Protocol

TD-P Revision 3.0

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Affinity His-Tag Purification
Utilizing Chelating Agarose Beads

Introduction

The purification of recombinant target proteins can be achieved using a powerful polyhistidine tagging system that allows binding of target proteins labeled with a tag consisting of histidine residues to resins/agarose beads containing immobilized metal ions (Co^{2+}, Ni^{2+}, Cu^{2+}, or Zn^{2+}). Use of Nickel NTA magnetic beads makes this process easier and faster. Tagged proteins are first incubated with the magnetic beads. Then, the beads are separated from the supernatant, followed by washing of non-specifically bound protein. Finally, His-tagged proteins are eluted from the magnetic beads. Purified proteins can then be analyzed using various methods including SDS-PAGE and Western blotting, or used in protein-protein interaction studies. Here, we present a protocol for purification of His-tagged protein that includes the packing of a column, purification of the protein, and a chelating resin regeneration procedure.

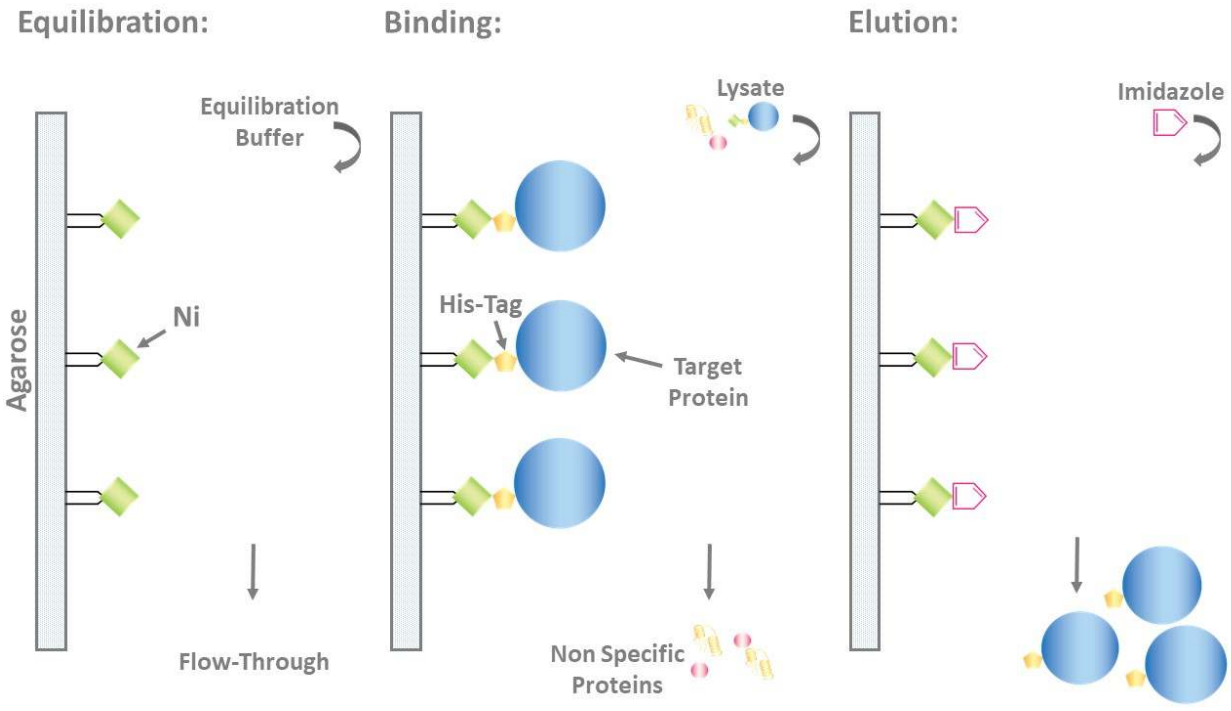

Figure 1. Purification of His-tagged target proteins using a Ni resin.

Gold Biotechnology
St. Louis, MO
Ph: (800) 248-7609
Web: www.goldbio.com
Email: contactgoldbio86@goldbio.com

Materials

- Agarose Beads
  - Cobalt Agarose Beads High Density (GoldBio Catalog # H-310)
  - Copper Agarose Beads Low Density (GoldBio Catalog # H-311)
  - Nickel Agarose Beads (GoldBio Catalog # H-320 or # H-321)
  - Nickel NTA Agarose Beads (GoldBio Catalog # H-350)
  - Nickel NTA HTC Agarose Beads (GoldBio Catalog # H-355)
  - Nickel NTA Magnetic Agarose Beads (GoldBio Catalog # H-351)
  - Zinc Agarose Beads (GoldBio Catalog # H-330 or # H-331)
  - Metal Free Agarose Beads (GoldBio Catalog # H-300 or # H-301)
- Distilled water
- Binding Buffer: 50mM acetate or phosphate (10-150mM) with imidazole (10-40mM) to increase binding selectivity
- 0.5M Imidazole (GoldBio Catalog # I-901 or # I-902)
- See Table 1 for suitable reagents for specific studies
- GoldBio Plastic Columns (GoldBio Catalog # P-301)

Method

These resins are adapted for purification performed under native or denaturing conditions and are compatible with batch processing or column purification with gravity flow. The following procedure describes packing a column with resin and purification of His-tagged proteins under native conditions. The binding strength of the protein to the resin will depend on the type of resin used (both the number of chelate groups and the chelating metal), accessibility of the His-tag, pH, and buffer composition. This product is supplied as a suspension in 20% ethanol.

Recombinant proteins can form insoluble aggregates or inclusion bodies that require solubilizing by purifying the tagged protein under denaturing conditions using reagents such as urea or guanidine chloride at specific stages (see Table 1).

Table 1. Reagent classes and concentrations for His-Tag resin.

Studies	Reagents
Chemical Stability	HCl 0.01M, NaOH 0.1M, Ethanol 20%, Sodium Acetate pH 4.0, SDS 2% 2-propanol, NaOH 1M, HAc 70%
Denaturing Agents	Urea 8M, Guanidine-HCl 6M
Detergents	Triton X-100 2%, Tween 20 2%, Chaps 1%
Additives	Imidazole 2.0M, Ethanol 20% + Glycerol 50%, Na_2SO_4 100mM, NaCl 1.5M, EDTA 1mM, EDTA 1mM + $MgCl_2$ 10mM, Citrate 60mM,

	Citrate 60mM + $MgCl_2$ 80mM
Reducing Agents[a]	Reduced Glutathione 10mM, B-mercaptoethanol 20mM, DTE 5mM, DTT 5mM
Buffers	Na_2HPO_4 50mM pH 7.5, Tris-HCl 100mM pH 7.5, MOPS 100mM pH 7.5, Tris-acetate 100mM pH 7.5, HEPES 100mM pH 7.5

[a] Under extended treatments with reducing agents, or in processes where high concentrations of these reagents are used, reduction of the metal ion may result. This will affect the binding capacity of the resin, so these agents should be avoided. The reagents described in the table are compatible with Nickel Activated Agarose Beads (Nickel is most commonly used) under the conditions and concentrations indicated in the table.

Column Packing

1. Manually shake the bottle to obtain a homogenous suspension of Chelate Agarose beads. Refer to Table 2 for recommended working conditions.

Table 2. Recommended working conditions.

Linear Flow Rate	26 cm/hour
Recommended Flow Rate	0.5-1.0 ml/minute
Max. Pressure	2.6 psi (0.18 Bar)

2. Place a funnel in the head of the column and slowly run the suspension down the walls of the column.

Note: It is advisable to make the addition slowly to avoid the formation of bubbles. The product may also be degassed before being added to the column.

3. Decant the product by passing it through the column or pipetting it from the top of the column and discard most of the leftover liquid, leaving 1 cm above the column head to prevent the column from drying out.

4. Repeat previous steps until the desired column height is obtained.

5. Insert the adapter gently on the column head until it begins to displace the liquid.

Note: Make sure no air is trapped under the net.

6. Add distilled water to the purification stream until a constant height (corresponding to the height of the column) is achieved.

Note: If the desired height is not achieved, repeat steps 1 through 6.

7. When a constant height has been obtained, maintain the flow with the addition of 5 volumes of distilled water to completely eliminate the preservative.

Note: In the case of Metal Free Beads (GoldBio Catalog # H-300 and # H-301), the corresponding metal must be added before use, as described in the following steps:

a. For Metal Adsorption: Prepare a 0.1M acidic or neutral solution of the salt (chloride or sulphate) of the required metal (Zn^{2+}, Ni^{2+}, Cu^{2+}, Co^{2+}), by adding 5 column volumes of this salt solution.

b. For elimination of the Nonretained Metal: Wash the beads with 5-10 column volumes of distilled water to eliminate the non-retained metal.

Purification

1. Add 5-10 column volumes of binding buffer to equilibrate the column.

Note: The choice of buffer depends on the particular properties of the protein as well as the type of chelate used. The buffers used most frequently are acetate (50mM) or phosphate (10-150mM). The pH of binding buffers is generally neutral (pH 7.0-8.0) but can vary over a pH 5.5-8.5 range. To avoid ionic interchange, add 0.15-0.5M NaCl. Binding buffer is used during equilibration, washing, and elution steps, unless otherwise indicated.

Note: In some cases, to increase the selectivity of binding of a target protein, it is necessary to add a small concentration of imidazole (10-40mM) to the binding buffer. High purity imidazole must be used to avoid affecting the optical density (O.D.) at 280 nm.

Note: Avoid the presence of agents like EDTA or citrate at all times.

2. Once the resin is equilibrated, add the sample containing the fused protein for purification. In some cases, a slight increase of contact time may facilitate binding.

Note: Binding capacity can be affected by several factors such as sample concentration, binding buffer or the flow rate during sample application.

3. Wash the resin with binding buffer until the O.D. at 280 nm reaches the baseline level.

4. The tagged protein can be eluted in different ways:
    a. Addition of a competitive ligand (usually imidazole), which allows elution of the tagged protein. In general, 0.5M imidazole is sufficient for elution. It is also possible to use a concentration gradient of imidazole (0-0.5M). Most proteins

are eluted with ~250mM imidazole. Other reagents that can be used as competitive ligands are histidine and ammonium chloride.

Note: Generally, the subsequent elimination of imidazole is not necessary. However, if imidazole elimination is necessary, it may be done by dialysis, precipitation with ammonium sulfate or ultrafiltration.

b. Reduction of pH (between pH 3.0 and 4.0), with or without a gradient, also allows elution of the target protein.
c. Chelating agents like EDTA or EGTA (0.05M) cause elution of both the protein and the chelating metal as a complex.

Note: In some cases, an excessively drastic elution can be avoided by using different activated beads (another chelate cation with lower affinity or fewer groups) that might have weaker interactions with the target protein.

Note: Most applications do not require removal of the His-tag after elution. However, specific applications including X-ray crystallography or NMR, where the tag may interfere with structure or function of the purified protein, do require cleavage of the His-tag. Then, the tag can be cleaved at a protease cleavage site between the tag and the protein.

5. Store the purified protein at 2-8°C. Do not freeze.

Standard Chelating Resin Regeneration Procedure

Long-term use of resin can lead to decreased binding capacity and diminished protein yield due to protein retention. In addition, the use of the same resin to purify different types of protein can cause contamination of the eluted protein. To avoid these effects, the column should be regenerated (cleaned) when a change in protein yield is observed. The column should also be regenerated before using it in the isolation of a different protein to obtain stable and pure yields. Regeneration of chelating resins consists of cleaning the resin to completely eliminate the metal and retained protein. Then, the resin should be loaded with metal for future use (Figure 2).

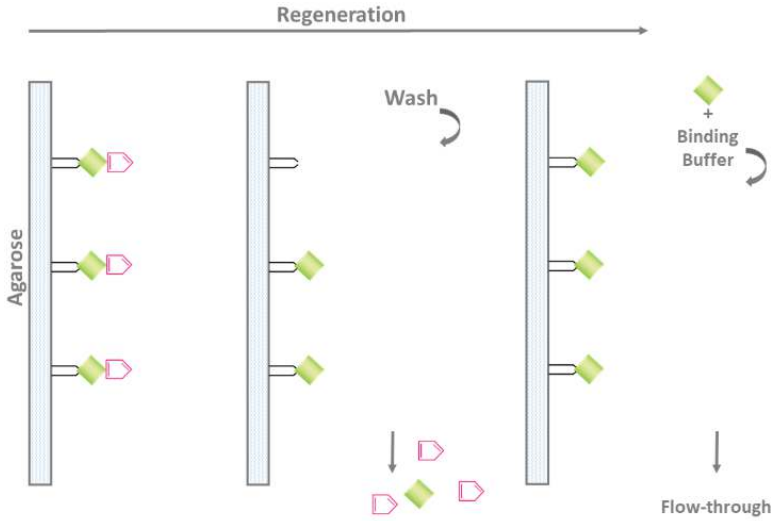

Figure 2. Regeneration of a chelating resin (Ni-resin shown here).

Standard Regeneration

1. To eliminate metal from the resin, wash with 10 column volumes of 20mM sodium phosphate containing 0.5M NaCl and 50mM EDTA at pH 7.0.

2. To eliminate residual EDTA before reloading the resin with the corresponding metal, wash with 5 column volumes of distilled water.

Note: More drastic regeneration steps may be necessary to eliminate denatured protein or lipids. If lipids or proteins remain after performing step 2 of the Standard Regeneration described here, perform a drastic regeneration as follows:

- **a. To eliminate ionic interactions, wash in batch for approximately 20 minutes in 1.5M NaCl. Wash with 10 column volumes of distilled water to eliminate ions.**
- **b. To eliminate precipitated proteins, which may be responsible for column pressure changes, wash in batch for at least 2 hours with 1.0M NaOH. Wash with 10 column volumes of distilled water to eliminate residual NaOH.**
- **c. To eliminate strong hydrophobic interactions, resuspend the resin in batch with 30% isopropanol for ~20 minutes. Wash with 10 column volumes of distilled water to eliminate the isopropanol.**
- d. **Wash in batch for 2 hours with a 0.5% non-ionic detergent in 0.1M acetic acid. Rinse away the detergent with 70% ethanol (approximately 10 column volumes).**

3. To load the column with the corresponding metal, add 5 column volumes of 0.1M metal solution (normally chlorides or sulphates are used).

4. To eliminate excess metal, wash with 5 column volumes of distilled water.

5. To prepare the column for the next purification cycle, add 5 column volumes of binding buffer.

Note: If the resin is not going to be used for a while, replace the last step with the addition of a preservative (20% ethanol). It is also recommended to include a regeneration step when beginning to purify a new protein.

Tips

- To prevent aggregation (nonspecific binding of the target protein with Histidine residues in adjacent proteins), add 10mM 2-mercaptoethanol to loading, washing, and elution buffers.
- Addition of Triton X-100 or Tween-20 to the elution buffer might reduce disulfide bonds with other proteins.
- Salt and imidazole concentrations might require optimization for the specific target protein.
- To prevent nonspecific hydrophobic interactions, add salt (500mM maximum), glycerol (20% maximum), or ethanol (20% maximum) during elution.
- Buffer components might require optimization for the specific target protein.
- Perform cell lysis during sample preparation in a buffered solution, pH 8.0.
- Consider saving the flow-throughs from washing and binding steps since they might contain your target protein, if the target protein did not efficiently bind the resin.
- Perform column packing and purification procedures in a cold room or at 4°C to prevent denaturing of the protein.

Associated Products

- Cobalt Agarose Beads (GoldBio Catalog # H-310)
- Copper Agarose Beads (GoldBio Catalog # H-311)
- Nickel Agarose Beads (GoldBio Catalog # H-320 or # H-321)
- Zinc Agarose Beads (GoldBio Catalog # H-330 or # H-331)
- Metal Free Agarose Beads (GoldBio Catalog # H-300 or # H-301)
- Guanidine HCl (GoldBio Catalog # G-211)
- L-Glutathione, Reduced (GoldBio Catalog # G-155)
- MOPS (GoldBio Catalog # M-790 or # M-791)
- DTT (GoldBio Catalog # DTT)
- Tris Acetate (GoldBio Catalog # T-090)
- This HCl (GoldBio Catalog # T-095)
- HEPES (GoldBio Catalog # H-400 or # H-401)

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