

Theory and Introduction: Reactive Dye Affinity Chromatography is a form of affinity chromatography. Unlike affinity chromatography, where a small molecule is used as a ligand for a stationary phase Reactive Dye Chromatography uses mimics of natural protein ligands to act as a pseudo-affinity chromatography. Reactive dyes are textile dyes that are immobilized to a solid support such as agarose. The dyes act as a competitive inhibitor for a protein's normal ligand (co-enzyme, substrate...). There are a wide variety of dyes that can be used in large scale as affinity chromatographies. The dyes serve as an inexpensive and stable alternative to true affinity chromatography. There are literature reports of up to 60% of the proteins in a crude lysates binding to one or more dyes. Many of the dyes bind to proteins that use NADH, NADPH or ATP, although there are plenty of examples of other proteins that can bind reactive dyes. Lactate Dehydrogenase and Malate Dehydrogenase have both been purified by dye chromatography. Typically one would conduct several small-scale tests with a number of dyes to determine which dye binds the protein analyte.

Important Points to Consider For Reactive Dye Affinity Chromatography

- **Preparation of resin** - Resins are supplied as a dry powder or pre-swollen in solution. The resin for this lab will already be prepared. Please remember to save your unused resin. There is a container for the used resin in the refrigerator.
- **Column and Sample Preparation** - Use a one or two ml column volume and prepare by resuspending a 50% slurry and pipette 2-4 ml into your column. Simple gravity flow in the plastic column should suffice although you can use a 2 ml flow rate for the glass columns and adaptors but slow down the rate to 0.5 ml/min when loading the protein onto the column. The slower flow rate allows weaker binding proteins the time to equilibrate with the solid phase.
- **Buffer selection** - The protocol below is very specific for buffer selection.
 - Use a pH in the range of 6.5 to 8.5. The dyes are stable but the pH should be stable for your protein. For MGH, chose a buffer with a concentration of 10 to 25 mM and a pH of 8.0.
 - The affinities for most proteins are diminished when the salt concentration is near or greater than 200 mM NaCl. Ensure that your solution has less than 50 mM salt by dilution with a buffer or dialysis prior to loading onto the column.
 - Use a final wash with a buffer that includes 1M NaCl to remove any tightly bound proteins.
- **Elution** - You can experiment with the concentration of NADH or / and salt to achieve an optimal purification. This chromatography can easily be conducted using gravity flow or using the glass columns with column adaptor and pump. Your choice. If using gravity, use the plastic column.

General Protocol for MGH Purification Using Reactive Dye Affinity Chromatography -

1. Sample Preparation - Save a sample of the lysate for later analysis. Freeze in a microfuge tube. After saving the fraction, add ensure the protein load is at an appropriate salt concentration to bind to the dye (see above).
2. Resuspend the Reactive Blue Dye resin by inverting the bottle several times. The resin comes in a 50% slurry in 20% ethanol and can be found in the refrigerator.
3. Pipette 2-4 ml of slurry (1-2 ml of resin) into the column and wash 10 ml of equilibration buffer through the resin. It is your choice if you wish to pump buffer through the column or use a gravity flow method.
4. If using gravity flow to run your column, drain the remaining buffer until it reaches the top of the resin bed and gently add the protein load. Do not let the column resin swirl, use a pipettor to slowly drip the solution down the side of the column. Do not let the resin bed dry out. Save the flow through as one fraction.
5. Wash the column once with 5 column volumes of wash buffer. Collect one ml fractions.
6. Elute the protein with 3 column volumes of elution buffer. Collect one ml fractions.
7. Wash the column with the high salt buffer. Use 5 column volumes and collect one ml fractions.
8. Analyze each tube for the protein for total protein concentration (Bradford assay) and MGH (Fluorescence Assay).
9. Prepare a chromatograph showing both total protein concentration and MGH concentration for the samples.
10. Pool fractions as indicated in the purification handout.

Dye Equilibration buffer (50 ml)
10 mM Tris-Cl Buffer pH 8.0

Dye Wash Buffer (20 ml)
10 mM Tris-Cl Buffer pH 8.0
50 mM NaCl

Dye Elution Buffer (10 ml)
10 mM Tris-Cl Buffer pH 8.0
0.5 mM NADH
- (powder in freezer) Let warm to room temp before opening
50 mM NaCl

Dye High Salt Buffer (20 ml)
10 mM Tris-Cl Buffer pH 8.0
1M mM NaCl

Note: Prepare enough Dye Equilibration buffer for each buffer and prepare the specific wash and elution buffer using that stock. This will save you time!