Macro-Prep® Hydrophobic Interaction Chromatography Supports

Instruction Manual

BIO-RAD

Table of Contents

Section 1
Introduction

Hydrophobic interaction chromatography (HIC), like reversed phase chromatography (RPC), is a technique for separating biomolecules by their degree of hydrophobicity. It is a powerful technique for resolving complex sample mixtures. Although RPC is successful for peptides, samples containing proteins may be irreversibly denatured by the organic solvents, resulting in the loss of biological activity. Since HIC uses a decreasing salt gradient as the mobile phase, the biological activity of the purified proteins is usually preserved. HIC is ideally suited for use after salt precipitation or ion exchange of a biomolecule because of the desalting effects of the decreasing salt gradient.

Section 2
Technical Description

The Macro-Prep HIC supports are rigid acrylic beads for lab and process scale purification of biomolecules.

They are shipped fully hydrated in a 20% (v/v) ethanol solution. They are offered in two different degrees of hydrophobicity: methyl (weakly hydrophobic) and t-butyl (mildly hydrophobic). The methyl support is suitable for purification of compounds which have few or weakly hydrophobic regions, while the t-butyl support is appropriate for purification of intermediate to weakly hydrophobic molecules. Table 1 lists the properties of the two matrices.

Table 1. Properties of the Macro-Prep HIC Supports

	Methyl	t-Butyl
Type of support	weakly hydrophobic	mildly hydrophobic
Functional ligand	$-CH_3$	$-C(CH_3)_3$
Protein capacity*	≥25 mg/ml	≥15 mg/ml
Nominal particle size	50 µm	50 µm
Nominal pore diameter	1,000 Å	1,000 Å
Recommended maximum linear flow rate	3,000 cm/h	3,000 cm/h
Autoclavable (121 °C for 30 min)	yes	yes
pH stability	2-14	2-14

*determined with human serum albumin (HSA) in 2 M $(NH_4)_2SO_4$.

The number of interactive, hydrophobic groups covering the surface of the bead for the methyl matrix is higher than the t-butyl matrix, resulting in a higher protein capacity for the methyl matrix. However, the more hydrophobic t-butyl matrix will bind the sample more tightly, which may result in higher resolution of less hydrophobic proteins. The degree of interaction with the support, and hence the relative retention on the column, is given by a hydrophobicity index of proteins for HIC supports. Table 2 lists the hydrophobicity index of various proteins on the Macro-Prep HIC supports.

Table 2. Hydrophobicity Index* of Proteins on Macro-Prep HIC Supports

	Methyl	t-Butyl
Myoglobin (void volume)	0.0	0.0
Cytochrome c	0.0	0.1
Bovine serum albumin	0.3	0.5
Soybean trypsin inhibitor	0.7	0.9

$$\text{* Hydrophobicity Index} = \frac{\text{(retention time of protein - retention time of void volume)}}{\text{(gradient running time - retention time of void volume)}}$$

The hydrophobicity of a biomolecule is associated with the content of exposed hydrophobic amino acids.

Table 3 lists the relative hydrophobicity of the hydrophobic amino acids in decreasing order.[1] Although a biomolecule may contain a large percentage of hydrophobic amino acids throughout the molecule, only the ones that are exposed on the surface contribute to its hydrophobic nature.

Table 3. Relative Hydrophobicity of Amino Acids

tryptophan > norleucine > phenylalanine > tyrosine > leucine > valine > methionine > alanine

Ammonium sulfate ($(NH_4)_2SO_4$) is the most commonly used salt, but other salts such, as KCl, NaCl, $NH_4CH_3CO_2$, and Na_2SO_4 may also be used. The initial concentration of salt used when applying the sample onto the HIC column may affect the binding capacity.

Section 3
Chemical Stability

The Macro-Prep HIC supports are able to withstand treatment in acid, base, chaotropes, and detergents, while retaining high protein capacities. These conditions are commonly employed for cleaning, regeneration, or

elution. Table 4 illustrates the different conditions tested and the minimal effects of exposure.

Table 4. Chemical Stability of the Macro-Prep HIC Supports (percentage of original protein binding capacity)*

	Methyl	t-Butyl
1% SDS, 24 hours	100%	100%
8 M Guanidine-HCl, 24 hours	95%	100%
1 M NaOH, 48 hours	100%	100%
1 M HCl, 48 hours	100%	100%

*evaluated with bovine serum albumin

Section 4
Instructions for Use

4.1 Preparation

If the packing buffer precipitates in 20% ethanol, the support must be rinsed with distilled deionized water before introducing the buffer. If precipitation is not an issue, go directly to the column packing procedure.

1. Slurry the support in the ethanol solution by carefully swirling the sealed container or by gently rolling the container.
2. Rinse the support with 4-5 bed volumes of deionized water either in a Buchner funnel or in the column.
3. Resuspend in the packing buffer.

4.2 Column Packing

1. Slurry a mixture of the matrix 1:1 (v/v) in the application buffer and degas if necessary.
2. Close the outlet of the column and fill approximately 10% of the column with degassed starting buffer. Remove any air bubbles that might be trapped in the bed support or column end piece.
3. Add an appropriate amount of the matrix in a homogeneous slurry to the column.
4. Fill the remainder of the column with buffer.
5. Connect the flow adaptor to the pump, fill it with buffer, and remove any trapped air bubbles.

6. Attach the flow adaptor to the column. Inserting it at a slight angle makes it easier to avoid trapping air bubbles.
7. Open the column outlet and pump at least 4-5 bed volumes through the column at a flow rate approximately 20% higher than the flow rate to be used in the application.
8. Switch off the pump and close the column outlet. Remove the inlet tubing from the buffer reservoir, release the pump pressure plate, and adjust the flow adaptor until it is in contact with gel surface. During this step, buffer will back-flow through the flow adaptor.
9. Re-insert the inlet tubing in the buffer reservoir and remove any air trapped in the tubing. Tighten the pump pressure plate, open the column outlet, and run 4-5 more column volumes of start buffer. Repeat steps 7, 8, and 9 for a final adjustment of the flow adaptor. Optimal column packing can be achieved with approximately 10 bed volumes of packing buffer.

10. After equilibration in the starting buffer, the column is ready for sample application. Typically, samples are applied in a high salt buffer such as 1.5 to 2.0 M $(NH_4)_2SO_4$. It is recommended to load a sample of Bio-Rad's HIC Protein Standard on the column prior to applying the sample of interest, to check the efficiency of the column packing. The chromatographic profile should look similar to Figure 1 or 2.
11. Elution of the sample may be accomplished with a decreasing linear or step gradient of the chaotropic buffer.

Conditions

Media: Macro-Prep methyl HIC support
Column: 1.0 cm ID, 10 cm bed height
Sample: 50 µl Bio-Rad's HIC Protein Standard II
1. 1.0 mg/ml cytochrome c
2. 2.0 mg/ml bovine serum albumin
3. 2.0 mg/ml soybean trypsin inhibitor

Eluant: Buffer A: 0.1 M sodium phosphate, 1.7 M ammonium sulfate, pH 7.0
Gradient: 0-75% Buffer B in 30 minutes
Flow rate: 1.5 ml/min
Detection: A_{280} nm

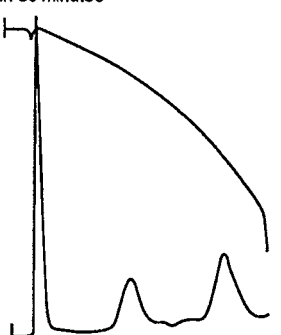

Fig. 1. HIC Protein Standard on the Macro-Prep methyl HIC support.

Conditions

Media: Macro-Prep t-butyl HIC support

Column: 1.0 cm ID, 10 cm bed height

Sample: 50 µl Bio-Rad's HIC Protein Standard II
1. 1.0 mg/ml cytochrome c
2. 2.0 mg/ml bovine serum albumin
3. 2.0 mg/ml soybean trypsin inhibitor

Eluant: Buffer A: 0.1 M sodium phosphate, 1.7 M ammonium sulfate, pH 7.0

Gradient: 0-100% Buffer B in 40 minutes

Flow rate: 1.5 ml/min

Detection: A_{280} nm

Fig. 2. HIC Protein Standard on the Macro-Prep t-butyl HIC support.

4.3 Regeneration

After several cycles of use, or when the resolution starts to decrease and becomes inconsistent, regeneration of the support may be necessary to remove bound hydrophobic contaminants. Use of Bio-Rad's HIC standard may be helpful in determining whether regeneration is necessary.

1. In many instances, contaminants may be removed simply by washing the support bed with low ionic strength salt buffer (i.e., 0.1 M sodium phosphate), and then equilibrating with the starting buffer before sample loading.
2. Tightly bound contaminants may be removed by washing with an organic polar solvent such as a 10-30% ethanol or methanol solution. Afterward, rinse with 3-8 bed volumes of deionized water and equilibrate with the starting buffer before sample loading.

Section 5
Optimization

Factors affecting an HIC separation include pH, temperature, type of chaotropic salt, gradient profile, addition of organic modifiers, and concentration of the mobile phase.

- Adjustments in pH can alter the hydrophobic/hydrophilic properties of a protein, so that it has more hydrophilic (hydrophobic) characteristics. This results in decreased (increased) retention by the HIC matrix.
- Lowering the temperature facilitates desorption of the sample from the hydrophobic matrix.
- Although various types of salts such as $CaSO_4$ and Na_2SO_4 may be used with the Macro-Prep HIC supports, $(NH_4)_2SO_4$ is most commonly used.
- The gradient profile may be adjusted to yield optimal binding and purification. For example, if the protein of interest elutes from the column toward the end of a decreasing salt gradient, then the gradient may be steep during the early stages of the purification. As the ionic strength of the buffer approaches elution of the sample of interest, the gradient may be flattened to increase resolution.
- The addition of small amounts of organic modifiers such as SDS or ethylene glycol may be added to the elution buffers, resulting in decreased retention by the HIC matrix. However, a drift in the baseline from the UV monitor may result due to the absorbance of the organic modifiers.
- In general, the higher the salt concentration of the mobile phase, the higher the degree of interaction of the sample with the support and therfore the higher the the binding capacity. However, if sample elution occurs toward the end of a decreasing salt gradient, the initial concentration of the chaotropic salt in the application buffer may be lowered. By lowering the initial chaotropic salt concentration, some contaminants that bind to the HIC matrix in high salt concentrations will elute in the void volume, increasing the number of available ligand sites for binding the protein of interest to the matrix.

Table 5 lists the amount of human serum albumin (HSA) bound to the Macro-Prep HIC supports in 1 M $(NH_4)_2SO_4$ and in 2 M $(NH_4)_2SO_4$.

Table 5. Human Serum Albumin Binding to the Macro-Prep HIC Supports

	1 M $(NH_4)_2SO_4$	2 M $(NH_4)_2SO_4$
Methyl	> 10 mg/ml	> 29 mg/ml
t-Butyl	> 6 mg/ml	> 19 mg/ml

Section 6 Storage

For long term storage, the Macro-Prep HIC supports should be prepared by rinsing with at least 10 bed volumes of deionized water, and stored in a bacteriostatic solution such as 20% v/v ethanol solution or 0.05% NaN_3. The supports may also be stored for short periods (2-3 weeks) in a low ionic strength buffer.

Section 7 Shelf Life

The Macro-Prep HIC supports are stable for at least 1 year when stored sealed in the original container at room temperature. The shelf life may be extended if stored at 4 °C.

Section 8 Technical Assistance

All of the Macro-Prep supports are produced under strict quality control and documentation to meet the demands of the biotechnology industry. For additional information and technical assistance, contact your local Bio-Rad representative, or in the USA, call 1-800-4BIORAD.

Section 9
Ordering Information

Catalog Number	Product Description
156-0080	**Macro-Prep Methyl HIC Support,** 100 ml
156-0081	**Macro-Prep Methyl HIC Support,** 500 ml
156-0082	**Macro-Prep Methyl HIC Support,** 5 liters
156-0083	**Macro-Prep Methyl HIC Support,** 10 liters
156-0090	**Macro-Prep t-Butyl HIC Support,** 100 ml
156-0091	**Macro-Prep t-Butyl HIC Support,** 500 ml
156-0092	**Macro-Prep t-Butyl HIC Support,** 5 liters
156-0093	**Macro-Prep t-Butyl HIC Support,** 10 liters
125-0559	**HIC Protein Standard II,** 6

Section 10
References

1 Deutscher, M. P., Guide to Protein Purification, *Methods in Enzymology*, **182,** 340 (1990).

Bio-Rad Laboratories, 2000 Alfred Nobel Dr., Hercules, CA 94547
LIT486 Rev B