His•Bind® Kits

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About the Kits

Product	Size	Cat. No.
His•Bind® Resin	10 ml	69670-3
	50 ml	69670-4
	100 ml	69670-5
His•Bind Resin, Ni-charged	10 ml	71035-3
	25 ml	71035-4
	100 ml	71035-5
His•Bind Columns	pkg/5	70971-3
	pkg/25	70971-4
His•Mag™ Agarose Beads	2 × 1 ml	71002-3
	10 × 1 ml	71002-4
His•Bind Buffer Kit	1 kit	69755-3
His•Bind Purification Kit	1 kit	70239-3
His•Bind Quick Columns	pkg/12	70159-3
	pkg/60	70159-4
His•Bind Quick 300 Cartridges	pkg/10	70155-3
	pkg/50	70155-4
His•Bind Quick 900 Cartridges	pkg/10	70156-3
	pkg/50	70156-4
His•Bind Quick Buffer Kit	1 kit	70665-3
His•Bind Fractogel®	25 ml	70693-3
BugBuster® His•Bind Purification Kit	1 kit	70793-3
PopCulture® His•Mag Purification Kit	1 kit	71114-3

Description

His•Bind Resin and Buffer Kit

His•Bind Resin is used for rapid one-step purification of proteins containing His•Tag® sequence by metal chelation chromatography. The His•Tag sequence binds Ni^{2+} cations that are immobilized on His•Bind resin using Charge Buffer supplied in the His•Bind Buffer Kit. After unbound proteins are washed away, target protein is recovered by elution with imidazole. The His•Bind Resin can be regenerated and reused many times. The versatile system allows proteins to be purified under gentle, non-denaturing conditions, or in the presence of either 6 M guanidine or urea. Up to 20 mg target protein can be purified on a single 2.5-ml column.

His•Bind Resin, Ni-charged

His•Bind Resin, Ni-charged is a pre-charged version of the popular His•Bind Resin used for purification of His•Tag fusion proteins by metal affinity chromatography. The Ni^{2+} ions are held by chelation with iminodiacetic acid (IDA). The IDA support can be recycled many times with no loss in performance. His•Bind Resin, Ni-charged is supplied as 50% slurry and has binding capacity of up to 8 mg/ml settled resin. The resin is compatible with up to 1.0 mM THP, a reducing agent that is more stable and effective than dithiothreitol (DTT). Use His•Bind Resin, Ni-charged with His•Bind Quick Buffer Set, or Ni-NTA Buffer Kit.

His•Bind® Columns

His•Bind Columns are packed with 1.25 ml Ni^{2+}-charged His•Bind resin. The binding capacity is 10 mg of target protein per column. Top and bottom frits ensure uniform buffer flow and minimal disturbance of the bed when loading and running the column. The His•Bind Columns can be used under native conditions, or in the presence of either 6 M guanidine or urea. Reuse is not recommended.

His•Bind Quick Cartridges and Columns

His•Bind Quick Cartridges and Columns are precharged with Ni^{2+} and use a large diameter cellulose matrix that has flow rates 5–50 times faster than agarose resins. Using these formats, target proteins can be purified from crude lysates under native or denaturing conditions in as little as five minutes.

His•Bind Quick 300 and 900 Cartridges are designed for operation with a syringe. His•Bind Quick Columns are designed for use with the Novagen Vacuum Manifold (Cat. No. 70147), which enables processing of up to 12 columns simultaneously.

Product	Approx. capacity/run	Method
His•Bind Quick 300 Cartridge	0.5 mg protein	Syringe
His•Bind Quick 900 Cartridge	2.0 mg protein	Syringe
His•Bind Quick Column	5.0 mg protein	Vacuum

Please note that because His•Bind Quick Resin is cellulose-based, it is not recommended for use with proteins encoding a cellulose binding domain, in addition to the His•Tag® sequence.

His•Mag™ Agarose Beads

His•Mag Agarose Beads are 3-µm diameter beads pre-charged with Ni^{2+}, and are ideal for rapid purification of multiple samples with minimal handling. His•Mag Agarose Beads have a binding capacity of 5 mg target protein per 1 ml beads. The beads are compatible with magnetic separation based high throughput applications. Proteins can be purified under non-denaturing conditions or in the presence of either 6 M guanidine or urea.

BugBuster® His•Bind Purification Kit

BugBuster His•Bind Purification Kit combines His•Bind Resin, His•Bind Buffer Kit, Benzonase® Nuclease, and BugBuster Protein Extraction Reagent for convenient preparation of soluble cell extracts, and affinity purification of His•Tag fusion proteins. BugBuster Protein Extraction Reagent is formulated for the gentle disruption of the *E. coli* cell wall, resulting in the liberation of soluble protein. BugBuster® Protein Extraction Reagent provides a simple, rapid alternative to mechanical methods, such as French Press or sonication, for releasing expressed target proteins in preparation for purification. The proprietary formulation utilizes a mixture of non-ionic detergents that is capable of cell wall perforation without denaturing soluble protein. Benzonase® Nuclease is a genetically engineered endonuclease from *Serratia marcescens* (1–2). This promiscuous endonuclease attacks and degrades all forms of DNA and RNA (single stranded, double stranded, linear and circular), and is effective over a wide range of operating conditions (3). Cells are harvested by centrifugation, followed by suspension in BugBuster Protein Extraction Reagent. Extracts prepared using BugBuster Protein Extraction Reagent and Benzonase Nuclease are fully compatible with all of the Novagen His•Bind® supports.

Note: BugBuster Protein Extraction Reagent is supplied in a Tris-HCl-based buffer. For applications requiring other buffers, Novagen offers BugBuster (primary amine free, Cat. No. 70923) Protein Extraction Reagent, which contains a PIPPS buffer, and BugBuster 10X Protein Extraction Reagent (Cat. No. 70921), which does not contain added buffer.

PopCulture® His•Mag Purification Kit

The PopCulture His•Mag Purification Kit is ideally suited for high throughput (HT) protein extraction and purification with His•Mag Agarose Beads in the original culture tube or multiwell

plate, without the need for centrifugation or columns. PopCulture Reagent is a Tris-HCl buffered mixture of concentrated detergents formulated to perforate the *E. coli* cell wall, and extract proteins directly in the culture medium without denaturing soluble protein. To further enhance the purification procedure when using PopCulture Reagent, rLysozyme™ Solution is included in this kit. Lysozyme cleaves a peptidoglycan bond in the *E. coli* cell wall, enhancing cell lysis and increasing protein yield(4–5).

His•Bind Fractogel® Resin

His•Bind Fractogel Resin is 40–90 µm tentacle methacrylate beads, and is ideal for low pressure chromatography, or FPLC under higher flow rates and pressures. His•Bind Fractogel Resin is supplied as uncharged resin. For best results, charge His•Bind Fractogel Resin with Cu^{2+} cations using $CuSO_4$ solution. Alternatively, His•Bind Fractogel Resin may be charged with Ni^{2+} cations using a $NiSO_4$ solution. His•Bind Fractogel Resin can be regenerated and reused many times. Proteins can be purified under gentle, non-denaturing conditions, or in the presence of either 6 M guanidine or urea. Greater than 10 mg target protein can be bound per 1 ml resin.

Components

His•Bind Buffer Kit

The His•Bind Buffer Kit contains the following components sufficient to run a minimum of ten 2.5-ml columns:

- 2 × 80 ml 8X Binding Buffer (8X = 4 M NaCl, 160 mM Tris-HCl, 40 mM imidazole, pH 7.9)
- 25 ml 8X Wash Buffer (8X = 4 M NaCl, 480 mM imidazole, 160 mM Tris-HCl, pH 7.9)
- 50 ml 4X Elute Buffer (4X = 4 M imidazole, 2 M NaCl, 80 mM Tris-HCl, pH 7.9)
- 50 ml 4X Strip Buffer (4X = 2 M NaCl, 400 mM EDTA, 80 mM Tris-HCl, pH 7.9)
- 20 ml 8X Charge Buffer (8X = 400 mM NiSO4)

His•Bind Purification Kit

- 10 ml His•Bind Resin, 10 ml settled volume (20 ml of a 50% v/v suspension)
- 1 His•Bind Buffer Kit
- pkg/4 Chromatography Columns (2.5-ml resin capacity)

His•Bind Quick Buffer Kit

- 2 × 80 ml 8X Binding Buffer (8X = 4 M NaCl, 160 mM Tris-HCl, 40 mM imidazole, pH 7.9)
- 25 ml 8X Wash Buffer (8X = 4 M NaCl, 480 mM imidazole, 160 mM Tris-HCl, pH 7.9)
- 50 ml 4X Elute Buffer (4X = 4 M imidazole, 2 M NaCl, 80 mM Tris-HCl, pH 7.9)
- 50 ml 4X Strip Buffer (4X = 2 M NaCl, 400 mM EDTA, 80 mM Tris-HCl, pH 7.9)

BugBuster His•Bind Purification Kit

- 2 × 100 ml BugBuster Protein Extraction Reagent
- 10,000 U Benzonase Nuclease, Purity > 90%
- 10 ml His•Bind Resin, 10 ml settled volume (20 ml of a 50% v/v suspension)
- 1 His•Bind Buffer Kit
- pkg/4 Chromatography Columns

PopCulture® His•Mag Purification Kit

- 15 ml PopCulture Reagent
- 300 KU rLysozyme™ Solution
- 1 ml rLysozyme Dilution Buffer
- 3 × 1 ml His•Mag™ Agarose Beads, 1 ml settled volume (2 ml of a 50% v/v suspension)
- 80 ml 8X Binding Buffer (8X = 4 M NaCl, 160 mM Tris-HCl, 40 mM imidazole, pH 7.9)
- 2 × 25 ml 8X Wash Buffer (8X = 4 M NaCl, 480 mM imidazole, 160 mM Tris-HCl, pH 7.9)
- 50 ml 4X Elute Buffer (4X = 4 M imidazole, 2 M NaCl, 80 mM Tris-HCl, pH 7.9)

Storage

Store BugBuster® Protein Extraction Reagent, His•Bind Quick Columns and Cartridges, and PopCulture® Reagent at room temperature. Store His•Bind® Resin, His•Bind Resin, Ni-charged, His•Bind Columns, His•Mag™ Agarose Beads, His•Bind Fractogel®, His•Bind Buffer Kit and His•Bind Quick Buffer Kit at 4°C. Store rLysozyme™ Solution, rLysozyme Dilution Buffer and Benzonase® Nuclease at –20°C.

Note: Storage of BugBuster Protein Extraction Reagent and PopCulture Reagent at temperatures below 4°C may cause precipitation of detergents. Incubate BugBuster Protein Extraction Reagent or PopCulture Reagent in room temperature water bath with gentle swirling or inversion to redissolve.

Note: Storage of rLysozyme Solution at –70°C may result in precipitation and loss of activity. Dilutions of rLysozyme Solution with rLysozyme Dilution Buffer of 1:100 or less are stable at 4°C for one week. DO NOT store diluted rLysozyme at –20°C because freezing will result in precipitation and/or loss of activity.

Overview

This technical bulletin describes methods for *E. coli* cell extract preparation, and procedures for purification of protein with a His•Tag® sequence using the His•Bind supports. These procedures begin with cell culture that has been induced for target protein production. The fusion protein should contain a sequence of at least 6 consecutive histidine residues (His•Tag sequence) at the N-terminus, C-terminus, or internal to the polypeptide. A detailed discussion of target protein induction using the Novagen pET System can be found in the pET System Manual User Protocol TB055, which accompanies pET vectors and systems, and is also available at www.novagen.com.

For proteins expressed using the pET System, a yield of 20 mg target protein per 100 ml culture is not unusual. Like other affinity chromatography methods, the highest purity of target proteins is achieved when using amounts of extract and resin such that the resin will be near its binding capacity. Thus, we recommend obtaining an estimate of the quantity of target protein present in the extract. SDS-PAGE, Western blot, S•Tag™ Rapid Assay, FRETWorks™ S•Tag Assay, or other protein-specific assays can estimate the mass of target protein in crude extracts. Once an estimate of the quantity of target protein has been determined, prepare a corresponding amount of resin, or choose the appropriately sized pre-packed column or cartridge.

While some target proteins remain soluble in the cytoplasm, other proteins form insoluble aggregates, or inclusion bodies in *E. coli*. In addition to the target protein, inclusion bodies also contain contaminating bacterial proteins and nucleic acids. It is possible to solubilize the protein from purified inclusion bodies and perform purification using His•Bind products under denaturing conditions. Modifications required for purification under denaturing conditions are described for each resin format.

Cell Extract Preparation

Considerations before you begin:

- Consider using PopCulture® Reagent or BugBuster® Protein Extraction Reagent with Benzonase® Nuclease and rLysozyme™ Solution, or BugBuster Protein Extraction Reagent with Lysonase™ Bioprocessing Reagent to extract soluble proteins, and/or prepare inclusion bodies. These products greatly simplify the process because mechanical disruption is avoided.
- Avoid 2-mercaptoethanol, DTT, and EDTA in solutions that will be used with His•Bind® supports. The reducing reagents react with Ni^{2+} to form brown precipitate. EDTA will chelate Ni^{2+} and, thereby, strip the column of the active affinity group.
- If target protein requires a reducing environment, 0.5 M THP Solution (Cat. No. 71194) can be used. THP [Tris(hydroxypropyl)phosphine] is a ready-to-use, water soluble, odorless, pH-neutral reducing agent that is more stable and effective than DTT as a sulfhydryl reductant. THP is more resistant to air oxidation than DTT, and is compatible at a concentration of 1 mM with His•Bind Resin.
- If necessary, protease inhibitors may be added to buffers to protect against degradative enzymes. We recommend initially proceeding without protease inhibitors, as the addition of these reagents is often unnecessary.
- Serine protease inhibitors should be used with caution if the target protein is to be treated with Thrombin (Cat. No. 69671), Factor Xa (Cat. No. 69036), Recombinant Enterokinase (rEK, Cat. No. 69066), or HRV 3C (Cat. No. 71493); any active inhibitor carried through purification may affect cleavage reactions. If proteolytic degradation of target protein is problematic, try adding the following: AEBSF (10–100 µM; Cat. No. 101500); Pepstatin A (1 µM; Cat. No. 516482); Leupeptin (10–100 µM; Cat, No. 108975); Aprotinin (2 µg/ml; Cat. No. 616398); Benzamidine (15 µg/ml; Cat. No. 324890); or Protease Inhibitor Cocktail Set III (without EDTA; Cat. No. 539134). Although it is likely that the inhibitors will be removed, and/or inactivated during purification, we recommend including a dialysis or gel filtration step prior to proteolytic cleavage with Thrombin, rEK, Factor Xa, or HRV 3C.
- Protease inhibitor cocktails that include EDTA are not compatible with His•Bind Resin.

Mechanical disruption method

Soluble fraction

Prepare 1X Binding Buffer for procedures below by diluting the 8X supplied stock or prepare according to buffer compositions provided on page 4.

rLysozyme Solution (Cat. No. 71110) is available for efficient bacterial cell lysis.

Benzonase Nuclease (Cat. No. 70664) degrades all forms of nucleic acid, eliminating viscosity and reducing processing time

1. Harvest cells by centrifugation at 10,000 × g for 10 min. Decant supernatant. Allow cell pellet to drain as completely as possible. Resuspend cells in 10 ml ice-cold 1X Binding Buffer, or 1X Fractogel Binding Buffer per 100-ml culture volume. If desired, up to 0.1% NP-40, or another non-ionic detergent, can be added to reduce non-specific binding. If resuspension is difficult, a Dounce homogenizer, blender, or sonicator can be used to break up cell pellet.
2. Sonicate sample in tube on ice, or in a salt-ice bath. Sonication conditions are not specified here because results are dependent on a number of factors including the type of sonicator probe, power setting, and shape and size of vessel containing cells. Avoid long sonication times to prevent sample heating; instead, divide sonication into bursts, allowing cooling between treatment. Sonicate until sample is no longer viscous. If DNA is not sheared by sonication, the viscosity of the extract will cause the column to clog, and the flow rate will be reduced. Larger cell masses may be treated in 1X Binding Buffer by alternative methods, such as French Press.

Optional:

a) Add rLysozyme Solution to a final concentration of 45–60 KU/gram cell paste. Mix by pipetting up and down. Incubate at 30°C for 15 min prior to sonication.

Note: *rLysozyme™ Solution can be diluted in rLysozyme Dilution Buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, and 0.1% Triton® X-100]. Dilutions should be used as soon as possible and stored on ice, or at 4°C until use. Do not store diluted rLysozyme Solution at –20°C as freezing may result in loss of activity.*

b) Add 1 µl (25 units) Benzonase® Nuclease per 1 ml lysis buffer used for resuspension.

Note: *Benzonase Nuclease is not recommended for nuclease-free preparations. Protein purification may not remove Benzonase.*

c) Alternatively, add 3 µl Lysonase™ Bioprocessing Reagent (Cat. No. 71230), per 1 ml lysis buffer. Lysonase Bioprocessing Reagent is an optimized, ready-to-use mix of rLysozyme Solution and Benzonase Nuclease.

d) Add protease inhibitors. Protease inhibitors are compatible with BugBuster® Protein Extraction Reagent and Benzonase Nuclease.

Note: *Serine protease inhibitors should be avoided if the target protein will be treated with Thrombin (Cat. No. 69671), Factor Xa (Cat. No. 69036), rEK (Cat. No. 69066), or HRV 3C (Cat. No. 71493). Although purification may remove active inhibitors, dialysis and gel filtration are recommended prior to cleavage. Protease inhibitor cocktails that include EDTA are not compatible with His•Bind® Resin.*

3. Centrifuge lysate at 14,000 × g for 20 min to remove debris. Filter post-centrifugation supernatant though a 0.45-µm membrane to prevent clogging of resins (syringe-end filters are convenient for this purpose).

Inclusion body purification

The following protocol describes isolation and washing of inclusion bodies from *E. coli* to remove contaminating proteins, followed by suspension in 1X Binding Buffer plus either 6 M guanidine-HCl or 6 M urea to solubilize target protein.

1. Prepare 1X Binding Buffer by diluting 8X supplied stock with deionized water, or according to buffer compositions provided on page 4 (without denaturant).
2. Harvest cells by centrifugation at 10,000 × g for 10 min. Decant supernatant. Allow cell pellet to drain as completely as possible. Resuspend cells in 40 ml 1X Binding Buffer per 100 ml culture volume.
3. Sonicate briefly (see “Mechanical disruption method,” page 6) to resuspend pellet thoroughly and shear DNA.
4. Centrifuge at 5,000 × g for 15 min to collect inclusion bodies and cellular debris while leaving other proteins in solution.
5. Remove supernatant. Resuspend pellet in 20 ml 1X Binding Buffer per 100 ml culture volume (without denaturant). Repeat Step 3. Sonication may be necessary to resuspend pellet.

Note: *Repeating this step several times may release more trapped proteins.*

6. Remove supernatant from final centrifugation and resuspend pellet in 5 ml 1X Binding Buffer containing either 6 M guanidine-HCl or 6 M urea per 100 ml culture volume. See the appropriate section in the following pages for buffer preparation under denaturing conditions.
7. Incubate on ice for 1 h to completely solubilize protein. Remove insoluble material by centrifugation at 16,000 × g for 30 min. Filter supernatant through a 0.45-µm membrane prior to performing purification with His•Bind products.

Note: *rLysozyme Solution may be added at Step 5 for processing insoluble protein fractions, although addition is not required. Lysozyme has been shown to improve the purity of inclusion body preparations by digesting cell wall debris. Add rLysozyme to the resuspended material in 1X Binding Buffer using a final concentration of 1 KU/ml. Vortex gently to mix. Incubate for 5–10 min. Proceed with centrifugation.*

Cell extract preparation using BugBuster® Reagent

BugBuster Protein Extraction Reagent is a simple, rapid alternative to mechanical methods of protein extraction, formulated for the gentle disruption of *E. coli* cell wall to liberate active proteins. Cells are harvested by centrifugation, resuspended in BugBuster Protein Extraction Reagent, and incubated briefly at room temperature. Following clarification by centrifugation, the supernatant, containing soluble protein, is ready for purification with His•Bind® products. BugBuster Protein Extraction Reagent can also be used for the preparation of highly purified inclusion bodies prior to processing insoluble target proteins.

BugBuster Protein Extraction Reagent plus Benzonase® Nuclease is an efficient combination for gently releasing target proteins and reducing extract viscosity prior to downstream processing. Cells are harvested by centrifugation, followed by suspension in BugBuster Protein Extraction Reagent and Benzonase Nuclease treatment. During a brief incubation at room temperature, soluble proteins are released, and nucleic acids are digested. Insoluble protein and cell debris is easily removed by centrifugation. The resulting low viscosity, clarified extract contains soluble protein ready for purification with His•Bind products. Soluble extract can be loaded directly onto any His•Bind Resin or His•Mag™ Agarose Beads.

For more information regarding BugBuster Protein Extraction Reagent, see User Protocol TB245.

Cell extract preparation using PopCulture® Reagent

PopCulture Reagent is a buffered mixture of concentrated detergents formulated to efficiently extract proteins from *E. coli* cells directly in their culture medium without cell harvest. The entire procedure, from culturing through purification, can be performed in one day. Therefore it is ideal for small culture volumes ($\leq$ 50 ml) or multiwell plate cultures. An induced *E. coli* culture is treated with PopCulture Reagent for 10 min at room temperature. Proteins in this extract preparation can be assayed directly, or purified using equilibrated His•Bind Resin or His•Mag Agarose Beads. To further enhance the effectiveness of PopCulture Reagent-mediated cell lysis and protein extract preparation, rLysozyme™ Solution (Cat. No 71110) and/or Benzonase® Nuclease (Cat. No. 70746) may be added to PopCulture Reagent prior to use.

If isolation of insoluble protein is desired, centrifugation would be required. After combining the PopCulture total cell extract with the His•Bind support, the supernatant could be centrifuged (10,000 × g) to pellet the inclusion body fraction. Process inclusion bodies according to the "Inclusion body purification" on page 7.

For more information regarding PopCulture Reagent, see User Protocol TB323.

His•Bind Resin Chromatography

The following protocol is recommended for rapid purification of soluble target protein under native conditions using His•Bind Resin. The highest purity of target proteins can be achieved when using amounts of extract and resin such that the resin will be near its binding capacity. For batch purification from PopCulture Reagent extracts, use 50 µl 50% slurry His•Mag Agarose Beads per 1 ml cell culture. Modifications required for denaturing conditions are described on page 11.

If minor *E. coli* protein contaminants remain bound to the column after standard washing with 1X Wash Buffer containing 60 mM imidazole, additional washes using 100 mM or intermediate imidazole concentrations may be used. However, some fusion proteins containing a His•Tag® sequence may partly or entirely elute with 100 mM imidazole. Generally, proteins with six consecutive histidines elute at lower imidazole concentrations than those with ten consecutive histidines. The Binding Buffer and Elute Buffer can be mixed in various ratios to obtain various imidazole concentrations. For example, 100 mM imidazole buffer can be obtained by combining 13.5 ml 1X Binding Buffer with 1.5 ml 1X Elute Buffer.

Buffer preparation

In the following protocol, one volume is equivalent to the settled bed volume (e.g. 100 µl slurry yields 50µl resin for a settled bed volume of 50 µl).

1. Prepare 5 vol 1X Charge Buffer by diluting supplied stocks to 1X with deionized water, or according to buffer compositions provided on page 4.

Note: For purification from extracts prepared with PopCulture® Reagent, prepare 6 vol 1X Charge Buffer per 1 ml original culture volume.

2. Prepare 26 vol 1X Binding Buffer by diluting supplied stocks to 1X with deionized water, or according to buffer compositions provided on page 4.

Note: For purification from extracts prepared with PopCulture Reagent, prepare 20 vol 1X Binding Buffer per 1 ml original culture volume.

3. Prepare 6 vol 1X Wash Buffer by diluting supplied stocks to 1X with deionized water, or according to buffer compositions provided on page 4.

Note: For purification from extracts prepared with PopCulture Reagent, prepare 26 vol 1X Wash Buffer per 1 ml original culture volume.

4. Prepare 6 vol 1X Elute Buffer by diluting supplied stocks to 1X with deionized water, or according to buffer compositions provided on page 4.

Note: For purification from extracts prepared with PopCulture Reagent, prepare 2–6 vol 0.5X Elute Buffer per 1 ml original culture volume.

Column preparation

Small polypropylene columns, such as the Novagen Chromatography Columns (Cat. No. 69673), that hold 2.5 ml settled resin, can be used to purify up to 20 mg target protein.

Note: Adding a few ml sterile, deionized water to the dry column, and gently pushing on the column top with a gloved finger, will wet the frit and start column flowing.

1. Gently mix bottle of His•Bind® Resin by inversion until completely suspended. Using a wide-mouth pipet, transfer desired amount of slurry to column (e.g. 100 µl slurry yields 50 µl resin for settled bed volume of 50 µl). Allow resin to pack under gravity flow. If batch method is preferred, follow the "Small-scale purification–batch method," on page 10.
2. When level of storage buffer drops to top of column bed, use the following sequence of washes to charge and equilibrate column:
   a) 3 vol sterile deionized water
   b) 5 vol 1X Charge Buffer
   c) 3 vol 1X Binding Buffer

Column chromatography

1. Allow 1X Binding Buffer to drain to top of column bed.
2. Load column with prepared extract.

Note: A flow rate of approximately 10 vol per hour is optimal for efficient purification. If flow rate is too fast, more impurities will contaminate eluted fraction.

3. Wash column with 10 vol 1X Binding Buffer.
4. Wash column with 6 vol 1X Wash Buffer.
5. Elute bound protein with 6 vol 1X Elute Buffer. Alternatively, 6 vol 1X Strip Buffer may be used to remove protein by stripping Ni^{2+} from column. The eluate may be captured in fractions (e.g. 1 ml fractions), if desired.

Small scale purification - batch method

Extract prepared without medium – mechanical or BugBuster® Reagent

His•Bind® Resin can also be used in batch format for small-scale purification. Settled bed volumes of 50–200 µl can be processed in 1.5-ml microcentrifuge tubes. In the following protocol, one volume is equivalent to the settled bed volume (e.g. 100 µl slurry yields 50 µl resin for a settled bed volume of 50 µl).

1. Transfer 100–400 µl slurry to 1.5-ml microcentrifuge tube. Centrifuge for 1 min at 400–1000 × g. Remove supernatant.
2. Use the following sequence of washes to charge and equilibrate resin. For each wash step, add appropriate buffer, invert tube several times to mix, and centrifuge for 1 min at 400–1000 × g.
   a) 2 times with 2 vol sterile deionized water
   b) 3 times with 2 vol 1X Charge Buffer
   c) 2 times with 2 vol 1X Binding Buffer.
3. Add cell extract to 1.5-ml microcentrifuge tube containing prepared resin. Mix gently by inverting tube several times. Incubate for 5 min. Centrifuge for 1 min at 400–1000 × g. Discard supernatant.
4. Wash resin 3 times with 3 vol 1X Binding Buffer.
5. Wash resin 2 times with 3 vol 1X Wash Buffer.
6. Elute bound protein 2 times with 3 vol 1X Elute Buffer. Alternatively, 1X Strip Buffer may be used to elute protein by stripping Ni^{2+} from resin.

Extract prepared with medium – PopCulture® Reagent

His•Bind Resin can also be used in batch format for small scale purification of extracts prepared with PopCulture Reagent. A settled bed volume of 50 µl is recommended per 1 ml original culture volume. In the following protocol, one volume is equivalent to the settled bed volume (e.g. 100 µl slurry yields 50 µl resin for a settled bed volume of 50 µl).

1. Transfer 100 µl slurry to a 1.5-ml microcentrifuge tube. Centrifuge for 1 min at 400–1000 × g. Remove supernatant.
2. Use the following sequence of washes to charge and equilibrate resin. For each wash step, remove supernatant by centrifuging for 1–5 min at 400–1000 × g. Carefully aspirate supernatant.
   a) 2 times with 2 vol sterile deionized water
   b) 3 times with 2 vol 1X Charge Buffer
   c) 2 times with 2 vol 1X Binding Buffer.
3. Resuspend resin in 1 vol 1X Binding Buffer.
4. Add equilibrated resin to prepared extract. Mix gently by pipetting. Incubate for 5 min with gentle mixing. Remove supernatant.

Note: The sample can be processed using a vacuum filter plate system, and transferred to a 0.5-cm diameter column, or a microcentrifuge tube. As required, centrifuge for 1 min at 400–1000 × g prior to removing supernatant in the following wash steps.

1. Wash resin with 20 vol 1X Binding Buffer.
2. Wash resin with 20 vol 1X Wash Buffer.
3. Elute bound protein with 1–3 vol 0.5 X Elute Buffer.
   Optional: Repeat elution again with 1–3 vol 0.5X Elute Buffer, and pool supernatants.

Purification under denaturing conditions

If target protein is found in inclusion body fraction, purification with His•Bind® products can be performed under denaturing conditions at room temperature.

1. The inclusion body fraction is solubilized in 1X Binding Buffer, including a denaturant (6 M guanidine-HCl or 6 M urea) according to protocols in "Cell Extract Preparation" section on page 7.
2. The His•Bind Resin is charged and equilibrated, as described previously using 1X Binding Buffer with denaturant.

Note: The Charge Buffer should not contain a denaturant.

3. Purification under denaturing conditions is identical to purification under native conditions, with the modification that Wash and Elution Buffers should contain a denaturant.

Note: A lower imidazole concentration (20 mM) should be used in wash buffer containing denaturant, as target proteins tend to elute at lower imidazole concentrations in the presence of 6 M urea or 6 M guanidine-HCl.

Buffer preparation for purification under denaturing conditions

1. Add solid guanidine-HCl or urea directly to an aliquot of concentrated buffers.
2. Bring to 90% final volume with deionized water. Stir until solid is dissolved.
3. Adjust pH to 7.9 with either HCl or NaOH. Bring to final volume with deionized water.

For example, to prepare 100 ml 1X Binding Buffer with 6M urea, combine 12.5 ml 8X Binding Buffer and 36 g urea. Bring volume up to 90 ml with deionized water. Once urea is dissolved, adjust pH to 7.9. Bring to final volume of 100 ml with deionized water. To prepare 20 mM imidazole Wash Buffer, combine 11 ml 1X Binding Buffer with 4.1 ml 1X Wash Buffer, both including denaturant.

Caution: Urea solutions must be made fresh and used promptly. Urea decomposes to form cyanate ions that can covalently modify primary amines on target protein.

Note: Samples in 6M urea may be mixed with sample buffer and loaded directly on an SDS polyacrylamide gel. Samples in 6M guanidine must be diluted 1:5 in water, or dialyzed, before running on an SDS polyacrylamide gel.

Resin regeneration

When elution is complete, His•Bind Resin can be regenerated for reuse. This process can be carried out many times. However, because some small amounts of protein may not be released with EDTA treatment, we recommend a different sample of resin for each different protein studied.

Routine reuse

Following the last elution step, wash column with 3 bed vol 1X Strip Buffer. The presence of 100 mM EDTA in Strip Buffer will prevent bacterial growth. Store column in this solution and recharge as in "Column preparation" on page 9 before use.

Resin regeneration

When the flow rate of a column slows noticeably or the resin does not turn a strong blue-green color when Charge Buffer is added, clean the resin more thoroughly. One volume is equivalent to the settled bed volume. In Step 13, it is important to use 3 vol water to completely remove EDTA.

1. 2 vol 6 M guanidine-HCl, 0.2 M acetic acid
2. 2 vol water
3. 1 vol 2% SDS
4. 1 vol 25% ethanol
5. 1 vol 50% ethanol
6. 1 vol 75% ethanol
7. 5 vol 100% ethanol
8. 1 vol 75% ethanol
9. 1 vol 50% ethanol
10. 1 vol 25% ethanol
11. 1 vol water
12. 5 vol 100 mM EDTA, pH 8.0
13. 3 vol water
14. 3 vol 20% ethanol
15. Store at 4°C.

His•Bind® Resin, Ni-charged, Chromatography

The following protocol is recommended for purification of soluble target protein under native conditions using His•Bind® Resin, Ni-charged. His•Bind Resin, Ni-charged is supplied as 50% slurry and has binding capacity of up to 8 mg/ml settled resin. The highest target protein purity can be achieved using amounts of extract and resin such that the resin is near its binding capacity. Soluble fractions prepared using BugBuster® Protein Extraction Reagent, PopCulture® Reagent, or mechanical means can be loaded on the His•Bind Resin, Ni-charged. Modifications required for denaturing conditions are described on page 11. The resin is compatible with up to 1.0 mM THP, a reducing agent that is more stable and effective than DTT. Use His•Bind® Resin, Ni-charged with His•Bind Quick Buffer Kit.

Note: The His•Bind Resin, Ni-charged can also be used with the Ni-NTA Buffer Kit (Cat. No. 70899).

Buffer preparation

In the following protocol, one volume is equivalent to the settled bed volume (e.g. 100 µl slurry yields 50µl resin for a settled bed volume of 50 µl).

4. Prepare 10 vol 1X Binding Buffer by diluting supplied stocks to 1X with deionized water, or according to buffer compositions provided on page 4.

Note: For purification from extracts prepared with PopCulture Reagent, prepare 20 vol 1X Binding Buffer per 1 ml original culture volume.

5. Prepare 6 vol 1X Wash Buffer by diluting supplied stocks to 1X with deionized water, or according to buffer compositions provided on page 4.

Note: For purification from extracts prepared with PopCulture Reagent, prepare 20 vol 1X Wash Buffer per 1 ml original culture volume.

6. Prepare 6 vol 1X Elute Buffer by diluting supplied stocks to 1X with deionized water, or according to buffer compositions provided on page 4.

Note: For purification from extracts prepared with PopCulture Reagent, prepare 2–5 vol 0.5X Elute Buffer per 1 ml original culture volume.

Column preparation

Small polypropylene columns, such as the Novagen Chromatography Columns (Cat. No. 69673), can be used.

1. Assemble column according to manufacturer instructions. Cap bottom outlet.
2. Gently mix bottle of His•Bind Resin, Ni-charged by inversion until completely resuspended. Using a wide-mouth pipet, transfer desired amount of slurry to column (e.g. 100 µl slurry yields 50 µl resin for settled bed volume of 50 µl).
3. Allow resin to settle under gravity flow.
4. Equilibrate column with 3 vol sterile deionized water.
5. Equilibrate column with 3 vol 1X Binding Buffer.

Column chromatography

Refer to page 9.

Small scale purification - batch method

Refer to page 10.

Purification under denaturing conditions

If target protein is found in inclusion body fraction, purification can be performed with His•Bind Resin, Ni-charged under denaturing conditions. Refer to page 11.

His•Bind® Column Chromatography

The following protocol is recommended for purification of soluble target protein under native conditions using pre-charged His•BindColumn. Soluble fractions prepared using BugBuster® Protein Extraction Reagent, PopCulture® Reagent, or mechanical means can be loaded on the His•Bind Column. Modifications required for denaturing conditions are described on page 11. Each pre-packed column contains 1.25 ml His•Bind Resin and binds up to 10 mg target protein. Cell extract volumes are not specified due to variations in expression levels between individual target proteins. The highest target protein purity can be achieved using amounts of extract and resin such that the resin is near its binding capacity. These single-use columns are designed to fit into most 15- or 50-ml conical centrifuge tubes (e.g. Falcon Cat. No. 352097 or 352098) for convenient fraction collection.

Buffer preparation

1. Prepare 20 ml 1X Binding Buffer per column by diluting supplied stocks to 1X with deionized water, or prepare according to buffer compositions provided on page 4.
2. Prepare10 ml 1X Wash Buffer per column by diluting supplied stocks to 1X with deionized water, or prepare according to buffer compositions provided on page 4.
3. Prepare 5 ml 1X Elute Buffer per column by diluting supplied stocks to 1X with deionized water, or prepare according to buffer compositions provided on page 4.

Tip: *To avoid clogging and slower flow rates, treat cell extracts with Benzonase® Nuclease. Alternatively, sonicate briefly, clarify by high-speed centrifugation, and pass through a 0.45-µm syringe filter prior to loading. (see "Cell Extract Preparation" section on page 6).*

Column preparation

1. Remove His•Bind Column cap.
2. Pour off, or pipet, to remove storage buffer in upper chamber. Remove lower Luer plug.
3. Equilibrate column with 10 ml 1X Binding Buffer. Allow entire buffer volume to flow through column.

Note: *Failure to remove storage buffer and fully equilibrate column may cause a loss of resin affinity for target protein, and/or precipitation of purified target protein during purification or subsequent dialysis steps.*

Column chromatography

1. After Binding Buffer has drained, load column with prepared cell extract.
2. Wash column with 10 ml 1X Binding Buffer.
3. Wash column with 10 ml 1X Wash Buffer.
4. Elute protein from column with 5 ml 1X Elute Buffer. Alternatively, 5 ml 1X Strip Buffer may be used to elute protein by stripping Ni^{2+} from resin. Eluate may be captured in fractions (e.g., 1 ml fractions), if desired.

Purification under denaturing conditions

If target protein is found in inclusion body fraction, His•Bind Column purification can be performed under denaturing conditions. Refer to page 11.

His•Bind® Quick Column and Cartridges Protocols

The following protocol is recommended for purification of soluble target protein under native conditions using Ni^{2+}-charged His•Bind Quick Columns and Cartridges. Soluble fractions prepared using BugBuster® Extraction Reagent, PopCulture® Reagent, or mechanical means can be loaded on Ni^{2+}-charged His•Bind Quick Columns and Cartridges. Modifications required for denaturing conditions are described on page 11. Reuse is not recommended. Cell extract volumes are not specified due to variations in expression levels between individual target proteins. The highest target protein purity can be achieved using amounts of extract and resin such that the resin is near its binding capacity.

Tip: *To avoid clogging the columns and cartridges treat viscous cell extracts with Benzonase® Nuclease. Alternatively, sonicate briefly, clarify by high-speed centrifugation, and pass through a 0.45-µm syringe filter prior to loading. (see "Cell Extract Preparation" section on page 6).*

His•Bind Quick Columns

His•Bind Quick Columns are designed for the Novagen Vacuum Manifold (Cat. No. 70147), or other vacuum source.

Buffer preparation

1. Prepare 45 ml 1X Binding Buffer per column by diluting supplied stocks to 1X with deionized water, or prepare according to buffer compositions provided on page 4.
2. Prepare 15 ml 1X Wash Buffer per column by diluting supplied stocks to 1X with deionized water, or prepare according to buffer compositions provided on page 4.
3. Prepare 10 ml 1X Elute or Strip Buffer per column by diluting supplied stocks to 1X with deionized water or prepare according to buffer compositions provided on page 4.

Column preparation

1. Attach columns to the Vacuum Manifold.
2. Wet and equilibrate column with 15 ml 1X Binding Buffer. Apply vacuum, and adjust flow rate to 1–3 ml/min.

Column chromatography

1. Load cell extract onto column. Apply vacuum and adjust flow rate to 1–3 ml/min.

Note: *Passing flow-through over the same column or cartridge a second time may increase yields.*

2. Wash with 30 ml 1X Binding Buffer. Apply vacuum and adjust flow rate to 5–10 ml/min.
3. Wash with 15 ml 1X Wash Buffer.
4. Elute with 10 ml 1X Elute Buffer or 10 ml 1X Strip Buffer.

His•Bind Quick 900 Cartridges

Buffer preparation

1. Prepare 26 ml 1X Binding Buffer per cartridge by diluting supplied stocks to 1X with deionized water, or prepare according to buffer compositions provided on page 4.
2. Prepare 10 ml 1X Wash Buffer per cartridge by diluting supplied stocks to 1X with deionized water, or prepare according to buffer compositions provided on page 4
3. Prepare 4 ml 1X Elute or Strip Buffer per cartridge by diluting supplied stocks to 1X with deionized water, or prepare according to buffer compositions provided on page 4.

Cartridge chromatography

Attach cartridge to 20–30-cc syringe loaded with appropriate buffer. Push buffer through cartridge at a rate of approximately 2 drops/sec.

Note: Buffers can be loaded into syringe such that some air space exists between plunger and liquid. A small amount of air pushed through the resin has no negative effect, and allows for a more complete change of buffers, and more efficient elution of target protein.

1. Wet and equilibrate cartridge with 6 ml 1X Binding Buffer.
2. Load cell extract onto cartridge.
3. Wash with 20 ml 1X Binding Buffer.
4. Wash with 10 ml 1X Wash Buffer.
5. Elute with 4 ml 1X Elute Buffer or 4 ml 1X Strip Buffer.

His•Bind® Quick 300 Cartridges

Buffer preparation

1. Prepare 7 ml 1X Binding Buffer per cartridge by diluting supplied stocks to 1X with deionized water, or prepare according to buffer compositions provided on page 4.
2. Prepare 2.5 ml 1X Wash Buffer per cartridge by diluting supplied stocks to 1X with deionized water, or prepare according to buffer compositions provided on page 4.
3. Prepare 1 ml 1X Elute or Strip Buffer per cartridge by diluting supplied stocks to 1X with deionized water, or prepare according to buffer compositions provided on page 4.

Cartridge chromatography

Attach the cartridge to 5–10-cc syringe loaded with appropriate buffer. Push buffer through cartridge at a rate of approximately 2 drops/sec.

1. Wet and equilibrate column with 2 ml 1X Binding Buffer.
2. Load cell extract onto cartridge.
3. Wash with 5 ml 1X Binding Buffer.
4. Wash with 2.5 ml 1X Wash Buffer.
5. Elute with 1 ml 1X Elute Buffer or 1 ml 1X Strip Buffer.

Purification under denaturing conditions

If target protein is found in inclusion body fraction, His•Bind Quick Column and Cartridge purification can be performed under denaturing conditions. Refer to page 11.

His•Mag™ Agarose Beads

The following protocols describe purification of proteins containing a His•Tag® sequence with Ni^{2+}-charged His•Mag Agarose Beads. The binding capacity is 5 mg target protein per 1 ml (settled bed volume) of beads and is provided in 50% slurry. In general, the highest target protein purity is achieved when using amounts of extract and resin such that the resin is near its binding capacity. As a starting point, 50 µl slurry (25 µl resin) is recommended for each 1 ml of culture.

Extract prepared without medium – mechanical or BugBuster® Reagent

Buffer preparation

Note: One volume is equivalent to settled bed volume of beads.

1. Prepare 13 vol 1X Binding Buffer per sample by diluting supplied stocks to 1X with deionized water, or prepare according to buffer compositions provided on page 4.
2. Prepare 12 vol 0.5X Wash Buffer per sample by diluting supplied stocks to 1X with deionized water, and further diluting to 0.5X using 1X Binding Buffer, or prepare according to buffer compositions provided on page 4.
3. Prepare 6 vol 0.5X Elute Buffer per sample by diluting supplied stocks to 1X with deionized water, and further diluting to 0.5X using 1X Binding Buffer, or prepare according to buffer compositions provided on page 4.

Purification

1. Transfer the appropriate amount of resuspended His•Mag Agarose Beads to a 1.5-ml, 15-ml or 50-ml (e.g. Falcon) tube. Place tube in a Magnetight™ Separation Stand (Cat. No. 69964), or Magnetight Mulitube Rack (Cat. No. 70747) to collect beads.
2. Remove supernatant and wash beads 3 times each with 4 vol 1X Binding Buffer.

Note: For each wash, remove tube from rack, add buffer, and resuspend beads. Replace tube in magnetic rack, and remove supernatant. His•Mag Agarose Beads may also be centrifuged (1,000 x g, 3 min) for separation during wash procedures.

3. After final wash, resuspend beads in 1 vol 1X Binding Buffer.
4. Combine equilibrated beads with prepared cell extract. Mix well by inverting tube several times. Incubate for 5 min with occasional mixing.
5. Place tube in magnetic rack to collect beads. Remove supernatant.
6. Wash beads 3 times with 4 vol 0.5X Wash Buffer.

Note: For extracts prepared with PopCulture Reagent, Wash beads 3 times with 20–30 vol 0.5X Wash Buffer.

Note: For each wash, remove tube from rack, add buffer, and resuspend beads. Replace tube in magnetic rack, and remove supernatant.

7. After removing final wash, add 4 vol 0.5X Elute Buffer. Resuspend beads, and incubate for 5 min with occasional inversion to mix.
8. Place tube in magnetic rack to collect beads. Remove eluted protein to a fresh tube. **Optional:** Repeat elution step with a smaller volume of Elute Buffer. Combine supernatants.

Extracts prepared with medium – PopCulture® Reagent

For each 1 ml original culture prepared with PopCulture Reagent, use 50 µl 50% slurry His•Mag Agarose Beads.

Buffer preparation

Note: One volume is equivalent to settled bed volume of beads.

1. Prepare 13 vol 1X Binding Buffer per 1 ml sample by diluting supplied stocks to 1X with deionized water, or prepare according to buffer compositions provided on page 4.
2. Prepare 60–90 vol 0.5X Wash Buffer per 1 ml sample by diluting supplied stocks to 0.5X with deionized water, or prepare according to buffer compositions provided on page 4.
3. Prepare 4–8 vol 0.5X Elute per 1 ml sample by diluting supplied stocks to 0.5X with deionized water, or prepare according to buffer compositions provided on page 4.

Purification

Refer to page 16.

Purification under denaturing conditions

If target protein is found in inclusion body fraction, His•Mag™ Agarose Bead purification can be performed under denaturing conditions. Refer to page 11.

His•Mag Agarose Bead regeneration

When elution is complete, His•Mag Agarose Beads can be reused 2–3 times using the following regeneration protocol. However, because small amounts of protein remain bound, and may not be released with EDTA treatment, it is advisable to use a different aliquot of beads for each protein.

1. Following the last elution step, wash beads 5 times in 4 vol 1X Strip Buffer.
2. Resuspend and store beads in 20 mM phosphate buffer (NaH_2PO_4/NaOH), pH 7.0 containing 150 mM NaCl, 0.1% NaN_3 and 0.1% Triton® X-100.
3. Prior to re-use, wash beads with 3 vol deionized water.
4. Charge beads by washing 2 times with 3 vol 1X Charge Buffer.
5. Equilibrate beads by washing with 4 vol 1 X Binding Buffer prior to use.

His•Bind Fractogel® Chromatography

The following protocols describe purification of proteins containing a His•Tag® sequence using His•Bind Fractogel Resin. His•Bind Fractogel Resin is supplied as uncharged hydrated resin, and binds > 10 mg target protein per ml resin. His•Bind Fractogel Resin is charged with Cu^{2+} rather than Ni^{2+}. Soluble fractions prepared using BugBuster® Extraction Reagent or mechanical methods can be loaded on His•Bind Fractogel columns.

Note: The His•Bind Buffer Kit is not recommended for use with His•Bind Fractogel Resins because the Tris-HCl buffer may reduce the binding affinity for His•Tag fusion proteins.

Buffer preparation

In the following protocol, one volume is equivalent to the settled bed volume (e.g. 100 µl slurry yields 50µl resin for a settled bed volume of 50 µl).

Prepare the following solutions for use with His•Bind Fractogel resins. Prepare approximately 10 vol Fractogel Charge Buffer, 20 vol Fractogel Binding Buffer, and 10 vol Fractogel Elute Buffer (allowing for void volumes in column head space, tubing, and pump).

- 1X Fractogel Equilibration Buffer (500 mM NaCl, 20 mM Na Phosphate, pH 7.5)
- 1X Fractogel Rinse Solution (500 mM NaCl)
- 1X Fractogel Charge Buffer (250 mM $CuSO_4$)
- 1X Fractogel Binding Buffer (20 mM Na Phosphate, 1 mM imidazole, pH 7.5)
- 1X Fractogel Elution Buffer (200 mM imidazole, 20 mM Na Phosphate, pH 7.5)
- 1X Fractogel Strip Buffer (500 mM NaCl, 100 mM EDTA, 20 mM Na Phosphate pH 7.5).

Note: Fractogel Binding Buffer contains a very low concentration of imidazole to decrease background binding of proteins to the resin. If the level of background binding is too high, increase the concentration to 5 mM imidazole, and wash with 60 mM imidazole/20 mM Na phosphate pH 7.5 before elution. The Na Phosphate buffers are a combination of NaH_2PO_4 (monobasic) and Na_2HPO_4 (dibasic) solutions. For a 20 mM Na Phosphate buffer, combine 19.0 ml 0.1 M monobasic with 81.0 ml 0.1 M dibasic Na phosphate and other components as needed (see below), adjust the final volume to 500 ml with deionized water and adjust the pH to 7.5.

Resin preparation and column packing

Notes:

- His•Bind Fractogel resins can be used in low pressure glass columns, but higher flow rates can be attained with columns capable of withstanding pressures > 35 psi (~2.5 bar). Linear flow rates of 1.2–5 cm/min (corresponding to 1–4 ml/min for 1-cm diameter columns) are recommended initially. A pump should be used to control the flow rate with His•Bind Fractogel resin. A bed height of 5 cm is convenient in columns of dimension 5 × 1 cm, 5 × 1.6 cm, or 5 × 2.6 cm.
- Fines in resin slurry must be removed by decantation before packing column. Failure to remove resin fines will result in increased operating pressure and decreased flow rates. To remove fines, completely suspend settled resin in 4 vol sterile deionized water. Allow mixture to settle until a stable resin bed is formed. Decant the cloudy, fine-containing supernatant.
- Pack column with 50% (v/v) slurry de-fined resin suspended in sterile deionized water. Measure approximately 1.2 vol settled resin for desired column bed size to accommodate bed compression during packing.

Note: The formula for volume of resin required = $1.2 \times [(\pi) \times (r^2) \times (ht)]$, where r = column radius in cm, and ht = column height in cm.

1. Pack column using packing reservoir large enough to accommodate the entire resin slurry. Stepwise additions of slurry are not recommended as resin bed irregularities will affect

resolution of sample components during chromatography. For best results, the column packing velocity should be 4–6 times the desired linear flow rate operating velocity.

Note: Calculate the linear flow rate with the following formula:
linear flow rate (cm/h) = [(ml/min) × 60)] / [(cm^2 = (π) × (r^2)].

Higher flow rates and better resolution of complex mixtures are achieved when column is packed at constant velocity with pump control.

2. Charge and equilibrate column using the following sequence of washes. Use a linear flow rate of 1.2–5 cm/min for charging and equilibrating.
   a) 3 vol Fractogel® Equilibration Buffer
   b) 2 vol Fractogel Rinse Solution
   c) 1 vol Fractogel Charge Buffer
   d) 2 vol Fractogel Rinse Solution
   e) 3 vol Fractogel Binding Buffer

Column chromatography

1. Load column with prepared sample. Particulates must be removed from sample by centrifugation or filtration (0.45 µm) to prevent column fouling. A flow rate of about 10 vol per hour is optimal.
2. Wash column with 10 vol 1X Fractogel Binding Buffer.
3. For step gradient elution, elute bound protein by applying 6 vol Fractogel Elution Buffer. For linear imidazole gradient elution, use Fractogel Binding Buffer and Fractogel Elution Buffer with a gradient-forming pump. Alternatively, use a pH gradient, or Fractogel Strip Buffer to remove the protein by stripping the column of Cu^{2+}.

Note: Elution with imidazole can be performed with a linear imidazole gradient (1 mM–200 mM) or a step gradient. Higher concentrations of imidazole may be required to elute strongly bound His•Tag® fusion proteins.

Note: Alternatively, bound proteins may be eluted with a descending pH gradient (e.g., 500 mM NaCl, 20 mM Na Phosphate, pH 7.5 down to pH 3.0). A step or linear pH gradient may be used.

4. Wash column with 3 vol Fractogel Strip Buffer.

Purification under denaturing conditions

If target protein is found in inclusion body fraction, purification with His•Bind® Fractogel resin can be performed under denaturing conditions at room temperature.

5. The inclusion body fraction is solubilized in 1X Fractogel Binding Buffer, including a denaturant (6 M guanidine-HCl or 6 M urea) according to protocols in "Cell Extract Preparation" section on page 7.
6. The His•Bind Fractogel Resin is charged and equilibrated, as described previously, using 1X Fractogel Binding Buffer with denaturant.

Note: The Charge Buffer should not contain a denaturant.

7. Purification under denaturing conditions is identical to purification under native conditions, with the modification that Wash and Elution Buffers should contain a denaturant.

Note: A lower imidazole concentration (20 mM) should be used in wash buffer containing denaturant, as target proteins tend to elute at lower imidazole concentrations in the presence of 6 M urea or 6 M guanidine-HCl.

Resin regeneration

For routine regeneration of His•Bind Fractogel after protein elution, wash column with 3 vol Fractogel Strip Buffer. The presence of 100 mM EDTA in the solution will inhibit bacterial

growth. Store column in Fractogel® Strip Buffer. Recharge, as in "Resin preparation and column packing" on page 18 before use.

When column flow rate slows noticeably, or the resin does not bind Cu^{2+} efficiently, the column may be treated with NaOH to remove contamination from the resin. His•Bind® Fractogel is stable to alkali treatment; however, metal ions must first be completely stripped to prevent the formation of insoluble metal hydroxides or oxides within the resin.

1. After treatment with Fractogel Strip Buffer, wash column with 1 vol 0.5 M HCl solution at a slow flow rate (0.5ml/min for a 1-cm diameter column) to remove metal ions from column.
2. Wash column with 2 vol Fractogel Rinse Solution.
3. Wash column with 10 vol 0.5 M NaOH at a slow flow rate.
4. Wash column with 2 vol Fractogel Rinse Solution.
5. For storage, equilibrate column by washing with 10 vol Fractogel Strip Buffer.
6. For re-use, equilibrate column by washing with 10 vol Fractogel Equilibration Buffer. Proceed with charging, as detailed on page 18.

Processing Sample after Elution

After eluting purified protein from a His•Bind support, the sample can be concentrated, or the buffer changed by one of several methods. The storage buffer for your purified protein is often determined through an empirical process. Inappropriate storage buffer may lead to precipitation of the protein. If the protein was purified under denaturing conditions, removal of the denaturing agent may allow refolding of the protein, and reconstitution of activity, but it may also result in precipitation. Please refer to the Novagen Protein Refolding Kit User Protocol TB234 for additional information on protein refolding. Four alternative buffer exchange procedures follow:

1. Dialyze into buffer of choice. Sample will contain nickel; therefore, avoid reducing agents until nickel is removed. If refolding is desired, gradual removal of 6 M guanidine or urea is recommended (e.g., successive changes into 4 M, 2 M, and no denaturant). A three-fold concentration can be achieved by dialysis into storage buffer and 50% glycerol. Glycerol stabilizes protein for long-term storage. After dialysis, the sample may be concentrated by sprinkling solid polyethylene glycol (15,000–20,000 MW) or Sephdex G-50 (Pharmacia) on the dialysis tubing. Use dialysis tubing with an exclusion limit of 6,000 MW or less, and leave solid in contact with tubing until desired volume is reached, replacing with fresh solid, as necessary.
2. Use the Novagen D-Tube™ Dialyzers (see User Protocol TB422) for dialysis and sample concentration.
3. Use plastic disposable microconcentrator units (e.g., Centricon; Amicon), as directed by manufacturer, to both desalt and concentrate the sample by ultrafiltration.
4. Desalt sample by gel filtration on Sephadex (G-10, G-25, G-50; Pharmacia) or Bio-Gel (P6DGm P-10, P-30; Bio-Rad).

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