GE Healthcare

**Instructions 71-7097-00 AE** **Affinity Media**

# EAH Sepharose™ 4B

EAH Sepharose 4B is formed by covalent linkage of 1,6-diaminohexane to Sepharose 4B using an epoxy coupling method.

EAH Sepharose 4B has free amino groups at the end of 11-atom spacer arms, which are used to couple ligands containing carboxyl groups with the carbodiimide coupling method.

The long hydrophilic spacer arm makes EAH Sepharose 4B particularly suitable for immobilization of small molecules.

Sepharose

**Table 1. Matrix characteristics.**

| | |
|---|---|
| Active group: | amino group |
| Active group concentration: | 7–12 µmoles amino groups/ml drained matrix |
| Spacer: | 1,6-diaminohexane (11 atom) |
| Coupling capacity: | Application dependent |
| Bead structure: | 4% agarose |
| Bead size range: | 45–165 µm |
| Mean bead size: | 90 µm |
| Max linear flow rate*: | 75 cm/h at 25 °C, HR 16/10 column, 5 cm bed height |
| pH stability** | |
| Long term: | 3–14 |
| Short term: | 3–14 |
| Chemical stability: | Stable to all commonly used aqueous buffers |
| Physical stability: | Negligible volume variation due to changes in pH or ionic strength |

* Linear flow rate = $\frac{\text{volumetric flow rate (cm}^3\text{/h)}}{\text{column cross-sectional area (cm}^2\text{)}}$

** Data refer to the coupled product, provided that the ligand can withstand the pH or chemical environment. Please note the following:

*pH stability, long term* refers to the pH interval where the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance.

*pH stability, short term* refers to the pH interval for regeneration and cleaning procedures.

## Preparing the matrix

EAH Sepharose 4B is supplied preswollen in 20% ethanol. Decant the ethanol solution and wash the required amount of matrix on a sintered glass filter (porosity G3) with distilled water adjusted to pH 4.5 with HCl, followed by 0.5 M NaCl (80 ml) in aliquots/ml sedimented matrix.

## Coupling the ligand

The method for coupling is the carbodiimide method. Carbodiimides may be regarded as anhydrides of ureas. The N,N'-disubstituted carbodiimide promotes condensation between a free amino and a free carboxyl group to form a peptide link by acid catalyzed removal of water.

### Choice of carbodiimide

A water soluble carbodiimide should be used, for example N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydro-chloride (EDC) or N-cyclohexyl-N'-2-(4'-methyl-morpho-linium) ethyl carbodiimide-p-toluene sulphonate (CMC). These two carbodiimides have been used in a variety of experimental conditions and at a wide range of concentrations (Table 2). Observations in our laboratory indicate that EDC often gives better coupling yields than CMC.

**Table 2. Examples of conditions used during coupling via carbodiimides.**

| Coupled ligand | Carbodiimide | Conc. of carbodiimide mg/ml | pH | Reaction time |
|---|---|---|---|---|
| Methotrexate | EDC | 18 | 6.4 | 1.5 h |
| UDP-glucuronic acid | EDC | 32 | 4.8 | 24 h |
| p-amino-benzamidine | CMC | 2 | 4.75 | 5 h |
| Folic acid | EDC | 5 | 6 | 2 h |
| Mannosylamine | EDC | 19 | 4.5–6.0 | 24 h |

## Concentration of carbodiimide

A concentration of carbodiimide greater than the stoichometric concentration is required. The amount is usually 10–100 times greater than the concentration of spacer groups. A wide range of concentrations of carbodiimide has been used, but a final concentration of 0.1 M is recommended to start with. The state of purity is important. Old samples of carbodiimide should not be used

## pH dependence

The coupling reaction is performed in distilled water adjusted to pH 4.5–6.0 to promote the acid-catalyzed condensation reaction.

The pH value of the reaction mixture decreases during the first hour of the coupling. The pH must thus be adjusted during this time by addition of dilute sodium hydroxide solution.

## Coupling solution

Buffers are usually not employed but can be used provided no amino, phosphate or carboxyl groups, which would compete with the coupling reaction, are present.

Purified dioxane or ethylene glycol may be used in the coupling solution, up to a final concentration of 50%, if the ligand does not dissolve in water. In this case the pH should be measured using pH paper since organic solvents may damage electrodes.

### Temperature and time dependence

Coupling is completed within 1.5–24 hours at 4 °C – 25 °C.

Typically the reaction takes place overnight either in the cold or at room temperature.

### Ligand concentration dependence

The ligand should be at a concentration in excess of the concentration of spacer groups.

### Blocking excess remaining groups

It is not usually necessary to carry out a blocking reaction when an excess of ligand is used. Remaining groups may, however, be removed by using a blocking group in a further carbodiimide reaction. Acetic acid (1 M) is a suitable blocking agent.

Consideration must be given to subsequent effects on the affinity chromatography step of coupling such blocking groups.

### Washing the adsorbent

To remove the excess of uncoupled ligand that remains after coupling, the adsorbent is washed alternatively with high and low pH buffer solutions at least three times. Acetate or formate buffer (0.1 M, pH 4) and Tris buffer (0.1 M, pH 8.3) each containing 0.5 M NaCl are suitable. This procedure ensures that no free ligand remains ionically bound to the immobilized ligand.

If a mixture of aqueous solution and an organic solvent has been used, this mixture should be used to wash the final product.

The washing procedure should be followed by a wash with distilled water and then a wash with the buffer to be used in the affinity chromatography stage.

## A general ligand coupling procedure

1. Dissolve the ligand to be coupled in coupling solution. Adjust the pH to 4.5.
2. Add the ligand solution to the matrix. Use a matrix: ligand solution ratio between 1:0.5 to 1:1 to produce a suspension that is suitable for coupling.
3. Add the carbodiimide to the suspension. Carbodiimide can be added either as free flowing powder or drop-wise as an aqueous solution to a final concentration of 0.1 M.

   If the carbodiimide is added as an aqueous solution, dissolve the carbodiimide in water and adjust the pH to 4.5.

   Rotate the mixture end-over-end for between 1.5 and 24 hours, generally overnight in a cold room or at room temperature. Other gentle stirring methods may be employed.

   Do not use magnetic stirrers as they may disrupt the Sepharose matrix.
4. Adjust the pH of the reaction mixture during the first hour (pH may increase or decrease, a change that is ligand dependent) by adding 0.1 M sodium hydroxide or hydrochloric acid
5. Wash the product thoroughly with at least three cycles of alternating pH. Each cycle should consist of a wash with 0.1 M acetate buffer pH 4.0 containing 0.5 M NaCl followed by a wash with 0.1 M Tris-HCl buffer pH 8 containing 0.5 M NaCl.

   If organic solvent has been used to dissolve the ligand it is necessary to wash the medium with an organic solvent to remove unreacted ligand. Dioxane, ethylene glycol, ethanol, methanol or acetone may be used.

   If no organic solvent was used, wash with distilled water.

## Packing Sepharose 4B

Prepare a slurry with binding buffer in a ratio of 75% settled medium to 25% buffer. The binding buffer should not contain agents which significantly increase the viscosity. The column may be equilibrated with viscous buffers at reduced flow rates after packing is completed.

1. Equilibrate all material to the temperature at which the chromatography will be performed.
2. De-gas the medium slurry.
3. Eliminate air from the column dead spaces by flushing the end pieces with buffer. Make sure no air has been trapped under the column net. Close the column outlet with a few centimeters of buffer remaining in the column.
4. Pour the slurry into the column in one continuous motion. Pouring the slurry down a glass rod held against the wall of the column will minimize the introduction of air bubbles.
5. Immediately fill the remainder of the column with buffer, mount the column top piece onto the column and connect the column to a pump.
6. Open the bottom outlet of the column and set the pump to run at the desired flow rate. This should be at least 133% of the flow rate to be used during subsequent chromatographic procedures. However, the maximum flow rate, see Table 1, is typically employed during packing.

   **Note:** If you have packed at the maximum linear flow rate, do not exceed 75% of this in subsequent chromatographic procedures.
7. Maintain the packing flow rate for 3 bed volumes after a constant bed height is reached.

## Using an adapter

Adapters should be fitted as follows:

1. After the medium has been packed as described above, close the column outlet and remove the top piece from the column. Carefully fill the rest of the column with buffer to form an upward meniscus at the top.
2. Insert the adapter at an angle into the column, ensuring that no air is trapped under the net.
3. Make all tubing connections at this stage. There must be a bubble-free liquid connection between the column and the pump.
4. Slide the plunger slowly down the column so that the air above the net and in the capillary tubings is displaced by eluent. Valves on the inlet side of the column should be turned in all directions during this procedure to ensure that air is removed.
5. Lock the adapter in position on the medium surface, open the column outlet and start the eluent flow. Pass eluent through the column at the packing flow rate until the bed is stable. Re-position the adapter on the medium surface as necessary.

The column is now packed and equilibrated and ready for use.

## Binding

Conditions for adsorption depend on which ligand is used. Literature references and textbooks may give good guidelines.

The adsorption will depend upon parameters such as sample concentration, flow rate, pH, buffer composition and temperature.

General guidelines for adsorption are:

- Sample pH should be the same as that of the binding buffer. Filter the sample through a 0.22 µm or 0.45 µm filter to prolong the working life of the medium.
- After the sample has been loaded, wash the medium with binding buffer until the base line is stable.

## Elution

Conditions for elution of bound substances depend on which ligand is used. Literature references and textbooks may give good guidelines.

General guidelines are described below.

- **pH change:** A change in pH alters the degree of ionization of charged groups at the binding sites. Desorption is generally affected by a decrease in pH. The chemical stability of the matrix, ligand and adsorbed substances determines the limits of pH which may be used.
- **Ionic strength:** Elution with a continuous or step gradient of increasing ionic strength may be used to separate substances bound to the adsorbent. NaCl is most frequently used and enzymes usually elute at a concentration of 1 M NaCl or less. If the interaction has a very high affinity, a chaotropic salt may be required.
- **Competitive elution:** Competitive eluents are often used to selectively desorb substances from a group specific adsorbent. Selectively retained substances are usually displaced at low concentrations of eluting agents, often less then 10 mM. Either step or gradient elution may be used.
- **Reduced polarity:** Conditions which lower the polarity of the eluent promote desorption and may be used if they do not inactivate eluted substances. Dioxane (up to 10%) or ethylene glycol (up to 50%) may be used.
- **Deforming eluents:** If the elution methods described above fail to affect desorption, deforming agents, such as chaotropic salts, guanidine-HCl or urea, which alter the structure of the proteins, can be used.

## Regeneration

Conditions for regeneration depend on which ligand has been coupled. Literature references and textbooks may give good guidelines.

A general regeneration method is described below:

Wash the medium with 2–3 bed volumes of alternating high pH (0.1 M Tris-HCl, 0.5 M NaCl, pH 8.5) and low pH (0.1 M sodium acetate, 0.5 M NaCl, pH 4.5) buffers. This cycle should be repeated 3 times.

Re-equilibrate immediately by washing the medium with at least 5 bed volumes of binding buffer.

## Storage

EAH Sepharose 4B should be stored at 4–8 °C in presence of a bacteriostate, e.g. 20% ethanol. The medium must not be frozen.

## Further Information

Check www.gehealthcare.com/protein-purification for more information. Useful information is also available in the Affinity Chromatography Handbook, see Ordering Information.

## Ordering Information

| Product | Pack size | Code No. |
|---|---|---|
| EAH Sepharose 4B | 50 ml | 17-0569-01 |

| Literature | Pack size | Code No. |
|---|---|---|
| Affinity Chromatography Handbook, Principle and Methods | 1 | 18-1022-29 |

For local offoce contact information, visit
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