Poroszyme® Immobilized Trypsin Cartridge

Applied Biosystems

Operating Instructions

Your Poroszyme® Cartridge is Unique!

Applied Biosystems Poroszyme® Immobilized Trypsin cartridges perform online tryptic digests in a flow-through environment.

Benefits of the flow-through environment include:

- The cartridge can be connected directly to an HPLC or LC/mass spectrometry system for analysis of the digest.
- The degree of digestion can be optimized by changing flow rate or temperature.
- Sample handling is reduced because of the direct connection to the analysis device.

Poroszyme Immobilized Trypsin cartridges contain the enzyme attached to POROS® media (a hydrophilic polystyrene support). Additional advantages provided by using POROS media as the support include:

- Autodigestion of the enzyme is minimized, because the enzyme is immobilized instead of free in solution.
- The cartridge is stable over a range of pH's and organic solvents and can withstand high flow rates and pressures.
- Fast flow rates allow quick, efficient washings.

1 Product Description

Poroszyme Immobilized Trypsin cartridges are packed with POROS media consisting of trypsin covalently bound to POROS 20 µm beads. The solid support consists of cross-linked poly(styrene-divinylbenzene) flow-through particles with a patented bimodal pore size distribution for rapid mass transport of substrate to the enzyme immobilized within the pores of the POROS media particles.

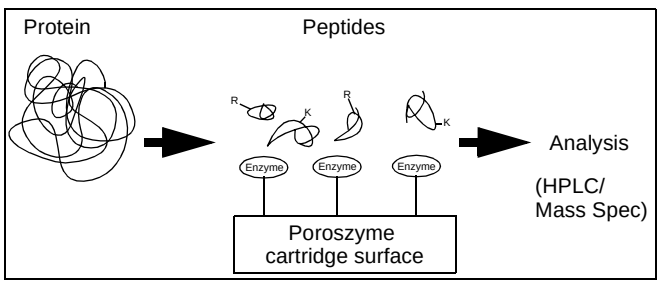

Figure 1 Poroszyme Trypsin Digestion

Poroszyme Trypsin cartridges are designed for protein characterization work. Trypsin [EC 3.4.21.4] cleaves peptide bonds on the carboxy side of arginine and lysine. It has a pH optima of 8.0. The Trypsin is treated with TPCK (L-1-Tosylamide-2-phenylethyl chloromethyl ketone) to inhibit chymotrypic activity, and is isolated from bovine pancreas.

Materials Provided

Poroszyme Immobilized Trypsin cartridge packages include the following:

- Packed cartridge (2.1 mmD/30 mmL) with sealing end caps
- Product *Operating Instructions*

Materials Recommended But Not Provided

- INTEGRAL® Micro-Analytical Workstation, BioCAD® Workstation or BioCAD SPRINT™ System. If these systems are not available, use an HPLC system with 4-solvent capability, UV detector and injection valve. A system capable of tandem column work greatly facilitates automation.
- Acetonitrile or methanol
- Digestion buffer (50mM Tris, 10 mM calcium chloride, pH 8.0)
- 2 M HCl or NaOH to adjust the pH of the digestion buffer
- Column heater
- Reversed-phase column, if peptide separation is desired
- Aqueous reversed-phase buffer to equilibrate the reversed-phase column
- Concentrated organic solvent to elute and wash the reversed-phase column

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2 Overview of Digestion Process

To enhance digestion, protein can be reduced or alkylated.

Digestion on a Poroszyme Trypsin cartridge involves:

- **Equilibration**—The Poroszyme Trypsin cartridge and the reversed-phase column are equilibrated with the appropriate buffers.
- **Injection**—The protein is injected onto the Poroszyme Trypsin cartridge.
- **Digestion**—The protein is digested by cleavage at arginine and lysine residues. Flow rate and temperature can be adjusted to control digestion. Effluent from the Poroszyme Trypsin cartridge flows onto the reversed-phase column.

After digestion, peptides can be analyzed using reversed-phase HPLC, mass spectrometry, or other analytical method.

3 General Considerations

Temperature and flow rate

Different proteins digest at different rates. Temperature and flow rate are two variables you can modify to control the degree of sample digestion and achieve optimal digestion conditions. Different temperatures and flow rates may generate different size fragments and allow monitoring of intermediate species.

- **Temperature**—Digestion can be carried out between 25°C and 67°C. However, the recommended temperature for reproducible digestion is 37°C. Increase the temperature to increase digestion. Decrease the temperature to decrease digestion.

  Improved protein digestion may be observed at temperatures up to 60°C. However, elevated temperatures decrease the lifetime of the cartridge, particularly if used in combination with organic solvents.
- **Flow rate**—Decrease the flow rate to increase digestion (increases the amount of time the protein is in contact with the enzyme). This usually results in enhanced digestions, but longer digestion times. Increase the flow rate to decrease digestion.

  You can also stop the flow when the protein solution is inside the cartridge to increase digestion.

  The INTEGRAL and BioCAD Workstations include flow rate templates that can help you easily determine the optimum flow rate for your digestion process.

Recovery of hydrophobic peptides

Peptide recovery is enhanced if 5 percent methanol or acetonitrile is added to the digestion buffer. The 5 percent organic reduces binding of hydrophobic peptides to the Poroszyme Trypsin cartridge without compromising peptide binding on the reversed-phase column.

***Note**: If you are not using a reversed-phase column to capture peptide fragments, you can increase acetonitrile or methanol concentration to more than 5 percent.*

Protein treatment to enhance digestion

Proteolytic digestion of most proteins is facilitated by denaturation and removal of disulfide bridges before digestion. “Reductive Alkylation” is a general term that describes a sequence of steps that:

- Denatures the protein
- Reduces disulfide bonds
- Alkylates the free -SH groups to prevent their reassociation

Reductive Alkylation yields a protein that digests more rapidly, and generates peptide fragments that are free of disulfide bridges.

***Note**: A reductively alkylated protein may be less soluble in aqueous buffers than it was initially. You may need to add reagents such as organic solvents, urea, or guanidine to keep the protein soluble for digestion in the Poroszyme cartridge.*

Your Poroszyme Trypsin cartridge digests proteins that have been reduced and alkylated with 4-vinyl pyridine, iodoacetamide, or iodoacetic acid. After alkylation, scavenge excess alkylating agent with a molar excess of soluble thiol, such as cysteine or thiomalic acid. This prevents alkylation of primary amines (such as lysine residues).

Typical steps involved in reductive alkylation include:

- Incubation of protein with a 40-fold molar excess of dithiothreitol (DTT) in 3 M urea for 15 minutes at 50°C.
- Addition of excess alkylating agent.
- Incubation for 15 minutes.
- Addition of excess thiol.

You can inject the entire mixture of protein and reagents in urea directly onto your Poroszyme Trypsin cartridge for digestion.

Each protein can be tested to determine conditions sufficient to completely reduce and alkylate the disulfides without reacting with primary amines.

Automating the process

In traditional methods, reductive alkylation has been a manual procedure performed offline before enzymatic digestion. Digestion has then been carried out in a second procedure, and reversed-phase separation and analysis of peptide fragments in a third procedure.

The INTEGRAL Microanalytical Workstation allows you to automate and combine these procedures. Consult your Applied Biosystems representative for details.

Solvent compatibility

Your Poroszyme Trypsin cartridge is compatible with many of the common solvents used to keep proteins in solution including 3 M urea, 2M guanidine hydrochloride, and high percentages (up to 90 percent) of methanol or acetonitrile.

***Note**: When using organics in combination with elevated temperatures (>40°C), you will see a gradual loss of enzymatic activity and a shorter cartridge lifetime.*

For maximum cartridge lifetime, use the following solvents and conditions when possible:

- Use methanol instead of acetonitrile
- Use urea instead of guanidine hydrochloride
- Keep temperatures below 37°C when using organics

4 Preparing Digestion Buffer

1. Prepare a 1M Tris(hydroxymethyl) aminomethane (Tris®) stock solution. Use high-quality water to prepare the stock.
2. Add 50 ml of 1M Tris stock to a 1 liter beaker and dilute to about 600 ml with high-quality water.
3. Add 1.1g of $CaCl_2$ and stir to dissolve.
4. Adjust the solution to pH 8.0 with a 2 M solution of hydrochloric acid or sodium hydroxide as required.
5. Bring the volume up to 1 liter in a graduated cylinder. Filter before use.

5 Digesting the Protein

Initial Cartridge Equilibration

The first time you use the Poroszyme Trypsin cartridge, wash it with 20 column volumes of 50/50 digestion buffer/acetonitrile to equilibrate and pretreat the cartridge.

Digesting without a reversed-phase column

1. Set the column heater temperature to 37°C (or the optimum temperature for your protein) and allow temperature to equilibrate.
2. Wash and activate the Poroszyme Trypsin cartridge with 10 column volumes of 95 percent digestion buffer, 5 percent acetonitrile at 3 to 5 ml/min.
3. Inject the protein.
4. Starting with a flow rate of 50 µl/min, wash the protein through the cartridge with at least 3 column volumes of the digestion/ acetonitrile buffer. If your system does not run at low flow rates, use the lowest flow rate at which your system can run.
5. Collect the material from the Poroszyme Trypsin cartridge in an polypropylene tube for subsequent analysis.
6. Wash the Poroszyme Trypsin cartridge with 10 column volumes of 1:1 digestion buffer:acetonitrile at 3 to 5 ml/min.

***Note**: If this is the last run on the cartridge, wash with an additional 10 column volumes of digestion buffer.*

7. Repeat step 2 through step 6 for subsequent samples.

Digesting with a reversed-phase column

1. Set the column heater temperature to 37°C (or the optimum temperature for your protein) and allow temperature to equilibrate.
2. Take the reversed-phase column out of line.
3. Wash and activate the Poroszyme Trypsin cartridge with 10 column volumes of 95 percent digestion buffer, 5 percent acetonitrile at 3 to 5 ml/min. Take the cartridge out of line.
4. Place the reversed-phase column inline. Equilibrate with aqueous buffer.
5. Place the Poroszyme Trypsin cartridge and the reversed-phase column inline. Inject the protein.
6. Starting with a flow rate of 50 µl/min, wash the protein through the cartridge with at least 3 column volumes of the digestion/ acetonitrile buffer. If your system does not run at low flow rates, use the lowest flow rate at which your system can run.
7. Take the Poroszyme Trypsin cartridge out of line.
8. Elute the peptides from the reversed-phase column with appropriate gradient conditions at 1 ml/min.
9. Wash the reversed-phase column with a high concentration of organic solvent. Take the reversed-phase column out of line.
10. Place the Poroszyme Trypsin cartridge inline and wash with 10 column volumes of 1:1 digestion buffer:acetonitrile at 3 to 5 ml/min.

***Note**: If this is the last run on the cartridge, wash with an additional 10 column volumes of digestion buffer.*

11. Repeat step 2 through step 10 for subsequent samples.

Optimizing Digestion

Analyze the digest to determine whether the desired degree of digestion has occurred. Every protein is different, and you may need to adjust temperature and flow rate to optimize digestion:

To increase digestion	• Increase temperature • Decrease flow rate or stop flow • Reductively alkylate the protein
To decrease digestion	• Decrease temperature • Increase flow rate

Testing your cartridge for enzyme activity

You can quantify the amount of enzyme activity remaining on your Poroszyme Trypsin cartridge using a synthetic substrate. The substrate, L-Benzoyl-Arginine-para-nitroanilide, BAPNA, (Boehringer Mannheim catalog number 775-819) is a chromogenic substrate, which when hydrolyzed by Trypsin:

- Yields a yellow para-nitroanaline (pNA) hydrophobic product
- Is retained on the Poroszyme Trypsin cartridge. It can be recovered from the Poroszyme Trypsin cartridge in 90 percent methanol.

To test the amount of enzyme activity:

1. Dissolve the L-BAPNA in a small volume of 1:1 acetonitrile:water.
2. Dilute it to a working solution of 1 mg/ml in digestion buffer.
3. Connect your Poroszyme Trypsin cartridge to your HPLC system.
4. Configure digestion buffer as the mobile phase and set the UV detector to 410 nm.
5. Start flow at 1 ml/min and inject 25 µl of L-BAPNA.
6. Inject 1 ml 90 percent methanol:water.

The methanol elutes the liberated pNA product, which is detected by the UV detector.

It is good practice to check the enzyme activity of your cartridge when it is new, and periodically check activity over time. Compare subsequent peak areas to the original peak area to determine the relative amount of enzyme activity remaining on the cartridge.

6 Storing the Cartridge

Short-term Storage

Store the cartridge at 4°C in digestion buffer when not in use.

Store the cartridge with the endcaps in place, carefully sealed to prevent drying.

Long-term Storage

Store the cartridge in 0.02 percent sodium azide as a preservative.

***Caution**: Sodium azide is toxic. Follow precautions and decontamination procedures recommended by the National Institute for Occupational Safety and Health.*

7 Technical Support

Applied Biosystems is dedicated to helping you use Perfusion Chromatography®, ImmunoDetection®, immobilized enzymes, and POROS media to the fullest extent possible. Our biochromatographers, bioprocess engineers, and applications development laboratories are available for support ranging from telephone consultation to full-scale method development.

Applied Biosystems also offers a full line of other POROS media for Perfusion Chromatography in the reversed-phase, ion exchange, affinity, and other chromatographic modes. We also offer ImmunoDetection and other immobilized enzyme products. Please contact your Applied Biosystems representative for technical and ordering information.

Applied Biosystems publishes a continuing series of Application and Technical Notes, highlighting specific purification problems and technical aspects of Perfusion Chromatography. Please contact Applied Biosystems directly for a publication list.

For further details or for answers to questions on Poroszyme cartridges, POROS media, Perfusion Chromatography, ImmunoDetection, or other products, please contact Applied Biosystems. Refer to the back page of this document for contact information.

8 Accessories, Spare Parts, and Ordering Information

These accessories are available for your Poroszyme columns:

Table 1 Poroszyme Cartridge Accessories

Description	Quantity	Part Number
Poroszyme Immobilized Trypsin Cartridge, 2.1 mmD/30 mmL	1	2-3128-00
Poroszyme Immobilized Trypsin Bulk Media	0.1 ml	2-3127-00
POROS R2 Column for Reversed-Phase Chromatography 2.1 mmD/100 mmL PEEK	1	1-1112-16
Frits, PEEK, 2.1mmD	Package of 5	1-9124-05
EZ™ Grip Fittings (SS)	Package of 5	5-1011-05
Fitting Adaptor Kit	1	1-9532-00
The Fitting Adaptor Kit lets you connect POROS columns to M-6 (FPLC®) and 1/4-28 low pressure fitting systems. Includes two 10-32 fittings, two low pressure ferrules, two M-6 nuts, two 1/4-28 nuts and 1/16-inch OD PEEK tubing. Kit is included with all F- and P-Series columns.		

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Subtractive Assay technology, enabled by the use of ImmunoDetection (ID) Sensor Cartridges and the INTEGRAL Micro-Analytical Workstation, is covered by U.S. patent 5,234,586. Other patents pending.

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