

# METHODS IN ENZYMOLOGY

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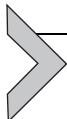
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# PREFACE

These volumes of *Methods in Enzymology* contain the protocols that made up the online *Methods Navigator*. Our philosophy when we selected the protocols to include in the *Navigator* was that they should be for techniques useful in any biomedical laboratory, regardless of the system of the lab studies. Each protocol was written by researchers who use the technique routinely, and in many cases by the people who actually developed the procedure in the first place. The protocols are very detailed and contain recipes for the necessary buffers and reagents, as well as flowcharts outlining the steps involved. Many of the chapters have accompanying videos demonstrating key parts of the procedures. The volumes are broken into distinct areas: DNA methods; cell-based methods; lipid, carbohydrate, and miscellaneous methods; RNA methods; and protein methods. Our goal is that these protocols will be useful for everyone in the lab, from undergraduates and rotation students to seasoned postdoctoral fellows. We hope that these volumes will become dog-eared and well-worn in your laboratory, either physically or electronically.

Professor JON R. LORSCH  
National Institute of General Medical Sciences



## CHAPTER ONE

# Purification of His-Tagged Proteins

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## Abstract

Ni-NTA affinity purification of His-tagged proteins is a bind-wash-elute procedure that can be performed under native or denaturing conditions. Here, protocols for purification of His-tagged proteins under native, as well as under denaturing conditions, are given. The choice whether to purify the target protein under native or denaturing conditions depends on protein location and solubility, the accessibility of the His tag, and the desired downstream application. His-tagged proteins can be purified by a single-step affinity chromatography, namely immobilized metal ion affinity chromatography (IMAC), which is commercially available in different kinds of formats, Ni-NTA matrices being the most widely used. The provided protocols describe protein purification in the batch binding mode and apply gravity-assisted flow in disposable columns; this procedure is simple to conduct and extremely robust. IMAC purification can equally be performed in prepacked columns using FPLC or other liquid chromatography instrumentation, or using magnetic bead-based methods (Block et al., 2009).

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## 1. THEORY

The purification of recombinant proteins is a prerequisite for a lot of downstream applications such as functional and structural studies. Recombinant proteins are commonly expressed with an affinity tag fused to the N- or C-terminus to facilitate purification and detection. One of the most commonly used fusion tags for recombinant protein expression and purification is the His tag, which contains six or more consecutive histidine residues (Waugh, 2005). Due to its small size (0.84 kDa in the case of a hexahistidine tag) and the fact that it is uncharged at physiological pH, the His tag does usually not affect folding and in most cases does not interfere with the structure or function of the fusion protein (Carson et al., 2007). It

has a very low immunogenicity and it is compatible with most downstream applications.

Purification of His-tagged proteins by IMAC is based on the affinity of histidine residues for immobilized metal ions (e.g.,  $\text{Ni}^{2+}$  or  $\text{Cu}^{2+}$ ). The metal ions are immobilized on chromatographic matrices by a chelating ligand, most commonly nitrilotriacetic acid (NTA) or iminodiacetic acid (IDA). While IDA has only three metal-chelating sites, NTA contains four and hence binds the metal ions more stably resulting in reduced ion leaching ([Hochuli, 1989](#); [Block et al., 2009](#)). Elution conditions for IMAC of His-tagged proteins are mild and flexible (100–500 mM imidazole, pH 5.9–4.5, or EDTA).

Since the affinity of the His tag toward the Ni-NTA depends only on its primary structure, His-tagged proteins can be purified under native or denaturing conditions ([Hochuli et al., 1987](#)). While many recombinant proteins can be produced in a soluble form in *E. coli*, a lot of other proteins aggregate to insoluble inclusion bodies when expressed at high levels and can be efficiently purified only using denaturing purification conditions. Strong denaturants such as 8 M urea or 6 M guanidinium hydrochloride are usually used to dissolve protein aggregates for IMAC purification. Generally, the binding of His-tagged proteins to the Ni-NTA matrix improves under denaturing conditions since the His tag is fully exposed and the potential of untagged proteins to co-purify is decreased.

Generally, the level of co-purifying contaminants is higher for samples from eukaryotic expression systems, because of the higher abundance of endogenous proteins containing consecutive histidines or metal-binding motifs. In many instances, contaminants that co-purify can be removed using more stringent binding and washing conditions, for example, increasing imidazole concentration, increasing the salt concentration up to 2 M NaCl, or the addition of low concentrations of detergent (for more information on detergents see Explanatory Chapter: Choosing the right detergent). However, a parameter with a very high impact on the purity of the protein preparation is the ratio of Ni-NTA resin to His-tagged protein. To prevent co-purification of untagged proteins with a certain affinity to Ni-NTA, the binding capacity of the resin should be adjusted to the amount of His-tagged protein in the sample ([Structural Genomics Consortium, 2008](#)).

Due to the relatively high affinity and specificity of the His-tag, a single IMAC purification step in most cases results in an efficient purification with a reasonably high purity of the target protein preparation, which is sufficient

for many applications. However, in some cases optimization of the purification process is required, especially if poorly expressed proteins are to be purified. In this regard, it has been recently reported that purification yields of very low abundance His-tagged proteins from *E. coli* lysates can be greatly increased by the removal of the periplasmic material prior to cell lysis (Magnusdottir et al., 2009). Alternative approaches to increasing purity include the use of two affinity tags in a double-tag purification procedure (Cass et al., 2005) or the combination of His-tag removal with a reverse IMAC purification step (Block et al., 2008; see [Proteolytic affinity tag cleavage](#)).

Although recombinant His-tagged proteins can be expressed and purified from various expression systems, the following protocols focus on the purification of proteins from *E. coli* as it is the most widely used expression system. The amounts given in the following protocols usually give good results for the purification of His-tagged proteins showing an expression level of 10–50 mg per liter culture volume. However, due to the scalability of the purification procedure, the protocols can be readily scaled up or down depending on the required amount of target protein and the individual expression level of the His-tagged protein. Moreover, the protocols can be easily adapted to other protein expression systems by adjusting the amount of processed culture volume and the cell lysis method (e.g., replacing lysozyme with 1% Igepal CA-630 for insect and mammalian cells. For an example of a lysis protocol, see [Lysis of mammalian and Sf9 cells](#)).

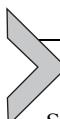
Since it is difficult to provide a general protocol for optimal purification for any His-tagged protein, the protocols provided should be used as guidelines and might need to be optimized for a specific target. The buffers given in the protocols can be supplemented to suit the specific requirements of a protein (for an overview see Block et al., 2009), for example, to stabilize a protein by addition of glycerol, to generate reducing conditions or by providing cofactors. However, due to the incompatibility with Ni-NTA resins, strong chelators such as EDTA can be used only in low concentrations and also strong denaturants (e.g., DTT) and ionic detergents (e.g., SDS) are compatible with IMAC only to a certain extent.



## 2. EQUIPMENT

- Refrigerated centrifuge
- End-over-end shaker
- Disposable gravity flow columns

Micropipettors  
 Pipettes  
 Pipettor tips  
 15-ml conical polypropylene centrifuge tubes  
 1.5-ml microcentrifuge tubes



### 3. MATERIALS

Sodium phosphate monobasic ( $\text{NaH}_2\text{PO}_4$ )  
 Sodium chloride (NaCl)  
 Imidazole  
 Sodium hydroxide (NaOH)  
 Tris base  
 Urea  
 Hydrochloric acid (HCl)  
 Lysozyme (e.g., Roche, Cat. # 10837059001)  
 Protease inhibitor cocktail without EDTA (e.g., Roche, Complete, EDTA-free, Cat. # 11836170001)  
 Benzonase (Novagen, Cat. # 70664) (or RNase A and DNase I)  
 IMAC resin (e.g., Ni-NTA Agarose, QIAGEN, Cat. # 30210, or Ni Sepharose HP, GE Healthcare, Cat. # 17-5318-01)

#### 3.1. Solutions & buffers

##### Step 1a Basis Buffer NPI-10

Component	Final concentration	Stock	Amount
$\text{NaH}_2\text{PO}_4$	50 mM	1 M	50 ml
NaCl	300 mM	1 M	300 ml
Imidazole	10 mM	1 M	10 ml

Mix in 550 ml water. Adjust the pH to 8.0 using NaOH. Add water to 1 l

##### Step 2a Wash Buffer NPI-20

Component	Final concentration	Stock	Amount
$\text{NaH}_2\text{PO}_4$	50 mM	1 M	50 ml
NaCl	300 mM	1 M	300 ml
Imidazole	20 mM	1 M	20 ml

Mix in 550 ml water. Adjust the pH to 8.0 using NaOH. Add water to 1 l

### Elution Buffer NPI-500

Component	Final concentration	Stock	Amount
NaH <sub>2</sub> PO <sub>4</sub>	50 mM	1 M	50 ml
NaCl	300 mM	1 M	300 ml
Imidazole	500 mM	1 M	500 ml

Add water to 950 ml. Adjust the pH to 8.0 using NaOH. Add water to 1 l

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**Tip** If reducing conditions are required during protein purification, add 1–5 mM Tris(2-carboxyethyl) phosphine (TCEP) or β-mercaptoethanol (β-ME) to the buffers prior to use. Concentrations up to 20 mM can be used

---

### Step 1b Basis Buffer B

Component	Final concentration	Stock	Amount
NaH <sub>2</sub> PO <sub>4</sub>	100 mM	1 M	100 ml
Tris base	10 mM	1 M	10 ml
Urea	8 M		480 g

Dissolve in ~500 ml of water, and then bring the volume up to 950 ml. Adjust the pH to 8.0 using concentrated HCl. Add water to 1 l. **Note:** Due to the dissociation of urea, the pH of Basis Buffer B should be adjusted immediately before use

### Step 2b Wash Buffer C

Component	Final concentration	Stock	Amount
NaH <sub>2</sub> PO <sub>4</sub>	100 mM	1 M	100 ml
Tris base	10 mM	1 M	10 ml
Urea	8 M		480 g

Dissolve in ~500 ml of water, and then bring the volume up to 900 ml. Adjust the pH to 6.3 using concentrated HCl. Add water to 1 l. **Note:** Due to the dissociation of urea, the pH of Wash Buffer C should be adjusted immediately before use

### Elution Buffer E

Component	Final concentration	Stock	Amount
NaH <sub>2</sub> PO <sub>4</sub>	100 mM	1 M	100 ml
Tris base	10 mM	1 M	10 ml
Urea	8 M		480 g

Dissolve in ~500 ml of water, and then bring volume up to 900 ml. Adjust the pH to 4.5 using concentrated HCl. Add water to 1 l. **Note:** Due to the dissociation of urea, the pH of Elution Buffer E should be adjusted immediately before use



## 4. PROTOCOL

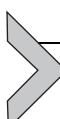
### 4.1. Preparation

Express the His-tagged target protein in a suitable *E. coli* strain and store a cell pellet from 200 ml of expression culture at  $-80^{\circ}\text{C}$  until use.

### 4.2. Duration

Preparation	2–3 days
Protocol	About 3 h

See Fig. 1 for the flowchart of the complete protocol.



## 5. PROTOCOL A: PURIFICATION OF HIS-TAGGED PROTEINS UNDER NATIVE CONDITIONS

### 5.1. Tip

When working under native conditions, keep the cells and protein solutions at  $0\text{--}4^{\circ}\text{C}$  at all times to prevent protein degradation.

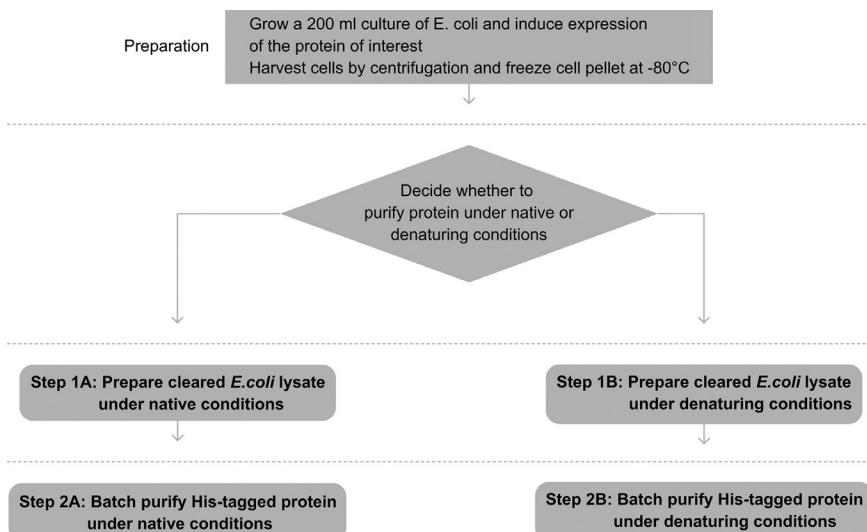
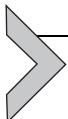


Figure 1 Flowchart of the complete protocol, including preparation.



## 6. STEP 1A PREPARATION OF A CLEARED *E. COLI* LYSATE UNDER NATIVE CONDITIONS

### 6.1. Overview

During this step, a cleared lysate from an *E. coli* cell pellet is generated for the purification of His-tagged proteins under native conditions.

In this protocol, cell lysis is carried out using lysozyme, since it is inexpensive and very efficient for cells that had been frozen. However, lysis methods based on physical disruption (e.g., sonication or homogenization) or detergents (e.g., CHAPS) can also be applied.

### 6.2. Duration

1.5 h

- 1.1a Thaw the *E. coli* cell pellet on ice.
- 1.2a Resuspend the cell pellet completely in 10 ml Basis Buffer NPI-10 supplemented with 1 mg ml<sup>-1</sup> lysozyme. Optionally, add EDTA-free protease inhibitor cocktail.
- 1.3a Add 600 U Benzonase (3 units ml<sup>-1</sup> bacterial culture) to the lysate to reduce viscosity caused by genomic DNA.
- 1.4a Incubate for 30 min on ice.
- 1.5a Centrifuge the lysate for 30 min at 10 000 × g and 2–8 °C.
- 1.6a Collect the supernatant (containing the soluble proteins).

### 6.3. Tip

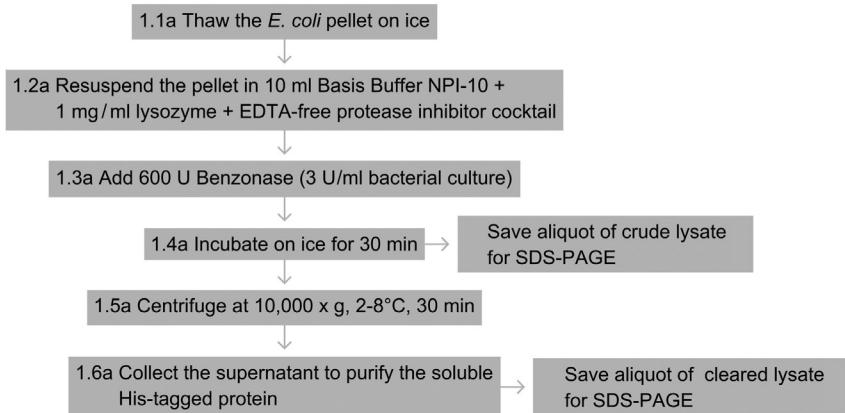
*Freezing an E. coli cell pellet at –20 or –80 °C for at least 30 min increases cell lysis by lysozyme.*

### 6.4. Tip

*The amount of basis buffer NPI-10 used for cell lysis depends on the number of cells and the expression level of the protein. The amount given in this protocol applies to 200 ml of an IPTG-induced bacterial culture of a well-expressed protein (~10–50 mg l<sup>-1</sup>) and might require adaptation to changes in processed culture volume or in cases of higher or lower expressed proteins.*

### 6.5. Tip

*The basis buffer contains 10 mM imidazole to prevent untagged proteins from binding to the column. If the His-tagged protein does not bind under these conditions, reduce the imidazole concentration in the Basis Buffer to 1–5 mM.*

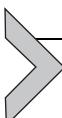
**Step 1A: Prepare cleared *E.coli* lysate under native conditions****Figure 2** Flowchart of Step 1 of Protocol A.**6.6. Tip**

*Save an aliquot of the crude lysate for SDS-PAGE analysis (see One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE)). The crude lysate represents the total cellular protein.*

**6.7. Tip**

*Save an aliquot of the cleared lysate for SDS-PAGE analysis.*

See Fig. 2 for the flowchart of Step 1 of Protocol A.



## 7. STEP 2A BATCH PURIFICATION OF HIS-TAGGED PROTEINS FROM *E. COLI* UNDER NATIVE CONDITIONS

### 7.1. Overview

During this step, the His-tagged target protein is purified under native conditions from the cleared *E. coli* lysate prepared in Step 1 using IMAC resin in a bind-wash-elute procedure. In this protocol binding is performed in batch mode (in contrast to on-column binding), since it is the most efficient method, especially when the target protein is present only at low concentrations or when the His-tag is not fully accessible.

## 7.2. Duration

1.5 h

- 2.1a** Resuspend the IMAC resin by repeated gentle inversion of the bottle and transfer 1 ml of the resin to a 15-ml conical centrifuge tube. The resin is supplied as a 50% slurry, which corresponds to a 0.5 ml bed volume (bv). Allow the resin to settle by gravity and remove the supernatant.
- 2.2a** Add 2.5 ml (5 bv) of Basis Buffer NPI-10 and gently resuspend the slurry to equilibrate the resin. Allow the resin to settle by gravity and remove 2 ml of the supernatant.
- 2.3a** Add the 10 ml cleared lysate prepared in Step 1 to the equilibrated IMAC resin and incubate at 4 °C for 1 h, rotating end-over-end.
- 2.4a** Pour the binding suspension into a disposable gravity flow column with a capped bottom outlet.
- 2.5a** Remove the bottom cap of the column and collect the flow-through.
- 2.6a** Wash the column with 5 ml (10 bv) Wash Buffer NPI-20.
- 2.7a** Elute the His-tagged protein five times with 0.5 ml Elution Buffer NPI-500 (total of 5 bv). Collect each eluate in a separate tube and determine the protein concentration of each of the fractions.

## 7.3. Tip

*To speed up equilibration, the IMAC resin can be pelleted at 500–1000 × g.*

## 7.4. Tip

*Alternatively, batch binding can also be performed directly in a gravity flow column with closed bottom and top outlets.*

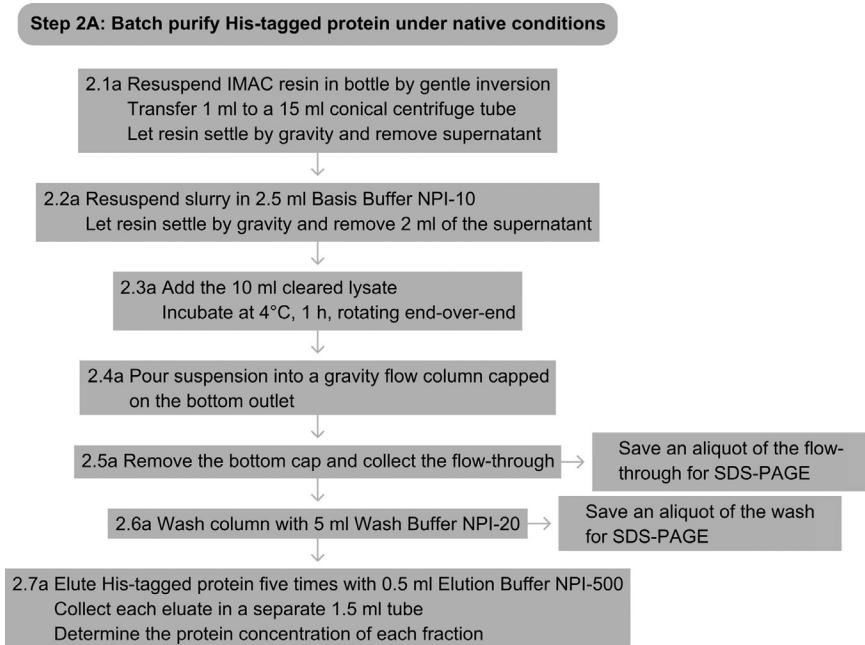
## 7.5. Tip

*After transferring the suspension to the gravity flow column, use Basis buffer NPI-10 to rinse the centrifuge tube and to remove resin that sticks to the wall.*

## 7.6. Tip

*Save small samples at the different steps for subsequent analysis of the purification process by SDS-PAGE (see One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE)).*

See Fig. 3 for the flowchart of Step 2 of Protocol A.



**Figure 3** Flowchart of Step 2 of Protocol A.

## 8. STEP 1B PREPARATION OF A CLEARED *E. COLI* LYSATE UNDER DENATURING CONDITIONS

### 8.1. Overview

During this step, a cleared lysate from an *E. coli* cell pellet is generated for the purification of His-tagged proteins under denaturing conditions. Cells are lysed by a high concentration of urea, which also serves to dissolve insoluble protein aggregates.

### 8.2. Duration

1.5 h

- 1.1b** Thaw the *E. coli* cell pellet on ice.
- 1.2b** Resuspend the cell pellet completely in 10 ml Basis Buffer B.
- 1.3b** Incubate the lysate at room temperature for 30 min, rotating end-over-end.
- 1.4b** Centrifuge the lysate at  $10\ 000\times g$  for 30 min at room temperature.
- 1.5b** Collect the supernatant (containing the chaotropic-soluble proteins).

### 8.3. Tip

The amount of Basis Buffer B used for cell lysis depends on the number of cells and expression rate of the protein. The amount given in this protocol applies to 200 ml of an IPTG-induced bacterial culture of a well expressed protein ( $\sim 10\text{--}50 \text{ mg l}^{-1}$ ) and might require adaption to changes in processed culture volume in cases of higher or lower expressed proteins.

### 8.4. Tip

In some cases 8 M urea is not sufficient to completely solubilize inclusion bodies. In these cases the urea in Basis Buffer B can be replaced by 6 M guanidinium hydrochloride (Gu-HCl). Note: Samples that contain Gu-HCl cannot be directly applied to SDS-PAGE, but need to be diluted or subjected to a precipitation step (see TCA Precipitation or Salting out of proteins using ammonium sulfate precipitation) prior to SDS-PAGE analysis.

### 8.5. Tip

If lysate viscosity is to be reduced by addition of Benzonase (3 units  $\text{ml}^{-1}$  bacterial culture), the urea concentration in Basis Buffer B must be decreased, because Benzonase is active only at urea concentrations of 7 M or below. Note: In contrast to urea, Gu-HCl inactivates Benzonase even at low concentrations.

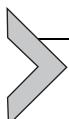
### 8.6. Tip

Save an aliquot of crude lysate for SDS-PAGE analysis (see One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE)). The crude lysate represents the total cellular protein.

### 8.7. Tip

Save an aliquot of cleared lysate for SDS-PAGE analysis.

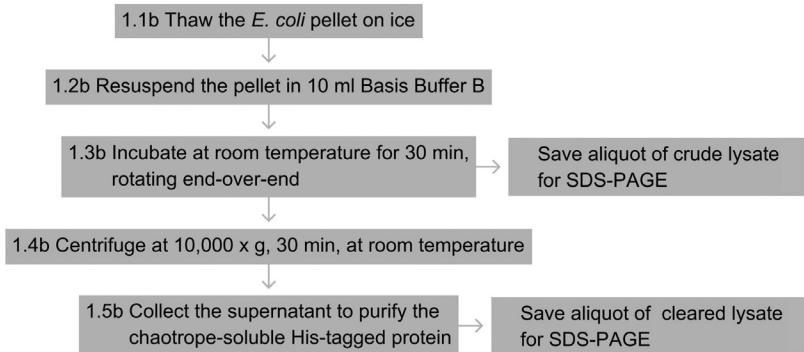
See Fig. 4 for the flowchart of Step 1 of Protocol B.



## 9. STEP 2B BATCH PURIFICATION OF HIS-TAGGED PROTEINS FROM *E. COLI* UNDER DENATURING CONDITIONS

### 9.1. Overview

During this step the His-tagged target protein is purified under denaturing conditions from the *E. coli* cleared lysate prepared in Step 1 using IMAC resin in a bind-wash-elute procedure. Binding is performed in batch mode

**Step 1B: Prepare cleared *E.coli* lysate under denaturing conditions**


**Figure 4** Flowchart of Step 1 of Protocol B.

because it is most efficient, especially when the target protein is present at low concentrations.

## 9.2. Duration

1.5 h

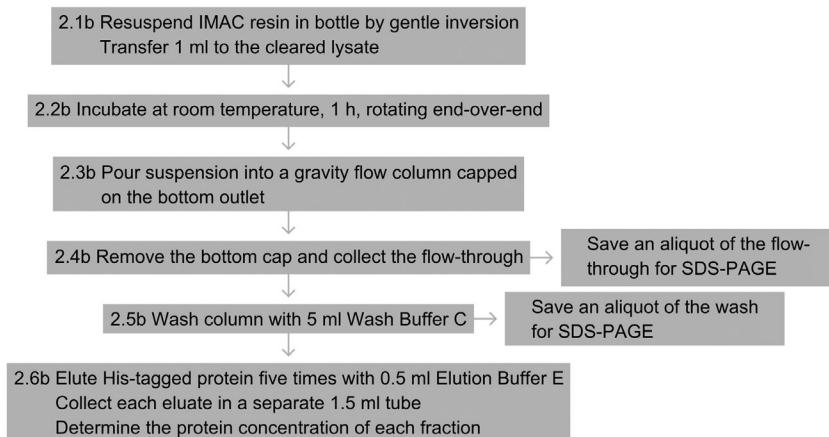
- 2.1b Resuspend the IMAC resin by repeated gentle inversion of the bottle and transfer 1 ml of the resin (supplied as a 50% slurry, which corresponds to 0.5 ml bed volume [bv]) to the cleared lysate prepared in Step 1.
- 2.2b Incubate the lysate–resin mixture at room temperature, rotating end-over-end for 1 h.
- 2.3b Transfer the binding suspension to a disposable gravity flow column with a capped bottom outlet.
- 2.4b Remove the bottom cap of the column and collect the flow-through.
- 2.5b Wash the column with 5 ml (10 bv) Wash Buffer C.
- 2.6b Elute the His-tagged protein five times using 0.5 ml of Elution Buffer E (total of 5 bv). Collect each eluate in a separate tube and determine the protein concentration of each fraction.

## 9.3. Tip

*Alternatively, batch binding can also be performed directly in a gravity flow column with closed bottom and top outlet.*

## 9.4. Tip

*Use Basis Buffer B to rinse the 15-ml centrifuge tube and to remove resin that sticks to the wall.*

**Step 2B: Batch purify His-tagged protein under denaturing conditions**


**Figure 5** Flowchart of Step 2 of Protocol B.

### 9.5. Tip

Save small samples at the different steps for subsequent analysis of the purification process by SDS-PAGE (see One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE)).

### 9.6. Tip

If the target protein is acid-labile, elution can also be performed using 250–500 mM imidazole.

See Fig. 5 for the flowchart of Step 2 of Protocol B.

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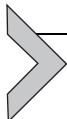
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Lysis of mammalian and Sf9 cells.

One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE).

TCA Precipitation.

Salting out of proteins using ammonium sulfate precipitation.



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## CHAPTER TWO

# Affinity Purification of a Recombinant Protein Expressed as a Fusion with the Maltose-Binding Protein (MBP) Tag

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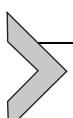
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## Abstract

Expression of fusion proteins such as MBP fusions can be used as a way to improve the solubility of the expressed protein in *E. coli* (Fox and Waugh, 2003; Nallamsetty et al., 2005; Nallamsetty and Waugh, 2006) and as a way to introduce an affinity purification tag. The protocol that follows was designed by the authors as a first step in the purification of a recombinant protein fused with MBP, using fast protein liquid chromatography (FPLC). Cells should have been thawed, resuspended in binding buffer, and lysed by sonication or microfluidization before mixing with the amylose resin or loading on the column. Slight modifications to this protocol may be made to accommodate both the protein of interest and the availability of equipment.

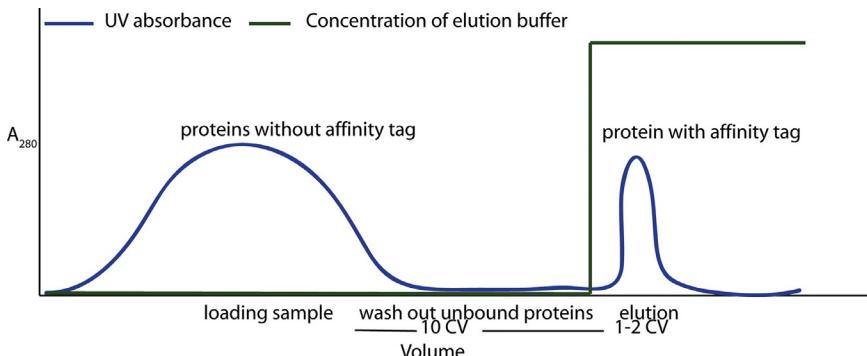
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## 1. THEORY

Affinity chromatography separates proteins on the basis of an interaction between a protein and a specific ligand. The binding of the protein to a ligand attached to a matrix is reversed by either competition or by decreasing the affinity with pH and/or ionic strength. Affinity chromatography is an ideal first purification step due to its selectivity and high capacity. It exploits biological protein functions such as antibody–antigen recognition (e.g., protein A), lectin–polysaccharide binding, nucleic acid–heparin interactions, and recombinant fusion tags of proteins, such as maltose-binding protein and glutathione S-transferase (see [Purification of GST-tagged proteins](#)), or metal chelators such as 6 $\times$  histidine tags (see [Purification of His-tagged proteins](#)). Affinity chromatography can also be used as a way to remove serine proteases such as thrombin and Factor X, utilizing their affinity for benzamidine sepharose (see other methods for affinity purification of proteins on Hydroxyapatite Chromatography: Purification Strategies for Recombinant Proteins, [Protein Affinity Purification using Intein/Chitin Binding Protein Tags](#), [Immunoaffinity purification of proteins](#) or [Strep-tagged protein purification](#)).

Affinity chromatography of MBP fusion proteins can be performed on an FPLC system with an amylose column or batch-wise using amylose agarose resin, followed by a step elution ([Fig. 1](#)). The purification of an MBP-fusion protein exploits the natural affinity of MBP for  $\alpha$ -(1–4) maltodextrin.



**Figure 1** Chromatograph of a purification of an MBP-tagged fusion protein using amylose beads.

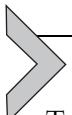
However, the MBP fusion tag should be considered mainly as a way to improve the solubility of a protein rather than as an affinity tag for the column to ease purification (for other ways to improve solubility, see Explanatory Chapter: Troubleshooting protein expression: what to do when the protein is not soluble). Only two buffers are required: a binding buffer and an elution buffer. They only differ in the presence of 10 mM maltose in the latter.

Many vectors are available to construct fusion proteins with MBP. Some of them target the fusion protein to the cytoplasm and others to the periplasmic space. Usually, higher yields of expression have been achieved in the cytoplasm than in the periplasm. Many of these vectors are available with a variety of sites for protease cleavage and may be obtained through academic laboratories ([Fox and Waugh, 2003](#); [Kapust and Waugh, 1999](#); [Geisbrecht et al., 1998](#)) and commercial vendors (e.g., New England Biolabs and Invitrogen).



## 2. EQUIPMENT

- FPLC (capable of reading UV absorbance at 280 nm)
- Amylose column (or amylose resin)
- Sidearm filtering flask
- Filter holder
- 0.22  $\mu\text{m}$  syringe filters
- 0.22  $\mu\text{m}$  filters (for vacuum filtration assembly)



### 3. MATERIALS

Tris base

Hydrochloric acid (HCl)

Sodium chloride (NaCl)

Dithiothreitol (DTT)

EDTA

Amylose resin (or amylose column)

#### 3.1. Solutions & buffers

##### Step 1 Binding buffer

Component	Final concentration	Stock	Amount
Tris-HCl, pH 8.0	50 mM	1 M	50 ml
NaCl	200 mM	1 M	200 ml
EDTA	1 mM	0.5 M	2 ml

Add water to 1 l and pass through a 0.22 µm filter

Optional: 0.1-mM Na azide, or reducing agents such as 1 mM DTT or β-mercaptoethanol can be added

##### Step 4 Elution buffer

Component	Final concentration	Stock	Amount
Tris-HCl, pH 8.0	50 mM	1 M	50 ml
NaCl	200 mM	1 M	200 ml
EDTA	1 mM	0.5 M	2 ml
Maltose	10 mM	0.5 M	20 ml

Add water to 1 l and pass through a 0.22 µm filter

Any reducing agents used in the binding buffer should also be used in the elution buffer

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**Tip** Other buffers such as HEPES or MOPS are well tolerated. Buffers that contain concentrations of up to 10% glycerol, 5% ethanol, or acetonitrile are compatible with the beads

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**Tip** EDTA should be removed by dialysis before cleaving with a protease that requires a divalent cation for activity (e.g.,  $\text{Ca}^{+2}$ )

---



## 4. PROTOCOL

### 4.1. Duration

Preparation	Varies
Protocol	About 2 h

### 4.2. Preparation

Grow bacteria and induce for expression of the MBP fusion protein according to the expression system used (see Small-scale Expression of Proteins in *E. coli*). Harvest the cells by centrifugation and freeze the cell pellet at –80 °C (optional). Resuspend the cell pellet in about 10-ml binding buffer per gram of cells, lyse the cells (e.g., by sonication or French press), and centrifuge at 9000  $\times$  g to remove the cell debris. Keep in mind that typical binding capacities for amylose agarose columns are 3 mg of protein per milliliter of column resin.

Determine the maximum and optimal flow rates for the column and the FPLC.

Prepare the collection tubes to save fractions from Steps 2–4 for further analysis.

See [Fig. 2](#) for the flowchart of the complete protocol.



## 5. STEP 1 EQUILIBRATION OF THE COLUMN

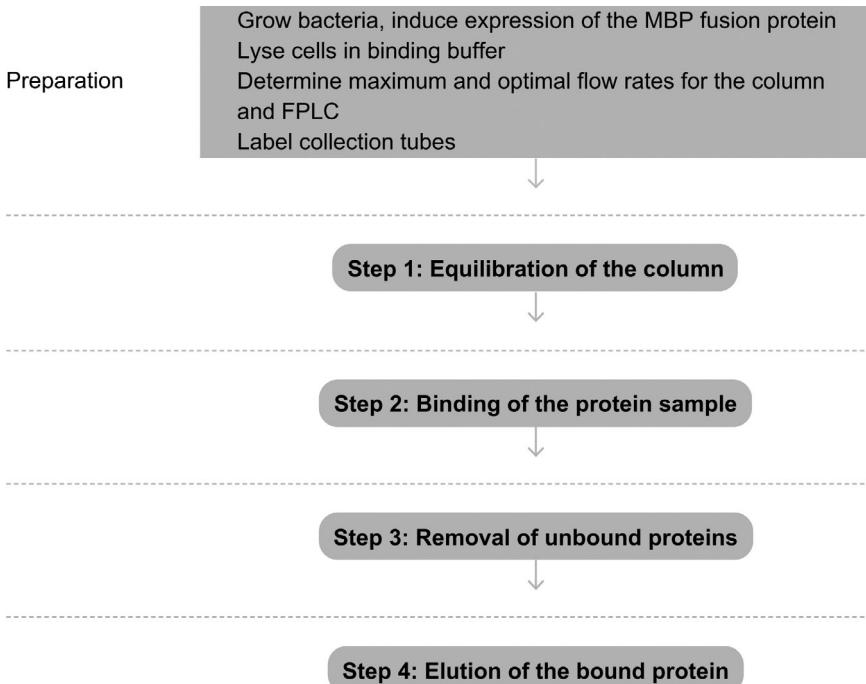
### 5.1. Overview

The column will be prepared for the binding of the protein.

### 5.2. Duration

15 min

- 1.1 Follow the manufacturer's instructions for removing the storage buffer of the column and placing the column in water.
- 1.2 Equilibrate the column by running at least 8–10 column volumes of binding buffer over the beads. Check whether the UV absorbance reading (at 280 nm) is stable before proceeding.
- 1.3 Set the UV absorbance reading to zero.



**Figure 2** Flowchart of the complete protocol, including preparation.

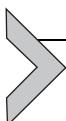
### 5.3. Tip

If the protein of interest does not contain tryptophan, it will not absorb appreciably at 280 nm. Choose another wavelength, such as 260 nm for tyrosine, to monitor the protein.

### 5.4. Tip

If the UV absorbance reading has many spikes or is unusually high and DTT is present in the equilibration buffer, this may indicate that the DTT is oxidized. If DTT is used, it should be added immediately before purification. Do not use buffers containing DTT that are more than 1 day old.

See [Fig. 3](#) for the flowchart of Step 1.



## 6. STEP 2 BINDING OF THE PROTEIN SAMPLE

### 6.1. Overview

The protein will be applied to the column.

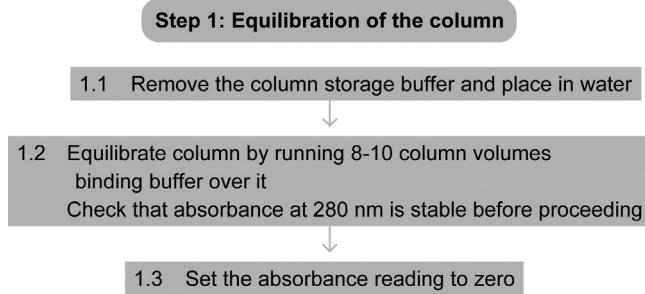


Figure 3 Flowchart of Step 1.

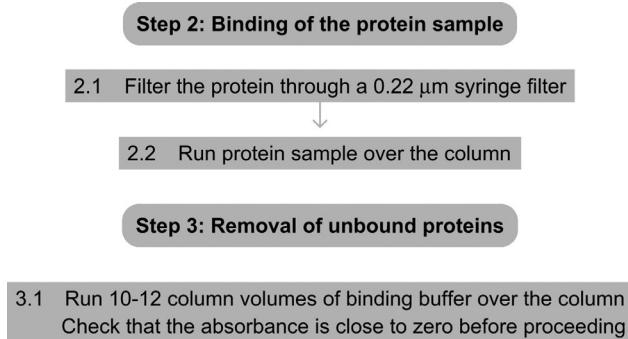


Figure 4 Flowchart of Steps 2 and 3.

## 6.2. Duration

30 min

2.1 Filter the protein through a 0.22-μm syringe filter.

2.2 Run the protein sample over the column.

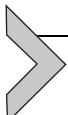
## 6.3. Tip

If the protein sample contains many aggregates, the syringe filter may clog during filtering. If this occurs, simply use multiple filters to filter the sample.

## 6.4. Tip

If the binding between amylose and the MBP fusion protein is weak, consider alternative constructs with additional tags for affinity purification (see [Purification of GST-tagged proteins](#), [Purification of His-tagged proteins](#), [Protein Affinity Purification using Intein/Chitin Binding Protein Tags](#), [Strep-tagged protein purification](#)).

See Fig. 4 for the flowchart of Steps 2 and 3.



## 7. STEP 3 REMOVAL OF UNBOUND PROTEINS

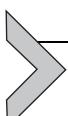
### 7.1. Overview

Any remaining weakly interacting or nonbinding proteins will be removed.

### 7.2. Duration

10 min

- 3.1** Run at least 10–12 column volumes of binding buffer over the beads. Check whether the UV absorbance is zero or close to zero before proceeding.



## 8. STEP 4 ELUTION OF THE BOUND PROTEIN

### 8.1. Overview

An isocratic elution using elution buffer (containing 10 mM maltose) will be performed to elute proteins with MBP tags.

### 8.2. Duration

1 h

- 4.1** Run 2-column volumes of elution buffer to elute the protein of interest. Collect fractions from the column.
- 4.2** Regenerate the beads according to the manufacturer's protocol.
- 4.3** Analyze fractions (loaded sample, flow through, washes, eluted protein) by SDS-PAGE (see One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE)). Pool peak fractions containing the purified protein.
- 4.4** Dialyze pooled fractions in the buffer required for protease cleavage.
- 4.5** If the fusion protein will be stored at  $-80^{\circ}\text{C}$ , either flash-freeze in liquid nitrogen and/or add 10% glycerol.

### 8.3. Tip

*Most MBP fusion proteins elute in the first column volume; the suggested size of the fractions collected is 1/5 of the column volume.*

### 8.4. Tip

*To improve binding of the fusion protein to the amylase resin, consider removing all low-ionic detergents such as Triton X-100 and Tween-20. These have been shown to interfere with binding in some cases (see also Explanatory Chapter: Choosing the right detergent).*

## 8.5. Tip

The affinity of amylose for MBP fusion constructs requires that the MBP portion of the protein be properly folded. The presence of denaturants such as urea and guanidinium chloride abolishes binding since they unfold MBP.

## 8.6. Tip

If the MBP tag will be cleaved (see [Proteolytic affinity tag cleavage](#)), do not subject the cleavage reaction to the column to separate the cleaved and the uncleaved proteins. Residual maltose will interfere with binding. Ion exchange chromatography (see [Using ion exchange chromatography to purify a recombinantly expressed protein](#)) is the preferred method to separate the cleaved and uncleaved species. *E. coli* MBP has a pI of 4.9 and binds efficiently to anion exchange resins at pH values higher than its pI. It has been observed that some proteins precipitate after being cleaved from MBP.

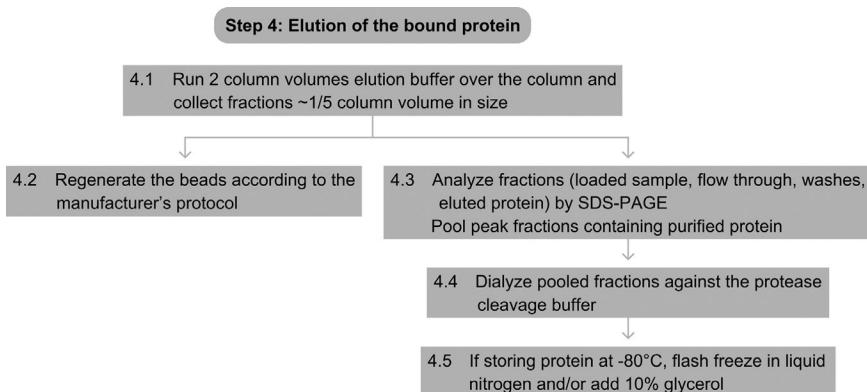
## 8.7. Tip

If the MBP fusion protein is insoluble, try expressing it at a lower temperature (e.g., 30 °C or 15 °C. See also [Explanatory Chapter: Troubleshooting protein expression: what to do when the protein is not soluble](#)).

## 8.8. Tip

Some, but not all, proteins require the addition of glycerol for freezing.

See [Fig. 5](#) for the flowchart of Step 4.



**Figure 5** Flowchart of Step 4.

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Strep-tagged protein purification.

Explanatory Chapter: Troubleshooting protein expression: what to do when the protein is not soluble.

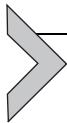
Small-scale Expression of Proteins in *E. coli*.

One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE).

Explanatory Chapter: Choosing the right detergent.

Proteolytic affinity tag cleavage.

Using ion exchange chromatography to purify a recombinantly expressed protein.



# Immunoaffinity Purification of Proteins

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## Abstract

This protocol entails a single-step, high-affinity purification of proteins using an immobilized antibody column.

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## 1. THEORY

Antibodies typically recognize antigens with a high degree of specificity and avidity. These characteristics make antibodies an excellent affinity matrix for protein purification. In addition, there is usually a high recovery of the antigen from the column. Combined, these characteristics make immunoaffinity purification a highly attractive step in the purification of a protein.

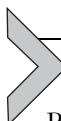
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## 2. EQUIPMENT

Refrigerated centrifuge  
Nutator mixer or Platform rocker  
Dialysis tubing or Slide-A-Lyzer dialysis units  
50-ml polypropylene tube  
Gravity-flow column

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## 3. MATERIALS

Protein-specific antibody  
Tris base  
Hydrochloric acid (HCl)  
Sodium chloride (NaCl)  
Glycine  
Sepharose

### 3.1. Solutions & buffers

#### Step 1 Binding Buffer

Component	Final concentration	Stock	Amount
Tris-HCl, pH 8.0	25 mM	1 M	25 ml
NaCl	300 mM	5 M	60 ml

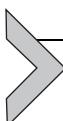
Add water to 1 l

#### Step 4 Elution Buffer

Add 25 ml 1 M glycine, pH 2.7–225 ml of water to give a final concentration of 100 mM

#### Neutralization Buffer

1 M Tris-HCl, pH 9.0



## 4. PROTOCOL

### 4.1. Duration

Preparation	2 days
Protocol	2 days

### 4.2. Preparation

Couple antibody to Sepharose beads (see Coupling Antibody to Cyanogen Bromide-Activated Sepharose).

Dialyze protein into binding buffer.

Centrifuge the protein solution at  $16000 \times g$ , 4 °C, for 15 min to remove insoluble and aggregated materials.

### 4.3. Tip

*Reducing agents cannot be used during any step in this protocol because they will reduce the disulfide bonds in the antibody and diminish its activity.*

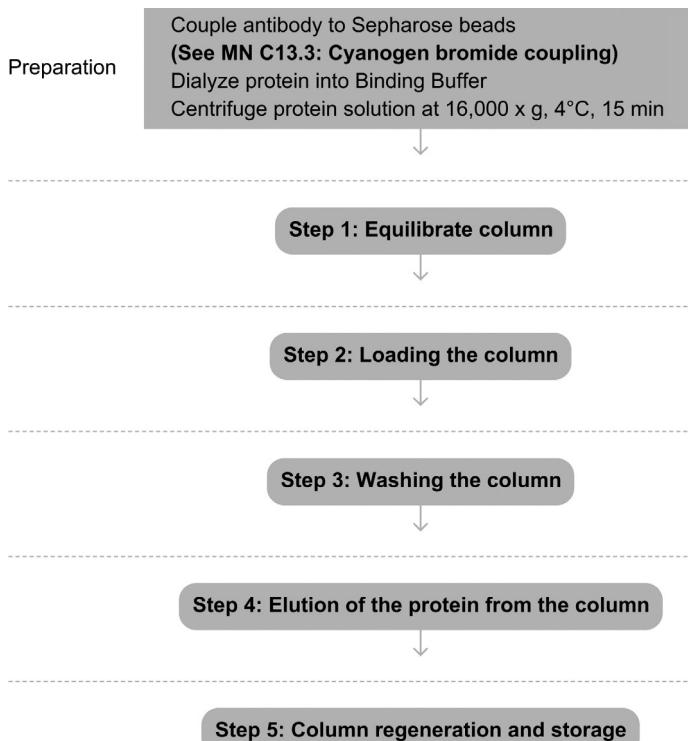


Figure 1 Flowchart of the complete protocol, including preparation.

## 4.4. Tip

To retain protein activity and avoid proteolysis, all steps should be performed at 4 °C.

See Fig. 1 for the flowchart of the complete protocol.



## 5. STEP 1 EQUILIBRATE COLUMN

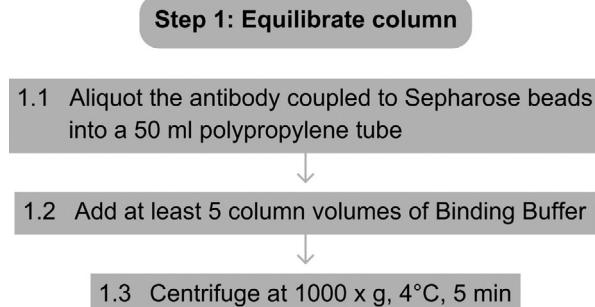
### 5.1. Overview

Column is washed with the same buffer used as a diluent for the protein.

### 5.2. Duration

30 min

- 1.1 Aliquot the antibody coupled to Sepharose beads into a 50-ml polypropylene tube.
- 1.2 Add a minimum of 5 column volumes of Binding Buffer to the resin.
- 1.3 Spin the resin at 1000 × g at 4 °C, for 5 min and decant the supernatant.



**Figure 2** Flowchart of Step 1.

### 5.3. Tip

*The amount of resin needed depends on the amount of protein to be purified and the efficacy of the antibody. Initially, use at least a two- to fivefold molar excess of antibody to protein purified.*

### 5.4. Tip

*The protein to be purified should be stable in binding buffer. If not, the binding buffer should be modified as needed.*

See Fig. 2 for the flowchart of Step 1.



## 6. STEP 2 LOADING THE COLUMN

### 6.1. Overview

The resin and protein solution are incubated to allow the antibody to bind the protein.

### 6.2. Duration

Overnight

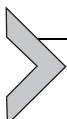
- 2.1 Save a small sample of the protein to be purified before mixing with resin for later gel analysis.
- 2.2 Mix the protein solution and the antibody resin.
- 2.3 Incubate the mixture on a nutator overnight at 4 °C.

### 6.3. Tip

*Remove an aliquot of the slurry supernatant after an hour and analyze by SDS-PAGE (see One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE)) or Western blotting (see Western Blotting using Chemiluminescent*

*Substrates) to see whether binding is complete. If so, next time shorten the column loading time to 1 h.*

See Fig. 3 for the flowchart of Step 2.



## 7. STEP 3 WASHING THE COLUMN

### 7.1. Overview

Unbound material and nonspecific proteins are washed off of the column.

### 7.2. Duration

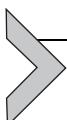
1–2 h

- 3.1 Pour the slurry of the protein bound to the antibody beads into an empty gravity-flow column.
- 3.2 Collect the flow through and remove a sample to analyze by SDS-PAGE (see One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE)).
- 3.3 Wash the column with 10–25 column volumes of Binding Buffer.
- 3.4 Collect the washes and remove a sample for SDS-PAGE.

### 7.3. Tip

*If needed, more stringent washes can be performed at this step to remove nonspecific proteins thereby increasing the purity of the protein. More stringent washing conditions can include increasing the salt concentration (e.g., to 0.5 M NaCl) or adding a small amount of detergent (e.g., 0.1% NP-40).*

See Fig. 4 for the flowchart of Step 3.



## 8. STEP 4 ELUTION OF THE PROTEIN FROM THE COLUMN

### 8.1. Overview

The protein is eluted from the column under acidic conditions. The eluted fractions must be neutralized quickly to minimize any deleterious effects on the protein's structure and function.

### 8.2. Duration

2 h

- 4.1 Prepare elution collection tubes by adding 200 µl of Neutralization Buffer per ml of eluate to be collected.
- 4.2 Add 10 column volumes of Elution Buffer to the column.

## Step 2: Loading the column

2.1 Have ready the protein to be purified

2.1 Remove a sample for SDS-PAGE

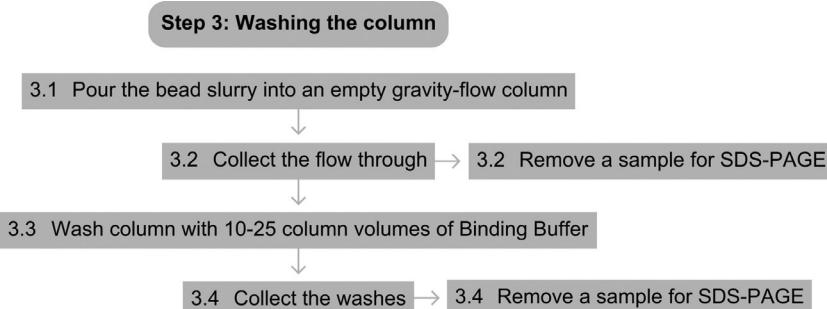


2.2 Mix the protein solution and the antibody resin



2.3 Incubate at 4°C, O/N, with mixing

Figure 3 Flowchart of Step 2.



**Figure 4** Flowchart of Step 3.

- 4.3 Manually collect five 2-column-volume fractions into the prepared collection tubes. Remove a sample of each fraction for SDS-PAGE analysis.
- 4.4 Quickly mix each fraction to neutralize the solution.
- 4.5 Analyze the efficiency of purification by running samples on an SDS-PAGE gel (see One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE)) and analyze by staining (see Coomassie Blue Staining) or Western blotting (see Western Blotting using Chemiluminescent Substrates), as appropriate.

### **8.3. Tip**

*If there is a large amount of protein in the flow through, increase the column size. If there is still protein bound to the column after elution, try harsher elution conditions. If the eluted protein is not clean, try more stringent wash conditions.*

### **8.4. Tip:**

*If elution is not complete, the protein can be eluted in a larger volume, or additives can be added to the elution buffer to disrupt the antigen–antibody interaction such as high salt (1 M NaCl) or detergent.*

### **8.5. Tip:**

*If acidic conditions interfere with protein quality, other elution conditions can be tried such as using a basic pH 9 elution, incubation with a small molecule that competes for the antigen binding site, high ionic conditions such as 1 M NaCl, or high concentrations of propylene glycol.*

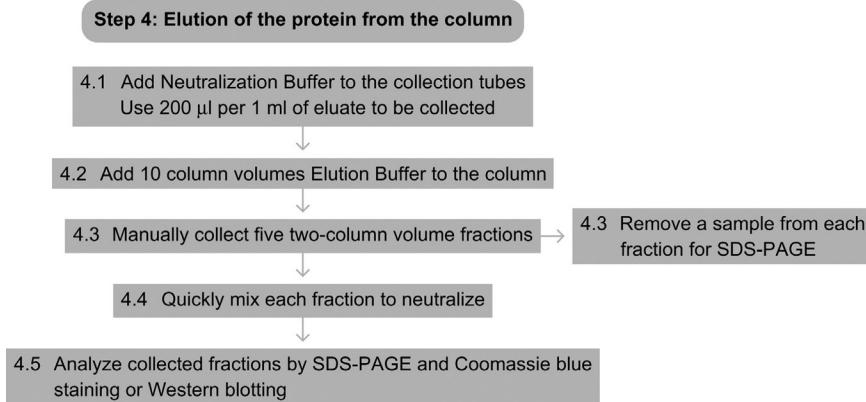


Figure 5 Flowchart of Step 4.

## 8.6. Tip:

If the antigen being recognized is an affinity tag, the protein of interest can be cloned so that the affinity tag is removable by protease cleavage. In this case, the protein can be liberated from the immunoaffinity column by incubation with the appropriate protease. This method is gentler than the acidic elution conditions outlined here.

See Fig. 5 for the flowchart of Step 4.

# 9. STEP 5 COLUMN REGENERATION AND STORAGE

## 9.1. Overview

Immunoaffinity columns can be recharged for future use.

## 9.2. Duration

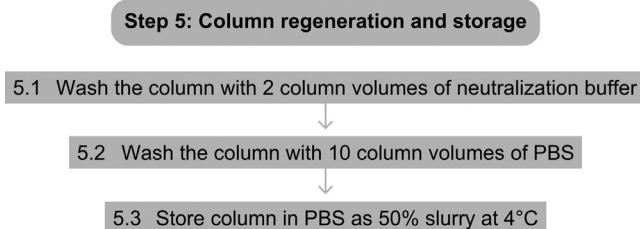
1 h

- 5.1 Wash the column with 2 column volumes of neutralization buffer.
- 5.2 Wash the column with 10 column volumes of PBS.
- 5.3 Store the column in PBS as 50% slurry at 4 °C.

## 9.3. Tip

Immunoaffinity columns can often be reused multiple times. However, this should be determined empirically for each specific antibody column.

See Fig. 6 for the flowchart of Step 5.



**Figure 6** Flowchart of Step 5.

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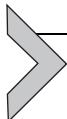
Referenced Protocols in Methods Navigator

Coupling Antibody to Cyanogen Bromide-Activated Sepharose.

One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE).

Western Blotting using Chemiluminescent Substrates.

Coomassie Blue Staining.



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## CHAPTER FOUR

# Affinity Purification of Protein Complexes Using TAP Tags

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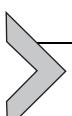
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## Abstract

This protocol is used for the isolation and analysis of protein complexes using the tandem affinity purification (TAP) tag system. The protocol describes the purification of a protein fused to a TAP tag comprised of two protein A domains and the calmodulin binding peptide separated by a TEV cleavage site. This is a powerful technique for rapid purification of protein complexes and the analysis of their stoichiometric composition, posttranslational modifications, structure, and functional activities.



## 1. THEORY

This is a two-step purification protocol that allows for the identification of specific protein complexes with a low background of contaminating proteins (Rigaut et al., 1999; Puig et al., 2001). The strategy has been used successfully for purification and identification of complexes from *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and mammalian cells (Tanny et al., 2004; Verdel et al., 2004; Chen et al., 2001). The TAP-tagged protein is purified, first, by binding of its protein A tag to an IgG resin. After cleavage with TEV (tobacco etch virus) protease, the remaining calmodulin-binding peptide (CBP) fusion protein is bound to a calmodulin resin. The target protein is then eluted by the addition of a calcium chelator, resulting in purified material that is free of peptide or IgG, which is ideal for identifying interacting partners using tandem mass spectrometry approaches. However, since the TAP tag is quite large, not all proteins are functional when fused with this tag. It is critical to test functionality prior purification. In addition, weak or transient interactions may not be detected using this two-step purification method and therefore, a one-step purification approach may also need to be tried. Other combinations of tags have also been successfully tried (Bürckstümmer et al., 2006; Gloeckner et al., 2007).

TAP purifications have emerged as ideal methods for the isolation and characterization endogenous proteins or complexes that are not particularly

abundant. The two purification steps provide sufficient purity for the biochemical and proteomic analysis of most complexes under study in our laboratory. This protocol can easily be scaled up or down depending on protein expression level and desired protein yield.



## 2. EQUIPMENT

Refrigerated centrifuge  
Bead beater (BioSpec Bead Beater, model 1107900)  
0.5-mm glass beads (BioSpec)  
Refrigerated microcentrifuge  
SDS-PAGE gel rig  
Platform rotator/mixer  
Poly-prep columns (BioRad)  
Poly-prep column caps and tip closures (BioRad)  
Square stand (ring stand)  
Stand clamps  
50-ml polypropylene conical tubes  
15-ml polypropylene conical tubes  
Pipet-aid  
Pipettes  
Micropipettors  
Pipettor tips  
1.7-ml polypropylene microcentrifuge tubes



## 3. MATERIALS

Tris base  
Sodium chloride (NaCl)  
Calcium chloride ( $\text{CaCl}_2$ )  
EDTA  
EGTA  
Magnesium acetate ( $\text{Mg(OAc)}_2$ )  
Sodium phosphate dibasic ( $\text{Na}_2\text{HPO}_4$ )  
Sodium phosphate monobasic monohydrate ( $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ )  
Sodium fluoride (NaF)  
 $\text{Na}_2\text{VO}_4$   
Imidazole  
**NP-40**

Trichloroacetic acid  
 2-Mercaptoethanol (BME)  
 Glycerol  
 Potassium acetate (KOAc)  
 1,3-Dithiothreitol (DTT)  
 Leupeptin  
 Benzamidine  
 Phenylmethylsulfonyl fluoride (PMSF)  
 EDTA-free complete protease inhibitor tablet (Roche)  
 IgG-Sepharose (GE Healthcare)  
 Calmodulin-Sepharose (GE Healthcare)  
 TEV protease, recombinant (Invitrogen)  
 Ethanol  
 Acetone  
 Anti-TAP antibody (Thermo Fisher)  
 Bromophenol Blue  
 Sodium dodecyl sulfate (SDS)

### 3.1. Solutions & buffers

#### Step 1 Lysis Buffer

Component	Final concentration	Stock	Amount
Na <sub>2</sub> PO <sub>4</sub>	6 mM	–	0.85 g
NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O	4 mM	–	0.55 g
NP-40	1%	100%	10 ml
NaCl	150 mM	–	8.8 g
EDTA	2 mM	0.5 M	4 ml
EGTA	1 mM	0.5 M	2 ml
NaF	50 mM	–	2.1 g
Leupeptin	4 µg ml <sup>-1</sup>	–	4 mg
Na <sub>2</sub> VO <sub>4</sub>	0.1 mM	0.5 M	0.2 ml
Glycerol	5%	100%	50 ml

Add water to 1 l

Filter-sterilize, wrap in foil and store at 4 °C until use

Aliquot 50 ml immediately prior to use and add 1 complete protease inhibitor cocktail tablet (Roche) – or – pepstatin, bestatin, and aprotinin to 1 µg ml<sup>-1</sup>;

130 µl of 0.5 M benzamidine, freshly prepared in 100% ethanol

500 µl of 100 mM PMSF, freshly prepared in 100% ethanol

**Step 2 Wash Buffer**

<b>Component</b>	<b>Final concentration</b>	<b>Stock</b>	<b>Amount</b>
Tris-HCl, pH 8.0	10 mM	1 M	1 ml
NaCl	150 mM	5 M	3 ml
NP-40	0.1%	10%	1 ml

Add water to 100 ml

**Step 3 TEV Cleavage (TEV-C) Buffer**

<b>Component</b>	<b>Final concentration</b>	<b>Stock</b>	<b>Amount</b>
Tris-HCl, pH 8.0	10 mM	1 M	0.5 ml
KOAc	150 mM	5 M	1.5 ml
NP-40	0.1%	10%	0.5 ml
EDTA	0.5 mM	0.5 M	50 µl
DTT*	1 mM	1 M	50 µl

\*Add DTT immediately before use

Add water to 50 ml

**Step 4 Calmodulin Binding (CAM-B) Buffer**

<b>Component</b>	<b>Final concentration</b>	<b>Stock</b>	<b>Amount</b>
Tris-HCl, pH 8.0	10 mM	1 M	1 ml
NaCl	150 mM	5 M	3 ml
Mg(OAc) <sub>2</sub>	1 mM	1 M	0.1 ml
Imidazole	1 mM	1 M	0.2 ml
CaCl <sub>2</sub>	2 mM	1 M	0.2 ml
BME*	14.3 M	14.3 M	69.7 µl

\*Add BME immediately before use

Add water to 100 ml

**Calmodulin Elution (CAM-E) Buffer**

<b>Component</b>	<b>Final concentration</b>	<b>Stock</b>	<b>Amount</b>
Tris-HCl, pH 8.0	10 mM	1 M	100 µl
NaCl	150 mM	5 M	300 µl
Mg(OAc) <sub>2</sub>	1 mM	1 M	10 µl

Imidazole	1 mM	1 M	10 µl
EGTA	10 mM	0.5 M	200 µl
BME*	10 mM	14.3 M	6.9 µl

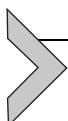
\*Add BME immediately before use

Add water to 10 ml

### Step 5 2× Sample Buffer

Component	Final concentration	Stock	Amount
Tris-HCl, pH 6.8	100 mM	1 M	100 µl
SDS	4%	25%	160 µl
Glycerol	20%	50%	400 µl
DTT	200 mM	1 M	200 µl
PMSF	2 mM	100 mM	20 µl
Bromophenol blue	0.2%		2 mg

Add water to 1 ml



## 4. PROTOCOL

### 4.1. Preparation

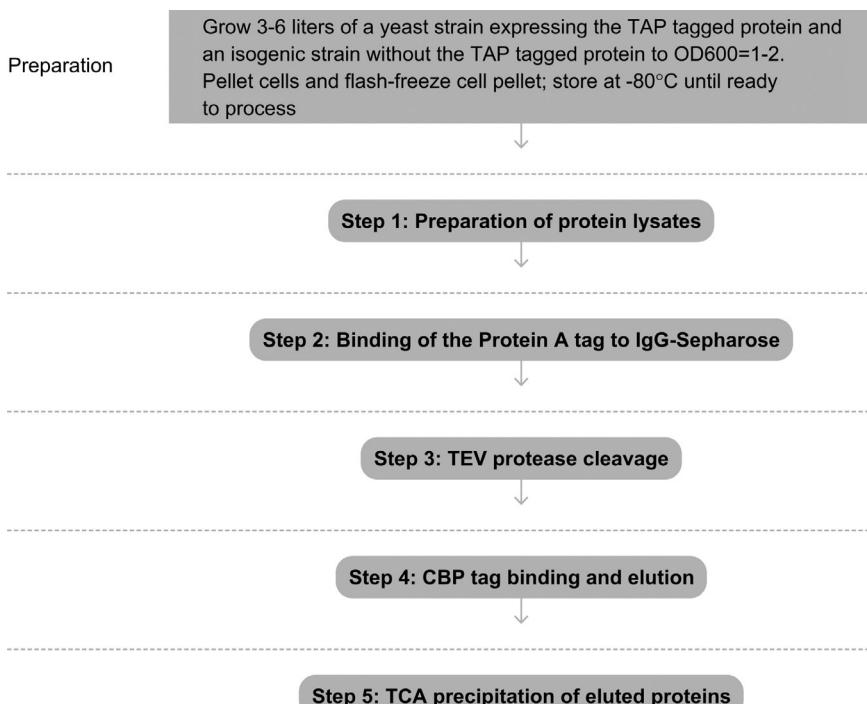
Grow 3–6 l of yeast to  $OD_{600} = 1–2$ . Pellet the cells by centrifugation and flash-freeze the cell pellet in liquid nitrogen. The pellets are usually about 5–10 g. Store all pellets at  $-80^{\circ}\text{C}$ .

For each purification, include an untagged strain isogenic to the strain expressing the TAP-tagged protein. During proteomics analysis, the untagged strain will be used as a comparison for nonspecific background binding.

### 4.2. Duration

Preparation	About 1 day
Protocol	About 6–10 h

See Fig. 1 for the flowchart of the complete protocol.



**Figure 1** Flowchart of the complete protocol, including preparation.

## 5. STEP 1 PREPARATION OF PROTEIN LYSATES

### 5.1. Overview

Pelleted, frozen yeast cells will be lysed by bead beating and the resulting lysates will be cleared by centrifugation to remove cell debris before beginning the purification. Other lysis methods, which generate less heat than bead beating, may be more suitable for some protein purifications and should be considered. These include lysis in a coffee grinder in the presence of dry ice (Schultz et al., 1997), lysis under liquid nitrogen using a mortar and pestle, or lysis in a liquid nitrogen-cooled steel chamber (e.g., Retsch Cryomill).

### 5.2. Duration

1–1.5 h

**1.1** Add frozen yeast pellets to the medium BioSpec bead beating chamber (89 ml) filled about half way with 0.5-mm glass beads (so that the beads

just cover the screw that holds the rotor in place). Then add lysis buffer with freshly added protease inhibitors until the chamber is completely filled.

- 1.2 Fill the outer chamber with ice water. Pulse the bead beater 7–10 s up to 8 times with 1 min between each pulse.
- 1.3 Using a pipet-aid and pipette, transfer the lysate to a 50-ml conical tube, leaving behind the glass beads. Wash the beads with 5 ml of lysis buffer containing protease inhibitors and add the wash to the same 50-ml tube.
- 1.4 Spin in a refrigerated centrifuge (such as a Sorvall RC5, with SH-3000 rotor) for 10 min at  $1500 \times g$ .
- 1.5 Transfer the supernatant to a clean conical tube, 15 or 50 ml, depending on the lysate volume. Remove 50  $\mu$ l of each supernatant (supernatant fraction, S1), to be analyzed later (see Step 6.1).

### 5.3. Tip

*Filling the bead beating chamber completely prevents trapping air bubbles, which promote protein denaturation during lysis.*

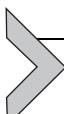
### 5.4. Tip

*The larger 250-ml lysis chamber can also be used for pellets ranging in size from about 10 to 100 g of cells. The larger chamber appears to generate less heat during lysis; however, with smaller pellets, this results in a less concentrated supernatant.*

### 5.5. Tip

*To check for efficient lysis, a small aliquot can be taken from the bead beater and visualized under a microscope. >50% lysis is good.*

See Fig. 2 for the flowchart of Step 1.

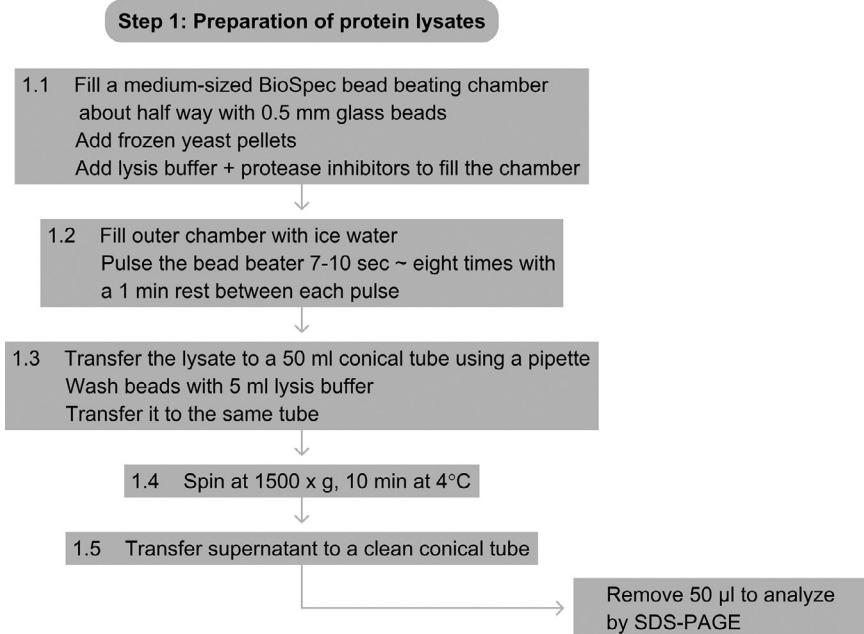


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## 6. STEP 2 BINDING OF THE PROTEIN A TAG TO IgG-SEPHAROSE

### 6.1. Overview

This is the first binding step of the tandem affinity tag purification. Each supernatant will be incubated with IgG-Sepharose beads to capture the target protein. After this binding reaction, the beads will be washed before proceeding with the purification. All steps are carried out in the cold room (at 4 °C).

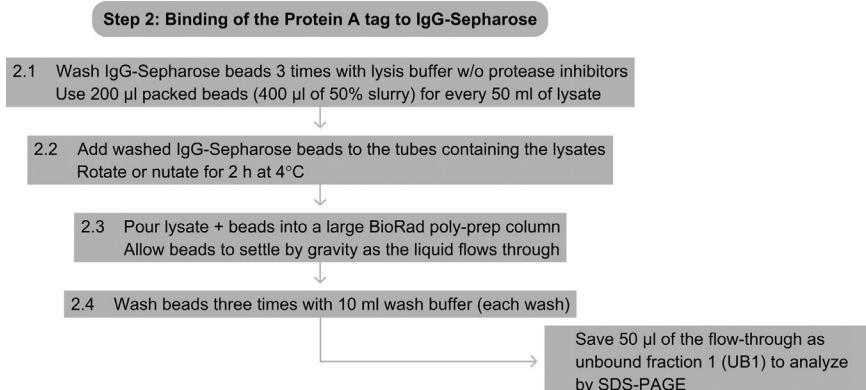


**Figure 2** Flowchart of Step 1.

## 6.2. Duration

2.5–3 h

- 2.1 For every 50 ml of lysate, remove 400 µl of 50% slurry of IgG-Sephaorse beads (200 µl of packed beads). Wash beads 3 times in lysis buffer (without protease inhibitors added), spinning the beads at 500 × g between washes.
- 2.2 Add the washed beads to the conical tubes containing each supernatant and put all tubes on a rotating or nutating platform for 2 h at 4 °C.
- 2.3 Pour the supernatant and beads from the conical tube into a large BioRad poly-prep column. Allow the beads to settle by gravity as the unbound supernatant flows through. Save 50 µl of the flow-through (unbound fraction, UB1), to be analyzed later (see Step 6.1).
- 2.4 Wash the beads 3 times with 10 ml of wash buffer.



**Figure 3** Flowchart of Step 2.

### 6.3. Tip

*Using a vacuum aspirator during bead washing can lead to accidental bead loss. Therefore, use a pipetor instead.*

### 6.4. Tip

*When working with Sepharose or agarose beads, always cut the tip of your pipette using a clean razor blade prior to pipetting the beads. Otherwise the beads will clog the tip during pipetting leading to the uptake of more buffer and fewer beads.*

### 6.5. Tip

*Be gentle with the IgG-Sepharose beads. Never spin the beads at >500 × g, and always resuspend the beads by gently pipetting, never vortexing.*

### 6.6. Tip

*Do not add more than the recommended amount of beads for a given purification. The beads have a high binding capacity, and increasing bead volume does not lead to more target protein binding, but will increase background binding.*

See Fig. 3 for the flowchart of Step 2.

## 7. STEP 3 TEV PROTEASE CLEAVAGE

### 7.1. Overview

The TAP tag is composed of two protein A domains and a CBP domain separated by a TEV protease cleavage site. During this step, the target protein, now bound to IgG beads, is eluted by cleavage using the TEV protease before proceeding to the second binding step.

## 7.2. Duration

2 h

- 3.1 Add 10 ml of TEV-C buffer to the column of washed beads from Step 2.4 and allow to flow through the column.
- 3.2 Close the bottom of the column with a tip closure and move it to room temperature.
- 3.3 Add 1 ml of TEV-C buffer containing the recommended amount of recombinant TEV protease (300–500 U ml<sup>-1</sup> for Invitrogen TEV protease, or as described by manufacturer). Incubate for 1–1.5 h at room temperature. Every 20 min, gently resuspend the beads by tapping the tube or by pipetting up and down.
- 3.4 Align the column with a fresh poly-prep column (with the bottom capped). Carefully remove the stopper and allow the eluate to drain into the new column.
- 3.5 Wash out the old column with 0.5 ml of TEV cleavage buffer, and drain into the new column containing the eluate.
- 3.6 Save a small amount of the IgG-Sepharose beads (IgG beads fraction, B1), to analyze later (see Step 6.1).

## 7.3. Tip

Alternatively, the TEV cleavage can be done overnight at 4 °C on an end-over-end rotator.

See Fig. 4 for the flowchart of Step 3.

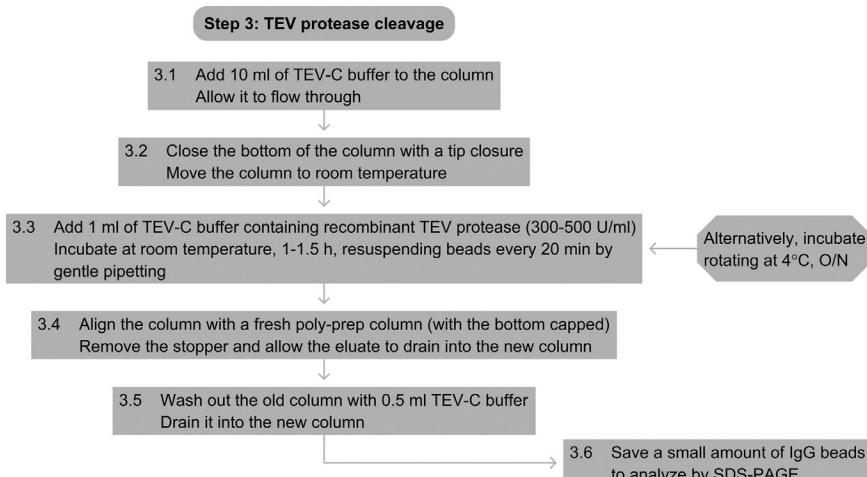


Figure 4 Flowchart of Step 3.



## 8. STEP 4 CBP TAG BINDING AND ELUTION

### 8.1. Overview

This is the second binding step of the tandem affinity tag purification. The target protein, now cleaved by TEV protease, has the CBP tag remaining and will be incubated with Calmodulin-Sepharose beads. After this binding reaction, the beads will be washed and the protein will be eluted and will be ready for analysis. All steps are performed at 4 °C.

### 8.2. Duration

2 h

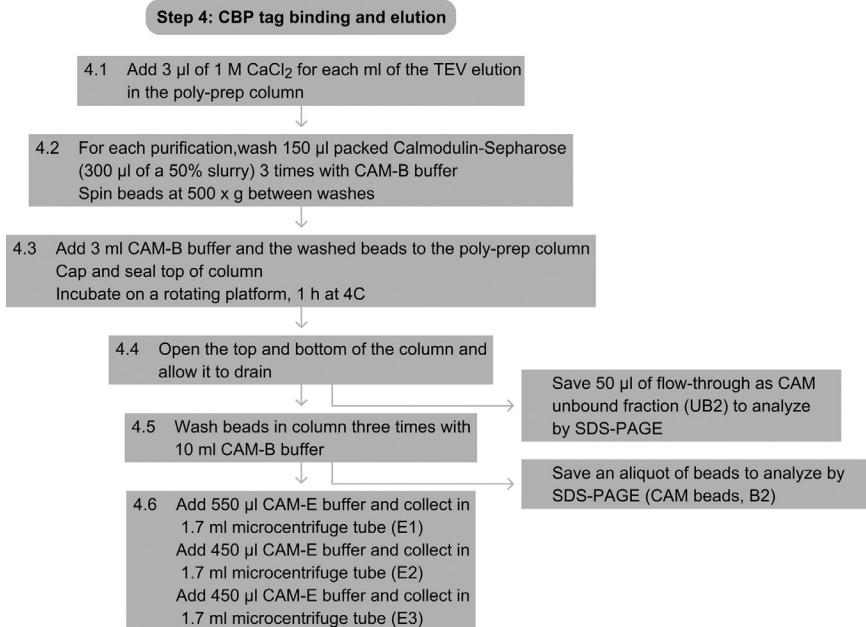
- 4.1 To the poly-prep column containing the TEV eluate (from Step 3.5), add 3 µl of 1 M CaCl<sub>2</sub> for each ml of the TEV elution (e.g., 4.5 µl 1 M CaCl<sub>2</sub> to 1.5 ml of eluate, adjust as necessary for other volumes).
- 4.2 For each purification, remove 300 µl of 50% slurry of Calmodulin-Sepharose beads (150 µl of packed beads). Wash the beads 3 times in CAM-B buffer, spinning the beads at 500 × g between washes.
- 4.3 Add 3 ml of CAM-B buffer and the washed beads to the poly-prep column. Cap and seal the top of the column, and incubate on a rotating platform at 4 °C for 1 h.
- 4.4 After binding, allow the column to drain, but keep an aliquot of the flow-through (CAM unbound fraction, UB2), to analyze later (see Step 6.1).
- 4.5 Wash the beads in the column 3 times 10 ml with CAM-B buffer. Keep an aliquot of the beads to analyze later (CAM beads, B2).
- 4.6 Elute the protein into 1.7-ml microcentrifuge tubes. Obtain three fractions by gently pipetting 550 µl (E1), 450 µl (E2), and 450 µl (E3) of CAM-E buffer to the top of the column bed and allowing it to drain into the collection tubes.

### 8.3. Tip

*To ensure effective washes, for each wash, allow for the entire 10 ml to flow through the column before adding the next wash.*

### 8.4. Tip

*Keep a close eye on your column during the washes. Waiting too long between washes can cause the beads to dry out.*



**Figure 5** Flowchart of Step 4.

## 8.5. Tip

*To get the last drop of each elution fraction, carefully touch the tip of the column to the side of the collection tube while pressing the top of the column with your gloved thumb.*

## 8.6. Tip

*Step 4.6 suggests collecting three fractions, but this can be adjusted to obtain a more concentrated peak fraction if necessary.*

See Fig. 5 for the flowchart of Step 4.



## 9. STEP 5 TCA PRECIPITATION OF ELUTED PROTEINS

### 9.1. Overview

The purified proteins often need to be concentrated by TCA precipitation prior to gel electrophoresis followed by Coomassie Blue staining or silver staining or analysis by mass spectrometry.

## 9.2. Duration

1–3 h

- 5.1 Split each eluate fraction equally into two microcentrifuge tubes. Adjust the volume with 100% TCA to a final concentration of 20% TCA for each eluate. Vortex well and keep on ice for 20 min.
- 5.2 Spin in a microcentrifuge at maximum speed ( $\sim 15\,000 \times g$ ) at 4 °C for 20 min.
- 5.3 Carefully remove the supernatant with a pipettor and discard. Add 1 ml of 10% TCA, cap the tube, invert once to wash the sides of the tube, and spin for 10 min at maximum speed at 4 °C. Carefully remove the supernatant with a pipettor and discard.
- 5.4 Add 1 ml cold acetone (stored at –20 °C), cap the tube, invert, and spin at maximum speed at 4 °C for 10 min. Carefully remove the supernatant with a pipettor and discard. Briefly spin again, and remove the residual liquid with a pipettor. Allow the pellet to air-dry at room temperature for 5–10 min.
- 5.5 To one of the TCA pellets, add 15–20 µl of 1× SDS sample buffer. Leave the tubes at room temperature for at least 30 min, vortexing vigorously every 5 min or so, heat at 65 °C for 10 min, and resolve the proteins by SDS-PAGE (see One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE)). Stain the gel with either colloidal Coomassie Blue or silver to visualize purified proteins (see Coomassie Blue Staining or Silver Staining of SDS-polyacrylamide Gel).
- 5.6 The second TCA pellet can be used for analysis by mass spectrometry, which can also be performed on the whole mixture of proteins or excised bands from silver or Coomassie stained gels (Haas et al., 2006; Shevchenko et al., 1996).

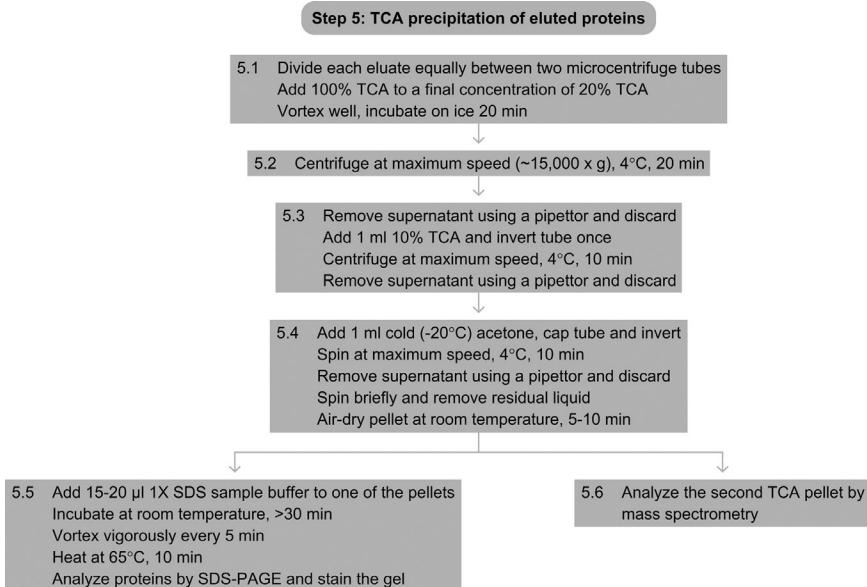
## 9.3. Tip

*Many times there will be no visible pellet after the TCA precipitation, so be careful to gently remove washes with a pipettor, not a vacuum aspirator.*

## 9.4. Tip

*Large TCA pellets may take longer than 30 min to dissolve in sample buffer. It may be necessary to leave the tubes at room temperature overnight prior to loading the gel to ensure that the pellets have completely dissolved.*

See Fig. 6 for the flowchart of Step 5.



**Figure 6** Flowchart of Step 5.



## 10. STEP 6 TROUBLESHOOTING

### 10.1. Overview

In the event that the purification fails to yield the desired protein, the following steps can be taken to troubleshoot.

- 6.1** The fractions that were collected throughout the procedure can help diagnose the step that is problematic. For instance, some proteins can stick to the IgG beads even after TEV cleavage. Performing a Western blot on the collected fractions would indicate that the protein remained on the beads. Take fractions, S1, UB1, B1, UB2, B2, and an aliquot of each eluate and add 2× sample buffer to a final concentration of 1×. See Chapter Western Blotting using Chemiluminescent Substrates for a detailed protocol for Western blotting. After resolving the fractions by SDS-PAGE, probe the membrane with an anti-TAP antibody (Thermo Fisher, binds the CBP portion of the TAP tag).
- 6.2** Proteins with very low expression levels may result in a low yield, in which case you can scale up the procedure to increase yield. For some proteins, we have had to use 100 g of yeast cells instead of the standard 5–10 g described above (Hong et al., 2005; Motamedi et al., 2004).

- 6.3** For proteins that are prone to degradation, alternative lysis methods that prevent heat generation during cell breakage are more desirable. These include lysis in a coffee grinder with dry ice (Schultz et al., 1997), grinding by mortar and pestle under liquid nitrogen, or using a cooled steel mill device (e.g., Retsch Cryomill).

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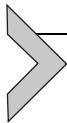
### Referenced Protocols in Methods Navigator

One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE).

Coomassie Blue Staining.

Silver Staining of SDS-polyacrylamide Gel.

Western Blotting using Chemiluminescent Substrates.



# Strep-Tagged Protein Purification

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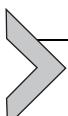
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## Abstract

The Strep-tag system can be used to purify recombinant proteins from any expression system. Here, protocols for lysis and affinity purification of Strep-tagged proteins from *E. coli*, baculovirus-infected insect cells, and transfected mammalian cells are given. Depending on the amount of Strep-tagged protein in the lysate, a protocol for batch binding and subsequent washing and eluting by gravity flow can be used. Agarose-based matrices with the coupled Strep-Tactin ligand are the resins of choice, with a binding capacity of up to  $9\text{ mg ml}^{-1}$ . For purification of lower amounts of Strep-tagged proteins, the use of Strep-Tactin magnetic beads is suitable. In addition, Strep-tagged protein purification can also be automated using prepacked columns for FPLC or other liquid-handling chromatography instrumentation, but automated purification is not discussed in this protocol. The protocols described here can be regarded as an update of the Strep-Tag Protein Handbook (Qiagen, 2009).



## 1. THEORY

Recombinant expressed and purified proteins are a prerequisite for a wide range of downstream applications such as activity assays, interaction analysis, and crystallization approaches. To allow simple and fast purification, recombinant proteins can be fused to different affinity purification tags (see [Purification of His-tagged proteins](#), [Affinity purification of a recombinant protein expressed as a fusion with the maltose-binding protein \(MBP\) tag](#), [Purification of GST-tagged proteins](#), [Protein Affinity Purification using Intein/Chitin Binding Protein Tags](#), and [Affinity Purification of Protein Complexes Using TAP Tags](#)). Together with His- and GST-tags, Strep-tag is one of the most frequently used tags for expression and purification of recombinant proteins. The Strep-tag is one of the systems of choice if native and active proteins should be purified from eukaryotic or prokaryotic cell lysates in a single step since affinity chromatography takes place under physiological conditions. The tag itself is a small, biologically nearly inert peptide with rare influence on protein function, structure and immunogenicity ([Schmidt and Skerra, 2007](#)). The tag can be positioned at the N- or C-terminus and fusion proteins can also be purified in a high-throughput format.

The principle of Strep-tag purification is based on the streptavidin–biotin interaction, an interaction that is known to be among the strongest noncovalent bindings between molecules. Engineering of both streptavidin and the Strep-tag resulted in the Strep-tag system. Compared to the original streptavidin tetramer, Strep-Tactin shows an increased binding capacity

for the eight amino acid peptide ‘WSHPQFEK’ known as Strep-tag II. Strep-Tactin can be immobilized on matrices to allow affinity chromatography of proteins fused to the Strep-tag II under physiological conditions. Due to the high binding affinity of Strep-Tactin and Strep-tag II ( $K_d = 1 \mu\text{M}$ ), single-step purification from (crude) cell lysates can be performed. After a short washing step, gentle elution can be performed with biotin or desthiobiotin. Desthiobiotin is a stable, reversible binding analog of biotin, the natural ligand of streptavidin. Due to the reversible binding feature, it is possible to regenerate the Strep-Tactin resin after elution with desthiobiotin (Schmidt and Skerra, 2007; Skerra and Schmidt, 2000).

The Strep-tag system is especially suited for analysis of native and active proteins, because the purification procedure can be carried out under physiological conditions. On the one hand, this allows the isolation of sensitive proteins in a native state. On the other hand, it is also possible to purify intact protein complexes. Hetero-oligomeric complexes can even be purified if just one subunit carries the Strep-tag (Junttila et al., 2005).

The Strep-tag system is compatible with a variety of different additives that are commonly used in affinity purifications to enhance solubility and yield of recombinant proteins. The reagents according to [Table 1](#) have been used successfully up to the concentration given.

In case ultrapure protein is required, a Two-Step Affinity Purification based on the Strep-tag II and 6xHis tag can result in the simple and efficient purification of such protein (Cass et al., 2005). Recombinant proteins carrying both tags are purified sequentially on Ni-NTA (see [Purification of His-tagged proteins](#)) and Strep-Tactin matrices. Ultrapure protein (>98%) can be obtained from two affinity purifications without the need for protein-specific purification protocol development and optimization. The order of purifications can be reversed and no buffer exchange is required after elution from the first column. Two-step purification is not a subject of this protocol; for further information, please refer to the Strep-Tag Protein Handbook ([QIAGEN, 2009](#)).

For expression in *E. coli*, the amount of recombinant protein is usually high enough to use Strep-Tactin Superflow resins. 50 ml bacteria culture can be processed for proteins expressing at high levels (10–50 mg of tagged protein per liter of bacteria culture). For lower expressing proteins (1–5 mg of tagged protein per liter of bacteria culture), a 200 ml cell culture should be processed. Some recombinantly expressed proteins might remain insoluble and are located in the pellet after lysis and

**Table 1** Reagents compatible with the Strep-tag/Strep-Tactin interaction

Nonionic detergents	2% Triton X-100 2% Tween-20 2% <i>N</i> -octyl- $\beta$ -D-glucopyranoside 0.2% <i>N</i> -nonyl- $\beta$ -D-glucopyranoside 2% Igepal CA-630 (Nonidet P40) 0.12% C10 E5 (Decylpentaoxyethylene) 0.03% C10 E6 0.005% C12 E8 0.023% C12 E9 (Dodecylnonaoxyethylene; Thesit) 0.35% DM (Decyl- $\beta$ -D-maltoside) 0.007% LM ( <i>N</i> -dodecyl $\beta$ -D-maltoside)
Ionic and zwitterionic detergents	2% <i>N</i> -lauryl-sarcosine 0.1% SDS 1.3% <i>N</i> -octyl-2-hydroxy-ethylensulfoxide 0.3% CHAPS 0.1% dodecyl- <i>N,N</i> -dimethylamine- <i>N</i> -oxide 0.034% DDAO <i>N</i> -decyl- <i>N,N</i> -dimethylamin- <i>N</i> -oxide
Reducing agents	50 mM DTT 1 mM Phenylmethylsulfonyl fluoride (PMSF)
Salts	5 M Sodium chloride 2 M Ammonium sulfate 1 M Calcium chloride
Others	25% Glycerol 1 M Guanidine-Hydrochloride

centrifugation. For a more complete recovery of the recombinant protein, lysis of the pellet can be repeated with a denaturing lysis buffer (1 M guanidine-hydrochloride, Gu-HCl).

Expression levels in insect cells (see Recombinant protein expression in baculovirus-infected insect cells) are typically lower than those obtained in bacterial systems (see Small-scale Expression of Proteins in *E. coli*). The recombinant protein is expressed at levels ranging between 10 µg and 10 mg per 10<sup>7</sup> cells. This amount of insect cells corresponds to 1 semiconfluent T75 flask. For proteins expressed at high levels in insect cells, Strep-Tactin Superflow resins are also suitable. In case of low level expression in insect cells (<50 µg), it is strongly recommended that Strep-Tactin Magnetic beads be used.

Expression levels of recombinant proteins in transiently transfected mammalian cells (see Single Cell Cloning of a Stable Mammalian Cell Line)

are protein and cell line dependent, but usually 30–300 times lower than in insect cells and range between 0.3 and 30 µg per  $10^7$  cells. Therefore, it is highly recommended that Strep-Tactin Magnetic beads be used to purify Strep-tagged proteins from mammalian cells.



## 2. EQUIPMENT

- Sonicator (optional)
- Refrigerated high-speed centrifuge
- Microcentrifuge
- End-over-end rotator
- Magnetic separator
- Platform rotator
- Disposable gravity flow columns (with capped bottom outlet)
- 15-ml conical centrifuge tubes
- Micropipettors
- Micropipettor tips
- 1.5-ml microcentrifuge tubes
- Cell scraper
- Narrow-gauge blunt-ended syringe needle (optional)



## 3. MATERIALS

- Sodium phosphate monobasic ( $\text{NaH}_2\text{PO}_4$ )
- Sodium chloride ( $\text{NaCl}$ )
- Tris base
- Glycerol
- Sodium dodecyl sulfate (SDS)
- Bromophenol blue
- Dithiothreitol (DTT)
- Lysozyme
- Benzonase nuclease (alternatively RNase A and DNase I)
- Phosphate buffered saline (PBS)
- Igepal CA-630
- Tween-20
- Triton X-100
- Desthiobiotin (Sigma Cat. # D1411)
- HABA (4-Hydroxyazobenzene-2-carboxylic acid; Sigma Cat. # H 5126)

Strep-Tactin Superflow resin, 10 ml (Qiagen Cat. # 30003; IBA Cat. # 2-1206-010); Strep-Tactin Superflow Plus or high-capacity resin (Qiagen Cat. # 30004; IBA Cat. # 2-1208-010)

### 3.1. Solutions & buffers

#### Step 1a Lysis buffer

Component	Final concentration	Stock	Amount
NaH <sub>2</sub> PO <sub>4</sub>	50 mM	0.5 M	100 ml
NaCl	300 mM	5 M	60 ml

Add water to 1 l. Adjust pH to 8.0 using NaOH

#### 2× SDS-PAGE sample buffer

Component	Final concentration	Stock	Amount
Tris-HCl, pH 6.8	0.09 M	1 M	900 µl
Glycerol	20%	98%	2.04 ml
SDS	2%	10%	2 ml
Bromphenol blue	0.02%	10%	20 µl
DTT	0.1 M	2 M	500 µl

Add water to 10 ml

#### Step 1b and 1c PBS Buffer

Component	Final concentration	Stock	Amount
NaH <sub>2</sub> PO <sub>4</sub>	50 mM	0.5 M	100 ml
NaCl	300 mM	5 M	60 ml
Ipegal CA-630	1%		10 ml

Add water to 1 l. Adjust pH to 8.0 using NaOH

#### Step 2a Washing Buffer

Component	Final concentration	Stock	Amount
NaH <sub>2</sub> PO <sub>4</sub>	50 mM	0.5 M	100 ml
NaCl	300 mM	5 M	60 ml

Add water to 1 l. Adjust pH to 8.0 using NaOH

## Elution buffer

Component	Final concentration	Stock	Amount
NaH <sub>2</sub> PO <sub>4</sub>	50 mM	0.5 M	100 ml
NaCl	300 mM	5 M	60 ml
Desthiobiotin	2.5 mM		0.54 g

Add water to 1 l. Adjust pH to 8.0 using NaOH

## Regeneration buffer

Component	Final concentration	Stock	Amount
NaH <sub>2</sub> PO <sub>4</sub>	50 mM	0.5 M	100 ml
NaCl	300 mM	5 M	60 ml
HABA	1 mM		0.24 g

Add water to 1 l. Adjust pH to 8.0 using NaOH

**Step 2b** Magnetic Beads Washing Buffer

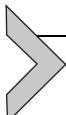
Component	Final concentration	Stock	Amount
NaH <sub>2</sub> PO <sub>4</sub>	50 mM	0.5 M	100 ml
NaCl	300 mM	5 M	60 ml
Tween-20	0.05%	10%	5 ml

Add water to 1 l. Adjust pH to 8.0 using NaOH

## Magnetic Beads Elution Buffer

Component	Final concentration	Stock	Amount
NaH <sub>2</sub> PO <sub>4</sub>	50 mM	0.5 M	100 ml
NaCl	300 mM	5 M	60 ml
Desthiobiotin	2.5 mM		0.54 g
Tween-20	0.05%	10%	5 ml

Add water to 1 l. Adjust pH to 8.0 using NaOH



## 4. PROTOCOL

### 4.1. Preparation

Express the Strep-tagged fusion protein in your system of choice (see Small-scale Expression of Proteins in *E. coli*, Recombinant protein expression in baculovirus-infected insect cells or Single Cell Cloning of a Stable Mammalian Cell Line). For *E. coli*, induce expression of the Strep-tagged fusion protein in a 50 or 200 ml culture and harvest the cells by centrifugation. You can use the pellet fresh or freeze it at  $-80^{\circ}\text{C}$  (freezing *E. coli* cell pellets increases lysis efficiency by lysozyme). For baculovirus-infected insect cells, use a T75 flask of infected cells ( $1\text{--}2 \times 10^7$  cells). For transfected mammalian cells, use a T75 flask of adherent cells or the pellet from suspension cells ( $1\text{--}2 \times 10^7$  cells).

### 4.2. Duration

Preparation	2–3 days
Protocol	3–4 h

See Fig. 1 for the flowchart of the complete protocol.

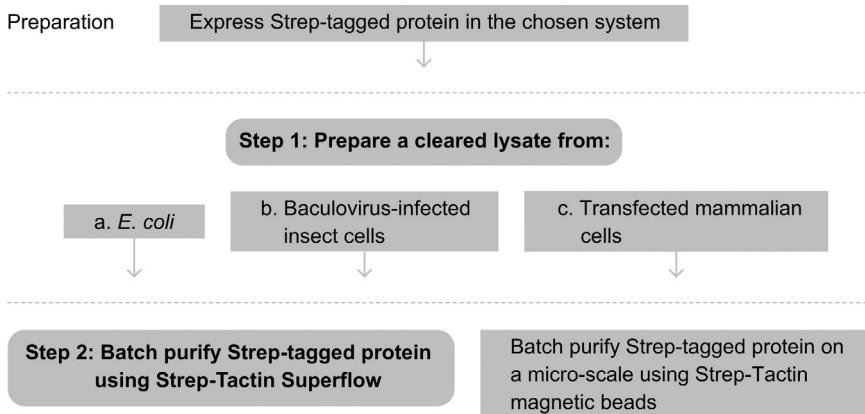


Figure 1 Flowchart of the complete protocol, including preparation



## 5. STEP 1A PREPARATION OF CLEARED *E. COLI* LYSATES UNDER NATIVE CONDITIONS

### 5.1. Overview

In this step, prepare cleared lysates of an *E. coli* cell pellet under native conditions with Lysis buffer. 50 or 200 ml *E. coli* cell suspension should be used to obtain a pellet depending of the expression level of the tagged protein.

### 5.2. Duration

1.5–2 h

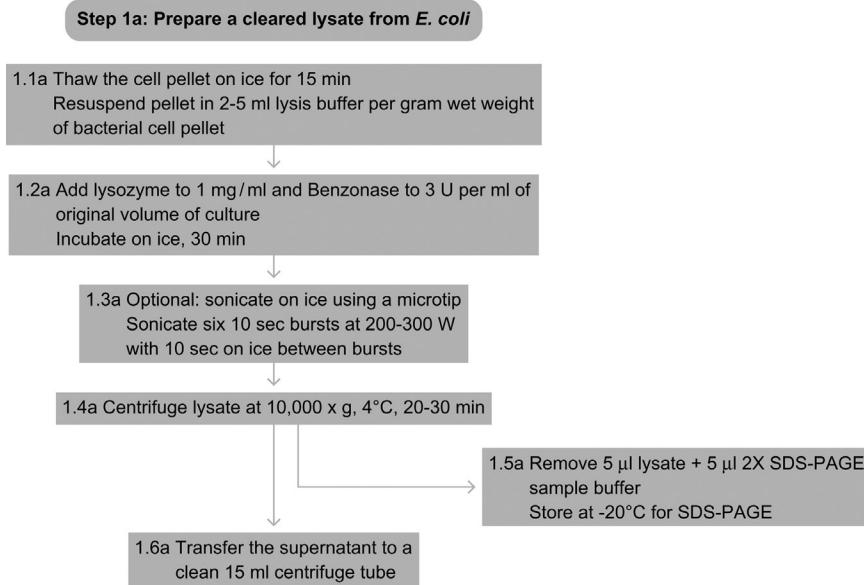
- 1.1a Thaw the cell pellet on ice for 15 min and resuspend the cells in lysis buffer. Use 2–5 ml buffer per gram wet weight.
- 1.2a Add lysozyme to 1 mg ml<sup>-1</sup> (50 000 units ml<sup>-1</sup>) and Benzonase nuclease (3 U per ml of original cultured volume processed) and incubate 30 min on ice.
- 1.3a Optional: sonicate on ice using a sonicator equipped with a microtip. Use six 10-s bursts at 200–300 W with a 10 s cooling period on ice between each burst of sonication.
- 1.4a Centrifuge the lysate at 10 000 × g for 20–30 min at 4 °C to pellet cellular debris and proceed with the supernatant.
- 1.5a Remove 5 µl of the lysate and add 5 µl of 2× SDS-PAGE sample buffer. Store the sample at –20 °C for SDS-PAGE analysis.
- 1.6a Transfer the supernatant to a clean 15-ml centrifuge tube.

### 5.3. Tip

The amount of cells required depends on the expression level of the Strep-tagged protein. The binding capacity of Strep-Tactin resin is protein-dependent and normally lies around 3 mg ml<sup>-1</sup> for Strep-Tactin Superflow and 9 mg ml<sup>-1</sup> for Strep-Tactin Superflow Plus/high capacity. The choice of resin and therefore the amount needed for purification can be adjusted to the expression level of the protein of interest.

### 5.4. Tip

Nucleases are suitable to reduce viscosity of cell lysates caused by genomic DNA. Instead of using Benzonase, add RNase A (10 µg ml<sup>-1</sup>) and DNase I (5 µg ml<sup>-1</sup>) to the cells in lysis buffer and incubate on ice for 10–15 min. Alternatively, draw the lysate through a narrow-gauge blunt-ended syringe needle several times.



**Figure 2** Flowchart of Step 1a.

## 5.5. Tip

If recombinant protein remains insoluble in the pellet fraction after lysis and centrifugation, repeat lysis of the pellet with additives (detergents, Gu-HCl or reducing agents) shown in Table 1 (additionally, see Explanatory Chapter: Troubleshooting protein expression: what to do when the protein is not soluble and Explanatory Chapter: Choosing the right detergent).

See Fig. 2 for the flowchart for Step 1a.

## 6. STEP 1B PREPARATION OF CLEARED LYSATES FROM BACULOVIRUS-INFECTED CELLS

### 6.1. Overview

In this step, cleared lysates are prepared from baculovirus-infected insect cells under native conditions.  $1-2 \times 10^7$  cells ( $\sim 1$  T75 flask) are lysed with 0.5–1% nonionic detergents.

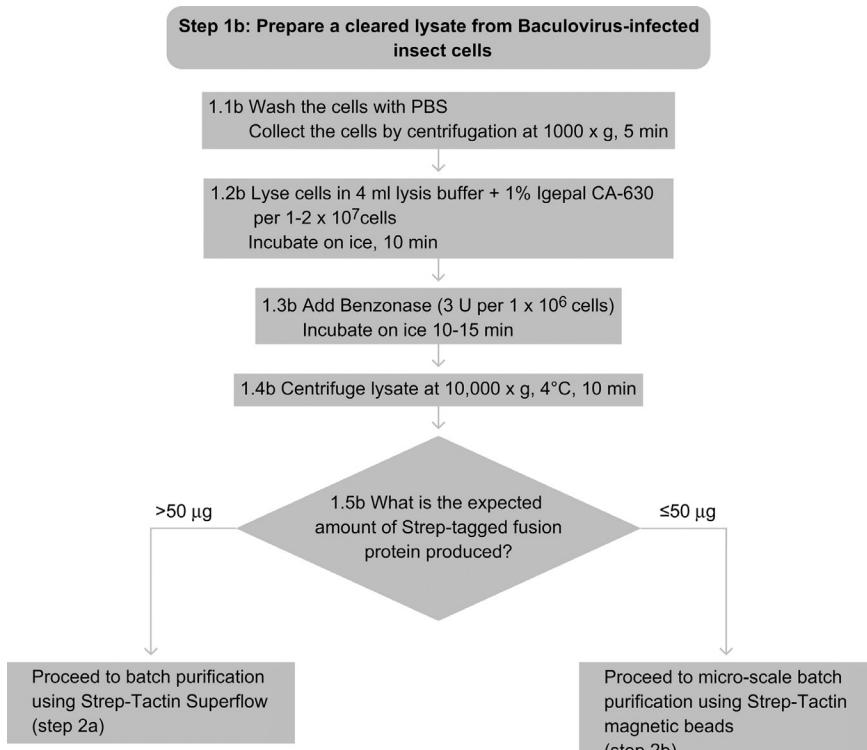
### 6.2. Duration

45–60 min

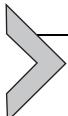
**1.1b** Wash the baculovirus-infected cells with PBS and collect cells by centrifugation at  $1000 \times g$  for 5 min.

- 1.2b** Lyse cells in lysis buffer supplemented with 1% Igepal CA-630 by incubating on ice for 10 min. Use 4 ml lysis buffer for  $1\text{--}2 \times 10^7$  cells.
- 1.3b** If the lysate is very viscous, add Benzonase nuclease (3 U per  $1 \times 10^6$  cells) and incubate on ice for an additional 10–15 min. Alternatively, draw the lysate through a blunt-ended narrow gauge syringe needle several times.
- 1.4b** Centrifuge the lysate at 10 000 g at 4 °C for 10 min to pellet cellular debris and proceed with the supernatant.
- 1.5b** If the expected yield of Strep-tagged fusion protein is  $>50 \mu\text{g}$ , use the batch purification protocol (Step 2a). If the expected yield of Strep-tagged fusion protein is  $\leq 50 \mu\text{g}$ , use the magnetic bead protocol (Step 2b).

See Fig. 3 for the flowchart of Step 1b.



**Figure 3** Flowchart of Step 1b.



## 7. STEP 1C PREPARATION OF LYSATES FROM TRANSFECTED MAMMALIAN CELLS

### 7.1. Overview

In this step, cleared lysates from  $\sim 10^7$  transfected mammalian cells, either grown in suspension or adherently, are prepared. For cell lysis, the lysis buffer contains 0.5–1% nonionic detergent.

### 7.2. Duration

1.5 h

**1.1c** For adherent cells: Wash the adherent transfected cells with PBS and incubate with 0.5 ml lysis buffer per  $10^7$  cells for 10 min on ice on a platform rotator. Collect the cells with a cell scraper.

For suspension cells: Spin down the cells growing in suspension culture and incubate with 0.5 ml lysis buffer per  $10^7$  cells for 10 min on an end-over-end rotator at 4 °C.

**1.2c** If the lysate is very viscous, add Benzonase nuclease (3 U per  $1 \times 10^6$  cells) and incubate on ice for an additional 10–15 min.

**1.3c** Centrifuge the lysate at  $10\,000 \times g$  for at 4 °C for 10 min to pellet cellular debris and proceed with the supernatant using the microscale purification protocol (Step 2b).

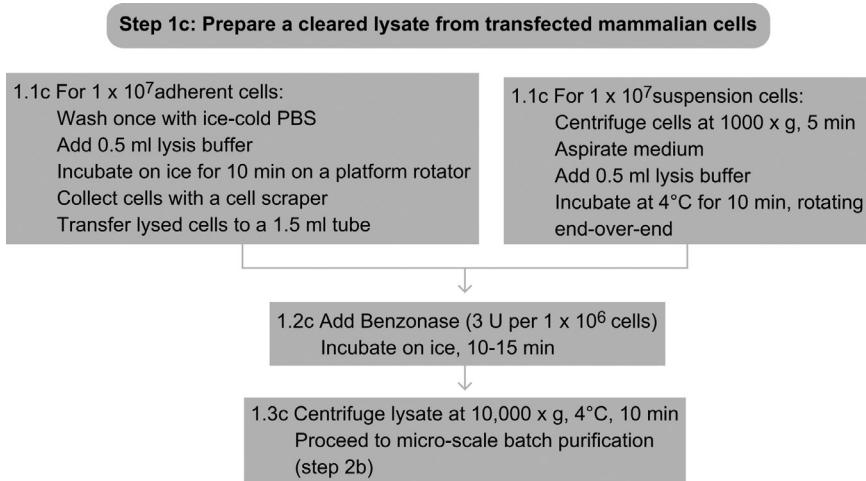
### 7.3. Tip

0.5–1% nonionic detergents such as Tween-20, Triton X-100, Igepal CA-630 and CHAPS are compatible with Strep-tag purification (additionally see Explanatory Chapter: Choosing the right detergent). The detergents can be used in lysis, wash, and elution buffers. Alternatively, the cells can be lysed by sonication on ice or by three consecutive freeze–thaw cycles.

### 7.4. Tip

Nucleases are suitable to reduce viscosity of cell lysates caused by genomic DNA. Instead of using Benzonase, draw the lysate through a narrow-gauge blunt-ended syringe needle several times.

See Fig. 4 for the flowchart of Step 1c.



**Figure 4** Flowchart of Step 1c.



## 8. STEP 2A BATCH PURIFICATION OF STREP-TAGGED PROTEINS

### 8.1. Overview

During this step, Strep-tagged protein is purified under native conditions from cell lysates prepared from *E. coli* or baculovirus-infected insect cells. Binding to Strep-Tactin Superflow resin is done in a batch mode and elution is subsequently done by gravity flow. Finally, the Strep-Tactin Superflow resin is regenerated.

### 8.2. Duration

1.5 h

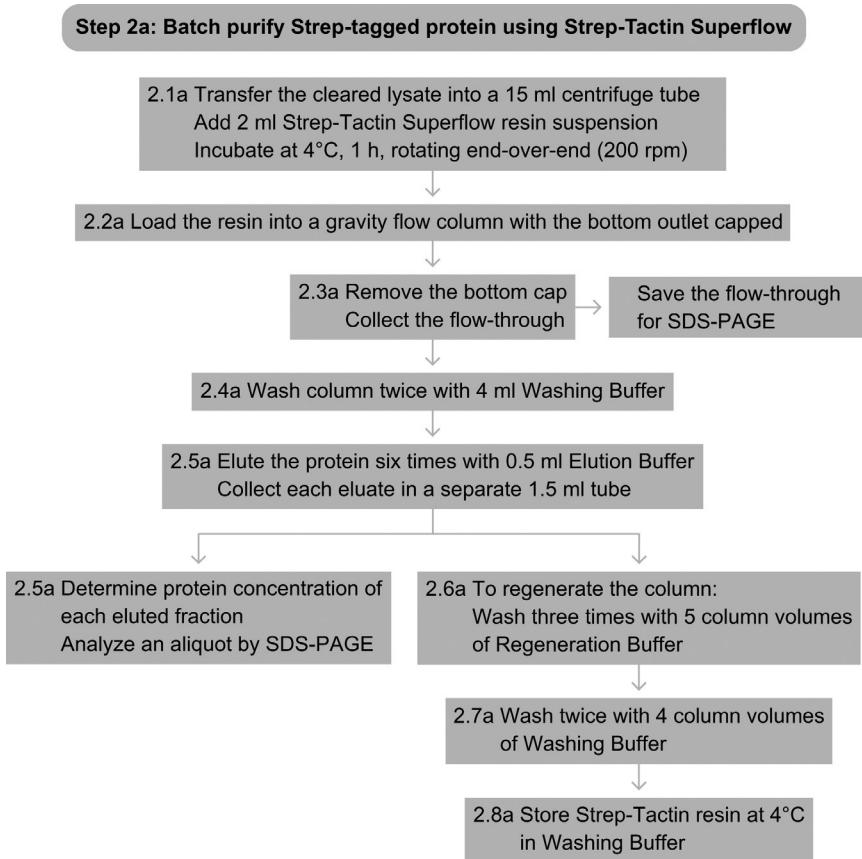
- 2.1a** Pipette the cleared lysate into a 15-ml conical centrifuge tube and add 2 ml Strep-Tactin Superflow resin suspension. Mix gently using an end-over-end rotator (200 rpm) at 4 °C for 1 h.
- 2.2a** Load the Strep-Tactin Superflow resin into a gravity flow column with the bottom outlet capped.
- 2.3a** Remove the bottom cap and collect the flow-through. Save the flow-through for SDS-PAGE analysis.
- 2.4a** Wash the column two times with 4 ml Washing Buffer.

- 2.5a** Elute the protein six times with 0.5 ml Elution Buffer. Collect each eluate in a separate 1.5-ml microcentrifuge tube. Determine the protein concentration of each fraction (see Quantification of Protein Concentration using UV absorbance and Coomassie Dyes) and analyze an aliquot by SDS-PAGE (see One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE)).
- 2.6a** To regenerate the column, wash it three times with 5 column volumes of regeneration Buffer.
- 2.7a** Wash the column twice with 4 column volumes of Washing Buffer.
- 2.8a** Store the Strep-Tactin resin in Washing Buffer at 4 °C.

### 8.3. Tip

The color change from white to red indicates that the column has been regenerated by the displacement of desthiobiotin.

See Fig. 5 for the flowchart of Step 2a.



**Figure 5** Flowchart of Step 2a.



## 9. STEP 2B MICROSCALE PURIFICATION OF STREP-TAGGED PROTEINS USING STREP-TACTIN MAGNETIC BEADS

### 9.1. Overview

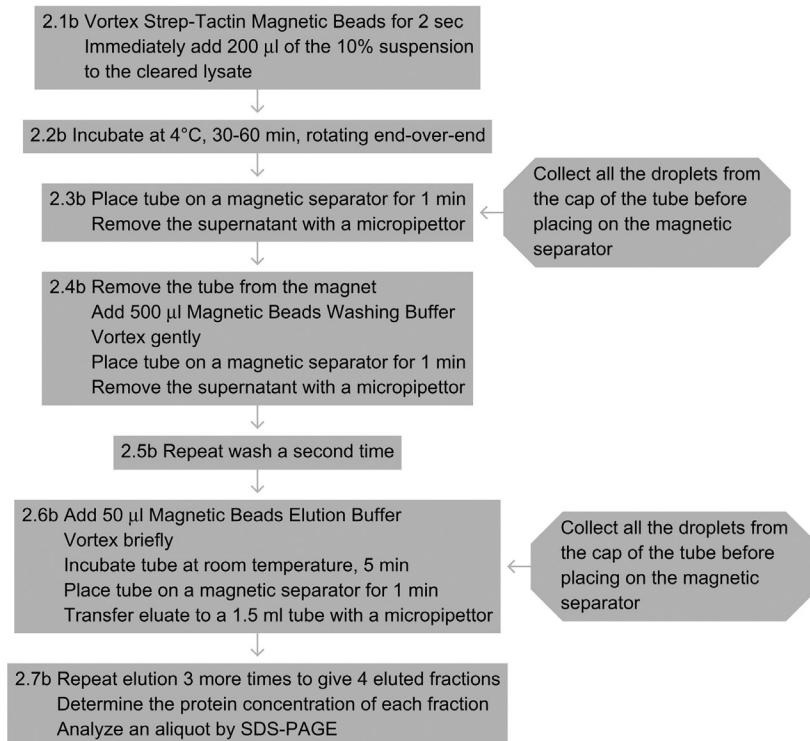
During this step, Strep-tagged protein is purified under native conditions from cell lysates prepared from transfected mammalian cells. Binding to Strep-Tactin Magnetic beads is done in a batch mode to ensure efficient binding of the Strep-tagged protein even at low concentrations. Washing and elution steps are done in a magnetic separator where small volumes are easy to handle.

### 9.2. Duration

1.5 h

- 2.1b** Resuspend Strep-Tactin Magnetic Beads by vortexing for 2 s and then immediately add 200  $\mu$ l of 10% Strep-Tactin Magnetic Bead suspension to the cleared lysate.
- 2.2b** Mix the suspension gently on an end-over-end rotator for 30–60 min at 4 °C.
- 2.3b** Place the tube on a magnetic separator for 1 min and remove the supernatant with a micropipettor. Make sure to collect all the droplets of suspension from the tube cap before placing on the separator.
- 2.4b** Remove the tube from the magnet, add 500  $\mu$ l Magnetic Beads Washing Buffer, gently vortex the suspension, place the tube on a magnetic separator for 1 min, and carefully remove the buffer.
- 2.5b** Repeat wash a second time.
- 2.6b** Add 50  $\mu$ l Magnetic Beads Elution Buffer, gently vortex the suspension, and incubate the tube for 5 min at room temperature. Place the tube on a magnetic separator for 1 min and collect the eluate in a clean 1.5-ml microcentrifuge tube. Make sure to collect all droplets from the tube cap before placing the tube in the magnetic separator.
- 2.7b** Repeat elution three times to give four elution fractions. Determine the protein concentration of each eluted fraction (see Quantification of Protein Concentration using UV absorbance and Coomassie Dyes) and analyze by SDS-PAGE (One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE)).

**Step 2b: Batch purify Strep-tagged protein on a micro-scale using Strep-Tactin magnetic beads**



**Figure 6** Flowchart of Step 2b.

### 9.3. Tip

Care is necessary to ensure that constant amounts of beads are pipetted. Cut the end off of the pipette tip to make a larger opening. 200 µl of Strep-Tactin Magnetic bead suspension has a binding capacity of 40–60 µg of protein. The volume of bead suspension can be adapted to the amount of Strep-tagged protein present in the lysate but using volumes less than 10 µl are not recommended because of associated handling problems.

See Fig. 6 for the flowchart of Step 2b.

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Affinity purification of a recombinant protein expressed as a fusion with the maltose-binding protein (MBP) tag.

Purification of GST-tagged proteins.

Protein Affinity Purification using Intein/Chitin Binding Protein Tags.

Affinity Purification of Protein Complexes Using TAP Tags.

Recombinant protein expression in baculovirus-infected insect cells.

Small-scale Expression of Proteins in E. coli.

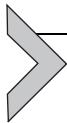
Single Cell Cloning of a Stable Mammalian Cell Line.

Explanatory Chapter: Troubleshooting protein expression: what to do when the protein is not soluble.

Explanatory Chapter: Choosing the right detergent.

Quantification of Protein Concentration using UV absorbance and Coomassie Dyes.

One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE).



# Proteolytic Affinity Tag Cleavage

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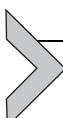
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## Abstract

Here, we present protocols describing the use of the dipeptidyl-aminopeptidase-1 (DPP1, DAPase) exoprotease-based TAGZyme system and the endoprotease, Factor Xa. Both enable the recovery of proteins free of any amino acids encoded by the vector and/or protease recognition site. They also provide the possibility of removing the proteases from the preparation of the target protein by a simple subtractive chromatography step. TAGZyme enzymes contain an uncleavable His tag for removal by Immobilized Metal Ion Affinity Chromatography (IMAC). Factor Xa can be removed using Xa Removal Resin.

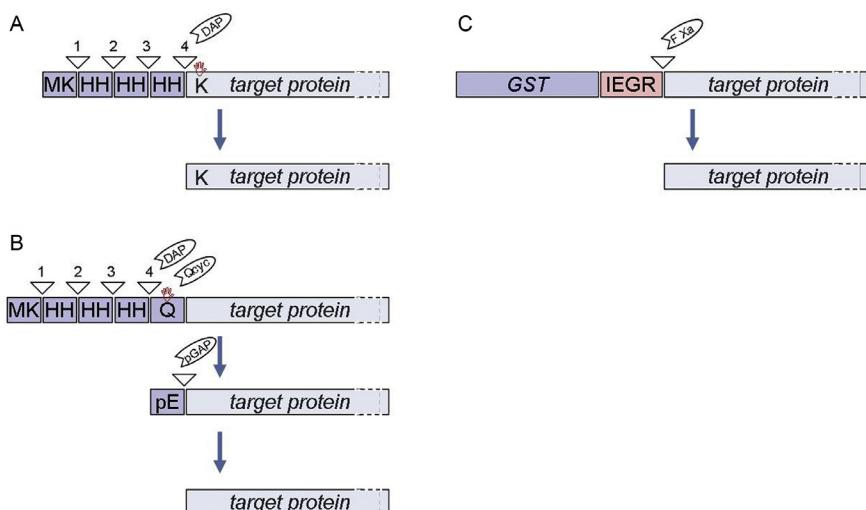


## 1. THEORY

Proteinaceous tags such as the His tag and the GST tag are widely used to purify recombinant proteins by a single and simple affinity chromatography step (Smith and Johnson, 1988; Block et al., 2008; see also [Purification of His-tagged proteins](#) and [Purification of GST-tagged proteins](#)). The removal of affinity tags can be required if the tag interferes with protein structure or function. In order to exclude any influence of the tag on structure or function, tags are often cleaved using proteases, prior to performing activity assays or determining the protein structure by NMR or X-ray crystallography. As affinity tags fused to the target protein might have an immunogenic effect when administered to humans or animals, tags are usually cleaved from proteins that are used as biopharmaceuticals. Furthermore, tag-cleaving proteases are used as a tool in protein purification (e.g., tandem affinity purification, TAP, Günzl and Schimanski, 2009). Affinity tags are usually and most precisely removed using proteases which specifically cleave polypeptide chains at their recognition sites – amino acid motifs 4 to 8 residues in length (endoproteases), or which cleave proteins exoproteolytically starting either at the N- or at the C-terminus by sequentially removing amino acids until they reach a stop point. The endoprotease recognition site and the exoprotease stop point are provided for in the vectors used to clone the cDNA coding for the protein of interest (Smith and Johnson, 1988; Schäfer et al., 2002).

A large number of proteases have been reported in the literature and are the subjects of several recent reviews (Nilsson et al., 1997; Jenny et al., 2003; Arnau et al., 2006). It is noteworthy that most procedures remove N-terminal tags. Precise cleavage of C-terminal tags, for example, by carboxypeptidases (summarized in Kenig et al., 2006) or by chemical cleavage (Nilsson et al., 1997) is problematic and still awaits a robust commercial solution.

The removal of large tags, such as the 25-kDa glutathione S-transferase, requires an endoproteolytic cleavage. In some proteins, the recognition site may occur naturally at other positions and unwanted cleavage will result. Also, nonspecific cleavage by endoproteases has been reported (Jenny et al., 2003). For endoproteolytic cleavage by Factor Xa, the IEGR recognition motif must be located upstream of the target protein. Shorter tag sequences, up to 50 residues in length, can be removed by using the exoproteolytic approach; nonspecific cleavage has not been observed here (Arnaud et al., 2006; Block et al., 2008). Exoproteolytic tag removal requires that the epitope to be cleaved is free of DAPase stop points. Fig. 1 describes the mode of action and shows the structure of the resulting untagged target protein after cleavage of a His tag by TAGZyme



**Figure 1** Exo- and endoproteolytic tag cleavage. (a) Exoproteolytic cleavage of a N-terminal His<sub>6</sub> tag from a target protein that provides a stop point (K), e.g., K in position 1) in its native amino acid sequence. Dipeptides are cleaved sequentially from the N-terminus at positions marked  $\nabla$ . DAP, DAPase. Other stop points (lower case: position relative to target protein N-terminus) are R<sub>1</sub>, I<sub>2</sub>, P<sub>2</sub>, and P<sub>3</sub>. (b) Exoproteolytic cleavage of an N-terminal His<sub>6</sub> tag from a target protein that does not contain a stop point. In this case the tag is extended by a glutamine (Q), which serves as the stop point (glutamine is cyclized to pyroglutamate, pE, in the presence of Qcyclase, Qcyc). The resulting pyroglutamate cannot be cleaved by DAPase and is removed by pyroglutamyl aminopeptidase (pGAP) in a subsequent step. (c) endoproteolytic cleavage of a GST tag by Factor Xa, which cleaves between its recognition site, IEGR, and the target protein sequence.

**Table 1** Vectors for the expression of His- or GST-tagged proteins for tag cleavage by TAGZyme or Factor Xa (the list may not be complete)

Tag	Enzyme	Vector	Comment
His tag	TAGZyme	TAGZyme pQE-2, pQE-T7-1, pQE-TriSystem-5 <sup>a</sup>	For target proteins containing an intrinsic DAPase stop
His tag	TAGZyme	TAGZyme pQE-1, pQE-TriSystem-5 <sup>a</sup>	For target proteins not containing an intrinsic DAPase stop; stop point Q provided
His tag	Factor Xa	pQE-30 Xa, pET-16b, pET30 Xa, pET32-Xa	
GST tag	Factor Xa	pGEX-3X and -5X series	

<sup>a</sup>Check for compatibility with the TAGZyme system using the TAGDesigner webtool if other vectors are used or other N-termini have been designed: <http://www1.qiagen.com/products/protein/tagdesigner/default.aspx>

and of a GST tag by Factor Xa. **Table 1** provides examples of vectors suitable for expressing His- and GST-tagged target proteins with subsequent cleavage by TAGZyme and Factor Xa.



## 2. EQUIPMENT

Water bath

Heating block

Desalting column (e.g., PD-10 column, GE Healthcare cat. # 17-0851-01)

Filter units (0.2 µm)

Tricorn 5/20 or Tricorn 5/50 columns (GE Healthcare) (Protocol 1)

Disposable gravity flow columns (1 ml, e.g., QIAGEN cat. # 34924) (Protocol 2)

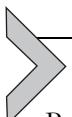
1.5-ml microcentrifuge tubes

Micropipettors

Micropipettor tips

Polyacrylamide gel electrophoresis equipment

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### 3. MATERIALS

#### Protocol 1

Sodium chloride (NaCl)  
Sodium phosphate monobasic ( $\text{NaH}_2\text{PO}_4$ )  
Sodium hydroxide (NaOH)  
EDTA  
Imidazole  
Dipeptidyl-aminopeptidase-1 enzyme (DAPase; 10 U  $\text{ml}^{-1}$ , QIAGEN cat. # 34362)  
Glutamine-cyclotransferase enzyme (Qcyclase; 50 U  $\text{ml}^{-1}$ , QIAGEN cat. # 34342)  
Pyroglutamyl-aminopeptidase enzyme (pGAPase; 25 U  $\text{ml}^{-1}$ , QIAGEN cat. # 34342)  
Cysteamine-HCl (20 mM, contained in QIAGEN cat. # 34362)  
Ni-NTA Agarose IMAC resin (QIAGEN cat. # 30210)  
Alternative: TAGZyme kit containing the enzymes and resins needed (QIAGEN cat. # 34300)

#### 3.1. Solutions & buffers

##### Step 1.1 1× TAGZyme buffer

Component	Final concentration	Stock	Amount
$\text{NaH}_2\text{PO}_4$	20 mM	0.2 M	100 ml
NaCl	150 mM	1.5 M	100 ml

Mix with 750 ml water. Adjust the pH to 7.0 using NaOH. Add water to 1 l. Filter through a 0.2- $\mu\text{m}$  filter unit and store at 2–8 °C

#### Protocol 2

Sodium chloride (NaCl)  
Tris base  
Calcium chloride ( $\text{CaCl}_2$ )  
Hydrochloric acid (HCl)  
Glycerol  
Sodium dodecyl sulfate (SDS)  
Bromophenol blue  
Dithiothreitol (DTT)

Factor Xa protease (QIAGEN cat. # 33223, or Novagen cat. # 69037-3)

Glutathione Affinity Resin (QIAGEN cat. # 30900, or GE Healthcare cat. # 17-5279-01)

Xa Removal Resin (QIAGEN cat. # 33213, or Novagen cat. # 69037-3)

## 3.2. Solutions & buffers

### Step 2.1 TNC buffer

Component	Final concentration	Stock	Amount
Tris-HCl	20 mM	1.0 M	20 ml
NaCl	50 mM	1.0 M	50 ml
CaCl <sub>2</sub>	1 mM	0.1 M	10 ml

Mix in 870 ml water. Adjust the pH to 7.5 using HCl. Add water to 1 l. Filter through a 0.2-μm filter unit and store at 2–8 °C. *Note:* This buffer must not contain protease inhibitors!

### 5× SDS-PAGE buffer

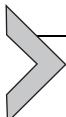
Component	Final concentration	Stock	Amount
Tris-HCl, pH 6.8	225 mM	1.0 M	2.25 ml
Glycerol	50% (v/v)	100%	5 ml
SDS	5% (w/v)		0.5 g
DTT	250 mM	1.0 M	2.5 ml
Bromophenol blue	0.05% (w/v)		5 mg

Add water to 10 ml

### Step 2.3 TN buffer

Component	Final concentration	Stock	Amount
Tris-HCl	20 mM	1.0 M	20 ml
NaCl	50 mM	1.0 M	50 ml

Mix in 880 ml water. Adjust the pH to 7.2 using HCl. Add water to 1 l. Filter through a 0.2-μm filter unit and store at 2–8 °C



## 4. PROTOCOL 1 EXOPROTEOLYTIC CLEAVAGE OF HIS TAGS FROM PROTEINS USING TAGZyme

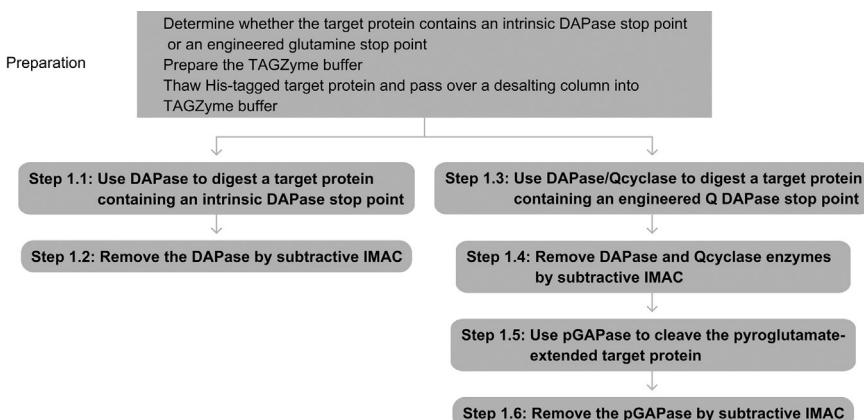
### 4.1. Preparation

For a protein containing an intrinsic DAPase stop point, follow Steps 1.1 and 1.2. For a protein with an engineered glutamine stop point, follow Steps 1.3–1.6. Prepare the 1× TAGZyme buffer. Thaw the cysteamine solution and store it on ice until use. Store the enzyme solutions on ice until use. Thaw the target protein solution containing 1 mg of His-tagged protein and add EDTA to 5 mM (final concentration). Pass over a PD-10 gel filtration column into 1× TAGZyme buffer. The final concentration after desalting should be  $\geq 0.3 \text{ mg ml}^{-1}$  (see Gel filtration chromatography (Size exclusion chromatography) of proteins).

### 4.2. Duration

Preparation	About 1 h
Protocol	About 1 h (intrinsic DAPase stop point)
	About 3 h (engineered Q stop point)

See Fig. 2 for the flowchart of protocol 1.



**Figure 2** Flowchart of Protocol 1, including preparation.



## 5. STEP 1.1 DAPase DIGESTION OF A TARGET PROTEIN CONTAINING AN INTRINSIC DAPase STOP POINT

### 5.1. Overview

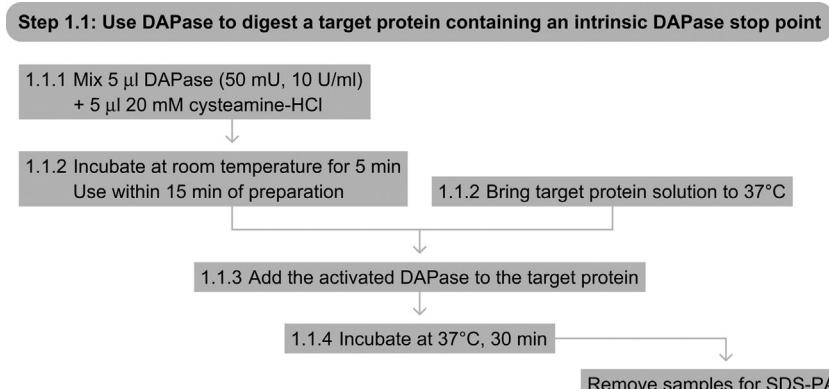
N-terminal amino acids are cleaved exoproteolytically from 1 mg of target protein using DAPase enzyme under standard conditions. Reaction conditions (buffer additives, amount of enzyme required, incubation time and temperature) may be optimized or adjusted according to the needs of the target protein or to maximize process economy.

### 5.2. Duration

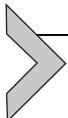
45 min

- 1.1.1 Activate the DAPase by mixing 5 µl of DAPase enzyme solution (50 mU, 10 U ml<sup>-1</sup>) with 5 µl of 20 mM cysteamine-HCl.
- 1.1.2 Incubate for 5 min at room temperature. The enzyme mixture should be used within 15 min of preparation. During DAPase enzyme preparation, bring the target protein solution to the incubation temperature of 37 °C.
- 1.1.3 Add the activated enzyme to the temperature-adjusted protein solution to start the digestion reaction.
- 1.1.4 Incubate at 37 °C for 30 min. If desired, remove samples during the reaction for SDS-PAGE analysis.

See [Fig. 3](#) for the flowchart of Step 1.1.



**Figure 3** Flowchart of Step 1.1.



## 6. STEP 1.2 REMOVAL OF DAPase ENZYME BY SUBTRACTIVE IMAC

### 6.1. Overview

The His-tagged DAPase enzyme is removed from the untagged target protein preparation.

### 6.2. Duration

30 min

- 1.2.1 Resuspend the Ni-NTA agarose suspension by repeated inversion of the bottle and pipette 1 ml of the 50% suspension (corresponding to 0.5 ml bed volume) into a disposable gravity flow column.
- 1.2.2 Equilibrate the column with 2.5 ml (5 bed volumes) of 1X TAGZyme buffer.
- 1.2.3 Pass the digestion reaction mixture through the column and collect the flow-through. *Important:* The flow-through fraction contains the processed target protein!
- 1.2.4 Wash the column with 2 ml aliquots of 1X TAGZyme buffer, until the flow-through no longer contains protein. Collect fraction(s). *Important:* The flow-through fraction contains the processed target protein!
- 1.2.5 Combine the flow-through fraction from Step 1.2.3 and the protein-containing fractions from Step 1.2.4. Discard the Ni-NTA resin used in this subtractive step. The native target protein is ready for further applications.

### 6.3. Tip

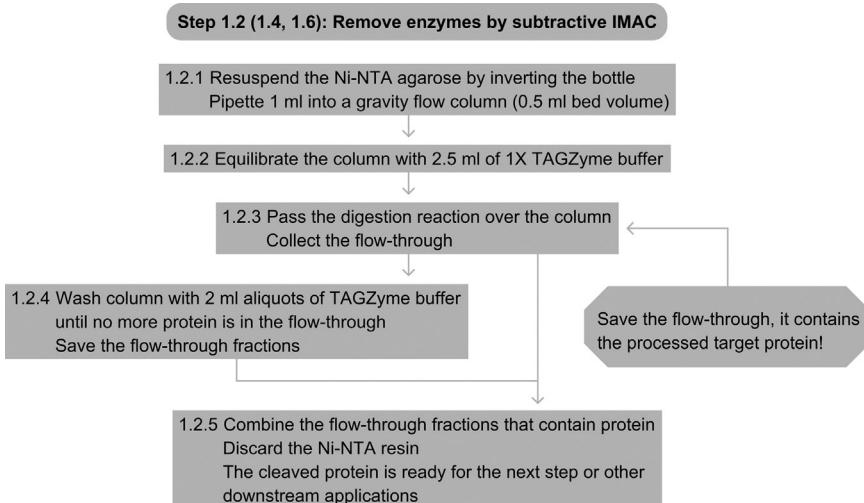
*Alternatively, pack a LC or FPLC column with 0.5 ml bed volume of Ni-NTA agarose.*

### 6.4. Tip

*The column can be prepared while the target protein is being digested.*

### 6.5. Tip

*A Tricorn 5/20 or Tricorn 5/50 column (GE Healthcare, inner diameter 5 mm) is suitable for this volume of matrix. In general, the bed volume height should be at least two times the inner diameter of the column.*



**Figure 4** Flowchart of subtractive IMAC (Steps 1.2, 1.4, and 1.6).

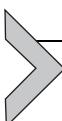
## 6.6. Tip

*When using a chromatography system, do not exceed a flow rate of  $0.5 \text{ ml min}^{-1}$  ( $\sim 150 \text{ cm h}^{-1}$ ) to ensure quantitative binding of the His-tagged DAPase enzyme.*

## 6.7. Tip

*If you would like to analyze the protein captured on the Ni-NTA Agarose column, elute it by applying 2 ml of 1× TAGZyme buffer containing 500 mM imidazole and analyze a fraction by SDS-PAGE.*

See Fig. 4 for the flowchart for subtractive IMAC (Steps 1.2, 1.4 and 1.6).



## 7. STEP 1.3 DAPase/QCYCLASE DIGESTION OF A TARGET PROTEIN CONTAINING AN ENGINEERED GLUTAMINE (Q) DAPase STOP POINT

### 7.1. Overview

This step describes the exoproteolytic cleavage of tags from proteins without an intrinsic DAPase stop point. N-terminal amino acids are cleaved exoproteolytically from 1 mg of target protein using DAPase enzyme and an excess of Qcyclase enzyme under standard conditions. Qcyclase cyclizes the glutamine stop point to form pyroglutamate after DAPase cleaves the upstream dipeptides. Reaction conditions (buffer additives,

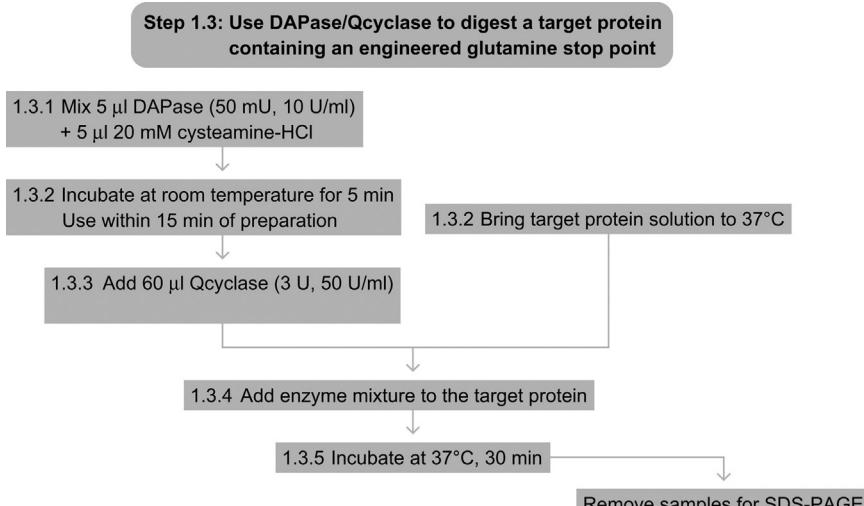
amount of enzyme required, incubation time and temperature) can be optimized or adjusted according to the needs of the target protein or to maximize process economy (*Note:* the DAPase/Qcyclase ratio must be kept constant).

## 7.2. Duration

40 min

- 1.3.1 Mix 5 µl of DAPase enzyme solution (50 mU, 10 U ml<sup>-1</sup>) with 5 µl of 20 mM cysteamine-HCl to activate the enzyme.
- 1.3.2 Incubate for 5 min at room temperature. The enzyme mixture should be used within 15 min of preparation. During DAPase enzyme preparation, bring the target protein solution to the incubation temperature of 37 °C.
- 1.3.3 Add 60 µl of Qcyclase (3 U, 50 U ml<sup>-1</sup>) to the prepared DAPase.
- 1.3.4 Add the enzyme mixture to the temperature-adjusted target protein solution to start the digestion.
- 1.3.5 Incubate for 30 min at 37 °C. If desired, remove samples during the reaction for SDS-PAGE analysis.

See Fig. 5 for the flowchart of Step 1.3.



**Figure 5** Flowchart of Step 1.3.



## 8. STEP 1.4 REMOVAL OF DAPase AND QCYCLASE ENZYMES BY SUBTRACTIVE IMAC

### 8.1. Overview

The His-tagged DAPase and Qcyclase enzymes are removed from the pyroglutamate-extended target protein. See [Fig. 4](#) for the flowchart for subtractive IMAC.

### 8.2. Duration

30 min

- 1.4.1 Resuspend the Ni-NTA Agarose suspension by repeated inversion and pipette 1 ml of the 50% suspension (corresponding to 0.5 ml bed volume) into a disposable gravity flow column.
- 1.4.2 Equilibrate the column with 2.5 ml (5 column volumes) of 1× TAGZyme buffer.
- 1.4.3 Pass the digestion reaction mixture through the column and collect the flow-through. *Important:* The flow-through fraction contains the pyroglutamate-extended target protein!
- 1.4.4 Wash the column with 2 ml aliquots of 1× TAGZyme buffer, until the flow-through no longer contains protein. Collect fraction(s).  
*Important:* The flow-through fraction contains the pyroglutamate-extended target protein!
- 1.4.5 Combine the flow-through from Step 1.4.3 and the protein-containing fractions from Step 1.4.4. Discard the Ni-NTA resin used in this subtractive step. The recovered target protein is ready for the removal of pyroglutamate by the action of pGAPase enzyme.

### 8.3. Tip

*The column can be prepared while the target protein is being digested.*

### 8.4. Tip

Alternatively, pack a LC or FPLC column with 0.5 ml bed volume of Ni-NTA agarose.

### 8.5. Tip

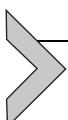
*A Tricorn 5/20 or Tricorn 5/50 column (GE Healthcare, inner diameter 5 mm) is suitable for this volume of matrix. In general, the bed volume height should be at least two times the inner diameter of the column.*

## 8.6. Tip

When using a chromatography system, do not exceed a flow rate of  $0.5 \text{ ml min}^{-1}$  ( $\sim 150 \text{ cm h}^{-1}$ ) to ensure quantitative binding of the His-tagged DAPase and Qcyclase enzymes.

## 8.7. Tip

If you would like to analyze the protein captured on the Ni-NTA Agarose column, elute it by applying 2 ml of 1× TAGZyme buffer containing 500 mMimidazole and analyze a fraction by SDS-PAGE (see One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE)).



## 9. STEP 1.5 pGAPase DIGESTION OF A PYROGLUTAMATE-EXTENDED TARGET PROTEIN

### 9.1. Overview

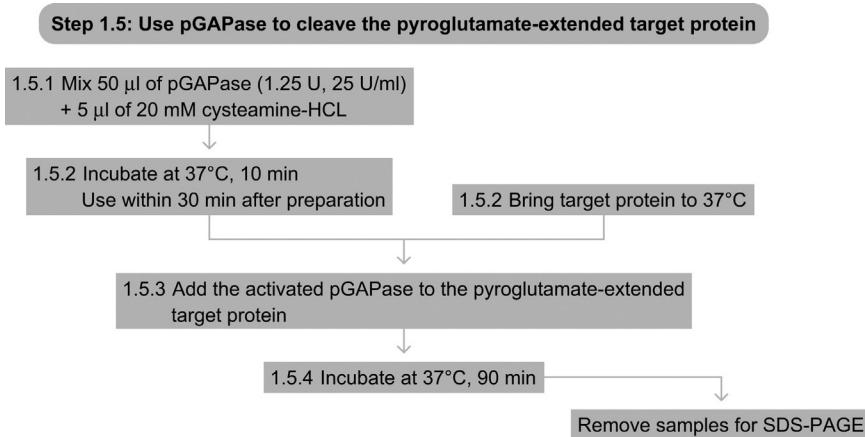
The N-terminal pyroglutamate is cleaved from the DAPase/Qcyclase-treated target protein. Reaction conditions (buffer additives, amount of enzyme required, incubation time and temperature) may be optimized or adjusted according to the needs of the target protein or to maximize process economy.

### 9.2. Duration

1 h 15 min

- 1.5.1 Mix 50 µl of pGAPase enzyme solution (1.25 U, 25 U ml<sup>-1</sup>) with 5 µl of 20 mM cysteamine-HCl to activate the pGAPase.
- 1.5.2 Incubate at 37 °C for 10 min. The enzyme mixture should be used within 30 min of preparation. During DAPase enzyme preparation, bring the target protein solution to the incubation temperature of 37 °C.
- 1.5.3 Add the activated pGAPase enzyme to the pyroglutamate-extended target protein.
- 1.5.4 Incubate for 90 min at 37 °C. If desired, remove samples during the reaction for SDS-PAGE analysis.

See Fig. 6 for the flowchart of Step 1.5.



**Figure 6** Flowchart of Step 1.5.



## 10. STEP 1.6 REMOVAL OF pGAPase ENZYME BY SUBTRACTIVE IMAC

### 10.1. Overview

The His-tagged pGAPase enzyme is removed from the untagged target protein. See Fig. 4 for the flowchart for subtractive IMAC.

### 10.2. Duration

30 min

- 1.6.1 Resuspend the Ni-NTA agarose suspension by repeated inversion and pipette 1 ml of the 50% suspension (corresponding to 0.5 ml bed volume) into a disposable gravity flow column.
- 1.6.2 Equilibrate the column with 2.5 ml (5 column volumes) 1× TAGZyme buffer.
- 1.6.3 Pass the digestion reaction mixture through the column and collect the flow-through. *Important:* The flow-through fraction contains the processed target protein!
- 1.6.4 Wash the column with 2 ml aliquots of 1× TAGZyme buffer, until the flow-through no longer contains protein. Collect fraction(s). *Important:* The flow-through fraction contains the processed target protein!
- 1.6.5 Combine the flow-through fraction from Step 1.6.3 with the protein-containing fractions from Step 1.6.4. Discard the Ni-NTA

resin used in this subtractive step. The recovered target protein is ready for further applications.

### 10.3. Tip

*The column can be prepared while the target protein is being digested.*

### 10.4. Tip

*Alternatively, pack a LC or FPLC column with 0.5 ml bed volume of Ni-NTA Agarose.*

### 10.5. Tip

*A Tricorn 5/20 or Tricorn 5/50 column (GE Healthcare, inner diameter 5 mm) is suitable for this volume of matrix. In general, the bed volume height should be at least two times the inner diameter of the column.*

### 10.6. Tip

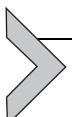
*When using a chromatography system, do not exceed a flow rate of  $0.5 \text{ ml min}^{-1}$  ( $\sim 150 \text{ cm h}^{-1}$ ) to ensure quantitative binding of the His-tagged pGAPase enzyme.*

### 10.7. Tip

*If you would like to analyze the protein captured on the Ni-NTA Agarose column, elute it by applying 2 ml of 1× TAGZyme buffer containing 500 mM imidazole and analyze a fraction by SDS-PAGE.*

### 10.8. Tip

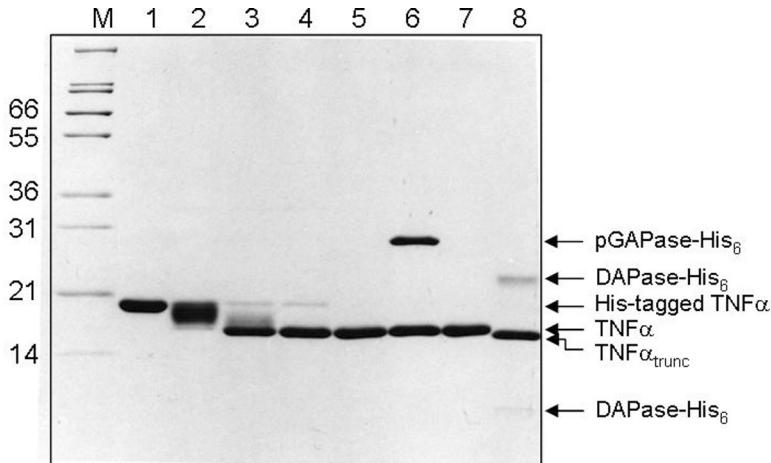
*To test how efficiently the pGAPase has removed the pyroglutamate, subject an aliquot of the processed target protein to proteolytic digestion by an excess of DAPase and analyze the reaction by SDS-PAGE (Fig. 7; upon removal of the pyroglutamate, a target protein lacking an intrinsic DAPase stop point is susceptible once again to exoproteolytic cleavage. See One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE)). Alternatively, analyze the N-terminus by mass spectrometry.*



## 11. PROTOCOL 2 ENDOPROTEOLYTIC TAG CLEAVAGE BY FACTOR XA

### 11.1. Preparation

Prepare the GST fusion protein to be cleaved. The protein can be in solution (follow Steps 2.1–2.3) or bound to glutathione agarose



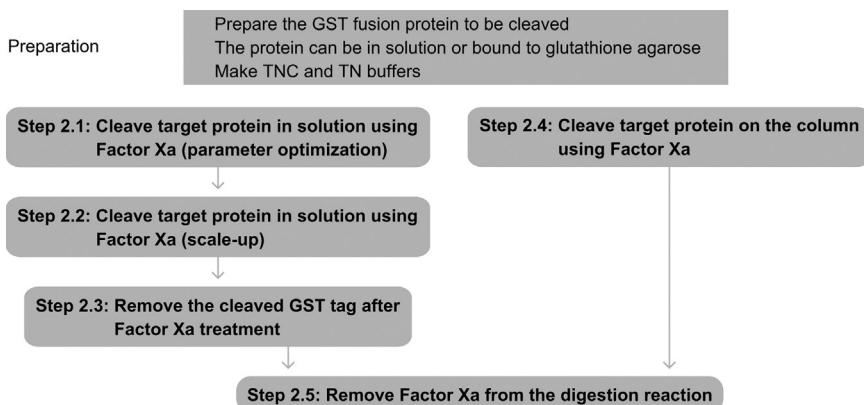
**Figure 7 Removal of the His tag from His-tagged TNF- $\alpha$  using the TAGZyme system.** M, marker; 1, purified His-tagged TNF- $\alpha$ ; 2–4, aliquots taken after 10, 20 and 30 min during DAPase/Qcyclase digestion; 5, pyroglutamate-extended TNF- $\alpha$  obtained after first subtractive IMAC (Step 2.5); 6, aliquot from the pGAPase reaction; 7, processed TNF- $\alpha$  obtained after second subtractive IMAC (Step 4.5); 8, an aliquot of the processed target protein was incubated with excess DAPase (0.125 U/mg TNF- $\alpha$ ) for 2 h; a complete shift to a faster migrating protein band (TNF- $\alpha_{trunc}$ ) is indicative of complete pyroglutamate removal; two DAPase subunits are visible in lane 8. All samples were subjected to SDS-PAGE and the gel stained by Coomassie blue.

(follow Step 2.4). Place the Factor Xa protease on ice. Prepare buffers TNC, TN, and 5× SDS-PAGE buffer.

## 11.2. Duration

Preparation	1–2 days
Protocol (setup)	About 15 min
(digestion)	Up to 16 h
(SDS-PAGE)	About 3 h
(Factor Xa removal)	About 45 min

See Fig. 8 for the flowchart of Protocol 2.



**Figure 8** Flowchart of Protocol 2, including preparation.

## 12. STEP 2.1 FACTOR XA IN-SOLUTION DIGESTION OF A TARGET PROTEIN (PARAMETER OPTIMIZATION)

### 12.1. Overview

An N-terminal GST-tag is removed from a GST fusion protein by endoproteolytic cleavage using Factor Xa protease. As endoproteolytic cleavage efficiency can vary depending on the target protein, the cleavage steps include a titration of incubation time and enzyme concentration to find the optimal conditions. Endoproteases might cleave nonspecifically; therefore, the lowest concentration necessary to cleave the fusion protein should be used.

### 12.2. Duration

Setup: 15 min

Incubation: 16 h

SDS-PAGE: 3 h

- 2.1.1 Prepare 4 solutions, each one containing 10 µg of the target GST fusion protein to be cleaved in TNC buffer. The protein concentration should be at least 0.25 µg µl<sup>-1</sup>.
- 2.1.2 Prepare serial dilutions of Factor Xa protease in 1× reaction buffer (TNC) with concentrations of 1.0, 0.2, and 0.05 U µl.
- 2.1.3 Add 1 µl of each Factor Xa dilution to one solution of the protein to be cleaved, and adjust the reaction volume to 40 µl using TNC buffer. Adjust the volume of the fourth protein solution to 40 µl

using TNC buffer; this sample will serve as a negative control to monitor the progress of cleavage.

- 2.1.4 Incubate the reactions at room temperature (15–25 °C). Remove an 8- $\mu$ l aliquot from each reaction after 3, 6, 9, and 16 h. Add 2  $\mu$ l of 5× SDS-PAGE buffer to each aliquot and mix thoroughly.
- 2.1.5 Analyze the efficiency of cleavage in each sample by SDS-PAGE (see One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE)).

### 12.3. Tip

*Since Factor Xa protease is sensitive to various buffer components, it is recommended that the protein to be cleaved is prepared in TNC buffer before cleavage. If your individual protein requires other specific buffering conditions, please see Glutathione Affinity Handbook, QIAGEN (2009), for the compatibility of some commonly used buffer components with Factor Xa.*

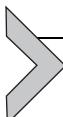
### 12.4. Tip

*Mix the dilutions of Factor Xa completely before use to avoid localized differences in enzyme concentration. Use the protease immediately after diluting it.*

### 12.5. Tip

*Immediately mix the aliquots removed from the reaction with SDS-PAGE buffer to completely stop Factor Xa protease activity.*

See Fig. 9 for the flowchart of Step 2.1



## 13. STEP 2.2 FACTOR XA IN-SOLUTION DIGESTION OF A TARGET PROTEIN (SCALE-UP)

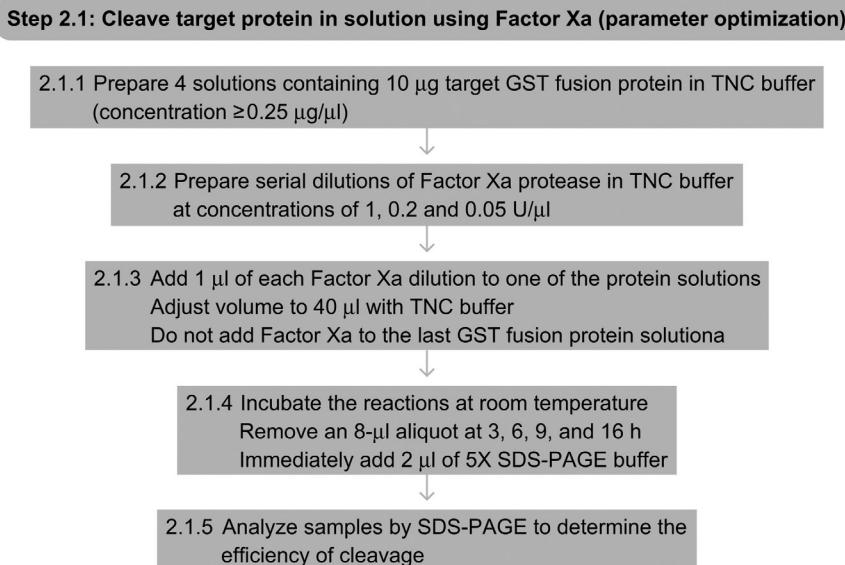
### 13.1. Overview

An N-terminal GST tag is cleaved endoproteolytically from 1 mg of GST fusion protein using Factor Xa protease under the conditions found to be optimal in Step 2.1.

### 13.2. Duration

As determined to be optimal in Step 2.1

- 2.2.1 Dilute 1 mg of the target GST fusion protein to be cleaved in TNC buffer. The solution should have a protein concentration of at least 0.25  $\mu$ g  $\mu$ l<sup>-1</sup>.



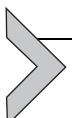
**Figure 9** Flowchart of Step 2.1.

- 2.2.2** Add Factor Xa protease to the target GST fusion protein. The amount needed is scaled up linearly from the ratio of protease to GST fusion protein found to be optimal in Step 2.1.
- 2.2.3** Incubate the reaction at room temperature (15–25 °C) for the length of time found to be optimal in Step 2.1. If desired, remove samples during the reaction for SDS-PAGE analysis.

### 13.3. Tip

*If there is a need to reduce the total reaction volume, perform small-scale experiments in which the total reaction volume is varied while the ratio of the protease to GST fusion protein and the incubation conditions are kept constant.*

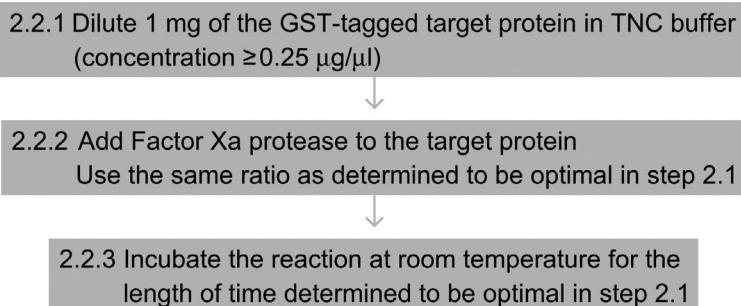
See Fig. 10 for the flowchart of Step 2.2



## 14. STEP 2.3 REMOVAL OF THE CLEAVED GST TAG AND OF UNDIGESTED GST FUSION PROTEIN AFTER FACTOR XA TREATMENT

### 14.1. Overview

The cleaved Glutathione S-Transferase tag and residual uncleaved GST fusion protein is separated from the preparation of cleaved target protein.

**Step 2.2: Cleave target protein in solution using Factor Xa (scale-up)**

**Figure 10** Flowchart of Step 2.2.

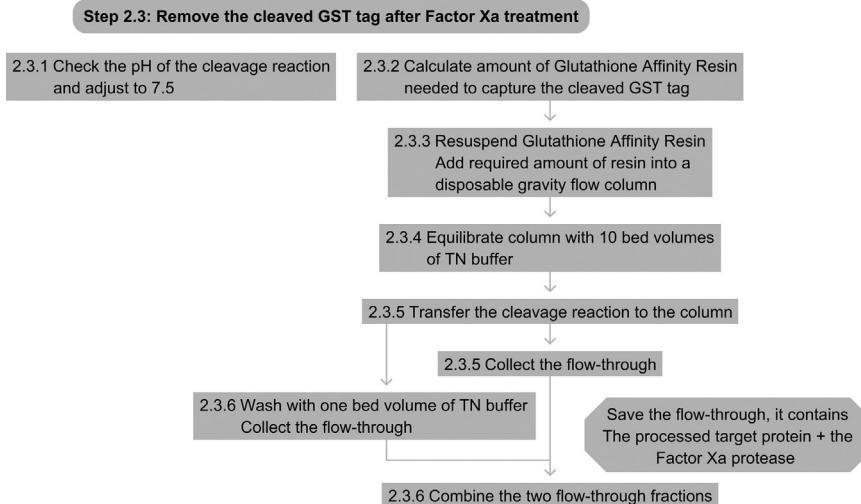
## 14.2. Duration

30 min

- 2.3.1 Check the pH of the cleavage reaction mixture and, if necessary, adjust it to 7.5.
- 2.3.2 Calculate the amount of Glutathione Affinity Resin needed to capture the GST tag and residual uncleaved GST fusion protein.
- 2.3.3 Resuspend the Glutathione Affinity Resin completely by gently inverting the bottle and then immediately transfer the required amount of slurry into a disposable gravity flow column.
- 2.3.4 Equilibrate the column with 10 bed volumes of TN buffer.
- 2.3.5 Transfer the pH-adjusted cleavage reaction mixture to the equilibrated resin and collect the flow-through. *Important:* The flow-through fraction contains the processed target protein (plus the Factor Xa protease)!
- 2.3.6 Wash with one bed volume of TN buffer and collect the flow-through. Combine it with the flow-through fraction from Step 2.3.5. *Important:* The flow-through contains the processed target protein (plus the Factor Xa protease)!

## 14.3. Tip

If the cleavage reaction had been performed at pH 6.5, add  $\sim 1/100$  volume of 1 M Tris-Cl, pH 8.0 to adjust the pH to 7.5. Factor Xa protease cleavage and removal are optimal at pH values between 6.5 and 7.5.



**Figure 11** Flowchart of Step 2.3.

#### 14.4. Tip

1 ml bed volume (usually 2 ml slurry) is usually sufficient to bind 5–10 mg GST protein. For optimal performance, the binding capacity of the Glutathione Affinity resin used for removal should match the total amount of GST-tagged protein that was subjected to Factor Xa protease cleavage.

See Fig. 11 for the flowchart of Step 2.3.

## 15. STEP 2.4 FACTOR XA ON-COLUMN DIGESTION OF A TARGET PROTEIN

### 15.1. Overview

The GST tag is cleaved from a GST fusion protein while bound to the Glutathione Affinity Resin. The protocol described here is designed for cleaving protein from ~1 ml bed volume of Glutathione Affinity Resin. The cleavage starts immediately after protein purification (binding and washing, see [Purification of GST-tagged proteins](#)). Do not elute the GST-tagged protein from the glutathione affinity resin using reduced glutathione!

## 15.2. Duration

About 3 h (or overnight)

- 2.4.1 Equilibrate/wash the glutathione affinity resin (with the GST-tagged fusion protein bound) using 10 ml (10 bed volumes) of TNC buffer. This step serves to provide optimal reaction conditions for Factor Xa cleavage. Factor Xa activity is reduced in the presence of phosphate.
- 2.4.2 Prepare Factor Xa protease cleavage solution: Mix 80 µl (160 U) of protease with 920 µl of TNC buffer in a 1.5-ml microcentrifuge tube by gently inverting the tube. This amount of Factor Xa protease is sufficient to cleave approximately 10 mg of GST fusion protein on the column.
- 2.4.3 Load the Factor Xa protease cleavage solution onto the column
- 2.4.4 Close the inlet and the outlet of the column.
- 2.4.5 Incubate the column at room temperature (15–25 °C) for 2–16 h.
- 2.4.6 Open both ends and elute the target protein by applying 3 ml (3 bed volumes) of TNC buffer. Save the flow-through. *Important:* The flow-through fraction contains the processed target protein (plus the Factor Xa protease)!

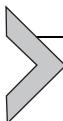
## 15.3. Tip

*If using a gravity flow column, let the resin drain completely by gravity flow until no buffer stands above the resin. Do not force additional liquid out of the resin by applying pressure! Pipette 1 ml of the cleavage solution onto the resin, close the column outlet using the cap provided, seal the inlet opening with Parafilm, and incubate upright.*

## 15.4. Tip

*If using a Glutathione Affinity prefilled cartridge, load 1 ml of the cleavage solution onto the cartridge using a syringe. Close the outlets using the plugs provided. Elute the target protein by applying 3 ml (3 bed volumes) of TNC buffer at a flow rate of 155 cm h<sup>-1</sup> (~1 ml min<sup>-1</sup>; 1 ml cartridge). Collect the flow-through.*

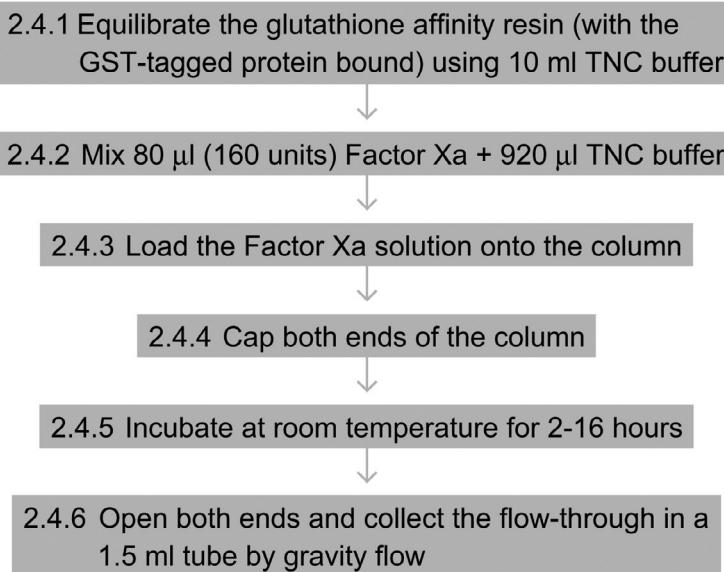
See Fig. 12 for the flowchart of Step 2.4.



## 16. STEP 2.5 REMOVAL OF FACTOR XA

### 16.1. Overview

After protease digestion, Factor Xa protease can be removed by affinity chromatography using Xa Removal Resin. The Xa Removal Resin binds

**Step 2.4: Cleave target protein on the column using Factor Xa**

**Figure 12** Flowchart of Step 2.4.

the protease in the reaction mixture while the cleaved target protein remains in solution. After the resin is pelleted by centrifugation, the cleaved target protein is recovered in the supernatant.

## 16.2. Duration

About 45 min

- 2.5.1 Calculate the amount of Xa Removal Resin needed to capture the Factor Xa protease present in the cleavage reaction. A 100 µl of the slurry (50-µl bed volume) is sufficient to bind 4 U of Factor Xa protease in TNC reaction buffer.
- 2.5.2 Resuspend the Xa Removal Resin completely by gently inverting and then immediately transfer the required amount of slurry into a centrifuge tube of appropriate size.
- 2.5.3 Centrifuge the beads for 5 min at 1000 × g and discard the supernatant.
- 2.5.4 Resuspend the beads in 10 bed volumes of TN buffer and mix gently. Centrifuge for 5 min at 1000 × g and discard the supernatant.

- 2.5.5 Add the flow-through fraction(s) containing the cleaved target protein and Factor Xa to the equilibrated resin. Mix gently to resuspend the resin and incubate for 10 min at room temperature (15–25 °C). Rotate end-over-end to keep the beads in suspension.
- 2.5.6 Centrifuge the beads at 1000 × g for 5 min to pellet the resin. Collect the supernatant that contains the cleaved target protein (Factor Xa protease remains bound to the resin).

### 16.3. Tip

*Using slurry volumes less than 25 µl is not recommended due to associated handling problems, for example, during centrifugation.*

### 16.4. Tip

*If the target protein requires cleavage buffer other than the recommended TNC buffer, consider that the protease capture step may be sensitive to the use of other buffers. Binding of Factor Xa protease to the Xa Removal Resin is unaffected by increasing the pH to 7.5, the presence of 20–100 mM Tris–HCl, and up to 1% Triton X-100 or Nonidet P-40. High salt concentrations will reduce binding capacity. For example, increasing NaCl concentration from 50 mM to 500 mM will result in a 20–40% reduction in binding. The recommended TN buffer supports high-efficiency cleavage and capture.*

### 16.5. Tip

*The beads will quickly settle out of solution. Use a wide-mouth pipette to transfer the beads.*

### 16.6. Tip

*Equilibration of the beads with buffer is necessary for maximum capture efficiency and prevents contamination of the cleaved target protein with resin storage buffer. Use Xa Removal Resin immediately after equilibration.*

### 16.7. Tip

*If the cleaved target protein is temperature-sensitive, binding can be performed at 2–8 °C without any loss of binding efficiency.*

See Fig. 13 for the flowchart of Step 2.5.

**Step 2.5: Remove Factor Xa from the digestion reaction**

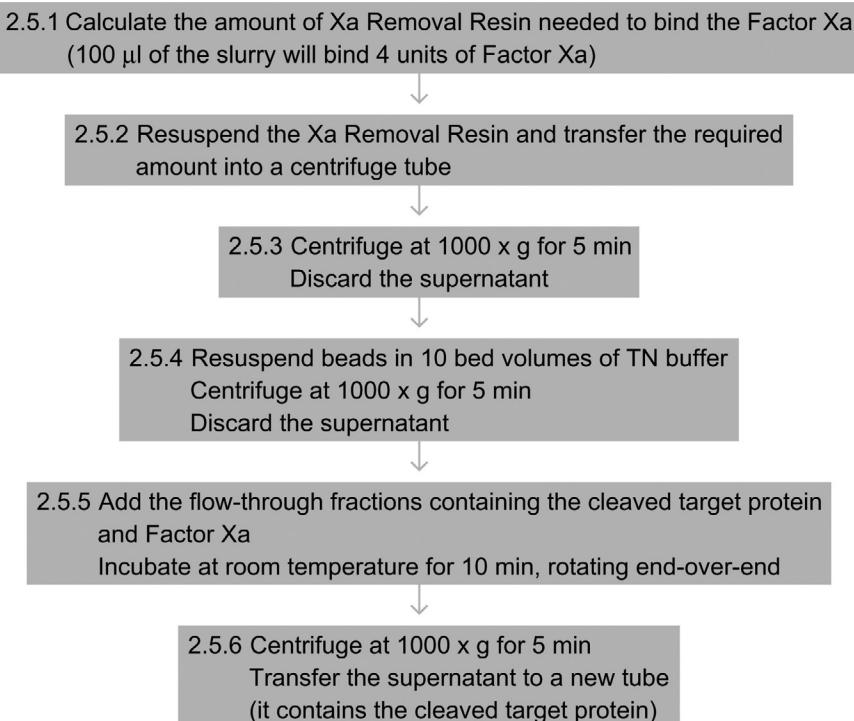


Figure 13 Flowchart of Step 2.5.

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TAGZyme handbook for exoproteolytic cleavage of N-terminal His tags, (2003) Hilden, Germany: QIAGEN.

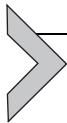
### Referenced Protocols in Methods Navigator

[Purification of His-tagged proteins](#).

[Purification of GST-tagged proteins](#).

Gel filtration chromatography (Size exclusion chromatography) of proteins.

One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE).



# Affinity Pull-Down of Proteins Using Anti-FLAG M2 Agarose Beads

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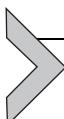
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## Abstract

FLAG is an affinity tag widely used for rapid and highly specific one-step protein purification. Native elution of protein from anti-FLAG antibody resins allows the identification of protein and nucleic acid binding partners and functional analysis using biochemical activity assays.



## 1. THEORY

$3\times$  FLAG, a small tag of only 25 amino acids, has been successfully fused with many proteins. The FLAG tag allows highly specific pull-downs that contain low nonspecific background. This protocol describes isolation of a FLAG-tagged target protein in one step and is therefore relatively quick and simple. FLAG-tags have been used to pull down recombinant proteins made in bacteria, or using a baculovirus system, as well as proteins expressed in *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and mammalian cells (reviewed in [Einhauer and Jungbauer, 2001](#)).

FLAG-tagged proteins are recognized and bound by the anti-FLAG M2 antibody and efficiently pulled-down using M2-conjugated agarose beads. The pulled-down protein is then eluted from the beads by competition with a  $3\times$  FLAG peptide. Anti-FLAG antibodies specifically recognize the target protein within lysates with relatively low cross-reactivity with other cellular proteins; however, the M2 antibody can react with native protein epitopes in mammalian cells ([Schäfer and Braun, 1995](#)) as well as in *S. pombe* ([Buker et al., 2007](#)).

We outline a small-scale pull-down; however, the protocol can be scaled up for larger preparations of protein. The protein isolated using this method is suitable for use in a variety of techniques, such as functional assays, analysis by proteomics approaches, such as mass spectrometry to identify binding partners, or to assess associated nucleic acids ([Buker et al., 2007](#)). FLAG tags are also widely used for testing if two proteins coimmunoprecipitate (e.g., [Gerace et al., 2010](#)) and for chromatin immunoprecipitations (ChIP) (e.g., [Buker et al., 2007](#)). Please refer to Sections Co-Immunoprecipitation of proteins from yeast and Chromatin Immunoprecipitation and Multiplex Sequencing (ChIP-Seq) to identify global transcription factor binding sites in the nematode *Caenorhabditis elegans* for detailed protocols on coimmunoprecipitation and ChIP, respectively.



## 2. EQUIPMENT

Centrifuge (refrigerated)  
Microcentrifuge (refrigerated)  
Bead beater (e.g., BioSpec Mini-beadbeater 8)  
Micropipettors  
Pipettor tips  
1.7-ml polypropylene microcentrifuge tubes  
1.7-ml low retention microcentrifuge tubes  
2-ml screw-capped microcentrifuge tubes  
5-ml polypropylene round-bottom tubes  
21 gauge needles  
0.5-mm glass beads (BioSpec)  
End-over-end rotator



## 3. MATERIALS

HEPES  
Sodium chloride (NaCl)  
Magnesium chloride ( $MgCl_2$ )  
EDTA  
Glycerol  
Dithiothreitol (DTT)  
Triton X-100  
Complete EDTA-free Protease Inhibitor Cocktail tablets (Roche)  
Phenylmethylsulfonyl fluoride (PMSF)  
Anti-FLAG M2 agarose beads (Sigma)  
HA peptide (Sigma)  
 $3 \times$  FLAG peptide (Sigma)

### 3.1. Solutions & buffers

#### Step 1 2× Buffer G

Component	Final concentration	Stock	Amount
Na-HEPES, pH 7.5	50 mM	1 M	50 ml
NaCl	150 mM	5 M	30 ml
EDTA	1 mM	0.5 M	2 ml

Add water to 500 ml. Filter-sterilize and store at 4 °C

**1× Lysis Buffer**

<b>Component</b>	<b>Final concentration</b>	<b>Stock</b>	<b>Amount</b>
2× Buffer G	1×	2×	25 ml
Triton X-100	0.5%	20%	1.25 ml
Glycerol	10%	50%	10 ml
DTT	0.5 mM	1 M	25 µl
PMSF	1 mM	100 mM	500 µl
Protease Inhibitor Cocktail			1 tablet

Add water to 50 ml

**Step 3 Wash Buffer 1**

<b>Component</b>	<b>Final concentration</b>	<b>Stock</b>	<b>Amount</b>
2× Buffer G	1×	2×	25 ml
Triton X-100	0.5%	20%	1.25 ml
Glycerol	5%	50%	5 ml
DTT	0.5 mM	1 M	25 µl
PMSF	1 mM	100 mM	500 µl

Add water to 50 ml

**Wash Buffer 2**

<b>Component</b>	<b>Final concentration</b>	<b>Stock</b>	<b>Amount</b>
2× Buffer G	1×	2×	5 ml
Glycerol	5%	50%	1 ml
PMSF	1 mM	100 mM	100 µl

Add water to 10 ml

**Elution Buffer**

<b>Component</b>	<b>Final concentration</b>	<b>Stock</b>	<b>Amount</b>
Na-Hepes, pH 7.5	25 mM	1 M	2.5 ml
NaCl	100 mM	5 M	2 ml

Add water to 100 ml. Filter-sterilize and store at 4 °C

**HA buffer**

<b>Component</b>	<b>Final concentration</b>	<b>Stock</b>	<b>Amount</b>
Elution Buffer			1 ml
HA peptide	100 µg	10 mg ml <sup>-1</sup>	10 µl
PMSF	1 mM	100 mM	10 µl

**Step 4 FLAG buffer**

<b>Component</b>	<b>Final concentration</b>	<b>Stock</b>	<b>Amount</b>
Elution Buffer			1 ml
3× FLAG peptide	200 µg	5 mg ml <sup>-1</sup>	40 µl
PMSF	1 mM	100 mM	10 µl

**4. PROTOCOL****4.1. Duration**

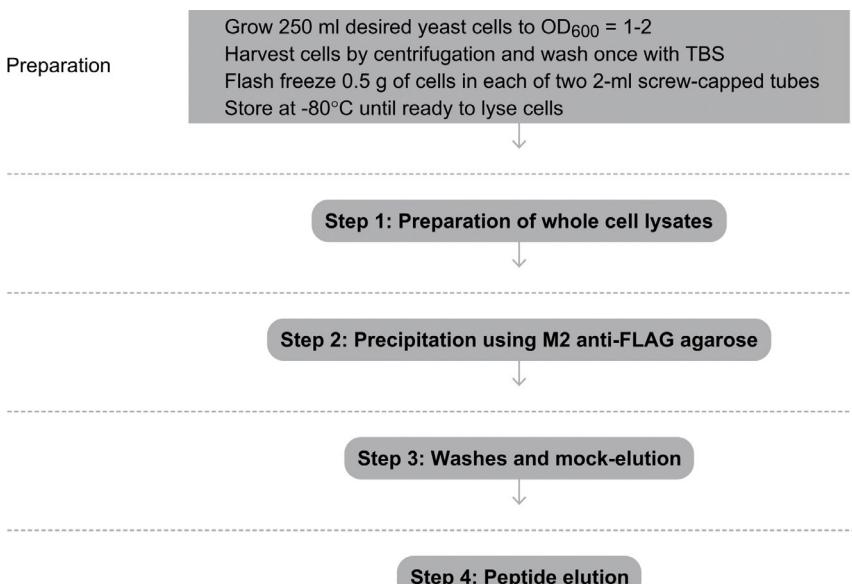
Preparation	About 1 day
Protocol	About 5–6 h

**4.2. Preparation**

Grow 250 ml of yeast cells to  $OD_{600}=1\text{--}2$ . Pellet cells and wash once with 1× TBS. For each strain, flash-freeze 0.5 g of cells in each of two 2-ml screw-capped tubes (total of 1 g for each strain). Tubes can be stored at  $-80^{\circ}\text{C}$  until ready to begin the experiment.

Whether the pulled-down protein will be used to find protein–protein or nucleic acid interactions or will be used in functional assays, be sure to include an isogenic untagged strain to serve as a control for background binding.

See Fig. 1 for the flowchart of the complete protocol.



**Figure 1** Flowchart of the complete protocol, including preparation.

## 5. STEP 1 PREPARATION OF WHOLE CELL LYSATES

### 5.1. Overview

Prepare cell lysates to be added to each pull-down reaction. Cell pellets are thawed, lysed by bead beating, and then cleared by centrifugation to remove cell debris.

### 5.2. Duration

45 min

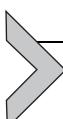
- 1.1 To the frozen pellets, add 0.1 ml of ice-cold, freshly prepared lysis buffer for each 0.1 g of cells. Thaw the cells by gently flicking and inverting the tubes. Once completely thawed, keep all tubes on ice.
- 1.2 Add 400 µl of 0.5-mm glass beads to each tube. Pulse the tubes in a bead beating instrument (e.g., BioSpec Mini-beadbeater-8) for 45 s at homogenizing intensity. Place all tubes on ice for 5 min before a second 45-s pulse. Again, place the tubes on ice.
- 1.3 Puncture the bottom of each tube with a 21-gauge needle. Carefully place each punctured tube into an appropriately sized collection tube, such as a 5-ml polypropylene round-bottom tube.

- 1.4 Spin samples in a centrifuge (such as Eppendorf 5702R or Sorvall RC5C) at 4 °C for 5 min at  $200 \times g$ .
- 1.5 Gently resuspend the flow-through with a pipettor and transfer the entire volume to a 1.7-ml microcentrifuge tube. Combine the flow-throughs from identical strains into one microcentrifuge tube.
- 1.6 Spin the tubes in a microcentrifuge at 4 °C for 5 min at maximum speed ( $\sim 20\,000 \times g$ ).
- 1.7 Pipet the resulting supernatants to clean the microcentrifuge tubes. This is the whole cell lysate.

### 5.3. Tip

Larger cell pellets can be lysed using a BioSpec bead beating chamber. In this case, other lysis methods, which generate less heat than bead beating, may be more suitable for some protein purifications, and should be considered. These involve lysis in a coffee grinder in the presence of dry ice (Schultz et al., 1997), lysis under liquid nitrogen using a mortar and pestle, or lysis in a liquid nitrogen-cooled, steel chamber (e.g., the Retsch Cryomill).

See Fig. 2 for the flowchart of Step 1.



## 6. STEP 2 IMMUNOPRECIPITATION USING ANTI-FLAG TAG ANTIBODY

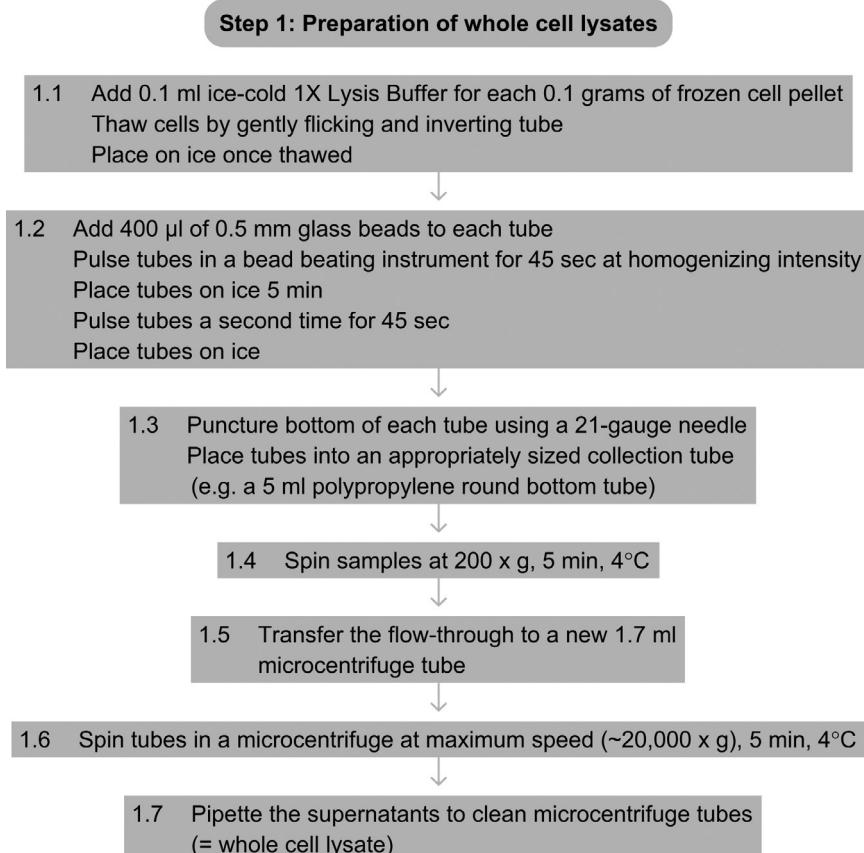
### 6.1. Overview

The cell lysates will be incubated with anti-FLAG M2 agarose to pull down the FLAG-tagged target protein.

### 6.2. Duration

2 h 20 min

- 2.1 For each immunoprecipitation, remove 30 µl of the 50% slurry of anti-FLAG M2 agarose (15 µl of packed beads) and place in a low-retention microcentrifuge tube. Wash the beads 5 times in 1× Lysis Buffer. Gently resuspend the beads in 1 ml lysis buffer using a cut pipette tip. Between each wash, spin the tubes in a microcentrifuge at  $500 \times g$  for 1 min. Carefully remove the supernatant with a pipettor.
- 2.2 Resuspend the washed beads in lysis buffer, in a volume of 100 µl for each pull-down, and aliquot the beads into the required number of low-retention microcentrifuge tubes.



**Figure 2** Flowchart of Step 1.

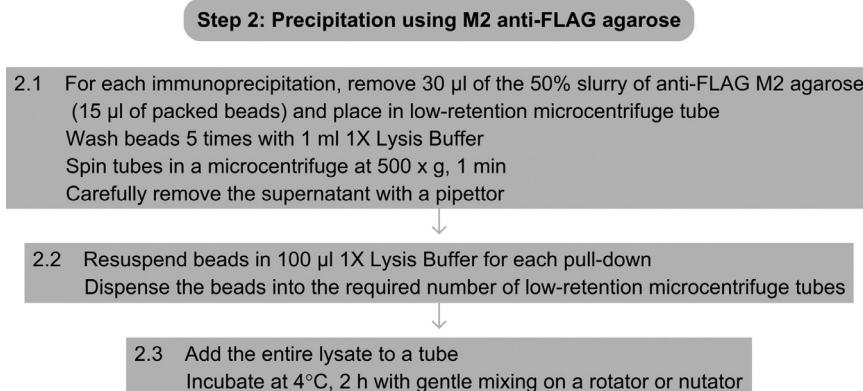
**2.3** Add the entire lysate from Step 1.7 to a tube of washed M2 agarose beads. Incubate at 4 °C for 2 h on an end-over-end rotator or equivalent.

### 6.3. Tip

*The target protein will be efficiently immunoprecipitated with 15 µl of packed bead volume for 1 g of cells.*

### 6.4. Tip

*Using a vacuum aspirator during bead washing can lead to accidental bead loss. Therefore, use a pipettor to remove the wash buffer.*



**Figure 3** Flowchart of Step 2.

## 6.5. Tip

*When scaling up this protocol to purify from a greater number of cells, use 50 µl of packed M2 agarose beads for lysates made from 10 g of cells. These beads have a high capacity, and using more beads will not necessarily increase protein yield, but will definitely increase the background binding.*

## 6.6. Tip

*When working with agarose beads, always cut the end of the pipettor tip using a clean razor blade prior to pipetting the beads. Otherwise, the beads will clog the tip during pipetting, leading to the uptake of more buffer and fewer beads.*

See Fig. 3 for the flowchart of Step 2.



## 7. STEP 3 WASHES AND MOCK-ELUTION

### 7.1. Overview

After the M2 beads have incubated with the lysate for 2 h, they will be washed several times. Prior to elution, the beads will be mock-eluted using the HA peptide. This step will eliminate any proteins that would elute in the presence of any peptide not specific to 3× FLAG.

### 7.2. Duration

30 min

**3.1** Spin samples in a microcentrifuge at  $500 \times g$  for 2 min at 4 °C. Gently remove the supernatant with a pipettor.

- 3.2 Add 1 ml of ice-cold Wash Buffer 1 to each tube, and resuspend all the beads by gently inverting each tube several times. Make sure all the beads have been completely resuspended. Spin the tubes in a microcentrifuge at  $500 \times g$  for 1 min at 4 °C. Carefully remove the supernatant with a pipettor.
- 3.3 Repeat the wash two more times.
- 3.4 Wash a final time using 1 ml of ice-cold Wash Buffer 2.
- 3.5 Spin the tubes again briefly to make sure any of the excess wash buffer does not remain on the sides of the tubes. Use a P20 pipettor to remove all of the excess wash buffer, making sure not to remove any of the beads.
- 3.6 Prepare and add 1 ml of HA Buffer for each pull-down sample. Incubate at 4 °C on an end-over-end rotator or equivalent for 15 min.
- 3.7 Spin the tubes in a microcentrifuge at  $500 \times g$  for 1 min at 4 °C. Carefully remove the supernatant with a pipettor. Spin briefly and use a P20 pipettor to remove all the excess buffer, making sure not to remove any of the beads.

See Fig. 4 for the flowchart of Step 3.

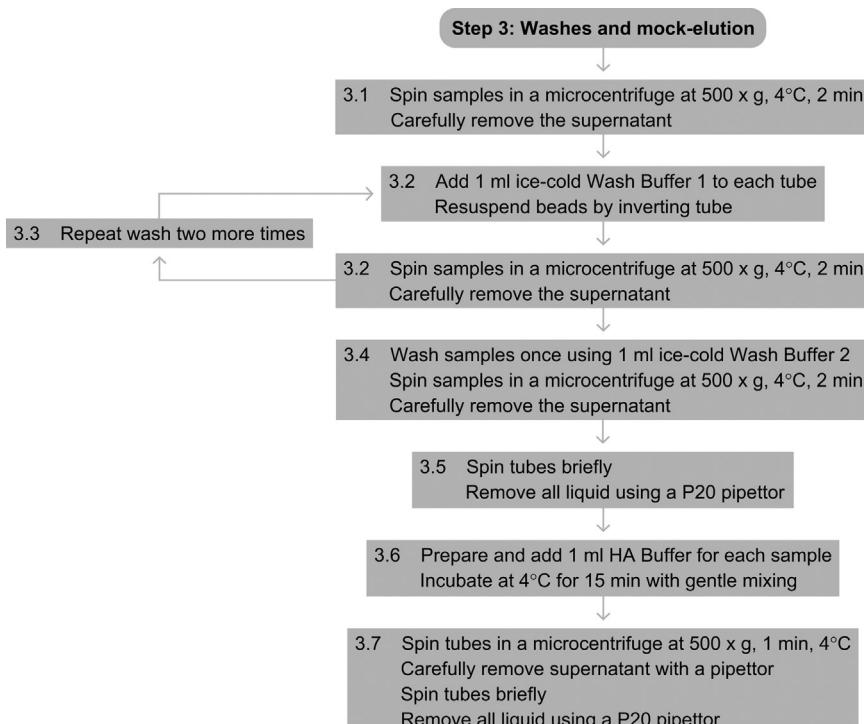


Figure 4 Flowchart of Step 3.



## 8. STEP 4 PEPTIDE ELUTION

### 8.1. Overview

During this step, the FLAG-tagged target protein will be eluted from the beads. This is accomplished by adding an excess of  $3\times$  FLAG peptide, which will compete for the binding sites on the M2 agarose beads, releasing the protein into the eluate.

### 8.2. Duration

45 min

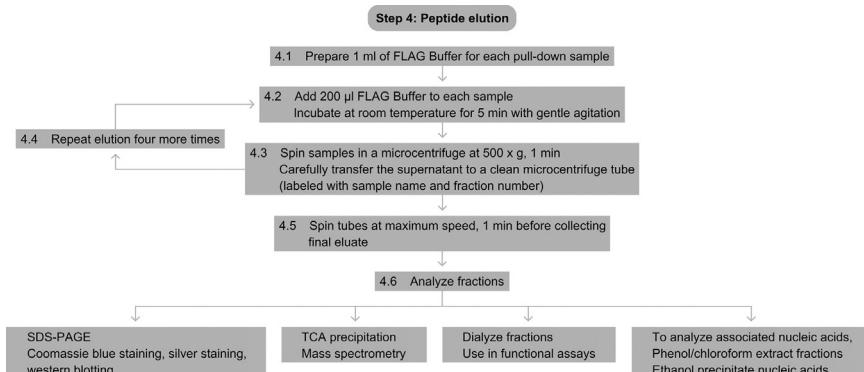
- 4.1 Prepare 1 ml of FLAG Buffer for each pull-down sample.
- 4.2 Add 200  $\mu$ l of FLAG Buffer to each tube of washed, mock-eluted beads from Step 3.7. Incubate at room temperature on a nutator or shaking rack (such as an Eppendorf Thermomixer) for 5 min.
- 4.3 Spin the samples at  $500\times g$  in a microcentrifuge for 1 min. Carefully collect the supernatant with a pipettor and transfer it to a clean microcentrifuge tube appropriately labeled with the sample and fraction number.
- 4.4 Repeat Steps 4.2 and 4.3 four more times.
- 4.5 After the final incubation, spin the tubes at maximum speed ( $\sim 20\,000\times g$ ) before collecting the fifth fraction.
- 4.6 The fractions can now be separated by SDS-PAGE (see One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE)) and analyzed by Coomassie Blue staining (see Coomassie Blue Staining), silver staining (see Silver Staining of SDS-polyacrylamide Gel), or Western blotting (see Western Blotting using Chemiluminescent Substrates). Alternatively, the fractions can be TCA-precipitated (see TCA Precipitation) and analyzed by mass spectrometry, or dialyzed and used in functional assays.

For analysis of associated nucleic acids, a phenol/chloroform extraction followed by an ethanol precipitation can be performed on the elution fractions (Halic and Moazed, 2010).

### 8.3. Tip

*This protocol outlines collecting five fractions of 200  $\mu$ l, but this can be adjusted as necessary.*

See Fig. 5 for the flowchart of Step 4.



**Figure 5** Flowchart of Step 4.

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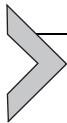
One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE).

Coomassie Blue Staining.

Silver Staining of SDS-polyacrylamide Gel.

Western Blotting using Chemiluminescent Substrates.

TCA Precipitation.



## CHAPTER EIGHT

# Protein Affinity Purification using Intein/Chitin Binding Protein Tags

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## Abstract

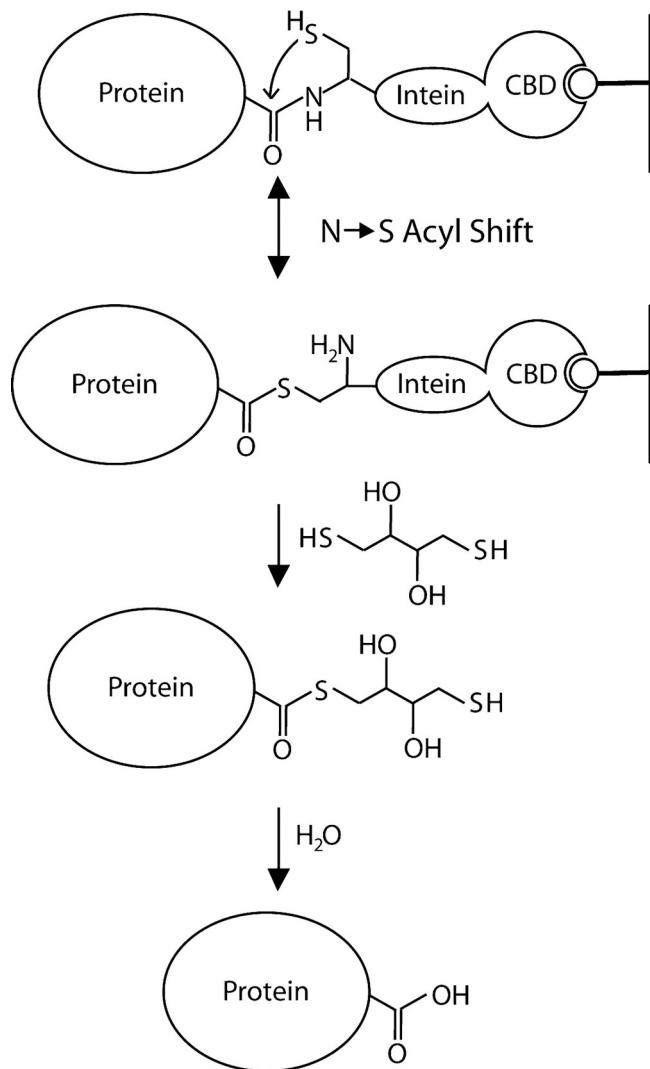
Isolation of highly purified recombinant protein is essential for a wide range of biochemical and biophysical assays. Affinity purification in which a tag is fused to the desired protein and then specifically bound to an affinity column is a widely used method for obtaining protein of high purity. Many of these methods have the drawbacks of either leaving the recombinant tag attached to the protein or requiring the addition of a protease which then must be removed by further chromatographic steps. The fusion of a self-cleaving intein sequence followed by a chitin-binding domain (CBD) allows for one-step chromatographic purification of an untagged protein through the thiol-catalyzed cleavage of the intein sequence from the desired protein. The affinity purification is highly specific and can yield pure protein without any undesired N- or C-terminal extensions. This protocol is based on the IMPACT<sup>TM</sup>-System (**i**ntein **m**ediated **p**urification with an **a**ffinity **c**hitin-binding **t**ag) marketed by New England Biolabs.



### 1. THEORY

Inteins are protein sequences that catalyze their own removal from a longer protein, splicing together the two surrounding protein halves (exteins) in the process (Perler et al., 1994). Since their discovery in 1990 (Kane et al., 1990), a great deal of mechanistic insight has been gained into the chemistry of the intein reaction. The first step involves an N→S acyl shift in which the thiol group of a cysteine in the intein attacks the carbonyl carbon of the amide bond on the C-terminal residue of the N-terminal extein. The result is a thioester intermediate. A Cys at the N-terminus of the C-terminal extein then attacks the thioester, creating a branched peptide intermediate, from which the intein sequence is then removed through the formation of a succinimide from its C-terminal Asp residue. An S→N acyl shift creates a peptide bond between the two extein sequences creating a continuous, linear polypeptide chain (David et al., 2004). For over a decade, this chemistry has been utilized for a variety of biotechnical applications including protein purification.

In order to utilize this chemistry for protein purification and other applications, inteins have been modified so that their cleavage can be turned off in vivo and later activated in vitro. The mutation of the intein C-terminal Asp to Ala is one such mutation (Chong et al., 1996). This mutation prevents the intein sequence from splitting from the C-terminal extein preventing the fusion of the two extein sequences (Chong et al., 1997). The addition of a thiol-containing compound such as DTT, βME, or cysteine catalyzes the



**Figure 1** An illustration of the chemistry involved in purification using the intein/chitin protein tag method.

cleavage of the N-terminal extein sequence from the thioester intermediate formed with the intein sequence (Fig. 1). Following hydrolysis, a completely untagged protein remains. This technology has been utilized in protein purification by the fusion of an affinity tag, the chitin-binding domain (CBD), C-terminal to the modified intein sequence in the IMPACT™-System

available from New England Biolabs. Today they offer a variety of vectors that can fuse the intein sequence and affinity tag to either the N- or C-terminal side of the target protein. Sandwiching an intein sequence between a highly expressed sequence and the target protein may increase protein expression (Chong et al., 1998). Some vectors allow the intein cleavage reaction to be catalyzed without the addition of thiol-containing compounds (pTWIN plasmids). The protocol described here is for use with the vectors that require a thiol-containing compound for cleavage (pTYB and pTXB vectors). This protocol can also be modified for use with pKYB vectors which function much as pTYB vectors but contain a kanamycin resistance cassette rather than an ampicillin resistance gene. A detailed discussion of available vectors and the ideal conditions for expression and purification using each has been published (Xu et al., 2000).

The high-affinity, high-specificity interaction between the CBD and chitin allows for the isolation of protein of high purity and the use of the intein cleavage system produces a protein of native or near-native sequence without the use of a separate protease to remove the affinity tag. The ability to circumvent protease treatment is advantageous for several reasons. First, the use of a protease creates the risk of unwanted proteolysis at additional sites within the target protein. Second, many proteases require incubation at a high temperature, at which some proteins are not stable. Third, additional chromatographic steps are needed to remove the protease. Fourth, the tertiary structure of the fusion protein may prevent the protease from accessing the cleavage site. Intein-based protein purification allows for one-step affinity purification of untagged proteins under relatively gentle conditions.

Despite its utility, this type of protein purification is not without some occasional problems of its own. The amino acids surrounding the intein sequence can influence the rate of cleavage, either reducing cleavage upon addition of DTT, or increasing the rate of cleavage *in vivo*. Both changes can greatly reduce the yield of the protein. If possible, a single amino acid can be added to the protein in order to optimize cleavage. This method is employed by the pTYB2 plasmid, which inserts a Gly residue at the C-terminus of the protein just before the intein sequence. Lists of recommended amino acids for the N- and C-terminal ends of specific inteins have been published (Xu et al., 2000). The three-dimensional structure of the protein may also interfere with cleavage. However, it is less likely to do so when using a small molecule such as DTT to catalyze cleavage than when using a macromolecular protease.

In addition, cleavage is less efficient at lower temperatures, such that cleavage occurs during an overnight incubation at room temperature, but may require a longer incubation at 4 °C. If the protein is unstable, this can present a problem.

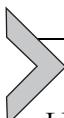
A variety of parameters may be varied during this procedure. In order to optimize purification of a specific protein, it may be necessary to do so, though this may also cause a reduction in yield. To avoid *in vivo* cleavage, induction of protein expression should be performed at a low temperature (12–16 °C). For many proteins this will increase the final yield. If a higher temperature is required, a shorter induction may be recommended. The reaction works most efficiently at higher pH (7.0–8.5, dependent upon intein and vector choice). As mentioned earlier, temperature has an effect on the rate of cleavage, but increasing incubation time can offset this. The interaction between the CBP and chitin is stable against low levels of detergents, high salt concentrations (up to 5 M), and low concentrations of denaturants (<2 M Guanidinium HCl or urea) (Chong et al., 1997; Chong et al., 1998). A very low concentration of DTT or βME (1–2 mM) may also be added prior to cleavage without greatly decreasing yield in some cases (Chong et al., 1997). Varying these buffer ingredients may reduce aggregation, which can cause the protein to remain stuck to the column during elution.



## 2. EQUIPMENT

- Refrigerated shaking incubator
- Centrifuge
- SS-34 rotor or equivalent
- UV/vis spectrophotometer
- Polyacrylamide gel electrophoresis equipment
- Pipet-Aid
- Micropipettors
- French press
- Ring stand
- Oak Ridge polycarbonate centrifuge tubes
- 2.5-l Baffled Flasks
- 25-ml pipettes
- Micropipettor tips

~ 12-ml sterile snap-cap tube  
0.8- $\mu$ m syringe filters  
30-ml or larger disposable syringe  
30-ml disposable column



### 3. MATERIALS

LB media  
Carbenicillin  
Isopropyl  $\beta$ -D-1 thiogalactopyranoside (IPTG)  
HEPES  
Potassium chloride (KCl)  
EDTA  
Triton X-100  
Potassium acetate (KOAc)  
Dithiothreitol (DTT)  
Chitin beads  
Bradford reagent  
Bovine serum albumin (BSA)  
SDS Loading buffer  
Tris base  
SDS polyacrylamide gel electrophoresis materials  
Complete EDTA-free protease inhibitor tablet (Roche)  
Aprotinin  
Leupeptin  
Pepstatin A  
Benzamidine  
4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF)

#### 3.1. Solutions & buffers

##### Step 2 Intein Lysis Buffer

Component	Final concentration	Stock	Amount
HEPES•KOH, pH 7.4	20 mM	1 M	20 ml
KCl	0.5 M	2 M	250 ml
EDTA, pH 8.0	1 mM	0.5 M	2 ml
Triton X-100	0.1%		1 ml

Add water to 1 l

**Step 3 Intein Wash Buffer**

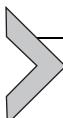
Component	Final concentration	Stock	Amount
HEPES•KOH, pH 7.4	20 mM	1 M	20 ml
KCl	1 M	2 M	500 ml
EDTA, pH 8.0	1 mM	0.5 M	2 ml
Triton X-100	0.1%		1 ml

Add water to 1 l

**Intein Cleavage Buffer**

Component	Final concentration	Stock	Amount
HEPES•KOH, pH 8.0	20 mM	1 M	20 ml
KCl	0.5 M	2 M	250 ml
EDTA, pH 8.0	1 mM	0.5 M	2 ml

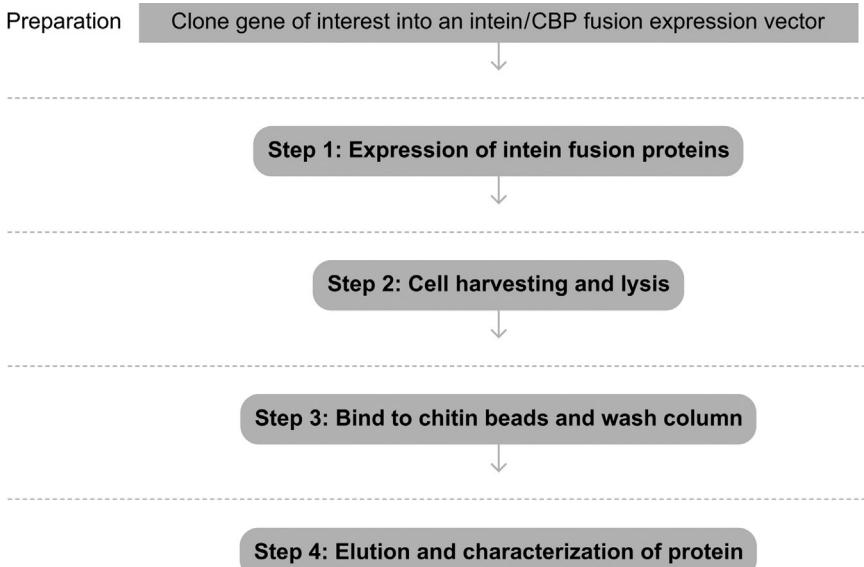
Add water to 1 l

**Tip***Print out recipes of all stock solutions needed to create the buffers online***4. PROTOCOL****4.1. Duration**

Preparation	Several days
Protocol	About 2.5 days

**4.2. Preparation**

Clone your desired gene into the appropriate vector (see Molecular Cloning) to produce an intein-CBP fusion. A variety of vectors can be found in the IMPACT™-System from New England Biolabs. A detailed description of this cloning is available ([Xu et al., 2000](#)). This protocol is appropriate for use with the pTYB or pTXB series of vectors. Transform an appropriate expression strain of *E. coli* cells containing a chromosomal copy of the T7



**Figure 2** Flowchart of the complete protocol, including preparation.

RNA polymerase gene under the control of the lac promoter with your plasmid (see Transformation of Chemically Competent *E. coli* or Transformation of *E. coli* via electroporation).

Prepare the solutions listed earlier and bring them to 4 °C.

See Fig. 2 for the flowchart of the complete protocol.

## 5. STEP 1 EXPRESSION OF INTEIN FUSION PROTEINS

### 5.1. Overview

Induce expression of your desired protein fused to the intein and CBP domains (see Small-scale Expression of Proteins in *E. coli*).

### 5.2. Duration

36 h

**1.1** Inoculate 5 ml of LB media supplemented with 50 µg ml<sup>-1</sup> carbenicillin (or an appropriate antibiotic if the IMPACT™ System is not used) with a single colony of the desired strain. Grow with shaking at 250 rpm overnight at 37 °C.

- 1.2 Inoculate 1 l of LB media supplemented with 50  $\mu\text{g ml}^{-1}$  carbenicillin with the 5 ml overnight culture. Grow with shaking at 250 rpm at 37 °C until the culture reaches an OD<sub>600</sub> of 0.5–0.6.
- 1.3 Incubate for 15 min on ice while cooling shaking incubator to 16 °C.
- 1.4 Add 0.5 ml 1 M IPTG to the culture.
- 1.5 Return the flask to the shaker. Shake at 250 rpm, overnight, at 16 °C.

### 5.3. Tip

*Induction can be done at 12–16 °C with minimal in vivo cleavage of the intein sequence. Higher temperature induction may be possible but should be done for a shorter period of time and may decrease yield because of in vivo cleavage. Some vectors with the intein fused N-terminally to the protein of interest have less in vivo cleavage at higher temperatures, but a low temperature of induction is still recommended for proper folding of the intein segment.*

See Fig. 3 for the flowchart of Step 1.

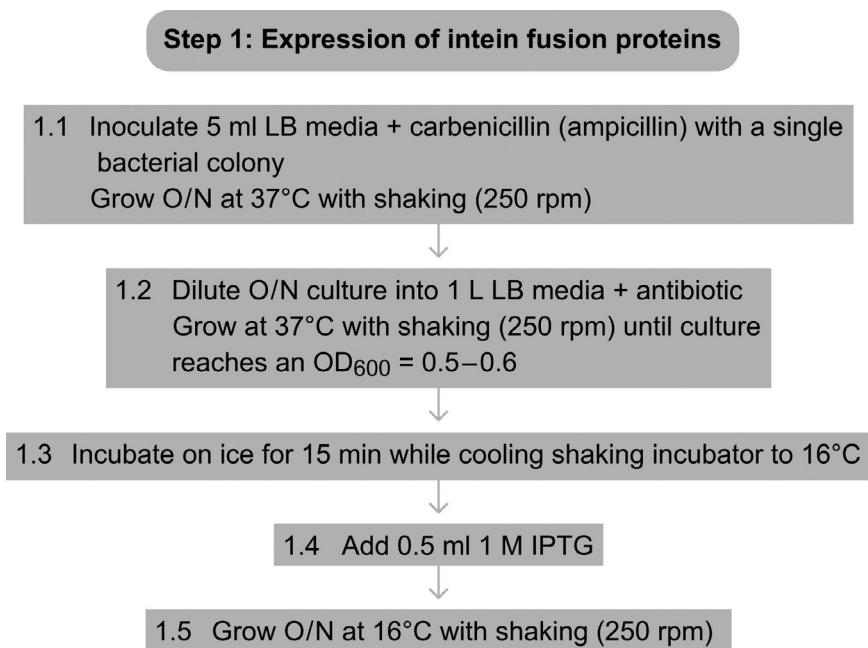
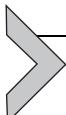


Figure 3 Flowchart of Step 1.



## 6. STEP 2 CELL HARVESTING AND LYSIS

### 6.1. Overview

In this step you harvest your cells from the media, lyse them using a French press, and clarify the lysate.

### 6.2. Duration

2 h

- 2.1** Pellet cells at 5000 rpm for 20 min.  
**2.2** To 50 ml of Intein Lysis Buffer add:

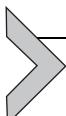
1 Complete EDTA-free protease inhibitor tablet (Roche)  
10 µl 10 mg ml<sup>-1</sup> aprotinin  
10 µl 10 mg ml<sup>-1</sup> leupeptin  
50 µl 1 mg ml<sup>-1</sup> pepstatin A  
50 µl of 1 M benzamidine  
50 µl of 1 M AEBSF

- 2.3** Resuspend the cells in 25 ml of Intein Lysis Buffer supplemented with protease inhibitors. Keep cells on ice.  
**2.4** Lyse cells using a French press.  
**2.5** Clarify the lysate by pouring it into an Oak Ridge tube and spinning in an SS-34 (or equivalent) rotor at 12 000 rpm at 4 °C for 30 min.  
**2.6** Filter the clarified lysate through the 0.8-µm syringe filter and place on ice.

### 6.3. Tip

*Be sure to keep the lysate cold by incubating on ice between rounds of lysis in the French press. Other methods of lysis, such as sonication, may also be employed if the lysate remains chilled throughout. Warming the lysate may cause the intein to cleave prematurely.*

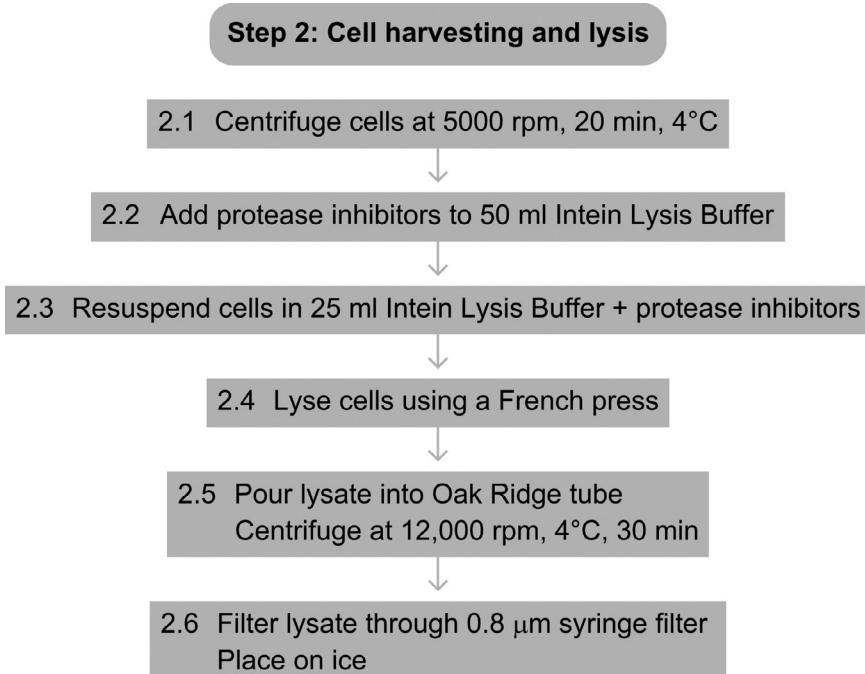
See Fig. 4 for the flowchart of Step 2.



## 7. STEP 3 BIND TO CHITIN BEADS AND WASH COLUMN

### 7.1. Overview

In this step you bind the CBD domain fused to the intein sequence to chitin beads, wash with a high salt buffer to remove nonspecifically bound proteins, and then add DTT to catalyze the intein cleavage reaction.



**Figure 4** Flowchart of Step 2.

## 7.2. Duration

1.5 h active time, overnight incubation

- 3.1 Prepare a chitin column by placing 2 ml of chitin bead slurry in a disposable 30-ml column to make a 1-ml column. Rinse the beads with 50 ml of deionized water followed by 25 ml of Intein Lysis Buffer and then the remaining 25 ml of Intein Lysis Buffer supplemented with protease inhibitors. Cap the column with a small layer of buffer above the level of the column bed.
- 3.2 Pour the filtered, clarified lysate onto the beads.
- 3.3 Close the top of the column and parafilm both ends.
- 3.4 Gently rock or rotate the column at 4 °C for 1 h to allow the protein to bind.
- 3.5 Attach the column to a ring stand. Remove the parafilm from the column and open both ends, allowing the lysate to drip out by gravity flow.
- 3.6 Wash the column with 60 ml of Intein Wash Buffer.
- 3.7 Equilibrate the column by running 25 ml of Intein Cleavage Buffer over the column.
- 3.8 Make Intein Cleavage Buffer with 75 mM DTT by adding 375 µl of 1 M DTT to 4.6 ml of Intein Cleavage Buffer and mixing well.

- 3.9** Drain the remaining Intein Cleavage Buffer out of column. Add 1 ml of Intein Cleavage Buffer with 75 mM DTT and allow the buffer to drain until the miniscus is near the column bed. Plug the bottom of the column. Add an additional 5 ml of Intein Cleavage Buffer with 75 mM DTT. Mix well to distribute the DTT. Cap the top of the column.
- 3.10** Incubate the column at room temperature overnight.

### 7.3. Tip

*Do not let the column surface dry out after the lysate has been added to the column.*

### 7.4. Tip

*As with any protein purification protocol, it is wise to keep the flowthrough and washes until the purified protein has been observed on a gel.*

### 7.5. Tip

*All steps until the overnight incubation may be done at 4 °C. The overnight incubation may also be performed at 4 °C, but this may lead to a reduction in the efficiency of the cleavage reaction. If incubating at 4 °C, consider increasing the duration of the incubation.*

See Fig. 5 for the flowchart of Step 3.

---



## 8. STEP 4 ELUTION AND CHARACTERIZATION OF PROTEIN

### 8.1. Overview

The cleaved protein, now in solution, will be eluted, leaving the intein and CBD on the column. The protein will then be analyzed using SDS-PAGE electrophoresis (see One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE)).

### 8.2. Duration

1.5 h

- 4.1** Open the column and allow the eluent to drip into four 1.5-ml tubes.
- 4.2** Drip 1 ml of cold Intein Cleavage Buffer over the column and collect in a 1.5-ml tube. Repeat 3 times.
- 4.3** Perform a Bradford assay on all fractions collected. If the last fraction collected has a significant amount of protein in it, collect several more

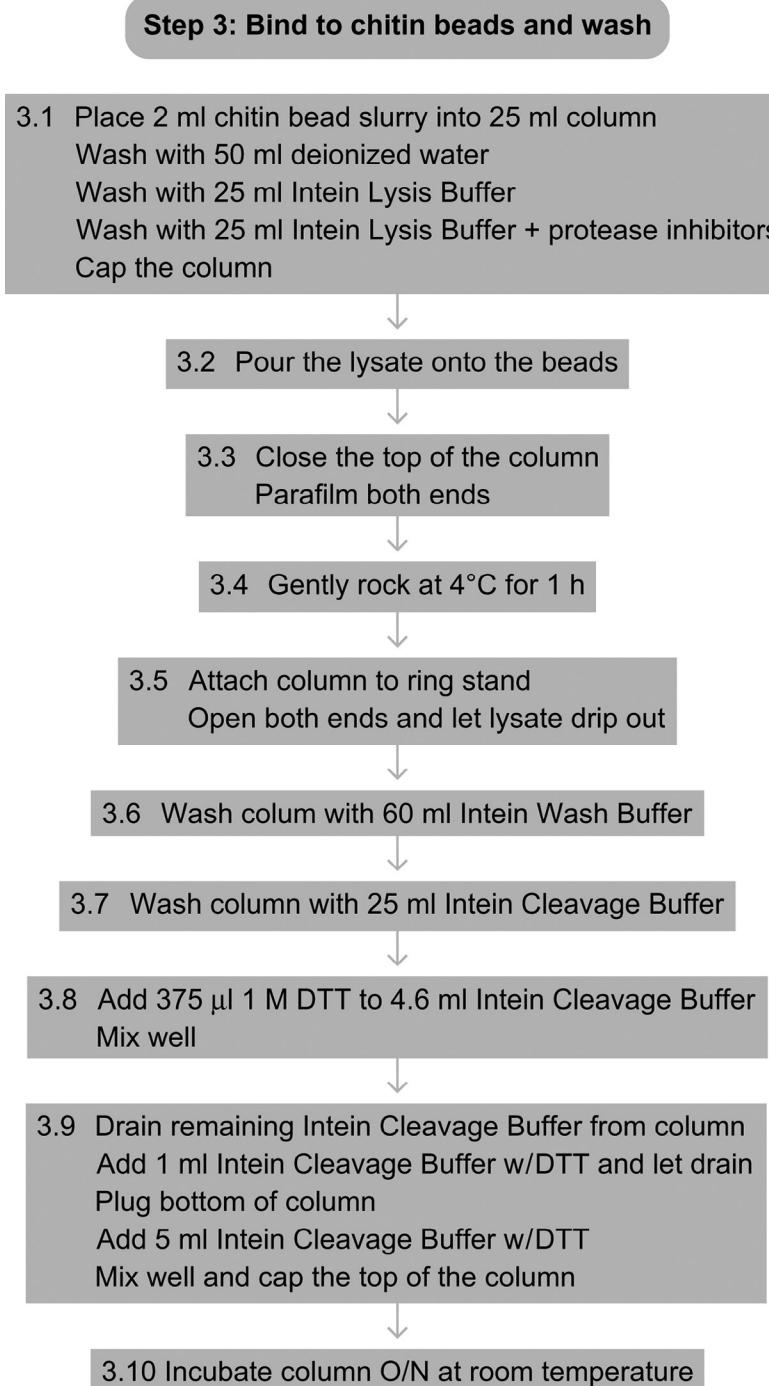


Figure 5 Flowchart of Step 3.

fractions and repeat the Bradford assay (see Quantification of Protein Concentration using UV absorbance and Coomassie Dyes).

- 4.4 Analyze the purity of the protein using SDS-PAGE.
- 4.5 Additional chromatography steps may be added if the protein is not pure enough. If using a construct in which the protein of interest has been fused C-terminal to the intein sequence, dialysis (or further chromatography) will be necessary to remove the small (1.2 kD) peptide formed when the N-terminal extein is cleaved.

### 8.3. Tip

*If no protein has eluted, it may have precipitated on the column, or cleaved in vivo, separating it from the CBD.*

### 8.4. Tip

*Samples may be collected at earlier stages (cells, lysate, flowthrough) and from the beads prior to cleavage and after elution to better analyze the purification. If running these fractions on a gel, make sure to use a loading buffer without DTT or  $\beta$ ME; otherwise, the protein will cleave during sample preparation and no uncleaved product will be visible on the gel.*

See Fig. 6 for the flowchart of Step 4.

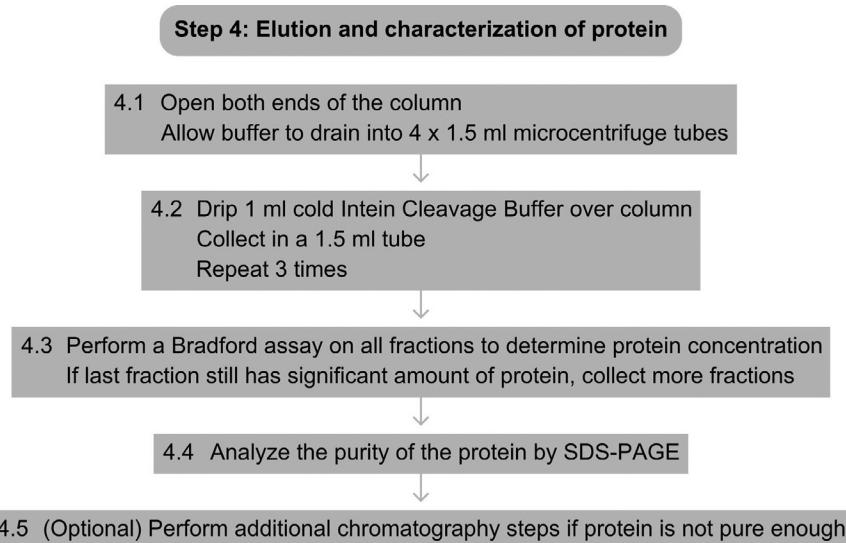


Figure 6 Flowchart of Step 4.

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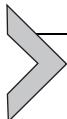
Transformation of Chemically Competent *E. coli*.

Transformation of *E. coli* via electroporation.

Small-scale Expression of Proteins in *E. coli*.

One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE).

Quantification of Protein Concentration using UV absorbance and Coomassie Dyes.



# Purification of GST-Tagged Proteins

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## Contents

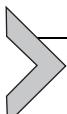
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## Abstract

This protocol describes the purification of recombinant proteins fused to glutathione S-transferase (GST, GST-tagged proteins) by Glutathione Affinity purification. The GST

tag frequently increases the solubility of the fused protein of interest and thus enables its purification and subsequent functional characterization. The GST-tagged protein specifically binds to glutathione immobilized to a matrix (e.g., agarose) and can be easily separated from a cell lysate by a bind-wash-elute procedure. GST-tagged proteins are often used to study protein–protein interactions, again making use of glutathione affinity in a procedure called a GST pull-down assay.

The protocol is designed to process 200 ml of *E. coli* culture expressing intermediate to high amounts of a GST-tagged protein ( $\sim 25 \text{ mg l}^{-1}$ ). Depending on the expression rate or the available culture volume, the scale can be increased or decreased linearly. The protocol can also be used to purify GST-tagged proteins from other expression systems, such as insect or mammalian cells. Tips are provided to aid in modifying certain steps if proteins shall be recovered from alternative expression systems.



## 1. THEORY

Glutathione Affinity is an efficient method for single-step purification of proteins fused to a GST (glutathione S-transferase) tag. GST protein from various sources, both native and recombinantly expressed in *Escherichia coli* and other host cells, can be purified by affinity chromatography on immobilized glutathione, followed by competitive elution with excess reduced glutathione (Smith et al., 1988). Smith and Johnson (1988) made use of this observation when they fused the coding region of GST from *Schistosoma japonicum* to a protein of interest, expressed the GST-tagged protein, and purified it by subsequent Glutathione Affinity chromatography.

GST can be expressed as a soluble protein in the *E. coli* cytoplasm in high amounts and with full enzymatic activity. Furthermore, many eukaryotic proteins that are insoluble when expressed in *E. coli* have been shown to be at least partially soluble when expressed as a GST fusion protein (Smith and Johnson, 1988). When fused to the N-terminus of another protein, enzymatic activity is usually retained. *S. japonicum* GST is a protein with a molecular weight of 26000. The crystal structure of the recombinant protein has been resolved (McTigue et al., 1995) and shown to undergo dimerization, similar to that observed in nature (Parker et al., 1990; Ji et al., 1992; Maru et al., 1996). To generate constructs that express GST fusion proteins, the sequences coding for the protein of interest can be inserted into commercially available vectors, such as the pGEX (GE Healthcare) or pET (Novagen) series of plasmids, using standard cloning techniques.

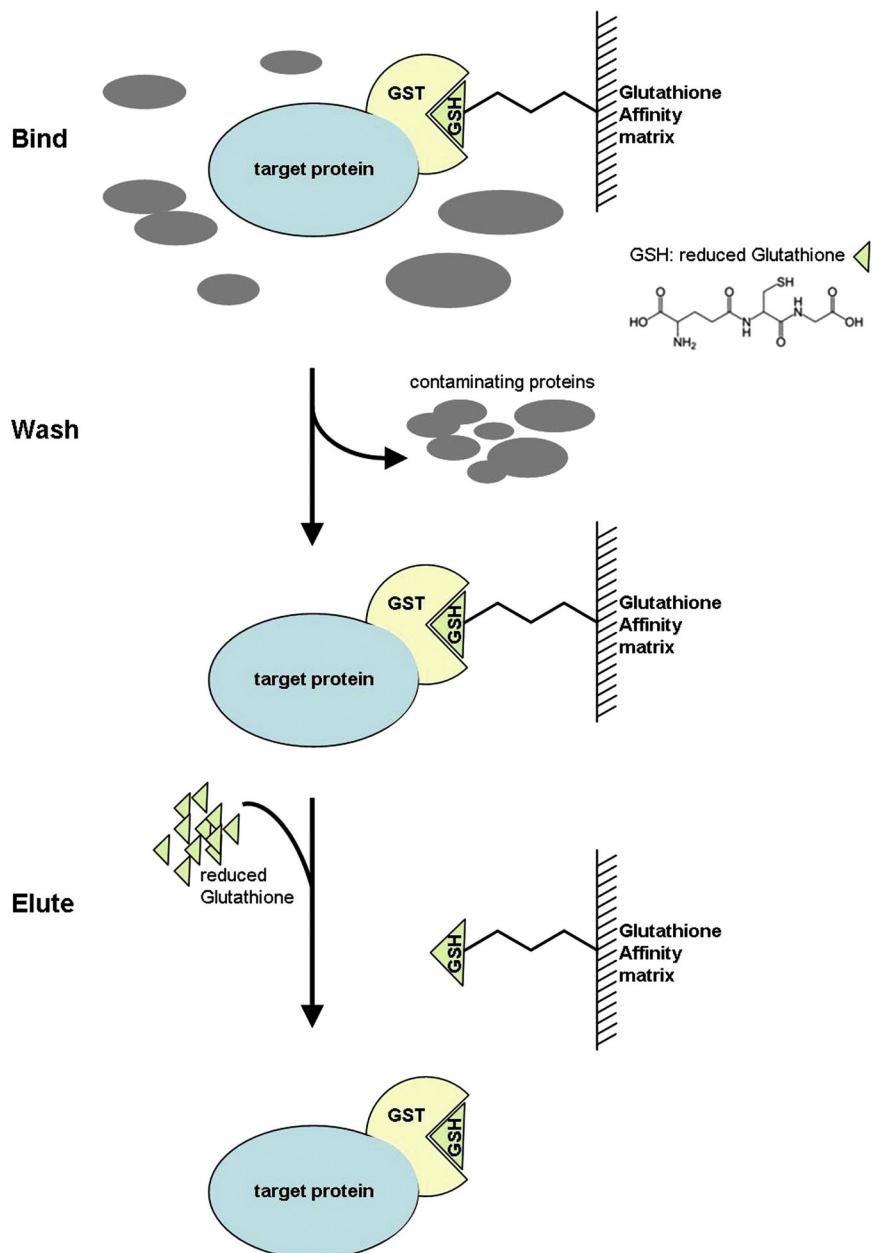
Glutathione Affinity resins are available from various commercial sources for purification of GST and GST fusion proteins via their strong and specific binding to the tripeptide ligand, glutathione, which is stably immobilized on bead-based supports. The simple bind-wash-elute workflow of Glutathione Affinity purification is shown in the flowchart ([Fig. 1](#)).

In addition to its use for affinity purification, the GST tag is frequently utilized in so-called pull-down experiments to investigate protein–protein interactions. The pull-down assay is an *in vitro* method used to determine the physical interaction between two or more proteins. Pull-down assays are useful both for confirming the existence of a protein–protein interaction predicted by other research techniques (e.g., co-immunoprecipitation) and as an initial screening assay for identifying previously unknown protein-binding partners. The minimum requirement for a pull-down assay is the availability of a purified and tagged protein (the bait), which will be used to capture and ‘pull down’ a protein-binding partner (the prey). The Glutathione Affinity resins listed in the Materials section are suitable for GST pull-down experiments.

The scale of purification of GST-tagged proteins is dependent on the amount of protein in the preparation. A column size and total binding capacity should be chosen to approximately match the amount of protein to be purified in order to save resin and to suppress binding of unwanted proteins. Very few nontagged proteins are usually retained on the resin if the target protein occupies nearly all of the available glutathione-binding sites. If too much matrix is used, other proteins may bind nonspecifically to unoccupied sites and elute as contaminants.

The listed Glutathione Affinity resins are suitable for batch purification, gravity flow chromatography, and packed-bed column chromatography (cartridges or custom-packed columns), as well as for high-throughput applications in vacuum- ([Carter and Homes, 2005](#)) or centrifugation-based protocols. Reagents known to be compatible with Glutathione Affinity purification are listed in [Table 1](#).

Following purification, it may be desirable to remove the GST moiety from the protein of interest. Most vectors for expression of GST fusion proteins, such as the pGEX plasmid series, encode for an endoprotease site for cleavage between GST and the protein of interest. Endoprotease cleavage may also be performed on-column. Protocols for Factor Xa endoprotease cleavage and subsequent protease removal from the target protein are included in Methods Navigator (see [Proteolytic affinity tag cleavage](#)).



**Figure 1** Schematic presentation of Glutathione Affinity chromatography. GST fusion proteins contained in a cleared lysate bind to immobilized glutathione (GSH, Bind). Non-binding proteins are washed from the matrix (Wash) and bound GST fusion proteins eluted from the support by the addition of excess reduced glutathione (Elute).

**Table 1** Reagents compatible with Glutathione Affinity purification (may not be complete and concentrations may not be the compatible maxima)

Reagent	Concentration tested	Reagent	Concentration tested
<b>Detergents</b>		<b>Denaturants</b>	
Triton X-100	Up to 2% (v/v)	Urea	Up to 4 M
Tween-20	Up to 2% (v/v)	Guanidinium hydrochloride (GuHCl)	Up to 3 M
SDS	Up to 0.03% (w/v)		
CTAB	Up to 1%	<b>Reducing reagents</b>	
DDM	0.1% (w/v; ~10× cmc)	DTT	Up to 10 mM
CHAPS	1% (w/v; ~10× cmc)	<b>Salts</b>	
β-OG	5% (w/v; ~10× cmc)	NaCl	1 M
C <sub>12</sub> E <sub>8</sub>	0.05% (w/v; ~10× cmc)		
Brij-35	0.1% (w/v; ~10× cmc)		
NP-40 (Igepal CA-630)	0.2% (v/v; ~10× cmc)		
Cholate	5% (v/v; ~0.3× cmc)		
Deoxycholate	2% (w/v; ~0.2× cmc)		



## 2. EQUIPMENT

Centrifuge

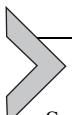
End-over-end rotator

UV/vis spectrophotometer

Micropipettors

Micropipettor tips

Disposable gravity flow columns  
50-ml polypropylene tubes  
1.5-ml polypropylene tubes



### 3. MATERIALS

Sodium phosphate monobasic ( $\text{NaH}_2\text{PO}_4$ )  
Sodium chloride ( $\text{NaCl}$ )  
Protease inhibitors (Roche Complete cocktail tablets, Cat. # 05 056 489 001)  
Benzonase nuclease (Novagen Cat. # 72106-3)  
Lysozyme  
EDTA  
Dithiothreitol (DTT)  
Tris-hydrochloride (Tris-HCl, powder)  
Sodium hydroxide ( $\text{NaOH}$ )  
Glutathione Affinity resin (e.g., Glutathione Superflow, QIAGEN, Cat. #30930; or Glutathione Sepharose 4 FF, GE Healthcare, Cat. # 175132-02)  
Reduced glutathione (L-glutathione, reduced, e.g., Sigma Cat. # G4251 5G)  
Triton X-100  
ATP  
Magnesium sulfate ( $\text{MgSO}_4$ )  
Glycerol  
Sodium dodecyl sulfate (SDS)  
Bromophenol blue  
Igepal CA-630 (Nonidet P40, NP-40) (optional)

#### 3.1. Solutions & buffers

##### Step 1 PBS basis buffer

Component	Final concentration	Stock	Amount
$\text{NaH}_2\text{PO}_4$	50 mM	0.5 M	100 ml
$\text{NaCl}$	150 mM	1.0 M	150 ml

Dissolve in 850 ml water. Adjust pH to 7.2 with NaOH and add water to 900 ml

**PBS-L (*E. coli* lysis buffer)**

<b>Component</b>	<b>Final concentration</b>	<b>Stock</b>	<b>Amount</b>
PBS basis buffer, pH 7.2			90 ml
Protease inhibitors	1 ×		2 tablets
Benzonase	3 U per ml of bacterial culture		600 Units
Triton X-100	1% (v/v)		1.0 ml
DTT	1 mM	1.0 M	0.1 ml
EDTA	1 mM	0.5 M	0.2 ml
Lysozyme	1 mg ml <sup>-1</sup>	100 mg ml <sup>-1</sup>	1.0 ml

Add water to 100 ml and stir until the protease inhibitor tablets are completely dissolved  
 Prepare fresh and use within 30 min of preparation (the PBS basis containing NaH<sub>2</sub>PO<sub>4</sub> and NaCl can be prepared in larger amounts and stored at room temperature)

**5× SDS-PAGE buffer**

<b>Component</b>	<b>Final concentration</b>	<b>Stock</b>	<b>Amount</b>
Tris-HCl, pH 6.8	225 mM	1.0 M	2.25 ml
Glycerol	50% (v/v)	(100%)	5 ml
SDS	5% (w/v)		0.5 g
DTT	250 mM	1.0 M	2.5 ml
Bromophenol blue	0.05% (w/v)		5 mg

Add water to 10 ml

**Step 2 PBS-EW (Glutathione Affinity equilibration and wash buffer)**

<b>Component</b>	<b>Final concentration</b>	<b>Stock</b>	<b>Amount</b>
PBS basis buffer, pH 7.2			90 ml
DTT	1 mM	1.0 M	0.1 ml
EDTA	1 mM	0.5 M	0.2 ml

Add water to 100 ml

## ATP buffer (optional)

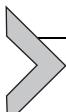
Component	Final concentration	Stock	Amount
Tris-HCl, pH 7.4	50 mM	1 M	5.0 ml
ATP	2 mM	100 mM	2.0 ml
MgSO <sub>4</sub>	10 mM	1.0 M	1.0 ml

Add water to 100 ml

## TNGT (Glutathione Affinity elution buffer)

Component	Final concentration	Stock	Amount
Tris-HCl, pH 8.0	50 mM	1.0 M	5.0 ml
NaCl	100 mM	1.0 M	10.0 ml
Triton X-100	0.1% (v/v)		0.1 ml
Reduced glutathione	50 mM	1 M	5 ml
DTT	1 mM	1.0 M	0.1 ml

Add water to 100 ml and stir until the glutathione is completely dissolved. Prepare fresh



## 4. PROTOCOL

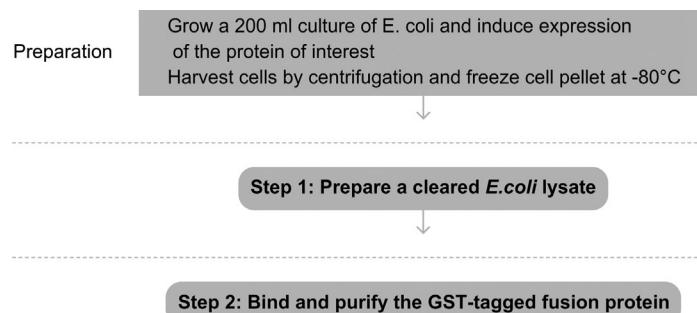
### 4.1. Preparation

Express the GST-tagged target protein in a suitable *E. coli* strain (see Small-scale Expression of Proteins in *E. coli*) and store a cell pellet derived from 200 ml of culture at -80 °C until use.

### 4.2. Duration

Preparation	2–3 days
Protocol	About 3 h

See Fig. 2 for the flowchart of the complete protocol, including preparation.



**Figure 2** Flowchart of the complete protocol, including preparation.



## 5. STEP 1 PREPARATION OF A CLEARED *E. COLI* LYSATE

### 5.1. Overview

This protocol step describes the generation of a cleared lysate from *E. coli* cells under native conditions. The protocol may be modified depending on initial results which may have revealed (partial) insolubility of the GST-tagged protein (for troubleshooting see also Explanatory Chapter: Troubleshooting protein expression: what to do when the protein is not soluble) or contamination of the preparation with unwanted proteins.

### 5.2. Duration

1.5 h

- 1.1 Thaw the pellet on ice for 15 min and resuspend a pellet derived from 200 ml of *E. coli* culture in 20 ml of buffer PBS-L.
- 1.2 Pour into a 50 ml conical centrifuge tube and incubate on an end-over-end rotator at room temperature (15–25 °C) for 30 min.
- 1.3 Remove a 5 µl sample, mix it with 1 µl of 5× SDS-PAGE buffer, and store at –20 °C for SDS-PAGE analysis. This total lysate control can be used to analyze the expression of the GST-tagged protein.
- 1.4 Centrifuge the lysate at 10000 × g, at room temperature for 30 min.
- 1.5 Carefully collect the supernatant (the cleared lysate) without touching the pellet. Remove a 5 µl sample from the supernatant, mix it with 1 µl of 5× SDS-PAGE sample buffer, and store at –20 °C. This contains the soluble GST-tagged fusion protein and can be compared with the total lysate control to determine the solubility of the fusion protein.

### 5.3. Tip

Freezing an *E. coli* cell pellet at  $-20$  or  $-80\text{ }^{\circ}\text{C}$  for at least 30 min increases the efficiency of lysozyme-mediated cell lysis.

### 5.4. Tip

If the GST-tagged protein localizes to a membrane, refer to Kubicek et al. (see Expression and purification of membrane proteins) for a protocol to generate a membrane fraction. Perform a screen to determine the most suitable detergent to solubilize the membrane protein (see Explanatory Chapter: Choosing the right detergent).

### 5.5. Tip

If the GST-tagged protein is not soluble under the recommended native buffer conditions (PBS-L) and is mostly or completely in the insoluble fraction, or if it does not bind to the Glutathione Affinity matrix, you may try to destabilize conformation of the GST-tagged protein by adding the denaturant urea. Up to 4 M urea has been successfully applied in the lysis and binding step and is, in principle, compatible with GST binding to immobilized glutathione. The concentration of urea or other reagents that is compatible with the target fusion protein may vary and must be determined empirically since the activity or structure of the protein might be affected by the inclusion of chaotropes or detergents. [Table 1](#) lists a selection of reagents and their highest concentrations that have been successfully used in Glutathione Affinity applications.

### 5.6. Tip

If protein shall be purified from insect or mammalian cells, wash the cell pellet with PBS prior to lysing the cells.

### 5.7. Tip

For lysis of insect or mammalian cells, omit the lysozyme and Triton X-100 from the lysis buffer and supplement it with 1% (*v/v*) Igepal CA-630. Use 4 ml of lysis buffer per  $1\text{--}2 \times 10^7$  cells.

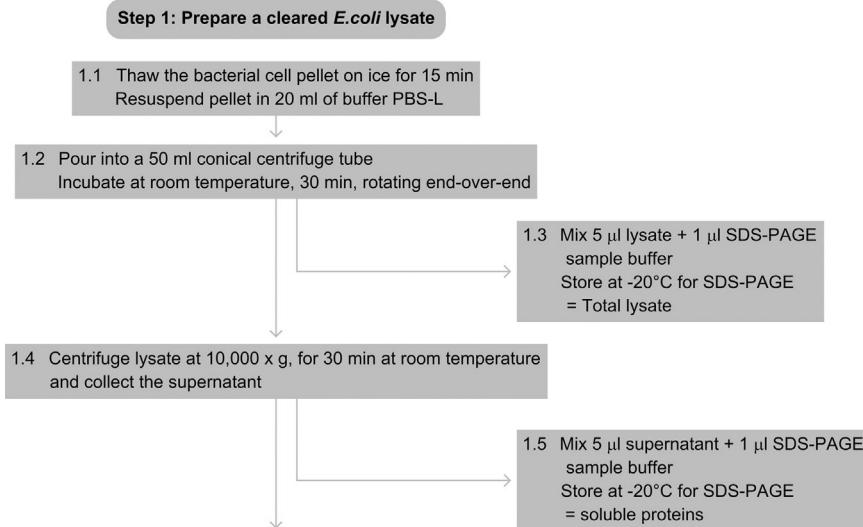
See [Fig. 3](#) for the flowchart of Step 1.



## 6. STEP 2 PURIFICATION OF GST-TAGGED PROTEINS

### 6.1. Overview

This protocol step describes the purification of GST-tagged proteins and gives good results in most cases. If purity and recovery are compromised, results may be improved by the addition of suitable reagents (e.g., detergents or reducing agents, see [Table 1](#) for compatibility).



**Figure 3** Flowchart of Step 1.

## 6.2. Duration

1.5 h

- 2.1 Resuspend the Glutathione Affinity resin by inverting the bottle until the slurry is homogeneous. Transfer 1 ml of the resin (supplied as a 50% slurry, which corresponds to a 500 µl bed volume [bv]) into an empty gravity flow column and drain the storage buffer.
- 2.2 Equilibrate the settled resin with 5 ml of buffer PBS-EW (10 bv) and drain the buffer by gravity flow.
- 2.3 Load the cleared lysate from Step 1.5 to the equilibrated resin and collect the column flow-through.
- 2.4 Wash twice with 2.5 ml of buffer PBS-EW (5 bv).
- 2.5 Elute the GST-tagged protein by addition of 0.5 ml of TNGT elution buffer (1 bv) and collect the eluate (elution fraction 1).
- 2.6 Repeat the elution step another four times, for a total of five elutions (5 bv).
- 2.7 Determine the protein concentration of each of the elution fractions. Analyze all of the fractions by SDS-PAGE (see One-dimensional SDS-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE)).

## 6.3. Tip

*Cut the tip off of a 1-ml pipette tip and pipette the resin slurry immediately to ensure transfer of the desired amount of resin bed volume.*

## 6.4. Tip

Save the flow-through for SDS-PAGE analysis.

## 6.5. Tip

Collect wash fractions for SDS-PAGE analysis.

## 6.6. Tip

In some cases, a 65–70 kDa protein band from E. coli co-elutes with the target GST fusion protein from Glutathione Affinity resins. This well-known contaminant is most likely the chaperone DnaK. If an additional wash step is performed in the presence of ATP and MgSO<sub>4</sub> (wash with 1 bv of ATP buffer), this protein is reliably and specifically removed, leaving the highly pure target fusion protein bound to the resin.

## 6.7. Tip

Incubate the resin in elution buffer for 15 min before collecting the eluate; this can increase the protein concentration of the elution fraction. Apply 500 µl of elution buffer (1 bv) and immediately seal the column with the bottom outlet cap and incubate for

### Step 2: Bind and purify the GST-tagged fusion protein

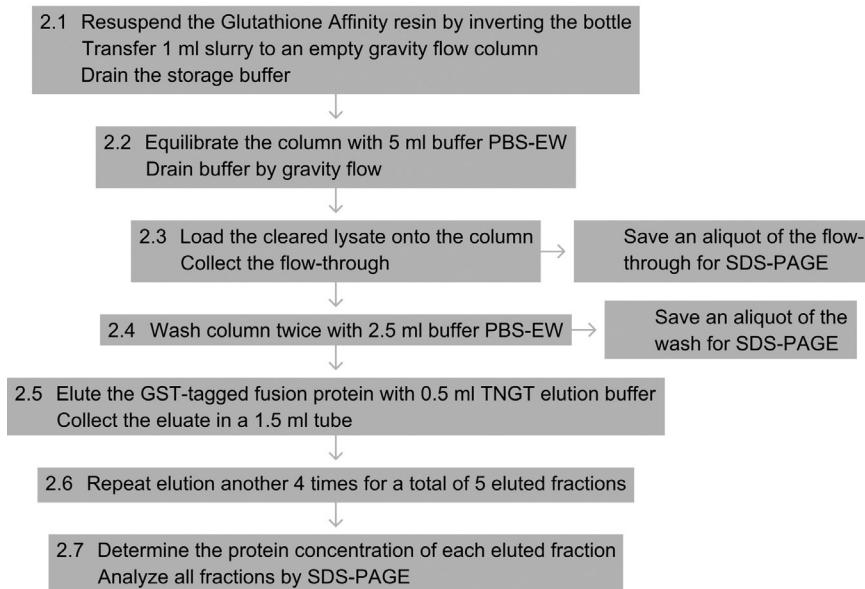


Figure 4 Flowchart of Step 2.

15 min at room temperature (15–25 °C); open the column and collect the eluate containing the GST-tagged protein.

## 6.8. Tip

The number of elution steps required to completely elute the GST-tagged protein from the Glutathione Affinity resin can vary with the nature and amount of bound protein.

See Fig. 4 for the flowchart of Step 2.

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[Small-scale Expression of Proteins in E. coli](#).

[Explanatory Chapter: Troubleshooting protein expression: what to do when the protein is not soluble](#).

[Expression and purification of membrane proteins](#).

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