Agilent Macroporous Reversed-Phase C18 Column (mRP-C18)

Data Sheet

General Description

The Macroporous Reversed-Phase C18 Column (mRP-C18) is a unique silica-based C18 column packing. The unique features of this column include high protein recoveries*, consistent reproducibility, increased column loads, and enhanced resolution compared to conventional silica-based RP HPLC columns. The special silica support is designed to reduce or eliminate strong adsorption of proteins and is based on ultrapure, less acidic 5-µm silica particles. Columns made with this material are especially useful for carrying out gradient separations of macromolecules because of the favorable kinetic properties of the silica support. The material is packed in PEEK column hardware to minimize protein adsorption to the column surface. Columns are loaded to provide a uniform bed density using a proprietary high-pressure slurry-loading technique to give optimum column efficiency.

The Agilent mRP-C18 column is designed specifically for the separation of complex protein mixtures for protein/polypeptide fractionation prior to downstream analysis. The mRP-C18 column performs optimally under gradient separations at low pH, with mobile phases such as acetonitrile/aqueous trifluoroacetic acid combinations widely used for protein and peptide reversed-phase separations. This column, used with the recommended gradient and elevated temperatures between 70 °C and 80 °C, will provide excellent reproducibility, high protein mass recovery and high resolution.

HPLC equipment used with this column should have the capability to operate effectively for rapid, low-volume peaks with minimum instrument dead volume that would decrease the column resolution. Satisfactory results have been obtained with an Agilent Technologies Model 1100 liquid chromatograph fitted with a microcell, microsampling valve, a thermostatted column compartment and an analytical scale fraction collector. Conventional autosamplers generally do not provide optimum sampling because of conditions that can lead to extra column band broadening and mixing, thereby reducing resolution.

*Greater than 98% recoveries have been observed with immunodepleted serum using several high-sensitivity protein recovery methods.

Safety Considerations

- All points of connection in liquid chromatographic systems are potential sources of leaks. Users of liquid chromatographic equipment should be aware of the toxicity or flammability of their mobile phases.
- Because of its small particle size, dry mRP-C18 packings are respirable. Columns should be opened only in a well-ventilated area.

Operational Guidelines

The mRP-C18 column is designed for high stability at low pH (for example, pH <5). All silica-based packings have some solubility in pH >6 aqueous mobile phases. Therefore, it is not recommended to use the column with solvent systems with pH >6 for a prolonged period of time.

- The direction of flow is marked on the column.
- While generally not harmful to the column, reversed flow should be avoided except to attempt removal of inlet frit plugging (see “Column Care” section).
- A new column contains a mixture of methanol and water. Initially, care should be taken not to pass any mobile phase through the column that might cause a precipitate.
- mRP-C18 material is compatible with water and all common organic solvents, including N, N-dimethyl-formamide (DMF), and dimethylsulfoxide (DMSO).
- Maximum protein loading (4.6 × 50 mm mRP-C18 column) is 380 µg.
- Avoid use of this column below pH 0.9 or above pH 8.0.
- Maximum operating pressure is 250 bar (4000 psi).
- Maximum operating temperature at pH <5 is 80 °C, for pH 5–8 is 40 °C.

Column Care

The inlet frit on these columns has a nominal porosity of 2 µm. Samples that contain particulate matter larger than 2 µm will plug the column inlet frit. If solvent flow appears to be restricted (high column back-pressure), check first to see that solvent flow is unobstructed up to the column inlet. If the column flow is restricted, there may be particulate matter on the inlet frit. Make an initial attempt to remove any inlet debris by backflushing 10–15 mL of mobile phase through the column.

Mobile Phase Selection

The nonpolar bonded stationary phase is best used with mobile phases such as acetonitrile/water or methanol/water mixtures. Increasing the concentration of the organic modifier generally reduces the sample retention. Initial gradient elution separations typically use 5% aqueous acetonitrile or methanol as the initial solvent, and 100% organic as the final solvent. Separations of proteins and polypeptides often use trifluoroacetic or formic acid modifier for pH control and/or as an ion-pairing additive for desired retention and selectivity.

Recommended mRP-C18 Applications

The separation characteristics of the Agilent mRP-C18 column make it a robust proteomic tool to fractionate complex protein samples. High protein recovery, enhanced resolution, high column loading, and run-to-run reproducibility make the mRP-C18 column ideal for proteomic sample fractionation and/or desalting/concentration. Complex proteomic samples, such as serum, should be immunodepleted to remove high-abundant proteins.

Due to the potential irreversible binding characteristics resulting from albumin, immunodepletion is highly recommended prior to use of the mRP-C18 column. The effects of nonimmunodepleted serum on the mRP-C18 column will result in poor recovery, resolution, and reproducibility.

Sample Preparation and mRP-C18 Column Loading for Protein Fractionation and Desalting

To 1 mL of flow-through fraction from an Agilent Multiple Affinity Removal Column (for example, a 4.6 × 100 mm Hu-6 human serum column, part number 5185-5985, where 1 mL of the flow-though fraction contains ~300 µg of protein):

1. Dissolve 0.48 g of urea pellets in 1-mL flow-through fraction (final concentration 6M).
2. Add 13 µL of neat glacial acetic acid (final concentration 1.0%).
3. Load immunodepleted serum sample directly on the 4.6-mm id × 50-mm mRP-C18 column.

The flow-through fraction from any Agilent Multiple Affinity Removal column (for human or mouse serum high-abundant protein removal) can be used, as long as the mass of protein injected onto the mRP-C18 column does not exceed the column capacity of 380 µg.

Sample can be loaded directly with the use of a large volume injector loop (900 µL) or may be sequentially loaded with several smaller injections. For sequential loading of smaller sample volumes, an isocratic loading method is required. To load the mRP-C18 column, the method involves the injection of the desired volume, followed by an isocratic run of 97% aqueous mobile phase for 3 minutes (proteins will retain on the column and not elute until a higher percent organic is introduced). This "smaller loading" can be repeated several times until desired load or column capacity is reached. When column is loaded, proteins may then be separated by the start of the gradient. **See Figures 1 and 2 for the HPLC run conditions for protein fractionation and desalting.**

Protein Fractionation Application

Samples of human serum were depleted of the six most abundant proteins with the Agilent Multiple Affinity Removal Column. The flow-through proteins were then separated on the mRP-C18 column shown in Figure 1. During the separation, column fractions were collected, treated, and investigated by LC/MS/MS. Use of the mRP-C18 column at elevated temperatures, with a linear multisegment gradient, enhanced peak resolution increased column load and improved protein recovery (>98%), while permitting robust and reproducible operation of the separation [1].

WARNING: Alteration of the recommended sample and fractionation

Protein Fractionation Run Conditions

		Gradient	
Flow	0.75 mL/min	Time (min)	% B
Stoptime	58 min	0	3
Posttime	20 min	1	3
Col. temp	80 °C	6	30
Detection	UV, 280 nm	39	55
Starting solvent composition		49	100
Solvent A	97% (Water/0.1% TFA)	53	100
Solvent B	3% (ACN/0.08% TFA)	58	3
Pressure limit	250 bar		

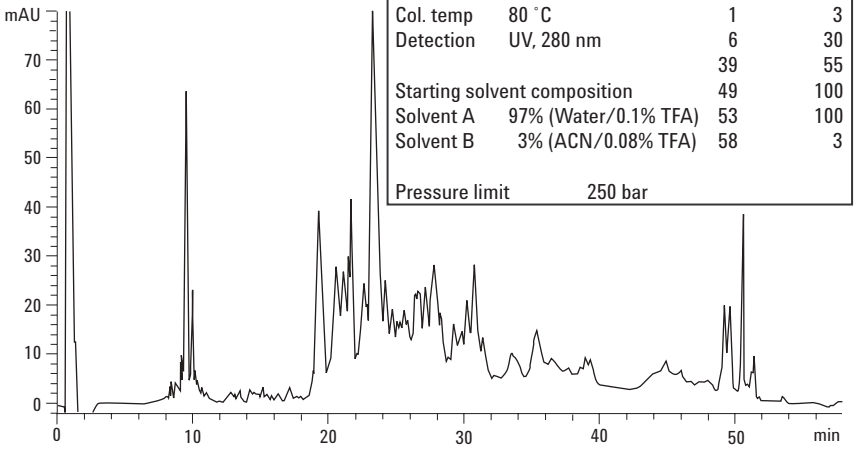

Figure 1. Immunodepleted human serum proteins (300 µg) separated on a 4.6-mm id × 50 mm mRP-C18 column at 80 °C under optimized conditions. Note that the recommended maximum amount of immunodepleted serum protein that can be loaded on this column in gradient separations is 380 µg.

conditions can result in decreased performance from those stated.

Further optimization of samples derived from other sources may require different sample preparation and gradient elution conditions, which are the sole responsibility of the end user. Please check the Agilent Web site http://www.agilent.com/chem/bioreagents for specific application details and notes.

Protein Desalting Application

Samples of human serum were depleted of the six most abundant proteins with the Agilent Multiple Affinity Removal Column, followed by a fast gradient program on the mRP-C18 column to retain and desalt/concentrate all polypeptides and proteins (Figure 2). The conditions enabled high protein recoveries with increased column loads while preventing loss of polypeptides of less than 5000 Daltons when using 5KDa MWCO spin concentrators [2].

Using an aqueous solvent of 0.1% TFA (trifluoroacetic acid) and an organic buffer of acetonitrile and 0.08% TFA is recommended for both protein fractionation as well as desalting.

WARNING: Alteration on the recommended sample and desalting conditions can result in decreased performance from those stated.

Further optimization of samples derived from other sources may require different sample preparation and gradient elution conditions, which are the sole responsibility of the end user.

Storage Recommendations

Long term storage of silica-based, bonded-phase columns should be in a pure organic solvent, preferably an aprotic liquid such as 100% acetonitrile. If the column was previously used with a buffered mobile phase, the buffer should first be removed by purging the column with 20–30 column volumes of a 50/50 mixture of methanol or acetonitrile and water, followed by 20–30 column volumes of the pure solvent. Before storing the column, the end-fittings should be tightly capped with end-plugs to prevent the packing from drying out. Columns may be stored safely for short periods in most mobile phases. However, to protect equipment, it is desirable to remove salts from the instrument and column by purging the column with the same mobile phase without the buffer (for example, using 60/40 ACN/H_2O to remove a 60/40 ACN/0.02 M phosphate buffered mobile phase). Re-equilibration is rapid with the original mobile phase when using this approach, and any danger of corrosion from salts is eliminated.

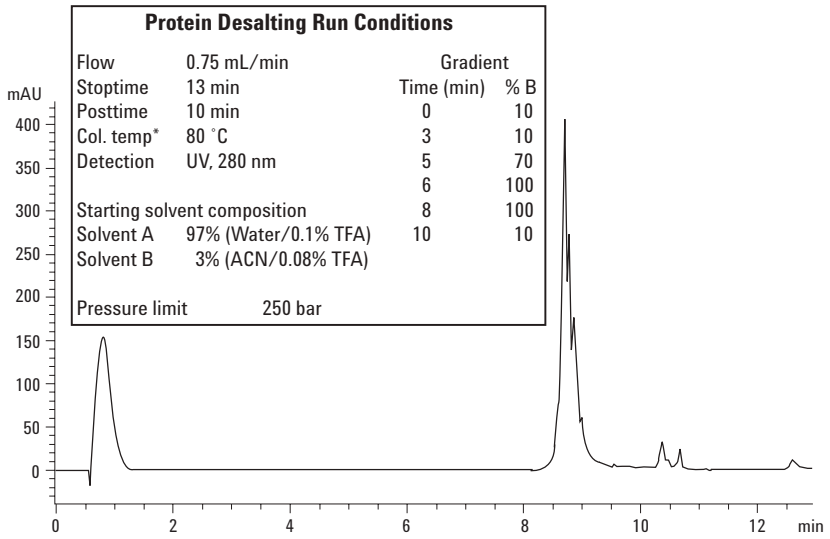

Figure 2. Immunodepleted serum proteins (57 µg), desalted/concentrated with optimized gradient and temperature conditions as listed with the mRP-C18 column. *The column can be used between 70–80 °C.

Ordering Information

Column	Agilent part number
mRP-C18 Column (5 µm) 4.6 mm id × 50 mm	5188-5231

Visit www.agilent.com/chem/bioreagents for more details on the mRP-C18 Column and for application notes. You will also find information here about the Agilent Multiple Affinity Removal System for depleting highly abundant proteins from biological fluids, as well as information about related accessories such as spin concentrators, sample filters, and C18 Cleanup pipette tips and spin tubes.

For More Information

For more information on our products and services, visit our Web site at www.agilent.com/chem/bioreagents

References

1. Comparative Analysis of Serum Samples with the Agilent mRP-C18 Column. Agilent Technologies, publication 5989-2505EN www.agilent.com/chem
2. A Rapid Method to Desalt and Concentrate Proteomic Samples. Agilent Technologies, publication 5989-2506EN www.agilent.com/chem

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