

**Theory and Introduction: Ni-Affinity Chromatography** uses the ability of His to bind nickel. Six histadine amino acids at the end of a protein (either N or C terminus) is known as a 6X His tag. Nickel is bound to an agarose bead by chelation using nitroloacetic acid (NTA) beads. Several companies produce these beads as His Tagged proteins are some of the most used affinity tags in today's market. See the website for links to the handouts to Qiagen and Pharmacia, two commercial sources of NTA-Agarose resins. The general method is to batch absorb the protein onto the column, by mixing the beads with the sample, then pouring the slurry of NTA beads and protein into a column, where low concentrations of phosphate and imidazole are used to remove low affinity bound proteins. If needed, the imdazole can be increased to 20 mM before most His tagged proteins are eluted. Finally, higher concentrations of imidazole is used to elute the protein from the NTA-beads.

### Important Points to Consider For Ni-Affinity Chromatography

- Preparation of resin - The NTA-Agarose beads are very expensive and need to be saved and recycled. As long as the beads have a light blue tint to them, there is still nickel bound to the beads. The blue is due to the metal (think of the general chemistry lab where you precipitated nickel).
- Column and Sample Preparation - For this purification you should use the plastic column and a 50 ml falcon tube. No pump is necessary; simply allow gravity to draw the buffer and sample through the column.
- Buffer selection - The protocol below is very specific for buffer selection.
  - Increasing the pH of the equilibration buffer from 7 to 8 will increase the binding of your protein to the NTA beads, but will also increase non-specific binding of other proteins. Start using a pH 7.0 buffer unless you are having a difficult time getting the protein to bind to the beads.
  - Adding imidazole to your buffer, will change the pH of the solution. Double check the pH of the solution after adding imidazole.
  - If there is a high level of contaminant, the imidazole in the equilibration buffer can be increased to 50 - 75 mM.
  - Imidazole has a higher affinity for the metal than does histadine. Look at the structure of histadine and imidazole... they are basically the same functional group.
  - NOTE: free imidazole has been known to denature protein when freezing and thawing. Therefore, it is important to continue to the next chromatography before freezing sample.
  - If you are using this chromatography in the first step of a purification, you should dialyze the sample against a buffer without imidazole before freezing (1X PBS is a good choice).
- Elution - You can experiment with the concentration of imidazole and salt to achieve an optimal purification.
- Regeneration - Return the resin to the container in the hood. The beads can be regenerated with 10 column volumes of the following: 1) MES Buffer wash at pH 5.0, 2) wash with water, 3) 20% EtOH. Store the beads in 20% EtOH.

**General Protocol for MGH Purification Using Ni-Affinity Chromatography -**

1. Sample Preparation - ensure your protein solution is clear and not cloudy. Cloudy solution forms from precipitated protein. Remove ppt by centrifugation in the centrifuge at the front of the room or filter the solution through a .45 mm syringe filter. Save a sample of the lysate for later analysis. Freeze in a microfuge tube. After saving the fraction, add 10 ml of equilibration buffer to the lysates or sample from an earlier chromatography.
2. Resuspend the resin by inverting the bottle several times. The resin comes in a 50% slurry in 20% ethanol. The resin is 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. The resin has about a 3 mg of protein / ml of resin binding capacity.
4. Transfer the beads and load solution to a 50 ml tube. Incubate at room temp for 30 - 60 min with rocking to keep the beads in suspension.
5. Transfer the beads and solution to the column with the bottom closed and let the beads settle.
6. Drain the remaining buffer until it reaches the top of the resin bed. Do not let the resin bed dry out. Save the flow through as one fraction.
7. Wash the column once with 5 column volumes of wash buffer. The wash buffer has a low concentration of imidazole which helps wash off non-specifically bound proteins. Collect one ml fractions.
8. Elute the protein with 5 column volumes of elution buffer. Collect one ml fractions. Most of the protein will elute in the second and third fraction.
9. Analyze each tube for the protein for total protein concentration (Bradford assay) and MGH (Fluorescence Assay). Imidazole may interfere with the assay giving a false-positive reading.
10. Prepare a chromatograph showing both total protein concentration and MGH concentration for the samples.
11. Pool fractions as indicated in the purification handout.

**6XHis Wash Buffer (25 ml)**

50 mM Phosphate Buffer pH 7.0  
300 mM NaCl  
1 mM Imidazole  
Adjust pH to 7.0 w/NaOH

**6X His Elution Buffer (10 ml)**

50 mM Phosphate Buffer pH 7.0  
300 mM NaCl  
150 mM Imidazole  
Adjust pH to 7.0 w/NaOH