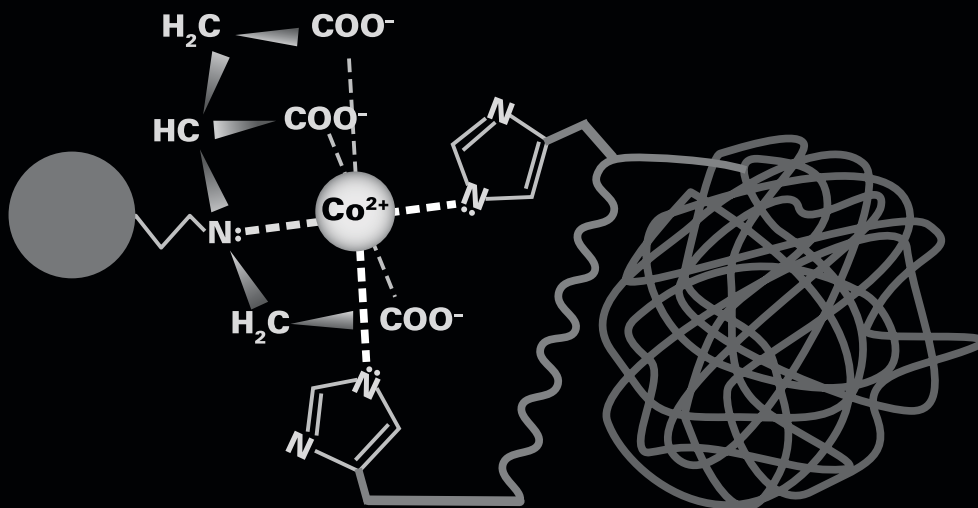


# Clontech Protein Purification Products

Protein Expression & Purification



From Screening to Production—a complete platform



Clontech

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## List of Keywords

Activity

Antibodies

Batch

Baculovirus

$\beta$ -Mercaptoethanol

Compatibility

Denature

Direct Capture

Expanded bed chromatography

Expression Systems

FPLC

Glutathione S-transferase

Polyhistidine-tags

Inclusion bodies

Metal ion leakage

Mammalian

Negative adsorption

pH Gradient

Proteolysis

Regeneration

Solubility

Spin Columns

Immunoglobulins

Yeast

# Protein Purification Introduction

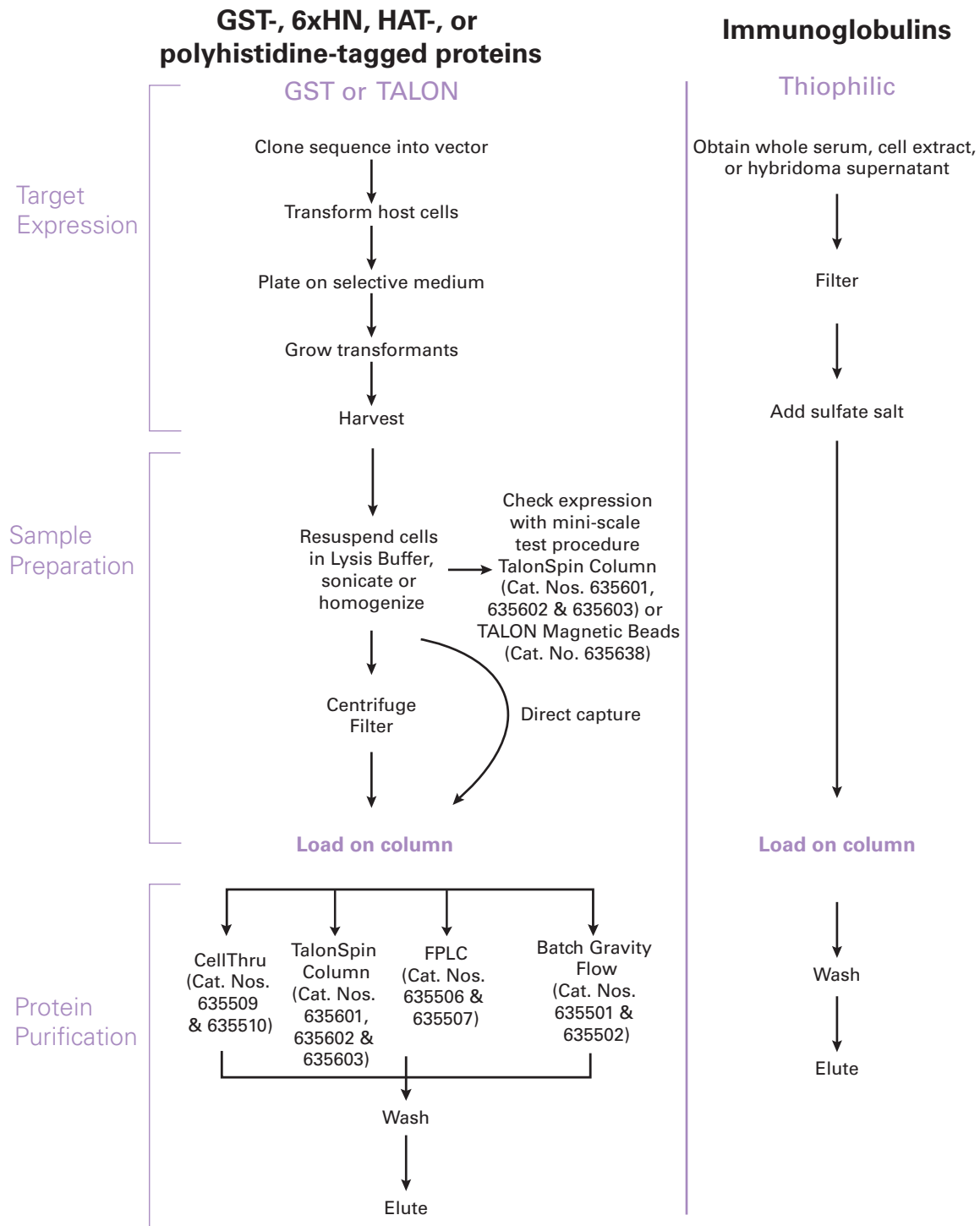


Figure 1. Recombinant protein purification flowchart.

# Protein Purification Introduction

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 proteins, phosphoproteins, phosphopeptides, 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:

- gravity-flow purification
- magnetic-beads based purification
- spin-column purification
- batch purification
- high-throughput 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.

Table I: Protein Purification Products from Clontech

Application	Polyhistidine-tagged proteins	Immunoglobulins Thiophilic Resins	GST-tagged proteins	Phosphoproteins
Small- or medium-scale production	TALON Resins TALON Single Step Columns	Thiophilic Uniflow Resin	Glutathione Resins	Phosphoprotein Enrichment Kit
Low-pressure gravity flow	TALON Resin	Thiophilic Superflow Resin	Glutathione Uniflow Resin	Phosphoprotein Enrichment Kit
Large- or production-scale (FPLC)	TALON Superflow Resin	n/a	Glutathione Superflow Resin	Phosphoprotein Enrichment Kit*
Analytical scale	TALONspin Columns	n/a	n/a	n/a
From crude cell lysate	TALON CellThru	n/a	n/a	n/a
Microscale Purification	TALON Magnetic Beads	n/a	n/a	TALON PMAC Magnetic Phospho Enrichment Kit
High-Throughput Purification	TALON Magnetic Beads TALON HT 96-Well Plate	n/a	n/a	n/a

\* Phosphoprotein resin can be packed in FPLC-compatible columns.

# TALON® Products

For polyhistidine-tagged protein purification

Product	Application
<p><b>TALON Metal Affinity Resin</b></p> <p>Resin ready for loading in columns for small or medium-scale purification of polyhistidine-tagged proteins. Purify &gt; 5 mg protein using 1 ml of resin.</p>	For purification of most cytosolic and secreted polyhistidine-tagged proteins by small-scale or batch/gravity flow, under native or denaturing conditions
<p><b>TALON Superflow Resin</b></p> <p>Specially designed for quick and effective purification of polyhistidine-tagged proteins at high flow rates and medium pressure (up to 150 psi).</p>	For FPLC, medium-pressure chromatography, or scale-up for production applications
<p><b>TALON CellThru</b></p> <p>Novel IMAC resin designed for quick purification of polyhistidine-tagged proteins by direct capture.</p>	For rapid purification of polyhistidine-tagged proteins from crude cell lysates, sonicates, and fermentation harvests.
<p><b>TALONspin Columns</b></p> <p>Ready-made spin columns containing TALON-NX resin for the simultaneous purification of several polyhistidine-tagged proteins in parallel in only 30 minutes.</p>	For small-scale single-use applications such as verifying positive transformants for polyhistidine-tagged protein expression levels, or trial-level purification protocols.
<p><b>TALON Purification Kit</b></p> <p>Convenient kit containing TALON resin, columns, and all the buffers necessary to extract, wash, and elute polyhistidine-tagged proteins.</p>	This kit provides the ideal place to start when using TALON in your applications.
<p><b>TALON Disposable Columns (empty columns)</b></p> <p>Two different types of disposable, single-use columns to be packed by user with TALON Resin and TALON CellThru Resin, respectively.</p>	For use with TALON Resin for regular polyhistidine-tagged protein purification and with TALON CellThru Resin for purification from crude cell lysates.
<p><b>TALON Buffer Kit</b></p> <p>Supplemental kit containing concentrated forms of optimized buffers.</p>	For extracting, washing, and eluting proteins.
<p><b>TALON Magnetic Beads</b></p> <p>Combine TALON chemistry with magnetic bead separation.</p>	For quick and easy microscale purification and high-throughput small-scale purification (25 µg of protein/100 µl of suspension) from cleared crude cell lysates or cultures.
<p><b>TALON HT 96-Well Plate</b></p> <p>A 96-well filtration plate preloaded with TALON Superflow Resin.</p>	For high-throughput analytical scale (0.5-1 mg) purification of up to 96 polyhistidine-tagged proteins in 30 minutes.
<p><b>TALON HT Single Step Columns</b></p> <p>Ready-to-use, disposable columns which combine xTractor Buffer with TALON Resin in a dehydrated format to lyse bacterial cells and bind polyhistidine-tagged protein in one step.</p>	For rapid purification and preliminary characterization of polyhistidine-tagged proteins directly from bacterial expression cultures.

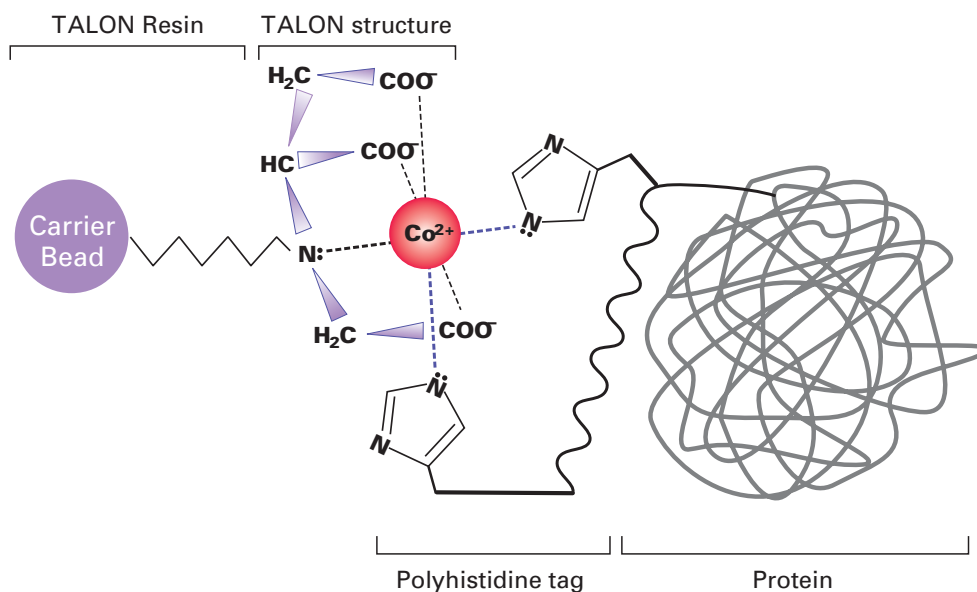
# TALON<sup>®</sup> Metal Affinity Resin

TALON Resins are durable, cobalt-based IMAC resins designed to purify recombinant polyhistidine-tagged proteins (Bush *et al.*, 1991). These resins are compatible with many commonly used reagents, 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-scale batch screening, large-scale batch preparations, and methods using gravity-flow columns, spin columns, and FPLC.

## 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 applying generic 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 allows the protein of interest to 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.



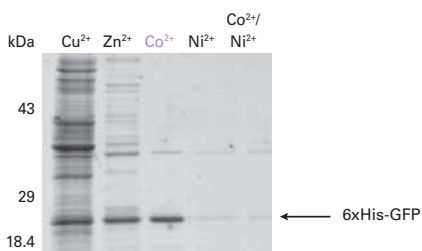
**Figure 2. Molecular mechanism of polyhistidine binding to TALON Resin.** Our patented tetradentate chelator binds the cobalt ion with strong affinity, resulting in improved binding to polyhistidine tags.

## 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).

## Polyhistidine-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 two to 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 alternative polyhistidine-purification tags, including 6xHN and HAT (Table VII). These tags possess characteristics favorable for binding to IMAC resins and can improve protein solubility and yield (see page 25).



**Figure 3. The cobalt ion has higher affinity and specificity for polyhistidine-tagged proteins.** The indicated metals were immobilized onto Sepharose<sup>®</sup> CL-6B (GE Healthcare) using TALON's unique tetradentate chelator. 20  $\mu$ l of eluate from the indicated resin was electrophoresed on a 12% polyacrylamide gel and stained with Coomassie blue.

# Unique Properties of TALON<sup>®</sup> Resin

## Reactive core contains cobalt

TALON has a remarkable affinity and specificity for polyhistidine-tagged proteins (Figures 3, 6, 8, 9 & 10). 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 polyhistidine-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 leakage of the nickel ion from the resin.

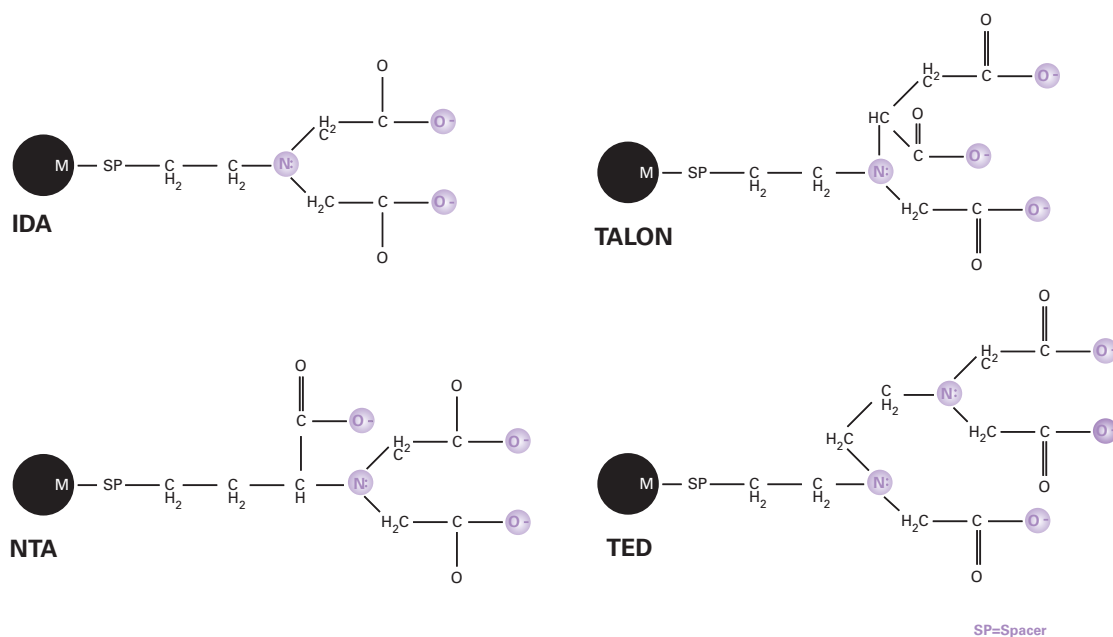


Figure 4. Chemical structures of chelating ligands used in IMAC. Binding groups are colored purple. SP = spacer. M = matrix.

# Unique Properties of TALON<sup>®</sup> Resin...continued

## 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 (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—closer to neutral pH or lower imidazole concentration—than with nickel IMAC resins. Elution occurs when the imidazole nitrogen (pKa of 5.97) is protonated, disrupting the coordination bond with the immobilized ion. 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 leakage is detrimental to protein purification

During protein purification, metal separates from the chelating core of the purification resin and flows down the column. This is called metal leakage. When it occurs, metal leakage reduces the number of reactive sites available for protein binding on the column, therefore reducing the amount of purified protein obtained. Free metal ions also have a detrimental effect on protein activity.

All metals will leak out of a resin, but nickel leaks out 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 chelating core rather than nickel.

Table II: Comparison of TALON Resin with Ni-NTA Resin

	TALON Resin	Ni-NTA Resin
Metal	Cobalt	Nickel
Metal Ion Complex	Strong; lower metal ion leakage	Weak; metal ion leakage results in lower yields of polyhistidine-tagged protein and contamination by nonspecific proteins
Sensitivity to $\beta$ -mercaptoethanol	Low to negligible sensitivity when concentration < 30 mM	High, resulting in low yields of polyhistidine-tagged protein
Performance under denaturing conditions	+++	+
Performance under native conditions	++	+
Reusability	++	+
Nonspecific protein binding	None	++

<sup>1</sup> See McMurry & Macnab, January 2004 *Clontechiques*.

# Protein Purification with TALON<sup>®</sup>

## Denaturing vs. native conditions

### Purification conditions

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

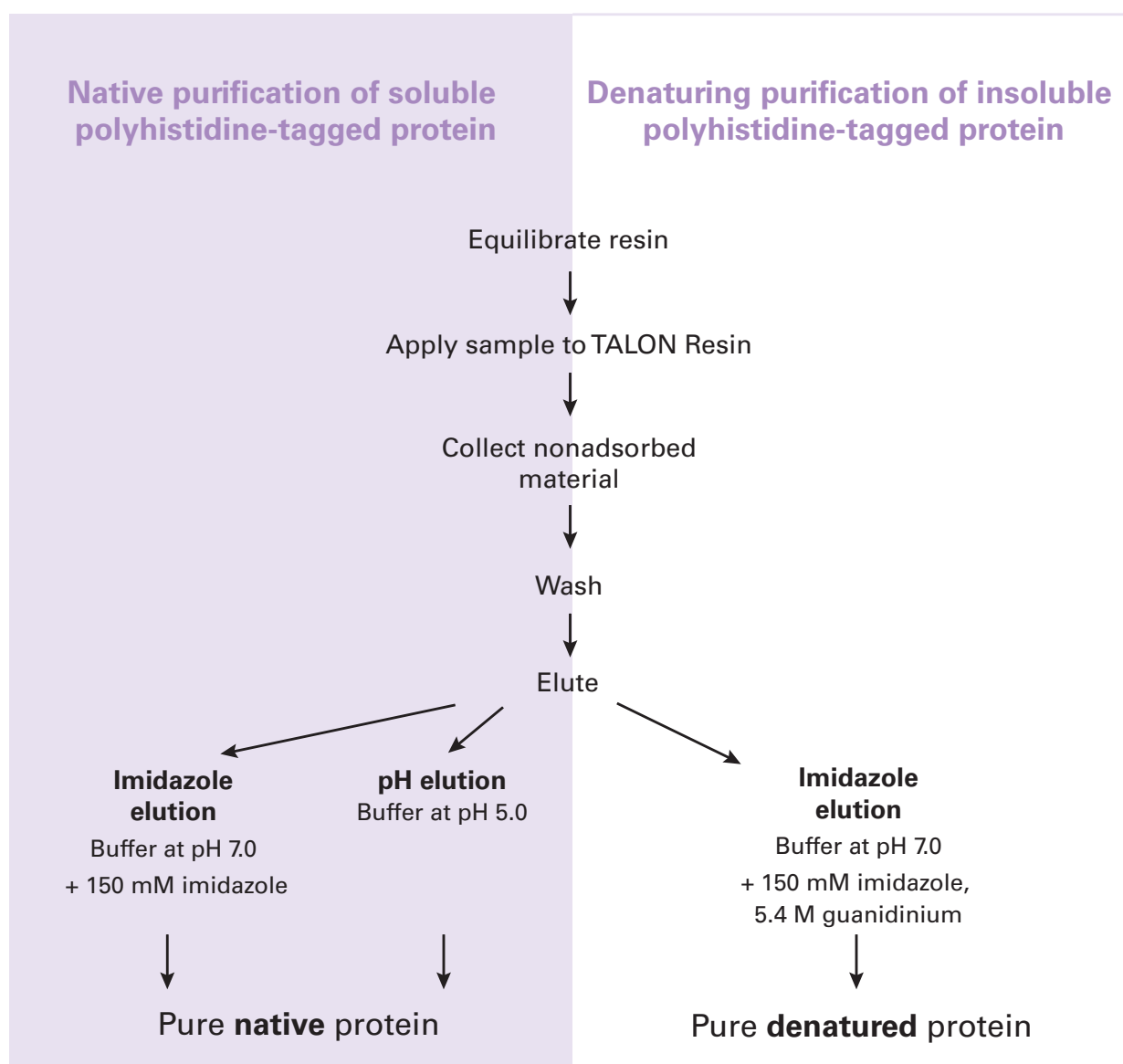
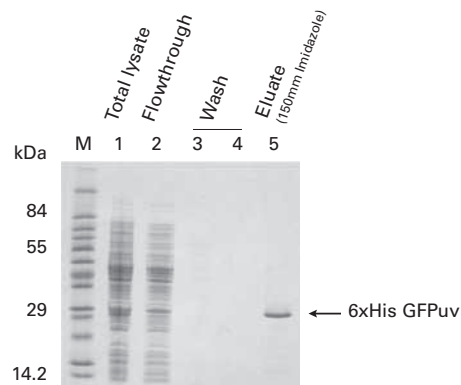


Figure 5. Native vs. denaturing purification procedures.

## 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 polyhistidine-tagged proteins. Under denaturing conditions, the polyhistidine tag on a protein will be fully exposed so that binding to the matrix will improve, and the potential for nonspecific binding will be greatly reduced.

Polyhistidine-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 6. 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 reducing the level of expression. Alternatively, switching from a 6xHis tag to a HAT tag may help to increase protein solubility (see page 25 for details). Sometimes switching to a eukaryotic expression system increases the solubility of expressed protein because eukaryotes have the ability to add posttranslational modifications or utilize chaperone-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.

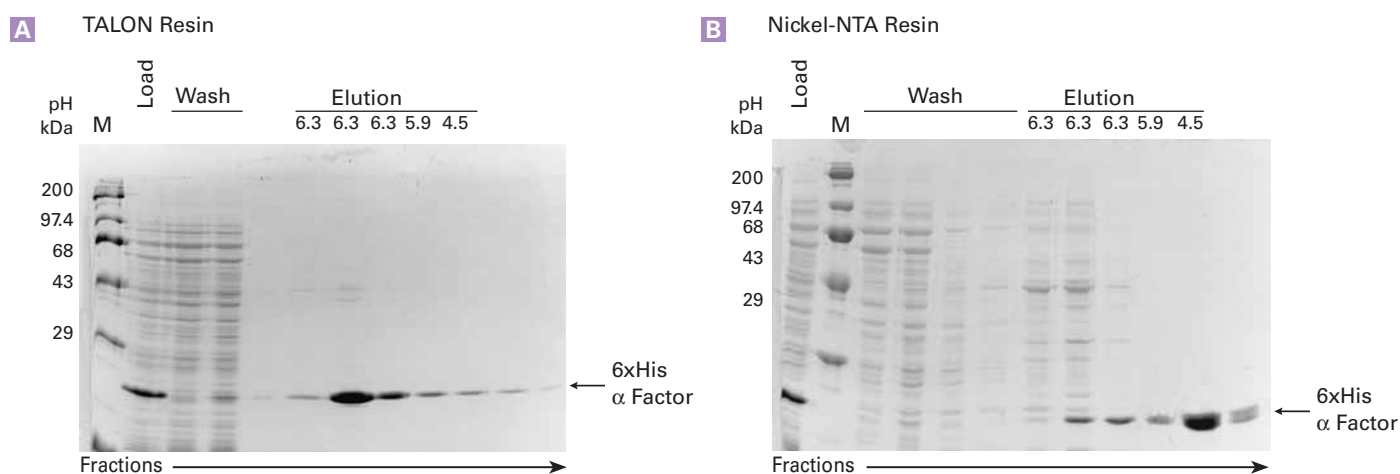
# Protein Purification with TALON<sup>®</sup>...continued

## 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, there is usually significant loss of activity (Rudolph & Lilie, 1996).

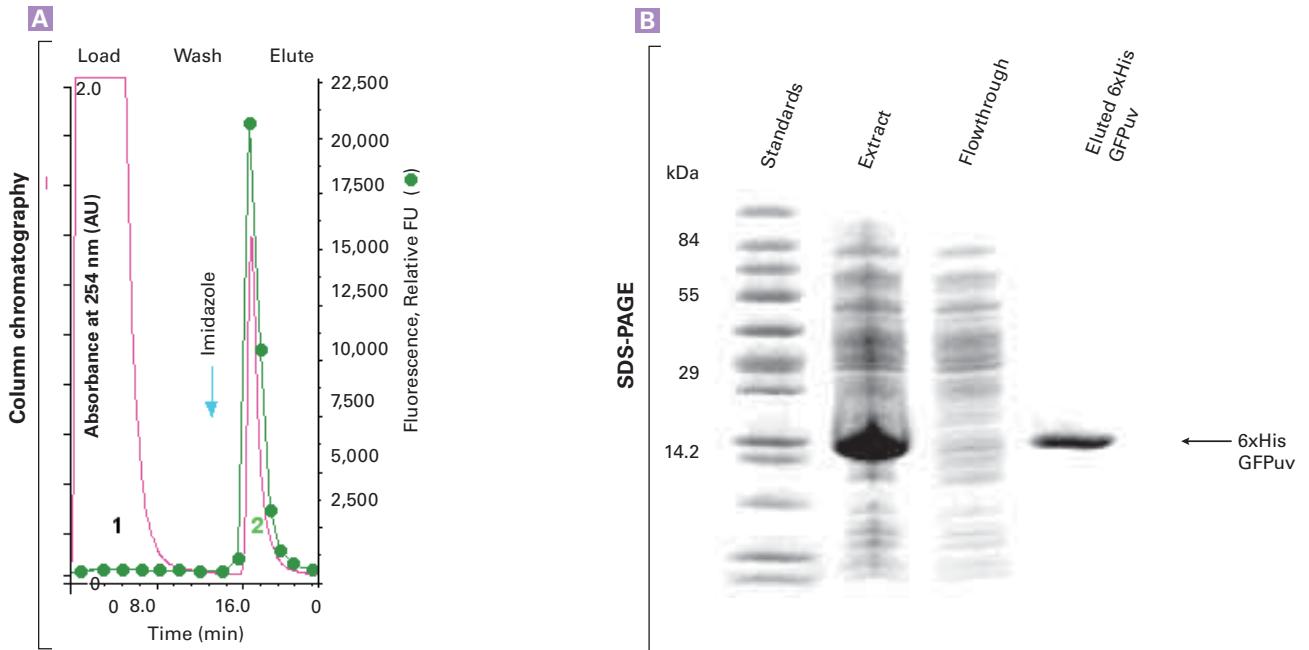
One disadvantage of using native conditions is that unrelated, nontagged proteins are more likely to be nonspecifically bound to the TALON Resin than with denaturing conditions. However, the nonspecific binding can be reduced by including a low concentration of imidazole (5–20 mM) in the wash buffer.

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 Table VII).



**Figure 7. Purification of polyhistidine-tagged proteins under native conditions compared to purification using Ni-NTA.** In comparison with Ni-NTA resin, TALON is more specific for polyhistidine-tagged proteins. These proteins can be eluted from TALON at more neutral conditions (pH = 6.3) than from Ni-NTA resins (pH = 4.5). 6xHis-tagged prepro- $\alpha$ -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 quantitatively enriches the target proteins. Figure 8 shows that the fluorescent signal of green fluorescent protein (GFPuv) is completely enriched by TALON Superflow Resin.

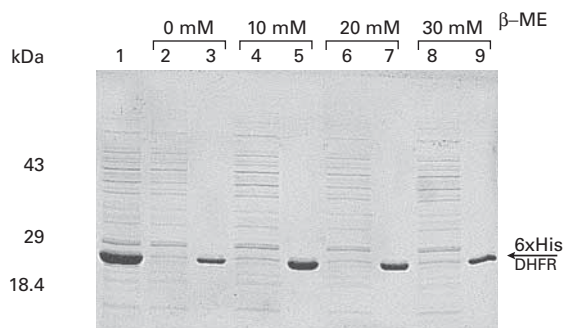


**Figure 8. Native purification with TALON preserves biological activity of proteins.** Fresh cells (0.5 g) expressing 6xHis-GFPuv were extracted in 5 ml of 50 mM sodium phosphate; 0.3 M NaCl, pH 7.0 **Panel A.** Elution profile of GFP which was loaded, washed with the same buffer, and eluted with a step gradient of imidazole (150 mM). **Panel B.** Fractions were analyzed by SDS-PAGE.

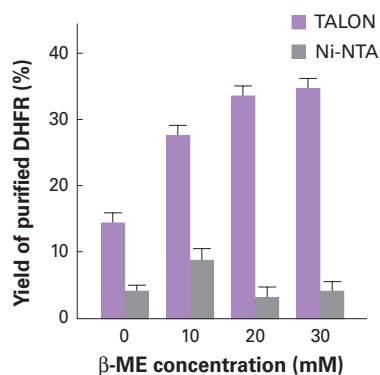
# Purification with $\beta$ -Mercaptoethanol

## Why use $\beta$ -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  $\beta$ -mercaptoethanol helps to preserve those -SH groups during purification.



**Figure 9. Native purification of 6xHis protein in the presence of  $\beta$ -mercaptoethanol.** 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  $\beta$ -mercaptoethanol. Even lanes: 20  $\mu$ l of nonadsorbed material. Odd lanes: 5  $\mu$ l of eluate.



**Figure 10. Yields of purification in the presence of  $\beta$ -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<sup>®</sup> Resin

## Physicochemical properties

Table III: Physicochemical Properties of TALON Resins

Features	TALON Resin	TALON Superflow Resin	TALON CellThru Resin	TALONspin 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)	5–15	5–18	5–10	2–4
Matrix	Sepharose 6B-CL (6% cross-linked agarose)	Superflow (6% cross-linked agarose)	Uniflow (4% cross-linked agarose)	Sepharose 6B (6% agarose beads)
Bead size (µm)	45–165	60–160	300–500	45–165
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	n/a
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 x 10 <sup>7</sup>	4 x 10 <sup>6</sup>	2 x 10 <sup>7</sup>	n/a

\*For washing and elution only.

# 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 yields efficient binding of polyhistidine-tagged proteins, most notably when the polyhistidine 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<sup>2</sup> 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

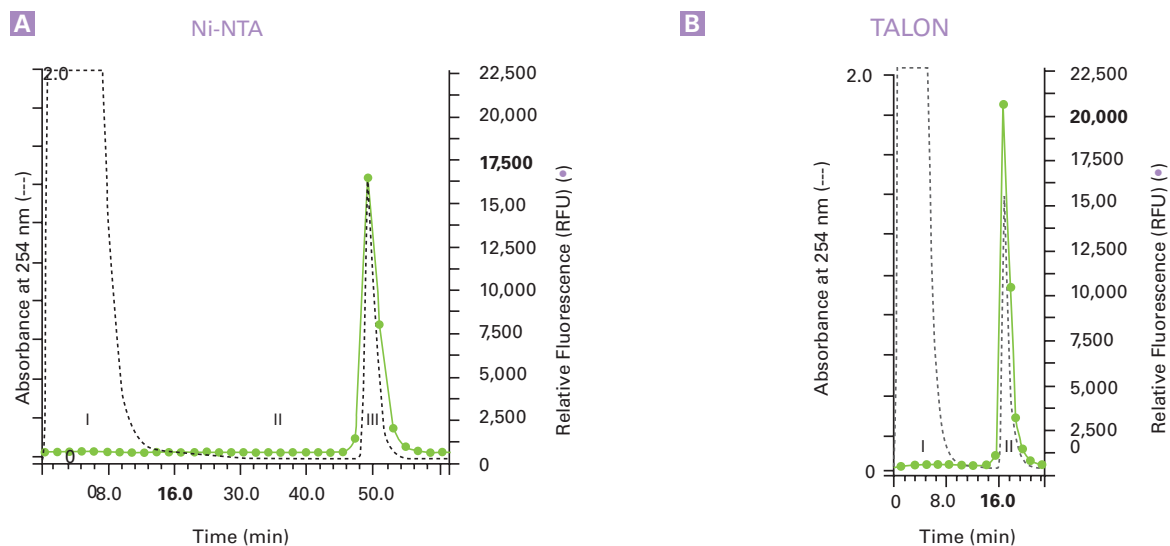
Spin-column purification is intended for very small-scale, analytical-grade protein purification. This method is employed when purifying only small amounts of protein from many different samples.

## TALON Magnetic Beads

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

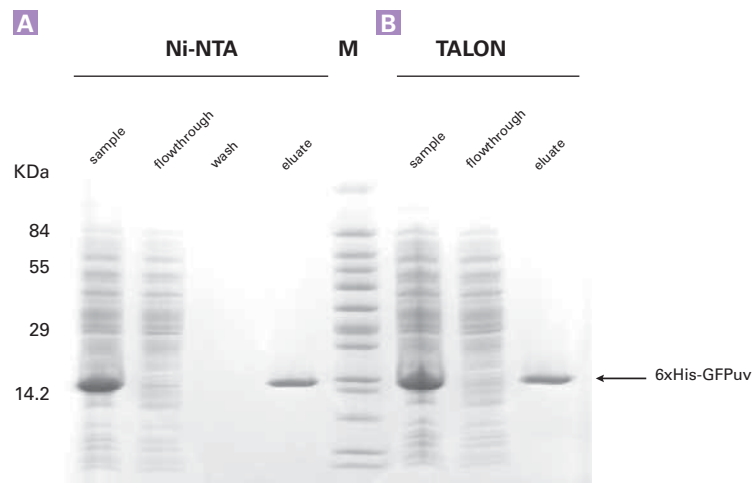
## Purification using FPLC

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 to 150 psi (1 MPa). TALON Superflow can be used at high linear flow rates—up to 5 ml/min/cm<sup>2</sup>. 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 11. 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...continued



**Figure 12. 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 IV: TALON-Compatible Reagents**

Reagent	Acceptable Concentration
$\beta$ -Mercaptoethanol	30mM (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 [ $\beta$ -amino-ethyl ether])

# Purification from Crude Cell Lysates

TALON CellThru is a novel IMAC resin for purifying poly-histidine-tagged proteins from crude cell lysates, sonicates, and fermentation liquids. The large bead size of TALON CellThru (300–500  $\mu\text{m}$ ) 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, and 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, phosphatases, and glycosidases released from the lysed cells can degrade or modify 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 polyhistidine-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  $\mu\text{m}$ ) 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 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.

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.

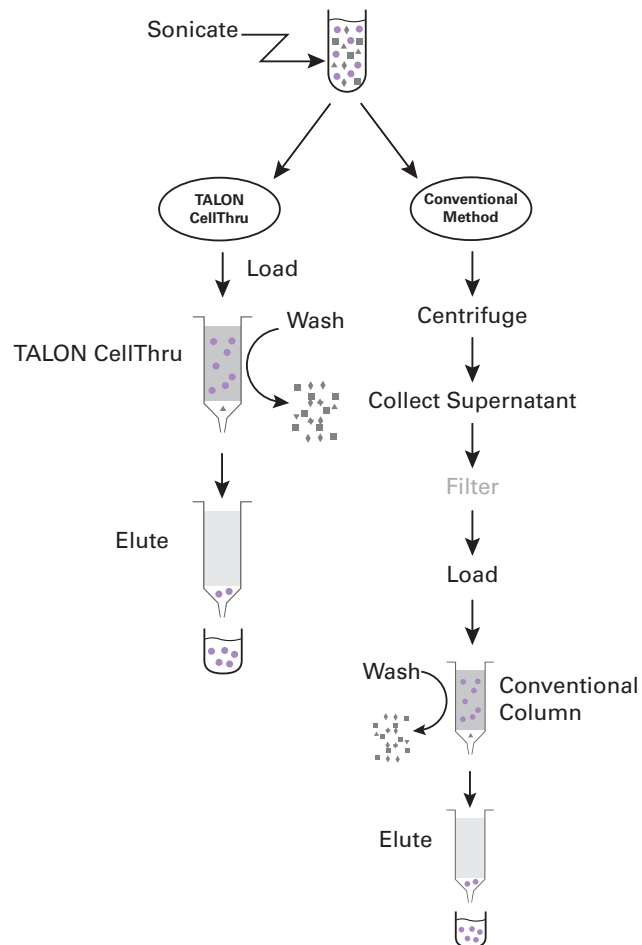
# Purification from Crude Cell Lysates

## CellThru purifies membrane-bound proteins and multiprotein complexes

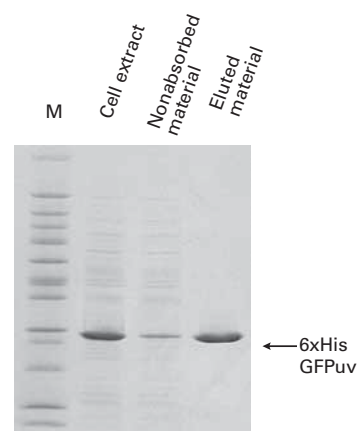
Some proteins are not as easy to access as soluble cytosolic proteins. For example, certain recombinant proteins may interact with proteins embedded in the cell membrane (membrane-bound or membrane-associated), while others may be compartmentalized within subcellular organelles. When performing SDS-PAGE 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. In addition, the tag used for purification must not be obscured by the membrane or protein complex.



**Figure 13. TALON CellThru purifies protein from crude cell lysates faster than conventional methods.**



**Figure 14. 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.

# Purification using TALON Magnetic Beads

## TALON Magnetic Beads

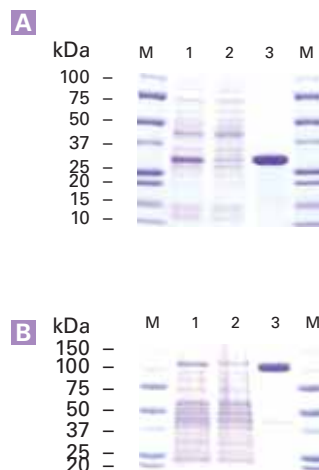
Combine the advantage of our highly selective TALON chemistry with magnetic bead separation. Magnetic particles in the beads facilitate quick and easy separation of microscale quantities of protein when placed on a magnetic separator. The beads, which are precharged with  $\text{Co}^{2+}$ , have a higher specificity for polyhistidine-tagged proteins than nickel-based resins.  $\text{Co}^{2+}$  is bound to the beads using TALON's unique tetradentate metal chelator, which binds cobalt at four sites, virtually eliminating metal leakage during purification.

## Highly Specific Binding & Elution

TALON Magnetic Beads bind polyhistidine-tagged proteins ranging from low to high molecular weight with high specificity as shown in Figure 15 and Table V. Purified proteins are eluted in small volumes (50–200  $\mu\text{l}$ ), resulting in concentrated sample (up to 3 mg/ml). TALON Magnetic Beads are supplied as a 5% suspension with a demonstrated binding capacity of 750  $\mu\text{g}$  of protein per ml of suspension.

## Microscale Screening

Microscale purification with TALON Magnetic Beads can be used for screening expression levels or for protein-protein interaction studies. In addition, the use of TALON chemistry allows for seamless scaling-up of purification of target proteins using our standard TALON Resin.



**Figure 15. Purification of polyhistidine-tagged proteins using TALON Magnetic Beads.** Proteins were expressed in BL21 *E. coli* cells and extracted in TALON Extractor Buffer (Cat. No. 635623). Then 200  $\mu\text{l}$  of a 5% suspension of TALON Magnetic Beads was washed with water to remove storage buffer and equilibrated with loading buffer (50 mM sodium phosphate, 0.3 M NaCl, pH 7.2). The extract was mixed with TALON Magnetic Beads for 30 min at room temperature (6xHN-AcGFP) or 60 min at 4°C (6xHN-LacZ). The beads were washed with loading buffer, followed by washing with 10 mM imidazole in loading buffer. The protein was eluted with 250 mM imidazole in the loading buffer. **Panel A.** SDS PAGE analysis (4–20% gradient gel) of 6xHN-AcGFP purification using TALON Magnetic Beads. Lane 1: Starting *E. coli* extract. Lane 2: Nonadsorbed material. Lane 3: Eluted protein. **Panel B.** SDS-PAGE analysis (4–20% gradient gel) of 6xHN-LacZ purification using TALON Magnetic Beads. Lane 1: Starting *E. coli* extract. Lane 2: Nonadsorbed material. Lane 3: Eluted protein. Lane M: Molecular weight markers. The SDS-PAGE results for both constructs indicate a high level of purification.

**Table V. Protein Purification Results for 6xHN-Tagged AcGFP and LacZ Using TALON Magnetic Beads**

Protein	Sample loaded		Flowthrough + wash		Eluate	
	Protein (mg)	Fluorescence <sup>1</sup> (RFU)	Protein (mg)	Fluorescence <sup>1</sup> (RFU)	Protein (mg)	Fluorescence <sup>1</sup> (RFU)
6xHNAcGFP2	1.34	15,425	1.18	3,015	0.15	10,750
6xHN LacZ2	1.23	—	1.16	—	0.05	—

<sup>1</sup> Relative Fluorescence Units (RFU) for 6xHNAcGFP.

<sup>2</sup> 200  $\mu\text{l}$  of a 5% suspension of TALON Magnetic Beads was used for each purification.

# 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 selected for positive colonies. Bacteria grow rapidly and express high levels of recombinant proteins. In addition, the bacterial genome is relatively well-characterized. Clontech offers several bacterial expression systems: TALON Express, HAT™, PRO™ Tet, and Creator™-compatible 6xHN-Vectors. However, when expressed in bacterial systems, some recombinant proteins become insoluble and are trapped in inclusion bodies. Another drawback is that proteins expressed in bacteria will not contain any eukaryotic posttranslational modifications.

### Yeast

Yeast expression systems are a good alternative when a bacterial expression system will not be adequate because you obtain expression levels ranging up to several milligrams per liter of culture with most eukaryotic posttranslational modifications. Some recombinant proteins that are insoluble when expressed in bacteria will be soluble when expressed in yeast because protein processing is more complex in yeast. 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 polyhistidine-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 baculoviruses infect and multiply 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 posttranslational 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.

### Mammalian cells

Mammalian cells usually provide the best system for generating recombinant eukaryotic proteins because they produce necessary posttranslational 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 generally 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 VI: Comparison of Expression Systems

Expression System	Advantages	Disadvantages	Clontech Vectors
Bacteria	Easy, fast, can produce a large quantity of protein rapidly using the TALON Express, Pro Tet, and HAT expression systems	No posttranslational modifications, no phosphorylation or glycosylation	TALON Express, PRO Tet, HAT, 6xHN Vectors
Yeast	Some posttranslational modifications, faster than mammalian	Handling may be difficult	Matchmaker Vectors (HA + Myc tags)
Baculovirus	Large quantities of proteins, some posttranslational modifications, secreted and tagged forms	Difficult to handle large vectors	BacPak Vectors
Mammalian	Posttranslational modifications	Slow, more complex, expensive	Tet, Retroviral, Adenoviral, CMV Vectors

# Protein Expression Systems..continued

## Polyhistidine-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 the electron density of the imidazole nitrogen with the electron-deficient orbitals of transition metals (Figure 2). Although a tag of three histidines may bind transition metals under certain conditions, a six-histidine tag reliably binds 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)<sub>6</sub>.” 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.

### Polyhistidine 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 VII) 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 (US Patent No. 7,176,298) is a histidine-rich peptide that has similar solubility and binding characteristics to 6xHis, 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 polyhistidine 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 when fused to high molecular weight proteins.

Table VII: Polyhistidine 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..continued

## TALON Express Bacterial Expression & Purification System

The TALON Express Bacterial Expression & Purification Kits provide a simple and robust system for efficient cloning, expression, and purification of polyhistidine-tagged proteins using TALON Express Bacterial Expression Vectors and TALON purification technology (Figure 16). The TALON Express system offers the following benefits:

- **Choice of cloning formats**

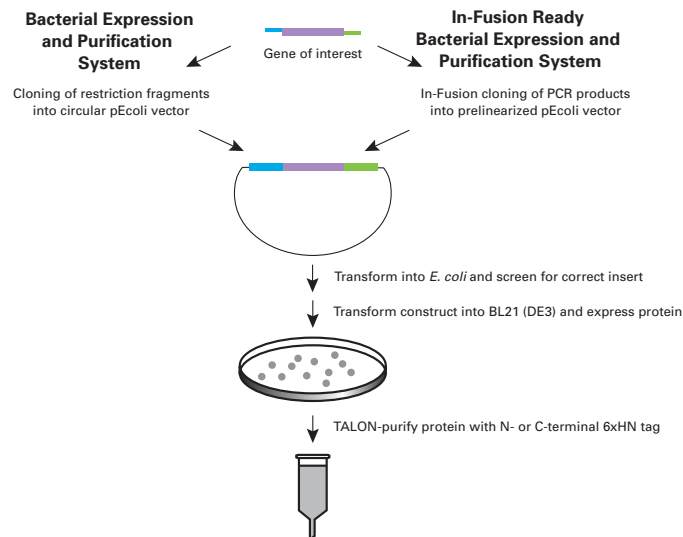
The kits are available in two formats, each of which provides both N- and C-terminal 6xHN-tagged vectors. The TALON Express Bacterial Expression & Purification Kit (Cat. No. 635639) contains circular vectors for traditional restriction enzyme cloning. The In-Fusion™ Ready TALON Express Bacterial Expression & Purification Kit (Cat. No. 635640) contains prelinearized vectors and In-Fusion Dry-Down Mix for easy, precise directional cloning of PCR products without the need for restriction enzyme digestion or *in vitro* ligation.

- **Tightly regulated, high-level expression**

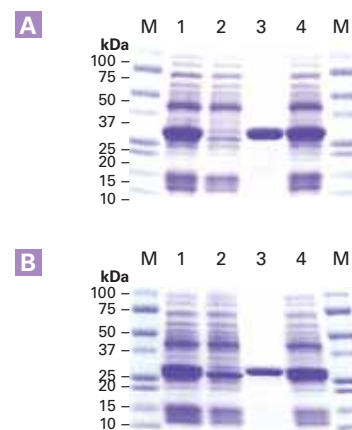
The TALON Express Vectors, which are based on the pET system, (Moffat & Studier, 1986) produce very high levels of protein under tight regulation using the IPTG-inducible T7 polymerase gene and promoter. These vectors are compatible with our BacPAK™ and Living Colors® In-Fusion Ready Vectors, as well as with the wide variety of pET-related products, including those designed to address such issues as protein folding and toxicity.

- **Easy purification**

The TALON Express Vectors are designed to incorporate 6xHN tags at the amino- or carboxy-terminus of the protein of interest. This allows for convenient purification under native or denaturing conditions using TALON resin and the appropriate buffers, which are also included in the kits (Figure 17).



**Figure 16. The TALON Express Bacterial Expression and Purification Kits protocol.** A gene of interest is cloned into a pEcoli vector, a pET-based TALON Express Bacterial Expression Vector, to generate an N- or C-terminal-tagged construct. The TALON Express Bacterial Expression and Purification System allows a choice of cloning sites, while the In-Fusion Ready TALON Express Bacterial Expression and Purification System provides the option of easy, precise PCR cloning to yield a 6xHN-tagged construct. After transformation and growth in *E. coli*, the expressed N- or C-terminal-tagged protein is efficiently purified using TALON technology



**Figure 17. Rapid Purification of N- and C-terminal 6xHN-tagged AcGFP using the In-Fusion Ready Bacterial Expression and Purification Kit.** The In-Fusion Ready Bacterial Expression and Purification Kit was used to generate BL21(DE3) *E. coli* expressing N-terminal 6xHN-tagged AcGFP (6xHN-AcGFP) and C-terminal 6xHN-tagged AcGFP (AcGFP-6xHN) and to purify these proteins (5). The SDS-PAGE analysis results (using a 4–20% gradient gel) for each purification are shown in Panel A (for 6xHN-AcGFP) and Panel B (for AcGFP-6xHN). Lanes 1 and 4: Starting BL21(DE3) *E. coli* extract. Lane 2: Nonadsorbed material. Lane 3: Eluted 6xHN-tagged AcGFP. A high level of purification was achieved for each tagged protein.

## PRO Tet Expression System

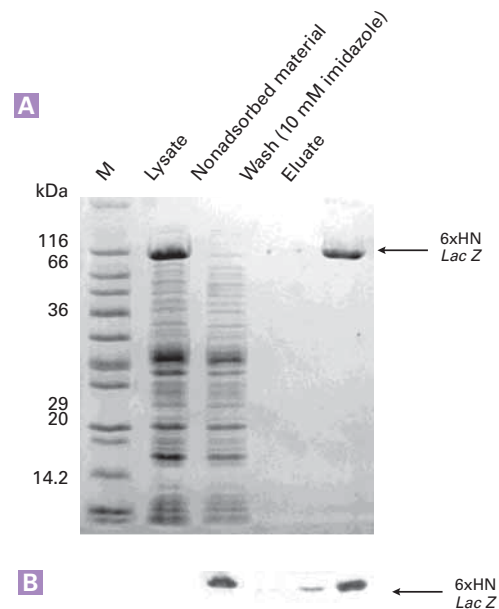
The PRO Bacterial Expression System is based on a set of tetracycline-inducible prokaryotic expression vectors that allow tightly controlled gene expression over a broad range of induction levels. These vectors combine features that let you:

- **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 PRO Tet 6xHN Vectors encode an N-terminal polyhistidine affinity tag. The 6xHN tag allows proteins to be easily and efficiently purified. All PRO Tet 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 PRO Tet 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 PRO Tet 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](http://www.clontech.com).



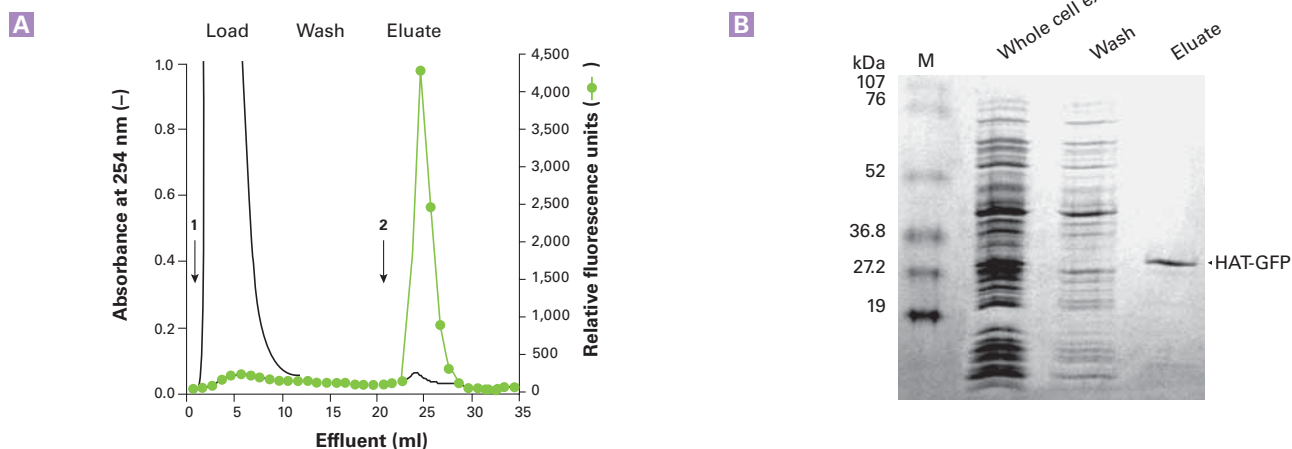
**Figure 18. Bacterially expressed 6xHN-tagged protein purified with TALON Superflow. Panel A.** Coomassie stain of 6xHN-LacZ purification fractions. **Panel B.** Western blot of fractions detects fusion with the 6xHN Polyclonal Antibody. M= molecular weight marker.

# Protein Expression Systems..continued

## 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.



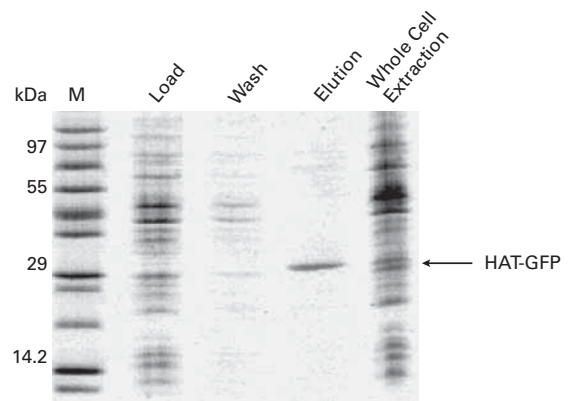
**Figure 19. FPLC purification of HAT-tagged protein using TALON Superflow.** *E. coli* cells were extracted in 50 mM sodium phosphate, 0.3 M NaCl, 5 mM imidazole, pH 7.0 and eluted in 150 mM imidazole. **Panel A.** Cell lysate was purified with a TALON Superflow column. **Panel B.** SDS-PAGE analysis of the procedure. M=molecular weight markers.

## HAT Protein Purification

The HAT System offers advantages for native protein purification compared to polyhistidine-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-polyhistidine-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 polyhistidine-tagged (or HAT-tagged) proteins. TALON requires only low stringency washes to remove nonspecific proteins. We recommend using the HAT tag with TALON resins rather than 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.

Table VIII: 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



**Figure 20. 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=molecular weight markers.

## Polyhistidine-Tag Antibodies

These antibodies detect polyhistidine-tagged recombinant proteins in Western blot, ELISA, and immunocytochemical assays. They are highly sensitive and specific for proteins bearing the polyhistidine 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 21, 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

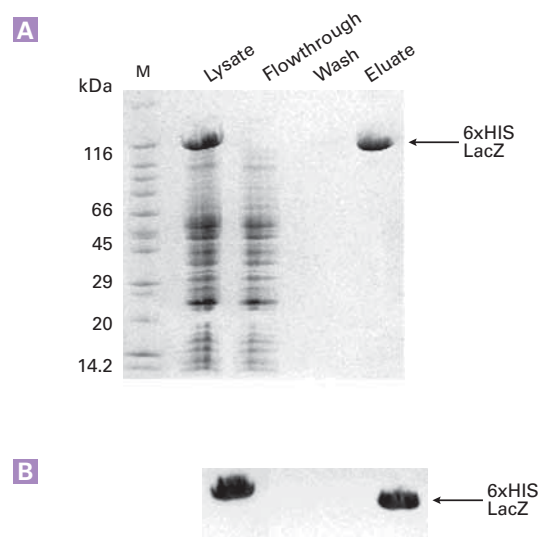
This is the same antibody as above and conjugated to horseradish peroxidase (HRP). The HRP conjugate can be used to detect and visualize 6xHis-tagged proteins using chemiluminescent, colorimetric, or fluorometric substrates without requiring a secondary antibody.

### 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 21. 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.

# Western Detection

## Universal His Western Detection Kit 2.0

The Universal His Western Blot Kit 2.0 is the most specific Western blot kit for detection of polyhistidine-tagged proteins, including 6xHis, our Histidine Affinity Tag (HAT), and the 6xHN tag for which there is no specific antibody (Table VII).

### No Antibodies

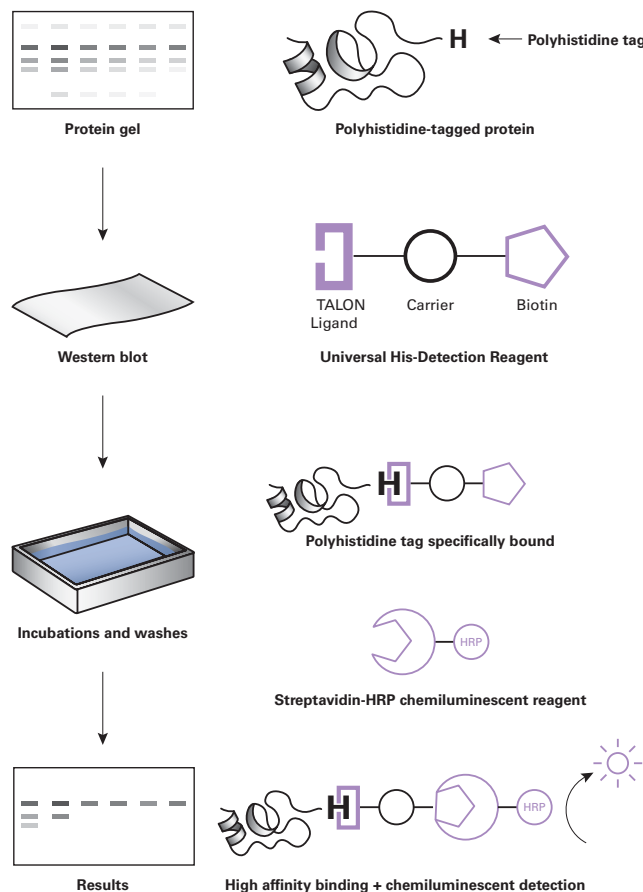
The basis of the detection method is our TALON Metal Affinity Resin, which combines high specificity and affinity for polyhistidine-tagged proteins. After incubation and washing, the detection reagent is bound by streptavidin conjugated to horseradish peroxidase (HRP) and unbound reagent is again washed away. Addition of the enhanced chemiluminescent substrate, which reacts with the bound HRP conjugate, allows visualization of the polyhistidine-tagged protein. The chemiluminescent signal can be detected using autoradiography or a phosphorimager. The entire procedure takes less than four hours after blocking. If desired, it can be modified to use colorimetric detection as an alternative (Figure 22).

### Improvements over Traditional Western Blots

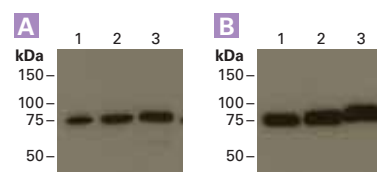
The Universal His Western Blot Kit 2.0 is more specific than antibody-based Western blot methods for detecting polyhistidine tags (January 2007, *Clontechiques*). Traditional Western detection techniques require the use of a secondary antibody, which can contribute to higher nonspecific background. Since our detection reagent binds to a variety of polyhistidine tags, you can use this reagent with any polyhistidine tag and even detect proteins bearing different polyhistidine tags on the same Western blot.

Our kit allows you to detect as little as 0.5 ng of purified protein—less than other Western blot methods that typically require 2–4 ng of purified protein per lane. When compared to other kits on the market in terms of level of sensitivity, range of tags detected, and reduced background levels, none matched its overall performance (Table IX). Our chemiluminescent detection reagents and novel background reducer yield highly sensitive results with low background, even with crude extracts (Figure 23). Film exposure times for these reagents range from 10 seconds to 10 minutes with relatively constant signal intensity over a six hour period, thus allowing for multiple exposures.

Use the Universal His Western Blot Kit 2.0 with any of the polyhistidine tags that are available with our many Protein Expression Vectors.



**Figure 22. Schematic overview of the Universal His Western method.**



**Figure 23. The Universal His Western Blot Kit 2.0 detects purified 6xHN-tagged proteins and 6xHN-tagged proteins in crude extracts.** Panel A. Lanes 1–3: 6xHN ClonScript purified protein (10, 25, and 50 ng). Panel B. Lanes 1–3: 5, 25, and 50 µg of total bacterial lysate, from *E. coli* expressing 6xHN ClonScript protein.

**Table IX: His Detection Kit Comparison**

	Sensitivity	Background	Detects a Wide Range of Tags
<b>Universal His Western Blot Kit 2.0</b>	<b>High</b>	<b>Low</b>	<b>Yes</b>
HisDetector™	Low	Low	ND
HisProbe™-HRP	Medium	Low	ND
Penta-His HRP™	High	High	No <sup>a</sup>

ND = Not Determined

<sup>a</sup> This kit is antibody-based and does not detect HAT or 6xHN tags.

# Phosphoprotein Enrichment

## Phosphoprotein Enrichment Kit

The Phosphoprotein Enrichment Kit provides a rapid and specific affinity-based procedure for isolating phosphorylated proteins from mammalian cells and tissues. It may be used for enrichment of both cytosolic and membrane-bound phosphoproteins regardless of the amino acid modified—including serine, tyrosine, or threonine. Since only a small percentage of all cellular proteins are phosphorylated at any given time (Alberts *et al.*, 1994, Ficarro *et al.*, 1992), phosphoprotein enrichment prior to analysis increases the likelihood of detecting rare and novel phosphoproteins.

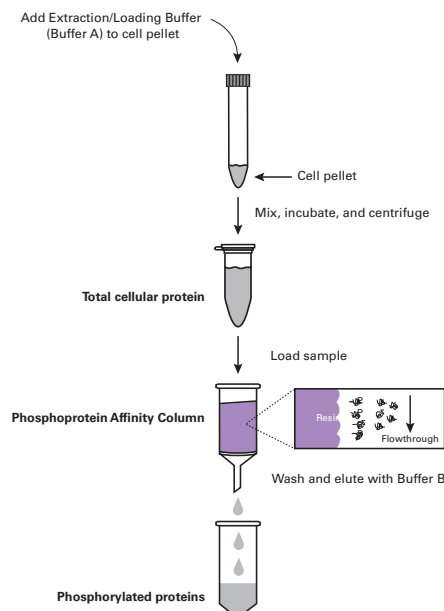
### Advantages of the Enrichment Procedure

The Phosphoprotein Enrichment Procedure is fast; with an average cell-to-sample purification time of less than 2 hours. It is also straightforward, consisting of four main steps (Figure 24): adding Extraction/Loading Buffer to the cell or tissue pellet to extract total cellular protein, loading the extract on an affinity column, washing, and finally eluting the bound phosphoprotein with a detergent-free Elution Buffer. A single buffer—Extraction/Loading Buffer—is used for both the protein extraction and affinity column steps, making buffer exchange unnecessary. This saves time and prevents sample loss. Each column has a maximum binding capacity of ~4 mg of phosphorylated protein, and the procedure is nondenaturing, so phosphoproteins remain folded throughout the process, even during the extraction and elution steps.

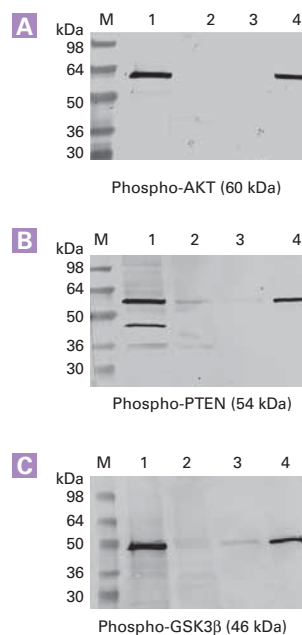
### Highly Selective Enrichment of Phosphoproteins

The Phosphoprotein Enrichment Kit may be used with any mammalian cell type. Cell lines tested include NIH 3T3, HEK 293, HeLa, Cos-7, and Jurkat. The enrichment procedure is highly efficient as demonstrated by Western blotting analyses (Figure 25). Using a colorimetric phosphate detection method, we found the majority of the phosphoprotein in the eluate; negligible traces were detected in the wash fraction.

Phosphoprotein Affinity Columns yield a concentrated solution of phosphoprotein that can be analyzed by several different methods, including mass spectrometry, analysis of phosphorylation and two-dimensional polyacrylamide gel electrophoresis (2D-PAGE).



**Figure 24. Overview of the Phosphoprotein Enrichment Procedure.** Extraction/Loading Buffer contains a mild, non-ionic detergent for efficient, nondenaturing extraction of cellular protein



**Figure 25. Highly effective enrichment of phosphorylated proteins.** A Phosphoprotein Affinity Column was loaded with ~3 mg of total protein from HEK 293 cells. The extract (Lanes 1), flowthrough (Lanes 2), wash (Lanes 3), and eluate (Lanes 4) were then analyzed by Western blotting using antibodies specific for phosphorylated AKT (Panel A), PTEN (Panel B), and GSK3 $\beta$  (Panel C) proteins. The phosphorylated proteins were clearly detected in the eluate fraction. Samples were not diluted, nor concentrated before loading on the gel.

# Phosphoprotein Enrichment

## TALON PMAC Magnetic Phospho Enrichment Kit

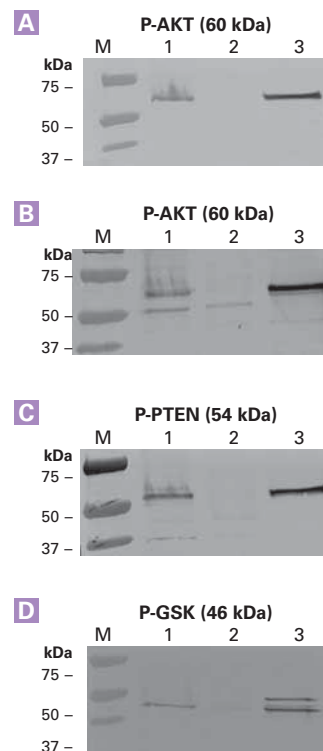
The TALON PMAC Magnetic Phospho Enrichment Kit combines the phosphospecificity of our TALON based Phosphoprotein Enrichment Kit with the convenience of magnetic bead separation to provide a simple, rapid, metal affinity-based method for isolating microgram quantities of phosphorylated proteins from mammalian cells and tissues (Andersson & Porath, 1986; Rishi *et al.*, 2006; Muszynska *et al.*, 1992). Magnetic particles in the beads facilitate quick and easy purification of microscale quantities of phosphoproteins when placed on a magnetic separator.

### Microscale Isolation of Cytosolic & Membrane-Bound Phosphoproteins

The kit provides a complete set of buffers along with our Phospho Magnetic Beads for group-specific enrichment of all types of phosphoproteins, both cytosolic and membrane-bound, that contain a phosphorylated amino acid side chain—including serine, tyrosine, or threonine. Phosphoproteins can be eluted in small volumes (50–200  $\mu$ l) to yield concentrated samples. Different amounts of beads (binding capacity = 400  $\mu$ g of  $\alpha$ -casein per ml of suspension) may be used, depending on the initial sample concentration.

### Highly Selective Enrichment of Phosphoproteins

Only a small percentage of all cellular proteins are phosphorylated, so it is often necessary to enrich for this fraction before beginning an analysis. The TALON PMAC Magnetic Phospho Enrichment Kit can be used with any mammalian cell type or tissue sample. The yield of phosphorylated protein varies with different cell lines (Table X). The enrichment procedure is highly efficient, as demonstrated by Western blotting (Figure 26).



**Figure 26. Highly effective enrichment of phosphorylated proteins using the TALON PMAC Magnetic Phospho Enrichment Kit.** Proteins extracted from HEK 293 cells in Extraction/Loading Buffer containing protease inhibitors and a phosphatase inhibitor (10 mM sodium fluoride), were mixed with 200  $\mu$ l of a 5% suspension of Phospho Magnetic Beads at room temperature for 30 min, followed by washing and elution. The extract (Lanes 1), flowthrough (Lanes 2), and eluates (Lanes 3) were then analyzed by Western blotting using antibodies specific for phosphorylated AKT (Ser 473) in Panel A, P-AKT (Thr 308) in Panel B, P-PTEN (Ser 380) in Panel C, and P-GSK3 $\beta$  (Ser 9) in Panel D; the band at 51 kDa is due to cross-reactivity of the antibody with P-GSK3a.

**Table X: Yields of Phosphoproteins Obtained with the TALON PMAC Magnetic Phospho Enrichment Kit**

Cell Line	Protein Loaded (mg)	Flowthrough + Washes (mg)	Eluate (mg)	Percentage of Protein Eluted
HEK 293	0.192	0.146	0.034	18%
Jurkat	0.183	0.133	0.033	18%
COS	0.150	0.113	0.025	17%
NIH 3T3	0.176	0.132	0.027	15%
HeLa	0.174	0.139	0.023	13%

<sup>1</sup> 200  $\mu$ l of a 5% suspension of Phospho Magnetic Beads was used for each purification.

Total protein in each fraction was determined with BCA Protein Assay Reagent (Pierce Biotechnology, Cat. No. 23225).

# Phosphopeptide Enrichment

## Magnetic Phosphopeptide Enrichment Kit

Tryptic peptides generated from low-abundance phosphoproteins are often challenging to detect and study. Our Magnetic Phosphopeptide Enrichment Kit uses Phospho Magnetic Beads to enhance recovery of phosphorylated peptides that might otherwise be difficult to purify and analyze (Liao, P. *et al.*, 1994). The kit can be used to rapidly enrich phosphopeptides containing phosphotyrosine, phosphoserine, or phosphothreonine and is compatible with various elution buffers to provide samples in formats that are ideal for different downstream applications such as matrix-assisted laser desorption ionization (MALDI), mass spectroscopy, or liquid chromatography (LC).

### Quick Separation

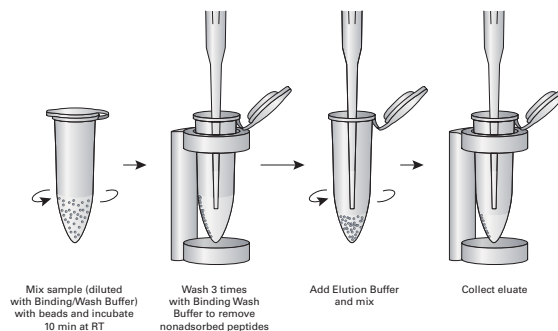
Phospho Magnetic Beads bind phosphopeptides through the specific interaction of phosphate groups with immobilized ferric ions on the surface of the beads. Magnetic particles in the beads facilitate quick and easy separation of the bead-peptide complexes from solutions using either magnetic force or gravity (Figure 27). Any magnetic separator that accommodates 1.5 ml microcentrifuge tubes can be used. Alternatively, a microcentrifuge can be used to separate the particles.

### Optimized Buffers Minimize Nonspecific Binding

The buffers in the kit are detergent-free and have been optimized for specific phosphopeptide binding and elution (Figure 28). The improved Binding/Wash Buffer minimizes the nonspecific binding of peptides containing acidic residues that can complicate immobilized metal affinity chromatography (IMAC) technologies. The kit includes 100 mM ammonium bicarbonate elution buffer (pH 9.0–9.5), however bound phosphopeptides can also be eluted into other buffers, such as 100 mM sodium carbonate buffer (pH 9.3) or phosphate buffered saline, to accommodate different applications.

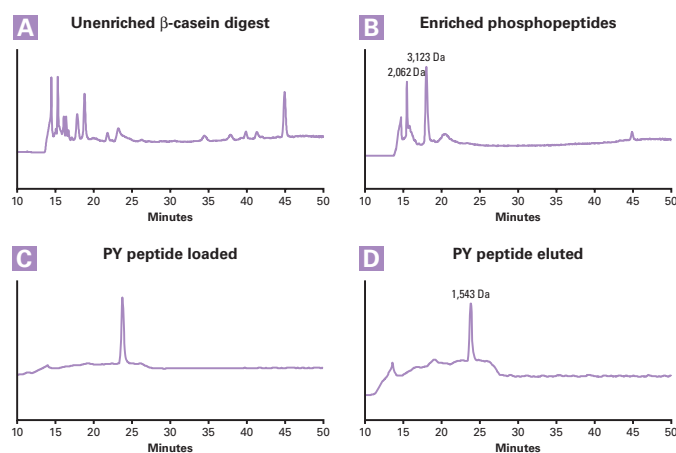
### Nanoscale Purification in Small Elution Volumes

Phospho Magnetic Beads are supplied as a 5% suspension, and have a binding capacity of approximately 1–2 pmol of phosphate per  $\mu\text{g}$  of Phospho Magnetic Beads. Different amounts of beads may be used, depending on the initial sample concentration. Phosphopeptides can be eluted in volumes as small as 20  $\mu\text{l}$  to yield concentrated samples.



**Figure 27. The Magnetic Phosphopeptide Enrichment Kit Protocol.**

Phosphopeptide enrichment is carried out in a single microfuge tube, using a simple 30 min protocol. Phospho Magnetic Beads that have been equilibrated with Binding/Wash Buffer are mixed with the sample (diluted with Binding/Wash Buffer) for 10 min at room temperature (RT). Then the bead mixture is placed on a magnetic separator in order to remove the buffer, which contains nonadsorbed peptides. The beads are washed three times with Binding/Wash Buffer and once with distilled water, using the separator to remove the remaining nonadsorbed material. Phosphopeptides are eluted in as little as 20  $\mu\text{l}$  of Elution Buffer (provided with the kit).



**Figure 28. Phosphopeptide purification with Magnetic Phosphopeptide Enrichment Kit.**  $\beta$ -casein protein digested with immobilized trypsin was diluted with Binding/Wash Buffer and mixed with Phospho Magnetic Beads at room temperature for 10 min. After washing, the enriched fraction was eluted with Elution Buffer. Reverse phase (RP)-HPLC analysis was performed on a Waters Breeze HPLC, XTerra® RP18 column (5 m, 4.6 x 150 mm) with UV detection at 215 nm (Solvent A: 0.1% TFA in water [v/v], Solvent B: 0.1% TFA in acetonitrile [v/v]). Reverse phase HPLC (RP-HPLC) data are shown for the crude protein digest in Panel A and for the eluate (enriched phosphopeptides) in Panel B. Synthetic pY peptide dissolved in Binding/Wash Buffer (Panel C) was mixed with Phospho Magnetic Beads. After washing, bound phosphopeptide was eluted with Elution Buffer (Panel D).

# Phosphopeptide Enrichment

## Phosphopeptide Enrichment Spin Columns & Buffer Kit

Our Phosphopeptide Enrichment Spin Columns and Phosphopeptide Enrichment Buffer Kit can enhance detection of phosphorylated peptides that would otherwise be undetectable. The columns enrich for any type of phosphopeptide—phosphotyrosine, phosphoserine, or phosphothreonine. The straightforward protocol enriches your protein digests, providing increased sensitivity in analysis by mass spectroscopy or HPLC.

### Convenient Prepackaged Spin Columns

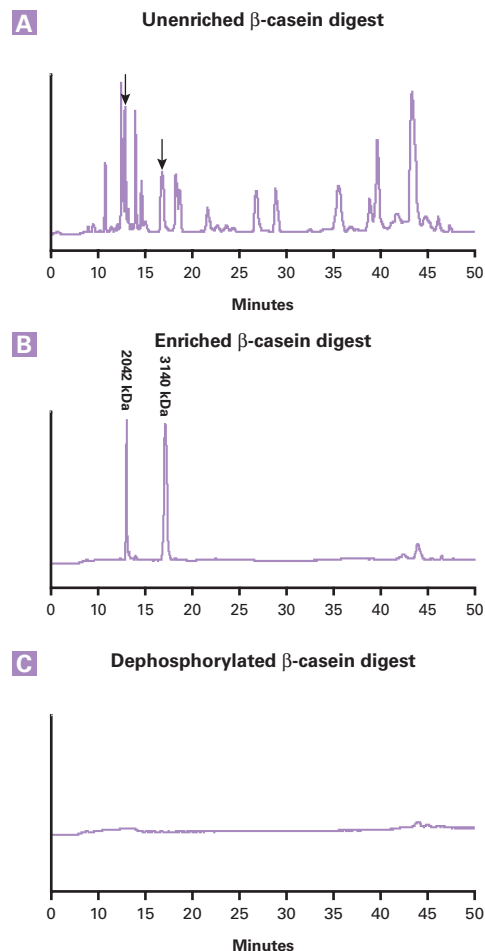
The spin columns contain a unique immobilized metal affinity chromatography resin that binds phosphopeptides. These columns have the capacity to bind up to 250  $\mu\text{g}$  of phosphopeptide and can accommodate up to an 850  $\mu\text{l}$  sample volume. The columns fit into most microcentrifuges. There is no need to pre-equilibrate—simply spin out the storage buffer.

### Optimized Buffer Kit Available

Our Phosphopeptide Enrichment Buffer Kit provides optimized buffers for specific phosphopeptide binding and elution using the spin columns and is recommended for optimal results. Each buffer kit includes enough buffer for use with 25 spin columns and saves time when compared to making your own buffer.

### Effective, Specific Phosphopeptide Enrichment

Our Phosphoprotein Enrichment Spin Columns and Buffer Kit provide an effective affinity-based procedure for isolating phosphorylated proteins from mammalian cells and tissues. To demonstrate the kinds of results that can be obtained with the phosphopeptide enrichment spin columns, we analyzed  $\beta$ -casein protein digest samples with reverse phase HPLC (Figure 29). First,  $\beta$ -casein was digested with trypsin (Figure 29, Panel A). This digestion generates 16 peptides, two of which are known to be phosphorylated (1). After running the sample over our spin column, the two phosphorylated peptides were resolved in the eluted fraction demonstrating that the column specifically bound the phosphorylated peptides (Figure 29, Panel B). The specificity of the binding by the phosphopeptides was further demonstrated by dephosphorylating  $\beta$ -casein; after treatment with phosphatase, neither of the peptides bound to the column (Figure 29, Panel C).



**Figure 29. Phosphopeptides of  $\beta$ -casein purified using Phosphopeptide Enrichment Spin Columns.**  $\beta$ -casein protein was digested with trypsin (sequencing grade). The protein digest was diluted at a ratio of 1:1 with Loading Buffer and run on a Phosphopeptide Enrichment Spin Column. The enriched fraction was eluted with Elution Buffer, then freeze-dried and reconstituted in 0.1% TFA in water (v/v). Reverse phase HPLC (RP-HPLC) data is shown for the crude protein digest (Panel A), eluate (purified phosphopeptides) (Panel B) and eluate of a dephosphorylated sample (Panel C). The eluted fractions were separated by RP-HPLC on an XTerra RP18 column (5  $\mu\text{m}$ , 4.6 x 150 mm) with UV detection at 215 nm (Solvent A: 0.1% TFA in water [v/v], Solvent B: 0.1% TFA in acetonitrile [v/v]). The two phosphopeptide fractions collected in Panel B were analyzed by MALDI.

# 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 30).

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 nondegrading conditions is necessary in order to minimize sample loss. GST loses its ability to bind Glutathione Resin when denatured, so do not use strong denaturants such as guanidinium or urea in the purification buffers. Check reagent compatibilities (Appendix A) when designing your purification scheme.

## Glutathione Resins

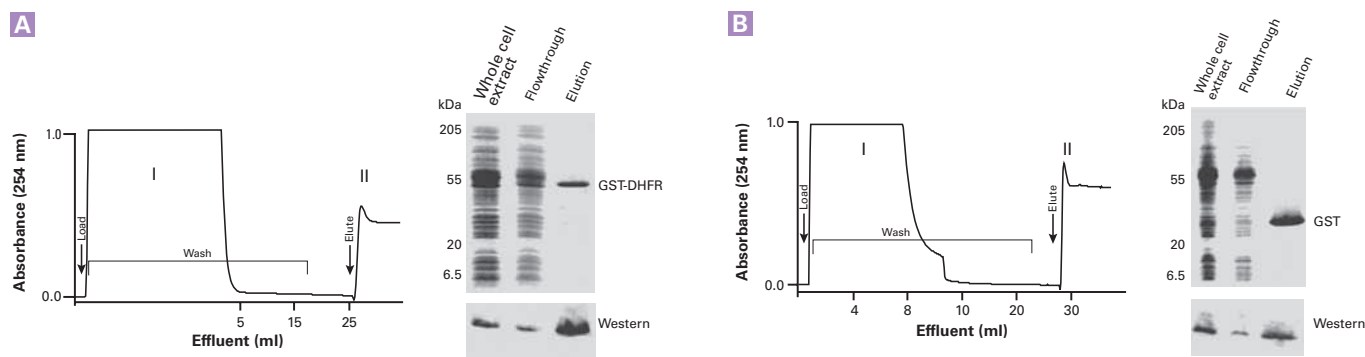
Glutathione-Superflow and -Uniflow Resins allow rapid affinity purification of GST-tagged proteins. These resins are based on 6% and 4% crosslinked agarose, respectively, with glutathione covalently bound to the resins. Both resins possess superior structural and flow characteristics for efficient purification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

## 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<sup>2</sup>/min. Alternatively, the Glutathione-Uniflow Resin, with a maximum linear flow rate of 2 cm<sup>2</sup>/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 (Cat. No. 635619) 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.



**Figure 30. 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.

# 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 immunoglobulins and  $\alpha_2$ -macroglobulins. The term “thiophilic” refers to the affinity that proteins have for sulfone groups that lie in close proximity to thioether groups (Figure 31; 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

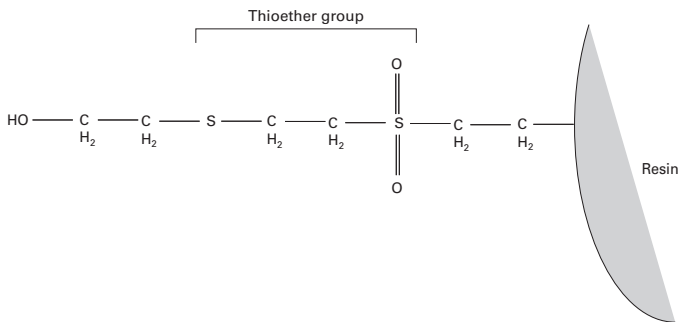


Figure 31. Structure of Thiophilic Resin.

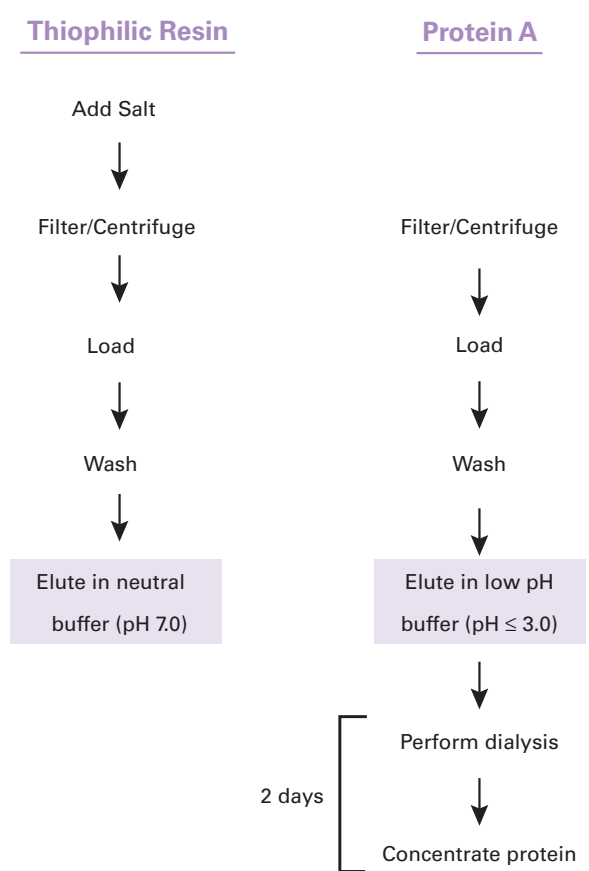


Figure 32. Thiophilic Resin purifies antibodies at neutral pH and more quickly than Protein A.

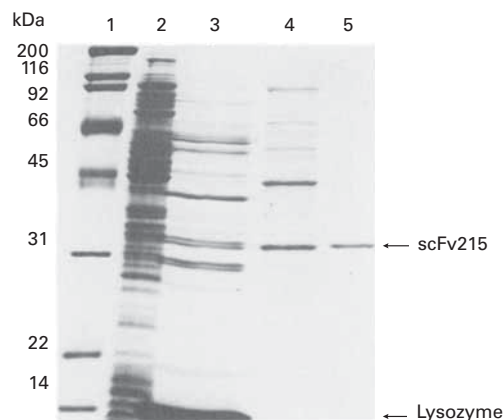
## Purification of Immunoglobulins..continued

### 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 immunoabsorbents, 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.

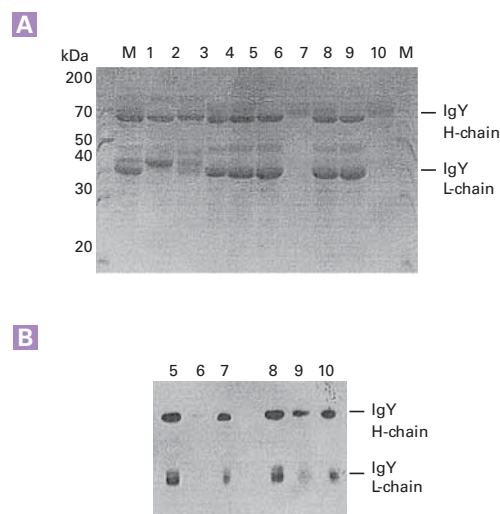


**Figure 33. Single-chain antibody purification with Thiophilic Resin.** SDS-PAGE analysis of the following samples: Bacterial lysate expressing scFv215 (lane 2), Periplasmic fraction (lane 3), peak fraction from Ni-NTA (lane 4) and peak fraction from thiophilic resin (lane 5). Shultze, *et al.* 1994. Permission to reprint obtained.

# Purification of Immunoglobulins..continued

## 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.

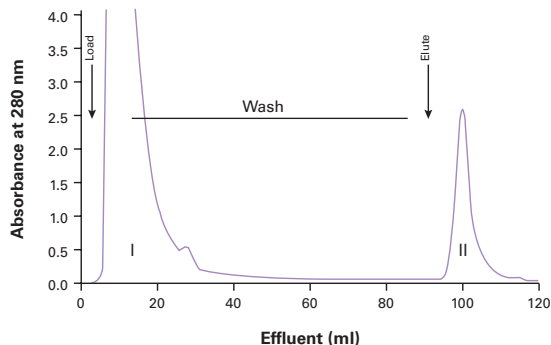


**Figure 34. Purification of IgY from chicken egg using Thiophilic Resin.** **Panel A.** SDS-PAGE analysis of fractions from purification of chicken egg immunoglobulins. In lanes 7 & 10, 10 mg of protein was loaded, and all other lanes, 25 mg was loaded. Lane 1: supernatant of egg yolk extract. Lane 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).

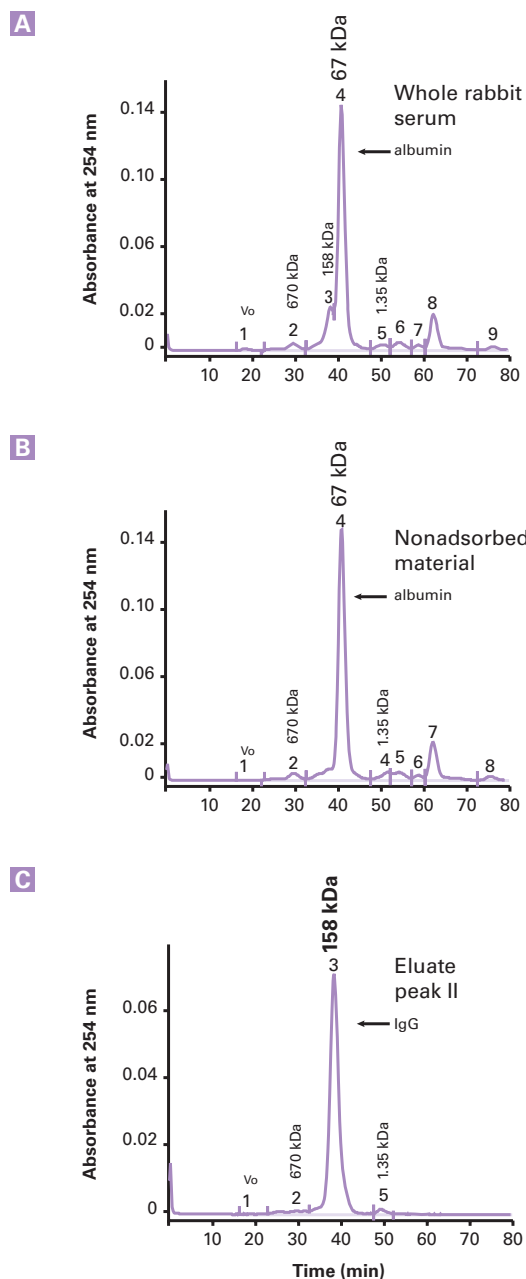
## Purification of Immunoglobulins..continued

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 XI compares the features of the two different resins.

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 crosslinked 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 35.** 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).



**Figure 36.** 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 nonadsorbed fraction from whole rabbit serum (**Panel A**) and wash (**Panel B**). Then, the intact IgG from Peak II is eluted (**Panel C**).

**Table XI: Properties of Thiophilic-Uniflow & -Superflow Resins**

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	Crosslinked agarose	Crosslinked 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).

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# TALON Resin Protocols

## I. List of Components

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

Store TALON Resins, TALONspin Columns and TALON Buffers at 4°C unless otherwise indicated. Do not freeze TALON resins.

- **TALON Metal Affinity Resin**
- **TALON Superflow Metal Affinity Resin**

<u>Cat. No.</u>	<u>Size</u>	<u>Cat. No.</u>	<u>Size</u>
635501	10 ml	635506	25 ml
635502	25 ml	635507	100 ml
635503	100 ml		
635504	250 ml		

- **TALONspin Columns** (Cat. Nos. 635601, 635602 & 635603)

These columns contain 0.5 ml of TALON-NX Resin as a 50% suspension in nonbuffered 20% ethanol.

- **TALON CellThru**

<u>Cat. No.</u>	<u>Size</u>
635509	10 ml
635510	100 ml

- **TALON CellThru Disposable Columns**

10 ml column (Cat. No. 635513)

- **TALON Purification Kit** (Cat. No. 635515)

10 ml	TALON Metal Affinity Resin
160 ml	5X Equilibration/Wash Buffer (250 mM sodium phosphate, 1.5 M sodium chloride, pH 7)
160 ml	5X Equilibration 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** (Cat. No. 635514)

160 ml	5X Equilibration/Wash Buffer (250 mM sodium phosphate, 1.5 M sodium chloride, pH 7)
160 ml	5X Equilibration 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** (Cat. No. 635606)

- **TALON Single Step Columns** (5 ml, Cat. Nos. 635628 & 635631; & 20 ml, Cat. No. 635632)

These columns contain a dry mixture of TALON CellThru resin and xTractor Buffer to extract and bind polyhistidine-tagged proteins in one step.

- **TALON HT 96-Well Plate** (Cat. No. 635622)

- 1 TALON 96-Well Plate
- 1 Plate Top Seal
- 1 Plate Base Seal
- 1 Collection Plate

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- **TALON Magnetic Beads** (Cat. Nos. 635636 & 635637)

<b>Cat. No.</b>	<b>Amount</b>
635636	2 x 1 ml
635637	6 x 1 ml

- **TALON Magnetic Beads Buffer Kit** (Cat. No. 635638)

60 ml 5X Equilibration/Wash Buffer  
15 ml 4X Elution Buffer  
30 ml 1X xTractor Buffer

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

Store DNase I at  $-20^{\circ}\text{C}$ .

If a precipitate has formed in the lysozyme solution, allow the tube to warm at room temperature and gently invert the tube.

The solution may remain turbid after this procedure.

200 ml 1X xTractor Buffer  
2.5 ml 50X Lysozyme  
400  $\mu\text{l}$  DNase (1 unit/ $\mu\text{l}$ )

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

500 ml 1X xTractor Buffer

## II. Buffers for TALON Purification

### A. Choosing Buffers

If you have not purchased the TALON Purification Kit (Cat. No. 635515) the TALON Buffer Kit (Cat. No. 635514), or the TALON xTractor Buffer Kit (Cat. No. 635623), we recommend preparing the following buffers for purifying polyhistidine-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 (Appendix A) to evaluate resin compatibility.

To decrease the amount of nonspecifically bound protein, we recommend using the Equilibration/Wash Buffer at pH 7.0 during purification; however, if your target protein is more stable at pH 8.0, or if it does not adsorb at pH 7.0, use the Equilibration Buffer at pH 8.0 (in place of the Equilibration/Wash Buffer) during all extraction and wash steps. Note that at elevated pH values, amino acids other than histidine, as well as the peptide bond, contribute to protein adsorption. Thus, proteins without a polyhistidine tag can also adsorb to 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 Equilibration/Wash Buffer** (pH 7.0)

50 mM sodium phosphate  
300 mM NaCl

- **1X Equilibration 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		

# TALON Resin Protocols

- HT 96-Well Plate Wash Buffer (pH 7.0)\*

83 mM	sodium phosphate
500 mM	NaCl
10 mM	imidazole

- TALON Magnetic Beads 1X Elution Buffer  
–Imidazole Elution (pH 7.0)

50 mM	sodium phosphate
300 mM	NaCl
250 mM	imidazole

\* See Sambrook & Russell, 2001 or your standard protocol for preparing sodium phosphate buffer.

## C. Denaturing Buffers

Denaturants, such as 6 M guanidinium, enhance protein solubility. Because overexpressed 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 Equilibration/Wash Buffer (pH 7.0)

50 mM	sodium phosphate
6 M	guanidine-HCl
300 mM	NaCl

- 1X Equilibration Buffer (pH 8.0)

50 mM	sodium phosphate
6 M	guanidine-HCl*
300 mM	NaCl

- 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
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- 5X SDS PAGE Sample Buffer

5%	$\beta$ -mercaptoethanol ( $\beta$ -ME)
5%	SDS
50%	glycerol
1.5%	bromophenol blue

- Phosphate Buffered Saline (PBS; pH 7.5)

	<b>Final conc.</b>	<b>To prepare 2 L of solution</b>
$\text{Na}_2\text{HPO}_4$	58 mM	16.5 g
$\text{NaH}_2\text{PO}_4$	17 mM	4.1 g
NaCl	68 mM	8.0 g

Dissolve the above components in 1.8 L of deionized  $\text{H}_2\text{O}$ . Adjust to pH 7.5 with 0.1 N NaOH. Add deionized  $\text{H}_2\text{O}$  to final volume of 2 L. Store at room temperature

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

# TALON Resin Protocols

## III. TALON Kits Premade Buffers

If you have purchased the TALON Purification (Cat. No. 635515) or Buffer Kits (Cat. No. 635514), prepare buffers as described below. To decrease the amount of nonspecifically bound protein, we recommend using the Equilibration/Wash Buffer at pH 7.0 during purification; however, if your target protein is more stable at pH 8.0, or if it does not adsorb to the resin at pH 7.0, use the Equilibration Buffer (pH 8.0) in place of the Equilibration/Wash Buffer during all extraction and wash steps. See page 45 and 48 for more information on choosing a buffer.

### A. Equilibration Buffer

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

### B. Elution Buffer

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

### C. Denaturing Buffer

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

**Note:** Perform all steps during the purification procedure in the presence of 6 M guanidinium. 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 Equilibration/Wash Buffer at pH 7.0 to wash nonadsorbed proteins. If the protein is not stable at pH 7.0, then use the Equilibration Buffer at pH 8.0 with 5–10 mM imidazole.
- If your host cell line produces unwanted multi-histidine proteins, incorporate a more stringent wash:

Dilute 5X Elution Buffer in either 1X Equilibration/Wash Buffer or 1X Equilibration Buffer for a final concentration of 5–10 mM imidazole (1:300–1:150 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 Equilibration/Wash Buffer (pH 7.0). If your target protein is more stable at pH 8.0, or if it does not adsorb at pH 7.0, use the Equilibration Buffer at pH 8.0 (instead of the Equilibration/Wash Buffer) during extraction and wash steps.
3. A reducing agent, such as 10 mM  $\beta$ -ME, or a protease inhibitor, such as PMSF, in the Equilibration/Wash Buffer (pH 7.0), may improve the structural stability of fragile proteins during sample preparation. See Reagent Compatibility Table (Appendix A) 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 Equilibration/Wash Buffer (pH 7.0) to inhibit metalloproteases during the extraction. **However, before applying the sample to TALON resin, remove EDTA using a gel filtration column (PD-10, GE Healthcare) equilibrated with the Equilibration/Wash Buffer.** In some cases, the host cell produces low molecular weight chelators that can also be removed using gel filtration.

Chelators can be detected 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.

# TALON Resin Protocols

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 & Russell, 2001). See Appendix A: 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 fivefold with Equilibration/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 VI).

## 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 Equilibration and/or Equilibration/Wash Buffers minimizes nonspecific binding and reduces the amount of contaminating proteins. Thus, we recommend first purifying polyhistidine-tagged proteins using an imidazole gradient. However, imidazole and polyhistidine-tagged proteins absorb at 280 nm and elution peaks may be difficult to detect spectrophotometrically, especially if you are purifying small amounts of polyhistidine-tagged proteins. In these cases, collect the leading edge of the imidazole breakthrough peak and check for polyhistidine-tagged proteins by a protein specific assay (Bradford, 1976) and SDS-PAGE. In some cases, imidazole may have an effect on the biological activity of target proteins (e.g., metalloproteins). 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 polyhistidine-tagged protein. See Section VIII for important information on washing, storing, and reusing TALON Resins.

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## E. TALON CellThru Considerations

The procedure for purifying polyhistidine-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  $\geq 7.0$ , 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 unadsorbed protein is an insoluble material which is normally removed during high-speed centrifugation. The amount of nonadsorbed target protein will vary as a function of sonication efficiency.

### 3. Chromatography Considerations

TALON CellThru Beads have a diameter of 300–500  $\mu\text{m}$ ; therefore, use a column with a filter pore size of 90–130  $\mu\text{m}$  to adequately pass cellular debris. We recommend using our CellThru 10 ml Disposable Columns (Cat. No. 635513).

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. Sample Preparation

### A. Native Proteins

This procedure can be used with any TALON Resin and TALON Superflow Resin. For CellThru Sample preparation, see Section V.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  $\times g$  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 Equilibration/Wash Buffer (4°C) per 25 ml of culture  $\leq 100$  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 Equilibration/Wash Buffer for a concentration of 0.75 mg/ml. To reduce the chance of introducing proteases, use the highest purity lysozyme available.
4. Incubate at room temperature for 20–30 min.

**Note:** Incubations at room temperature result in elevated proteolytic activities. Alternatively, you can use lysozyme at 4°C with lower efficiency. If this treatment hydrolyzes the target protein, use the method described in Step 6. Alternatively, disrupt the cells by repeated freeze/thaw cycles; that is, flash-freezing the cell suspension in a dry ice-ethanol bath and thawing in chilled H<sub>2</sub>O.

5. If your sample is  $\leq 50$  ml, sonicate it 3  $\times$  10 sec, with a pause for 30 sec on ice between each burst. If your sample is  $\geq 200$  ml, sonicate it 3  $\times$  30 sec, with a 2 min pause on ice between each burst. Proceed to Step 7.

**Note:** Excessive sonication can destroy protein functionality.

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

**Note:** If there is a high level of proteolytic activity in the cell lysate, we recommend adding 1 mM EDTA (final concentration) to the Equilibration/Wash Buffer in order to inhibit metalloproteases during the extraction. Before application of the sample to the TALON adsorbent, EDTA must be removed by gel filtration chromatography (PD-10, GE Healthcare) equilibrated with the Equilibration/Wash Buffer for IMAC.

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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 Equilibration/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 Section A, Step 6.
2. Resuspend the cell pellet by vortexing in 2 ml of chilled 1X Equilibration/Wash Buffer (4°C) per 25 ml of culture ≤100 ml. For cultures >1 L, resuspend the pellet in 1–2% of the original culture volume.

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

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

**Note:** Incubations at room temperature result in elevated proteolytic activities. Alternatively, you can use lysozyme at 4°C with lower efficiency. If this treatment hydrolyzes the target protein, use the method described in Section A, 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<sub>2</sub>O.

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 Equilibration/Wash Buffer (pH 7.0) per 20–25 ml of culture.
3. Gently agitate or stir the sample until it becomes translucent.
4. Set aside a small portion of the clarified sample for SDS-PAGE analysis. Then proceed with the appropriate purification protocol (Section VII).

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

## VI. Test Expression Levels and Purification Strategy

### A. Mini-scale purification

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

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

You can also use a TALONspin Column (Cat. No. 635601) with this procedure (Section VI.B).

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 polyhistidine-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 Equilibration Buffer (pH 8.0).
  5. Vortex until cell pellet is completely dissolved.
  6. Centrifuge at 14,000 rpm for 5 min to pellet any insoluble debris.
  7. Set aside 50  $\mu$ l of the supernatant for later analysis. Transfer the remainder of the supernatant that was prepared as described in Section V.B.1–6 to a clean 1.5 ml tube containing 50  $\mu$ l of prewashed TALON Resin, . Start with 100  $\mu$ l 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  $\mu$ l for later analysis. A high protein concentration in this sample indicates a problem with protein binding.
  11. Add 1 ml of Denaturing Equilibration Buffer.
  12. Vortex for a few seconds.
  13. Centrifuge at 14,000 rpm for 1 min to pellet resin.
  14. Remove the supernatant and set aside 50  $\mu$ l (“first wash”) for later analysis. Discard the remainder of the supernatant.
  15. Repeat Steps 11–14. Set aside 50  $\mu$ l for analysis.
  16. Elute bound polyhistidine-tagged protein by adding 50  $\mu$ l 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  $\mu$ l of 100 mM EDTA (pH 8.0) and vortex briefly.

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- b. Centrifuge briefly at 14,000 rpm.
- c. Carefully remove the supernatant containing the polyhistidine-tagged 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  $\mu$ l 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 mini-scale procedure (Section VI.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 polyhistidine-tagged protein in the lysate is very dilute, use one column to enrich the protein from several 0.6–1-ml lysate aliquots. Simply repeat Steps 7–13 (below) until the desired amount of lysate has been processed. Alternatively, concentrate the polyhistidine-tagged protein by reducing the sample volume.
- The centrifugation rotor and speed may affect your results. Ideally, centrifuge TALONspin Columns in a swinging bucket rotor to allow the sample to pass through the resin uniformly. However, a fixed angle rotor or a microcentrifuge is also acceptable.
- Centrifugation speeds higher than 700 x g may cause irregularities in the flow of solution through the resin bed and thus, decrease the performance of the column.
  1. Hold the TALONspin Column upright and flick it until all resin falls to the bottom of the column.
  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 Equilibration/Wash Buffer and mix briefly to pre-equilibrate the resin.
  8. Recentrifuge 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 polyhistidine-tagged protein binding. Do not vortex.
  15. Remove both caps from column and place column inside the 2 ml microcentrifuge tube.

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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 Equilibration/Wash Buffer. Close the column with the clear top-cap.
21. Allow the buffer to passively wet the resin bed for 30 sec.
22. Agitate or vortex briskly for a few 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  $\mu$ l of Elution Buffer.

**Note:** Alternatively, use 100 mM EDTA (pH 8.0) if it does not interfere with downstream applications of the protein. Samples eluted with EDTA will also contain cobalt.

31. Allow 1 min for Elution Buffer to passively wet the resin bed.
32. Briefly agitate or vortex to resuspend the resin.
33. Place a fresh 2 ml collection tube into centrifuge rotor.
34. Remove both caps and place column into the 2 ml collection tube.
35. Centrifuge sample at 700 x g for 2 min.
36. Repeat Steps 30–35.

**Note:** The polyhistidine-tagged protein can usually be recovered in 800–1,200  $\mu$ l 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.

## C. TALON Magnetic Beads Sample Preparation & Purification Protocol

### 1. General Considerations

TALON Magnetic Beads can be used for microscale purification of polyhistidine-tagged proteins. Procedures are provided here for preparing clarified and crude lysates and purifying proteins under native and denaturing conditions. Buffers are provided in the TALON Magnetic Beads Buffer Kit for purification under native conditions.

- a. Buffers for protein purification under native conditions
  - 1X Equilibration/Wash Buffer (50 mM sodium phosphate, 300 mM NaCl, pH 7.0)
  - 1X Elution Buffer (50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole, pH 7.0)
  - TALON xTractor Buffer

# TALON Resin Protocols

- b. Buffers for denatured proteins are the same as above except for addition of 6 M guanidine-HCl to each of the above buffers and adjustment of the pH to 7.0.

## 2. Standard Sample Preparation to Isolate Native Proteins

- a. Add 0.5 ml of xTractor Buffer per 25 mg of cell pellet. The volume of xTractor Buffer can be increased or decreased depending on the size of the cell pellet.
- b. **[Optional]**: Add 1  $\mu$ l of 1 unit/ $\mu$ l DNase I solution.
- c. Mix gently, pipetting up and down several times.
- d. Incubate with gentle shaking for 10 min at room temperature, or at 4°C, if desired.
- e. Centrifuge the sample at 10,000–12,000 x g for 20 min at 4°C to remove any insoluble material.
- f. Carefully transfer the supernatant to a clean tube without disturbing the pellet. Set aside a small portion of this clarified sample at 4°C or on ice for protein assays and SDS-PAGE analysis and proceed with the TALON Magnetic Beads purification protocol (Section 5).

**Note:** The uncentrifuged crude cell lysate can also be applied to TALON Magnetic Beads. However, the lysate may have to be diluted further or require more DNase I to decrease the viscosity of the solution.

## 3. Standard Sample Preparation to Isolate Denatured Proteins

- a. Add 0.5 ml of Denaturing 1X Equilibration/Wash Buffer per 25 mg of cell pellet. The volume of the buffer can be increased or decreased depending on the size of the cell pellet.
- b. Gently agitate or stir the sample until it becomes translucent.
- c. Centrifuge the sample at 10,000–12,000 x g for 20 min at 4°C to remove any insoluble material.
- d. Carefully transfer the supernatant to a clean tube without disturbing the pellet. Set aside a small portion of this clarified sample at 4°C or on ice for protein assays and SDS-PAGE analysis and proceed with the TALON Magnetic Beads purification protocol (Section 5).

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

## 4. Sample Preparation Directly from Overnight Cultures

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

## 5. Protein Purification under Native or Denaturing Conditions

- a. Aliquot 100–200  $\mu$ l of beads into a 1.5 ml microfuge tube.
- b. Place the tube on a magnetic separator for 1 min and remove storage buffer.
- c. Remove the tube from the magnetic separator and add 0.5 ml of deionized water to the beads.
- d. Mix the liquid and the beads thoroughly using a pipette.
- e. Place the tube on a magnetic separator and remove the supernatant.
- f. To equilibrate the beads, add 0.5 ml of 1X Equilibration/Wash Buffer.
- g. Repeat Steps d and e.
- h. Add the cell lysate (from Sections 2, 3, or 4) to the beads.

**Note:** If the cell lysate volume is less than 200  $\mu$ l, add sufficient 1X Equilibration/Wash Buffer to bring the volume up to at least 200  $\mu$ l. This is necessary to ensure thorough mixing of beads with the cell lysate, for optimal binding.

- i. Mix on a rotary shaker for 30 min at room temperature.

**Note:** If the protein is vulnerable to degradation at room temperature, incubate at 4°C for 1 hr. Protease inhibitors that do not contain EDTA can also be added during the incubation.

- j. Place on a magnetic separator and collect the supernatant.
- k. Remove the tube from the magnetic separator and add 0.5 ml of 1X Equilibration/Wash Buffer.
- l. Mix thoroughly and let it stand for 1 min before placing on a magnetic separator and collecting the first wash.
- m. Repeat Steps k and l twice to collect the second and third washes, respectively.
- n. **[Optional]:** If necessary, repeat Steps k and l under more stringent conditions using 0.5 ml of 5–10 mM imidazole in 1X Equilibration/Wash Buffer.
- o. To elute the protein, add 50  $\mu$ l of Elution Buffer. The volume of Elution Buffer can be varied depending on the amount of beads used. 50  $\mu$ l of Elution Buffer can be used for eluting from 200  $\mu$ l of bead suspension. Most of the protein will elute in this fraction. Smaller volumes, such as 25  $\mu$ l, can be used if a concentrated sample is needed. Volumes below 25  $\mu$ l may be difficult to handle.
- p. Mix for 5 min and collect Eluate 1.
- q. Add another 50  $\mu$ l of Elution Buffer.
- r. Mix for 1 min and collect Eluate 2.
- s. If necessary, Steps q and r can be repeated twice to ensure that protein recovery is maximized. In a specific instance, when using 200  $\mu$ l of bead suspension, 60% of the total protein was eluted in the first 50  $\mu$ l fraction, 20% in the second, 10% in the third, and 5% in the fourth.
- t. Use spectrophotometric and SDS-PAGE analyses to determine which fractions contain the bulk of the polyhistidine-tagged protein.

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

# TALON Resin Protocols

## 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 Equilibration/Wash Buffer and mix briefly to pre-equilibrate the resin.
6. Recentrifuge at 700 x g for 2 min to pellet the resin. Discard the supernatant.
7. Repeat Steps 5 and 6.
8. Add the clarified sample from Section V to the resin.
9. Gently agitate at room temperature or on ice for 20 min on a platform shaker to allow the polyhistidine-tagged protein to bind the resin.
 

**Note:** Incubation on ice will decrease proteolysis.
10. Centrifuge at 700 x g for 5 min.
11. Carefully remove as much supernatant as possible without disturbing the resin pellet.
12. Wash the resin by adding 10–20 bed volumes of 1X Equilibration/Wash Buffer. Gently agitate the suspension at room temperature 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 Equilibration/Wash Buffer to the resin, and resuspend by vortexing.
 

**Note:** Steps 17–22 can be performed on ice or at room temperature, but incubation on ice will decrease proteolysis.
17. Transfer the resin to a 2 ml gravity-flow column with an end-cap in place, and allow the resin to settle out of suspension.
18. Remove the end-cap and allow the buffer to drain until it reaches the top of the resin bed, making sure no air bubbles are trapped in the resin bed.
19. Wash column once with 5 bed volumes of 1X Equilibration/Wash Buffer.
20. **[Optional]:** If needed, repeat Step 19 with more stringent conditions using 5–10 mM imidazole in 1X Equilibration/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 polyhistidine-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 Equilibration/Wash Buffer, and then measure their UV absorbance at 280 nm.

# TALON Resin Protocols

## 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  $\mu\text{m}$ .
3. Apply a vacuum to the filter to remove excess ethanol.
4. Add 5 bed volumes of deionized water to the resin and apply vacuum.
5. Add 5 bed volumes of 1X Equilibration/Wash Buffer to the resin and apply vacuum.
6. Repeat Step 5 two times.
7. Add crude lysate (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 Equilibration/Wash Buffer. Gently agitate the suspension at room temperature for 10 min on a platform shaker to promote thorough washing.
10. Apply vacuum to remove buffer.
11. Repeat the above wash (Steps 9–10) 2–3 times.
12. **[Optional]**: If necessary, repeat Step 11 under more stringent conditions using 5 mM imidazole in 1X Equilibration/Wash Buffer (Section 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 analysis (absorbance at 280 nm) or a Bradford protein assay 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<sup>2</sup>. 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 Equilibration/Wash Buffer. Do not exceed a 5 ml/min/cm<sup>2</sup> flow rate. Monitor the eluant at 280 nm; the baseline should be stable after washing with 5–10 column volumes.

# TALON Resins Protocol

7. Apply the clarified sample to the column after filtering it through a 0.22  $\mu\text{m}$  filter and wash with Equilibration/Wash Buffer until the baseline (280 nm) is stable. Monitor column backpressure during sample application. Start collecting fractions.

**Note:** If the sample is very viscous, the column pressure may 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<sup>2</sup> 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<sup>2</sup> 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 Equilibration/Wash Buffer, or until the baseline at 280 nm is stable. If necessary, wash with 5–10 mM imidazole in Equilibration/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<sub>2</sub>O.
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<sub>2</sub>O.
3. Store resin at 4°C in 20% nonbuffered ethanol containing 0.1% sodium azide.

### C. Regeneration of TALON Superflow Columns

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

### D. Complete Regeneration

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<sub>2</sub>O. Charge the resin with 50 mM CoCl<sub>2</sub> solution (10 bed volumes). Again, wash with 10 bed volumes of Milli-Q H<sub>2</sub>O to remove excess cobalt metal ions. Equilibrate the resin with Equilibration/Wash buffer (10 bed volumes).

## E. Regeneration and reuse of TALON and TALON Superflow resins\*

The regeneration and reuse 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<sub>2</sub>O.
3. Wash the column with five column volumes of 25% ethanol<sup>†</sup>.
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<sub>2</sub>•6H<sub>2</sub>O.
8. Wash the column with ten column volumes of deionized H<sub>2</sub>O.
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 be 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.

<sup>†</sup> 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 ( $\pi r^2$ ) in cm<sup>2</sup>, where  $\pi = 3.1416$  and r is the radius of the cross section of the column in cm.

# Glutathione Resins Protocol

## I. List of Components

Store all components at 4°C.

Glutathione-Superflow Resin		Glutathione-Uniflow Resin	
<u>Cat. No.</u>	<u>Size</u>	<u>Cat. No.</u>	<u>Size</u>
635607	10 ml	635610	10 ml
635608	100 ml	635611	100 ml
635609	2.5 ml	635612	2.5 ml

### Glutathione S-Transferase (GST) Purification Kit (Cat. No. 635619)

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<sub>2</sub>HPO<sub>4</sub>; 18 mM KH<sub>2</sub>PO<sub>4</sub>, 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:

- **Equilibration Buffer (loading):**

140 mM NaCl; 10 mM Na<sub>2</sub>HPO<sub>4</sub>; 1.8 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.5).

- **Elution buffer:**

33 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).

Buffer 3: 140 mM NaCl; 10 mM Na<sub>2</sub>HPO<sub>4</sub>; 1.8 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.5).

- **Alumina (Sigma, Cat. No. A-2039)**

- **Polypropylene tubes**

- **Centrifuge (prechilled to 4°C)**

- **TALON 2-ml Disposable Gravity Columns (Cat. No. 635606)**

- **Deionized H<sub>2</sub>O**

- **Ice**

- **Column (GE Healthcare Tricorn 10/20 or Tricorn 10/100; see Section IV. B. 1)**

- **Mortar/pestle (for alumina-based protein extraction)**

- **0.45 µm filter (for FPLC applications)**

# Glutathione Resins Protocol

## 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 (Cat. No. 635619), 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 (Appendix A) 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 Equilibration 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 Equilibration 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, prechilled 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 (Cat. No. 635606). 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<sub>2</sub>O 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 prechilled 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 prechilled Equilibration Buffer to the column. Do not disturb the resin.
6. Repeat (Step 5) three times.

# Glutathione Resins Protocol

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 Equilibration 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, Cat. No. A-2039) for 2–3 min, until the composition of the mixture is paste-like.
4. Add 2 ml of the precooled Equilibration 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 prechilled 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 GE Healthcare's Tricorn 10/20 (Cat. No. 18-1163-13) or Tricorn 10/100 (Cat. No. 19-7402-01), 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 ( $\pi r^2$  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/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  $\mu$ m 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.

# Glutathione Resins Protocol

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.

## 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.

# Thiophilic Resins Protocol

## I. List of Components

Store all components at 4°C.

Thiophilic-Uniflow Resin		Thiophilic-Superflow Resin	
<u>Cat. No.</u>	<u>Size</u>	<u>Cat. No.</u>	<u>Size</u>
635613	10 ml	635616	10 ml
635614	100 ml	635617	100 ml
635615	2.5 ml	635618	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** (Cat. No. 635606)
- **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**

# Thiophilic Resins Protocol

## 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  $\geq 5$  ml.

#### 1. Dilution Method

- Dilute 1 ml of whole serum with 9 ml of Sample Buffer.
- To extend the life of the adsorbent, filter sample through a 0.45  $\mu\text{m}$  filter.
- Store on ice.

#### 2. Saturation Method

- Add 355 mg of anhydrous sodium sulfate to 5 ml of whole serum.
- Dissolve the salt crystals by gently inverting the container.
- To extend the life of the adsorbent, filter sample through a 0.45  $\mu\text{m}$  filter.
- 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.

- Thoroughly resuspend Thiophilic Resin.
- Transfer 10 ml of resin suspension to a 50 ml filtration bottle and allow resin to settle.
- Remove supernatant and add 5 ml of deionized  $\text{H}_2\text{O}$ .
- Thoroughly resuspend resin.
- Degas resin by applying a vacuum to the filtration bottle.
- Gently resuspend resin.
- Immediately transfer 10 ml of resin suspension to a low-pressure chromatography column.
- Fill column with deionized water.
- Allow resin to settle.
- 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.
- 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.

# Thiophilic Resins Protocol

l. Equilibrate column with  $\geq 10$  column volumes of Equilibration Buffer. Use a flow rate of  $\leq 2$  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  $\sim 1$  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  $\sim 0.030$  AU, switch to Elution Buffer, and collect fractions. In general,  $\leq 10$  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 (Cat. No. 635606).

c. Allow resin to settle.

d. Wash column with five bed volumes of deionized water.

e. Equilibrate column by washing with  $\geq 10$  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 sodium sulfate 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  $\sim 0.030$  AU, switch to Elution Buffer, and collect fractions. In general,  $\leq 10$  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  $\geq 5$  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  $\mu\text{m}$  cellulose acetate 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.

## Thiophilic Resins Protocol

- c. To extend the life of the adsorbent, filter sample through a 0.45  $\mu\text{m}$  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 GE Healthcare's Tricorn 10/20 (Cat. No. 18-1163-13) or Tricorn 10/100 (Cat. No. 19-7402-01) 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 ( $\pi r^2$  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  $\mu\text{m}$  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.

#### Mini-scale 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  $\geq 80\%$  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.

# Thiophilic Resins Protocol

## 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 higher sulfate salt concentrations ( $\leq 4.1$  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.

# Troubleshooting Guide

## A. Protein Expression

Problem	Possible Causes	Solution
1. No expression	• Bad vector construct	Check sequence of the vector to make certain that the protein is expressed in the correct reading frame.
	• 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 overexpressed protein	Use denaturing extraction and purification conditions, or reduce expression levels by lowering the amount of inducer.
	• Unsuitable expression conditions	Check cell growth and inducer concentration; check for wild-type (nontransformed) or antibiotic-resistant cells.
	• Protein is secreted	Use fermentation liquid or serum (for Thiophilic Resin) as starting sample for purification after proper buffering.

## B. Loading/Washing

Problem	Possible Causes	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.  <i>For example, slightly increase the pH of the wash buffer, lower its imidazole concentration, or increase the sulfate concentration (for Thiophilic Resin).</i>
	• Protein degraded during extraction	a) Perform initial purification step more quickly. b) Use mild extraction conditions in the presence of protease inhibitors (e.g., $\beta$ -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 Appendix A: 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.
2. High back pressure during load of sample	• Tag is not accessible under native conditions	a) <b>TALON Resin only:</b> If the under native conditions protein fails to bind under native conditions, treat a small aliquot (<1 ml) with 6 M guanidinium and bind to 50 $\mu$ l of TALON. Then follow the mini-scale procedure in Section VI.  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) <b>GST only:</b> GST is denatured.
	• High viscosity due to presence of DNA	a) Use DNase I or b) Dilute sample fivefold before loading on column.

# Troubleshooting Guide

C. Elution		
Problem	Possible Causes	Solution
1. High amount of coeluted impurities	• Insufficient wash	Use larger volumes of Equilibration/Wash Buffer
	• Buffer compositions are not optimal	a) Check buffers used for sample preparation and wash steps. b) Check pH. The Equilibration/Wash Buffer should be pH 7.0. Contaminants will coelute 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) <b>TALON Resins only:</b> add 1–5 mM imidazole to the Equilibration/Wash Buffer and use it as a wash step immediately before elution.
	• Resin was used in large excess	Estimate the fusion protein content of your lysate and use only as much resin as required. (See Table III for binding capacities of TALON resins.)
	• Proteolytic product	Use mild extraction conditions in presence of protease inhibitors (e.g., $\beta$ -ME and EDTA) at 4°C. <b>Remove EDTA before applying to TALON.</b>
	• Covalent attachment (Cys-Cys, disulfide bonds) of impurities to the protein	Use 5–10 mM of $\beta$ -ME in the Equilibration/Wash Buffer. ( <b>Not for Thiophilic Resin</b> )
	• Copurifying histidine-rich (for TALON) or sulfone-rich (for thiophilic) proteins	a) For HAT- or polyhistidine-tagged proteins, use enterokinase to remove HAT tag and rerun IMAC with the mixture. Target protein will pass through the column, while impurities and tag will be adsorbed.  <i>Note: Remove chelating ligands by gel filtration before loading the proteolytic mixture onto TALON Resin.</i> 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 Equilibration/Wash Buffer and load as two aliquots. c) Increase the number of 1 ml washes. d) Use Equilibration/Wash Buffer (pH 7.0). e) Add 1–5 mM imidazole to Equilibration Buffer, pH 8.0 and use it as an intermediate wash step before elution. f) To repurify a TALONspin sample, perform the following after performing TALON Purification Section VI.B: Step 37. <ol style="list-style-type: none"> <li>1) Add 4 volumes of Equilibration/Wash Buffer to the semipurified sample.</li> <li>2) Load the sample onto another TALONspin Column.</li> <li>3) Wash twice with 1 ml of Equilibration/Wash Buffer.</li> <li>4) Elute as before (Section VI.B.30–35).</li> </ol>

# Troubleshooting Guide

C. Elution ...continued		
Problem	Possible Causes	Solution
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, Cat. No. D-6277) in the Equilibration/Wash Buffer.
	• The lower resin bed support may be clogged with cellular debris	a) Remove resin from clogged column and resuspend. Then wash it in a batch format and transfer to a fresh column. b) Use a syringe filled with wash buffer or reverse the pump on the column to gently run the column backwards. In addition, test for tubing blockages in a similar manner. Apply gentle pressure. Do not exceed a 1 drop/sec flow rate.
4. Polyhistidine-tagged proteins do not elute	• Elution Buffer is not optimal	a) Elute with 150 mM imidazole or pH 4.0 buffer. b) <b>TALON only:</b> 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) <b>Not for Thiophilic Resin:</b> Add 1–5 mM β-ME to reduce disulfide linkages. Supplement buffer with 1% nonionic detergent. d) Purify polyhistidine-tagged protein under denaturing conditions.
D. Changes in Resin		
Problem	Possible Causes	Solution
1. Loss of Co <sup>2+</sup> (TALON only)	• Presence of chelators in sample	Remove chelators from sample by gel filtration and regenerate adsorbent as described in TALON Resin Section VIII.D.
2. Gray or brown resin	• TALON Resin was overexposed 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. 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. e) Avoid long-term storage of resin in denaturants.

# Troubleshooting Guide

E. Analysis		
Problem	Possible Causes	Solution
1. High background on silver-stained gels	<ul style="list-style-type: none"> <li>Nucleic acid contaminant</li> </ul>	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	<ul style="list-style-type: none"> <li>Protein was damaged by sonication</li> </ul>	a) Conduct a time-course assay to determine the minimum sonication time needed to disrupt the cells while maintaining the native protein/enzyme function.  <i>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 <math>A_{280}</math> of each sample.</i> b) Perform the lysis or sonication procedure on ice.
	<ul style="list-style-type: none"> <li>Protein has degraded</li> </ul>	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.
	<ul style="list-style-type: none"> <li>Protein may not be folded properly</li> </ul>	Try purifying protein under denaturing conditions, then try refolding (Lin <i>et al.</i> , 2007)
F. Resin Reuse		
Problem	Possible Causes	Solution
1. Binding drops below original capacity	<ul style="list-style-type: none"> <li>Lysate contains naturally occurring reducing agent or a nonspecific polyanion may be obscuring the metal binding sites.</li> </ul>	a) Use a larger volume of the previously-used resin. b) Replace used resin with fresh resin. c) <b>TALON only:</b> Wash resin with 6 M guanidinium (pH 5.0) and 1% Triton X-100 or SDS, and re-equilibrate before use.
	<ul style="list-style-type: none"> <li>Resin is dirty or has not been fully regenerated.</li> </ul>	a) Resin has been damaged or has worn out. These resins are reusable with proper handling and regeneration. However, they do not last indefinitely. TALON Resins can be reused at least 3–5 times. b) Thiophilic Resin can be reused more than 10 times if properly maintained. c) Glutathione Resins can be reused at least 5–10 times.
	<ul style="list-style-type: none"> <li>Protein may require a posttranslational modification to be active</li> </ul>	In this case, it may be necessary to change the expression system (e.g., switch from <i>E. coli</i> to insect or mammalian cells.)

- Alberts, B., Bray, D., Lewis, K., Raff, M., et al. (1994) In *Molecular Biology of the Cell*, 3rd ed., (Garland Publishing, NY), pp. 195–222.
- Andersson, L. & Porath, J. (1986) Isolation of phosphoproteins by immobilized metal (Fe<sup>3+</sup>) affinity chromatography. *Anal. Biochem.* **154**(1):250–254.
- 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.
- Ficarro, S. B., McClelland, M. L., Stukenberg, P. T., Burke, D. J., Ross, M. M., Shabanowitz, J., Hunt, D. F. & White, F. M. (2002) Phosphoproteome analysis by mass spectrometry and its application to *Saccharomyces cerevisiae*. *Nature Biotechnol.* **20**:301–305.
- 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) *Clontechiques* **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. *Bio/Techniques* **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.
- Liao, P. C., Leykam, J., Andrews, P.C., Gage, D.A. & Allison, J. (1994) An approach to locate phosphorylation sites in a phosphoprotein: mass mapping by combining specific enzymatic degradation with matrix-assisted laser desorption/ionization mass spectrometry. *Anal. Biochem.* **219**(1):9–20.
- Lin, Z., Lei, H. & Cao, P. (2007) Expression, purification, and *in vitro* refolding of soluble tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). *Protein Expr. Purif.* **51**(2):276–82.
- 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.
- McMurry, J. L. & Macnab, R. M. (January 2004) BD TALON™ Resin Dies Not Bind E. coli SlyD, a Common Contaminant in Ni-NTA IMAC. *Clontechiques* **XIX**(1):16–17.
- Moffatt, B.A. & Studier, F.W. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**(1):113–130.
- Muszynska, G., Dobrowolska, G., Medin, A., Ekman, P. & Porath, J. O. Model studies on iron(III) ion affinity chromatography. II. Interaction of immobilized iron(III) ions with phosphorylated amino acids, peptides and proteins. (1992) *J. Chromatogr.* **604**(1):19–28.
- 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 mercaptopropylidene-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.
- Rishi, A. K., Zhang, L., Yu, Y., Jiang, Y., Nautiyal, J., Wali, A., Fontana, J.A., Levi, E. & Majumdar, A. P. (2006) Cell cycle- and apoptosis-regulatory protein-1 is involved in apoptosis signaling by epidermal growth factor receptor. *J. Biol. Chem.* **281**(19):13,188–13,198.
- 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.
- Rudolph, R. & Lilie, H. (1996) *In vitro* folding of inclusion body proteins. *FASEB J.* **10**(1):49–56.
- 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. & Russell, D.W. (2001) *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.
- Shultz, 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.
- Universal His Western Blot Kit 2.0 (January 2007) *Clontechiques* **XXII**(1): 24–25.
- 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., Verkhovskiy, 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

# Appendix A: Reagent Compatibilities

Table XII: Reagent Compatibilities

Reagent	TALON Resin	Glutathione Resin	Thiophilic Resin
$\beta$ -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

# Appendix B: TALON & Related Product List

TALON Products	Size	Cat. No.
TALON Metal Affinity Resin	10 ml	635501
	25 ml	635502
	100 ml	635503
	250 ml	635504
TALONspin Columns	10 columns	635601
	25 columns	635602
	50 columns	635603
TALON 2-ml Disposable Gravity Column	50 columns	635606
TALON Superflow Metal Affinity Resin	25 ml	635506
	100 ml	635507
TALON CellThru	10 ml	635509
	100 ml	635510
CellThru 10-ml Disposable Columns	20 columns	635513
TALON xTractor Buffer Kit	500 ml	635625
TALON Buffer Kit	each	635514
TALON Purification Kit	each	635515
TALON HT 96-Well Purification Plate	each	635622
TALON Magnetic Beads	2 x 1 ml	635636
	6 x 1 ml	635637
TALON Magnetic Beads Buffer Kit	each	635638

Phospho Purification Products	Size	Cat. No.
TALON PMAC Magnetic Phospho Enrichment Kit	each	635641
Magnetic Phosphopeptide Enrichment Kit	each	635643
Phosphoprotein Enrichment Kit	6 preps	635624
Phosphoprotein Kit – Buffer A	500 ml	635626
Phosphopeptide Enrichment Spin Columns	25 columns	635634
Phosphopeptide Enrichment Buffer Kit	each	635635

Bacterial Expression Systems Products	Size	Cat. No.
TALON Express Bacterial Expression & Purification Kit	each	635639
	each	635640
TALON Express In-Fusion Ready Bacterial Expression & Purification Kit	each	631205
HAT Protein Expression & Purification System	each	631203
PRO Tet 6xHN Bacterial Expression System	each	631203
	Creator-Compatible PRO Tet 6xHN Bacterial Expression System	each

Polyhistidine Detection Products	Size	Cat. No.
Universal His Western Detection Kit 2.0	1 kit	635642
6XHis mAb-HRP Conjugate	100 µl	631210
6xHis Monoclonal Antibody (Albumin Free)	200 µg	631212
6xHN Polyclonal Antibody	200 µl	631213

Thiophilic Resin Products	Size	Cat. No.
Thiophilic-Uniflow Resin	10 ml	635613
	100 ml	635614
Thiophilic-Superflow Resin	10 ml	635616
	100 ml	635617

Glutathione Resin Products	Size	Cat. No.
Glutathione-Superflow Resin	10 ml	635607
	100 ml	635608
Glutathione-Uniflow Resin	10 ml	635610
	100 ml	635611
GST Purification Kit	5 purifications	635619











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