



AcroSep™ HEA and PPA HyperCel™ Columns **Mixed-Mode Chromatography Columns for Protein Separation**

Description

- HEA and PPA HyperCel resins; represent new “mixed-mode” chromatography, sharing some features of hydrophobic interaction chromatography (HIC) combined with some features of ion exchange chromatography (IEX).
 - Separation is based on hydrophobicity and protein isoelectric point (pI).
 - Bi-dimensional separation mechanism can be used to separate proteins with very close isoelectric points.
 - Buffer choices influence the degree to which each mode contributes to separation.
- Two different robust synthetic ligands, aliphatic (HEA – hexylamine), and aromatic (PPA – phenylpropylamine) exist to offer different selectivity options.
 - PPA is more hydrophobic than HEA resin.
 - The hydrophobic portion of the ligand is generally the dominant mode for protein capture and requires less salt than typical hydrophobic interaction chromatography media.
 - Ionizable amine groups are utilized to promote elution via electrostatic repulsion.
 - These ligands are generally effective at physiologic pH and salt concentration, although they are functional at a broad range of pH and salt concentration.
- HEA and PPA HyperCel resins are novel industry-scaleable chromatography resins designed for protein capture and impurity removal.

Description *(continued)*

- Versatile use
 - Fully automated in combination with an automated chromatography instrument.
 - Semi-automated in combination with pumps.
 - Manual operation in combination with a syringe.

Ordering Information

Part Number	Description	Color	Column Volume	Pkg
20250-C001	HEA HyperCel	Black	1 mL	5/pkg
20060-C001	PPA HyperCel	Yellow	1 mL	5/pkg

Table of Contents

Section	Page
Specifications	2
Technical Overview	4
Working Conditions	5
Instructions for Use – Automated or Pumped Chromatography Systems	8
Instructions for Use – Manual Use with Syringe	9
Procedure for the Determination of Target Protein DBC Using an Automated Chromatography System	10
Cleaning and Maintenance	11
Storage Recommendations	11
Adapter Recommendations	12
Warning	12

Note: *The procedures herein are intended only as a guide. Users should always verify product performance with their specific applications under actual use conditions. If you have questions about the information presented in this guide, please contact Pall Life Sciences technical service.*

Specifications

Materials of Construction

Column Housing, Cap, Plug, and Adapter: Polypropylene
Column Frit: Polyethylene

HEA and PPA HyperCel Resin Properties

Particle Size: 80-100 μm

Bead Composition: High porosity, cross-linked cellulose

HEA Ligand: Hexylamine (Aliphatic)

PPA Ligand: Phenylpropylamine (Aromatic)

pKa both Ligand: ~ 8

Typical Working Pressure: < 1 bar (100 kPa, 14.5 psi)

Dynamic Binding Capacity (DBC) BSA: 40-60 mg/mL for HEA and PPA resin[†]

Typical Adsorption pH: 7.0-9.0

Typical Elution pH: 7.0-2.6, gradient or step elution

Cleaning pH: 1-14

Column Geometry

Column Volume (CV): 1.04 mL

Bed Height: 1.48 cm (0.58 in.)

Bed Diameter: 0.94 cm (0.37 in.)

[†] Determined at 10% breakthrough using 5 mg/mL BSA in PBS, flow rate = 50 cm/hr, column dimension = 1.6 cm ID x 3.75 cm; residence time (Tr) = 4.51 min.

Specifications *(continued)***Device Dimensions**

Diameter: 1.6 cm (0.6 in.)

Length (Without Plugs): 4.8 cm (1.9 in.)

Connections

Inlet: Threaded female luer

Outlet: Rotating male luer locking hub

Flow Rates

Recommended Flow Rate: 0.2-1.0 mL/min
(Tr = 5.18-1.02 min)

Maximum Column Pressure

Back Pressure: 1 bar (100 kPa, 14.5 psi)

Storage

2-8 °C (36-46 °F); do not freeze

Technical Overview

HEA and PPA HyperCel resins operate on the basis of a mixed-mode mechanism, where both hydrophobic and ionic effects are present. Protein binding is predominantly hydrophobic while elution is driven by electrostatic repulsion. This dual interaction mechanism has several advantages over traditional hydrophobic chromatography methods (HIC or reverse phase). These ligands are more hydrophobic than traditional HIC chemistries, thus less salt is required for protein capture. The use of charge repulsion in the presence of low salt concentrations significantly improves the elution efficiency. Thus, purification methods using HEA or PPA resin rather than other hydrophobic separations are likely to result in less protein structure alteration (less extreme salt and pH conditions) and higher recovery. The aromatic PPA ligand is more hydrophobic than the aliphatic HEA ligand. The best choice for a specific protein separation must be determined and optimized on a case-by-case basis.

Protein binding on both ligands is typically achieved at physiologic salt and pH (PBS) in contrast to conventional HIC or IEX resins where adjustments to conductivity must be made. In certain cases, salt (e.g. sodium chloride, ammonium sulphate) may be added to act on either the hydrophobic or the ionic effects (salt enhances the hydrophobic component and decreases the ionic component). The amine group has a pKa of approximately 8. Thus, about 50% of the ligand is charged at pH 8, with increasing ligand charge as pH decreases. Proteins will also take on a positive charge as the pH nears then drops below their pKa. When the ligand and protein are both positively charged, repulsion occurs and prevents binding or promotes elution. In the absence of salt, electrostatic repulsion will occur at moderate pH for basic proteins, and lower pH for acidic proteins. Thus, buffer pH can be used to separate proteins of similar hydrophobicity but different pI.

Working Conditions

- Equilibration
 - Equilibrate the column in appropriate running buffer, typically PBS, pH 7.4 (5-10 CV). Alternatively, 50 mM Tris-HCl, pH 8.0 can be used. For very basic proteins, high pH buffers such as carbonate can be used to reduce charge repulsion and promote protein binding. Salt concentration and conductivity in the equilibrium buffer should match that in the sample loading solution.
- Protein binding
 - Protein binding generally occurs via hydrophobic interaction. At neutral pH, both resins will carry some positive charge. The more basic proteins in a sample are also positively charged in this pH range, and will typically not bind either ligand due to charge repulsion. In contrast, more acidic proteins will be retained (those with pI > 1 pH unit below binding buffer). If target proteins have a high pI and/or low hydrophobicity, either use a higher pH for binding (pH 9-10) in the presence of 0.5 M sodium chloride or ammonium sulphate, or stay at neutral pH and increase the salt concentration to 1 M (ammonium sulphate). Ensure that proteins in the sample will not precipitate at chosen pH and salt concentration. Intermediate conditions can also be used to maintain protein solubility and structure if appropriate.
 - Initially load sample at flow rates of 0.2 mL/min and increase velocity if needed. The sample can be loaded directly onto HEA or PPA HyperCel AcroSep columns without preliminary diafiltration or concentration. Typical residence times of 5-7 minutes are recommended for optimal capacity (< 0.2 mL/min). Testing with pure proteins shows a significant increase in DBC when the sample load flow rate is reduced from 0.5 to 0.2 mL/min.

Notes: Temperature – *The hydrophobic component of the mixed-mode mechanism is sensitive to temperature, and significant variations in capacity may be observed. Ensure that all buffers reach desired temperature prior to use to avoid variation.*

Chaotropic agents – *The presence of agents such as 4-8 M urea or CHAPS will alter the conformation of proteins and may modify their binding to HEA and PPA HyperCel resins.*

Working Conditions *(continued)*

- Elution is performed either by gradient or step elution. Elution generally occurs via electrostatic repulsion when the pH nears or is below the protein pI. For gradient elution, decrease the pH in mixed phosphate-citrate (or acetate) buffer – typically from pH 7.4 to 2.6. For example, use 5 CV of 0.2 M phosphate/0.1 M citrate, pH 7.0 to pH 2.6 in 60 minutes. Check that changes in pH and ionic strength during elution do not cause precipitation.
 - A decrease of the salt concentration can also contribute to elution as this reduces hydrophobic interaction.
- Regeneration is achieved with 0.5-1.0 M NaOH for 30 to 60 minutes. Re-equilibrate with 10 CV appropriate buffer before the next use.

Protein Separation Example with HEA and PPA HyperCel Resin

Method: Four proteins (below) were used to demonstrate mixed-mode separations with HEA and PPA HyperCel.

Protein	Isoelectric Point (pI)
Lysozyme	10 - 11
Chymotrypsinogen A	8.8 - 9.6
Ovalbumin	4.8
Bovine Serum Albumin	4.9

These proteins were eluted with stepwise pH decrease from loading pH to pH 7.0, then a further drop to pH 5.4. Then a gradient from pH 5.4 to 2.6 was used. All elution buffers were created by mixing 0.2 M phosphate and 0.1 M citrate buffers at appropriate ratios to reach desired pH. Water was added for a final buffer dilution of ~2 fold.

Note: *Throughout the elution sequence the conductivity decreases (increased citrate vs phosphate buffer ratio) and thus reduces hydrophobic interaction (conductivity trace shown in chromatograms).*

HEA HyperCel Resin: Binding of lysozyme and chymotrypsinogen to HEA HyperCel requires a pH 10 buffer (carbonate + 150 mM NaCl) to minimize charge repulsion (HEA ligand uncharged). Lysozyme elutes when the pH drops to 7.0 while chymotrypsinogen A does not elute until the pH decreases further. This

Working Conditions *(continued)*

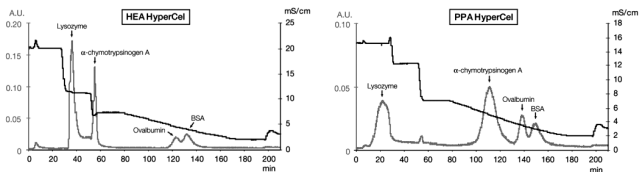
suggests that chymotrypsinogen A binding is maintained at pH 7.0 via hydrophobic interaction even in the presence of charge repulsion. Ovalbumin and BSA elute during the gradient and are separated based on differences in hydrophobicity rather than pI as their pI values are very similar.

PPA HyperCel Resin: Lysozyme does not bind to PPA at pH 7.4 (PBS) due to charge repulsion. However, chymotrypsinogen A binds to PPA at this pH in spite of charge repulsion, suggesting that the hydrophobic interaction is quite strong. The elution of chymotrypsinogen A occurs quite late with PPA, due to changes in hydrophobic interaction at lower pH combined with charge repulsion. Ovalbumin and BSA, which have very similar pI (4.8 vs 4.9), are separated on PPA HyperCel due to hydrophobicity differences.

Note: *Chymotrypsinogen A elution from HEA HyperCel occurs much earlier as compared to PPA HyperCel, indicating that this hydrophobic interaction is not as strong as the PPA HyperCel interaction. Ovalbumin and BSA also elute later from PPA HyperCel. These observations are consistent with expectation since the PPA ligand is more hydrophobic than the HEA ligand.*

Figure 1

Separation of a Protein Mix on HEA and PPA HyperCel Resin



Examples of protein separation using HEA and PPA HyperCel resin. Step elution with pH at 7.0 and 5.4 followed by gradient elution from 5.4 to 2.6 is shown (changes in conductivity correspond to changes in pH, phosphate and citrate buffers used for elution as described above). Protein loading on HEA occurs at pH 10, while protein loading with PPA is done at pH 7.4.

Instructions for Use – Automated or Pumped Chromatography Systems

Materials Required

- Chromatography System (ÄKTAdesign*, pump, or equivalent)
- Filtered, degassed buffers

Automated System Protocol

1. Attach column to pre-primed system. To prevent air from getting into the column, fill the neck of the column dropwise while system is running very slowly. Allow the buffer to flow through the column until all bubbles in the outlet of the column have been evacuated, prior to connecting downstream tubing.
2. Equilibrate with 5-10 CV of loading buffer.
3. Load desired amount of sample.
4. Wash column with 5-10 CV loading buffer.
5. Elute with chosen elution buffer, stepwise or gradient.
6. Strip with 5-10 CV of elution buffer.
7. Re-equilibrate with 5-10 CV loading buffer.

Instructions for Use – Manual Use with Syringe

Materials Required

- Syringes (5-30 mL) with luer lock fittings
- Filtered buffers

Syringe Protocol

Note: *It is important to avoid introducing air into the column. Remove air bubbles from fluid filled syringe before attachment to the column each time the syringe is changed.*

When pushing fluid through the syringe, maintain a relatively constant flow rate with minimal backpressure, typically 0.2-1.0 mL/min for protein capture.

1. Fill a syringe with loading buffer.
2. Equilibrate the column with 5-10 CV of loading buffer.
3. Fill a syringe with sample.
4. Load sample onto the column avoiding the introduction of air bubbles.
 - Collect all effluent for analysis.
5. Wash column with 5 CV of loading buffer to remove unbound proteins.
 - Collect all effluent for analysis.
6. Fill a syringe with elution buffer. Secure this syringe to the column luer connector. Check that there are no air bubbles at the site of attachment.
7. Run 10-20 CV of elution buffer through the column to elute bound protein. Repeat with additional elution buffers if desired.
 - Collect all effluent containing eluted protein(s) in appropriate sized fractions.
8. If the column will be reused, strip residual protein with 5 CV of low pH buffer.
9. Fill the syringe with loading buffer. Equilibrate the column with 5-10 CV of loading buffer.

Procedure for the Determination of Target Protein DBC Using an Automated Chromatography System

For the most accurate prediction of DBC during a purification scheme, choose conditions that closely match those to be used during target protein purification.

System Parameters

- Protein loading step
 - Flow rate: 0.2-0.5 mL/min (or intended flow rate)
 - Equilibrate: 10 CV loading buffer
 - Sample load: inject sufficient quantity of protein-containing sample to exceed column capacity
 - Wash: 10 CV loading buffer
 - Clean: 5-10 CV elution buffer
 - Re-equilibration: 5-10 CV loading buffer
- Void volume (V_0): To determine V_0 , either perform a run in the by-pass position (fluid by-passes the column) or run the procedure using conditions which prevent protein binding. For example, use an elution buffer instead of a loading buffer for the equilibration and sample loading steps.
- Formula: $DBC = C \times (V_L - V_0)$
 - C = Concentration of load
 - V_L = Volume at 10% or 50% breakthrough
 - V_0 = From the void volume determination described above, V_0 is the total volume passing through the system from the time of injection (0% deflection of OD_{280}) until protein is detected (increase in OD_{280}), determined as described above.

Note: To convert the column DBC to resin DBC, divide the DBC value above by the column volume (1.04 mL resin).

Cleaning and Maintenance

To avoid frequent regeneration and column fouling, use pre-filtered (0.2 μm) samples and buffers. The following regeneration procedures are recommended for general and specific cleaning challenges.

Situation	Recommendation
General cleaning-in-place	Wash with 0.5-1 M sodium hydroxide, 30-60 min contact time.
Adsorbed contaminants	Wash with 6 M guanidine (2-3 CV) or 8 M urea. Wash with 40% isopropanol (2-3 CV).

Storage Recommendations

- The column must be stored at 2-8 °C (36-46 °F) and cannot be frozen.
- Between runs, store the column at 2-8 °C (36-46 °F) in loading buffer.
- The storage buffer may also contain bacteriostatic agents such as 20% (v/v) ethanol and/or 1 M NaCl.

Adapter Recommendations

AcroSep pre-packed columns are made with a luer inlet and outlet for easy connection to syringes. The following table lists recommendations if adapters are needed to connect the columns to other types of tubing.

Connection To	Adapters (Upchurch Scientific*)
1/16" OD Teflon* and Tefzel* Tubing	1 kit (P-837) <i>Instructions provided with kit</i>
1/8" OD Teflon and Tefzel Tubing	1 kit (P-838) <i>Instructions provided with kit</i>
1/16" Stainless Steel Tubing	1 inlet fitting (P-658), 1 outlet fitting (P-655), 2 ferrules (P-259), 2 nuts (LT-115) <i>Instructions provided with fittings</i>
1/32" Stainless Steel Tubing	1 inlet fitting (P-658), 1 outlet fitting (P-655), 2 ferrules (P-248), 2 nuts (LT-115) <i>Instructions provided with fittings</i>

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Pall Life Sciences

600 South Wagner Road
Ann Arbor, MI 48103-9019 USA

800.521.1520	USA and Canada
(+1)800.PALL.LIFE	Outside USA and Canada
734.665.0651	phone
734.913.6114	fax

Visit us on the Web at www.pall.com/lab

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Australia, Cheltenham, VIC, 03 9584 8100
Austria, Wien, 00 1 49 192 0
Canada, Ontario, 905-542-0330
Canada, Québec, 514-332-7255
China, P.R., Beijing, 86-10-6780 2288
France, St. Germain-en-Laye, 01 30 61 32 32
Germany, Dreieich, 06103-307 333
India, Mumbai, 91 (0) 22 67995555
Italy, Buccinasco, +3902488870.1
Japan, Tokyo, 03-6901-5800
Korea, Seoul, 82-2-560-8711
Malaysia, Selangor, +60 3 5569 4892
Poland, Warszawa, 22 510 2100
Russia, Moscow, 5 01 787 76 14
Singapore, 65 6 389-6500
South Africa, Johannesburg, +27-11-2662300
Spain, Madrid, 91-657-9876
Sweden, Lund, (0)46 158400
Switzerland, Basel, 061-638 39 00
Taiwan, Taipei, 886 2 2545 5991
Thailand, Bangkok, 66 2937 1055
United Kingdom, Farlington, 02392 302600