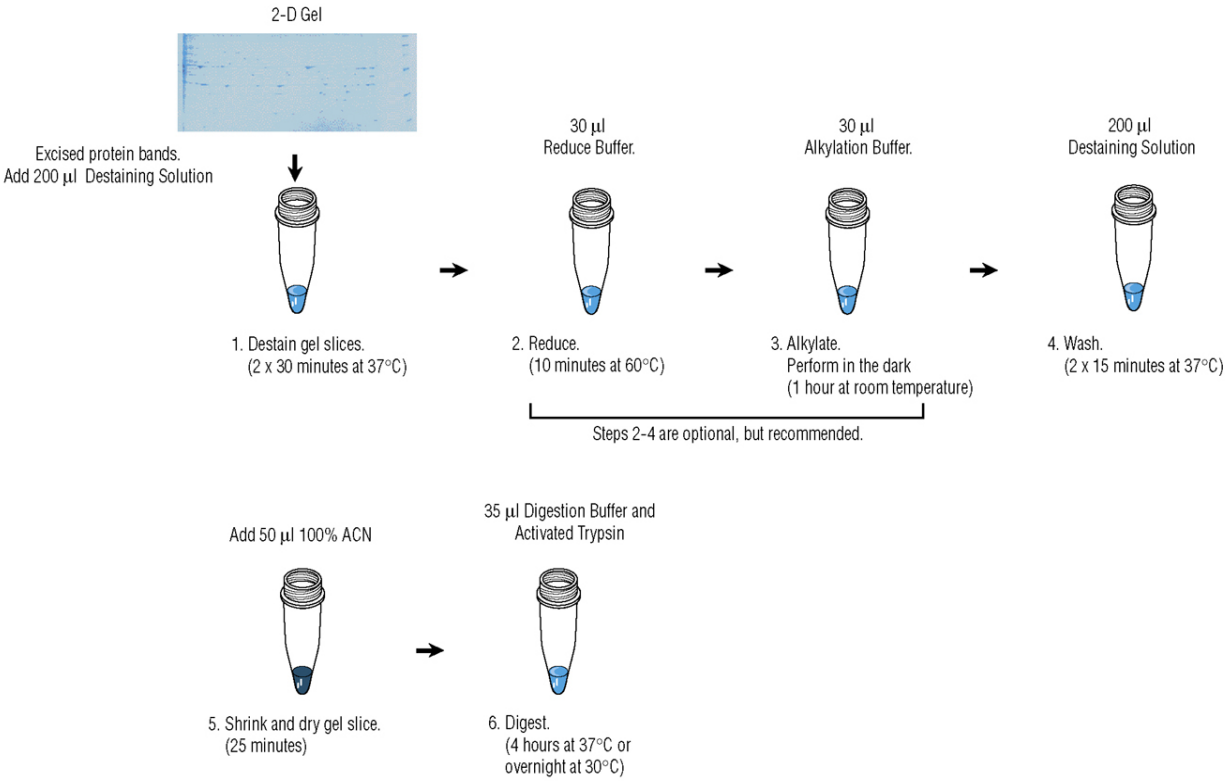
Introduction

In-gel digestion coupled with mass spectrometric analysis is a powerful tool for the identification and characterization of proteins.[1,2] The Thermo Scientific™ 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 Thermo Scientific™ Pierce™ Trypsin Protease, MS Grade, 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.

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 Pierce Trypsin Protease, MS Grade 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 In-Gel Tryptic Digestion Kit is designed for collodial 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.[3-4]
- For SDS-PAGE separations, use polyacrylamide gels of 1mm thickness. Gels of other thicknesses may result in reduced peptide recovery yield.[5]

- 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.[6] 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,[7-9] 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 2D 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.[7,10] Alkylation of sample before 2D electrophoresis is not required for proteins with a pI < 8.0.

Additional Materials Required

- 600µL microcentrifuge tubes
- 50mL capped bottle or equivalent
- 10mL storage bottle, tube or equivalent
- Ultrapure water [18 megaohm (MΩ) 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:	Pierce Trypsin Protease, MS Grade (20µg) is supplied lyophilized and may be stored in this form at -20°C for > 1 year without significant loss in activity. When required, prepare trypsin stock solution by hydrating the lyophilized trypsin with 20µL of the supplied Trypsin Storage Solution. This solution contains components that inactivate and protect the enzyme from autodigestion. To minimize freeze-thaw cycles and to increase storage stability, divide the hydrated Trypsin into four separate tubes of ~5µL 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).
Trypsin Working Solution:	When required, thaw a Trypsin Stock aliquot on ice. Dilute stock 10-fold by adding 45µL of ultrapure water. This solution may be stored at -20°C for 2 months without significant activity loss.
Destaining Solution:	Mix 80mg of ammonium bicarbonate with 20mL of acetonitrile (ACN) and 20mL 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.
Digestion Buffer:	Mix 10mg of ammonium bicarbonate with 5mL of ultrapure water (final concentration ~25mM). Digestion Buffer may be stored at 4°C for 2 months. This stock solution can be prepared three times with this kit. **Note:** An excess of Digestion Buffer is supplied to minimize the need for long-term storage and weighing minute quantities of ammonium bicarbonate.
Reducing Buffer:	Prepare just before use (Step B.1). Mix 3.3µL of TCEP with 30µL of Digestion Buffer for each digest to be performed. Final TCEP concentration is ~50mM. **Note: Do not store Reducing Buffer.**

Alkylation Buffer:	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 7mg of IAA in 70µL water to make a 5X stock (~500mM final concentration). Dilute 7µL of the 5X stock solution with 28µL 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. **Note: Do not store the Alkylation Buffer or stock solution.**
Activated Trypsin:	Shortly before use (Step C.3) dilute 1µL of Trypsin Working Solution with 9µL of Digestion Buffer for each sample being processed. Final concentration will be ~10ng/µL. Store Activated Trypsin on ice until use. **Note: Do not store Activated Trypsin.** **Note:** The recommended amount of trypsin used per digest is 100ng (see protocol). This amount of trypsin can be reliably used for a wide variety of protein concentration within an excised gel band. However, if protein band contains significantly less than ~20ng protein (~300fmol), 25ng of trypsin may be used per digest by diluting the Trypsin Working Solution an additional four-fold with Digestion Buffer.

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

A. Band Preparation and Destaining

Note: This procedure is for collodial coomassie or fluorescent dye-stained acrylamide gel slices. Alternative destaining procedures are required for silver- or zinc-stained protein bands. See Related Thermo Scientific Products Section for a listing of compatible protein stains and the Additional Information Section for alternative destaining procedures.

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

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

2. Add 200µL of 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 30µL 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 30µL 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 200µL 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 50µL 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 10µL is insufficient to cover and fully swell gel pieces, increase volume accordingly.
4. Add 25µL 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 10µL of 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 was 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 C18 sample prep device (Product No. 89870) **Note:** Limits vary considerably based on application and instrumentation
	Interfering agents	Clean-up digest with C18 sample prep device

Additional Information Available on Our Website

Tech Tip #50: Process stained polyacrylamide gel pieces for mass spectrometry

Related Thermo Scientific Products

24582	**Pierce Zinc Reversible Stain Kit**
24590	**GelCode™ Blue Stain Reagent,** 500mL
24600	**Pierce Silver Stain Kit for Mass Spectrometry**
89895	**In-Solution Tryptic Digestion and Guandination Kit**
89853	**Phosphopeptide Isolation Kit**
89870	**Pierce C18 Spin Columns,** 25/pkg

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