Affinity His-Tag Purification Chelating Agarose Beads (Bulk Resins)
Detailed Procedure for Use

Introduction
The resins are adapted to work mainly in native conditions.

The following summarized procedure is adapted for the purification of His-tagged proteins under native conditions. The strength of binding of the protein to the resin will depend on: the resin employed (both the number of chelate groups and the chelant metal), the accessibility of the His-tag, the pH and the buffer composition.

Associated Products
- Cobalt Agarose Beads (GoldBio Catalog # H-310)
- Copper Agarose Beads (GoldBio Catalog # H-311)
- Nickel Agarose Beads (GoldBio Catalog # H-320 or GoldBio Catalog # H-321)
- Zinc Agarose Beads (GoldBio Catalog # H-330 or GoldBio Catalog # H-331)
- Metal Free Agarose Beads (GoldBio Catalog # H-300 or GoldBio Catalog # H-301)

Note: All Agarose bead resins are supplied as a suspension in 20% ethanol.

Method
1. Elimination of the Preservative –
   Wash the beads with 5-10 column volumes of distilled water to eliminate the preservative.

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

   a. Metal adsorption: Prepare acid or neutral solution 0.1M 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 to the column.

   b. Elimination of non-retained metal: Wash the beads with 5-10 column volumes of distilled water to eliminate the non-retained metal.
2. Equilibration of the Resin –
Equilibrate the column with 5-10 column volumes of binding buffer.

**Binding buffer:** The choice of buffer depends on the particular properties of the protein as well as of the type of chelate used. The buffers used most frequently are acetate (50mM) or phosphate (10-150mM). The pH of binding buffers generally leads to neutrality (pH 7.0-8.0), but can vary over the range 5.5-8.5. To avoid ionic interchange, add 0.15 - 0.5M of NaCl.

**Note:** In some cases to increase the selectivity of the binding of target protein it is necessary to add to the binding buffer a small concentration of imidazole (10-40mM). It is important to use high purity imidazole to avoid affecting the O.D. 280 nm. It is important to avoid the presence of agents like EDTA or citrate at all times.

3. Application of the Sample –
Once the resin is equilibrated, the sample containing the fused protein for purification is applied. 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.

4. Washing of the Resin –
It will be washed with the binding buffer until the O.D. 280 nm reaches the baseline level.

5. Elution of the Pure protein –
The elution of the protein can be done in different ways:

a. Addition of a competitive ligand (generally imidazole), which allows the elution of the retained protein. In general, 0.5M of imidazole is enough to elute the protein. It is also possible to use concentration gradients of this reagent (0-0.5 M). Most proteins are eluted in concentrations around 250mM. Other reagents that can be used as competitive ligands are histidine and ammonium chloride.

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

b. Reduction of pH (with or without gradient), also allows the elution of the desired protein (between pH 3.0 and 4.0).

c. A more drastic method uses reagents like EDTA or EGTA (0.05M), which causes the elution of both the protein and chelating metal.
Note: In some cases, an excessively drastic elution can be resolved through the use of other activated beads (another chelate cation with lower affinity or fewer groups), where the interaction is weaker.

Note: For most of the applications it is not necessary to eliminate the His-tag. However, this elimination is necessary for certain applications such as X-ray crystallography or RMN, where the protein structure is to be determined later.

For these purposes, a His-tag is usually spliced to the protein, at a protease cleavage site.

6. Storage – Keep at 2-8°C. Do not freeze.

The recombinant proteins often form insoluble inclusion bodies. If so, these need to be rendered soluble by purification under denaturing conditions, using for example urea or guanidinium chloride at relevant stages (see table below).

<table>
<thead>
<tr>
<th>Studies</th>
<th>Reagents</th>
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<tbody>
<tr>
<td>CHEMICAL STABILITY</td>
<td>HCl 0.01M</td>
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<tr>
<td></td>
<td>NaOH 0.1M</td>
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<tr>
<td></td>
<td>Ethanol 20%</td>
</tr>
<tr>
<td></td>
<td>Sodium acetate, pH 4.0</td>
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<tr>
<td>DENATURING AGENTS</td>
<td>Urea 8M</td>
</tr>
<tr>
<td>DETERGENTS</td>
<td>Triton X-100 2%</td>
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<tr>
<td></td>
<td>Tween 20 2%</td>
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<tr>
<td>ADDITIVES</td>
<td>Imidazole 2.0M</td>
</tr>
<tr>
<td></td>
<td>Ethanol 20% + glycerol 50%</td>
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<tr>
<td></td>
<td>Na₂SO₄ 100mM</td>
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<tr>
<td></td>
<td>NaCl 1.5M</td>
</tr>
<tr>
<td>REDUCING AGENTS*</td>
<td>Reduced glutathione 10mM</td>
</tr>
<tr>
<td></td>
<td>β-mercaptoethanol 20mM</td>
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<tr>
<td>BUFFERS</td>
<td>Na₂HPO₄ 50mM, pH 7.5</td>
</tr>
<tr>
<td></td>
<td>Tris-HCl 100mM, pH 7.5</td>
</tr>
<tr>
<td></td>
<td>MOPS 100mM, pH 7.5</td>
</tr>
<tr>
<td></td>
<td>Tris-acetate 100mM, pH 7.5</td>
</tr>
<tr>
<td></td>
<td>HEPES 100mM, pH 7.5</td>
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</tbody>
</table>

* Note: 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 Packaging**

1. Manually shake the bottle to obtain a homogenous suspension of Chelate Agarose Beads/preservative. Place a funnel in the head of 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 added to the column.

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

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

3. Insert the adapter gently in the column head until it begins to displace the liquid.

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

4. 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 4.

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

6. Equilibrate the column with 5 to 10 column volumes of binding buffer.

   **Note:** It is advisable to previously de-gas all the solutions before adding to the column to avoid the formation of bubbles.

**Recommended Work Conditions**

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

During the life of the resin, it may lose binding points because some protein is retained. Hence a loss of the binding capacity may be observed in successive cycles. To return to the starting state, regeneration may be necessary. Regeneration consists of the complete elimination of the metal and therefore of the retained protein.

In general, column regeneration is always necessary when changing proteins. When continuing with the same protein it is recommended to do a regeneration when an appreciable diminution in the yield is observed. The frequency of these stages varies with the protein and the conditions used.

1. Elimination of the metal from the resin: It is necessary to wash the resin with 5 column volumes of a solution 20mM sodium phosphate containing 0.5M NaCl, 50mM of EDTA at pH 7.0.

2. Elimination of the excess EDTA: In order to eliminate the residual EDTA before reloading the resin with the corresponding metal, the column should be washed with 5 column volumes of distilled water.

   Note: In some special cases it is advisable to make more drastic intermediate stages of regeneration to eliminate denatured proteins or lipids:

   Drastic Regeneration Stage: denatured proteins and lipids elimination.

Use when denatured proteins or lipids may have been retained after step 2.

   a) Elimination of ionic interactions: Wash in batch for approximately 20 minutes in a solution with 1.5M NaCl. Later wash with 10 column volumes of distilled water to eliminate ions.

   b) Elimination of precipitated proteins (may be responsible for column pressure changes). Wash in batch at least 2 hours with a solution 1.0M NaOH. Eliminate the NaOH with 10 column volumes of distilled water.

   c) Elimination of strong hydrophobic interactions: resuspend the resin in batch with a solution of isopropanol 30% for approximately 20 minutes. Then wash with 10 column volumes of distilled water to eliminate the isopropanol.

   d) Wash in batch for two hours with a 0.5% non-ionic detergent in 0.1M acetic acid. Rinse the detergent with ethanol 70% (approximately 10 column volumes).

   e) Finally wash with 10 column volumes of distilled water to rinse out the ethanol.
3. Load the column with the corresponding metal: once the excess EDTA has been eliminated, add 5 volumes of the 0.1M metal solution (normally chlorides or sulfates are used).

4. Elimination of the excess of metal: wash with 5 column volumes of distilled water.

5. Preparation of the column: add 5 column volumes of the binding buffer.
   
   Note: If the resin is not going to be used for a while it is recommended to replace the last step by the addition of the preservative.
   
   Also it is recommended to include a regeneration step when beginning to purify a new protein.

References


