GE Healthcare

Instructions 71-7087-00 AG **Affinity media**

Epoxy-activated Sepharose™ 6B

Epoxy-activated Sepharose 6B is a pre-activated medium for immobilization of various ligands. Epoxy-activated Sepharose 6B can be used to couple sugars and other carbohydrates via stable ether linkages to hydroxyl groups. Other ligands can be coupled through hydroxyl, amino or thiol groups.

The medium has a long hydrophilic spacer arm which makes it particular suitable for immobilization of small molecules.

Epoxy-activated Sepharose 6B is formed by reacting Sepharose 6B with 1,4-*bis* (2,3-epoxy-propoxy-) butane.

Sepharose

Table 1. Medium characteristics.

Active group:	Epoxy group
Active group density:	19 to 40 µmole/ml drained medium
Spacer:	1,4-*bis*(2,3-epoxypropoxy-)butane (12 atom)
Bead structure:	6% agarose
Bead size range:	45 to 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:	2 to 14
Short term:	2 to 14
Chemical stability**:	Stable to all commonly used aqueous solutions. Can be used with non-ionic detergents, denaturing solvents, e.g. 8 M urea and 6 M guanidine hydrochloride, Stable in organic solvents, e.g. 50% dimethyl-formamide and 50% dioxane.
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 wothout adverse effects on its subsequent chromatographic performance.

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

1. Preparing the medium

Epoxy-activated Sepharose 6B is supplied freeze-dried in the presence of additives. These additives must be washed away at neutral pH before coupling the desired ligand.

Weigh out the required amount of freeze dried powder (1 g freeze-dried powder gives about 3.5 ml final volume of medium) and suspend it in distilled water. The medium swells immediately and should be washed for 1 hour on a sintered glass filter. Use approximately 200 ml distilled water per gram freeze-dried powder, added in several aliquots.

2. Coupling the ligand

General ligand coupling procedure

1. Weigh out the required amount of Epoxy-activated Sepharose 6B. Swell and wash the freeze-dried powder with distilled water as described above.
2. Dissolve the ligand to be coupled in coupling buffer. Organic solvent may be used. For small ligands add at least 200 µmoles ligand per ml medium.

   A medium: buffer ratio of 1:0.5 to 1:1 gives a suitable suspension for coupling
3. Mix the coupling solution containing the ligand with the medium in a stoppered vessel. Use a shaker in a water bath for 16 hours at 20°C to 40°C.

   Other gentle stirring methods may be employed.

   Do not use a magnetic stirrer.
4. Wash away excess ligand using coupling buffer.
5. Block any remaining active groups. Transfer the medium to 1 M ethanolamine pH 8.0. Let it stand for at least 4 hours or overnight at 40°C to 50°C.
6. 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.

3. Factors affecting the coupling efficiency

pH

The coupling reaction proceeds most efficiently in the pH range 9 to 13. Coupling to hydroxyl groups requires high pH and should therefore be performed around pH 13.

The stability of the ligand limits the maximum pH which can be used.

Coupling solution

Coupling should be performed in distilled water, carbonate, borate or phosphate buffers. Sodium hydroxide may be used for making solutions of high pH.

Tris and other buffer salts containing amino groups or other nucleophilic components should not be used since these will couple to the medium.

Organic solvents may be needed to dissolve the ligand. Dimethylformamide and dioxane may be used to up to 50% of the final mixture. The same concentration of organic solvents should be included in the coupling buffer. Always adjust the pH after dissolving the ligand, since organic solvent usually lowers pH.

Temperature

Coupling is performed in the range 20°C to 40°C, preferably using a shaker in a water bath. Direct heating and and magnetic stirrer should be avoided. The stability of the ligand limits the maximum temperature which can be used.

Time

The time for the reaction depends largely on the pH of the coupling solution, properties of the ligand and temperature of coupling. The coupling time

decreases at higher temperatures.

The efficiency of coupling is also pH and temperature dependent. 16 hours at 20°C to 40°C is most often used.

Ligand concentration

A very high ligand concentration can have adverse effects on affinity chromatography. Firstly, the binding efficiency of the adsorbent may be reduced due to steric hindrance between the active sites. Secondly, substances are more strongly bound to the immobilized ligand which may result in difficult elution. Thirdly, the extent of non-specific binding increases at high ligand concentrations.

As a general guideline; load 100 to 400 µmole ligand/ml drained medium (approximately 5 to 10 times concentration of active groups).

Blocking excess remaining groups

Remaining active groups on the gel should be de-activated or blocked after the coupling. Leave the medium in 1 M ethanol-amine for about 4 h at 20°C to 40°C or overnight at room temperature.

Washing the adsorbent

To remove excess uncoupled ligand after coupling, the medium should be thoroughly washed with coupling solution, including organic solvent if used for coupling. The medium should then be washed alternatively with high and low pH buffer solution at least three times. Acetate buffer (0.1 M, pH 4) and coupling buffer (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.

4. Packing Sepharose 6B

Prepare a slurry with binding buffer, see below, 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.

5. 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 starting 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 starting buffer until the base line is stable.

6. 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 limit of pH which may be used.
- **Ionic strength:** A buffer with increased ionic strength is used. Elution with a continuos or step-wise gradient may be used. A gradient of increasing

salt concentration can 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.

7. Regeneration

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

A general regeneration method is described below:

An affinity medium may be regenerated for re-use by washing it 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 followed by re-equilibration in binding buffer.

8. Storage

Freeze-dried Epoxy-activated Sepharose 6B should be stored below 8°C.

Swollen coupled medium should be stored in a solution that maintains the stability of the ligand and contains a bacteriostatic agent, for example, 20% ethanol in a suitable buffer. Do not freeze.

9. Further information

Check www.gelifesciences.com/protein-purification for more information. Useful information is also available in the Affinity Chromatography handbook, see ordering information.

10. Ordering information

Product	Pack size	Code No.
Epoxy-activated Sepharose 6B	15 g	17-0480-01
Literature		
Affinity Chromatography Handbook, Principles and Methods	1	18-1022-29
Affinity Columns and Media, Product Profile	1	18-1121-86

For local office contact information, visit
www.gelifesciences.com/contact

GE Healthcare Bio-Sciences AB
Björkgatan 30
751 84 Uppsala
Sweden

www.gelifesciences.com/protein-purification

GE Healthcare Europe GmbH
Munzinger Strasse 5
D-79111 Freiburg
Germany

GE Healthcare UK Ltd
Amersham Place
Little Chalfont
Buckinghamshire, HP7 9NA
UK

GE Healthcare Bio-Sciences Corp
800 Centennial Avenue
P.O. Box 1327
Piscataway, NJ 08855-1327
USA

GE Healthcare Japan corporation
Sanken Bldg. 3-25-1, Hyakunincho
Shinjuku-ku, Tokyo 169-0073
Japan

imagination at work

71-7087-00 AG 08/2010