GE Healthcare

Instructions 71-7086-00 AF **Affinity media**

CNBr-activated Sepharose™ 4B

CNBr-activated Sepharose 4B is a pre-activated medium for immobilization of ligands containing primary amines. It provides a very convenient way to immobilize ligands by the cyanogen bromide method. The coupling reaction is spontaneous, rapid and easy to carry out.

The application area covers immobilization of proteins, peptides and nucleic acids.

Sepharose

Table 1. Medium characteristics.

Bead structure:	4% agarose
Spacer:	None
Coupling capacity:	25–60 mg α-chymotrypsinogen/ml drained medium
Bead size range:	45–165 µm
Average 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:	3–11
Short term:	2–11
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, such as 50% dimethylformamide and 50% dioxane.
Autoclavable:	Not recommended

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

** The ranges given are estimates based on our knowledge and experience. Please note the following:
pH stability, long term refers to the pH interval where the gel 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, cleaning-in-place and sanitization procedures.

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

Contents

1. Preparing the medium

CNBr-activated Sepharose 4B is supplied lyophilized in the presence of additives. These additives must be washed away at low pH (pH 3) before coupling the desired ligand. The use of low pH (pH 3) preserves the activity of the reactive groups, which otherwise hydrolyze at high pH.

Weigh out the required amount of powder (1 g lyophilized powder gives about 3.5 ml final volume of medium) and suspend it in 1 mM HCl. The medium swells immediately and should now be washed for 15 minutes with 1 mM HCl on a sintered glass filter (porosity G3). Use approximately 200 ml 1 mM HCl per gram freeze-dried powder, added in several aliquots.

2. Coupling the ligand

General ligand coupling procedure

1. Dissolve the ligand to be coupled in coupling buffer, 0.1 M $NaHCO_3$ pH 8.3 containing 0.5 M NaCl. Use about 5 ml coupling solution/g lyophilized powder.

   About 5–10 mg protein per ml medium is recommended. For smaller ligands add 1–10 µmoles per ml medium.

2. Add the coupling solution containing the ligand with the prepared medium suspension in a stoppered vessel.

3. Rotate the mixture end-overend for 1 h at room temperature or overnight at 4 °C. Other gentle stirring methods may be employed.

   Do not use a magnetic stirrers as these can disrupt the Sepharose beads.

4. Wash away excess ligand with at least 5 medium (gel) volumes of coupling buffer.

5. Block any remaining active groups. Transfer the medium to 0.1 M Tris-HCl buffer, pH 8.0 or 1 M ethanolamine, pH 8.0. Let it stand for 2 hours.

6. Wash the medium with at least three cycles of alternating pH. Wash with at least 5 medium volumes of each buffer.

   Each cycle should consist of a wash with 0.1 M acetic acid/sodium acetate, pH 4.0 containing 0.5 M NaCl followed by a wash with 0.1 M Tris-HCl, pH 8 containing 0.5 M NaCl.

3. Factors effecting the coupling efficiency

pH

The coupling reaction proceeds most efficiently in the pH range 8–10 where the amino groups on the ligand are predominantly in the unprotonated form. A buffer at pH 8.3 is most frequently used for coupling proteins.

Coupling at low pH is less efficient but may be advantegeous if the ligand looses biological activity when it is fixed firmly by multi-point attachment or if steric hindrance between binding sites occurs when a large amount of high molecular weight ligand is immobilized. A buffer of approximately pH 6 is used for coupling at low pH.

Coupling solution

Coupling should be performed in bicarbonate or borate buffers. Tris and other buffer salts containing amino groups 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 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.

Salt

To minimize protein-protein adsorption and the formation of protein aggregates, it is recommended to have a high salt content, 0.5 M NaCl, in the coupling buffer.

Temperature

Coupling is completed within 2 hours at room temperature, 20–25 °C. If cold room temperatures are necessary, coupling can be carried out overnight.

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 and this may result in difficult elution. Thirdly, the extent of non-specific binding increases at high ligand concentrations.

For an efficient adsorbent, 1–10 µmoles ligand per ml medium is recommended. For protein ligands, 5–10 mg protein per ml medium is recommended.

Controlling the coupling efficiency

Sometimes it may be necessary to reduce the number of coupling groups on the matrix to preserve the structure of the binding site in a labile molecule, or to facilitate elution when high binding constants make elution difficult or when steric effects reduce the binding efficiency of a large ligand.

Reduced coupling activity may be achieved by controlled hydrolysis of the activated medium prior to coupling, or by coupling at a lower pH. Pre-hydrolysis reduces the number of active groups available for coupling and reduces the number of points of attachment between the protein and matrix as well as the amount of protein coupled. In this way a higher binding activity of the product is obtained. At pH 3, coupling activity is lost only slowly, whereas at pH 8.3 activity is lost fairly rapidly. A large molecule is coupled at only about half as many points after 4 h pre-hydrolysis at pH 8.3 as it is before the number of active groups was reduced.

Blocking excess remaining groups

Remaining active groups on the medium should be deactivated or blocked after the coupling. These can be hydrolyzed in a mildly alkaline pH (2 hours at room temperature or 16 h at 4 °C).

Alternatively, these can also be blocked by adding an excess of a small primary amine (e.g. Tris-HCl, ethanolamine, glycine) at approximately pH 8 (2 hours at room temperature or 16 h at 4 °C).

These blocking agents introduce a small number of charged groups into the medium. The effect of these charged groups is overcome by the use of a relatively high salt concentration (0.5 M NaCl) in the buffer for affinity chromatography.

Washing the adsorbent

To remove excess of uncoupled ligand after coupling, the adsorbent is washed alternatively with high and low pH buffer solutions 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 4B

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 medium 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 binding 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.

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. Elution 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:** A buffer with increased ionic strength is used. Elution with a continuous 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 elute substances from a group specific adsorbent and also when the affinities are relatively low. Selectively retained substances are usually displaced at low concentrations of eluting agents, often less then 10 mM. Either continuous or step-wise gradients may be used.

- **Reduced polarity:** Conditions which lower the polarity of the eluent to promote elution 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 elution, 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 the medium with 2–3 column 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

Lyophilized CNBr-activated Sepharose 4B should be stored below 8 °C.

Swollen coupled medium should be stored at 4–8 °C in presence of a bacteriostatic agent, e.g. 20% ethanol.

9. Further information

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

10. Ordering information

Product	Pack size	Code No.
CBNr-activated Sepharose 4B	15 g 250 g	17-0430-01 17-0430-02
Literature		
Affinity Chromatography Handbook, Principles and Methods	1	18-1022-29
Affinity Columns and Media, Product Profile	1	18-1121-86

For local offoce contact information, visit
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