USER GUIDE: AM-50 Blue resin

## Introduction:

Dye-ligands have been considered as one of the important alternatives to natural counterparts for specific affinity chromatography. Dye-ligands are able to bind most types of proteins, in some cases in a remarkably specific manner. They are commercially available, inexpensive and widely used in labs and in industries. Although dyes are all synthetic in nature, they are still classified as affinity ligands because they interact with the active sites of many proteins mimicking the structure of the substrates, cofactors, or binding agents for those proteins. A number of dyes have been successfully used for protein affinity purification. The interaction between the dye ligand and proteins can be by complex combination of electrostatic, hydrophobic, hydrogen bonding. Selection of the supporting matrix is the first important consideration in dye-affinity systems. There are several methods for immobilizations of dye molecules onto the support matrix, in which usually several intermediate steps are followed. Both the adsorption and elution steps should carefully be optimized or designed for a successful separation. Dye-affinity systems in the form of spherical sorbents or as affinity membranes have been used in protein separation. A wide variety of functional molecules, including enzymes, coenzymes, cofactors, antibodies, amino acids, oligopeptides, proteins, nucleic acids, and oligonucleotides may be used as ligands in the design of novel sorbents. These ligands are extremely specific in most cases. However, they are expensive, due to high cost of production and/ or extensive purification steps. In the process of the preparation of specific sorbents, it is difficult to immobilize certain ligands on the supporting matrix with retention of their original biological activity. Dye-ligands have been considered as one of the important alternatives to natural counterparts for specific affinity chromatography to circumvent many of their drawbacks, mentioned above.

Dye-ligands has been shown in many kinetic studies that triazinyl dyes interact with an enzyme in a way involving the binding site, the substrate or coenzyme binding site, or the active site. for a natural biological ligand, NADH, NADPH, NADq, NADPq, GTP, IMP, ATP, HMG-CoA, folate, etc.. of that enzyme so that this natural ligand cannot bind. Many form of inhibition, including competitive, non-competitive, and mixed inhibition have been observed in these interactions. AM-50 Blue developed by EBL scientists is Cibacron Blue 3G coupled to matrix via a coupling chemistry. As a member of the affinity family of media (resins), this product is a well-suited adsorbent for albumin, interferon and several at both laboratory and process scale. AM-50 Blue resin has been developed and tested in cooperation with leading large-scale manufacturers and is widely used in routine commercial production.

### EBL AM-50 Blue resin

AM-50 Blue resin is Cibacron Blue 3G covalently attached to matrix by the triazine coupling method, giving a highly stable medium with minimal nonspecific adsorption. The rigidity of the matrix, together with a high degree of substitution, enables rapid processing of large volumes at production scale. With excellent binding kinetics, AM-50 Blue is suitable for recovery and purification of albumin and interferon. Purification and concentration are achieved in one single step. The swollen medium has a Cibacron Blue content of > 20 mM dye/mL drained medium. The total binding capacity for human serum albumin is > 18 mg/ml drained medium.

## Key Features:

- High dynamic binding capacity
- High flow rates powered by the monodispersed polymer beads.
- Suitable for separation of albumin, interferon.
- Specially developed in cooperation with commercial manufacturers

**Specifications:**

| | |
|---|---|
| Particle Size (μm) | 50 |
| Pore Size (Å) | 800 |
| Ligand Density | > 20 nM Cibacron Blue 3G/mL |
| Target Protein Binding Capacity (g/ml) | ≥ 18 mg human serum albumin |
| Bead Matrix | Polyacrylate |
| Recommended Flow Rate (cm/h) | 150-700 |
| pH Stability | 4-12 |
| Pressure (MPa) | 10 |
| Chemical stability | 40°C for 7 days in:<br>70% ethanol, 6 M guanidine Hydrochloride,<br>8 M urea |
| Storage buffer | 20% ethanol |
| Storage temperature | +4–30 °C |

※ EBL AM-50 Blue has high thermal stability and is autoclavable at 121°C for 20 min.

**Operation instruction:**

Columns and flow velocities

Table 2 lists columns recommended for AM-50 Blue resin. When packed medium with a 5cm bed height, the recommended packing flow velocity is at least 700cm/h, with a 15cm bed height at least 300cm/h, and with a 30cm bed height at least 150cm/h. The working flow velocity should not exceed 80% of the packing flow velocity.

Table 2. Recommended columns and flow velocities for AM-50 Blue

| Column Model(mm) | | Bed Volume |
|---|---|---|
| Inside Bed | Height(cm) | |
| 50 | 100-150 | 100-300 mL |
| 100 | 300 | 2.4L |
| 200 | 300 | 9.4L |

Dynamic binding capacity

The dynamic binding capacity of a chromatography medium is a function of the flow rate

used for loading of the sample. The binding capacity increases with decreasing flow rate. In addition, individual samples differ in their affinity for AM-50 Blue. To obtain an optimized purification scheme with respect to capacity and time, the capacity for the specific sample to be purified must first be determined over a range of different flow rates. Once this is known, the flow rate for loading can be determined to maximize binding of the sample in minimum time.

## Sanitization

Sanitization is the reduction of microbial contamination of the medium. Two suggested protocols are listed below:

Protocol I:

1. Equilibrate the packed column with a buffer consisting of 2% Hibitane digluconate and 20% ethanol.
2. Allow to stand for 6 h.
3. Wash with at least 5 bed volumes of sterile buffer.

Protocol II:

1. Equilibrate the packed column with sterile filtered 70% ethanol.
2. Allow to stand for 12 h.
3. Wash with at least 5 bed volumes of sterile filtered buffer.

## Cleaning-in-place (CIP)

Cleaning-in-place is the removal from the purification system of precipitated or denatured substances generated in previous production runs. A suggested protocol is:

1. Wash the packed column with 0.1M NaOH.
2. Wash with 3 to 4 bed volumes of 2M potassium thiocyanate.
3. Wash immediately with at least 5 column volumes of sterile filtered binding buffer at pH8

The medium can also be washed with 0.5M NaOH at 4° C. Further information on process hygiene can be found from other sources.

## Operation

AM-50 Blue is supplied pre-swollen in 20% ethanol, 0.1 M $KH_2PO_4$, pH 8.0.

1. After column packing, the medium bed should be washed with at least 3 column volumes of starting buffer to remove preservative.
2. When loading the sample, bear in mind the following.
3. We recommend the sample pH to be the same as that of the starting buffer.

4. It was recommended that the sample is filtered through a 0.22 to 0.45 μm filter to prolong the working life of the medium.
5. After loading the sample, wash the medium with starting buffer until the base line is stable. The medium can be washed at high flow rates.
6. Elution conditions need to be specifically optimized for each sample to obtain high purity and throughput.

## Storage

For long-time storage, e.g. several weeks, recommend the medium to be stored at 4- 8°C in 20% ethanol, 0.1M $KH_2PO_4$, pH 8.0.

**Application Examples:**

In the last twenty years, dye-based affinity chromatography was extensively studied and dozens of dyes were found was useful in protein and enzyme purification in an affinity manner. Blue 3G is the most extensively studied one of them. For details of the dyes successfully used in affinity chromatography, please see the following reference. The most important application areas for AM-50 Blue are the purification of interferon and albumin, as well as albumin removal. The high capacity and high flow rates make the medium suitable for both laboratory and process scale separations.

## Interferon

Albumin and interferon β both are adsorbed to the medium, but eluted under different conditions. In the first step, albumin is eluted using a salt- containing buffer, while interferon β is eluted in the second step using a buffer containing both salt and ethylene glycol.

## Human serum albumin purification

The adsorption of human serum albumin to the medium is dependent on the pH in the starting buffer. As the pH of the starting buffer decreases from 7.0 to 4.0, more albumin binds to the medium and less elutes from the column. Controlling binding in this way can help you increase the efficiency of albumin purification.

Column: 2 mL AM-50 Blue

Sample: 1.5 mL human serum albumin (20 mg/mL) in starting buffer

Flow rate: 2.0 mL/min (153 cm/h)

Start buffer A: 0.05 M Tris, 0.1M KCl, pH8.0

Start buffer B: 0.05 M citric acid, 0.1 M $Na_2HPO_4$, pH from 7.0 to 4.0

Bound efficiency (%): >85%HSA at pH7.0-8

Elution buffer A: 0.05 M $KH_2PO_4$, 1.5 M KCl, pH 7.0

Elution buffer B: 0.05M $KH_2PO_4$, 2.5M KCl, 10% ethanol

Elution buffer C: 0.05 M $KH_2PO_4$, 0.5N NaSCN

The above two starting buffers and three elution buffers can be used in your work in any combination. But we suggest Starting Buffer A and Elution Buffer C work together. As shown in our lab, Elution C is the best one in all tested elution buffers, and up to > 98% of the bund HSA was eluted from the beads.
Please note that BSA and HSA have quite different binding and characteristics even though they are homologous proteins in mammals. The protocol for HSA is not suitable for BSA purification.

## Human immunoglobulin G

Human immunoglobulin G (IgG) is an important group of proteins present in human blood with a wide range of diagnosis and therapeutic applications. Blue dye affinity has been successfully used in purification of IgG from human and other mammals.
Gondim et al (2014) reported that Blue 3G was a good absorbent for human serum IgG which adsorbed up to 110.9 mg human IgG at pH 7.8 with Tris-HCl buffer, and the bund IgG was eluted with this buffer containing 1 M NaCl. It was demonstrated that this buffer a suitable buffer system because it allowed for the highest recovered IgG capacity in the elution step. According to literatures, IgG adsorption is influenced by the pH and the type of buffer. When HEPES was used as buffer at pH 6.8, the highest IgG amount adsorbed was reached (76.56 mg/ g). In a previous work (Gondim et al. 2012) the IgG adsorption onto E-Ch/Al-Cibacron was carried out using only sodium phosphate 25 mmol/ L as buffer at different pH values. At pH 6.0, the highest amount adsorbed was 43.34 mg/ g. Among all buffers, MES presented the lowest amount of adsorbed IgG as compared to MOPS, HEPES and Tris-HCl. However, the amount adsorbed was higher to that measured for sodium phosphate, as reported by Gondim et al. (2012). In this study, IgG adsorption capacity was superior to 43.34 mg/ g for all buffers.

## Lysozyme Purification

Loading and Washing Buffer: 100 mM phosphate buffer, sodium acetate, 0.1M NaCl (pH 7.0).
Binding capacity: 342 mg/g matrix
Elution Buffer: 0.1M Tris-HCl, pH8.0, 0.5M NaCl.