Agilent Protein In-Gel Tryptic Digestion Kit

Instructions

Kit Contents

The Protein In-Gel Tryptic Digestion Kit includes sufficient reagents for approximately 150 in-gel digestions.

Trypsin, Modified	20 µg
Trypsin Storage Solution	40 µL
Acetonitrile (ACN)	3 × 24 mL
Ammonium bicarbonate	300 mg
TCEP (Tris[2-carboxyethyl]phosphine)	500 µL
Iodoacetamide (IAA)	500 mg

Storage

Upon receipt store Trypsin, Modified at -20°C in a non-frost-free freezer. Store all other components at 4°C. Product is shipped on a gel pack. [See "Important Product Information" on page 2 for additional storage and handling information of each component upon receipt.]

Hazard Classifications

Refer to Table 1 for material safety classifications for this product. For Material Safety Data Sheets (MSDSs) and Certificates of Analysis, visit www.agilent.com/chem/msds.

Table 1 Hazard Classifications

Common name	Classification
USA	
Trypsin, modified	Sensitizing substance
Trypsin storage solution	–
Acetonitrile	Flammable liquid having a flash point lower than 37.8°C (100°F) Toxic Irritating substance
Ammonium bicarbonate	Irritating substance
TCEP	–
Iodoacetamide	Toxic Sensitizing substance
Europe	
Trypsin, modified	Xn
Trypsin storage solution	–
Acetonitrile	F,T
Ammonium bicarbonate	Xn
TCEP	C
Iodoacetamide	T

Introduction

In-gel digestion coupled with mass spectrometric analysis is a powerful tool for the identification and characterization of proteins [1,2]. The Agilent Protein 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 was designed to function with a wide range of protein band concentrations producing complete and accurate digests for dependable mass spectrometric (MS) analysis.

Procedure Summary

The following steps delineate the procedure.

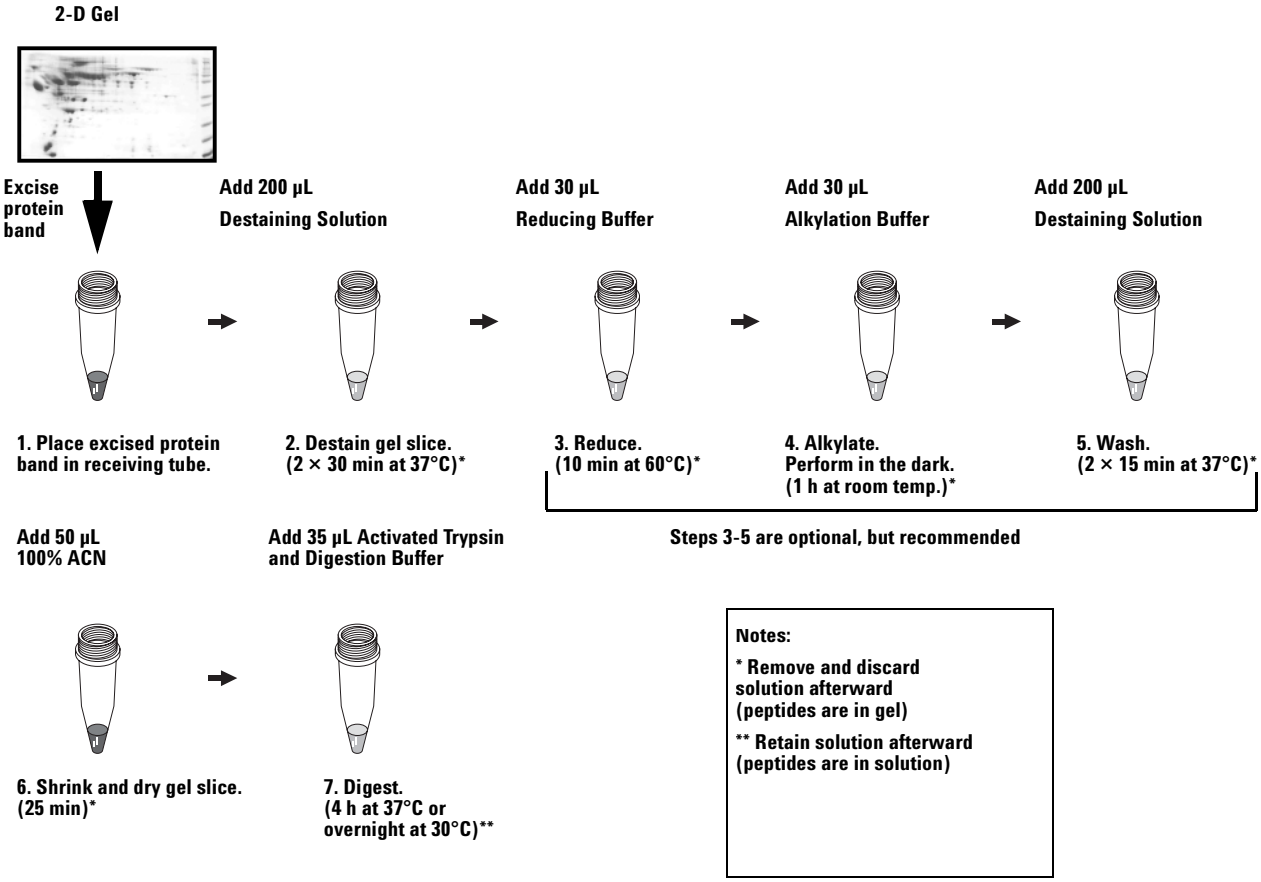

Figure 1 Protein In-Gel Tryptic Digestion Kit procedure.

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 proximal acidic, aromatic, or proline residues with 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. Trypsin fragments of mass 842.51 (*m/z*, M + H) and 2211.10 (*m/z*, M + H) will be the most common using standard conditions and can be used as internal standards.

The Protein In-Gel Tryptic Digestion Kit is designed for colloidal Coomassie or fluorescent dye-stained acrylamide gel slices. For protein bands stained with MS–compatible silver stains or reversible zinc staining, alternative destaining procedures will be required [3, 4].

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 [5]. A reliable and optimized method for reduction and alkylation, as part of the in-gel digestion protocol, is provided below. Nevertheless, alkylation can be performed in a variety of ways dependent on the application, [6–8] and no one method is optimal for all applications.

NOTE Alkylation with IAA 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 two-dimensional (2-D) gel electrophoresis using alkaline conditions (that is, pH >8), alkylate the sample before isoelectric focusing (IEF) or use an alternative reducing agent (for example, hydroxyethyl disulfide) to avoid spurious banding in the alkaline regions from disulfide bond formation [6,9]. Alkylation of samples before 2-D electrophoresis is not required for proteins with a pI <8.0.

Additional Materials Required

The list below includes required materials.

- 600-µL microcentrifuge tubes (receiver tubes)
- 50-mL capped bottle or equivalent
- 10-mL storage bottle, tube or equivalent
- Ultrapure water (18 megaohm equivalent)

CAUTION Use ultrapure water in the preparation of all materials.

Material Preparation

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

Trypsin Stock

Modified Trypsin (20 µg) 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 20 µL 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 ~5 µL each. Store each aliquot at -20°C in a non-frost-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 45-µL ultrapure water. This solution may be stored at -20°C for 2 months without significant loss of activity.

Destaining Solution

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.

Digestion Buffer

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.

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 as described in step 1 of the "Reduction and Alkylation (Optional)" procedure. Mix 3.3 µL of TCEP with 30 µL of Digestion Buffer for each digest to be performed. Final TCEP concentration is ~50 mM.

CAUTION Do not store Reducing Buffer.

Alkylation Buffer

Prepare just before use (as described in step 3 of the "Reduction and Alkylation (Optional)" procedure on page 5) in foil-wrapped tubes to avoid exposure to light. To avoid weighing submicrogram 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 is supplied with this kit.

CAUTION Do not store the Alkylation Buffer or stock solution.

Activated Trypsin

Shortly before use (step 3 of "Digestion" on page 6) 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.

CAUTION Do not store Activated Trypsin.

NOTE The recommended amount of trypsin used per digest is 100 ng (see protocol). This amount of trypsin can be reliably used for a wide variety of protein concentrations within an excised gel band. However, if a 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.

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

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.

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

CAUTION Take care to include only the stained region of the gel.

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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 2 and 3.

5 Proceed to "Reduction and Alkylation (Optional)" or "Digestion" on page 6.

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.

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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 4 and 5.

7 Proceed to "Digestion".

Digestion

1 Shrink gel pieces by adding 50 µL of ACN. Incubate sample for 15 minutes at room temperature.

2 Carefully remove ACN and allow gel pieces to air-dry for 5-10 minutes.

3 Prepare Activated Trypsin as described in "Material Preparation" on page 3. 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 (TFA) or 1% formic acid solution to digestion mixture and incubate for 5 minutes. Remove extraction solution and add to 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). For matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) or nanospray ionization mass spectrometry, additional processing/clean-up is required. Use Agilent Cleanup C18 Pipette Tips, Part no. 5188-5239.

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

Troubleshooting

If experiencing a problem, refer to Table 2 for possible causes and suggested solutions.

Table 2 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 C18 sample prep device (part no. 5188-5239) Note: Limits vary considerably based on application and instrumentation.
	Interfering agents	Clean-up digest with C18 sample prep device (part no. 5188-5239)

Related Agilent Products

Other available related Agilent products include the following:

Product number	Description
5188-5239	**Cleanup C18 Pipette Tips pk/96**, for purifying and concentrating peptides from in-gel digests
5188-2750	**Peptide Cleanup C18 Spin Tubes pk/50**, for cleanup of peptides from in-solution digests before analysis
5188-2747	**Lys Tag 4H Kit for MALDI-MS**, mass tagging reagent that improves sensitivity and mass spectrum quality for Lysine-containing peptides in MALDI-MS analysis
5188-2748	**Mass Tagging Accessories Kit**, kit containing products used in conjunction with mass tagging reagents for preparing samples for MS analysis (includes Cleanup C18 Pipette Tips, Tryptic Digestion Kit, MALDI matrix, and a peptide standard)
G2037A	**Alpha-Cyano-4-Hydroxycinnamic Acid**, MALDI Matrix, 3 x 3 mL
5185-5984	**Multiple Affinity Removal Column 4.6 x 50 mm**, depletes six high-abundance proteins from human serum samples, 15-20–µL serum capacity per injection
5185-5985	**Multiple Affinity Removal Column 4.6 x 100 mm**, 30-40–µL serum capacity per injection
5185-5986	**Multiple Affinity Removal System Reagent Kit**, starter reagent kit containing buffers, spin filters, and spin concentrators for use with Multiple Affinity Removal Columns.

References

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Notices

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Agilent Technologies, Inc.
2850 Centerville Road
Wilmington, DE 19808-1610 USA

Safety

CAUTION

A **CAUTION** notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in damage to the product or loss of important data. Do not proceed beyond a **CAUTION** notice until the indicated conditions are fully understood and met.

WARNING

A WARNING notice denotes a hazard. It calls attention to an operating procedure, practice, or the like that, if not correctly performed or adhered to, could result in personal injury or death. Do not proceed beyond a WARNING notice until the indicated conditions are fully understood and met.