GE Healthcare
Life Sciences

Instructions 28-9955-29 AB **Affinity Chromatography**

Capto™ Blue

Capto Blue is an affinity medium for purification of many proteins, such as albumin, interferon, lipoproteins and blood coagulation factors. It also binds several enzymes including kinases, dehydrogenases, and most enzymes requiring adenyl-containing cofactors e.g., NAD^+.

Capto Blue is more chemically stable and has a more rigid agarose base matrix than Blue Sepharose™ 6 Fast Flow. This allows the use of faster flow rates and larger sample volumes, leading to higher throughput and improved process economy.

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1 BioProcess™ Media

BioProcess media are developed and supported for production scale chromatography. All BioProcess media are produced with validated methods and are tested to meet manufacturing requirements. Secure ordering and delivery routines give a reliable supply of media for production-scale. Regulatory Support Files (RSF) are available to assist process validation and submissions to regulatory authorities. BioProcess media cover all purification steps from capture to polishing.

2 Properties of Capto Blue

Capto Blue medium is based on a highly rigid agarose base matrix. The Cibacron™ Blue ligand is attached to the base matrix via a hydrophilic spacer and is immobilized with a stable amine bond.

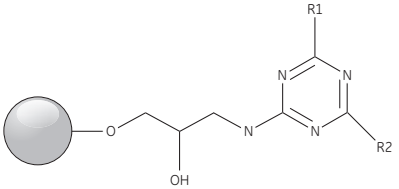

Fig 1. Capto Blue, the base matrix, spacer, and ligand.

Capto Blue is a medium for affinity chromatography that can be used in the same way as Blue Sepharose 6 Fast Flow. Higher flow rates can be used with Capto Blue during equilibration, wash and regeneration.

The Cibacron Blue ligand contains sulfonic groups that can take part in ion exchange interactions and also groups that can bind to the target molecule by hydrophobic interactions.

Depending on the target molecule, the effect of these groups can be enhanced or weakened by the choice of buffer salt and conductivity. To increase yield or to regenerate the chromatography medium, elution with salt can be complemented by adding an organic solvent such as ethanol or by changing pH.

Table 1. Characteristics of Capto Blue.

Matrix	Highly cross-linked agarose
Particle size, d_{50v}[1]	75 µm
Ligand	Cibacron Blue
Ligand density	≈ 13 µmol/ml
Total binding capacity	24 mg/ml human serum albumin at 4 min residence time
Recommended liquid velocity[2]	150 to 300 cm/h
Maximum liquid velocity	At least 600 cm/h in a 1 m column with 20 cm bed height at 20°C using process buffers with the same viscosity as water; corresponds to a residence time of 2 min.
pH stability[3] **- short term** **- long term**	 2 to 13 2 to 13
Storage	2°C to 8°C in 0.1 M KH_2PO_4, pH 8.0 in 20% ethanol

[1] d_{50v} is the average particle size of the cumulative volume distribution.

[2] Water at room temperature.
Note: For viscous buffers and samples - the liquid velocity must be optimized and starting with a low flow rate is recommended in order not to exceed pressure limits.

[3] Short term pH: pH interval where the medium can be subjected to cleaning- or sanitization-in-place without significant change in function.
Long term pH: pH interval where the medium can be operated without significant change in function.

3 Scale-up

After optimizing the method at laboratory scale, the process can be scaled up. Scale-up is typically performed by keeping bed height and linear liquid velocity constant while increasing bed diameter and volumetric flow rate. However, since optimization is preferentially performed with small column volumes, in order to save sample and buffer, some parameters such as the dynamic binding capacity may be optimized using shorter bed heights than those being used in the final scale. As long as the residence time is kept constant, the binding capacity for the target molecule remains the same.

Other factors, such as clearance of critical impurities, may change when column bed height is modified and should be validated using the final bed height. The residence time is approximated as the bed height (cm) divided by the linear liquid velocity (cm/h) applied during sample loading.

Select the bed volume according to required binding capacity. Keep sample concentration and gradient slope constant.

The larger equipment used when scaling up may cause some deviations from the method optimized at small scale. In such cases, check the buffer delivery system and monitoring system for time delays or volume changes. Different lengths and diameters of outlet tubing can cause zone spreading on larger systems.

4 Packing columns

Recommended columns

Table 2. Recommended columns for Capto Blue.

Column	Inner diameter (mm)	Bed volume[1]	Bed height (cm)
Lab scale			
HiScale™ 16/20	16	20–40 ml	max 20
HiScale 16/40	16	20–70 ml	max 35
HiScale 26/20	26	53–106 ml	max 20
HiScale 26/40	26	53–186 ml	max 35
HiScale 50/20	50	196–393 ml	max 20
HiScale 50/40	50	196–687 ml	max 35
Production scale			
AxiChrom™	50–200	0.2–12.5 l	max 40
AxiChrom	300–1000	7–314 l	max 40

[1] Bed volume range calculated from 10 cm bed height to maximum bed height.

All large-scale columns can be supplied as variable bed height columns. Do not choose large diameter columns if the bed height is low.

For practical instructions in good packing techniques, see the CD-ROM *Column Packing - The Movie* (18-1165-33).

For more details about packing HiScale columns, see instructions *HiScale™ columns (16, 26, 50) and accessories* (28-9674-70).

Packing HiScale columns

Packing preparations

Materials needed

Capto Blue

HiScale column

HiScale packing tube (depending on bed height)

Plastic spoon or spatula

Glass filter G3

Vacuum suction equipment

Filter flask

Measuring cylinder

20% ethanol with 0.4 M NaCl

Equipment

ÄKTA™ systems, or a stand-alone pump such as Pump P-900, depending on the flow rate required, can be used for packing.

Equilibrate all materials to room temperature.

Definitions

The bed height of a gravity settled bed differs from the bed height of a bed settled at a given flow (consolidated). Therefore, the compression factor (CF) has to be separated from the packing factor (PF).

$L_{settled}$	Bed height measured after settling by gravity.
L_{cons}	Consolidated bed height Bed height measured after settling the medium at a given liquid velocity.
L_{packed}	Packed bed height
CF	Compression factor $CF = L_{settled}/L_{packed}$
PF	Packing factor $PF = L_{cons}/L_{packed}$
A_C	Cross sectional area of the column
V_C	Column volume $V_C = L_{packed} \times A_C$
C_{slurry}	Concentration of the slurry

Preparation of the slurry

To measure the slurry concentration, let the media settle in 20% ethanol at least overnight in a measuring cylinder or use the method for slurry concentration measurement described in application note 28-9259-32. This method can also be used with HiScale columns.

Washing the medium

Mount a glass filter funnel onto a filtering flask. Suspend the medium by shaking and pour into the funnel and wash according to the following instructions:

- 5 times with 5 ml 20% ethanol with 0.4 M NaCl/ml medium
- Gently stir with a spatula between additions.
- Move the washed medium from the funnel into a beaker and add 20% ethanol with 0.4 M NaCl to obtain a 50% slurry concentration.

Packing the column

Table 3. Main features of the packing method for HiScale 16/20 and HiScale 16/40

Column	HiScale 16/20	HiScale 16/40
Bed height (cm)	10	20
Slurry/ packing solution	20% ethanol with 0.4 M NaCl	
Slurry concentration (%)	50	50
Packing factor (PF)	1.10	1.10
Packing velocity (cm/h)	750	750
Packing flow rate (ml/min)	25	25
Flow condition (cm/h)	750	750
Flow condition (ml/min)	25	25

Table 4. Main features of the packing method for HiScale 26/20 and HiScale 26/40

Column	HiScale 26/20	HiScale 26/40
Bed height (cm)	10	20
Slurry/ packing solution	20% ethanol with 0.4 M NaCl	
Slurry concentration (%)	50	50
Packing factor (PF)	1.15	1.10
Packing velocity (cm/h)	750	750
Packing flow rate (ml/min)	66	66
Flow condition (cm/h)	750	750
Flow condition (ml/min)	66	66

Table 5. Main features of the packing method for HiScale 50/20 and HiScale 50/40

Column	HiScale 50/20	HiScale 50/40
Bed height (cm)	10	20
Slurry/ packing solution	20% ethanol with 0.4 M NaCl	
Slurry concentration (%)	50	50
Packing factor (PF)	1.15	1.15
Packing velocity (cm/h)	750	750
Packing flow rate (ml/min)	250	250
Flow condition (cm/h)	750	750
Flow condition (ml/min)	250	250

Packing procedure

1. Assemble the column according to the column instructions (HiScale columns (16, 26, 50) and accessories, code no 28-9674-70).
2. Mount the column tube in a stand.
3. Connect the bottom adapter unit to the pump or a syringe and prime the bottom net with a slow flow of packing solution. This is easiest done if the nets are dry but if air is trapped under the net it can be removed by a light suction with a syringe.
4. Mount the bottom adapter unit in the bottom of the column tube and tighten the o-ring.
5. Fill the column with approximately 1 cm packing liquid using the pump/syringe. Disconnect the pump/syringe and put a stop plug on the outlet.
6. Mount the packing tube on top of the column tube.
7. Connect the top adapter to the pump and prime it with a slow downward flow. The net needs to be facing the roof as this is done. If air is trapped under the net it can be removed by a light suction with a syringe.
8. Fill the column with slurry suspended in packing solution. If needed, top up the slurry with extra packing solution so the top adapter dips into the slurry to avoid air under the net.
9. Mount the top adapter unit on top of the packing tube. Tighten the o-ring firmly and remove the bottom stop plug.
10. Start a downward flow with packing velocity according to Table 3, 4 and 5.
11. Let the flow run until the bed has consolidated.
12. Use the scale on the column to measure the bed height. There might be a build up of media at the column wall after the bed is consolidated and to easier see where the top of the bed is, a light source can be used.
13. Calculate the final bed height by dividing the consolidated bed height with the desired packing factor.
    $L_{packed} = L_{cons}/PF$
14. Turn off the flow and put a stop plug in the bottom.

15 Dismount the top adapter from the packing tube.

16 Over a beaker or a sink, detach the packing tube from the column.

17 Remount the top adapter in the column tube. Make sure no air is trapped under the net and lower the adapter down to 1 to 2 cm above the bed, making sure the surface is not disturbed.

18 Tighten the O-ring on the adapter. Remove the bottom stop plug and carefully start turning the end cap down. While spilling out liquid through the bottom, proceed turning until the calculated final bed height is reached.

19 Make sure that the pressure peaks that occur during turning the end knob down do not exceed the pressure specifications of the media.

20 Start a downward flow to flow condition the bed. The flow rate is shown in Table 3, 4 and 5.

21 Let the flow run for about 10 column volumes. The column is ready to be tested.

Testing the packed column

See Chapter *Evaluation of column packing*.

5 Evaluation of column packing

Test the column efficiency to check the quality of packing. Testing should be done after packing, at regular intervals during the working life of the column or when separation performance is seen to deteriorate. The best method of expressing the efficiency of a packed column is in terms of the height equivalent to a theoretical plate (HETP) and the asymmetry factor (A_s). These values are easily determined by applying a sample such as 1% acetone solution to the column. Sodium chloride can also be used as a test substance. Use a concentration of 0.8 M NaCl in water with 0.4 M NaCl in water as eluent.

For more information about column efficiency testing, consult the application note *Column efficiency testing* (28-9372-07).

Note: *The calculated plate number will vary according to the test conditions and it should only be used as a reference value. It is important that test conditions and equipment are kept constant so that results are comparable. Changes of solute, solvent, eluent, sample volume, liquid velocity, liquid pathway, temperature, etc. will influence the results.*

For optimal results, the sample volume should be at maximum 2.5% of the column volume and the liquid velocity 30 cm/h. If an acceptance limit is defined in relation to column performance, the column plate number can be used as one of the acceptance criteria for column use.

Method for measuring HETP and A_S

Calculate HETP and A_S from the UV curve (or conductivity curve) as follows:

$$HETP = \frac{L}{N}$$

$$N = 5.54 \times \left(\frac{V_R}{W_h}\right)^2$$

L = bed height (cm)

N = number of theoretical plates

V_R = volume eluted from the start of sample application to the peak maximum

W_h = peak width measured as the width of the recorded peak at half of the peak height

V_R and W_h are in the same units

The concept of reduced plate height is often used for comparing column performance.

The reduced plate height, h, is calculated as follows:

$$h = \frac{HETP}{d_{50v}}$$

d_{50v} = mean diameter of the beads (cm)

As a guideline, a value of < 3 is very good.

The peak should be symmetrical, and the asymmetry factor as close to 1 as possible
(a typical acceptable range could be $0.8 < A_S < 1.8$).

A change in the shape of the peak is usually the first indication of bed deterioration due to excessive use.

Peak asymmetry factor calculation:

$$A_s = \frac{b}{a}$$

a = ascending part of the peak width at 10% of peak height

b = descending part of the peak width at 10% of peak height

Figure 2 shows a UV trace for acetone in a typical test chromatogram from which the HETP and A_s values are calculated.

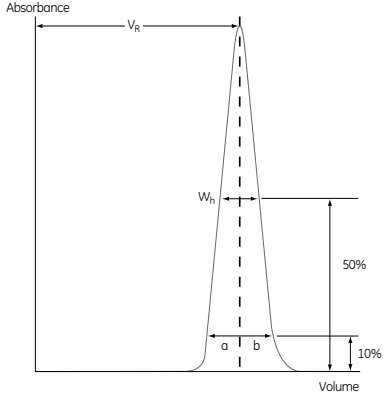

Fig 2. A typical test chromatogram showing the parameters used for HETP and A_s calculations.

6 Operation

Binding

Different substances differ in their affinity for Capto Blue. The available capacity will depend upon parameters such as flow rate, pH, buffer composition and temperature.

1. 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.
2. After the sample has been loaded, wash the medium with binding buffer until the base line is stable.

Elution

1. Elution conditions vary with the sample. Elution may be accomplished by a change in pH, polarity (e.g., ethylene glycol) or ionic strength of the buffer. Enzymes can often be eluted at less than 1 M NaCl.
2. Competitive elution with low concentrations of the cofactor is required for very specifically bound proteins. Either step or continuous gradients may be used.

7 Maintenance

For the best performance from Capto Blue over a long working life, follow the procedures described below.

Equilibration

After packing, and before a chromatographic run, equilibrate with start buffer by washing with at least 5 bed volumes, or until the column effluent shows stable conductivity and pH values.

Regeneration

Depending on the nature of the sample, reversibly bound material can be eluted with 4 to 5 washing cycles of alternate 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, followed by re-equilibration in binding buffer.

In some applications, substances like denaturated proteins or lipids do not elute in the regeneration procedure. These can be removed by Cleaning-In-Place.

Cleaning-In-Place (CIP)

CIP removes very tightly bound, precipitated or denatured substances from the medium. If such contaminants are allowed to accumulate, they may affect the chromatographic properties of the packed column, reduce the capacity of the medium and, potentially, come off in subsequent runs. If the fouling is severe, it may block the column, increase back pressure and reduce flow rate. CIP should be performed regularly to prevent the build-up of contaminants and to maintain the capacity, flow properties and general performance of packed columns.

It is recommended to perform a CIP:

- When an increase in back pressure is seen.
- If reduced column performance is observed.
- Before first time use or after long term storage.
- Between runs when the same column is used for purification of different proteins to prevent possible cross-contamination.

- After every run with real feed.

CIP protocol

The packed column can be cleaned by the following CIP procedures:

- Remove precipitated proteins by
    - washing the column with 4 column volumes (CV) of 0.5 M NaOH at 40 cm/h, followed by washing the column with 3–4 CV of 70% ethanol or 2 M potassium thiocyanate.
    - Alternatively, wash the column with 2 CV of 6 M guanidine hydrochloride.
    - In both cases wash immediately with at least 5 CV filtered binding buffer, pH 8.0.
- Remove strongly bound hydrophobic proteins, lipoproteins and lipids by
    - washing the column with 3–4 CV of up to 70% ethanol or 30% isopropanol.
    - Alternatively, wash the column with 2 CV detergent in a basic or acidic solution, e.g., 0.1% non-ionic detergent in 1 M acetic acid. Wash at a flow rate of 40 cm/h. Remove residual detergent by washing with 5 CV of 70% ethanol.
    - In both cases wash immediately with at least 5 CV filtered binding buffer, pH 8.0.

Sanitization

Capto Blue can be sanitized by autoclaving, which is particularly appropriate if microbial contamination is suspected.

Note: *If the medium is packed in a column, check if the column can be autoclaved.*

Storage

Capto Blue is supplied preswollen, in a solution of 0.1 M KH_2PO_4 and 20% ethanol, pH 8. The medium should be stored in this solution at 2°C to 8°C.

8 Ordering information

Product	Pack size	Code No
Capto Blue[1]	25 ml	17-5448-01
	500 ml	17-5448-02
	5 l	17-5448-04

[1] Capto Blue is supplied preswollen, in a solution of 0.1 M KH_2PO_4 and 20% ethanol, pH 8. For additional information, including data file, please contact your local GE Healthcare representative.

Related product	Quantity	Code No
HiScreen™ Capto Blue	1 x 4.7 ml	28-9924-74
HiScale 16/20	1	28-9644-41
HiScale 16/40	1	28-9644-24
HiScale 26/20	1	28-9645-14
HiScale 26/40	1	28-9645-13
HiScale 50/20	1	28-9644-45
HiScale 50/40	1	28-9644-44

Related literature		Code No
Movie	Column Packing - The Movie	18-1165-53
Handbook	Affinity Chromatography Handbook	18-1022-29
Data file	Capto Blue and Capto Blue (high sub)	28-9369-60
Instructions	HiScale columns (16, 26, 50) and accessories	28-9674-70

9 Further information

Visit us at:

www.gelifesciences.com/capto

For technical support,
visit: www.gelifesciences.com/techsupport

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