Clontech® Laboratories, Inc.

HAT™ Protein Expression and Purification System User Manual

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I. Introduction

In order to perform such a diverse array of functions, proteins have evolved very complex structures. As a result, their physicochemical properties vary greatly, posing difficulties for the development of purification protocols with wide applicability. One way to circumvent this problem is to incorporate a purification tag into the primary amino acid sequence of a protein of interest, thus constructing a recombinant protein with a general binding site that allows purification under general conditions. The HAT Sequence is one such purification tag. It is a histidine-rich sequence that confers to the protein an affinity for immobilized di-metal ions such as cobalt, nickel, and zinc.

Immobilized Metal Affinity Chromatography (IMAC)

Immobilized Metal Ion Affinity Chromatography (IMAC) was introduced in 1975 by Porath *et al*. (Porath *et al*., 1975) as a group-specific affinity principle for separating proteins. The principle is based on the reversible interaction between some amino acid side chains and immobilized metal ions. Depending on the type of 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 the binding of proteins to immobilized transition metal ions and zinc (Porath, 1985; Sulkowski, 1985; Hemdan & Porath, 1985a; Hemdan & Porath, 1985b; Zhao *et al*., 1991).

The chelating ligand used for immobilization of the metal ions can influence the selectivity, capacity, and strength of immobilization of the metal ions on the matrix. Chelating ligands such as iminodiacetate (IDA) and dipicolylamine (DPA) (forming three coordination bonds with the metal ion), TALON® ligand and nitrilotriacetate (NTA) (forming four coordination bonds with the metal ion), and tris(carboxymethyl)ethylenediamine (TED) (forming five coordination bonds with the metal ion), have found numerous applications for the purification of native proteins (Porath, 1990; Wong *et al*., 1991; Arnold, 1991; Andersson, 1992).

Widespread application of recombinant genetic technologies has fostered the production of recombinant proteins containing polyhistidine tags on their N- or C-termini (Hochuli *et al*., 1987; Hochuli *et al*., 1988). The HAT (histidine affinity tag) is one such tag. The HAT Protein Expression and Purification System is a complete system containing vectors designed for the bacterial expression of HAT fusion proteins, and reagents for purification of HAT fusion proteins.

The HAT sequence is a novel IMAC affinity tag derived from a unique natural protein sequence in chicken lactate dehydrogenase. It contains six histidines unevenly interleaved by other amino acid residues (see Figure 2 in Appendix A). The novel tag does not have the excessive positive charge characteristic of the commonly used 6 x histidine tag, thus HAT-fusion proteins have better solubility and similar affinity towards immobilized transition metal ions and zinc. HAT-fusion proteins can be adsorbed in the absence of imidazole at neutral pH. As a result the alkaline proteases present in cell lysates are less active, and therefore most proteins are more stable.

The core of the HAT system is the set of pHAT Vectors for protein expression in *Escherichia coli*. Three vectors—pHAT10, pHAT11, and pHAT12—contain the multiple cloning site (MCS) in all three frames to allow easy cloning of the cDNA of interest for fusion to the HAT tag. Another vector, pHAT20, provides alternative restriction sites. The presence of a conveniently located enterokinase proteolytic site between the HAT sequence and the MCS provides the means for removing the affinity tag and obtaining the wild-type protein. See Appendix A for more information.

Properties of the HAT Protein Expression and Purification System

- A positive charge is evenly distributed throughout the affinity tag. Lack of an excessive positive charge allows proteins to elute more easily from the column.
- The affinity tag is based on a unique natural sequence, resulting in a lower risk of toxicity of the recombinant proteins to the host cell.
- Loading and purification occur at physiological pH 7.0 (using imidazole).
- Purification is carried out in one chromatographic step with two buffers—load sample, then wash and elute.
- Elution is carried out at pH 6.0 (a mild change in pH from the loading conditions at pH 7.0).
- The HAT tag does not contribute to protein instability and/or aggregation

Cloning in the pHAT Vectors

The successful expression of HAT-tagged proteins in *E. coli* with the pHAT vectors is accomplished through use of the lacZ promoter. The pHAT Vectors are derived from the pUC19 vector and fully utilize its promoter-translation system.

The HAT amino acid sequence derives from the N-terminus of chicken muscle lactate dehydrogenase—a sequence that is unique among reported protein sequences. This sequence has remarkable affinity towards transition metal ions and zinc, a property that has been utilized for the successful purification of chicken lactate dehydrogenase in one chromatographic step from crude cell extract. The sequence responsible for binding was identified after cleavage of the enzyme and subsequent purification of the peptide mixture under the conditions used for purification of the native enzyme.

TALON IMAC Resins

TALON resins are agarose-based IMAC resins utilizing the high specificity of immobilized Co2+ ions for purification of polyhistidine recombinant proteins. Adsorption selectivity in IMAC increases in the following order: $Cu^{2+} < Ni^{2+} < Zn^{2+} < Co^{2+}$ (Porath, 1992). The ligand used for immobilization of the metal ion is a tetradentate chelator that retains Co^{2+} ions strongly, thus eliminating problems stemming from metal ion leakage that could be detrimental to the biological activity of the proteins being purified (in stark contrast to IDA- based adsorbents). Since immobilized Co2+ ions possess the highest possible specificity for polyhistidine-tagged proteins while adsorbing very low amounts of unwanted proteins (unlike Ni^{2+} based adsorbents), TALON resin is the best tool for purification of polyhistidine-tagged proteins.

Overview of TALON Resins & Prepacked Columns

The following is a list of different formats for various purification needs. See www.clontech.com for more information.

- **TALON Metal Affinity Resin** is useful for batch and low-pressure chromatographic applications. TALON resin utilizes Sepharose CL-6B (Pharmacia LKB Biotechnology), 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.
- **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 cm2 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.
  TALON Superflow resin utilizes Superflow-6 beads (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 crosslinking reaction that allows flow rates up to 10 times higher than are possible with regular crosslinked beads.
- **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 CellThru resin (300–500 µm) permits cellular debris to flow through the column, eliminating the need for high-speed centrifugation. With CellThru resin, destabilizing factors are removed more quickly than with other resins, because the number of steps is reduced. CellThru 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. These columns are suitable for 5–10 ml bed-volumes.
- **TALON Magnetic Beads** provide the selective chemistry of TALON immobilized metal affinity chromatography (IMAC) resin in a magnetic bead format. The beads are designed for the microscale purification of high and low molecular weight recombinant polyhistidine-tagged proteins under native or denaturing conditions, when placed on a magnetic separator.
- **TALON Spin 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 Single Step Columns** are designed for quick and simple extraction and purification of polyhistidine-tagged protein from bacterial cultures. The columns contain dried TALON xTractor Buffer to lyse the bacterial cells, and TALON CellThru Resin to bind the histidine-tagged protein released from the cells. The columns are suitable for centrifuge as well as gravity flow purification procedures.
- **HisTALON Cartridges** are prepacked with TALON Superflow Resin. Use these cartridges for efficient purification of his-tagged proteins from a total soluble protein extract of bacterial, mammalian, or baculovirus-infected cells.
- **HisTALON Gravity Columns** are prepacked gravity columns containing TALON resin, for the purification of recombinant his-tagged proteins. These columns are suitable for gravity-flow purification under native or denaturing conditions.

All resins have a capacity of at least 12 µmol Co^{2+} per ml of bed-volume and are provided charged with the metal ion for ease of use. TALON adsorbents can also be regenerated (see Section VII.E).

When used together with the HAT tag, TALON adsorbents deliver the best possible performance under mild physiological conditions.

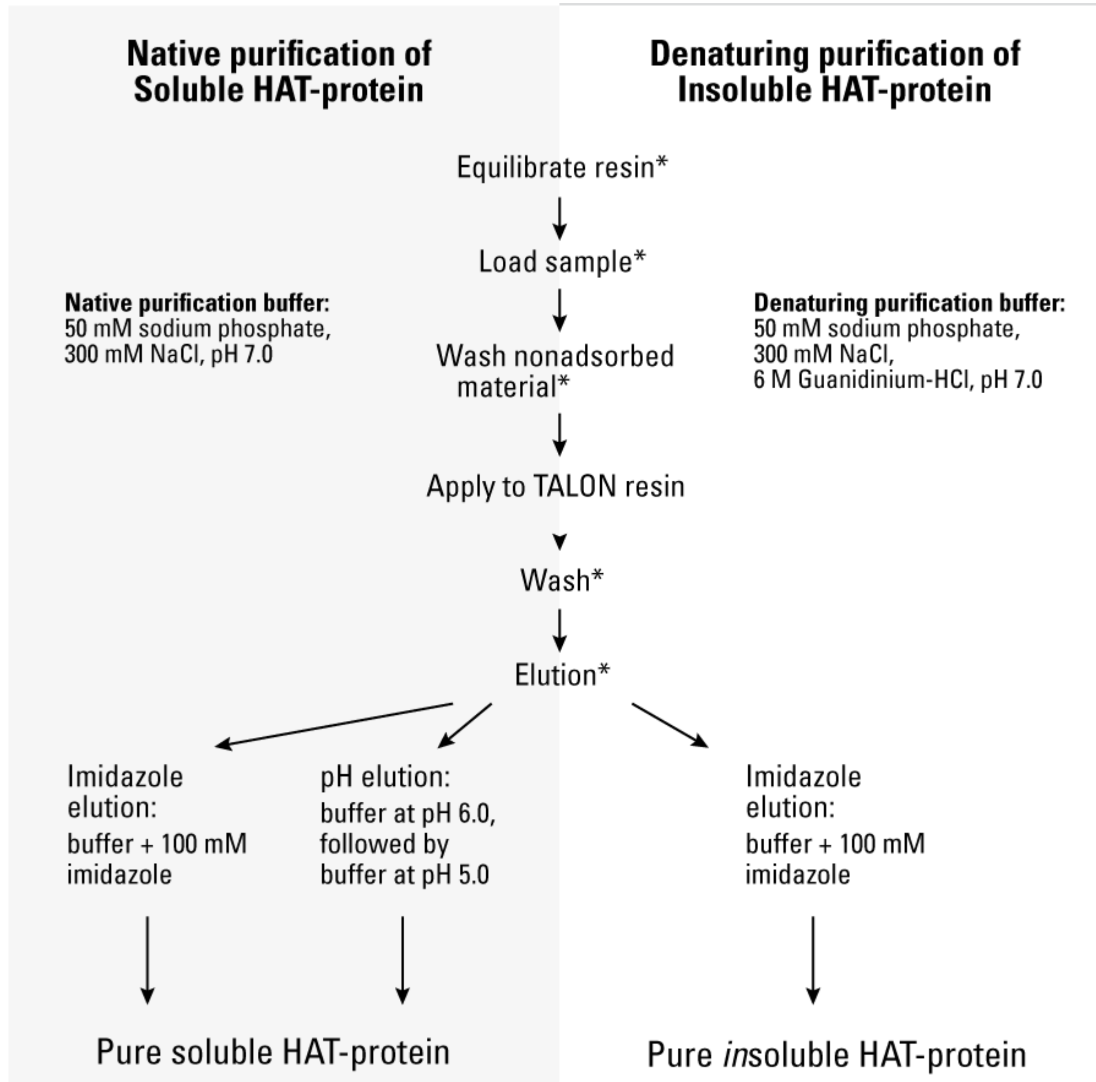

Figure 1. Overview of purification with the HAT System. This flowchart outlines the procedures for native and denaturing purification of HAT-tagged proteins. Steps denoted with an asterisk involve the indicated buffer: the native purification buffer for native HAT-protein purification (soluble proteins), or the denaturing purification buffer for denaturing HAT-protein purification (insoluble proteins). See the protocol in Section V for detailed procedures.

II. List of Components

Store vectors at –20°C. Store buffers and TALON Resin at 4°C. Store columns at room temperature.

- 5 µg pHAT10 Vector (0.5 µg/µl)
- 5 µg pHAT11 Vector (0.5 µg/µl)
- 5 µg pHAT12 Vector (0.5 µg/µl)
- 2 µg pHAT-DHFR Control Vector (0.5 µg/µl)
- 10 ml TALON Resin
- 70 ml Buffer A
- 10 ml Buffer B
- 10 ml Buffer C
- 40.1 g Guanidine HCl
- 10 Disposable Plastic Columns
- Vector Information Packet (PT3251-5)

Buffer Compositions

- Buffer A: 0.5 M sodium phosphate; 3.0 M NaCl pH 7.0 (10X)
- Buffer B: 1.0 M imidazole pH 7.0 (10X)
- Buffer C: 0.5 M sodium phosphate; 3.0 M NaCl pH 5.0 (10X)

The following kit components are also available separately:

- TALON Metal Affinity Resin (Cat. Nos. 635501, 635502, 635503 & 635504)
- TALON 2 ml Disposable Gravity Columns (Cat. No. 635606)

III. Additional Materials Required

The following items are required for use with the HAT System, but are not included in the kit.

A. For Cloning Recombinant Proteins

In-Fusion® is a revolutionary technology that greatly simplifies cloning.
For more information, visit www.clontech.com/infusion

Cat. No.	In-Fusion Cloning Kit
639645	In-Fusion HD Cloning System (10 rxns)
639646	In-Fusion HD Cloning System (50 rxns)
639647	In-Fusion HD Cloning System (100 rxns)

B. For Expression, Isolation, and Purification of HAT-Tagged Proteins

- Centrifuge
- Centrifuge tubes
- Spectrophotometer
- Electrophoretic system
- Imidazole (Sigma, Cat. No. I0250) for FPLC applications
- pH meter

C. For Care of the TALON Resin

- MES Buffer [20 mM 2-(N-morpholine)-ethanesulfonic acid, pH 5.0]
- PD-10 Gel Filtration Columns (GE Healthcare, Cat. No. 17-0851-01) for removing EDTA from a crude cell lysate sample before applying it to a TALON column. These columns are necessary only if EDTA is present in the extraction buffer during cell lysate preparation (see Sections V.A.3 & VI.A.6.Note).

IV. Cloning Recombinant HAT-Proteins

A. Use of the HAT Purification Sequence in Other Vectors

The HAT sequence can be easily transferred to any other vector using the HindIII and ClaI sites surrounding the HAT sequence. If desired, the HAT and enterokinase cleavage sites can be excised together using the HindIII site and a site in the MCS. If these sites are not convenient, the primers below can be used to amplify the HAT sequence with any desired terminal restriction sites (incorporated in the primers at the X):

5' primer: 5' - X AGCTTGAAGGATCATCTCAT - 3'
3' primer: 5' - X TCTTGTTGTGGGCATGAGCG - 3'

To amplify the HAT sequence and EK site, use the 3' primer below:

3' primer: 5' - X AAACAGTAGCAGTAGCTAGA - 3'

B. Transformation of Host Cells with HAT Expression Vectors

The following protocol is only an example for chemically induced transformation of *E. coli* competent cells. Any standard procedure, including electroporation, can be used for transformation. Perform control transformations with the Control Vector and without vector DNA in parallel.

Note: Use Stellar™ Competent Cells (Cat. Nos. 636763 or 636764) or another lac-inducible cell line to see induction of expression.

1. On ice, thaw a tube containing 100 µl of 0.5 M 2-mercaptoethanol (2-ME) and one 50 µl tube of frozen *E. coli* competent cells for each ligation/transformation.
2. Dispense 2 µl of 0.5 M 2-ME into each tube of competent cells and mix.
3. Dispense 2 µl of each ligation reaction directly into the mixture from Step 2.
4. Incubate the tubes on ice for 30 min.
5. Heat shock for exactly 30 sec in the 42°C water bath.
6. Remove the tubes from the 42°C water bath and place on ice for 2 min.
7. Add 250 µl of SOC medium (at room temperature) to each tube.
8. Shake the tubes horizontally at 37°C for 1 hr at 225 rpm in a rotary shaking incubator.
9. Spread all the transformation mixtures onto LB-ampicillin (50 µg/ml) agar plates. Incubate the plates at 37°C overnight.
10. Pick colonies, make small plasmid preparations of each, and sequence the region of the plasmid containing the HAT sequence and the sequence of interest with an M13/pUC reverse sequencing primer.

V. HAT System Protocol: General

PLEASE READ ENTIRE PROTOCOL BEFORE STARTING.

A. General Information

1. All manipulations as well as the centrifugation for removal of cell debris should be carried out at 4–8 °C in order to improve the protein stability and yield.
2. The addition of a reducing agent such as β2-mercaptoethanol (up to 10 mM) or a protease inhibitor such as PMSF to the sonication buffer may improve the structural stability of fragile proteins during sample preparation. See Appendix C for compatibility information.

   Note: Depending on the concentration and volume of additive you wish to use, you may need to remake the buffers to preserve the recommended concentrations of NaCl and buffering agent. **DTT and DTE are not compatible with this protocol in any concentration.**

3. If there is a high level of proteolytic activity in the cell lysate, we recommend adding 1 mM EDTA to the extraction buffer to inhibit metalloproteases during the extraction. Before application of the sample to the TALON resin, EDTA must be removed by gel filtration on a column (PD-10, GE Healthcare; see Section III) equilibrated with Extraction/Loading Buffer. In some cases, the host cell produces low molecular weight chelators that also must be removed by gel filtration prior to application of the sample to the TALON column.
   The presence of such chelators can be detected easily by application of your sample to a small column packed with the TALON adsorbent. If you observe that the top of the column is losing its characteristic pink color and the colorless front moves in the direction of the flow, or if you obtain pink colored fractions during batch adsorption, the sample needs to be equilibrated with a gel filtration column.
4. Overexpression of some recombinant proteins can lead to their accumulation in insoluble form as inclusion bodies. In order to determine optimal extraction/purification conditions, the distribution of the protein of interest in soluble and insoluble form must be determined. A preliminary SDS/PAGE analysis of the protein extracts obtained under native conditions followed by extraction of the residual proteins under denaturing conditions should be performed. One should take care to use the same extraction volumes for both the native and denaturing extracts and run the cell extract before induction as a control in one of the lanes in order to identify the protein of interest. Use of denaturing conditions is recommended only if the biological activity of the protein of interest has no relevance. It is preferable to use native conditions for extraction even if only 5 to 10% of the protein of interest is soluble.
5. The volumes given for the extraction buffers in the procedures below have been optimized for purification of the HAT-DHFR protein from 20–25 ml of an overnight *E. coli* culture. The volume of extraction buffer for overnight culture used may need to be adjusted for other proteins, dependent on the expression level and anticipated yield.
6. If you are purifying protein from harvested eukaryotic cells, lyse the cells in an appropriate lysis buffer containing a mild detergent (Sambrook & Russell, 2001). See Appendix C for compatible buffer additives. Note that if EDTA or EGTA are included in the lysis buffer, they must be removed by gel filtration prior to loading the sample on the Talon resin, since these metal chelators will strip the cobalt from the resin. See Step 3 for more information.
7. Carefully check the appearance of the sample after lysis or sonication. Bacterial samples often remain viscous from incomplete shearing of genomic DNA. Complete DNA fragmentation improves the HAT-protein recovery and allows efficient removal of cellular debris during centrifugation. It is

possible to decrease the viscosity of the sample 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). An alternative method is to dilute the sample fivefold with Equilibration/Wash Buffer before applying it to the resin. This should not significantly affect recovery.

B. Protein Expression

1. Grow an overnight culture of *E. coli* transformed with the plasmid encoding the HAT-protein of interest. If the amount of the protein that can be isolated from this culture is sufficient, proceed to Step 3 after removing a 1 ml sample for electrophoretic analyses. Centrifuge the 1 ml sample at 1,000–3,000 x g for 15 min at 4°C and store the cell pellet at –20°C after removal of the supernatant.

   Note: If there is a need for a large-scale preparation of the protein, proceed to Step 2.

2. Use the overnight culture to inoculate a larger volume of medium if you need a greater quantity of the protein of interest (use 20 ml of overnight culture per 1 L of medium). Incubate with shaking for another 1 to 2 hr, until the culture has attained an absorbance (OD_{600}) of approximately 0.6 measured against the starting medium. 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 addition of an appropriate inducer (the lac promoter in the pHAT vectors 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.

C. Buffers for Extraction and Purification of HAT-tagged Proteins

1. Buffers for native purification of HAT-tagged proteins

We suggest the following buffers for the purification of HAT recombinant proteins under nondenaturing conditions; however, other compatible buffers may be used. Imidazole-based purifications performed at pH 7.0 are generally recommended (eluted material is less dilute) especially when the HAT-tagged protein of interest cannot tolerate pH changes. We recommend adding 300 mM NaCl to reduce electrostatic interactions that result in nonspecific binding of unwanted proteins to the adsorbent. Alternative salt additives may provide better results for certain HAT-proteins. Before planning buffer compositions, please consult Appendix C.

Note: After storage at 4°C, you may observe a precipitate in one or more of the provided 10X buffers. If this occurs, warm the buffer to room temperature to redissolve the precipitate and continue as indicated below.

Equilibration/Wash Buffer [a,b]

50 mM sodium phosphate; 300 mM NaCl, pH 7.0 (final pH)
Dilute 5 ml of Buffer A with 45 ml of deionized water. Check and adjust pH if necessary.

Note: This buffer is also used as an extraction buffer for isolating proteins under native conditions using sonication (see Section VI.A).

Elution Buffer

- Imidazole elution:
  50 mM sodium phosphate; 300 mM NaCl; 100 mM imidazole, pH 7.0 (final pH)
  Add 1 ml of Buffer B to 1 ml of Buffer A and dilute with 8 ml of deionized water. Check and adjust pH if necessary.

- pH elution:
  For monomers: 50 mM sodium phosphate; 300 mM NaCl, pH 6.0 (final pH)
  Mix 0.5 ml of Buffer A with 0.5 ml of Buffer C and dilute with 9 ml of deionized water. Check and adjust pH if necessary.
  For dimers: 50 mM sodium phosphate; 300 mM NaCl, pH 5.0 (final pH)
  Dilute 1 ml of Buffer C with 9 ml of deionized water. Check and adjust pH if necessary.

2. Buffers for denaturing purification of HAT-tagged proteins

Equilibration/Wash Buffer [a,b]

50 mM sodium phosphate; 300 mM NaCl; 6 M guanidine-HCl, pH 7.0 (final pH)
Dissolve the 40.1 g of guanidine-HCl in 7 ml of Buffer A and 10 ml of deionized water. Mix until the guanidine-HCl is completely dissolved (~1 hr). Check the pH. Add deionized water to a final volume of 70 ml.

Note: This buffer is also used as an extraction buffer for isolating proteins under denaturing conditions (see Section VI.B).

Elution Buffer:

- Imidazole elution:
  45 mM sodium phosphate; 270 mM NaCl; 5.4 M Guanidine-HCl; 100 mM imidazole, pH 7.0 (final pH)
  Add 0.5 ml of Buffer B to 4.5 ml of Equilibration/Wash Buffer for denatured HAT-proteins. Check the pH.

Notes

[a] All volumes given are calculated for approximately one protein purification from 25 ml of culture using 1 ml of TALON adsorbent.

[b] If an intermediate wash before elution is necessary, use the following wash buffers:

- For native purification:
  Mix 1 ml of Buffer A with 50 µl of Buffer B and dilute with 8.95 ml of deionized water. Check the pH.
- For denaturing purification:
  Mix 50 µl of Buffer B with 9.95 ml of Extraction/Loading Buffer for denatured HAT-proteins. Check the pH.

VI. HAT System Protocol: Isolation

These are general guidelines. Some modifications may be required, particularly for eukaryotic expression or fragile enzyme systems. Chilling samples on ice before and during extraction may be necessary to preserve protein functionality. Extraction buffer volumes may also need to be adjusted according to cell pellet size and anticipated protein yield. As a starting point for scaling up, use 2 ml of extraction buffer per 20–25 ml of culture.

A. Isolation of Native HAT-tagged 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 the yield is low, use the mild extraction method described in Step 6.
2. Resuspend the cell pellet by vortexing in 2 ml of chilled extraction buffer (4°C) per 25 ml of culture for small preparations (less than 100 ml). Use 1–2 % of the volume of the culture for large preparations (1 L or more).
3. (Steps 3 and 4 may be omitted if lysozyme treatment interferes with the functionality of your protein). Add lysozyme to the extraction buffer to 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. (Incubations at room temperature result in elevated proteolytic activities.) Alternatively, lysozyme can be used at 4°C with lower efficiency. If the protein of interest is hydrolyzed by this treatment, use the method described in Step 6. Alternatively, disrupt the cells by repeated freeze/thaw cycles, (i.e., flash-freezing the cell suspension in a dry ice-ethanol bath and thawing in chilled H_2O).
5. Thoroughly sonicate the cells using a series of short, repetitive bursts. Use the minimum time necessary to disrupt the cells (3 x 10 sec with 30 sec pauses on ice for small preparations (≤ 50 ml) or 3 x 30 sec with 2 min pauses on ice for large preparations (≥ 200 ml). 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 of Alumina (Sigma, Cat. No. A-2039) for 2–3 min until a paste-like composition forms. Add chilled (4°C) extraction buffer (2 ml per 25 ml culture). Proceed to Step 7.

   Note: If there is a high level of proteolytic activity in the cell lysate, we recommend adding 1 mM EDTA (final concentration) to the extraction 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 on a column (PD-10, GE Healthcare, see Section III) equilibrated with Equilibration/Wash Buffer (Section V.C).

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. Set aside a small portion of the clarified sample at 4°C to run on an analytical SDS/PAGE gel in parallel with the column-purified sample, to estimate yield and purity.
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 small portion via SDS/PAGE in parallel with known amounts of protein standards to estimate the amount of HAT-protein in the clarified sample. Once satisfactory expression is observed, proceed with the appropriate purification protocol (see Section VII).

B. Isolation of Denatured HAT-tagged Proteins

These are general guidelines for using denaturing conditions with bacterial expression cultures. Some modifications may be required for eukaryotic expression systems. Extraction buffer volumes may also need to be adjusted according to cell pellet size and anticipated protein yield.

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 extraction buffer per 20–25 ml of culture.
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 pellet any insoluble material.
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 at 4°C to analyze via SDS/PAGE in parallel with the column-purified sample, to estimate yield and purity.

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

VII. HAT System Protocol: Purification

A. General Information

Choice of purification conditions

The following general guidelines can be used for purification of a HAT-tagged protein from transformed *E. coli* cultures. The buffers and purification conditions (see Section V.C and Figure 1) should work well for most soluble, monomeric proteins expressed in *E. coli*. Each different expression system and HAT-protein should be tested initially in a small-scale batch purification to determine expression levels and to optimize the protocol. If you have any difficulties with the procedure, please refer to the Troubleshooting Guide (Appendix B).

Choosing the buffers: pH gradient versus imidazole gradient

TALON purification schemes typically use either a pH gradient or an imidazole gradient for washing and elution. The presence of imidazole in the wash and/or loading buffer minimizes nonspecific binding and reduces the amount of contaminating proteins. Thus, purification using an imidazole gradient is the generally preferred procedure, and should be tried first. However, both imidazole and HAT-proteins absorb at 280 nm, so elution peaks may be difficult to detect spectrophotometrically, especially when purifying small amounts of HAT-protein. In these cases, the leading edge of the imidazole breakthrough peak should be collected and checked for the presence of HAT-protein by a protein specific assay (Bradford, 1976) and SDS/PAGE analysis. Alternatively, a pH gradient may be used instead of imidazole for purification of HAT-proteins that are stable in the pH range of 6.0–7.0. Buffers containing EDTA or EGTA will elute all HAT-proteins, but will also strip the metal off the resin. Thus, the purified protein will contain cobalt (in addition to EDTA or EGTA), which can inhibit protein function and lead to precipitation. See Section V.A for more information about removing EDTA from samples prior to purification.

Elution strategy: linear versus step 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.

Reusing TALON Resin

Used TALON Resin may be stored and reused up to 3–4 times before discarding or complete regeneration (see Section VII.E); the exact number of uses varies depending on the applications. To avoid possible cross-contamination, use a particular aliquot of resin only for the purification of a single type of HAT-protein.

B. Batch/Gravity Flow Column Purification

1. Thoroughly resuspend the TALON Resin to achieve a homogeneous 50% suspension of resin in the storage solution (it will settle during shipping and storage). TALON resin is shipped as a 20% ethanol suspension.
2. Immediately transfer the required amount of resin suspension to a sterile tube that will accommodate 10–20X the resin bed-volume. Use 2 ml of resin suspension per ~3 mg of anticipated HAT-protein. Note that 2 ml of homogeneously resuspended resin will provide 1 ml (bed-volume) of TALON Resin.
3. Centrifuge at 700 x g for 2 min to pellet the resin.
4. Remove and discard the 20% ethanol supernatant.
5. Add 10 bed-volumes of Equilibration/Wash Buffer and mix briefly to pre-equilibrate the resin.
6. Recentrifuge at 700 x g for 2 min to pellet the resin, and discard the supernatant.
7. Repeat Steps 5 and 6.
8. Add the clarified sample from Section VI to the resin.
9. Gently agitate the suspension at room temperature for 20 min on a platform shaker to allow the HAT-protein to bind to 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 bed-volumes of Equilibration/Wash Buffer (pH 7.0). 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 the above wash (Steps 11–14).
16. Add one bed-volume of 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 Equilibration/Wash Buffer.
20. (Optional) If necessary, an intermediate wash may be performed under more stringent conditions using 5 mM imidazole in Equilibration/Wash Buffer (see Section V, footnote b for intermediate wash buffer compositions).
21. Elute the HAT-protein by adding 5 bed-volumes of Elution buffer (100 mM imidazole in Equilibration/Wash Buffer for imidazole elution or pH 6.0 buffer for pH elution) to the column. Collect the eluate in 500 µl fractions.

    Note: Under most conditions, a majority of the HAT-protein will be recovered in the first two bed-volumes.

22. (Optional) In order to ensure that all HAT-tagged protein is recovered, elute with 5 bed-volumes of stronger elution buffer (200 mM imidazole in Equilibration/Wash Buffer for imidazole elution or pH 5.0 buffer for pH elution). Collect the eluate in 500 µl fractions.
23. Use spectrophotometric and SDS/PAGE analyses to determine which fraction(s) contains the majority of the HAT-protein.

C. Large-Scale Batch Purification

Pure HAT-protein is obtained much faster with this method, compared to using gravity-flow columns. However, batch washes are somewhat less efficient at removing impurities than are gravity-flow columns. Therefore, larger wash buffer volumes are needed to obtain pure HAT-protein. Very little unwanted protein should bind to TALON Resin at pH 7.0. It is generally sufficient to wash the resin-bound HAT-protein complex 3–4 times with 10 bed-volumes of pH 7.0 Equilibration/Wash Buffer and proceed with elution. If additional stringent washes are needed to achieve the desired purity level, include Step 17.

1. Thoroughly resuspend the TALON Resin to achieve a homogenous 50% suspension of resin in the storage solution (it will settle during shipping and storage). TALON resin is shipped as a 20% ethanol suspension.
2. Immediately transfer the required amount of resin suspension to a sterile tube that will accommodate 10–20X the resin bed-volume. Use 2 ml of resin suspension per ~3 mg of anticipated HAT-protein. 2 ml of homogeneously resuspended resin will provide 1 ml (bed-volume) of TALON resin.
3. Centrifuge at 700 x g for 2 min to pellet the resin.
4. Remove and discard the 20% ethanol supernatant.
5. Add 5 bed-volumes of Equilibration/Wash Buffer (pH 7.0) and mix briefly to pre-equilibrate the resin.
6. Recentrifuge at 700 x g for 2 min to pellet the resin, and discard the supernatant.
7. Repeat Steps 5 and 6.
8. Add the clarified sample from Section VI to the resin.
9. Gently agitate the suspension at room temperature for 20 min on a platform shaker to allow the HAT-protein to bind to 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-protein complex by adding 10 bed-volumes of Equilibration/Wash Buffer (pH 7.0).
13. Gently agitate the suspension at room temperature for 10 min on a platform shaker to promote thorough washing.
14. Centrifuge at 700 x g for 5 min.
15. Remove and discard the supernatant.
16. Repeat the above wash (Steps 12–14) 2–3 times.
17. (Optional) If more stringent washing is needed to achieve the desired purity, add 5 bed-volumes of wash buffer (5 mM imidazole in Equilibration/Wash Buffer; see Section V, footnote b) and gently agitate the suspension at room temperature for 10 min. Centrifuge at 700 x g for 5 min to pellet resin. Remove and discard the supernatant.
18. Elute the HAT-protein by adding 1 bed-volume of Elution buffer (100 mM imidazole in Equilibration/Wash Buffer for imidazole elution or pH 6.0 buffer for pH elution).
19. Gently agitate the suspension at room temperature for 10 min.
20. Centrifuge at 700 x g for 5 min.
21. Collect supernatant.
22. Repeat Steps 17–20 three times, collecting four separate, 1-bed-volume fractions.
23. (Optional) In order to ensure that all HAT-tagged protein is recovered, elute with 4 bed-volumes of stronger elution buffer (200 mM imidazole in Equilibration/Wash Buffer for imidazole elution or pH 5.0 buffer for pH elution) by repeating Steps 18 through 20.
24. Use spectrophotometric and SDS/PAGE analyses to determine which fraction(s) contains the majority of the HAT-protein.

D. Medium-Pressure (FPLC) Column Purification (for TALON Superflow resin only)

1. Consult the manufacturer's instructions for FPLC column assembly.
2. Thoroughly resuspend the TALON Superflow resin to achieve a homogeneous 50% resin suspension in the storage buffer. Slowly pour the slurry into the column, taking care to avoid introducing air bubbles.
3. Allow the resin to settle. This process can be accelerated by allowing the buffer to flow through the column with a peristaltic pump. 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 in Equilibration/Wash Buffer and repack the column.
4. Insert and adjust the top adaptor and connect the column to the chromatography system according to FPLC system specifications.

   Note: Avoid trapping air between the adaptor and the resin surface.

5. Equilibrate the column with 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 (from Section VI) 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 binding of the HAT-tagged protein to the resin. If the protein does not bind, the flow rate should be reduced further. The flow rate can be increased later for washing and protein elution.
   If the protein of interest is not very stable at room temperature, the chromatography can be performed at 4°C. Also, higher flow rates of up to 5 $ml/min/cm^2$ can be used to wash and elute the protein: Recovery may decrease by 10–15%, but a chromatography run will take less time (15–20 min average elution).
7. Wash column with Equilibration/Wash Buffer followed by wash buffer (5–10 mM imidazole in Equilibration/Wash Buffer) until the baseline at 280 nm is stable (usually 10–20 column-volumes).
8. Elute the protein using the Elution Buffer (100–200 mM imidazole gives best results). Five column-volumes are usually sufficient for elution. The HAT-protein usually elutes in the second and third column-volumes.
9. Use spectrophotometric and SDS/PAGE analyses to determine which fraction contains the majority of the HAT-protein.

E. Resin Washing, Reuse, Regeneration, and Storage

Generally, you can reuse TALON resins 3–4 times before discarding or fully regenerating. The exact number of uses varies among preparations 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 HAT-tagged protein.

Important precautions

- 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 2-(N-morpholine)-ethanesulfonic acid (MES) buffer (pH 5.0) before reuse to remove the bound imidazole.

A. Stringent Wash (optional)
   a. Wash resin with four bed-volumes of 6 M guanidinium (pH 5.0) + 1% nonionic detergent.
   b. Rinse resin with five bed-volumes of distilled H_2O.
   c. Store resin at 4°C in 20% nonbuffered ethanol containing 0.02% azide.

B. Removing Imidazole
   a. Wash resin with five bed-volumes of 20 mM MES buffer (pH 5.0) containing 0.1 M NaCl.
   b. Rinse resin with five bed-volumes of distilled H_2O.
   c. Store resin at 4°C in 20% nonbuffered ethanol containing 0.02% azide.

C. Regeneration of TALON Superflow Columns
   Purification of HAT-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
   Strip the resin of cobalt ions by washing with 10 bed-volumes of 0.2 M EDTA (pH 7.0). Wash excess EDTA with an additional 10 bed-volumes of Milli-Q H_2O. Charge the resin with 50 mM $CoCl_2$ solution (10 bed-volumes). Again, wash with 10 bed-volumes of Milli-Q H_2O to remove excess cobalt metal ions. Equilibrate the resin with Equilibration/Wash buffer (10 bed-volumes).

VIII. References

Andersson, L. (1992) New developments in protein isolation, purification, and characterization. *Cancer Invest*. **10**(1):71–84.

Arnold, F. H. (1991) Metal-Affinity Separations: A New Dimension in Protein Processing. *Biotechnology* **9**:151–156.

Bradford, M. (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the principle of Protein-Dye binding. *Analytical Biochemistry* **72**:248–254.

Hemdan, E. S. & Porath, J. (1985a) Development of Immobilized Metal Affinity Chromatography II. Interaction oaf amino acids with immobilized nickelimionodiacetate. *Journal of Chromatography* **323**:255–264.

Hemdan, E. S. & Porath, J. (1985b) Development of Immobilized Metal Affinity Chromatography III. Interaction of oligopeptides with immobilized nickelimionodiacetate. *Journal of Chromatography* **323**:265–272.

Hochuli, E., Bannwarth, W., Doebeli, H., Gentz, R. & Stueber, D. (1988) Genetic approach to facilitate purification of recombinant proteins with a novel metal chelate adsorbent. *Bio/Technology* **6**(11):1321–1325.

Hochuli, E., Dobeli, H. & Schacher, A. (1987) New Metal Chelate Adsorbent Selective for Proteins and Peptides Containing Neighboring Histidine Residues. *Journal of Chromatography* **411**:177–184.

Porath, J. (1985) Immobilized Metal Ion Affinity Chromatography - a Powerful Method for Protein Purification. In H. Tschelsche (Ed.), Modern Methods in Protein Chemistry (pp. 85–95). Berlin & NY: Walter de Gruyter & Co.

Porath, J. (1990) Amino acid side chain interaction with chelate-liganded crosslinked dextran, agarose and TSK gel. A minireview of recent work. *J. Mol. Recognit*. **3**(3):123–127.

Porath, J. (1992) Immobilized Metal Ion Affinity Chromatography. *Protein Express. Purif*. **3**:263–281.

Porath, J., Carlsson, J., Olsson, I. & Belfrage, G. (1975) Metal chelate affinity chromatography, a new approach to protein fractionation. *Nature* **258**:598–599.

Sambrook, J. & Russell, D. W. (2001) Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory Press (Cold Springs Harbor, NY).

Sulkowski, E. (1985). Purification of proteins by IMAC. *Trends in Biotechn*. **3**:1–7.

Wong, J. W., Albright, R. L. & Wang, N. H. L. (1991) Immobilized metal ion affinity chromatography (IMAC) - chemistry and bioseparation applications. *Sep. Purif. Methods* **20**(1):49–106.

Zhao, Y.-J., Sulkowski, E. & Porath, J. (1991) Surface topography of histidine residues in lysozymes. *Eur. J. Biochem*. **202**:1115–1119.

Appendix A. Vector Information

Figure 2. pHAT10/11/12 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 a lower overall charge than tags with consecutive histidine residues, such as the 6 x histidine tag. As a result, HAT-protein fusions exhibit solubilities that more closely resemble those of wild-type proteins while still possessing a 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 into other vectors.

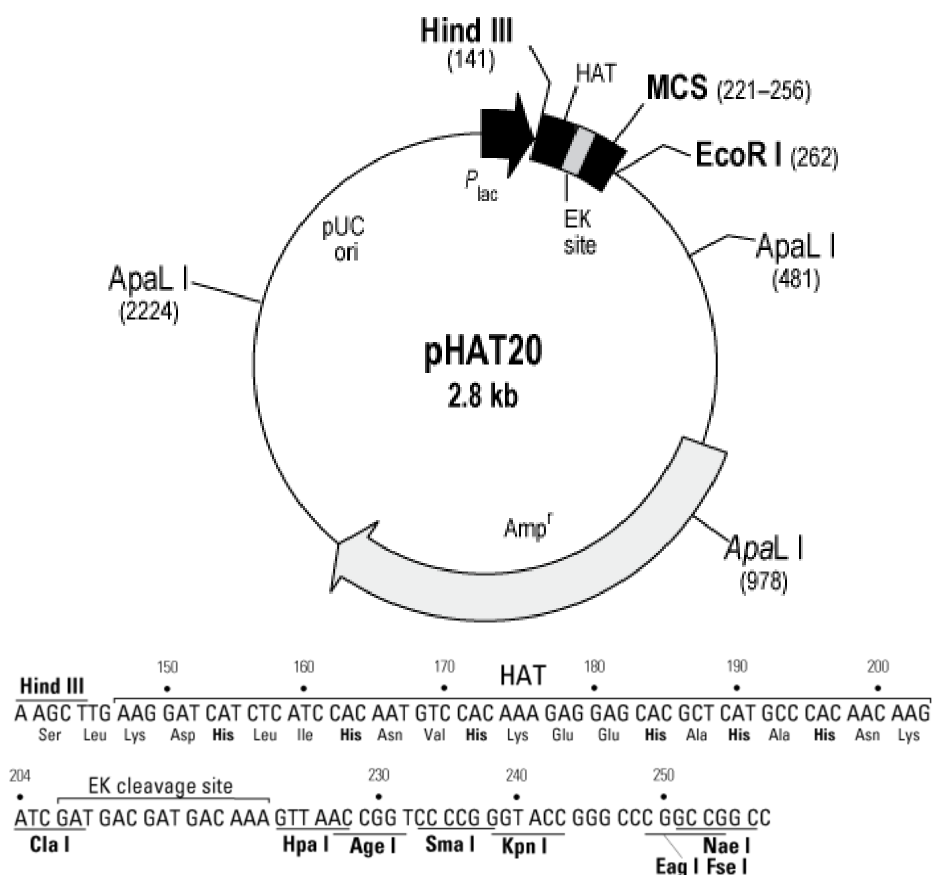

Figure 3. pHAT20 Vector Map and MCS. Unique restriction sites are in bold. The sequence of pHAT20 is shown. This vector encodes a novel Histidine Affinity Tag (HAT) that enables purification of expressed proteins at neutral pH. The pHAT Vectors allow protein purification under both native and denaturing 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 a lower overall charge than tags with consecutive histidine residues, such as the 6 x histidine tag. As a result, HAT-protein fusions exhibit solubilities that more closely resemble those of wild-type proteins while still possessing a 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 into other vectors.