TALON®
Metal Affinity Resins
User Manual

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I. Introduction

Proteins have evolved very complex structures in order to perform a diverse array of functions. As a result, their physicochemical properties vary greatly, posing difficulties for developing versatile purification protocols. One way to circumvent this problem is to incorporate a purification tag into the primary amino acid sequence of a target protein, thus constructing a recombinant protein with a binding site that allows purification under well-defined, generic conditions.

Immobilized Metal Affinity Chromatography (IMAC)

IMAC was introduced in 1975 as a group-specific affinity technique for separating proteins (Porath *et al.*, 1975). The principle is based on the reversible interaction between various amino acid side chains and immobilized metal ions. Depending on the immobilized metal ion, different side chains can be involved in the adsorption process. Most notably, histidine, cysteine, and tryptophan side chains have been implicated in protein binding to immobilized transition metal ions and zinc (Figure 1, Porath, 1985; Sulkowski, 1985; Hemdan & Porath, 1985a; Hemdan & Porath, 1985b; Zhao *et al.*, 1991).

TALON® IMAC Resins

TALON® Resins are durable, cobalt-based IMAC resins designed to purify recombinant polyhistidine-tagged proteins (Bush *et al.*, 1991). These resins are compatible with many commonly used reagents (Appendix A), and allow protein purification under native or denaturing conditions. They can be used with all prokaryotic and eukaryotic expression systems in a variety of formats, including small- (or mini-) scale batch screening, large-scale batch preparations, and methods using gravity-flow columns and spin columns. In addition, protocols used with Ni^{+2}-based IMAC columns usually work with TALON® resins.

TALON Magnetic Beads are agarose beads utilizing our patented TALON technology. The beads combine the advantage of highly selective TALON chemistry with magnetic bead separation. Magnetic particles in the beads facilitate quick and easy purification of proteins at microscale level using a magnetic separator. Microscale purification with TALON Magnetic Beads can be used for screening of expression levels or for protein-protein interaction studies.

Tetradentate metal chelator

To overcome the problem of metal leakage encountered with other IMAC resins, TALON® Resin utilizes a special tetradentate metal chelator for purifying recombinant polyhistidine-tagged proteins (U.S. Patent No. 5,962,641). This chelator tightly holds the electropositive metal in an electronegative pocket (Figure 1), which is ideal for binding metal ions such as cobalt. The binding pocket is an octahedral structure in which four of the six metal coordination sites are occupied by the TALON Resin ligand. This process enhances the protein binding capacity of TALON Resin by making the bound metal ion accessible to surrounding polyhistidine-tagged proteins. The tetradentate metal binding means that no metal loss occurs during protein purification under rec-

I. Introduction *continued*

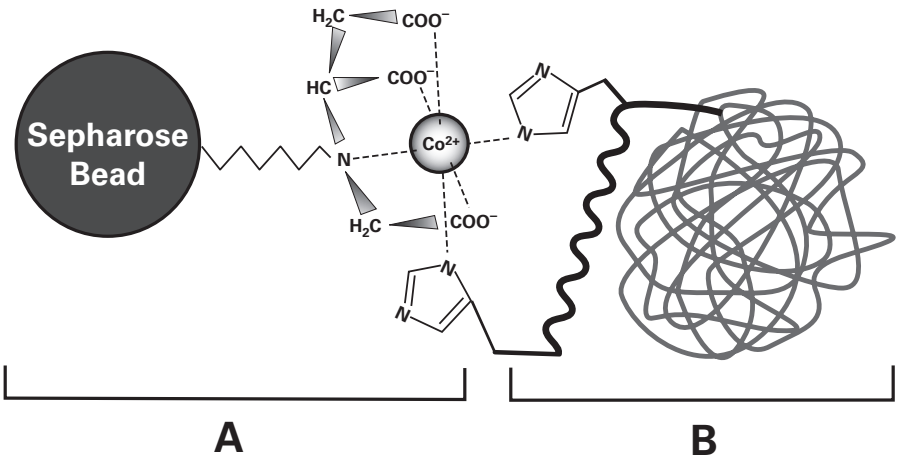

Figure 1. Schematic diagram of the TALON® IMAC System. Part A. TALON Metal Affinity Resin; A Sepharose bead bearing the tetradentate chelator of the Co^{2+} metal ion. **Part B.** The polyhistidine-tagged recombinant protein binds to the resin.

ommended conditions, even in the presence of strong denaturants such as 6 M guanidinium. Such durability allows TALON Resin to be reused (See Section IX).

Cobalt IMAC Resin permits milder elution conditions

TALON Resin exhibits subtle yet important differences in binding of polyhistidine-tagged proteins when compared with nickel IMAC resins. For example, nickel-based IMAC resins often exhibit an undesirable tendency to bind unwanted host proteins containing exposed histidine residues (Kasher *et al.*, 1993). While TALON Resin binds polyhistidine-tagged proteins with enhanced selectivity over nickel-based resins, it exhibits a significantly reduced affinity for host proteins. This behavior offers two practical advantages. First, virtually no background proteins are bound to the resin when the sample is applied; consequently, cumbersome washing procedures are not generally required before protein elution. Second, polyhistidine-tagged proteins elute from the resin under slightly less stringent conditions—a slightly higher pH or lower imidazole concentration—than with nickel IMAC resins. Elution occurs when the imidazole nitrogen (pKa of 5.97) is protonated (Figure 2), generating a positively charged ammonium ion, which is repelled by the positively charged metal atom. Alternatively, the bound polyhistidine-tagged protein can be competitively eluted by simply adding imidazole to the elution buffer, because imidazole is identical to the histidine side chain.

I. Introduction *continued*

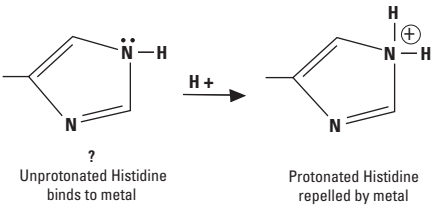

Figure 2. Elution mechanism of recombinant polyhistidine-tagged proteins from TALON® Resin. Elution occurs when the imidazole nitrogen (pKa = 5.97) is protonated, generating a positively charged ammonium ion which is repelled by the positively charged metal ion. Alternatively, the bound polyhistidine-tagged protein can be competitively eluted by adding imidazole to the elution buffer.

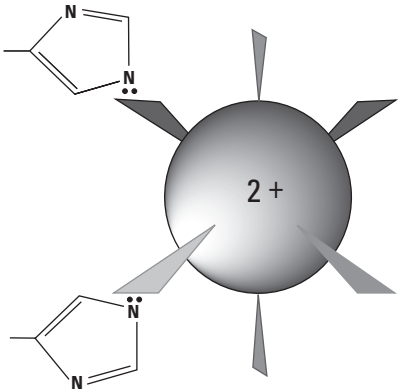

Figure 3. Binding of histidines to the TALON® Resin metal ion. Under conditions of physiological pH, histidine binds by sharing imidazole nitrogen electron density with the electron-deficient orbitals of the metal ion.

Polyhistidine affinity tags

Histidines exhibit highly selective coordination with certain transition metals and have great utility in IMAC. Under conditions of physiological pH, histidine binds by sharing electron density of the imidazole nitrogen with the electron-deficient orbitals of transition metals (Figure 3). Although three histidines may bind transition metals under certain conditions, six histidines reliably bind transition metals in the presence of strong denaturants such as guanidinium (Hochuli *et al.*, 1987). Such protein tags are commonly referred to as "6 x histidine," "hexaHis," or "$(His)_6$."

HAT—a novel IMAC affinity tag

With the advent of recombinant genetic technologies, the design and production of recombinant proteins containing novel polyhistidine tags on their N- or C-termini has become more straightforward (Hochuli *et al.,* 1987; Hochuli *et al.,* 1988). The HAT sequence (patent pending) is a novel IMAC affinity tag derived from a unique natural protein sequence (Chaga *et al.*, 1999). It contains six histidines unevenly interleaved by other amino acid residues (see Appendix C). The HAT amino acid sequence is derived from the N-terminus of chicken muscle lactate dehydrogenase—a sequence that is unique among reported protein sequences. The novel tag does not have the excessive positive charge characteristic of the commonly used 6 x histidine tag, thus contributing to better solubility of HAT-fusion proteins and similar affinity towards immobilized transition metal ions and zinc. Clontech offers the HAT Protein Expression and Purification System (Cat. No. 631205)—a complete system containing reagents and vectors

I. Introduction *continued*

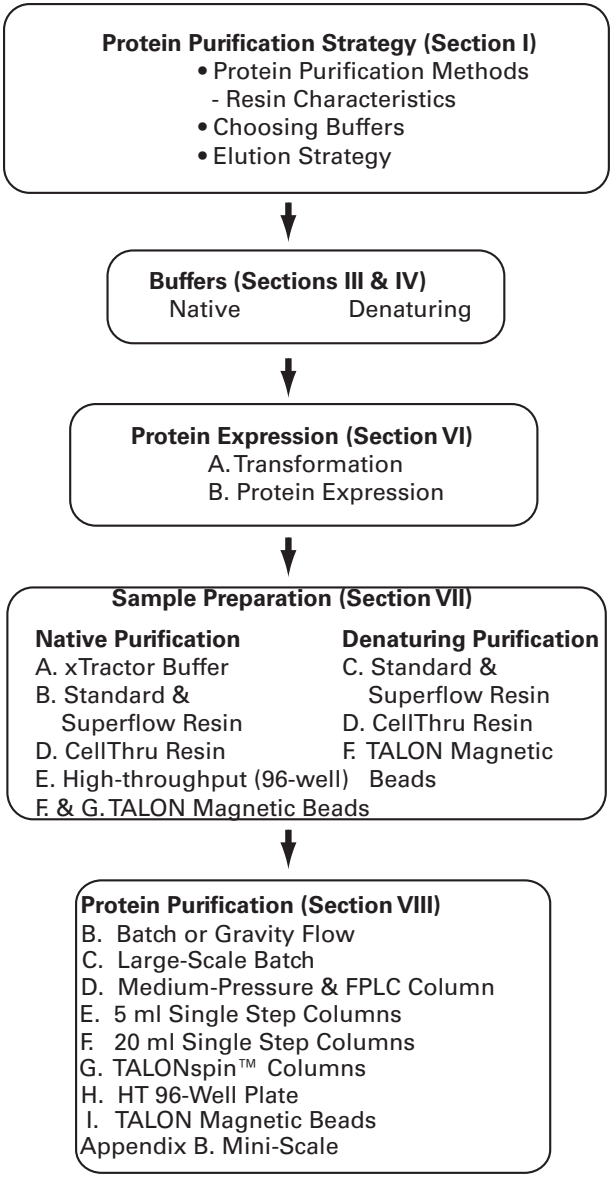

Figure 4. Using the TALON® Metal Affinity Resins User Manual. Overview of the procedures.

I. Introduction *continued*

designed for bacterial expression and purification of HAT (histidine affinity tag) proteins. Each of the three vectors—pHAT10, pHAT11, and pHAT12—contain a multiple cloning site (MCS) in all three frames to allow cloning of target cDNA. (For vector map and MCS, see Appendix C of this User Manual.) A conveniently located enterokinase proteolytic site between the HAT sequence and the MCS provides a means for removing the affinity tag.

For more information, see the HAT Expression & Purification System User Manual (PT3250-1), which can be downloaded from our web site at **www.clontech.com**.

TALON® Express Bacterial Expression and Purification Kits

TALON Express Bacterial Expression and Purification Kits are designed for the cloning, expression, and purification of polyhistidine-tagged proteins using *E. coli*. The kits contain two separate bacterial expression vectors encoding N- or C-terminal 6xHN fusion tags. These IPTG-inducible, pET-based vectors provide high levels of protein expression. The expressed proteins are ready for quick and easy purification using the TALON resin and buffers provided in the kits.

Overview of TALON® Resins

The following is a list of different resin formats to meet your purification needs.

- **TALON® Metal Affinity Resin** is useful for batch and low-pressure chromatographic applications. This resin utilizes Sepharose CL-6B (GE Healthcare), a durable substrate that performs very well under native and denaturing conditions in centrifuge-mediated purification schemes. The large pore size resin has a high-binding capacity. This resin is also available pre-packed in 2 ml gravity columns.
- **TALON® Superflow Resin** is useful for a range of applications, including medium pressure applications with FPLC systems at back pressures of up to 150 psi (1 MPa) and high flow rates up to 5 ml per cm^2 per min. This resin is recommended if short purification times are essential, or if purification protocols developed at bench scale will be scaled up for larger volumes.

  This resin utilizes Superflow-6 (Sterogene Bioseparations, Inc.), an agarose-based medium featuring a unique polysaccharide composition that resists biological degradation. Superflow-6 beads are also stabilized by a chemical cross-linking reaction that allows flow rates up to 10 times higher than are possible with regular cross-linked beads.

  The Talon® Superflow Resin is also present in the high throughput (HT) 96-well plate.

I. Introduction *continued*

- **TALON® CellThru Resin** is a novel IMAC resin for purifying polyhistidine-tagged proteins from crude cell lysates, sonicates, and fermentation liquids. The larger bead size of TALON CellThru Resin (300–500 µm) permits cellular debris to flow through the column, eliminating the need for high-speed centrifugation. Destabilizing factors are removed more quickly with this resin than with other IMAC resins, because the number of steps are reduced.

  CellThru 2 ml & 10 ml Disposable Columns have a large filter pore size (90–130 µm) that allows cellular debris to flow through the column during the purification process. The 2 ml columns are suitable for 1–2 ml bed volumes, while the 10 ml columns are suitable for 5–10 ml bed volumes.

- **TALONspin™ Columns** are ideal for rapidly and simultaneously purifying small amounts of polyhistidine-tagged proteins. These columns are recommended for single-use applications or for use as mini gravity-flow columns. Each column contains 0.5 ml of TALON-NX Resin, which is optimized for performance in a spin column. Each column will yield 2–4 mg of polyhistidine-tagged protein; exact yields will vary with conditions used and polyhistidine-tagged protein characteristics. In addition, yield and purity will depend upon expression level and lysate concentration. Beginning with the clarified sample, the entire procedure takes approximately 30 min.

- **TALON® Magnetic Beads** are useful for microscale purification of polyhistidine-tagged proteins under native or denaturing conditions. The beads can also be used to purify proteins directly from cleared (centrifuged) or crude cell lysates. For screening of expression levels, proteins can be purified directly from overnight cultures as small as 0.5 ml (depending on the expression level). The use of TALON chemistry allows for seamless scaling-up to large-scale purification of target proteins using our standard TALON resin.

  TALON Magnetic Beads are supplied as a 5% suspension in 25% ethanol, available in either a 2 x 1 ml or 6 x 1 ml format. The beads have a binding capacity of 750 µg of 6xHN-tagged AcGFP per 1 ml of suspension. When performing assays in single tubes, 100–200 µl of beads are sufficient for each assay. Smaller amounts of beads may be used, but there may be difficulties in handling the beads in small buffer volumes.

I. Introduction *continued*

TABLE I. PROTEIN PURIFICATION USING TALON® RESINS

Method	Application	Key Benefit
TALON® Metal Affinity Resin or TALON® Superflow Resin		
Mini-Scale (Appendix B)	• Check for presence of tagged protein • Estimate expression levels • Test buffer conditions	• Fast • Requires only 1 ml of cell culture + 1 ml of resin
Batch/Gravity Flow Column (Sec. VIII.B)	• Purify ≥5 mg of tagged protein using 1 ml of resin	• Very high purity • Does not require pressurized column equipment
Large-Scale (Sec. VIII.C & D)	• Large- and production-scale purification; easy to scale up	• Faster than protocols that use gravity-flow columns • Higher purity than using batch process alone
TALON® CellThru Resin		
Batch/Gravity Flow Column & Large-Scale (Sec. VIII.B & C)	• For purifying proteins from nonclarified cell lysates, sonicates, or fermentation liquids	• Fast • Does not require high-speed centrifugation
TALON® Single Step Columns (5 ml, 20 ml)		
Miniprep (Sec. VIII.E & F)	• Process several different samples simultaneously • Lyse bacterial cells and bind histidine-tagged protein in one step • Obtain 0.2–0.6 mg (5 ml column) or 0.5–4 mg (20 ml column).	• Fast (~30–40 min)[1] • Uses unlysed cell culture • Simplifies screening of multiple proteins • Ready-to-use columns
TALONspin™ Columns		
Spin Column (Sec. VIII.G)	• Process several different samples simultaneously • Obtain 2–4 mg of purified protein per spin column	• Fast (~30 min)[2] • Uses only 0.6–1 ml of cell culture lysate • Ready-to-use columns
TALON® HT 96-Well Plates		
96-Well Plates (Sec. VIII.H)	• High-throughput processing of samples • Obtain up to 1.0 mg of purified protein per well	• Fast(<30 min)[2] • Uses up to 2 ml of crude lysate per load
TALON® Magnetic Beads		
Magnetic Beads (Sec. VII.F & G and Sec. VIII.I)	• Microscale purification • Check for presence of tagged protein • Estimate expression levels • Purify from crude uncleared cell lysates or cultures	• Fast • Requires 0.5 ml culture • Does not require high-speed centrifugation • Amenable to high-throughput

[1] Includes time for sample prep and purification.

[2] Starting with clarified lysate; does not include time to prepare samples.

I. Introduction *continued*

Protein Purification Methods Using TALON® Resin

The following general guidelines are used for purifying polyhistidine-tagged protein from transformed *E. coli* cultures. Figure 4 and Table I provide an overview of TALON Resin protein purification methods and applications. Choose a method that best suits your research needs.

- Use 2 ml of resin suspension per ~3 mg of anticipated polyhistidine-tagged protein. 2 ml of homogeneously resuspended resin will provide 1 ml (bed volume) of TALON Resin.
- The buffers and purification conditions should work well for most soluble, monomeric proteins expressed in *E. coli*.
- Initially, test each different expression system and polyhistidine-tagged protein in small-scale batch purification to determine expression levels and to optimize the protocol. TALON Single Step Columns are designed for this type of analysis (Section VIII.E & F). Alternatively, A mini-scale batch purification protocol is provided in Appendix B; or you can use a TALONspin Column (Section VIII.G).
- Purification methods that work with nickel or zinc-based IMAC resins should also work with these resins. However, some optimization may be required.

  Note: TALON resin has been optimized and should only be used with the buffer formulations outlined in this user manual for optimal performance.

Choosing the Buffers: Imidazole Versus pH Gradient

TALON Resin purification schemes typically use either an imidazole or a pH gradient for washing and elution. Imidazole in the Equilibration and/or Equilibration/Wash Buffers minimizes nonspecific binding and reduces the amount of contaminating proteins. Thus, we recommend first purifying polyhistidine-tagged proteins using an imidazole gradient. However, imidazole and polyhistidine-tagged proteins absorb at 280 nm and elution peaks may be difficult to detect spectrophotometrically, especially if you are purifying small amounts of polyhistidine-tagged proteins. In these cases, collect the leading edge of the imidazole breakthrough peak and check for polyhistidine-tagged proteins by a protein specific assay (Bradford, 1976) and SDS/PAGE. Alternatively, use a pH gradient to purify polyhistidine-tagged proteins that are stable from pH range 5.0–7.0. See Section III for buffer compositions.

Elution Strategy: Step Versus Linear Gradients

In most cases, step gradients are preferred over linear gradients, because linear gradients lead to broad elution peaks, which can dilute the product and make detection difficult. Scaling-up step gradients is also less complicated than scaling-up linear gradients.

I. Introduction *continued*

TABLE II. TALON® RESIN CHARACTERISTICS

Feature	TALON® Resin	TALON® Superflow	TALON® CellThru	TALONspin™ Column
Capacity[1] (mg protein/ml resin)	5–15	5–20	5–10	2–4
Matrix	Sepharose CL-6B	Superflow	Uniflow	Sepharose 6B
Bead size (µm)	45–165	60–160	300–500	16–24
Max. Linear flow rate (cm/hr)	75–150	3,000	800	n/a[2]
Max. Volume flow rate[3] (ml/min)	0.5–1.0	50	13	n/a
Max. Pressure	2.8 psi 0.2 bar 0.02 MPa	140 psi 10 bar 0.97 MPa	9 psi 0.62 bar 0.06 MPa	n/a n/a n/a
pH stability	2–14 (2 hr) 3–14 (24 hr)	2–14 (2 hr) 3–14 (24 hr)	2–14 (2 hr) 3–14 (24 hr)	2–8.5 (2 hr) 2–7.5 (24 hr)
Protein exclusion limit (Da)	4×10^7	4×10^6	2×10^7	n/a

[1] The binding capacity for individual proteins may vary. Each of the above mentioned TALON products has different applications. Please refer to Table I for applications and benefits.
[2] n/a = not applicable
[3] Determined on a 5 x 1 cm column.

II. List of Components

TALON Resin, TALON Superflow Resin, and TALON CellThru Resin are supplied as 50% (w/v) slurries in nonbuffered 20% ethanol. Please note that during shipping and storage, the resin will settle; thus, we recommend that you thoroughly resuspend it before aliquotting. 2 ml of homogeneously resuspended resin will provide 1 ml of TALON Resin with a binding capacity of at least 5 mg of polyhistidine-tagged protein.

Store all of these resins, columns and buffers at 4°C unless otherwise indicated. **Do not freeze TALON® Resins.**

- **TALON® Metal Affinity Resin**

Cat. No.	Amount
635501	10 ml
635502	25 ml
635503	100 ml
635504	250 ml

- **TALON® Superflow Resin**

Cat. No.	Amount
635506	25 ml
635507	100 ml

- **TALON® Single Step Columns** (5 ml, Cat. Nos. 635628 & 635631; & 20 ml, Cat. No. 635632)
  These columns contain a dry mixture of TALON CellThru resin and xTractor Buffer to extract and bind histidine-tagged proteins in one step.
- **TALONspin™ Columns** (Cat. Nos. 635601, 635602, 635603)
  These columns contain 0.5 ml of TALON-NX resin as a 50% suspension in nonbuffered 20% ethanol.
- **TALON® HT 96-Well Plate** (Cat. No. 635622)
  1 TALON 96-Well Plate
  1 Plate Top Seal
  1 Plate Base Seal
  1 Collection Plate
- **TALON® Magnetic Beads** (Cat. Nos. 635636 & 635637)

Cat. No.	Amount
635636	2 x 1 ml
635637	6 x 1 ml

- **TALON® Magnetic Beads Buffer Kit** (Cat. No. 635638)
  60 ml 5X Equilibration/Wash Buffer
  15 ml 4X Elution Buffer
  30 ml 1X xTractor Buffer

II. List of Components *continued*

- **TALON® CellThru Resin**

Cat. No.	Amount
635509	10 ml
635510	100 ml

- **TALON® CellThru Disposable Columns**

Cat. No.	Size
635512	50 x 2 ml column
635513	20 x 10 ml column

- **TALON® 2 ml Disposable Gravity Columns** (Cat. No. 635606)
- **TALON® Purification Kit** (Cat. No. 635515)

  10 ml TALON® Metal Affinity Resin
  160 ml 5X Equilibration/Wash Buffer (250 mM sodium phosphate, 1.5 M NaCl, pH 7)
  160 ml 5X Equilibration Buffer (250 mM sodium phosphate, 1.5 M NaCl, pH 8)
  25 ml 10X Elution Buffer (1.5 M imidazole, pH 7)
  5 2 ml Disposable Gravity Columns
  1 10 ml Disposable Gravity Column

- **TALON® Buffer Kit** (Cat. No. 635514)

  160 ml 5X Equilibration/Wash Buffer (250 mM sodium phosphate, 1.5 M NaCl, pH 7)
  160 ml 5X Equilibration Buffer (250 mM sodium phosphate, 1.5 M NaCl, pH 8)
  25 ml 10X Elution Buffer (1.5 M imidazole, pH 7)

- **TALON® xTractor Buffer Kit** (Cat. No. 635623)

  Store DNase I at –20°C.

  If a precipitate has formed in the lysozyme solution, allow the tube to warm at room temperature and gently invert the tube. The solution may remain turbid after this procedure.

  200 ml 1X xTractor Buffer
  2.5 ml 50X Lysozyme
  400 µl DNase (1 unit/µl)

- **TALON® xTractor Buffer** (Cat. No. 635625)

  500 ml 1X xTractor Buffer

III. Additional Materials Required

See Section IV for preparing buffers with the TALON® Purification Kit (Cat. No. 635515) or the TALON® Buffer Kit (Cat. No. 635514). If you have **not purchased** those kits, we recommend preparing the following buffers for purifying polyhistidine-tagged proteins under native or denaturing conditions. Before preparing other buffer compositions, please consult Appendix A to evaluate resin compatibility. For TALON Magnetic Beads (see Section V), use the same Equilibration/Wash Buffer as with the resins, and elute with an imidazole-based elution buffer containing a higher concentration of imidazole than that used to elute from the resins, as described in Section V.D.

Choosing Buffers

To decrease the amount of nonspecifically bound proteins, we recommend using the **Equilibration/Wash Buffer** at pH 7.0 during purification; however, if your target protein is more stable at pH 8.0, or if it does not adsorb at pH 7.0, use the Equilibration Buffer at pH 8.0 (in place of the Equilibration/Wash Buffer) during all extraction and wash steps. Note that at elevated pH values, amino acids other than histidine, as well as the peptide bond, contribute to protein adsorption. Thus, proteins without a polyhistidine tag can also adsorb to IMAC resins, which decreases resin capacity and the final purity of your target protein. You may choose to use either native or denaturing buffer conditions, depending on the solubility of your protein. Figure 5 outlines the purification procedure.

A. Native Buffers

Native protein purification regimens use buffer conditions that preserve the native, three-dimensional structure and surface charge characteristics of a selected soluble protein during harvest from an expression host. The low affinity of TALON Resin for nonpolyhistidine-tagged proteins minimizes contaminant carryover. In addition, increasing buffer ionic strength can minimize nonspecific interactions. Regardless of the conditions used and the nature of the polyhistidine-tagged protein being purified, most applications will benefit from the presence of 100–500 mM NaCl in the IMAC buffer. In many cases, adding glycerol or ethylene glycol neutralizes nonspecific hydrophobic interactions. Small amounts of nonionic detergent may also dissociate weakly bound species.

- **1X Equilibration/Wash buffer** (pH 7.0)*
  50mM sodium phosphate
  300 mM NaCl
- **1X Equilibration buffer** (pH 8.0)*
  50 mM sodium phosphate
  300 mM NaCl

III. Additional Materials Required *continued*

- **1X Elution buffer***
  - **Imidazole Elution** (pH 7.0)
    50 mM sodium phosphate
    300 mM NaCl
    150 mM imidazole
  - **pH Elution** (pH 5.0)[1]
    50 mM sodium acetate
    300 mM NaCl
    [1] Prepare fresh before use.
- **HT 96-Well Plate Wash buffer** (pH 7.0)*
  83 mM sodium phosphate
  500 mM NaCl
  10 mM imidazole
- **TALON Magnetic Beads 1X Elution buffer***
  - **Imidazole Elution** (pH 7.0)
    50 mM sodium phosphate
    300 mM NaCl
    250 mM imidazole

B. Denaturing Buffers

Denaturants, such as 6 M guanidinium, enhance protein solubility. Because proteins overexpressed in prokaryotic systems are sometimes insoluble, you may need to purify proteins under denaturing conditions. When purifying proteins under denaturing conditions, we recommend preparing the buffers indicated below.

In the presence of 6 M guanidinium, *the resin's color will change from a pinkish-mauve to violet due to a change in metal ion hydration in response to the chaotrope*. After removal of the chaotrope, the resin will return to a pinkish-mauve color. The change to violet does not reflect any change in the physical or chemical properties of the resin. In fact, the color change can be useful for indicating the buffer in which the resin is suspended, and for following the movement of guanidinium through the resin bed.

- **1X Equilibration/Wash Buffer** (pH 7.0)*
  50 mM sodium phosphate
  6 M guanidine-HCl
  300 mM NaCl
- **1X Equilibration Buffer** (pH 8.0)*
  50 mM sodium phosphate
  6 M guanidine-HCl
  300 mM NaCl

* See Sambrook, Appendix B.21, or your standard protocol for preparing sodium phosphate buffer.

III. Additional Materials Required *continued*

- **1X Imidazole Elution Buffer** (pH 7.0)
  45 mM sodium phosphate
  5.4 M guanidine-HCl
  270 mM NaCl
  150 mM imidazole
- **TALON Magnetic Beads 1X Elution Buffer***
  –Imidazole Elution (pH 7.0)
  45 mM sodium phosphate
  5.4 M guanidine-HCl
  270 mM NaCl
  250 mM imidazole

C. Additional Buffers & Reagents

- **MES Buffer**
  20 mM 2-(N-morpholine)-ethanesulfonic acid (MES), pH 5.0
- **5X SDS PAGE sample buffer**
  15% β-Mercaptoethanol (β-ME)
  15% SDS
  50% Glycerol
  1.5% Bromophenol blue
- **Imidazole** (Sigma, Cat. No. I0250) Also suitable for FPLC applications
- **Bio-Rad Protein Assay** (Bio-Rad, Cat. No. 500-0001)

D. Additional Materials required for TALON® CellThru Resin

- CellThru 2 ml Disposable Columns (Cat. No. 635512)
- CellThru 10 ml Disposable Columns (Cat. No. 635513)

E. Additional Materials for TALON® HT 96 Plate

Vacuum Purification

- QIAVac 96 (QIAGEN, Cat. No. 19504), NucleoVac (MACHEREY-NA-GEL, Cat. No. 740630), or similar vacuum manifold
- (Extra) Collection 96-Deep Well Titer Plates (Whatman Cat. No. 7701-5200 or Evergreen Cat. No. 240-8556-030)

Centrifugation

- Centrifuge with a rotor for centrifugation of microtiter plates, such as the Allegra 6R Centrifuge (Beckman Coulter) with the GH 3.8; GH 3.8A; or JS 4 Beckman rotors.

III. Additional Materials Required *continued*

F. Additional Materials for TALON® Single Step Columns

- 1 X Equilibration/Wash Buffer* (50 mM sodium phosphate, 300 mM NaCl, pH 7.0)
- Wash-2 Buffer* (50 mM sodium phosphate, 300 mM NaCl, 7.5 mM imidazole, pH 7.0)
- 1X Elution Buffer* (50 mM sodium phosphate, 300 mM NaCl, 150 mM imidazole, pH 7.0)
- 15 ml screw-cap tubes (5 ml columns) or 50 ml screw-cap tubes (20 ml columns), receiving tubes for fraction storage
- [Optional] BCA Protein Assay Kit (Pierce, Cat No. 23226)

*The buffers used for the Single Step Columns can be prepared by dilution of the buffers in our TALON Buffer Kit (Cat. No. 635514). See Section IV for preparation details.

G. Additional Materials for TALON® Magnetic Beads

- Magnetic separator (colorless or white for best visibility, since the beads are black)
- 1.5 ml and 0.5 ml microfuge tubes
- DNase I

Note: Although TALON xTractor Buffer is included in the TALON Magnetic Beads Buffer Kit (Cat. No. 635638), if more is needed, it is available as Cat. No. 635623.

III. Additional Materials Required *continued*

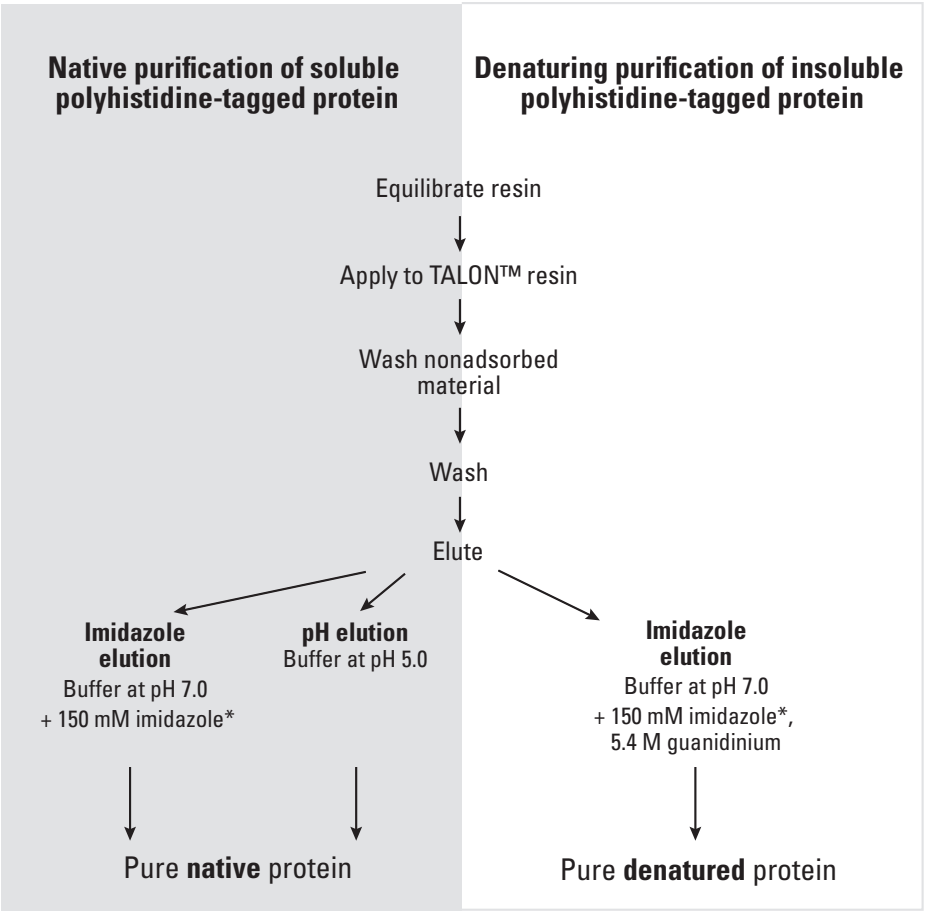

Figure 5. Purification of polyhistidine-tagged proteins using TALON® Resin. The protocols in this User Manual are designed using the Equilibration/Wash Buffer at pH 7.0. If your target protein is more stable at pH 8.0, or if it does not adsorb at pH 7.0, use the Equilibration Buffer (pH 8.0) instead of the Equilibration/Wash Buffer during the extraction and wash steps.

*Use 250 mM imidazole instead of 150 mM imidazole when eluting from TALON Magnetic Beads.

IV. Buffers for TALON® Purification and Buffer Kits

If you have purchased the TALON® Purification or Buffer Kits, prepare buffers as described below. To decrease the amount of nonspecifically bound proteins, we recommend using the **Equilibration/Wash Buffer** at pH 7.0 during purification; however, if your target protein is more stable at pH 8.0, or if it does not adsorb to the resin at pH 7.0, use the Equilibration Buffer (pH 8.0) in place of the Equilibration/Wash Buffer during all extraction and wash steps. Note that at elevated pH values, amino acids other than histidine, as well as the peptide bond, can be adsorbed by TALON Resins; Thus, under high pH conditions (pH>8.0), proteins without a polyhistidine tag can be adsorbed, decreasing resin capacity and the final purity of your target protein.

Note: If a precipitate is observed in the buffers, warm them at 37°C, and stir or shake to dissolve precipitate prior to diluting and using the buffers.

A. TALON xTractor Buffer: No preparation necessary except optional addition of DNase I or Lysozyme (see Section VII.A).

B. Equilibration Buffers

1. Dilute one part of the 5X Equilibration/Wash Buffer or 5X Equilibration Buffer with four parts of deionized water.
2. Check and correct pH if necessary. The 1X Equilibration/Wash Buffer should be pH 7.0, while the 1X Equilibration Buffer should be pH 8.0.

C. Elution Buffer

Dilute one part of the 10X Elution Buffer with nine parts of 1X Equilibration/Wash Buffer (pH 7.0) (or 1X Equilibration Buffer [pH 8.0], depending on the solubility of your protein) prepared in Step A.

D. Denaturing Conditions

Add 6 M guanidinium to the Equilibration/Wash Buffer (pH 7.0), or Equilibration Buffer (pH 8.0), and the Elution Buffer prepared in Steps A and B, respectively.

Note: Perform all steps during the purification procedure in the presence of 6 M guanidinium. Protein samples containing high guanidinium concentrations form a precipitate when loaded on SDS polyacrylamide gels. Therefore, dialyze the sample overnight in a buffered solution containing 8 M urea before loading it onto the gel.

E. Wash Buffers

- In general, use the Equilibration/Wash Buffer at pH 7.0 to wash nonadsorbed proteins. If the protein is not stable at pH 7.0, then use the Equilibration Buffer at pH 8.0 with 5–10 mM imidazole.
- If your host cell line produces unwanted multi-histidine proteins, incorporate a more stringent wash:

  Dilute 10X Elution Buffer in either 1X Equilibration/Wash Buffer or 1X Equilibration Buffer for a final concentration of 5–10 mM imidazole (1:300–1:150).

V. Buffers for TALON® Magnetic Beads

If you have purchased the TALON® Magnetic Beads Buffer Kit (Cat. No. 635638), prepare buffers as described below.

A. TALON xTractor Buffer: No preparation necessary except optional addition of DNase I or Lysozyme (see Section VII.A).

B. Equilibration Buffers

1. Dilute one part of the 5X Equilibration/Wash Buffer with four parts of deionized water.
2. Check and correct pH if necessary. The 1X Equilibration/Wash Buffer should be pH 7.0.

C. Elution Buffer

1. Dilute one part of the 4X Elution buffer with three parts of 1X Equilibration Buffer.
2. Check and correct pH if necessary. The 1X Elution Buffer should be pH 7.0.

D. Wash Buffers

- In general, use the 1X Equilibration/Wash Buffer at pH 7.0 to wash non-adsorbed proteins.
- If your host cell line produces unwanted histidine-rich proteins, incorporate a more stringent wash with 10 mM imidazole in 1X Equilibration/Wash Buffer:

  Dilute 4X Elution Buffer (1M imidazole) in 1X Equilibration/Wash Buffer for a final concentration of 5–10 mM imidazole (1:200–1:100).

E. Denaturing Conditions

Add 6 M guanidinium to the Equilibration/Wash Buffer (pH 7.0) and the Elution Buffer prepared in Steps B and C, respectively.

Note: Perform all steps during the purification procedure in the presence of 6 M guanidinium. Protein samples containing high guanidinium concentrations form a precipitate when loaded on SDS polyacrylamide gels. Therefore, dialyze the sample overnight in a buffered solution containing 8 M urea before loading it onto the gel.

VI. Transformation & Protein Expression

A. Transformation of Host Cells with Expression Vectors

The following protocol is for chemically-induced transformation of *E. coli* competent cells. Perform control transformations in parallel.

Note: Use JM109 or another lac-inducible cell line to see induction of expression. For tighter control of expression levels, use our PROTet 6xHN Bacterial Expression System—especially recommended for expression of cytotoxic proteins.

1. On ice, thaw a tube containing 100 µl of 0.5 M β-mercaptoethanol (β-ME) and one 50 µl tube of frozen *E. coli* competent cells for each ligation/transformation.
2. Dispense 2 µl of 0.5 M β-ME into each tube of competent cells and mix.
3. Dispense 2 µl of DNA directly into the mixture from Step 2.
4. Incubate tubes on ice for 30 min.
5. Heat shock for exactly 30 sec in a 42°C water bath.
6. Remove tubes from water bath and place on ice for 2 min.
7. Add 250 µl of SOC medium to each tube at room temperature.
8. Shake the tubes horizontally at 37°C for 1 hr at 225 rpm in a rotary shaking incubator.
9. Spread transformation mixtures onto LB-ampicillin (50 µg/ml) agar plates [containing X-gal (75 µg/ml) and IPTG (1 mM) if blue-white selection is desired]. Incubate the plates at 37°C overnight.

B. Protein Expression

1. Grow an overnight culture of *E. coli* transformed with your expression plasmid. If you can isolate a sufficient amount of protein from this culture, proceed to Step 3 after taking a 1 ml sample for electrophoretic analysis. Centrifuge the sample at 1,000–3,000 x g for 15 min at 4°C, remove the supernatant, and store the cell pellet at –20°C.

   Note: If a large-scale preparation of the protein is required, proceed to Step 2.
2. If you need a greater quantity of the target protein, use 20 ml of overnight culture to inoculate 1 L of medium. Incubate with shaking for another 1–2 hr, until the culture has an absorbance of ~0.6 OD_{600}. Remove a 1 ml sample of the culture, centrifuge at 1,000–3,000 x g for 15 min at 4°C, remove the supernatant and store the cell pellet at –20°C for electrophoretic analysis.
3. Induce expression by adding an appropriate inducer. For example, the *lac* promoter in the pHAT10 expression vector can be induced with 1 mM IPTG. Continue the incubation for another 3–5 hr.
4. Remove a 1 ml sample of the culture, centrifuge at 1,000–3,000 x g for 15 min at 4°C, remove the supernatant, and store the cell pellet at –20°C for electrophoretic analysis.
5. Proceed with sample preparation (Section VII).

VII. Sample Preparation

A. TALON® xTractor Buffer Sample Preparation

This procedure has been optimized for extraction of native proteins from fresh or frozen bacterial cell pellet using TALON xTractor Buffer (Cat. No. 635623). The volumes of this extraction can be adjusted, as long as 20 ml of xTractor Buffer are used per 1 g of cell pellet.

1. Add 20 ml of xTractor Buffer to 1 g of bacterial cell pellet. Mix gently. Pipet the mixture up and down to fully resuspend the pellet.

   Notes

   - For TALON HT 96-Well Plate (Cat. No. 635622), resuspend 40–100 mg of bacterial cell pellet in 2 ml of xTractor Buffer.
   - A log-phase culture of *E. coli* (O.D.=0.6–0.8) when induced for 2–4 hours, would be expected to provide ~20–40 mg bacterial pellet from 2 ml of the culture.

2. [Optional]: Add 40 µl of 1 unit/µl DNase I solution and 200 µl of 50X lysozyme solution.

   Notes

   - DNase I reduces the viscosity of the lysate, allowing for more efficient removal of cellular debris. DNase can be used without lysozyme. However, if you are you are treating cells with lysozyme, then you **must** treat cells with DNase I as well.
   - Lysozyme helps to fully disrupt bacterial walls, and thus it has been demonstrated to be highly beneficial in extraction of high molecular weight proteins (>40 KDa). However, lysozyme should be omitted for mammalian extraction procedures as well as when lysozyme interferes with your protein's functionality.

3. Mix gently, pipetting up and down several times.
4. Incubate with gentle shaking for 10 min at room temperature. (You may incubate the solution at 4°C, if desired).

   Note: At the end of this incubation period, there should be no visible particles. If cell pellet fragments are present, resuspend them by pipetting solution up and down and incubating for additional 1–2 min.

5. The resulting cell lysate can now be applied directly to a TALON CellThru Column, or the lysate supernatant can be applied to any other TALON Resin column after centrifuging at 10,000–12,000 x g for 20 min at 4°C.

B. Standard Sample Preparation to Isolate Native Proteins

1. Harvest the cell culture by centrifugation at 1,000–3,000 x g for 15 min at 4°C. Remove the supernatant. If yield is low, use the mild extraction method described in Step 6, below.
2. Resuspend the cell pellet by vortexing in 2 ml of chilled 1X Equilibration/Wash Buffer (4°C) per 25 ml of culture ≤100 ml. For cultures >1 L, resuspend the pellet in 1–2% of the original culture volume.

   Note: You may omit Steps 3–4 if lysozyme treatment interferes with your protein's functionality.

VII. Sample Preparation *continued*

3. Add lysozyme to the 1X Equilibration/Wash Buffer for a concentration of 0.75 mg/ml. To reduce the chance of introducing proteases, use the highest purity lysozyme available.
4. Incubate at room temperature for 20–30 min.

   Note: Incubations at room temperature result in elevated proteolytic activities. Alternatively, you can use lysozyme at 4°C with lower efficiency. If this treatment hydrolyzes the target protein, use the method described in Step 6 (below). Alternatively, disrupt the cells by repeated freeze/thaw cycles; that is, flash-freezing the cell suspension in a dry ice-ethanol bath and thawing in chilled H_2O.
5. If your sample is ≤50 ml, sonicate it 3 x 10 sec, with a 30 sec pause on ice between each burst. If your sample is ≥ 200 ml, sonicate it 3 x 30 sec, with a 2 min pause on ice between each burst. Proceed to Step 7.

   Note: Excessive sonication can destroy protein functionality.
6. **[Optional]: High-yield, mild extraction method.** Transfer the cells to a chilled mortar and grind 1 part cells with 2.5 parts alumina (Sigma Cat. No. A-2039) for 2–3 min or until the composition of the mixture becomes paste-like. Add 2 ml chilled 1X Equilibration/Wash Buffer (4°C) per 25 ml culture.

   Note: If there is a high level of proteolytic activity in the cell lysate, we recommend adding 1 mM EDTA (final concentration) to the Equilibration/Wash Buffer in order to inhibit metalloproteases during the extraction. Before application of the sample to the TALON Resin, EDTA must be removed by gel filtration chromatography (PD-10, GE Healthcare) equilibrated with the Equilibration/Wash Buffer for IMAC.
7. Centrifuge the cell extract at 10,000–12,000 x g for 20 min at 4°C to pellet any insoluble material.
8. Carefully transfer the supernatant to a clean tube without disturbing the pellet. This is the clarified sample.
9. Reserve a small portion of the clarified sample at 4°C for SDS/PAGE analysis.
10. If this is the first time you have prepared clarified samples from cells expressing a particular recombinant protein, we recommend that you estimate the protein's expression level in that host strain. To do so, perform a small-scale purification, and then analyze a portion by SDS/PAGE in parallel with protein standards. Once expression is observed, proceed with the appropriate purification protocol (see Section VIII).

C. Standard Sample Preparation to Isolate Denatured Proteins

1. Harvest 20–25 ml of cell culture by centrifugation at 1,000–3,000 x g for 15 min at 4°C.
2. Resuspend the pellet in 2 ml of **denaturing** 1X Equilibration/Wash Buffer (pH 7.0) per 20–25 ml of culture.
3. Gently agitate orr the sample until it becomes translucent.
4. Centrifuge the sample at 10,000–12,000 x g for 20 min at 4°C to pellet any insoluble material.

VII. Sample Preparation *continued*

5. Carefully transfer the supernatant to a clean tube without disturbing the pellet. This is the clarified sample.
6. Set aside a small portion of the clarified sample for SDS/PAGE analysis. Then proceed with the appropriate purification protocol (see Section VIII).

   Note: Samples containing 6 M guanidinium must be dialyzed overnight against buffer containing 8 M urea before loading on a gel.

D. Standard Sample Preparation for TALON® CellThru Resin

Sample Preparation to Isolate Native Proteins

1. Harvest the cell culture by centrifugation at 1,000–3,000 x g for 15 min at 4°C. Remove the supernatant.
2. Resuspend the cell pellet by vortexing in 2 ml of chilled 1X Equilibration/Wash Buffer (4°C) per 25 ml of culture ≤100 ml. For cultures >1 L, resuspend the pellet in 1–2% of the original culture volume.

   Note: You may omit Steps 3–4 if lysozyme treatment interferes with your protein's function.
3. Add lysozyme to the 1X Equilibration/Wash Buffer for a concentration of 0.75 mg/ml. To reduce the chance of introducing proteases, use the highest purity lysozyme available.
4. Incubate at room temperature for 20–30 min.

   Note: Incubations at room temperature result in elevated proteolytic activities. Alternatively, you can use lysozyme at 4°C with lower efficiency. If this treatment hydrolyzes the target protein, use the method described in Step 6. Alternatively, disrupt the cells by repeated freeze/thaw cycles; that is, flash-freezing the cell suspension in a dry ice-ethanol bath and thawing in chilled H_2O.
5. If your sample is ≤50 ml, sonicate it 3 x 10 sec, with a pause for 30 sec on ice between each burst. If your sample is ≥ 200 ml, sonicate it 3 x 30 sec, with a 2 min pause on ice between each burst.

   Note: Excessive sonication can destroy protein functionality.
6. Store a small portion of the clarified sample at 4°C for SDS/PAGE analysis.

Sample Preparation to Isolate Denatured Proteins

1. Harvest 20–25 ml of cell culture by centrifugation at 1,000–3,000 x g for 15 min at 4°C.
2. Resuspend the pellet in 2 ml of **denaturing** 1X Equilibration/Wash Buffer (pH 7.0) per 20–25 ml of culture.
3. Gently agitate or stir the sample until it becomes translucent.
4. Set aside a small portion of the clarified sample for SDS/PAGE analysis. Then proceed with the appropriate purification protocol, below (see Section VIII).

   Note: Samples containing 6 M guanidinium must be dialyzed overnight against buffer containing 8 M urea before loading on a gel.

VII. Sample Preparation *continued*

E. Standard HT 96-Well Sample Preparation

1. Grow cells in appropriate format for high-throughput analysis.
2. Centrifuge if necessary and remove supernatant.
3. If the target proteins are secreted in the medium, utilize the supernatant as a starting material and proceed to Step 6. If the target proteins are intracellular, proceed to the next step.
4. Disrupt the cells in presence of 1X Equilibration/Wash Buffer (use 2 ml of buffer per 200 mg of cells per purification well).

   Note: [Optional] Use TALON xTractor buffer for better extraction efficiency.
5. Centrifuge extracts and collect the supernatant to be used as a starting material.
6. Remove 50 µl of each sample for protein concentration analyses.

F. Standard Sample Preparation for TALON Magnetic Beads

When purifying proteins, more effective binding is achieved when running clarified lysates. For screening of expression levels, crude lysates derived from overnight cultures can be added directly to the beads (see Section VII.G).

Sample Preparation to Isolate Native Proteins

1. Harvest the cell culture by centrifugation at 1,000–3,000 x g for 15 min at 4°C. Remove the supernatant. The pellet can either be used immediately or stored frozen at –70°C.
2. Add 0.5 ml of xTractor Buffer per 25 mg of cell pellet. The volume of xTractor Buffer can be increased or decreased depending on the size of the cell pellet.
3. **[Optional]:** Add 1 µl of 1 unit/ml DNase I solution.
4. Mix gently, pipeting up and down several times.
5. Incubate with gentle shaking for 10 min at room temperature, or at 4°C, if desired.
6. **[Optional]: High-yield, mild extraction method.** Transfer the cells to a chilled mortar and grind 1 part cells with 2.5 parts alumina (Sigma Cat. No. A-2039) for 2–3 min or until the composition of the mixture becomes paste-like. Add 1 ml chilled 1X Equilibration/Wash Buffer (4°C) per 25 mg of cell pellet.
7. Centrifuge at 10,000-12,000 x g for 20 min at 4°C.

   Note: The uncentrifuged crude cell lysate can also be applied to TALON Magnetic Beads. However, the lysate may have to be diluted further or require more DNase to prevent the beads from failing to migrate to the magnet because of the high viscosity of the solution.
8. Carefully transfer the supernatant to a clean tube without disturbing the pellet. This is the clarified sample.

VII. Sample Preparation *continued*

9. Reserve a small portion of the clarified sample at 4°C or on ice for protein assays and SDS-PAGE analysis. Then proceed with the TALON Magnetic Beads purification protocol (Section VIII.I).

Sample Preparation to Isolate Denatured Proteins

1. Harvest the cell culture by centrifugation at 1,000–3,000 x g for 15 min at 4°C. Remove the supernatant. The pellet can either be used immediately or stored frozen at –70°C.
2. Add 0.5 ml of **denaturing** 1X Equilibration/Wash Buffer per 25 mg of cell pellet. The volume of the buffer can be increased or decreased depending on the size of the cell pellet.
3. Gently agitate or stir the sample until it becomes translucent.
4. Centrifuge the sample at 10,000–12,000 x g for 20 min at 4°C to remove any insoluble material.
4. Carefully transfer the supernatant to a clean tube without disturbing the pellet. This is the clarified sample.
5. Set aside a small portion of the clarified sample at 4°C or on ice for protein assays and SDS-PAGE analysis. Then proceed with the TALON Magnetic Beads purification protocol (Section VIII.I).

   Note: Samples containing 6 M guanidine must be dialyzed overnight against buffer containing 8 M urea before loading on a gel.

G. Sample Preparation Directly from Overnight Cultures for TALON Magnetic Beads

If screening transformants for expression levels, pick a single colony from the plate and inoculate 4.5 ml medium. Incubate the culture at 37°C until the OD600 reaches ~0.6–0.8 AU (mid-log phase). Then induce protein expression with the recommended concentration of inducer agent (depending on your expression strain and the expression plasmid being used). Continue to grow the culture with rigorous shaking at 37°C for another 4 hr or overnight. Alternatively, follow your standard induction or expression protocol.

1. Dilute overnight culture 1:1 with xTractor Buffer and add DNase to a concentration of 1 unit/ml of culture. (For example, dilute 0.5 ml of an overnight culture with 0.5 ml of xTractor Buffer and add 1 unit of DNase.)
2. Mix thoroughly at 4°C for 30 min.
3. **[Optional]** If the culture is still too viscous, dilute it with sufficient 5X Equilibration/Wash Buffer to obtain a final concentration of 1X Equilibration/Wash Buffer.
4. Check pH to ensure it falls between 7–8 for optimal binding and proceed with the TALON Magnetic Beads purification protocol (Section VIII.I).

VIII. Protein Purification Protocols

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

A. General Information

1. Perform all manipulations at 4–8°C in order to maintain protein stability and improve yield.
2. **This protocol is designed using the Equilibration/Wash Buffer (pH 7.0).** If your target protein is more stable at pH 8.0, or if it does not adsorb at pH 7.0, use the Equilibration Buffer at pH 8.0 (instead of the Equilibration/Wash Buffer) during extraction and wash steps.
3. A reducing agent, such as 10 mM β-ME, or a protease inhibitor, such as PMSF, in the Equilibration/Wash Buffer (pH 7.0), may improve the structural stability of fragile proteins during sample preparation. See Appendix A for compatibility information.

   Note: Depending on the concentration and volume of the additive you wish to use, you may need to remake the buffers to preserve the recommended concentration of NaCl and buffering agent. **DTT and DTE are not compatible with this TALON protocol in any concentration.**
4. If the cell lysate contains a high level of proteolytic activity, we recommend adding 1 mM EDTA to the Equilibration/Wash Buffer (pH 7.0) to inhibit metalloproteases during the extraction. **However, before applying the sample to the resin, remove EDTA using a gel filtration column (such as PD-10, GE Healthcare) equilibrated with the Equilibration/Wash Buffer.** In some cases, the host cell produces low molecular weight chelators that can also be removed using gel filtration.

   Chelators can be detected by applying your sample to a small column packed with TALON Resin. If the top of the column loses its characteristic pink color, and the colorless front moves in the direction of the flow, or if you obtain pink fractions during batch adsorption, you must equilibrate the sample using a gel filtration column.
5. Overexpressed recombinant proteins can accumulate in insoluble inclusion bodies. In order to determine optimal extraction/purification conditions, you must determine the distribution of the protein in soluble and insoluble forms. Perform a preliminary SDS/PAGE analysis of protein extracts obtained under native conditions, followed by extraction of the residual proteins under denaturing conditions. Take care to use the same extraction volumes for both native and denaturing extracts, and run the cell extract before induction as a control in one lane to identify the target protein. Use of denaturing conditions is recommended only if the biological activity of the target protein would not be affected by denaturation or is unimportant. It is preferable to use native conditions for extraction even if only 5–10% of the target protein is soluble.

VIII. Protein Purification Protocols *continued*

6. The buffer volumes in the following protocols were optimized for purifying the HAT-DHFR fusion protein from 20–25 ml of *E. coli* culture. Depending on the expression level and anticipated yield, you may need to adjust the buffer volumes for other proteins. As a starting point, use 2 ml of buffer per 20–25 ml of culture.
7. If you are purifying protein from harvested eukaryotic cells, lyse the cells in an appropriate buffer containing a mild detergent (Sambrook & Russell, 2001). See Appendix A for compatible buffer additives. *Note that EDTA and EGTA are not compatible with these protocols because these metal-chelating reagents strip the cobalt from the resin.*
8. Carefully check the sample's appearance after lysis or sonication. Bacterial samples often remain viscous from incomplete shearing of genomic DNA. Complete DNA fragmentation improves protein yields and allows efficient removal of cellular debris during centrifugation. You may decrease the sample's viscosity by digestion for 20–30 min at room temperature with 2.5 µg/ml of DNase I. Remember that proteolytic activity is much higher at room temperature. Alternatively, dilute the sample fivefold with Equilibration/Wash Buffer before applying it to the resin. This procedure should not significantly affect recovery.

Notes on Protein Purification methods using TALON® Resin

The following general guidelines are used for purifying polyhistidine-tagged protein from transformed *E. coli* cultures. Table I provides an overview of TALON® Resin protein purification methods and applications. Choose a method that best suits your research needs.

- Use 2 ml of resin suspension per ~3 mg of anticipated polyhistidine-tagged protein. 2 ml of homogeneously resuspended resin will provide 1 ml (bed volume) of TALON Resin.
- The buffers and purification conditions should work well for most soluble, monomeric proteins expressed in *E. coli*.
- Initially, test each different expression system and polyhistidine-tagged protein in small-scale batch purification to determine expression levels and to optimize the protocol. A mini-scale batch purification protocol is provided in Appendix B; alternatively, you can use a TALON spin column.
- Purification methods that work with nickel or zinc-based IMAC resins should also work with these resins. However, some optimization may be required.

TALON® CellThru Considerations

The procedure for purifying polyhistidine-tagged proteins using TALON CellThru Resin is essentially the same as other TALON Resins with the following significant differences.

VIII. Protein Purification Protocols *continued*

1. Extracellular Proteins

If there are no chelating agents in the fermentation liquid and the pH is ≥7.0, you can apply sample directly onto a prepacked column. Otherwise, a desalting/equilibration step is necessary (such as ultrafiltration or gel filtration with Sephadex G25).

2. Intracellular Proteins

For purifying intracellular proteins, apply the sonicated sample containing your target proteins, directly onto a prepacked column. There is no need for centrifugation. Electrophoresis will reveal that some of the target protein has passed through the column without adsorption. To a large extent the material passing through the column is insoluble protein, which would normally have been removed during high-speed centrifugation. The amount of non-adsorbed target protein will also vary as a function of sonication efficiency.

3. Chromatography Considerations

TALON CellThru Beads have a diameter of 300–500 µm; therefore, use a column with a filter pore size of 90–130 µm to adequately pass cellular debris. We recommend using our CellThru 2 ml & 10 ml Disposable Columns (Cat. No. 635512 & 635513). The 2 ml columns are suitable for 1–2 ml bed volumes, while the 10 ml columns are suitable for 5–10 ml bed volumes. Because the column filters have a larger pore size and permit higher flow rates, you may need to incubate your sample with the resin for 5 min before letting it flow through. If necessary, pass the sample through the column a second time.

The technique of expanded bed chromatography works well with these resins as the material can flow through the resin more effectively. Flow rates may have to be adjusted to get the maximum binding efficiency when using this technique.

B. Batch/Gravity-Flow Column Purification

For column IMAC using TALON Resins, we recommend a hybrid batch/gravity-flow procedure. This method combines the speed and convenience of a batch procedure with the exceptionally high purity of the gravity-flow column method. In this hybrid procedure, the binding and initial washing steps are performed in a batch format to save time, eliminate extraneous debris, and avoid column clogging. After the initial washes, the resin is transferred to a column for additional washing and protein elution.

1. Thoroughly resuspend the TALON Resin.
2. Immediately transfer the required amount of resin suspension to a sterile tube that will accommodate 10–20 times the resin bed volume.
3. Centrifuge at 700 x g for 2 min to pellet the resin.

VIII. Protein Purification Protocols *continued*

4. Remove and discard the supernatant.
5. Add 10 bed volumes of 1X Equilibration/Wash Buffer and mix briefly to pre-equilibrate the resin.
6. Recentrifuge at 700 x g for 2 min to pellet the resin. Discard the supernatant.
7. Repeat Steps 5 and 6.
8. Add the clarified sample from Section VI.A, B, or C to the resin.
9. Gently agitate at room temperature for 20 min on a platform shaker to allow the polyhistidine-tagged protein to bind the resin.
10. Centrifuge at 700 x g for 5 min.
11. Carefully remove as much supernatant as possible without disturbing the resin pellet.
12. Wash the resin by adding 10–20 bed volumes of 1X Equilibration/Wash Buffer. Gently agitate the suspension at room temperature for 10 min on a platform shaker to promote thorough washing.
13. Centrifuge at 700 x g for 5 min.
14. Remove and discard the supernatant.
15. Repeat Steps 12–14.
16. Add one bed volume of the 1X Equilibration/Wash Buffer to the resin, and resuspend by vortexing.
17. Transfer the resin to a 2 ml gravity-flow column with an end-cap in place, and allow the resin to settle out of suspension.
18. Remove the end-cap, and allow the buffer to drain until it reaches the top of the resin bed, making sure no air bubbles are trapped in the resin bed.
19. Wash column once with 5 bed volumes of 1X Equilibration/Wash Buffer.
20. **[Optional]:** If necessary, repeat Step 19 under more stringent conditions using 5–10 mM imidazole in 1X Equilibration/Wash Buffer (Section IV.D).
21. Elute the polyhistidine-tagged protein by adding 5 bed volumes of Elution Buffer to the column. Collect the eluate in 500 µl fractions.

    Note: Under most conditions, the majority of the polyhistidine-tagged protein will be recovered in the first two bed volumes.
22. Use spectrophotometric and SDS/PAGE analyses to determine which fraction(s) contain(s) the bulk of the polyhistidine-tagged protein.

    Note: Use a Bradford protein assay (Bradford, 1976) or UV absorbance at 280 nm. Use UV absorbance only if you are eluting sufficient protein to exceed the absorbance of the imidazole at 280 nm. Alternatively, dialyze the fractions overnight against the Equilibration/Wash Buffer, and then measure their UV absorbance at 280 nm.

VIII. Protein Purification Protocols *continued*

C. Large-Scale Batch Purification

This method purifies polyhistidine-tagged proteins faster than gravity-flow columns; however, batch washes remove impurities less efficiently than gravity-flow columns. Therefore, they require larger wash buffer volumes to obtain pure polyhistidine-tagged proteins.

1. Thoroughly resuspend TALON Resin.
2. Transfer required amount of resin to a glass filter with a pore size of 10–20 µm.
3. Apply a vacuum to the filter to remove excess ethanol.
4. Add 5 bed volumes of deionized water to the resin, and apply vacuum.
5. Add 5 bed volumes of 1X Equilibration/Wash Buffer to the resin, and apply vacuum.
6. Repeat Step 5 two times.
7. Add crude lysate (CellThru Resin) or clarified sample (other than CellThru Resin) to the resin, and mix for 3–5 min.
8. Apply vacuum and collect the filtrate.
9. Wash the resin by adding 10–20 bed volumes of 1X Equilibration/Wash Buffer. Gently agitate the suspension at room temperature for 10 min on a platform shaker to promote thorough washing.
10. Apply vacuum to remove buffer.
11. Repeat the above wash (Steps 9–10) 2–3 times.
12. **[Optional]:** If necessary, repeat Step 11 under more stringent conditions using 5 mM imidazole in 1X Equilibration/Wash Buffer (Section IV.D).
13. Elute the polyhistidine-tagged protein by adding 5 bed volumes of Elution Buffer.
14. Gently agitate suspension at room temperature for 5 min.
15. Apply vacuum, and collect the purified polyhistidine-tagged protein.
16. Repeat Steps 13–15 two times, collecting separate fractions.
17. Use spectrophotometric and SDS/PAGE analyses to determine which fraction(s) contain(s) the majority of the polyhistidine-tagged protein.

    Note: Samples containing 6 M guanidinium must be dialyzed overnight against buffer containing 8 M urea before loading on a gel.

VIII. Protein Purification Protocols *continued*

D. Medium-Pressure Column Purification

1. Assemble column according to the manufacturer's instructions.
2. Thoroughly resuspend TALON Superflow Resin. Slowly pour the slurry into the column, and avoid introducing air bubbles.
3. Allow resin to settle. Accelerate this process by allowing the buffer to flow through the column with a peristaltic pump attached to the output of the column. Do not exceed a flow rate of 5 ml/min/cm^2. Do not allow the resin to dry out. If this occurs, resuspend the resin and repack the column.
4. Insert and adjust the top adaptor and connect the column to the chromatography system according to manufacturer's instructions.

   Note: Avoid trapping air between the adaptor and the resin surface.
5. Equilibrate the column with 1X Equilibration/Wash Buffer. Do not exceed a 5 ml/min/cm^2 flow rate. Monitor the eluant at 280 nm; the baseline should be stable after washing with 5–10 column volumes.
6. Apply the clarified sample to the column after filtering it through a 0.22-µm filter and wash with Equilibration/Wash Buffer until the baseline (280 nm) is stable. Monitor column backpressure during sample application. Start collecting fractions.

   Note: If the sample is very viscous, the column pressure may exceed the recommended value (150 psi, 1.0 MPa). Reduce the flow rate or dilute the sample to bring the pressure into an acceptable range.

   Load the sample at a flow rate of 0.5–1.0 ml/min/cm^2 to ensure that the polyhistidine-tagged protein will bind to the resin. If the protein does not bind, reduce the flow rate further. If desired, increase the flow rate for washing and protein elution.

   If the target protein is unstable at room temperature, perform the chromatography at 4°C. Alternatively, use flow rates up to 5 ml/min/cm^2 to load, wash, and elute the protein. Capacity will decrease by 10–15%, but on average, a chromatography run should only take 15–20 min.
7. Wash column with 10–20 column-volumes of Equilibration/Wash Buffer, or until the baseline at 280 nm is stable. If necessary, add 5–10 mM imidazole to the Equilibration/Wash Buffer.
8. Elute the polyhistidine-tagged protein with 5–10 column-volumes of Elution Buffer. The polyhistidine-tagged protein usually elutes in the second and third column volumes.
9. Use spectrophotometric and SDS/PAGE analyses to determine which fraction(s) contain(s) the majority of the polyhistidine-tagged protein.

   Note: Samples containing 6 M guanidinium must be dialyzed overnight against buffer containing 8 M urea before loading on a gel.
10. If you plan to store, regenerate, and reuse a resin-packed column, see Section IX.C.

VIII. Protein Purification Protocols *continued*

E. 5 ml TALON® Single Step Column Purification

These protocols are designed for use with TALON Single Step Columns for gravity flow or centrifuge purification.

- For purifications of less than 10 samples, we recommend using the gravity flow procedure. For high-throughput purification of more than 10 samples, the centrifuge procedure should be faster.
- The buffers used in this procedure can be easily prepared by dilution from the stock buffers from our TALON Buffer Kit (Cat. No. 635514). See Section IV for preparation details.

1. Sample preparation and lysis
   a. TALON Single Step Columns can be used for purification of any histidine-tagged protein from an *E. coli* culture. For example, if screening transformants for expression levels, pick a single colony from the plate and inoculate 4.5 ml medium. Incubate the culture at 37°C until the OD_{600} reaches ~0.6–0.8 AU (mid-log phase). Then induce protein expression with the recommended concentration of inducer agent (depending on your expression strain and the expression plasmid being used). Continue to grow the culture with rigorous shaking at 37°C for another 4 hr or overnight. Alternatively, follow your standard induction or expression protocol. [Optional: remove 200 µl of the expression culture for SDS/PAGE analysis.]
   b. Place the bottom closure firmly on a TALON Single Step Column, remove the top cap, add 4.5 ml culture and then replace the top cap. Mix the suspension either on a carousel shaker for 20–30 min at room temperature or by inverting the tube every 2 min for a total of 30 min.
   c. Remove the top cap and the bottom closure and place the TALON Single Step Column into a Receiving Tube. Proceed with either the gravity flow or the centrifuge procedure.
2. Gravity Flow Procedure
   a. Let the extract drain by gravity flow. Remove the column from the receiving tube and replace the bottom closure. **[Optional:** remove 200 µl of non-adsorbed material from the Receiving Tube for SDS/PAGE and Protein Assay analysis (Step 4).]
   b. Add 4.5 ml 1X Equilibration/Wash Buffer, replace the top cap and resuspend the resin by inverting the column. Remove the bottom closure and put the column into a Receiving Tube. Allow the wash to drain by gravity flow. **[Optional:** remove 200 µl of Wash-1 from the Receiving Tube for SDS/PAGE and Protein Assay analysis.]
   c. **[Optional]** For improved purity of target protein, repeat Step b twice.

VIII. Protein Purification Protocols *continued*

d. Replace the bottom closure, then add 4.5 ml Wash-2 Buffer. Replace the top cap and resuspend the resin by inverting the column. Remove the bottom closure and put column into a Receiving Tube. Let the buffer drain by gravity flow. **[Optional:** remove 200 µl of Wash-2 from the Receiving Tube for SDS/PAGE and Protein Assay analysis.]

e. **[Optional]** For improved purity of the target protein, repeat the wash in Step d twice.

f. Add 1.0 ml Elution Buffer and resuspend the resin by inverting the columns for 2 min.

g. Remove the bottom closure and put the column into a Receiving Tube. Allow the elution fraction to drain by gravity flow. Proceed with Step 4. Protein Analysis.

3. Centrifuge Procedure

a. Centrifuge at 700 x g for 2 min. Take the column from the Receiving Tube and replace the bottom closure. **[Optional:** remove 200 µl of non-adsorbed material collected in the Receiving Tube for SDS/PAGE and Protein Assay analysis.]

b. Add 4.5 ml 1X Equilibration/Wash Buffer, replace the top cap and resuspend the resin by inverting the column. Remove the bottom closure and put the column into a Receiving Tube. Centrifuge at 700 x g for 2 min. Remove the column from the Receiving Tube and replace the bottom closure. [Optional: remove 200 µl of Wash-1 for SDS/PAGE and Protein Assay analysis.]

c. **[Optional]** For improved purity of target protein, repeat Step b twice.

d. Add 4.5 ml Wash-2 Buffer, replace the top cap and resuspend the resin by inverting the column. Remove the bottom closure and put the Single Step Column into a Receiving Tube. Centrifuge at 700 x g for 2 min. Remove the column from the Receiving Tube and replace the bottom closure. **[Optional:** remove 200 µl of Wash-2 from the Receiving Tube for SDS/PAGE and Protein Assay analysis.]

e. **[Optional]** For improved purity of the target protein, repeat the wash in Step d twice.

f. Add 1 ml Elution Buffer and resuspend the resin by inverting the column for 2 min.

g. Remove the bottom closure and put the TALON® Single Step Column into a Receiving Tube. Centrifuge the column in the tube at 700 x g for 2 min. Proceed with Step 4. Protein Analysis.

VIII. Protein Purification Protocols *continued*

4. Protein Analysis

   Determine amount of protein in a 1:10 dilution of the non-adsorbed fractions and the amount of protein in the (undiluted) elution fraction by performing a Bio-Rad Protein Assay. Analyze the samples by SDS/PAGE to determine the purity of the target protein (Elution fraction).

   Note: A BCA Protein Assay (see Section III) can be performed on an undiluted sample of the non-adsorbed fraction, if desired.

F. 20 ml TALON® Single Step Column Purification

These protocols are designed for use with the 20 ml TALON Single Step Columns for gravity flow or centrifuge purification.

- For purifications of less than 10 samples, we recommend using the gravity flow procedure. For high-throughput purification of more than 10 samples, the centrifuge procedure should be faster.
- The buffers used in this procedure can be easily prepared by dilution from the stock buffers from our TALON Buffer Kit (Cat. No. 635514). See Section IV for preparation details.

1. Sample preparation and lysis

   a. TALON Single Step Columns can be used for purification of any histidine-tagged protein from an *E. coli* culture. For example, if screening transformants for expression levels, pick a single colony from the plate and inoculate 25 ml medium. Incubate the culture at 37°C until the OD_{600} reaches ~0.6–0.8 AU (mid-log phase). Then induce protein expression with the recommended concentration of inducer agent (depending on your expression strain and the expression plasmid being used). Continue to grow the culture with rigorous shaking at 37°C for another 4–6 hr or overnight. Alternatively, follow your standard induction or expression protocol. **[Optional: r**emove 200 µl of the expression culture for SDS/PAGE analysis.]

   b. Ensure that the end cap is firmly on the TALON Single Step Column, remove the top cap, add 20 ml culture and then replace the top cap. Mix the suspension either on a carousel shaker for 20–30 min at room temperature or by inverting the tube every 2 min for a total of 30 min.

   c. Remove the top cap and the end cap and place the TALON Single Step Column back into the Receiving Tube. Proceed with either the gravity flow or the centrifuge procedure.

2. Gravity Flow Procedure

   a. Let the extract drain by gravity flow. Remove the column from the receiving tube and replace the end cap. [Optional: remove 200 µl of non-adsorbed material from the Receiving Tube for

VIII. Protein Purification Protocols *continued*

SDS/PAGE and Protein Assay analysis (Step 4).]

b. Add 20 ml 1X Equilibration/Wash Buffer, put the column into a fresh Receiving Tube, replace the top cap and resuspend the resin by inverting the column. Remove the end cap and put the column back into the Receiving Tube. Allow the wash to drain by gravity flow. **[Optional:** remove 200 µl of Wash-1 from the Receiving Tube for SDS/PAGE and Protein Assay analysis.]

c. **[Optional]** For improved purity of target protein, repeat Step b twice.

d. Replace the end cap, put the column into a fresh receiving tube, then add 20 ml Wash-2 Buffer. Replace the top cap and resuspend the resin by inverting the column. Remove the end cap and replace the column into the Receiving Tube. Let the buffer drain by gravity flow. **[Optional:** remove 200 µl of Wash-2 from the Receiving Tube for SDS/PAGE and Protein Assay analysis.]

e. **[Optional]** For improved purity of the target protein, repeat the wash in Step d twice.

f. Replace the end cap, add 2.0 ml Elution Buffer, place the 20 ml column into a fresh receiving tube, and resuspend the resin by inverting the column for 2 min. For an additional 10–15% of purified protein, repeat elution with an additional 2.0 ml Elution Buffer.

g. Remove the end cap and replace the column into the Receiving Tube. Allow the elution fraction to drain by gravity flow. Proceed with Step 4. Protein Analysis.

3. Centrifuge Procedure

a. Centrifuge the column at 700 x g for 2 min. Take the column from the Receiving Tube and replace the end cap. **[Optional:** remove 200 µl of non-adsorbed material collected in the Receiving Tube for SDS/PAGE and Protein Assay analysis.]

b. Place the column in a fresh receiving tube, then add 20 ml of 1X Equilibration/Wash Buffer, replace the top cap and resuspend the resin by inverting the column. Remove the end cap and replace the column into the Receiving Tube. Centrifuge at 700 x g for 2 min. **[Optional:** remove 200 µl of Wash-1 for SDS/PAGE and Protein Assay analysis.]

c. **[Optional]** For improved purity of target protein, repeat Step b twice.

d. Remove the column from the Receiving Tube and replace the end cap. Place the column in a fresh receiving tube, add 20 ml Wash-2 Buffer, replace the top cap and resuspend the resin by inverting the column. Remove the end cap and replace the Single Step Column into the Receiving Tube. Centrifuge at 700 x g for 2 min. **[Optional:** remove 200 µl of Wash-2 from the Receiving Tube for SDS/PAGE and Protein Assay analysis.]

VIII. Protein Purification Protocols *continued*

e. **[Optional]** For improved purity of the target protein, repeat the wash in Step d twice.

f. Remove the column from the Receiving Tube and replace the end cap. Add 2 ml Elution Buffer, place the column in a fresh Receiving tube, close the top cap and resuspend the resin by inverting the column for 2 min. For an additional 10–15% of purified protein, repeat elution with an additional 2.0 ml Elution Buffer.

g. Remove the end cap and replace the TALON Single Step Column into the Receiving Tube. Centrifuge the column in the tube at 700 x g for 2 min. Proceed with Step 4. Protein Analysis.

4. Protein Analysis

Determine amount of protein in a 1:10 dilution of the non-adsorbed fractions and the amount of protein in the (undiluted) elution fraction by performing a Bio-Rad Protein Assay. Analyze the samples by SDS/PAGE to determine the purity of the target protein (Elution fraction).

Note: A BCA Protein Assay (see Section III) can be performed on an undiluted sample of the non-adsorbed fraction, if desired.

G. TALONspin™ Column Purification

Important Points

- Before proceeding with purification, determine the concentration of polyhistidine-tagged protein in your sample using the mini-batch screening protocol (Appendix B). Alternatively, run a sample of the clarified lysate directly on SDS/PAGE, and estimate the amount of polyhistidine-tagged protein by band intensity.
- Avoid excessively concentrated or viscous lysates. See Troubleshooting (Section IX.B.2) for tips on reducing sample viscosity.
- If the concentration of polyhistidine-tagged protein in the lysate is very dilute, use one column to enrich the protein from several 0.6–1 ml lysate aliquots. Simply repeat Steps 11–16 (below) until the desired amount of lysate has been processed. Alternatively, concentrate the polyhistidine-tagged protein by reducing the sample volume.
- The centrifugation rotor and speed may affect your results. Ideally, you should centrifuge TALONspin Columns in a swinging bucket rotor to allow the sample to pass through the resin uniformly. However, a fixed angle rotor or a microcentrifuge is also acceptable. Centrifugation speeds higher than 700 x g may cause irregularities in the flow of solution through the resin bed, and thus, decrease the performance of the column.

1. Hold the TALONspin Column upright and flick it until all resin falls to the bottom of the column. Then, snap off the breakaway seal.

   Note: Save white end-cap for later use.

2. Place column in the 2 ml microcentrifuge tube.

VIII. Protein Purification Protocols *continued*

3. Remove the clear top-cap and centrifuge column at 700 x g for 2 min to remove the storage buffer from the resin bed.
   Note: The resin bed will appear semi-dry after centrifugation.
4. Remove column from centrifuge, and place the white end-cap over the male luer fitting.
5. Add 1 ml 1X Equilibration/Wash Buffer and mix briefly to pre-equilibrate the resin.
6. Recentrifuge at 700 x g for 2 min to pellet the resin. Discard the supernatant.
7. Repeat Steps 7 and 8, twice.
8. Add the clarified sample from Section VII.A, B or C to the resin.
9. Add 0.6–1 ml of sample to the column, and replace the clear top-cap.
10. Allow sample to passively wet the resin bed for 30 sec.
11. Mix or vortex contents briskly for 1–2 sec, completely resuspending the resin in the lysate.
14. Gently agitate the suspension for 5 min to allow polyhistidine-tagged protein binding. **Do not vortex.**
15. Remove both caps from column and place column inside the 2 ml microcentrifuge tube.
16. Centrifuge at 700 x g for 2 min.
17. Remove the column and microcentrifuge tube from the centrifuge rotor, making sure that all of the sample has passed through the resin bed.
    Note: Viscous samples may require additional centrifugation.
18. Save the 2 ml tube, but discard the flowthrough.
19. Place microcentrifuge tube in rotor.
20. Place white end-cap on the column, and add 1 ml of 1X Equilibration/Wash Buffer. Close the column with the clear top-cap.
21. Allow the buffer to passively wet the resin bed for 30 sec.
22. Agitate or vortex briskly for a few sec until the resin is completely resuspended.
23. Gently agitate for 5 min.
24. Remove both caps, and centrifuge at 700 x g for 2 min.
25. Repeat Steps 18–24. Repeat twice for particularly concentrated lysates, or if necessary, to improve purity.
26. Examine the resin bed to ensure that it appears semi-dry, and to ensure that all wash buffer has drained from the resin bed and the column end.
27. Discard the used 2 ml microcentrifuge tube.
28. If necessary, repeat the spin to remove all traces of wash buffer.

VIII. Protein Purification Protocols *continued*

29. Replace the white end-cap on the spin column.
30. Add 400–600 µl of Elution Buffer.

    Note: Alternatively, use 100 mM EDTA (pH 8.0) if it does not interfere with downstream applications of the protein. Samples eluted with EDTA will also contain cobalt.
31. Allow 1 min for Elution Buffer to passively wet the resin bed.
32. Briefly agitate or vortex to resuspend the resin.
33. Place a fresh 2 ml collection tube into centrifuge rotor.
34. Remove both caps and place column into the 2 ml collection tube.
35. Centrifuge sample at 700 x g for 2 min.
36. Repeat Steps 30–35.

    Note: The polyhistidine-tagged protein sample can generally be recovered in 800–1,200 µl of Elution Buffer, but it may be necessary to use a larger Elution Buffer volume or repeat Steps 30–35.
37. Determine polyhistidine-tagged protein yield using gel or spectrophotometric analysis.

    Note: If the purity of the polyhistidine-tagged protein preparation is unsatisfactory, refer to the procedure in the Troubleshooting Guide Section X.C.2.

H. TALON® HT 96-Well Purification Protocol

Each well of the TALON HT 96-Well Plate has a capacity of up to 1.0 mg of polyhistidine tagged protein. In order to obtain the maximum yield of pure protein, do not attempt to load more than 1.0 mg of polyhistidine-tagged protein/well. Also, observe the following guidelines:

- When using pipette tips to mix the resin, use wide-bore tips, or cut the tips to make the opening wider. This will reduce mechanical damage to proteins as well as resin.
- TALON Resin is designed to permit buffer to flow through freely. Therefore, when the HT 96-Well Plate is not on the vacuum manifold or over a Collection Plate, we recommend that it is kept on the Base Seal that acts as a temporary stopper.
- The amount of sample applied to a well should not exceed the capacity of 1.0 mg/well.
- Avoid overdrying the resin under the vacuum. For the best results, keep the resin wet.
- When using vacuum manifold, adjust the vacuum to obtain a flow rate of 1–2 drops per sec (~100–200 mm Hg or 2–4 psi).

1. Unpacking and removal of seals

   HT 96-Well plates come with solid plate seals to prevent resin from leaking during transportation. Before removing the upper seal, we recommend performing a 2 min centrifugation step at 500 x g to pack resin that might have adhered to the silicon lid during transportation.

VIII. Protein Purification Protocols *continued*

After this procedure, the upper seal can be removed and the steps outlined in the purification protocols can be performed.

If you do not desire to use all 96 wells, the plate seals can be cut so that only the wells that are needed are exposed. After chromatography, the removed portion of the plate seals can be replaced and the plate can be stored at 4°C until the remaining wells are used. When stored, the resin in unused wells should be covered in 20% ethanol.

2. HT 96-Well Plate Equilibration
   a. Remove the top and bottom seals from the plate.
   b. Place the plate on the manifold and apply vacuum to remove storage solution or centrifuge 5 min at 700 x g.
   c. Add 1 ml of deionized water to each well of the plate. Apply vacuum or centrifuge to drain the water from the wells. Repeat twice.
   d. Add 1 ml of 1X Equilibration/Wash Buffer to each well of the plate and apply vacuum or centrifuge to drain the buffer from the well. Repeat twice.
3. Vacuum Purification

   When performing vacuum purification, adjust the vacuum to obtain a flow rate of 1–2 drops per sec (~100–200 mm Hg or 2–4 psi). In addition, avoid overdrying the resin which introduces air bubbles and reduces performance.

   a. Apply 1.5 ml of the starting sample (See Section VII.E) per well. Mix the sample with the resin shortly by vortexing the plate or pipetting up and down inside the wells. Leave the plate on ice for 5–10 min mixing samples every 2 min.
   b. Place the plate on the vacuum manifold, apply vacuum and let the excess liquid drain into the manifold. Firmly press all four sides of the plate to the rubber gasket of the vacuum manifold. Ensure by observation that all wells have been drained of buffer.
   c. Repeat Steps 3.a and 3.b if additional loading is necessary.
   d. Add 1 ml of 1X Equilibration/Wash Buffer and suspend the resin by vortexing the plate or pipetting up and down inside the wells.
   e. Place the plate on the vacuum manifold, apply vacuum and let the excess liquid drain into the manifold. Firmly press all four sides of the plate to the rubber gasket of the vacuum manifold. Ensure by observation that all wells have been drained of buffer.
   f. Repeat Steps 3.d and 3.e twice.
   g. Add 1 ml of HT 96-Well Plate Wash Buffer (See Section III) to each well and suspend the resin by vortexing the plate or pipetting up and down inside the wells.

VIII. Protein Purification Protocols *continued*

h. Place the plate on the vacuum manifold, apply vacuum and let the excess liquid drain into the manifold. Firmly press all four sides of the plate to the rubber gasket of the vacuum manifold. Ensure by observation that all wells have been drained of buffer.

i. Repeat Steps 3.g and 3.h five times.

j. Remove the HT 96-Well Plate from the vacuum manifold. Drain the collected filtrate from the vacuum manifold.

k. Place a Collection Plate inside the vacuum manifold and place the HT 96-Well Plate on the vacuum manifold.

Note: Before eluting, place the plate over a Collection Plate or on the base seal.

l. Add 200 µl of 1X Elution buffer (Section III.A) and suspend the resin by vortexing the plate or pipetting up and down inside the wells.

m. Place the HT 96-Well Plate on the vacuum manifold, apply vacuum, and let the eluate drain into the Collection Plate.

n. Repeat elution (Steps 3.m and 3.n) twice.

o. Determine amount of loaded and adsorbed protein in each well by Bradford Assay (Bradford, 1976).

4. Centrifuge Purification

As a variety of rotors and centrifuges can be used, the following instructions are only general guidelines for successful purification:

- Do not utilize centrifugal force higher than 700 x g.
- Ensure proper balance of the HT 96-Well Plate/Collection Plate inside the rotor.
- When performing the centrifuge procedure below, extra Collection Plates are recommended. See Additional Materials Required for information on obtaining compatible plates.

a. Add 1.5 ml of the starting sample per well (See Section VII.E). Mix the sample with the resin briefly by vortexing the plate or pipetting up and down inside the wells. Leave the plate on ice for 5–10 min mixing samples every 2 min.

b. Centrifuge the plate for 5 min. Ensure by observation that all wells have been drained of buffer.

c. Repeat Steps 4.a and 4.b if additional loading is necessary.

d. Add 1 ml of 1X Equilibration/Wash Buffer and suspend the resin by vortexing the plate or pipetting up and down inside the wells.

e. Centrifuge the plate for 5 min. Ensure by observation that all wells have been drained of buffer.

f. Repeat Steps 4.d and 4.e twice.

g. Add 1 ml of HT 96-Well Plate Wash buffer and suspend the resin by vortexing the plate or pipetting up and down inside the wells.

VIII. Protein Purification Protocols *continued*

h. Centrifuge the HT 96-Well Plate for 5 min. Ensure by observation that all wells have been drained of buffer.

i. Repeat Steps 4.g and 4.h five times.

j. Drain the collected filtrate from the Collection Plate or use a fresh Collection Plate (See Section III.E).

k. Place the HT 96-Well Plate on the Collection Plate in the rotor and centrifuge 5 min. Ensure by observation that all wells have been drained of buffer.
Note: Before eluting, ensure that the plate is over a Collection Plate or on the base seal.

l. Add 200 µl of 1X Elution buffer (Section III.A) and suspend the resin by vortexing the plate or pipetting up and down inside the wells.

m. Centrifuge the HT 96-Well Plate on the Collection Plate for 5 min. Ensure by observation that all wells have been drained of buffer.

n. Repeat elution (Steps 4.l and 4.m) twice.

o. Determine amount of loaded and adsorbed protein in each well by Bradford Assay (Bradford, 1976).

I. TALON® Magnetic Beads Purification Protocol

This protocol provides instructions for carrying out the TALON Magnetic Beads purification in a single tube. The buffers used in this procedure are easily prepared by dilution from the stock buffers in our TALON Magnetic Beads Buffer Kit (Cat. No. 635638). See Section V for general buffer preparation guidelines.

1. Buffer preparation

   a. Prepare 5 ml of 1X Equilibration/Wash Buffer

      - If the 5X stock buffer is precipitated, place bottle at 37°C for 5 min, then shake until the precipitate dissolves.
      - Dilute 1ml of 5X stock with 4 ml of H_2O, confirm that final pH is 7.0 and correct pH if necessary.

   b. Prepare 0.5 ml of Elution Buffer

      Add 0.125 ml of 4X Elution Buffer to 0.375 ml of 1X Equilibration/Wash buffer and confirm that the final pH is 7.0. Any unused diluted buffer can be stored and used later.

2. General considerations for working with magnetic beads

   a. Use a pipette to mix buffer thoroughly with the beads.

   b. If needed, magnetic beads can be mixed using a vortexer.

   c. If there is a great deal of liquid/buffer adhering to the sides of the tube, centrifuge the tubes in a microfuge before placing them on a magnetic separator.

   d. Ensure that the beads are adhering to the sides of the magnet before removing the supernatant.

VIII. Protein Purification Protocols *continued*

3. Protein purification under native or denaturing conditions
   a. Aliquot 100–200 µl of beads into a 1.5 ml microfuge tube.
   b. Place the tube on a magnetic separator for 1 min and remove storage buffer.
   c. Add 0.5 ml of deionized water to the beads.
   d. Mix the liquid and the beads thoroughly using a pipette.
   e. Place the tube on a magnetic separator and remove the supernatant.
   f. To equilibrate the beads, add 0.5 ml of 1X Equilibration/Wash Buffer.
   g. Repeat steps d and e.
   h. Add the cell lysate (from Sections VII.F or VII.G) to the beads.

      Note: If the cell lysate volume is less than 200 µl, add sufficient 1X Equilibration/Wash Buffer to bring the volume up to at least 200 µl. This is necessary to ensure thorough mixing of beads with the cell lysate, for optimal binding.
   i. Mix on a rotary shaker for 30 min at room temperature.

      Note: If the protein is vulnerable to degradation at room temperature, incubate at 4°C for 1 hr. Protease inhibitors that do not contain EDTA can also be added during the incubation.
   j. Place on a magnetic separator and collect the supernatant.
   k. Add 0.5 ml of 1X Equilibration/Wash Buffer.
   l. Mix thoroughly and let it stand for 1 min before placing on a magnetic separator and collecting the first wash.
   m. Repeat steps k and l twice to collect the second and third washes, respectively.
   n. **[Optional]:** If necessary, repeat steps k and l under more stringent conditions using 0.5 ml of 5–10 mM imidazole in 1X Equilibration/Wash Buffer (section V.D)
   o. To elute the protein, add 50 µl of Elution Buffer. The volume of Elution Buffer can be varied depending on the amount of beads used. 50 µl of elution buffer can be used for eluting from 200 µl of bead suspension. Most of the protein will elute in this fraction. Smaller volumes, such as 25 µl can be used if a concentrated sample is needed. Volumes below 25 µl may be difficult to handle.
   p. Mix for 5 min and collect Eluate 1.
   q. Add another 50 µl of Elution Buffer
   r. Mix for 1 min and collect Eluate 2.

VIII. Protein Purification Protocols *continued*

s. If necessary, steps q and r can be repeated twice to ensure that protein recovery is maximized. In a specific instance, when using 200 µl of bead suspension, 60% of the total protein was eluted in the first 50 µl fraction, 20% in the second, 10% in the third & 5% in the fourth.

t. Use spectrophotometric and SDS-PAGE analyses to determine which fractions contain the bulk of the polyhistidine-tagged protein.

Note: A Bradford protein assay is recommended for measuring protein yields. Since the detergents in the xTractor Buffer may interfere with the Bradford assay, it is advisable to run the original lysate and non-adsorbed fraction at a 1:5 dilution or use a BCA assay for undiluted samples.

IX. Resin Washing, Reuse, and Storage

Generally, reuse TALON® Resins 3–4 times before discarding or complete regeneration. The exact number of uses varies among preparations and application because of differences in redox potential, organic complexity, and debris content. To avoid possible cross-contamination, use a particular aliquot of resin to purify a single type of polyhistidine-tagged protein.

Important precautions

- TALONspin™ Columns are not reusable.
- Do not store TALON Resin in denaturants such as 6 M guanidinium.
- Do not store TALON Resin with bound imidazole: the resin should be washed with MES Buffer (pH 5.0) described in Section III, which is required before reuse to remove the bound imidazole.

A. Stringent Wash (optional)

1. Wash resin with four bed volumes of 6 M guanidinium (pH 5.0) + 1% nonionic detergent.
2. Rinse resin with five bed volumes of distilled H_2O.
3. Store resin at 4°C in 20% nonbuffered ethanol containing 0.1% azide.

B. Removing Imidazole

1. Wash resin with five bed volumes of 20 mM MES Buffer (pH 5.0) containing 0.1 M NaCl.
2. Rinse resin with five bed volumes of distilled H_2O.
3. Store resin at 4°C in 20% nonbuffered ethanol containing 0.1% azide.

C. Regeneration of Superflow Columns

Purification of polyhistidine-tagged proteins using imidazole gradients will cause the column to take on a purplish hue. Washing the column with 5–10 column volumes of 20 mM MES Buffer (pH 5.0) will restore the normal pink color and bring the absorbance at 280 nm back to the original baseline level. After equilibrating the column with Equilibration/Wash Buffer, the column is ready for reuse.

D. Complete Regeneration

1. Strip the resin of cobalt ions by washing with 10 bed volumes of 0.2 M EDTA (pH 7.0).
2. Wash excess EDTA from the resin with an additional 10 bed volumes of double distilled H_2O (ddH_2O).
3. Charge the resin with 50 mM $CoCl_2$ solution (10 bed volumes).
4. Wash resin with 7 bed volumes of ddH_2O followed by 3 bed volumes of 300 mM NaCl, and 3 bed volumes of ddH_2O to remove excess cobalt metal ions.

IX. Resin Washing, Reuse, and Storage *continued*

5. Equilibrate the resin with Equilibration/Wash buffer (10 bed volumes).
6. If you plant to use β-mercaptoethanol in subsequent buffers/procedures, then re-equilibrate the resin as follows before proceeding with futher purifications:
   a. Wash the resin with at least two bed volumes of Equilibration/Wash Buffer.
   b. Re-equilibrate the resin with Equilibration/Wash Buffer containing β-mercaptoethanol.

E. TALON Magnetic Beads

TALON Magnetic Beads are for single use only. They cannot be regenerated.

X. Troubleshooting Guide

A. Protein Expression

1. No expression

- **Bad vector construct**
  Check sequence of the vector.
- **Bad transformation**
  Make a plasmid miniprep and confirm sequence.
- **No inducing agent added to culture to induce expression**

2. Apparent low expression

- **Insoluble overexpressed protein**
  Use denaturing extraction and purification conditions or reduce expression levels by lowering the amount of inducer.
- **Unsuitable expression conditions**
  Check cell growth and inducer concentration; check for wild-type (nontransformed) or antibiotic resistant cells.
- **Protein is secreted**
  Use fermentation liquid as starting sample for IMAC after proper buffering.

B. Loading/Washing

1. Polyhistidine-tagged protein elutes in the wash buffer

- **Problems with vector construction**
  Ensure that protein and tag are in frame.
- **Buffer is not optimal**
  Check the pH and composition of all buffers. Use a lower stringency wash buffer for all washing steps. For example, slightly increase the pH of the wash buffer or lower its imidazole concentration.
- **Protein degraded during extraction**
  a. Use mild extraction conditions in the presence of protease inhibitors (e.g., β-ME and EDTA) at 4°C. **Be sure to remove EDTA before applying to TALON® Resin.**
  b. Make C-terminal construct.
  c. Work quickly at 4°C to reduce the time for initial purification steps.
- **Reagent interferes with binding**
  See Appendix A for reagent compatibilities. Dilute an aliquot of lysate (1:10), or sonicate, and check binding on a small scale. Try using a different polyhistidine-tagged protein as a control.
- **Tag is not accessible under native conditions**
  If the protein fails to bind under native conditions, treat a small

X. Troubleshooting Guide *continued*

aliquot (<1 ml) with 6 M guanidinium and bind to 50 µl of resin. Then follow the mini-scale procedure in Appendix B. If the target protein binds to the resin under the denaturing conditions, then try to move the tag to the other terminus of the protein where it may be more exposed.

2. High back pressure during load of sample
   - **High viscosity due to presence of DNA**
     Use DNase I or dilute sample fivefold (Section VIII.A.8).

C. Elution

1. High amount of co-eluted impurities
   - **Insufficient wash**
     Use larger volumes of Equilibration/Wash Buffer.
   - **Buffer compositions are not optimal**
     a. Check buffers used for sample preparation and wash steps.
     b. Check pH. The Equilibration/Wash Buffer should be pH 7.0. Contaminants will co-elute in buffers <pH 7.0.
     c. Increase volume of wash buffer and continue to wash resin bed until the A_{280} drops to zero.
     d. Increase counterion concentration up to 0.5 M NaCl or KCl to inhibit nonspecific ionic interactions.
     e. Add ethylene glycol or glycerol to inhibit nonspecific hydrophobic interactions.
     f. Add small amounts of nonionic detergent(s); this is particularly important when isolating proteins from a eukaryotic expression system.
     g. Add 5–10 mM imidazole to the Equilibration/Wash Buffer and use it as an intermediate wash step before elution.
   - **Proteolytic product**
     Use mild extraction conditions in presence of protease inhibitors (e.g., β-ME and EDTA) at 4°C. **Remove EDTA before applying to TALON®Resin.** Proteins can be extracted in presence of protease inhibitors specially designed for purification of histidine-tagged proteins as they do not contain EDTA.
   - **Covalent attachment (Cys-Cys) of impurities to the protein**
     Use 5–10 mM of β-ME in the Equilibration/Wash of Buffer.
   - **Co-purifying histidine rich proteins**
     a. For HAT proteins, use enterokinase to remove HAT tag and rerun IMAC with mixture. Target protein will pass through the column, while impurities and tag will be adsorbed.

X. Troubleshooting Guide *continued*

Note: Remove chelating ligands by gel filtration before loading the proteolytic mixture onto TALON Resin.

b. Use second purification principle, such as size exclusion, ion exchange, hydrophobic, or thiophilic chromatography.

- **Protein sample is too concentrated and/or viscous**
  Dilute sample 1:5 or 1:10 with additional buffer and centrifuge again before proceeding. Also, see the note on reducing sample viscosity after sonication in Section VII.A.8.

2. Excessive background after TALONspin™ Column procedure

- **Sample is too viscous**
  a. Reduce the viscosity of the sample (Section VIII.A.8).
  b. Dilute clarified sample with an equal volume of Equilibration/Wash Buffer and load as two aliquots.
  c. Increase number of 1 ml washes.
  d. Use Equilibration/Wash Buffer (pH 7.0).
  e. Add 1–5 mM imidazole to Equilibration Buffer, pH 8.0 and use it as an intermediate wash step before elution.
  f. To re-purify a sample, perform the following after Step 37 in Section VIII.G:
     1. Add 4 volumes of Equilibration/Wash Buffer to semi-purified sample.
     2. Load sample onto another TALONspin™ Column.
     3. Wash twice with 1 ml of Equilibration/Wash Buffer.
     4. Elute as before (Steps VIII.G.30–35).

3. Column ceases to flow

- **Filter is clogged with subcellular debris**
  Change column filters and centrifuge sample at 12,000 x g for 20–30 min at 4°C.
- **Proteins precipitated on the column**
  Use a mild detergent such as Decanoyl-N-methylglucamide (MEGA-10, Sigma Cat. No. D6277) in the Equilibration/Wash Buffer.
- **The lower resin bed support may be clogged with cellular debris**
  a. Remove resin from clogged column and resuspend. Then wash it in a batch format and transfer to a fresh column.
  b. Use a syringe filled with wash buffer or reverse the pump on the column to gently run the column backwards. In addition, test for tubing blockages in a similar manner. Apply gentle pressure. Do not exceed a 1 drop/sec flow rate.

X. Troubleshooting Guide *continued*

4. Polyhistidine-tagged proteins do not elute
   - **Elution Buffer is less than optimal**
     a. Elute with 150 mM imidazole or pH 4.0 buffer.
     b. For really tough elution problems, you can strip off the protein using 100 mM EDTA (pH 8.0); however, doing so will remove the cobalt from the resin and deposit it in your protein sample.
     c. Add 1–5 mM β-ME to reduce disulfide linkages. Supplement buffer with 1% nonionic detergent.
     d. Purify polyhistidine-tagged protein under denaturing conditions.

D. Changes in Resin

1. Resin changes from pink to white—Loss of Co^{2+}
   - **Presence of chelators in sample**
     Remove chelators from sample by gel filtration
     Regenerate resin as described in Section IX.D.
2. Gray or brown resin
   - **TALON® Resin exposed to reducing agents or high concentration of β-ME**
     Completely remove reducing agents, such as DTE or DTT, or if possible, by gel filtration with β-ME. Reduce β-ME concentration (≤5 mM).
3. Resin particles aggregate or exhibit change in consistency
   - **DNA cross-linking**
     a. Increase ionic strength of the buffers by using ≤ 500 mM NaCl or KCl.
     b. Vigorously sonicate sample to shear DNA.
     c. Pretreat sample with 100 µg/ml DNase I at 30°C for 30 min.
     d. Dilute sample 1:5–1:10 with buffer, and repeat.
     e. Avoid long-term storage in denaturants.

E. Analysis

1. High background on silver-stained gels
   - **Nucleic acid**
     a. Supplement buffer with 0.2–0.5 M NaCl or KCl. Repeat purification.
     b. Shear DNA more vigorously.
     c. Use DNase I in the extraction procedure.

X. Troubleshooting Guide *continued*

2. Nonfunctional protein
   - **Protein was damaged by sonication**
     a. Conduct a time-course assay to determine the minimum sonication time needed to disrupt the cells while maintaining the native protein/enzyme function. For example, sonicate samples at a medium-high setting for 0, 20, and 30 sec. Then perform protein or enzyme function assays and measure the A_{280} of each sample.
     b. Perform the lysis or sonication procedure on ice.

F. Reuse

1. Binding drops below original capacity
   - **Lysate contains naturally occurring reducing agent or a nonspecific polyanion may be obscuring the metal binding sites.**
     a. Use a larger volume of previously used resin.
     b. Replace used resin with fresh resin.
     c. Wash resin with 6 M guanidinium (pH 5.0) + 1% Triton X-100 or SDS, and re-equilibrate before use.

G. Application of samples prepared from overnight cultures to TALON Magnetic Beads.

1. No protein binds to the beads when using overnight culture. Check the pH and ensure that it is between 7–8.
2. The beads fail to migrate to the magnet, due to the high viscosity of the solution.
   a. Add sufficient DNase (1unit/ml of culture) and mix thoroughly before adding beads.
   b. Dilute the sample further with 5X Equilibration/Wash Buffer to obtain a final concentration of 1X Equilibration/Wash Buffer.

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Appendix A. Reagent Compatibilities and Incompatibilities

A. Compatible reagents

Table III shows the maximum concentrations of each reagent tested at Clontech. Higher levels may be acceptable, but they should be tested before use. Note that some of these reagents may partially or completely denature your protein.

TABLE III. REAGENT COMPATIBILITY

Reagent	Acceptable Concentration
β-Mercaptoethanol[a]	10 mM (with caution)
CHAPS[b]	1% (with caution)
Ethanol[c]	30%
Ethylene glycol	30%
HEPES	50 mM
Glycerol	20%
Guanidinium[a]	6 M
imidazole[d]	200 mM at pH 7.0–8.0, for elution
KCl	500 mM
MES	20 mM
MOPS	50 mM
NaCl	1.0 M
NP-40	1%
SDS[b]	1% with caution
TRIS[e]	50 mM
Triton-X 100	<1%
Urea	8 M

[a] Use resin immediately after equilibrating with buffers containing these reagents. Otherwise, the resin will change color. Do not store resin in buffers containing these reagents.

[b] Ionic detergents like CHAPS (3-[(30Cholamidopropyl)-dimethylammonio]-1-propane-sulfonate), SDS (sodium dodecyl sulfate), and sarkosyl are compatible up to 1%. However, due to their charged nature, you should anticipate interference with binding.

[c] Ethanol may precipitate proteins, causing low yields and column clogging.

[d] Imidazole cannot be used at concentrations higher than 5–10 mM for loading polyhistidine-tagged proteins, because it competes with the histidine side chains (imidazole groups) for binding to the immobilized metal ions.

[e] TRIS coordinates weakly with metal ions, causing a decrease in capacity.

Appendix A. Reagent Compatibilities and Incompatibilities

B. Incompatible reagents

These reagents are incompatible at any concentration:

- DTT (dithiothreitol) and DTE (dithioerythritol)

  Note: Use of strong reducing agents will interfere with the binding of the cobalt metal ions to the resin.

- EDTA (ethylenediaminetetraacetic acid) and EGTA (ethylene glycol-bis([β-amino-ethyl ether])

  Note: Although you can use EDTA at indicated points, it must be removed from the sample by gel filtration prior to applying it to TALON® Resins.

Appendix B. Mini-Scale Protein Purification Protocol

Mini-scale protein purification is ideal for any of the following:

(a) checking for a polyhistidine-tagged protein

(b) determining expression levels

(c) testing buffer conditions

You can use a TALON® Single Step (Cat. No. 635628 or 635631) for protein miniprep, or you can use a TALONspin™ Column (Cat. No. 635601) with this procedure.

We recommend that you set aside a sample after each critical step of the procedure, and analyze all samples by SDS/PAGE.

Important

- This protocol is not intended for obtaining highly purified polyhistidine-tagged protein samples. Furthermore, protein samples eluted with EDTA (Step 19, below) will contain cobalt and EDTA, which may seriously inhibit enzyme activity and may cause the protein to precipitate.
- This protocol was optimized using denaturing conditions at pH 8.0. If you wish to obtain native samples, then substitute buffers accordingly. You may also need to use lysozyme (0.75 mg/ml of native buffer) to completely disrupt the cells in Step 5.

1. Transfer 1 ml of expression culture to a 1.5 ml microcentrifuge tube.
2. Centrifuge at 14,000 rpm for 2 min.
3. Remove and discard supernatant.
4. Add 0.5 ml of Denaturing Equilibration Buffer (pH 8.0).
5. Vortex until cell pellet is completely dissolved.
6. Centrifuge at 14,000 rpm for 5 min to pellet any insoluble debris.
7. Set aside 50 µl of the supernatant for later analysis. Transfer the remainder of the supernatant to a clean 1.5 ml tube containing 50 µl of prewashed TALON® Resin, prepared as described in Section VIII.B. Steps 1–7. Start with 100 µl of resuspended slurry.
8. Agitate sample at room temperature for 10 min.
9. Centrifuge at 14,000 rpm for 1 min to pellet protein/resin complexes.
10. Carefully remove the supernatant and set aside 50 µl for later analysis. A high protein concentration in this sample indicates a problem with protein binding.
11. Add 1 ml of Denaturing Equilibration Buffer.
12. Vortex for a few sec.
13. Centrifuge at 14,000 rpm for 1 min to pellet resin.
14. Remove the supernatant and set aside 50 µl ("first wash") for later analysis. Discard the remainder of the supernatant.

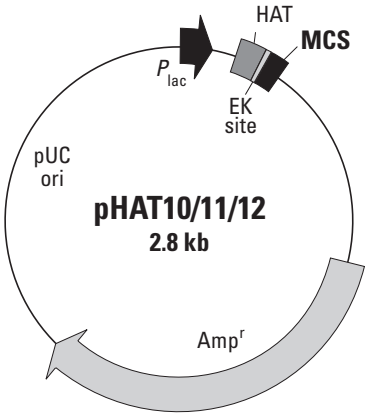
Appendix B. Mini-Scale Protein Purification...*continued*

15. Repeat Steps 11–14. Set aside 50 µl for analysis.
16. Elute bound polyhistidine-tagged protein by adding 50 µl of Elution Buffer to the resin/protein pellet and briefly vortexing.
17. Centrifuge briefly at 14,000 rpm.
18. Carefully remove the supernatant containing the polyhistidine-tagged protein.
19. Repeat the Steps 16–18. Alternatively, if you only intend to determine the concentration of polyhistidine-tagged protein in your sample, you can achieve a more complete elution, and thus, a more accurate protein quantification by eluting with EDTA as follows:
    a. Add 50 µl of 100 mM EDTA (pH 8.0) and vortex briefly.
    b. Centrifuge briefly at 14,000 rpm.
    c. Carefully remove the supernatant containing the 6 x histidine-tagged protein.

    Note: EDTA removes bound metal from the resin: the protein sample will contain cobalt, and the TALON® Resin cannot be reused.
20. Add 12 µl of 5X SDS/PAGE sample buffer to each of the saved samples.

    Note: The sample buffer will reduce multimers to monomers; thus, only a single band will be visible on an SDS/PAGE gel, even for naturally homologous multimeric proteins.
21. Heat samples at 95–98°C for 5 min.
22. Load samples and analyze on an SDS/PAGE gel.

Appendix C: Vector Information



***Hin*d III** HAT

A AGC TTG AAG GAT CAT CTC ATC CAC AAT GTC CAC AAA GAG GAG CAC GCT CAT GCC CAC AAC AAG

Ser Leu Lys Asp **His** Leu Ile **His** Asn Val **His** Lys Glu Glu **His** Ala **His** Ala **His** Asn Lys

EK cleavage site *

ATCGATGACGATGACAAAGTCGACGGATCCCCGGGTACCGAGCTCGTAATTAGCTGAATTC

***Cla* I** ***Sal* I** ***Bam*H I** ***Sma* I** ***Kpn* I** ***Sac* I** ***Eco*R I**

Figure 6. pHAT10/11/12 combined vector map and MCS. Unique restriction sites are in bold. The sequence of pHAT10 is shown. The asterisk indicates the insertion point of additional bases in pHAT11 (G) and pHAT12 (GG) that alter the reading frame of the MCS. These vectors encode a novel polyhistidine epitope tag that enables purification of expressed proteins at neutral pH. The pHAT Vectors allow protein purification under both native and denaturing conditions. The HAT epitope is a naturally occurring, 19-amino-acid sequence from the chicken lactate dehydrogenase protein. This sequence of nonadjacent histidine residues has lower overall charge than tags with consecutive histidine residues, such as the 6 x histidine tag. As a result, HAT-protein fusions exhibit solubility that more closely resembles wild-type proteins while still possessing strong affinity for immobilized metal ions. The unique binding characteristics of the HAT sequence allow both imidazole- and pH-gradient purification of proteins under native conditions at neutral pH (7.0), as well as under denaturing conditions. The HAT sequence and an enterokinase (EK) cleavage site have been incorporated into the pUC19 backbone. The EK site allows for optional removal of the HAT sequence from the purified protein by treatment with enterokinase. Restriction sites allow excision of the HAT sequence, with or without the EK site, for cloning in other vectors.

Appendix C: Vector Information *continued*

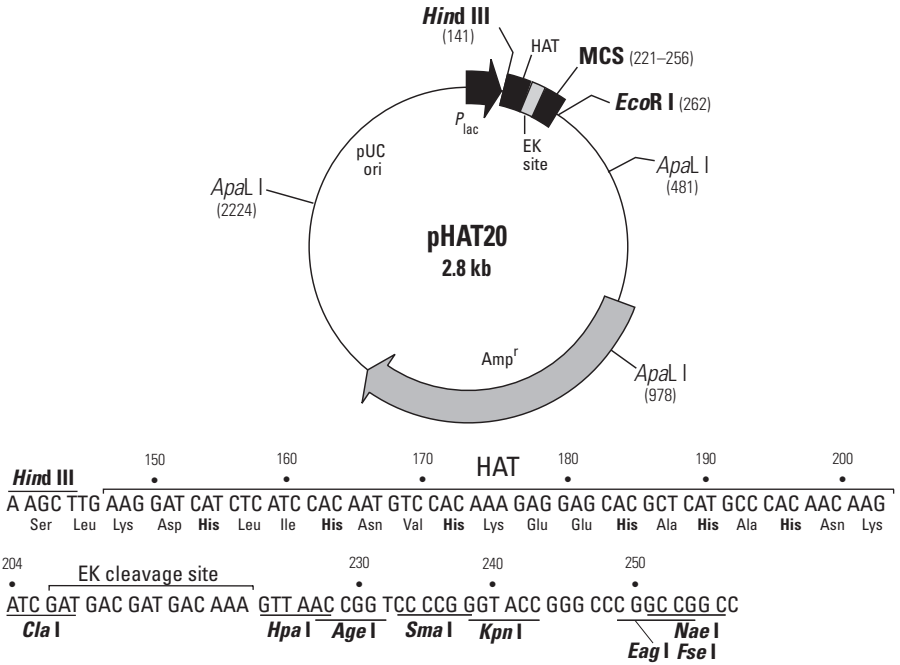


Figure 7. pHAT20 combined vector map and MCS. Unique restriction sites are in bold. The sequence of pHAT20 is shown. This vector encodes a novel Histidine Affinity Tag (HAT) that enables purification of expressed proteins at neutral pH. The pHAT Vectors allow protein purification under both native and denaturing conditions. The HAT epitope is a naturally occurring, 19-amino-acid sequence from the chicken lactate dehydrogenase protein. This sequence of nonadjacent histidine residues has lower overall charge than tags with consecutive histidine residues, such as the 6 x histidine tag. As a result, HAT-protein fusions exhibit solubility that more closely resembles wild-type proteins while still possessing strong affinity for immobilized metal ions. The unique binding characteristics of the HAT sequence allow both imidazole- and pH-gradient purification of proteins under native conditions at neutral pH (7.0), as well as under denaturing conditions. The HAT sequence and an enterokinase (EK) cleavage site have been incorporated into the pUC19 backbone. The EK site allows for optional removal of the HAT sequence from the purified protein by treatment with enterokinase. Restriction sites allow excision of the HAT sequence, with or without the EK site, for cloning in other vectors.