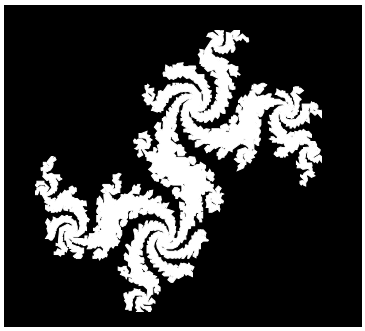


PepMap C18™ Columns for Reversed-Phase Chromatography of Peptides

Operating Instructions

1 Product Description

PepMap C18™ columns are high-resolution columns designed for reversed-phase chromatography of peptides and similar biomolecules. The column packing consists of 5 µ spherical silica particles with a mean pore diameter of 300 Å. The silica is derivatized with a monofunctional octadecylsilane and the remaining silanols are end-capped. The surface chemistry is optimized for high resolution separation of complex mixtures of peptides.

The PepMap C18 column package includes the following items:

- Packed column, with sealing end caps
- Product *Operating Instructions*
- Column Test Certificate
- EZ™ Grip stainless steel fittings

Table 1 Product Characteristics

Support Matrix	Silica
Surface Functionality	C18, end-capped
Particle size	5 µm
Recommended flow rate	See Table 3
Maximum pressure drop	350 bar (5,000 psi, 35 MPa)

Table 2 Chemical Resistance

pH Range	2–7 ***Note**: The PepMap C18 column can be exposed to a pH of 7–9. Aqueous samples of 1 to 2 column volumes can be used at flow rates recommended for the column but must be removed immediately from the column by washing with about 2 column volumes of the starting eluent.*
Additives	Reagents commonly used for reversed-phase separation of peptides, including acids, buffers, detergents, and ion-pairing agents.
Solvents	Water, 0–100% alcohols, acetonitrile, other common organic solvents
Operating Temperature	10–60°C

Table 3 Typical Flow Rates and Pressure

Column size (mmD/mmL)	Volumetric Flow (ml/min)	Linear Velocity (cm/hr)	Pressure drop* (Psi)
4.6 mmD/250 mmL	1.00	361	2,600
4.6 mmD/150 mmL			1,500
4.6 mmD/100 mmL			1,000
2.1 mmD/250 mmL	0.21	364	2,600
2.1 mmD/150 mmL			1,500
2.1 mmD/100 mmL			1,000

* Pressure measured with 0.1% (v/v) TFA in water as eluent at ambient temperature (22°C).

2 Connecting and Preparing the Column

The column fitting is Parker 10-32 female.

PepMap C18 columns come with EZ Grip stainless steel fittings that are designed to be tightened by hand.

Extra EZ Grip fittings are available from PerSeptive Biosystems. See Section 9, Accessories, Spare Parts, and Ordering Information.

Connecting the Column

Use the following tubing and the EZ Grip fittings provided to connect the column. Use the shortest tubing lengths possible without crimping tubing:

- 2.1 mmD columns—0.005-inch I.D. red PEEK tubing
- 4.6 mmD columns—0.010-inch I.D. blue PEEK tubing

Section **Page**

Preparing the Column

PepMap C18 columns are shipped in 70 percent (V/V) acetonitrile/ water. Before the first injection, wash the column with at least 5 column volumes of 100% acetonitrile followed by 5 column volumes of starting mobile phase.

3 Selecting and Preparing the Mobile Phase

Regardless of the mobile phase you choose, it is always important to:

- Use mobile phases of the highest purity practical.
- Degas all mobile phases prior to use.
- Helium sparge or blanket all mobile phases.

3.1 Solvents

Keep this information in mind as you select the solvent for the mobile phase:

- Acetonitrile is the preferred solvent for reversed-phase chromatography.
- Solvents such as methanol, isopropanol, or acetonitrile can be used to improve chromatographic performance.

***Note**: Extended exposure of PepMap C18 columns to alkaline conditions (pH >7) can cause deterioration of column performance. However, PepMap C18 columns can be exposed to a pH of 7–9 for short periods to introduce large volumes (5 to 10 milliliters) of aqueous samples.*

3.2 Additives

Acidic compounds such as trifluoroacetic acid (TFA) are commonly added to the mobile phases for reversed-phase chromatography of peptides.

Other additives such as buffer salts, amines (mono-, di- or triethyl-amines, ethanolamines) or acidic compounds (phosphoric, formic, or acetic acids) may be used to further improve resolution of peptides.

***Note**: Formic and acetic acids exhibit significant absorption at 215 nm and may reduce sensitivity of detection.*

4 Preparing and Loading the Sample

To ensure efficient binding and prevent column plugging, it is important to:

1. Dissolve or exchange samples for PepMap C18 columns into the starting mobile phase.
2. Centrifuge or filter (0.22 or 0.45 µm) samples before injection to prevent column plugging.

5 Eluting the Sample

Peptide analysis is generally performed by gradient elution with increasing organic concentration.

Typical analysis time for peptide separation ranges from 1 to 2 hours, depending on the flow rate, column length, and complexity of the sample.

6 Cleaning Up and Regenerating the Column

In some applications, sample molecules may not fully elute or may precipitate on the column. Regenerate the column if these symptoms appear:

- Increased bandspreading
- Loss of binding capacity
- Loss of recovery
- Increased pressure drop
- Trace or “ghost” peaks occurring during blank gradient runs

***Note**: In any cleanup method, reversing the flow direction is recommended to help flush out particulates and to prevent contamination of the lower part of the bed. If you reverse the flow direction, reduce flow rates to 0.5 ml/min (4.6 mmD columns) or 0.1 ml/min (2.1 mmD/ columns) to minimize changes in the column bed structure. It is a good practice to disconnect the column from the detector during the cleanup method.*

In reversed-phase chromatography, the bound species may have very limited solubility in the organic solvent used to remove them from the surface. Therefore, regeneration solutions must be both strong solubilizing agents and strong eluents. These qualities are often mutually exclusive. To manage this situation:

1. Run rapid “sawtooth” gradients from 100% of a very strong solubilizer (such as 50% acetic or phosphoric acid, 1 to 3 M guanidine) to 100% of a strong eluent, (such as isopropanol or acetonitrile), and back to the solubilizer.

   Running a gradient helps achieve the correct blend of the two agents needed to remove the bound contaminant.
2. Take care to ensure that the solubilizer is miscible with the organic solvent selected.

Multiple Injections

It is possible to use multiple injections of regeneration solutions instead of pumping them directly. This method is recommended when using very aggressive or highly viscous solvents.

To clean by injections:

- Make the injection volume as large as possible.
- Use a low flow rate that exposes the column to the regeneration solution for several minutes.

7 Storing the Column

When you store your column, always be sure to:

- Wash the column with 2 column volumes of water to remove salts, if present, followed by an organic-rich solvent mixture containing acetonitrile or methanol:water (70:30).
- Avoid long-term storage of stainless steel columns with halide (Cl) salts, because frit corrosion may result.
- Store the column at ambient temperature.
- Store the column with the end plugs in place, carefully sealed to prevent drying.

8 Standard Test Protocols

8.1 Chromatographic Efficiency

The Column Test Certificate supplied with your column lists the chromatographic efficiency test method and initial test chromatogram for the column. The test uses small, retained molecules run at a low flow rate. Plate count is determined by the half height method.

8.2 Peptide Separation

PerSeptive Biosystems uses a reversed-phase peptide test standard (Sigma Chemical catalog# H-2016) to test the performance of PepMap C18 columns. The test standard is a mixture of five synthetic peptides designed for monitoring the performance of reversed-phase columns.

The test standard components are separated by a linear gradient of acetonitrile.

The test consists of these steps:

1. Dissolve the lyophilized sample mixture in 1 ml of Eluent A. Store unused reconstituted test mix frozen.
2. Filter the solution using a 0.45 or 0.22 micron filter before use.
3. Run the sample.

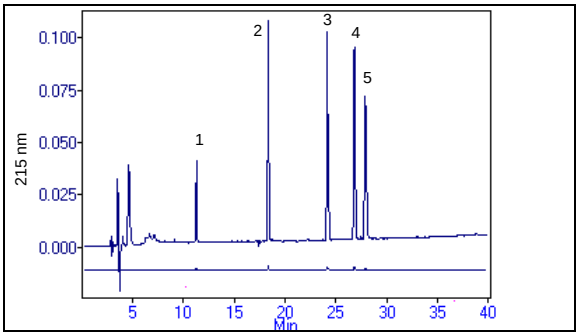
8.3 Conditions

Table 4 Protocol

These conditions are common to all column sizes:		
Eluent A	0.1% TFA in water	
Eluent B	95% acetonitrile, 5% water, 0.1% TFA	
Gradient	0–50% B in 40 minutes	
Detection	215 nm	
Sample (µl)	15	
Flow rate depends upon column diameter and are listed below:		
Column (mmD)	4.6	2.1
Flow (ml/min)	1.0	0.2

8.4 Results

The Reversed-Phase Peptide Test Standard should yield a profile similar to the one shown below.



1 Gly-Tyr
2 Val-Tyr-Val
3 Met Enkephalin (Tyr-Gly-Gly-Phe-Met)
4 Leu Enkephalin (Tyr-Ala-Gly-Phe-Leu)
5 Angiotensin II (Asp-Arg-Val-Tyr-ILeu-His-Pro-Phe)

Eluent A 0.1% TFA in H_20
Eluent B 95% ACN + 0.1% TFA in H_20
Gradient 0–50% B in 40 min
Flow rate 1 ml/min
Column 4.6 mmD/250 mmL

Figure 1 Reversed-Phase Separation of Peptide Test Standard

9 Accessories, Spare Parts, and Ordering Information

These accessories are available for your PepMap C18 columns:

Table 5 PepMap C18 Column Accessories

Description	Quantity	Part Number
PepMap C18 Column Kits for Reversed-phase Peptide Chromatography		
4.6 mmD/250 mmL	1	2-3141-00
4.6 mmD/150 mmL	1	2-3140-00
4.6 mmD/100 mmL	1	2-3139-00
2.1 mmD/250 mmL	1	2-3138-00
2.1 mmD/150 mmL	1	2-3137-00
2.1 mmD/100 mmL	1	2-3136-00

Column parts are available from PerSeptive Biosystems on special order. Please inquire.

10 Technical Support

For further details or for answers to questions on PepMap C18 columns, Perfusion Chromatography, or other products, please contact PerSeptive Biosystems.

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