INSTRUCTIONS

UltraLink™ Immobilized Carboxy

53148

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Introduction

UltraLink™ Immobilized Carboxy is prepared on the azlactone-activated support, 3M Emphaze™ Biosupport Medium AB 1, using a reaction mechanism that results in a stable linkage. The support is hydrophilic charge-free, high capacity and highly crosslinked, rigid, copolymeric and porous.[1-7] This means that the support has minimal non-specific interactions with the sample. The porosity, rigidity and the durability of this support are important considerations when working with large volumes of samples requiring fast-flow techniques and large scale bulk applications. Agarose supports are extremely useful for gravity flow procedures, however more rigid supports are required if flow rates result in pressures greater than 25 p.s.i. The 3M Emphaze™ Biosupport AB 1 matrix is useful for medium pressure techniques such as FPLC. *See* table 1 for further specific information regarding this support.

Table 1: Characteristics of 3M Emphaze™ Biosupport Medium AB 1

pH stability of matrix:	1-13
Particle size (average):	50-80 microns
Exclusion limit (proteins):	>2,000,000 daltons
Surface Area (average):	>250 ml/g of beads
Pore volume (average):	> 1.2 ml/g of beads (>60% of bead volume)
Pore size:	1000 A
Maximum pressure:	100 p.s.i.
Maximum linear velocity:	3000 cm/hour

***NOTE:** The indicated maximum pressure of 100 p.s.i. refers to the maximum pressure drop across a column that the support can withstand. It does not necessarily refer to the indicated system pressure shown on a liquid chromatography apparatus, since the system pressure may not be actually measuring the pressure drop across the column. Refer to pages 3-4 for additional information.

UltraLink™ supports are extremely valuable for use in medium pressure chromatography applications. When packed into a 3 mm inside diameter x 14 cm height glass column. UltraLink™ supports have been run to approximately 400 p.s.i. (system pressure) with no visual compression of the gel or adverse effects on chromatography. Typically these columns can be run at linear flow rates of 85-3000 cm/hour with excellent separation characteristics.

Solid-Phase Immobilization on UltraLink™ Immobilized Carboxy Support Using EDC

EDC is useful for coupling primary amine containing ligands to UltraLink™ Immobilized Carboxy supports. Ethylenediamine is coupled to this matrix and then succinylated to create a terminal carboxylic acid group. The extended spacer arm is useful for immobilizing small peptides which may otherwise be sterically hindered upon immobilization. The reaction times for coupling to supports are generally in the range of 1-3 hours. When coupling water-insoluble peptides, ethanol, methanol, DMSO or DMF may be employed as solvents. The haptens should be dissolved in the organic water-miscible solvent first and then added to the conjugation buffer. Up to 30% organic solvent may be used as long as the peptide is not denatured by the solvent.

EDC reacts with a carboxyl group first and forms an amine-reactive intermediate, an *O*-acylisourea. The intermediate is unstable in aqueous solutions and is therefore not useful in two-step conjugation procedures without stabilization of the intermediate using *N*-hydroxysuccinimide.[8] Failure to react with an amine will result in hydrolysis of the intermediate, regeneration of the carboxyl, and release of an *N*-substituted urea. A side reaction is the formation of an *N*-acylurea which is usually restricted to carboxyls located in the hydrophobic regions of proteins. The crosslinking reaction is very rapid between pH 4.5 and 5 requiring only a few minutes in many applications. At higher pH's the reaction rate will be comparatively slower. The hydrolysis of EDC is a competing reaction during coupling and is dependent on temperature, pH, and buffer concentration and composition.[8,9,10] 2-(*N*-Morpholino)ethane sulfonic acid (MES) is the buffer for the reaction. Phosphate buffers react with EDC, reducing conjugation efficiency but can be used if compensated for by an increased amount of crosslinker. Tris, glycine, and acetate buffers can react with EDC or the *O*-acylisourea intermediate and should not be used as conjugation buffers. Avoid compounds containing extraneous primary amines, carboxyls, or thiols in conjugation buffers as they will reduce crosslinking efficiency. Hydroxylamine a strong nucleophile greatly accelerates the loss of EDC in solution. Halides such as iodide, chloride and bromide have little effect on EDC between pH 5 and 7.[10]

Product Description

NUMBER	DESCRIPTION
53148	**UltraLink™ Immobilized Carboxy,** *10 ml* This product is provided with a Disposable Column Trial Kit. This kit contains 2 of each of 2, 5, and 10 ml gel bed capacity columns and accessories for packing these two-disc columns. A 25 ml column extender is provided to allow application of larger volumes of wash and sample than is otherwise possible. The extender fits all three columns. Store this gel at 4°C.

Instructions for the Immobilization of Peptides Using EDC to UltraLink™ Immobilized Carboxy

Described below is a general method for the immobilization of peptides using EDC to UltraLink™ Immobilized Carboxy. The protocol must be optimized for your application to get the best results.

Materials

A. UltraLink™ Immobilized Carboxy.

B. EDC [1-Ethyl-3-(3-dimethyaminopropyl)carbodiimide].

C. Conjugation buffer: 0.1 M MES (*N*-Morpholinoethane sulfonic acid), 0.9% NaCl, pH 4.7.

D. Wash buffer: 1 M NaCl.

Preparation

Carefully pack the UltraLink™ Immobilized Carboxy gel into the appropriate column supplied by following the steps below:

a. Place the bottom cap on the end of one of the columns provided.

b. Place the column in a 16 x 125 mm test tube and add 6 ml of the degassed water to the column. Tap the end of the column sharply to dislodge any air bubbles.

c. Float one of the porous polyethylene discs on the top of the liquid within the column.

d. Use the reverse end of a serum separator or Pasteur pipette to push the disc to the bottom of the column. As the disc is pushed to the bottom, the air will be purged through the disc.

e. Drain the water from the column by removing the bottom cap. Replace the cap and add the desired quantity of the UltraLink™ Immobilized Carboxy gel as a slurry to the column and allow the gel to settle for 30 minutes.

Method

1. Equilibrate the gel by passing 5 column volumes of water followed by 5 column volumes of the conjugation buffer through the column.

2. After washing the column, drain the column until the liquid level reaches the top of the gel and replace the bottom cap.
3. Dissolve the primary amine containing peptide or protein in conjugation buffer at a concentration of 10 mg/ml. If the peptide or protein absorbs at 280 nm determine the OD at 280.
4. Apply the peptide or protein solution to the column (1 ml of the peptide or protein solution for every ml of the gel), put the top cap on the column. Mix the gel and the peptide solution on a shaker or a rotator.
5. Dissolve 60 mg of EDC per 0.5 ml of conjugation buffer immediately before use.
6. Add the EDC solution to the gel-peptide or protein slurry from step 4. (Add 0.25 ml of the EDC solution per every ml of gel). Put on the top cap.
7. Mix by shaking or rotating for 3 hours at room temperature.
8. Remove the top and bottom caps of the column sequentially and let the solution drain from the column into a tube. Measure the volume of this fraction.
9. When the liquid level reaches the top of the gel bed, move the column to a fresh tube and apply 1 ml of wash buffer the top of the column. Allow the solution to drain.
10. When the liquid level reaches the top of the gel replace the bottom cap. Transfer the column to a fresh tube and apply 1 ml of wash buffer to the top of the column.
11. Insert one of the supplied porous polyethylene discs into the column so that it is 1-3 mm above the top of the gel. Allow the buffer to drain.
12. Transfer the column to a fresh tube and apply 1 ml wash buffer to the top of the column.
13. Collect 1 ml-fractions. Determine the OD at 280 nm of the collected fractions. Collect fractions until the absorbance of the fractions reaches the baseline.

Calculate the total amount of peptide or peptide immobilized using the following method:

1. Determine the total A_{280} units of protein or peptide applied to the column using a cell with a 1 cm path.
2. Determine the total units that did not bind the column by adding the absorbance units of the 1 ml fractions.

   NOTE: This calculation method works because the fractions are 1-ml. If fractions collected are smaller or larger than 1ml appropriate adjustments will need to be made to the units. For example if 0.5 ml fractions are collected multiply the absorbance units by 0.5 and if 2 ml samples are collected multiply the absorbance units by 2.
3. Determine the units that bound by subtracting the values in 2 from 1.
4. Divide the bound units by the total units and multiply by the amount of peptide (mass) to obtain the total amount of immobilized peptide.
5. Divide the bound weight of peptide by the molecular weight of the peptide to determine the number moles bound to 2 ml of the gel.

Medium Pressure Chromatographic Applications

The 3M Emphaze™ Biosupport Medium can be used in Fast Liquid Chromatography Pharmacia® applications. The affinity chromatography protocols described in these instructions can be adapted for use with medium pressure chromatographic (MPC) applications. With MPC however, the critical factor for success is limiting the pressure drop across the column that the support experiences. Due to variations with the instrument, the indicated gauge pressure of an MPC apparatus may actually measure the pressure drop across the column.

A more universal criteria for MPC applications, therefore, would be to measure the linear velocity of the buffers through the column, a pressure-independent measurement. The linear velocity is defined as the velocity of the buffer front passing through the gel bed. Linear velocity is usually expressed in cm/hour, and in case of the 3M Emphaze™ Biosupport Medium AB 1, the maximum linear velocity should not exceed 3000 cm/hr.

The linear velocity through a cylindrical column can be calculated if the inside diameter of the column is known, and if the column effluent is collected and measured for a given time to determine the flow rate. The calculations for determining linear velocity are shown below:

Radius measured in cm = r

Column cross-sectional area in square centimeters = πr^2

1 ml = 1 cubic centimeter

Measured flow rate (ml/minute) = Column effluent collected in ml/minute

$$\text{Linear velocity per minute} = \frac{\text{(measured flow rate in ml/minute or cm}^3\text{/minute)}}{\text{cross-sectional area in cm}^2}$$

Linear velocity per hour = (linear velocity per minute)(60 minutes/hour)

$$\frac{\text{Measured flow rate per hour}}{\text{Column cross-sectional area}} = \frac{\text{(cm}^3\text{/minute)(60 minute/hour)}}{\pi r^2} = \text{Linear velocity expressed as cm/hour}$$

Sample Protocol for Medium Pressure Chromatography

The affinity chromatography protocols described in these instructions can be adapted for use with medium pressure chromatographic (MPC) applications.

Materials

A. UltraLink™ Immobilized Peptide/Protein (immobilized using EDC and UltraLink™ Immobilized Carboxy)

B. Anti-peptide/protein antibody

C. Binding buffer: 20 mM sodium phosphate buffer, pH 7.5, 500 mM NaCl

D. Elution buffer: 0.1 *M* glycine•HCl, pH 2.8

E. Column

Method

1. Gravity pack or pack under flow UltraLink™ Immobilized Peptide/Protein into a medium pressure chromatography column.
2. Store the column in 0.02% sodium azide if column will not be used immediately.
3. Equilibrate the column with at least 4 column volumes of the binding buffer.
4. Add or inject the antibody.
5. Wash the column with an additional 8 column volumes of binding buffer or until baseline is reached.
6. Elute the bound protein with 15 column volumes of the elution buffer or until baseline is reached.
7. Collect fractions. Monitor the elution of the protein at 280 nm.
8. Dialyze or desalt the eluted antibody.
9. Wash the UltraLink™ Immobilized Peptide/Protein column with 10-20 column volumes of 0.02% sodium azide in water or binding buffer.

References

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