Quick Start Guide

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AdvanceBio HIC Columns

In this document, Agilent applications chemists share their recommendations for an optimum LC system and its configuration for performing hydrophobic interaction chromatography (HIC). They offer guidance on generic methods for protein and monoclonal antibody separations, and for separation of antibody drug conjugates (ADCs). These methods can then be modified to meet your exact requirements.

Additional information is available at **www.agilent.com/chem/advancebio-hic**.

Introduction

Hydrophobic interaction chromatography (HIC) relies on the effect of "salting out" to cause proteins to absorb onto the weakly hydrophobic HIC column. The most common salt used in this technique is ammonium sulfate. This is readily soluble at a concentration of 2 M, which is required in the analysis of many proteins. It is necessary to use a regular buffer to maintain protein solubility and to stabilize pH. For this purpose, sodium phosphate at neutral pH in concentrations from 20 to 100 mM is recommended. Proteins are eluted from the column in order of increasing hydrophobicity using a gradient from high to low ammonium sulfate concentration.

Operating guidelines

An Agilent 1260 Infinity II Bio-inert LC is recommended due to the high salt concentrations used. Gradients should typically last from 10 to 20 column volumes for good resolution. Flushing and re-equilibration should last at least five column volumes. High salt concentrations can cause issues with some LC systems. Use of a fully bio-inert LC is recommended, ideally with a quaternary pump so that additional channels can be used for flushing. It is not advisable to leave 2 M ammonium sulfate solution in either the HIC column or the LC; flush the column with sodium phosphate buffer after use. Additional precautions, such as pump seal washing and needle washing, should be taken where possible.

Mobile phase solutions should be UV transparent and have little background absorption, allowing detection at low wavelengths for maximum sensitivity. However, it is advisable to use the highest solvent grade available. The Agilent AdvanceBio HIC column is available in two formats: 4.6 × 30 mm for fast separations, and 4.6 ×100 mm for higher resolution separations. For proteins, the optimum flow rate is typically around 0.4 to 0.5 mL/min. It is important to also take the viscosity of the mobile phase into consideration.

Maintaining temperature control is also vital: Samples should be kept in a refrigerated autosampler to avoid deterioration. Temperature control is also recommended during HIC separation: Many proteins are temperature sensitive, and changes in retention time and peak shape may be observed at different temperatures.

AdvanceBio HIC columns are shipped containing 100 % acetonitrile. **It is important that the columns are returned to 100% acetonitrile for storage after use.** Care must be taken not to mix 100 % acetonitrile with high salt mobile phase in case of precipitation. Please refer to the User Guide at **www.agilent.com/chem/advancebio-hic-userguide** for column conditioning, use, and storage.

Experimental

Reagents and chemicals

All reagents should be HPLC grade or higher.

Sample preparation

Dissolve samples in high concentration ammonium sulfate solution. A final concentration of 1 mg/mL should be sufficient for most needs.

Mobile phase preparation

Care should be taken to ensure that all salts are fully dissolved and the pH has been adjusted to its target value. It is necessary to filter all mobile phase solutions through a 0.22 µm membrane filter before use. Do not leave mobile phase on the instrument longer than necessary, and replace regularly.

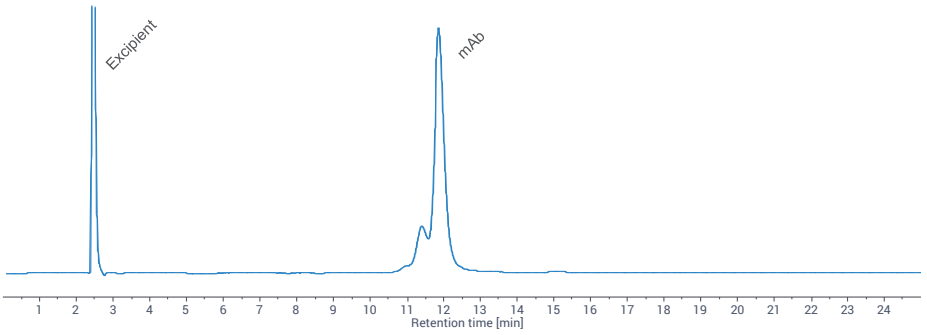
Instrumentation

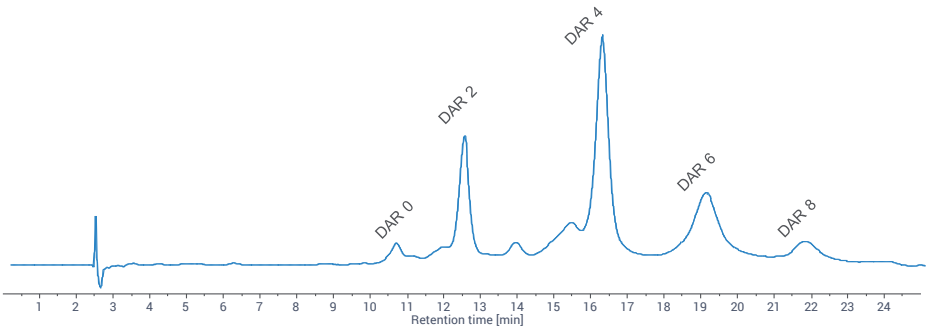
A 1260 Infinity II Bio-inert LC is recommended.

Gradients should typically last from 10 to 20 column volumes for good resolution. Flushing and re-equilibration should last at least five column volumes.

Suggested starting conditions

HPLC conditions	
Column	Agilent AdvanceBio HIC, 4.6 × 100 mm, 450 Å, 3.5 µm (p/n 685975-908)
Mobile phase	Eluent A: 50 mM sodium phosphate, pH 7.0 Eluent B: 2 M ammonium sulfate in 50 mM sodium phosphate, pH 7.0 Eluent C: propan-2-ol Eluent D: water (for flushing)
Flow rate	0.5 mL/min
Temperature	25 °C
Injection volume	1 to 10 µL





Gradient profile: Proteins

Time	%A	%B	%C
0	0	100	0
20	100	0	0
25	100	0	0
30	0	100	0
40	0	100	0

After all samples have been completed, flush the column with eluent A.

Gradient profile: Monoclonal antibodies (mAbs)

Time	%A	%B	%C
0	50	50	0
20	100	0	0
25	100	0	0
30	50	50	0
40	50	50	0

After all samples have been completed, flush the column with eluent A.

Gradient profile: Antibody drug conjugates (ADCs)

Time	%A	%B	%C
0	45	50	5
20	75	0	25
25	75	0	25
30	45	50	5
40	45	50	5

After all samples have been completed, flush the column with eluent A.

www.agilent.com/chem/advancebio

Printed in the USA, December 17, 2018
5991-9514EN