BD Biosciences Clontech
Protein Purification Products

From screening to production—a complete platform

BD Biosciences
Clontech
Discovery Labware
Immunocytometry Systems
Pharmingen

BD Biosciences Clontech
Protein Purification Products

Notice to Purchaser

The use of TALON™ products are covered under U.S. Patent #5,962,641.

HAT™, TALON™,TALONspin™, TALON-NX™, and ProTet™ are trademarks of Becton, Dickinson and Company.

Sepharose and Sephadex are registered trademarks of Amersham Biosciences Limited.

Triton® is a registered trademark of Rohm and Haas Co.

Superflow™, Uniflow™ and CellThru™ are trademarks of Sterogene Bioseparations, Inc.

FPLC® is a registered trademark of Amersham Pharmacia Biotech.

© 2002, Becton, Dickinson and Company

Table of Contents

Protein Purification Overview

Protein Purification Introduction 1
TALON™ Products 3
TALON™ Metal Affinity Resin 4
Unique Properties of TALON™ Resin 6
Protein Purification with TALON™ 8
Purification with β-Mercaptoethanol 12
Formats of TALON™ Resin 13
Protein Purification Procedures 14
Purification using FPLC 15
Purification from Crude Cell Lysates 17
Protein Expression Systems 20
His-tag Antibodies 26
Purification of Immunoglobulins 27
Purification of GST-tagged Proteins 32

Purification Protocols

TALON™ Resin Protocols 37
Thiophilic Resin Protocols 58
Glutathione Resin Protocols 66
Troubleshooting Guide 72
Reagent Compatibility Table 80

References 81

List of Tables

Protein Purification Products from BD Biosciences Clontech 2
Comparision of TALON™ Resin with Ni-NTA Resin 7
Physicochemical Properties of TALON™ Resins 13
TALON-Compatible Reagents 16
Comparison of Expression Systems 21
Histidine Tags 22
The HAT™ System 25
Properties of Thiophilic. 31
Reagent Compatibilities 80

List of Figures

Recombinant protein purification steps 1
Molecular mechanism of histidine binding to TALON™ Resin 4
Cobalt-based TALON has higher affinity and
specificity for his-tagged proteins 5
Hypothetical stuctures of Histidine Tags 5
Chemical structures of chelating ligands used in IMAC 6
Native vs. denaturing purification procedures 8
Purification of 6xHis-GFPuv under denaturing conditions 9
Purification of 6xHis proteins under native conditions compared to Ni-NTA. 10
Native purification on TALON™ preserves biological activity of proteins 11
Native Purification of 6xHis protein in the presence of β–mercaptoethanol. 12
Yields of purification in the presence of β–mercaptoethanol
compared to Ni-NTA Resin 12
FPLC purification of 6xHis-GFP with TALON Superflow 15
SDS-PAGE of FPLC fractions from 6xHis-GFP uv purification 16
TALON™ CellThru purifies protein from crude cell lysate faster than
conventional methods 18
SDS-PAGE of TALON™ CellThru purified proteins 19
6xHN-tagged protein purified with TALON™ Superflow 23
HAT-tagged protein purified with TALON™ Superflow 24
Batch purification of HAT-GFP by pH gradient 25
6xHis Monoclonal Antibody detects 6xHis-LacZ 26
Thiophilic Resin purifies antibodies at neutral pH and more quickly than Protein A 27
Single-chain antibody purification with Thiophilic Resin 28
Structure of Thiophilic Resin. 28
Purification of IgY using Thiophilic Resin 29
Thiophilic-Superflow Resin purifies IgG at high flow rates and neutral pH. 30
Analysis of purified IgG fractions 30
GST-tagged protein purification from whole cell extract. 32

List of Keywords

Activity 8, 10-11
Antibodies 26, 28-31
Batch 1,2,13-14
Baculovirus. 20-21
β-Mercaptoethanol 12, 16
Compatibility 16, 80
Denature. 9
Direct Capture 17-19
Expanded bed chromatography 17
Expression Systems. 20-25
FPLC 13, 14-16
Glutathione S-transferase 32-33
His-tags 22-25
Inclusion bodies 9
Leaching 6, 7
Mammalian 20-21
Negative adsorption 65
pH Gradient 10
Proteolysis 19, 33, 73, 74
Regeneration 56-57, 65, 71
Solubility 9, 19
Spin Columns 14
Immunoglobulins. 27-31
Yeast 21-22

TALON Product List

TALON Product List	Size	Cat. #
TALON Metal Affinity Resin	10 ml	8901-1
	25 ml	8901-2
	100 ml	8901-3
	250 ml	8901-4
TALONspin Columns	10 cols.	8902-1
	25 cols.	8902-2
	50 cols.	8902-3
	100 cols.	8902-4
TALON 2-ml Disposable Gravity Column	50 cols.	8903-1
TALON Superflow Metal Affinity Resin	25 ml	8908-1
	100 ml	8908-2
TALON CellThru	10 ml	8910-1
	100 ml	8910-2
CellThru 2-ml Disposable Columns	50 columns	8914-1
CellThru 10-ml Disposable Columns	20 columns	8915-1
TALON Buffer Kit	each	K1252-1
TALON Purification Kit	each	K1253-1
Talon-Dextran Trial Size	5 mg	8918-y

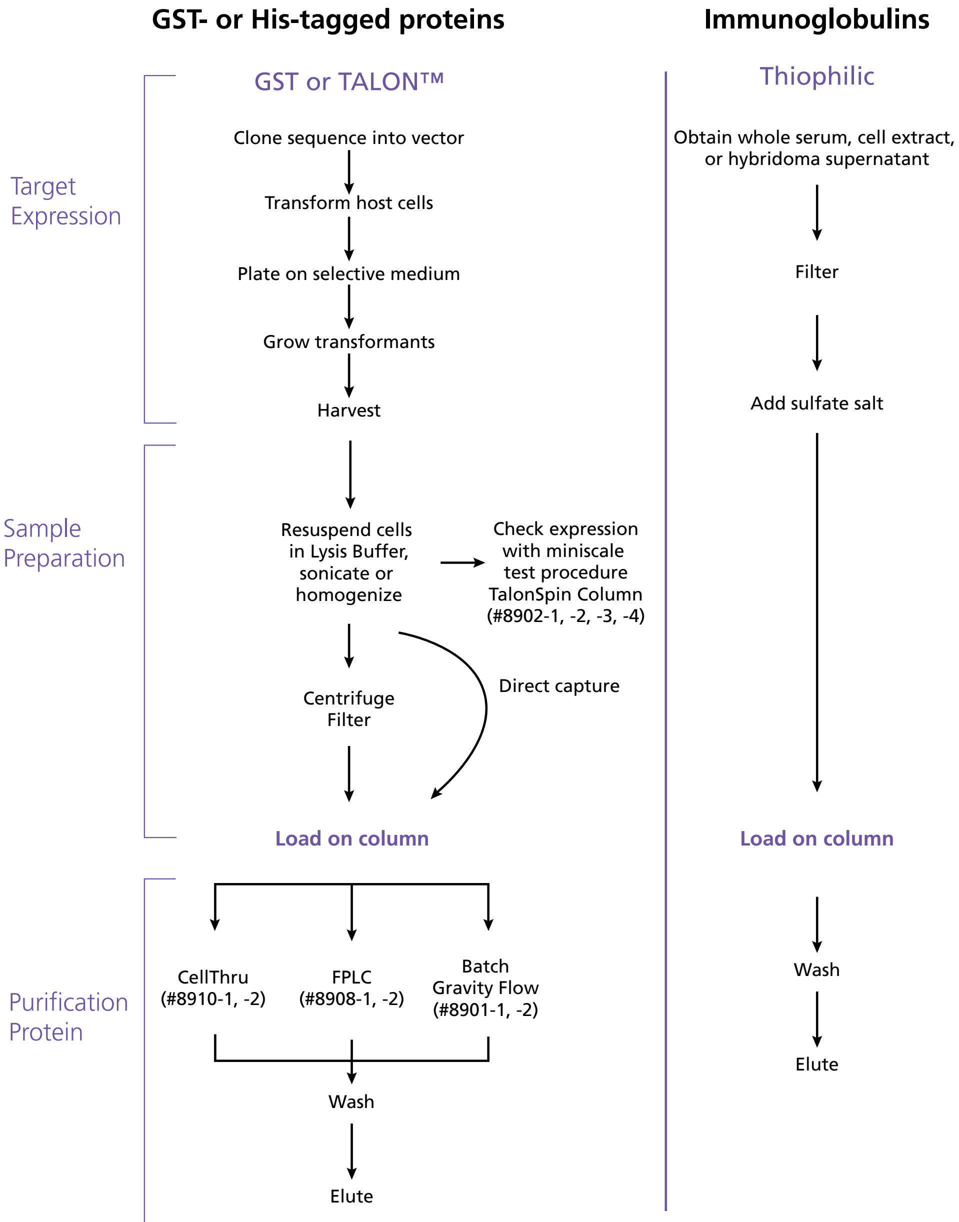
Thiophilic Resin Product List

Product	Size	Cat. #
Thiophilic-Uniflow Resin	10 ml	8913-1
	100 ml	8913-2
Thiophilic-Superflow Resin	10 ml	8917-1
	100 ml	8917-2

Glutathione Resin Product List

Product	Size	Cat. #
Glutathione-Superflow Resin	10 ml	8911-1
	100 ml	8911-2
Glutathione-Uniflow Resin	10 ml	8912-1
	100 ml	8912-2
GST Purification Kit	5 purifications	K1251-1

Recombinant Protein Purification Flowchart



Protein Purification Introduction

BD Biosciences Clontech's full line of protein purification products serves a broad spectrum of purification needs. These products purify a wide range of proteins, including polyhistidine-tagged (His-tagged) proteins, glutathione S-transferase-tagged (GST-tagged) proteins, and immunoglobulins. Our protein purification platform can help you reach a variety of purification goals—from isolating a small amount of protein for proteomics research to purifying large-scale quantities for production applications.

Multiple formats for multiple applications

Our high-quality purification products are available in a variety of formats to complement any technique, including:

- spin-column purification
- gravity-flow purification
- batch purification
- FPLC applications

All of our purification products generate high yields and high purity—the two most important factors in successful protein purification—no matter what type of protein you purify or which application you use.

Protein Purification Products from BD Biosciences Clontech

Application	His-tagged proteins	Immunoglobulins	GST-tagged proteins
Small- or medium-scale production	TALON™ Resins	Thiophilic Resins	Glutathione Resins
Low-pressure gravity flow	TALON™ Resin	Thiophilic Uniflow Resin	Glutathione Uniflow Resin
Large- or production-scale (FPLC)	TALON™ Superflow Resin	Thiophilic Superflow Resin	Glutathione Superflow Resin
Analytical scale	TALONspin™ Columns	n/a	n/a
From crude cell lysate	TALON™ CellThru	n/a	n/a

TALON™ Products

For polyhistidine-tagged protein purification

Product	Application
TALON™ Metal Affinity Resin Resin ready for loading in columns for small or medium-scale purification of His-tagged proteins. Purify > 5 mg protein using 1 ml of resin.	For purification of most cytosolic and secreted His-tagged proteins by small-scale or batch/gravity flow, under native or denaturing conditions
TALON™ Superflow Resin Specially designed for quick and effective purification of His-tagged proteins at high flowrates and medium-pressure (up to 150 psi).	For FPLC, medium-pressure chromatography, or scale-up for production applications
TALON™ CellThru Novel IMAC resin designed for quick purification of His-tagged proteins by direct capture.	For small-scale single-use applications such as verifying positive transformants for His-tagged protein expression levels, or trial-level purification protocols.
TALONspin™ Columns Ready-made spin columns containing TALON-NX™ resin for the simultaneous purification of several His-tagged proteins in parallel in only 30 minutes.	For small-scale single-use applications such as verifying positive transformants for His-tagged protein expression levels, or trial-level purification protocols.
TALON™ Purification Kit Convenient kit containing TALON resin, columns, and all the buffers necessary to extract, wash, and elute His-tagged proteins. This kit provides the ideal place to start when using TALON in your applications.	
TALON™ Disposable Columns Two different types of disposable columns—one for use with TALON Resin for regular His-tagged protein purification and one for use with CellThru Resin for purification from crude lysates.	
TALON™ Buffer Kit Supplemental kit containing concentrated forms of optimized buffers for extracting, washing, and eluting proteins.	

TALON™ Metal Affinity Resin

TALON Resins are durable, cobalt-based IMAC resins designed to purify recombinant poly-histidine-tagged proteins (Bush *et al.*, 1991). These resins are compatible with many commonly used reagents, and allow protein purification under native and denaturing conditions. They can be used with all prokaryotic and eukaryotic expression systems in a variety of formats, including small- (mini-) scale batch screening, large-scale batch preparations, and methods using gravity-flow columns and spin columns.

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 when developing versatile purification protocols. A host of purification methods have been developed that capitalize on the general physical properties of proteins. One of the quickest and easiest ways to purifying a protein is to use affinity chromatography since it is generally a more selective method of purification, which lets the protein of interest can be purified in one or two steps. However, many proteins have not been characterized sufficiently, or do not have any known strong binding properties that can be utilized for purification. 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.

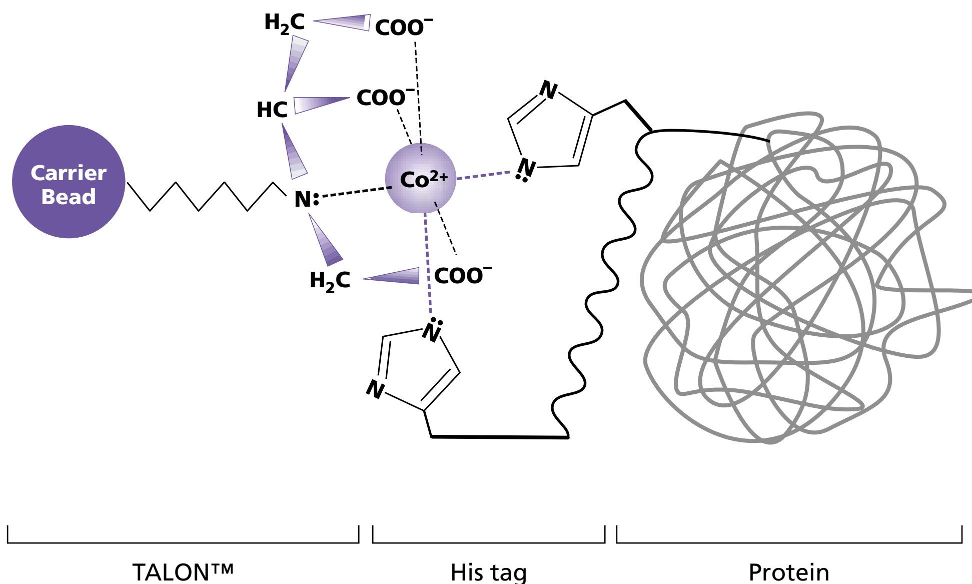


Figure 2. Molecular mechanism of histidine binding to TALON™ Resin.

TALON™ Metal Affinity Resin...*cont.*

IMAC technology

TALON Resin is an immobilized metal affinity chromatography (IMAC) resin based on our innovative, patented technology. IMAC was introduced in 1975 as a group-specific affinity technique for separating proteins (Porath *et al.*, 1975). This 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 (Porath, 1985; Sulkowski, 1985; Hemdan & Porath, 1985a; Hemdan & Porath, 1985b; Zhao *et al.*, 1991).

His-tag purification

Histidines exhibit highly selective binding to certain 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. Although only 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 6xHis tags. We have developed several other His-tag purification systems, including 6xHN and HAT. These tags possess characteristics favorable for binding to IMAC resins and improve protein solubility and yield (see page 22).

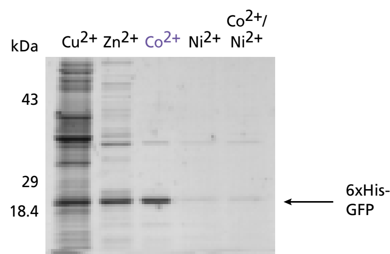


Figure 3. The cobalt ion has higher affinity and specificity for His-tagged proteins. The indicated metals were immobilized onto sepharose CL-6B (Pharmacia) using TALON's unique tetradentate chelator. 20 µl of eluate from the indicated resin was electrophoresed on a 12% polyacryl-amide gel and stained with Coomassie blue.

Sepharose is a registered trademark of Amersham Biosciences Limited.

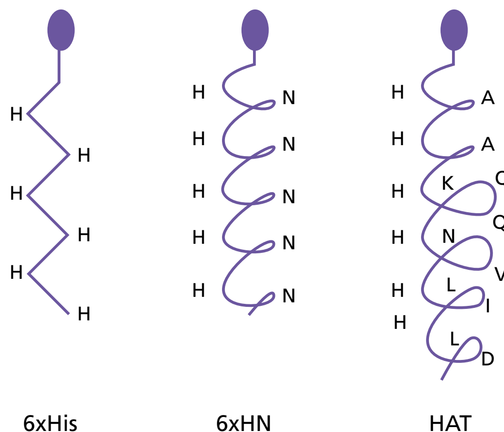


Figure 4. Hypothetical structures of commonly used histidine-affinity tags.

Unique Properties of TALON™ Resin

Reactive core contains cobalt

TALON has a remarkable affinity and specificity for His-tagged proteins (Figures 3, 7, 9, 10, & 11). The TALON reactive core, which contains cobalt, has strict requirements for the spatial positioning of histidines. Only adjacent histidines or specially positioned, neighboring histidines are able to bind cobalt in this reactive core. In nickel-based resins (i.e. Ni-NTA Resin), these spatial requirements are less strict. Therefore, nickel-based resins are also able to bind histidines located in places other than the protein's His-tag (Figure 3).

Uniform matrix

Cobalt-based resins have a more uniform structure than nickel-based resins. All reactive sites in TALON resin look like three-dimensional pockets, similar to the one drawn in Figure 2. In these pockets, cobalt is bound to three carboxyl groups and one nitrogen atom, and is able to bind to two other ligands, i.e. two histidines. In this configuration, cobalt is bound very tightly and does not leak out of the resin. Nickel-based resins are less homogeneous in structure because nickel ions can form two different coordination structures. One of them is a three-dimensional pocket, similar to TALON. The other structure is planar (flat). In this distorted, planar structure nickel is bound to only two carboxyl groups and one nitrogen atom. Since this binding is not very strong, planar reactive cores are not able to hold nickel ions very tightly. This leads to leaching of the nickel ion from the resin.

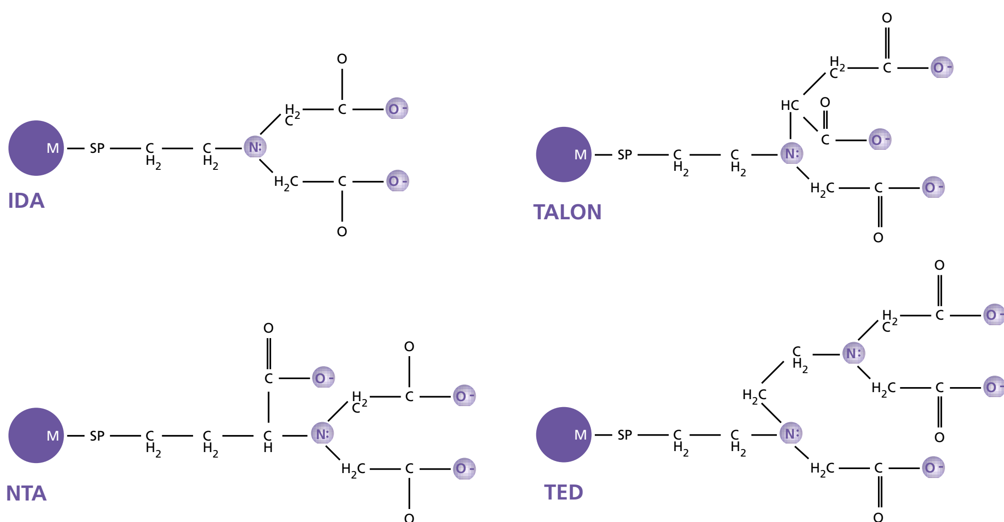


Figure 5. Chemical structures of chelating ligands used in IMAC. Binding groups are colored purple. SP = spacer. M = matrix.

Unique Properties of TALON™ Resin...*cont.*

Comparison with Ni-NTA resin

TALON exhibits subtle yet important differences in character 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). In contrast, TALON binds polyhistidine-tagged proteins with enhanced selectivity over nickel-based resins, and it also exhibits a significantly reduced affinity for host proteins (see page 6; Sulkowski, 1989). This characteristic offers two practical advantages. First, virtually no background proteins are bound to TALON when the sample is applied; consequently, cumbersome washing procedures are not generally required before protein elution. Second, polyhistidine-tagged proteins elute from TALON 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, generating a positively charged ammonium ion, which is repelled by the positively charged metal atom. Alternatively, simply adding imidazole to the elution buffer can competitively elute the bound polyhistidine-tagged protein because imidazole is structurally identical to the histidine side chain and therefore out-competes histidines for resin binding.

Why metal leaching is detrimental to protein purification

During protein purification, metal separates from the reactive core of the purification resin and flows down the column. This is called metal leaching. When metal leaching occurs, it reduces the number of reactive sites available for protein binding on the column, therefore reducing the amount of purified protein obtained.

All metals will leach out of a resin, but nickel leaches more readily than cobalt. Nickel can also precipitate proteins by forming salt bridges, can be toxic to cells and tissues, and can damage purified protein because of its nucleophilic properties. For these reasons, TALON Resin employs cobalt in its reactive core rather than nickel.

Table I: Comparison of TALON™ Metal Affinity Resin vs. Ni-NTA Resin

	TALON™ Resin	Ni-NTA Resin
Metal	Cobalt	Nickel
Metal Ion Complex	Strong	Weak, metal leakage results no metal leakage in lower yields of His-tagged protein and contamination by nonspecific proteins
Sensitivity to β-mercapto-ethanol	Low to negligible sensitivity when concentration < 30 mM	High, resulting in low yields of His-tagged protein
Performance under denaturing conditions	++	+
Performance under nondenaturing conditions	+	+
Reusability	++	+
Nonspecific protein binding	None	Significant

Protein Purification with TALON™

Denaturing vs. native conditions

Purification conditions

Deciding whether to use native or denaturing purification conditions depends on protein location, solubility, accessibility of the histidine tag, downstream applications, and preservation of biological activity. TALON Resin retains its protein binding specificity and yield in a variety of purification conditions. It is stable in both denaturing and native (nondenaturing) conditions.

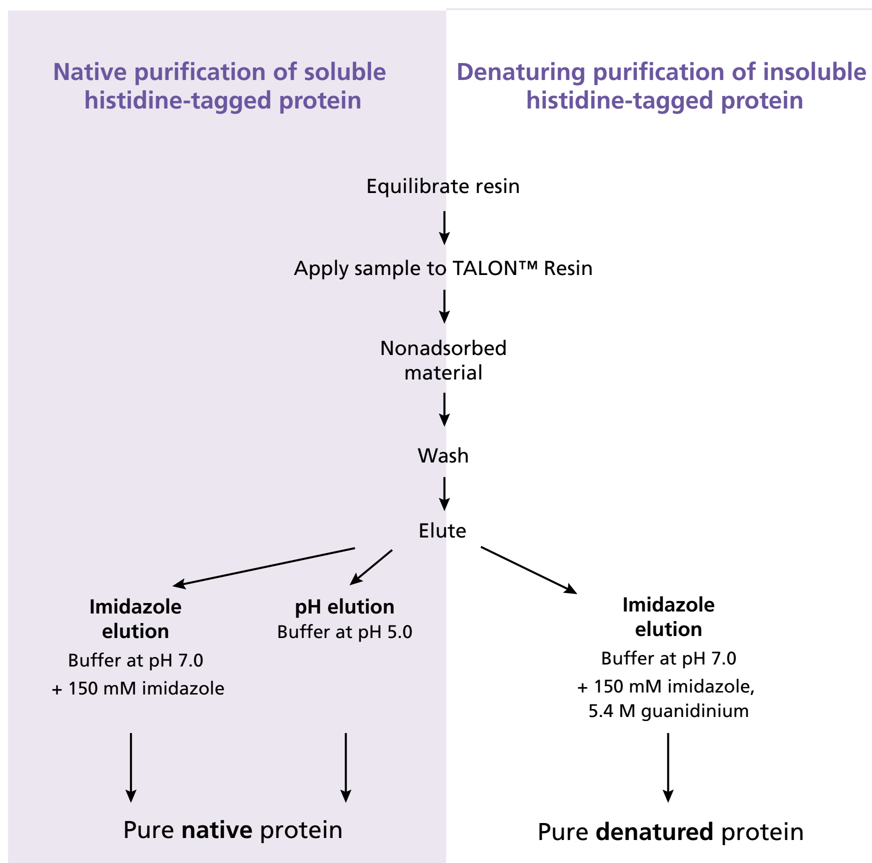


Figure 6. Native vs. denaturing purification procedures.

Why use denaturing conditions?

Denaturants, such as 6 M guanidinium, enhance protein solubility. Because proteins that are overexpressed in prokaryotic systems sometimes form insoluble aggregates called inclusion bodies, you may need to purify proteins under denaturing conditions. Strong denaturants such as 6 M guanidinium or 8 M urea completely solubilize inclusion bodies and 6xHis-tagged proteins. Under denaturing conditions, the 6xHis tag on a protein will be fully exposed so that biding to the matrix will improve, and the potential for nonspecific binding will be greatly reduced.

6xHis-tagged proteins purified under denaturing conditions can be used directly in subsequent applications, or may need to be renatured and refolded. Protein renaturation and refolding can be performed prior to elution from the column (Holzinger *et al*., 1996) or in solution (Wingfield *et al*., 1995). However, yields of recombinant proteins will be lower than under native conditions. This is because urea and guanidinium molecules compete with histidines for binding to metal.

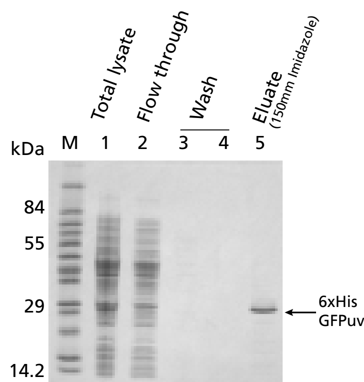


Figure 7. Purification of 6xHis-GFPuv under denaturing conditions. The fusion protein was purified in 8-M urea using TALON resin. M=molecular weight markers.

Protein Solubility

Protein solubility is largely dependent on two factors: the hydrophobicity of the amino acids in the polypeptide backbone, and the ability of the protein to fold correctly. Researchers can use a number of standard methods developed to influence protein solubility. At the level of protein expression, protein solubility can be changed by changing the level of expression. In *E. coli* Recombinant proteins that are overexpressed are frequently found to form protein aggregates called inclusion bodies. Such structures are believed to be masses of the expressed protein that have not folded correctly. Depending on your application, inclusion body formation can frequently be overcome by either reducing the level of expression. Alternatively, switching from 6xHis tag to HAT tag may help to increase protein solubility (see page 22 for details). Sometimes switching to a eukaryotic expression system helps the solubility of expressed protein because eukaryotes have the ability to add post-translational modifications or utilize chaperonin-assisted protein folding. At the level of protein purification, solubility can be increased by changing the temperature or salt concentration, or using reducing agents and denaturants in the method.

Protein Purification with TALON™...*cont.*

Why use native conditions?

Purifying a protein under native conditions is the most efficient method of retaining its biological activity. In order to use native conditions the protein must be soluble. Purification of proteins under native conditions is advantageous not only because you avoid the renaturation step at the end of the purification, but also because native purification will usually copurify enzyme subunits, cofactors, and associated proteins present in the cells (Le Grice, *et al.*, 1990; Flachmann & Khulbrandt, 1996). When renaturing protein after a denaturing purification, it is uncommon to regain more than 2–5% of the activity.

One disadvantage of using native conditions is that unrelated, nontagged proteins are more likely to be nonspecifically bound to the TALON Resin than with denaturing conditions. However, the nonspecific binding can be reduced by including a low concentration of imidazole (5–20 mM) in the wash buffer.

Sometimes the 6xHis tag is concealed by the tertiary structure of the soluble protein, so the protein must be denatured before it can be purified. If purification can only be performed under denaturing conditions, and this does not suit the downstream applications, an inaccessible tag can be moved to the other terminus of the protein. Alternatively, a larger tag like HAT or 6xHN can be used (see page 22).

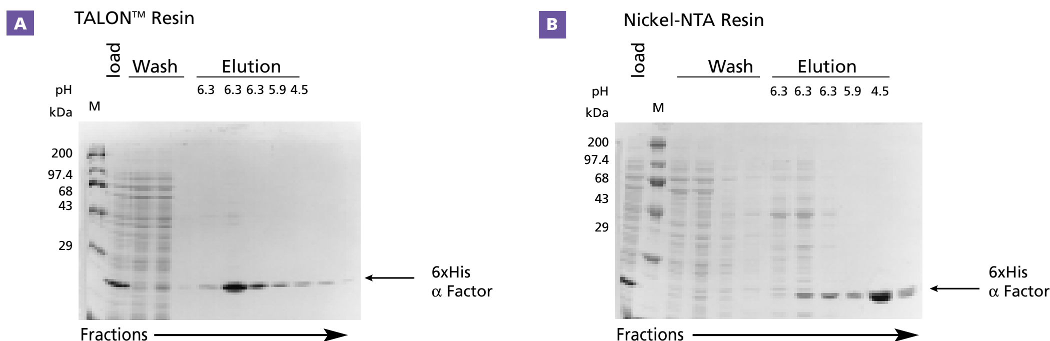


Figure 8. Purification of 6xHis proteins under native conditions compared to purification using Ni-NTA. In comparison with Ni-NTA resin, TALON is more specific for His-tagged proteins. His-tagged proteins can be eluted from TALON at more neutral conditions (pH = 6.3) than from Ni-NTA resins (pH = 4.5). 6xHis-tagged prepro-α-factor was expressed in *E. coli*, lysed and loaded onto each gravity flow column and eluted by a step-wise pH gradient. Purified fractions were analyzed by SDS-PAGE. M=molecular weight markers.

TALON resin preserves the native activity of purified proteins. Figure 9 shows that biological activity of green fluorescent protein (GFPuv) is preserved when purified using TALON Superflow Resin.

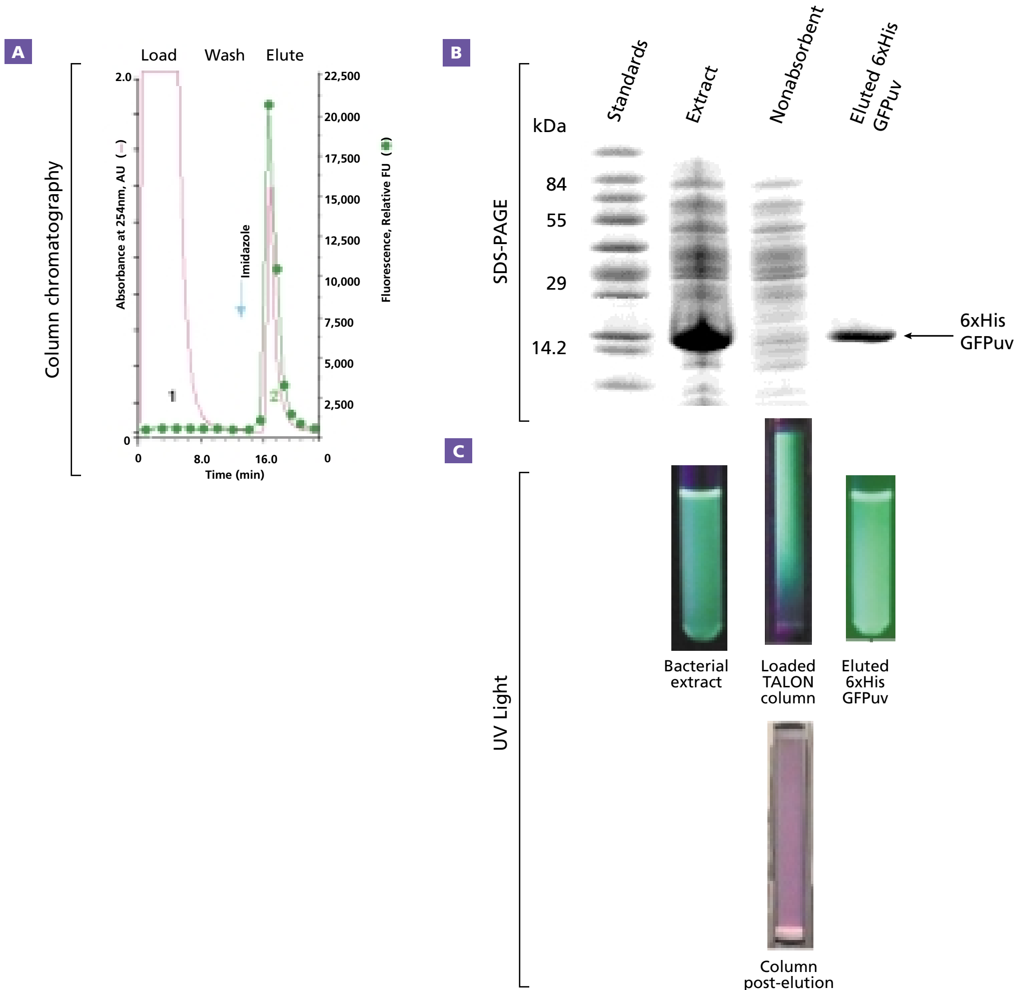


Figure 9. Native purification with TALON™ preserves biological activity of proteins. Fresh cells (0.5 g) expressing 6xHis-GFPuv were extracted in 5 ml of 50 mM sodium phosphate; 0.3 M NaCl, pH 7.0 **Panel A.** Elution profile of GFP which was loaded, washed with the same buffer, and eluted with a step gradient of imidazole (150 mM). **Panel B.** Fractions were analyzed by SDS-PAGE. **Panel C.** Active, intact GFP protein visualized under UV light.

Purification with β-Mercaptoethanol

Why use β-mercaptoethanol in protein purification?

Some intracellular proteins contain reduced sulfhydryl (-SH) groups that are important for the biological activity and structure of the protein. Adding β-mercaptoethanol helps to preserve those -SH groups during purification.

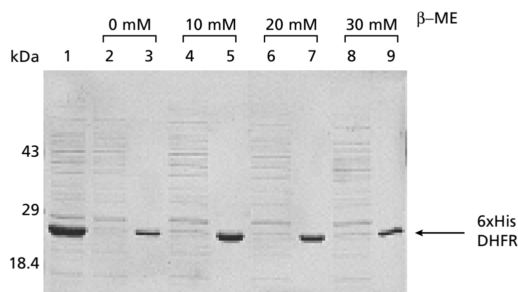


Figure 10. Native Purification of 6xHis protein in the presence of β-mercapto-ethanol. N-terminal 6xHis-tagged mouse DHFR (19.5 kDa) was expressed in *E. coli*. 2 ml of lysate was purified using gravity flow on TALON resin in increasing concentrations of β-mercaptoethanol. Even lanes: 20 µl of non-adsorbed material. Odd lanes: 5 µl of eluate.

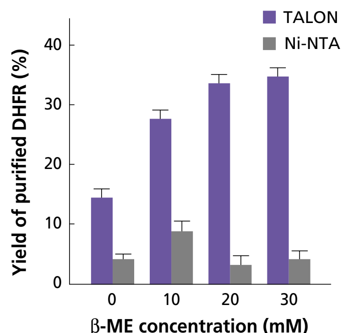


Figure 11. Yields of purification in the presence of β-mercaptoethanol compared to Ni-NTA resin. N-terminal 6xHis DHFR was expressed and purified under native conditions. Protein concentrations were determined by Bradford assay. Yields are expressed as a percentage of total protein in the cell lysate.

Formats of TALON™ Resin

Physicochemical properties

Table II: Physicochemical properties of TALON™ Resins				
Features	**TALON™ Superflow TALON™ Resin**	**TALON™ Resin**	**TALONspin™ CellThru**	**Columns**
Batch/gravity flow applications	Yes	Yes	Yes	No
FPLC applications	No	Yes	Yes	No
Scale	Analytical, preparative, production	Analytical, preparative, production	Preparative, production	Analytical
Capacity (mg protein/ml adsorbent)	5–10	5–8	5–10	2–4
Matrix	Sepharose 6B-CL (6% cross-linked agarose)	Superflow (6% cross-linked agarose)	Uniflow (4% cross-linked agarose)	Silica
Bead size (µm)	45–165	60–160	300–500	16–24
Maximum linear flow rate (cm/hr)*	30	3,000	800	n/a
Maximum volumetric flow rate (ml/min)*	0.5	50	13	n/a
Recommended volumetric flow rate (ml/min)	0.3	1.0–5.0	1.0–5.0	0.3
Maximum pressure	2.8 psi 0.2 bar 0.02 MPa	150 psi 10 bar 0.97 MPa	9 psi 0.62 bar 0.02 MPa	n/a
pH stability (duration)	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

*For washing and elution only.

Sepharose is a registered trademark of Amersham Biosciences Limited.

Protein Purification Procedures

Batch

In batch purification, the sample is applied to a tube containing resin. After incubation, the tube is centrifuged and the supernatant is discarded. The resin is washed with buffer and centrifuged. Then, elution buffer is added and the supernatant is collected after centrifugation.

Batch/gravity flow

Batch/gravity-flow purification means the protein is bound to the resin in solution and then the protein-resin mixture is applied to a column for washing and elution. This procedure gives efficient binding of 6xHis-tagged proteins, most notably when the 6xHis tag is not completely accessible or when the desired protein in the lysate is present in low concentration. By taking this approach, you optimize the time of contact between the resin and your sample. This method is also simpler and requires less equipment than other methods. Batch/gravity flow is usually intended for small-scale purification.

Standard column chromatography

In column purification, the protein binds the resin directly in the column, not in solution as with batch and gravity-flow purification. The resin is first packed into the column and equilibrated with lysis buffer. Then, the cell lysate is applied to the column. Washing and elution steps follow just as in the batch purification procedure. This method affords higher purity of the final product and is also faster than other methods.

FPLC (Fast Protein Liquid Chromatography)

FPLC is a protein purification technique utilizing inert materials, such as glass or plastic, to purify proteins without any metal leaching from the instruments into the protein sample. This method permits you to run chromatography purification at flow rates of 10 ml/min/cm^2 under medium pressure (up to 3 MPA). High flow rates are desirable because you obtain purified protein much more quickly. Fast purification limits the amount of time your protein spends in the presence of proteases (and other impurities) so you get a higher yield of purified product. However, in order to use such high flow rates, the resin must be able to withstand the associated pressure and maintain permeability. TALON Superflow Resin contains specially cross-linked agarose beads that are stronger than conventional agarose beads, so they can be used in FPLC applications. In addition, TALON Superflow beads have high permeability which results in decreased back pressure at elevated flowrates.

Spin column

Spin-column purification is intended for very small-scale, analytical-grade protein purification. This method is employed when purifying only small amounts of protein from many different samples.

Purification using FPLC

TALON™ Resin is available in the TALON™ Superflow format, which is useful for a variety of applications, including medium-pressure applications with FPLC systems at back pressures of up 150 psi (1 MPa). TALON Superflow can be used at high linear flow rates—up to 5 ml/min/cm^2. This resin is recommended if short purification times are essential, or if purification protocols developed for small or medium scale volumes scale need to be scaled up for larger volumes.

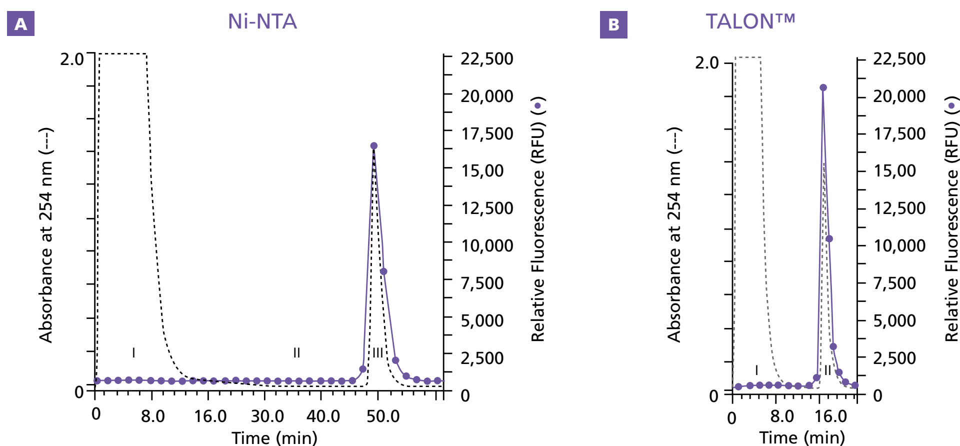


Figure 12. FPLC purification of 6xHis-GFPuv with TALON™ Superflow. Nickel-NTA (**Panel A**) requires longer washing and lower flow rates to purify 6xHis-GFPuv than TALON Superflow (**Panel B**). Protein was extracted in 50 mM sodium phosphate, 0.3 M NaCl, pH 7.0. **Panel A.** 3.2 ml culture filtrate was loaded at 0.5 ml/min. Then nonadsorbed material was washed in the same buffer with 10 mM imidazole. Protein was eluted with 20 mM imidazole (peak II) and 250 mM imidazole (peak III). **Panel B.** 3.2 ml culture filtrate was loaded at 1 ml/min. Then, nonadsorbed material was washed with the same extraction buffer and eluted with 150 mM imidazole (peak II).

Purification using FPLC*...cont.*

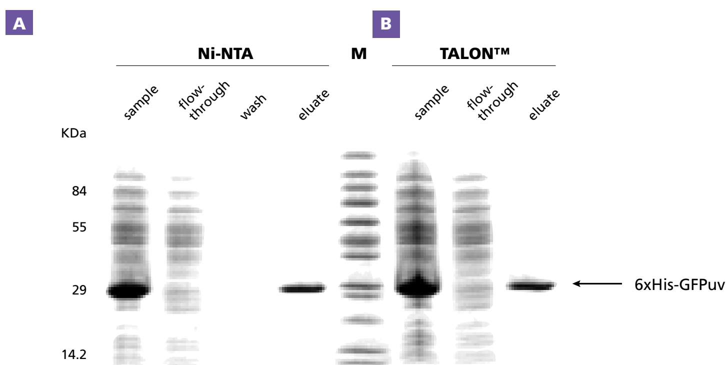


Figure 13. SDS-PAGE of FPLC fractions from 6xHis-GFPuv purification. FPLC purification fractions from the peaks in Figure 11. Purification with TALON Superflow requires less washing with exceptional results.

Table III: TALON™-compatible reagents

Reagent	Acceptable concentration
β-Mercaptoethanol	10 mM (with caution)
CHAPS	1% (with caution)
Ethanol	30% (only for storage)
HEPES	50 mM
Glycerol	20%
Guanidinium-HCl	6 M
Imidazole	200 mM at pH 7.0–8.0 for elution
KCl	500 mM
MOPS	50 mM
NaCl	1.0 M
NP-40	1%
SDS	1% (with caution)
Tris	50 mM
Urea	8 M

Incompatible reagents

- DTT (dithiothreitol)
- DTE (dithioerythritol)
- EDTA (ethylenediaminetetraacetic acid)
- EGTA (ethylene glycol-bis [β-amino-ethyl ether])

Purification from Crude Cell Lysates

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

Advantages of direct capture

Traditionally, obtaining protein from crude cell lysates, such as cell culture and fermentation harvests, requires two steps: isolation, followed by column or batch purification. In the isolation step, the removal of particulate material by centrifugation and/or microfiltration is followed by an initial volume reduction step (typically ultrafiltration). Since conventional chromatography columns are quickly clogged by particles such as cells, cell debris, precipitated proteins, the lysate must be particle-free prior to purification. Therefore, the load must be cleaned before applying it to the column.

However, these centrifugation and filtration steps can be time-consuming and expensive and can also compromise quality. Proteases and glycosidases released from the lysed cells can degrade the target protein, complicate purification, and increase purification costs. The longer the target protein is in the presence of the cell lysate, the more likely it is to be degraded.

One alternative to centrifugation and filtration before loading is a technique called **direct capture**. With direct capture, you can minimize protein degradation, improve product quality and yield, and save time and money. Also, the initial recovery procedure can be simplified if protein capture and debris removal are combined into a single operation. TALON CellThru allows you to purify His-tagged protein directly from crude cell lysates, including serum, tissue extracts, cell culture harvests, fermentation broth and other crude samples on resin-packed, standard low-pressure columns.

A large agarose bead adsorbent is packed into standard chromatography columns whose end-plate frits (filters) have large pores (190 µm) to prevent column blockage. Because of the large bead sizes, particulate material flows between the beads while the soluble product binds to the immobilized metal ions on TALON Resin. Residual particulate material can be removed from the column by using bidirectional high-speed wash pulses. The product is the eluted by normal elution methods.

Expanded bed chromatography vs. top-loading

TALON CellThru can be used in expanded bed chromatography. With this type of chromatography, the crude lysate is applied to the column in an upward rather than downward direction, resulting in increased distance between resin particles (Anspach, *et al*., 1999). Using the upward flow, the bed does not become clogged and a greater amount of protein is recovered.

Expanded bed chromatography integrates solid-liquid separation, volume reduction, and partial purification all into one step. The amount of cellular debris can be reduced up to five orders of magnitude. The combination of increased distance between particles and the large bead size of TALON CellThru allows for excellent protein adsorption without clogging the bed.

Purify Protein from Crude Cell Lysates...*cont.*

The yield from a particular expanded bed or CellThru application depends to a large extent on the efficiency of the extraction procedure in promoting interaction of the target proteins with the resin beads. Incomplete lysis will result in perceived losses of the target protein in the cell debris, which is removed by centrifugation.

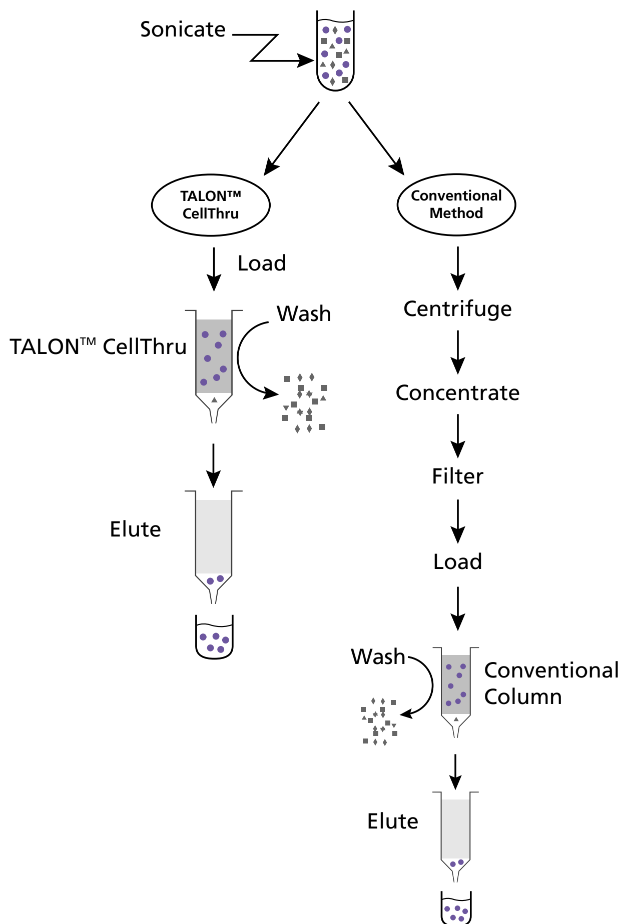


Figure 14. TALON™ CellThru purifies protein from crude cell lysate faster than conventional methods.

Purification from Crude Cell Lysates...*cont.*

CellThru purifies membrane bound proteins and multiprotein complexes

Some proteins are not as easy to access as soluble cytosolic proteins. For example, some recombinant proteins may interact with proteins embedded in the cell membrane (membrane-bound or membrane-associated), while others may be compartmentalized within subcellular organelles. When performing SDS-PAGE analysis, this is generally not apparent because the high SDS and salt concentrations in the sample buffer help solubilize the membranes. Thus, nearly all the proteins present in a cell lysate can be visualized when run on an SDS-PAGE gel.

Purifying membrane-associated proteins with standard TALON Resin is challenging because lysates must be clarified before application to the column. This centrifugation step will usually remove most of the membrane-associated proteins along with the cell membranes and subcellular organelles.

In contrast, with TALON CellThru Resin you can run the crude lysate on the column without centrifuging (direct capture). In this procedure all membranes and unbroken subcellular compartments pass through the column increasing the likelihood of capturing membrane-associated proteins. Therefore, when purifying multiprotein complexes or membrane-associated proteins, TALON CellThru Resin will provide better yields than conventional TALON. However, if a recombinant protein strongly interacts with the membrane or is contained within unbroken subcellular compartments, some proportion of the protein will not be adsorbed by TALON CellThru and will pass through in the wash fractions.

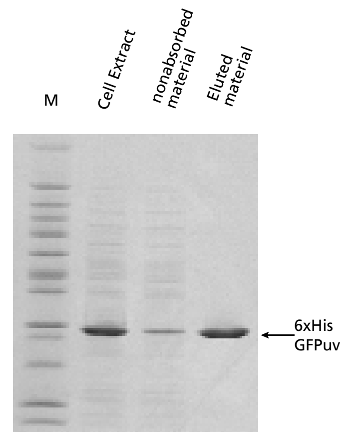


Figure 15. SDS-PAGE of TALON™ CellThru™ purified proteins. *E. coli* BL21 cells were sonicated in TALON wash buffer and run through a TALON CellThru column eluted in 150 mM imidazole. Note that some target protein is trapped in membrane fractions and does not get absorbed on the column. M=molecular weight standards.

Protein Expression Systems

Expression of Recombinant Proteins

Your choice of expression system depends on the downstream application of the purified protein.

Bacteria

Bacterial expression systems are the most popular means of expressing recombinant proteins. Bacteria can be easily transformed with versatile expression constructs and can be easily selected for positive colonies. Bacteria grow rapidly and express high levels of recombinant proteins. In addition, the bacterial genome is relatively well-characterized. BD Biosciences Clontech offers several bacterial expression systems: HAT™, PROTet™, and Creator™-compatible 6xHN-Vectors. However, when expressing in bacterial systems many recombinant proteins become insoluble and are trapped in inclusion bodies. Another drawback is that proteins expressed in bacteria will not have any eukaryotic post-translational modifications.

Yeast

Yeast expression systems are a good alternative when a bacterial expression system will not be adequate because you get expression levels ranging up to several milligrams per liter of culture with most of the eukaryotic post-translational modifications. Some recombinant proteins that are insoluble when expressed in bacteria will be soluble when expressed in yeast because the protein processing is more complex in yeast. BD Biosciences Clontech offers the YEASTMAKER™ Yeast Transformation System and the YEASTMAKER™ Yeast Plasmid Isolation Kit, as well as many types of yeast media and MATCHMAKER Yeast Two-Hybrid System 3. One drawback to using yeast expression; however, is that yeast cells may acidify the culture medium and both the cells and the medium may contain compounds that affect binding of His-tags to the resin. Also, transfecting and lysing yeast cells can be challenging processes.

Baculovirus

Baculovirus vectors are a viral system for expressing proteins in insect cells. Baculovirus systems rely on the principle that baculovirus infects and multiplies in cultured insect cells. This is advantageous because insect cells also recognize most mammalian protein-targeting sequences. Thus, they can express a variety of proteins. Insect cells can also perform many of the post-translational modifications performed in mammalian cells. Recombinant proteins can be produced either within the cells or secreted into the culture medium. We offer the BacPak™ Baculovirus Expression System, for efficient production of high yields of recombinant protein. Despite its many advantages, baculovirus expression can be challenging because baculovirus vectors are sometimes difficult to generate and use in infecting cells. Also, insect cells grow more slowly than bacterial and yeast cells.

Protein Expression Systems*...cont.*

Mammalian cells

Mammalian cells usually provide the best system for generating recombinant eukaryotic proteins because they produce necessary post-translational modifications and recognize the same synthesis and processing signals found in the original organism. We offer several mammalian expression systems, including multiple plasmid-based systems, inducible Tet-On™ and Tet-Off™ Expression Systems, the adenoviral Adeno-X™ System, the inducible adenoviral Adeno-X™ Tet-On™ and Tet-Off™ Systems, and multiple Retroviral Expression Systems.

Mammalian systems can have several drawbacks. The expression levels are sometimes low and mammalian cells grow much more slowly than bacteria or yeast. In addition, it is expensive to grow mammalian cells in large quantities. Mammalian cell transfections are generally less efficient, which contributes to lower overall expression levels in those systems.

Table IV: Comparison of Expression Systems

Expression System	Advantages	Disadvantages	BD Biosciences Clontech Vectors
Bacteria	Easy, fast, can produce a large quantity of protein rapidly	No post-translational modifications, no phosphorylation or glycosylation	PROTet, HAT, 6xHN Vectors
Yeast	Some post-translation modifications, faster than mammalian	Handling may be difficult	MATCHMAKER Vectors
Baculovirus	Large quantities of proteins, some post-translational modifications, secreted and tagged forms	Difficult to handle large vectors	BacPak Vectors
Mammalian	Post-translational modifications	Slow, more complex, expensive	Tet, Retroviral, Adenoviral, CMV Vectors

Protein Expression Systems*...cont.*

Histidine-tag expression systems

Widespread application of recombinant genetic technologies has fostered the production of recombinant proteins containing polyhistidine tags on either the N- or C-terminus (Hochuli *et al.*, 1987; Hochuli *et al.*, 1988). 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 2). 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 "6xHis," "hexaHis," or "(His)6." However, since development and widespread use of this tag, sometimes it was found that the 6xHis tag affects the solubility of expressed proteins. Other tags are now available that exploit the histidine binding, yet have better solubility characteristics.

Histidine tags

The HAT™ sequence (patent pending) is a novel IMAC affinity tag derived from a unique natural protein sequence in chicken lactate dehydrogenase. This tag contains six histidines unevenly interleaved by other amino acid residues (Table V) and does not have the excessive positive charge characteristic of the commonly used 6xHis tag. Thus, HAT-fusion proteins have better solubility and similar affinity towards immobilized transition metal ions and zinc. In addition, 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 HAT-tagged proteins are more stable.

The 6xHN tag is a histidine-rich peptide that has similar solubility and binding characteristics to 6xHis (see page 5), but is more useful than 6xHis when purifying high molecular weight proteins. Generally, it is more difficult for a resin to bind a high molecular weight protein than a low molecular weight protein because the bulk of the larger proteins can interfere with the resin's ability to bind to the histidine tag. The 6xHN tag tends to be more exposed on the surface of the protein than 6xHis, so it is easier for TALON resin to bind the 6xHN tag.

Table V: Histidine Tags

Tag	Amino acids
6xHis	His –His –His –His –His –His
6xHN	His –Asn –His –Asn –His –Asn –His –Asn –His –Asn–His –Asn
HAT	Lys –Asp –His –Leu –Ile –His –Asn –Val –His –Lys –Glu –His –Ala –His –Ala –His –Asn–Lys

Protein Expression Systems*...cont.*

Histidine-tag Expression Systems

The PRO™ Bacterial Expression System is based on a set of tetracycline-inducible prokaryotic expression vectors that allow tightly controlled gene expression over a broad range of induction levels. These vectors combine features that let you:

- **Precisely control expression.** The PRO Vectors produce extremely low background, high expression levels, and precise expression in response to the level of tetracycline inducers.
- **Reduce expression problems.** Controlled expression of a given protein greatly reduces the formation of insoluble inclusion bodies. It also allows the expression of toxic or growth-inhibitive proteins.
- **Purify expressed proteins quickly and easily.** The PROTet 6xHN Vectors encode an N-terminal polyhistidine affinity tag. The 6xHN tag allows proteins to be easily and efficiently purified. All PROTet Vectors contain an enterokinase (EK) site so that the tag can be removed from the protein of interest by proteolytic cleavage.
- **Modify the vectors to suit your expression needs.** The design of PRO Vectors allows them to be easily customized. Each vector consists of three main functional units separated by three unique restriction sites, allowing you to construct new vectors by substituting for any of the three modules independently.

The PROTet Vectors are available in a Creator™-compatible format. The Creator System is our comprehensive, integrated platform enabling gene transfer from a donor vector into multiple expression acceptor vectors without cloning. With Creator-compatible PROTet Vectors, you can easily express your target gene in a highly inducible bacterial expression system. For more information on the Creator System, visit **www.clontech.com**.

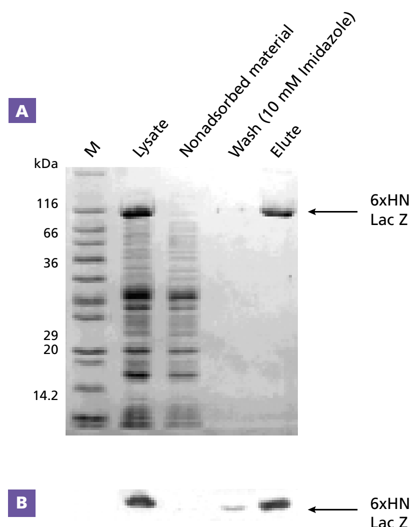


Figure 16. Bacterially expressed 6xHN-tagged protein purified with TALON™ Superflow. Panel A. Coomassie stain of 6xHN-LacZ purification fractions. **Panel B.** Western blot of fractions detects fusion with the 6xHN Polyclonal Antibody. M= molecular weight marker.

The HAT™ System

The HAT™ Protein Expression & Purification System (patent pending) provides a more convenient and efficient way to express and purify proteins. The HAT Vectors encode a novel polyhistidine epitope tag discovered in avian species that enables purification of protein expressed in bacteria under the mild conditions of neutral or physiological pH. The tag is based on a natural poly-histidine peptide, so it is less likely to result in inclusion body formation. The tag is also longer than 6xHis, which may be beneficial for expressing and purifying high molecular weight proteins because the HAT tag tends to be located on the outside of high molecular weight proteins. Therefore, resin can bind the histidine residues more easily than when they are buried within the structure of the protein. In concert with TALON Resin, the HAT Vectors facilitate simplified protein purification under either native or denaturing conditions.

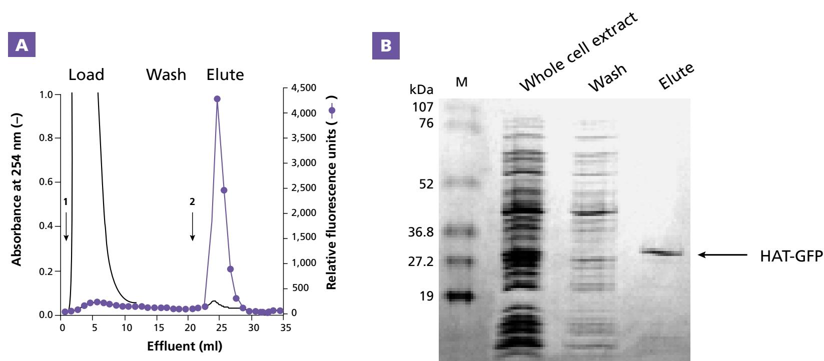


Figure 17. FPLC purification of HAT-tagged protein with TALON™ Superflow. *E. coli* cells were extracted in 50 mM sodium phosphate, 0.3 M NaCl, 5 mM imidazole, pH 7.0 and eluted in 150 mM imidazole. **Panel A.** Cell lysate was purified with a TALON Superflow column. **Panel B.** SDS-PAGE analysis of the procedure. M=molecular weight markers.

HAT Protein Purification

The HAT System offers advantages for native protein purification compared to 6xHis-tag purification protocols that require the use of alkaline buffers (pH 8). Purification at neutral pH is more efficient due to the reduction in binding and elution of impurities, such as non-His-tagged proteins. In addition, purification at neutral pH decreases the activity of basic proteases and generally results in higher protein stability. For proteins that exhibit lower solubility, the HAT System is also suitable for purification under denaturing conditions.

Proteins can be expressed with the HAT tag from pHAT, which contains an enterokinase cleavage site to obtain the native protein. For protein purification, the HAT tag is ideal for use with TALON resin, which selectively binds His-tagged (or HAT-tagged) proteins. TALON requires only low stringency washes to remove nonspecific proteins. We do not recommend using the HAT tag with nickel-based resins because high stringency conditions are required to remove nonspecifically-bound proteins from Ni-NTA columns. Additionally, the HAT tag can easily be incorporated into any other expression vector using PCR cloning.

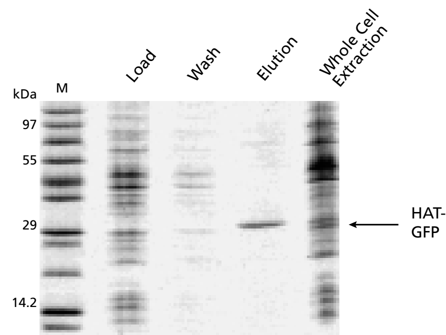


Figure 18. Batch purification of HAT-GFP by pH gradient. SDS-PAGE analysis of protein purification using pH 6.0 elution buffer. Sample was loaded in loading/washing buffer (50 mM sodium phosphate, 0.3 M NaCl, pH 7.0). After washing, the protein was eluted with the same buffer at pH 6.0. M=size markers.

Table VI: The HAT™ System

Features of the HAT™ System	Benefits
Longer tag	Best for high molecular weight proteins
Evenly distributed charge throughout the tag	Higher solubility
Based on unique natural sequence	Lower probability of toxicity to the host cell
Purification at physiological pH	No damage to the target protein

His-tag Antibodies

These antibodies detect histidine-tagged recombinant proteins in Western blot, ELISA, and immunocyto-histochemical assays. They are highly sensitive and specific for proteins bearing the histidine tag that they were raised against.

Albumin-free 6xHis Monoclonal Antibody

This antibody is an IgG2a isotype from mouse ascites fluid. Because this antibody is albumin-free, it provides a high signal-to-noise ratio, as shown in Figure 19, and can detect as little as 1 ng of 6xHis-tagged protein. It comes in a salt-free form for added stability. Antibody-protein complexes can be visualized using any labeled secondary anti-mouse antibody.

6xHis Monoclonal Antibody-HRP Conjugate

This is the same antibody as above and conjugated to horseradish peroxidase (HRP). The HRP conjugate can be used to detect and visualize 6xHis-tagged proteins using chemiluminescent, colorimetric, or fluorometric substrates without requiring a secondary antibody.

HAT™ Polyclonal Antibody

This antibody is raised in rabbits against the 19-amino-acid Histidine Affinity Tag (HAT). This antibody may be used in Western blotting, ELISA, and immunoprecipitation applications to detect or quantify HAT-proteins.

6xHN Polyclonal Antibody (Albumin Free)

An albumin-free rabbit polyclonal antibody for the detection of 6xHN-tagged recombinant protein is available. The antibody can be used for Western blotting and ELISA applications.

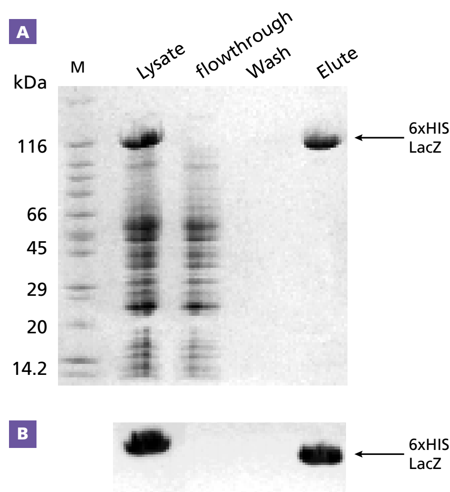


Figure 19. 6xHis Monoclonal Antibody detects 6xHis-LacZ. The protein was purified from lysate on TALON resin by washing in 10 mM imidazole, and then eluting in 150 mM imidazole buffer. **Panel A.** IMAC purification fractions of 6xHis-LacZ on TALON resin were analyzed by SDS-PAGE. **Panel B.** Albumin-free 6xHis mAB was used to detect the protein on a Western blot.

Purification of immunoglobulins

Protein A vs. Thiophilic Resin

Historically, Protein A has been the preferred method of immunoglobulin purification. However, there are certain types of antibodies, such as the single-chain antibodies IgE, IgY, and IgM, that cannot be purified using Protein A. An alternative method of immunoglobulin purification, thiophilic adsorption chromatography, is ideal for these types of applications, as well as immunoglobulin purification in general.

Thiophilic adsorption chromatography (TAC) was developed by Porath *et al.*(1984). TAC is a group-specific, salt-dependent purification technique with distinct adsorption affinity towards immuno-globulins and α_2-macroglobulins. The term "thiophilic" refers to the affinity that proteins have for sulfone groups that lie in close proximity to thioether groups (Figure 22; Porath *et al.*, 1985).

Advantages of Thiophilic Resin

- Broad selectivity for IgE, IgM, IgY and IgG
- Purify single-chain antibodies
- Reduce the number of purification steps
- High recovery rate
- Purification at neutral pH
- High flow rates of 5 cm/min

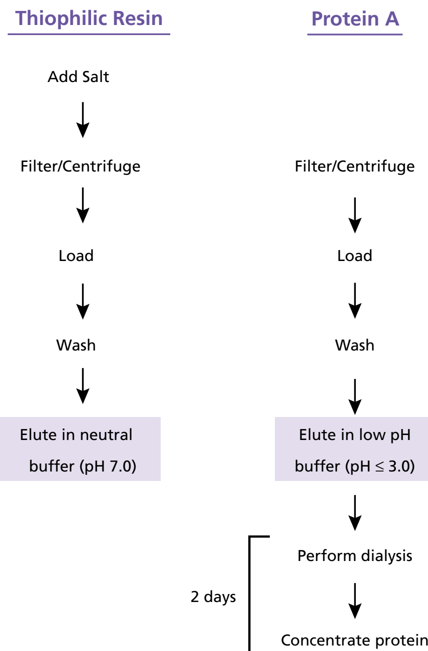


Figure 20. Thiophilic Resin purifies antibodies at neutral pH and more quickly than Protein A.

Purification of immunoglobulins...*cont.*

Purification of single-chain antibodies

Several sulfate salts can be used to promote the adsorption of target proteins. The most commonly used salts are potassium sulfate, sodium sulfate, and ammonium sulfate. In addition, salt concentration can differentially affect the adsorption kinetics of IgG, IgM, IgA, Fab and Fc fragments, and complement factors C3 and C4 (Lutomski *et al.*, 1995; Oscarsson *et al.*, 1992; Schulze *et al.*, 1994; Yurov *et al.*, 1994).

TAC is an economical technique for purifying immunoglobulins from whole serum and tissue cultures (Porath & Belew, 1987; Scoble & Scopes, 1997). In comparison to Protein A-based immunoadsorbents, thiophilic adsorbents have broader affinity towards immunoglobulins (Hutchens & Porath, 1986). Furthermore, >99% of total proteins are recovered using a thiophilic adsorbent in comparison to less than 92% for phenyl and 75% for octyl agarose adsorbents (Oscarsson *et al.*, 1995).

Recombinant, single-chain antibodies are becoming increasingly common in research use because they can be genetically manipulated to bind different proteins and to perform specific, desired functions. However, standard antibody purification methods such as Protein A and Protein G do not work well for single-chain antibodies because these antibodies lack the Fc domain that natural antibodies possess. Protein A usually binds to this Fc domain. Because Thiophilic Resin binds to a region other than the Fc domain on single chain antibodies, it is able to purify them.

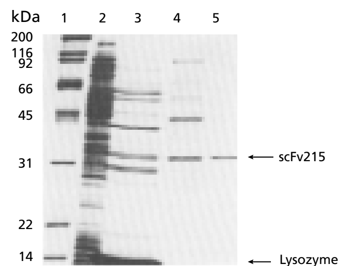


Figure 21. Single-chain antibody purification with Thiophilic Resin. SDS-PAGE analysis of the following samples: Bacterial lysate expressing scFv215 (lane 2), Periplasmic fraction (lane 3), peak fraction from Ni-NTA (lane 4) and peak fraction from thiophilic resin (lane 5). Shultze, *et al.* 1994. Permission to reprint obtained.

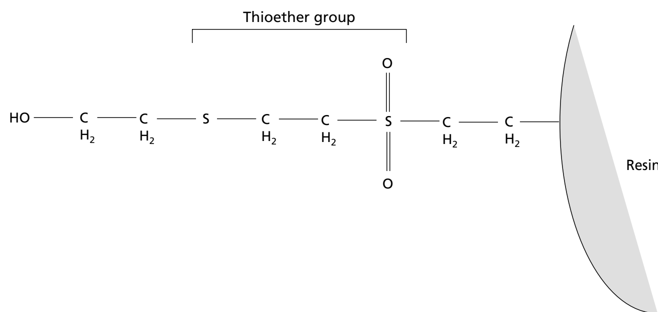


Figure 22. Structure of Thiophilic Resin.

Purification of immunoglobulins*...cont.*

Purification of IgY

Generating antibodies in chickens rather than rabbits is becoming a common method of immunoglobulin production. Antibodies produced in immunized chickens are transferred to the egg yolk, so antibodies can be collected daily from eggs rather than animal serum. Also, higher amounts of a specific immunoglobulin can be obtained from chicken egg yolk than from rabbit serum (Hansen *et al*., 1988). Standard immunoglobulin purification methods do not work well for purifying IgY because IgY does not adsorb to Protein A. In contrast, IgY does adsorb to Thiophilic Resin. Our Thiophilic Resin is ideal for this type of purification because it provides a fast, simple, and inexpensive way to obtain large amounts of purified IgY.

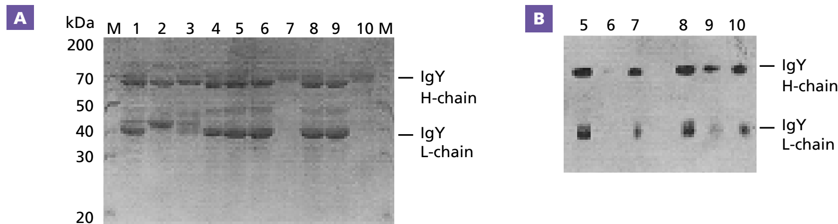


Figure 23. Purification of IgY from chicken egg using Thiophilic Resin. Panel A. SDS-PAGE analysis of fractions from purification of chicken egg immunoglobulins. In lanes 7 & 10, 10 mg of protein was loaded, and all other lanes, 25 mg was loaded. Lane 1: supernatant of egg yolk extract. Lane 2: supernatant after 60% SAS (Saturated concentration of Ammonium Sulfate). Lane 3: Wash with 60% SAS. Lane 4: Pellet after 60% SAS. Lane 5: column load. Lane 6: unbound material. Lane 7: eluted material. Lanes 8–10: purification using another type of thiophilic resin. **Panel B.** Immunoblot of Panel A results. One tenth of the material of the gel in panel A was loaded, then immunoblotted. The IgY was detected with polyclonal rabbit anti-chicken HRP-conjugate. M=molecular weight. (Hansen *et al.* 1998; permission to reprint obtained).

Thiophilic-Superflow and -Uniflow can both be used for batch and gravity-flow purification. Thiophilic-Superflow has greater physical strength, which makes it suitable for FPLC. Table VII (page 32) compares the features of the two different resins.

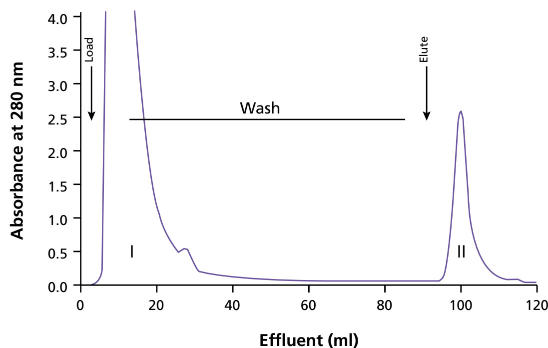


Figure 24. Thiophilic-Superflow Resin purifies IgG at high flow rate and neutral pH. Whole rabbit serum in 0.5 M sodium sulfate was purified on a Thiophilic-Superflow Resin column and eluted with 0.05 M sodium sulfate (peak II).

Thiophilic-Uniflow Resin is prepared using Uniflow 4 agarose cross-linked beads, which permit linear flow rates as high as 2 cm/min. Thiophilic-Superflow Resin is prepared using Superflow 6 agarose cross-linked beads, which permit linear flow rates as high as 5 cm/min. In both cases, the agarose beads were activated with divinylsulfone, and mercaptoethanol was coupled to the activated resin. Both Thiophilic-Uniflow and -Superflow can be regenerated and reused without detrimental effects on specificity and capacity.

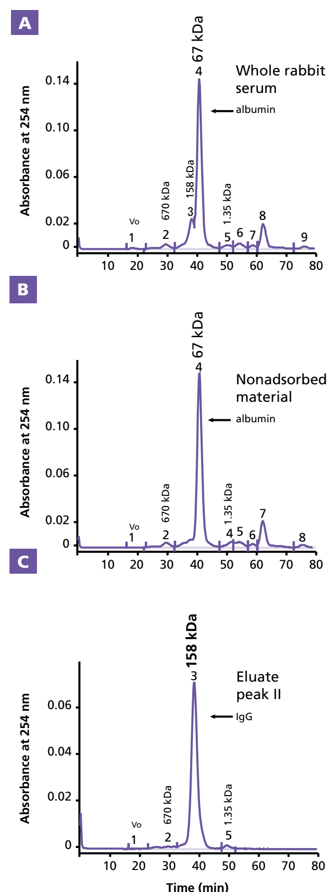


Figure 25. Analysis of purified IgG fractions. Analytical Size Exclusion Chromatography was performed on the purified fractions from Figure 24. Results indicate that the albumin, which constitutes 60–70% of the whole serum, is removed in the non-adsorbed fraction from whole rabbit serum (**Panel A**) and wash (**Panel B**). Then, the intact IgG from Peak II is eluted (**Panel C**).

Table VII: Properties of Thiophilic-Uniflow & -Superflow		
Feature	**Thiophilic-Uniflow**	**Thiophilic-Superflow**
Batch/gravity	yes	yes
FPLC	no	yes
Scale	Analytical	Analytical, preparative
Preparative production capacity (mg IgG/ml adsorbent)	20	25
Matrix	Cross-linked agarose	Cross-linked agarose
Maximum linear flow rate (cm/min)	2.0	5.0
Maximum volumetric flow rate (ml/min) At 5 x 1 cm.i.d	1.6	4.0
pH stability	2–10	2–10
Supplied as	bulk/slurry 50% in 25% ethanol	bulk/slurry 50% in 25% ethanol
Storage	4°C, do not freeze	4°C, do not freeze

Thiophilic adsorbents can also purify other types of proteins such as horseradish peroxidase (Chaga *et al.*, 1992), allergens (Goubran-Botros *et al.*, 1998), glutathione peroxidase (Huang *et al.*, 1994), procollagen (Pedersen & Bonde, 1994), acetolactate synthase (Poulsen & Stougaard, 1989), insect hemolymph proteins (Samaraweera *et al.*, 1992), serpins (Rosenkrands *et al.*, 1994), lactate dehydrogenase (Kminkova & Kucera, 1998), and tuberculosis antigen proteins (Rosenkrands *et al.*, 1998).

Purification of GST-tagged Proteins

GST Tags

Epitope tags such as glutathione-S-transferase (GST) are often used to label proteins for expression and purification applications. Glutathione transferases are abundant enzymes involved in cellular defense against electrophilic chemical compounds, which bind glutathione with high affinity and specificity. The strength and selectivity of this interaction enables glutathione-based affinity resins to effectively purify GST-tagged proteins. The glutathione resin selectively binds the GST-tagged protein under normal conditions, allowing the one protein of interest to be separated from whole cell extracts rapidly and efficiently. A high degree of purification can be achieved in just one chromatographic step (Figure 25).

GST is a 35-kDa protein that can be assayed biochemically as well as immunologically. This characteristic sets it apart from many epitope tags that are simply short peptides. However, the large size of GST results in a higher potential for degradation by proteases than other smaller tags. Therefore, performing GST-protein purification as quickly as possible under non-degrading conditions is necessary in order to minimize sample loss.

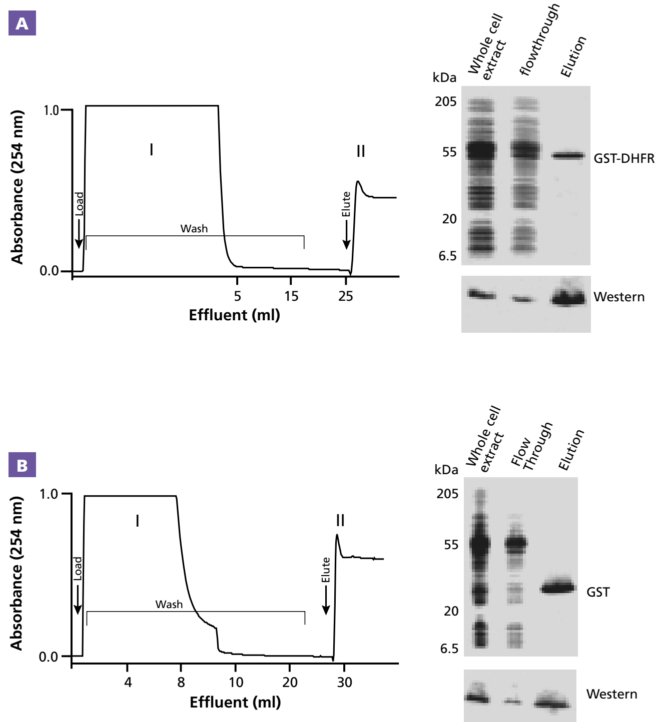


Figure 26. GST-tagged protein purification from whole cell extract. Whole cell extracts containing GST-DHFR (**Panel A**) and GST alone (**Panel B**) were loaded, washed and eluted from glutathione resin columns. Then, the resulting purification fractions were analyzed by SDS-PAGE (upper panels) and Western blot (lower panels) with an anti-GST IgG.

GST loses its ability to bind Glutathione resin when denatured, do not use strong denaturants such as guanidinium or urea in the purification buffers. Check Reagent Compatibilities when designing your purification scheme.

Purification of GST-tagged Proteins*...cont.*

Glutathione Resins

Glutathione-Superflow and -Uniflow Resins allow rapid affinity purification of GST-tagged proteins. These resins are based on 6% and 4% cross-linked agarose, respectively, with glutathione covalently bound to the resins. Both resins possess superior structural and flow characteristics for efficient purification of GST-tagged proteins with minimal degradation during processing (January, 1999 *CLONTECHniques*).

Why use Glutathione-Superflow Resin?

The Glutathione-Superflow Resin is suitable for FPLC applications. It can withstand higher flow rates and back pressure with flow rates as high as 15 ml/cm^2/min. Alternatively, the Glutathione-Uniflow Resin, with a maximum linear flow rate of 2 cm^2/min, is suitable for purification of large fusion proteins using batch/gravity-flow purification or standard chromatography applications.

Why use Glutathione-Uniflow Resin?

For greater convenience, the GST Purification Kit (#K1251-1) provides sufficient stock buffers and prepacked Glutathione-Uniflow Columns for performing five batch/gravity-flow purifications. Up to 10 mg of GST-tagged proteins per column can be purified using the GST Purification Kit.

Notes

Table of Contents
for Protein Purification Protocols

TALON™ Resin Protocols

I. List of Components 37
II. Buffers for TALON™ Purification 38
III. TALON™ Kits Premade Buffers 40
IV. General Considerations 41
    A. General Information 41
    B. Elution Strategy: Imidazole vs. pH Gradient 42
    C. Elution Strategy: Step vs. Linear Gradients 43
    D. Reusing TALON™ Resins 43
    E. TALON™ CellThru™ Considerations 43
V. Test Expression Levels and Purification Strategy 44
    A. Mini-Scale Protein Purification Protocol 44
    B. TALONspin™ Column Purification 45
VI. Sample Preparation 48
    A. Native Proteins 48
    B. Denatured Proteins 49
    C. TALON™ CellThru Sample preparation 49
VII. Protein Purification Protocols 51
    A. Batch/Gravity-Flow Column Purification 51
    B. Large-Scale Batch Purification 53
    C. Medium-Pressure Column (FPLC) Purification using TALON™ Superflow 54
VIII. TALON Regeneration, and Storage 56

Thiophilic Resin Protocols

I. List of Components 58
II. Additional Materials Required 58
III. Purification of IgG from Whole Serum 59
    A. Sample Preparation 59
    B. Low-Pressure Chromatography 59
    C. Batch/Gravity Flow Chromatography 61
IV. FPLC Purification using Thiophilic-Superflow Resin 62
    A. Sample Preparation 62
    B. Preparation of Thiophilic-Superflow Resin for FPLC Purification 62
    C. FPLC Purification 63

Table of Contents...*cont.*

Thiophilic Resin Protocols...cont.

V. General Protein Purification 64
VI. Column Regeneration and Storage 65

Glutathione Resin Protocols

I. List of Components 66
II. Additional Materials Required 66
III. Batch/Gravity-Flow Purification 68
- A. Preparation of Buffers 68
- B. Preparation of GST-fusion Protein Lysate 68
- C. Packing Glutathione Resin into Disposable Gravity Columns 68
- D. Equilibration of Glutathione Resin in the Gravity Column 69
- E. Batch/Gravity-flow Purification of GST Protein 69

IV. FPLC Purification using Glutathione-Superflow Resin 70
- A. Preparation of GST Fusion Protein Lysate 70
- B. Preparation of Glutathione Resin for FPLC Purification 70
- C. FPLC Purification of GST Protein 71

V. Regeneration and storage of Glutathione Resins 71

Appendices

Troubleshooting Guide 72
Reagent Compatibilities 80
References 81

TALON™ Resins

I. List of Components

TALON, TALON Superflow, and TALON CellThru 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 His-tagged protein.

Store TALON Resins, TALONspin Columns and TALON Buffers at 4°C. **Do not freeze.**

- **TALON™ Metal Affinity Resin**

Cat. #	Size
8901-1	10 ml
8901-2	25 ml
8901-3	100 ml
8901-4	250 ml

- **TALON™ Superflow Resin**

Cat. #	Size
8908-1	25 ml
8908-1	100 ml

- **TALONspin™ Columns** (#8902-1, -2, -3, -4)
  These columns contain 0.5 ml of TALON-NX™ Resin as a 50% suspension in nonbuffered 20% ethanol.

- **TALON™ CellThru**

Cat. #	Size
8910-1	10 ml
8910-2	100 ml

- **TALON™ CellThru Disposable Columns**
  2-ml column (#8914-1)
  10-ml column (#8915-1)

- **TALON™ Purification Kit** (#K1253-1)

10 ml	TALON Metal Affinity Resin
160 ml	5X Extraction/Wash Buffer (250 mM Sodium Phosphate, 1.5 M Sodium Chloride, pH 7)
160 ml	5X Extraction Buffer (250 mM Sodium Phosphate, 1.5 M Sodium Chloride, 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** (#K1252-1)

160 ml	5X Extraction/Wash Buffer (250 mM Sodium Phosphate, 1.5 M Sodium Chloride, pH 7)
160 ml	5X Extraction Buffer (250 mM Sodium Phosphate, 1.5 M Sodium Chloride, pH 8)
25 ml	10X Elution Buffer (1.5 M Imidazole, pH 7)

- **TALON™ 2-ml Disposable Gravity Columns** (#8903-1)

II. Buffers for TALON™ Purification

A. Choosing Buffers

If you have not purchased the TALON Purification Kit (#K1253-1) or the TALON Buffer Kit (#K1252-1), we recommend preparing the following buffers for purifying His-tagged proteins under native or denaturing conditions. See Section III for preparing buffers with TALON Purification Kit or Buffer Kit. Before preparing other buffer compositions, please consult Reagent Compatibility Table to evaluate resin compatibility.

To decrease the amount of nonspecifically bound protein, we recommend using the Extraction/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 Extraction Buffer at pH 8.0 (in place of the Extraction/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 TALON Resins, which decreases resin specific 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.

B. 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. TALON's low affinity 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 Extraction/Wash Buffer (pH 7.0)**
  - 50 mM Sodium Phosphate
  - 300 mM NaCl
- **1X Extraction Buffer** (pH 8.0)
  - 50 mM Sodium Phosphate
  - 300 mM NaCl
- **1X Elution Buffer**

Imidazole Elution (pH 7.0)		pH Elution (pH 5.0)	
50 mM	Sodium Phosphate	50 mM	Sodium Acetate
300 mM	NaCl	300 mM	NaCl
150 mM	Imidazole		

C. Denaturing Buffers

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

In the presence of 6 M guanidinium, TALON's color will change from a pinkish-mauve to violet due to a change in metal ion hydration in response to a change in the chaotrope. After removal of the guanidinium, TALON will return to a pinkish-mauve color. The change to violet does not reflect any change in the physical or chemical binding 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 Extraction/Wash Buffer** (pH 7.0)

50 mM	Sodium Phosphate
6 M	Guanidine-HCl
300 mM	NaCl

- **1X Extraction Buffer** (pH 8.0)

50 mM	Sodium Phosphate
6 M	Guanidine-HCl*
300 mM	NaCl

- **1X Imidazole Elution Buffer** (pH 7.0)

45 mM	Sodium Phosphate
5.4 M	Guanidine-HCl*
270 mM	NaCl
150 mM	Imidazole

D. 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

- **Phosphate Buffered Saline (PBS; pH 7.5)**

	Final conc.	To prepare 2 L of solution
Na_2HPO_4	58 mM	16.5 g
NaH_2PO_4	17 mM	4.1 g
NaCl	68 mM	8.0 g

Dissolve the above components in 1.8 L of deionized H_2O. Adjust to pH 7.5 with 0.1 N NaOH. Add deionized H_2O to final volume of 2 L. Store at room temperature

*Before SDS-PAGE analysis, guanidinium must be exchanged with 8 M urea.

III. TALON™ Kits Premade Buffers

If you have purchased the TALON Purification (#K1253-1) **or Buffer Kits** (#K1252-1), prepare buffers as described below. To decrease the amount of nonspecifically bound protein, we recommend using the Extraction/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 Extraction Buffer (pH 8.0) in place of the Extraction/Wash Buffer during all extraction and wash steps. See page 38 and 42 for more information on choosing a buffer.

A. Extraction Buffer

1. Dilute one part of the 5X Extraction/Wash Buffer or 5X Extraction Buffer with four parts of deionized water.

2. Check and correct pH if necessary. The 1X Extraction/Wash Buffer should be at pH 7.0, while the 1X Extraction Buffer should be at pH 8.0.

B. Elution Buffer

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

C. Denaturing Buffer

Add 6-M guanidinium to the Extraction/Wash Buffer (pH 7.0), or Extraction 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. Unfortunately, protein samples containing high guanidinium concentrations form a precipitate when loaded on SDS -PAGE gels. Therefore, dialyze the sample overnight in a buffered solution containing 8 M urea before loading it onto the gel.

D. Wash Buffer

- In general, use the Extraction/Wash Buffer at pH 7.0 to wash nonadsorbed proteins. If the protein is not stable at pH 7.0, then use the Extraction 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 5X Elution Buffer in either 1X Extraction/Wash Buffer or 1X Extraction Buffer for a final concentration of 5–10 mM imidazole (1:300–1:150 dilution).

Note: If a small amount of 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.

IV. General Considerations

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 Extraction/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 Extraction Buffer at pH 8.0 (instead of the Extraction/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 Extraction/Wash Buffer (pH 7.0), may improve the structural stability of fragile proteins during sample preparation. See Reagent Compatibility Table for more 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 at any concentration.

4. If the cell lysate contains a high level of proteolytic activity, we recommend adding 1 mM EDTA to the Extraction/Wash Buffer (pH 7.0) to inhibit metallo proteases during the extraction. **However, before applying the sample to TALON resin, remove EDTA using a gel filtration column (PD-10, Amersham, Pharmacia) equilibrated with the Extraction/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 easily 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 is not affected by denaturation. In that case, it is preferable to use native conditions for extraction even if only 5–10% of the target protein is soluble.

6. The buffer volumes in the following protocols were optimized for purifying the HAT-DHFR 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 *et al.*, 1989). See Reagent Compatibility Table for compatible buffer additives.
   Note that EDTA and EGTA are not compatible with the TALON Resins because these reagents strip the cobalt from the resin.

8. Carefully check the sample 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 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 five fold with Extraction/Wash Buffer before applying it to the resin. This procedure should not significantly affect recovery.

9. 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.

10. The buffers and purification conditions should work well for most soluble, monomeric proteins expressed in *E. coli*.

11. Initially, test each different expression system and polyhistidine-tagged protein in small-scale batch purification to determine expression levels and to optimize the protocol (See Section V).

B. Elution Strategy: Imidazole vs. pH Gradient Elution

TALON purification schemes typically use either an imidazole or a pH gradient for washing and elution. Imidazole in the Extraction and/or Extraction/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 II for buffer compositions.

C. Elution Strategy: Step vs. Linear Gradients

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

D. Reusing TALON™ Resins

TALON Resins may be stored and reused up to 3–4 times before discarding or complete regeneration; the exact number of uses depends on the application. To avoid possible cross-contamination, use a particular aliquot of resin for purifying a single type of His-tagged protein. See Section VIII for important information on washing, storing, and reusing TALON Resins.

E. TALON™ CellThru Considerations

The procedure for purifying His-tagged proteins using TALON CellThru is similar to methods for other TALON Resins with the following significant differences.

1. **Extracellular Proteins**
   If there are no chelating agents in the fermentation liquid and the pH is ≥7.0, you can apply the sample directly to a TALON CellThru-prepacked column. Otherwise, a desalting/equilibration step by ultracentrifugation or gel filtration with Sephadex G25 is necessary.

2. **Intracellular Proteins**
   For purifying intracellular proteins, apply the sonicated sample containing your target proteins directly to a TALON CellThru-prepacked column. There is no need for centrifugation. Electrophoresis may reveal that some of the target protein has passed through the column without adsorption. To a large extent, the unabsorbed protein is an insoluble material which is normally removed during high-speed centrifugation. The amount of non-adsorbed target protein will 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 (#8914-1 & #8915-1). 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 adsorbent for 5 minutes before letting it flow through. If necessary, pass the sample through the column a second time.

V. Test Expression Levels and Purification Strategy

A. Miniscale purification

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

- checking for a His-tagged protein
- determining expression levels
- testing buffer conditions

You can also use a TALONspin Column (#8902-1) with this procedure (p. 45).

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

Important Notes

- This protocol is not intended for obtaining highly purified His-tagged protein samples. Furthermore, protein samples eluted with EDTA (Step 19, below) will contain cobalt and EDTA, which may inhibit enzyme activity as well as 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 Extraction 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 that was prepared as described in Section VI.B.1–6 to a clean 1.5-ml tube containing 50 µl of prewashed TALON Resin, . Start with 100 µl of resuspended TALON Resin 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 Extraction Buffer.

12. Vortex for a few seconds.

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.

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 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 6xHis protein

    Note: EDTA removes bound metal from the resin; the protein sample will contain cobalt, and the TALON Resin cannot be reused unless completely regenerated as described in Section VIII.D.

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.

B. TALONspin™ Column Purification

Important

- Before proceeding with purification, determine the concentration of polyhistidine-tagged protein in your sample using the miniscale procedure (Section V. A). 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 Guide for tips on reducing sample viscosity.

- If the concentration of His-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 7–13 (below) until the desired amount of lysate has been processed. Alternatively, concentrate the His-tagged protein by reducing the sample volume.

- The centrifugation rotor and speed may affect your results. Ideally, 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 micro-centrifuge 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.
2. Snap off the breakaway seal.
3. Place column in the 2-ml microcentrifuge tube.
4. Save white end-cap for later use.
5. 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.
6. Remove column from centrifuge, and place the white end-cap over the male luer fitting.
7. Add 1-ml 1X Extraction/Wash Buffer and mix briefly to pre-equilibrate the resin.
8. Re-centrifuge at 700 x g for 2 min to pellet the resin. Discard the flowthrough.
9. Repeat Steps 7 and 8, twice.
10. Add the clarified sample from Section V.A or V.B to the resin.
11. Add 0.6–1 ml of sample to the column, and replace the clear top-cap.
12. Allow sample to passively wet the resin bed for 30 sec.
13. Mix or vortex contents briskly for a 1–2 sec, completely resuspending the resin in the lysate.
14. Gently agitate the suspension for 5 min to allow His-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. Discard the flowthrough, but save the 2-ml tube.
19. Place microcentrifuge tube in rotor.

20. Place white end-cap on the column, and add 1 ml of 1X Extraction/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 seconds 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 semidry, 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.

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 polyhisidine-tagged protein can usually be recovered in 800–1200 µl of Elution Buffer. If necessary, use a larger Elution Buffer volume or repeat Steps 30–35.

37. Determine polyhistidine-tagged protein yield using gel or spectrophotometric analysis.

VI. Sample Preparation

A. Native Proteins

This procedure can be used with any TALON Resin and TALON Superflow Resin. For CellThru Sample preparation, see Section VI. C.

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 mini-scale purification, and then analyze a portion by SDS-PAGE in parallel with protein standards. Once satisfactory expression is observed, proceed with the appropriate purification protocol.

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.

2. Resuspend the cell pellet by vortexing in 2 ml of chilled 1X Extraction/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.

3. Add lysozyme to the 1X Extraction/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. 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, #A-2039) for 2–3 min or until the composition of the mixture becomes paste-like. Add 2 ml chilled 1X Extraction/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 Extraction/Wash Buffer in order to inhibit metalloproteases during the extraction. Before application of the sample to the TALON adsorbent, EDTA must be removed by gel filtration chromatography (PD-10, Amersham, Pharmacia) equilibrated with the Extraction/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. Store a small portion of the clarified sample at 4°C for SDS-PAGE analysis.

B. 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 Extraction/Wash Buffer (pH 7.0) 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 for SDS-PAGE analysis. Then proceed with the appropriate purification protocol (below).

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

C. TALON™ CellThru Sample Preparation

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.

2. Resuspend the cell pellet by vortexing in 2 ml of chilled 1X Extraction/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 Extraction/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.

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 Extraction/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 (Section VII).

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

VII. Protein Purification Protocols

A. Batch/Gravity-Flow Column Purification

For IMAC column using TALON, we recommend a hybrid batch/gravity-flow procedure. This method combines the speed and convenience of a batch procedure with the higher 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.
4. Remove and discard the supernatant.
5. Add 10 bed volumes of 1X Extraction/Wash Buffer and mix briefly to pre-equilibrate the resin.
6. Re-centrifuge 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 to the resin.
9. Gently agitate at room temperature or on ice*for 20 min on a platform shaker to allow the His-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 Extraction/Wash Buffer. Gently agitate the suspension at room temperature or on ice 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 Extraction/Wash Buffer to the resin, and resuspend by vortexing.

    Note: Steps 17–22 can be performed on ice or at room temperature*.

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 Extraction/Wash Buffer.

20. **[Optional]:** If needed, repeat Step 19 with more stringent conditions using 5–10 mM imidazole in 1X Extraction/Wash Buffer (Section II.C).

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 His-tagged protein will be recovered in the first two bed volumes.

22. Use spectrophotometric and SDS-PAGE analysis to determine which fraction(s) contain(s) the majority 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, you might dialyze the fractions overnight against the Extraction/Wash Buffer, and then measure their UV absorbance at 280 nm.

* Incubation on ice will decrease proteolysis

B. 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 the TALON Resin.
2. Transfer the 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 Extraction/Wash Buffer to the resin and apply vacuum.
6. Repeat Step 5 two times.
7. Add crude lysate (TALON CellThru) or clarified sample (TALON & TALON Superflow) 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 Extraction/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 Extraction/Wash Buffer (Section II. C.).
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 analysis 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.

C. Medium-Pressure Column (FPLC) Purification Using TALON™ Superflow

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. Add deionized water to the top of the column to avoid trapping air between the adapter and the resin surface.

5. Insert and adjust the top adaptor. Then, connect the column to the chromatography system.

6. Equilibrate the column with 1X Extraction/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.

7. Apply the clarified sample to the column after filtering it through a 0.22-µm filter and wash with Extraction/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 start to exceed the recommended value (150 psi, 1.0 MPa). If this occurs, then 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.

8. Wash the column with 10–20 column volumes of Extraction/Wash Buffer, or until the baseline at 280 nm is stable. If necessary, wash with 5–10 mM imidazole in Extraction/Wash Buffer.

9. 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.

10. Use spectrophotometric and SDS-PAGE analysis 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.

11. If you plan to store, regenerate, and reuse a TALON Superflow-packed Column, see Section VIII.C.

VIII. TALON Resin Regeneration and Storage

Generally, reuse TALON Resins 3–4 times before discarding. 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 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 2-(N-morpholine)-ethanesulfonic acid (MES) buffer (pH 5.0) 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) and 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% sodium 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% sodium azide.

C. Regeneration of TALON™ Superflow Columns

Purification of His-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 Extraction/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 extraction/wash buffer (10 bed volumes).

E. Regeneration and re-use of TALON™ and TALON™ Superflow resins

The regeneration and re-use of TALON and TALON Superflow Resins depends on the nature and the presence of subcellular particles, hydrophobic proteins, and DNA/RNA in the samples that are applied to the resin. We recommend that this regeneration procedure is not repeated more than 5 times and is used for purification of only one type of protein.

1. Wash the column with five column volumes of 50 mM sodium phosphate; 0.25 M NaCl; 0.3 M imidazole pH 7.5.

2. Wash the column with three column volumes of deionized H_2O.

3. Wash the column with five column volumes of 25% ethanol†.

4. Wash the column with three column volumes of deionized water.

5. Wash the column with five column volumes of 0.2 M EDTA (pH 7.5).

6. Wash the column with 10 column volumes of deionized water.

7. Charge the resin with three column volumes of 100 mM $CoCl_2 \bullet 6H_2O$.

8. Wash the column with ten column volumes of deionized H_2O.

9. Equilibrate the column with 10 column volumes of the respective sonication/loading buffer. Check the pH of the flow through—it has to be the same as that of your buffer.

*All solutions for regeneration of FPLC columns have to filtered through a 0.22-µm filter and degassed before use. Only the 25% ethanol and, if used, the 30% n-propanol has to be degassed when used for regeneration of low pressure/gravity flow columns. The batch procedure can be performed using the same washing steps on a sintered glass filter.

† If the yield from the material balance from the previous chromatography run on the column is lower than 80%, we suggest that the column is washed additionally with 30% n-propanol after the wash with 25% ethanol. This will remove most of the hydrophobic proteins that were adsorbed nonspecifically to the resin. Repacking of the column might be necessary if air accumulates in the column bed during this step. FPLC columns packed with TALON Superflow can be degassed by washing them at elevated flow rates of up to 15 cm/min linear flow rate during Step 6 until all air bubbles are washed out. Do not exceed backpressure of 150 psi (1.0 MPa).

Linear flow rate (cm/min) is the volumetric flow rate (ml/min) divided by the cross section area of the column (πr^2) in cm^2, where $\pi = 3.1416$ and r is the radius of the cross section of the column in cm.

Thiophilic Resins

I. List of Components

Store all components at 4°C.

Thiophilic-Uniflow Resin

Cat. #	Size
8913-1	10 ml
8913-2	100 ml
8913-x	2.5 ml

Thiophilic-Superflow Resin

Cat. #	Size
8917-1	10 ml
8917-2	100 ml
8917-x	2.5 ml

II. Additional Materials Required

The following reagents are required, but not supplied.

- **Sample Buffer:**
  50 mM sodium phosphate; 0.55 M sodium sulfate (pH 7.0)
- **Equilibration Buffer:**
  50 mM sodium phosphate; 0.5 M sodium sulfate (pH 7.0)
- **Elution Buffer:**
  50 mM sodium phosphate
- **Regeneration Buffers:**
  Buffer 1: 50 mM sodium phosphate (pH 7.0; same as Elution Buffer)

  Buffer 2: 35 mM sodium phosphate; 30% n-propanol (pH 7.0)
- **Sodium sulfate**
- **Disposable Gravity Column, such as TALON™ 2-ml Columns (#8903-1)**
- **50-ml filtration bottle**
- **Deionized water**
- **25% Ethanol**
- **0.45-µm filter**
- **Low-pressure Chromatography Column**
- **Peristaltic pump (Must provide flow rates from 0.1–5 ml/min)**
- **Fraction collector**
- **FPLC column (for FPLC purification using Thiophilic-Superflow Resin)**
- **UV spectrophotometer**
- **Chart recorder**

III. Purification of IgG from Whole Serum

PLEASE READ THE ENTIRE PROTOCOL BEFORE BEGINNING

Before starting, prepare buffers as specified in Additional Materials Required (Section II).

The following protocol is optimized for purifying IgG from whole serum using low-pressure or batch/gravity flow chromatography. For FPLC purification, see Section IV. For purifying other immunoglobulins, use the following methods as a general starting point to determine optimal purification conditions. Section V provides a general protocol for purifying other types of proteins.

A. Sample Preparation

Below are two methods for preparing starting samples. The ***Dilution Method*** is recommended for use with small sample volumes and 1-ml columns. The ***Saturation Method*** is recommended for use with larger sample volumes and columns ≥5 ml.

1. Dilution Method

a. Dilute 1 ml of whole serum with 9 ml of Sample Buffer.
b. To extend the life of the adsorbent, filter sample through a 0.45-µm filter.
c. Store on ice.

2. Saturation Method

a. Add 355 mg of anhydrous sodium sulfate to 5 ml of whole serum.
b. Dissolve the salt crystals by gently inverting the container.
c. To extend the life of the adsorbent, filter sample through a 0.45-µm filter.
d. Store on ice.

B. Low-Pressure Chromatography

The following protocol provides general guidelines for packing a 5-ml column. Once packed, you can reuse the column several times. Use 1 ml Thiophilic Resin per 1–3 ml of whole serum or tissue culture supernatant.

Consult the manufacturer's instructions when assembling and packing a column for valuable information concerning column operation and pressures.

1. Column packing and equilibration

Note: Use deionized water to wet the bottom filter of the column. To decrease air bubble formation, leave ~0.5 cm of water inside the column before adding Thiophilic Resin.

a. Thoroughly resuspend Thiophilic Resin.
b. Transfer 10 ml of resin suspension to a 50-ml filtration bottle and allow resin to settle.
c. Remove supernatant and add 5 ml of deionized H_2O.
d. Thoroughly resuspend resin.
e. Degas resin by applying a vacuum to the filtration bottle.
f. Gently resuspend resin.

g. Immediately transfer 10 ml of resin suspension to a low-pressure chromatography column.
h. Fill column with deionized water.
i. Allow resin to settle.
j. To avoid trapping air between the top adaptor and the liquid surface, add adaptor at a 45° angle. Push adaptor gently down to the surface of the resin bed.
k. Run the pump at a flow rate of 0.5 ml/min to avoid trapping air inside the column pump and tubing during assembly. Fill peristaltic pump tube with Equilibration Buffer, and connect tube to column inlet.
l. Equilibrate column with ≥10 column volumes of Equilibration Buffer. Use a flow rate of ≤2 cm/min; therefore, for a column with an internal diameter of 1 cm, use a maximum flow rate of 1.56 ml/min.
m. Check pH (7.0) of the flowthrough to ensure the column is equilibrated.

2. Chromatography

a. Decrease flow rate to ~1 cm/min (e.g., 0.78 ml/min for a column with an internal diameter of 1 cm).
b. Load 1–3 ml of whole serum per 1 ml of Thiophilic Resin.
c. Extensively wash out nonadsorbed proteins with Equilibration Buffer. Collect fractions and measure their absorbance at 280 nm. When the absorbance decreases to ~0.030 AU, switch to Elution Buffer, and collect fractions. In general, ≤10 column volumes of Elution Buffer are needed to efficiently elute IgG.
d. If residual salt affects the activity or the functional assay of the eluted protein, dialysis may be required. Concentrations of ammonium sulfate that are greater than 1.0 M will affect electrophoretic analysis.

C. Batch/Gravity Flow Chromatography

1. Column packing and equilibration

The following protocol provides guidelines for packing a 2-ml gravity flow column.

a. Thoroughly resuspend Thiophilic Resin.
b. Ensure the column is plugged with a stopper. Immediately transfer 2 ml of resin suspension to a 2-ml Disposable Gravity Column (#8903-1).
c. Allow resin to settle.
d. Wash column with five bed volumes of deionized water.
e. Equilibrate column by washing with ≥10 column volumes of Equilibration Buffer.
f. Check pH (7.0) of the flowthrough to ensure the column is equilibrated.

2. Chromatography

a. Saturate the sample to 0.5 M $NaSO_4$ by any of the methods described in Section III. A. Load 1–3 ml of whole serum per 1 ml of Thiophilic Resin.
b. Extensively wash out nonadsorbed proteins with Equilibration Buffer. Collect fractions and measure their absorbance at 280 nm. When the absorbance decreases to ~0.030 AU, switch to Elution Buffer, and collect fractions. In general, ≤10 column volumes of Elution Buffer are needed to efficiently elute IgG.
c. If residual salt affects the activity or the functional assay of the eluted protein, dialysis may be required.

IV. FPLC Purification Using Thiophilic-Superflow Resin

Before starting, prepare buffers as specified in Additional Materials (Section II). For low-pressure or batch/gravity-flow purification, see Section III.

The Thiophilic-Uniflow Resin can be used for FPLC purification, but with linear flow rates lower than 2 cm/min.

A. Sample Preparation

Below are two methods for preparing starting samples. The ***Dilution Method*** is recommended for use with small sample volumes and 1-ml columns. The ***Saturation Method*** is recommended for use with larger sample volumes and columns ≥5 ml.

1. Dilution Method

a. Dilute 1 ml of whole serum with 9 ml of Sample Buffer.
b. To extend the life of the adsorbent, filter sample through a 0.45-µm filter.
c. Store on ice.

2. Saturation Method

a. Add 355 mg of anhydrous sodium sulfate to 5 ml of whole serum.
b. Dissolve the salt crystals by gently inverting the container.
c. To extend the life of the adsorbent, filter sample through a 0.45-µm filter.
d. Store on ice.

B. Preparation of Thiophilic-Superflow Resin for FPLC Purification

1. We recommend a column whose internal diameter is at least 1 cm. Columns similar to Pharmacia's HR 10/2 or HR 10/10 are convenient because a volumetric flow rate of 0.78 ml/min can be used during loading. We recommend a bed length of at least 3 cm.

2. Pack the column according to its manufacturer's specifications. We recommend a linear flow rate of at least 5 cm/min for packing. The linear flow rate is the volumetric flow rate, in ml/min, divided by the area of the cross section of the column (πr^2 where r is the column radius in cm.)

3. Due to the diffusion constraints of antibodies inside the pores of the resin, a relatively low flow rate must be used during loading. The flow rate for washing and eluting can be increased significantly, thus reducing purification time and increasing yield. At a loading linear flow rate of 0.5–1 cm/min, the capacity for antibodies from whole serum or filtered raw ascite fluid is approximately 20–25 mg/ml of resin. Equilibration with Equilibration Buffer can be performed at the same flow rate.

C. FPLC Purification

1. We recommend that you filter your sample through a 0.45-µm filter before FPLC purification. This action will extend the life of the column.

2. During the loading and washing steps, the linear flow rate should not exceed 1 cm/min; therefore, the flow rate should not exceed 0.8 ml/min if your column has an internal diameter of 1 cm. If antibody leakage occurs, the flow rate should be decreased. Once the sample is loaded and the absorbance of the nonadsorbed flowthrough material starts to decrease, you may increase the linear flow rate to 5 cm/min or to 4 ml/min for a column with 1-cm internal diameter. In general, the whole chromatographic purification should not take more than 60 min.

3. Elution can be performed at an elevated flow rate, unless the amount of eluted material is much less than the adsorbed material. Collect 1-ml fractions during chromatography and store them on ice.

4. Use a bicinchoninic acid (BCA) protein assay (Lowry, *et al.*, 1951) as well as SDS-PAGE gel-electrophoresis to identify fractions containing your eluted antibody.

V. General Protein Purification

Thiophilic Resin can purify a variety of proteins in addition to immunoglobulins. The recommendations given below are very general; therefore, you should determine the optimal conditions for each protein.

- It is best to develop an assay or method of detection of a protein, to aid in determining which fractions contain the purified protein of interest.
- Use a portion of the total sample to determine the optimal binding conditions. Then, purify the rest of the sample using those parameters.

Miniscale Trial Procedure

1. Obtain a 5 ml sample of cell extract containing a sufficient amount of target protein activity.
2. Divide extract into five aliquots.
3. Saturate each aliquot with increasing salt concentrations. For example, use 0.1, 0.2, 0.3, 0.4, and 0.5 M sodium sulfate.

Note: The optimal salt concentration should have minimal affect on protein activity.

4. Centrifuge samples at 5,000 x g for 25 min to clarify sample.
5. Collect the clear supernatant.
6. Determine residual activity of the target protein. For the chromatography trial, use the supernatant with the highest salt concentration that contains ≥80% of the initial activity.
7. Perform a chromatography trial. Test the binding affinity of the target protein of the sample to the resin using the protocol outlined in Section III.C.

Protein analysis

8. Using your assay or detection method, analyze the sample flowthrough to determine the distribution of the target protein.
9. If the target protein adsorbs to the Thiophilic Resin, optimize the washing and elution conditions.

   Determine the optimal elution conditions by doing a step-gradient elution. Decrease the salt concentration of the buffer in steps, washing with at least two column volumes with each decrease in salt concentration. Analyze the target protein distribution in each elution step.

   If the target protein has not adsorbed to the resin under any of the starting salt concentrations, switch the salt to ammonium sulfate. With ammonium sulfate, you can adsorb at a higher sulfate salt concentrations (≤4.1 M). Repeat the steps outlined above.

10. If the target protein still does not adsorb, it is possible to obtain significant purification using "negative adsorption"; that is when the majority of unwanted proteins are adsorbed to the resin, allowing the protein of interest to pass through the column unadsorbed (Chaga *et al.*, 1992).

VI. Column Regeneration and Storage

A. Wash column with five column volumes of Regeneration Buffer 1.

B. Wash column with five column volumes of Regeneration Buffer 2.

C. Store column in 25% ethanol at room temperature for 1–2 weeks; alternatively, store column at 4°C for >2 weeks.

Glutathione Resins

I. List of Components

Store all components at 4°C.

Glutathione-Superflow Resin

Cat. #	Size
8911-1	10 ml
8911-2	100 ml
8911-x	2.5 ml

Glutathione-Uniflow Resin

Cat. #	Size
8912-1	10 ml
8912-2	100 ml
8912-x	2.5 ml

Glutathione S-Transferase (GST) Purification Kit (#K1251-1)

Purchase of the GST Purification Kit provides sufficient reagents for performing five batch/gravity flow purifications of up to 10 mg of GST-tagged protein per column.

- **Five Glutathione-Uniflow Columns**
  Each column is prepacked with 1-ml Glutathione-Uniflow Resin.
- **5 x 100 mg of Glutathione** (reduced)
- **10X Extraction/Loading Buffer** (1.4 mM NaCl; 100 mM Na_2HPO_4; 18 mM KH_2PO_4, pH 7.5):

  To prepare the extraction/loading buffer, dilute 4 ml of 10X Extraction/Loading Buffer with 36 ml of deionized water. If necessary, warm the diluted buffer to room temperature to dissolve precipitated salts, and adjust the pH to 7.5. Prepare fresh.
- **Elution Buffer** (50 mM Tris-Base, pH 8.0):
  Dissolve one vial of 100 mg glutathione (reduced) in 10 ml of the elution buffer and adjust the pH to 8.0, if necessary. Prepare fresh.

II. Additional Materials Required

The following reagents are required but not supplied with the Glutathione-Superflow and -Uniflow Resins:

- **Extraction buffer (loading):**
  140 mM NaCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4 (pH 7.5).
- **Elution buffer:**
  10 mM Glutathione in 50 mM Tris-HCl (pH 8.0). Prepare fresh.
- **Regeneration buffers:**
  Buffer 1: 0.1 M Tris-HCl; 0.5 M NaCl (pH 8.5).
  Buffer 2: 0.1 M Sodium acetate; 0.5 M NaCl (pH 4.5).
  Buffer3: 140 mM NaCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4 (pH 7.5).
- **Alumina** (Sigma, #A-2039)

- **Polypropylene tubes**
- **Centrifuge** (pre-chilled to 4°C)
- **TALON™ 2-ml Disposable Gravity Columns** (#8903-1)
- **Deionized H_2O**
- **Ice**
- **Column** (Pharmacia, HR 10/2 or HR 10/10)
- **Mortar/pestle (for Alumina-based protein extraction)**
- **0.45-µm filter (for FPLC applications)**

III. Batch/Gravity-Flow Purification

A. Preparation of Buffers

Prepare the buffers as specified in Additional Materials (Section II). If you have purchased the GST Purification Kit (#K1251-1), dilute and dissolve the premade buffer solutions as specified in Section I.

B. Preparation of GST-fusion Protein Lysate

Note: Solutions containing GST must be kept at 4°C or on ice at all times.

The method given below is a generic one that is applicable for up to 50 g of *E. coli* cells. Other extraction methods can be used with varying recovery and yield. GST loses its ability to bind glutathione resin when denatured. Do not use strong denaturants such as guanidinium or urea in the purification buffers. Check the Reagent Compatibility Table when designing your purification scheme. The batch/gravity-flow protocol can be used with either Glutathione-Superflow or -Uniflow Resins. However, the FPLC purification protocol (Section IV) is only intended for use with the Glutathione-Superflow Resin.

1. Precool the mortar and pestle, centrifuge, and extraction buffer to 4°C. Place a small polypropylene tube on ice.

2. Transfer cells that express your GST-fusion protein to the precooled mortar. We recommend using 1 ml of resin for every 100–500 mg of cell lysate.

3. Grind 1 part cells with 2.5 parts Alumina for 2–3 min, until the composition of the mixture is paste-like.

4. Add 2 ml of the precooled extraction buffer per 100–500 mg of cells. Centrifuge the cell extract in the precooled centrifuge for 20 min at 10,000–12,000 x g. This procedure will pellet any insoluble material.

5. Carefully transfer the supernatant to the clean, pre-chilled tube. **Do not disturb the pellet.** The supernatant is your clarified sample. If you have purchased the GST Purification Kit, proceed to Section D; otherwise, proceed to Section C.

C. Packing of Glutathione Resin into Disposable Gravity Columns

1. Thoroughly resuspend the Glutathione Resin to achieve a homogenous 50% suspension of resin in the storage solution.

2. Immediately transfer 2 ml of resin suspension to a disposable gravity column (#8903-1). Ensure that the bottom of the column is plugged with a stopper.

3. Allow the resin to settle in the column.

D. Equilibration of Glutathione Resin in the gravity column

1. Remove the stopper and drain the storage solution from the column.
2. Add 4 ml of deionized H_2O to the top of the column and allow it to drain. **Do not disturb the resin.**
3. Repeat (Step 2) three times.
4. Equilibrate the column by adding 4 ml of loading buffer. **Do not disturb the resin.** Allow the buffer to drain.
5. Repeat (Step 4) three times.
6. Replace the column's top and bottom stoppers. Place it on ice to prechill the resin.

E. Batch/gravity-flow Purification of GST-fusion Protein

1. Add 1.5 ml of the clarified GST lysate (Section B.5) to the pre-chilled resin in the column.
   Important: Disperse the resin while you are adding the lysate. To do so, rapidly add the lysate directly to the resin or invert the column a few times after adding the lysate.
2. Place the column upright on ice for 20 min to allow the resin to settle in the column.
3. Remove the column from ice.
4. Discard the top and bottom stoppers and drain the nonadsorbed lysate from the column.
5. Wash the resin by adding 4 ml of pre-chilled extraction buffer to the column. **Do not disturb the resin.**
6. Repeat (Step 5) three times.
7. Elute your GST fusion protein by adding 6 x 1 ml of elution buffer to the column. Collect the eluate in 1-ml fractions on ice.
8. Because glutathione absorbs strongly at 280 nm and masks the absorbance of the eluted protein at low loads, use a Bradford protein assay (Bradford, 1976) as well as SDS-PAGE to identify fractions containing your eluted GST fusion protein.

IV. FPLC Purification using Glutathione-Superflow Resin

PLEASE READ THE ENTIRE PROTOCOL BEFORE BEGINNING

Before starting, prepare buffers as specified in Additional Materials Required (Section II). For the batch/gravity-flow purification protocol, see Section III.

The FPLC protocol cannot be used with Glutathione-Uniflow Resin.

A. Preparation of GST-fusion protein lysate

Note: Solutions containing GST must be kept on ice at all times.

The method given below is a generic one that is applicable for up to 50 g of *E. coli* cells. Other extraction methods can be used with varying recovery and yield.

1. Precool the mortar and pestle, centrifuge, and extraction buffer to 4°C. Place a small polypropylene tube on ice.
2. Transfer cells that express your GST fusion protein to the precooled mortar. We recommend using 1 ml of resin for every 100–500 mg of cell lysate.
3. Grind 1 part cells with 2.5 parts Alumina (Sigma A-2039) for 2–3 min, until the composition of the mixture is paste-like.
4. Add 2 ml of the precooled extraction buffer per 100–500 mg of cells. Centrifuge the cell extract in the precooled centrifuge for 20 min at 10,000–12,000 x g. This procedure will pellet any insoluble material.
5. Carefully transfer the supernatant to the clean pre-chilled tube. **Do not disturb the pellet.** The supernatant is your clarified sample. Proceed to Section B, below.

B. Preparation of Glutathione Resin for FPLC Purification

1. We recommend a column whose internal diameter is at least 1 cm. Columns similar to Pharmacia's HR 10/2 or HR 10/10 are convenient because a volumetric flow rate of 0.78 ml/min can be used during loading. We recommend a bed length of at least 3 cm.
2. Pack the column according to its manufacturer's specifications. We recommend a linear flow rate of at least 5 cm/min for packing. The linear flow rate is the volumetric flow rate, in ml/min, divided by the area of the cross-section of the column (πr^2 where r is the column radius in cm.)
3. Due to the slow binding kinetics of GST to glutathione, a relatively low flow rate must be used during loading. The flow rate for washing and eluting can be increased significantly thus, reducing purification time and increasing yield. At a loading linear flow rate of 1 cm/min, the capacity for GST-fusion proteins from whole cell lysates is approximately 1.5 mg per ml of resin. Equilibration with the extraction/loading buffer can be performed at the same flow rate.

C. FPLC Purification of GST-fusion Protein

1. We recommend that you filter your sample through a 0.45-µm filter before FPLC purification. This action will extend the life of the column.

2. During the loading and washing steps, the linear flow rate should not exceed 1 cm/min; therefore, a column with an internal diameter of 1 cm should not exceed a flow rate of 0.78 ml/min. If fusion protein leakage occurs, the flow rate should be decreased. Once the sample is loaded and the absorbance of the nonadsorbed flowthrough material starts to decrease, you may increase the linear flow rate to 5 cm/min or to 4 ml/min for a column with 1-cm internal diameter. In general, the whole chromatographic purification should not take more than 30–45 min.

3. Elution can be performed at an elevated flow rate, unless the amount of eluted material is much less than the adsorbed material. Collect 1-ml fractions during chromatography and store them on ice.

4. Use a Bradford protein assay (Bradford, 1976) as well as SDS-electrophoresis to identify the fractions containing your eluted GST fusion protein. Western blotting may also be used to identify GST-containing bands with GST Monoclonal Antibody (#3818-1).

V. Regeneration and Storage of Glutathione Resins

Note: If you will not be using the column immediately after regeneration of the resin, complete Steps 1–3, skip Step 4, and proceed directly to Step 5.

1. Wash the column/resin with approximately 10 resin volumes of Regeneration Buffer 1.

2. Wash the column/resin with approximately 10 resin volumes of Regeneration Buffer 2.

3. Repeat Steps 2 and 3 twice.

4. Equilibrate the column/resin with 10 resin volumes of Regeneration Buffer 3.

5. Store resin in a 20% ethanol slurry at 4°C.

A. Protein Expression

Problem	Possible Cause	Solution
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 before harvest	Add proper amount of inducing agent
2. Apparent low expression	• Insoluble over-expressed 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 or serum (for Thiophilic Resin) as starting sample for purification after proper buffering

B. Loading/Washing

Problem	Possible Cause	Solution
1. 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, lower its imidazole concentration, or increase the sulfate concentration (for Thiophilic Resin).*

B. Loading/Washing *...cont.*

Problem	Possible Cause	Solution
2. Protein elutes in the wash buffer ...cont.	• Protein degraded during extraction	a) Perform initial purification step more quickly. b) 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. c) For expressed proteins, make a C-terminal construct.
	• Reagent interferes with binding	a) Check Reagent Compatibilities b) Dilute an aliquot of lysate (1:10), or sonicate, and check binding on a small scale. c) Try using a different polyhistidine-tagged protein as a control.
	• Tag is not accessible under native conditions	a) *TALON Resin only:* If the protein fails to bind under native conditions, treat a small aliquot (<1 ml) with 6 M guanidinium and bind to 50 µl of TALON. Then follow the miniscale procedure in Section V. If the target protein is now bound to the resin, then try to move the tag to the other terminus of the protein where it may be more exposed under native conditions. b) *GST only:* GST is denatured.
2. High back pressure during load of sample	• High viscosity due to presence of DNA	a) Use DNase I or b) Dilute sample five-fold before loading on column.

C. Elution

Problem	Possible Cause	Solution
1. **High amount of co-eluted impurities**	• Insufficient wash	Use larger volumes of Extraction/Wash Buffer
	• Buffer compositions are not optimal	a) Check buffers used for sample preparation and wash steps. b) Check pH. The Extraction/Wash Buffer should be pH 7.0. Contaminants will co-elute in buffers with 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 small amounts of nonionic detergent(s); this is particularly important when isolating proteins from a eukaryotic expression system. f) *TALON Resins only:* Add ethylene glycol or glycerol to inhibit nonspecific hydrophobic interactions. g) *TALON Resins only:* add 1–5 mM imidazole to the Extraction/Wash Buffer and use it as a wash step immediately 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.**
	• Covalent attachment (Cys-Cys, disulfide bonds) of impurities to the protein	Use 5–10 mM of β-ME in the Extraction/Wash Buffer. (Not for Thiophilic Resin)

C. Elution...*cont.*

Problem	Possible Cause	Solution
1. High amount of co-eluted impurities...cont.	• Co-purifying histidine rich (for TALON) or sulfone rich (for Thiophilic) proteins	a) For HAT- or His-tagged proteins, use enterokinase to remove HAT tag and re-run IMAC with the mixture. Target protein will pass through the column, while impurities and tag will be adsorbed. *Note: Remove chelating ligands by gel filtration before loading the proteolytic mixture onto TALON Resin.*
		b) Buffer pH is not optimal. Refer to TALON Resin Section II.
		c) Use second purification scheme, such as size exclusion, ion exchange, hydrophobic chromatography, etc.
	• 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 TALON Resin Section IV.A.8.
2. Excessive background after TALONspin Column procedure	• Sample is too viscous	a) Treat sample with DNase I (TALON Section IV.A.8).
		b) Dilute clarified sample with an equal volume of Extraction/Wash Buffer and load as two aliquots.
		c) Increase the number of 1-ml washes.
		d) Use Extraction/Wash Buffer (pH 7.0).
		e) Add 1–5 mM imidazole to Extraction Buffer, pH 8.0 and use it as an intermediate wash step before elution.

C. Elution...*cont.*

Problem	Possible Cause	Solution
2. Excessive background after TALONspin Column procedure...cont.		re-purify a TALONspin sample, perform the following after performing TALON Purification Section V.B:Step 37. (1) Add 4 volumes of Extraction/Wash Buffer to semi-purified sample. (2) Load sample onto another TALONspin Column. (3) Wash twice with 1 ml of Extraction/Wash Buffer. (4) Elute as before (Section V.B.30–35).
3. Column ceases to flow	• Frit or 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, #D-6277) in the Extraction/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.

C. Elution...cont.

Problem	Possible Cause	Solution
4. polyhistidine-tagged proteins do not elute	• Elution Buffer is not optimal	a) Elute with 150 mM imidazole or pH 4.0 buffer. b) *TALON only*: For proteins that will not elute otherwise, 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) *Not for Thiophilic Resin*: Add 1–5 mM β-ME to reduce disulfide linkages. Supplement buffer with 1% nonionic detergent. d) Purify His-tagged protein under denaturing conditions.

D. Changes in Resin

Problem	Possible Cause	Solution
1. Loss of Co^{2+} (TALON only)	• Presence of chelators in sample	Remove chelators from sample by gel filtration and regenerate adsorbent as described in TALON Resin Section VII.D.
2. Gray or brown resin	• TALON Resin was over-exposed to reducing agents or high concentration of β-ME	Completely remove reducing agents, such as DTE or DTT, or by gel filtration chromatography in the presence of β-ME. Reduce β-ME concentration (≤5 mM).
3. Resin particles aggregate or exhibit change in consistency	• DNA crosslinking	a) Increase ionic strength of the buffers by using ≤ 500 mM NaCl or KCl. b) Vigorously sonicate samples before loading 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 before loading on column.

D. Changes in Resin *...cont.*

Problem	Possible Cause	Solution
3. Resin particles aggregate or exhibit change in consistency ...cont.		e) Avoid long-term storage of resin in denaturants.

E. Analysis

Problem	Possible Cause	Solution
1. High background on silver-stained gels	• Nucleic acid contaminant	a) Supplement buffer with 0.5 M NaCl or KCl. Repeat purification
		b) Shear DNA more vigorously.
		c) Use DNAse I in the extraction procedure.
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 functional assays and measure the* A_{280} *of each sample.*
		b) Perform the lysis or sonication procedure on ice.
	• Protein has degraded	a) Keep protein samples at 4°C during purification
		b) Reduce purification time for initial steps.
		c) Add some proteinase inhibitors. Try different proteinase inhibitors.

F. Resin Reuse

Problem	Possible Cause	Solution
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 the re-used resin. b) Replace used resin with fresh resin. c) TALON only: Wash resin with 6 M guanidinium (pH 5.0) and 1% Triton X-100 or SDS, and re-equilibrate before use.
	• Resin is dirty or has not been fully regenerated.	Resin has been damaged or has worn out. These resins are re-usable with proper handling and regeneration. However, they do not last indefinitely. TALON Resins can be reused at least 3–5 times. Thiophilic Resin can be reused more than 10 times if properly maintained. Glutathione Resins can be reused at least 5–10 times.

Reagent Compatibilities

Reagent	TALON™ Resin	Glutathione Resin	Thiophilic Resin
β-Mercaptoethanol, 10 mM	+/–	+ (elution only)	–
CHAPS, 1%	+/–	–	–
DTT	–	–	–
DTE	–	–	–
EDTA	–	+	+
EGTA	–	+	+
Ethanol, 30%	Regeneration only	Regeneration only	Regeneration only
Ethylene glycol, 30%	+	No data	–
HEPES, 50 mM	+	+	+
Glycerol, 20%	+	no data	–
Guanidinium, 6 M	+	–	–
Imidazole, 200 mM pH=7–8	+ (elution only)	+	+
KCl, 500 mM	+	+	+
MOPS, 50 mM	+	+	+
NaCl, 1.0 M	+	+	+
NP–40, 1%	+	+	–
SDS, 1%	+/–	–	–
Phosphate	+	+	+
Tris, 50 mM	+	+	+
Urea	+	–	–

+ = Compatible
– = Not compatible
+/– = Not recommended

Anspach, F.B., Curbelo, D., Hartmann, R., Garke, G., & Deckwer, W.D. (1999) Expanded-bed chromatography in primary protein purification. *J. Chromatogr. A.* **865**:129–44.

Bradford, M. M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254.

Bush, G. L., Tassin, A.-M., Friden, H. & Meyer, D. I. (1991) Secretion in yeast: purification and *in vitro* translocation of chemical amounts of prepro-alpha-factor. *J. Biol. Chem.* **266**:13811–13814.

Chaga, G., Andersson, L., Ersson, B. & Berg, M. (1992) Use of immobilized metal ions as a negative adsorbent for purification of enzymes: application to phosphoglycerate mutase from chicken muscle extract and horseradish peroxidase. *Biomed. Chromatogr.* **6**:172–176.

Flachmann R, Kuhlbrandt W. (1996) Crystallization and identification of an assembly defect of recombinant antenna complexes produced in transgenic tobacco plants. *Proc. Natl. Acad. Sci. U S A* **93(25)**:14966–71.

Glutathione-Uniflow Resin (January, 1999) *CLONTECHniques* **XIV**(1):29.

Goubran-Botros, H., Rabillon, J., Gregoire, C., David, B. & Dandeu, J. P. (1998) Thiophilic adsorption chromatography: purification of Equ c2 and Equ c3, two horse allergens from horse sweat. *J. Chromatogr. B. Biomed. Sci. Appl.* **710**:57–65.

Hansen, *et al.* (1998). Isolation and purification of immunoglobins from chicken eggs using thiophilic interaction chromatography. *J. Immun. Methods* **215**:1–7.

Hemdan, E. S. & Porath, J. (1985a). Development of immobilized metal affinity chromatography II: interaction of amino acids with immobilized nickel iminodiacetate. *J. Chromatogr*. **323**:255–264.

Hemdan, E. S. & Porath, J. (1985b). Development of immobilized metal affinity chromatography III: interaction of oligopeptides with immobilized nickel iminodiacetate. *J. Chromatogr.* **323**:265–272.

Hochuli, E., Döbeli, H. & Schacher, A. (1987) New metal chelate adsorbent selective for proteins and peptides containing neighboring histidine residues. *J. Chromatogr.* **411**:177–184.

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

Holzinger A., Phillips K. S. & Weaver T. E. (1996) Single-step purification/solubilization of recombinant proteins: application to surfactant protein B. *Biotechniques* **20(5)**:804–6.

Huang, K., Lauridsen, E. & Clausen, J. (1994) Selenium-containing peroxidases of germinating barley. *Biol. Trace Elem. Res*. **46**:91–102.

Hutchens, T. W. & Porath, J. (1986) Thiophilic adsorption of immunoglobulins—analysis of conditions optimal for selective immobilization and purification. *Anal. Biochem.* **159**:217–226.

Kasher, M. S., Wakulchik, M., Cook, J. A. & Smith, M. (1993) One-step purification of recombinant human papillomavirus Type 16 E7 oncoprotein and its binding to the retinoblastoma gene product. *BioTechniques* **14**:630–641.

Kminkova, M. & Kucera, J. (1998) Purification of carp (*Cyprinus carpio*) hepatopancreatic lactate dehydrogenase (EC 1.1.1.27). *Prep. Biochem. Biotechnol.* **28**:313–317.

Le Grice, S. F. & Gruninger-Leitch F. (1990) Rapid purification of homodimer and heterodimer HIV-1 reverse transcriptase by metal chelate affinity chromatography. *Eur. J. Biochem* **187(2)**:307–14.

Lowry, O.H., Rosenbrough, N. H., Farr, A. L. & Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.

Lutomski, D., Joubert-Caron, R., Bourin, P., Bladier, D. & Caron, M. (1995) Use of thiophilic adsorption in the purification of biotinylated Fab fragments. *J. Chromatogr. B. Biomed. Appl.* **664**:79–82.

Oscarsson, S., Angulo-Tatis, D., Chaga, G. & Porath, J. (1995) Amphiphilic agarose-based adsorbents for chromatography: comparative study of adsorption capacities and desorption efficiencies. *J. Chromatogr.* A **689**:3–12.

Oscarsson, S., Medin, A. & Porath, J. (1992) Kinetic and conformational factors involved in chemisorption and adsorption of proteins on mercaptopyridine-derivatized agarose. *J. Colloid Interface Sci.* **152**:114–124.

Pedersen, B. J. & Bonde, M. (1994) Purification of human procollagen type I carboxyl-terminal propeptide cleaved as *in vivo* from procollagen and used to calibrate a radioimmunoassay of the propeptide. *Clin. Chem.* **40**:811–816.

Porath, J. (1985). Immobilized Metal Ion Affinity Chromatography—A powerful method for protein purification. In H. Tschelsche (Ed.), *Modern Methods in Protein Chemistry*, Berlin & NY: Walter de Gruyter & Co.85–95.

Porath, J. & Belew, M. (1987) 'Thiophilic' interaction and the selective adsorption of proteins. *Trends Biotechnol.* **5**:225–229.

Porath, J., Belew, M., Maisano, F. & Olin, B. (1984) Group specific protein adsorbents based on "thiophilic" interaction. In B. Rand (Ed.) *Physical Chemistry of Colloids and Macromolecules*, Uppsala, Sweden, 137–142.

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

Porath, J., Maisano, F. & Belew, M. (1985) Thiophilic adsorption—a new method for protein fractionation. *FEBS Letters* **185**:306–310.

Poulsen, C. & Stougaard, P. (1989) Purification and properties of *Saccharomyces cerevisiae* acetolactate synthase from recombinant *Escherichia coli*. *Eur. J. Biochem.* **185**:433–439.

Rosenkrands, I., Hejgaard, J., Rasmussen, S. K. & Bjorn, S. E. (1994) Serpins from wheat grain. *FEBS Letters* **343**:75–80.

Rosenkrands, I., Rasmussen, P. B., Carnio, M., Jacobsen, S., Theisen, M. & Andersen, P. (1998) Identification and characterization of a 29-kilodalton protein from *Mycobacterium tuberculosis* culture filtrate recognized by mouse memory effector cells. *Infect. Immun.* **66**:2728–2735.

Samaraweera, P., Porath, J. & Law, J. H. (1992) Separation of insect hemolymph proteins by cascade-mode multi-affinity chromatography. *Arch. Insect Biochem. Physiol.* **20**:243–251.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Schulze, R. A., Kontermann, R. E., Queitsch, I., Dubel, S. & Bautz, E. K. (1994) Thiophilic adsorption chromatography of recombinant single-chain antibody fragments. *Anal. Biochem.* **220**:212–214.

Scoble, J. A. & Scopes, R. K. (1997) Ligand structure of the divinylsulfone-based T-gel. *J. Chromatogr.* A **787**:47–54.

Shultze, *et al.* (1994). Thiophilic adsorption chromatography of recombinant single chain antibody fragments. *Anal. Biochem.* **220**:212–214.

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

Sulkowski, E. (1989) The saga of IMAC and MIT. *Bioessays* **10**:170–175.

Wingfield, P. T. (1995) Overview of the purification of recombinant proteins produced in *Escherichia coli*. In: Current protocols in protein science, vol. 1, Coligan, J. E., Dunn, B. M., Ploegh, H. L., Speicher, D. W. & Wingfield P. T. eds. John Wiley and Sons, New York, pp 6.1.1–6.1.22.

Yurov, G. K., Neugodova, G. L., Verkhovsky, O. A. & Naroditsky, B. S. (1994) Thiophilic adsorption: rapid purification of Fab2 and Fc fragments of IgG1 antibodies from murine ascitic fluid. *J. Immunol. Methods* **177**:29–33.

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

Related Products

For the latest and most complete listing of all our products, please visit **www.clontech.com**

Product	Cat. No.
• 6xHis Monoclonal Antibody	8904-1
• 6xHis mAb-AP Conjugate	8905-1
• 6xHis mAb-HRP Conjugate	8906-1
• 6xHis Polyclonal Antibody	8940-1
• ProTet™ 6xHN Bacterial Expression System	K1628-1
• HAT™ Protein Expression and Purification System	K6050-1
• HAT™ Polyclonal Antibody	8909-1
• pHAT20 Vector	8921-1
• pTRE-6XHN Vector	6246-1
• PROTet 6xHN Bacterial Expression System	K1628-1
• Creator-Compatible PROTet 6xHN Bacterial Expression System	K1676-1
• HAT Protein Expression & Purification system	K6050-1
• pHAT20 Vector	8921-1
• BacPAK Baculovirus Expression System	K1601-1
• MATCHMAKER two-Hybrid System 3	K1612-1
• YEASTMAKER Yeast Plasmid Isolation Kit	K1611-1
• Tet-Off Gene Expression System	K1620-1
• Tet-On Gene Expression System	K1621-1
• Adeno-X Expression System	K1650-1

For information or to place orders, contact your local BD office or distributor:

ADMINISTRATIVE OFFICES

BD Biosciences Clontech
1020 East Meadow Circle
Palo Alto, California 94303-4230
USA
Tel: 650 424 8222
Fax: 650 424 0133
URL: www.clontech.com

INTERNATIONAL BD OFFICES & DISTRIBUTORS

ARGENTINA
BD ARGENTINA S.R.L.
Tel: 54 11 4551 7100
Fax: 54 11 4551 7400

AUSTRALIA
BD Australia PTY Ltd.
Tel: 02 8875 7000
Fax: 02 8875 7200

AUSTRIA
BD Biosciences Austria
Tel: 43 1 310 6688
Fax: 43 1 310 7744

BELGIUM
BD Biosciences Benelux N.V.
(Belgium)
Tel: 32 5372 0550
Fax: 32 5372 0549

BRAZIL
BD Ind. Cirurgicas Ltda.
Tel: 55 51 5185 9945
Fax: 55 51 5185 9895

Sinapse Biotechnologia
Tel: 55 11 66940539
Fax: 55 11 292 1625

CENTRAL AMERICA & THE CARIBBEAN
BD CCA
Tel: 506 290 7318
Fax: 506 290 7318

CHILE
BD de Chile
Tel: 011 56 2 460 0380
Fax: 011 56 2 460 0306

Biosonda S. A.
Tel: 562 209 6770
Fax: 562 274 5462

CHINA
BD Asia Ltd. (China)
Tel: 86 10 6418 1608
Fax: 86 10 6418 1610

Genetimes, Inc.
Tel: 86 21 34240452
Fax: 86 21 62489823

Gene Co., Ltd.
Tel: 8621 6428 7163
Fax: 8621 6428 7165

COLOMBIA
BD de Colombia LTDA.
Tel: 5 71 345 0510
Fax: 5 71 255 6320

DENMARK
BD Biosciences Denmark
Tel: 45 43 43 45 66
Fax: 45 965 676

EASTERN EUROPE/ MIDDLE EAST/AFRICA
BD Biosciences EMA
Tel: 49 6221 305 161
Fax: 49 6221 305 418

EGYPT
m.p.t. Medicopharmatrade
Tel: 202 749 3734
Fax: 202 749 8311

FINLAND
BD Biosciences Finland
Tel: 358 98 870 780
Fax: 358 98 870 7817

FRANCE
BD Biosciences France
Tel: 33 4 7668 3636
Fax: 33 4 7668 3504

Ozyme
Tel: 33 1 34 602424
Fax: 33 1 34 609212

GERMANY
BD Biosciences Germany
Tel: 49 6221 3417 0
Fax: 49 6221 303 511

GREECE
Bio Sure
Tel: 301 973 5245
Fax: 301 970 3736

HONG KONG
BD ASIA Ltd.
Tel: 852 2575 8668
Fax: 852 2803 5320

Bio-Gene Technology Ltd.
Tel: 852 2646 6101
Fax: 852 2686 8806

HUNGARY
Novo-Lab Bt.
Tel: 36 1 280 3728
Fax: 36 1 282 3728

INDIA
BD India Private Ltd. (India)
Tel: 91 124 638 3219
Fax: 91 124 638 3225

ISRAEL
Biological Industries Co.
Tel: 972 4 9960595
Fax: 972 4 9968896

ITALY
BD Italia SPA (Italy)
Tel: 39 02 4824 0242
Fax: 39 02 4820 3336

JAPAN
BD Biosciences Japan
Tel: 81 3 5324 9609
Fax: 81 3 5324 9636

KOREA
Koram Biotech Corp.
Tel: 82 2 556 0311
Fax: 82 2 556 0828 or 0829

BD Biosciences Korea
Tel: 82 2 3404 3770
Fax: 82 2 557 4048

MALAYSIA
BD Sdn. Bhd. (Malaysia)
Tel: 603 7725 5517
Fax: 603 7725 4990

Brisk Resources
Tel: 603 777 2611
Fax: 603 777 2610

MEXICO
BD DE MEXICO S.A.
Tel: 52 5 5999 8200
Fax: 52 55 5999 8288

Apco Inc./UNIPARTS
Tel: 525 281 4718
Fax: 525 281 4722

NETHERLANDS
BD Biosciences Benelux N.V
(The Netherlands)
Tel: Orders: 31 20 582 94 20
Fax: 31 20 582 94 21

NORWAY& ICELAND
BD Biosciences Norway & Iceland
Tel: 46 8 775 5110
Fax: 46 8 775 5111

PERU
BD Peru
Tel: 58 212 472 3736
Fax: 58 212 442 4477

PHILIPPINES
BD Phils., Inc (Philippines)
Tel: 63 2 807 6073
Fax: 63 2 850 4774

POLAND
BD Biosciences Poland
Tel: 48 22 651 5300
Fax: 48 22 651 7924

PORTUGAL
Merck Lab. S.A. (Division Biocontec)
Tel: 351 21 361 36 20
Fax: 351 21 362 56 15

SINGAPORE
Becton Dickinson & Company
BD Medical (Singapore)
Tel: 65 860 1475
Fax: 65 860 1590

Biomed Diagnostics Private Ltd.
(Singapore)
Tel: 65 298 4347
Fax: 65 298 4723

SOUTH AFRICA
Southern Cross Biotechnology
Tel: (27 21) 6715166
Fax: (27 21) 6717734

SPAIN
BD Biosciences Spain
Tel: (34) 91 848 8185/22/83
Fax: (34) 91 848 8104

SWEDEN
BD Biosciences Sweden
Tel: 46 8 775 5110
Fax: 46 8 775 5111

SWITZERLAND
BD Biosciences AG
Tel: 41 61 48522 84
Fax: 41 61 48522 86

TAIWAN
BD Biosciences Taiwan
Tel: 886 2 2275 5660
Fax: 886 2 2725 1768

Unimed Healthcare Inc.
Tel: 886 22 720 2215/720 2216
Fax: 886 22 723 3666

THAILAND
BD Thailand Ltd.
Tel: 662 643 1371
Fax: 662 643 1381

Delta Laboratory Co., Ltd.
Tel: 66 2 530 7341 4
Fax: 66 2 559 3365

TURKEY
BD Italia SPA
Tel: 39 02 4824 0242
Fax: 39 02 4820 3336

UNITED KINGDOM
BD Biosciences UK
Tel: 44 1256 476500
Fax: 44 1256 476499

VENEZUELA
BD de Venezuela
Tel: 58 212 472 3736
Fax: 58 212 442 4477

BK21788
1850-9A