# Bio-Rad® Nuvia™ IMAC Resin

## Instruction Manual

Catalog numbers
780-0800
780-0801
780-0802

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

BIO-RAD

# Table of Contents

## 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, nitrilotriacetic acid (NTA) in this case, may be charged with transition metals such as $Cu^{2+}$, $Ni^{2+}$, $Co^{2+}$, or $Zn^{2+}$. This results in high selectivity for proteins with clustered histidine residues to be strongly retained on a porous chromatographic support.

The use of IMAC to separate an expressed recombinant protein fused with a hexahistidine 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 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 histidine-tagged proteins is frequently used for structural and functional studies of proteins.

# Section 2
# Product Information

## Nuvia™ IMAC Resins and UNOsphere™ Technology

Nuvia 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 high-capacity IMAC resin to exhibit excellent flow properties without compromising protein binding, recovery, or purity.

Nuvia IMAC uses NTA as its functional ligand. The tertiary amine and carboxylic acid side chains of NTA serve as the chelating ligands for divalent metal ions. The structure offers selective binding of recombinant histidine-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 open-pore structure of Nuvia IMAC beads result in superb mechanical strength with high stringency, low nonspecific effects, and the ability to provide separations at fast flow rates. These unique features of the UNOsphere base matrix lend a number of performance benefits to the Nuvia IMAC resin.

Nuvia IMAC is also stable across a wide pH range (2–14) 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 chromatography instrumentation, gravity flow columns, or sample-preparation spin columns.

**Note**: UNOsphere media, from which Nuvia 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 and will stay within the pressure limits of the column and chromatography system.

* U.S. patent 6,423,666.

Under optimized conditions, the binding capacity for 6x histidine-tagged proteins is >40 mg/ml resin (see Table 1). The product is a 50% (v/v) slurry of resin, which is suspended in a 20% ethanol solution. Nuvia IMAC resin is amenable to process and laboratory-scale use and is available precharged with $Ni^{2+}$ in bottles as well as prepacked into columns. Table 1 lists key characteristics of the resin, while Table 2 lists a variety of compounds compatible with Nuvia IMAC support.

## Chemical Interactions

Nuvia IMAC resin is composed of NTA groups coupled to a UNOsphere base matrix via a proprietary polymerization derivatization technology. It is well-suited to recombinant histidine-tagged purifications and results in high binding capacity and specificity for the target molecule.

Although the most commonly used metal ion for histidine-tagged purifications is $Ni^{2+}$, other metals may be used to increase efficacy of purification. Therefore, choosing another type of immobilized metal ion can change the selectivity of an IMAC resin.

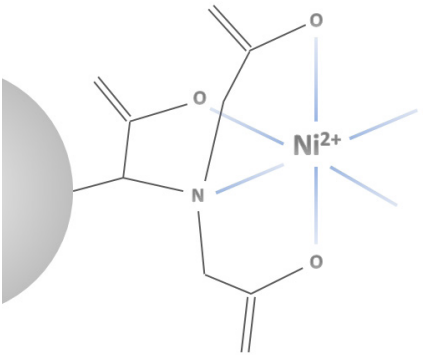


**Fig. 1. Partial structure of Nuvia Ni-charged IMAC resin.** Image illustrates UNOsphere base bead with coupled NTA functional ligand.

## Resin Characteristics

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

**Table 1. Characteristics of Bio-Rad Nuvia IMAC Resin.**

| | |
|---|---|
| Functional ligand | Nitrilotriacetic acid (NTA) |
| Base bead | UNOsphere base matrix |
| Form | 50% suspension in 20% ethanol or pre-packed into columns; comes precharged with $Ni^{2+}$ |
| Particle size | 38–53 µm |
| Mean particle size | 50 µm |
| Metal ion capacity | ≥18 µmol $Cu^{2+}$/ml Nuvia IMAC resin |
| Dynamic binding capacity* | ≥40 mg/ml resin |
| Recommended linear flow rate | <500 cm/hr at 25°C |
| Maximum operating pressure | 45 psi |
| pH stability, short-term/ cleaning | 2–14 |
| Chemical compatibility | See Table 2 |
| Storage | 4°C to ambient temperature |
| Shelf life in 20% ethanol | ≥3 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 (Q10% determination):
Column volume: 1 ml, 5.6 mm x 4 cm (ID x H)
Sample: 1.0 mg/ml 6x histidine-tagged pure protein (40 kD)

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

## Chemical Compatibilities

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

**Table 2. Chemical Compatibilities for Nuvia 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 can cause significant reduction in binding capacity |
| Sulfhydryl reagents | β-Mercaptoethanol | Reduces random disulfide bonds preventing protein aggregation during purification | ≤20 mM |
| | DTT, TCEP | Transition metals at the center of IMAC resin ($Ni^{2+}$) are susceptible to reduction | ≤10 mM DTT and 20 mM TCEP |
| Detergents | Nonionic detergents (Triton, Tween) | Removes background proteins and nucleic acids | ≤2% |
| | Zwitterionic detergents (CHAPS, CHAPSO) | Solubilizes membrane proteins | ≤1% |
| Denaturants | Guanidine HCl (GuHCl) | Solubilizes proteins | ≤6 M |
| | Urea | | ≤8 M |

**Table 2. Chemical Compatibilities for Nuvia IMAC Resins, Continued.**

| Reagent Group | Reagent | Comments | Stability |
|---|---|---|---|
| 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 | ≤5 mM (HEPES or Tris buffers should be used to prevent precipitation) |
| | Glycerol | Included to prevent hydrophobic interactions between proteins | ≤20% (backpressure may increase significantly, slower flow rates may be required) |
| | Ethanol | Included to prevent hydrophobic interactions between proteins | ≤20% |
| | Imidazole | Competes for binding sites with histidine-tagged residues by interaction with the metal residues | May be used in low concentrations in the wash buffer (<30 mM) to limit binding of undesired proteins; for elution, ≤500 mM may be used |

# Section 3
# General IMAC Procedures

## Protein Binding

Protein adsorption to immobilized ions is performed around neutral to slightly alkaline pH conditions (pH 7.0–8.0). To reduce nonspecific ionic effects, concentrations of up to 1 M NaCl may be added to the binding solution. Recombinant 6x histidine tags, located at either the amino or carboxyl terminus of the protein, can bind with high affinity to the matrix even when the 6x histidine 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 6x histidine tag is low. In this case, proteins are bound to the Nuvia™ 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 (0–15 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 histidine-tagged proteins to IMAC resins. For example, urea, GuHCl, nonionic detergents, and organic solvents (refer to Section 2, Table 2) are all valid options. Chelating agents, such as EDTA or citrate, should not be included. Reducing agents such as β-mercaptoethanol 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.

  Begin with: 50 mM sodium phosphate, 0.3 M NaCl, pH 8.0

## Washes

Stringency of 6x histidine-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 from a bacterial expression system, have fewer contaminant proteins that copurify along with the protein of interest. Endogenous protein contaminants are more abundant in eukaryotic expression systems and tend 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 5–30 mM. The optimal pH and/or imidazole concentration used in wash buffers is always protein dependent and should always be determined experimentally.

Recommended wash buffer:

- 5–30 mM imidazole; for example, 50 mM sodium phosphate, 0.3 M NaCl.

  Begin with: 5 mM imidazole, 50 mM sodium phosphate, 0.3 M NaCl, pH 8.0

## Elution

Desorption of the histidine-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 pre-equilibrate the column with low concentrations of imidazole (1 mM) and include the same concentration in the sample. This prevents adsorption of imidazole onto the resin from triggering a drop in pH, which might prematurely elute bound histidine-tagged proteins.

Lowering the pH of the elution buffer (pH 4.5–5.3) also releases bound histidine-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 neutralizing buffer such as 1 M Tris-HCl, pH 8.0 may be used to collect acidic eluates (100–200 µl eluate/ml neutralizing buffer). 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 histidine-tagged protein to elute as a protein-metal complex. This results in metal ions appearing in the protein fractions.

*Recommended elution buffer for Nuvia IMAC resin*

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

Recommended elution buffer:

- 20–500 mM imidazole; for example, 50 mM sodium phosphate, 0.3 M NaCl.

  Begin with: 0.5 M imidazole, 50 mM sodium phosphate, 0.3 M NaCl, 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 histidine-tagged protein. Usually proteins expressed as inclusion bodies are not in their native conformation, so high concentrations of denaturants may be used during lysate preparation 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.

## 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 established. Determine optimized conditions using a small amount of sample. 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 points in mind during lysate preparation and purification:

- Low concentrations of imidazole (1–30 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 (1–30 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 also found in the histidine-containing compound
- If the recombinant histidine-tagged protein does not bind under higher concentrations, the imidazole concentration can be reduced for binding and wash steps
- A gradient elution test may be used to determine 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 Nuvia™ IMAC resin with aid from a pump. For best results, use 5–50 mm ID columns and a bed height of 5–30 cm.

## Recommended Columns

Bio-Rad's Bio-Scale™ MT high-resolution columns may be used for the following column-packing procedure. These columns are empty and may be packed with the support of choice. Bio-Scale MT columns are convenient to use with Bio-Rad's NGC™ chromatography 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

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

## Resin Preparation

Nuvia IMAC Ni-charged resins come in a 20% ethanol solution, supplied for resin storage. The column can be slurry packed in this solution; simply resuspend the resin in the ethanol solution provided. Before applying sample, ensure that all ethanol is removed during the equilibration step.

## Method

1. Eliminate air from the column dead spaces. Attach the inlet of a peristaltic or other pump to the outlet of the MT column. Fill the column with distilled water to about 10% of its volume. Flush end pieces 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 when closing the outlet valve.
2. Suspend the 50% v/v slurry by gently swirling or stirring with a glass or plastic rod.
3. Carefully transfer about a third of the slurry down the side into the column using a glass or plastic rod to avoid introducing air bubbles.
4. Start the pump at a low flow rate (eg. 0.5 ml/min). The resin will begin to pack in the column. As the liquid level in the column drops, continue to transfer the rest of the slurry until the packed bed reaches 1 cm from the top. Stop or slow the pump flow rate as necessary.

   Gently add more distilled water down the side of the column to make sure the liquid does not fall below the resin level. Continue adding distilled water until the bed seems to have stabilized. Then gently fill the column to the top with distilled water.
5. Stop the pump and prepare to insert the top adapter.
6. Insert the top flow adaptor. Use care to avoid introducing any air bubbles. Insert the adaptor into the column at an angle. Make sure the exit tubing is open so that distilled water can flow out the top adaptor along with any air.

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

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

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

8. Reconnect the pump and equilibrate. Pass eluent (distilled water) through the column at the packing flow rate. During equilibration, the bed may compress even further. When a constant bed height is reached, mark the compression level at this flow rate. Again, adjust the adaptor to compress the bed an additional 0.1–0.5 cm past the level marked.

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

# Section 5
# Column Packing — Sample Preparation–Sized Columns

Use this method for packing Nuvia™ IMAC resin into sample preparation micro spin columns.

## Materials

### 1. Reagents

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

**Note**: Charging Nuvia 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 sample.

If needed, the procedure to charge Nuvia IMAC (uncharged) resin is explained in Section 6, Immobilizing Metal Ions.

### 2. Equipment

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

### Resin Preparation

Nuvia IMAC Ni-charged resins come in a 20% ethanol solution, supplied for resin storage. The column can be packed in this slurry or decanted into distilled water and packed.

## Method

1. Thoroughly suspend Nuvia IMAC resin.
2. Place the column into an appropriate collection vessel; for example, a 2 ml capless collection tube.
3. Using a pipet, transfer the appropriate amount of Ni-charged Nuvia IMAC resin to a microcentrifuge tube. If using a Micro Bio-Spin column, transfer 0.2 ml slurried Ni-charged Nuvia IMAC resin 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.

## Section 6
## Immobilizing Metal Ions

Efficacy of protein binding by IMAC depends 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 amino acid residues. Nuvia™ IMAC resin uses a quadridentate ligand (NTA), which leaves two 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 through the choice of metal ion used, understanding of 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. Nuvia IMAC resin, based on the polymeric UNOsphere™ technology, has been specifically formulated with an optimal number 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.
2. After slurry packing is complete (see Sections 4 and/or 5), the column is ready for the removal or addition of metal ions.
3. If necessary, strip any metal ions by washing with 10 column volumes of 50 mM sodium phosphate, 0.3 M NaCl, and 0.05–0.5 M EDTA, pH 7.5.
4. 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).
5. Apply 3–5 column volumes of the metal ion solution.
6. Wash with 5 column volumes of 50 mM sodium acetate, 0.3 M NaCl, pH 4.0. Remove excess ions by washing.
7. Wash with 10 column volumes of deionized water.
8. 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
## Medium-Pressure Column Purification of Histidine-Tagged Proteins

For this guideline, the sample is applied to a packed column and the proteins are eluted using a high concentration of imidazole. The guideline does not optimize the imidazole concentration, but instead provides for fast capture of the target protein that may be used as a quick check for protein expression levels. Higher levels of purity are achievable by optimizing imidazole concentrations, which improves protein separation. See Section 8, Medium-Column Purification – Using an Imidazole Gradient to Determine Optimal Purification of Histidine-Tagged Proteins.

### Materials

**1. Reagents**

Binding buffer

- 50 mM sodium phosphate ($NaH_2PO_4$)
- 300 mM NaCl
- Low concentrations imidazole* (0–15 mM)
- Adjust to pH 8.0

Wash buffer

- 50 mM sodium phosphate ($NaH_2PO_4$)
- 300 mM NaCl
- Low concentrations of imidazole* (0–30 mM)
- Adjust to pH 8.0

Elution buffer

- 50 mM sodium phosphate ($NaH_2PO_4$)
- 300 mM NaCl
- Higher concentrations of imidazole* (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 buffers, as well as elution buffers, be empirically established. Determine optimized conditions using a small amount of sample.

2. **Equipment**

   - IMAC column (as prepared in Section 4)

3. **Biological Sample**

   - Clarified lysate

   The binding capacity of the Nuvia™ IMAC resin is ~40 mg histidine-tagged protein per ml resin. Larger amounts of protein will require use of a larger column.

4. **Additional Materials**

   - Medium-pressure chromatography system (such as Bio-Rad's BioLogic™ or NGC™ 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 is recommended as a general starting buffer; for example, 50 mM sodium phosphate, 300 mM NaCl, 5 mM imidazole, pH 8.0. Binding of histidine-tagged protein on the Nuvia IMAC resin is optimal in the pH range of 7–8.

   The column may be run at flow rates up to 500 cm/hr. Higher binding of histidine-tagged proteins will be achieved at lower flow rates. Average binding capacity of the Nuvia IMAC resin is approximately 40 mg histidine-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 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 2 ml fractions, or approximately 0.2 column volumes each.

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

7. Repeat elution steps 2 to 4 more times.

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

*IMAC Purification under Denaturing Conditions*

In some cases it may be necessary to use denaturants such as urea to solubilize inclusion bodies, which are not generally in their native conformation. To perform this, up to 8 M urea (or 6 M guanidine HCl) may be used in the binding, wash, and elution buffers listed above. Elution is still achieved by increasing the imidazole concentration.

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

Often the protein can be restored to its native form. To do this, the denaturant used to lyse and purify the sample must be removed using 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. Guidelines for on-column renaturation are suggested on the following page.

## Materials for On-Column Renaturation

### 1. Reagents

Refolding buffer

- 20 mM sodium phosphate ($NaH_2PO_4$) (pH 8.0)
- 20 mM imidazole
- 300 mM NaCl
- 1 mM β-mercaptoethanol

Urea binding buffer (refolding buffer with 8 M urea)

Phosphate elution buffer (with high imidazole)

- 20 mM sodium phosphate ($NaH_2PO_4$) (pH 8.0)
- 500 mM imidazole
- 300 mM NaCl
- 1 mM β-mercaptoethanol

**Note**: The β-mercaptoethanol should be added to solutions only immediately before use.

## Method for On-Column Renaturation

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.
4. If necessary, add another chromatography step.

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

## Section 8
## Medium-Pressure Column Purification — Using an Imidazole Gradient to Determine Optimal Purification of Histidine-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 elution may be used for subsequent purification of larger sample volumes.

With step elution, 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 NGC™ system to establish a linear gradient.

### Materials

**1. Reagents**

Binding buffer

- 20 mM sodium phosphate ($NaH_2PO_4$)
- 300 mM NaCl
- 20 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

**2. Equipment**

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

3. **Biological Sample**

   - Clarified lysate

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

4. **Additional Materials**

   - Equipment for assessing protein purity and recovering of the histidine-tagged protein

## Method

### Part 1: Optimizing the Imidazole Concentration

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

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

2. Equilibrate the 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 from 0.2 to 0.5 column volumes.

4. Load sample and collect the flowthrough in fractions appropriate to the size of the column (as recommended above).

   **Note**: Monitor the backpressure while sample is being applied. If the sample is insufficiently clarified, 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 0–50% 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 that contain the histidine-tagged protein using an activity assay (such as one of Bio-Rad's protein assay kits), UV absorbance, SDS-PAGE, or western blot analysis with antihistidine antibodies or antibodies specific to the target protein.
10. Calculate the concentration of imidazole that corresponds to the elution peak of the histidine-tagged protein.

    **Note**: The delay due to column volume and the tubing in the chromatography system need to be considered when comparing the actual gradient trace to the programmed gradient.

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

The following tips are useful to keep in mind when designing the experiment:

- Maintain the concentration of imidazole in the binding (also called equilibration) buffer at 20 mM. If large amounts of contaminants are also adsorbed onto the resin, the concentration of imidazole in the sample and equilibration buffer may be increased. This may reduce the overall amount of target protein bound and should be carried out with care. However, it will also increase the column's binding capacity for the target protein due to the reduction in contaminating proteins
- Include a wash step with 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 histidine-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

### Part 2: Using an Optimized Imidazole Concentration for Purification

1. Prepare 500 ml binding buffer and 500 ml elution buffer for a 5 ml column.

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

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

3. Begin collecting fractions.

   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 flowthrough 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 that contains imidazole in a quantity that will not elute the target protein.

7. Elute the histidine-tagged protein with 5 column volumes of elution buffer with an optimized imidazole concentration.

8. Wash the column with 5 column volumes of elution buffer. Stop collecting fractions.

9. Re-equilibrate the column with 10 column volumes of equilibration (binding) buffer.

10. Assess the purity and recovery of the target protein using an activity assay (such as one of Bio-Rad's protein assay kits), UV absorbance, SDS-PAGE, or western blot analysis with antihistidine antibodies or antibodies specific to the target protein.

# Section 9
# Sample Preparation–Sized Spin-Column Purification of Histidine-Tagged Proteins

## Materials

### 1. 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 8)

- 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 8, Medium-Pressure Column Purification — Using an Imidazole Gradient to Determine Optimal Purification of Histidine-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

**Note**: If necessary, up to 8 M urea may be added to these buffers in order to solubilize proteins in inclusion bodies. See Section 7 for more information.

### 2. Equipment

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

3. **Biological Sample**

   - Clarified lysate

   The binding capacity of Nuvia™ IMAC resin is ≥40 mg histidine-tagged protein per ml resin. Larger amounts of protein will require a larger column

4. **Additional Materials**

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

## Method

Reserve a small amount of lysate prior to loading sample onto the column. This will serve as sample for the lysate lane for later analysis with, for example, SDS-PAGE.

### Part 1: Binding the 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. Pre-equilibrate 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 histidine-tagged protein on the Nuvia™ 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

1. Insert micro spin column into new collection vessel.
2. 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, you may use an optimized concentration of imidazole that is slightly less than the concentration necessary to elute the target protein. See Section 8.
3. 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 Histidine-Tagged Protein

1. Insert micro spin column into a new, clean collection vessel.
2. 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 Nuvia IMAC resin.
3. Analyze fractions from above steps by $A_{280}$, SDS-PAGE, ELISA, etc.

## Section 10
## Regenerating, Cleaning, Sanitizing, and Storage

Nuvia™ 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 they need to be recharged with metal ions. When it becomes necessary, regenerate metal-charged Nuvia IMAC resins by first 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 if 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 can be run at 2–5 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:

   a. 1% acetic acid
      - This solution may be used as a cleaning, sanitizing, and storage solution with Nuvia IMAC resins
      - 10–15 min exposure time
      - Rinse with 10 column volumes of distilled water

   b. 2 M NaCl (removes ionic contaminants)
      - 10–15 min exposure time
      - Rinse with 10 column volumes of distilled water

   c. 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

   d. 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
   - Ensure the eluate is ~pH 8 and the UV signal has returned to 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 the following solution:

- 1 M NaOH

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

## Storage

Nuvia IMAC resin is stable at room temperature across a broad pH range (2–14). The medium may also be stored in either of the following solutions:

- 2% benzyl alcohol
- 20% ethanol

# Section 11
# 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 adding Benzonase nuclease (1.7 U/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 | Prevent 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, flowthrough, eluted fraction, and pellet upon centrifugation. Use western blotting with anti-6x histidine 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 |
| | 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 3, and 7) |
| | Target protein is found in the flowthrough | 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 histidine-tagged sequence placed at the opposite terminus |

| Problem | Possible Cause | Solution |
|---|---|---|
| No protein is eluted (continued from previous page) | Target protein is found in the flowthrough (continued from previous page) | Proteolytic cleavage during fermentation or purification has caused the histidine tag to be removed. Add protease inhibitors or make a new construct with histidine tag attached to other terminus |
| | Elution conditions are too mild or protein may be in an aggregated or multimer form | Elute with pH or imidazole step elution |
| Protein precipitates during purification | Temperature is too low | Perform the purification at room temperature |
| | Aggregate forms | Add solubilization agents to 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 flowthrough | See recommendations in No protein is 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 flowthrough | 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 |
| Histidine-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 Nuvia™ 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. Exercise caution if using DTT |

| Problem | Possible Cause | Solution |
|---|---|---|
| Histidine-tagged protein is not pure (continued from previous page) | Association between the histidine-tagged protein and protein contaminant | Add nonionic detergent or alcohol (that is, ≤2% Triton X-100, 2% Tween 20, or ≤20% 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 in lysis buffer to reduce partial degradation |
| | Contaminants exhibit similar affinity to target protein | Add a chromatography step; that is, ion exchange, hydrophobic interaction, or size exclusion |

## Section 12
## Ordering Information

**Nuvia IMAC™ Ni-Charged Resin**

| Catalog # | Description |
|---|---|
| 780-0800 | **Nuvia IMAC Ni-Charged Resin**, 25 ml bottle |
| 780-0801 | **Nuvia IMAC Ni-Charged Resin**, 100 ml bottle |
| 780-0802 | **Nuvia IMAC Ni-Charged Resin**, 500 ml bottle |

**Bio-Scale™ Mini Nuvia™ IMAC Ni-Charged Cartridges**

| Catalog # | Description |
|---|---|
| 780-0811 | **Bio-Scale Mini Nuvia IMAC Ni-Charged Cartridge**, 1 x 5 ml column |
| 780-0812 | **Bio-Scale Mini Nuvia IMAC Ni-Charged Cartridge**, 5 x 5 ml columns |

## Section 13
## References

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

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

## Section 14
## Legal Notices


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