# MDH Column Purification Protocol:

## *His Tag-Recombinant Protein Purification*

**Theory and Introduction**: **Ni-Affinity Chromatography** uses the ability of His to bind nickel. Six histidine 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 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 in the correct oxidation state.
- **Average binding capacity is about 5-10 mg** of His tagged protein per ml of beads. Some companies claim their media can bind up to 100 mg of protein per ml of beads. Depending on many factors protein expression can range from 2 - 100 mg of His tagged protein per liter of medium.
- **Expression level** – Of course the actual yield of His-tagged MDH depends on conditions and actual clones. Most of the MDH clones are cut into pET28 vectors which are strong promotor/expression systems. wgMDH is cut into a pQE vector (also a strong expression system). wgMDH, hMDH1, hMDH2 are all high to medium high expressors with 20-100 mg of purified protein from a 1000 ml culture. Use this information to adjust column bed size to meet individual experimental needs..
- Column and Sample Preparation – For this purification you should use the plastic column. 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. Lowering pH to 6.8 or adding 10-50 mM imidazole can reduce binding of endogenous proteins but reduce binding capacity
- 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 histidine. Look at the structure of histidine and imidazole... they are basically the same functional group.
- Fee 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.

***Cell lysate Preparation***: The key to preparing a lysate (broken cells) is to perform this as quickly as possible while keeping the preparation at low temperature to minimize protease degradation and protein denaturation. A good cell lysate will be clear (no particulate and yellow/tan in color) and not viscous. Viscosity is due to genomic DNA and will cause many problems during chromatography. Pre-Chill buffers, centrifuge heads and other equipment. Keep your solutions and cell pellets IN the ice not ON the ice. For proteins that aggregate or membrane proteins, additional additives include a final concentration of 10% glycerol (aggregation) 0.1% Triton X-100 (aggregation and membrane proteins).

***Lysis Instructions:*** Follow lysis protocol for instructions. Freeze/thaw cycles of lysate will result in precipitation proteins.

***Column Basics***

- 1 liter culture will need 20 ml of beads (use less for low expressors, adjust bed volume based on culture size)
- In general, scale the washes and elution using the information below.
- A rule of thumb is to wash with 10-20 column volumes (volume of packed beads) for the wash buffer.

- Elute in a total of 5-10 column volumes of elution buffer.
- Divide the elution into 8-12 total fractions. High expressing proteins may need more elution buffer.

**Important Note: A quick check of wash and elution fractions with a bradford assay (20 ul sample mixed with ~1 ml of 1X bradford) will inform you the relative amount of protein in the fraction.**

***Purification Instructions (per 1000 ml culture adjust to the appropriate volumes as per information above):***

- ***Preparation of Ni-Agarose Beads/Resin***:
  - Prepare 20 ml of beads by transferring 50 ml of a 50% slurry of beads equilibrate into a clean column. Wash and equilibrate the column by running 200 ml of His Elution Buffer followed by 500 ml of His Binding Buffer through the column. This SHOULD be done ahead of time! Store prepared beads with a few ml of His Binding buffer at RT.
- **Purification**:
  - Save 100 ul of lysate. Add clarified lysate (if frozen, check for ppt material. If there is any clumpy or ppt material or if the lysate is cloudy, centrifuge and keep the supernatant) to the washed beads.
  - *Batch Binding* - if protein expression is low or the His-tagged protein binds with low affinity then use a batch purification method.
    - Combine the washed beads and lysate onto a drained and capped column or a 50 ml falcon tube for smaller volumes. Use a spatula and/or a transfer pipette to suspend the beads. Tightly cover with parafilm and incubate rocker for 30 min at room temp.
    - Replace the column on the stand and allow most of the beads settle then open column. Add frit back to column. ***Reapply the flow thru***. This is the non-binding protein. Continue with purification
  - *Column Binding* - Flow the clarified lysate through the column. Save the eluate as flow-through in one fraction. If there is a concern with binding efficiency the flow-through can be reapplied. Continue with the purification.

After binding

- Wash beads ~ ***250 ml of Ni-Column His Binding Buffer***. Save as flow through wash in one fraction.
- Wash the column with ***~500 ml of His Wash Buffer***. *This will remove some of the weakly binding protein. (check flow through for protein with quick bradford). Continue with the His wash buffer until no detectible protein is found in eluate.
- Elute the protein with ***20 x 15 ml of His Elution Buffer***. Save ALL fractions. Stop eluting only when protein is no longer detected in the eluate. Check for eluted protein with Quick Bradford*.
- Check each fraction for total protein and determine purity by SDS-PAGE – Coomasie stain

*Quick Bradford. ~0.5 ml of 1X bradford plus ~20 ul or one drop of eluate or sample. If the dye turns blue-ish protein is present

| ***His-Binding and lysis Buffer:*** | ***His-Wash Buffer:*** | ***His-Elution Buffer:*** |
|---|---|---|
| • 50 mM Tris-Cl (pH8.0)<br>• 1 mM Imidazole<br>• 100 mM NaCl<br>• 0.1 mM EDTA<br>• * include 1 mM PMSF made fresh | • 50 mM Tris-Cl (pH8.0)<br>• 300 mM NaCl<br>• 10 mM Imidazole<br>• 0.1 mM EDTA<br>• include 1 mM PMSF made fresh | • 50 mM Tris-Cl (pH8.0)<br>• 50 mM NaCl<br>• 300 mM Imidazole<br>• 0.1 mM EDTA<br>• include 1 mM PMSF made fresh |

NOTE: Some use 50 mM Na Phosphate buffer in place of Tris buffer.

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