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Process stained polyacrylamide gel pieces for mass spectrometry

TR0050.2

Introduction

Gel electrophoresis of protein samples, trypsin digestion of target proteins and analysis of the resulting peptide fragments by mass spectrometry (MS) comprise a powerful method for protein identification and characterization. A fluorescent, coomassie or silver stain is necessary to visualize proteins that have been separated in 1-D or 2-D gels. Processing such samples for mass spectrometry necessitates first excising the protein spot of interest, removing the stain, and digesting and eluting the protein in the gel piece using an in-gel tryptic digestion procedure.

Several Thermo Scientific Pierce Protein Research Products facilitate electrophoresis and sample processing for MS workflows. The In-Gel Tryptic Digestion Kit (Product No. 89871) includes all necessary components and a detailed procedure for processing gel pieces that have been detected with coomassie-based stains such as GelCode™ Blue Stain Reagents (Product No. 24590, 24954) and Imperial™ Protein Stain (Product No. 24615). GelCode Blue Stains are colloidal coomassie G-250 stains that are able to detect most proteins at ~25 ng per spot. Imperial Protein Stain uses coomassie R-250 dye to detect as few as three nanograms of protein per spot. This Tech Tip provides a general procedure for processing coomassie-stained gels.

The In-Gel Tryptic Digestion Kit can also be used for certain types of silver-stained gels, although different steps are required to destain the gel piece(s). In addition, most silver stains include either formaldehyde or glutaraldehyde that can permanently fix proteins in the gel matrix, thereby limiting protein/peptide recovery. The Pierce® Silver Stain Kit (Product No. 24612) is a convenient, highly-sensitive, formaldehyde-based silver stain for protein polyacrylamide gels. This second procedure described in this Tech Tip has been validated for use with the Pierce Silver Stain Kit. For best results, use the Pierce Silver Stain Kit for Mass Spectrometry (Product No. 24600), which includes an optimized procedure and all the necessary reagents for staining gels and destaining gel pieces before in-gel trypsin digestion and MS.

Procedure for Gels Stained with Coomassie Dye (GelCode Blue or Imperial Stain)

Note: The following procedure may be used for coomassie or fluorescent dye-stained polyacrylamide gel pieces. If not certain of the quality of the available reagents and trypsin, use the In-Gel Tryptic Digestion Kit (Product No. 89871).

Material Preparation

Destaining Solution:	25 mM ammonium bicarbonate in 50% acetonitrile. Mix 80 mg of ammonium bicarbonate with 20 ml of acetonitrile and 20 ml of ultrapure water. Store this solution at 4°C for up to 2 months.
Digestion Buffer:	25 mM ammonium bicarbonate in water. Mix 10 mg of ammonium bicarbonate with 5 ml of ultrapure water. Store Digestion Buffer at 4°C for up to 2 months.

A. Excise and Destain Gel Piece

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

   Note: Reduction and alkylation of the protein sample are optional but recommended if high-sequence coverage is desired. Refer to instructions for the In-Gel Tryptic Digestion Kit (Product No. 89871) for a detailed protocol for reduction and alkylation.

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B. Shrink Gel Pieces

6. Shrink gel pieces by adding 50 µl of acetonitrile. Incubate sample for 15 minutes at room temperature.
7. Carefully remove acetonitrile and allow gel pieces to air-dry for 5-10 minutes.

C. Trypsinize Proteins and Recover Fragments

8. Prepare a 1 µg/0.1 ml solution of high-quality trypsin in ultrapure water. Do not attempt to store this solution.
9. Add 10 µl of preprared trypsin solution to the tube containing the shrunken gel pieces; incubate at room temperature for 15 minutes to allow gel pieces to swell and absorb the trypsin solution.

   Notes:
   a) Using 100 ng of trypsin per digest is effective for a wide variety of protein concentrations 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.

   b) If 10 µl is not sufficient to cover and fully swell gel pieces, increase volume of trypsin solution accordingly.
10. Add 25 µl of Digestion Buffer to the tube. Incubate sample at 37°C for 4 hours or at 30°C overnight with shaking.
11. Remove digestion mixture and place in a clean tube.
12. (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 6). This step also serves to inactivate trypsin, stopping additional enzymatic activity. A second extraction generally results in only a minor increase in peptide recovery.
13. Sample is now ready for liquid chromatographic separation and electrospray ionization mass spectrometry (LC-ESI MS). Additional processing/clean-up by C-18 resin (Product No. 89870) is required for matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) or nanospray ionization mass spectrometry.

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

Procedure for Gels Stained with Silver (Pierce Silver Stain Kit)

Note: The following procedure is based on standard methods cited in the literature (see References). For best results, use Pierce Silver Stain for Mass Spectrometry (Product No. 24600), which contains optimized reagents and protocol.

Material Preparation

Destaining Solution:	Prepare separate 100 mM sodium thiosulfate and 30 mM potassium ferricyanide solutions, then mix them in a 1:1 (v:v) ratio. For example, dissolve 15.8 mg of sodium thiosulfate in 1 ml water and dissolve 9.9 mg potassium ferricyanide in 1 ml water, then mix the two solutions. The two separate solutions may be prepared in advance and stored indefinitely at room temperature (store potassium ferricyanide protected from light).
Wash Solution:	25 mM ammonium bicarbonate in 50% acetonitrile. Dissolve 10 mg ammonium bicarbonate in 2.5 ml ultrapure water and 2.5 ml acetonitrile.
Digestion Buffer:	25 mM ammonium bicarbonate in water. Mix 10 mg of ammonium bicarbonate with 5 ml of ultrapure water. Store Digestion Buffer at 4°C for up to 2 months.

A. Modifications to the default Pierce Silver Stain Procedure

1. During the staining procedure, incubate gel for 5 minutes instead of 30 minutes in the Silver Stain Working Solution, as described in the Pierce Silver Stain Instructions.
2. After the development step, be sure to incubate stained gel in 5% acetic acid for at least 5 minutes to ensure complete termination of development.
3. Wash gel extensively in ultrapure water for 15 minutes. Repeat this step twice to ensure that all the acetic acid is removed and the gel is completely rehydrated.

B. Band Isolation and Destaining

1. Isolate protein bands with a spot picker and transfer gel piece into a 0.5 ml microcentrifuge tube.
2. Add 0.1 ml of Destaining Solution to gel piece. (Add a sufficient volume to completely cover gel piece.)
3. Incubate sample at room temperature for 15 minutes with shaking.
4. Decant and discard destain solution. Add 0.2 ml of Wash Solution to the sample and incubate with gentle shaking for 10 minutes. Repeat this step twice.

C. Trypsinize Proteins and Recover Fragments

Use the In-Gel Tryptic Digestion Kit (Product No. 89871) or follow the procedure in Section C of the Procedure for Gel Stained with Coomassie Dye.

Related Thermo Scientific Products

24590, 24592	**GelCode Blue Stain Reagent,** 500 ml or 3.5 L, respectively, colloidal coomassie stain for gels
24594, 24596	**GelCode Blue Safe Protein Stain,** 500 ml or 3.5 L, respectively, colloidal coomassie stain for gels
24615, 24617	**Imperial Protein Stain,** 1 L or 3 L, respectively, R250 coomassie stain for gels
46629, 46630	**Krypton Fluorescent Protein Stain,** 100 ml and 500 ml, respectively
24600	**Pierce Silver Stain for Mass Spectrometry,** sufficient to stain 20 mini-gels and process > 500 gel pieces for subsequent in-gel digestion and analysis by mass spectrometry
24612	**Pierce Silver Stain Kit,** sufficient to stain 20 mini-gels
24582	**Pierce Zinc Reversible Stain Kit,** sufficient to stain 20 mini-gels
89870	**Pierce C-18 Spin Columns,** 25/pkg
89871	**In-Gel Tryptic Digestion Kit,** sufficient for 150 in-gel digestions
89895	**In-Solution Tryptic Digestion and Guanidination Kit,** sufficient for 90 digestions
89853	**Phosphopeptide Isolation Kit,** 30 spin columns

References

Processed from gels stained with GelCode Blue Stain Reagent:

Aulak, K.S., *et al.* (2001) Proteomic method identifies proteins nitrated in vivo during inflammatory challenge. *PNAS* **98:**12056-61.

Hilton, J.M., *et al.* (2001) Phosphorylation of a synaptic vesicle-associated protein by an inositol hexakisphosphate-regulated protein kinase. *J. Biol Chem.* **276:**16341-7.

Lim, J., *et al.* (2002) Metastable macromolecular complexes containing high mobility group nucleosome-binding chromosomal proteins in HeLa nuclei. *J. Biol Chem.* **277:**20774-82.

Tani, M., *et al.* (2000) Purification and characterization of a neutral ceramidase from mouse liver. *J. Biol Chem.* **275:**3462-8.

Processed from gels stained with formaldehyde-based silver stain:

Gharahdaghi, F., *et al.* (1999) Mass spectrometry identification of proteins from silver-stained polyacrylamide gel: A method for the removal of silver ions to enhance sensitivity. *Electrophoresis* **20:**601-5.

Current versions of product instructions are available at *www.thermo.com/pierce*. For a faxed copy, call 800-874-3723 or contact your local distributor.