Thermo Scientific

PepSwift and ProSwift Capillary Monolith Columns for Bioseparations

Product Manual

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Thermo SCIENTIFIC

Product Manual

for

PepSwift and ProSwift Capillary Monolith Columns for BioSeparations

PepSwift Monolith Columns	
PepSwift Monolithic Nano Column, 100 µm ID × 5 cm, nanoViper	164584
PepSwift Monolithic Nano Column, 100 µm ID x 25 cm, nanoViper	164543
PepSwift Monolithic Capillary Column, 200 µm ID x 5 cm, nanoViper	164557
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For Research Use Only. Not for use in diagnostic procedures.

Revision History:

Revision 01, March., 2014 – Original Publication.

Revision 02, February, 2015 – Updated manual with ProSwift C4 RP-5H products and associated figures.

Safety and Special Notices

Make sure you follow the precautionary statements presented in this guide. The safety and other special notices appear in boxes.

Safety and special notices include the following:

 **SAFETY**	***Indicates a potentially hazardous situation which, if not avoided, could result in death or serious injury.***
 **WARNING**	***Indicates a potentially hazardous situation which, if not avoided, could result in damage to equipment.***
 **CAUTION**	***Indicates a potentially hazardous situation which, if not avoided, may result in minor or moderate injury. Also used to identify a situation or practice that may seriously damage the instrument, but will not cause injury.***
 **NOTE**	***Indicates information of general interest.***
IMPORTANT	***Highlights information necessary to prevent damage to software, loss of data, or invalid test results; or might contain information that is critical for optimal performance of the system.***
Tip	***Highlights helpful information that can make a task easier.***

Contents

1. Introduction

PepSwift® and ProSwift® reversed-phase monolithic columns are specifically designed to provide fast, high-resolution and high efficiency separations of proteins, peptides, and other biomolecules.

1.1 Morphology of Polymer Monoliths

PepSwift and ProSwift reversed-phase media are based on polymeric monoliths prepared by an *in situ* polymerization process. Since 2008 they have become an established family of separation products that are uniquely designed and engineered for the separation of biomolecules. The monolith is a single cylindrical polymeric rod containing an uninterrupted, interconnected network of through pores (channels). Its unique morphology, pore structure, and pore size distribution offer optimum performance for separation of proteins, peptides, oligonucleotides, and other biomolecules.

The morphologies of the PepSwift and ProSwift monoliths are shown in the figure below. The monoliths consist of aggregates of globules which form a continuous, yet porous polymer bed. The open spaces among the large aggregates form large flow-through channels, allowing liquid flow without generating high back pressure. The spaces among the smaller globules are the open or through pores allowing fast access of the samples to the functionalized surface of the media. The mass transfer of the samples is primarily driven by convective flow through these open pores instead of much slower molecular diffusion. These pores are large enough for large molecules to flow through freely. Most of the small globules are engineered to be less than 500 nm in size. Therefore, the path lengths for mass transfer through these small globules are much shorter than the path lengths in conventional bead-based chromatographic phases. In addition, the globules are mainly non-porous based on BET measurements and SEM examinations. Since they are non-porous, these globules minimize diffusion-controlled mass transfer. This is in contrast to porous beads where diffusion-controlled mass transfer predominates.

In summary, flow-through pores, short mass transfer paths, and non-porous globules are characteristics unique to PepSwift and ProSwift monolith morphology. These monolith features enable much faster mass transfer of large biomolecules compared to porous beads.

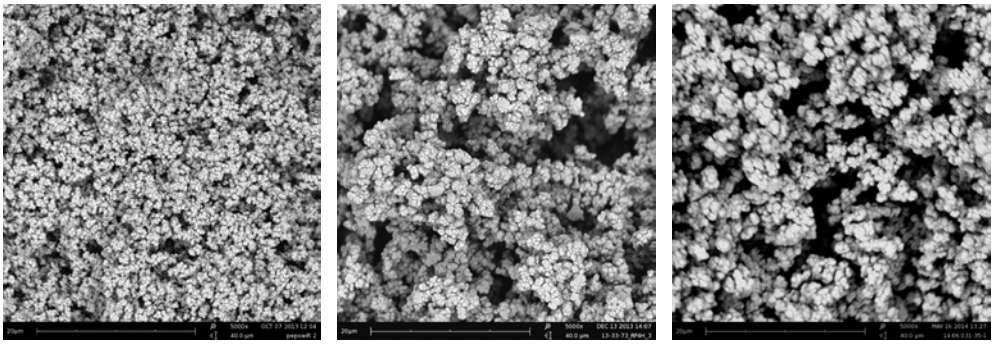


Figure 1: Scanning electron microscope images of a PepSwift (left), ProSwift RP-4H (center), and ProSwift C4 RP-5H column (right).

1.2 Backpressures and Pore Size Distributions

Monolithic materials typically exhibit high permeability with pore volumes around 60% of the column volume, which is much higher than that of porous beads. Pores are defined as macro (>50 nm), meso (2 – 50 nm) or micro (< 2 nm). Typically the materials used for chromatographic applications contain two types of pores; macropores (700 – 6000 nm) through which eluent flows and mesopores where separations can take place. The pore size of these materials is stated as the modal pore size, the size at which there are the greatest number of pores within the material. The pore size of each material is listed in the table below as well as the permeability of the materials. The permeability determines column backpressure and is affected by both the overall pore distribution and pore volume of the material. From the pore distribution figure below, it should be noted that even though the ProSwift RP-4H monolith has a smaller modal pore size, it also has a greater volume contribution of larger pores resulting in a 10-fold increase in permeability. The result is a lower operating backpressure with excellent efficiency for large proteins. The absence of these larger pores in the PepSwift material makes this the best choice for the separation of smaller proteins and peptides, however this results in a higher operational backpressure. In contrast, the ProSwift C4 RP-5H has a narrow pore distribution resulting in a slightly higher backpressure than the ProSwift RP-4H.

Table 1: Pore distribution of PepSwift and ProSwift polymer monoliths

Product	**Modal Pore Size, nm**	**Permeability, m^2**
PepSwift	375	7.04×10^{-15}
ProSwift RP-4H	159	6.82×10^{-14}
ProSwift C4 RP-5H	1630	4.74×10^{-14}

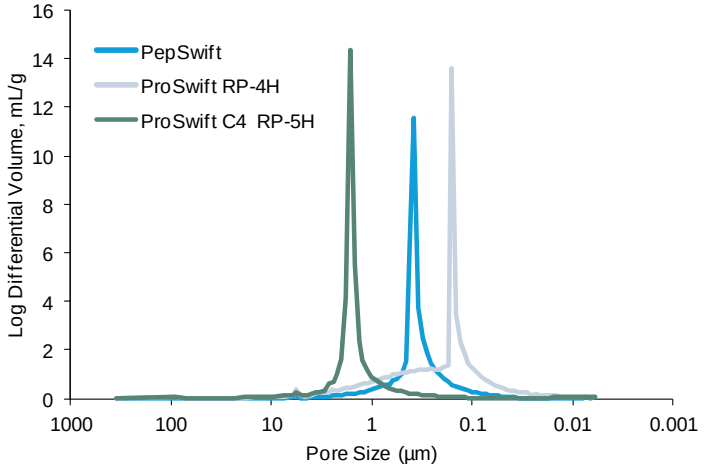


Figure 2: Pore size distribution of the ProSwift RP-4H and the PepSwift media.

The column permeability determines the column backpressure. The figure below shows the backpressure generated at different flow rates and different temperatures for the PepSwift, ProSwift RP-4H and ProSwift C4 RP-5H columns in a 100 µm x 25 cm format.

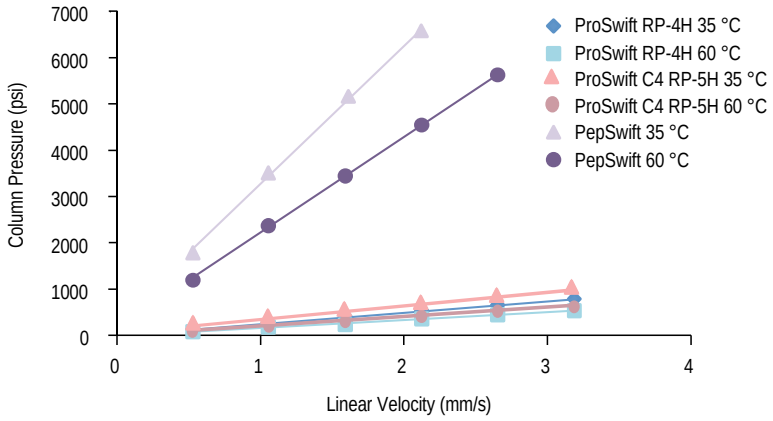


Figure 3: Column backpressure as a function of eluent flow rate at 35 °C and 60 °C for a ProSwift RP-4H column, a ProSwift C4 RP-5H, and a PepSwift column. All column formats are 100 µm x 25 cm, Eluent: 90.5/9.5 H_2O/MeCN + 0.1% TFA.

1.3 Resolution and Speed of Separation

The uniquely designed morphology of PepSwift and ProSwift monoliths allows fast analyte mass transfer. This fast mass transfer minimizes band broadening as flow rate increases, allowing faster separations with less loss of resolution compared to typical porous bead based media. This is especially true for large molecules whose diffusivities are much lower than small molecules. In combination with low backpressure, PepSwift and ProSwift monoliths offer excellent separation at low and high flow rates which improves operational versatility and productivity.

At increased flow rates the effect of band broadening by diffusion is minimal therefore the loss of resolution at elevated flow rates is minimal. Using standard protein mixtures the figures below show how not only throughput can be increased by increasing flow rate but also resolution. For direct comparison of gradients, the figures below show how resolution is maintained at increased flow rates.

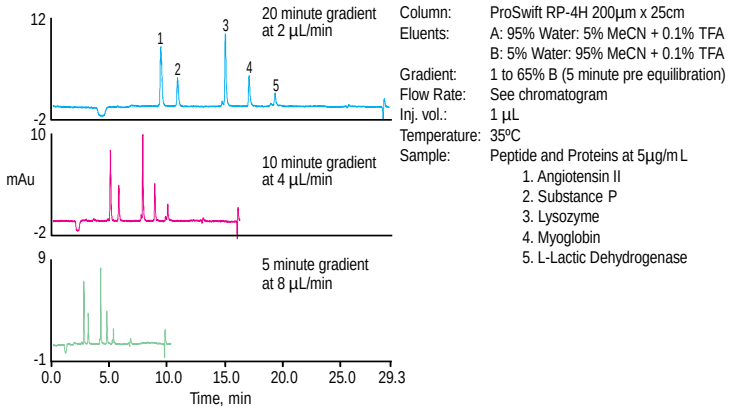


Figure 4: The effect of increased flow rate at equivalent column volume gradients on the ProSwift RP-4H 200 µm x 25 cm monolith column.

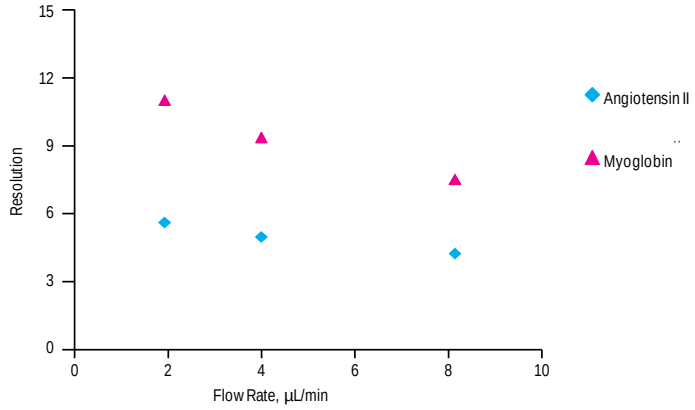


Figure 5: Separation of a 5 peptide/protein mixture on the ProSwift RP-4H 200 µm x 25 cm column. Data extracted from the figure above.

At elevated flow rates, one can take advantage of the fast mass transfer. By maintaining the gradient time, effectively extending the gradient length in terms of column volume, the resolution can be increased. In the example below, higher resolution is achieved at 8 µL/min. This is due to a dominance of convective mass transfer through the pores. This resolution maximum will be dependent on conditions and analyte size. When productivity is the most important factor in your separation, higher linear velocities can be employed. With ProSwift columns, when resolution is the most important factor in your separation, optimization of your gradient and flow rate is recommended. You can often take advantage of the fast mass transfer by using a shallower gradient at elevated flow rates.

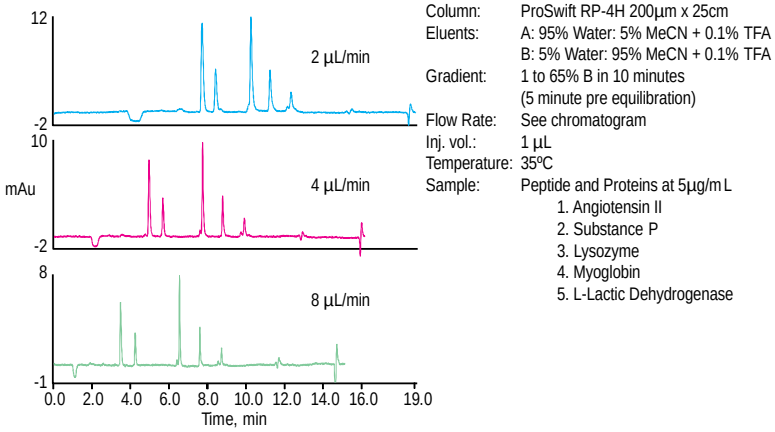


Figure 6: The effect of increased flow rate using the same gradients on the ProSwift RP-4H, 200 µm x 25 cm monolith column.

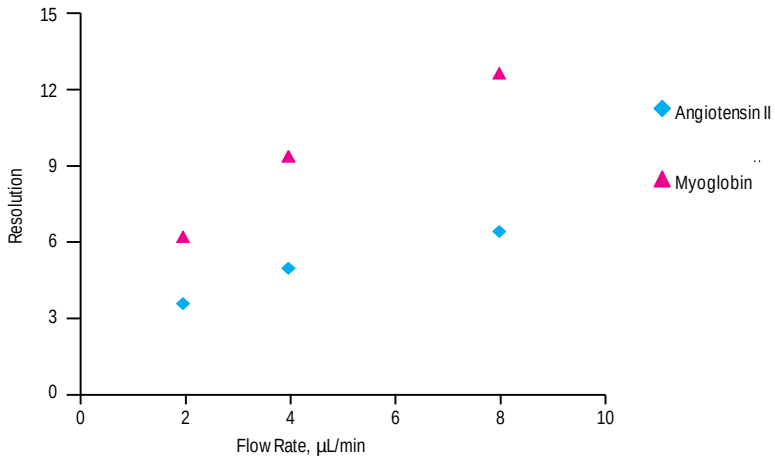


Figure 7: Separation of a 5 peptide/protein mixture on the ProSwift RP-4H, 200 µm x 25 cm column. Data extracted from the figure above.

1.4 Temperature Effects

The polymers used to prepare the PepSwift and ProSwift monoliths are highly cross linked making them extremely stable to elevated temperatures. By increasing the temperature, backpressure can be reduced allowing the use of increased flow rates. Additionally, changes in analyte retention time and selectivity may be observed providing an additional parameter for optimizing separations or increasing productivity.

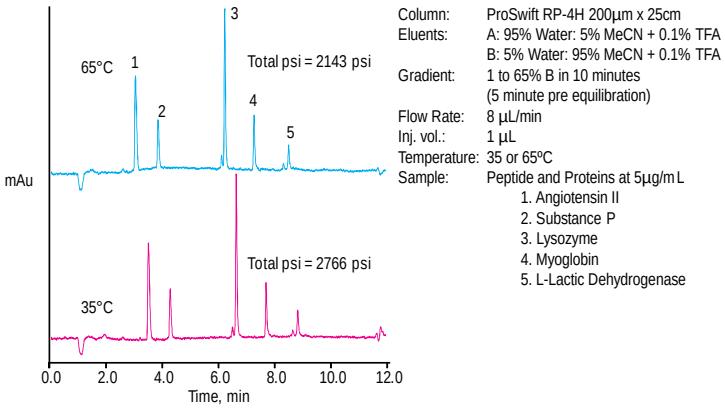


Figure 8: Separation of a 5 protein mixture on a ProSwift RP-4H column at 35 °C and 65 °C demonstrating a decrease in retention time of all sample species with increasing temperature.

At elevated temperatures the PepSwift columns exhibit excellent resolution of peptides and medium sized proteins. At these elevated temperatures, the diffusivity of peptides increases resulting in faster mass transfer to the stationary phase to provide increased interaction with the PepSwift monolith. Operating at 60 °C, the PepSwift column can separate a peptide mixture with peak widths at half height of only 1.6 – 3.5 seconds and base line resolution of each peptide using only a 7 minute gradient.

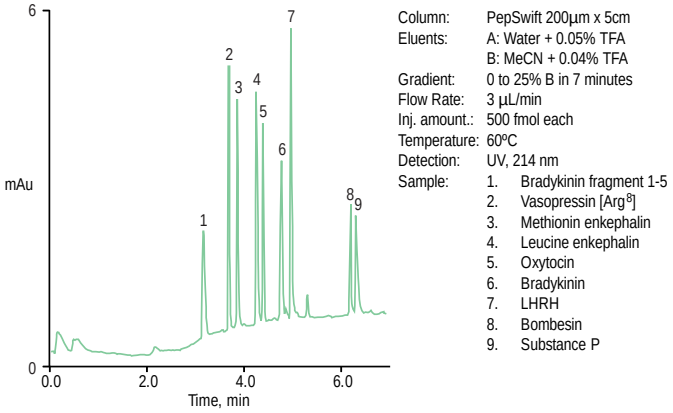


Figure 9: Separation of a 9 peptide mixture on a PepSwift column at 60 °C.

1.5 Robustness and Run Stability

PepSwift and ProSwift columns are durable and robust. These monoliths exhibit stability and reproducibility for hundreds of runs. The ProSwift columns retain high resolution and high run stability over 500 cycles of operation with complex samples. The low operational backpressure makes it more robust to backpressure increase due to column fouling from dirty samples*. Excellent run to run stability is also observed with the PepSwift capillary columns even at elevated temperatures.

* Sample preparation should always be carried out to remove particulates that may block the column and system components.

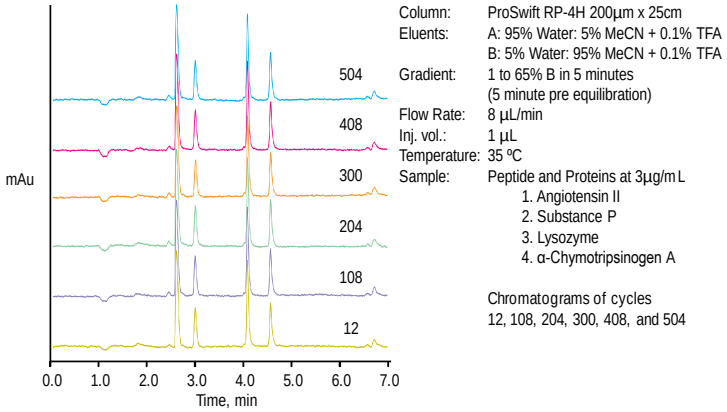


Figure 10: Ruggedness and stability of a ProSwift RP-4H, 200 µm x 25 cm column

1.6 pH Stability

Typical operating conditions using monolithic columns prepared in fused silica are limited to pH 1 – 10 for cleaning in place with a recommended operational limit from pH 2 – 8. The use of extreme pH may cause hydrolysis of the monolith-fused silica bonds which anchor the stationary phase to the column housing. This may result in critical failure of the column and potential damage to the system due to blockage.

Under normal operating conditions the columns are stable from 100's to 1000's of injections depending on your sample.

For certain samples, column washing may be required to remove particularly stubborn components. Regeneration of the column may be achieved using 1 µL injections of dilute (1mM, pH 10) sodium hydroxide using solvent without causing any detrimental effects to the column. Note that it is important to ensure complete removal of the sodium hydroxide before introduction of high solvent concentrations that may cause precipitation.

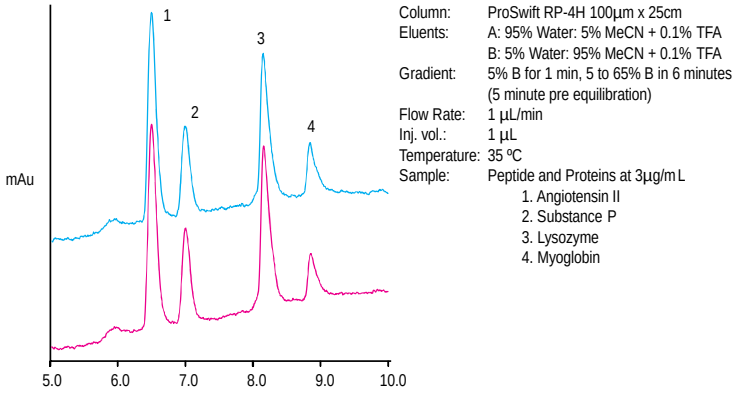


Figure 11: Separation of a simple peptide-protein mixture on a ProSwift RP-4H column before (bottom) and after (top) treatment with pH 10 NaOH.

1.7 Batch-To-Batch Reproducibility

1.7.1 Lot and batch definitions

Columns produced using monolith technology are manufactured by a patented *in situ* synthetic process that does not require additional sieving, coating, multiple surface modifications, or packing processes. This synthetic process is markedly simpler than the multiple steps required to make packed bead columns. As a result, the variation in chromatographic performance from one batch to another is smaller than that for conventional resin and silica columns. A benefit of this simpler manufacturing process is greater reproducibility in the chromatographic performance across the different production batches.

	Packed Bed Materials	**Monolith columns**
Batch	Unique synthesis to prepare resin in bulk. Additional functionalization of the batch may take place. Each individual lot is unique with respect to the combination of chemicals and processing. Batch is qualified for selectivity	This term is interchangeable with Lot. Each Batch or Lot of columns is prepared at one time. This essentially refers to the polymerization mixture used to prepare the monolith in combination with the process used.
Lot	A unique lot number is assigned to a set of columns packed at the same time using a portion of resin from a single batch. This is the number visible on the box and can be used to trace the batch.	Each lot is unique based on the Batch processing. Batch and Lot are interchangeable terminology.
Serial	Every column packed from each lot is assigned a unique identifier.	Each column is assigned a unique identifier. Due to the nature of the manufacturing, each column can essentially be considered unique as the material is polymerized in-situ where the column body is the reaction vessel.
Typical Lot size	>50 columns depending on column size/demand	5 – 30 columns depending on product and dimension

When carrying out lot testing, a smaller number of columns may be available from a monolith column lot.

1.8 Specifications

The following tables summarize the specifications of the PepSwift and ProSwift monolithic columns.

Table 2: PepSwift and ProSwift Capillary Column Specifications

Columns	PepSwift and ProSwift RP-4H	ProSwift C4 RP-5H
Base matrix material	Poly(ethylvinylbenzene-*co*-styrene-*co*-divinylbenzene)*	Poly(ethylene dimethacrylate-*co*-butyl methacrylate)
Surface chemistry	Phenyl	Butyl
pH range	1–10	
Max Temperature**	90 °C	
Solvent compatibility	Most common organic solvents	

*The PepSwift and ProSwift RP-4H polymer matrices differ with respect to crosslink and co-monomer ratio, which may result in different selectivity under comparable conditions.

** For EASY-Spray maximum operating temperature, please refer to the EASY-Spray product brochure for specifications.

Table 3: PepSwift and ProSwift Capillary Column Operating Parameters

Parameter	*Column dimension*	*Recommended flow rate, µL/min*	*Maximum Pressure, psi/Mpa*	*Dynamic binding capacity per mL of monolith*
PepSwift	100 µm x 5 cm	0.7-1.0	4350/30	1.6 µg/mL of tryptic cytochrome C digest
	100 µm x 25 cm			
	200 µm x 5 cm	2.0-3.0		
	200 µm x 25 cm			
	500 µm x 5 cm	15-25		
ProSwift	100 µm x 25 cm	0.5-3.0	4950 (34.1)	59.9 µg/mL α-Chymotrypsinogen
	100 µm x 50 cm	0.5-3.0		
	200 µm x 25 cm	2.0-12.0		
	500 µm x 10 cm	12-80	2000 (13.8)	
ProSwift C4 RP-5H	50 µm x 25 cm	0.125 – 0.75	4950 (34.1)	40.0 µg/mL α-Chymotrypsinogen
	100 µm x 25 cm	0.5-3.0		
	100 µm x 50 cm	0.5-3.0		
	200 µm x 25 cm	2.0-12.0		
	500 µm x 10 cm	12-100	2000 (13.8)	
	500 µm x 25 cm	12-100		

Typical operating pressures at 35 °C for ProSwift RP-4H and ProSwift C4 RP-5H columns: 100 µm × 50 cm: <3300 psi @ 2 µL/min; 100 µm × 25 cm: <1650 psi @ 2 µL/min; 200 µm × 25 cm: <1650 psi @ 8 µL/min; 500 µm × 10 cm < 660 psi @ 50 µL/min; and ProSwift C4 RP-5H 50 µm x 25 cm <1650 psi @ 0.5 µL/min and 500 µm x 25 cm <1650 psi @ 50 µL/min

1.9 Column Formats

Columns are available in different formats designed to obtain the best column performance.

All formats are either complete with nanoViper fittings or compatible with nanoViper connection tubing. All columns are made of biocompatible materials.

1.10 Interfacing with Mass Spectrometry

The PepSwift and ProSwift columns are available in a range of column formats that operate at flow rates that are compatible with mass spectrometry for the detection of peptides and proteins. The flow rates on the smallest, 100 µm ID columns are well suited for top-down and bottom-up proteomics applications where separation of a large number of proteins is desirable using a high resolution mass spectrometer. 100 µm ID columns are also better suited for analyzing small sample sizes. This is especially desirable when analyzing samples of limited quantity such as organ, tissue, and blood samples collected from patients for clinical research. The flow rates used for the largest, 500 µm ID columns enable fast separations for high throughput analysis of multiple samples necessary for ballistic analyses to identify target proteins for tissue or bacterial specie identification. The 200 µm ID columns bridge the flow rate gap between the two other formats enabling faster throughput than the 100 µm ID columns while providing greater sample resolution than the 500 µm ID columns.

The columns are available with zero-dead volume NanoViper connections that enable quick and easy connection to the HPLC system and the mass spectrometer without compromising the separation of the sample of interest.

The PepSwift is also available as an EASY-Spray™ column for use with the Thermo Scientific EASY-Spray™ source. These columns require a single connection with the EASY-Spray™ source to eliminate connection dead volumes with integrated temperature control. These attributes provide a column with maximum reliability and reproducibility for mass spectrometry analyses. For more information on EASY-Spray™ sources and associated devices, see www.thermoscientific.com.

2. Installation

For further information on application specific installation and set up please refer to the Thermo Scientific Dionex UltiMate™ 3000 RSLCnano Standard Applications Manual which can be found at www.thermoscientific.com

2.1 System Requirements

For optimal performance capillary monolith columns should be run on a nano or micro HPLC system, such as Thermo Fisher Scientific HPLC systems. Each of the possible configurations offers multiple sampling options.

Considerations for set up include:

- Flow rate and system void volume
- Tubing I.D.
- Flow cell volume
- Trap columns

For optimum system performance we recommend the use of the applicable nanoViper installation kit. More information can be found in the Thermo Scientific Dionex UltiMate™ 3000 RSLCnano Standard Applications Manual.

CAUTION	***There may be an increase in back pressure when using smaller internal diameter tubing. The upstream back pressure generated after the column outlet (including the connecting tubing between the column outlet and detector cell inlet, the cell, and cell waste line) MUST not exceed the maximum allowable pressure limits*** ***Excessive back pressure after the column may cause irreversible damage to the column.***

2.1.1 Flow rate

The flow rate used for a specific column is limited by the maximum allowable pressure of your system pump.

When changing from one column diameter to another, the equation below can be used to determine the appropriate flow rate required for the new column,

$$\nu_1 = \nu_2 \left(\frac{D_1}{D_2}\right)^2$$

where ν and D represent flow rate, and column diameter, respectively.

For example, when the internal porosity is assumed to be the same for two columns and one uses a flow rate of 1 µL/min for column 2 with an ID of 100 µm, the appropriate flow rate for column 1 with an ID of 200 µm is 4 µL/min.

2.1.2 Tubing i.d.

The choice of connective tubing will affect the system pressure with smaller ID tubing resulting in higher backpressures. The system backpressure at the required flow rate for your specific application will determine the choice of tubing diameter used. Smaller internal diameter tubing should be used as pressure allows in order to minimize gradient delay volumes post column and band broadening post column.

In a direct injection set up for 200 µm i.d. and smaller format monoliths, the best results will be obtained when all connecting tubing between the pump and the detector is 20 - 50 µm ID nanoViper tubing. Each length of tubing should be added one at a time and the increase in system pressure monitored. For columns with an internal diameter of 500 µm, the best results will be obtained when all connecting tubing between the pump and the detector is 50 - 75 µm ID nanoViper tubing depending on the flow rate used for the application.

Before connecting the column, record the system pressure at intended flow with the column removed (do not connect the post column components for this initial measurement). This pressure is separate from that experienced by the column. When evaluating the back pressure on the column, this pressure value should be subtracted from the total pressure when all components are installed. Connect the post column tubing using a coupler. Measure the total system pressure. When calculating the column pressure, subtract this number from the total system pressure observed with the column in place.

Eluents containing salts may evaporate and clog the narrow bore tubing when the chromatograph is idle. This may produce very high pressure values when the system is restarted. Take the precaution of clearing all eluent lines before restarting the system with the column installed.

2.1.3 Flow cell volume

Consider using micro and nano flow cells. Typically standard analytical flow cells enclose illuminated volumes of 9-11 µL. Peaks from 100 µm columns operating at 1-1.5 µL/min may elute in a fraction of that volume. Hence, the peak dispersion in a standard flow cell will broaden and otherwise deform a peak eluting from a capillary column.

2.1.4 Injection loop volume

The injection loop size may affect the delay time (for example, a 1 µL injection loop will introduce a 1 minute delay when operating at 1 µL/min). Full loop injections will minimize delay volumes compared to injecting the same volume as a partial injection with a larger loop size.

Use a sample loop according to your sample injection volume requirement. For manual injections Thermo Fisher Scientific recommends full-loop injections. For autosamplers, follow the manufacturer’s recommendation.

2.1.5 MS interface tubing

The tubing between the column outlet and the MS interface should be as short as possible with a narrow ID to minimize peak dispersion that can compromise detection performance. Additionally, using wider tubing to minimize backpressure can have a similar detrimental effect.

2.1.6 Trap columns

Trap columns can be used before the column to either concentrate the sample onto the column or retain materials that may cause damage to the system or column. Information on how to set up your system using a trap column can be found in the Thermo Scientific Dionex UltiMate™ 3000 RSLCnano Standard Applications Manual.

2.2 System Void Volumes

2.2.1 Gradient delay

All HPLC systems have a gradient delay. This is the time between when the pumps start pumping at a certain mobile phase composition and the time it takes for that solvent composition to reach the column. This should be taken into consideration when developing your gradient in order to ensure all peaks of interest have eluted prior to the equilibration cycle.

2.2.2 System void volume

The system void volume is the total volume occupied by mobile phase. This includes the tubing from the outlet of the HPLC pump to the column, the interstitial space in the column, and the subsequent tubing to the detector cell or mass spectrometer. This volume can be determined by both calculating and summing the volumes of the individual components, or by determining the elution time of a non-retained analyte. The void volume is a commonly used correction factor.

The system void volume should be minimized without generating excessive backpressure in order to decrease analysis time as well as minimize dispersive effects.

2.3 Column Installation

Connect the column with the direction of flow as indicated by the arrow on the column label. Ensure the connections are made correctly so as to not introduce dead volume that will cause peak dispersion.

3. Operation

3.1 General Operating Conditions

Table 4: General Operating Conditions for PepSwift and ProSwift Monolith Columns

Parameter	Recommendation			
Column:	ID (µm)	PepSwift	ProSwift RP-4H	ProSwift C4 RP-5H
Eluent Flow Rate (µL/min):	50 µm	-	-	0.125 – 0.75
	100 µm	0.7 – 1.0	0.5 – 3	0.5 – 3
	200 µm	2 – 3	2 – 12	2 – 12
	500 µm	15 – 25	12 – 80	12 – 100
Typical Sample Volume:	1 µL for 50 µm, 100 µm, and 200 µm ID 1 - 5 µL for 500 µm ID			
Eluent:	1–100% solvent			
Detector:	UV, Fluorescence, MS, etc.			
Storage Solution:	100% Acetonitrile			

3.2 ProSwift and PepSwift Operation Precautions

CAUTION

Filter and Degas Eluents
Filter Samples
Eluent pH between 1 and 10
Sample pH between 1 and 10
Do not exceed Maximum Operating Flow Rate
Do not exceed the Maximum Column Pressure

3.3 Chemical Purity Requirements

Obtaining reliable, consistent and accurate results requires eluents that are free of impurities. Chemicals, solvents and deionized water used to prepare eluents must be the highest purity available. Thermo Fisher Scientific cannot guarantee proper column performance when the quality of the chemicals, solvents, and water used to prepare eluents has been compromised.

When using solvents, HPLC or LCMS Grade products or equivalent should be used to prepare eluents.

3.3.1 Deionized water

The deionized water used to prepare eluents should be Type I Reagent Grade Water with a specific resistance of 18.2 megaohm-cm. The deionized water should be free of ionized impurities, organics, microorganisms and particulate matter larger than 0.2 µm.

3.3.2 Solvents

The PepSwift and ProSwift columns are cross-linked polymeric monoliths with very hydrophobic surfaces. These columns should be operated so that the eluent being pumped over the column contains minimally 1% solvent to ensure that the hydrophobic surfaces are “wetted” and maintain maximum column performance.

The PepSwift and ProSwift columns can withstand all common HPLC solvents listed in the table below. However, solvents and degassed water should be premixed in concentrations that allow proper mixing by the gradient pump and minimize out-gassing. Therefore, the columns should have an operational organic solvent concentration range of 1 to 95% to ensure proper chromatographic system performance.

Avoid creating high viscosity pressure fronts that may compress the monolith when the eluent is changed. To do this, equilibrate the column for about 10 minutes with an eluent containing only 5% of the current solvent type (e.g., methanol) Exchange this eluent for an eluent with 5% of the new solvent type (e.g., acetonitrile) and then equilibrate the column and allow the system to stabilize (about 10 minutes more). Next, run a 15-minute gradient from 5% of the new solvent type to the highest percentage used during the new analysis protocol.

The solvents used must be free of ionic impurities. However, since most manufacturers of solvents do not test for ionic impurities, use the highest grade of solvents available. Currently, several manufacturers are making ultrahigh purity solvents that are compatible for HPLC and spectrophotometry applications. These ultrahigh purity solvents will usually ensure that the results are not affected by ionic impurities in the solvent. At Thermo Fisher Scientific, we have obtained consistent results using High Purity Solvents such as Optima® Solvents from Fisher Scientific.

When using a solvent in an ionic eluent, column generated back pressures will depend on the solvent used, concentration of the solvent, the ionic strength of the eluent, and the flow rate used. Also, the column back pressure will vary as the composition of water and solvent mixture varies.

Ensure that all of the inorganic chemicals are soluble in the highest solvent concentration to be used during the analysis.

Table 5: HPLC Solvents for Use with PepSwift and ProSwift Columns

Solvent	Maximum Concentration
Acetonitrile	100%
Methanol	100%
2-Propanol	100%
Tetrahydrofuran	80%

3.4 Preparing Eluents Containing Solvents

Remember to mix solvents with water on a volume to volume basis. If a procedure requires an eluent of 95% acetonitrile, prepare the eluent by adding 950 mL of acetonitrile to an eluent reservoir. Then, add 50 mL of deionized water or eluent concentrate to the acetonitrile in the reservoir. Using this procedure to mix solvents with water will ensure that a consistent true volume/volume (v/v) eluent is obtained. Premixing water with solvent will minimize the possibility of out-gassing.

For increased eluent repeatability preparation to preparation, the weight of the mobile component should either be measured upon first preparation or calculated using density.

When purging or degassing eluents containing solvents, do not purge or degas the eluent excessively. Volatile solvents may evaporate.

Always degas and store all eluents in glass or plastic eluent bottles pressurized with helium. Nitrogen is soluble in solvent containing eluents, helium is recommended to purge and degas these eluents.

Acetonitrile (CH_3CN) hydrolyzes to ammonia and acetate when left exposed to basic solutions. To prevent eluent contamination from acetonitrile hydrolysis, always add acetonitrile to basic aqueous eluents by proportioning the acetonitrile into the basic eluent with the gradient pump. Keep the acetonitrile in a separate eluent bottle which contains only acetonitrile and water. Never add the acetonitrile directly to the basic solutions, e.g., sodium hydroxide in the eluent bottles.

3.4.1 Eluents for gradients

Gradient applications are straightforward as long as solvents and water are premixed in concentrations that allow mixing by the gradient pump to give the required gradient ramp for your chromatography. For example, if you want to build a solvent gradient from 5% solvent to 95% solvent, make the following eluents:

- Eluent A: 5% solvent/95% water
- Eluent B: 95% solvent/5% water

Then, by programming the gradient pump properly, you can go from 100% Eluent A to 100% Eluent B. This will avoid out-gassing and refractive index problems associated with mixing neat solvents with water.

4. Example Applications

4.1 General Use and Applications

For columns with similar chemistry (e.g. phenyl based phases), the differences in the structure of the monolith determine the suitability of the columns for various types of biomolecule separations. The PepSwift column has a smaller average pore size making it more suited to peptides, digests and smaller proteins. It also operates at a higher backpressure than the ProSwift column due to the smaller pore size. Separations on the PepSwift column typically employ elevated temperatures in order to reduce the operational backpressure. The larger pore size of the ProSwift columns allow room temperature operation for thermally sensitive samples.

Due to the higher surface area, PepSwift columns should be employed when the sample contains primarily lower molecular weight components such as peptides and low to medium molecular weight proteins (e.g. ≤ 30 kDa). ProSwift columns should be employed for samples containing higher molecular weight proteins (e.g. > 20 kDa) such as monoclonal antibodies and cell lysates. Fast convective mass transfer makes these phases ideal for larger molecules that suffer greatly from diffusion limitations in traditional porous media. Due to the large pore size and lower pressure, the ProSwift columns are less prone to fouling when analyzing high molecular weight proteins and complex, unrefined cell lysates. This benefit results in an extended column life time without compromised column performance or poor reproducibility.

Additional column applications can be found on the respective product pages at www.thermoscientific.com.

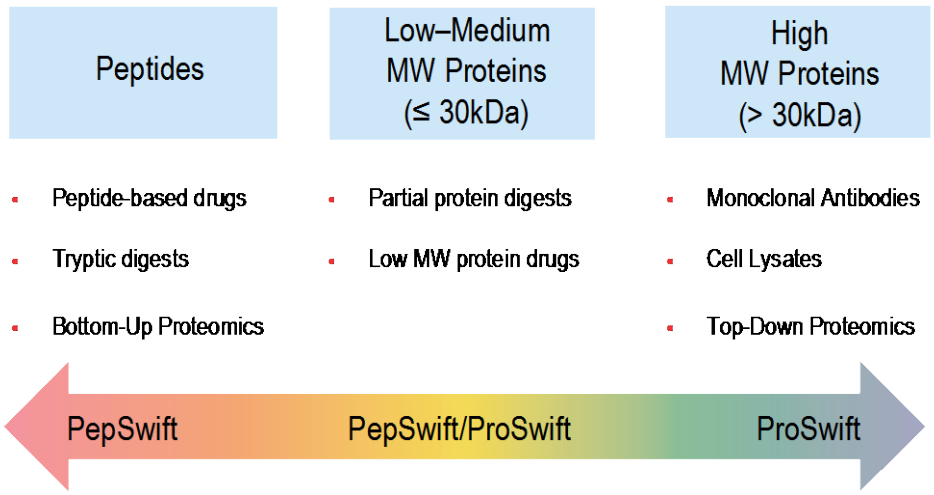


Figure 12: Examples of applications and the appropriate monolithic column for achieving optimal results.

The following applications are considered general. They have been performed using either the PepSwift or ProSwift columns depending on application. Many of these applications can be performed using either the PepSwift or ProSwift monoliths. However, for mixed peptide/protein separations, these applications will assist you in choosing the best column for your application.

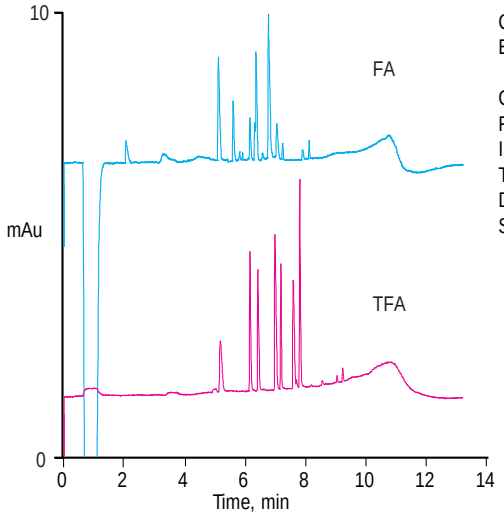
4.1.1 Sample Preparation

Generally when analyzing peptide and protein samples, performance is greatly influenced by how the analyte initially binds to the stationary phase in the starting eluent.

The closer in composition to the initial gradient conditions the sample matrix is, generally the better the performance. In cases where sample dilution is prepared, using eluent A as the diluents may help to improve performance. Adjusting the pH of the sample to close to that of the initial gradient composition will greatly improve interaction of the analyte with the stationary phase. This is especially important when the sample matrix is such that the analyte is neutral when often ionization is required for efficient binding.

4.1.2 Mass spectrometry: trifluoroacetic acid vs formic acid

The capillary formats of the PepSwift and ProSwift columns make them well suited for applications using mass spectrometry for detection. For many peptide and protein separations, trifluoroacetic acid (TFA) is an excellent ion pairing agent and generally results in better peak shape and resolution. However, when used for mass spectrometry applications, TFA creates an ion pair with basic groups of peptides and proteins resulting in suppressed ionization and reduced MS signal. In place of TFA, formic acid (FA) is commonly used as an ion pairing agent at a concentration (0.1 – 0.2%) approximately twice the normal TFA concentration used (0.05 – 0.1%). The figure below compares chromatograms of the same sample using eluents with TFA and FA as ion pairing agents and a UV detector. This figure shows slightly broader peaks, lower resolution and a different selectivity demonstrating the effect of replacing TFA with FA as the ion pairing agent.



Column:	PepSwift 200µm x 5cm
Eluents:	A: Water + 0.05% TFA
	B: MeCN + 0.04% TFA
Gradient:	0 to 25% B in 7 minutes
Flow Rate:	3 µL/min
Inj. amount.:	500 fmol each
Temperature:	60ºC
Detection:	UV, 214 nm
Sample:	1. Bradykinin fragment 1-5
	2. Vasopressin [Arg^8]
	3. Methionin enkephalin
	4. Leucine enkephalin
	5. Oxytocin
	6. Bradykinin
	7. LHRH
	8. Bombesin
	9. Substance P

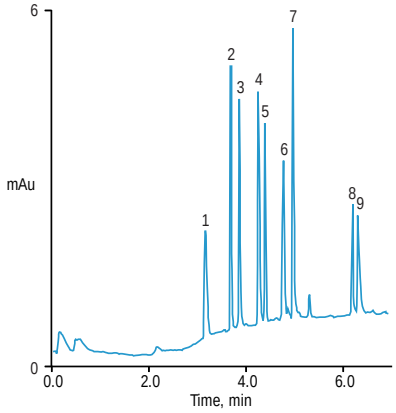
Figure 13: Effect of ion pairing agent FA (formic acid) vs. TFA (trifluoroacetic acid) on the separation of the simple peptide test mixture.

4.2 PepSwift Applications

The PepSwift monolith columns are well-suited for the separation of both simple and complex peptide mixtures using detection methods ranging from UV detection to mass spectrometry analysis. Additionally they show excellent performance for the separation of protein digests and low to medium molecular weight protein mixtures. The following applications are considered to be relevant or more specific to analyses conducted using the PepSwift columns.

4.2.1 Simple peptide sample sets

The analysis of peptide sets is important in a wide range of applications including column validation, procedure validation, quantitative analysis, and QA/QC. For instance, separation on the PepSwift column can be used to confirm purity, yield, functionalization, and/or the accuracy of the final peptide product produced by liquid-phase or solid-phase peptide synthesis (e.g., testing for protecting groups used during synthesis or the improper sequencing of amino acids in the peptide chain). Separation on reverse phase columns can also be used to confirm or quantify the binding of peptides to other chemical species (biological, organic, or inorganic) and peptide aggregation. As an example of the separation power of the PepSwift column for a set of peptides, the figure below shows the separation of a peptide test mixture using UV absorbance at 214 nm for detection. The narrow PWHH of the different peptides ranges from 1.6 – 3.5 seconds resulting in excellent resolution of each individual peptide.



Peptide #	Retention Time min	PWHH sec
1) Bradykinin fragment 1-5	3.3	3.5
2) Vasopressin [Arg^8]	3.8	1.6
3) Methionin Enkephalin	4.0	1.9
4) Leucin Enkephalin	4.4	2.3
5) Oxytocin	4.6	1.6
6) Bradykinin	4.9	2.5
7) LHRH	5.1	1.9
8) Bombesin	5.1	1.9
9) Substance P	6.4	2.6

Figure 14: Separation of a 9 peptide mixture on a PepSwift column. See Figure 9 for conditions.

4.2.2 Analysis of protein digests

The analysis of tryptic digests is important in a wide range of applications including column validation, procedure validation, quantitative analysis, QA/QC, and bottom-up proteomics. Simple analyses such as the testing of a single protein digest can be used to validate a new column or procedure prior to performing high value analyses. Digests of proteins can also be used for QA/QC at specific stages of production or of the final protein product. The Figure below shows the separation of a tryptic digest of cytochrome C on a 100 µm ID monolithic PepSwift column. A gradient from 0-45% acetonitrile (0.04% $TFA_{(aq)}$) was performed in 8 minutes resulting in the fast separation of each peptide with baseline resolution. This example illustrates the excellent separation and high throughput capabilities of PepSwift columns for the analysis of a relatively simple single protein digest. Although this example is carried out using UV detection, mass spectrometry can easily be used for detection and the identification of specific peptide fragments as shown below.

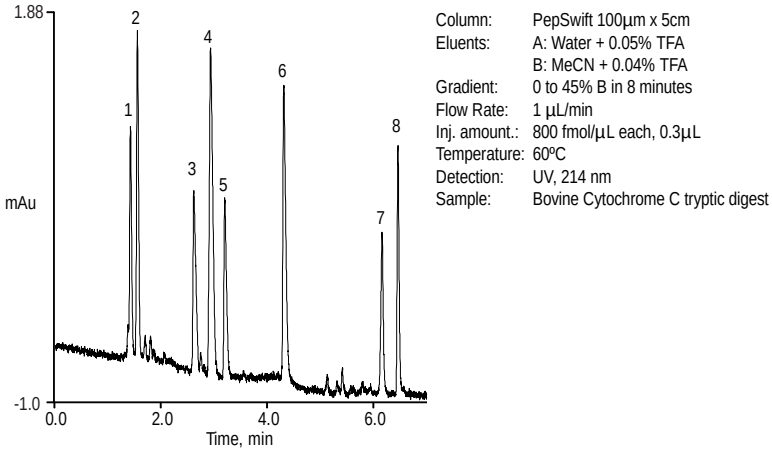
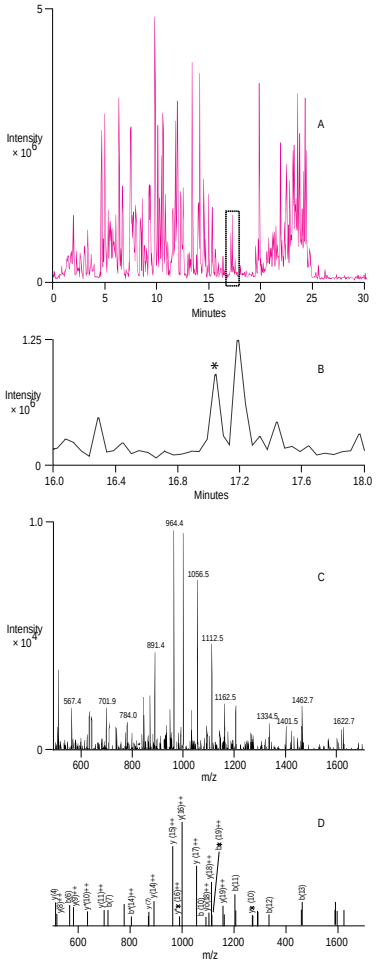


Figure 15: Nano LC separation of a tryptic cytochrome C digest on a 100 µm ID PepSwift column using a 0.3 µL injection of an 800 fmol/µL sample.

PepSwift monoliths are also well-suited to the separation of more complex mixtures of peptides derived from the digest of many proteins in bottom-up proteomics applications (e.g. a digest of proteins from a cell lysate). Figure 16 on the next page shows the separation and MS analysis of a tryptic digest of a protein mixture composed of cytochrome C, lysozyme, alcohol dehydrogenase, BSA, apotransferrin, and ß-galactosidase with molecular weights ranging from 11 to 135 kDa. The gradient consists of (A) 0.1% formic acid and (B) 80% acetonitrile and 0.08% formic acid, from 0 to 50% B in 25 min. Figure 16A shows the base peak chromatogram (BPC), illustrating the high peak capacity of the monolithic column for the separation of the complex sample. Figure 16B shows a close-up of the BPC between 16.0 and 18.0 minutes with two minor tryptic peptides eluting at 17.0 – 17.2 minutes. From the tryptic peptide eluting at 17.0 minutes, the MS-MS spectrum is shown in Figure 16C with the corresponding Mascot® database search (Figure 16D). This peptide has the sequence GLVLIAFSQYLQQCPFDEHVK and was unambiguously identified as a BSA fragment.



Column:	PepSwift 200µm x 5cm
Eluents:	A: Water + 0.1% Formic Acid
	B: 80% MeCN + 0.08% Formic Acid
Gradient:	0 to 50% B in 25 minutes
Flow Rate:	2.5 µL/min
Inj. amount.:	100 fmol
Temperature:	60ºC
Detection:	MS
Sample:	Tryptic digest mixture of
	Cytochrome c
	Lysozyme
	alcohol dehydrogenase
	Bovine serum albumin
	Apotransferrin
	ß-galactosidase

Figure 16: (A) Base peak chromatogram of a tryptic digest of 6 proteins separated on a PepSwift column. The dashed lines indicate the region of the chromatogram enlarged in (B) to highlight an arbitrary peptide peak (indicated by the star) analyzed using MS and (C) MS/MS and identified using a (D) Mascot® search.

4.2.3 Low molecular weight protein samples

In addition to peptides, many low molecular weight proteins can be separated on the PepSwift columns. Generally, proteins below a molecular weight of ~30 kDa can be separated without compromising the performance of the column due to fouling leading to peak broadening and decreased resolution. Applications include column or procedure validation, QA/QC, investigation of low molecular weight protein-based drugs, and top-down proteomics analysis of low-molecular weight protein fractions. The figure below shows the separation of a simple protein set of 4 low molecular weight proteins and one high molecular weight protein on a 200 µm ID PepSwift column using a gradient of 20-50% acetonitrile in water and 0.05% TFA over 15 minutes.

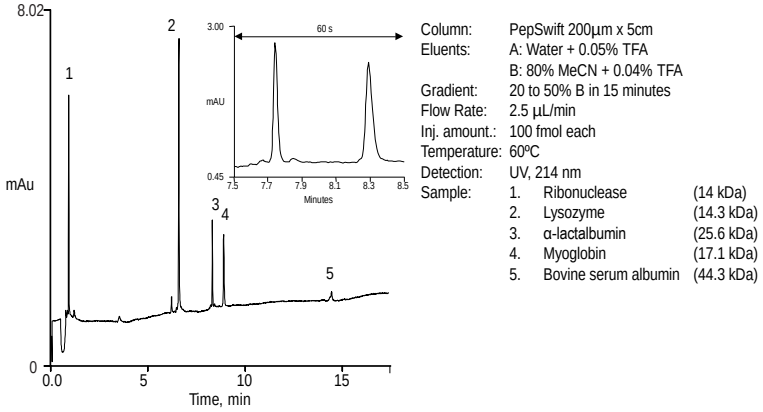


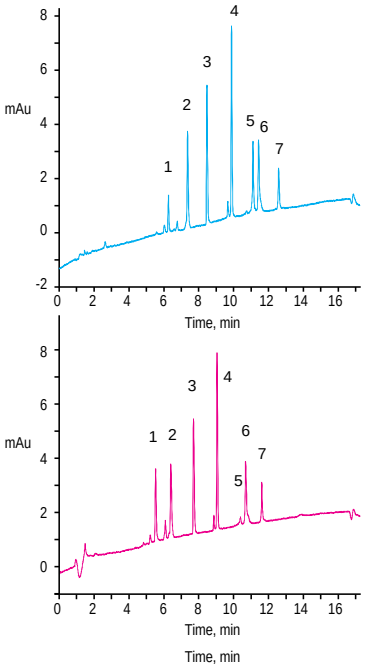
Figure 17: (Left) Separation of a simple set of 5 proteins on a 200 µm ID PepSwift column, and (right) an enlarged region of the chromatogram showing the symmetrical shape and narrow peak widths of peaks 3 and 4.

4.3 ProSwift Applications

The ProSwift monolith columns are specifically designed for the separation of higher molecular weight proteins (MW ≥30 kDa). More specifically, the properties of the monolith are engineering to offer low back pressure to enable coupling to a mass spectrometer and to reduce column fouling resulting in impaired chromatographic separation due to peak broadening and decreased resolution. For these reasons, the ProSwift columns are well-suited for the separation of samples ranging from simple protein mixtures to complex cell lysates and GELFrEE fractions. The following applications are considered to be specific to analyses conducted using the ProSwift columns.

4.3.1 High molecular weight proteins

The analysis of protein sets is important in a wide range of applications including column validation, procedure validation, quantitative analysis, and QA/QC. Separation on the ProSwift columns can be used to confirm purity, yield, functionalization, and/or the accuracy of a final protein product produced in bacteria, yeast, or mammalian cells and subsequently purified from the complex cell lysate. The figure below shows the analysis of individual proteins ranging in molecular weight from 5.8 kDa to 66 kDa using (top) a ProSwift RP-4H column and (bottom) a ProSwift C4 RP-5H column both in 200 µm x 25 cm formats. The absence of a strong correlation between protein molecular weight and retention time indicates that the hydrophobic properties of the protein determine their retention times and resolution with the absence of a secondary interaction through size exclusion. The difference in selectivity is also shown for the two columns with the less hydrophobic ProSwift C4 RP-5H having shorter retention times than the ProSwift RP-4H column. In comparison to the ProSwift C4 RP-5H, the protein trypsin inhibitor is observed to denature on the ProSwift RP-4H column as evidenced by the large denatured protein peak (5) eluting near porcine albumin.



Column:	Top: ProSwift RP-4H 200µm x 25cm		
	Bottom: ProSwift C4 RP-5H 200µm x 25cm		
Eluents:	A: Water + 0.05% TFA		
	B: 20% Water: 80% MeCN + 0.04% TFA		
Gradient:	5 to 70% B in 15 minutes		
	(5 minute pre equilibration)		
Flow Rate:	8 µL/min		
Inj. vol.:	1 µL		
Temperature:	35ºC		
Sample:	1. Trypsin Inhibitor	(20.1 kDa)	15µg/mL
	2. Ribonuclease A	(14 kDa)	9µg/mL
	3. Insulin	(5.8 kDa)	9µg/mL
	4. Lysozyme	(14.3 kDa)	9µg/mL
	5. Trypsin Inhibitor (Denatured)		
	6. Porcine serum albumin	(66 kDa)	9µg/mL
	7. Thermolysin	(34.6 kDa)	9µg/mL

Figure 18: Separation of low and high molecular weight proteins (5.8 – 66 kDa) on a ProSwift RP-4H column (Top) and a ProSwift C4 RP-5H column (bottom).

4.3.2 Cell lysates and GELFrEE fractions

The analysis of cell lysates and GELFrEE fractions (a portion of cell lysate fractionated for a specific molecular weight range) is extremely important in the areas of top-down proteomics and clinical research of healthy and diseased tissues. Often, these samples are very complicated in nature due to the presence of thousands of proteins and many other potential artifacts from the cell lysate and other sample prep purification processes. The high dynamic loading capacity and separation capabilities of the ProSwift RP-4H capillary columns enables the separation and MS detection of a large number of proteins when analyzing these complex samples. The figure below shows the separation of GELFrEE fractions from human HeLa cells (MW range: ~11 – 23 kDa and 20 – 52 kDa) using a 100 µm x 50 cm ProSwift RP-4H column with MS detection. This data illustrates the ability to separate a large number of proteins in both low and high molecular weight samples.

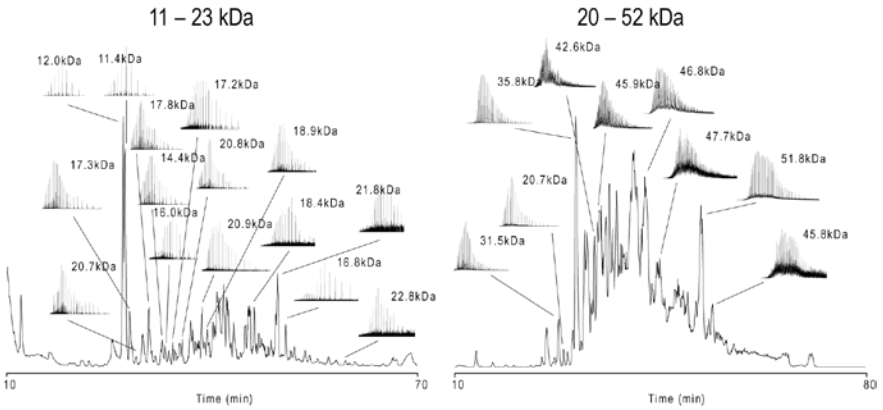


Figure 19: Separation of GELFrEE fractions from HeLa cell lysates on a 100 µm x 50 cm ProSwift RP-4H using mass spectrometry for protein detection and identification. (Data Courtesy of Prof. Neil Kelleher, Northwestern University, Evanston, IL)

For a given internal diameter, the length of the column used can significantly affect the loading capacity and resolution of proteins. For these reasons, it is important to select a column format that is best suited for your specific application. The figure below highlights the differences in separation power of two 100 µm ID ProSwift RP-4H columns of different lengths (25 cm and 50 cm) when analyzing GELFrEE fractions of two different molecular weight ranges (12 – 15 kDa and 25 – 35 kDa). From this data, it is clear that the longer column format is better suited to identifying a greater number of gene products and proteoforms for both low and higher molecular weight GELFrEE fractions of proteins. As the molecular weight range of the sample protein fraction increases, the advantage that the longer 50 cm column offers becomes greater as evidenced by comparing the number of gene product and proteoform identifications for each fraction. From this data, it is clear that longer columns are better suited for top-down proteomics applications since they offer greater loading capacity and improved resolution of proteins. However, for clinical research and other applications aimed at identifying target proteins, the user may benefit from the shorter column especially for high throughput applications.

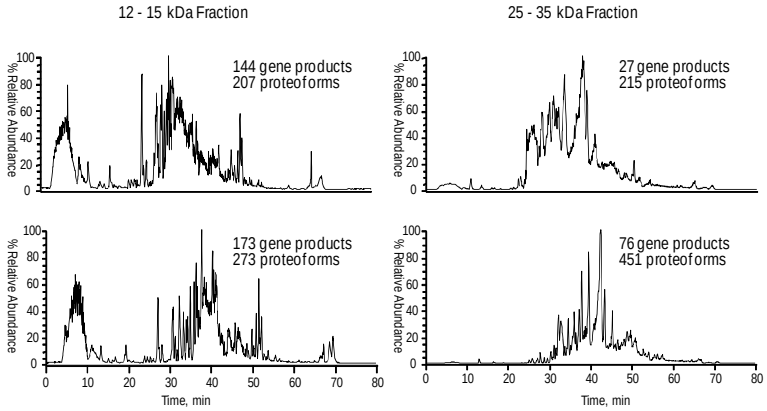
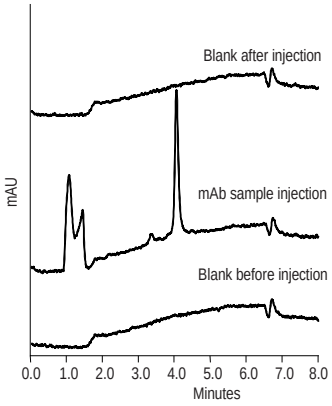


Figure 20: Effect of column length (25 cm, top chromatograms, vs 50 cm, bottom chromatograms) on the separation of proteins in two different HeLa cell GELFrEE fractions. The GELFrEE fractions were analyzed using 100 µm ID ProSwift RP-4H monoliths. (Data Courtesy of Prof. Neil Kelleher, Northwestern University, Evanston, IL)

4.3.3 Monoclonal antibodies

The analysis of antibodies, is of great interest in both academic and pharmaceutical applications including analysis of product purity and structural confirmation for the design of antibody-drug conjugates (ADCs). The molecular weight of antibodies is generally on the order of ~150 kDa. Proteins of this size can often bind strongly to the column and cause carryover from injection to injection. As shown below, the ProSwift C4 RP-5H capillary monolith columns are capable of separating antibodies without significant carryover of the antibody from one run to another. When coupled to MS, the narrow peak widths of antibodies on these columns (8 – 15 seconds) can result in higher resolution and thus improve MS analysis.



Column:	ProSwift C4 RP-5H 200µm x 25cm
Eluents:	A: Water + 0.05% TFA
	B: 20% Water: 80% MeCN + 0.04% TFA
Gradient:	5 to 70% B in 5 minutes
	(5 minute pre equilibration)
Flow Rate:	8 µL/min
Inj. vol.:	1 µL
Temperature:	35ºC
Sample:	Monoclonal antibody, 10 µg/mL in eluent A

Figure 21: Analysis of a monoclonal antibody on a 200 µm x 25 cm ProSwift C4 RP-5H column demonstrating no protein carryover between runs.

The chemical properties of an antibody determine how the protein will elute from a column. The figure below compares the elution of two antibodies with different isoelectric points (pI) when analyzed using different column temperatures. The more basic mAb with a pI in the range of 9 – 10 elutes with a sharp peak at both low and high temperatures. However, the more acidic mAb with a pI of 6 – 8 elutes with a poorly defined broad peak at 35 °C, which becomes sharper with increasing temperature. The broad tailing of the more acidic mAb is potentially due to additional weak hydrophobic interactions between the mAb and monolith solid phase at lower temperatures. This peak shape can be improved by increasing the temperature to disrupt these weaker interactions.

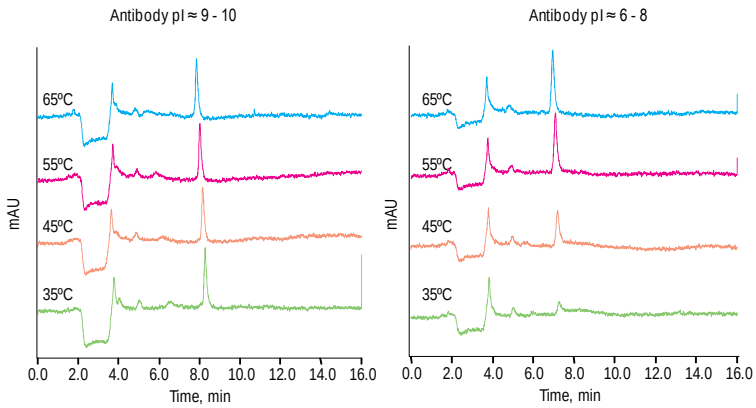


Figure 22: Effect of column temperature on the peak shape of monoclonal antibodies with two different isoelectric points (pI 9 – 10 vs pI 6 – 8)

The analysis of a mAb and associated variants (e.g., glycosylated forms), aggregates, and degradation products is extremely important to the development of these biotherapeutic compounds for both production and efficacy. Relative to the ~150 kDa size of the mAb, glycosylation and other protein modifications are relatively small and may not significantly change the overall properties of the mAb. Due to the similarity of the structures, separation of the main mAb protein from the variants can be difficult to achieve for detection and characterization by mass spectrometry. Figure 23 below shows the analysis of a mAb on a 500 µm x 10 cm ProSwift C4 RP-5H column using a flow rate of 150 µL/min at a temperature of 85°C. The increased temperature reduces the potential for protein carryover, improves peak shape due to increased mass transfer, and also reduces the column backpressure. The left chromatogram in the figure below shows the main mAb peak with a PWHH of only 11 seconds and the detection of minor peaks both before and after the main peak. The right chromatogram shows an enlarged region at the base of the main peak illustrating the separation of other components including potential minor structural variants or degradation products. Under these separation conditions, five minor peaks are observed before the main mAb peak and three minor peaks are observed after the main mAb peak.

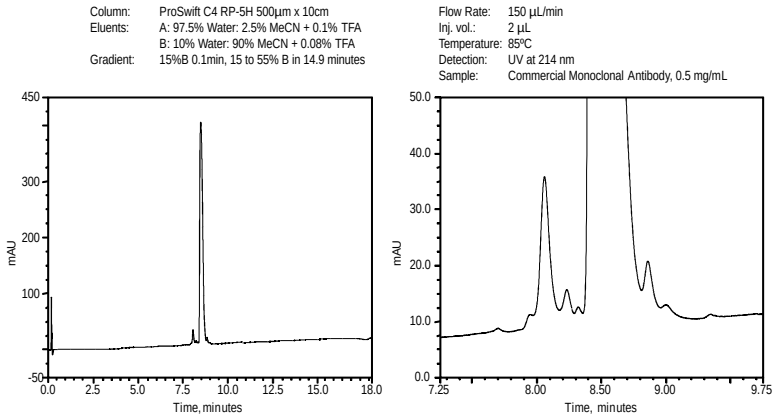


Figure 23: mAb analysis on a 500 µm x 10 cm ProSwift C4 RP-5H monolith column (left) the full mAb peak and (right) an expanded region at the base of the peak.

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5. Appendix

5.1 QuickStart

5.1.1 Overview

The ProSwift RP-4H monolith columns offer superior separation of biomolecules ranging from low to high molecular weight proteins including monoclonal antibodies. Conditioning of the column bed is **required** prior to initial use and after long-term storage. This QuickStart is intended to help first-time users quickly get started and also ensure extended column lifetime and reproducibility.

5.1.2 Preparation

A. Eluent Preparation:
The following eluents are recommended, but the column may be used with any eluent appropriate for analysis. Typically, Eluent A is a mobile phase with low organic content, and Eluent B is a mobile phase with high organic content.

- **Eluent A**: 0.1% TFA in water (Typical 95:5 v/v Water:CH_3CN)
- **Eluent B**: 0.1% TFA in CH_3CN (Typical 95:5 v/v CH_3CN:Water)

B. Column Installation:
Install the column on the instrument in the correct flow direction.

WARNING

Sudden increases in flow rates may damage monolithic columns. Always increase the flow rate slowly using a linear flow gradient or stepwise increments in flow rate.
If the eluent composition generates back pressure in excess of the maximum operating pressure, reduce the flow rate to ensure the upstream back pressure is less than the maximum operating pressure.

5.1.3 Flow rate start-up

Using a linear or stepwise flow gradient, increase the flow rate of **Eluent B** starting from 0.00 µL/min to the desired flow rate over 5 minutes.

5.1.4 Column conditioning

Use the guidelines below to determine the proper startup conditions:

A. Removal of Storage Solution:
   a. Using the desired flow rate, run a binary gradient from 100% B to starting conditions so that 10 column volumes pass through the column during the gradient. Use the table below to determine the total gradient volume necessary for your column format.
   b. Pump another 10 column volumes of starting eluent through the column.

Use the table below to determine the amount of eluent (10 column volumes) to flush through the column for the removal of storage solution in the above steps. Multiply the length of the column in cm by the volume factor to determine the 10 column volume in µL.
For example, for a 200 µm x 25 cm column, 10 column volumes = 25 cm X 1.88 = 47 µL.

Column Diameter	Volume Factor
50 µm	0.118
100 µm	0.471
200 µm	1.88
500 µm	11.8

B. Column Equilibration:
   a. Run the column at the desired starting eluent composition for 3 minutes.
   b. Then, run a binary gradient to 100% B over 1 minute.
   c. Hold at 100% B for 5 minutes.
   d. Next, run a binary gradient to your desired starting eluent composition over 1 minute.
   e. Repeat a – d until a reproducible background signal is obtained.

C. Pre-injection Equilibration
   a. Equilibrate the column with 5 to 10 column volumes prior to sample injection.

5.1.5 Storage

A. Store the column in 100% CH_3CN.