Profinity™ IMAC Resins

Instruction Manual

Please read these instructions prior to using Profinity IMAC resins. If you have any questions or comments regarding these instructions, please contact your Bio-Rad Laboratories representative.

BIO-RAD

Table of Contents

Section 1 **Introduction** 1

Section 2 **Product Description** 1

Profinity™ IMAC Resins and UNOsphere™ Technology ... 1

Chemical Interactions 2

Resin Characteristics 3

Chemical Compatibilities 4

Section 3 **General IMAC Procedures** 5

Protein Binding 5

Washes 5

Elution 6

Purification Under Denaturing Conditions 6

Purification Under Nondenaturing Conditions 7

Imidazole Concentrations 7

Section 4 **Column Packing — Medium-Pressure Columns** 9

Slurry Packing an IMAC Column 9

Recommended Columns 9

Materials 9

Resin Preparation 10

Method 10

Section 5 **Column Packing — Sample Preparation-Sized Columns** 12

Materials 12

Resin Preparation 12

Method 12

Section 6 **Immobilizing Metal Ions** 14

Section 7 **Preparation of Clarified *E. coli* Lysate Using Nondenaturing Conditions** 15

Materials 15

Method 16

Section 8 **Preparation of Clarified *E. coli* Lysate Using Denaturing Conditions** 18
Materials 18
Method 19
Section 9 **Medium-Pressure Column Purification of Histidine-Tagged (His-Tagged) Proteins Using Nondenaturing Conditions**....21
Materials 21
Method 22
Section 10 **Medium-Pressure Column Purification — Using an Imidazole Gradient to Determine Optimal Purification of His-Tagged Proteins**....23
Materials 23
Method 24
Section 11 **Medium-Pressure Column Purification of His-Tagged Proteins Using Denaturing Conditions**....27
Materials 27
Method 29
Section 12 **Sample Preparation-Size Spin-Column Purification of His-Tagged Proteins Using Nondenaturing Conditions**....31
Materials 31
Method 32
Section 13 **Sample Preparation-Size Spin-Column Purification of His-Tagged Proteins Using Denaturing Conditions** 34
Materials 34
Method 35
Section 14 **Batch-Mode Purification of Histidine Proteins — Gravity Flow**....37
Recommended Columns....37
Materials 37
Resin Preparation....38
Method 38
Section 15 **Regenerating, Cleaning, Sanitizing, and Storage** 40

Regenerating the Medium 40
Cleaning-in-Place 40
Sanitization.....41
Storage 42
Section 16 **Troubleshooting Guide** 43
Section 17 **Ordering Information** 47
Section 18 **References**.....47
Section 19 **Legal Notices**.....47

Section 1
Introduction

Immobilized metal affinity chromatography (IMAC) is a powerful purification technique that relies on a molecule's affinity for certain metals immobilized onto a chelating surface. The chelating ligand, iminodiacetic acid (IDA) in this case, may be charged with transition metals such as Cu^{2+}, Ni^{2+}, Co^{2+}, or Zn^{2+}. This results in the high selectivity of proteins with clustered histidine residues to be strongly retained onto a porous chromatographic support. Jerker Porath and colleagues first identified the role of IMAC in protein purification in the 1970s, which incorporated the use of IDA as its chelating ligand (Porath et al. 1975). The use of IMAC to separate an expressed recombinant protein fused with a hexa-histidine peptide tag was demonstrated by Hochuli (1988) to yield a highly purified protein in a single chromatographic step under both denaturing and native conditions. The strong affinity of a histidine-tagged (His-tagged) molecule for metal ions often makes extensive optimization unnecessary while also allowing chromatography under conditions that denature proteins. For this reason, expression and IMAC purification of His-tagged proteins is frequently used for structural and functional studies of proteins.

Section 2
Product Description

Profinity™ IMAC Resins and UNOsphere™ Technology

Profinity IMAC resin, a unique affinity support, is based on Bio-Rad's innovative UNOsphere beads, which use proprietary polymerization and derivatization technologies.* The UNOsphere technology enables the polymeric Profinity IMAC to exhibit excellent flow properties without compromising protein binding, recovery, or purity.

Profinity IMAC uses IDA as its functional ligand. The tertiary amine and carboxylic acid side chains of IDA serve as the chelating ligands for di- or trivalent metal ions. The structure offers selective binding of recombinant His-tagged proteins when this resin is charged with Ni^{2+} or other transition metals. As a result, the desired proteins can often be purified close to homogeneity in a single step.

Structural characteristics such as the polymeric nature, optimized ligand density, and the open pore structure of the Profinity IMAC bead result in superb mechanical strength with high stringency, low nonspecific effects, and the ability to perform separations at extremely fast flow rates. These unique features of the UNOsphere base matrix lend a number of performance benefits to the Profinity IMAC resin.

*US patent 6,423,666.

Profinity IMAC is also stable across the entire pH range (1–14) and is compatible with most reagents commonly used in protein purifications, such as denaturants, detergents, and reducing agents. It is amenable to separations under native or denaturing conditions, using liquid chromatographic instrumentation, gravity flow columns, or sample-preparation spin columns.

Note: UNOsphere media, from which Profinity IMAC is derived, was designed to achieve the highest productivity (grams of drug or target per operational hour per liter of support) possible. UNOsphere media may be run at the highest rates and loading capacities while staying within the pressure limits of the column and chromatography system.

Under optimized conditions, the binding capacity for 6xHis tagged proteins is **>15 mg/ml resin** (see Table 1). The product is a 50% (v/v) slurry of resin, suspended in a 20% ethanol solution. Profinity IMAC is amenable to process and laboratory-scale use and is available bottled in two forms: uncharged and precharged with Ni^{2+}. Table 1 lists key characteristics of the resin, while Table 2 lists a variety of compounds compatible with Profinity IMAC.

Chemical Interactions

Profinity IMAC resin is comprised of IDA groups coupled to a UNOsphere base matrix via a proprietary polymerization derivatization technology. It is well suited for recombinant His-tagged purifications, and results in high binding capacity and specificity of the target molecule.

In addition to the number of histidine, tryptophan, and cysteine residues available for binding on a protein's surface, efficacy of protein purification by IMAC is also dependent on the number of coordination sites of the immobilized metal that are not occupied by the chelating ligand, which are then free to interact with proteins (Figure 1). In the case of Profinity IMAC Ni-charged resin, the chelating ligand is IDA. Ni^{2+} ions, which have six coordination sites bound to this tridentate chelator, will expose three coordination sites to the environment.

Although the most commonly used metal ion for His-tagged purifications is Ni^{2+}, other metals may be used to increase efficacy of purification. Therefore, a concerted choice on the type of immobilized metal ions used will dictate the selectivity of an IMAC resin.

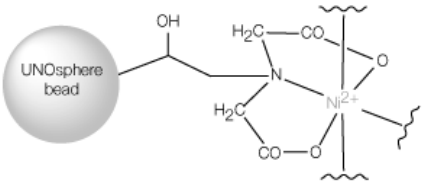

Fig. 1. Partial structure of Profinity Ni-charged IMAC resin.
Illustration indicates UNOsphere base bead with coupled IDA functional ligand. Wavy lines indicate available binding sites.

Resin Characteristics

The characteristics of Profinity IMAC resin are detailed in Table 1.

Table 1. Characteristics of Profinity IMAC Resin

Functional ligand	Iminodiacetic acid (IDA)
Base bead	UNOsphere base matrix
Form	50% suspension in 20% EtOH; comes precharged with Ni^{2+} or uncharged
Particle size	45–90 µm
Mean particle size	60 µm
Metal ion capacity	12–30 µmol Cu^{2+}/ml Profinity IMAC resin
Dynamic binding capacity*	≥15 mg/ml resin
Recommended linear flow rate	<600 cm/hr at 25°C
Maximum operating pressure**	109 psi
pH stability, uncharged resin* (up to 200 hr)**	1–14
Chemical compatibility	See Table 2
Storage	4°C to ambient temperature
Shelf life in 20% EtOH	>1 year at ambient temperature
Operational temperature	4–40°C
Autoclaving conditions	0.1 M sodium acetate at 120°C for 30 min

*** Dynamic binding capacity conditions ($Q_{10\%}$ determination):**

Column volume:	1 ml, (ID x H), 7 mm x 2.6 cm
Sample:	1.8 mg/ml 6xHis-tagged pure protein (32 kD)
Flow rate:	1 ml/min loading, and 2 ml/min washing and eluting
Loading buffer:	50 mM sodium phosphate, 300 mM NaCl, 5 mM imidazole, pH 8.0
Washing buffer:	50 mM sodium phosphate, 300 mM NaCl, 10 mM imidazole, pH 8.0
Elution buffer:	50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole, pH 8.0

Note: Dynamic binding capacity will vary from protein to protein.

**** Maximum pressure test:**

Profinity IMAC resin packed in a 1.1 x 30 cm Amicon column to a bed height of 20 cm with 20 mM sodium phosphate buffer up to 43 psi (3 bar). Flow rates were increased stepwise by 200 cm/hr and held for 2 min at each step. The pressure-flow curve for Profinity IMAC becomes nonlinear at pressures above 109 psi.

***** pH stability:** Ligand density and protein BC are essentially unchanged after resin is treated with pH 1–14 solutions/buffers for up to 200 hr.

Chemical Compatibilities

The chemical characteristics of Profinity IMAC resin are detailed in Table 2.

Table 2. Chemical Compatibilities for Profinity IMAC Resins

Reagent Group	Reagent	Comments	Stability
Buffer reagents	Tris, HEPES, MOPS	Used with proteins more stable in nonphosphate buffers	≤50 mM secondary and tertiary amines
	Sodium or potassium phosphate		50 mM sodium or potassium phosphate are recommended as starting buffers
Chelating reagents	EDTA, EGTA	Strips nickel ions from the resin	≤0.1 mM successfully used to remove trace metal contaminants (>1 mM causes significant reduction in binding capacity
Sulfhydryl reagents	β-Mercaptoethanol	Reduces random disulfide bonds; preventing protein aggregation during purification	≤30 mM
	DTT, TCEP	Transition metals at the center of IMAC resin (Ni^{2+}) are susceptible to reduction	≤5 mM DTT and 10 mM TCEP
Detergents	Nonionic detergents (Triton, Tween, NP-40)	Removes background proteins and nucleic acids	≤5%
	Cationic detergents (CTAB)	Improves solubility of membrane/lipid associating proteins or proteins with hydrophobic domains	≤1% but care must be taken to avoid protein precipitation
	Zwitterionic detergents (CHAPS, CHAPSO)	Solubilizes membrane proteins	≤5%
	Anionic detergents (SDS, Sarkosyl)	Selectively solubilizes membrane proteins; in higher concentrations anionic functionalities might cause stripping of metal ion	≤1% may be used; solubility of Sarkosyl in 50 mM potassium phosphate/300 mM NaCl is better than solubility of SDS
Denaturants	Guanidine HCl (GuHCl) Urea	Solubilizes proteins	≤6 M ≤8 M
Other additives	NaCl	Deters nonspecific protein binding due to ionic interactions	≤2 M (at least 300 mM NaCl should be included in buffers)
	$MgCl_2$	Essential component for purification of Ca^{2+} binding proteins	≤100 mM (HEPES or Tris buffers should be used to prevent precipitation)
	$CaCl_2$	Essential metal cofactor for nucleases	≤10 mM (HEPES or Tris buffers should be used to prevent precipitation)
	Glycerol	Included to prevent hydrophobic interactions between proteins	≤20%
	Ethanol	Included to prevent hydrophobic interactions between proteins	≤20%
	Imidazole	Competes for binding sites with His-tagged residues by interaction with the metal residues	May be used in low concentrations in the wash buffer (<25 mM) to limit binding of undesired proteins; for elution, ≤500 mM may be used
	Citrate	Carboxylic side chains could potentially serve as chelation site for Ni^{2+}, causing metal leakage	≤80 mM

Section 3
General IMAC Procedures

Protein Binding

Protein adsorption to immobilized ions is performed around neutral to slightly alkaline pH conditions (pH 7.0–8.5). To reduce nonspecific ionic effects, concentrations of up to 1 M NaCl may be added to the binding solution. Recombinant 6xHis tags, located at either the amino or carboxyl terminus of the protein, can bind with high affinity to the matrix even when the 6xHis tag isn't completely accessible. In general, the fewer the number of accessible histidine residues, the weaker the protein binding is to the affinity matrix. Untagged proteins that have naturally occurring and noncontiguous histidine residues also bind to IMAC resins, but with much lower affinity.

Batch mode binding is a good alternative if proteins are expressed at low levels or if the overall concentration of the recombinant 6xHis tag is low. In this case, proteins are bound to the Profinity™ IMAC resin in solution prior to packing the protein-resin complex into a liquid chromatography column for wash and elution steps. Altering the imidazole concentration of the lysis buffer may also optimize binding. Low concentrations such as 0–20 mM imidazole are recommended and will aid in reducing nonspecific binding of weakly interacting proteins.

Many additives can be used without affecting the binding of His-tagged proteins to IMAC resins, for example, urea, GuHCl, nonionic detergents, and organic solvents (refer to Section 2, Table 2). Chelating agents, such as EDTA or citrate, should not be included. Though not recommended, reducing agents such as β-mercapto-ethanol and DTT may be used at low concentrations.

Potassium phosphate or sodium phosphate buffers are recommended solutions for equilibration and binding.

Recommended binding buffer

- 20–50 mM sodium or potassium phosphate, containing up to 1.0 M NaCl, **e.g., 50 mM sodium phosphate, 0.3 M NaCl, pH 8.0**

Washes

Stringency of 6xHis tag binding can be effectively increased by 1) including low concentrations of imidazole in the binding and wash solutions, or 2) reducing the pH. Generally, highly expressed proteins, such as those expressed using a bacterial expression system, have fewer contaminant proteins that copurify along with the protein of interest. Abundance of endogenous protein contaminants is higher in eukaryotic expression systems and tends to bind to the IMAC adsorbent more weakly. In these instances, nonspecific binding of proteins containing neighboring histidine residues becomes a problem. These endogenous species

may be washed from the resin by either lowering the pH to 6.3 or adding imidazole to binding and wash solutions in concentrations of 5–20 mM. The optimal pH and/or imidazole concentration used in wash buffers is always protein-dependent and should always be determined experimentally.

Elution

Desorption of the His-tagged protein may be accomplished in one of three ways: introduction of a competitor ligand, reduction of the pH, or stripping of the immobilized metal.

In competitive elution, a step or gradient elution with ligands such as imidazole, histidine, histamine, or glycine may be carried out. When using a gradient elution with imidazole, it is important to ensure that the column has been preequilibrated with low concentrations of imidazole (that is, 1 mM) with the same concentration being included in the sample. This action avoids a drop in pH from occurring (caused by significant adsorption of imidazole onto the resin), which might prematurely elute bound His-tagged proteins.

Lowering the pH of the elution buffer (pH 4.5–5.3) also releases bound His-tagged proteins. In this case, the histidine residues become protonated and are unable to bind to the immobilized ion. Protein sensitivity to low pH ranges, however, must be taken into consideration. If lower pH is used to elute bound proteins, tubes filled with a strong neutral buffer such as 1 M Tris-HCl, pH 8.0 may be used to collect acidic eluates (that is, 100–200 µl/ml eluate). The recommended range is pH 3–5 with acetate buffers being a preferred choice. Weakly bound contaminants may be washed off in an intermediate wash at around pH 5.5–6.5.

Strong chelating agents such as EDTA and EGTA strip immobilized ions from the column and cause the bound His-tagged protein to elute as a protein-metal complex. This results in metal ions appearing in the protein fractions.

Recommended elution buffer for Profinity IMAC resin

Though a number of conditions can be used to elute the target protein from the Profinity resin, adjusting the concentration of imidazole is recommended.

- 20–500 mM imidazole, **e.g., 50 mM sodium phosphate, 0.3 M NaCl, 0.5 M imidazole, pH 8.0**

Purification Under Denaturing Conditions

When overexpressed in *E. coli*, some proteins may aggregate, forming what are known as inclusion bodies. These inclusion bodies need to be solubilized in strong denaturants such as 6 M guanidine HCl or 8 M urea in order to purify the His-tagged protein. Usually proteins expressed as inclusion bodies are not in their native conformation, thereby allowing the use of high concentrations of denaturants during the preparation of lysate and protein purification.

In order to restore the native conformation and activity of the protein, the denaturant must be removed by dilution, dialysis, or size exclusion chromatography. Renaturation of the protein while it is still bound to the IMAC column is a good alternative and offers several advantages. Aggregation may be kept to a minimum if the protein refolds on the column when the denaturant is removed. Higher concentrations of the refolded protein may therefore be collected. Finally, the use of a liquid chromatography system ensures that the adjustment of denaturants, detergents, salts, and pH will be effectively controlled.

Sections 8 and 11 discuss preparation of lysates and purification of His-tagged protein using denaturants.

Purification Under Nondenaturing Conditions

Purification under nondenaturing conditions might be preferred to denaturing conditions in instances when restoration of a protein's structure and activity are difficult to achieve. However, the potential to bind background contaminants might be higher under nondenaturing conditions. In these instances, a number of parameters may be optimized to improve purification results. The addition of nonionic detergents or glycerol might improve recovery by reducing nonspecific hydrophobic interactions. Additionally, low amounts of imidazole in the lysis and wash buffers are suggested to minimize levels of contaminating proteins that bind to the IMAC adsorbent. Finally, ensuring that all buffers have sufficient ionic strength, with the addition of NaCl, will minimize nonspecific electrostatic interactions.

Imidazole Concentrations

For optimal protein purification results, it is crucial that the imidazole concentrations in lysis, binding, and wash buffers as well as elution buffers be empirically determined. Optimized conditions should be determined using a small amount of sample and a 1 ml IMAC column. These conditions may then be used to design the purification protocol for larger samples on the same column or on a larger column. As each protein behaves differently, it is helpful to keep the following elements in mind during lysate preparation and purification:

- Low concentrations of imidazole (0–20 mM) in lysis, binding, and wash buffers are recommended if the potential for background contaminants exists. The ability for nontagged, contaminating proteins to bind to the resin is generally higher under nondenaturing conditions than under denaturing conditions
- Low concentrations of imidazole (0–20 mM) help minimize nonspecific binding of proteins containing noncontiguous histidine residues by competing with them for available binding sites on the transition metal. Competition occurs because the imidazole ring is found also in the histidine-containing compound
- If binding of the recombinant His-tagged protein does not occur under higher concentrations, the imidazole concentration can be reduced

- A gradient elution test may be used to determine suitable concentrations of imidazole for wash and elution steps. Once the imidazole concentration to elute the protein is established, large samples and/or columns may be used

Section 4
Column Packing — Medium-Pressure Columns

Slurry Packing an IMAC Column

Slurry packing is preferred for small columns. This method describes packing a Profinity™ IMAC resin with aid from a pump. For best results, 5 to 50 mm ID columns and a bed height of 5 to 30 cm should be used.

Recommended Columns

Bio-Rad's Bio-Scale™ MT high-resolution columns may be used for the following column-packing procedure. These columns are empty but may be packed with the support of choice. Bio-Scale MT columns are convenient to use with Bio-Rad's BioLogic™ system, or any medium- or high-pressure system:

- Bio-Scale MT2 column (7 x 52 mm) for bed volumes up to 2 ml
- Bio-Scale MT5 column (10 x 64 mm) for bed volumes up to 5 ml
- Bio-Scale MT10 column (12 x 88 mm) for bed volumes up to 10 ml
- Bio-Scale MT20 column (15 x 113 mm) for bed volumes up to 20 ml

Materials

Reagents

- 0.1 M $NiSO_4$ (or other suitable metal salt solution)

Note: Charging of Profinity IMAC nickel-charged resin (Ni-charged resin) is not required for initial use. For subsequent uses it is recommended that the resin be cleaned of all contaminants, stripped of metal ions, and recharged with proper metal ions prior to loading the next sample.

The procedure to charge Profinity IMAC (uncharged resin) is explained in Section 6, Immobilizing Metal Ions.

Equipment

- Empty column (1–5 cm ID x 30 cm) with flow adaptors, inlet and outlet ports
- Glass filter
- IMAC resin (Profinity IMAC or Profinity IMAC Ni-charged resin)
- Packing reservoir
- Pump

Resin Preparation

Profinity IMAC and Profinity IMAC Ni-charged resins come supplied in a 20% ethanol solution for resin storage. Before the column can be slurry packed, the resin storage solution must be replaced with distilled water.

1. **For easy removal of storage solution, transfer an appropriate amount of resin slurry to a graduated container, such as a Bio-Rad Econo-Pac® column (bed volume up to 20 ml).**
2. **Apply vacuum to the column for rapid removal of the storage solution.**

   The storage solution can be removed rapidly by applying a vacuum to the column.
3. **Wash the column with 3 column volumes of distilled water.**
4. **Add enough distilled water to make a 50% slurry.**
5. **The resin is now ready to be packed.**

Alternatively, if larger volumes of Profinity IMAC or Ni-charged IMAC resins are being used, simply resuspend the resin in the ethanol solution provided. Proceed with the method below and during step 6, ensure that all ethanol is removed during the equilibration step.

Method

1. **Use a column that is 2.5 times longer than the required bed height.**
2. **Eliminate air from the column dead spaces.**

   Fill the column with distilled water to about 10% of its volume. Flush endpieces with distilled water to ensure that the bottom of the bed support is fully saturated and free of air bubbles. Allow a few centimeters of distilled water to remain before closing the outlet valve.
3. **Suspend the 50% v/v Profinity slurry by gently swirling or stirring with a glass or plastic rod.**
4. **Pour slurry into the column.**

   Carefully pour the slurry down the side of the column using a glass or plastic rod so as to not introduce air bubbles.

   Note: If using Profinity IMAC uncharged resin, proceed with Immobilizing Metal Ions procedure (Section 6) once slurry packing is accomplished.
5. **Insert the top flow adaptor.**

   Use care to not introduce any air bubbles. Insert the adaptor at an angle into the column. Make sure that the exit tubing is open to allow distilled water to flow out of the top adaptor along with any air.

6. **Adjust the adaptor to sit directly on top of resin bed.**

   Open the column outlet and pump distilled buffer through the column at a packing flow rate of ~400–600 cm/hr for 5 to 10 min or at the maximum pressure allowed by the column hardware and resin. While packing at high flow rates, the resin bed will be compressed down. **Mark this compression level with a pen.**

   Stop the flow. At this point, the resin bed height might readjust and rise up. If this happens, adjust the flow adaptor to compress the bed an additional 0.1–0.5 cm down past the level marked with the pen.

7. **Reconnect the pump and equilibrate.**

   Pass eluent (distilled water) through the column at the packing flow rate. During equilibration, the bed might be compressed down even further. After a constant bed height is reached, mark the compression level at this flow rate. Again, adjust the adaptor to **further compress** the bed an additional 0.1–0.5 cm past the level marked.

8. **Proceed with Immobilizing Metal Ions procedure (Section 6) if using uncharged Profinity IMAC resin or if recharging Profinity IMAC Ni-charged resin.**

   Note: Chromatographic steps during purification should not be run greater than 75% of the packing flow rate.

Section 5
Column Packing — Sample Preparation-Sized Columns

Use this method for packing Profinity™ IMAC into small-sized sample preparation micro spin columns.

Materials

Reagents

- 0.1 M $NiSO_4$ (or other suitable metal salt solution)

Note: Charging of Profinity™ IMAC nickel-charged resin (Ni-charged resin) is not required for initial use. For subsequent uses it is recommended that the resin be cleaned of all contaminants, stripped of metal ions, and recharged with proper metal ions prior to loading the next sample.

The procedure to charge Profinity IMAC (uncharged resin) is explained in Section 6, Immobilizing Metal Ions.

Equipment

- Sample preparation-sized column (for example, Micro Bio-Spin™ columns, catalog #732-6204)
- Plasticware, 2 ml capped and 2 ml capless tubes
- IMAC resin (Profinity IMAC or Profinity IMAC Ni-charged resin)
- Tabletop centrifuge
- 1 ml pipet with wide-bore pipet tips

Resin Preparation

Profinity IMAC and Profinity IMAC Ni-charged resins come supplied in a 20% ethanol solution for resin storage. Before the column can be slurry packed, the resin storage solution must be replaced with distilled water. See procedure on page 10.

Method

1. **Thoroughly suspend Profinity IMAC resin.**
2. **Place the column into an appropriate collection vessel and spin;** for example, 2 ml capless collection tube.
3. **Using a pipet, transfer enough uncharged or Ni-charged Profinity IMAC to a microcentrifuge tube.**

   If using a Micro Bio-Spin column, transfer **0.2 ml** slurried uncharged or Ni-charged Profinity IMAC to the column. This is equivalent to ~100 µl of a packed resin bed.

4. **Remove storage solution by centrifugation.**

   Centrifuge at 1,000 x g for 15 sec to pack resin.

5. **Wash column with at least 5 column volumes (or ~500 µl) of distilled water.**

   Centrifuge at 1,000 x g for 15 sec to pack resin.

6. **If using Ni-charged resin, equilibrate the column with at least 5 column volumes of binding buffer.**

   The column is now ready for separation.

   Note: Proceed to sections for preparation of lysates and column purification only if using the Ni-charged Profinity IMAC resin.

7. **If using uncharged resin, proceed to Section 6, Immobilizing Metal Ions.**

Section 6
Immobilizing Metal Ions

Efficacy of protein binding by IMAC is dependent on two factors — the number of available histidine, cysteine, and tryptophan residues on a protein's surface, and the number of coordination sites on the immobilized ion that are not occupied by the chelating ligand and thus available to bind the amino acid residues. Profinity IMAC uses a tridentate ligand (IDA), which leaves three of the six coordination sites on the nickel ion accessible to the protein of interest.

Although the most commonly used ion is Ni^{2+}, protein selectivity may be increased by the choice of metal ion used, understanding the structure of the metal-chelate complex and its interaction with the protein, knowledge of the protein's expression level, and the ligand density of the IMAC adsorbent. While high ligand density usually means higher binding capacity, it can also translate into lower target protein selectivity. Profinity IMAC, based on the polymeric UNOsphere™ technology, has specifically been formulated with an optimal amount of chelating ligands on the resin's surface and pores, to deliver both good capacity and excellent protein purity.

1. **Equilibrate the column with 5 column volumes of 50 mM sodium acetate, 0.3 M NaCl, pH 4.0.**

   After slurry packing is complete (see Sections 4 and 5), the column is ready for application of metal ions.

2. **Make a 0.1–0.3 M solution of the metal ion of choice.**

   For best results, the pH of the solution should be <7 (neutral to weakly acidic).

3. **Apply 3–5 column volumes of the metal ion solution.**

4. **Wash with 5 column volumes of 50 mM sodium acetate, 0.3 M NaCl, pH 4.0.**

   Remove excess ions by washing.

5. **Wash with 10 column volumes of deionized water.**

6. **Equilibrate with at least 5 column volumes of starting buffer, for example, 50 mM sodium phosphate, 0.3 M NaCl, pH 7–8.**

   The column is now ready for separation.

Section 7
Preparation of Clarified *E. coli* Lysate Using Nondenaturing Conditions

This guideline covers a **general method** for preparation of a clarified lysate from bacterial cells using nondenaturing conditions. Extract purification is necessary in order to release the target His-tagged protein from cells and separate out insoluble material that would otherwise foul and contaminate the column. To reduce viscosity of the cell lysate, Lysonase bioprocessing reagent (rLysozyme solution plus Benzonase nuclease) can be used to break up bacterial DNA. Protease inhibitors such as phenylmethane-sulfonyl fluoride (PMSF) may also be included in the lysis buffer to prevent proteolysis from occurring. In the absence of strong denaturants, proteins may be subject to degradation during the cell harvest and lysis steps.

An optional lysis step in the protocol is included when equipment for mechanical disruption may not be available. This uses B-PER bacterial protein extraction reagent for extraction of recombinant proteins from *E. coli.*

Always work quickly and keep cells at 4°C at all times during this procedure.

Materials

Reagents

- Lysis buffer (pH 8.0)
  - 50 mM sodium phosphate (NaH_2PO_4)
  - 300 mM NaCl
  - 5 mM imidazole
  - Add Lysonase (Novagen catalog #71230-4), or PMSF just prior to use in step 4
- Optional lysis buffer

  B-PER bacterial protein extraction reagent (Pierce catalog #78243), add just prior to use in step 4

Equipment

- Filter apparatus
- Sonicator

Biological Sample

- *E. coli* with respective recombinant protein

Additional Materials

- Equipment for determining total protein concentration within the lysate

Method

1. **Harvest *E. coli* from an appropriate volume of bacterial culture by centrifugation at 4,000–8,000 x g for 5–10 min at 4°C.**

2. **Discard supernatant and place centrifuge bottles on ice.**

   Alternatively, the pellet may be stored at –70°C for later use. In this case, it is recommended that the pellet be stored in flat containers or bags to allow for easier thawing.

3. **Determine weight of pellet.**

   This is easily done by subtracting the weight of an identical, **empty** container from the weight of the one containing the *E. coli* pellet.

4. **Resuspend pellet in 1:10 ratio (w/v) in lysis buffer and add Lysonase bioprocessing reagent (20 µl/g of cell paste).**

   Thoroughly resuspend the pellet by vortexing.

   Following lysis, the lysate often becomes very viscous due to the release of genomic DNA into the solution. It is very important to reduce viscosity using a homogenization method (step 5), as a viscous heterogeneous solution may clog the column.

   Optional lysis step:

   Resuspend pellet in 1:10 ratio (w/v) in B-PER/5 mM DTT. After resuspending, add Lysonase bioprocessing reagent, 20 µl/g of cell paste and shake at 150 rpm at room temperature for 20 min. Proceed to step 6 below.

   B-PER may be used when equipment required for mechanical disruption is unavailable in the laboratory.

5. **Sonicate the cell suspension/lysate 4 times at 1 min intervals each. Sonicate on ice at all times.**

   Decrease interval time if the sample becomes warm. Keep samples cold at all times. Check for clarity and increase sonication if needed.

6. **Centrifuge the homogenized lysate at 12,000 x g for 20 min at 4°C to clarify sample. Save supernatant.**

   Centrifugation times and speeds may need to be altered in order to remove all cell debris. If supernatant is still not completely clear, lysate should be filtered through a 0.45 µm filter to prevent column backpressure from occurring. The target protein is now in the supernatant.

Note: Some portion of the target protein will remain insoluble and will localize in the pellet. To properly release the His-tagged protein from the pellet, it will need to be subjected to denaturing conditions. See Section 8, Preparation of Clarified *E. coli* Lysate Using Denaturing Conditions.

7. **Analyze the concentration of the target protein using a small amount of supernatant.**

   Use SDS-PAGE to get a rough determination of the protein's concentration. This may help determine the appropriate size of the IMAC column needed.

8. **Continue with purification protocols using nondenaturing conditions.** Proceed with protocols using nondenaturing conditions.

Section 8
Preparation of Clarified *E. coli* Lysate Using Denaturing Conditions

This guideline describes a **general method** for the preparation of inclusion bodies from *E. coli*. After cell lysis, inclusion bodies are separated from cell debris and then resuspended/washed in a solution containing urea. For purification, inclusion bodies must be solubilized in a high concentration of denaturant.

To reduce viscosity of the cell lysate, Lysonase bioprocessing reagent (rLysozyme solution plus Benzonase nuclease) can be used to break up bacterial DNA. Protease inhibitors, such as phenylmethane-sulfonyl fluoride (PMSF) may also be included in the lysis buffer to prevent proteolysis from occurring.

An optional lysis step in the protocol is included when equipment for mechanical disruption may not be available. This uses B-PER bacterial protein extraction reagent, which may be used for both soluble and inclusion body purification of recombinant proteins from *E. coli*.

Always work quickly and keep cells at 4°C at all times during this procedure.

Materials

Reagents

- Denaturing lysis buffer, pH 8.0 (urea-based)
  - 50 mM sodium phosphate (NaH_2PO_4)
  - 300 mM NaCl
  - 5 mM imidazole
  - Up to 8 M urea
  - Add Lysonase (Novagen catalog #71230-4), or PMSF just prior to use in step 4
- Optional lysis buffer
  B-PER bacterial protein extraction reagent (Pierce catalog #78243), add just prior to use in step 4
- Resuspension/wash buffer for inclusion bodies: 1x PBS, 8 M urea

Equipment

- Filter apparatus (0.45 µm)
- Sonicator

Biological Sample

- *E. coli* with respective recombinant protein

Method

1. **Harvest *E. coli* from an appropriate volume of bacterial culture by centrifugation at 4,000–8,000 x g for 5–10 min at 4°C.**

2. **Discard supernatant and place centrifuge bottles on ice.**

   Alternatively, the pellet may be stored at –70°C for later use. In this case, it is recommended that the pellet be stored in a flat container or bag to allow easier thawing.

3. **Determine weight of pellet.**

   This is easily done by subtracting the weight of an identical, **empty** container from the weight of the one containing the *E. coli* pellet.

4. **Resuspend pellet in 1:10 ratio (w/v) in lysis buffer and add Lysonase bioprocessing reagent (20 µl/g of cell paste).**

   Thoroughly resuspend the pellet by vortexing.

   Following lysis, the lysate often becomes very viscous due to the release of genomic DNA into the solution. It is very important to reduce viscosity using a homogenization method (step 5), as a viscous heterogeneous solution may clog the column.

   Optional Lysis Step:

   Resuspend pellet in 1:10 ratio (w/v) in B-PER/5 mM DTT. After resuspending, add Lysonase, 20 µl/g of cell paste and shake at 150 rpm at room temperature for 20 min. Proceed to step 6 below.

   B-PER may be used when equipment required for mechanical disruption is unavailable in the laboratory.

5. **Sonicate the cell suspension/lysate 4 times at 1 min intervals each. Sonicate on ice at all times.**

   Decrease interval time if the sample becomes warm. Keep samples cold at all times. Check for clarity and increase sonication if needed.

6. **Centrifuge the homogenized sample at 12,000 g for 20 min at 4°C to pellet the inclusion bodies.**

   Discard supernatant.

7. **Resuspend the inclusion body pellet in 1:10 ratio (w/v) in 1x PBS/8 M urea, pH 7.5.**

8. **Sonicate, as needed, to redissolve the pellet.**

9. **Steps 5 and 6 may need to be repeated several times in order to achieve optimal protein purity.**

   If the sample needs to be clarified further, filter sample through a 0.45 µm filter.

10. **Analyze the concentration of the target protein using a small amount of supernatant.**

    Use SDS-PAGE to get a rough determination of the protein's concentration. This will help determine the appropriate size of the IMAC column needed.

11. **Continue with purification protocols under denaturing conditions.**

Section 9
Medium-Pressure Column Purification of Histidine-Tagged (His-Tagged) Proteins Using Nondenaturing Conditions

For this guideline, the sample is applied to a packed column and the proteins are eluted using a high imidazole concentration. The guideline **does not optimize** the imidazole concentration, but instead provides for fast capture of the target protein and may be used as a quick check for protein expression levels.

Higher levels of purity are achievable by optimizing imidazole concentrations, which improve protein separation. See Section 10, Medium-Column Purification – Using an Imidazole Gradient to Determine Optimal Purification of His-Tagged Proteins.

Materials

Reagents

- Binding buffer
  - 50 mM sodium phosphate (NaH_2PO_4)
  - 300 mM NaCl
  - Low concentrations imidazole* (such as 0–10 mM)

  Adjust to pH 8.0.

- Wash buffer
  - 50 mM sodium phosphate (NaH_2PO_4)
  - 300 mM NaCl
  - Low concentrations of imidazole* (such as 0–20 mM)

  Adjust to pH 8.0.

- Elution buffer
  - 50 mM sodium phosphate (NaH_2PO_4)
  - 300 mM NaCl
  - Higher concentrations of imidazole* (such as 250–500 mM)

  Adjust to pH 8.0.

  * For optimal protein purification results, it is crucial that the imidazole concentrations in lysis, binding, and wash as well as elution buffers be empirically determined. Optimized conditions should be determined using a small amount of sample and a 1 ml IMAC column.

Equipment

- IMAC column (as prepared in Section 4)

Biological Sample

- Clarified lysate (as prepared in Section 7)

The binding capacity of the Profinity™ IMAC resin is ~15 mg His-tagged protein per ml resin (please refer to Table 1 for determination of dynamic binding capacity conditions). Larger amounts of protein will require use of a larger column.

Additional Materials

- Chromatography system (such as the Bio-Rad BioLogic DuoFlow™ system)
- Equipment for determining total protein concentration within the lysate.

Method

1. **Equilibrate the column with at least 5 column volumes of binding buffer.**

2. **Add or dilute sample in binding buffer and load onto the column using a desired flow rate.**

   The choice of binding buffer will vary based on the properties of the sample to be purified. Sodium or potassium phosphate are recommended as general starting buffers; for example, 50 mM sodium phosphate, 300 mM NaCl, 5 mM imidazole, pH 8.0. Binding of His-tagged protein on the Profinity IMAC resin is optimal in the pH range of 7–8.

   The column may be run at flow rates up to 600 cm/hr. Higher binding of His-tagged proteins will be achieved at lower flow rates. Average binding capacities of the Profinity IMAC resin range between 10 and 15 mg His-tagged protein/ml resin.

3. **Collect fractions.**

   These fractions represent unbound proteins.

4. **Wash the resin with at least 5 column volumes of wash buffer to remove unbound sample.**

   Wash out remaining unbound solutes. Repeat wash steps as necessary for the A_{280} to be at or near the baseline.

5. **Collect fractions from wash steps.**

   Pool recovered unbound proteins with fractions collected in step 3.

6. **Elute bound proteins with 5 column volumes of elution buffer. Collect 1 ml fractions.**

   The choice of elution buffer will vary depending on the procedure used. For example, a range of imidazole (100–500 mM) may be used to elute bound protein from the Profinity IMAC resin.

7. **Repeat elution steps 2 to 4 more times.**

   Save the eluates for further analysis; for example, A_{280}, SDS-PAGE, ELISA, etc.

Section 10
Medium-Pressure Column Purification — Using an Imidazole Gradient to Determine Optimal Purification of His-Tagged Proteins

Gradient elution tests are useful because they do not require optimization of imidazole concentrations, but instead may be used to determine suitable imidazole concentrations for wash and elution steps. Once a suitable concentration has been determined using a gradient elution such as the one outlined below, often an easier protocol using step elutions may be used for subsequent purification of larger sample volumes.

With step elutions, the protein can be collected in smaller volumes and at higher concentrations. Using this protocol, the concentration of imidazole that elutes the target protein may be calculated and used for a step protocol.

This protocol requires the use of a gradient mixer coupled to a chromatography system, such as Bio-Rad's BioLogic DuoFlow™ system, to establish a linear gradient.

Materials

Reagent

- Binding buffer
  - 20 mM sodium phosphate (NaH_2PO_4)
  - 300 mM NaCl
  - 20 mM imidazole
- Elution buffer
  - 50 mM sodium phosphate (NaH_2PO_4)
  - 300 mM NaCl
  - 500 mM imidazole

Equipment

- Chromatography system with dual pump and gradient capability
- Fraction collector
- IMAC column (as prepared in Section 4)

Biological Sample

- Clarified lysate (as prepared in Section 7)

Note: Keep the sample as small as possible during optimization of binding and elution conditions.

Additional Materials

- Equipment for assessing protein purity and recovery of the His-tagged protein

Method

Part 1: Optimizing the Imidazole Concentration

1. **Purge the entire flow path of the chromatography system with water according to manufacturer's instructions.**

   Connect the column and wash it with 10 column volumes of water. Disconnect the column by valve switching or manually. Purge the flow path before the column with elution buffer from inlet B, and with the column off the line, then with binding buffer from inlet A. Purge the entire system with binding buffer. Reconnect the column to the system.

2. **Equilibrate column with 10 column volumes of binding buffer (0% B).**

3. **Begin collecting fractions of 1 column volume.**

   If a 1 ml IMAC column is used, 1 ml fractions are recommended. For larger columns, reduce the fractions collected to amounts ranging from 0.2 to 0.5 column volumes.

4. **Load sample and collect the flow-through in fractions appropriate to the size of the column (as recommended above).**

   Note: Monitor the backpressure while the sample is being applied. If the sample is insufficiently clarified, the backpressure will increase.

5. **Wash the unbound material with 10 column volumes of binding buffer (0% B).**

6. **Elute the sample with a linear gradient of 0% to 50% elution buffer.**

7. **Wash the column with 100% of the elution buffer for 5 column volumes.**

8. **Equilibrate the column with 10 column volumes of binding buffer (0% B). Stop collecting fractions.**

9. **Identify the fractions containing the His-tagged protein.**

   Use an activity assay (Bio-Rad's protein assay kit), UV absorbance, SDS-PAGE, or western blot analysis with anti-histidine antibodies or antibodies specific to the target protein.

10. **Calculate the concentration of imidazole corresponding to the elution peak of the His-tagged protein.**

    Note: Delay due to the column dead volume and chromatography system needs to be considered compared to the programmed gradient.

11. **Based on calculated imidazole concentration, a stepwise experiment may now be designed.**

The following are useful items to keep in mind during the design of the experiment:

- Maintain the concentration of imidazole in the binding (also called equilibration) buffer at 20 mM. If high amounts of contaminants are also adsorbed onto the resin, the concentration of imidazole in the sample and equilibration buffer may be increased. This measure might reduce the overall amount of target protein bound and should be carried out with care. However, it will also increase the column binding capacity for the target protein due to a reduction of contaminating proteins.
- Include a wash step containing an imidazole concentration slightly lower than the concentration necessary to elute the target protein. This will increase purity by removing unbound contaminants, without eluting the bound His-tagged protein. The optimized wash step should include 50 mM sodium phosphate, 0.3 M NaCl, and an appropriate concentration of imidazole.
- The elution buffer should contain a concentration of imidazole greater than the calculated concentration corresponding to the eluted peak of target protein.
- Perform a trial run (see Section 11).

Part 2: Using an Optimized Imidazole Concentration for Purification

1. **Prepare 200 ml binding buffer and 200 ml elution buffer for a 1 ml column.**

   Purge the pumps with the fresh buffers. Use suitable buffer volumes for larger scale purifications.

2. **Equilibrate the column with 10 column volumes of equilibration/binding buffer.**

3. **Begin collecting fractions of 1 column volume.**

   If a 1 ml IMAC column is used, 1 ml fractions are recommended. For larger columns, reduce the fractions collected to amounts ranging from 0.2 to 0.5 column volumes.

4. **Load sample and collect the flow-through in fractions appropriate to the size of the column (as recommended above).**

   Note: Monitor the backpressure while the sample is being applied. If the sample is insufficiently clarified, the backpressure will increase.

5. **Wash the column with a minimum of 5 column volumes of binding buffer to remove unbound contaminants.**

6. **Wash the column with a minimum of 5 column volumes of binding or starting buffer, containing some amount of imidazole that will not elute the target protein.**
7. **Elute the His-tagged protein with 5 column volumes of elution buffer with optimized imidazole concentration.**
8. **Wash the column with 5 column volumes of elution buffer. Stop collecting fractions.**
9. **Reequilibrate the column with 10 column volumes of equilibration (binding) buffer.**
10. **Assess the purity and recovery of the target protein.**

    Use an activity assay (Bio-Rad's protein assay kit), UV absorbance, SDS-PAGE, or western blot analysis with anti-histidine antibodies or antibodies specific to the target protein.

Section 11
Medium-Pressure Column Purification of His-Tagged Proteins Using Denaturing Conditions

This guideline uses denaturants such as urea to solubilize inclusion bodies, which are not generally in their native conformation. Elution is achieved by increasing the imidazole concentration. The protocol also recommends how to restore the protein to its native form. In this case, the denaturant used to lyse and purify the sample must be removed using dilution, dialysis, or size exclusion chromatography. The guideline **does not optimize** the imidazole concentration, but instead allows fast capture of the target protein and may be used as a quick check for protein expression levels.

Renaturation of the protein while it is still bound to the IMAC column is a good alternative and offers several advantages. Aggregation may be kept to a minimum if the protein refolds on the column when the denaturant is removed. Higher concentrations of the refolded protein may therefore be collected. Finally, with the use of a liquid chromatography system, denaturing agents, detergents, salts, and pH can be adjusted and effectively controlled.

Note: If using guanidine HCl (GuHCl), it must be removed from purified samples prior to loading onto SDS-PAGE gels due to precipitation that occurs. Proteins that have been lysed and adsorbed onto the column with guanidine HCl may be washed and eluted with a urea-based buffer.

A gradient elution protocol (see Section 10) may be used with small sample volumes to optimize the imidazole concentration needed in the stepwise elution protocol.

Materials

Reagents for Protein Binding and Elution

- Binding buffer (urea-based)
  - 50 mM sodium phosphate (NaH_2PO_4)
  - 300 mM NaCl
  - Low concentration of imidazole* (such as 0–10 mM)
  - Up to 8 M urea

  Adjust to pH 8.0.
- Wash buffer
  - 50 mM sodium phosphate (NaH_2PO_4)
  - 300 mM NaCl
  - Low concentration of imidazole* (such as 0–20 mM)
  - Up to 8 M urea

  Adjust to pH 8.0.

- Elution buffer
  - 50 mM sodium phosphate (NaH_2PO_4)
  - 300 mM NaCl
  - Higher concentration of imidazole* (such as 250–500 mM)
  - Up to 8 M urea

  Adjust to pH 8.0.

  * For optimal protein purification results, it is crucial that the imidazole concentrations in lysis, binding, and wash as well as elution buffers be empirically determined. Optimized conditions should be determined using a small amount of sample and a 1 ml IMAC column.

Reagents for on-column refolding

- Refolding buffer
  - 20 mM imidazole
  - 300 mM NaCl
  - 1 mM β-mercaptoethanol
  - 20 mM sodium phosphate (NaH_2PO_4) (pH 8.0)
- Phosphate elution buffer (with high imidazole)
  - 500 mM imidazole
  - 300 mM NaCl
  - 1 mM β-mercaptoethanol
  - 20 mM sodium phosphate (NaH_2PO_4) (pH 8.0)
- Urea binding buffer** (refolding buffer with 8 M urea)

  Prepare the urea binding buffer by first weighing out the urea. Add 2 M imidazole, 1 M sodium phosphate (pH 8.0), and water to approximately 90% of the final volume into a container with a stirbar. Place the container into a water bath at 45°C as addition of the denaturant causes the solution to cool. **Use caution when warming urea solutions. Breakdown occurs rapidly above 28°C.**

  Stir the solution to dissolve the denaturant. Bring the solution to room temperature before adjusting the pH. Adjust the pH, add the β-mercaptoethanol, and add water to the final volume. Filter the solution.

  Note: The solution must be room temperature before pH adjustment and heat causes pH to fluctuate. The β-mercaptoethanol should only be added to the solution immediately before use.

  ** The urea binding buffer may be used to wash and elute proteins prepared with 6 M guanidine HCl.

Equipment

- Chromatography system with dual pump and gradient capability
- IMAC column (as prepared in Section 4)

Biological Sample

- Denatured protein sample as prepared in Section 8

Additional Materials

- Equipment for assessing protein purity and recovery of the His-tagged protein

Method

Part 1: Protein Binding

1. **Purge the instrument with binding buffer and then elution buffer.**
2. **Equilibrate the column with 5 column volumes of binding buffer (0% B) at 1 ml/min (or according to the manufacturer's instructions).**
3. **Begin collecting 1 ml fractions.**
4. **Load lysate at desired flow rate.**

   To increase yield, the lysate can be loaded at lower flow rates.

   Note: Monitor the backpressure, which will increase during sample application.

5. **Wash the column with 10 column volumes of wash buffer (0% B) to elute unbound sample material.**

   Note: If the target protein will be eluted, continue onto Part 2. If the protein will be renatured on-column, skip to Part 3.

Part 2: Eluting the Denatured Target

1. **Elute the bound sample with 5–10 column volumes of elution buffer at desired flow rate.**
2. **Equilibrate the column with 10 column volumes of binding buffer. Stop collecting fractions.**
3. **Assess protein purity and recovery of fractions containing His-tagged protein.**

   Use an activity assay (Bio-Rad's protein assay kit), SDS-PAGE, or western blot analysis with anti-histidine antibodies or antibodies specific to the target protein.

Part 3: Purification and On-Column Renaturation of Proteins

1. **Wash the column (containing the bound protein) with 10 column volumes of urea binding buffer.**
2. **Apply a linear gradient from 100% urea-binding buffer to 100% refolding buffer over 60 min at 0.5 ml/min. Refolding is initiated by a descending gradient from 8 to 0 M urea.**

3. **Apply a linear gradient from 100% refolding buffer to 100% phosphate elution buffer, with high imidazole.**

   Note: Elution is done by a gradient from 20 mM to 500 mM imidazole.

4. **Access protein purity and recovery of fractions containing His-tagged proteins.**

   Use an activity assay (Bio-Rad's protein assay kit), SDS-PAGE, or western blot analysis with anti-histidine antibodies or antibodies specific to the target protein.

5. **If necessary, add an additional chromatography step.**

   Size exclusion chromatography may be a good choice because aggregates of un-refolded protein can be removed and the buffer composition of the purified material can be changed simultaneously.

Section 12
Sample Preparation-Size Spin-Column Purification of His-Tagged Proteins Using Nondenaturing Conditions

Materials

Reagents

- Binding/wash buffer
  - 50 mM sodium phosphate (NaH_2PO_4)
  - 300 mM NaCl
  - 5 mM imidazole

  Adjust to pH 8.0.

- Optimized wash buffer with imidazole (optional, see Section 10)
  - A wash buffer containing slightly less imidazole than necessary to elute the target protein may be used to increase the stringency of the wash step. Refer to Section 10, Medium-Pressure Column Purification — Using an Imidazole Gradient to Determine Optimal Purification of His-Tagged Proteins.
  - Once the concentration of imidazole in the wash step is determined using medium-pressure column chromatography, a stepwise elution step may be carried out as indicated in this protocol.

- Elution buffer
  - 50 mM sodium phosphate (NaH_2PO_4)
  - 300 mM NaCl
  - 500 mM imidazole

  Adjust to pH 8.0.

Equipment

- Sample preparation-sized IMAC spin column (as prepared in Section 5) (for example, Micro Bio-Spin™ columns, catalog #732-6204)
- Plasticware, 2 ml capped, 2 ml capless tubes

Biological Sample

- Clarified lysate (as prepared in Section 7)

  The binding capacity of the Profinity IMAC resin is ≥15 mg His-tagged protein per ml resin. Larger amounts of protein will require a larger column.

Additional Materials

- Equipment for assessing protein purity and recovery of the His-tagged protein

Method

Reserve a small amount of lysate (as prepared in Section 7) prior to loading sample onto the column. This will serve as sample for the "lysate" lane for later analysis, for example, SDS-PAGE.

Part 1: Binding of Sample

1. **Start with a prepacked spin column, charged with the metal ion of choice.**

   See Section 5, Column Packing — Sample Preparation-Sized Columns for protocol.

2. **Place prepacked spin column in an appropriate spin collection tube.**

3. **Preequilibrate the spin column with 5 column volumes of binding buffer.**

   The choice of binding buffer will vary based on the properties of the sample to be purified. Potassium phosphate and sodium phosphate are recommended as general starting buffers, for example, 50 mM sodium or potassium phosphate, 300 mM NaCl, pH 8.0. Binding of His-tagged protein on the Profinity™ IMAC resin is optimal in the pH range of 7–8.

4. **Add an appropriate amount of lysate (≤0.5 ml) to the micro spin column.**

5. **Mix by pipetting up and down 5 times.**

   Incubate for up to 5 min in micro spin column.

6. **Centrifuge at 1,000 x g for 1 min.**

   Remove the unbound proteins by centrifuging.

Part 2: Washing the Resin

7. **Insert micro spin into new collection vessel.**

8. **Wash the resin with at least 5 column volumes of binding buffer containing imidazole.**

   Pipet up and down at least 5 times.

   Note: If previously determined, an optimized concentration of imidazole may be used that is slightly less than the concentration necessary to elute the target protein. See Section 10.

9. **Centrifuge at 1,000 x g for 1 min.**

   Remove remaining unbound proteins by centrifuging. The wash step can be repeated if necessary.

Part 3: Eluting the His-Tagged Protein

10. Insert micro spin column into new, clean collection vessel.

11. Elute bound proteins with 5 column volumes of elution buffer.

Pipet up and down at least 5 times and incubate for up to 5 min.

The choice of elution buffer will vary depending on the procedure used. For example, a range of imidazole concentrations (150–500 mM) may be used to elute bound protein from the Profinity IMAC resin.

Analyze fractions from above steps by A_{280}, SDS-PAGE, ELISA, etc.

Section 13
Sample Preparation-Size Spin-Column Purification of His-Tagged Proteins Using Denaturing Conditions

Materials

Reagent

- Binding buffer (urea-based)
  - 50 mM sodium phosphate (NaH_2PO_4)
  - 300 mM NaCl
  - 5 mM imidazole
  - Up to 8 M urea

  Adjust to pH 8.0.

- Wash buffer
  - 50 mM sodium phosphate (NaH_2PO_4)
  - 300 mM NaCl
  - 10 mM imidazole
  - Up to 8 M urea

  Adjust to pH 8.0.

- Elution buffer
  - 50 mM sodium phosphate (NaH_2PO_4)
  - 300 mM NaCl (pH 6.8 or higher)
  - 250 mM imidazole
  - Up to 8 M urea

  Adjust to pH 8.0.

- Optimized wash buffer (optional, see Section 10)

  A wash buffer containing slightly less imidazole than necessary to elute the target protein may be used to increase the stringency of the wash step. Refer to Section 10, Medium-Pressure Column Purification — Using an Imidazole Gradient to Determine Optimal Purification of His-Tagged Proteins.

  Once the concentration of imidazole in the wash step is determined using medium-pressure column chromatography, a stepwise elution step may be carried out as indicated in this protocol.

Equipment

- Sample preparation sized IMAC spin column (as prepared in Section 5) (for example, Micro Bio-Spin column™, catalog #732-6204)
- Plasticware, 2 ml capped, and 2 ml capless tubes

Biological Sample

- Denatured protein sample as prepared in Section 8

Additional Materials

- Equipment for assessing protein purity and recovery of the His-tagged protein

Method

Reserve a small amount of lysate (as prepared in Section 8) prior to loading sample onto the column. This will serve as sample for the "lysate" lane for later analysis, for example, SDS-PAGE.

Part 1: Binding of Sample

1. **Start with a prepacked spin column, charged with the metal ion of choice.**

   See Section 5, Column Packing — Sample Preparation-Sized Columns for protocol.

2. **Place prepacked spin column in an appropriate 2 ml spin collection tube.**

3. **Preequilibrate the spin column with 5 column volumes of binding buffer.**

4. **Add an appropriate amount of lysate (≤0.5 ml) to the micro spin column.**

   See Section 8, Preparation of Clarified *E. coli* Lysate Using Denaturing Conditions for protocol.

5. **Mix by pipetting up and down 5 times.**

   Incubate in micro spin column for up to 5 min.

6. **Centrifuge at 1,000 x g (700 x g for 2 min) for 15 sec.**

   Remove the unbound proteins by centrifuging. Save flow-through and label it "unbound 1".

Part 2: Washing the Resin

7. **Insert micro spin column into new collection vessel.**

8. **Wash the resin with at least 5 column volumes of wash buffer.**

   Pipet up and down at least 5 times.

   Note: If previously determined, an optimized concentration of imidazole may be added to the contents of the guanidine binding buffer that is slightly less than the concentration necessary to elute the target protein. See Section 10.

9. **Centrifuge at 1,000 x g for 15 sec (or 700 x g for 2 min).**

Remove remaining unbound proteins by centrifuging. Save flow-through and pool with unbound fraction from step 6. Label “unbound 2”. The wash step can be repeated if necessary.

Part 3: Eluting the His-Tagged Protein

10. **Insert micro spin column into new, clean collection vessel.**

11. **Elute bound proteins with 5 column volumes of elution buffer.**

    Pipet up and down at least 5 times and incubate for 5 min.

12. **Centrifuge at 1,000 x g for 1 min to recover His-tagged protein.**

    Analyze fractions from above steps by A_{280}, SDS-PAGE, ELISA, etc.

Section 14
Batch-Mode Purification of Histidine Proteins — Gravity Flow

The following guideline is a **general procedure** for batch-mode binding, which is an effective means for purification when the recombinant His-tagged protein is either expressed at low levels or when the tag itself may be inaccessible.

Recommended Columns

The following Bio-Rad columns may be used for gravity-flow chromatography. They are empty and can be filled with a support of choice.

- Poly-Prep® columns, for bed volumes up to 2 ml of chromatography support and 10 ml sample
- Econo-Pac® columns, for bed volumes up to 20 ml
- Glass Econo-Column® columns, 5–170 cm long and 0.5–5.0 cm in diameter

Materials

Reagents

- Binding/wash buffer
  - 50 mM sodium phosphate (NaH_2PO_4)
  - 300 mM NaCl
  - 5 mM imidazole

  Adjust to pH 8.0.

- Elution buffer
  - 50 mM sodium phosphate (NaH_2PO_4)
  - 300 mM NaCl
  - 500 mM imidazole

  Adjust to pH 8.0.

Equipment

- IMAC resin
- Sample preparation-sized IMAC spin column (as prepared in Section 5) (for example, Micro Bio-Spin columns, catalog #732-6204)
- Plasticware, 2 ml capped, 2 ml capless tubes

Biological Sample

- Clarified lysate (as prepared in Section 7)

  The binding capacity of the Profinity™ IMAC resin is ≥15 mg His-tagged protein per ml resin. Larger amounts of protein will require a larger column to be used.

Additional Materials

- Equipment for assessing protein purity and recovery of the His-tagged protein

Resin Preparation

Profinity IMAC and Profinity IMAC Ni-charged resins come supplied in a 20% ethanol solution for resin storage. Before purification, the resin storage solution provided must first be replaced with distilled water.

1. **For easy removal of storage solution, transfer an appropriate amount of resin slurry to a graduated container, such as a Bio-Rad Econo-Pac column (bed volume up to 20 ml).**
2. **Apply vacuum to the column for rapid removal of the storage solution.**

   Exert caution to not allow the resin to dry out.
3. **Wash the column with 3 column volumes of distilled water.**
4. **Add enough distilled water to make a 50% slurry.**
5. **The resin is now ready to be packed.**

Method

1. **Prepare the resin.**

   If using uncharged Profinity IMAC resin, charge the resin as recommended in Section 6, Immobilizing Metal Ions.

   If using Profinity IMAC Ni-charged resin, ensure that all ethanol has been thoroughly washed away before proceeding to step 2.
2. **Add an appropriate amount of the prepared resin slurry (see Resin Preparation above) to an appropriate amount of clarified lysate.**
3. **Swirl mixture gently in an appropriate container. Incubate the resin-lysate mixture at 4°C for up to 30 min.**
4. **Load the resin-lysate mixture into an appropriate-sized column. Cap the bottom outlet of the column.**
5. **Collect column flow-through.**

   Save the flow-through for SDS-PAGE analysis.
6. **Wash column with at least 5 column volumes of binding/washing buffer.**

   Collect wash fractions. Pool with fractions collected in step 5.

7. **Elute the protein with at least 5 column volumes of elution buffer.**

   Save the eluates for further analysis, for example, A_{280}, SDS-PAGE, ELISA, etc.

8. **Repeat elution step if necessary.**

Section 15
Regenerating, Cleaning, Sanitizing, and Storage

Profinity™ IMAC columns are well suited for reuse. The polymeric nature and open pore structure of the resin allow the column to be run at high flow rates during regeneration, cleaning, and sanitizing steps. Protein separations are unaffected, even after numerous cycles, as reproducibility is extremely high.

Unless otherwise stated, the following steps may be carried out at 2 ml/min.

Regenerating the Medium

Regeneration cleans the medium adequately to start the next cycle. In general, IMAC columns may be used a number of times before it becomes necessary to recharge them with metal ions. If this is the case, regenerate metal-charged Profinity IMAC resins by stripping with an EDTA solution. Wash the column with 10 column volumes of 20 mM sodium phosphate, up to 1 M NaCl, and 0.2 M EDTA, pH 7.4. Ensure that residual EDTA is completely removed from the column by washing it with 3–5 column volumes of binding buffer followed by 3–5 column volumes of distilled water.

Recharge the IMAC resin as recommended in Section 6, Immobilizing Metal Ions, or proceed with cleaning-in-place measures (below).

Cleaning-in-Place

A column used to purify protein from cell extract usually contains some soluble substances and cell debris that are nonspecifically adsorbed onto the matrix. Cleaning-in-place eliminates material not removed by regeneration and prevents progressive buildup of contaminants. If the column is to be reused, these contaminants should be cleaned from the column, as they were not completely removed during the sample clarification steps.

The following steps may be followed to clean IMAC columns. This protocol also includes a regeneration step. For optimal results, the column should be run at 2 ml/min.

1. **Strip metal ions.**

   Wash with 10 column volumes of 50 mM sodium phosphate, 0.3 M NaCl, and 0.05–0.5 M EDTA, pH 7.5.

2. **Wash the column with one of the following solutions at 2 ml/min:**

   - 1% acetic acid with 0.12 M phosphoric acid, pH 1.5
     - This solution may be used as a cleaning, sanitizing and storage solution with Profinity IMAC resins
     - 10–15 min exposure time
     - Rinse with 10 column volumes of distilled water
   - 2 M NaCl (removes ionic contaminants)
     - 10–15 min exposure time
     - Rinse with 10 column volumes of distilled water
   - 1 M NaOH up to 3 hr (removes precipitated, hydrophobic, and lipoproteins)
     - Exposure time is usually 1–3 hr
     - Rinse with 10 column volumes of distilled water
   - 70% ethanol or 30% isopropyl alcohol (removes precipitated, hydrophobic, and lipoproteins)
     - 15–20 min exposure time
     - Alternatively, use 0–30% gradient isopropyl alcohol over 5 column volumes, followed by 2 column volumes 30% isopropyl alcohol
     - Rinse with 10 column volumes of distilled water

3. **Remove cleaning solution(s) from column by rinsing with 10 column volumes of binding buffer.**

   - Rinse column with 50 mM sodium phosphate, 0.3 M NaCl, pH 8.0
   - Ensure the eluate is ~pH 8 and the UV signal has returned to the baseline

4. **Recharge the column with metal ion of choice.**

   - Refer to Section 6, Immobilizing Metal Ions

Sanitization

Sanitization inactivates microorganisms and prevents buildup of endotoxins. For optimal results, the column should be run at 300 cm/hr.

The column may be washed with either of the following two solutions:

- 1% acetic acid and 0.12 M phosphoric acid, pH 1.5
- 1 M NaOH

Rinse solution from column with 3–5 column volumes of distilled water. Reequilibrate the column with 3–5 column volumes of binding buffer.

Storage

The Profinity IMAC resin is stable at room temperature across a broad pH range (1–14). The medium may also be stored in any of the following solutions:

- 1% acetic acid and 0.12 M phosphoric acid, pH 1.5 (up to 200 hr)
- 2% benzyl alcohol
- 20% ethanol

Section 16
Troubleshooting Guide

Problem	Possible Cause	Solution
Sample is too viscous	High concentration of host nucleic acids in lysate	Viscosity of extract can be reduced by addition of Benzonase nuclease (1.7 units/ml) with 1 mM $MgCl_2$ to fragment bacterial DNA. Incubate on ice for 15 min
	Insufficient amount of homogenization buffer	Dilute sample by adding more homogenization buffer
Sample application causes column to clog	Insufficient clarification of sample	Reduce cell debris from clogging the column by increasing the centrifugation speed or filtering the sample
No protein is eluted	Expression of target protein in extract is very low and is not found in the eluate	Check expression level of protein by estimating the amount in the extract, flow-through, eluted fraction, and pellet upon centrifugation. Use western blotting with anti-6xHis antibodies, target-protein-specific antibodies, ELISA, or enzyme activity determination Apply larger sample volume Minimize contact with hydrophobic surfaces (that is, polystyrene tubes). Proteins at low concentration may bind to the surface of the tube

Problem	Possible Cause	Solution
No protein is eluted (continued from previous page)	Target protein is found in inclusion bodies or possible insufficient lysis	Increase intensity/duration of disruption and homogenization If protein is insoluble, use 6 M guanidine HCl or 8 M urea to lyse denatured proteins (see Sections 8, 11, and 13)
	Target protein is found in the flow-through	Reduce imidazole concentration in sample, binding, and wash buffers. An imidazole gradient may be used to determine optimal amounts for wash and elution conditions Check pH levels of sample. A decrease in pH may result during the homogenization step or during growth of the culture medium. Adjust pH to 7–8 The histidine tag may not be accessible. Use denaturing conditions to purify protein or reclone the plasmid construct with the His-tagged sequence placed at the opposite terminus Proteolytic cleavage during fermentation or purification has caused the histidine tag to be removed. Add protease inhibitors or make a new construct with His-tag attached to other terminus

Problem	Possible Cause	Solution
No protein is eluted (continued from previous page)	Elution conditions are too mild or protein may be in an aggregated or multimer form	Elute with pH or an imidazole step-elution
Protein precipitates during purification	Temperature is too low	Perform the purification at room temperature
	Aggregate formation	Add solubilization agents to the samples and/or buffers: 0.1% Triton X-100, Tween-20, 20 mM β-mercaptoethanol and ≤20% glycerol to maintain protein solubility
Poor recovery of target protein	Protein is found in the flow-through	See recommendations in "No protein eluted" section
	Binding capacity of the column has been exceeded	Increase the column size or reduce the sample volume application
	Target protein was not detected in the flow-through	Capillary sample loop is too small
	Strong nonspecific adsorption of the target protein to the matrix	Reduce hydrophobic adsorption by including detergents, or organic solvents, or by increasing the concentration of NaCl

Problem	Possible Cause	Solution
His-tagged protein is not pure	Contaminants elute with target protein	Make binding and wash steps more stringent. Include 10–20 mM imidazole in binding and wash buffers Prolong the wash step containing imidazole Column is too large; reduce amount of Profinity™ IMAC resin used
	Strongly bound contaminants elute with protein	Very high concentrations of imidazole will cause strongly bound contaminants to elute as well. Reduce the imidazole concentration during the elution
	Association of contaminating proteins with target protein via disulfide bonds	Include ≤30 mM β-mercaptoethanol. Exert caution if using DTT
	Association between the His-tagged protein and protein contaminant	Add nonionic detergent or alcohol (that is, ≤2% Triton X-100, 2% Tween 20, or ≤50% glycerol) to reduce hydrophobic interactions. Concentration of NaCl may be increased to minimize electrostatic interactions
	Potential degradation of fusion protein by proteases	Include 1 mM PMSF or other protease inhibitor to lysis buffer to reduce partial degradation
	Contaminants exhibit similar affinity to target protein	Add an additional chromatography step, that is, ion exchange, hydrophobic interaction, or size exclusion

Section 17
Ordering Information

Catalog #	Description
156-0121	Profinity IMAC Resin, 10 ml
156-0123	Profinity IMAC Resin, 50 ml
156-0125	Profinity IMAC Resin, 500 ml
156-0127	Profinity IMAC Resin, 1 L
156-0131	Profinity IMAC Ni-Charged Resin, 10 ml
156-0133	Profinity IMAC Ni-Charged Resin, 25 ml
156-0135	Profinity IMAC Ni-Charged Resin, 100 ml
156-0137	Profinity IMAC Ni-Charged Resin, 500 ml

Section 18
References

Hochuli E, Large-scale chromatography of recombinant proteins, J Chromatogr 444, 293–302 (1988)

Porath J et al., Metal chelate affinity chromatography, a new approach to protein fractionation, Nature 258, 598–599 (1975)

Section 19
Legal Notices

Bio-Rad Laboratories, Inc.
2000 Alfred Nobel Dr., Hercules, CA 94547 USA
510-741-1000

10001677 Rev B