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Richard J. Giannone
Andrew B. Dykstra *Editors*

# Protein Affinity Tags

## Methods and Protocols

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## Methods and Protocols

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# Preface

In this post-genomic era, emphasis continues to be placed on the derivation of genetic information from the simplest to the most complex of organisms. Recent advancements in DNA sequencing technology, specifically with regard to speed, cost, and overall availability, promise a future that is awash with novel genomic information—information that will not only expand our knowledge of the processes that govern life, but also fuel innovation that will undoubtedly conquer many of today's most salient health and environmental challenges. Though emphasis is often placed on the "gene," it is the encoded product—the "protein"—that actually serves any real biochemical function. Compared to genes, which themselves hold information and more so describe potential function, proteins are the actual tangible components of the cellular machinery, carrying out the multitude of functions that support life, whether biochemical or structural. Thus, to fully understand and appreciate any biological process, one must take a hard look at the proteins involved, taking note of the exquisite functional interplay between themselves and amongst other biomolecules.

Given the intrinsic DNA–protein link and coupled with the routine use of molecular cloning/recombinant DNA technology, today's researchers can easily alter the amino acid sequence of any given protein. This powerful technology allows for precise engineering of specific proteins and enables unprecedented inquiry into both their structures and function. A common technique utilized by many molecular biologists interested in probing protein function involves genetically grafting a protein or short peptide sequence onto one or both terminal ends of a given protein of interest. By virtue of the appended protein/peptide sequence, this new recombinant, "tagged" protein can be easily purified by one of several routine methods. Aptly named, protein affinity tagging employs the use of known protein binding interactions in order to "fish out" a protein of interest or, with more advanced tags, a protein complex.

The intent of this collection is to provide researchers with the necessary information, tools, and strategy needed to properly interrogate a given protein's function. The development and use of recombinant affinity tags has been paramount in this endeavor, providing an efficient means to purify a protein of interest for downstream functional assessment. This volume is not intended, however, to be an all-inclusive listing of affinity tags but rather attempts to highlight the general strategies employed by researchers who use this technology on a daily basis. In fact, more emphasis has been placed on advanced affinity tagging procedures that introduce novel purification techniques. As follows, purposefully missing are chapters that focus on the one-step purification of proteins employing commonly used affinity tags *without* additional novelty (e.g., visualization, solubility enhancements, or enhanced tag removal strategies). These simple affinity tags have been covered *ad nauseam* in the literature and most are optimized, detailed, and readily available through commercial means. Furthermore, single tag purification methodologies can be easily extrapolated from the included chapters as many include these often used affinity tags packaged into more advanced constructs.

With regard to the volume at hand, the included chapters are roughly divided into tag constructs that feature one purification event and those that feature two or more. Though

engineering proteins with a single affinity tag is routine and generally useful for purifying ample amounts of recombinant protein, issues with proper folding, solubility, and localization often arise. Many of the chapters included here provide means to circumvent these shortcomings. Likewise, single tag-based strategies are sometimes afflicted with purity issues. Often, this is of no consequence. When purity does matter (i.e., certain sensitive applications such as LC-MS/MS-based identification of interacting protein partners) tagging proteins with multiple affinity motifs/domains provides enhanced specificity but at the expense of reduced protein recovery. Finally, readers will notice that many affinity-tag constructs include other features that aid in the purification process. These features can be grouped into three major categories: tags that enhance protein solubility, tags that include motifs/proteins/domains that allow for protein visualization, and tags that can be removed from the final purified product, either via the targeting of proteases or those that are self-cleavable.

Whatever the needs of the researcher, there is most likely an affinity tag available to accomplish the task at hand—they come in virtually every flavor, differing in size, complexity, binding/bait capture affinities, and types of interaction (i.e., antibody–antigen/epitope, enzyme–substrate, receptor–ligand, coordination of amino acid residues and metals, etc.). It is our hope that the collection of methodologies listed here will be an invaluable resource to those seeking to utilize protein affinity tags to study their biological system of interest.

*Oak Ridge, TN, USA* — *Richard J. Giannone*
*West Greenwich, RI, USA* — *Andrew B. Dykstra*

# Contents

*Preface* . . . . . *v*
*Contributors* . . . . . *ix*

1 1D4: A Versatile Epitope Tag for the Purification and Characterization of Expressed Membrane and Soluble Proteins . . . . . 1
***Laurie L. Molday and Robert S. Molday***

2 Affinity Purification of Heme-Tagged Proteins . . . . . 17
***Wesley B. Asher and Kara L. Bren***

3 Purification of a Recombinant Protein with Cellulose-Binding Module 3 as the Affinity Tag . . . . . 35
***Dongmei Wang and Jiong Hong***

4 Purification of *E. coli* Proteins Using a Self-Cleaving Chitin-Binding Affinity Tag . . . . . 47
***Michael J. Coolbaugh and David W. Wood***

5 Simplified Protein Purification Using an Autoprocessing, Inducible Enzyme Tag . . . . . 59
***Aimee Shen***

6 SUMO as a Solubility Tag and In Vivo Cleavage of SUMO Fusion Proteins with Ulp1 . . . . . 71
***Dennis Kuo, Minghua Nie, and Albert J. Courey***

7 Rescuing Aggregation-Prone Proteins in *Escherichia coli* with a Dual $His_6$-MBP Tag . . . . . 81
***Danielle Needle and David S. Waugh***

8 Expression, Purification, and Immobilization of Recombinant Tamavidin 2 Fusion Proteins . . . . . 95
***Yoshimitsu Takakura, Naomi Oka, and Masako Tsunashima***

9 Use of Tandem Affinity Chromatography for Purification of Cannabinoid Receptor $CB_2$ . . . . . 107
***Silvia C. Locatelli-Hoops and Alexei A. Yeliseev***

10 Detection of Protein–Protein Interactions Using Tandem Affinity Purification . . . . . 121
***Ian Goodfellow and Dalan Bailey***

11 An Improved In Vivo Biotinylation Strategy Combined with FLAG and Antibody Based Approaches for Affinity Purification of Protein Complexes in Mouse Embryonic Stem Cells . . . . . 135
***Francesco Faiola, Arven Saunders, Baoyen Dang, and Jianlong Wang***

12 Purification of Recombinant Proteins with a Multifunctional GFP Tag . . . . . 151
***Takashi Murayama and Takuya Kobayashi***

13 Targeted Purification of SnAvi-Tagged Proteins . . . . . 163
***Ursula Schäffer, Ralf Baumeister, and Ekkehard Schulze***

14 An Efficient Fluorescent Protein-Based Multifunctional Affinity Purification Approach in Mammalian Cells . . . . . 175
***Hanhui Ma, Janel R. McLean, Kathleen L. Gould, and Dannel McCollum***

15 Bimolecular Affinity Purification: A Variation of TAP with Multiple Applications . . . . . 193
***Petro Starokadomskyy and Ezra Burstein***

*Index* . . . . . *211*

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# Chapter 1

# 1D4: A Versatile Epitope Tag for the Purification and Characterization of Expressed Membrane and Soluble Proteins

**Laurie L. Molday and Robert S. Molday**

## Abstract

Incorporation of short epitope tags into proteins for recognition by commercially available monoclonal or polyclonal antibodies has greatly facilitated the detection, characterization, localization, and purification of heterologously expressed proteins for structure–function studies. A number of tags have been developed, but many epitope–antibody combinations do not work effectively for all immunochemical techniques due to the nature of the tag and the specificity of the antibodies. A highly versatile, multipurpose epitope tag is the 9 amino acid C-terminal 1D4 peptide. This peptide tag together with the Rho1D4 monoclonal antibody can be used to detect proteins in complex mixtures by western blotting and ELISA assays, localize proteins in cells by immunofluorescence and immunoelectron microscopic labeling techniques, identify subunits and interacting proteins by co-immunoprecipitation, and purify functionally active proteins including membrane proteins by immunoaffinity chromatography. In this chapter we describe various immunochemical procedures which can be used for the detection, purification and localization of 1D4-tagged proteins for structure–function studies.

**Key words** 1D4 epitope tag, Rho1D4 antibody, 1D4-tagged proteins, Immunoaffinity chromatography, Immunocytochemical localization, Co-immunoprecipitation, Membrane protein purification, Protein expression

## 1 Introduction

The expression of proteins in heterologous cell systems including mammalian, yeast, insect, and bacterial cells is widely used to identify, characterize, localize, and purify proteins for structure–function analysis. This has been greatly facilitated by engineering a short epitope tag within the protein for recognition by highly specific, commercially available monoclonal or polyclonal antibodies [1]. Epitope tags can be of any length and in principle inserted anywhere within the protein sequence. However, in most cases the preferred length is between 6 and 12 amino acids with the tag placed at the N or C terminus of the protein where it is less likely to affect

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protein structure and function and can be readily cleaved if required by inserting an additional protease-specific sequence between the protein and tag. In some applications epitope tags are generated as multiple copies to enhance binding or used with other protein affinity tags such as His-tags to facilitate protein purification and characterization of binding partners [2].

Some of the commonly used epitope tags include Flag (DYKDDDDK), HA (YPYDVPDYA), Myc (EQKLISEEDL), and 1D4 (TETSQVAPA). In principle these epitope tags can be used with a wide variety of immunochemical techniques to detect, localize, purify, and characterize proteins and their interacting partners. In practice, however, the epitope–antibody combinations may only work well with specific techniques due to the affinity and specificity of the anti-epitope antibody and amino acid characteristics of the epitopes. For example, HA and Myc tags work well for the detection of tagged proteins by western blotting and ELISA assays, localization of proteins in transfected cells by immunocytochemical techniques, and analysis of protein–protein interactions by co-immunoprecipitation, but in general these tags are inefficient for the purification of native proteins for structure–function studies. The Flag tag has been widely used with western blotting, ELISA assays, co-immunoprecipitation and protein purification, but is limited in some applications due to relatively high nonspecific background labeling by many anti-Flag antibodies. Furthermore, the lysine residues in the Flag sequence can react with chemical fixatives (aldehydes) commonly used in sample preparations for immunocytochemistry and the tyrosine residue is susceptible to posttranslational modification reducing the immunoreactivity of the Flag tag [3, 4].

One of the most versatile tags is the 9 amino acid 1D4 epitope derived from the C-terminus of bovine rhodopsin [5, 6]. This tag together with the monoclonal antibody Rho1D4, an IgG1, has been used with essentially all immunochemical techniques. These include (1) the detection of expressed proteins in cell extracts by western blotting (Fig. 1) [7], (2) analysis of protein–protein and subunit–subunit complexes by co-immunoprecipitation (Fig. 2) [8, 9], (3) localization of proteins in cells by immunocytochemical techniques (Fig. 3) [7, 10], (4) ELISA-based assays [6, 11, 12], and (5) purification of heterologously expressed proteins for structure–function analysis [13–17].

The 1D4 epitope has a number of advantages. The amino acid sequence is only present in rhodopsin and related photoreceptor proteins and is essentially devoid of charged residues which can result in nonspecific ionic interactions. The Rho1D4 immunoreactivity is insensitive to chemical fixatives due to the absence of reactive lysine residues and hence has been used with immunocytochemical labeling techniques for fluorescent and electron microscopy [7, 10, 18]. Since the Rho1D4 monoclonal antibody binds

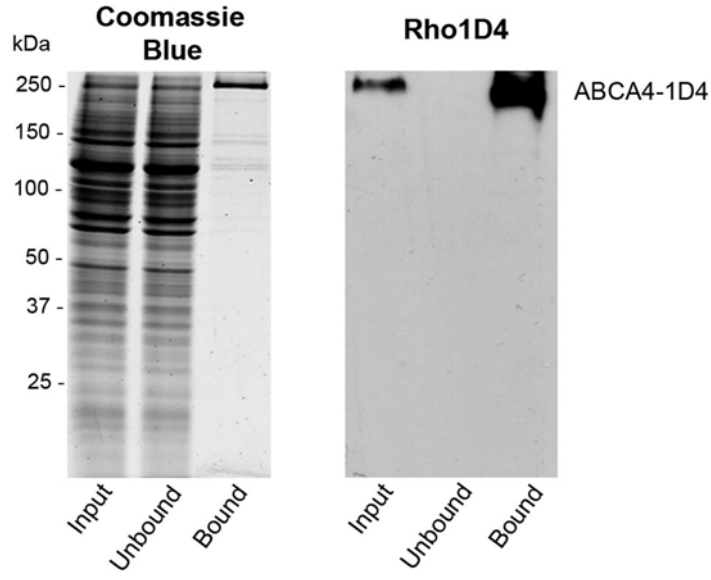


**Fig. 1** Detection and purification of the 1D4-tagged ABC transporter ABCA4-1D4. HEK293T cells were transfected with pcDNA3 plasmid harboring the *ABCA4-1D4* cDNA. The cells were solubilized with 1 % Triton X-100 and incubated with Rho1D4 immunoaffinity matrix. After collecting the unbound fraction, the matrix was washed and the bound protein was eluted with the 1D4 peptide. The input, unbound, and bound fractions were analyzed on SDS gels stained with Coomassie Blue and a western blot labeled with the Rho1D4 antibody. This procedure produced a highly purified preparation of ABCA4-1D4

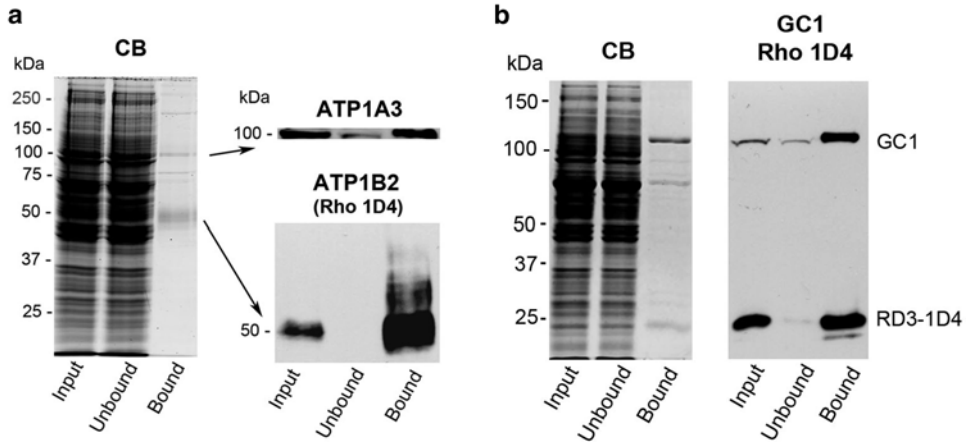


**Fig. 2** Co-expression and co-immunoprecipitation of multisubunit proteins and interacting proteins from co-transfected HEK293T cells. (**a**) HEK293T cells were co-transfected with pcDNA3 plasmids containing the cDNAs encoding the Na/K ATPase subunits ATP1A3 and the tagged ATP1B2-1D4. The cells were solubilized in Triton X-100 and applied to a Rho1D4 immunoaffinity matrix. The input, unbound, and bound (1D4 peptide-eluted) fractions were analyzed on SDS gels and western blots labeled with an ATP1A3 polyclonal antibody and Rho 1D4 monoclonal antibody for the detection of ATP1B2-1D4. (**b**) HEK293T cells co-expressing RD3-1D4 and guanylate cyclase 1 (GC1) were solubilized in Triton X-100 and applied to a Rho1D4 immunoaffinity matrix. The input, unbound, and bound fractions were analyzed on SDS gels and western blots labeled with Rho1D4 antibody (RD3-1D4 detection) and anti-GC1 antibody (GC1 detection)

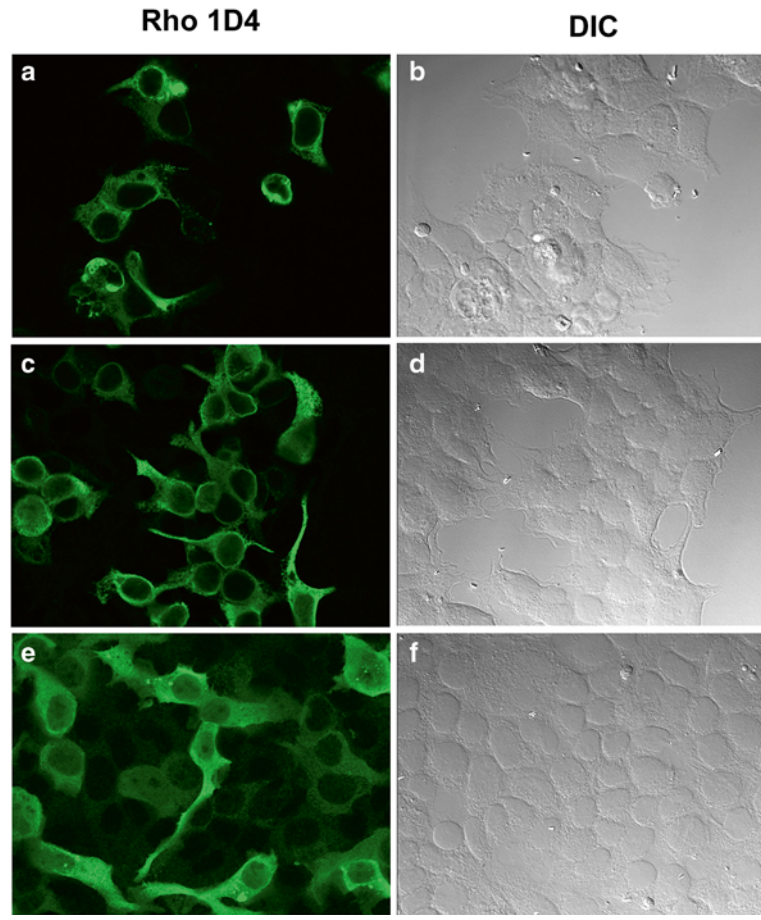


**Fig. 3** Immunofluorescence microscopy of HEK293T cells expressing ABCA4-1D4 (**a**, **b**); co-expressing ATPase subunits ATP1A3 and ATP1B2-1D4 (**c**, **d**); and interaction proteins RD3-1D4 and guanylate cyclase GC1 (**e**, **f**). The cells were labeled with the Rho1D4 antibody for immunofluorescence imaging. The total number of cells was visualized using differential interference contrast (DIC) imaging. Not all cells expressed the ATPase subunits and hence serve as internal control for antibody specificity

with high affinity to the 1D4 epitope, it is not necessary to insert multiple copies of the tag as commonly required for Flag tags. Furthermore, the Rho1D4 antibody is highly specific showing little if any nonspecific binding by immunofluorescence or western blotting techniques (Figs. 1 and 3). The binding properties of the Rho1D4 monoclonal antibody to its epitope have been systematically studied [5, 6]. The contribution of each amino acid residue within the epitope to Rho1D4 antibody binding has been evaluated by substituting each amino acid in the sequence with alanine for analysis by competitive ELISA assays.

The 1D4 tag has been particularly valuable for the purification and characterization of membrane proteins since the binding of the Rho 1D4 antibody to its epitope is insensitive to mild detergents such as Triton X-100, CHAPS, octylglucoside, and dodecylmaltoside widely used to solubilize membrane proteins. Importantly, the 1D4 peptide can be used to efficiently elute the 1D4-tagged protein from the immunoaffinity matrix under nondenaturing conditions. Examples of membrane proteins purified by the Rho1D4 immunoaffinity technique for structure–function analysis include members of the ABC transporters, P4-ATPases, tetraspanins, G-protein coupled receptors, and various channels [13, 16, 17, 19–21]. Importantly, this affinity tag has also been used to purify membrane proteins in sufficient quantities from native and expressed systems for high resolution X-ray crystallography [14, 16, 22]. Finally, the Rho 1D4 antibody can be efficiently produced and purified in large quantities and as a result the purified antibody is available to investigators at a reasonable cost (Flintbox http://www.rho1d4.com/).

A limitation of the 1D4 tag, however, is that it has to be placed at the C-terminus of a protein. This is because the Rho1D4 monoclonal antibody requires a free carboxylate group for high affinity binding [6]. Amidation of the carboxyl group lowers its immunoreactivity to the Rho1D4 antibody by over 100-fold.

Here, we describe in detail the methods used to purify, characterize, and localize 1D4-tagged membrane proteins expressed in HEK293T cells using a Rho1D4–Sepharose immunoaffinity matrix. The procedures are for small batch preparations, but can be readily adapted for the purification of large quantities of 1D4-tagged soluble or membrane proteins from any of a variety of cells including yeast, bacteria, or insect cells.

## 2 Materials

All solutions were prepared with distilled and deionized water using analytical-grade reagents unless specified otherwise. 1D4-tagged protein is generated by PCR and established cloning procedures using a typical reverse primer containing appropriate restriction sites for cloning as follows: [restriction site/TTA/GGC AGG CGC CAC TTG GCT GGT CTC TGT/- - - - -], where the underlined is the stop codon and the dashed line represents the nucleotide sequence for the protein of interest.

### 2.1 Transfection of HEK293T Cells

1. Mammalian expression plasmid containing 1D4-tagged gene purified in $ddH_2O$.
2. HEK293T cells (or another highly transfection efficient mammalian cell line).

3. DMEM (Dulbecco's Modified Eagle's Medium) with 4.5 g/l D-glucose, 0.584 g/l glutamine, 3.7 g/l sodium bicarbonate, and 110 mg/l sodium pyruvate.
4. HyClone Bovine Growth Serum (BGS) or Fetal Calf Serum (FCS).
5. Cellgro Antibiotic-Antimycotic solution (Manassas, Virginia).
6. 0.5 % Trypsin-EDTA (10×) (Gibco Life Technologies).
7. Phosphate buffered saline: 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.75 mM $KH_2PO_4$, pH 7.4.
8. 100 mm diameter × 20 mm sterile tissue culture plates.
9. Sterile BBS: BES-buffered saline pH 6.96-HCl (50 mM BES Free Acid, ULTRO Grade (Calbiochem, San Diego, CA)), 280 mM sodium chloride, and 1.5 mM monobasic sodium phosphate.
10. Sterile 1.0 M calcium chloride.

### 2.2 Preparation of Membrane Fractions

1. Tris-buffered saline (TBS): 20 mM Tris–HCl pH 7.4, 0.1 M sodium chloride.
2. Complete Protease Inhibitor (Roche) Dilute as suggested by manufacturer.
3. Sucrose solution 60 % w/v in TBS.
4. Beckman Ultra-Clear 11 × 34 mm tubes and 13 × 56 mm tubes (Beckman, Palo Alto, CA).

### 2.3 Solubilization of Membrane Proteins

1. 2 % Triton X-100, 18 mM CHAPS, or other mild detergents in TBS from Subheading 2.2 with a complete protease inhibitor.

### 2.4 Preparation of a Rho1D4–Sepharose Matrix

1. Purified Rho1D4 monoclonal antibody (*see* **Note 1**).
2. Sepharose 2B (not CL) or CNBr-activated Sepharose 4B (GE Healthcare BioSciences).
3. 0.15 g of cyanogen bromide (CNBr).
4. 2.0 l of 20 mM sodium borate pH 8.0.
5. TBS: 20 mM Tris–HCl pH 8.0, 0.15 M NaCl, 0.01 % $NaN_3$, and another TBS solution with 50 mM glycine.

### 2.5 Purification of the 1D4-Tagged Protein

1. Ultra-Free-MC Centrifugal Filters (Millipore).
2. Rho1D4–Sepharose matrix (50–100 μl per two 100 mm × 20 mm plates of transfected HEK293T cells).
3. Membranes prepared from two 100 mm × 20 mm plates of transfected HEK293 cells.
4. TBS-T: TBS from 2.4 with 0.2 % Triton X-100 or TBS-CHAPS: TBS with 10 mM CHAPS, or other mild detergents.
5. 1D4 peptide (Ac-TETSQVAPA) (0.2 mg/ml) in TBS/0.2 % Triton X-100 or TBS-CHAPS.

### 2.6 Analysis of the Purified 1D4-Tagged Proteins by SDS-Gels and Western Blot

1. 6.5–12 % Laemmli SDS page gel or a purchased pre-made SDS-PAGE.
2. Laemmli gel buffers:
   (a) Lower gel buffer: 1.5 M Tris–HCl, 0.4 % SDS pH 8.8.
   (b) Stack gel buffer: 0.5 M Tris–HCl, 0.4 % SDS pH 6.8.
   (c) 30 % Acrylamide in $H_2O$.
   (d) 4× Loading buffer: 0.7 g SDS, 4 ml glycerol, 5 ml stack gel buffer and 1.0 ml prepared 1 % bromophenol blue.
   (e) Gel running buffer: dissolve 25 mM Tris–HCl, 192 mM glycine, 0.1 % SDS in 1 l $H_2O$; pH should be 8.3 without adjustment.
3. Transfer buffer for western blot: 25 mM Tris–HCl, 192 mM glycine; pH 8.3 without adjustment.
4. Nitrocellulose or polyvinylidene fluoride (PVDF) membrane for protein transfer.
5. Methanol at 10–20 %.
6. PBS-T: PBS with 0.5 % Tween 20.
7. Stock milk solution: 5 % powdered milk in $ddH_2O$.
8. Rho1D4 antibody (Stock solution 1 mg/ml (dilute 1:5,000 for western blots)).
9. Chemiluminescence (ECL) detection solutions or a LiCor Odyssey infrared imager.
10. Anti-mouse secondary antibody either conjugated to IR Dye 680 or 800 for imaging on Odyssey (LiCor) or conjugated to horseradish peroxidase for ECL imaging (Sigma A4416).

### 2.7 Detection and Localization of 1D4-Tagged Proteins by Immunofluorescence Microscopy

1. Sterile poly-L-lysine-hydrogen bromide (0.02 mg/ml in $H_2O$).
2. 0.1 M Sterile Sorensen's Phosphate Buffer, pH 7.4 (PB).
3. 4 % Paraformaldehyde in PB.
4. Blocking solution: 10 % normal goat serum (NGS), 0.2 % Triton X-100 in PB.
5. Labeling solution: 2.5 % NGS, 0.1 % Triton X-100 in PB.
6. Rho1D4 monoclonal antibody (Stock solution 1 mg/ml (dilute 1:1,000) (*see* **Note 1**).
7. Secondary antibody conjugated to a fluorescent dye.
8. Mowiol 4-88 mounting media (Polysciences Inc. Warrington PA).
9. Dihydrochloride DAPI nuclear stain (Invitrogen, Burlington, ON).

## 3 Methods

### 3.1 Preparation of Rho1D4–Sepharose Immunoaffinity Matrix

This recipe makes approximately 8 ml of activated beads. Use 2 mg antibody per ml of activated beads. This prep of activated beads should preferably be used directly for antibody coupling. Due to inactivation, the activated beads should be used within 6 h.

1. Dialyze purified Rho1D4 for a minimum of 8 h in 3 × 500 ml 20 mM sodium borate pH 8.0 at 4 °C. Check concentration of antibody after dialysis (mg antibody/ml = Absorbance (at 280 nm) ÷ 1.3).
2. Wash 8 ml Sepharose 2B with several changes of $ddH_2O$ by low speed centrifugation. After the final wash, resuspend the beads in 8 ml $ddH_2O$ and pour the beads in a 50 ml glass beaker containing a small stir bar.
3. Set up pH meter in fume hood. Place the beads on a magnetic stirrer and while gently stirring at room temperature add small amounts of 0.2 N NaOH until solution is stable at pH 10.
4. In the fume hood weigh out 0.15 g CNBr and add to beads with continuous gentle stirring while maintaining the pH between 10 and 11 with 0.2 N NaOH for about 30 min at which time the pH should become relatively stable.
5. Add 40 ml of ice-cold 20 mM sodium borate buffer to the beads. Wash by low speed centrifugation with cold 40 ml borate four times to remove any excess CNBr.
6. Resuspend beads in 15 ml sodium borate buffer and keep on ice for 5 min. Centrifuge down beads and determine the approximate amount of packed beads present (Since some of the beads get lost during the activation and centrifugation steps, the amount of beads present may be less than the starting amount). Add RhoD4 antibody (2 mg/ml beads) to the beads with gentle mixing (*see* **Note 2**). It is best to perform this incubation in a tube that holds approximately the volume of beads and antibody combined to keep beads from drying out. Gently rock beads for 4 h at 4 °C. Do not leave beads incubating overnight as this will cause the antibody to aggregate resulting in a loss in column efficiency.
7. To determine percentage of Rho1D4 bound to beads measure the absorbance of the supernatant at a wavelength of 280 nm after low speed centrifugation of the beads. The absorbance should be reduced by 80–95 % indicating efficient coupling of the antibody to the beads (*see* **Note 3**).
8. The coupling reaction is stopped by the addition of TBS pH 8.0 containing 0.05 M glycine followed by low speed centrifugation. The immunoaffinity matrix is washed twice with the same buffer and once in TBS alone. The matrix is stored in

TBS pH 8.0, 0.01 % $NaN_3$ at 4 °C. The matrix should not be frozen as this can cause an inactivation of the antibody and disruption of the matrix structure.

### 3.2 Expression of 1D4-Tagged Protein HEK293T Cells

1. Prepare complete growth medium by adding 8 % BGS and 10 ml/l antibiotic-antimycotic solution to DMEM.
2. At the end of day 1, trypsinize exponentially growing cells and add $5 \times 10^5$ cells to each of two 100 mm dia. × 20 mm plates in a final volume of 9 ml medium/plate.
3. On the morning of day 2, in a 1.5 ml Eppendorf tube add plasmid DNA 2–20 μg (*see* **Note 4**) to $H_2O$ to a final volume of 0.372 ml for each plate of cells. Then add 0.123 ml 1.0 M calcium chloride and mix well. Slowly add 0.495 ml BBS while vortexing at low speed or by flicking tube with a finger. Incubate at room temperature for 10–20 min. Calcium phosphate–DNA solution is then added dropwise to the cells while gently swirling medium in plate and subsequently the cells are placed in an incubator. After 8–16 h replace the growth medium with fresh medium and incubate for an additional 16–35 h.

### 3.3 Preparation of the Membrane Extract from HEK293T Cells

1. The cells are removed from the two plates by pipetting or scraping and centrifuged in a 15 ml conical tube at 1,500 × *g* for 4 min. The medium is removed and the cells are resuspended in 1.0 ml TBS containing complete inhibitor. Incubate on ice for 20–60 min. During this time the cell homogenate is forced through a 22 gauge needle 2–4 times to break open any remaining cells. At end of incubation the homogenate is passed through a 28 g needle 12× and then layered on top of 60 % sucrose/TBS in an 11 × 34 mm tube and spun in a Beckman TLS55 rotor at 45,000 × *g* for 30 min.
2. Carefully remove the membrane fraction at the top of 60 % sucrose, dilute with 2 volumes of TBS, and centrifuge in a 13 × 56 mm tube at 37,000 × *g* for 20 min in the TLA 100 rotor. Resuspend the pellet in TBS containing complete inhibitor. The membranes can be directly used for protein purification as discussed below. Alternatively, the membranes can be frozen in 5 % glycerol and stored at −30 °C.

### 3.4 Solubilization of 1D4-Tagged Membrane Protein from HEK293T Cells or Membrane Extracts

1. Prepare 0.6 ml 2 % Triton X-100 or 36 mM CHAPS in TBS with complete protease inhibitor in a 13 × 56 mm centrifuge tube containing a flea magnetic stir bar. Many membrane proteins require phospholipids to maintain their activity. In such cases it is important to include phospholipids during solubilization and immunoaffinity chromatography. We typically use 0.2 mg/ml of brain polar lipid (Avanti Polar Lipids) or other suitable phospholipids.

2. Add 0.6 ml of cells or membranes in TBS to an equal volume of the detergent solution at room temperature to result in a final detergent concentration of 1 % Triton X-100 or 18 mM CHAPS. Continue stirring at 4 °C for 10–20 min.
3. Remove the stir bar and centrifuge solubilized membranes in TLA 100 rotor at 65,000 × *g* for 10 min at 4 °C to remove any aggregated material. Carefully remove the supernatant fraction containing the solubilized proteins and place the solution on ice. You may later want to analyze the pellet on SDS page to monitor for insolubilized proteins.

### 3.5 Purification of 1D4-Tagged Membrane Protein on a Rho1D4–Sepharose Matrix

1. Prepare 20 ml TBS-T or other detergent solution.
2. Add 50 μl Rho1D4 Sepharose (packed volume) to an equal volume of TBS-T buffer and add to the Ultra-Free-MC filter. Wash the matrix three times with 0.5 ml TBS-T by a short centrifugation (~6 s) in a microcentrifuge being careful not to dry out beads (*see* **Note 5**). If solubilization is carried out with CHAPS, then substitute TBS-T with TBS/10 mM CHAPS in all subsequent procedures.
3. Retain a small aliquot (~50 μl) of the solubilized protein for analysis by SDS-PAGE and western blotting. Add 0.55 ml of the solubilized proteins to the beads and incubate at 4 °C for 20–30 min preferably rocking continuously or rocking by hand every 10 min. After first incubation, centrifuge in a microfuge for about 6 s. Retain the flow through solution or unbound (UB) fraction and add second 0.6 ml of solubilized protein to the matrix. Incubate as above and spin down again. Combining the two UB fractions and retain for analysis by SDS gel electrophoresis and western blotting.
4. Wash the beads by centrifugation a minimum of six times each with 0.5 ml TBS-T. During the washing procedure, be careful not to dry out beads.
5. Briefly spin the beads dry and immediately add 50 μl of 0.2 mg/ml 1D4 peptide in TBS/0.2 % Triton X-100 or TBS/10 mM CHAPS. Incubate shaking for 20–30 min at 18–20 °C. Spin down bound fraction. Add another 50 μl peptide and incubate for 10 min. It may take 2–4 elutions to remove all the protein bound to the antibody. To determine if significant amounts of protein is still bound, the matrix can be incubated in 100 μl of 4 % SDS solution in the absence of reducing agent and eluted for analysis by SDS gel electrophoresis and western blotting. If this is carried out then the matrix should not be regenerated. Typically, 60–85 % of the 1D4-tagged protein is recovered in the 1D4-peptide eluted fraction with minimal protein present in the subsequent SDS eluted fraction (*see* **Note 6**).

### 3.6 Analysis of Expression and Purification by SDS Gel Electrophoresis and Western Blotting

1. Prepare SDS page gel with appropriate acrylamide concentration for analysis of proteins of interest. Pre-made SDS gradient gels can also be used.
2. SDS sample preparation: Add one part fraction to two parts SDS gel loading buffer containing 5 % 2-mercaptoethanol. Heating the sample is not generally recommended for membrane proteins as it can generate multimeric proteins. In some instances, it is useful to compare the protein profiles for heated and unheated samples.
3. Sample analysis: Typically, 3 fractions from the immunoaffinity matrix are analyzed: input, unbound, and peptide eluted fractions (Figs. 1 and 2). Accordingly, a SDS gel may have the following lanes: Molecular weight standards (colored), input (precolumn) fraction, unbound fraction, and bound (peptide-eluted) fraction. The set of lanes are repeated at least twice with a spacer lane separating the sets to facilitate cutting. One set is directly stained with Coomassie Blue or Silver Stain to analyze the protein components in the fractions and evaluate the purity of the 1D4-eluted fractions relative to the input. The 1D4-eluted fraction can also be subjected to mass spectrometry for analysis of possible unknown interacting proteins. The other sets are transfer to polyvinylidene fluoride (PVDF) or nitrocellulose membranes for western blotting with the Rho1D4 antibody and other antibodies of interest as required. In addition to the input, unbound, and bound fractions, it is often of interest to analyze the pellet fraction after detergent solubilization to determine the extent to which tagged proteins are solubilized and the SDS eluted fraction post peptide elution to determine the efficiency of elution of the tagged protein by the peptide.
4. Protein transfer for western blotting. Before transferring low molecular weight proteins (10–40 kDa), the gel should be soaked in transfer buffer containing 20 % methanol for at least 15 min. High molecular weight proteins transfer better in 10 % methanol and with only a brief wash in transfer buffer. As the transfer settings are dependent on the transfer apparatus used, the investigators need to consult the manual.
5. After transfer, the protein transfer blots are rinsed with $ddH_2O$ and left to dry at room temperature or used directly.
6. For PVDF, the blots must be briefly immersed in methanol and then rinsed in PBS before labeling. For nitrocellulose, the blots only have to be rinsed in PBS.
7. Blocking step. The blots are blocked with 1–2 % milk in PBS for 1 h at room temperature.
8. Labeling with Rho1D4. Remove the blot from blocking solution and apply Rho1D4 antibody diluted 1:5,000 in PBS

containing 0.2 % milk; incubate on a rocker for 1 h at room temperature.

9. Washing step. Remove the Rho1D4 antibody from the blot and wash by incubating with PBS-T for 15 min. Repeat washing 3 times.
10. Labeling with secondary antibody. Apply secondary anti-mouse antibody diluted in PBS-T with 0.1 % milk and incubate rocking for 20–40 min at room temperature. (Anti-mouse IgG IR dyes 680 and 800 can be diluted 1–10,000 and Sigma horseradish peroxidase anti mouse IgG can be diluted 1–5,000 or higher.)
11. Washing step. Remove the blot from the secondary antibody solution and wash three times by immersing the blot in PBS-T for 15 min per wash.
12. Blots are ready for imaging by the LiCor Odyssey Imager or by ECL as per manufacturer's instructions.

### 3.7 Localization of Expressed 1D4-Tagged Protein by Immunofluorescence Microscopy

1. Coat sterile glass coverslips for minimum of 20 min with sterile poly-L-lysine/dd$H_2O$ by floating the coverslips face down in the poly-L-lysine. Wash the coverslips with PB. These coverslips can be stored at 4 °C for a few days.
2. Just before plating HEK293T cells, place coated coverslips face up in the plate containing medium. The transfection (*see* Subheading 3.2) can be done in a 100 mm dia. × 20 mm TC plate with multiple coverslips or in a 6-well TC plate. (Divide the transfection reagent by three when transfecting in a 6-well plate).
3. After the transfection incubation time is completed, remove the coverslips from TC plate and immerse in 4 % paraformaldehyde/PB with cells facing up for 15 min.
4. Being careful not to disturb cells, gently wash the cells three times in PB with 10 min incubation for each wash.
5. Block and permeabilize cells with 0.2 % Triton X-100, 10 % goat serum in PB for 15–30 min.
6. Remove blocking buffer and add Rho1D4 antibody (dilute stock 1 mg/ml solution 1:1,000 in labeling solution (PB containing 0.1 % Triton X-100 and 2.5 % NGS) and incubate 1–2 h at room temperature.
7. Carefully wash three times in PB buffer with a 10 min incubation for each wash.
8. Add fluorescent dye conjugated secondary anti-mouse antibody diluted in labeling buffer containing DAPI nuclear stain and incubate 30–60 min at room temperature.
9. Carefully wash three times in PB buffer with a 10 min incubation for each wash.

10. Mount by lightly covering the cells with Mowiol mounting media (or similar mounting medium) and turn coverslip face down on microscope slide. Seal the coverslip by outlining with nail polish.
11. Visualize by confocal scanning or conventional fluorescence microscopy.

## 4 Notes

1. There are several commercial suppliers of the authentic Rho1D4 antibody. For purification of 1D4-tagged proteins, the Rho1D4 antibody can be purchased at a reasonable price through UBC-UILO Flintbox (http://www.rho1d4.com/). For western blotting and immunocytochemistry where smaller quantities are needed, the preferred suppliers are Millipore (MAB5356), Thermo Fisher Scientific (MA1-722), Santa Cruz (sc-57432), Stress Marq (SMC-177C or D), Phospho-Solutions (1840 RHO). Some companies including Abcam, Sigma, Novus, and others list different 1D4 monoclonal and polyclonal antibodies in their catalogue. These appear to differ in their specificity, affinity, and application.
2. It is important to stir the Sepharose matrix gently so that disruption of the beads does not occur since this can result in an inefficient immunoaffinity matrix.
3. Coupling should be complete within 2–4 h at 4 °C. Overnight coupling should be avoided as this can cause aggregation of the beads resulting in nonspecific binding of proteins to the matrix and inefficient protein purification. If the beads are disrupted, some of the absorbance at 280 nm may be due to light scattering. For the absorbance at 280 nm to reliably reflect protein concentration, then the absorbance at 260 nm should be considerable less than at 280 nm. If the absorbance at 260 nm is higher than 280 nm, this likely reflects a significant amount of light scattering from fractured beads.
4. For co-expression studies, plasmids for expression of each subunit or protein are added together in equal amounts or in amounts to be determined for optimal expression of both proteins.
5. It is important that the immunoaffinity matrix is not centrifuged to complete dryness as this can irreversibly denature the protein resulting in low functional protein recovery. The amount of sulfhydryl reducing agents such as dithiothreitol (DTT) in the buffers should be minimized (less than 1 mM) since these reagents can cause some of the immunoglobulin light and heavy chains to dissociate resulting in contamination

of the purified protein. Immunoglobulin dissociation is particularly evident when SDS containing reducing agents is used to elute proteins from the matrix.

6. In purification procedures employing small amounts of Rho1D4 affinity matrix (<100 μl), it is, in general, not worth regenerating the matrix. In purification procedures employing larger amounts, the Rho1D4 immunoaffinity matrix can be regenerated by washing the matrix three times in 0.1 M acetate buffer, pH 4 over a period of 5 min., and then washing the matrix in TBS until the pH is 7.4. Although in principal, the Rho1D4 affinity matrix can be regenerated after each use, in practice the efficiency of the column will be reduced since some bound protein may not be removed with the peptide in the elution step. We have found that the regenerated matrix has lost about 25 % of its immunoreactivity after this treatment.

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# Chapter 2

# Affinity Purification of Heme-Tagged Proteins

**Wesley B. Asher and Kara L. Bren**

## Abstract

Protein affinity purification techniques are widely used for isolating pure target proteins for biochemical and structural characterization. Herein, we describe the protocol for affinity-based purification of proteins expressed in *Escherichia coli* that uses the coordination of a peptide tag covalently modified with heme *c*, known as a heme-tag, to an L-histidine immobilized Sepharose resin. This approach provides an affinity purification tag visible to the eye, facilitating tracking of the protein. In addition, we describe methods for specifically detecting heme-tagged proteins in SDS-PAGE gels using a heme-staining procedure and for quantifying the proteins using a pyridine hemochrome assay.

**Key words** Affinity protein purification, Affinity tag, Heme-tag, Visible-tag, L-histidine immobilized Sepharose chromatography, Protein quantification, Visible tracking

## 1 Introduction

Protein purification using affinity chromatography is an indispensible tool in biochemistry and molecular biology [1–3]. The addition of affinity tags to the amino- (N) or carboxyl- (C) terminus of a target protein for purification purposes can dramatically reduce the amount of preparation time, reduce the number of purification steps needed, and increase the yield of pure protein [1–4]. Recently, we developed peptide fusion tags covalently modified with heme *c* chromophores, known as heme-tags, that purify proteins expressed in *Escherichia coli* using an L-histidine immobilized Sepharose (HIS) affinity resin [5]. The heme-tag-HIS method combines the ease of affinity-based purification with the convenience of visible detection for protein tracking purposes, as the heme chromophore grants the fusion protein with an intense red or red-brown color. Visible detection significantly reduces the time and effort associated with protein expression and handling during all purification and chromatographic steps [4]. Because of these

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**Table 1**
**Heme-binding peptides used to mature heme-tags for HIS affinity purification of the test protein *Pseudomonas aeruginosa* azurin**

| Heme-binding peptides | Amino acid sequence |
|---|---|
| Hm14 | GATSCAACHADSER |
| Hm16 | RESDACAACHSRGSTG |
| Hm17 | GATSERDCAACHADSER |

advantages, fusion tags have been developed for visual tracking purposes [4, 6], but unlike heme-tags, cannot be used for affinity purification procedures.

Mature heme-tags are composed of a heme-binding peptide (HBP) that is linked to the heme chromophore by two stable thioether bonds [7]. *See* Table 1 for the amino acid sequences of HBPs used for HIS affinity purification. HBPs are typically small, consisting of 14–20 amino acid residues. The critical element of a HBP is a Cys-X-X-Cys-His (CXXCH) heme-binding motif within the sequence, with X representing any amino acid residue [8]. The CXXCH motif is recognized by the *E. coli* cytochrome *c* (cyt *c*) maturation (Ccm) apparatus, which facilitates the covalent addition of the Cys-thiol groups from the CXXCH motif to the two vinyl groups of heme [9]. In our tag designs, the heme iron is coordinated by four pyrrole nitrogens of the porphyrin ring and by the histidine residue of the CXXCH motif (serves as a fifth coordinating ligand), leaving the six coordination site open for coordinating exogenous ligands (Fig. 1a) [5]. The basis of the heme-tag-HIS affinity purification method is the reversible coordination between the heme-iron open coordination site and the side chain of histidine immobilized on Sepharose beads (Fig. 1b). After purification, the protein can be eluted from the column using an imidazole-containing buffer, a low pH (≤5) buffer, or a high pH (≥8) buffer (*see* **Note 1**) [5].

The Ccm system is responsible for attaching heme to the HBP and is composed of eight membrane proteins (Ccm *A-H*) that reside in the *E. coli* cytoplasmic membrane facing the periplasmic space where the heme attachment reaction occurs (Fig. 1c) [9]. Therefore, maturation of proteins fused with HBPs by the Ccm system must also contain an N-terminal signal sequence peptide for recognition by the secretory system and transport to the periplasm (*see* **Note 2**) [7]. Secretory peptidase removes the signal peptide after the protein is translocated to the periplasm [10]. Table 2 lists several signal sequences used for exporting recombinant proteins expressed in *E. coli* to the periplasmic space. We have used pelB, malE, and Pa-Azu signal peptides to successfully mature heme-tagged proteins using the Ccm system for heme attachment (*see* **Note 3**).

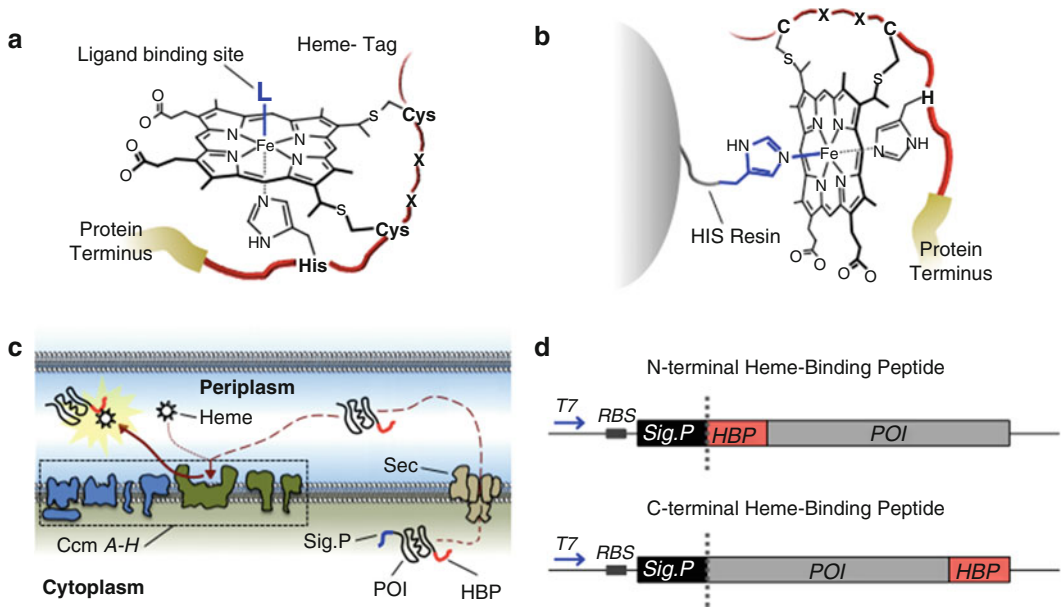


**Fig. 1** Schematic overview of the maturation and HIS affinity purification of heme-tagged proteins. (**a**) Mature heme-tag emphasizing the open coordination site for binding exogenous ligands (L). (**b**) Immobilized heme-tagged protein on the HIS affinity resin. (**c**) Transport of a POI containing a C-terminal heme-binding peptide (*red*) by sec translocase (Sec) from the cytoplasm to the periplasm where heme attachment occurs by the Ccm A-H system. The N-terminal signal sequence is shown in blue and is removed from the POI by seceratory peptidase in the periplasmic space. (**d**) DNA segments of the pET expression vectors showing the general coding region for POIs (*gray*) fused with N-terminal (*top*) and C-terminal (*bottom*) heme-binding peptide (HBP, *red*) sequences. Both designs include the N-terminal signal sequence peptide (Sig. P, *black*) and the point of cleavage (*gray dotted line*) by secretory peptidase. T7, *blue arrow*: T7 promoter, RBS, *square*: ribosomal binding site

**Table 2**
**List of representative signal sequences for protein translocation to the periplasm**

| Signal sequence | Amino acid sequence | References |
|---|---|---|
| pelB (pectate lyase B) | MKYLLPTAAAGLLLLAAQPAMA | [10] |
| ompA (outer-membrane protein A) | MKKTAIAIAVALAGFATVAQA | [10] |
| CSP (synthetic signal sequence) | MKKKLLALALLALLFNGAQ | [11] |
| StII (heat-stable enterotoxin 2) | MKKNIAFLLASMFVFSIATNAYA | [10] |
| MalE (maltose binding protein) | MKIKTGARILALSALTTMMFSASALA | [10] |
| Pa-Azu (*P. aeruginosa* azurin) | MLRKLAAVSLLSLLSAPLLA | [12] |
| Modified-cyt $c_{550}$ (*P. versutus*) | MKISIYATLAALSLALPAGA | [13] |

Herein, we outline the maturation of heme-tagged proteins by the concurrent overexpression of HBP-tagged proteins (*see* Fig. 1d for general construct designs) using the pET-expression vector series (Novagen) with the overexpression of the *Ccm A-H* cassette

from the pEC86 [14] vector. Using the pET17b vector for expression, we have successfully expressed and purified the test protein azurin from *Pseudomonas aeruginosa* fused at the C-terminus with the Hm14 and Hm17 HBP sequences, and at the N-terminus with the Hm16 sequence (*see* Table 1 for HBP sequences). In all three constructs we use the Pa-Azu signal sequence [12] for translocation of the protein to the periplasm. These plasmid constructs have the general design as shown in Fig. 1d for C- and N-terminally tagged proteins of interest (POIs), and are available upon request (*see* **Note 4**). Several cloning strategies are available for fusing the HBP's in Table 1 and the signal peptides in Table 2 to a POI in a variety of *E. coli* expression vectors. However, it is beyond the scope of this chapter to describe detailed molecular cloning strategies and instead we refer the reader to protocols focusing on these methods [15].

In this chapter, we outline the procedures used for expressing heme-tagged proteins in *E. coli* using the Ccm system and for their subsequent affinity purification using the HIS method. Likewise, we describe the protocol for synthesizing the HIS medium from functionalized Sepharose beads. In addition to expression and purification, we describe methods for SDS-PAGE detection of heme-tagged proteins using standard Coomassie Blue staining and a procedure for specifically detecting in-gel heme *c* proteins, referred to as heme staining [16]. The heme staining method can reveal heme-tagged proteins in non-purified samples and confirms that the target protein contains covalently bound heme. Lastly, we detail a spectrophotometric approach for quantifying heme-tagged proteins using the pyridine hemochrome assay (PHA) [17].

## 2 Materials

Prepare all solutions and buffers using ultrapure water unless stated otherwise. Consult the materials safety data sheets for all chemicals used and follow the procedures for safe handling and disposal. The pEC86 vector coding the *Ccm A-H* gene cassette can be requested from Prof. Linda Thöny-Meyer (EMPA Materials Science and Technology, St. Gallen, Switzerland).

### 2.1 Expression of Heme-Tagged Protein in E. coli

#### 2.1.1 Protein Expression in E. coli

1. pET-17b or other suitable expression plasmid encoding the POI tagged with an HBP (*see* Table 1 for sequences) and with an N-terminal signal sequence (*see* Table 2) following the general design shown in Fig. 1d.
2. pEC86 vector (chloramphenicol resistant) coding Ccm *A-H*.
3. Chemically competent One Shot® BL21 Star™ (DE3) *Escherichia coli* (Invitrogen) or other suitable *E. coli* expression host (*see* **Note 4**).

4. Liquid Luria–Bertani (LB) medium: add 10 g Bacto-Tryptone, 5 g Bacto-Yeast extract, and 10 g NaCl to 900 mL of ultrapure water. Adjust pH to 7.3 with NaOH if needed. Adjust to final volume of 1 L. Sterilize by autoclaving and store at 4 °C.
5. LB agarose plates: add 10 g Bacto-Tryptone, 5 g Bacto-Yeast Extract, 10 g NaCl, and 18 g Bacto-Agar to 500 mL of ultrapure water. Adjust pH to 7.3 with NaOH if needed. Adjust to final volume of 1 L. Sterilize by autoclaving, cool to ~45–50 °C, and add ~25 mL of LB agarose to 8 cm plates and allow to solidify. Store the plates at 4 °C.
6. 50 mg/mL ampicillin (Amp) solution: Filter-sterilize and aliquot to a desired volume. Store aliquots at –20 °C. Once an aliquot of Amp solution is thawed, store at 4 °C and discard after 2 weeks. Use at 50 μg/mL. Note: prepare, store, and use antibiotic solutions according to literature standards if using an expression vector with antibiotic resistance other than Amp.
7. 50 mg/mL chloramphenicol (CM) solution: Dissolve in ethanol. Prepare CM solution as needed. Store at 4 °C and use at 50 μg/mL within 2 weeks.
8. Amp supplemented LB agar plates: add 30 μL of 50 mg/mL Amp on top of the agar and spread over the entire surface aseptically.
9. Amp-CM supplemented LB agar plates: add 30 μL of 50 mg/mL Amp on top of the agar and spread over the entire surface aseptically. Follow by adding 30 μL of 50 mg/mL CM in ethanol and spread over the entire surface aseptically.
10. Incubated orbital shaker.
11. 100 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG): Add 2.38 g of IPTG to 100 mL of ultrapure water, filter-sterilize, and store at 20 °C in the dark (*see* **Note 5**).
12. Refrigerated (4 °C) centrifuge.
13. Ultraviolet–Visible (UV–Vis) spectrophotometer.
14. Plastic or quartz cuvettes.

### *2.1.2 Osmotic Shock Procedure*

1. Sucrose buffer: 50 mM Tris–HCl, 1 mM EDTA, 20 % sucrose, pH 7.5–8. Prepare a 0.5 M stock solution of EDTA, pH 8.0 in ultrapure water and adjust pH with NaOH. Use the EDTA stock solution to prepare the sucrose buffer.
2. Osmotic shock buffer: Ice-cold (0–4 °C) 5 mM $MgSO_4$ in water.
3. Method for concentrating protein in solution. Ex. EMD Millipore Amicon® Ultra centrifugal filters (0.5–15 mL capacity) or Amicon® stirred cells (4–400 mL capacity).
4. PD-10 desalting and buffer exchange columns (GE Healthcare).

*2.1.3 Whole Cell/ Cytoplasm Lysis Procedure*

1. Cytoplasmic extraction buffer: 50 mM Tris–HCl, 4 mg/mL chicken egg white lysozyme (EMD Millipore).
2. DNAase I solution (NEB).
3. Triton™ X-100, molecular biology grade.
4. Method for concentrating protein in solution. Ex. EMD Millipore Amicon® Ultra centrifugal filters (0.5–15 mL capacity) or Amicon® stirred cells (4–400 mL capacity).
5. PD-10 desalting and buffer exchange columns (GE Healthcare).

***2.2 Preparation of L-Histidine Immobilized Sepharose Resin***

1. 25 mL *N*-hydroxysuccinimide (NHS)-Activated Sepharose™ 4 Fast Flow (GE Healthcare).
2. 2.5 × 10 cm glass chromatography column. Ex. Econo-Column® Bio-Rad glass columns (*see* **Note 6**).
3. L-histidine coupling buffer: 10 mg/mL L-histidine, 200 mM $NaHCO_3$, 500 mM NaCl, pH 8.3. Prepare ~15 mL and store at room temperature.
4. 1 mM HCl in water: Prepare ~500 mL of the HCl solution for use and chill on ice before procedure (0–4 °C).
5. pH indicator strips (pH range 1–14).
6. Open air orbital shaker.
7. Bottom top 0.1 μm filtration unit, ≥250 mL capacity (e.g., Thermo Scientific Nalgene MF75™ 0.1 μm PES Filter Units).
8. Blocking buffer: 500 mM ethanolamine, 500 mM NaCl, pH 8.3. Prepare ~15 mL of block buffer for use.
9. High pH wash buffer: 100 mM Tris–HCl, pH 8–9.
10. Low pH wash buffer: 100 mM sodium acetate buffer, 500 mM NaCl, pH 4–5.

***2.3 HIS Affinity Purification of Heme-Tagged Protein***

1. Wash/binding buffer: 50 mM sodium phosphate ($NaP_i$), pH 7.0 (*see* **Note 7**). Prepare 1 L of buffer and store at 4 °C until use.
2. Imidazole elution buffer: 300 mM Imidazole, 50 mM $NaP_i$, pH 7.0. Prepare 500 mL of solution and store at 4 °C.
3. Low pH elution buffer: 50 mM $NaP_i$, pH 4–5.
4. High pH elution buffer: 50 mM Tris–HCl, pH 8–9.
5. Method for concentrating protein in solution. Ex. EMD Millipore Amicon® Ultra centrifugal filters (0.5–15 mL capacity) or Amicon® stirred cells (4–400 mL capacity).
6. PD-10 desalting and buffer exchange columns (GE Healthcare).

***2.4 SDS-PAGE Analysis of Heme-Tagged Protein***

1. SDS-PAGE gel electrophoresis unit. Ex. Hoefer SE 250 or 260 Mighty Small II mini gel electrophoresis unit and dual gel caster.

*2.4.1 SDS-PAGE*

2. 30 % acrylamide/bis-acrylamide solution (Bio-Rad).

3. 1.5 M Tris–HCl, pH 8.8.
4. 1.0 M Tris–HCl, pH 6.8.
5. 10 % Sodium dodecyl sulfate (SDS) solution.
6. 10 % ammonium persulfate (APS) solution. Store at 4 °C and use within 1 month.
7. *N,N,N′,N′*-tetramethyl-ethyleneldiamine (TEMED).
8. 10× Tris-glycine reservoir/running buffer: 30 g Tris-base, 144 g glycine, 10 g SDS per 1 L of solution. Add 100 mL of 10× running buffer to 900 mL ultrapure water to prepare 1 L of 1× running buffer.
9. SDS sample buffer: 1.6 mL ultrapure water, 625 μL 1.0 M Tris–HCl (pH 6.8), 5 mL 50 % glycerol, 2.0 mL 10 % SDS solution, 250 μL 0.5 % Bromophenol Blue. Add 50 μL β-mercaptoethanol (βME) to 950 μL of SDS sample buffer before use (*see* **Note 8**).
10. Molecular weight markers: Pre-stained Bio-Rad Precision Plus Protein™ all blue standards (10–250 kDa).

#### *2.4.2 Heme Staining*

1. Gel fixing solution: 12.5 % trichloroacetic acid (TCA) solution. Prepare ~150–200 mL of the TCA solution and store at 4 °C. The solution can be reused multiple times.
2. Heme staining solution: dissolve 0.2 g *o*-dianisidine in 15–20 mL glacial acetic acid (*see* **Note 9**). Add the dissolved *o*-dianisidine solution to 20 mL of 500 mM sodium citrate buffer (pH 4.4), 400 μL 30 % hydrogen peroxide, 160 mL ultrapure water (~200 mL total volume of heme staining solution).
3. Open air orbital shaker.

#### *2.4.3 Coomassie Blue Staining*

1. Coomassie blue staining solution: 1 % Coomassie brilliant blue R250, 40 % methanol, 10 % glacial acetic acid. Store at room temperature.
2. Destaining solution: 40 % methanol, 10 % glacial acetic acid. Store at room temperature.

### *2.5 Pyridine Hemochrome Assay for Protein Quantification*

1. Pyridine–base Solution: 100 mM NaOH (dissolve NaOH pellets in 50 mM $NaP_i$), 20 % pyridine. Prepare solution just before use.
2. Saturated dithionite solution: ≥1.0 M sodium dithionite (dissolved in 50 mM $NaP_i$). Prepare immediately before use.
3. UV–Vis spectrophotometer.
4. Quartz or plastic cuvettes.

## 3 Methods

### 3.1 Expression of Heme-Tagged Protein in E. coli

The procedure for expressing heme-tagged proteins below is based on the maturation of heme-tagged azurins using the pET17b expression vector [5]. The *E. coli* expression hosts used with the pET expression vectors (ex. pET17b) are lysogens of bacteriophage DE3 and contain a chromosomal copy of a DNA fragment containing the *lacI* gene coding for the *lac* repressor and the T7 RNA polymerase (T7 RNAP) gene regulated by an isopropyl-β-D-thiogalactopyranoside (IPTG) inducible *lacUV5* promoter. When supplemented in the growth media, IPTG binds and deactivates the *lac* repressor, allowing for increased production of T7 RNAP in the cell, which in turn binds to the strong T7 promoter of the pET vector controlling the overexpression of the HBP-tagged protein. It is important to note that expression schemes may vary depending on the protein being tagged. Several expression variables: temperature, shaking speeds, aeration, and the amount of IPTG used for induction, may need to be optimized to determine which conditions are most suitable for a given POI. In addition, the predicted solubility of the heme-tagged protein should be analyzed (*see* **Note 10**), as the target protein may form inclusion bodies in the cytoplasm, preventing transport to the periplasmic space. We highly recommend following the general expression strategies outlined in the Novagen pET systems manual before developing an optimized expression scheme for heme-tagged protein production (*see* **Note 11**).

#### 3.1.1 Protein Expression in E. coli

1. Transform both the pET expression plasmid coding the heme-tagged protein and the pEC86 plasmid coding Ccm A-H into One Shot® BL21 Star™ (DE3) *E. coli*. Add 5–10 ng of both plasmids per one aliquot of *E. coli* cells for the transformation. Use LB agar plates supplemented with Amp and CM for producing single colonies overnight.
2. Inoculate 5–10 mL of LB broth containing Amp and CM with a single colony of transformed *E. coli* and grow for ~8 h in an orbital shaker at 37 °C and 200–230 rpm.
3. Inoculate 1 L of liquid LB containing Amp and CM with the 5–10 mL of starter growth. Place foil over the top of the growth flask to reduce aeration (*see* **Note 12**). Grow for a desired amount of time before induction with IPTG. For most POIs, the growth is allowed to reach logarithmic phase (optical density at 600 nm of ~0.5) before induction with IPTG. However, this is dependent on the POI. Consult the Novagen pET systems manual for more details.
4. Grow (foil covered flask) for ~16 h uninduced at 25–28 °C with shaking at 125 rpm. Add 4 mL 100 mM IPTG and grow for an additional 4–6 h at the same conditions (*see* **Note 13**).

5. Harvest the cells by centrifugation at 10,000 × *g* for 20 min at 4 °C. Pour off the supernatant. The pellet should exhibit a dark brown/red color, immediately confirming that expression of the heme-tagged protein occurred (*see* **Note 14**).

### 3.1.2 Osmotic Shock Procedure

The *E. coli* cell pellets can be lysed using several standard protocols. Here, we describe two procedures: an osmotic shock procedure for obtaining only proteins from the periplasmic space (where the heme-tagged protein resides) and a whole cell/cytoplasmic lysis procedure, both of which will allow for purification of the heme-tagged protein by the HIS method. In some cases, the whole cell/cytoplasmic lysis results in a higher heme-tagged protein yield (but with a higher level of contamination) than the osmotic shock procedure.

1. For periplasmic lysis, resuspend the pellet in 25–35 mL of sucrose buffer. Slowly stir the solution at room temperature for 15–20 min until the pellet fully resuspends. Centrifuge the solution at 10,000 × *g* for 20 min at 4 °C to collect the cells. Carefully remove the supernatant (*see* **Note 15**).
2. Resuspend the pellet in 25–35 mL of cold osmotic shock buffer and stir gently at 4 °C for 30 min to disrupt the outer membrane of the cells, releasing the periplasm.
3. Centrifuge the solution to pellet the shocked cells and carefully isolate the periplasmic fraction/supernatant containing the heme-tagged protein. The supernatant should have a dark brown-red color. Concentrate the periplasmic fraction to a final volume of less than 10 mL (preferably lower if possible). Use a PD-10 desalting column to exchange the periplasmic fraction into 50 mM $NaP_i$, pH 7.0. Keep the periplasmic sample at 4 °C.

### 3.1.3 Whole Cell/ Cytoplasmic Lysis Procedure

1. For whole cell/cytoplasmic lysis, resuspend the pellet in 25–35 mL of cytoplasmic lysis buffer containing ~20 μL of Triton™ 100-X. After thorough resuspension, add ~2 units of DNAase I to the solution and mix by gently inverting the centrifuge tubes containing the resuspended cells 3–4 times (*see* **Note 16**).
2. Incubate for 1–1.5 h at 30 °C and gently invert the tubes to mix the solutions every 15–20 min during incubation. Centrifuge the solution to pellet the ruptured cells and carefully isolate the supernatant containing the heme-tagged protein. The supernatant should have a dark brown-red color.
3. It is recommended to partially clarify the lysate obtained using the whole cell/cytoplasm lysis procedure before any further chromatography steps. In the case for heme-tagged azurin, we apply the lysate to an ion-exchange resin and wash with an

appropriate buffer before eluting with a one-step NaCl buffer [5]. Concentrate the partially clarified lysate to a final volume of less than 10 mL (preferably lower if possible). Use a PD-10 desalting column to exchange the clarified lysate buffer to 50 mM $NaP_i$, pH 7.0. Keep the clarified lysate sample at 4 °C.

### 3.2 Preparation of L-Histidine Immobilized Sepharose Resin

The procedure for preparing the HIS resin is largely adapted from the manufacturer's instructions for reacting primary amine ($–NH_2$) containing ligands with NHS-activated Sepharose™ 4 Fast Flow (GE Healthcare Life Sciences Instructions 71-5000-14AD). The NHS-ester of the medium reacts specifically with primary amines forming a stable covalent linkage between the Sepharose beads and the ligand. Because histidine is a small ligand with an unobstructed $–NH_2$ group, the coupling efficiency will be >90 %. Thus, quantitative determination of the coupling efficiency (often done when large proteins are coupled to the resin) is not necessary.

1. Pour the 25 mL NHS-Activated Sepharose™ 4 Fast Flow suspended in isopropanol from the manufactures container onto the membrane of a 250 mL capacity bottom top 0.1 μm filtration unit.
2. Vacuum-filter to remove the isopropanol (*see* **Note 17**) and immediately resuspend the beads in ~150 mL of ice-cold 1 mM HCl in the absence of vacuum suction. Use a small stir rod to suspend the beads in the HCl solution. Vacuum-filter to remove the HCl solution and repeat the HCl wash step two additional times.
3. After the last wash step with HCl, immediately resuspend the beads in 12.5 mL of the histidine coupling buffer (to achieve the recommended 0.5:1 coupling solution to medium volume ratio) and pour the suspension into a sterile 100–125 mL beaker. Adjust the pH of the resuspended medium to ~8.0–8.3 using NaOH. Use pH indicator strips to measure approximate pH. Cover the beaker and gently agitate the coupling buffer/medium suspension using an open-air orbital shaker for 3 h. After coupling is complete, pour the newly synthesized HIS medium onto a new filter device and apply vacuum to remove the coupling buffer.
4. Resuspend the HIS medium in 12.5 mL of blocking buffer to block any remaining NHS-ester groups. Follow the same protocol for incubation as described with the L-histidine coupling buffer. After the blocking reaction, remove the blocking buffer by vacuum filtration.
5. To wash the HIS resin, resuspend the medium in 100 mL high pH wash buffer, vacuum-filter, and then resuspend the medium in 100 mL low pH. Vacuum-filter and repeat the high pH/low pH cycle two additional times.

6. Decant or slowly pour the washed HIS medium into a 2.5 × 10 cm glass chromatography column. Wash the HIS column three times with 20 % ethanol and store in the same solution at room temperature.

### 3.3 HIS Affinity Purification of Heme-Tagged Protein

1. Pre-equilibrate the HIS column with 100 mL 50 mM $NaP_i$, pH 7.0. Carefully load the concentrated periplasmic fraction or partially clarified lysate containing the heme-tagged protein on the HIS column (*see* **Note 18**). After the lysate has loaded on the resin, carefully add 2 column volumes (CV, ~50 mL) of wash buffer onto the resin (*see* **Note 19**). After 0.5 CV of buffer elutes, a bright red band will form at the top of the resin and a green band will begin migrating down the column (*see* Fig. 2, **Note 20**). The red band is the heme-tagged protein coordinated to the HIS resin, and the green band contains a degraded heme by-product associated with overexpressed heme-containing proteins. The green by-product does not coordinate the HIS resin and therefore migrates with the *E. coli* containing proteins during the wash step.
2. After 1 CV (~25 mL) of wash buffer elutes from the HIS resin, the green band will be at the bottom of the column and in the process of eluting. Collect a small fraction of the green band as it begins to elute from the column (~0.9–1 CV of wash buffer), and a fraction after the addition of 2.0 CV of wash buffer for analysis.
3. After washing the column with a total of 2 CV of wash buffer, add 50 mL of imidazole-, low pH-, or high pH-elution buffer. The red band at the top of the column will immediately begin to elute from the column. Collect the entire red fraction from the column. Concentrate the pure heme-tagged protein after elution and use a PD-10 desalting column to exchange the fraction into an appropriate buffer (ex. 50 mM $NaP_i$, pH 7.0).

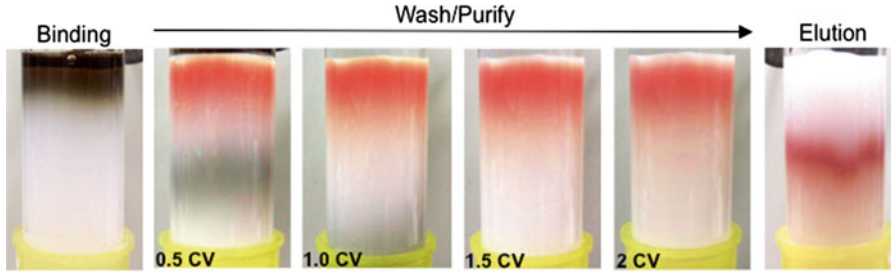


**Fig. 2** Affinity purification of azurin tagged at the N-terminus with the Hm16 sequence using the HIS resin. Loading of partially clarified lysate containing Az-Hm16 on the HIS resin (Binding), Az-Hm16 binding to the HIS resin after the addition of 0.5, 1.0, 1.5, and 2 CV (~12.5, 25, 37.5, and 50 mL, respectively) of wash buffer (Wash/Purify), and after ~1.5 CV of binding buffer containing 300 mM imidazole (Elution). A *green band*, representing the contaminating proteins, migrates down the column as shown after 0.5 CV wash buffer, and begins to elute at ~1 CV

### 3.4 SDS-PAGE Analysis of Heme-Tagged Protein

Here, we outline SDS-PAGE using the Hoefer electrophoresis unit; however, any commercial system can be used. The protocol for using the Hoefer duel gel caster and the assembly of the gel sandwich stack consisting of a glass and alumina plate separated by two spacers is thoroughly outlined in the SE 245 duel gel caster user's manual (*see* **Note 21**).

#### 3.4.1 SDS-PAGE

1. Use the Hoefer duel gel caster manual to properly set up the caster for pouring the gels.
2. Using a small ruler, measure 3 cm below the top of the glass plate and draw a line at this point.
3. To prepare the 12 % resolving gel, combine 1.6 mL of ultrapure water, 1.3 mL of 1.5 M Tris–HCl pH 8.8, 2.0 mL 30 % acrylamide/bis-acrylamide solution, 50 μL 10 % SDS, and 50 μL 10 % APS in a 15 mL conical tube. Mix by inverting the tube several times.
4. Next, add 2 μL of TEMED to the mixture, invert the tube to mix, and quickly pour the resolving gel solution up to the marked line (made in **step 2**) in the gel sandwich stack and overlay with ultrapure water. Allow 20–25 min for the resolving gel to polymerize, at which point the water can be poured off the top of the gel.
5. Prepare the 5 % stacking gel by combining 1.4 mL ultrapure water, 0.25 mL 1.0 M Tris–HCl pH 6.8, 0.33 mL 30 % acrylamide/bis-acrylamide solution, 20 μL 10 % SDS, and 20 μL 10 % APS in a 15 mL conical tube. Add 2 μL of TEMED to the mixture, quickly invert the tube to mix, and pour the stacking gel solution on top of the polymerized resolving gel up to the top of the plates. Immediately insert the comb and allow 30 min to polymerize.
6. After the stacking gel has polymerized, carefully remove the comb and assemble the gel into the Hoefer SE 250 or 260 Mighty Small II mini gel electrophoresis unit. Add 1× electrophoresis running buffer to the upper and lower chambers. Dilute aliquots of fractions collected during the HIS purification process 1:2 with SDS-sample buffer. Heat the samples at 95–100 °C for 5 min. Load the samples and the pre-stained molecular weight standard solution onto the gel. Run the gel for ~30–45 min at 200 mV, or until the bromophenol blue runs off the bottom of the gel.
7. Remove the gel stack from the electrophoresis unit and disassemble the gel sandwich stack by carefully separating the plates. Remove the gel between the plates and immerse in 200 mL of ultrapure water with gentle agitation using an orbital shaker for 30 min.

*3.4.2 Heme Staining*

1. Immerse the washed gel in 200 mL of gel fixing solution and gently agitate for 30 min.
2. After the TCA wash, repeat the 30 min water wash in **step 6** from Subheading 3.4.1.
3. Immerse the gel in heme staining solution with agitation. Bright green bands will appear in the gel within 15–30 min. Remove the gel and wash in ultrapure water before handling and imaging. *See* Fig. 3 for an example of expected results.

*3.4.3 Coomassie Blue Staining*

1. Prepare an identical gel with the same samples using **steps 1–6** from Subheading 3.4.1 for Coomassie blue staining. Immerse the gel in Coomassie blue stain for 30 min with agitation.
2. Immerse the gel in distaining solution with agitation for 20 min. Remove the stain, add fresh distaining solution, and agitate the gel for 2 h or until the background is completely removed. *See* Fig. 3 for an example of expected results.

### *3.5 Pyridine Hemochrome Assay for Protein Quantification*

1. Prepare samples for the pyridine hemochrome assay as follows: add 80 μL of HIS purified heme-tagged protein in 50 mM $NaP_i$ to 910 μL pyridine/base solution and mix by vortexing.
2. Prepare the saturated sodium dithionite solution and immediately add 10 μL of this solution to the samples prepared in **item 1** of Subheading 2.5 (*see* **Note 22**). Quickly mix by vortexing, transfer to a quartz cuvette and obtain the UV–Vis absorption spectrum of the samples at room temperature using a spectrophotometer (*see* **Note 23**).

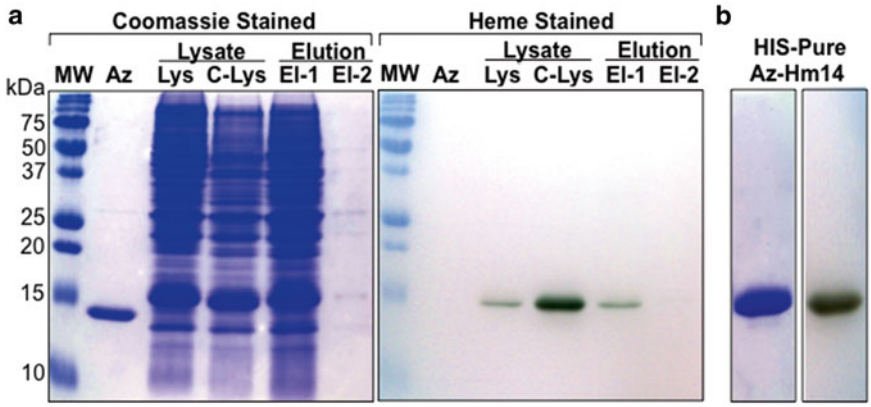


**Fig. 3** 12 % SDS-PAGE gel showing results from the HIS purification of azurin (Az) tagged at the C-terminus with Hm14 (Az-Hm14). (**a**) Both gels contain identical samples. The *left* gel was stained with Coomassie blue and the *right* using heme stain. *MW*: pre-stained molecular weight markers (10–250 kDa). *Az*: purified wild type Az (~14 kDa) not fused with any tag sequence. *Lys*: non-clarified lysate containing Az-Hm14. *C-Lys*: lysate clarified using an initial anion-exchange chromatography step. *El-1*: Fraction collected after ~0.9 CV of wash buffer had eluted from the column during HIS purification of Az-Hm14. *El-2*: Fraction taken after 2.0 CV of wash buffer eluted. (**b**) Coomassie and heme-stained gels showing pure Az-Hm14 after elution from the HIS resin

3. Repeat **steps 1–2** of Subheading 3.5 for the same sample to obtain data in triplicate.
4. Using the Beer-Lambert law ($A = \varepsilon \cdot l \cdot c$, where $A$ is absorbance, $\varepsilon$ is the extinction coefficient, $l$ is cuvette path length, and $c$ is concentration), the concentration of the heme-tagged protein solution can be calculated using the extinction coefficient of 30.27 $mM^{-1}$ $cm^{-1}$ at 550 nm for heme *c* containing proteins.

## 4 Notes

1. The imidazole-containing elution buffer displaces the histidine residues of the HIS resin. We propose that the low pH (≤5) elution buffer protonates the histidine residues preventing coordination, and the hydroxide ions of the high pH (≥8) elution buffer displace the histidine residues of the resin [5].
2. We recently developed a new strategy for maturing heme-tagged proteins in the *E. coli* cytoplasm by the enzyme cytochrome *c* heme lyase (CCHL), eliminating the need for transporting the protein to the periplasmic space [18]. We are currently developing HBPs for maturation by CCHL that can be used for affinity purification by the HIS method.
3. There is no rule for choosing a signal sequence that will guarantee or maximize the level of transport to the periplasmic space for any specific target protein [10]. Several signal peptide sequences exist, yet the *pelB* and *ompA* sequences are frequently used in commercial vectors for the purpose of targeting a protein to the periplasm. In our constructs, we check the likelihood that the sequence we choose will be recognized for periplasmic transport and of proper cleavage by signal peptidase using the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/) [19] and optimize the constructs accordingly.
4. If using an expression vector with a T7 promoter (as is the case for the pET series vectors), the *E. coli* host used for expression must contain a chromosomal copy of T7 RNAP. The host RNAP native to the organism will bind bacterial promoters (as in pEC86) but not the T7 promoter.
5. Solid IPTG should be stored in the absence of light in a desiccator at −20 °C.
6. Different column brands, sizes and types with a desired volume of HIS resin can be used. The 25 mL HIS resin packed in the 2.5 × 10 cm column is optimized for purifying protein from the 1 L scale of bacterial growth. After a purification run, the column can be washed with 5 CV of 50 mM $NaP_i$, 500 mM NaCl, pH 7.0 and stored in 20 % ethanol. After 10–15 purification runs, or until the flow rate is visibly slower than usual,

the column can be washed with 100 mM NaOH for cleaning purposes. Note that repeated washes with NaOH will reduce the binding capacity of the resin, as some histidine residues will be removed from the Sepharose. We typically dispose of the HIS resin after 1 year of use.

7. Wash buffers other than $NaP_i$ that are not known to chelate/bind metals and that are near neutral pH may be used with the HIS resin.
8. βME reduces the activity of the heme stain solution. Thus, it is recommended that βME be omitted from the sample buffer if the POI is known to not form disulfide bonds in the native and unfolded state.
9. Allow ~5 min for *o*-dianisidine to dissolve with stirring using a glass rod.
10. Use bioinformatics programs for predicting solubility. Ex. PROSO sequence-based protein solubility evaluator (http://mips.helmholtz-muenchen.de/proso/proso.seam) [20].
11. The 11th edition of the pET systems manual is available from www.novagen.com under the Novagen technical bulletins category.
12. We have observed that reduced aeration increases the yield of heme-tagged proteins for unknown reasons [5]. Although the bacterial pellet size will be smaller, the yield of heme-tagged protein may significantly change. For example, we obtain ~1.5–2.0 mg/L bacterial culture for heme-tagged azurin with aeration (open container) versus 7.0–8.0 mg/L when foil is placed over the growth flask. It is advised to test both conditions when expressing any new heme-tagged protein.
13. This is the optimal time we found for heme-tagged azurins; optimal conditions may vary with POI.
14. As a negative control, the expression protocol used for a heme-tagged protein can be followed for the same protein in the absence of a HBP sequence. The pellet may have a green color (due to degraded heme species) if strategies to increase oxygenation (high shaking speeds, baffled flasks, open growth container) are used (*See* **Note 12**).
15. After incubation with the sucrose buffer, the pelleted cells will be loosely attached to the side of the container.
16. DNAase I can be denatured when vortexing or shaking vigorously.
17. Continued exposure to vacuum after the beads are dry could damage the resin. The Sepharose beads should form a pellet on the filter membrane that appears wet. Avoid pellets that look dry and cracked after the solutions are removed.

18. It is critical that the pH of the loaded lysate and the buffer in the pre-equilibrated column be near neutral for binding.
19. The lysate containing the heme-tagged protein will be dark brown in color (*see* Fig. 2, binding step) before adding the wash buffer. The brown color is indicative of an open coordination site at the heme-iron of the tags. After binding the HIS resin, the heme-tagged protein will change from brown to a bright red color (*see* Fig. 2, wash steps) due to the histidine residues of the HIS resin coordinating the tags.
20. The dark brown colored lysate will migrate down the column and elute at ~1 CV of wash buffer if the heme-tagged protein does not bind the HIS column.
21. The Hoefer SE 245 user's manual is online at http://www.hoeferinc.com under the mini vertical units section.
22. Add 15 μL of saturated sodium dithionite solution to an identical sample. The heme-tag should be fully reduced if the spectra are identical.
23. Sodium dithionite is a strong reductant that reacts with air immediately upon exposure. Minimize the time between adding the sodium dithionite solution to the protein samples and taking the absorption spectra.

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# Chapter 3

# Purification of a Recombinant Protein with Cellulose-Binding Module 3 as the Affinity Tag

**Dongmei Wang and Jiong Hong**

## Abstract

Easy-to-perform and low-cost protein purification methods are in high demand for the mass production of commonly used enzymes that play an important role in bioeconomy. A low-cost and rapid recombinant protein purification system was developed using CBM3 (family 3 cellulose-binding module) as affinity tag. This protocol describes the purification of CBM3-fusion protein and tag-free protein expressed in *Pichia pastoris* using CBM3 as an affinity tag.

**Key words** Cellulose-binding module, Affinity tag, Protein purification, Intein, Cellulose

## 1 Introduction

The purification of a given target protein can be significantly enhanced by genetically fusing it to an affinity tag. To date, affinity chromatography with various affinity tags on various resins is widely used in laboratories and biotechnology applications [1–4]. Most of the current affinity chromatography matrix such as Ni-NTA agarose and glutathione are very expensive [4], thus prompting the need for developing economical methods for protein production. Cellulose binding module (CBM) has been identified as a potential tool for the rapid and low-cost manufacture of recombinant proteins [1, 5].

CBM is an attractive affinity tag for protein purification because of its high capacity and specific adsorption to cellulose. In addition, it can be efficiently adsorbed to cellulose in most buffers and eluted under non-denaturing conditions. The features of low production cost, excellent physical properties, low nonspecific affinity to most proteins, and stability in most buffers make cellulose an ideal matrix for large-scale affinity protocols. In addition, cellulose is easily procured and safe for many pharmaceutical and human applications.

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The CBM tag has been used for recombinant protein purification of commercial cellulose matrix or powder (Avicel microcrystalline cellulose, Sigmacell amorphous cellulose) in different capacities [6–13], and these were reviewed by Shoseyov et al. [7, 14, 15].

Affinity tags are robust tools for protein purification. However, in some cases, the tag needs to be removed, such as those in pharmaceutical proteins. Inteins can excise themselves and/or rejoin 2 fragments together by changing the pH or thiol reagent concentration of the solution. To avoid using costly peptide-specific proteases and to simplify the purification process, self-cleaving inteins are often used [4, 12, 16, 17, 18–21, 22].

The method of purification of the recombinant protein with a CBM3 tag was developed in both *Escherichia coli* and yeast, and has been proven successful in purifying several proteins [12, 17, 18]. This study introduced the recombinant protein expressed in yeast. EGFP was used to demonstrate the purification procedure, step by step. A self-cleaving intein-based tag removal method for CBM3 was also introduced. Figure 1 shows the procedure of CBM3-tagged protein purification, which includes centrifugation and column routes.

## 2 Materials

Prepare all solutions with ultrapure water and analytical grade reagents. Store all reagents at appropriate conditions.

### 2.1 Preparation of Regenerated Amorphous Cellulose

1. 85 % $H_3PO_4$, analytical grade.
2. 2 M $Na_2CO_3$.
3. Microcrystalline cellulose (FMC PH-105) (*see* **Note 1**).

### 2.2 Expression of CBM3-Fused Recombinant Protein

1. YPD medium: 1 % yeast extract, 2 % peptone, 2 % dextrose (for yeast cell preculture in recombinant protein expression).
2. Buffered Glycerol-complex (BMGY) medium: 1 % yeast extract, 2 % peptone, 100 mM potassium phosphate (pH 6.0), 1.34 % Yeast Nitrogen Base without Amino Acids (YNB), $4 \times 10^{-5}$ % biotin, 1 % glycerol. Prepare according to instructions described in the EasySelect *Pichia* Expression Kit (Invitrogen).
3. Buffered methanol-complex (BMMY) medium: 1 % yeast extract, 2 % peptone, 100 mM potassium phosphate (pH 6.0), 1.34 % YNB, $4 \times 10^{-5}$ % biotin with 0.5 % methanol.
4. Sorbitol, analytical grade.
5. Zeocin™ (Invitrogen).
6. Methanol, analytical grade.

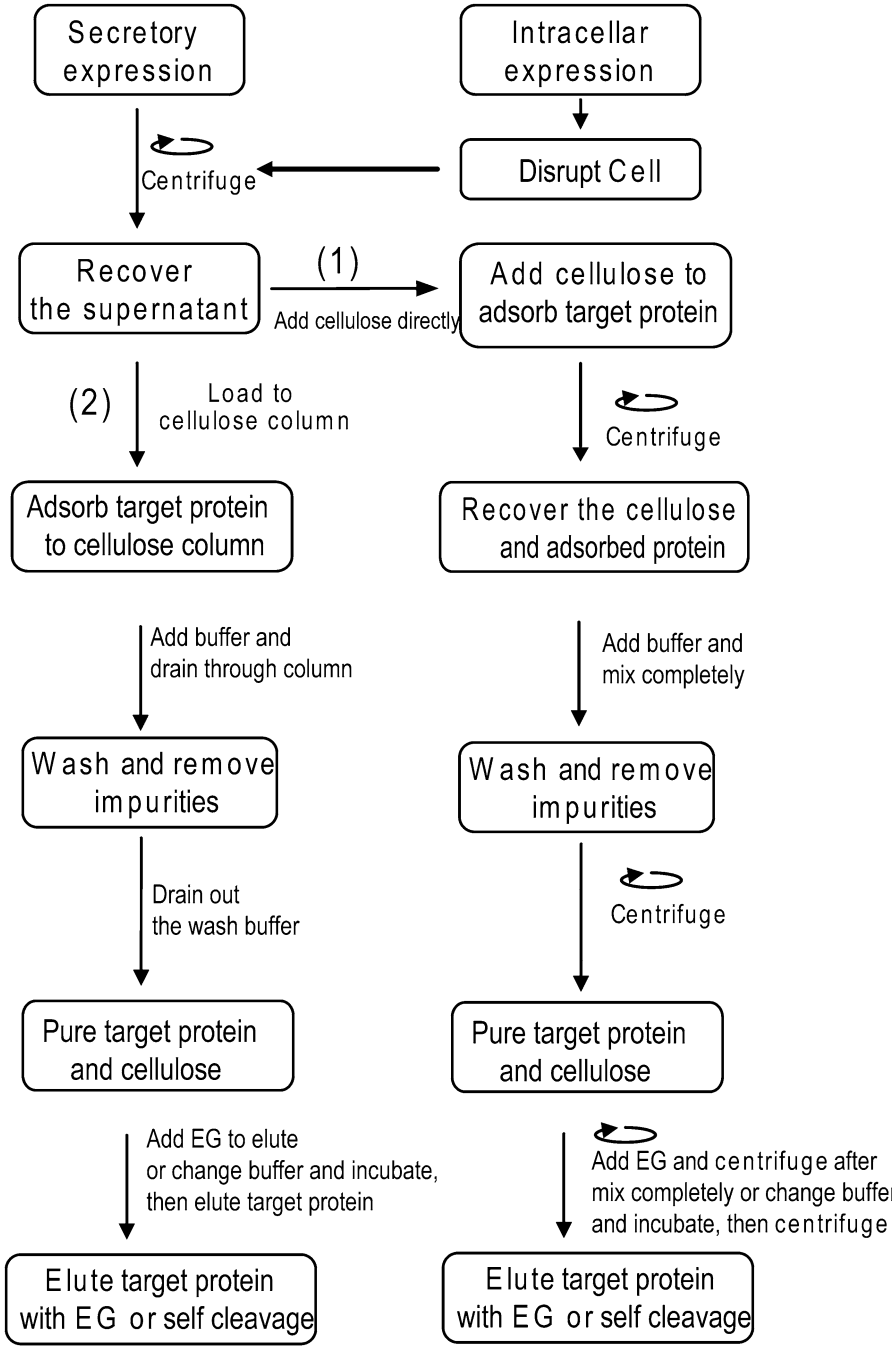


**Fig. 1** Schematic representation of CBM3-tagged protein purification with regenerated amorphous cellulose (RAC) adsorption: (1) centrifugation route, (2) column route

7. Expression plasmids pPCG and pGIC, for expression of CBM3-GFP and GFP-intein-CBM3, respectively, as described elsewhere [18] (available upon request).
8. *Pichia pastoris* KM71H, for recombinant protein expression.
9. Erlenmeyer flask (1 L, 2 L).
10. *Sac*I restriction enzyme (New England Biolabs, Waverly, MA).

### 2.3 Purification of CBM3-Fused Recombinant Protein

1. Ethylene glycol (EG), analytical grade.
2. Regenerated amorphous cellulose (RAC), prepared from microcrystalline cellulose (FMC PH-105) (*see* **Note 1**).
3. 50 mM Tris–HCl buffer (pH 8.0).
4. 50 mM Tris–HCl buffer (pH 6.5), containing 0.5 M NaCl and 1 mM EDTA.
5. Fisher Scientific Sonic Dismembrator Model 500 (*see* **Note 2**).
6. Slide-A-Lyzer™ dialysis cassettes (Thermo Fisher Scientific Inc. Rockford, IL USA) or dialysis tubing.
7. Cell lysis buffer: 20 mM Tris–HCl buffer (pH 8.5), 500 mM NaCl, and 1 mM EDTA.

### 2.4 Electrophoresis

For SDS-polyacrylamide gel electrophoresis (PAGE), analytical grade chemicals are used with the buffer system, according to Laemmli [23]. A 12 % resolving gel is used in this study.

## 3 Methods

Both of the recombinant proteins expressed in *E. coli* and yeast can be purified with the CBM3 affinity tag. This protocol introduces the purification of recombinant protein expressed in yeast. EGFP is used to demonstrate the purification procedure, step by step. A self-cleaving intein-based tag removal method is used to remove CBM3.

### 3.1 RAC Preparation [17]

1. Add 0.2 g of microcrystalline cellulose (FMC PH-105) to a 50-mL centrifuge tube and then add 0.6 mL distilled water to wet the cellulose powder and develop cellulose-suspended slurry.
2. Slowly add 10 mL of ice-cold 86 % $H_3PO_4$ to the slurry with vigorous stirring. Let the solution stand for 1 h on ice with occasional stirring.
3. Add 40 mL of ice-cold water at a rate of approximately 5 mL per addition with vigorous stirring between additions, resulting in a whitish, cloudy precipitate.
4. Centrifuge the precipitated cellulose at 3,000 × *g* for 10 min at 4 °C, using a swing-bucket rotor to remove the supernatant containing phosphoric acid.

5. Resuspend the pellet in ice-cold water and then centrifuge at 3,000 × *g* for 10 min at 4 °C, using a swing-bucket rotor to remove the supernatant containing phosphoric acid again.
6. Repeat **step 5** four times.
7. Resuspend the cellulose pellet with 0.5 mL of 2 M $Na_2CO_3$ and 45 mL of ice-cold distilled water.
8. Centrifuge the solution at 3,000 × *g* for 10 min at 4 °C, using a swing-bucket rotor. Resuspend the pellet in distilled water.
9. Repeat **step 8** once or until the solution reaches pH 5–7 (*see* **Note 3**).
10. Store the RAC slurry as a 10 g RAC/L suspension solution at 4 °C (*see* **Note 4**).

### 3.2 Purification of CBM3 Fused Recombinant Protein (CBM3-GFP)

#### 3.2.1 Expression of CBM3-GFP

1. Linearize the expression plasmid pPCG with *Sac*I and transform into *P. pastoris* KM71H by electroporation, as described in the manual of EasySelect *Pichia* Expression Kit with minor modifications [24]. Spread the transformed cells on YPDS (YPD medium containing 1 M sorbitol) plates containing 100 μg/mL Zeocin™.
2. Incubate the cultures for 3 days at 30 °C, then pick 96 colonies and inoculate onto an YPDS plate containing 1,000 μg/mL Zeocin™ to select for multicopy integrated strains.
3. Inoculate the selected multicopy integrated strains into 500 mL of BMGY medium in a 2-L Erlenmeyer flask and incubate for 24 h at 30 °C, with constant shaking at 250 rpm.
4. Recover the cells by centrifugation at 4,000 × *g* for 10 min at room temperature. Resuspend the cell pellet in 100 mL BMMY medium in a 1-L Erlenmeyer flask. Incubate the culture for 5 days at 30 °C, with constant shaking at 250 rpm. To maintain cell induction, add 0.5 mL of 100 % methanol every 24 h.
5. Harvest the culture supernatant by centrifugation at 4 °C and directly proceed to the purification step (*see* **Note 5**).
6. Roughly estimate the amount of target protein (CBM3-EGFP) in the supernatant by SDS-PAGE.

#### 3.2.2 Purification of CBM3-GFP

After centrifugation, the supernatant is ready for purification. It can be purified through two different purification routes (Figs. 1 and 2):

*Centrifugation route (Fig. 2a)*:

1. Mix the supernatant with the RAC slurry. Estimate the RAC amount by using the ratio of 200 mg of CBM3-GFP to 1 g of RAC (i.e., RAC was in excess to adsorb >90 % of the target protein) (Fig. 2a-2) (*see* **Note 6**).
2. After adsorption at 4 °C for 10–15 min (*see* **Note 7**) and centrifugation, resuspend the RAC pellet in a fivefold RAC volume of 50 mM Tris–HCl buffer (pH 8.0)(Fig. 2a-4) (*see* **Note 8**).

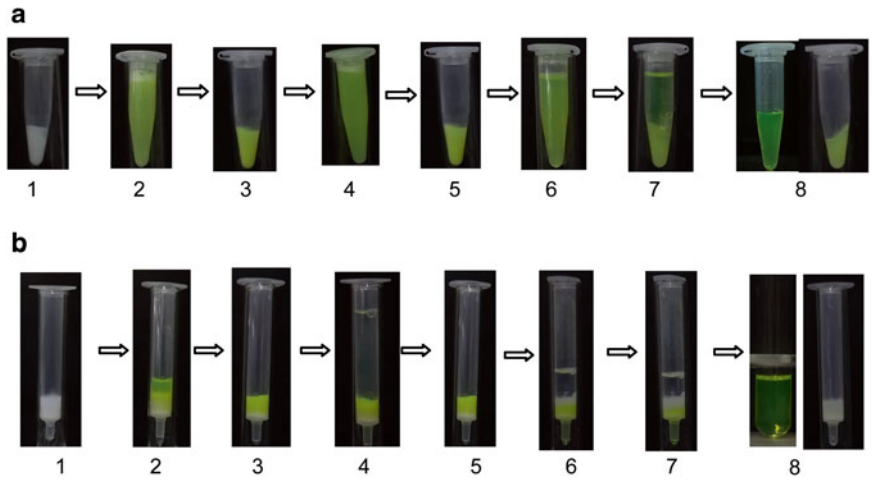


**Fig. 2** Purification of CBM3-EGFP with regenerated amorphous cellulose (RAC) (*see* **Note 9**). (**a**) Purification of CBM3-EGFP through the centrifugation route. *1* RAC, *2* supernatant + RAC, *3* RAC pellet with the adsorbed target protein, *4* RAC pellet resuspended in 50 mM Tris–HCl wash buffer (pH 8.0), *5* washed RAC pellet, *6* RAC pellet with adsorbed protein resuspended in 80 % EG, *7* centrifugation to separate the RAC and eluted CBM3-EGFP, *8* RAC pellet after EG elution and the purified CBM3-tagged protein. (**b**) Purification of CBM3-EGFP through the column route. *1* Column filled with RAC, *2* partly loaded column with supernatant, *3* fully loaded column with supernatant, *4* washing off of impurities by using Tris–HCl buffer (100 mM, pH 8.0), *5* draining out the wash buffer, *6* addition of 80 % EG to elute CBM3-EGFP, *7* CBM3-EGFP elution, *8* purified CBM3-EGFP and eluted RAC column

3. After another round of centrifugation (to remove impure proteins in the RAC matrix), resuspend the RAC pellet (containing the adsorbed CBM3-target protein) in 4 RAC pellet volumes of 100 % ethyl glycol (EG) to obtain 80 % (v/v) of the final EG concentration (Fig. 2a-6) (*see* **Note 10**).
4. Elute the purified CBM3-EGFP protein by centrifugation 12,000 × *g* and recover the pure protein in the supernatant (Fig. 2a-7). The purified protein can be stored in EG solution at −20 °C (*see* **Note 11**).

*Column route (Fig. 2b)* (*see* **Note 12**):

1. Load the supernatant onto an RAC column as in most affinity chromatography protocols. Adsorb the target protein (CBM3-EGFP) to the column (Figs. 2b-2 and 3).
2. After loading the sample and allowing adsorption onto the RAC column, use a fivefold RAC volume of 50 mM Tris–HCl buffer (pH 8.0) to wash the column and remove impurities in the RAC matrix (Fig. 2b-4).
3. Elute the target protein by using a fourfold RAC volume of 80 % ethyl glycol (EG). Collect the eluted fraction in several tubes, similar to routine chromatographic protocols (Fig. 2b-6,7). The fraction containing CBM3-GFP can be detected by the presence

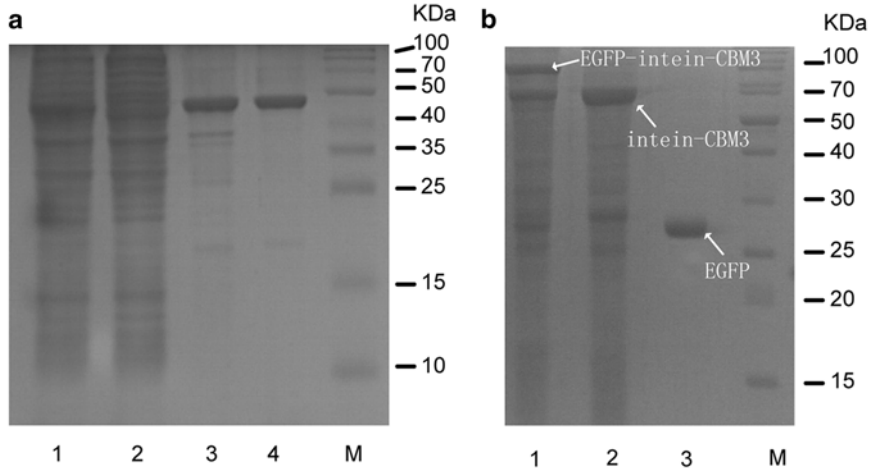


**Fig. 3** SDS-PAGE analysis of protein purification with CBM3 tag. (**a**) CBM3-EGFP eluted by EG. *Lane 1* crude supernatant, *lane 2* supernatant after RAC adsorption, *lane 3* CBM3-EGFP adsorbed to RAC (on RAC), *lane 4* pure CBM3-EGFP eluted by EG. (**b**) Tag-free EGFP eluted through intein self-cleavage. *Lane 1* EGFP-intein-CBM3 adsorbed onto RAC, *lane 2* after self-cleavage, the CBM3-intein was retained on RAC, *lane 3* pure tag-free EGFP eluted through intein self-cleavage

of green fluorescence, as described in Subheading 3.4, **step 1** (*see* **Notes 13** and **14**) and analyze using SDS-PAGE (Fig. 3a).

4. Store the purified protein in EG solution at −20 °C (*see* **Note 11**).

### *3.3 Purification of Tag-Free Protein with CBM3 as Affinity Tag (EGFP-Intein-CBM3)*

#### *3.3.1 Expression of EGFP-Intein-CBM3*

1. Linearize the expression plasmid (pGIC) with *Sac*I and transform into *P. pastoris* KM71H by electroporation.
2. Select the strains with multiple copies of the *GIC* gene on a YPD plate containing 1,000 μg/mL Zeocin™ and express as described in Subheading 3.2.1.
3. Harvest the cells by centrifugation at 4 °C and wash once with cell lysis buffer (20 mM Tris–HCl buffer pH 8.5, 500 mM NaCl, and 1 mM EDTA). Resuspend the pellet in ~30 mL of cell lysis buffer.
4. Disrupt the cells in an ice bath by means of ultrasonication (Fisher Scientific Sonic Dismembrator Model 500) (*see* **Note 15**). After centrifugation, obtain the supernatant of lysate (GIC) and proceed to the purification step (*see* **Note 16**).

#### *3.3.2 Purification of the Tag-Free Protein (EGFP) (See **Note 17**)*

The tag-free protein also can be purified using two different purification routes.

*Centrifugation route*

1. Mix the cell lysate with the RAC slurry. Estimate the RAC amount by using a ratio of 400 mg of GIC to 1 g of RAC (i.e., RAC was in excess to adsorb >90 % of the target protein) (*see* **Note 6**).

2. After adsorption for 10–15 min at 4 °C (*see* **Note 7**) followed by centrifugation, resuspend the RAC pellet in a fivefold RAC volume of 50 mM Tris–HCl buffer (pH 8.0) (*see* **Note 8**).
3. After another round of centrifugation (to remove impurities in the RAC matrix), resuspend the RAC pellet (adsorbed with EGFP–intein–CBM3 protein) in a fourfold RAC pellet volume of 50 mM Tris–HCl buffer (pH 8.0) containing 0.5 M NaCl, 1 mM EDTA, and 50 mM L-cystine or 100 mM DTT (*see* **Note 18**).
4. Incubate the solution for 12 h at 37 °C (Fig. 3b) (*see* **Note 19**).
5. Recover the cleaved EGFP in 1.5 mL tube by centrifugation at 12,000 × *g* at 4 °C for 5 min using angle rotor or in other bigger tube at 3,000 × *g* for 10 min using a swing-bucket rotor. The tag-free EGFP is in the supernatant.
6. Store the purified protein at −20 °C.

*Column route* (*see* **Note 12**):

1. Load the cell lysate onto an RAC column similar to most affinity chromatography protocols. Allow the target protein (EGFP-intein-CBM3) to adsorb onto the column.
2. After loading the sample onto the RAC column, use a fivefold RAC volume of 50 mM Tris–HCl buffer (pH 8.0) to wash the column, in order to remove impurities in the RAC matrix.
3. Add a threefold RAC volume of 50 mM Tris–HCl buffer (pH 8.0) containing 0.5 M NaCl, 1 mM EDTA, and 50 mM L-cystine or 100 mM DTT until the buffer is drained out (*see* **Note 18**), then seal the column with a cap.
4. Incubate the column for 12 h at 37 °C (Figs. 1 and 3b, *see* **Note 19**).
5. Open the cap and elute the tag-free EGFP with a threefold RAC volume of 50 mM Tris–HCl buffer (pH 8.0). Collect the eluted fraction in several tubes similar to general chromatography protocols. The fraction containing EGFP can be detected by the presence of green fluorescence, as described in Subheading 3.4, **step 1** and analyze the fraction by SDS-PAGE (Fig. 3b, *see* **Notes 20** and **14**).

### 3.4 EGFP Fluorescence Detection and Protein Analysis

1. Determine EGFP fluorescent intensity (excitation at 485 nm, emission at 528 nm) by using a SpectraMax M5 Multi-Mode Microplate Readers (Molecular Devices Inc., Sunnyvale, CA; *see* **Note 21**).
2. Determine the protein concentration by using the Bradford method, with bovine serum albumin as standard [25].
3. Perform SDS-PAGE with a gel containing 12 % acrylamide and 0.1 % SDS (w/v) and Tris–glycine buffer [23]. Following separation, visualize the resolved proteins with Coomassie Brilliant Blue R-250 staining.

## 4 Notes

1. Sigmacell or other cellulose powders can be used to replace FMC PH105.
2. Any other cell disruptors can also be used.
3. No detectable amount of cellulose (<1 % wt.) was lost during the treatment at most times. In general, more than 0.19 g RAC can be prepared from 0.2 g Avicel.
4. It can be stored in the presence of 0.2 % (w/v) sodium azide for an extended period (e.g., 1 year).
5. CBM3-GFP was expressed under α-factor signal sequence and is secreted into the medium.
6. Other proteins can be calculated by using 3.0 μmol to 1 g RAC.
7. Diluted sample needs longer time (e.g., 30 min or 1 h).
8. Repeat this step to increase the purity.
9. In this figure, 1.5-mL Eppendorf tubes were used. For a larger volume of a sample, a larger centrifuge tube or column can be used instead.
10. To obtain higher concentrations of the protein, alternatively, iterative elution with a small volume of EG (e.g., 1:1 volume of the RAC pellet/elution solution) can be used.
11. EG can be removed through dialysis and the diluted protein can be either re-concentrated by ultrafiltration, using centrifugal tubes or used directly. Although glycerol can be used to elute the purified protein instead of EG, its high viscosity makes it less ideal for this method.
12. In the column route, the procedure will take a longer time and thus will not be convenient for a larger volume sample; however, a higher concentration, smaller volume of target protein, and greater recovery rate can be obtained.
13. As an alternative, mix the EG with RAC and centrifuge the column in a bigger tube which is compatible with a centrifuge rotor for elution. The speed is dependent on the tolerance of the tube and column. To increase the yield and the concentration of protein, a twofold volume of RAC EG elution is recommended.
14. Other proteins can be detected by SDS-PAGE, ultraviolet light absorption at 280 nm, or enzymatic activity.
15. Set Fisher Scientific Sonic Dismembrator Model 500 at 5-s pulses for a total 10 min, at 40 % strength.
16. The GIC was intracellularly expressed.
17. To obtain the tag-free protein through the intein-*Sce* VMA after impurities were removed, the cellulose-bound target

protein was resuspended in a buffer containing thiols (DTT or cysteine) to induce intein self-cleavage, and the target protein was cleaved from CBM3-intein through intein itself. The tag-free protein was then eluted through the general elution procedure or centrifugation.

18. The concentration of L-cystine or DTT can be changed on the basis of the characteristics of the protein of interest.
19. Higher temperature and longer time can enhance cleavage; however, denaturation of the target protein should be avoided.
20. As an alternative, mix 1 volume of RAC elution buffer (50 mM Tris–HCl buffer [pH 8.0] containing 0.5 M NaCl, 1 mM EDTA, and 50 mM L-cystine or 100 mM DTT) with RAC and centrifuge the column in a bigger tube which is compatible with the centrifuge rotor.
21. Any other fluorescence photometers or microplate reader that can measure fluorescence intensities can also be used.

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# Chapter 4

# Purification of *E. coli* Proteins Using a Self-Cleaving Chitin-Binding Affinity Tag

**Michael J. Coolbaugh and David W. Wood**

## Abstract

The use of affinity tags to purify recombinant proteins is ubiquitous in molecular biology. However, tag removal after purification still remains a challenge. The most commonly used method, proteolytic digestion, has several drawbacks that make the process complex and costly. One alternative to the use of proteolytic digestion is the use of self-cleaving purification tags. Here, we describe a system that combines a chitin-binding domain (CBD) tag with the ΔI-CM intein to yield a self-cleaving purification tag. A protein gene of interest is genetically fused downstream of the tag, generating a fusion protein that can be rapidly and easily purified using a chitin resin. Intein self-cleavage is then induced by a simple pH and temperature shift, liberating the free target protein. This system can be used to readily purify any recombinant protein that can be expressed in *E. coli*, and has the potential to be applied to a wide variety of additional tags and expression hosts.

**Key words** Protein purification, Intein, Affinity chromatography, Self-cleaving tag, *E. coli*, Purification platform, Chitin binding domain

## 1 Introduction

One of the most effective methods for laboratory scale purification of recombinant protein is the use of affinity tags [1–4]. This method provides a rapid, simple, and general means of protein purification that can be used with any well-expressed protein of interest. These features have made the use of affinity tags, and the polyhistidine tag in particular, ubiquitous in molecular biology laboratories throughout the world [5]. There are some drawbacks to the use of this technology, however, which have limited its applicability in some cases. One of the major drawbacks is the difficulty in removing the tag after initial purification. The most commonly used method for tag removal is proteolytic cleavage using a highly specific exogeneous endopeptidase [6]. Difficulties associated with this step include nonspecific cleavage of the target protein, incomplete cleavage of the tag, and the requirement that the tag, endopeptidase, and uncleaved target protein be separated from the

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cleaved target before use. Further, proteolytic treatment can be costly, which has effectively prevented its use at large scale. These drawbacks have been recently addressed through the development of a variety of self-cleaving protein modules, which can be combined with conventional affinity tags to make them effectively self-cleaving [7, 8]. Several of these self-cleaving modules are based on inteins, and can be induced to self-cleave through the addition of thiol compounds (N-terminal cleaving), or through changes in pH and temperature (C-terminal cleaving). These methods provide a compelling alternative to proteolytic tag removal for the delivery of an untagged native target protein. The method described here uses a chitin-binding domain (CBD) tag developed by NEB, which has been incorporated into their commercially available IMPACT system [9, 10]. This system relies on a CBD tag fused to a thiol-inducible intein, thus generating a self-cleaving tag module. However, the use of thiol to induce intein self-cleavage has complicated the use of the IMPACT system for the purification of proteins containing disulfide bonds. For this reason, we have combined the CBD tag with the pH-inducible ΔI-CM intein developed in our own laboratories, which exhibits rapid and highly controllable C-terminal cleaving activity [11, 12]. This intein has been combined with a variety of tags to produce a robust and versatile self-cleaving purification module [13–17]. The target gene of interest is cloned downstream of the self-cleaving tag module and then overexpressed in *E. coli* using the T7 promoter system. The fusion protein can then be readily purified using a chitin resin, where the tight binding affinity of the CBD delivers a highly purified target. A simple pH shift induces on-column intein cleavage, which allows elution of pure target protein. This allows for rapid and simple purification of any protein that can be expressed in *E. coli*.

## 2 Materials

### 2.1 Cloning of Target Protein Gene

1. Luria–Bertani (LB) medium: 1 % tryptone, 0.5 % yeast extract, 1 % NaCl (w/v) at pH 7.0, sterilize by autoclaving for 20 min at 121 °C. Store at room temperature.
2. 50 mg/ml Ampicillin, sterilized by filtration. Store in 1 ml aliquots at −20 °C.
3. 5× Phusion HF Buffer (New England Biolabs (NEB), Ipswich, MA, USA). Store at −20 °C (*see* **Note 1**).
4. 10 μM forward PCR Primer. Store at −20 °C (*CRITICAL*: *see* **Notes 2** and **3** for proper primer design).
5. 10 μM reverse PCR Primer. Store at −20 °C (*see* **Note 4**).
6. 2 mM each deoxynucleotide triphosphates (dNTP mix) (NEB). Store at −20 °C.

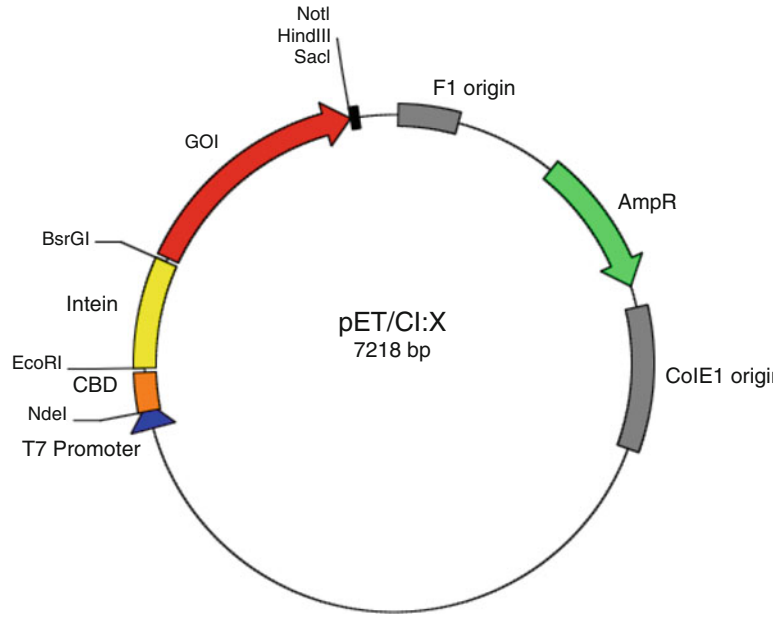


**Fig. 1** Plasmid map for the pET/CI:X expression plasmid. Note that the target gene of interest (GOI) can be cloned using a unique BsrGI site upstream and either SacI, HindIII, or NotI downstream

7. Phusion High-Fidelity DNA Polymerase (NEB). Store at –20 °C (*see* **Note 5**).
8. Seakem LE Agarose (or equivalent), 1× TAE running buffer, GelRed (Phenix Research, Candler, NC, USA) (*see* **Note 6**).
9. PCR Purification Kit (Qiagen, Valencia, CA, USA). Follow manufacturer's instructions. Store at room temperature (*see* **Note 7**).
10. Gel Extraction Kit (Qiagen). Follow manufacturer's instructions. Store at room temperature (*see* **Note 8**).
11. Restriction Enzymes and associated buffers (NEB). Typically, we use BsrGI and HindIII. Store at –20 °C (*see* **Notes 2** and **4**).
12. Z-competent *E. coli* transformation kit (Zymo Research Corporation, Irvine, CA, USA). Used to make *z*-competent cells. Store at 4 °C (*see* **Note 9**).
13. Z-competent *E. coli* DH5α. Store at -80 °C (*see* **Note 10**).
14. Z-competent *E. coli* BLR(DE3). Store at -80 °C (*see* **Note 11**).
15. pET CBD intein expression vector, pET/CI:X (contact authors for availability) (Fig. 1).

### 2.2 Expression of CI:X Fusion Protein

1. Luria–Bertani (LB) medium: 1 % tryptone, 0.5 % yeast extract, 1 % NaCl (w/v) at pH 7.0, sterilize by autoclaving for 20 min at 121 °C. Store at room temperature.

2. 2× LB Medium: 2 % Tryptone, 1 % Yeast Extract, 1 % NaCl (w/v) at pH 7.0, sterilize by autoclaving for 20 min at 121 °C. Store at room temperature (*see* **Note 12**).
3. 50 mg/ml Ampicillin, sterilized by filtration. Store in 1 ml aliquots at −20 °C.
4. 0.1 M IPTG (isopropyl-beta-D-thiogalactopyranoside) in deionized water, sterilize by filtration. Store at −20 °C.
5. Column Buffer, pH 8.5: 20 mM AMPD, 20 mM PIPES, 1 mM EDTA, 500 mM NaCl, 1 mM DTT. Titrated to pH 8.5 with HCl. Store at 4 °C (*see* **Note 13**).

### 2.3 Packing Chitin Column

1. Chitin resin (NEB). Suspended in 20 % ethanol. Store at 4 °C.
2. Disposable Chromatography Columns (Bio-Rad, Hercules, CA, USA) (*see* **Note 14**).
3. Column Buffer, pH 8.5.

### 2.4 Purification of Target Protein

1. Column Buffer, pH 8.5.
2. Column Buffer, pH 6.5: 20 mM AMPD, 20 mM PIPES, 1 mM EDTA, 500 mM NaCl, 1 mM DTT. Titrated to pH 6.5 with HCl. Store at 4 °C.
3. 1 % SDS (w/v). Store at 25 °C.
4. 20 % Ethanol (v/v). Store at 4 °C.

## 3 Methods

### 3.1 Cloning of Target Protein Gene (See Note 15)

1. Amplify the target gene using a 50 µl PCR reaction. The PCR reaction will contain 26.5 µl sterile $H_2O$, 10 µl 5× Phusion HF Buffer, 5 µl Forward PCR Primer, 5 µl Reverse PCR Primer, 2 µl template (~2 ng/µl), 1 µl dNTP mix, 0.5 µl Phusion-HF Polymerase (*see* **Note 6**).
2. The PCR reaction should consist of the following steps: 60 s at 98 °C, 25 cycles at 10 s at 98 °C, 15 s at 50 °C, 30 s/kb at 72 °C, then finally 10 min at 72 °C (*see* **Notes 16** and **17**).
3. Analyze the PCR reaction on a 1 % agarose gel containing GelRed (*see* **Note 6**).
4. Purify PCR product using PCR Purification Kit (Qiagen), according to manufacturer's instructions (*see* **Note 7**).
5. Estimate the concentration of PCR product and pET/CI:X plasmid by measuring the absorbance at 260 nm using a spectrophotometer. An absorbance of 1 corresponds to a concentration of 50 µg/µl double stranded DNA.
6. Digest approximately 1 µg each of the amplified target gene (insert) and the pET/CI:X plasmid (vector), according to

manufacturer's instructions for the chosen restriction enzymes. Typically, a 40 µl digest reaction will include 20 µl H2O, 4 µl 10× NEB Buffer, 4 µl 10× BSA (if required), 10 µl DNA, 1 µl each of restriction enzyme. Incubate at 37 °C for 3 h.

7. Separate the digested DNA bands by electrophoresis on a 1 % agarose gel containing GelRed. Excise the insert and vector bands from the gel using a UV gel box and a clean razor blade.
8. Purify the DNA from the gel using the Gel Extraction Kit (Qiagen), according to manufacturer's instructions (*see* **Note 8**).
9. Ligate the insert and vector in a 5:1 molar ratio of insert–vector. Typically, a 10 µl reaction contains 2.5 µl $H_2O$, 5 µl purified insert, 1 µl purified vector, 1 µl 10× ligase buffer, 0.5 µl T4 DNA ligase.
10. Incubate at 25 °C for 1 h or at 16 °C for 3 h.
11. Transform 1–10 µl of the ligation mixture into Z-competent DH5α or other appropriate cloning strain as per manufacturer's instructions. For Z-competent cells, thaw cells on ice, then add up to 5 % total volume of DNA and flick to mix. A typical transformation includes a single 100 µl aliquot of cells and up to 5 µl of ligation mixture. Incubate on ice for 5 min after DNA has been added (*see* **Note 18**).
12. Spread transformation mixture on an LB-agar plate containing 100 µg/ml ampicillin. Incubate overnight at 37 °C.
13. Screen colonies by digesting miniprep DNA with BsrGI and HindIII/NotI/SacI enzymes to visualize the inserted target protein gene by DNA electrophoresis (*see* **Note 19**).
14. Transform 1 µl of confirmed miniprep DNA (~50–100 ng) into BLR(DE3) Z-competent cells or other appropriate expression strain. Spread on LB-agar plate containing 100 µg/ml ampicillin. Incubate overnight at 37 °C.

### 3.2 Expression of CI:X Fusion Protein

1. Use a single colony of transformed BLR(DE3) to inoculate a 5-ml culture of LB medium supplemented with 100 µg/ml ampicillin in a test tube. Incubate at 37 °C overnight with agitation (*see* **Note 20**).
2. Dilute the overnight culture 1:100 into 2×-LB with 100 µg/ml that has been prewarmed to 37 °C. Typical expression culture size is 50–100 ml (*see* **Note 21**).
3. Incubate with shaking at 37 °C till cells are in mid-log phase, as indicated by an optical density at 600 nm wavelength (OD600) of about 0.4–0.6. This typically takes between 2.5 and 3 h (*see* **Note 22**).
4. Shift the culture temperature to 16 °C and equilibrate for 15–30 min (*CRITICAL: see* **Note 23**).

5. Take a 1-ml sample of the culture before induction (for **Pre-Induction** sample, see below). Harvest this 1-ml sample by centrifugation, 5,000 × *g* for 5 min at 4 °C. Discard supernatant and resuspend sample in 100 µl Column Buffer, pH 8.5. Freeze this sample at –20 °C until cell lysis step below.
6. Induce protein expression by addition of IPTG, final concentration 0.5–1 mM. Express at 16 °C for 8–24 h (*see* **Note 24**).
7. After expression, harvest cells by centrifugation, 5,000 × *g* for 10–30 min at 4 °C. From this point on, keep samples at 4 °C unless noted otherwise. Centrifugation time is dependent on culture size.
8. Discard the supernatant and resuspend the cells in 10 % of the original culture volume of ice-cold Column Buffer, pH 8.5 (*see* **Note 25**).

### 3.3 Packing Chitin Column

1. Chitin beads are stored at 4 °C in a slurry of 20 % ethanol. Shake well to resuspend the beads, and then add 1.5 times the desired column bed volume of slurry to disposable chromatography column. Typically, a 4-ml bed volume column is sufficient for a 100-ml culture. Note that references to column volume (CV) from this point forward refer to the bed volume (*see* **Note 26**).
2. Make sure that column is level, and then allow excess slurry volume to drain by gravity with valve fully open.
3. Wash the column with 8 CV's Column Buffer, pH 8.5, with valve fully open. Let the column run almost dry to ensure that the bed is properly packed, and then add a small amount of buffer to the top of the column. Cap the column to prevent buffer leakage.

4 Store the packed column at 4 °C with an excess of buffer above the resin until ready for use.

### 3.4 Purification of Target Protein

1. Lyse cells by sonication on ice. Typical sonicator settings are ~4–5 W RMS (*see* **Note 27**). Take sample, **Whole Lysate**, for SDS-PAGE (*see* **Notes 28** and **29**).
2. Also lyse the pre-induction sample and take for SDS-PAGE analysis. Take sample, **Pre-Induction**, for SDS-PAGE.
3. Clarify lysate by centrifugation at 14,000 × *g* for 10–30 min at 4 °C. If clarifying larger volumes, centrifuge at 10,000 × *g* for 20–40 min. Take sample, **Clarified Lysate**, for SDS-PAGE.
4. Wash the column with 10 CV's column buffer, pH 8.5, with valve fully open. Do not let the column run dry after this point.
5. Dilute clarified lysate 1:5 in Column Buffer, pH 8.5. Place a collection tube under the column to collect flowthrough. Make sure the valve is closed, and then add the sample to the

top of the column. Adjust the valve to give a flow rate of approximately one drop every 10 s. Adjust flow rate halfway through to account for loss of head volume. Take sample, **Load Flowthrough**, for SDS-PAGE.

6. Wash with 12 CV's column buffer, pH 8.5, with valve fully open and collect in single tube. Take sample, **Wash pH 8.5**, for SDS-PAGE.
7. Wash with 8 CV's column buffer, pH 6.5, with valve fully open and collect in a single tube. Take sample, **Wash pH 6.5**, for SDS-PAGE.
8. Stop column flow, remove the column from the cold room and add a small amount of buffer, pH 6.5, to the top of the column.
9. Take a pre-cleaving sample of the resin (*see* **Note 30**).
   (a) Take sample, **Resin 1**, for SDS-PAGE.
10. Incubate the column at 25 °C for 20–24 h to allow the intein to cleave (*see* **Note 31**).
11. Take a post-cleaving sample of the resin (*see* **Note 30**).
    (b) Take sample, **Resin 2**, for SDS-PAGE.
12. Elute cleaved target protein from column with 2 CV's Column Buffer, pH 6.5. Collect in single fraction or multiple 1-ml fractions. Take sample, **Elution(s)**, for SDS-PAGE.
13. Wash the column with 3 CV's 1 % SDS with valve fully open to regenerate. Incubate at 25 °C for 30 min.
14. Wash the column with an additional 7 CV's 1 % SDS and then wash with 20 CV's deionized water.
15. Finally wash with 5 CV's Column Buffer, pH 8.5. Column can be regenerated 4–5 times (*see* **Note 32**).
16. Analyze all samples using SDS-PAGE by the standard methods [18]. Typical results are shown in Fig. 2.

## 4 Notes

1. High-Fidelity buffer generally works well. However, GC buffer can also be used for GC rich amplicons.
2. Cloning into the intein vectors is typically done using a unique BsrGI site that has been engineered into the C terminus of the intein. However, the BsrGI site does NOT include the final 4 bases of the intein (CAAC). So, to maintain the proper reading frame and intein activity, forward primers should include the sequence **TGTACA**CAAC, (BsrGI in bold) followed by the N-terminal sequence of the target protein. An example is shown in Fig. 3.

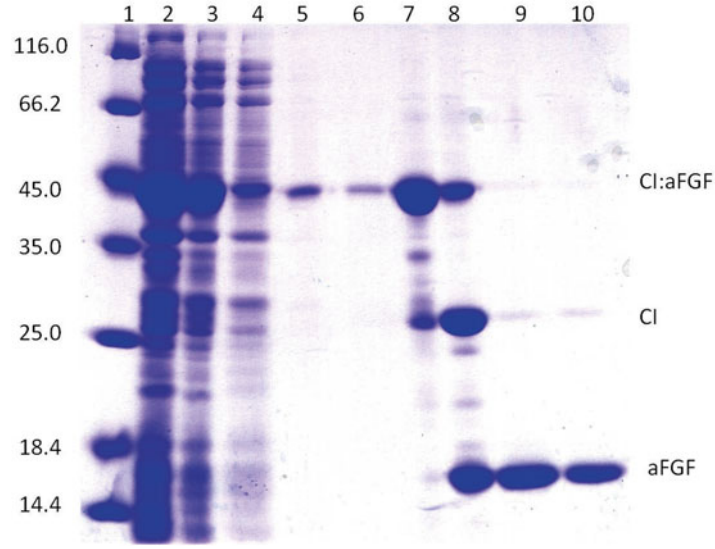


**Fig. 2** Typical purification results. Acidic fibroblast growth factor (aFGF) is purified as an example protein. *Lanes*: *1* = Marker; *2* = **Whole Lysate**; *3* = **Clarified Lysate**; *4* = **Load Flowthrough**; *5* = **Wash**, **pH 8.5**, *6* = **Wash**, **pH 6.5**; *7* = **Resin 1**; *8* = **Resin 2**; *9* = **Elution 1**; *10* = **Elution 2**. Note the presence of predominantly uncleaved precursor in the lysate and pre-cleaving resin sample, and then the appearance of cleaved precursor and product in the post-cleaving resin sample. Finally, note the presence of pure target protein in the elutions. The elutions correspond to 1 CV each. There was no pre-induction sample loaded onto this gel

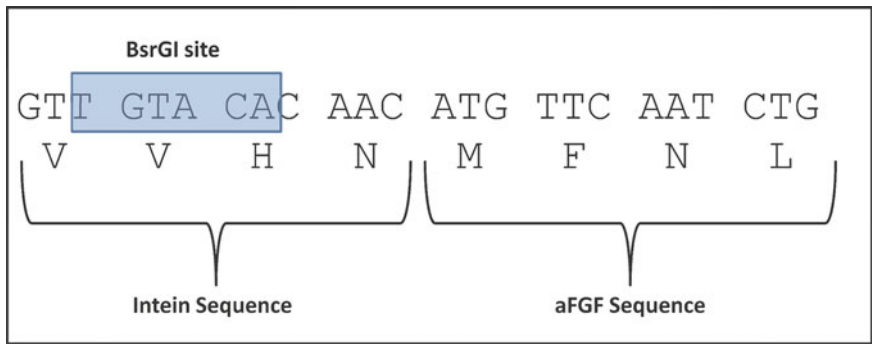


**Fig. 3** Coding sequence for the intein–target protein junction, with aFGF as a representative target protein. Note that the sequence "CAAC" must be appended downstream of the BsrGI site to maintain the proper reading frame

3. All of our work has involved target proteins that begin with methionine. Although this residue is not directly involved in the cleaving reaction, the identity of this residue does affect the cleaving reaction rate and it is recommended that at least one control clone be constructed for each target where a methionine residue immediately follows the intein.

4. The target protein can be cloned with a HindIII, NotI, or SacI site downstream. Remember to include a stop codon immediately downstream of the target protein on the reverse primer to prevent read through. All primers can be synthesized or ordered from a vendor. We order our primers from Sigma-Aldrich.
5. Any polymerase can be used for the PCR step. We have had good results using Phusion.
6. Any dye, such as Ethidium Bromide, can be used in place of GelRed.
7. The PCR Purification Kit can be used if there is a single band on the gel. If there are multiple bands, the correct band should be excised and the Gel Extraction Kit should be used.
8. It is typical to have significant product losses during Gel Extraction, up to 75 %. Several steps can be taken to improve yield, including prewarming elution buffer to 55 °C and passing eluate from column several times. Also, make sure not to load too much gel onto each spin column.
9. Any conventional method for producing competent cells is compatible with this system. The Z-competent cell kit from Zymo is a very simple method that yields competent cells with transformation efficiencies routinely about 10^7 CFU/ug for DH5a. Note that calcium-competent cells may not be sufficient for cloning, but are probably sufficient for transformation of miniprep DNA.
10. Any of the standard *E. coli* cloning strains can be used, including XL1-Blue, TOP10, etc.
11. Any expression strain will typically work, including BL21, Rosetta, Origami, etc., but it must contain a chromosomal copy of the T7 polymerase gene in order to work with the pET system.
12. Typically any expression media will work, including minimal or supplemented minimal media.
13. The composition of the buffer is flexible. Any buffering system that has strong buffering capacity at pH 8.5 and pH 6.5 can be used. The salt concentration used here is recommended by the manufacturer to discourage nonspecific binding to the resin, but can be adjusted as well. The use of DTT in the buffer is also optional.
14. Any column suitable for gravity-flow chromatography can be substituted.
15. This is a description of how a typical cloning is performed in our lab. Any equivalent protocol can be used.
16. The annealing temperature can be optimized to improve yield. We typically run a gradient on the PCR block to determine the

optimal annealing temperature. Lower temperatures allow for higher yields, but also higher risk of low molecular weight amplicons.

17. Always mix PCR mixtures on ice and preheat the PCR block to the initial denaturation temperature before adding the tubes.
18. The amount of DNA necessary for transformation will vary. This is typical for our lab. To ensure that there are separated individual colonies on the plate, we typically split the transformed cells between two plates, and spread 10 μl of cells on one and 90 μl on the other.
19. It is also possible to confirm that the clone is correct by sequencing. We recommend sequencing any clone that has an insert that was generated using PCR, to ensure that no mutations were introduced.
20. 12–18 h is optimal for the overnight time. The cells should be turbid, but not in death phase.
21. We have had the best results using baffled flasks for expression, which allows for better aeration of the culture. Also, the culture size should be no more than 20 % of the flask size.
22. If using baffled flasks, the agitation rate should be lower than with a typical Erlenmeyer flask, to prevent foaming.
23. This step is *CRITICAL*. The culture must be cooled to at least room temperature prior to IPTG addition to prevent premature intein cleavage. The actual expression temperature can vary from 15 to 25 °C, but the culture must be chilled before induction. Also, do not chill on ice.
24. Yields are typically higher with longer expression times. However, there is also a higher risk for premature cleavage with longer expression. 18–20 h is a good starting point. If there is too much premature cleavage, try lowering the temperature or reducing expression time.
25. Cell lysis can be aided by at least one freeze–thaw cycle. Also, cells can be stored frozen after being resuspended in lysis buffer for up to 12-weeks at –20 °C or 1 year at –80 °C.
26. For initial work, we recommend a 2-ml column. The flowthrough can be analyzed to see if precursor protein is overloaded. If there is a significant amount of precursor in the flowthrough, increase column size or decrease flow-rate.
27. Sonication causes samples to heat up, so be sure to sonicate on ice. Also, use multiple short sonication cycles, followed by cooling. For example, use three 15-s cycles with at least 30 s on ice in between each.
28. The SDS-PAGE samples in bold face in the text correspond to the samples in the representative data in Fig. 2.

29. Typically, we dilute whole lysate, clarified lysate, and flowthrough samples 1:1 in 2× sample buffer (30 µl of each). All other samples are dilute in 4× sample buffer, 10 µl of buffer to 40 µl of sample. All samples are vortexed to mix, then boiled at 95 °C for 3 min and stored at −20 °C until the gel is run.
30. To take a resin sample, we cut the tip of a p1000 tip, pipet up ~100 µl of sample and then add to a 1.5 ml tube with about 50 µl of 4× buffer. Vortex well to mix and then boil as with other samples. Finally, centrifuge the tube at max speed for 2 min to remove the resin, and take the supernatant as the SDS-PAGE sample.
31. If the column is not going to be used for several days, it should be stored in 20 % ethanol to prevent bacterial growth.
32. The intein self-cleavage reaction is temperature dependent. The reaction can also be carried out at 37 °C for 4–6 h.

## Acknowledgement

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# Chapter 5

# Simplified Protein Purification Using an Autoprocessing, Inducible Enzyme Tag

**Aimee Shen**

## Abstract

The development of affinity tags has greatly simplified protein purification procedures. A variety of affinity tags are now available to improve expression, solubility, and/or tag removal. In this chapter, we describe a method for purifying recombinant proteins expressed in *Escherichia coli* that uses a highly specific, inducible, C-terminal autoprocessing protease tag. This method streamlines affinity purification, cleavage, and tag separation into a one-step purification procedure, avoiding the need to remove fusion tags from target proteins with exogenous proteases. In addition to accelerating protein purification, we show that this method can enhance the expression, stability, and solubility of select proteins.

**Key words** Protein affinity tag, Autoprocessing, Inducible, Protein purification, Tag cleavage, Protein stability, Protein solubility, Protein expression

## 1 Introduction

The development of affinity tags such as poly-His and glutathione transferase (GST) has greatly simplified recombinant protein purification, while the development of fusion tags has improved the expression, solubility, and stability of target proteins [1, 2]. Despite these advances, tags can alter the biological activity of target proteins and interfere with protein crystallization studies. To overcome this problem, fusion tags are often coupled to protease cleavage sites so that the tag can be removed from target proteins upon addition of site-specific proteases [1, 2]. Unfortunately, these endoproteases can be costly, poorly soluble, and inefficient, which can lead to spurious cleavage and require additional chromatography steps to remove these proteases.

This report describes the use of an inducible, self-cleaving protease tag (CPD) to simplify and accelerate the purification of recombinant proteins from bacteria (Fig. 1, [3]). The CPD tag consists of the *Vibrio cholerae* MARTX toxin cysteine protease domain (CPD) [4] fused to a C-terminal hexahistidine tag ($His_6$).

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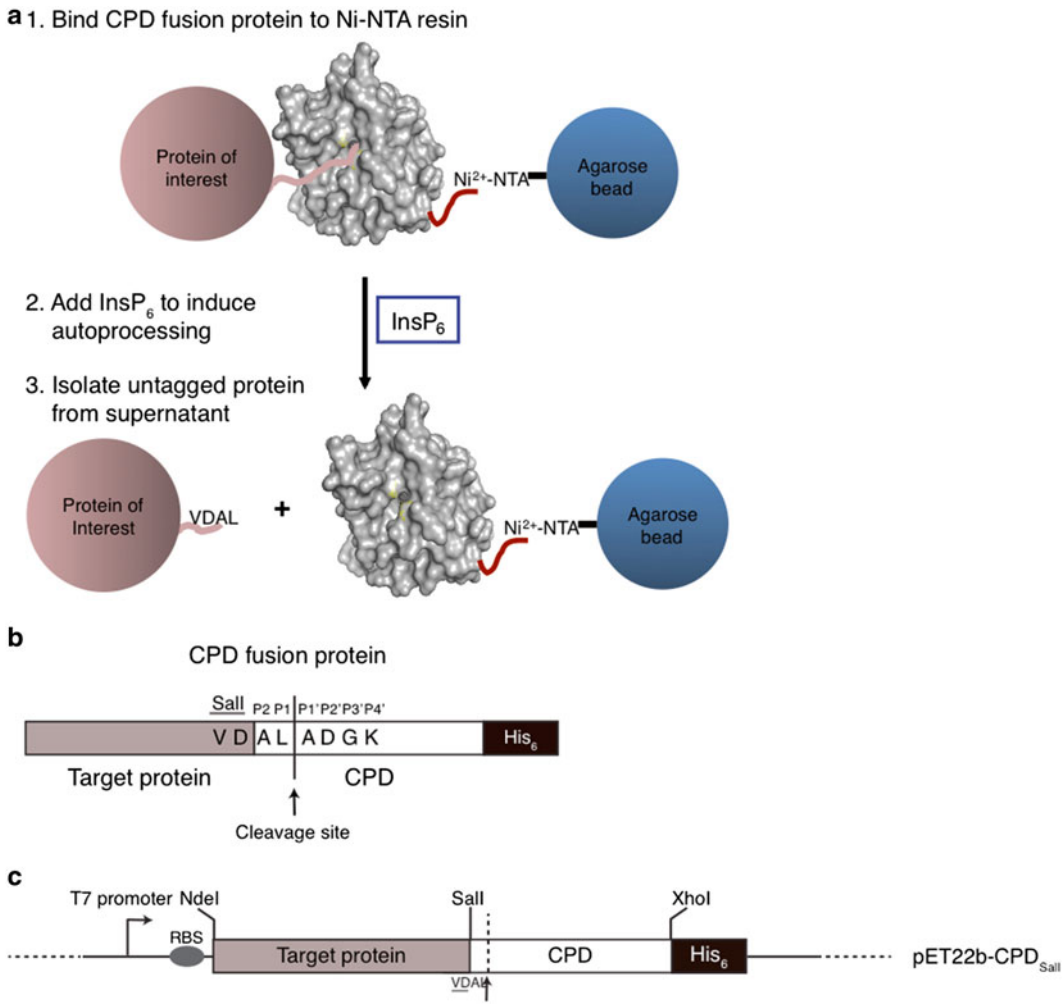


**Fig. 1** CPD autoprocessing purification system. (**a**) Schematic of purification scheme as described in text. (**b**) Schematic of CPD fusion protein and resulting protein post-CPD autoprocessing. (**c**) Schematic of pET-based cloning vector

The CPD is a highly specific protease that cleaves exclusively after Leu residues upon activation by the eukaryotic-specific small molecule inositol hexakisphosphate ($InsP_6$) [5, 6]. Since $InsP_6$ is absent from bacterial cells [7, 8], full-length CPD-$His_6$ fusion proteins are purified from bacterial lysates in a protease-inactive form by way of immobilized metal affinity chromatography (IMAC) via the $His_6$ region of the tag. Addition of $InsP_6$ to the immobilized, C-terminally $His_6$-tagged fusion protein induces autoprocessing after a Leu residue within the engineered target protein-CPD junction (Fig. 1). This processing event releases the untagged target protein into the supernatant, while the C-terminally $His_6$-tagged CPD remains immobilized on the $Ni^{2+}$-NTA resin.

We have previously used the CPD tag to purify a wide range of both soluble and insoluble proteins; in a number of cases, we showed that the CPD can increase the expression, solubility, and stability of fusion proteins [3]. Indeed, Wright et al. recently used the CPD to purify recombinant antimicrobial peptides, which can be difficult to produce in large quantities because of their intrinsic toxicity to *E. coli* [9]. Here, we describe the use of the CPD tag to purify CspBA, a serine protease from the Gram-positive pathogen *Clostridium difficile*, and mouse macrophage metalloelastase (MMP-12) from *E. coli*. Although both these proteins exhibit poor solubility when expressed as a C-terminal $His_6$-tag fusion, when fused to the CPD autoprocessing tag, they can be purified as soluble proteins (unpublished data and [3, 10], Fig. 2). We also use the CPD tag to improve the purification of biotin ligase from *Escherichia coli*, which is used to biotinylate recombinant proteins at engineered sites [3, 11].

## 2 Materials

Prepare all solutions using ultrapure water (deionized water purified to a sensitivity of 18 MΩ) and molecular biology-grade reagents. Prepare and store all reagents at room temperature unless specified otherwise.

To facilitate the production of CPD fusion proteins, we previously constructed a suite of pET-based expression vectors that allow the linker peptide to be varied and also permit the addition of C-terminal HA-tag to the protein of interest. These vectors are readily available from Addgene (Cambridge, MA, USA). In this report, we provide a detailed protocol for purifying proteins using the pET22b-$CPD_{SalI}$ expression vector.

### 2.1 Cloning

1. Cloning primers: The forward primer should carry a 5′ NdeI restriction site sequence (CATATG) diluted to 25 μM in water; the reverse primer should carry a 5′ SalI restriction site sequence (*see* **Note 1**).
2. Deoxyribonucleotide (dNTP) mix: 10 mM stock diluted in water.
3. TE Buffer: 10 mM Tris pH 8.0, 0.5 mM EDTA.
4. Phusion DNA polymerase (New England Biolabs, Ipswich, MA, USA).
5. NdeI Restriction Endonuclease (New England Biolabs, Ipswich, MA, USA) with supplied buffers.
6. XhoI Restriction Endonuclease (New England Biolabs, Ipswich, MA, USA) with supplied buffers.
7. Antarctic Phosphatase (New England Biolabs, Ipswich, MA, USA).

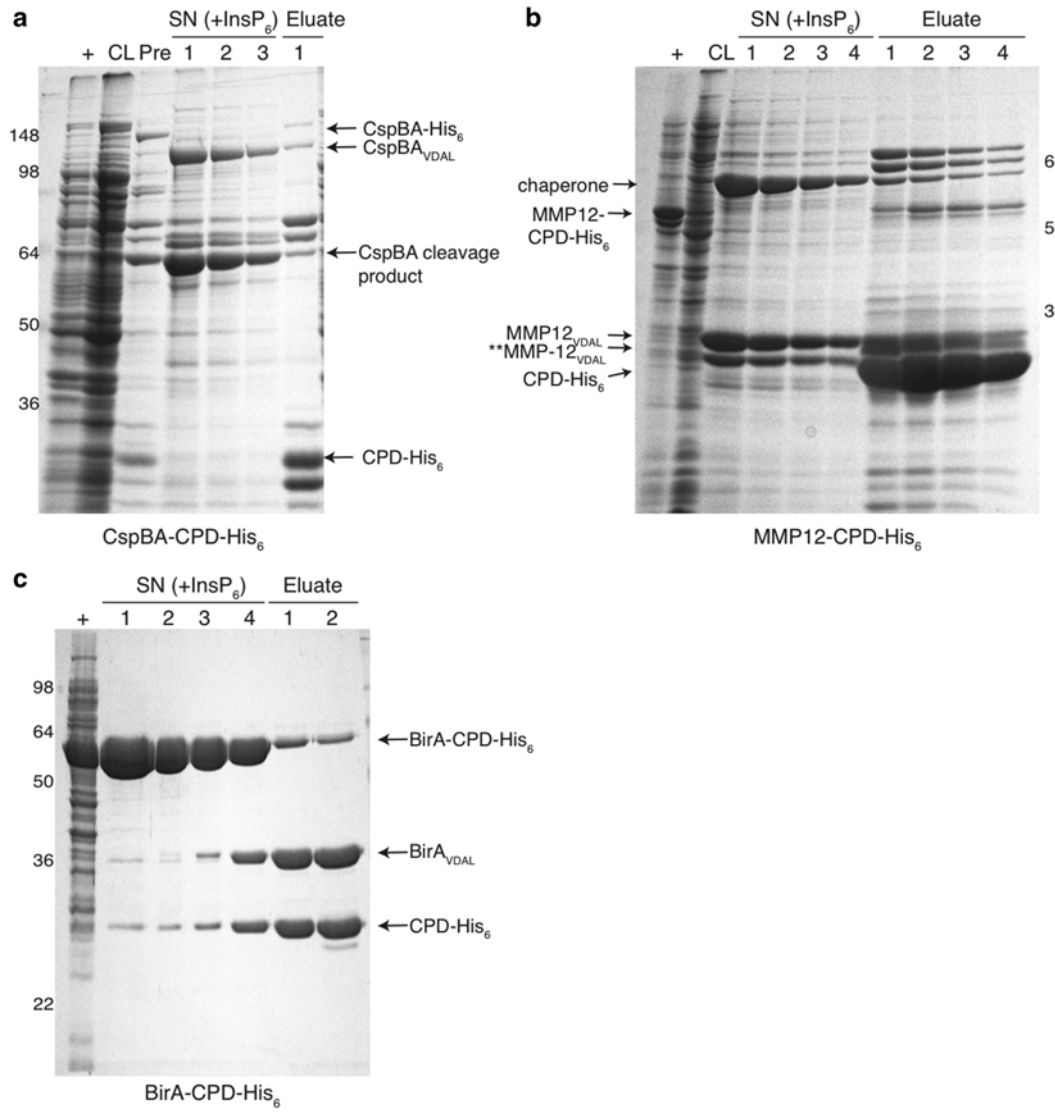


**Fig. 2** Purification of recombinant proteins using the CPD fusion tag. SDS-PAGE analysis CPD fusion tag-purified proteins using Coomassie staining. (**a**) *Clostridium difficile* CspBA (CD2247). When expressed as a C-terminally $His_6$-tagged protein in *E. coli*, CspBA exhibits poor solubility (A. Shen, unpublished observations); when expressed as a CPD-$His_6$-tagged protein in *E. coli*, CspBA can be purified in a soluble form. (**b**) mouse macrophage metalloelastin (MMP-12). MMP-12 is insoluble as a $His_6$-tagged protein in *E. coli* [10]. When fused to the CPD, active, untagged MMP-12 can be readily purified from *E. coli* [3]. *Double asterisk* indicates the autoprocessed, active form of MMP-12. (**c**) *E. coli* biotin ligase BirA. $His_6$-tgged proteins were bound to Ni-NTA resin then incubated with 50 μM $InsP_6$ for 1 h at room temperature; the resin was washed 2–3 times, followed by elution of $Ni^{2+}$-bound proteins by 200 mM imidazole. +IPTG-induced culture, *Pre* imidazole elution prior to $InsP_6$-mediated cleavage, *CL* cleared lysate, *SN* supernatant fraction resulting from $InsP_6$-induced autoprocessing by the CPD

8. T4 DNA ligase (New England Biolabs, Ipswich, MA, USA) with supplied buffers.
9. IPTG-inducible expression vector containing CPD-$His_6$ fusion tag (available from Addgene, Cambridge, MA, USA).
10. 10× Tris–borate-EDTA buffer (TBE) stock: 108 g Tris Base, 55 g boric acid, 40 mL 0.5 M EDTA pH 8.0 (or 9.3 g $Na_2$-EDTA) in 1 L $H_2O$ (final volume). 1× TBE (working stock) is 0.1 M Tris pH 8.0, 90 mM boric acid, 1 mM EDTA (*see* **Note 2**).
11. NaOH pellets.
12. 0.5 M EDTA stock solution: Dissolve 186.6 g EDTA in water (bring volume up to 800 mL). Add ~20 g NaOH pellets, stir to dissolve, and add more NaOH as needed to bring pH to 8.0. Add water to give final volume of 1 L. Autoclave to sterilize. Autoclave to sterilize (*see* **Note 3**).
13. 0.75 % agarose gel in TBE: 1 g molecular biology grade agarose in 100 mL TBE buffer. Microwave to melt and add 100 μL Sybr SAFE (Invitrogen, Carlsbad, CA, USA).
14. PCR Product Purification Kit.
15. Plasmid Mini Prep Kit.

### 2.2 E. coli Expression

1. LB Media: 5 g NaCl, 5 g yeast extract, 10 g tryptone per liter $H_2O$.
2. 2YT Media: Measure 5 g NaCl, 10 g yeast extract, 15 g tryptone into a 2.8 L Fernbach flask and then add 1 L water. Autoclave to sterilize.
3. Ampicillin, 1,000× stock: 100 mg/mL solution in water. Filter-sterilize and store at 4 °C (*see* **Note 4**).
4. 1 M Isopropyl β-D-1-thiogalactopyranoside (IPTG) in water: Measure 1.19 g IPTG in a 15 mL conical tube. Add $H_2O$ up to final volume of 5 mL. Filter-sterilize and store at 4 °C (or −20 °C) (*see* **Note 5**).
5. DH5α chemically competent cells: prepared in house or purchased from preferred vendor.
6. BL21(DE3) chemically competent cells: prepared in house or purchased preferred vendor.
7. 4× FSB (final sample buffer): for 50 mL stock, in a fumehood, mix 20 mL glycerol, 10 mL 1 M Tris–HCl pH 6.8 (SDS-PAGE stacking buffer), 10 mL β-mercaptoethanol, 6 g sodium dodecylsulfate (SDS, use mask to weigh and avoid aerosolizing, *see* **Note 6**), 20 mg bromophenol blue. Add water to final volume of 50 mL and dissolve. Aliquot and store in freezer for long-term storage (*see* **Note 7**).

### 2.3 Protein Purification

1. 5 M NaCl solution: Weigh 292.1 g of NaCl and pour into 2 L beaker. Bring to 1 L with water and stir to dissolve. Autoclave to sterilize (*see* **Note 8**).
2. 1 M Tris–HCl stock solution, pH 7.5: Dissolve 121.1 g Tris Base in 800 mL water. Add 65 mL concentrated HCl. Adjust pH to reach 7.5 and add water until final volume reaches 1 L. Autoclave to sterilize or filter-sterilize (*see* **Note 9**).
3. 1 M Imidazole stock solution, pH 8.0: Dissolve 68.08 g imidazole in 0.8 L $H_2O$, and add NaOH until pH reaches 8.0; bring $H_2O$ to 1 L. Filter-sterilize and store at room temperature or 4 °C.
4. Lysis buffer (LIB): 0.5 M NaCl, 50 mM Tris–HCl, pH 7.5, 15 mM imidazole, 10 % glycerol (*see* **Note 10**). Store at 4 °C. To minimize degradation of fusion proteins, add protease inhibitor cocktail at a 1:100 dilution (*see* **Note 11**).
5. Ni-NTA agarose beads.
6. 25 mM Inositol hexakisphosphate ($InsP_6$) stock solution: Dissolve 25 mg $InsP_6$ (also called phytic acid) in 1.5 mL sterile $H_2O$. Aliquot into 100 μL fractions and freeze (*see* **Note 12**).
7. Elution buffer (HIB): 0.5 M NaCl, 50 mM Tris–HCl, pH 7.5, 250 mM imidazole, 10 % glycerol ± protease inhibitor cocktail (*see* **Notes 10** and **11**).

## 3 Methods

### 3.1 Clone Gene Encoding Target Protein into CPD Fusion Vector

1. Design cloning primers to amplify target gene sequence (*see* **Note 1**).
2. Prepare working stocks of primers. Resuspend primers from lyophilized stocks by spinning down primers at 13,000 × *g* for 30 s and adding TE buffer (*see* **Note 13**) to give final concentration of 250 μM. Let primers hydrate for 1–2 min before vortexing them vigorously for 10–15 s. Quick spin to collect primer stock at bottom of tube. Dilute primer stock 1:10 in water to give a 25 μM working stock.
3. PCR amplify target gene sequence following manufacturer's directions for Phusion DNA polymerase.
4. Run PCR product on 0.75 % agarose gel in TBE (*see* **Note 2**).
5. Purify PCR product using PCR purification kit according to manufacturer's instructions. Elute in 50 μL water.
6. Add 6.3 μL of buffer 4 and 6.3 μL 10× bovine serum albumin (both supplied with restriction enzymes) to purified PCR product. Vortex briefly, quick spin, and then add 1 μL NdeI and 1 μL SalI and digest for minimum of 1–2 h at 37 °C.

7. Add 6.3 μL of buffer 4 and 6.3 μL 10× bovine serum albumin to 25–50 μg of pET22b-$CPD_{SalI}$ vector in 50 μL water. Vortex briefly, quick spin, and then add 1 μL NdeI and 1 μL SalI and digest for a *minimum* of 1–2 h. Add 1:10 dilution of 10× Antarctic phosphatase buffer, mix, and add 1 μL of Antarctic phosphatase to remove 5′ phosphate groups for 0.5–1 h at 37 °C.
8. PCR purify restriction digests using PCR purification kit according to manufacturer's instructions. Elute in 50 μL water.
9. Mix 0.5–1.0 μg of digested pET22b-$CPD_{SalI}$ plasmid with digested PCR product (amount will vary depending on size of insert) and 2 μL 10× T4 DNA Ligase buffer in a total volume of 19 μL. Vortex briefly, quick spin, then add 1 μL of T4 DNA ligase and incubate at 4–16 °C for a minimum of 4 h.
10. Add 50 μL of DH5α chemically competent cells directly to the ligation and transform according to manufacturer's directions.
11. After 20–24 h growth, pick transformants and inoculate 3.5 mL of LB + Amp 100 μg/mL for miniprep plasmid isolation.
12. Identify transformants containing gene of interest in pET22b-CPDSalI by restriction digest (*see* **Note 14**).
13. Sequence positive transformants and transform resulting plasmid into BL21(DE3) expression cells (*see* **Note 15**).

### 3.2 Purification of Target Proteins Using CPD Autoprocessing Fusion Tag

Carry out all procedures at room temperature unless otherwise specified.

1. Inoculate 2.5 mL overnight culture LB media containing 100 μg/mL ampicillin with BL21(DE3) CPD expression construct (*see* **Note 16**).
2. Dilute overnight culture 1:500 into 1 L 2YT media containing 100 μg/mL ampicillin (*see* **Note 17**).
3. Grow with vigorous shaking (225–250 rpm) at 37 °C until $OD_{600}$ of 0.6–1.0 is reached (between 3 and 4 h).
4. *Optional*: Remove a 1 mL sample for uninduced whole cell lysate. Pellet sample at 13,000 × *g* for 1 min and remove supernatant. Resuspend pellet in 50–80 μL 1× FSB. Boil at 95 °C for 3 min and save for SDS-PAGE.
5. Add IPTG to 250 μM (250 μL of 1 M IPTG stock, *see* **Note 18**).
6. Grow induced cultures overnight shaking (225–250 rpm) at 18–20 °C for 10–16 h.
7. *Optional*: Remove 1 mL sample for +IPTG whole cell lysate prep. Pellet sample at 13,000 × *g* for 1 min and remove supernatant.

Resuspend pellet in 100–200 μL 1× FSB. Boil at 95 °C for 3 min and save for SDS-PAGE (Fig. 2).

8. Pellet 1 L culture at 4,000 × *g* in 1 L screw cap bottle for 20 min.
9. Discard supernatant and partially resuspend pellet in 25 mL LIB by first scraping pellet with 10 mL pipet. Resuspend slurry by pipetting up and down with 10 mL pipet.
10. Transfer cells to 50 mL conical tube and flash-freeze in liquid nitrogen (*see* **Note 19**).
11. Thaw sample in water then lyse by sonication (Branson sonifier 250, three cycles of 45 s at 40 % amplitude with 3–5 min rest on ice between cycles using a ½″ tip, *see* **Note 20**).
12. Transfer lysate from 50 mL conical to round Sorvall tube and spin at 15,000 × *g* for 30 min to clear lysate (*see* **Note 21**).
13. During spin, wash 1.0 mL Ni-NTA agarose bead slurry with LIB buffer in 1.5 mL tube (pellet at 9,000 × *g* for 2 min, remove supernatant, add 0.5–1.0 mL lysis buffer) (*see* **Note 22**).
14. Decant cleared lysate into 50 mL conical tube. Remove 30 μL cleared lysate sample and add to 10 μL 4× FSB for SDS-PAGE analysis (Fig. 2).
15. Transfer Ni-NTA agarose beads to cleared lysate and nutate or place on shaker (low speed) for 2 h to overnight at 4 °C (*see* **Note 23**).
16. Pellet binding reaction at 2,000 × *g* for 2 min at 4 °C.
17. Wash beads in LIB (10–12 mL) and transfer to a 15 mL conical (*see* **Note 24**). Wash out 15 mL conical with LIB buffer to minimize bead loss.
18. Pellet binding reaction at 2,000 × *g* for 2 min at 4 °C.
19. Transfer beads to 1.5 mL tube (wash out 15 mL conical with LIB buffer to minimize bead loss).
20. Wash beads 1× with 1 mL LIB (add 1 mL LIB, pellet binding reaction at 8,000 × *g* for 2 min at room temperature or 4 °C, and remove supernatant).
21. Repeat wash **step 2** more times.
22. Add 300 μL of LIB to beads and resuspend by inversion; add $InsP_6$ to give a final concentration of 50 μM (1:500 dilution, *see* **Note 25**).
23. Incubate shaking at room temperature for 1–2 h (*see* **Note 26**).
24. Spin down beads and remove supernatant (containing cleaved protein of interest, *see* **Note 27**). Reserve a 30 μL fraction for SDS-PAGE analysis (supernatant fraction 1, Fig. 2).
25. Wash beads with 2–3 times more with 300 μL LIB (nutate for 5–10 min, pellet at 8,000 × *g* for 2 min, transfer supernatant to

a fresh tube) (*see* **Note 28**). Remove 30 μL of each supernatant fraction for SDS-PAGE analysis (Fig. 2).

26. To assess the efficiency of cleavage, elute the $His_6$-tagged CPD attached to the Ni-NTA beads by adding 300 μL of HIB buffer and repeating **steps 24** and **25**. The elution time can be reduced to 5–10 min (*see* **Note 29**). Remove 30 μL samples from the elution fractions for SDS-PAGE analysis (Fig. 2).
27. To analyze the purification, add 10 μL of 4× FSB to the 30 μL fractions removed during the purification (+IPTG, cleared lysate, supernatant fractions 1–4, and elution fractions). Boil at 95 °C and run on a 12 % SDS-PAGE gel. Analyze by Coomassie staining according to manufacturer's direction (Fig. 2, *see* **Note 30**).

## 4 Notes

1. We usually add an additional 4 nt upstream of the restriction site sequence to ensure efficient digestion. To construct a CPD fusion in pET22b-$CPD_{SalI}$, the forward primer should carry an NdeI restriction site, while the reverse primer should carry a SalI restriction site. This will produce a fusion protein carrying a VDAL linker sequence; CPD-mediated autoprocessing will leave a VDAL C-terminal tag on the target protein. CPD expression vectors are available from Addgene that provide alternate 5′ (NcoI) and 3′ cloning sites (SacI, BamHI). Changes in the 3′ restriction site will alter the C-terminus of the target protein produced upon CPD autoprocessing.
2. TBE can be used at 1× or 0.5× for gel electrophoresis.
3. EDTA will not dissolve at high concentrations unless it is ~pH 8.0.
4. Store filter-sterilized ampicillin at 4 °C. It is stable for 1–2 months.
5. IPTG is stable for at least 1 month at 4 °C. IPTG can also be aliquoted in 0.5 mL volumes and stored at −20 °C.
6. SDS is an irritant and should not be inhaled.
7. Storage of FSB at room temperature will result in oxidation of the reducing agent over time. We store FSB at room temperature for a maximum of 2 weeks.
8. We find that heating the solution to 65 °C ensures that all the salt dissolves.
9. The pH of Tris solutions is temperature dependent. We prepare our Tris solutions at room temperature.
10. Due to the high viscosity of the glycerol, we use a 50 % glycerol stock to make up the LIB buffer. For applications where reducing conditions are necessary, add ß-mercaptoethanol to 2 mM.

11. We typically make a 1 L lysis buffer stock; for a given purification, we add protease inhibitor to 50 mL of lysis buffer. The protease inhibitor will retain activity for a few days, but we use lysis buffer with freshly added inhibitor. Note - commercially available protease inhibitors, such as Halt from Pierce (Rockford, IL, USA) do not affect CPD autoprocessing.
12. $InsP_6$ will tolerate multiple freeze–thaw cycles.
13. For a quick TE solution, we usually add 0.5 M EDTA to the elution buffer supplied in the PCR purification kits (i.e., Fermentas PCR kit [Glen Burnie, MD, USA]).
14. We typically screen transformants using restriction digest with NdeI and XhoI, which will produce a product that consists of the gene of interest fused to the CPD (645 bp).
15. It may be necessary to optimize the *E. coli* expression strain used to avoid problems such as toxicity (e.g., transform BL21(DE3) pLysS cells instead to reduce leaky expression prior to induction) or poor expression due to rare codon usage (e.g., transform into Rosetta strains to supply rare tRNAs).
16. The culture only needs to be in stationary phase. We often inoculate from a freshly transformed plate in the morning and grow the culture for a minimum of 4–5 h until it is in stationary phase before back-diluting into 2YT media.
17. We often use pre-warmed 2YT media to reduce the time it takes for the expression culture to read OD ~0.8. It is important to use a wide bottom flask to ensure maximum aeration of the culture and more efficient growth. Yields will be reduced if cultures are grown under oxygen-limiting conditions. We typically perform initial purifications on a 1 L scale to determine whether small or large scale (>2 L) preps are needed to obtain sufficient amounts of protein. The 1 L scale of preps allows us to purify 0.1–0.3 mg/L of poorly expressed proteins.
18. We find that we get better yields when inducing at a higher OD, e.g., 1.0. However, it is sometimes desirable to cool down the cultures on ice prior to adding IPTG. This slows down translation and protein folding which can aid in the solubility of difficult-to-express proteins.
19. The flash-freeze step improves bacterial cell lysis.
20. It is important not to over-sonicate the sample (the pitch of the sonication will increase sharply if this occurs) or have the sample heat up too much. If sample temperature increases significantly, increase the incubation on ice or decrease the sonication time.
21. Transfer the cleared lysate immediately to a 50 mL conical to minimize transfer of unlysed cells and other debris.

22. Ensure that the Ni-NTA agarose slurry is fully resuspended: the beads settle quickly even after resuspension.
23. The longer the incubation time with the beads, the greater the yield. If the cleared lysate is not immediately transferred to the 50 mL conical tube, unlysed bacteria can sometimes be transferred and grow in the lysate during prolonged incubation. The cleared lysate will then appear cloudy when the beads are removed by centrifugation.
24. Leave sufficient buffer above beads to avoid aspirating Ni-NTA agarose beads during washes.
25. If cleavage is observed to be inefficient, it is possible to add $InsP_6$ up to 100 μM.
26. We have observed for some proteins that cleavage at 4 °C decreases their solubility. The CPD will autoprocess at a wide range of temperatures (4–37 °C), so the temperature used at this step depends on your specific application. In addition, we have performed the autoprocessing reaction in a wide range of buffer concentrations, including in the absence of salt (50 mM Tris pH 8.0). The composition of buffer used during the autoprocessing step should be user-determined based on the downstream applications for the purified protein.
27. **Steps 23–25** can be repeated. The efficiency of protease cleavage depends on the accessibility of the linker region to the CPD. Some proteins that are highly folded at the C-terminus are hampered by this problem. In these cases, it may be necessary to extend the length of the linker region, for example by addition of an HA-tag using the HA-CPD constructs.
28. To quickly assess the efficiency of autoprocessing, we usually test the supernatant fractions for the presence of protein by adding 1 μL of the supernatant fraction to 100 μL of Bradford reagent (Pierce, Rockford, IL, USA) and observing the color change. The amount of protein detected in the supernatant will decrease with each successive wash.
29. Immobilization of the CPD onto Ni-NTA agarose beads can reduce the efficiency of autocleavage. To avoid this issue, you can also elute the fusion protein with HIB buffer and then perform the $InsP_6$-induced autoprocessing reaction. Pool the collected fractions and buffer exchange using Amicon concentrators (EMD Millipore, Billerica, MA, USA) cleave with $InsP_6$, then perform a subtractive Ni-NTA pull-down to remove the $His_6$-tagged CPD (post-cleavage). Your protein of interest will be in the supernatant fraction.
30. The percentage SDS-PAGE gel that should be run depends on the size of the protein of interest. The CPD fusion tag will run ~24 kDa. We use GelCode Blue (Pierce, Rockford, IL, USA) to perform Coomassie staining, which has enhanced detection over traditional Coomassie staining.

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# Chapter 6

# SUMO as a Solubility Tag and In Vivo Cleavage of SUMO Fusion Proteins with Ulp1

**Dennis Kuo, Minghua Nie, and Albert J. Courey**

## Abstract

Expression of proteins in *E. coli* is often plagued by insolubility of the protein of interest. A solution to this problem is the expression of proteins as fusions to solubility tags such as the SUMO protein. SUMO fusion proteins can be cleaved to remove the SUMO moiety using SUMO-specific proteases such as Ulp1. Here, we describe the use of vectors for the expression of recombinant proteins in *E. coli* as fusions to the Drosophila SUMO protein. This includes a vector that encodes not only the SUMO tagged protein of interest but also SUMO-tagged Ulp1. Coexpression of these two proteins results in the in vivo cleavage of the protein of interest from the SUMO tag, while still leaving the protein of interest in a form that can be purified from a soluble cell lysate by nickel affinity chromatography.

**Key words** SUMO, Ulp1, Solubility tag, Ni-NTA resin, Affinity chromatography

## 1 Introduction

When expression of recombinant proteins in *E. coli* works well, the protein of interest is often the single-most abundant protein in the cell lysate. As a result, it is frequently possible to purify milligrams of recombinant protein from culture volumes of a few hundred milliliters. However, a major barrier to even more widespread use of this system is protein solubility. In many cases, proteins expressed in *E. coli* fail to fold properly as they exit the ribosome, and the nonnative proteins then aggregate and precipitate as inclusion bodies [1].

In some cases, it is possible to solubilize inclusion bodies with chaotropic agents such as urea, guanidine-HCl, or detergents and then induce folding into the native conformation upon removal of the chaotrope [1]. However, this approach is frequently unsuccessful as the protein often reassumes the nonnative conformation and therefore reprecipitates upon denaturant removal.

Recombinant protein insolubility could be due to the presence in the coding region of codons that are read inefficiently by the *E. coli*

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translational apparatus or to other differences between the bacterial and eukaryotic ribosomes that lead to inappropriate rates of translation. It is also likely that the absence in *E. coli* of appropriate folding partners or chaperones normally required for proper folding in eukaryotic cells is part of the problem. Finally, there may simply be a limit to the solubility of some proteins, even in their native state. Approaches targeting all these possibilities have been developed to increase the likelihood that a protein will assume its native conformation upon ribosome exit. While these approaches are useful, none represents a sure fire solution to the insolubility problem for all or even most proteins.

Another approach that has been used with some success is to generate gene chimeras encoding the protein of interest fused to another protein that renders the overall polypeptide soluble even if the protein of interest, when expressed alone, is insoluble [2]. Popular solubility tags include glutathione-S-transferase (GST), maltose binding protein (MBP), thioredoxin, and SUMO. Several of these tags (e.g., GST, MBP) contain well-characterized small molecule ligands that can be used as affinity reagents to purify the fusion proteins. In other cases, a separate affinity tag (e.g., a His tag) can be added to the fusion protein along with the solubility tag to allow affinity purification of the fusion proteins. Since it is often desirable to remove the solubility tag after expression of the protein, a cleavage site for a site-specific protease such as TEV protease or thrombin is often engineered into the fusion protein between the protein of interest and the solubility tag.

The mechanism by which solubility tags work is not clear. One possibility is that the combination of a highly soluble tag with an insoluble protein of interest results in a level of solubility adequate to allow for expression and purification of the fusion protein under native conditions. Alternatively, it is possible that the solubility tag functions as a chaperone to allow a protein of interest to which it is fused to fold into its native and therefore soluble conformation before it can aggregate.

One solubility tag that has gained popularity in recent years is SUMO (small ubiquitin-related modifier) [3–8]. Like other proteins in the ubiquitin family, SUMO becomes covalently conjugated to other proteins via an amide linkage (also termed an isopeptide bond) between the C-terminal carboxyl group in SUMO and an amino group in a lysine side chain on the other protein [9]. This conjugation process involves three distinct steps (Fig. 1), and recognition of SUMO by the conjugation machinery requires a Gly–Gly sequence at the extreme C-terminal end of the mature SUMO protein. Once formed, the isopeptide bond can be cleaved by ubiquitin deconjugating enzymes, such as *Drosophila* Ulp1, which recognize the diglycine motif and hydrolyze the amide linkage.

The SUMO coding region contains additional codons beyond those encoding the diglycine motif, and thus the primary translation

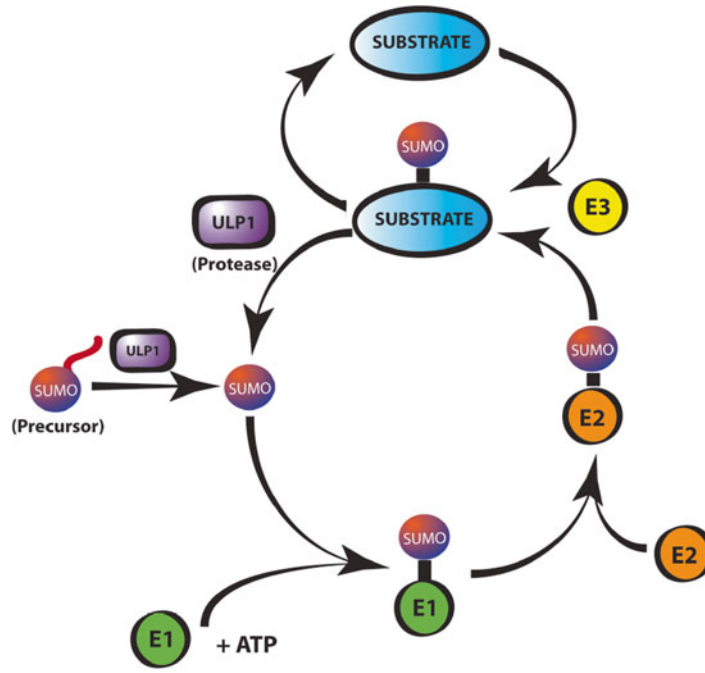


**Fig. 1** The SUMO cycle. Mature SUMO is generated by endoproteolytic cleavage of the SUMO precursor. Cleavage is catalyzed by SUMO-specific proteases such as Ulp1. Mature SUMO then becomes conjugated to lysine side chains in substrate proteins via the action of an E1 activating enzyme, an E2 conjugating enzyme, and E3 ligases. The resulting isopeptide bond between the substrate and SUMO can be hydrolyzed by SUMO-specific proteases including Ulp1 to regenerate mature SUMO and the unmodified substrate

product of the SUMO gene contains additional amino acids at the C-terminus that must be removed by endoproteolytic cleavage before SUMO conjugation can occur. The length of this C-terminal extension is species specific (in *Drosophila* it is just two amino acids long). The same enzymes that catalyze the deconjugation reaction, including Ulp1, catalyze this proteolytic cleavage. The efficiency of cleavage appears to be insensitive to the amino acid sequence following the diglycine motif.

The ability of Ulp1 to cleave peptide bonds at the C-terminus of SUMO means that Ulp1 can be used to efficiently cleave N-terminal SUMO fusion proteins releasing the unfused protein. Typically, this is achieved by isolating the SUMO fusion protein from cell lysates and then treating it in vitro with Ulp1 (which is commercially available).

In this chapter, we describe vectors for the expression of SUMO-His-tagged fusion proteins in *E. coli*. One such vector has been designed to encode not only the protein of interest fused to SUMO but also a SUMO-Ulp1 fusion protein. Expression of this "duet" vector results in the in vivo cleavage of both SUMO fusion

proteins by Ulp1. The protein of interest can be subsequently purified via nickel affinity chromatography. One protein produced in this way, the Groucho Q (GroQ) domain, was found to remain soluble even after in vivo cleavage even though GroQ is completely insoluble when expressed without a solubility tag. This suggests that SUMO may have a chaperone-like function to assist in the proper folding of its fusion partner, which therefore remains soluble even after cleavage with Ulp1 (Fig. 2).

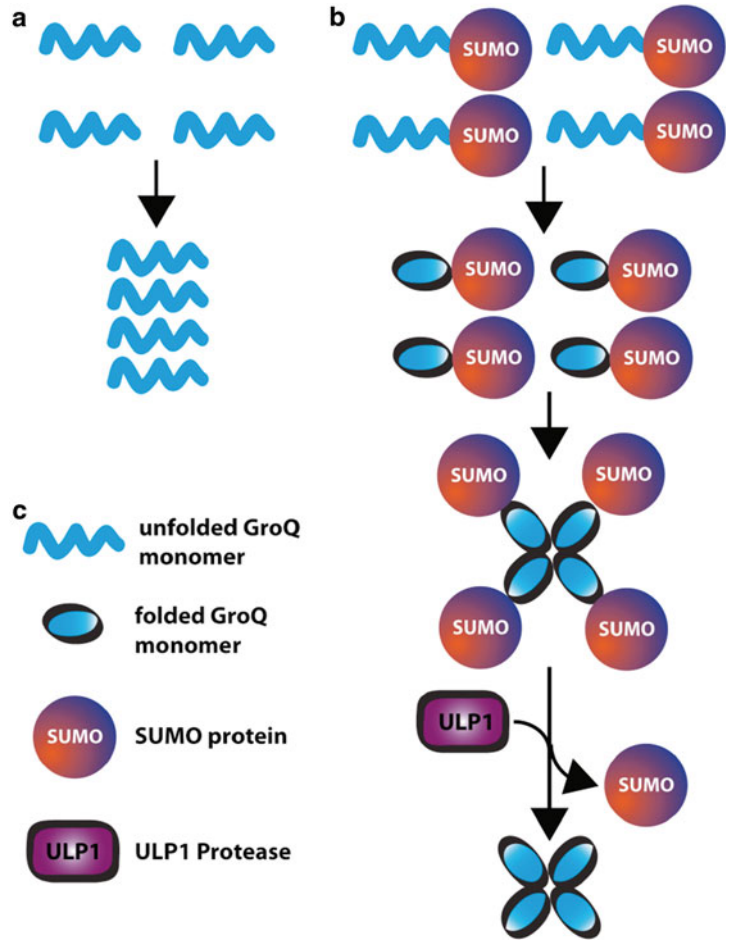


**Fig. 2** Mechanism by which SUMO may prevent aggregation of proteins such as GroQ. (**a**) In the absence of a solubility tag, proteins such as the GroQ domain often fail to fold into their native conformation when expressed at high levels and therefore aggregate and precipitate as inclusion bodies. (**b**) When fused to solubility tags such as SUMO, the tag could serve to prevent aggregation of domains such as the GroQ domain until the domain can assume its native tertiary and quaternary structure. In the case of GroQ, folding involves the formation of native tetramers [8]. Once the protein is folded, removal of the SUMO moiety by cleavage with Ulp1 proceeds without precipitation of the protein of interest. (**c**) Legend showing the *symbols* used for the folded and unfolded GroQ domain, SUMO, and Ulp1

## 2 Materials

### *2.1 Expression Vector Construction Components*

1. For expression of proteins fused at their N-terminus to SUMO followed by a 6×His tag, we have constructed plasmid pMP-SUMO-H6 (Fig. 3a). This plasmid is available upon request.
2. For coexpression of SUMO fusion proteins with a SUMO-Ulp1 fusion protein, we have constructed plasmid pMP-SUMO-H6/SUMO-Ulp1 (Fig. 3b). This plasmid is available upon request.
3. Enzymes and PCR primers required for subcloning DNA sequences encoding proteins of interest into the multiple cloning site (Fig. 3c) of either of the above two vectors are available from multiple commercial sources.

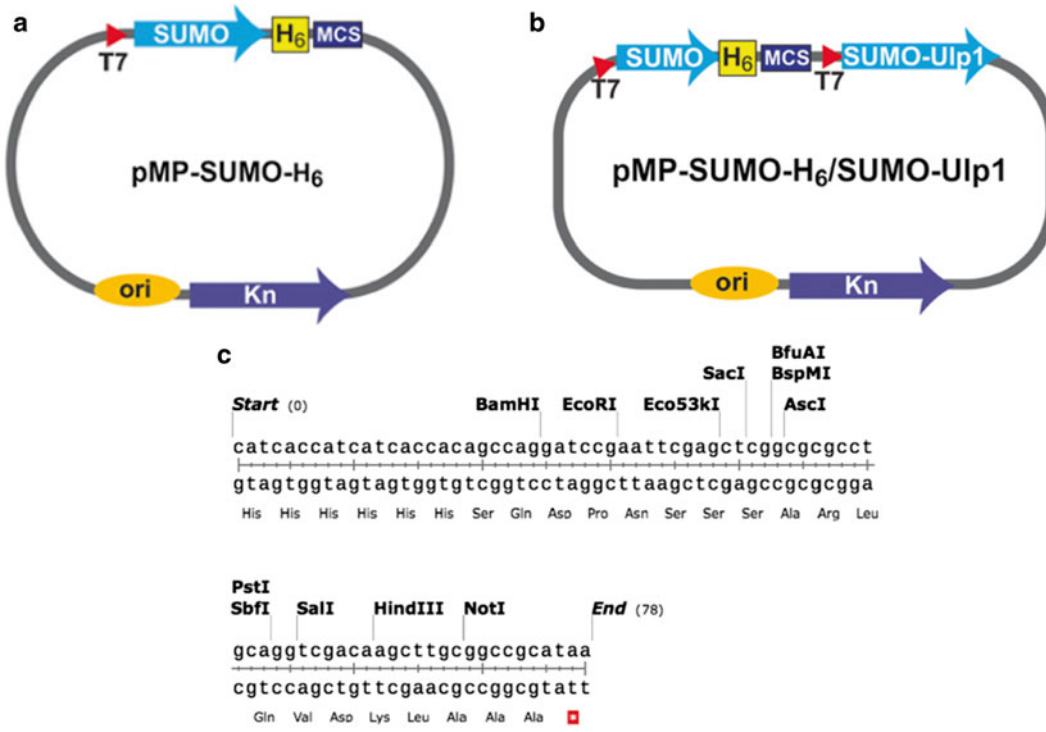


**Fig. 3** Vectors for expressing SUMO fusion proteins. (**a**) Expression vector pMP-SUMO-H6. This vector, which is based on the pRSFDuet-1 expression vector (Novagen) is used to express SUMO fusion proteins under the control of the T7 promoter. It contains sequences encoding *Drosophila* SUMO and a 6xHis tag as well as a multiple cloning site, which can be used to create in frame fusions to a protein of interest. (**b**) Expression vector pMP-SUMO-H6/SUMO-Ulp1. This vector is similar to pMP-SUMO-H6 except that it also contains sequences encoding a SUMO-Ulp1 fusion protein under the control of a separate T7 promoter. It is used to coexpress the SUMO-fused protein of interest with SUMO-Ulp1 leading to cleavage of both SUMO fusion proteins in vivo prior to cell lysis. (**c**) Sequence of the multiple cloning site found in both vectors. Panels (**a**) and (**b**) reproduced with the publisher's permission from Kuo et al. [8]

### 2.2 Cell Growth, Auto-induction, and Cell Lysis Components

1. Media for cell growth and auto-induction including non-inducing medium ZYM-505 and auto-inducing medium ZYM-5052 were prepared as detailed previously [10].
2. Lysis Buffer: 50 mM $NaH_2PO_4$, 300 mM NaCl, 10 % (w/v) glycerol, 0.1 % Igepal CA-630, pH 8.0. Weigh out 3.45 g $NaH_2PO_4$, 8.8 g NaCl and add to a dry beaker. Dissolve in 300 ml of water. Add 50 g of glycerol and 500 μl of Igepal CA-630. Mix and adjust pH with HCl. Make up to 500 ml with water. Store at room temperature.

### 2.3 Protein Purification Components

1. 5 M imidazole: Weigh 3.4 g of imidazole and make up to 10 ml with water. Store at room temperature.
2. Wash Buffer A: 50 mM $NaH_2PO_4$, 300 mM NaCl, 10 % (w/v) glycerol, pH 8.0, 30 mM imidazole. Weigh out 3.45 g $NaH_2PO_4$, 8.8 g NaCl and add to a dry beaker. Dissolve in 300 ml of water and add 50 g of glycerol. Mix and adjust pH with HCl. Make up to 500 ml with water. Store at room temperature. Before use, add 5 M imidazole stock to adjust the imidazole concentration to 30 mM.
3. Wash Buffer B: 50 mM $NaH_2PO_4$, 300 mM NaCl, 10 % (w/v) glycerol, pH 8.0, 50 mM imidazole. Weigh out 3.45 g $NaH_2PO_4$, 8.8 g NaCl and add to a dry beaker. Dissolve in 300 ml water and add 50 g of glycerol. Mix and adjust pH with HCl. Make up to 500 ml with water. Store at room temperature. Before use, add 5 M imidazole stock to adjust the imidazole concentration to 50 mM.
4. Elution Buffer "Zero": 50 mM Tris–HCl, 100 mM NaCl, 5 % (w/v) glycerol, pH 8.0. Weigh out 0.61 g Tris, 0.58 g NaCl and add to a dry beaker. Dissolve in 75 ml water and add 5 g glycerol. Mix and adjust pH with HCl. Make up to 100 ml with water. Store at room temperature.
5. Elution Buffer "Max": 50 mM Tris–HCl, 100 mM NaCl, 5 % (w/v) glycerol, 500 mM imidazole, pH 8.0. Weigh out 0.61 g Tris–HCl, 0.58 g NaCl, and 3.4 g imidazole and add to a dry beaker. Dissolve in 75 ml water and add 5 g glycerol. Mix and adjust pH with HCl. Make up to 100 ml with water. Store at room temperature.
6. Elution Buffer A (150 mM imidazole): Mix 1.2 ml of Elution Buffer "Max" with 2.8 ml of Elution Buffer "Zero".
7. Elution Buffer B (200 mM imidazole): Mix 1.6 ml of Elution Buffer "Max" with 2.4 ml of Elution Buffer "Zero".
8. Elution Buffer C (250 mM Imidazole): Mix 2 ml of Elution Buffer "Max" with 2 ml of Elution Buffer "Zero".

9. Nickel nitrilotracetic acid (Ni-NTA) resin was purchased from Pierce.
10. Plastic disposable column apparatus: We use Bio-Rad Poly-Prep chromatography columns.

## 3 Methods

### 3.1 Expression Vector Construction

Expression vector pMP-SUMO-H6 is used to express a protein of interest fused at its N-terminus to SUMO followed by a 6×His tag and yields the intact fusion protein (Fig. 3a). Expression vector pMP-SUMO-H6/SUMO-Ulp1 is used to coexpress a SUMO fusion protein with a SUMO-Ulp1 fusion protein and is used to achieve in vivo cleavage of the SUMO fusion protein (Fig. 3b). Coding sequences for the protein of interest are cloned into the multiple cloning sequences (Fig. 3c) of one of these two vectors to generate an in-frame fusion with the His tag by standard procedures, using restriction fragments, PCR products, or double-stranded synthetic oligonucleotides in which the codons have been optimized for expression in *E. coli*.

### 3.2 Cell Growth, Auto-induction, and Lysis

1. Introduce the expression vector into *E. coli* BL21 (DE3) and select transformants on LB agarose plates containing 100 μg/ml kanamycin.
2. Use a single colony to inoculate a test-tube containing 1 ml of non-inducing medium ZYM-505 with 100 μg/ml kanamycin.
3. Allow growth at 37 °C with shaking until the culture reaches an OD600 of 0.6.
4. Transfer the cultured cells into a 1-l baffled flask containing 250 ml auto-inducing medium ZYM-5052 with 100 μg/ml kanamycin and grow overnight with vigorous shaking at 30 °C until the OD600 is at least 10 (*see* **Note 1**).
5. Centrifuge at 4,000 × *g* to pellet the cells. Discard the supernatant (*see* **Note 2**).
6. Resuspend the cells in 25 ml of ice-cold lysis buffer. Make sure to disperse cell clumps by shaking and/or pipetting up and down. Carry out all subsequent manipulations at 4 °C or on ice. Work quickly to minimize opportunity for proteolysis.
7. Lyse the cells by two passages through a French press (*see* **Note 3**).
8. Centrifuge the sample at 15,000 × *g* at 4 °C to pellet insoluble debris. Check by SDS-PAGE to ensure that the bulk of the protein of interest is in the soluble fraction (the supernatant) and to estimate the yield of the protein of interest (Fig. 4a, *see* **Note 4**).

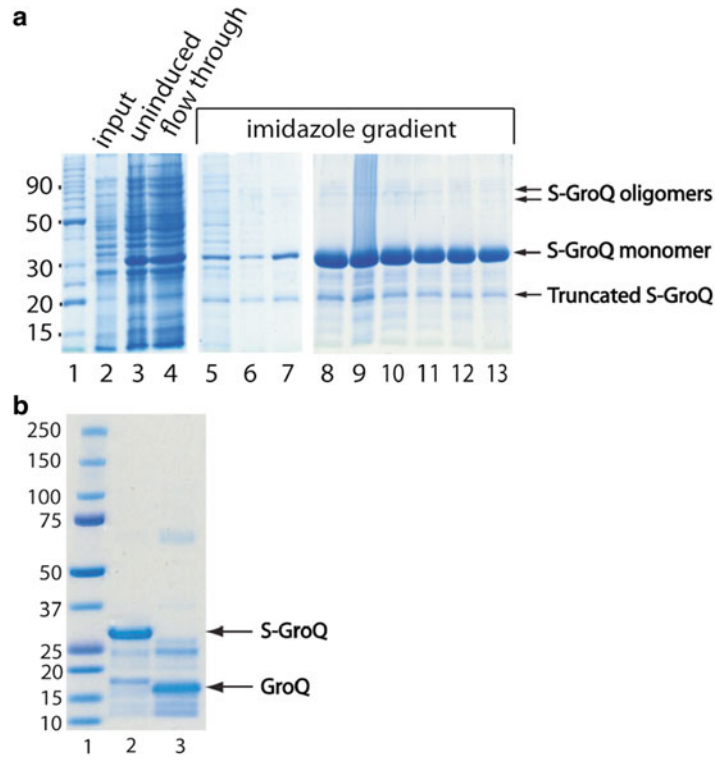


**Fig. 4** Expression of GroQ as a fusion to SUMO. (**a**) Purification of the fusion protein after expression using pMP-SUMO-H6 as monitored by 12 % SDS-PAGE. The lanes are: (*1*) markers (sizes labeled in kilodaltons); (*2*) uninduced culture; (*3*) induced culture; (*4*) flow through; and (*5–13*) imidazole washes and elutions carried out as described in Subheading 3 using the following concentrations of imidazole: 30 mM (*lanes 5* and *6*), 50 mM (*lane 7*), 150 mM (*lanes 8* and *9*), 200 mM (*lanes 10* and *11*), and 250 mM (*lanes 12* and *13*). Identity of bands as shown on the *right* was confirmed by mass spectrometry after in-gel trypsinolysis (not shown). S-GroQ is the SUMO-GroQ fusion protein. (**b**) Results of coexpression of SUMO-GroQ fusion protein and SUMO-Ulp1 fusion protein. The SUMO-GroQ fusion protein was expressed without (*lane 2*) or with (*lane 3*) SUMO-Ulp1. His tagged proteins were purified by Ni-NTA affinity chromatography. The *arrows* indicate the unprocessed fusion protein (S-GroQ) and the Ulp1-cleaved fusion protein (GroQ). *Lane 1* shows size markers (sizes labeled in kilodaltons). Reproduced with the publisher's permission from Kuo et al. [8]

### 3.3 Protein Purification

1. Add 1 ml of nickel nitrilotriacetic acid (Ni-NTA) resin to the supernatant. Allow binding of the protein to nickel by continuously mixing on a rotator for 2 h at 4 °C (*see* **Note 5**).
2. Pour the slurry into a small plastic disposable column apparatus and collect the flow through.
3. Wash the resin twice with 20 ml of Wash Buffer A.
4. Wash the resin twice with 2 ml of Wash Buffer B.

5. Elute the His-tagged protein from the beads with elution buffer as follows: Elute the column two times with 1 ml of Elution Buffer A, and collect the flow through. Repeat this elution with Elution Buffer B, followed by Elution Buffer C.
6. Assess the success of the purification by SDS–PAGE (Fig. 4a). Evaluate the concentration of the protein by the Bradford assay using BSA as a standard (*see* **Note 6**).

## 4 Notes

1. 30 °C is a good growth temperature for expression of many SUMO fusion proteins. If the fusion protein is insoluble when produced at this temperature, it may be useful to explore lower temperatures (down to 15 °C).
2. The cell pellet can be store in the freezer for later use.
3. Alternative methods for cell lysis include sonication and passage through a microfluiditor. However, we have found sonication to be inferior to the other techniques often resulting in reduced protein solubility.
4. It is usually possible to obtain 1 mg or more of protein per gram of wet *E. coli*, such that the protein of interest is the single most abundant protein in the soluble fraction.
5. Ni-NTA resin has a capacity of at least 10 mg of protein per milliliter of resin, and thus, this amount of resin should be more than sufficient to adsorb all the His-tagged protein. Be sure that the resin is kept well suspended in the mixture to maximize the binding. It may be worth optimizing the amount of resin for the particular protein of interest. This can be done on small scale by adding increasing amounts of resin to aliquots of the starting material, incubating with mixing for 2 h, and then removing the beads by centrifugation at $1{,}000 \times g$ for 2 min before checking the supernatant for depletion of the protein of interest by SDS-PAGE.
6. The purity of the final material can be optimized by carefully optimizing the imidazole concentration in the wash and elution buffers to find wash conditions that remove contaminants while leaving the bulk of the protein of interest bound to the beads.

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# Chapter 7

# Rescuing Aggregation-Prone Proteins in *Escherichia coli* with a Dual $His_6$-MBP Tag

**Danielle Needle and David S. Waugh**

## Abstract

Insolubility of recombinant proteins in *Escherichia coli* is a major impediment to their production for structural and functional studies. One way around this problem is to fuse an aggregation-prone protein to a highly soluble partner. *E. coli* maltose-binding protein (MBP) is widely recognized as a premier solubilizing agent. In this chapter, we describe how to construct dual $His_6$-MBP-tagged fusion proteins by Gateway® recombinational cloning and how to predict their yield and solubility. We also describe a simple and rapid procedure to test the ability of a $His_6$-MBP fusion protein to bind to Ni-NTA resin and to be digested by tobacco etch virus (TEV) protease, along with a method to assess the solubility of the target protein after it has been separated from $His_6$-MBP.

**Key words** Maltose-binding protein, MBP, Fusion protein, Solubility enhancer, TEV protease, Tobacco etch virus protease, Hexahistidine tag, His-tag, $His_6$-MBP, Gateway® cloning, Recombinational cloning

## 1 Introduction

When successful, producing recombinant proteins in *Escherichia coli* is relatively inexpensive and straightforward for most molecular biology laboratories. However, many potentially interesting proteins aggregate into an insoluble and unusable form when produced via simple expression schemes [1]. Before abandoning bacterial expression in favor of more complicated and costly eukaryotic systems, we suggest employing a simple strategy that combines the solubility-enhancing benefit conferred by *E. coli* maltose-binding protein (MBP) [2, 3] with the powerful advantage of immobilized metal affinity chromatography (IMAC) [4], made possible by the use of a polyhistidine tag in a tandem configuration with MBP ($His_6$-MBP) [5]. In this chapter, we describe how to construct $His_6$-MBP fusion proteins and conduct a few simple pilot experiments that are reliable predictors of protein production success prior to extensive resource investment.

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## 2 Materials

### 2.1 Construction of Expression Vectors by Recombinational Cloning

1. The Gateway® destination vector pDEST-HisMBP (*see* Fig. 1).
2. PCR Reagents, including thermostable DNA polymerase (*see* **Note 1**).
3. Synthetic oligodeoxyribonucleotide primers for PCR amplification (*see* Fig. 2).
4. TE buffer: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA.
5. Agarose, buffer, and an apparatus for submarine gel electrophoresis of DNA (*see* **Note 2**).
6. MinElute Gel Extraction Kit (Qiagen, Valencia, CA) for the extraction of DNA from agarose gels.
7. Chemically competent *ccdB* Survival™ 2 T1$^{R}$ cells (Life Technologies, Grand Island, NY) for propagating pDEST-HisMBP, pDONR201, or any vector with a Gateway® cloning cassette.

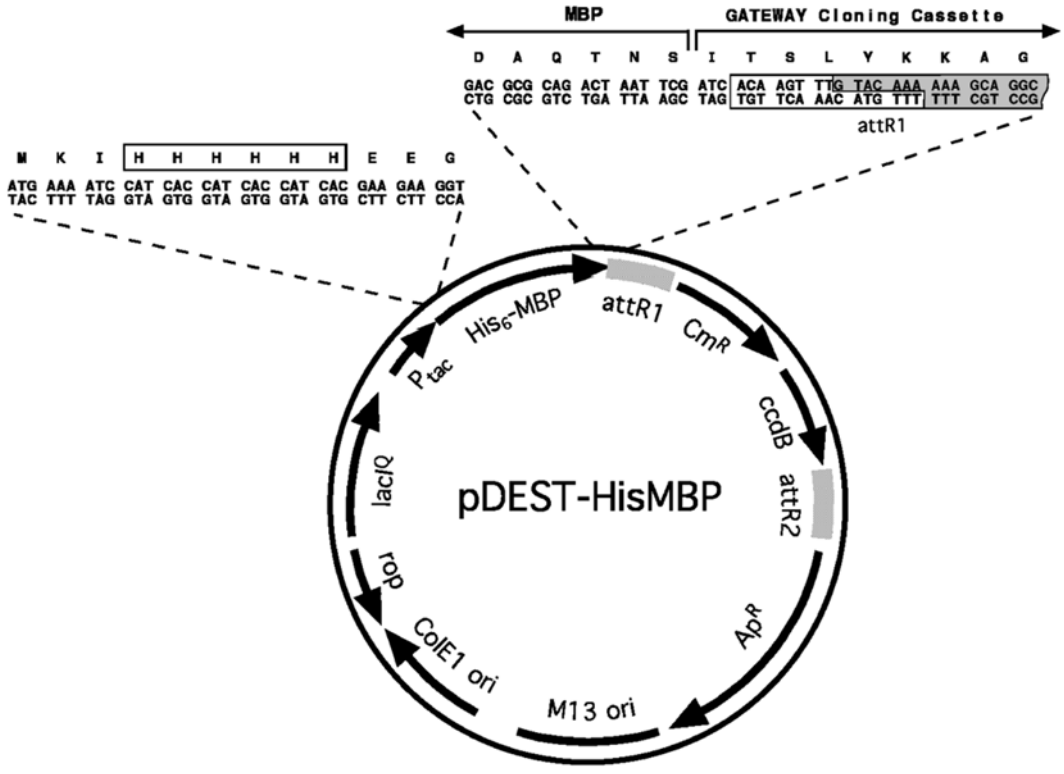


**Fig. 1** Schematic representation of the Gateway® destination vector pDEST-HisMBP. This vector can be recombined with an entry vector that contains an ORF of interest, via the LR reaction, to generate a $His_6$-MBP fusion protein expression vector

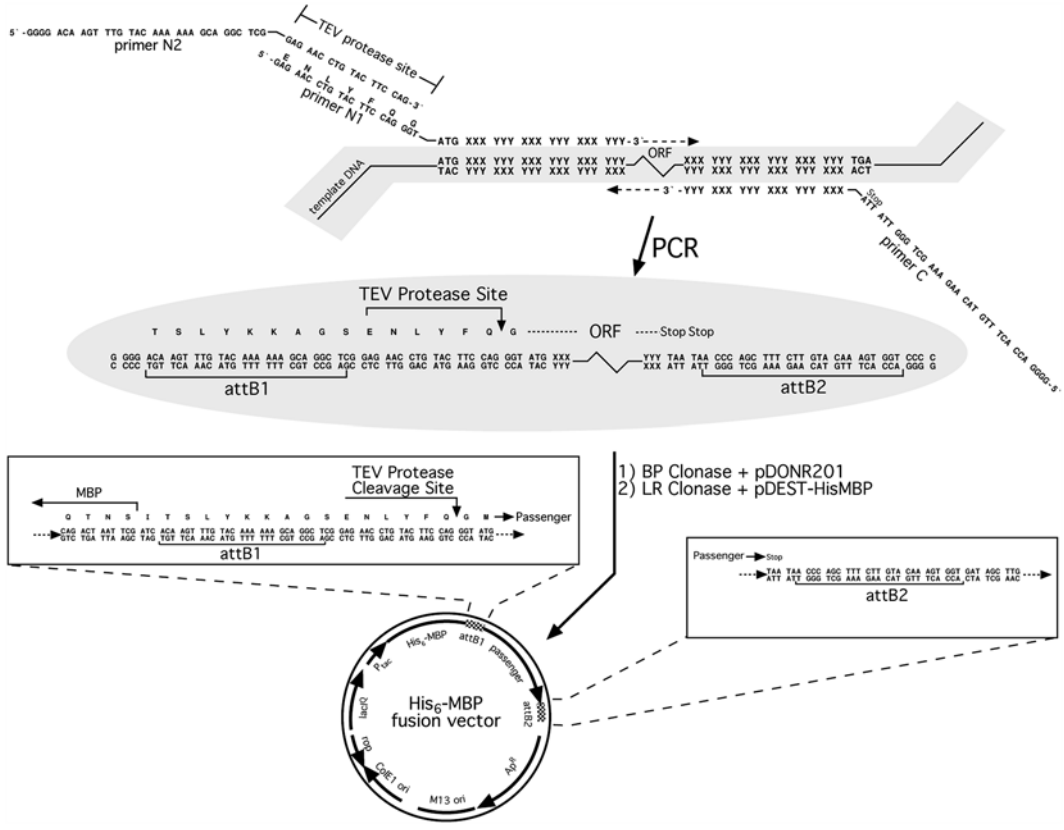


**Fig. 2** Construction of a $His_6$-MBP fusion vector using PCR and Gateway® cloning technology. The ORF of interest is amplified from the template DNA by PCR, using primers N1, N2, and C. Primers N1 and C are designed to base-pair to the 5′ and 3′ ends of the coding region, respectively, and contain unpaired 5′ extensions as shown. Primer N2 base pairs with the sequence that is complementary to the unpaired extension of primer N1. The final PCR product is recombined with the pDONR201 or pDONR221 vector to generate an entry clone, via the BP reaction. This entry clone is subsequently recombined with pDEST-HisMBP using LR Clonase to yield the final $His_6$-MBP fusion vector

8. Competent *gyrA*$^+$ cells (e.g., DH5α, MC1061, HB101) (*see* **Note 3**).
9. Gateway® PCR Cloning System (Life Technologies).
10. LB medium and LB agar plates containing ampicillin (100 μg/ml). LB medium: Add 10 g Bacto Tryptone, 5 g Bacto Yeast Extract, and 5 g NaCl to 1 l of $H_2O$ and sterilize by autoclaving. For LB agar, also add 12 g of Bacto Agar before autoclaving. To prepare plates, allow medium to cool until flask or bottle can be held in hands without burning, then add 1 ml ampicillin stock solution (100 mg/ml in $H_2O$, filter sterilized), mix by gentle swirling, and pour or pipet ca. 30 ml into each sterile petri dish (100 mm dia.).
11. Reagents for small-scale plasmid DNA isolation (*see* **Note 4**).
12. An incubator set at 37 °C.

### 2.2 Pilot Expression, Protease Cleavage, and Solubility Testing

1. Competent Rosetta™ 2(DE3) (EMD Millipore, Billerica, MA) (*see* **Notes 5** and **6**).
2. A derivative of pDEST-HisMBP that produces a $His_6$-MBP fusion protein with a TEV protease recognition site in the linker between MBP and the passenger protein (*see* Subheading 3.1).
3. LB agar plates and broth containing both ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml). Prepare a stock solution of 30 mg/ml chloramphenicol in ethanol. Store at −20 °C for up to 6 months. (*See* Subheading 2.1, **item 10** for LB broth, LB agar, and ampicillin stock solution recipes.) Dilute antibiotics 1,000-fold into LB medium or molten LB agar.
4. Isopropyl-thio-β-D-galactopyranoside (IPTG), Dioxane-free, Ultra Pure. Prepare a stock solution of 200 mM in $H_2O$ and filter-sterilize. Store at −20 °C.
5. Shaker/incubator.
6. Sterile baffled-bottom flasks.
7. B-PER with Enzymes Bacterial Protein Extraction Kit (Pierce Protein Biology Products, Thermo Fisher Scientific, Rockford, IL).
8. Ni-NTA Agarose.
9. AcTEV protease (Life Technologies), or TEV protease produced and purified as described [6].
10. Two IMAC-compatible buffers that contain 25 mM (for Buffer A) and 500 mM (for Buffer B) imidazole. For example, Buffer A: 25 mM Tris–HCl, 200 mM NaCl, 25 mM imidazole, pH 7.2; Buffer B: 25 mM Tris, 200 mM NaCl, 500 mM imidazole, pH 7.2.
11. 4× SDS-PAGE sample buffer (Life Technologies) and 2-mercaptoethanol.
12. SDS-PAGE gel, electrophoresis apparatus, and running buffer (*see* **Note 7**).
13. Gel stain (e.g., Gelcode® Blue from Pierce Protein Biology Products, Thermo Fisher Scientific, or PhastGel™ Blue R from GE Healthcare Life Sciences, Piscataway, NJ).
14. Spectrophotometer.
15. 1.5 ml microcentrifuge tubes.

## 3 Methods

### 3.1 $His_6$-MBP Fusion Vector Recombinational Cloning

The Gateway® recombinational cloning system is based on the site-specific recombination reactions that mediate the integration and excision of bacteriophage lambda into and from the *E. coli* chromosome, respectively. For detailed information about this system, the investigator is encouraged to consult the technical literature supplied by Invitrogen, Inc. (http://www.invitrogen.com).

### 3.1.1 pDEST-HisMBP

To utilize the Gateway® system for the production of $His_6$-MBP fusion proteins, one must first construct or obtain a suitable "destination vector." An example of a destination vector that can be used to produce $His_6$-MBP fusion proteins (pDEST-HisMBP), which is available from the authors or the Addgene plasmid repository (www.addgene.org), is shown in Fig. 1. pDEST-HisMBP was constructed by inserting an in-frame hexahistidine-coding sequence between codons 3 and 4 of MBP in pKM596 [4].

The Gateway® cloning cassette in pDEST-HisMBP carries a gene encoding the DNA gyrase poison CcdB, which provides a negative selection against the destination vector and the donor vector so that only the desired recombinant is obtained when the end products of the recombinational cloning reaction are transformed into *E. coli* and grown in the presence of ampicillin. pDEST-HisMBP and other vectors that carry the *ccdB* gene must be propagated in a host strain with a *gyrA* mutation (e.g., *E. coli* DB3.1) that renders the cells immune to the action of CcdB or, alternatively, in a strain that produces the CcdB antidote CcdA (e.g., *ccdB* Survival™ 2 $T1^R$ cells).

### 3.1.2 Gateway® Cloning Protocol

To construct a $His_6$-MBP fusion expression vector, we amplify the target open reading frame (ORF) by PCR, incorporating into the primers elements necessary for Gateway® cloning and downstream protein production, then perform successive BP and LR reactions. The 3′ ends of the primers include a sufficient number of nucleotides that are complementary to the template sequence to result in a 69 °C melting temperature (by modified Breslauer's method, *see* http://www.thermoscientificbio.com/webtools/tmc/). This enables 2-step PCR cycling using 72 °C as both the annealing and extension temperature. Proximal to the ORF-specific part of the forward primer, we add a sequence that encodes a TEV protease cleavage site preceded by an attB1 site to enable recombination. Because shorter primers are less expensive and because the TEV- and attB1-containing sequences are common to many of our experimental designs, we often use two overlapping forward primers, only one of which is ORF-specific (Fig. 2). An attB2 recombination site is added to the 5′ end of the ORF-specific portion of the reverse PCR primer. During early rounds of cycling, the inner, ORF-specific forward primer acts with the reverse primer to create a template amplified by N2 and the same reverse primer in later rounds. To favor full-length product accumulation, the concentration of N1 is 20-fold lower than that of N2 and C (*see* **Note 8**).

1. The PCR reaction mix is prepared as follows (*see* **Note 9**): 1 pg–10 ng template DNA, 10 μl 2× Phusion Flash PCR Master Mix (contains all necessary reaction components except primers and template, 0.025 μM primer N1, 0.5 μM primer N2, 0.5 μM primer C, $H_2O$ (to 20 μl total volume).

2. The reaction is placed in a thin-walled tube in a thermal cycler with an appropriate program, such as the following: initial denaturation for 3 min at 98 °C; 30 cycles of 98 °C for 10 s and 72 °C for 15 s, and final extension at 72 °C for 60 s (*see* **Note 10**); hold at 4 °C.
3. Purification of the PCR amplicon by agarose gel electrophoresis (*see* **Note 2**) is recommended.
4. To create the $His_6$-MBP fusion vector, the PCR product is recombined first into a donor vector, such as pDONR221 or pDONR201, to yield an entry clone intermediate (BP reaction), and then into pDEST-HisMBP (LR reaction; *see* **Note 11**).
   (a) Add to a microcentrifuge tube: 100 ng of the PCR product in 1–5 μl TE or $H_2O$, 1.3 μl of 150 ng/μl pDONR vector DNA, and enough TE to bring the total volume to 12 μl. Mix well.
   (b) Thaw BP Clonase II enzyme mix on ice (2 min) and then vortex briefly (2 s) twice (*see* **Note 12**).
   (c) Add 3 μl of BP Clonase II enzyme mix to the components in (a) and vortex briefly; incubate the reaction at room temperature for at least 4 h (*see* **Note 13**).
   (d) Add to 10 μl of BP reaction: 2 μl of 150 ng/μl destination vector (pDEST-HisMBP) and 3 μl of LR Clonase II enzyme mix (*see* **Note 12**). Mix by vortexing briefly.
   (e) Incubate the reaction at room temperature for 2 h.
   (f) Add 2 μl of the proteinase K stop solution and incubate for 10 min at 37 °C.
   (g) Transform 1 μl of the reaction into 50 μl of appropriate competent *E. coli*, such as electrocompetent DH5α cells (*see* **Note 3**).
   (h) Spread the cells on an LB agar plate containing ampicillin (100 μg/ml), the selective marker for pDEST-HisMBP (*see* Fig. 1). Incubate the plate at 37 °C overnight (*see* **Note 14**).
5. Plasmid DNA is isolated from saturated cultures started from individual ampicillin-resistant colonies and screened by sequencing putative clones to ensure that there are no PCR-induced mutations.

### *3.2 Pilot Fusion Protein Expression, Small-Batch Purification, TEV Protease Cleavage, and Solubility Testing*

Before investing time and resources on the large-scale expression and purification of a protein, we perform a series of pilot experiments to assess protein production, fusion protein-IMAC resin binding, TEV protease cleavage, and target protein solubility. First, we transform the sequence-verified expression plasmid into an appropriate expression strain and induce production of the fusion protein. Following chemical disruption of the cells, we confirm

that the $His_6$-MBP fusion protein is present in the soluble fraction of the crude cell lysate. After passing this checkpoint, we model the purification steps in a microcentrifuge tube to establish successful binding of fusion protein to Ni-NTA resin. Following elution in this small-batch format, we test for successful cleavage from $His_6$-MBP and sustained solubility of the protein of interest following its liberation. A problem at any of these steps can be addressed before scale-up.

### 3.2.1 Protein Expression

1. Transform competent Rosetta™ 2(DE3) (*see* **Notes 5** and **6**) with the $His_6$-MBP fusion protein expression vector and spread them on an LB agar plate containing ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml). Incubate the plate overnight at 37 °C.
2. Inoculate 5 ml of LB medium containing ampicillin (100 μg/ml) and chloramphenicol (30 μg/ml) in a culture tube with a single colony from the plate. Grow to saturation overnight at 37 °C with shaking.
3. The next morning, inoculate 50 ml of the same medium in a 250 ml baffled-bottom flask with 0.5 ml of the saturated overnight culture.
4. Grow the cells at 37 °C with shaking to mid-log phase ($OD_{600nm} \sim 0.5$).
5. Adjust the temperature to 30 °C (*see* **Note 15**) and add IPTG (1 mM final concentration).
6. After 4 h, measure the $OD_{600nm}$ of the cultures (dilute cells 1:10 in LB to obtain an accurate reading). An $OD_{600nm}$ of about 3–3.5 is normal, although lower or higher densities are possible. If the density of the culture is much lower than this, it may be necessary to adjust the volume of the samples that are analyzed by SDS-PAGE.
7. Transfer 10 ml to a 15 ml conical centrifuge tube and pellet the cells by centrifugation ($4{,}000 \times g$) at 4 °C.
8. Store the cell pellet at –80 °C. Alternatively, the cells can be disrupted immediately and the procedure continued without interruption, as described below.

### 3.2.2 B-PER Chemical Solubilization and Sample Preparation

1. Thaw the cell pellet on ice.
2. Chemically disrupt the cells with B-BER Bacterial Protein Extraction Reagent according to the manufacturer's instructions. Briefly, resuspend the pellet in 500 μl B-PER plus 1 μl lysozyme and 1 μl DNaseI. Incubate the suspension at room temperature for 15 min.
3. Prepare a sample of the total intracellular protein ("T") for SDS-PAGE by mixing 50 μl of the B-PER suspension with

50 µl of 2× SDS-PAGE sample buffer containing 10 % (v/v) 2-mercaptoethanol.

4. Pellet the insoluble cell debris and proteins by centrifuging the B-PER suspension at 15,000 × *g* in a microcentrifuge for 5 min.
5. Prepare a sample of the soluble intracellular protein ("S") for SDS-PAGE by mixing 50 µl of the supernatant from **step 4** with 50 µl of 2× SDS-PAGE sample buffer containing 10 % (v/v) 2-mercaptoethanol.

### 3.2.3 Small-Batch Purification and TEV Protease Cleavage

1. Centrifuge 250 µl of Ni-NTA Agarose slurry at 4,000 × *g* for 1 min in a microcentrifuge tube; remove and discard the supernatant.
2. Wash the resin by adding 400 µl of Buffer A; mix by pipetting up and down. Centrifuge at 4,000 × *g* for 1 min; remove and discard the supernatant. Repeat wash.
3. Bind protein by adding B-PER lysate to Ni-NTA; mix by pipetting up and down. Incubate for 1 h at 4 °C with rocking.
4. Centrifuge at 4,000 × *g* for 1 min; remove the supernatant and retain it as the flow-through ("FT") fraction (by analogy with column chromatography; represents unbound proteins).
5. Wash the resin 3 times with 400 µl Buffer A; centrifuge at 4,000 × *g* for 1 min each time and retain the supernatants from each wash separately for electrophoretic analysis (samples "W1," "W2," and "W3").
6. Elute bound protein from the Ni-NTA resin with 3 separate 50-µl volumes of Buffer B. Centrifuge at 4,000 × *g* for 1 min each time and retain the supernatants from each separately as samples "E1," "E2," and "E3."
7. After removing aliquots for electrophoretic analysis, combine fractions E1-E3 for pilot TEV protease cleavage. Add approximately 0.1 mg of TEV protease per 15 mg of fusion protein. Mix and remove an aliquot for overnight incubation at room temperature; incubate remaining reaction at 4 °C overnight.

### 3.2.4 SDS-PAGE

We typically use precast NuPAGE gradient gels for SDS-PAGE to assess the yield and solubility of MBP fusion proteins (*see* **Note 7**). The investigator may choose any appropriate SDS-PAGE formulation appropriate for the protein size and laboratory preference.

1. Prepare samples from each step of the small-batch purification by mixing an aliquot of each with an equal volume of 2× SDS-PAGE sample buffer containing 10 % (v/v) 2-mercaptoethanol.
2. Heat the T, S, and purification samples at 90 °C for about 5 min and then spin them at maximum speed in a microcentrifuge for 5 min.

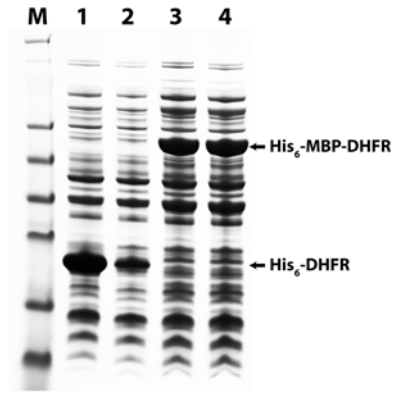


**Fig. 3** Comparison of the solubility of $His_6$-tagged and $His_6$-MBP-tagged *Yersinia pestis* dihydrofolate reductase (DHFR). *Lanes 1–4* represent protein extracted from Rosetta 2(DE3) cells expressing either $His_6$-tagged DHFR or $His_6$-MBP-DHFR from the appropriate plasmid. *Lane M*: SeeBlue Plus2 pre-stained marker standards. *Lane 1*: $His_6$-DHFR total protein. *Lane 2*: $His_6$-DHFR soluble protein. *Lane 3*: $His_6$-MBP-DHFR total protein. *Lane 4*: $His_6$-MBP-DHFR soluble protein

3. Assemble the gel in the electrophoresis apparatus, fill it with SDS-PAGE running buffer, load the samples (4–20 μl) and carry out the electrophoretic separation according to standard lab practices. T and S samples are loaded in adjacent lanes to allow easy assessment of solubility. Molecular weight standards may also be loaded on the gel, if desired.
4. Stain the proteins in the gel with GelCode® Blue reagent, PhastGel™ Blue R, or a suitable alternative.

### 3.2.5 Interpreting the Results

The overexpressed MBP fusion should be apparent as the predominant protein present on the gel. Examining the heaviest band relative to a molecular weight standard should confirm that the fusion is about the size of the protein of interest plus 42 kDa, the approximate size of MBP. Placing the total and soluble fractions next to each other on the gel allows for easy comparison and determination of the fusion's solubility.

Figure 3 illustrates the benefit of using MBP as a solubility enhancer. Lane 1 indicates that upon induction, the Rosetta 2(DE3) expression strain was able to produce *Yersinia pestis* dihydrofolate reductase (DHFR) from a plasmid encoding the $His_6$-tagged protein. However, lane 2 reveals that most of the $His_6$-tagged protein is not found in the soluble fraction. In contrast, lanes 3 (total protein) and 4 (soluble protein) clearly demonstrate that virtually all of the $His_6$-MBP-DHFR fusion protein is soluble when produced in the same strain.

Performing a small-batch purification using Ni-NTA agarose in a microcentrifuge tube can establish whether or not the soluble $His_6$-MBP fusion protein will bind to a similar column during

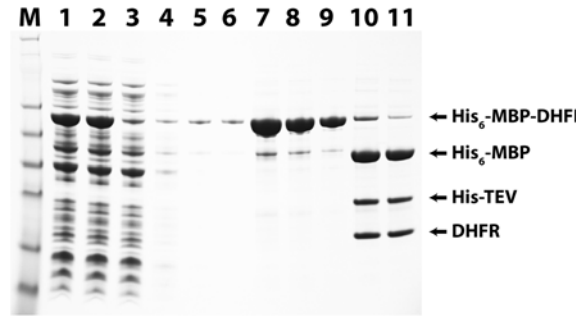


**Fig. 4** Small-scale pilot expression, purification, and digestion of fusion protein with TEV protease. *Yersinia pestis* DHFR was expressed from a derivative of pDEST-HisMBP in Rosetta 2(DE3) cells as described (*see* Subheading 3.2). *Lane M*: SeeBlue Plus2 pre-stained marker standards. *Lane 1*: $His_6$-MBP-DHFR total protein. *Lane 2*: $His_6$-MBP-DHFR soluble protein. *Lane 3*: Unbound/"flow-through" fraction. *Lanes 4–6*: Buffer A washes 1–3. *Lanes 7–9*: Elution fractions 1–3. *Lane 10*: TEV protease digest, total protein. *Lane 11*: TEV protease digest, soluble protein (*see* Subheadings 3.2, **items 4** and **5**)

scale-up, thereby enabling the investigator to identify at an early stage those rare cases in which the $His_6$-tag is inaccessible and therefore unavailable for binding. Figure 4 shows the results of such a pilot purification, again using *Y. pestis* DHFR. The total and soluble intracellular proteins from cells overproducing the $His_6$-MBP-DHFR fusion protein are shown in lanes 1 and 2, respectively. After incubation of the soluble fraction with Ni-NTA agarose, nearly all of the fusion protein was bound to the resin and removed by centrifugation at this stage (lane 3; "flow-through"). Had the $His_6$-MBP-DHFR fusion protein failed to bind to the resin, a heavy band migrating at the size of the fusion would have appeared in the "flow-through" lane, predicting potential trouble with nickel column binding during scale-up. Most other intracellular proteins were separated from the resin during the three wash steps (lanes 4–6). The predominant band in the three elution fractions (lanes 7–9) is the $His_6$-MBP fusion. Note that the protein is not absolutely pure at this stage, but it is significantly enriched relative to the starting material.

The product of the small-batch purification trial is sufficiently pure to test its ability to be cleaved by TEV protease in vitro. In lanes 10 and 11 of Fig. 4, which correspond to total and soluble products of the cleavage reaction, the band representing the fusion protein has largely disappeared, and three significant bands have materialized: a 42-kDa band for $His_6$-MBP, a 28-kDa band for His-TEV protease, and an 18-kDa band migrating at the expected size of DHFR. The nearly identical intensities of the DHFR bands in the total and soluble samples indicate that DHFR remains soluble after cleavage from MBP, suggesting that it is probably

properly folded. Had the TEV protease failed to cleave the fusion or had the target protein become insoluble after cleavage, troubleshooting would have been necessary. Otherwise, having successfully passed these diagnostic tests, the production and purification of the protein may now be scaled up as described [4].

#### 3.2.6 Troubleshooting

Not every MBP fusion protein will be highly soluble. However, solubility usually can be increased by reducing the temperature of the culture from 30 to 18 °C during the time that the fusion protein is accumulating in the cells (i.e., after the addition of IPTG). In some cases, the improvement can be quite dramatic. It may also be helpful to reduce the IPTG concentration to a level that will result in partial induction of the fusion protein. Under these conditions, longer induction times (18–24 h) are required to obtain a reasonable yield of fusion protein.

If the fusion protein is a poor substrate for TEV protease in the small batch experiment, then the same is likely to be true during scale-up. However, in most cases it is still possible to obtain a sufficient quantity of the pure passenger protein by scaling up production (e.g., 4–6 l of cells or more). In especially problematic cases, the efficiency of the protease digest can be improved by inserting additional amino acid residues between the TEV protease recognition site and the N-terminus of the passenger protein. We have used both polyglycine and a FLAG-tag epitope in this position with good results [7].

Occasionally, a passenger protein may accumulate in a soluble but biologically inactive form after intracellular processing of an MBP fusion protein. Exactly how and why this occurs is unclear, but we suspect that fusion to MBP somehow enables certain proteins to evolve into kinetically trapped folding intermediates that are no longer susceptible to aggregation. Therefore, although solubility after intracellular processing is generally a useful indicator of a passenger protein's folding state, it is not absolutely trustworthy. For this reason, we strongly recommend that a biological assay be employed (if available) at an early stage to confirm that the passenger protein is in its native conformation.

## 4 Notes

1. We recommend a processive, high-fidelity polymerase such as *Phusion* (Thermo Fisher, Waltham, MA or New England Biolabs, Ipswich, MA, USA) to reduce cycling times and minimize the occurrence of mutations during PCR.
2. We typically purify fragments by horizontal electrophoresis in 1–2 % Certified Molecular Biology Agarose (Bio-Rad, Hercules, CA) gels run in sodium boric acid [8] using standard

submarine equipment. DNA fragments are extracted from slices of the ethidium bromide-stained gel using a MinElute Gel Extraction Kit (Qiagen) in accordance with the instructions supplied with the product.

3. Any *gyrA*$^+$ strain of *E. coli* can be used. We prefer ElectroMAX™ DH5α-E™ Competent Cells (Life Technologies) because they are easy to use and very efficient.
4. We prefer the QIAprep™ Spin miniprep kit (Qiagen), but similar kits can be obtained from a wide variety of vendors.
5. Chemically competent cells are transformed according to the manufacturer's instructions. Electrocompetent cells can be purchased or prepared: briefly, the cells are grown in 1 l of LB medium (with antibiotics, if appropriate) to mid-log phase ($OD_{600} \sim 0.5$) and then chilled on ice. The cells are pelleted at 4 °C, resuspended in 1 l of ice-cold $H_2O$ and then pelleted again. After several such washes with $H_2O$, the cells are resuspended in 3–4 ml of 10 % glycerol, divided into 50 μl aliquots, and then immediately frozen in a dry ice–ethanol bath. Competent cells are stored at −80 °C. Electrotransformation procedures can be obtained from the electroporator manufacturers (e.g., Bio-Rad, BTX, Eppendorf). Immediately prior to electrotransformation, the cells are thawed on ice and mixed with 10–100 ng of DNA (e.g., a plasmid vector or a Gateway® reaction). The mixture is placed into an ice-cold electroporation cuvette and electroporated according to the manufacturer's recommendations (e.g., a 1.5 kV pulse in a cuvette with a 1 mm gap). 1 ml of SOC medium [9] is immediately added to the cells and they are allowed to grow at 37 °C with shaking (ca. 250 rpm) for 1 h. 5–200 μl of the cells is then spread on an LB agar plate containing the appropriate antibiotic(s).
6. If the open reading frame encoding the passenger protein contains codons that are rarely used in *E. coli* (http://www.doe-mbi.ucla.edu/cgi/cam/racc.html), this can adversely affect the yield of an MBP fusion protein. In such cases, it is advisable that the expression strain carry an additional plasmid that codes for the cognate tRNA genes for rare codons. The pRIL plasmid (Stratagene, La Jolla, CA) is a derivative of the p15A replicon that carries the *E. coli argU*, *ileY*, and *leuW* genes, which encode the cognate tRNAs for AGG/AGA, AUA, and CUA codons, respectively. pRIL is selected for by resistance to chloramphenicol. In addition to the tRNA genes for AGG/AGA, AUA, and CUA codons, the pRARE accessory plasmid in the Rosetta™ host strain (Novagen, Madison, WI) also includes tRNAs for the rarely used CCC and GGA codons. Like pRIL, the pRARE plasmid is a chloramphenicol-resistant derivative of the p15A replicon. Both of these tRNA accessory plasmids are compatible with derivatives of pDEST-HisMBP.

7. We find it convenient to use precast SDS-PAGE gels, running buffer, molecular weight standards and electrophoresis supplies from Life Technologies.
8. Alternatively, the PCR reaction can be performed in two separate steps, using primers N1 and C in the first step and primers N2 and C in the second step. The PCR amplicon from the first step is used as the template for the second PCR. All primers are used at the typical concentrations for PCR in the two-step protocol.
9. The PCR reaction can be modified in numerous ways to optimize results, depending on the nature of the template and primers. *See* [9] (Vol. 2, Chapter 8) for more information.
10. PCR cycle conditions can also be varied based on reagents and consumables chosen, template complexity, and length of gene. For example, when using Phusion Flash High-Fidelity PCR Master Mix, extend for 15 s/kb of DNA. Consult the directions provided by the manufacturer of your thermostable polymerase.
11. This "one-tube" Gateway® protocol bypasses the isolation of an "entry clone" intermediate. However, the entry clone may be useful if the investigator intends to experiment with additional Gateway® destination vectors, in which case the BP and LR reactions can be performed sequentially in separate steps; detailed instructions are included with the Gateway® PCR kit. Alternatively, entry clones can easily be regenerated from expression clones via the BP reaction, as described in the instruction manual.
12. Clonase enzyme mixes should be thawed according to the manufacturer's directions.
13. At this point, we remove a 5 μl aliquot from the reaction and add it to 0.5 μl of proteinase K stop solution. After 10 min at 37 °C, we transform 2 μl into 50 μl of competent DH5α cells (*see* **Note 3**) and spread 100–200 μl on an LB agar plate containing kanamycin (25 μg/ml), the selective marker for pDONR201. From the number of colonies obtained, it is possible to gauge the success of the BP reaction. Additionally, entry clones can be recovered from these colonies in the event that no transformants are obtained after the subsequent LR reaction.
14. If very few or no ampicillin-resistant transformants are obtained after the LR reaction, the efficiency of the process can be improved by incubating the BP reaction overnight.
15. We have found that decreasing the induction temperature to 30 °C increases the quality and solubility of the fusion protein without significantly decreasing the yield, especially in the presence of glucose.

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# Chapter 8

# Expression, Purification, and Immobilization of Recombinant Tamavidin 2 Fusion Proteins

**Yoshimitsu Takakura, Naomi Oka, and Masako Tsunashima**

## Abstract

Tamavidin 2 is a fungal avidin-like protein that binds biotin with high affinity. Unlike avidin or streptavidin, tamavidin 2 in soluble form is produced at high levels in *Escherichia coli*. In this chapter, we describe a method for immobilization and purification of recombinant proteins with the use of tamavidin 2 as an affinity tag. The protein fused to tamavidin 2 is tightly immobilized and simultaneously purified on biotinylated magnetic microbeads without loss of activity.

**Key words** Affinity tag, Biotin, *Escherichia coli*, Immobilization, Purification, Tamavidin

## 1 Introduction

Avidin from egg white and its bacterial analogue, streptavidin, are tetrameric proteins that bind biotin (vitamin H) with remarkably high affinity; the interactions between avidin or streptavidin and biotin are the strongest known noncovalent bonds to be formed between two biomolecules [1]. Consequently, the system of avidin or streptavidin–biotin association has been exploited for various biotechnology applications [2]. Avidin and streptavidin can be used conveniently as affinity tags for protein purification [3, 4] and for protein immobilization [5, 6] when fused with another protein. Although the *Escherichia coli* expression system is inexpensive and easy to work with, avidin and streptavidin and their respective fusion proteins are expressed as insoluble inclusion bodies in *E. coli* in most cases; therefore, renaturation and tedious downstream processing are required for the preparation of active proteins [7–10]. Even when the fusion proteins are expressed in soluble form, their expression levels are very low [6, 11]: for instance, the expression level of trypsin–streptavidin fusion protein in *E. coli* was only 1/10 that of trypsin alone in *E. coli* [6]. These difficulties have restricted the practical use of avidin- and streptavidin-containing fusion proteins.

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Tamavidins are avidin-like biotin-binding proteins from a mushroom, *Pleurotus cornucopiae*. Recently, we reported high-level production of tamavidins (tamavidin 1 and tamavidin 2) in soluble form in *E. coli* [12]. Here, we discuss a strategy for efficient purification and immobilization of recombinant proteins with the use of tamavidin 2 as an affinity tag. The gene encoding a protein of interest (the fusion partner) is fused to that encoding tamavidin 2 via a linker sequence, and the resultant fusion gene is expressed in *E. coli*. The crude extract from the bacteria, which contains the fusion protein, is incubated with biotin bound to a support (e.g., biotinylated magnetic microbeads). The partner protein is immobilized onto the support via the tamavidin 2-biotin interaction. Since tamavidin 2 binds biotin with a very high affinity, scarcely any tamavidin 2 fusion protein dissociates from the support during the washing procedure. Thus, the protein immobilized on the support is purified efficiently.

The use of the tamavidin 2 fusion technology, as described here, has been successfully demonstrated for several partner proteins. Further modification and optimization of experimental steps and factors specific to other partner proteins will likely maximize the production of functional fusion proteins immobilized onto magnetic microbeads.

## 2 Materials

### 2.1 Construction of a Fusion Gene Encoding a Partner Protein and Tamavidin 2

The genes encoding a partner protein and tamavidin 2 are fused according to standard molecular cloning methods and procedures. See a general book of molecular cloning (e.g., ref. 13) for the required materials.

Because tamavidin 2 serves as an affinity tag, an expression vector suitable for protein production in *E. coli* without another affinity tag can be used: e.g., pTrc99A manufactured by Amersham Biosciences (Piscataway, NJ, USA).

### 2.2 Expression of a Tamavidin 2 Fusion Protein in E. coli

1. Conical flasks or equivalent, suitable for culture of *E. coli*.
2. Shaking incubator at 30 °C, suitable for culture of *E. coli*.
3. Spectrophotometer or equivalent, suitable for monitoring the growth of *E. coli*.
4. Centrifuge with a swing rotor and conical tubes (50 mL) with caps that can be used at a centrifugal force of 3,000 × *g*.
5. Host *E. coli*, BL21 [$F^-_B$, *omp* T, *hsd* S($r_B^-$, $m_B^-$), *gal*, *dcm*], which is suitable for protein over-expression and is available from several manufacturers, including GE Healthcare UK (Buckinghamshire, England).

6. Luria–Bertani (LB) medium: 10 g tryptone, 5 g yeast extract, and 10 g NaCl per liter water (deionized), adjusted to pH 7.0 with NaOH, and sterilized by autoclaving.
7. 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) in water, stored at −20 °C.
8. *See* ref. 13 for other materials commonly used for culture of *E. coli*.

### 2.3 Confirmation of the Expression of a Fusion Protein

1. Centrifuge and centrifuge tubes that can be used at a centrifugal force of 15,000 × *g* or greater.
2. Ultrasonic homogenizer, or another apparatus, suitable for disruption of *E. coli* cells.
3. Apparatus for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). *See* ref. 13.
4. Imaging analyzer or a densitometer suitable for analyzing protein bands visualized by SDS-PAGE.
5. Apparatus for immunoblot analysis including a polyvinylidine fluoride (PVDF) membrane. *See* ref. 13.
6. 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), adjusted to pH 7.5 with KOH.
7. Reagents for measuring protein concentration, e.g., Bradford's dye reagent, commercially available from Thermo Fisher Scientific (Waltham, MA, USA).
8. Reagents for SDS-PAGE including a molecular marker (e.g., low-molecular-weight electrophoresis calibration sample from Amersham Biosciences). We used a 15 % SDS-polyacrylamide gel to separate the denatured monomer protein from other proteins, and Coomassie Brilliant Blue R-250 (CBB) from Sigma (St Louis, MO, USA) for protein staining.
9. Reagents required for immunoblot analysis (*see* ref. 13) including tamavidin 2 antibody [12], goat anti-rabbit IgG alkaline phosphatase conjugate (e.g., from Bio-Rad Laboratories, Hercules, CA, USA), and a substrate for alkaline phosphatase (e.g., alkaline phosphatase substrate kit II from Vector Laboratories, Burlingame, CA, USA).
10. Biotinylated peroxidase and a peroxidase substrate, commercially available from several manufacturers, including Vector Laboratories.
11. Tris-buffered saline (TBS): 50 mM Tris buffer, pH 7.5 with 150 mM NaCl.
12. TBS containing 0.5 % (w/v) defatted skim milk.
13. TBS containing 3 % (w/v) bovine serum albumin (BSA).
14. Reagents for measuring the activity of the protein of interest.

### 2.4 Immobilization and Purification of a Tamavidin 2 Fusion Protein on Biotinylated Solid Support

1. 15 mL conical tube with a cap (e.g., from Corning, Acton, MA, USA).
2. 1.5 mL conical tube with a cap (e.g., from Eppendorf, Hamburg, Germany).
3. Magnetic microbeads, commercially available from several manufacturers: e.g., Dynabeads M-270 Amine from Dynal Biotech (Oslo, Norway).
4. A magnet for separation of the magnetic microbeads from liquid sample matrices: e.g., DynaMag-2 and DynaMag-15 manufactured by Invitrogen (Carlsbad, CA, USA).
5. Phosphate-buffered saline (PBS): 10 mM phosphate buffer (pH 7.0) with 150 mM NaCl.
6. PBS containing 0.1 % (w/v) BSA and 0.01 % (v/v) Tween 20.
7. PBS containing 0.2 % (v/v) Tween 20.
8. *N*-hydroxysuccinimide ester of biotin (EZ-Link NHS-LC-biotin [linker length, 22.4 Å] or NHS-LC-LC-biotin [linker length, 30.5 Å], available from Pierce Biotechnology Inc., Rockford, IL, USA).
9. 2× SDS gel-loading buffer: 100 mM Tris–HCl, pH 6.8, 200 mM dithiothreitol (DTT) or β-mercaptoethanol, 4 % (w/v) SDS (electrophoresis grade), 0.2 % (w/v) bromophenol blue, and 20 % (v/v) glycerol.
10. Extraction buffer: 100 mM HEPES-KOH, pH 7.5.

## 3 Methods

### 3.1 Construction of a Fusion Gene Encoding a Partner Protein and Tamavidin 2

To minimize steric hindrance between the partner protein and tamavidin 2 in the fusion protein, it is necessary to insert a short linker between the proteins. For example, a peptide of Gly-Gly-Gly-Gly-Ser-Gly is a structurally flexible, versatile linker. For a partner protein with a monomer molecular mass of 30 kDa or more, longer linkers, such as 3–5 repeats of (Gly-Gly-Gly-Gly-Ser), are recommended.

Polymerase chain reaction (PCR) is a convenient method for creation of a fusion gene without extra vector sequences, such as unnecessary restriction sites, which may be introduced by conventional subcloning. In our trials, expression of fusion proteins in soluble form was higher when the partner proteins were connected to the N-terminus of tamavidin 2 than when the proteins were connected in the other orientation (*see* **Note 1**). Below we describe our procedure for construction of a fusion gene exemplified by the use of expression vector pTrc99A. For manipulation and cloning of DNA, see also a standard protocol book: e.g., ref. 13.

1. Amplify the DNA for the partner protein by performing a PCR with the primers P1 and P2 (PCR1 in Fig. 1). Because the 5′

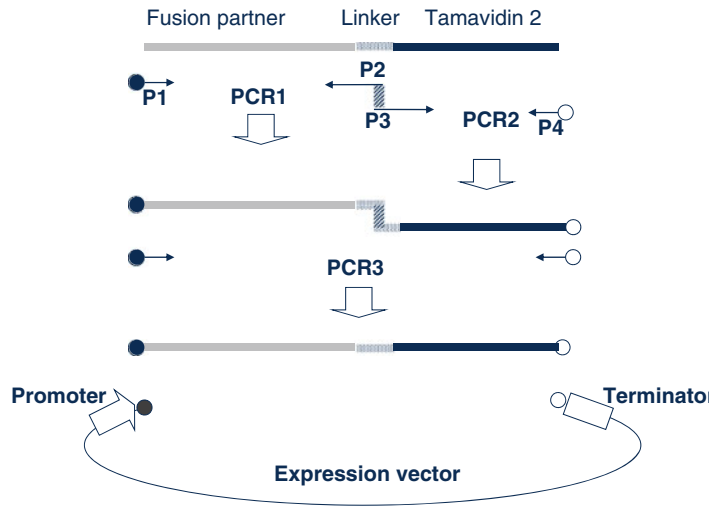


**Fig. 1** Gene construction scheme for tamavidin 2 fusion protein by using PCR

end of the coding sequence is later connected to the *Nco*I site of the promoter in the expression vector, a restriction site compatible with *Nco*I, such as *Nco*I (CCATGG), *Bsp*LU11I (ACATGT), or *Rca*I (TCATGA) (*see* **Note 2**), is added to P1. A part of the linker sequence is added to P2, whose 3′-terminal sequence of between 20 and 40 nucleotides is complementary to the 5′ end of P3 in the next step.

2. Amplify the DNA for tamavidin 2 by performing a PCR with primers P3 and P4 (PCR2 in Fig. 1). A part of the linker, including the complementary sequence described above, is added to P3. A restriction site, the recognition sequence for *Bam*HI or another enzyme compatible with *Bam*HI, is added just behind the stop codon of tamavidin 2 in P4.
3. Perform preparative agarose gel electrophoresis so that the PCR products from **steps 1** and **2** are separated from the templates and primers.
4. Excise and purify the products from the agarose gel. The QIAEX II kit from QIAGEN Inc. (Valencia, CA, USA) is suitable for this step.
5. Join the two PCR products from **step 3** by performing a PCR with primers P1 and P4 (PCR3 in Fig. 1).
6. Digest the resultant PCR product from **step 5** with the restriction enzymes that recognize the sequences at the both ends of the product, and conduct preparative agarose gel electrophoresis.
7. Excise and purify the digested product from the gel.
8. Clone the product into the expression vector pTrc99A predigested with *Nco*I and *Bam*HI. For methods for DNA ligation and transformation of *E. coli* strain BL21, *see* ref. 13.

9. Verify the DNA sequence of the cloned fragment.
10. Grow *E. coli* strain BL21 that carries the resultant expression vector on LB agar supplemented with 100 μg/mL ampicillin overnight at 37 °C.

### 3.2 Expression of a Tamavidin 2 Fusion Protein in E. coli

1. Pick up a single colony from a fresh plate produced in **step 10** of Subheading 3.1, Inoculate 2 mL of LB broth supplemented with 100 μg/mL ampicillin, and incubate the culture overnight at 30 °C with vigorous shaking.
2. Add 1 mL of the overnight culture from **step 1** to 50 mL of LB broth supplemented with 100 μg/mL ampicillin in a 200 mL flask, and incubate at 30 °C with vigorous shaking.
3. Add 1 mM (final concentration, *see* **Note 3**) IPTG when the absorbance at 600 nm ($A_{600}$) of the culture reaches the range between 0.4 and 0.6.
4. Incubate the culture further for 5 h at 30 °C with vigorous shaking (*see* **Note 3**).
5. Transfer the bacterial culture to a 50 mL tube and centrifuge at 2,300 × *g* in a swing rotor for 20 min.
6. Discard the supernatant and store the bacterial pellet at −80 °C until use.

### 3.3 Detection of the Expression of an Active Fusion Protein

The expression of the fusion protein is confirmed by SDS-PAGE and/or immunoblot analysis.

1. Add 3 mL of extraction buffer to the bacterial pellet obtained in **step 6** of Subheading 3.2, in the original 50-mL tube.
2. Dissolve the pellet well by pipetting for 1 min, and then extract the total soluble protein by sonication. Keep the tube on ice, insert the stainless probe of an ultrasonic homogenizer directly into the bacterial suspension, and apply ultrasonic waves for a total of 15 min with three intervals (between the pulses) of 1 min each to disrupt the bacterial cells completely.
3. Transfer the extract to 1.5 mL tubes, centrifuge at 15,000 × *g* for 20 min, and recover the supernatant to 1.5 mL new tubes.
4. Measure the protein concentration. Kits for this purpose are commercially available from several manufacturers, such as Thermo Fisher Scientific. BSA is recommended as a standard.
5. Separate the total soluble protein by SDS-PAGE and stain with CBB. Adjust the concentration of the gel to between 10 and 15 % acrylamide, depending on the size of the fusion protein. In panel A of Fig. 2, an example of SDS-PAGE analysis of the expression of a tamavidin 2 fusion protein is shown.
6. Blot the separated proteins onto a PVDF membrane (*see* ref. 13).

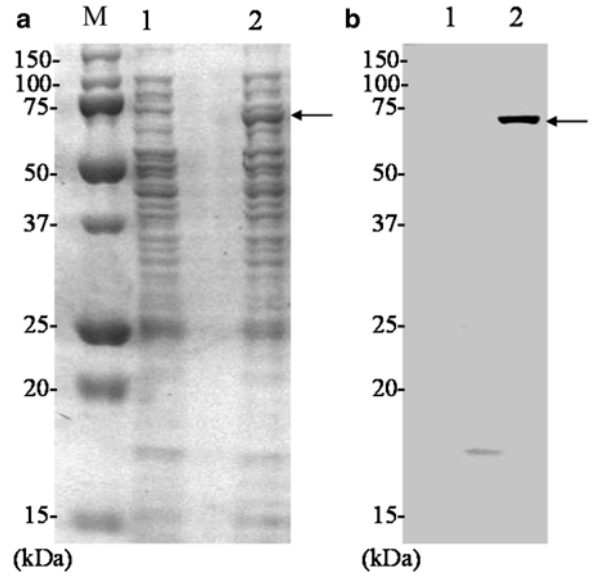


**Fig. 2** Expression of tamavidin 2 fusion proteins in *E. coli*. (**a**) Total soluble proteins from *E. coli* harboring pTrc99A empty vector (*lane 1*), or pTrc99A containing a gene for a bacterial sialyltransferase-tamavidin 2 fusion protein (*lane 2*) were separated by SDS-PAGE and stained with CBB. (**b**) The fusion protein was detected by immunoblotting with tamavidin 2 antibody. M denotes size markers. *Arrows* indicate the expressed fusion protein. Because the molecular mass of sialyltransferase is 56.7 kDa and that of tamavidin 2 is 15.5 kDa under denaturing conditions, we considered the 70-kDa protein to be a fusion protein. The expression level of the fusion proteins reached approximately 80 mg/L *E. coli* culture. (Reproduced from ref. 14 with permission from Elsevier)

7. Incubate the membrane with an antibody for tamavidin 2 [12] or for the partner protein diluted to 1:1,000 in 10 mL of TBS containing 0.5 % skim milk overnight with gentle shaking.
8. Incubate the membrane with a second antibody, such as goat anti-rabbit IgG alkaline phosphatase conjugate, and visualize immunoreactive molecules with a substrate suitable for the enzyme conjugated with the 2nd antibody. In panel B of Fig. 2, an example of an immunoblot analysis for the detection of a tamavidin 2 fusion protein is shown.
9. Measure the amount of the expressed fusion protein by using an imaging analyzer or a densitometer with either the recombinant tamavidin 2 available from Wako Pure Chemicals (Osaka, Japan) or the purified fusion partner protein as a calibration standard.
10. Optionally, the biotin-binding activity may be measured as follows (*see* **Note 4**). The total soluble protein is mixed with an equal amount of 2× SDS gel-loading buffer and loaded onto an

SDS-PAGE gel with an acrylamide concentration of between 7.5 and 10 %, depending on the size of the fusion protein. In this case, the usual heat pretreatment at 95 °C is omitted so that the quaternary structure of the fusion protein is retained. The separated protein is blotted onto a PVDF membrane and incubated with a biotinylated peroxidase in TBS buffer containing 3 % BSA at room temperature for 1 h. After washing the membrane three times, the biotin-binding activity on the membrane is visualized by using a suitable substrate for peroxidase. The activity is roughly estimated by comparing the signal strength with that of recombinant tamavidin 2 from Wako Pure Chemicals, which is loaded as a control onto the same gel.

### 3.4 Immobilization and Purification of the Fusion Protein on Biotinylated Microbeads

As solid supports for large amounts of protein, microspheres are more suitable than microplates because the former have a much larger surface area than the latter. Magnetic microbeads are commercially available from several manufacturers, including Dynal Biotech (Oslo, Norway). The fusion proteins we have tested so far formed tetramers (*see* **Note 5**). To minimize steric hindrance and increase the binding affinity between tamavidin 2 and biotin, we recommend that the linker between the fusion protein and the microbeads is made longer than 22.4 Å, preferably between 30 and 60 Å.

1. Biotinylate magnetic microbeads, Dynabeads M-270 Amine, with *N*-hydroxysuccinimide ester of biotin (NHS-LC-LC-biotin, linker length 30.5 Å) in a 1.5 mL tube according to the manufacturer's instruction.
2. Collect the biotinylated magnetic microbeads (3 mg; $2 \times 10^8$ beads) by placing the tube on a magnet, such as DynaMag-2, for 4 min and then removing the supernatant.
3. Add 400 μL of PBS containing 0.1 % BSA and 0.01 % Tween 20 to the beads.
4. Resuspend the beads well by pipetting for 1 min. Avoid foaming.
5. Place the tube again on the magnet for 4 min. Carefully pipette off the supernatant, leaving the beads undisturbed.
6. Repeat **steps 3–5**.
7. Add 400 μL of PBS to the beads, resuspend the beads well by pipetting for 1 min, and transfer the suspension to a 15 mL tube. At this stage, biotinylation of the microbeads is complete.
8. Suspend 20 mg (*see* **Note 6**) of total soluble protein (obtained from the 25 mL culture of *E. coli* expressing the fusion protein; *see* **step 4** in Subheading 3.3) in 10 mL HEPES-KOH pH 7.5, and add to the beads.
9. Resuspend the beads well by pipetting for 1 min. Avoid foaming.

10. Incubate the mixture with tilting or rotation for 1 h at room temperature to allow the tamavidin 2 moiety of the fusion protein to bind the biotin bonded to the beads.
11. Place the tube on a magnet, such as DynaMag-15, for 4 min. Carefully pipette off the supernatant, leaving the beads undisturbed.
12. Add 1 mL of PBS containing 0.2 % Tween 20 to the beads.
13. Resuspend the beads well by pipetting for 1 min. Avoid foaming.
14. Transfer the mixture to a new 1.5 mL tube.
15. Place the tube on a magnet, such as DynaMag-2 for 4 min so that the beads are collected at the tube wall.
16. Carefully pipette off the supernatant, leaving the beads undisturbed.
17. Wash the beads three times with 1 mL of PBS containing 0.2 % Tween 20.
18. Carefully pipette off the supernatant, leaving the beads undisturbed.
19. Add 200 μL of 0.02 % (w/v) sodium azide in PBS to the beads.
20. Resuspend the beads well by pipetting for 1 min. Avoid foaming.
21. Store the beads at 4 °C before use.
22. The purity of the fusion protein immobilized on the beads is estimated as follows. An aliquot of the beads is incubated with 2× SDS gel-loading buffer at 99 °C for 10 min. The fusion protein thus eluted in the buffer is separated by using SDS-PAGE and stained by CBB (Fig. 3). The intensity of staining is measured with the use of an imaging analyzer. The purity of the fusion protein is calculated as the ratio between the intensity of staining of the band specific to the fusion protein and the intensity of staining of the entire lane and is expressed as a percentage.
23. Characterize the nature and function of the partner protein immobilized with tamavidin 2 (*see* **Note 7**).

## 4 Notes

1. In cases where a short peptide like a hexa-histidine is the fusion partner, fusion to the N- or C-terminus of tamavidin 2 would probably both yield high levels of soluble protein.
2. The choice of the restriction enzyme depends on the nucleotide just downstream of ATG of the gene encoding the fusion partner protein. It is a good idea to add three more nucleotides, such as AAA at the 5′ end of the primer.

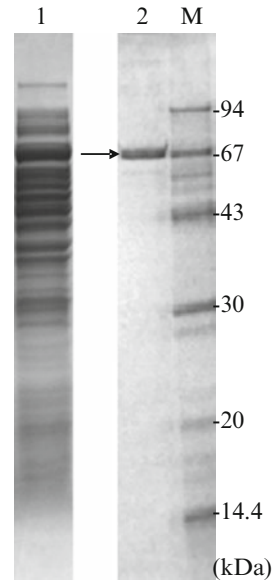


**Fig. 3** Purification of the tamavidin 2 fusion protein immobilized on magnetic microbeads. Crude protein extract (*lane 1*) from *E. coli* expressing a bacterial sialyltransferase–tamavidin 2 fusion protein was incubated with biotinylated microbeads (linker length: 30.5 Å) and washed three times; the immobilized fusion protein (*lane 2*) was eluted by heating in the presence of SDS. The proteins were separated by using SDS-PAGE and stained with CBB. M denotes size markers. The *arrow* indicates the fusion protein. (Reproduced from ref. 14 with permission from Elsevier)

3. For the induction, the ideal concentration of IPTG (between 0.1 and 1 mM), temperature (between 25 and 37 °C), and period of incubation (between 4 and 24 h) depends on the fusion partner and may be optimized experimentally.
4. This procedure for measuring biotin-binding activity is suitable only if the size of the native form of the fusion protein is within the range of separation of SDS-PAGE. For more accurate determination of the biotin-binding activity, the fusion protein is further purified by, for example, ion-exchange chromatography, and the biotin-binding activity is measured by using a surface plasmon resonance analyzer, such as BIAcore.
5. The integration of quaternary structure of the fusion protein seems to be primarily driven by the tamavidin 2 moieties, which associate with each other very stably. However, it is likely that this is not always the case. Therefore, we recommend that the subunit association of a particular fusion protein is determined experimentally by using gel filtration chromatography. No matter how the protein is structured, the linker should be long enough so that contact between the biotin

bonded to the beads and the biotin-binding pocket of the tamavidin 2 fusion protein is made freely.

6. The ratio of the protein to microbeads and the ratio of fusion protein to total soluble proteins would critically affect the purity of the end products. The expression level of a tamavidin 2 fusion protein would be affected by the fusion partner. Thus, fine tuning of these ratios case by case is essential.
7. The activity of the fusion partner is scarcely affected by its immobilization by the tamavidin 2-fusion technology, whereas its activity is often reduced by as much as 90 % by covalent bonding to beads (*see* ref. 14). A possible explanation for the difference is that, whereas the protein covalently bonded to the beads tends to be arranged randomly, the fusion protein immobilized to the beads by tamavidin 2-biotin binding could be present in a more organized manner due to the stable subunit association of tamavidin 2 moieties.

## Acknowledgements

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# Chapter 9

# Use of Tandem Affinity Chromatography for Purification of Cannabinoid Receptor $CB_2$

**Silvia C. Locatelli-Hoops and Alexei A. Yeliseev**

## Abstract

Tandem affinity purification has been increasingly applied to isolation of recombinant proteins. It relies on two consecutive chromatographic steps that take advantage of the affinity tags placed at opposing ends of the target protein. This allows for efficient removal of contaminating proteins, including products of proteolytic degradation of the fusion that lack either N- or C-terminal tags. Here, we describe the use of two small affinity tags, a poly-histidine tag and a Strep-tag for expression and purification of the human cannabinoid receptor $CB_2$, an integral membrane G protein-coupled receptor.

**Key words** His-tag, Strep-tag, Dual affinity tags, Membrane protein, $CB_2$ receptor, StrepTactin, Ni-NTA

## 1 Introduction

Affinity tags have become increasingly popular for purification of recombinant proteins.

We describe here the use of a combination of two small affinity tags, the polyhistidine tag [1] and a Strep-tag [2] for expression and purification of human cannabinoid receptor $CB_2$ [3], a class-A G protein-coupled receptor. The purification is performed in the presence of the nonionic detergent dodecyl maltoside (DDM), the zwitterionic detergent CHAPS as well as a derivative of cholesterol, cholesteryl hemisuccinate, required for stabilization of $CB_2$ in micelles. The necessity to perform purification in the presence of detergents constitutes a big challenge since they may affect the affinity and specificity of interaction between the tag and the resin by covering the tag and decreasing its accessibility [1].

The polyhistidine, or His-tag, usually contains 5–6 and, in some instances, as many as 10–14 consecutive histidine residues that bind to metal ions such as $Ni^{+2}$ or $Co^{+2}$ incorporated in different types of resins, allowing for purification of recombinant proteins via immobilized-metal affinity chromatography (IMAC)

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(Fig. 1a). The procedure described in this chapter is based on the Ni-NTA technology (Qiagen) [4]. The tetradentate chelant nitrilotriacetic acid occupies four valencies of the $Ni^{+2}$ ion, with two remaining valencies available for interaction with imidazole rings of histidine residues.

The binding step is usually performed at a pH of 7–8 to ensure that all histidine residues are deprotonated and, consequently, capable of interacting with nickel ions. The elution takes place in the presence of imidazole, which competes with histidine for the metal ions. While the high affinity binding of the $His_6$-tag to Ni-NTA resin has been reported ($K_D = 14$ nM [5]), both the affinity and specificity of binding can vary dramatically depending on the particular target protein, location of the tag, composition of the binding buffer, accessibility of the tag and the number of chelating residues. To improve the efficiency of interaction with the resin, the tag is usually placed at either N- or C-terminus of the target protein, where (in many cases) it is also less likely to interfere with the biological activity of the recombinant protein.

The second chromatographic step of our protocol involves purification on a StrepTactin resin using the repeat sequence of Strep-tag II (Fig. 1b). Strep-tag II is an 8-amino-acid polypeptide consisting of Trp-Ser-His-Pro-Gln-Phe-Glu-Lys that exhibits affinity ($K_D$ for the binding is 1 μM) for an engineered streptavidin termed StrepTactin [6, 7]. Because of its small size, the Strep-tag usually does not interfere with the biological activity of its fusion partner. Furthermore, the purification is performed under mild conditions that preserve the bioactivity of most proteins. The elution of the purified recombinant protein from the resin by desthiobiotin, an analog of biotin, is specific since only Strep-tagged proteins are displaced from the interaction with the StrepTactin resin. The resin can be reused after regeneration with the HABA (hydroxy-azophenyl-benzoic acid, pH 8.0) which, when provided in excess, displaces desthiobiotin from the resin.

In this chapter we present protocols for expression of peripheral cannabinoid receptor $CB_2$ in *E. coli*, its extraction from membranes, the solubilization and stabilization of the recombinant receptor in detergent micelles, its affinity purification using His-tag followed by proteolytic removal of fusion partners by TEV protease, and its final purification via Strep-tag. To achieve efficient purification, a C-terminal $His_{10}$ as well as two identical Strep-tag sequences bridged by a 4-amino acid linker were attached to the N-terminus of $CB_2$ to increase the binding affinity to their respective resin [3]. Purification is performed under mild conditions to maintain the functional fold of the receptor. The purity of the resulting $CB_2$ can be determined by SDS-PAGE followed by Coomassie Blue staining or western blot, by assessing its biophysical properties in micelles through a variety of techniques including circular dichroism spectroscopy and NMR, or by evaluating its

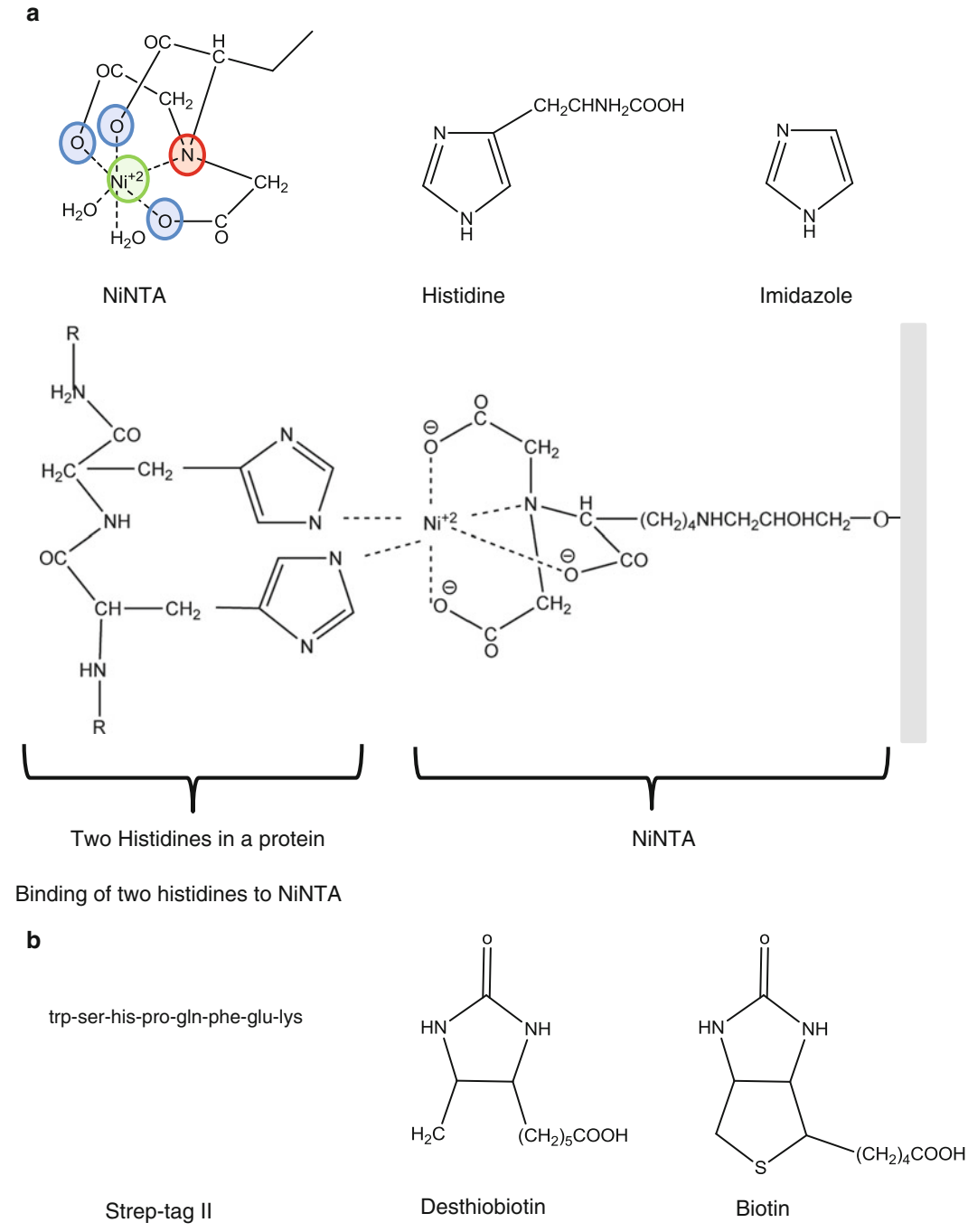


**Fig. 1** Ni-NTA affinity chromatography (**a**) and StrepTactin affinity chromatography (**b**). Chemical formulas of compounds required for chromatographic purification are shown

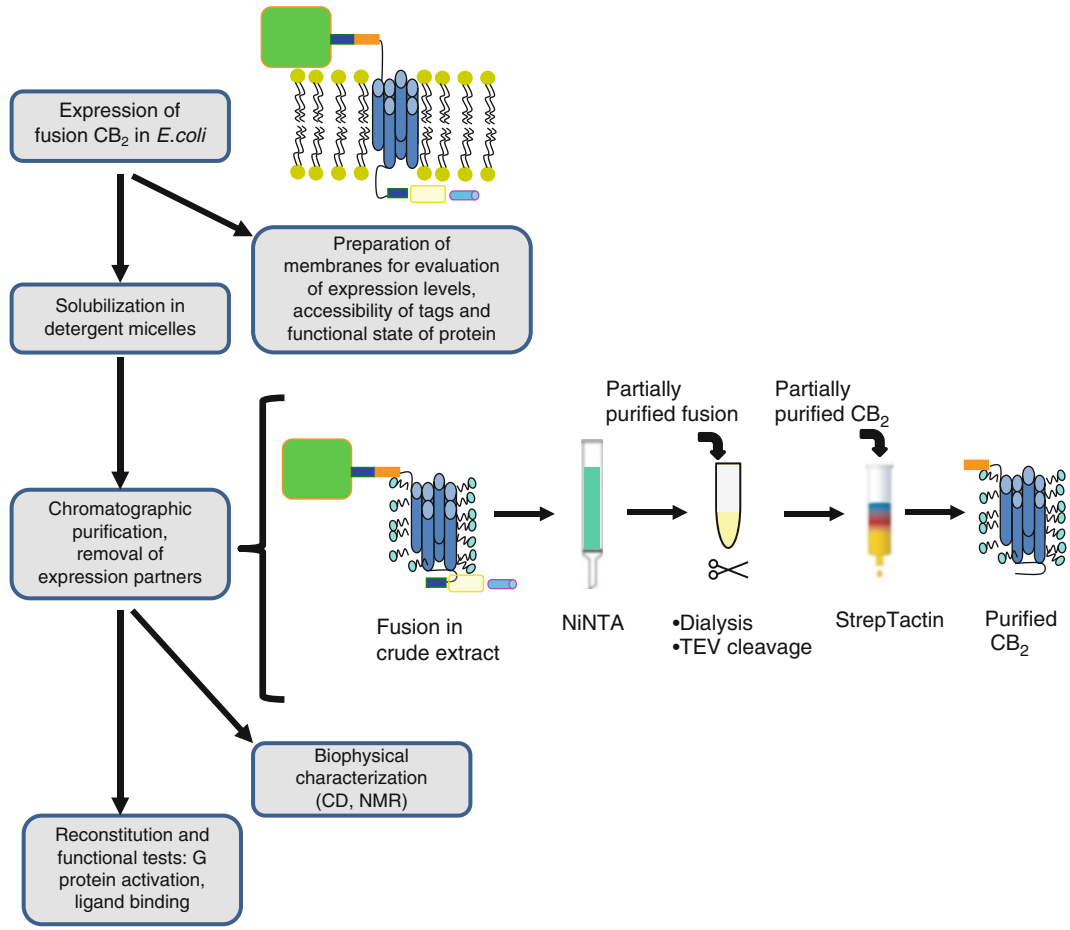


**Fig. 2** Flow chart of procedures for expression, purification and characterization of $CB_2$

functionality by binding of radioligands and measuring its ability to activate cognate G proteins upon reconstitution into proteoliposomes (Fig. 2).

## 2 Materials

### *2.1 Expression of $CB_2$ Fusion Protein and Preparation of Membranes*

1. Plasmid pAY-125 for expression of $CB_2$.
2. BL21(DE3) competent cells.
3. Shaker-incubator with a temperature range capability between 20 and 37 °C.
4. 50 mg/mL Ampicillin stock solution: Dissolve ampicillin in water and filter the solution though a 0.22 μm filter.
5. 0.5 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) stock solution: Dissolve IPTG in water and filter through a 0.22 μm filter.

6. 20 % (w/v) Glucose stock solution: To 250 mL water slowly add 100 g of glucose while stirring, complete to 500 mL with water and sterilize by autoclaving.
7. Luria-Bertani broth (LB) medium: 10 g/L casein digest, 5 g/L yeast extract, 5 g/L NaCl. Dissolve powder in water and sterilize by autoclaving.
8. Baffled, 125-mL shake flasks. Sterilize by autoclaving.
9. Double-strength YT-medium (2×YT): 16 g/L casein digest, 10 g/L yeast extract, 5 g/L NaCl. Autoclave 500 mL medium in 2 L baffled shake flasks.
10. Phosphate-buffered saline (PBS): 10 mM sodium phosphate, 150 mM NaCl, pH 7.4.
11. "Complete" protease inhibitor cocktail tablets (Roche).
12. Membrane storage solution: 10 mL of PBS adjusted to 20 % sucrose (2 g sucrose/10 mL PBS) with 1 tablet of "complete" protease inhibitor cocktail.
13. French press.
14. Handheld glass homogenizer.

### 2.2 Extraction, Solubilization and Stabilization of $CB_2$ in Detergent Micelles

1. Tris-buffered saline (TBS): 25 mM Tris–HCl, 130 mM NaCl, 2.7 mM KCl, pH 7.5.
2. EDTA-free protease inhibitor cocktail tablets (Roche).
3. DNAse I stock solution: 5 mg/mL DNAse I in water, approximately 1,000 U/mL.
4. 1 M $MgCl_2$ stock solution.
5. Anti foam A (Sigma).
6. 2× Solubilization buffer: 100 mM Tris–HCl, 400 mM NaCl, 60 % glycerol, pH 7.5.
7. 12× 3-[(Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)/cholesteryl hemisuccinate (CHS) stock solution: 6 % CHAPS, 1.2 % CHS, store at 4 °C (*see* **Note 1**).
8. 10 % *n*-dodecyl-β-D-maltoside (DDM) stock solution, store at 4 °C.
9. 20 mM CP-55,940 stock solution: Dissolve CP-55,940 in ethanol. Store at –20 °C.
10. Cell homogenizer (Avestin) or a sonicator.

### 2.3 Purification

1. Ni-NTA agarose (Qiagen).
2. Buffer A: 50 mM Tris–HCl, 200 mM NaCl, 0.5 % [w/v] CHAPS, 0.1 % [w/v] CHS, 1 % [w/v] DDM, and 30 % glycerol, pH 7.5.
3. Buffer B: 50 mM Tris–HCl, 200 mM NaCl, 0.5 % [w/v] CHAPS, 0.1 % [w/v] CHS, 1 % [w/v] DDM, and 30 % glycerol, 250 mM imidazole, pH 7.5.

4. Buffer C: 50 mM Tris–HCl, 100 mM NaCl, 15 % glycerol, 0.5 % [w/v] CHAPS, 0.1 % [w/v] CHS, 1 % [w/v] DDM, pH 7.5.
5. TEV protease: Either commercially available or in-house-prepared recombinant tobacco etch virus (TEV) protease can be used. We express and purify the recombinant poly-His-poly-Arg-tagged protease according to established protocols [8–10]. Expression in BL21(DE3) harboring the expression plasmid and purification via Ni-NTA affinity chromatography followed by cation exchange chromatography are described in [11].
6. StrepTactin Poros column (EMD Biosciences).
7. 5 μM Desthiobiotin in Buffer A: Weigh the required amount of powder desthiobiotin (EMD Biosciences) in a small Eppendorf tube and carefully dissolve in 100–200 μL of 0.1 N NaOH. Mix with buffer A to achieve a final concentration of 5 μM (*see* **Note 2**).
8. Centrifugal spin concentrators with a 30–70 kDa molecular mass cutoff.

## 3 Methods

### *3.1 Expression of Fusion $CB_2$ in Shaker Flasks*

The expression and purification protocols described here were developed for purification of the peripheral cannabinoid receptor $CB_2$, expressed as a fusion with several tags [3, 12] (*see* **Note 3**). The corresponding DNA construct, $CB_2$-125 is represented below (Fig. 3).

1. Inoculate 25 mL of LB medium supplemented with 25 μL of 50 mg/mL ampicillin in 125-mL baffled flask with *E. coli* BL21(DE3) cells harboring the expression plasmid (*see* **Note 4**).
2. Incubate overnight (ON) at 37 °C and 230 rpm agitation.
3. Prepare expression medium by adding 5 mL glucose solution and 500 μL ampicillin solution to the 500 mL of double-strength YT-medium flask immediately before inoculation. Use an appropriate number of flasks assuming the yield of ~400 μg of purified $CB_2$ protein from each flask.
4. Add 1–2 mL of the ON culture to each 2 L flask and incubate at 37 °C under agitation for 3–4 h until the optical density of the culture at 600 nm reached 0.4–0.5 U.
5. Lower the temperature of incubation to 20 °C (*see* **Note 5**).
6. Add 500 μL of IPTG solution to induce synthesis of the recombinant protein.

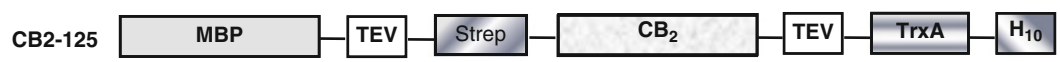


**Fig. 3** Fusion protein $CB_2$-125

7. Add 62.5 μL of a solution of CP-55,940 ligand (20 mM in Ethanol, *see* **Note 6**).
8. Incubate for additional 38–40 h (*see* **Note 5**).
9. Collect cells by centrifugation at 4,000 × *g* for 30 min at 4 °C.
10. Resuspend and wash cells once in ice-cold PBS.
11. Collect cells by centrifugation as above and store pellet at −80 °C.

### 3.2 Preparation of Membranes

We recommend testing the expression levels of the target protein in cell-membrane preparations obtained from small-scale cultures before proceeding with the large scale fermentation and purification. The following protocol is based on a previously published method for $CB_2$ expression [13]. Carry out all steps of membrane preparation on ice or at 4 °C.

1. Perform expression as described in Subheading 3.1, and collect cells from 25 to 50 mL of culture in a conical tube by centrifugation at 4,000 × *g* for 10 min. Alternatively, perform expression in a smaller scale format using 250 mL flasks and 50 mL of culture medium.
2. To 20 mL of cold PBS add 1 tablet of "complete" protease inhibitor cocktail.
3. Resuspended cells in a small volume (3–5 mL) of the cold PBS prepared in **step 2**.
4. Disrupt cells by passing the cell paste twice through a French Press.
5. Remove unbroken cells and cell debris by centrifugation at ~20,000 × *g* for 30 min at 4 °C.
6. Subject supernatant to a high-speed centrifugation at ~250,000 × *g* for 1 h at 4 °C.
7. Wash the resulting membrane pellet with cold PBS and resuspended in a small volume of membrane storage solution using a handheld glass homogenizer (*see* **Note 7**).
8. Flash-freeze small aliquots of the membrane suspension in liquid nitrogen and store at −80 °C until needed.
9. Proceed with evaluation of expression levels and functional activity of the target protein (*see* **Note 8**).

### 3.3 Extraction, Solubilization, and Stabilization of $CB_2$ from E. coli Cells

Starting from 10 L of cell culture (~80 g of wet biomass), typically 600 mL of crude extract can be obtained. Volumes of added solutions were calculated based on that number—adjust accordingly if necessary. Perform all the following procedures on ice or at 4 °C.

1. Transfer cell pellet (80 g) with a large spatula to a 1 L blender (*see* **Note 9**).
2. Add 100 mL of ice-could TBS buffer into the blender.

3. Add 2–3 tablets of EDTA-free protease inhibitor cocktail solubilized in 10 mL water.
4. Homogenize cell suspension in blender for 20–30 s.
5. Transfer the suspension on ice and start stirring.
6. Add 50 μL of DNAse I solution.
7. Add 3 mL of 1 M $MgCl_2$ to achieve a final concentration of 5 mM.
8. Add Anti-foam A until the foam disappears (20–40 μL).
9. Pass the suspension two times through a cell homogenizer (*see* **Note 10**).
10. Place the homogenized suspension in a 1,000-mL beaker on ice and start stirring.
11. Add 300 mL of 2× solubilization buffer (*see* **Note 11**).
12. Add 50 mL CHAPS/CHS 12× solution (*see* **Note 11**).
13. Add 60 mL of a 10 % DDM solution (*see* **Note 11**).
14. Add 300 μL of 20 mM ligand CP-55,940 solution (*see* **Note 11**).
15. Continue stirring for 40–60 min. Ensure that the final composition of the buffer in which $CB_2$ is solubilized is 50 mM Tris–HCl at pH 7.5, 200 mM NaCl, 0.5 % [w/v] CHAPS, 0.1 % [w/v] CHS, 1 % [w/v] DDM, 10 μM CP-55,940, 5 mM $MgCl_2$, and 30 % glycerol, supplemented with DNAse I (0.5 μg/mL) and EDTA-free protease-inhibitor cocktail.
16. Remove cell debris by centrifugation at a ~200,000 × *g* for 1 h at 4 °C.
17. Pass supernatant through a 0.45-μm filter and proceed with purification.

### 3.4 Ni-NTA Chromatography

All buffers used for purification should be supplemented with CP-55,940 at a final concentration of 10 μM. All procedures should be performed on ice or at 4 °C. Remember to take samples at each step to follow the purification process by SDS-PAGE and western blot (*see* **Note 12**).

1. Pack 8 mL of Ni-NTA resin to a suitable column compatible with an available chromatography work station (we use AKTA Purifier 100 [GE Healthcare]) allowing gradient-based chromatography and automated fraction collection.
2. Equilibrate the resin using at least 5 column volumes (CV) of buffer A + CP-55,940.
3. Load the filtered $CB_2$ solution at a flow rate of 0.5 μL/min. Maintain this flow rate for the entire purification.

4. Wash the resin with 20 CV of buffer A + CP-55,940 supplemented with 40 mM imidazole.
5. Elute protein with 10 CV of buffer B + CP-55,940 and collect 4 mL fractions (*see* **Note 13**).
6. Select the $CB_2$-containing fractions by SDS-PAGE followed by Coomassie Blue staining or by dot blot using anti $CB_2$ mAb or anti His-tag Ab (*see* **Note 14**).

### 3.5 Removal of Fusion Partners by Tobacco Etch Virus (TEV) Protease

1. Combine $CB_2$-containing fractions (typically 20 mL) and concentrate ~2.5-fold in centrifugal spin concentrators with a 30 kDa molecular mass cutoff (*see* **Note 12**).
2. Dialyze the concentrated sample against two exchanges of buffer C + CP-55,940 at 4 °C (*see* **Note 12**).
3. Add 0.5–1 mg of purified TEV protease to the dialyzed sample (5:1, $CB_2$: TEV, mol/mol) and allow digestion for at least 4 h or overnight at 4 °C (*see* **Note 15**).
4. Proceed with final step of purification or perform an intermediate purification step (*see* **Note 16**) to increase purity of the final product.

### 3.6 Purification via StrepTactin Affinity Tag

1. Pack a 3 mL-StrepTactin Poros column and operate it by gravity.
2. Equilibrate the resin with 5 CV of buffer C + CP-55,940.
3. Load the products of the TEV protease cleavage reaction (8 mL) onto the StrepTactin column.
4. Wash with 4 CV of buffer C + CP-55,940.
5. Elute $CB_2$ with 3 CV of buffer C + CP-55,940 supplemented with 5 μM desthiobiotin (*see* **Note 17**).
6. Combine elution fractions and concentrate in centrifugal spin concentrators with a 30 kDa molecular mass cutoff.
7. Dialyze against buffer A + CP-55,940 at 4 °C overnight.
8. Determine protein concentration with the Bio-Rad DC kit (*see* **Note 18**).
9. Aliquot the concentrated protein into Eppendorf tubes.
10. Freeze aliquots in liquid nitrogen and store at −80 °C until needed.
11. Evaluate purity of the preparation (*see* **Note 19**).
12. Characterize functional state of the purified protein (*see* **Note 20**).
13. The purified protein can be analyzed by a range of biophysical techniques (*see* **Note 21**).

## 4 Notes

1. CHS will not solubilize when resuspended in water directly—it has to be dissolved in a concentrated CHAPS solution. For preparation of 400 mL of the 12× stock solution of 6 % CHAPS and 1.2 % CHS we recommend the following protocol:
   - Take 4.8 g CHS and resuspend in 200 mL of water in a 250-mL beaker under vigorous stirring. Continue stirring through the entire solubilization procedure.
   - Dissolve 24 g CHAPS in 150 mL of water with stirring.
   - Add CHS suspension drop by drop to the CHAPS solution while stirring, until the solution is clear.
   - Adjust volume to 400 mL with water. Solution can be stored at 4 °C for at least 2–3 months.
2. 15–20 mL of elution buffer is usually enough to accomplish a single StrepTactin purification.
3. $CB_2$-125 includes the following fusion partners:
   - N-terminal Maltose-binding protein (MBP): facilitates proper folding of $CB_2$ as well as the insertion of the hydrophobic polypeptide into the cytoplasmic membrane of *E. coli*.
   - Tobacco etch virus protease recognition site (TEV): is a sequence recognized by TEV protease and is required for cleavage of the fusion protein. We use the recognition sequence E-N-L-Y-F-Q-S which is cleaved in between the Q and S residues, leaving the following non-native sequences attached to $CB_2$: S (the remainder of the TEV site) followed by WSHPQFEK (Strep-tag), GSGGAS (linker) followed by WSHPQFEK (2nd Strep-tag) followed by GGGS (linker) upstream of $CB_2$, and $A_3N_5G_3S$ (linker) followed by ENLYFQ (remainder of TEV site) downstream of $CB_2$.
   - Strep-tag (Strep): required for affinity chromatography on a StrepTactin-agarose (final chromatographic step in our protocol).
   - Thioredoxin A (Trx A): enhances the expression level of functional $CB_2$ receptor in the plasma membrane of *E. coli*.
   - Polyhistidine tag ($His_{10}$): required for affinity purification of the recombinant protein on a Ni-NTA resin (first chromatographic step in our protocol).
4. Work under sterile conditions and wear gloves on all occasions where cultivation of cells or handling of potentially harmful chemicals is involved.

5. Expression conditions were optimized previously [12]. While cells can be grown at 37 °C until they reach $OD_{600}$ of 0.5, expression of the recombinant protein is performed at 20 °C to facilitate production of functional receptor. Incubation proceeds for 40 h after induction because the accumulation of cellular biomass reaches a maximum at this time.
6. The addition of CP-55,940 to the growth medium enhances levels of expression and stabilizes functional $CB_2$ receptor [14].
7. Use a volume of 400–600 μL to resuspend the pellet. Transfer the partially resuspended pellet to a handheld glass homogenizer with an appropriate transfer pipette. Homogenize and transfer to an Eppendorf tube. Repeat the process with half of the original volume. Combine both fractions. Avoid formation of air bubbles to prevent losing membranes.
8. We recommend testing effects of tags on expression levels and functional state of the target protein before proceeding with a large-scale expression and purification. For $CB_2$, the expression levels of various constructs and functionality of the resulting recombinant fusion $CB_2$ are typically evaluated by western blot analysis, ligand binding assays, and G-protein activation assay performed on membrane preparations [12]. Incorporation of the N-terminal Strep-tag and C-terminal $His_{10}$ tag does not significantly affect the expression levels of the $CB_2$ as shown by western blot analysis using anti-$CB_2$, anti-His, and anti-Strep-tag antibodies [3, 12].

   The ability to detect small affinity tags on fusion $CB_2$ in membrane preparations subjected to SDS-PAGE and western blot, by assessing interaction with their respective antibodies does not guarantee that these same tags will be accessible for interaction with the chromatographic resin. However, we observe that the western blot analysis is usually an accurate predictor of the accessibility of a tag (including poly-hisitidine- and Strep-tag) for interaction with the respective resin, for subsequent chromatographic purification of $CB_2$ (unpublished observations).
9. Some pre-warming of the cell pellet (at room temperature for 20–30 min) may be required to facilitate transfer to the blender.
10. Alternatively, if a cell homogenizer is not available, the mixture can be subjected to sonication for 15 min (Branson Sonifier 250, 1/2-in. flat tip, output 6, duty cycle 50 %) [12]. Make sure that the solution is not overheated, and place it on ice and stir during sonication.
11. In order to stabilize the $CB_2$ and minimize loses of active protein during purification, several parameters are critical [14]. Temperature has to be maintained at 4 °C, and the entire purification procedure should take no longer than 3 days to

achieve the yield of functional protein ≥90 %. Solubilization of the fusion protein is performed in a mixture of CHAPS (0.5 %), DDM (1 %), and CHS (0.1 %) in the buffer (triple detergent buffer, TD), as described previously [12]. The presence of 0.1 % CHS during solubilization and purification is critical to maintain the functional structure of $CB_2$ in detergent micelles. In addition, the buffer contains 30 % glycerol. Supplementation with the high affinity ligand such as CP-55,940 during purification was also shown to significantly increase the recovery of active protein. We recommend 10 μM CP-55,940 in all purification buffers.

12. Take samples from load, flowthrough, washes, elution fractions (separated or combined) for each chromatographic step as well as before and after TEV cleavage. In order to prepare $CB_2$ for SDS-PAGE it is important not to subject the sample to boiling or even high-temperature treatment since this will result in aggregation of the protein. We recommend mixing the protein sample with equal volume of the 2× Laemmli sample buffer and incubating at 37 °C for 30 min. Detection with several antibodies is recommended, as some (such as anti-$CB_2$ antibody) will allow visualization of $CB_2$ in all steps whereas others (such as anti MBP or anti His-tag antibodies) will detect fusion or cleavage products before or after TEV treatment respectively.
13. For regeneration of the Ni-NTA resin, wash extensively with imidazole buffer and remove imidazole by extensive washing with binding buffer before use, according to the manufacturer's instructions.
14. This step can be omitted once the pattern of elution of the target protein is determined. We typically observe elution of $CB_2$ in fractions 3–7.
15. Conditions for efficient cleavage by TEV protease were established previously [3]. Since detergents, salts, imidazole and glycerol at concentrations present in buffer B inhibit TEV protease cleavage, we recommend concentrating the sample no more than 2 to 2.5-fold and dialyzing for 2 h against buffer C prior to TEV cleavage.
16. In order to increase the purity of the final product, removal of His-tagged TEV and His-tagged cleavage product can be performed by Ni-NTA chromatography as an intermediate step.
    - Pack a column with 1 mL Ni-NTA agarose and equilibrate it with 5 CV of buffer C containing 20 mM imidazole.
    - Pass the reaction mixture after TEV cleavage though the small Ni-NTA column by gravity to retain His-tagged products of cleavage as well as TEV protease, and proceed with purification of the flowthrough.

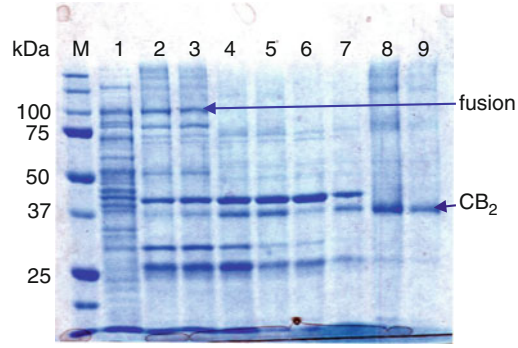


**Fig. 4** Purification of $CB_2$-125. Coomassie Blue staining of a typical gel is presented. *Lane 1*, crude extract before 1st Ni-NTA chromatography; *lane 2*, concentrated fractions after 1st Ni-NTA chromatography; *lane 3*, fractions after dialysis against buffer B; *lane 4*, proteins after TEV protease digest; *lane 5*, combined fractions (flowthrough and wash) from the 2nd Ni-NTA column; *lane 6*, flowthrough from the StrepTactin column; *lane 7*, wash fraction from the StrepTactin column; *lanes 8* and *9*, eluate fractions from StrepTactin column, after concentration (5 and 2.5 μg of protein per lane, respectively)

17. To regenerate the StrepTactin resin, use the HABA reagent (EMD Biosciences) according to manufacturer's instructions.
18. Concentrating the protein solution fourfold to fivefold on a centrifugal spin-concentrator results in a proportional co-concentration of detergents. Typically we obtain a concentrated 1.5–2 mg/mL solution of $CB_2$ that also contains ~0.5 % DDM, 2.5 % CHAPS, and 0.5 % CHS. Determination of protein concentration can be performed by Bio-Rad DC assay kit or similar system suitable for measurement of protein concentration in the presence of detergents.
19. The purity of the final preparation can be evaluated by Coomassie Blue staining (Fig. 4). Be aware of the fact that the efficiency of staining of highly hydrophobic membrane proteins (like $CB_2$) may be much lower than that of soluble proteins, resulting in a weaker than expected signal in Coomassie-stained gels.
20. To evaluate the functional state of the purified $CB_2$ protein, reconstitution in liposomes is required [14, 15]. Then, a G-protein activation assay with proteoliposomes can be performed [14]. Reaction conditions were optimized for 3–10 ng of $CB_2$ per reaction to ensure that less than 30 % of the available [$^{35}$S] GTPγS is consumed.
21. Purified $CB_2$ in detergent micelles is suitable for characterization by circular dichroism spectroscopy [3, 16], high-resolution NMR (ligand binding studies [12] and diffusion experiments [16]) or other biophysical techniques.

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# Chapter 10

# Detection of Protein–Protein Interactions Using Tandem Affinity Purification

**Ian Goodfellow and Dalan Bailey**

## Abstract

Tandem affinity purification (TAP) is an invaluable technique for identifying interaction partners for an affinity tagged bait protein. The approach relies on the fusion of dual tags to the bait before separate rounds of affinity purification and precipitation. Frequently two specific elution steps are also performed to increase the specificity of the overall technique. In the method detailed here, the two tags used are protein G and a short streptavidin binding peptide; however, many variations can be employed. In our example the tags are separated by a cleavable tobacco etch virus protease target sequence, allowing for specific elution after the first round of affinity purification. Proteins isolated after the final elution step in this process are concentrated before being identified by mass spectrometry. The use of dual affinity tags and specific elution in this technique dramatically increases both the specificity and stringency of the pull-downs, ensuring a low level of background nonspecific interactions.

**Key words** Protein–protein interactions, Tandem affinity purification, TAP tagging, Affinity purification, Affinity tags, Proteomics, Protein purification

## 1 Introduction

The identification of the protein–protein interactions that take place in the cell is a common approach for the characterization of protein function and is a fundamental aspect of most modern molecular and biochemical research. There are numerous molecular techniques available to identify such interactions including the use of affinity tags. Often single epitope or affinity purification tags (such as His, GST, HA, or GFP) are fused to a protein of interest (bait), and the fusion protein expressed in cells is affinity-purified. The subsequent precipitates are analyzed by mass spectrometry in order to determine a network of possible cellular interaction partners for any specific bait protein. However, this single step affinity purification is somewhat crude and the results can contain contaminants due to the low stringency and specificity of the

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interactions. Recently these affinity tag approaches have been optimized to increase their applicability for modern research. One such approach is tandem affinity purification (TAP)—a system initially developed in yeast [1, 2] but which has now been optimized for the eukaryotic system as well [3]. The TAP tagging technique detailed here demonstrates a highly specific and stringent method for isolating the binding partners of bait proteins in eukaryotic cells. We have previously used this system to identify interaction partners for the well-characterized eukaryotic translation initiation factor eIF4E [4]; however, this approach has also been applied to proteins from nonmammalian organisms such as yeast, viruses, plants, and parasites [5–9].

In the method detailed below, we have employed the dual protein G and streptavidin binding peptide tag developed by Burckstrummer et al. [3]. These two tags are separated by a cleavable peptide sequence. To briefly summarize the protocol, the tagged bait protein, expressed in a suitable target cell line, is initially purified from a cell lysate using rabbit IgG agarose beads. A specific elution is then performed using tobacco etch virus protease, which cleaves at a specific peptide sequence between the protein G domains and the streptavidin binding peptide, releasing the bait protein, along with any associated interacting partners, into the supernatant. A second affinity purification is then performed which targets the streptavidin binding peptide with streptavidin coated beads, allowing the bait to again be "pulled out" of solution. The bait is finally eluted from the beads, again specifically, using biotin, which has a high affinity for Streptavidin and displaces the bait. The advantage of these two specific purifications and elution steps is an overall increase in stringency and specificity. In simpler terms the final elution is less likely to contain nonspecific proteins, something which was evident during our previous analysis of eIF4e [4]. This technique is also summarized in Fig. 1.

This approach is not limited to the use of this tag, and many other combinations of affinity tag have, and can be, employed [9, 10, 11]. Recently, the use of stable isotope labelling by amino acids in cell culture (SILAC) has been combined with TAP to provide even greater assurances about the specificity of any identified interactions [12]. The use of SILAC also enables relative quantification to be obtained, which may provide useful when examining the effect of specific mutations known to affect protein function, on the binding partner profile. The increasing prevalence and use of TAP approaches in modern molecular research highlights the robustness of this approach and the relative ease of its application to various biological questions.

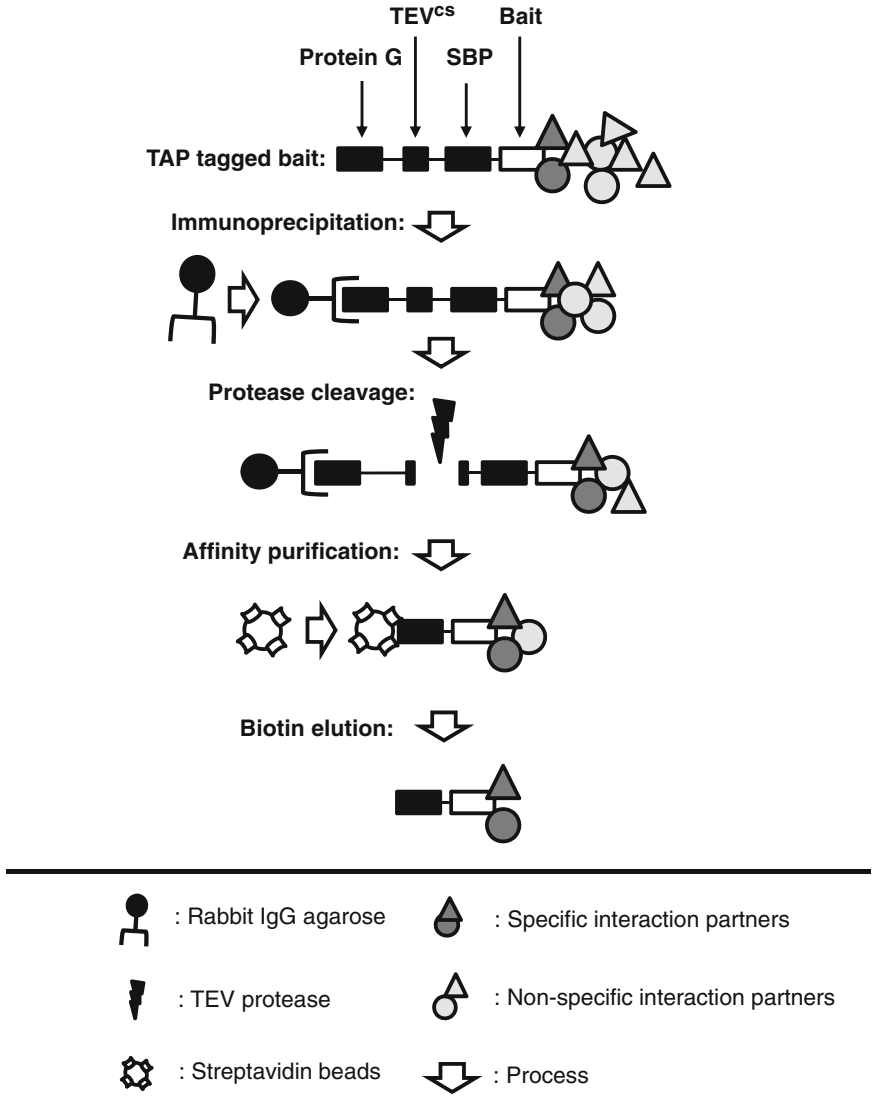


**Fig. 1** Overview of the TAP tagging procedure. The TAP tagged bait is immunoprecipitated from lysed cells using rabbit IgG agarose. The protein G domains are then cleaved off at the Tobacco Etch Virus cleavage site ($TEV^{CS}$) by TEV protease. This cleaved bait is affinity-purified from solution using Streptavidin beads before being specifically eluted again, this time using Biotin. The whole process ensures efficient isolation of specific bait interaction partners, as well as the removal of nonspecific contaminants

## 2 Materials

All reagents should be prepared using high grade filtered water. Extreme care should be taken to reduce contamination of any of the reagents with keratin from environmental skin, hair, and dust.

### *2.1 Core Reagents*

1. TAP lysis buffer: 50 mM Tris–HCl (pH 7.5), 125 mM NaCl, 5 % Glycerol, 0.2 % NP-40, 1.5 mM $MgCl_2$, 25 mM NaF, 1 mM $Na_3VO_4$, and protease inhibitors.

2. PBS: 1.06 mM $KH_2PO_4$, 155.17 mM NaCl, and 2.97 mM $Na_2HPO_4$ $7H_2O$, pH 7.4.
3. TEV-protease wash buffer: 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, and 0.2 % NP-40.
4. TEV cleavage mix: 467.5 μl $H_2O$, 25 μl 20x TEV Buffer (1 M Tris–HCl, (pH 8.0), 10 mM EDTA), 5 μl 0.1 M DTT and 2.5 μl (25 U) TEV Protease (shipped in 50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 5 mM DTT, 50 % (v/v) glycerol, 0.1 % (w/v) Triton® X-100). (AcTEV Protease, Invitrogen).
5. Rabbit IgG-agarose in saline solution.
6. Streptavidin beads: normally supplied as 50 % agarose slurry (UltraLink).
7. Immobilized Streptavidin Plus agarose beads (Pierce).
8. PBS and biotin mix: 1 mM D-biotin in PBS.

### 2.2 Additional Reagents and Apparatus

1. Siliconized Pre-lubricated tubes (Costar).
2. Protein/SDS sample buffer (LaneMarker 5× reducing sample buffer, Fisher).
3. Vivaspin Low molecular weight (<5 KDa) spin columns (Vivascience).
4. SilverQuest Silver staining and Colloidal Blue Coomassie staining kits (Invitrogen).
5. Novex system (Invitrogen).
6. Protease inhibitors (Pierce).
7. Additional equipment required: Narrow gauge blunt needles, syringes, sterile scalpels or gel band pickers, dry ice and ethanol, liquid nitrogen, refrigerated centrifuges (for 50 and 1.5 ml tubes), 15 ml and 50 ml sterile tubes (suitable for centrifugation), duck-billed flattened tips, SDS-PAGE and western blot equipment, tissue culture reagents.

## 3 Methods

General points: The overall objective of this technique is to isolate intact protein–protein interactions between the TAP tagged bait and "unknown" cellular interaction partners. These interactions are sensitive to disassociation especially if the lysates and precipitations are allowed to warm to room temperature. Therefore, where possible, all reactions, incubations, and washes should be performed on ice and using chilled centrifuges to maintain the integrity of the protein–protein interactions. Furthermore, because the final elution will be likely be analyzed by mass-spectrometry, it is essential that environmental contamination (especially from

Keratin) be kept to a minimum. A number of samples (A–H) should be taken during this procedure. These should all be immediately stored at –80 °C.

### 3.1 Preliminary Stages: Generation of Stable Cell Lines

1. Clone the gene of interest into a suitable plasmid. There are a number of expression systems available that can be employed (*see* **Note 1**).
2. Generate a stable eukaryotic cell line expressing the bait protein fused to the protein G/Streptavidin binding peptide tandem affinity purification (TAP) tag (*see* **Note 2**).
3. Confirm expression of the TAP tagged bait in the stable cell lines by western blot (*see* **Note 3**).
4. Prepare ten confluent 175 $cm^2$ flasks of the bait producing cells. Depending on the expression levels, this amount of starting material will ensure sufficient material is available for mass spectrometry analysis at the end of the protocol.

### 3.2 Cell Lysate Preparation

1. Trypsinize the cells (or scrape into ice-cold PBS) and centrifuge the cell suspension at 1,200 × *g* for 5 min. Resuspend the cell pellets in a small volume of ice-cold PBS and combine the cells into a single 50 ml tube. Centrifuge again at 1,200 × *g* for 5 min to pellet the cells. Repeat this PBS wash step three times to remove cell debris and trypsin (*see* **Note 4**).
2. Lysis of the cells should be performed using 5 ml of the TAP lysis buffer (*see* **Note 5**) Pipette the lysate up and down until the pellet is fully resuspended (approximately 10 times) and leave on ice for 5 min. To ensure efficient lysis, the cells should then be repeatedly syringed through a narrow gauge blunt needle before being left on ice for 5–10 min (*see* **Note 6**).
3. When the lysis is complete, the samples should be snap-frozen and then thawed. This can be achieved using liquid $N_2$ or dry ice and ethanol; however, do not allow the temperature of the sample to get above 4 °C when thawing the samples (*see* **Note 7**). After the thawing step, the samples can be aliquoted into appropriate tubes for clarification by centrifugation (10 min at 4 °C, 16,000 × *g*). This ensures the removal of unlysed cells and debris (*see* **Note 8**).
4. Combine the supernatants from the individual tubes (used for high speed centrifugation) and remove a 50 µl aliquot, labelling it Sample A (*see* **Note 9**).

***From this point all centrifugation steps should be performed at 1,200×g (4 °C for 1 min) unless otherwise stated.***

### 3.3 Rabbit IgG-Agarose Immunoprecipitation

1. Resuspend the rabbit IgG-agarose and remove 380 μl of the solution into a 15 ml tube (*see* **Note 10**). Remove the preservative solution by centrifugation and wash the beads three times in cold lysis buffer using centrifugation after each step to gently pellet the beads (*see* **Note 11**).
2. Afterwards combine the cleared cell lysate to the washed rabbit-IgG agarose and incubate, with continual rotation, overnight at 4 °C (*see* **Note 12**). The following day centrifuge the solution for 5 min at 4 °C and remove all the supernatant, labelling it Sample B (*see* **Note 13**).

### 3.4 TEV Protease Cleavage of the Protein G Domains

1. Wash the retained beads three times in chilled lysis buffer using centrifugation to gently pellet the beads (*see* **Note 14**). Due to the subsequent use of TEV protease, any inhibitors should now be removed from the solution. In order to achieve this, wash the beads twice with TEV-protease wash buffer using centrifugation to gently pellet the beads. Remove as much of the remaining buffer as possible from the remaining beads and keep on ice.
2. Each sample should now be incubated with 500 μl of the TEV cleavage mix and transferred to a microcentrifuge tube (*see* **Note 15**). However, before incubation remove a small 30 μl aliquot, labelling it Sample C (*see* **Note 16**).
3. Incubate the protease reaction (with rotation) overnight at 4 °C (*see* **Note 17**).
4. Gently pellet the rabbit IgG agarose beads in the TEV cleavage reaction by centrifugation (5 min at 1,200 × *g*/4 °C). Remove a small 20 μl aliquot of the clarified supernatant and label Sample D (*see* **Note 18**). Transfer the remaining supernatant to a new microcentrifuge tube and leave on ice.
5. Return to the remaining rabbit IgG agarose and add 500 μl of chilled TAP lysis buffer to the beads and mix by inversion. Gently pellet the beads before removing the supernatant and combining it with the previous supernatant, already on ice (*see* **Note 19**).
6. The rabbit IgG agarose beads should be labelled Sample E and retained for subsequent analysis (*see* **Note 20**).

### 3.5 Affinity Purification of the Streptavidin Binding Peptide (SBP) Tag

1. Resuspend the streptavidin agarose beads and remove a 70 μl aliquot into a microcentrifuge tube (*see* **Note 21**). Remove the shipping solution and wash, as detailed for the protein G agarose beads.
2. Combine the TEV-protease cleavage supernatant with the washed streptavidin beads and incubate at 4 °C, with rotation, for 3 h (or overnight—*see* **Note 22**).

3. Afterwards gently pellet the streptavidin beads for 5 min at 1,200 × *g*/4 °C. Remove the supernatant and label as Sample F (*see* **Note 23**). To remove any additional nonspecific contaminants, wash the streptavidin beads three times in chilled lysis buffer. After the final wash, remove as much wash buffer as possible using a flattened pipette tip to minimize contamination.

### 3.6 Biotin Elution of the Bait

1. The bait (and any directly associated proteins) should be specifically eluted from the streptavidin beads using a mixture of PBS and biotin. Add 500 μl of this mixture to the packed streptavidin beads and incubate at 4 °C for 3 h (with rotation).
2. Gently pellet the streptavidin beads by centrifugation (5 min at 1,200 × *g*/4 °C) and remove the eluted sample (labelled Sample G) to a clean microcentrifuge tube (*see* **Note 24**). Repeat this elution procedure and combine the second elution with the first (*see* **Note 25**). The remaining streptavidin beads should be retained for subsequent analysis (Sample H) (*see* **Note 26**).

### 3.7 Protein Concentration of the Biotin Elution

The large volume of the final eluate (Sample G) is impractical for mass-spectrometry analysis, western blot, or direct staining due to the its low protein concentration. To concentrate the protein, use low molecular weight (<5 KDa) spin columns and reduce the volume to less than 100 μl. After concentration add SDS sample buffer to the proteins and boil for 5 min at 90 °C before storing at −80 °C (*see* **Note 27**).

### 3.8 Analysis

Analyze a small percentage (approximately 10 %) of Sample G by 1D or 2D SDS PAGE and perform silver staining of the gel (*see* **Note 28** and Fig. 2). Compare this gel to the negative control (*see* **Note 1**) to determine the presence of specific interactions. For mass spectrometry, analyze >50 % of Sample G by SDS PAGE and stain the gel with Coomassie. Excise bands, or spots, of interest and store at 4 °C in 1 % Acetic Acid before analysis (*see* **Notes 29** and **30** and Fig. 2).

### 3.9 Troubleshooting

As detailed briefly in Subheading 4, samples A-H can be analyzed by western blot to determine the individual efficiencies of each step of the TAP protocol. Always perform these analyses when undertaking TAP purification of new baits. Certain baits may not purify as well as others due to their molecular weight, subcellular localization, the presence of transmembrane domains, etc. It may therefore be necessary to increase the detergent concentration of the lysis buffer or modify the salt conditions to improve the purification. This should be determined empirically by the user; however, harsh conditions are likely to reduce the efficient pull-down of interaction partners. The availability of bait-specific antibodies increases the scope of these analyses.

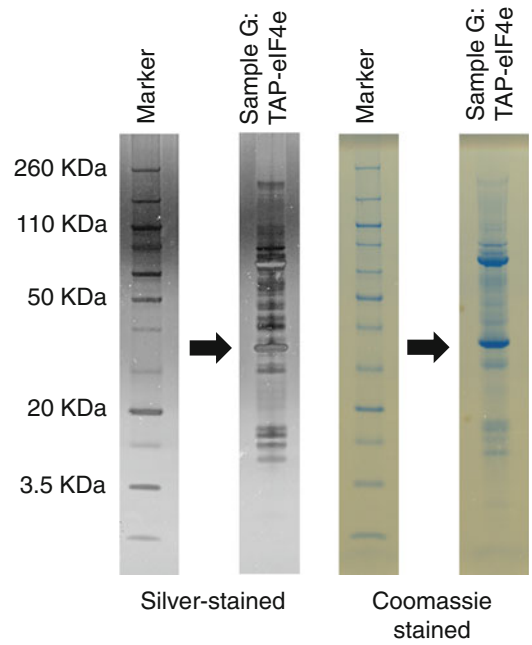


**Fig. 2** Protein interaction partners identified for the TAP tagged eIF4e protein. Concentrated sample G was analyzed on pre-cast 4–12 % gradient SDS-PAGE gels along with an unstained marker. The silver stain analysis was performed with 10 % of the concentrated final eluate (Sample G). The colloidal Coomassie stain was performed with 60 % of the eluate before the bands were excised and analyzed by tandem mass spectrometry. The tagged eIF4e bait is evident (indicated by the *black arrows*). The other protein bands represent the canonical translation initiation factors normally found associated with this protein (e.g., eIF4G)

It also very informative to analyze small aliquots of samples A–H on SDS-PAGE gels with subsequent silver or Coomassie staining (*see* Fig. 3). The TAP tag bait is normally evident from Sample B onwards (on silver stain gels), and this analysis can provide additional information about the efficiency and stringency of the TAP purification. It is advisable to run the two bead containing samples (E and H) on a separate gel (*see* Fig. 3).

## 4 Notes

1. We have used plasmids that allow inducible expression of the TAP tagged bait in order to reduce any toxicity to the cell, however, this is not entirely necessary. Plasmids we have successfully used include the episomally maintained plasmid (pMEP4), which allows inducible expression from a metallothionein promoter, (induced with 10 μM $CdCl_2$ for 16 h) as well as the Doxycycline inducible pcDNA4-TO system from

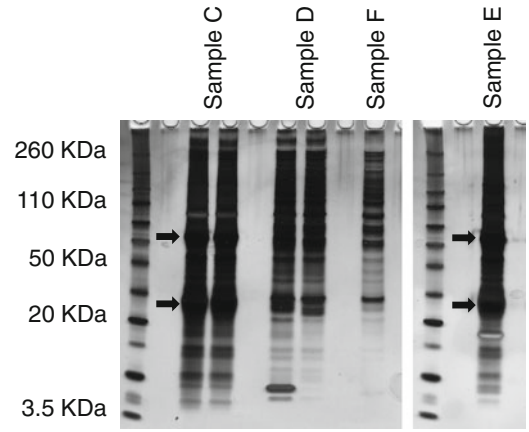


**Fig. 3** Control gels generated during the optimization stage of TAP tagging. Samples C, D, E, and F taken from two separate TAP purifications were analyzed on pre-cast 4–12 % gradient SDS-PAGE gels along with unstained marker. The proteins were visualized using the standard silver staining protocol. Large background quantities of the Rabbit IgG agarose heavy and light chains can be detected in Samples C and E (indicated by the *black arrows*). A reduction in the number of affinity-purified proteins occurred during the procedure, evidenced by comparison of the relative complexity of samples C and F

Invitrogen. Another alternative could be a lentivirus expression system. If possible generate negative control plasmids as well. For example, express only the TAP tag, the bait or, if at all possible, a mutant form of the bait protein that is no longer functional. This can be used to determine the presence of non-specifics in the final elution.

2. Expression of the tagged bait can be tested by transient transfection experiments prior to the generation of cell lines. Dependant on the system used, it may be necessary to select and expand individual clones.
3. The protein G domain of the TAP tag binds to most secondary antibody species, so it is not entirely necessary to have a bait specific antibody. Our experience would indicate that a substantial proportion of the protein will refold on the membrane during the blotting procedure and bind to secondary antibody. This is a useful approach to determining the relative solubilization and binding efficiencies up to the point of TEV cleavage; however, this affinity tag is then cleaved so the ability to detect the bait is lost. Also, if functional assays are available for the bait protein, the tagged bait should be tested to ensure the protein maintains its known function.
4. The initial cell pellet from ten large flasks will have an approximate volume of 2–4 $cm^3$. It is normal for this volume to reduce by about half after the repeated washing steps.

5. The NaF, $Na_3VO_4$, and protease inhibitors should be added immediately prior to use. Of note, for certain proteins the solubilization and release of the protein from the cells may require the optimization of the lysis buffer and lysis method. For example, proteins typically found associated with chromatin may require additional sonication and/or increased salt concentrations. This can be tested on a small scale initially prior to scaling up for the final purification.
6. This syringing of the lysate is initially quite difficult depending on the concentration of the starting material. This process should be repeated until any cell clumping is removed. The introduction of air and bubbles into the reaction should be minimized.
7. Snap freezing the lysate is an efficient way to fully lyse the cells. Once the lysate is frozen, it can be stored at −80 °C until required.
8. Normally a small proportion of cells remain unlysed in this protocol. This is due to the large amount of starting material.
9. A number of samples should be taken throughout the TAP purification to allow for experimental investigation of the efficiency and quality of the lysis, pull-downs, and enzymatic reactions being performed. Sample A can be used for protein quantification, western blot, and silver/Coomassie staining analysis.
10. Agarose bead solutions should always be aliquoted using low retention tips. It is also advisable to cut off the end of each tip (prior to use) to widen the opening—this will reduce any damage to the beads caused by pipetting.
11. The total volume of packed beads will be around 250 μl at this point.
12. This incubation step can be shortened to 3 h without a great reduction in immunoprecipitation efficiency; however, it should still be performed at 4 °C.
13. The TAP tagged bait will now be associated with the beads and should not be present, in large quantities, in Sample B. Comparison of sample A and B (by western blot) at this point can be used to calculate the efficiency of the rabbit IgG agarose immunoprecipitation. Due to the large quantity of starting material, it is normal to see some bait remaining in sample B. Densitometry analysis of the western blot (taking into account volumes loaded on the gel) can provide approximate "percentage immunoprecipitation" values.
14. Take care not to remove any beads during the washing steps. It is advisable to use duck-billed/flattened tips for this stage.

15. If possible use pre-lubricated tubes to reduce bead retention/loss at later steps in the protocol.
16. Comparison of sample B and C by western blot can provide useful information about the loss of bait during washing. Remember to factor in the different starting volumes prior to densitometric analysis.
17. The TEV protease cleavage reaction can be shortened; however, the efficiency will be reduced. This enzymatic reaction is slow (when incubated at 4 °C, compared to 37 °C) so it should be given time to run to completion. The efficiency of cleavage is also likely to be affected by the accessibility of the TEV cleavage site on the bait, so any optimization of this step should be empirically determined by the end user.
18. It is important to note that the cleaved bait is now present in the supernatant and not associated with the rabbit IgG agarose beads. Samples C and D can be compared by western blot to compare the efficiency of the TEV cleavage. If possible use a bait specific antibody for this step as the protein G tag has now been removed. It is normal to see cleavage efficiencies of over 95 % if the incubation was left overnight.
19. This additional "rinse" of the beads is to extract any cleaved bait still associated with the pelleted beads. This supernatant should be combined with the previously extracted one before continuing with the protocol.
20. It is not advisable to run large volumes of the beads on an SDS-PAGE gel (for direct staining or western blot) because the IgG heavy and light chains cause a lot of background (*see* Fig. 3). However it can be informative to analyze these samples to troubleshoot any problems with the TEV cleavage and first affinity purification. A small volume of beads should be boiled in reducing sample buffer prior to analysis.
21. The streptavidin beads that we have used (UltraLink Immobilized Streptavidin Plus agarose beads (Pierce)) are very small and should be handled with extreme care. Where possible use low-retention tips and pre-lubricated tubes to avoid damage and loss of the beads. It is normal for a 70 µl aliquot of these beads to reduce in size to around 45–50 µl of packed beads after washing.
22. Again this incubation can be performed overnight. It is possible that this protocol can be condensed into a 1 day protocol, or extended over 3–4 days. The relative merits of these two approaches should be determined empirically. In essence this represents a balance between maximizing the affinity purification and cleavage efficiencies and minimizing the loss of protein–protein interactions.

23. Sample F can be examined by western blot if a bait-specific antibody is available. This will provide information about the efficiency of the Streptavidin bead affinity purification.
24. Sample G is the final elution from the TAP purification and should contain the majority of the bait together with any associated proteins. This sample can be analyzed by western blot (and compared to earlier samples) to determine the efficiency of the TAP purification.
25. This second elution is performed to ensure the maximum yield of bait. In practice this step can be removed to save time since, in our experience, the majority of the bait is eluted during the first elution. However, this should be determined by the end user for each bait analyzed.
26. The streptavidin beads can be analyzed by western blot to determine the efficiency of the biotin elution. These beads do not generate as much background as the rabbit IgG agarose; however, they should still be run on separate gels, or in distinct wells.
27. It is advisable to boil this solution immediately in SDS sample buffer to reduce the risk of protein degradation.
28. Initial examination of the TAP elution should be run on gradient SDS PAGE gels to ensure the greatest range of proteins can be visualized. Silver staining is recommended at this stage because it provides high sensitivity. However, in our experience it is not always possible to identify the protein in a silver stained band slice by mass-spectrometry, which is why we advise running the majority of the final sample on a Coomassie gel instead (*see* Fig. 2).
29. We would highly recommend the use of pre-cast commercially available gels, stains, and reagents for this stage of the TAP purification (*see* Figs. 2 and 3). This will markedly reduce the environmental contamination of the sample, especially from Keratin. It is advisable to perform all the gel staining in a laminar flow cabinet or fume hood to reduce such risks. We routinely use disposable sleeve protectors as well.
30. Some commercial mass spectrometry services may run gels and excise relevant bands as part of their service. This may be advisable if the lab is not set up for keratin-free analysis.

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# Chapter 11

# An Improved In Vivo Biotinylation Strategy Combined with FLAG and Antibody Based Approaches for Affinity Purification of Protein Complexes in Mouse Embryonic Stem Cells

**Francesco Faiola, Arven Saunders, Baoyen Dang, and Jianlong Wang**

## Abstract

The proteome in mouse embryonic stem cells has not been extensively studied in comparison to other cellular systems, limiting our understanding of multi-protein complex functions in stem cell biology. Several affinity purification techniques followed by mass spectrometry analysis have been designed and validated to identify protein–protein interaction networks. One such approach relies on in vivo biotinylation of a protein of interest and subsequent pull-down of its interacting partners using streptavidin-conjugated agarose beads. This technique takes advantage of the high affinity between biotin and streptavidin, allowing for high affinity purification of protein complexes without the use of antibodies. Here, we describe an improved large-scale purification of multi-protein complexes in mouse embryonic stem cells by in vivo biotinylation, complemented with standard antibody and/or FLAG based affinity captures. This combined strategy benefits from the high efficiency of the streptavidin pull-down and the validation of the most highly confident interacting partners through the two alternative approaches.

**Key words** ESC, Biotinylation, Streptavidin, Multi-protein complexes, Purification, Mass spectrometry, BirA

## 1 Introduction

Mouse embryonic stem cells (mESCs) have the capacity to self-renew indefinitely and to differentiate into all cell types [1]. They exert these properties through many ESC specific proteins, such as the core pluripotency factors Nanog, Sox2, Oct4, and many other ubiquitous factors, including Polycomb proteins and epigenetic modifiers [2–4]. They form many multi-protein complexes to regulate functions such as transcription, translation, cell cycle, DNA replication, and so on. Therefore, to truly comprehend the many aspects of ESC biology, purification, identification, and analysis of these multi-protein complexes are fundamental.

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In recent years, the enormous technical improvements of mass spectrometry (MS) techniques and the availability of numerous proteomic datasets have significantly facilitated the determination of the protein content of a given sample [5]. However, the purification of such multi-protein complexes remains relatively challenging and still represents a bottleneck for executing proteomic studies [6].

Several different affinity purification strategies have been utilized over the past few decades [6–8]. These include (1) epitope tagging of a protein of interest with tags such as HA or FLAG, (2) antibody based affinity purification, provided that an antibody against the protein of interest is available, (3) ion exchange and gel filtration chromatography for the sequential fractionation of nuclear extracts to preserve and separate multi-protein complexes, and (4) biotinylation of a protein of interest by the biotin ligase BirA at a biotinylatable peptide sequence tag, described herein (Fig. 1). All these approaches have advantages and disadvantages, and since an optimal universal technique has not yet been designed, successful purification and identification of multi-subunit complexes may be achieved only by a combination of at least two of these approaches, for instance, the epitope tagging of a transcription factor with two different epitopes to perform a two-step (or tandem) affinity purification [9]. However, tandem affinity purification, although very efficient in reducing background, has the drawback of possible loss of peripheral and weakly interacting proteins due to the prolonged purification process. One solution to this is the iterative tagging of a different subunit of the multi-protein complex identified in the first affinity purification, and then performing a second independent affinity purification [9]. This second procedure greatly improves the identification of *bone fide* components of multi-protein complexes, but is much more lengthy and costly. Alternatively, double tagging of a single protein of interest, followed by two independent affinity purifications coupled, whenever possible, with antibody based immunoprecipitation (IP)/Co-IP, also improves the analysis of multi-protein complexes (Fig. 1) [10]. Everything considered, successful purification and identification of protein complexes relies on the improvements of each single affinity purification step. Here, we describe an improved biotin-based affinity purification approach for the identification of transcription factor associated protein complexes in mESCs. This strategy uses biotinylation of a bio-tagged [11] protein of interest by the bacterial enzyme BirA, followed by purification using streptavidin (SA)-conjugated agarose beads (Fig. 1) [12]. This technique offers several advantages compared to the traditional affinity purifications based on tags such as FLAG and HA. The high affinity between biotin and SA ($K_d \sim 10^{-15}$ M) allows for more efficient and stable capture of the protein of interest and associated factors. In addition, in ESCs there are only a few endogenously biotinylated proteins such as

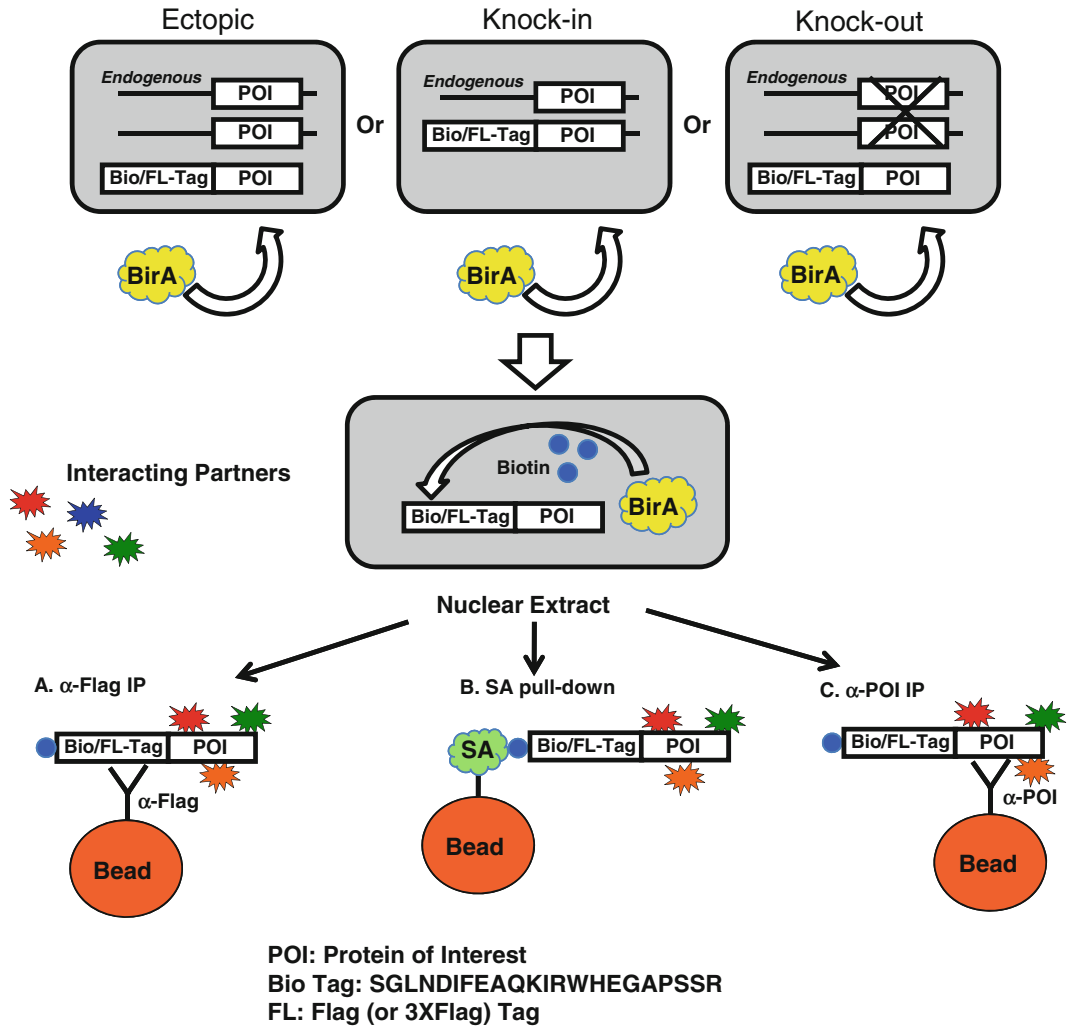


**Fig. 1** Strategies for affinity purification of the protein of interest (POI) and associated complexes. The transgene expressing the POI with a Bio and FLAG dual tag, is incorporated by stable transfection in either wild type cells or when possible, knockout cells,. Alternatively, the tag is engineered on the endogenous allele(s) of the gene of interest. The BirA transgene is always present to biotinylate the POI at the Bio tag. Upon nuclear extract preparation, the biotinylated POI and its associated factors are IPed with either a FLAG antibody, or pulled-down by SA conjugated beads, or captured with an antibody against the POI

some carboxylases and ribosomal proteins, which have already been characterized and can be excluded in the final list of interacting partners [9]. This approach also avoids using antibodies for the affinity capture, which significantly reduces nonspecific binding due to antibody cross-reactivity. Significant improvements over our previously published method [12, 13] have been outlined in this current protocol. Since mESCs are very sensitive to the expression levels of several key factors such as Oct4 and Sox2 [14, 15], we rely whenever possible on over-expressing our bio-tagged

protein of interest at physiological levels, and often in lines where the endogenous protein is knocked out. In the latter case, if the cells do not present any abnormal behavior, it means that the tagged ectopic version of the protein of interest is expressed at physiological levels and can substitute for the endogenous one. This also eliminates spurious complex formation and allows for more efficient and complete purification of the protein of interest and its associated multi-protein complexes. Alternatively, we sometimes engineer knock-in lines where the protein of interest is endogenously bio-tagged and the only transgene stably integrated in the genome is the BirA biotin ligase (Fig. 1). Once again, the normal phenotype of the knock-in cells will assure that knocked-in allele(s) functions similarly to the wild type. We now also employ transposon-based integration vectors over standard over-expression plasmids to facilitate the generation and screening of transgenic lines. In addition, to ensure detection of more peripheral and/or weakly interacting factors, we utilize lower salt and detergent conditions during the affinity purification. We also add a FLAG (or 3XFLAG) tag to the protein of interest to have the option of performing a large scale FLAG based affinity purification, and when possible a capture with an antibody raised against the protein of interest, to complement the SA pull-down. In conclusion, although very confident about the many advantages of our biotin-SA based affinity purification, we believe that a combination of a few techniques performed independently significantly improves the identification of *bone fide* multi-protein complexes. Indeed, in our recently published article, we purified an extended Nanog interactome in mESCs by utilizing a combination of SA, anti-FLAG, and anti-Nanog based affinity purifications [16]. This technique, where two or more independent affinity approaches are employed in parallel to identify an interactome (Interactomes by Parallel Affinity Capture (iPAC)), has already been shown to improve the identification of bona fide interacting partners [17].

## 2 Materials

All solutions, buffers, and media are sterilized by filtration unless otherwise specified.

1. Difco gelatin: 1 % (v/v) gelatin in $H_2O$ (autoclaved). Dilute 1:10 in $H_2O$ to gelatinize dishes. Gelatinized 3.5 cm, 10 cm, 15 cm, 245 mm × 245 mm square, and 6-well dishes.
2. ESC Medium: DMEM high glucose, 15 % (v/v) FBS, 0.1 mM β-mercaptoethanol, 2 mM L-glutamine, 0.1 mM nonessential amino acids, 1 % (v/v) nucleoside mix, 50 U/mL penicillin–streptomycin, 1,000 U/mL recombinant LIF.
3. STOP medium: DMEM, 10 % FBS.

4. Dulbecco's Eagle Medium (DMEM) with high glucose.
5. Fetal bovine serum (FBS).
6. Penicillin–streptomycin solution.
7. Phosphate buffered saline (PBS).
8. pPiggyBac-BirA-V5-HGR plasmid vector.
9. pPiggyBac-bio-FLAG-[cDNA of interest]-BSD plasmid vector.
10. pBASE (PiggyBac transposase).
11. Lipofectamine 2000 (Life Technologies).
12. Opti-MEM I (Gibco).
13. 0.05 % Trypsin–EDTA.
14. Hygromycin b solution at a concentration of 50 mg/mL.
15. Blasticidin S HCl.
16. Streptavidin agarose (SA) beads (Life Technologies).
17. Anti-FLAG M2 agarose beads (Sigma).
18. FLAG peptide (or 3XFLAG peptide) (Sigma).
19. Anti-POI antibody: antibody raised against protein of interest (POI).
20. IgG control antibody.
21. 2x Laemmli sample buffer.
22. Buffer A: 10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT (add fresh), 0.2 mM PMSF (add fresh), 1× protease inhibitor cocktail (add fresh).
23. Protease inhibitor cocktail, 1,000× (Sigma).
24. Buffer C: 20 mM HEPES pH 7.9, 25 % (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT (add fresh), 0.2 mM PMSF (add fresh), 1× protease inhibitor cocktail (add fresh).
25. Dialysis Buffer D: 20 mM HEPES pH 7.9, 20 % (v/v) glycerol, 100 mM KCl, 1.5 mM MgCl, 0.2 mM EDTA, 0.5 mM DTT (add fresh), 0.2 mM PMSF (add fresh).
26. Slide-A-Lyzer dialysis cassette 15 or 3 mL (Thermo Scientific).
27. Benzonase (Novagen).
28. Protein G agarose beads (Roche).
29. IP Buffer DNP: Dialysis Buffer D supplemented with 0.02 % NP-40.
30. Protein LoBind 1.5- or 2-mL safe-lock microcentrifuge tubes (Eppendorf) or other low protein binding tubes.
31. Amicon Ultra centrifugal 3 K filters (Millipore) or other compatible spin filters.
32. 4–20 % gradient polyacrylamide gels.

33. BupH Tris-HEPES-SDS running buffer (Thermo scientific).
34. Gel code blue safe protein stain (Thermo scientific) or other similar gel stain.
35. Dounce homogenizer with type B pestle (or 3–10 mL syringes with 16G1½ or 20G1½ needles).

## 3 Methods

### 3.1 Bio Cell Line Generation, Cell Culture, and Pilot Scale Affinity Purification

All mESC lines are grown on gelatinized dishes and maintained in standard ES medium at 37 °C in 5 % $CO_2$. The following procedures apply to control lines as well as lines with tagged proteins of interest.

1. Grow mESCs (parental ESC lines such as J1, or specific knockout/knock-in lines) in a 3.5 cm dish to 70–80 % confluence.
2. Pre-incubate 1 μg BirA and 2 μg pBASE plasmids diluted in 250 mL Opti-MEM I Medium, with 3 μL Lipofectamine 2000 diluted in 250 mL Opti-MEM I medium for 10 min at room temperature.
3. Meanwhile, wash cells once with PBS and add 1 mL 0.05 % Trypsin–EDTA for 5 min at 37 °C or at room temperature.
4. Detach cells by gently tapping the plate and then resuspend them by gently pipetting up and down.
5. Add 1 mL STOP medium, mix, harvest, and pellet the cells by spinning for 5 min at 300 × *g* at 4 °C.
6. Aspirate medium and resuspend cells in the 500 μL DNA/Lipofectamine 2000 mixture. Let the cells rest for 10 min at room temperature.
7. Add cells dropwise to a 10 cm dish containing 10 mL of ESC medium. Incubate overnight at 37 °C.
8. The next day, replace the medium and add the drug Hygromycin b at a concentration of 200 μg/mL for positive selection of transfectants.
9. Select the cells for at least 1 week by replacing medium supplemented with the selection drug every other day (*see* **Note 1**).
10. When discernible colonies appear (*see* **Note 1**), pick 6–12 of them and expand on a 6-well dish (*see* **Note 2**).
11. When the cells reach 70–80 % confluence, trypsinize each clone, freeze one half (in at least two vials; keep one at −80 °C for the next several days), and run SDS-PAGE with the other half of the cells to check for BirA expression (*see* **Note 3**).
12. Choose one clone in which BirA is expressed and thaw the corresponding vial in one well of a 6-well dish (*see* **Note 4**).

13. Repeat **steps 2–7** using a PiggyBac vector encoding the FLAG-bio-tagged version of the protein of interest.
14. Select as in **step 8** but use 20 μg/mL of Blasticidin S.
15. Repeat **steps 9–11** above for the protein of interest (for western blot we use an endogenous antibody and/or a streptavidin-HRP antibody).
16. Choose one clone in which BirA and the FLAG-bio-tagged protein of interest are expressed and thaw the corresponding vial (thaw also BirA-only control cells or any other negative control line) in one well of a 6-well dish.
17. Expand to a 10 cm dish until cells are 80–90 % confluent.
18. When confluence is reached, harvest the cells and replate 10 % of them in a 15 cm dish with ESC medium supplemented with selection drugs.
19. Resuspend the leftover 90 % of cells in 1 mL Buffer A (*see* **Note 5**). Transfer to a microcentrifuge tube and spin at 10,000 × *g* for 10 min at 4 °C.
20. Discard the supernatant cytosolic extract and repeat **step 19**.
21. Discard the second supernatant cytosolic extract and resuspend the nuclei in 1 mL of Buffer C (supplemented with 0.02 % NP-40). Pipette up and down several times, and then rotate at 4 °C for about 30 min.
22. Centrifuge at maximum speed for 30 min at 4 °C and then transfer the supernatant nuclear extract (NE) into a new Eppendorf microcentrifuge tube.
23. Repeat **step 22** once more.
24. Measure the protein concentration of the NE and then slowly dilute 500 μL of NE in a 1:1 ratio with Buffer C (without NaCl but supplemented with 0.02 % NP-40), to adjust the salt concentration to about 200 mM. Mix and spin as in **step 22**.
25. Meanwhile, cut off the tip of a 200 μL pipette tip and pipette 40 μL of streptavidin-agarose (SA) beads (50 % slurry; equivalent to 20 μL of solid beads) into two different Eppendorf tubes (one for the control line) (*see* **Note 6**).
26. Wash the beads twice by adding 1 mL of Buffer C, resuspend them, and spin at 1,000 × *g* for 15–30 s at 4 °C. Remove most of the supernatant after each spin without touching the beads.
27. Perform a pilot-scale affinity purification by adding 900 μL of the diluted and spun NE in the Eppendorf tube with the 20 μL of pre-equilibrated streptavidin-agarose beads, and rotate at 4 °C for a minimum of 3 h-overnight (*see* **Note 7**).
28. Transfer the supernatant (unbound) to a different tube and save for later.

29. Wash the beads four times with Buffer C (with 200 mM NaCl and supplemented with 0.02 % NP-40) as in **step 26**. After the last wash add 10–15 μL of 2× Laemmli sample buffer, heat at 95 °C for 3–5 min, spin for a few seconds at maximum speed, and transfer the supernatant with a gel loading tip to a new Eppendorf tube.
30. Run SDS-PAGE with 1–5 % input extracts, pull-down samples, and 1–5 % unbound extracts. Probe with streptavidin-HRP, or an antibody against the protein of interest (*see* **Note 8**).
31. Once biotinylation and capture of the protein of interest are verified, and the amount of SA beads to use per mg of NE is estimated, proceed to the large scale pull-down.
32. The 15 cm dishes where the 10 % fraction of cells was plated should be confluent by now (*see* **Note 9**). Wash with 5 mL of PBS and add 5 mL of 0.05 % Trypsin–EDTA. Resuspend the cells by pipetting up and down several times and neutralize the trypsin with 5 mL of STOP medium. Collect cells in a 15 mL conical tube and rinse plate with an additional 5 mL of STOP medium. Collect and pool into the same conical tube.
33. Spin at 300 × *g* for 5 min at 4 °C, remove supernatant, and resuspend cells in ESC medium. Plate cells in four 15 cm dishes (*see* **Note 10**).
34. Grow until 70–80 % confluent (*see* **Note 11**). Then split the four dishes into twenty 15 cm dishes.
35. Grow for an additional 2–3 days and change medium accordingly.
36. When cells are near confluence, wash with 10 mL of PBS, add 5 mL of 0.05 % Trypsin–EDTA for about 5 min at room temperature, resuspend the cells, neutralize the trypsin with 5 mL of STOP medium, and harvest the cells into 250 mL plastic centrifuge bottles. Rinse the dishes with 5 additional mL of STOP medium and collect leftover cells.
37. Pellet the cells by spinning at 300 × *g* for 20 min at 4 °C. Carefully decant the supernatant.
38. Resuspend the cells in 50 mL of ice-cold PBS and transfer into a 50 mL conical tube.
39. Spin at 300 × *g* for 10 min at 4 °C and decant the supernatant. Proceed to nuclear extract preparation.

### 3.2 Nuclear Extract Preparation

1. Estimate the packed cell volume (PCV) (*see* **Note 12**) and resuspend in 5× PCV of ice-cold Buffer A.
2. Spin at 2,100 × *g* for 10 min at 4 °C and discard the supernatant by aspiration.
3. Add 3× PCV of ice-cold Buffer A to the pellet, resuspend, and let it sit for 5–10 min on ice.

4. Prechill a glass dounce homogenizer with type B pestle (the loose one) on ice and pre-rinse it with Buffer A. Add the resuspended pellet and slowly homogenize up and down for no more than 12 times. Keep everything on ice while homogenizing (*see* **Note 13**).
5. Transfer into a conical tube and spin at 4,300 × *g* for 10 min at 4 °C. Discard the supernatant (cytosolic extract) (*see* **Note 14**) and keep the pellet (nuclei).
6. Add 3–10 mL of ice-cold Buffer A and resuspend the nuclei. Transfer into a high speed centrifuge tube and spin at 25,000 × *g* for 20 min at 4 °C (*see* **Note 15**).
7. Remove the supernatant and add 3–10 mL of ice-cold Buffer C to the nuclei and resuspend (*see* **Note 16**).
8. Transfer into a glass dounce homogenizer with type B pestle (loose one) (*see* **Note 17**), and slowly homogenize on ice with no more than ten strokes (*see* **Note 13**).
9. Transfer homogenate into a 15 mL conical tube and rotate for 30 min at 4 °C.
10. Centrifuge at 4,300 × *g* for 10 min at 4 °C and transfer the supernatant nuclear extract to several Eppendorf tubes.
11. Centrifuge at maximum speed for 30 min at 4 °C.
12. Meanwhile, for each sample pour Dialysis Buffer D into a beaker (*see* **Note 18**). We use at least 100 volumes of dialysis buffer per volume of NE.
13. After spinning, transfer the supernatant into a dialysis cassette, pre-equilibrated according to the manufacturer's instructions, and incubate for 3–5 h at 4 °C with constant stirring to adjust the salt concentration to 100 mM.
14. Following dialysis, transfer the nuclear extract to a 15 mL conical tube and spin at 4,300 × *g* for 10 min at 4 °C (*see* **Note 19**).
15. After centrifugation, transfer the supernatant NE to several Eppendorf tubes and spin at maximum speed for 30 min at 4 °C in a microcentrifuge. Carefully transfer the supernatant to new Eppendorf tubes without touching the precipitate.
16. Repeat **step 15** once more and combine the supernatants.
17. Supplement the NE with 1× protease inhibitor cocktail, 0.02 % NP-40, and Benzonase (*see* **Note 20**).
18. For each sample, pipette 200 μL of Protein G agarose beads (400 μL of 50 % slurry) into a 15 mL conical tube (*see* **Note 6**).
19. Resuspend the beads with 10 mL of IP Buffer DNP (without protease inhibitor cocktail), spin at 1,000 × *g* for 1 min at 4 °C, and aspirate most of the supernatant without touching the beads.

20. Add the NE to the tube containing the equilibrated beads and pre-clear by rotating overnight at 4 °C.
21. The following morning, centrifuge at 1,000 × *g* for 1 min at 4 °C to pellet the beads.
22. Carefully remove most of the supernatant NE without touching the beads.
23. Aliquot the NE into several Eppendorf tubes and centrifuge for 30 min at maximum speed at 4 °C.
24. After spinning, combine the supernatants in a 15 mL conical tube and label as pre-cleared NE.

### 3.3 SA Affinity Purification

All steps are performed on ice or at 4 °C unless otherwise specified.

1. Measure protein concentrations and use the same amount (in mg) of NE for all samples for the affinity purification.
2. Equilibrate no more than 1 mL of 50 % slurry streptavidin agarose beads (500 μL of beads) per sample by pipetting the slurry into a 15 mL conical tube and resuspending with 10 mL of IP Buffer DNP (without protease inhibitor cocktail) (*see* **Note 21**).
3. Spin at 1,000 × *g* for 1 min at 4 °C to pellet the beads, and aspirate most of the supernatant without touching the beads.
4. Add the pre-cleared NE directly to the equilibrated beads (adjust the volume to 10 mL with IP Buffer DNP) and gently rotate for 3–5 h at 4 °C (*see* **Note 22**).
5. Centrifuge at 500 × *g* for 2 min at 4 °C and remove most of the supernatant (unbound material) without disturbing the beads (bound material). Transfer into a new 15 mL conical tube (aliquot also ~100–200 μL in an Eppendorf tube), flash-freeze in liquid nitrogen, and store at −80 °C (*see* **Note 23**).
6. Wash the beads and captured complexes by adding 10 mL of cold IP Buffer DNP (without protease inhibitor cocktail) and rotating 15 min at 4 °C. Afterwards, centrifuge at 500 × *g* for 2 min at 4 °C and discard most of the wash by aspirating without disturbing the beads.
7. Repeat **step 6** three more times.
8. After the last wash, leave behind about 200–300 μL of washing buffer and transfer the beads into a low protein binding Eppendorf tube (*see* **Note 24**).
9. Wash one more time by adding 1 mL of IP Buffer DNP (without protease inhibitor cocktail) and rotating 5–10 min at 4 °C.
10. Centrifuge at 1,000 × *g* for 1 min at 4 °C and aspirate most of the wash without touching the beads.

11. Elute captured protein complexes by adding 500 µL of 2× Laemmli sample buffer (*see* **Note 25**). Vortex briefly and heat at 95–100 °C for 4–5 min (vortex briefly from time to time), spin at 5,000 × *g* for 30 s at RT and transfer as much supernatant as you can with a gel loading tip into a new 2 mL Eppendorf tube.
12. Repeat **step 11** (this time use 1× Laemmli sample buffer: 50 % 2× Laemmli sample buffer and 50 % $H_2O$) twice and pool eluates (about 1.5 mL total volume). Spin once more and transfer supernatant to a new tube to get rid of any bead carryover (*see* **Note 26**).
13. Concentrate the eluate with Amicon ultra centrifugal filter according to the manufacturer's instructions (*see* **Note 27**). Keep a 50–100 µL aliquot of the unconcentrated eluate to check by western blot.

### 3.4 FLAG Affinity Purification

All steps are performed on ice or at 4 °C unless otherwise specified (*see* **Note 28**).

1. Perform **steps 1–10** as in Subheading 3.3 but use anti-FLAG M2 agarose beads instead of SA beads.
2. Elute captured protein complexes by adding 500 µL of 0.3 µg/mL FLAG peptide (or 3XFLAG peptide if your protein of interest has a 3XFLAG tag) in IP Buffer DNP (*see* **Note 29**). Rotate at least 1 h at 4 °C, then centrifuge at 1,000 × *g* for 1 min at 4 °C and transfer supernatant in a new 2 mL Eppendorf tube (*see* **Note 30**).
3. Repeat **step 2** three more times and combine all the eluates (*see* **Note 31**). Spin once more and transfer the eluate into a new tube to get rid of any bead carryover.
4. Perform **step 13** as in Subheading 3.3.

### 3.5 Anti-POI Affinity Purification

All steps are performed on ice or at 4 °C unless otherwise specified (*see* **Note 32**).

1. Equilibrate 150–200 µL of protein G agarose beads (300–400 µL of 50 % slurry) in IP Buffer DNP, as in **steps 25** and **26** in Subheading 3.1.
2. Pre-bind 30–50 µg of purified anti-POI antibody (or IgG raised in the same species) with the beads overnight at 4 °C by gentle rotation (*see* **Note 33**).
3. The morning after, incubate the pre-cleared and spun nuclear extract with the pre-bound antibody and rotate for about 3–4 h at 4 °C (*see* **Note 34**).
4. Perform **steps 5–13** as in Subheading 3.3 but wash in Eppendorf tubes, and use 150–200 µL 2× Laemmli sample buffer per elution.

### 3.6 SDS-PAGE/MS

Before proceeding to MS analysis, run SDS-PAGE to check the efficacy of the pull down. Use 10–20 μL of input pre-cleared NE (use the frozen aliquot you set aside in **step 5** of the affinity purification procedure), the same percentage of the unbound aliquot you set aside in **step 5** of the affinity purification, and 20–30 μL of the unconcentrated eluate aliquot set aside in **step 13** of the affinity purification. Also load on the gel the aliquots from the negative control affinity purification. Check by western blot with an antibody against the protein of interest or with a streptavidin-HRP antibody. Proceed to running the gel for MS (*see* **Note 35**).

1. Use a commercial pre-cast 4–20 % gradient polyacrylamide gel.
2. Load on the gel a protein ladder marker and the concentrated eluates (specific and control). You can use more than one well to load all the eluate for each sample. Keep at least one well empty between control and specific eluates.
3. Run at 60 V to 1/3 or 1/2 of the gel length and stain with gel code blue safe stain for at least 2 h or overnight, according to the manufacturer's instructions. De-stain with $H_2O$ several times.
4. Proceed according to the guidelines from the MS facility of choice.

## 4 Notes

1. If after selection too many cells survived and colonies are not separated, trypsinize the cells and replate around 10,000 cells. Wait for colonies to appear and then pick them.
2. Use a microscope and a P200 pipet to pick colonies. If they are well separated, you can pick them in the hood. We generally pick colonies in PBS and transfer them into individual wells of a 96-well round bottom plate containing 50 μL trypsin per well to dissociate the cells in each colony. When the cells are separated, we transfer them to wells of a 24-well dish, then to wells of a 6-well dish.
3. We use an anti-BirA antibody or anti-V5-HRP antibody for western blot.
4. In our experience, screening six clones will yield several BirA lines from which to choose. Thaw the vial stored at −80 °C.
5. We generally resuspend the cells and extract the cytosolic portion by simply pipetting up and down.
6. Make sure you cut the bottom of the tip to facilitate accurate pipetting of the 50 % slurry.
7. It is better to incubate the lysate with the beads only for a few hours, but it can be done overnight if you start late in the afternoon.

8. The biotinylated version of the protein of interest should migrate slower than the unmodified endogenous form. Therefore, when using an antibody against that protein you should see two bands in the input and unbound lanes (unless you are using a knockout or knock-in line), but only one (the heavier) in the pull-down lane. This SDS-PAGE is to validate the affinity capture and estimate the amount of beads to use per mg amount of nuclear extract (by comparing input and unbound).
9. You should have one line with BirA plus the bio-tagged protein of interest and at least one control line: BirA only or bio-tagged protein only. Try to avoid using parental lines without either BirA or the bio-tagged protein of interest.
10. Alternatively, one 245 mm × 245 mm square dish can be used in place of three to four 15 cm dishes.
11. It should take 2–3 days. Change medium every other day, or daily if it becomes yellow.
12. You should have around 5 mL of pelleted cells.
13. If a dounce homogenizer is not available, you can use a syringe with a 16G1½ or a 20G1½ needle.
14. The cytosolic extract can be kept in case the protein of interest is cytosolic.
15. Alternatively, you can aliquot in several Eppendorf tubes and spin in a microcentrifuge at maximum speed for at least 10 min.
16. The more Buffer C you use, the more protein you will extract and the more Eppendorf tubes you will have to handle later on. It will also depend on how many cells you start with.
17. As in **step 4** above, the homogenizer is prechilled on ice and pre-rinsed with Buffer C.
18. We do not supplement the dialysis buffer with protease inhibitor cocktail.
19. This step will pellet most, but not all proteins precipitated during the dialysis.
20. We generally use 225 U of Benzonase per five 15 cm dishes worth of cells.
21. The optimal amount of beads should have been estimated in the pilot scale experiment. Using too many beads will increase the background; therefore, in case it is impossible to estimate the right amount of beads, use 200 μL of beads.
22. For the least concentrated NE, use everything except a few hundred μL (you will need these aliquots for SDS-PAGE later). For the more concentrated NE, pipette accordingly. It is not recommended to incubate for longer than 5 h with the beads to avoid nonspecific binding.

23. We keep the unbound fraction in case something is wrong and we need to repeat the capture. The small aliquot is for SDS-PAGE and western blot.
24. Use a 1,000 mL tip from which you cut off the bottom to make a bigger aperture to resuspend the beads up and down, and transfer into a microcentrifuge tube (set the pipet to 150 μL). Do not discard the tip. With another pipet add 200 μL of IP Buffer DNP to the original 15 mL conical tube (make sure you wash down the leftover beads form the bottom wall of the conical tube). Collect the leftover beads with the original bottom-cut 1,000 μL tip and transfer into the microcentrifuge tube. Repeat the 200 μL wash and harvest at least one more time to completely collect the beads from the conical tube and the 1,000 μL pipet tip.
25. Elute with one volume of Laemmli sample buffer per volume of beads. Therefore, if you originally used 200 μL of beads, elute with 200 μL of SDS-LB.
26. The eluates can be frozen at this time at –20 or at –80 °C for later use.
27. You may need to repeat the 30 min 4,000 × *g* centrifuge step more than once to reduce the volume to 50–100 μL.
28. For simplicity, the BirA only line can be used as negative control here as well.
29. Do not use the FLAG peptide to elute 3XFLAG tagged proteins and vice versa otherwise the elution process will not work.
30. It is not necessary to use a gel loading tip and to remove the supernatant completely.
31. At the end of the last elution step, use a gel loading tip to collect the supernatant completely.
32. Any wild type mESC line can be used for this purification. In this case five to ten 15 cm dishes worth of cells are enough for each antibody IP. To make nuclear extract, use 3 mL of Buffer C.
33. Make sure you have at least 800–1,000 μL volume in each Eppendorf tube. Protein G agarose beads can be replaced with Protein A beads, according to the antibody used.
34. Alternatively, incubate the nuclear extract with the antibody first, for at least 6 h to a maximum of 12–14 h. Then, centrifuge the extract at maximum speed for about 30 min in the cold to remove precipitates, and transfer into a new tube. After that, capture the immune-complexes with 150–200 μL pre-equilibrated protein G beads for 2 h at 4 °C.
35. It is fundamental to keep a very clean working environment to reduce background later on during the mass spectrometry analysis.

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# Chapter 12

# Purification of Recombinant Proteins with a Multifunctional GFP Tag

**Takashi Murayama and Takuya Kobayashi**

## Abstract

Green fluorescent protein (GFP) is the most widespread fluorescent reporter for cellular localization and interaction of proteins. Because GFP itself is not the protein purification tag, protein purification is generally carried out with the aid of additional affinity tags. We have recently engineered a "multifunctional GFP" (mfGFP), a variant of enhanced GFP (EGFP), in which multiple affinity tags are inserted in tandem into an internal loop of EGFP. The mfGFP can be used as a fluorescent reporter and an affinity tag, and is compatible with various expression systems in prokaryotic and eukaryotic cells. Herein, we describe detailed procedures for the expression and purification of mfGFP fusion proteins in mammalian cells. A method for tandem affinity purification using two different tags within mfGFP is also described.

**Key words** Affinity tag, Green fluorescent protein (GFP), Immobilized metal affinity chromatography (IMAC), Multifunctional GFP (mfGFP), Protein complex, Protein purification, Streptavidin binding peptide (SBP)

## 1 Introduction

Green fluorescent protein (GFP) is a fluorescent protein from the jellyfish *Aequorea victoria* [1]. GFP and related fluorescent proteins are the most commonly used fluorescent tags for expression, localization and interaction of proteins of interest in vitro and in vivo [2, 3]. GFP itself, unfortunately, cannot be used as a protein purification tag. Although GFP is widely used as an immunoprecipitation tag, recovery of the functional protein from the immunoprecipitated complex under mild conditions (e.g., a FLAG-tag system with an antigen peptide [4]) has not been achieved to date. Purification of a GFP fusion protein is generally performed with affinity tags that are fused separately or in tandem with GFP. However, developing multi-tagged constructs is time-consuming and labor-intensive, especially when analyzing a large number of proteins.

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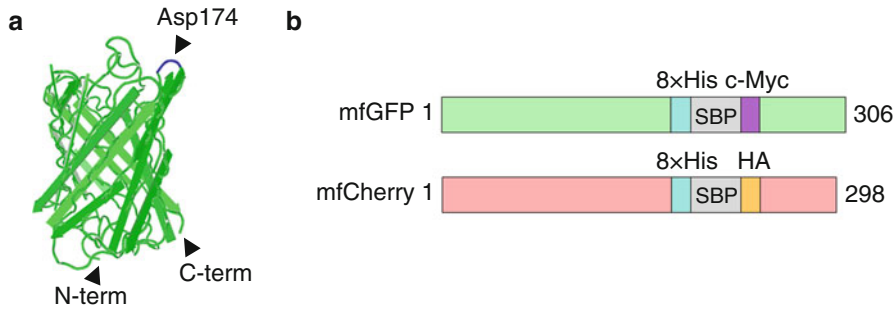


**Fig. 1** (**a**) Ribbon model of EGFP. Asp174 is located within a loop on the opposite side of the N- and C-termini. (**b**) Schematic representation of mfGFP and mfCherry. Octa-Histidine (8× His), streptavidin-binding peptide (SBP), and epitope tags (c-Myc for mfGFP, and HA for mfCherry) are inserted after Asp174. Short GS linkers (2–4 residues) are inserted between tag sequences

GFP forms a rigid and stable 11-stranded β-barrel structure with close proximity of the N- and C-termini (Fig. 1a). This enables internal fusion of GFP with the protein of interest, as well as fusion at its N- and C-termini. GFP is shown to be tolerant to the insertion of foreign peptides within certain loops between the β-strands [5] and thus can be a scaffold for short peptides [6, 7]. Using these properties, we have recently engineered a "multifunctional GFP" (mfGFP), in which multiple affinity tags, such as octa-histidine (8× His), streptavidin-binding peptide (SBP) [8] and c-Myc, were inserted in tandem after Asp174 in enhanced GFP (EGFP) [9] (Fig. 1b). By fusing to the protein of interest, mfGFP can then be used to monitor localization of the fusion protein in living cells and allow for purification of functional protein complexes with high purity [9, 10].

There are several advantages for using mfGFP as compared with other existing purification tags. Fluorescence from GFP is useful for several downstream applications, including in vitro fluorescence-based interaction assays (FRET/FLIM) [11], along with enabling quick detection of protein expression and purification. The GFP moiety provides a scaffold for the affinity tags, allowing better accessibility to the ligands. The affinity tags are located opposite the N- and C-termini of mfGFP (Fig. 1a), which minimizes steric hindrance produced by mfGFP and the protein of interest, especially in cases of internal fusion. Multiple tags in mfGFP provide several options for purification, including tandem affinity purification (TAP) [12]. Compatibility with existing and future tag technology would further expand options available to researchers. The mfGFP technology is also applicable to other fluorescent proteins. Multifunctional mCherry (mfCherry), a variant of the coral red fluorescent protein, has been successfully engineered [9]. All of these factors combined result in a saving of time and labor, with users able to easily make expression constructs for their protein of interest containing the new mfGFP, or replace an existing GFP with mfGFP.

In this chapter, we describe methods for the expression and purification of mfGFP fusion proteins in mammalian cells. Protein expression is done using an Flp-In T-REx HEK 293 cell system that enables stable and inducible expression of the recombinant proteins. Protein purification is carried out using the SBP tag, which allows for simple and rapid purification of the recombinant protein complex and results in a high level of purity. A method for TAP using the His and SBP tags within mfGFP is also described.

## 2 Materials

It is recommended that ultrapure water and chemicals of the highest quality be used for the preparation of all solutions.

### 2.1 Plasmids

There are no commercially available plasmids that express mfGFP or other multifunctional fluorescent proteins (*see* **Note 1**). The plasmids that we can provide encode mfGFP and mfCherry. The mfGFP plasmid is an EGFP variant in which 8× His, SBP, and c-Myc tags are inserted in tandem after Asp174. The mfCherry plasmid is an mCherry variant with 8× His, SBP, and hemagglutinin (HA) tags inserted in tandem at the corresponding site (after Asp174) (Fig. 1b).

1. mfGFP plasmid (available upon request).
2. pcDNA5/FRT/TO (Life Technologies).

### 2.2 Generation of Stable and Inducible HEK 293 Cell Lines

1. Flp-In T-REx HEK 293 (Life Technologies).
2. pOG44 plasmid for Flp recombinase (Life Technologies).
3. Dulbecco's Modified Eagle Medium (DMEM).
4. Fetal Bovine Serum (FBS), preferably One Shot® FBS (Life Technologies).
5. 200 mM L-glutamine.
6. 100× penicillin–streptomycin.
7. Culture medium: DMEM supplemented with 10 % One Shot FBS, 2 mM L-glutamine, and 1× penicillin–streptomycin.
8. Opti-MEM.
9. Lipofectamine LTX (Life Technologies/Invitrogen).
10. 10 mg/ml blasticidin S hydrochloride.
11. 10 mg/ml Zeocin
12. 50 mg/ml hygromycin B.
13. 2 mg/ml doxycycline: Dissolve 2 mg of doxycycline hyclate in 1 ml of water. Aliquot and store at −20 °C.

14. Phosphate-buffered saline (PBS).
15. Cell cryopreservation medium, such as Cellbanker (Zenoaq).
16. 0.05 % Trypsin-EDTA (1×), Phenol Red (Life Technologies).

### 2.3 Protein Purification

#### 2.3.1 Strep-Tactin Column Chromatography

1. Strep-Tactin column (StrepTrap HP, GE Healthcare) (*see* **Note 2**).
2. Triton X-100.
3. 100 mM ATP stock solution in ultrapure water.
4. Protease inhibitor cocktail, such as Complete Mini, EDTA-free (Roche).
5. Strep-Tactin buffer: 0.2 M NaCl, 50 mM Tris–HCl pH 7.5, 10 % sucrose, 5 mM $MgCl_2$, 1 mM dithiothreitol (*see* **Note 3**).
6. D-desthiobiotin.
7. Strep-Tactin elution buffer: Strep-Tactin buffer supplemented with 0.5 mM D-desthiobiotin. Place 5.4 mg of desthiobiotin into 10 ml of Strep-Tactin buffer, mix vigorously by vortexing or sonication, and stand for several minutes to completely dissolve.
8. 0.45-μm syringe filter.
9. Standard column chromatography system (*see* **Note 4**).
10. 0.5 M NaOH in ultrapure water.

#### 2.3.2 TAP: Immobilized Metal Affinity Chromatography (IMAC)

1. HisTrap HP nickel-nitrilotriacetic acid (Ni-NTA) column (GE Healthcare).
2. IMAC buffer: 0.2 M NaCl, 50 mM sodium phosphate pH 7.5, 10 % sucrose, 5 mM $MgCl_2$, 1 mM tris(2-carboxyethyl)phosphine (TCEP) (*see* **Note 5**).
3. 1 M imidazole: Dissolve 34.0 g of imidazole in 500 ml of deionized $H_2O$. Adjust pH to 7.5 with HCl.
4. Protease inhibitor cocktail, such as Complete Mini, EDTA-free (Roche) (*see* **Note 6**).
5. 0.45-μm syringe filter.
6. Standard column chromatography system.

#### 2.3.3 TAP: Strep-Tactin Column Chromatography

Materials are the same as for those listed in Subheading 2.3.1.

## 3 Methods

### 3.1 Plasmid Construction

1. Amplify the gene of interest by polymerase chain reaction (PCR) and clone into the multi-cloning site of the pcDNA5/FRT/TO vector using appropriate restriction enzymes. Use primers which have restriction enzyme recognition site

sequences before (forward primer) and after (reverse primer) the coding sequence. If mfGFP is to be fused at the C-terminus of the protein of interest, remove the stop codon from the reverse primer sequence.

2. Amplify the mfGFP gene by PCR and clone it into the above vector using restriction enzymes (*see* **Note 7**). To avoid steric hindrance, insert linkers (e.g., GGGS or longer) between mfGFP and the protein of interest.

### 3.2 Generation of Inducible HEK 293 Cell Lines Stably Expressing the mfGFP Fusion Protein

HEK 293 cells are widely used for expression of mammalian proteins. The Flp-In T-REx HEK 293 cell system is designed for generation of stable cell lines with tetracycline-inducible expression of a gene of interest. The gene of interest is integrated into a single specific genomic location (FRT site) by an Flp recombinase [13]. This allows for rapid and efficient generation of isogenic stable cell lines. Tetracycline-inducible expression is greatly advantageous especially for high levels of fusion protein expression, which can be toxic to cells.

1. Maintain Flp-In T-REx HEK 293 cells in a culture medium (*see* **Note 8**) in the presence of 15 μg/ml blasticidin and 100 μg/ml Zeocin.
2. The day before transfection, plate $5 \times 10^5$ Flp-In T-REx HEK 293 cells on a 35-mm dish in 1.5 ml of the above medium without antibiotics.
3. On the day of transfection, dilute 0.5 μg of plasmid DNA at a 9:1–3:1 ratio (pOG44:pcDNA5/FRT/TO carrying the mfGFP fusion protein gene) in 200 μl of Opti-MEM (*see* **Note 9**).
4. Add 2 μl of Lipofectamine LTX Reagent into the above solution, mix gently, and incubate for 30 min at room temperature.
5. Replace the cell medium with 1.3 ml of fresh medium, add the solution from **step 4**, and mix gently by rocking the plate back and forth.
6. Incubate the cells at 37 °C/5 % $CO_2$.
7. The day after transfection, trypsinize and plate the cells into two 100-mm dishes.
8. Two days after transfection, add blasticidin and hygromycin to the plates at a final concentration of 15 μg/ml and 100 μg/ml, respectively.
9. Replace the culture medium with fresh medium every 3 days until hygromycin-resistant foci can be identified.
10. Pick several foci and expand the cells in 12-well or larger plates (*see* **Note 10**). Following expansion, create stocks of the cells using cryopreservation medium and store aliquots in liquid nitrogen (*see* **Note 11**).

11. Check the inducible expression of the fusion protein. Culture the cells in 12-well plates to 50–70 % confluence. Incubate the cells in culture medium supplemented with doxycycline (0.01–1 μg/ml) for 24–48 h. GFP fluorescence on the plated cells can be visualized with the aid of a fluorescence microscope. Harvest the cells and assay for expression of the fusion protein by immunoblotting (*see* **Note 12**).

### 3.3 Purification of the mfGFP-Fusion Protein Using SBP-Tag

The SBP tag is a genetically engineered 38-amino acid sequence that binds to streptavidin with a high affinity ($K_D = 2.5$ nM) and is readily eluted with biotin under native conditions [8]. The SBP tag system allows simple and rapid purification of the recombinant protein complex with a high purity. Here, we describe purification of a 74 kDa intermediate chain (IC74) of cytoplasmic dynein, a minus end-directed microtubule molecular motor in eukaryotic cells [10].

1. Grow the stable HEK 293 cell lines in ten 100-mm dishes to 70–80 % confluence.
2. Replace the culture medium with fresh medium supplemented with doxycycline (0.01–1 μg/ml) to induce protein expression (24–48 h) (*see* **Note 13**).
3. Harvest the cells. HEK 293 cells are easily detached from the dishes by gently flushing the culture medium with a pipette. Collect the cell suspension in 50-ml conical tubes and centrifuge (1,000 × *g*, 5 min) to pellet the cells. Discard the supernatant and rinse the cells twice with PBS (*see* **Note 14**).
4. Suspend the cell pellet in 10 ml of Strep-Tactin buffer supplemented with 0.05 % Triton X-100, 0.1 mM ATP and a protease inhibitor cocktail (*see* **Note 15**). Transfer the suspension into a glass-Teflon homogenizer and homogenize with 10–15 strokes.
5. Sediment the cell debris by ultracentrifugation (100,000 × *g*, 30 min) and transfer the supernatant into a new tube.
6. Equilibrate the StrepTrap HP column (1 ml bed volume) with 10 ml of Strep-Tactin buffer at a flow rate of 0.5 ml/min (*see* **Note 16**).
7. Filter the supernatant from the ultracentrifugation step through a 0.45-μm syringe filter (*see* **Note 17**).
8. Load the filtered supernatant onto the column (*see* **Note 18**). Collect the flow-through fraction.
9. Wash the column with Strep-Tactin buffer (>10 ml) until the absorbance at 280 nm ($A_{280}$) reaches a baseline (*see* **Note 19**).
10. Elute the bound proteins with 5 ml of Strep-Tactin elution buffer (*see* **Note 20**). Collect 0.25 ml fractions.

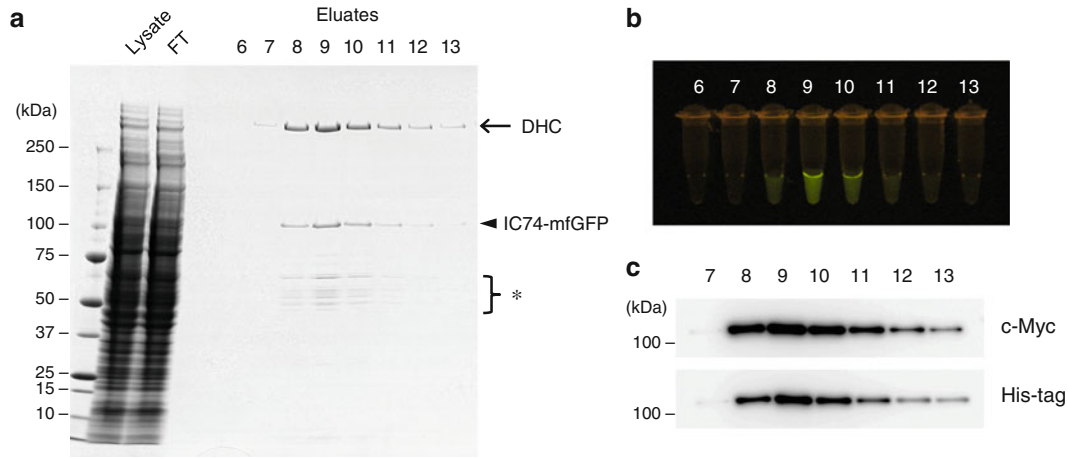


**Fig. 2** Purification of a cytoplasmic dynein complex with an IC74-mfGFP fusion protein using Strep-Tactin column chromatography. (**a**) Lysate, flow-through (FT), and eluates (from 6th to 13th) were subjected to SDS-PAGE with a 3–15 % linear gradient gel and stained with Coomassie Brilliant Blue. *Arrowhead* represents IC74-mfGFP (102 kDa). *Arrow* represents a dynein heavy chain (>400 kDa), the largest subunit of the dynein complex. Several bands around 50 kDa (indicated by an *asterisk*) may represent both dynein light intermediate chains and contaminating proteins. (**b**) Detection of GFP fluorescence in eluates with a blue light transilluminator. (**c**) Detection of mfGFP using antibodies against c-Myc and His. IC74-mfGFP is recognized by both antibodies

11. Detect GFP fluorescence of the fractions using a blue light transilluminator (Fig. 2b). Analyze aliquots of 10–20 μl from each fraction by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting (Fig. 2a, c).
12. Regenerate the column with successive washes of 10 ml of ultrapure water, 3 ml of 0.5 M NaOH, and 10 ml of ultrapure water (*see* **Note 21**).

### *3.4 TAP of mfGFP Fusion Proteins Using His and SBP Tags*

Because SBP tag purification provides highly pure proteins, further purification of an mfGFP fusion protein is not necessary for most downstream applications. For some applications that require highly purified proteins (e.g., crystallography and subunit analysis of the protein complexes), a TAP strategy, employing the His and SBP tags, can be used. The mfGFP fusion protein is first purified using the His tag via IMAC and then purified further with the aid of the SBP by Strep-Tactin chromatography. The combination of His and SBP tags allows for rapid and efficient purification of the fusion protein because no protease digestion step is included.

#### *3.4.1 IMAC with Ni-NTA Resin*

1. Grow and harvest the cells expressing the mfGFP fusion protein (**steps 1–3** in Subheading 3.3).
2. Suspend the cell pellet in IMAC buffer supplemented with 20 mM imidazole, detergent (e.g., Triton X-100), and a protease inhibitor cocktail (*see* **Note 22**). Transfer the suspension into the glass-Teflon homogenizer and homogenize using 10–15 strokes.

3. Sediment the cell debris by ultracentrifugation (100,000 × *g*, 30 min) and transfer the supernatant into a new tube.
4. Equilibrate the HisTrap HP column (1 ml bed volume) with 10 ml of IMAC buffer supplemented with 20 mM imidazole at a flow rate of 0.5 ml/min.
5. Filter the ultracentrifugation supernatant through a 0.45-μm syringe filter.
6. Load the filtered supernatant onto the column. Collect the flow-through fractions.
7. Wash the column with IMAC buffer supplemented with 20 mM imidazole (>10 ml) until the $A_{280}$ reaches a baseline.
8. Elute the bound proteins with 10 ml IMAC buffer supplemented with 300 mM imidazole. Collect 0.5 ml fractions.
9. Detect GFP fluorescence of the fractions using a blue light transilluminator. Analyze aliquots of 10–20 μl from each fraction via SDS-PAGE.

#### *3.4.2 Strep-Tactin Chromatography*

1. Equilibrate the StrepTrap HP column (1 ml bed volume) with 10 ml Strep-Tactin buffer at a flow rate of 0.5 ml/min.
2. Combine the IMAC eluates containing the mfGFP fusion protein and load onto the column (*see* **Note 23**). Collect the flow-through fractions.
3. Wash the column with Strep-Tactin buffer until the $A_{280}$ reaches a baseline (>10 ml).
4. Elute the bound proteins with 5 ml of elution buffer (Strep-Tactin buffer supplemented with 2.5 mM D-desthiobiotin). Collect 0.25 ml fractions.
5. Detect GFP fluorescence of the fractions using a blue light transilluminator. Analyze aliquots of 10–20 μl from each fraction by SDS-PAGE.

## 4 Notes

1. FLAG (DYKDDDDK) [4] and StrepTag II (WSHPQFEK) [14] are useful tags for the purification of recombinant proteins. We have not yet tested them for mfGFP. If desired, users can engineer their own multifunctional fluorescent proteins by combining affinity tags and fluorescent proteins. In our experience, a longer peptide sequence (>100 residues) was also successfully inserted in the loop of EGFP.
2. Other Strep-Tactin resins, such as those from IBA also work.
3. The composition of Strep-Tactin buffer depends upon the protein of interest. For optimum binding of the SBP tag to Strep-Tactin, the pH of the buffer should be greater than 7.0.

4. Purification can also be carried out using a syringe if a laboratory pump or liquid chromatography system is not available (please see the GE Healthcare StrepTrap Web site).
5. The composition of the IMAC buffer depends upon the protein of interest. A phosphate buffer is generally used for IMAC instead of Tris, as Tris may reduce the binding capacity of the Ni-NTA resin. Dithiothreitol may also reduce $Ni^{2+}$ from the Ni-NTA resin, which results in a loss of the binding capacity for the resin. Therefore, it is better to use TCEP as that does not affect NTA binding capacity [15]. Some Ni-NTA resins, such as HisTrap from GE Healthcare, are reported to be more stable even in the presence of Tris or dithiothreitol.
6. Do not use EDTA in IMAC. EDTA is a strong metal chelating reagent and reduces $Ni^{2+}$ in the Ni-NTA resins, causing a loss of binding capacity.
7. mfGFP can be fused internally or to the N- or C-terminus of the protein of interest, but the appropriate fusion site depends largely on the respective protein.
8. Use Tet-system approved FBS. Some FBS contains a trace amount of tetracycline, which induces leaky expression of the transgenes before induction and causes cell toxicity.
9. The manufacturer's protocol recommends a high pOG44:pcDNA5/FRT/TO ratio (approximately 9:1) to avoid nonspecific incorporation of the gene into the genome. For longer genes of interest (>5 kb), altering the ratio to around 3:1 may result in better recombination efficiency.
10. Because all of the hygromycin-resistant foci should be isogenic (i.e., pcDNA5/FRT/TO should integrate into the same genomic locus in every clone, therefore all clones should be identical), the hygromycin-resistant foci can be pooled as the "polyclonal" populations.
11. Cellbanker or a related cell cryopreservation medium is useful for easy and stable storage of cells. They show consistently high cell viability upon recovery of cells. Slow or programmable cooling is not absolutely necessary when these cryopreservation media are used.
12. mfGFP can be detected by antibodies against the various affinity tags (His, SBP, and c-Myc) or with an anti-GFP antibody (Fig. 2c). When using an anti-His antibody, choose one that can recognize internal His tags. Some His antibodies cannot, or only weakly, interact with the internal His tag.
13. Doxycycline concentration and induction period depends upon the protein of interest.
14. The cell pellet can be stored at −80 °C, after quickly freezing in liquid nitrogen, for future use.

15. Addition of ATP to the lysis buffer is optional for dynein, which is dissociated from microtubules by MgATP. To avoid protein degradation, use a protease inhibitor cocktail suitable for the cell types (bacterial, animal, or plant cells) employed.
16. The original method for SBP tag purification used a streptavidin column [8, 9]. However, it is difficult to regenerate the streptavidin column because of the strong binding of desthiobiotin. We found that the SBP tag binds to Strep-Tactin, a streptavidin variant engineered for the Strep-Tag II system [14], and is easily released by desthiobiotin [10]. Use of Strep-Tactin instead of streptavidin allows for reuse of the column.
17. Some particulates are present in the supernatant that could clog the column even after ultracentrifugation. It is therefore important to filter the sample just prior to loading. This effectively removes such particulate matter and is essential for reuse of the column.
18. The flow rate for sample loading somehow depends upon the protein of interest. Check the fusion proteins in the flow-through fraction and determine the optimum rate.
19. Washing the column with a buffer containing a high salt concentration, such as 0.5 M NaCl, may further decrease the presence of contaminants. However, it might also remove proteins that are weakly associated with the mfGFP fusion protein.
20. Desthiobiotin is weakly soluble at higher concentrations in water. We therefore directly dissolve desthiobiotin in the elution buffer.
21. Regeneration of the column can also be performed by washing with more than 25 ml of 1 mM 4-hydroxyazobenzene-2-carboxylic acid (HABA). This displaces desthiobiotin at the biotin binding site (please see the GE Healthcare StrepTrap Web site). The color of the resin should turn from yellow to red. HABA is also useful in confirming regeneration of the column.
22. Low concentrations of imidazole (10–30 mM) reduce nonspecific binding to the Ni-NTA resin without affecting specific binding of His-tagged proteins. Homo-multimeric proteins are more resistant to higher concentrations of imidazole (e.g., 100 mM), probably because of the presence of multiple His tags. Verify the imidazole concentration needed to elute the protein of interest.
23. IMAC eluates can be directly loaded onto the Strep-Tactin column. Imidazole does not affect SBP binding to the Strep-Tactin.

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# Chapter 13

## Targeted Purification of SnAvi-Tagged Proteins

**Ursula Schäffer, Ralf Baumeister, and Ekkehard Schulze**

### Abstract

Tandem affinity purification (TAP) is a powerful technique to identify protein complex members. The modular composition of TAP-tags allows two sequential protein enrichment steps and thereby drastically reduces the amount of contaminants. Here, we describe the application of the SnAvi-tag—a TAP-tag useful in different expression systems. Due to its modular composition, this tag is multifunctional and facilitates among others the in vivo visualization of tagged proteins and their cell type specific activation.

**Key words** Protein complex purification, Protein tag, Tandem affinity purification (TAP), Tissue specificity, Targeted in vivo biotinylation, Tag activation, In vivo visualization, Mass spectrometry, Protein interaction, Protein modification

### 1 Introduction

The application of systematic TAP-tag approaches had an enormous impact on our understanding of protein complexes and their function within cells. The primal TAP-tag strategy was developed and applied in yeast [1] and allowed the description of a tremendous amount of protein complexes and their members [2]; however, in several other expression systems, this tag was hardly functional or even failed [3, 4]. Therefore, a variety of other TAP-tags were developed to overcome these limitations (e.g., ref. 5–7).

Here we describe the usage of the synaptobrevin Avi purification-tag (SnAvi-tag)—our version of a TAP-tag [8]. Similar to other TAP-tags, the SnAvi-tag is composed of several genetically encoded modules (Fig. 1) which enable its application in different experimental contexts. The most important of those modules is the AviTag [9, 10], a short peptide sequence which is specifically and exclusively recognized by the *E. coli* biotin holoenzyme synthetase BirA. Only when BirA is expressed in the same cells as the SnAvi-tagged protein, the tag structure is biotinylated and thus activated for protein purification using (strept-)avidin coupled affinity matrixes. This feature is especially interesting for the application in

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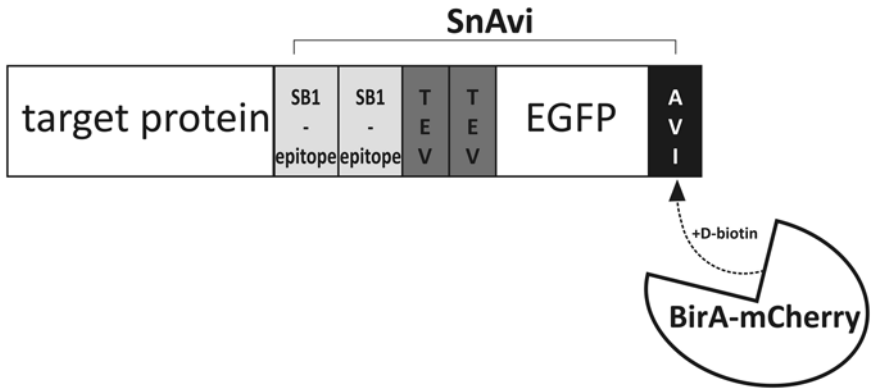


**Fig. 1** Schematic view of a SnAvi-tagged target protein. The target protein is fused to the SnAvi-tag that is composed of two epitopes for the SB1 antibody, two recognition motifs for the TEV protease, the coding sequence for a variant of the green fluorescent protein (EGFP), and the AviTag. In eukaryotic cells the AviTag is specifically and exclusively recognized and biotinylated by the *E. coli* biotin holoenzyme synthetase BirA that we express as fusion to the mCherry fluorophore

multicellular organisms, since it enables the expression of an endogenous gene under the regulation of its own promoter and the purification of the tagged-protein from a subset of cells which is defined by a selective co-expression of BirA. An additional advantage of the application of the AviTag for the first protein enrichment step in the sequential purification procedure is determined by the femtomolar dissociation constant of (strept-)avidin to biotin [11] which enables the enrichment of low-abundant proteins and stringent washing conditions to remove contaminants.

In order to detect the cellular or subcellular localization of the SnAvi-tagged proteins, the peptide sequence of the green fluorescent protein (GFP, [12]) was added. Two TEV-protease recognition motifs are also included in the tag structure. They are useful to specifically elute tagged proteins from the (strept-)avidin coupled affinity matrix after the first purification step [13], since the GFP-AviTag fusion and contaminants unspecifically bound to it can be removed by protease cleavage. To allow the concentration of the eluted protein complexes, two epitopes for the SB1 peptide antibody were introduced into the SnAvi-tag structure for the second step of the tandem purification strategy. The epitope of this antibody is derived from the synaptobrevin gene 1 (*snb-1*) of *C. elegans* and is detected in synthetic protein fusions in *C. elegans* as well as in cultivated mammalian cell lines with a high specificity. Thus, the SnAvi-tag is applicable for sequential protein purification in a broad variety of eukaryotic cells.

## 2 Materials

Prepare all solutions with ultrapure water (resistance > 18 MΩ) and reagents of analytical grade. Sterilize all solutions either by filtration or autoclaving. If not otherwise specified, store all buffers at room temperature.

### 2.1 Growth, Transfection and Lysis of Cultivated Mammalian Cells

1. Choose cell line for the expression of the SnAvi-tagged protein fusion.
2. Appropriate growth medium for the cell line to be transfected (*see* **Note 1**).
3. PBS: 8 g/l NaCl, 0.2 g/l KCl, 1.78 g/l $Na_2HPO_4 \times H_2O$, 0.24 g/l $KH_2PO_4$, adjust pH to 7.4 (*see* **Note 2**).
4. 1 mg/ml 25 kDa linear polyethylenimine (PEI, *see* **Note 3**); pH 7.2.
5. 1 M D-biotin (Sigma) stock solution in the appropriate cell culture medium.
6. Cell lysis buffer: 50 mM Tris (pH 7.4), 1 % Triton X-100, 137.5 mM NaCl, 1 % glycerol, 1 mM $NaO_4Va$, 0.5 mM EDTA, Protease Inhibitor Cocktail (Roche Applied Sciences).

### 2.2 Work with C. elegans

1. S basal buffer: 5.8 g NaCl, 50 ml 1 M $KH_2PO_4$ (pH 6.0; *see* **Note 4**), 950 ml $H_2O$, 1 ml Cholesterol stock solution (5 mg/ml in 95 % EtOH absolute; *see* **Note 5**).
2. 100× metal solution (500 ml): 0.346 g $FeSO_4 \times 7\ H_2O$, 0.098 g $MnCl_2 \times 4\ H_2O$, 0.144 g $ZnSO_4 \times 7\ H_2O$, 0.012 g $CuSO_4 \times 5\ H_2O$, 0.930 g $Na_2$ EDTA.
3. Nystatin stock solution: dissolve 1 g Nystatin (Sigma) in 50 ml EtOH (p. A.) and 50 ml 7.5 M $NH_4$Acetate.
4. Nematode growth medium (NGM)—plates : 3 g NaCl, 17 g agar, 2.5 g peptone (Bacto), fill up to 950 ml with $H_2O$; after autoclaving add 5 µg/ml Cholesterol, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM $KH_2PO_4$ (pH 6.0), 20 µg Nystatin, fill up to 1 l.
5. Liquid culture medium: supplement 50 ml S basal buffer with 150 µl 1 M $MgSO_4$, 150 µl 1 M $CaCl_2$, 0.5 ml 100× metal solution, 0.5 ml 1 M citrate (pH 6.0; adjust with concentrated KOH), 0.5 ml 100× Penicillin/Streptomycin stock solution (Gibco), 2 ml pelletized HB101 bacteria.
6. M9 buffer: 3 g/l $KH_2PO_4$, 6 g/l $Na_2HPO_4$, 5 g/l NaCl, adjust pH to 6.0 and autoclave; autoclave separately a 1 M MgSO4 stock solution; add 1 ml $MgSO_4$ solution per each l of the autoclaved buffer components.
7. 60 % ice-cold sucrose solution (w/w).

### 2.3 Tandem Affinity Purification

1. IPP150 lysis and washing buffer: 10 mM Tris HCl (pH 8), 150 mM NaCl, 0.1 % NP-40, 3× concentrated protease inhibitor cocktail (Roche Applied Sciences; *see* **Note 6**).
2. TetraLink Avidin Resin (Promega; *see* **Note 7**).
3. TEV cleavage buffer: 0.5 mM EDTA, 1 mM DTT, 150 mM NaCl, 50 mM Tris (pH 8, *see* **Note 8**).
4. AcTEV protease (Invitrogen).
5. Spin Cups—cellulose acetate filter (0.45 μm pore size; Thermo Scientific).
6. Crosslink IP kit (Thermo Scientific).
7. French Press (*see* **Note 9**).
8. Modified Laemmli buffer (6×): 300 mM Tris–HCl (pH 6.8), 12 mM EDTA, 6 % SDS (w/v), 30 % (v/v) glycerol.
9. Gels and buffers for sodium dodecyl sulfate-poly acrylamide gel electrophoresis (SDS-PAGE).

### 2.4 Antibodies and Conjugates

1. High Sensitivity Streptavidin coupled to horseradish peroxidase (HRP; Thermo Scientific) in combination with enhanced chemiluminescence substrates for the detection of biotinylated SnAvi-tagged proteins on western blot membranes (*see* **Note 10**).
2. Purified SB1 antibody recognizing the SNB-1 epitope in the SnAvi-tag (*see* **Note 11**) is used for immunoprecipitation or detection in western blot (*see* **Note 12**).

## 3 Methods

### 3.1 Expression of SnAvi-Tagged Proteins in Cultivated Mammalian Cells

1. Grow HEK293T or HeLa cells in the respective growth medium until they are about 80–90 % confluent.
2. Supplement D-biotin (100 μM final concentration) to the growth medium of the cells to increase the biotinylation level of SnAvi-tagged proteins 1 h before transfection.
3. Use 110 ng of the *birA* (*see* **Note 13**) encoding plasmid pBY2892 per $cm^2$ plate surface and 200–500 ng of the SnAvi-tag encoding plasmid for the co-transfection of adherently growing cells (*see* **Note 14**). Incubate in 200 μl medium w/o serum or antibiotics for 5 min. In parallel, incubate an appropriate PEI volume (ratio PEI volume to DNA mass: 3 μl PEI per μg plasmid DNA) in 200 μl medium w/o serum or antibiotics for 5 min.
4. Mix the DNA solution with the PEI solution. Incubate for another 20 min.
5. Pipet the DNA/PEI mixture dropwise to your cells.
6. Optionally: Exchange the growth medium of the cells 3 h after the DNA/PEI mixture was added.
7. Incubate cells for 24–48 h at 37 °C.

### 3.2 Preparation of Cell Lysates from SnAvi-Transfected Cells

1. Aspirate growth medium from the cells.
2. Carefully wash the cells once with ice-cold PBS.
3. Aspirate the PBS.
4. Add 16.5 µl of ice-cold cell lysis buffer per $cm^2$ growth surface.
5. Incubate on ice for 15–30 min.
6. Scrape cells from the cell culture dish. Transfer them into a polypropylene (PP) tube.
7. Clear the lysates by centrifugation (about 15,000 rcf, 4 °C, 15 min).
8. Transfer the supernatant to a fresh tube.
9. Proceed with "Tandem affinity purification of SnAvi-tagged proteins"

### 3.3 Expression of SnAvi-Tagged Proteins in C. elegans

1. Transform *C. elegans* either by microinjection transformation [14] or by biolistic transformation [15, 16], *see* **Note 15**) with the desired fusion of a gene and the SnAvi-tag coding sequence (*see* **Note 16**). Moreover, strains expressing the *birA* coding sequence must also be generated (*see* **Note 18**). When microinjection transformation is applied, the DNA for both can be co-transformed. If strains are made which carry only one of the two required transgenes, both traits have to be combined by genetic crossing in an additional step (*see* **Note 17**) before the SnAvi-Tag can be used.
2. Add 50 ml of liquid culture medium to a 250 ml Erlenmeyer flask (*see* **Note 18**).
3. Add the transgenic worms grown on ten NGM plates (10 cm diameter) seeded with the *E. coli* strain OP50 (wash animals from plates; collect the worms by centrifugation with 200 rcf, 4 °C, 1 min).
4. Incubate the Erlenmeyer flask in a shaking incubator (20 °C, 130 rpm) for several days until you have enough worm mass (usually 4–8 day).
5. Check every day if animals are starving. If dauer larvae are observed feed the animals with more HB101 bacteria (add 1–2 ml of bacterial pellet).
6. Pelletize the animals (200 rcf, 4 °C, 1 min).
7. Wash the worm pellet with ice-cold M9 buffer.
8. To separate living animals from dirt and dead worms transfer them to 50 ml PP-tubes.
9. Add 20 ml ice-cold M9 buffer.
10. Add 20 ml ice-cold 60 % sucrose solution w/w (40 g sucrose per 60 g water).
11. Mix shortly. Proceed instantly to the next step.

12. The sucrose floating of vital worms is established during centrifugation (860 rcf, 5 min, 4 °C). Carefully aspirate the floating worms in the upper phase with the help of a Pasteur pipette and transfer them to a fresh 50 ml PP tube.
13. Fill with ice-cold M9 buffer up to 50 ml.
14. Pelletize the worms.
15. Repeat the **steps 13** and **14** twice.
16. Aspirate the supernatant.
17. Either prepare worm lysates immediately or store the pellets at –80 °C.

### 3.4 Lysate Preparation from C. elegans

1. Combine 0.5 ml of worm pellet with 4.5 ml of IPP150 buffer.
2. Press the animals through a precooled French pressure cell (*see* **Note 3**) that is appropriate for the required volume. Apply 8,000 psi pressure.
3. Repeat this step twice.
4. Proceed with "Tandem affinity purification of SnAvi-tagged proteins".

### 3.5 Tandem Affinity Purification of SnAvi-Tagged Proteins

All steps are carried out at 4 °C if not stated otherwise. All centrifugation steps with TetraLink material are performed with 100 rcf, 4 °C, 1 min. All centrifugation steps with the SB1-coupled Protein A/G material are conducted with 2,000 rcf, 4 °C, 1 min. The cross-link of the SB1 antibody has to be accomplished with the "Crosslink IP kit" (Thermo Scientific) in advance according to the supplier's protocol. Protein A/G affinity matrix carrying the cross-linked antibody can be stored for several days.

1. Transfer TetraLink beads to a fresh PP-tube. Allow to settle. Use 80 µl affinity matrix per ml lysate (*see* **Notes 19** and **20**).
2. Wash TetraLink beads twice with IPP50 buffer (*C. elegans* lysates) or cell lysis buffer (lysates from mammalian cultivated cells).
3. Aspirate most of the washing buffer but leave approximately the same volume of buffer as the volume of affinity matrix.
4. Add your lysate.
5. Incubate on an "end over end rotator" at 4 °C for 30 min.
6. Wash 5× with IPP50 buffer (*C. elegans* lysates) or cell lysis buffer (lysates from mammalian cultivated cells).
7. Wash twice with TEV cleavage buffer.
8. After the last washing step, aspirate most of the buffer without drying the beads.

9. Add 0.5 U/µl AcTEV protease to a volume of TEV-cleavage buffer that is 3× higher than the volume of the affinity matrix material.
10. Incubate at 16 °C for 2 h (*see* **Note 21**).
11. Transfer the supernatant and the beads to spin cup—cellulose acetate filters.
12. Separate the supernatant from the affinity matrix material by centrifugation (7,000 rcf, 4 °C, 2 min, *see* **Note 22**).
13. Add settled Protein A/G affinity matrix which was cross-linked with the SB1 antibody before. Use 5 µl affinity matrix per ml lysate (*see* **Note 23**).
14. Incubate on an "end over end rotator" at 4 °C over-night.
15. Wash 3× with 500 µl TEV cleavage buffer.
16. Aspirate the TEV cleavage buffer.
17. Add sample buffer (e.g., per 10 µl affinity matrix add 15 µl 2× concentrated modified Laemmli buffer).
18. Elute the bound protein and its complex partners by incubation at 95 °C for 3–5 min.
19. Separate proteins by SDS-PAGE (*see* **Notes 24–26**).

## 4 Notes

1. We normally work with HEK293T or HeLa cells. Both are grown in MEM medium (PAA Laboratories) supplemented with 10 % fetal bovine serum (PAA), 50 U/ml Penicillin, 50 µg/ml Streptomycin (100 × stock, Gibco). The cells are cultivated with 5 % $CO_2$ at 37 °C.
2. We normally prepare a 10× PBS stock solution.
3. Heat the aqueous solution at 80 °C until the PEI (Polysciences Inc.) is completely dissolved.
4. Dissolve 136.1 g $KH_2PO_4$ in 800 ml $H_2O$; use concentrated KOH to adjust pH to 6.0; fill up to 1,000 ml.
5. Do not autoclave the cholesterol stock solution!
6. Since during the lysis of *C. elegans* a huge number of intestinal proteases are released, we always use a 3 times higher concentration of protease inhibitors as recommended by the company.
7. We tested several similar products from other companies. In our hands this affinity matrix had the best price/efficiency relation.
8. The TEV-protease is not sensitive for the Roche protease inhibitor cocktail when it is applied 1× concentrated. Therefore, this protease inhibitor cocktail can optionally be added.

9. Instead of using the French press, worm lysates can also be prepared by grinding the animals in liquid nitrogen or by sonication according to standard protocols. In the former case, the protein powder is transferred to polypropylene tubes before the IPP150 lysis buffer is added. In the latter case, the IPP150 lysis buffer is directly added before sonication. In both cases clear the lysates by centrifugation and use the supernatant for the following steps (16,100 rcf, 4 °C, 15 min).
10. Alternatively, we use Alexa680 coupled streptavidin (Invitrogen) for the detection of biotinylated SnAvi-tagged proteins with the Odyssey Scanner (LI-COR). This strategy has the advantage that a second detection can be done in parallel. We used this strategy for example to improve the efficiency of TEV-protease cleavage using the SB1 antibody for the detection of the SnAvi-tag after cleavage and Alexa 680 streptavidin for the detection of the unprocessed form.
11. The SB1 antibody developed by Michael Nonet and Gayla Hadwiger was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. It is publicly available. The peptide recognized by the antibody has the sequence PRPSNKRLQQ [8] and is specifically recognized in folded as well as denaturated proteins [8, 17]. We produced the antibody in CD hybridoma medium (Gibco) or enriched the antibody from serum supplemented DMEM medium with HPLC using HiTrap™ Protein G HP Columns (GE Healthcare). In the latter case, 0.5 M $Na_3$Citrate (pH 8.2) was used for washing, and 0.1 M $Na_3$Citrate (pH 3) was used for elution. 50 µl 1 M Tris (pH 9.5) per ml eluate were added for neutralization. A concentration of 4.6 µg/ml SB1 antibody was used for detection on western blot.
12. Alternatively to the SB1 antibody, commercially available α-GFP antibodies are useful to detect SnAvi-tagged proteins.
13. Alternatively, to the in vivo biotinylation of SnAvi-tagged proteins in *C. elegans* or in mammalian cell culture, SnAvi-tagged proteins can also be biotinylated in *E. coli* by the endogenous BirA protein, e.g., in the BL21 *E. coli* strain. However, we failed so far to express any protein in the AVB100 strain in which recombinant *birA* expression is induced in addition to the endogenous *birA*. Moreover, in vitro biotinylation of the SnAvi-tag is possible using the BirA enzyme from Avidity (Aurora, CO, USA).
14. Dependent on the gene-of-interest, different concentrations of plasmids encoding SnAvi-tagged proteins should be tested to identify the best expression conditions.

15. We prefer biolistically transformed rescued DP38 (*unc-119*(*ed3*)) lines, since stably integrated transgenes can be obtained with this method [15, 16].
16. SnAvi encoding vectors are available from Addgene (www.addgene.org). They are compatible either for expression in *C. elegans* (pBY2946), for expression exclusively in mammalian cells (pBY2727), or for expression in bacteria, insect and mammalian cells (pBY2807, pBY2887). To activate SnAvi-tagged proteins by biotinylation, the *E. coli* biotin holoenzyme synthetase gene *birA* has to be co-expressed in the same cells. The cDNA of this gene is available from Addgene as a fusion to the mCherry cDNA sequence (pBY2982). This vector can also be adapted for *birA*-mCherry expression in *C. elegans* by inserting *C. elegans* specific promoters 5′ to the start ATG of the *birA*-mCherry coding sequence.
17. For mass spectrometry analysis, a strain that carries only a SnAvi-tagged transgene but does not express BirA could be used as negative control in SILAC experiments [18].
18. We tried to grow high numbers of worms expressing SnAvi-tagged proteins on egg plates. However, even though the tandem-affinity purification should bias for SnAvi-tagged proteins and their complex partners, chicken avidin was still co-purified, reducing the sensitivity of mass spectrometry (MS) runs. We suggest therefore to exclusively use animals grown in liquid culture for the enrichment of SnAvi-tagged proteins followed by MS measurements. If the enriched proteins are analyzed with different methods, it might be useful to grow the worms on egg plates, since they are easier to handle than liquid worm culture. Composition of egg plates: prepare NGM plates (10 cm diameter, 30 ml volume) with agarose instead of agar; use one sterilized chicken egg per six plates (*see* **Note 25**), fill up with LB to 40 ml total volume, incubate at 60 °C for 1 h to inactivate the lysozyme in the egg, cool down to RT, add 5 ml bacterial suspension (best use the *E. coli* strain OP50), equally distribute to the plates, and allow to settle over-night. The next morning carefully aspirate the supernatant from the plates. Plates can be stored for several weeks at 4 °C.
19. You should be able to wash the affinity matrix in a washing buffer volume that is at least 30× more than the volume of your affinity matrix. The size of the PP-tube should be chosen accordingly. We normally use 15 ml PP-tubes for 0.4 ml settled affinity matrix and 5 ml lysate.
20. Although the SnAvi-Tag benefits from the femtomolar dissociation constant of its first affinity stage, one has to consider that endogenously biotinylated proteins which are produced in all species will occupy binding capacity on the streptavidin affinity matrix.

21. Dependent on the protein fused to the SnAvi-tag, other conditions (time, temperature, pH, varying AcTEV concentrations, etc.) for the AcTEV protease digestion can be tested.
22. In rare cases, the affinity matrix clogs the filter. In this case resuspend the material again in the residual buffer and centrifuge again or transfer the buffer/affinity matrix mixture to a fresh filter.
23. We also used BrCN Sepharose for cross-linking the antibody to the affinity matrix. This increased the number of detected peptides in mass spectrometry but also the number of contaminants. Nevertheless it could be considered to covalently bind the SB1 antibody.
24. After Coomassie Blue staining, we either cut specific bands from the SDS-PAGE for mass spectrometry or we ran the gel only shortly and cut all eluted proteins within a single band from the SDS-PAGE.
25. For trouble shooting in general and especially for the establishment of new SnAvi-Tag purification projects, it is very helpful to monitor the performance of the individual steps on a western blot. The amount of tagged BirA-biotinylated protein can be compared to the natively biotinylated proteins by probing a cell- or worm lysate with labeled streptavidin. After the first affinity purification, the amount of remaining tagged protein can be analyzed in the waste supernatant, and the loading of streptavidin beads can be tested with an anti-GFP-antibody. After TEV-cleavage the amount of remaining non-cleaved tagged protein should be tested by probing the eluate and the waste streptavidin matrix with the SB1 antibody. This is important for the optimization of TEV-protease cleavage conditions. For the second affinity step, the remaining protein can be tested in the waste-supernatant of the SB1-antibody pull-down.
26. Several SnAvi-tagged proteins degradation bands were detectable on western blots. However, in the first purification step using the Avidin coupled affinity matrix the full length protein is enriched preferentially.
27. Sterilize the shell of the chicken eggs in an aqueous NaOCl bath (approximately 0.12 %). Afterwards, wash twice in $H_2O$ for 10 min.

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# Chapter 14

# An Efficient Fluorescent Protein-Based Multifunctional Affinity Purification Approach in Mammalian Cells

**Hanhui Ma, Janel R. McLean, Kathleen L. Gould, and Dannel McCollum**

## Abstract

Knowledge of an individual protein's modifications, binding partners, and localization is essential for understanding complex biological networks. We recently described a fluorescent protein-based (mVenus) *m*ultifunctional *a*ffinity *p*urification (MAP) tag that can be used both to purify a given protein and determine its localization (Ma et al., Mol Cell Proteomics 11:501–511, 2012). MAP purified protein complexes can be further analyzed to identify binding partners and posttranslational modifications by LC-MS/MS. The MAP approach offers rapid FACS-selection of stable clonal cell lines based on the expression level/fluorescence of the MAP-protein fusion. The MAP tag is highly efficient and shows little variability between proteins. Here we describe the general MAP purification method in detail, and show how it can be applied to a specific protein using the human Cdc14B phosphatase as an example.

**Key words** Multifunctional affinity purification (MAP), TAP, Fluorescent protein Venus, FACS, Protein complex, MudPIT, LC-MS/MS, Proteomics

## 1 Introduction

Protein modifications, interactions, and dynamics play important roles in spatiotemporal regulation of cellular signaling. Therefore, identification of posttranslational modifications and protein interaction networks has become an integral part of modern biology. Various approaches have been developed for dissection of protein interactions. Among them, the tandem affinity purification (TAP) method, originally developed in yeast [1], has been used to successfully isolate native protein complexes with relatively high purity. The TAP tag has been used in mammalian cells; however, the overall recovery of native complexes is typically low [2]. Several modified TAP methods have improved recovery of native protein complexes by using optimal

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combinations of affinity tags [2–5]. Although these TAP systems have been quite useful, establishment of clonal cell lines expressing the fused protein is time-consuming and laborious, because individual clones must be selected, expanded, and then screened for fusion protein expression by western blotting. For this reason, affinity purification tags incorporating GFP were developed to allow FACS of individual cells expressing various levels of the fusion protein [6, 7]. However, we found that the efficiency of these approaches varied greatly between proteins (Ma and McCollum, unpublished observations).

To improve purification efficiency, but maintain FACS capability, we made an mVenus fluorescent protein-based multifunctional affinity purification (MAP) tag that allows fluorescence imaging of protein localization, easy selection of cell lines, and efficient affinity purification under either native or denaturing conditions [8]. Briefly, the MAP tag was created by insertion of multiple affinity tags (8×His, streptavidin binding peptide (SBP), and the FLAG epitope) into the surface loop between β8 and β9 of the mVenus fluorescent protein (Fig. 1). This modified version of mVenus both retains fluorescence and yields efficient protein recovery by two-step affinity purification. A schematic of the mVenus-MAP tag and some potential uses are shown in Fig. 1. We used the MAP tag to purify numerous proteins and showed that these preparations can be used to identify binding partners, and posttranslational modifications [8]. In addition, the MAP tag has been successfully applied to Chromatin IP (Personal communication from Lucy Chao and Kirsten Hagstrom) and RNA IP [9]. Although purification of proteins under native conditions is essential for identification of binding partners, it is not ideal for identification of posttranslational modification of proteins such as ubiquitination, which may be removed by ubiquitin proteases over the course of purification. To combat this, several modified TAP methods have been developed for purification of tagged proteins under denaturing conditions, where enzymes that might remove modifications have been inactivated [12, 13]. We showed that the MAP approach can be used to efficiently purify proteins under denaturing conditions [8]. In the following sections, the Cdc14B phosphatase will be used to illustrate the use of the MAP approach, including cloning and expression of the MAP-tagged protein, stable cell line generation, protein localization, protein purification, and identification of protein interactors and modifications using LC-MS/MS. Although here we focus on using the MAP tag with mammalian cell lines, the basic MAP methodology has been used successfully in genetically modified living organisms such as yeast, *C. elegans*, and *D. melanogaster* [8] and should presumably work in other systems.

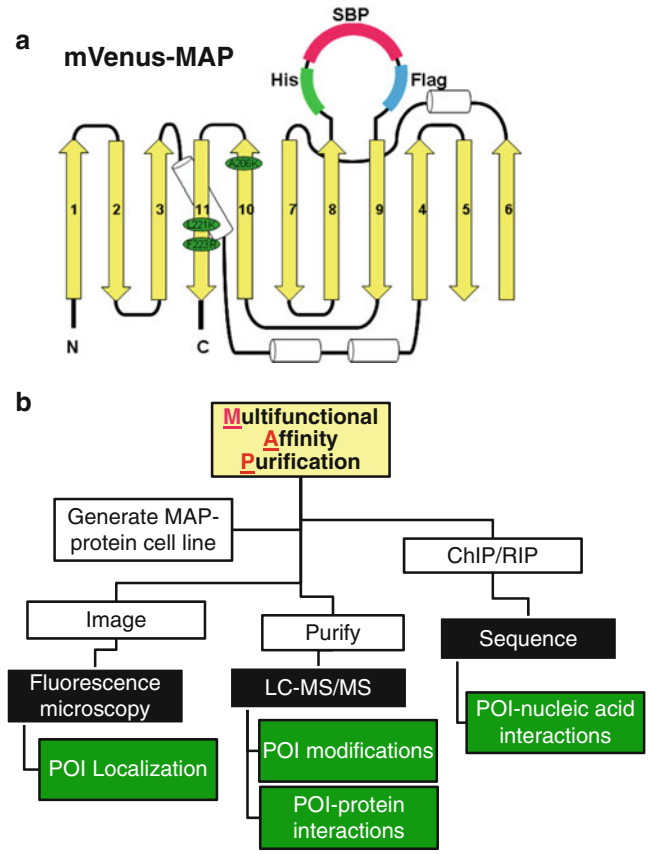



**Fig. 1** Overview of mVenus-based multifunctional affinity purification (mVenus-MAP). (**a**) Structure of mVenus-MAP. The A206K, L221K, and F223R mutations were made to reduce Venus dimerization [10, 11]. The MAP tag was inserted into the surface loop between β8 and β9 of Venus. (**b**) The MAP tag can be used for a variety of purposes including protein localization; MAP-LC-MS for identification of interacting proteins and posttranslational modifications; and MAP-ChIP/RIP-Seq for analysis of protein–DNA/RNA interactions. *LC-MS/MS* liquid chromatography tandem mass spectrometry, *ChIP* Chromatin immunoprecipitation, *RIP* RNA immunoprecipitation, *Seq* sequencing

## 2 Materials

### *2.1 Cloning Protein of Interest (POI) into pMAP*

1. Vector pMAP (Fig. 2) (*see* **Note 1**).
2. Herculase II Fusion DNA Polymerases (Stratagene).
3. Gene specific primers with restriction enzyme sites.
4. QIAquick gel extraction kit (Qiagen) or other similar product.
5. Restriction enzymes and T4 DNA ligase.
6. XL1-Blue or DH5α rubidium chloride competent cells (NEB).
7. LB (Luria Bertani) medium and LB-ampicillin agar plates.
8. QIAprep Spin Miniprep Kit (Qiagen) or other similar product.

**a**

**mVenus-MAP Tag Sequence:**

MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKLICTTGKLPVPWPTLVT
TLGYGLQCFARYPDHMKQHDFFKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIEL
KGIDFKEDGNILGHKLEYNYNSHNVYITADKQKNGIKANFKIRHNIEDGSLESAHHHHHHHHG
SAMDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREPGSADYKDDDDKGSASSGGGVQLA
DHYQQNTPIGDGPVLLPDNHYLSYQSKLSKDPNEKRDHMVLKERVTAAGITLGMDELYK

**b**

AflII/HindIII/KpnI BamHI

AAC TTA AGC TTG GTA CCG AGC TCG GAT CCA CTA GTC CAG TGT GGT GGA ATT CTG CAG

NotI MAP-Venus XbaI

ATA TCC AGC ACA GTG GGC GGC CGC GGC AGC AGC ATG GTG --- TAC AAG TAA TCT AGA

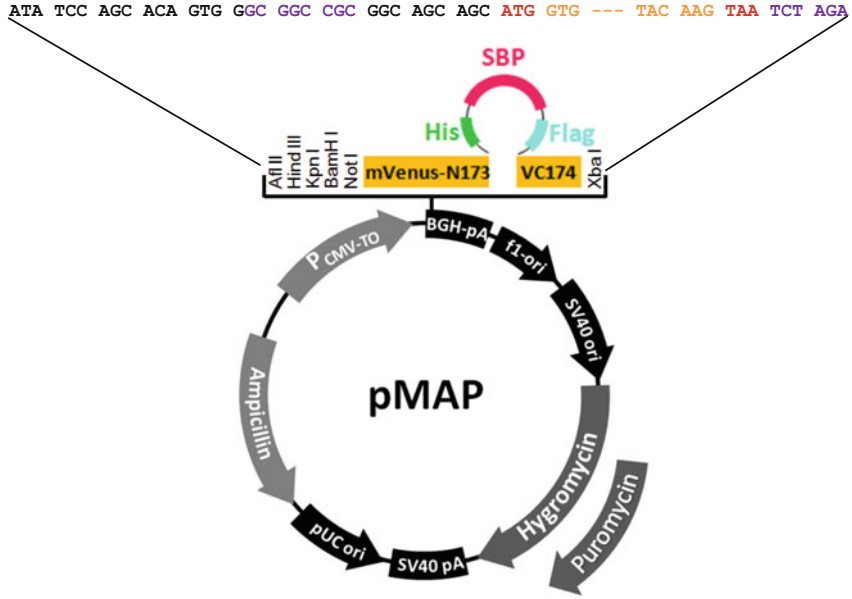



**Fig. 2** MAP tag sequence and expression Vector. (**a**) Sequence of mVenus-MAP tag. mVenus sequences on either side of the tag insertion site (1–173 and 174–239) is shown in *orange*. The 8×His-Tag sequence is shown in *green*. The SBP sequence is shown in *red*. The FLAG tag sequence is shown in *blue*, and linker sequences are shown in *black*. (**b**) pMAP plasmid. MAP and multiple cloning sites. pMAP contains either Hygromycin or Puromycin selection markers

### *2.2 Cell Culture and Transfection*

1. Dulbeco's Modified Eagle's Medium (DMEM).
2. Fetal bovine serum (FBS; heat-inactivated).
3. 0.05 % Trypsin–EDTA (1×) with Phenol Red.
4. Lipofectamine 2000 Transfection Reagent (Invitrogen).
5. Phosphate-buffered saline (PBS).
6. Serological pipettes, 25, 10, 5, and 1 ml.
7. Tissue culture flasks, Vented 25 and 75 $cm^2$.
8. 6-well plate and conical tubes 50 and 10 ml.

### 2.3 Stable Cell Line Generation

1. Hygromycin B at a concentration of 50 mg/ml.
2. 96-well plates.
3. FACS Aria Flow Cytometer (BD Biosciences).
4. Dimethyl sulfoxide (DMSO).
5. Cryo-Vials.
6. Cryo 1 °C freezing container.
7. Liquid nitrogen container.
8. Freezing medium: 20 % FBS, 8 % DMSO, in DMEM.

### 2.4 Cell Line Characterization

1. Lab-Tek 2-well Chamber glass (Nunc).
2. DMEM with 1 mM sodium pyruvate, 1 mM penicillin–streptomycin (Pen–Strep), and 10 % FBS.
3. Mineral oil.
4. Fluorescent Microscope with a YFP filter set.
5. Protease Inhibitor cocktail tablets, EDTA-free.
6. RIPA Buffer: 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 % NP-40, 0.5 % sodium deoxycholate, 0.1 % SDS, 5 mM EDTA.
7. BCA protein assay kit.
8. SDS-PAGE and western blotting reagents.
9. Immobilon-P membrane (Millipore) or other compatible product.
10. Monoclonal GFP antibody such as B-2 from Santa Cruz.

### 2.5 MAP Purification Under Native Condition

1. Lysis buffer A: 50 mM Tris–HCl, pH 7.5, 125 mM NaCl, 1 mM EDTA, 0.2 % NP-40, 5 % glycerol.
2. Anti-Flag M2 Affinity Agarose Gel (Sigma).
3. 1×Flag peptide (Sigma).
4. High capacity streptavidin agarose resin or Streptavidin UltraLink resin (Pierce).
5. Wash buffer A: 50 mM Tris–HCl, pH 7.5, 250 mM NaCl, 0.05 % NP-40.
6. Elution buffer A: 25 mM Tris–HCl, pH 7.5, 125 mM NaCl.
7. D-Biotin: 50 mg/ml in DMSO (~200 mM).
8. Trichloroacetic acid solution (TCA; 6.1 N).
9. Acetone.
10. Colloidal Blue Staining Kit (Invitrogen) or other compatible gel staining product.
11. Micro bio-spin chromatography column (Bio-Rad).
12. Amicon Ultracel-10 Centrifugal Filters (Millipore).

### 2.6 MAP Purification Under Denaturing Conditions

1. Cell scraper.
2. Lysis buffer B: 20 mM Tris–HCl, pH 7.5, 125 mM NaCl, 0.2 % NP-40, 8 M Urea.
3. Wash buffer B: 50 mM Tris–HCl, pH 7.5, 250 mM NaCl, 0.05 % NP-40.
4. Ni-NTA Agarose (Qiagen).
5. Imidazole.

## 3 Methods

### 3.1 Cloning of MAP Expression Constructs

We use PCR to add appropriate restriction sites for cloning the protein of interest (POI) into the MAP vector (Fig. 2, *see* **Note 1**). As an example, we describe below how Cdc14B was cloned into the vector pMAP-Hygro.

1. Design gene specific primers for the in-frame insertion of protein of interest (POI); the primer for Cdc14B as follows:
   (a) Forward: AAAA**GGTAC**CATGAAGCGGAAAAGCGAGC.
   (b) Reverse: AAA**GCGGCCGC**CACTACGCAAGACTGTT TTAGTCCTTG.
   Kpn I and Not I sites were used for cloning of Cdc14B (indicated in bold in primers).
2. Perform a PCR reaction (50 µl total volume per reaction):

| | |
|---|---|
| 5× Reaction Buffer | 10 µl |
| 10 mM dNTP | 1.5 µl |
| Herculase II (*see* **Note 2**) | 0.5 µl |
| 20 µM Forward primer | 1.5 µl |
| 20 µM Reverse primer | 1.5 µl |
| 1 ng/µl Template | 2.0 µl |
| $H_2O$ | 33 µl |

   PCR conditions: 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 2 min for 28 cycles.
3. Run the PCR product from above reaction on a 1 % agarose gel and purify the 1.5 kb Cdc14B DNA fragment using QIAquick Gel Extraction Kit, eluting the purified fragment in 50 µl dd$H_2O$.
4. Digest purified PCR product from **step 3** above (50 µl total volume):

| | MAP Vector | Insert |
|---|---|---|
| 10× NEB Buffer 4 | 5 µl | 5 µl |
| 25× BSA (2.5 mg/ml) | 2 µl | 2 µl |
| Kpn I-HF (20 U/µl) | 1 µl | 1 µl |
| Not I-HF (20 U/µl) | 1 µl | 1 µl |
| MAP Vector (250 ng/µl) | 8 µl | 41 µl of PCR products |
| $H_2O$ | 33 µl | – |

Incubate this digestion at 37 °C for 2 h. Then gel-purify the digested MAP vector (*see* **Note 3**) and PCR products, and elute them in 30 µl elution buffer.

5. Perform a ligation reaction with digested product (15 µl total volume):
6. Cdc14B Vector Control (*see* **Note 3**)

| | | |
|---|---|---|
| 10× Ligase Buffer | 1.5 µl | 1.5 µl |
| T4 DNA ligase | 1.0 µl | 1.0 µl |
| $H_2O$ | 8.5 µl | 10.5 µl |
| Vector (25 ng/µl) | 2.0 µl | 2.0 µl |
| Insert (15 ng/µl) | 2.0 µl | – |

7. Transformation ligated vector (with gene insert) into competent cells: Mix 2 µl of ligation reactions with 50 µl RuCl XL1-Blue competent cells and incubate at 42 °C for 2 min. Then directly spread to LB-Amp agar plates for overnight incubation at 36 °C.
8. Inoculate positively transformed cells: Pick 4 LB-Amp resistant colonies/clones and inoculate 3 ml LB-Amp media with each colony separately. Then incubate overnight at 36 °C (*see* **Note 4**).
9. Isolate amplified vector by DNA miniprep: Use QIAprep Spin Miniprep Kit as directed by the manufacturer. This process typically yields 10 µg plasmid DNA from 1 ml media.
10. Restriction digestion of newly acquired plasmid DNA (20 µl total volume):

| | |
|---|---|
| 10× NEB Buffer 4 | 2 µl |
| 25× BSA (2.5 mg/ml) | 2 µl |
| Kpn I-HF (20 U/µl) | 0.3 µl |
| Not I-HF (20 U/µl) | 0.3 µl |
| $H_2O$ | 11.4 µl |
| DNA (250 ng/µl) | 4 µl |

**Cell line generation flowchart:**

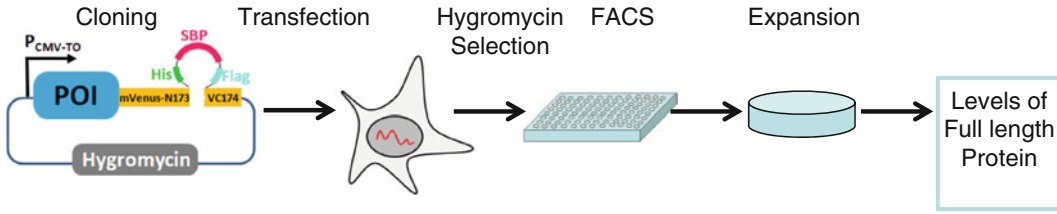



**Fig. 3** Schematic overview of cell line generation for MAP purification. The protein of interest (POI) is first cloned into the into pMAP expression vector. After transfection into host cells, cell lines are selected using hygromycin treatment for 2 weeks. Single cells are sorted into 96-well plates by FACS. Stable cell lines will be characterized for expression levels of full length protein by western blotting

Incubate reaction at 37 °C for 30 min. Then run digest on a 1 % agarose gel to verify presence of the insertion.

11. Sequence the digest by whatever means available to make sure no mutations were introduced during PCR.

### *3.2 Generation of Stable Cell Lines for MAP-Tagged Protein Expression*

The overview of cell line generation is shown in Fig. 3, including transfection, screening using Hygromycin or Puromycin, cell sorting into 96-well plates, and characterizing clonal cell lines for recombinant protein expression and function. The protocol is shown below for U2OS cells but could be adapted for other cell lines.

1. Day 1: Seed U2OS cells into 6-well plates ($2 \times 10^5$ cells/well).
2. Day 2: Transfection.
   (a) Tube 1: Mix 250 ng DNA (*see* **Note 5**) into 100 µl Opti-MEM and incubate at room temperature (RT) for 5 min.
   (b) Tube 2: Mix 2.5 µl Lipofectamine 2000 into 100 µl Opti-MEM and incubate at RT for 5 min.
   (c) Gently mix tube 1 and tube 2 and incubate at RT for 20 min.
   (d) Add DNA–Lipofectamine mixture to cells without changing the media for 16 hr (*see* **Note 6**).
3. Day 3-10: Selection:
   (a) Day 3: Passage 1 well of cells to a 75 $cm^2$ flask with DMEM containing 10 % FBS and 300 µg/ml Hygromycin.
   (b) Days 4–6: Replace with fresh media (DMEM containing 10 % FBS and 300 µg/ml Hygromycin) daily (*see* **Note 7**).
   (c) Day 11: Replace with fresh media (DMEM containing 10 % FBS and 300 µg/ml Hygromycin).

4. Day 14 FACS selection:
   (a) Prepare two of 96-well plates with fresh media (DMEM containing 10 % FBS and 300 μg/ml Hygromycin) and sort single cells (dispersed into a single cell suspension using Trypsin-EDTA) with various brightness levels of mVenus into single wells. Continue to culture for another 2 weeks. The proper localization and expression level of MAP-tagged protein can be examined by fluorescence microscopy (*see* **Note 8**).
   (b) Alternatively the remainder of the mVenus expressing cells can be sorted into one tube and passaged to 25 $cm^2$ flask for rapid expansion. Although these cells are not clonally derived, this approach allows large numbers of POI-MAP expressing cells to be isolated more quickly.
   (c) Choose six clones with the various levels of expression to split into 6-well plates and maintain them in DMEM containing 10 % FBS and 150 μg/ml Hygromycin.
5. Protein localization assessment.
   (a) Day 1: Seed U2OS cells stably expressing Cdc14B-MAP on Lab-Tek 2-well chambered coverglass (5 × 104 cells/well).
   (b) Day 2: For live cell imaging (Fig. 5a), keep the microscope and chamber at 37 °C while viewing. Change the media and add an equal volume of mineral oil on top to avoid evaporation and help maintain the pH. To assess MAP-tagged protein expression and localization, use a capable fluorescent microscope with imaging software (i.e., a Leica DM-IRB inverted microscope equipped with YFP filter sets and MetaMorph acquisition software (Molecular Devices)).
6. Expression levels of full length protein can be measured by the following procedure:
   (a) Lyse U2OS cells stably expressing Cdc14B-MAP on ice in RIPA buffer containing protease inhibitor cocktail. Determine the protein concentration of the lysate via BCA assay.
   (b) Separate protein lysate by SDS-PAGE and transfer to Immobilon-P membrane for Western blot analysis using an anti-GFP antibody at a 1:1,000 dilution. Expression of the full-length, MAP-tagged protein, in this case Cdc14B-MAP, is assessed by identifying a band at the proper molecular weight. In this case, a band at 92 kDa indicates that the stable cell line(s) express the full length Cdc14B fusion protein (*see* **Note 9**).
7. Choose one or two clones for expansion and preservation. Resuspend cells in freezing medium, place in the Cryo 1 °C freezing container and freeze at −80 °C for 24 h. Then transfer frozen cells to a liquid nitrogen tank for long term storage.

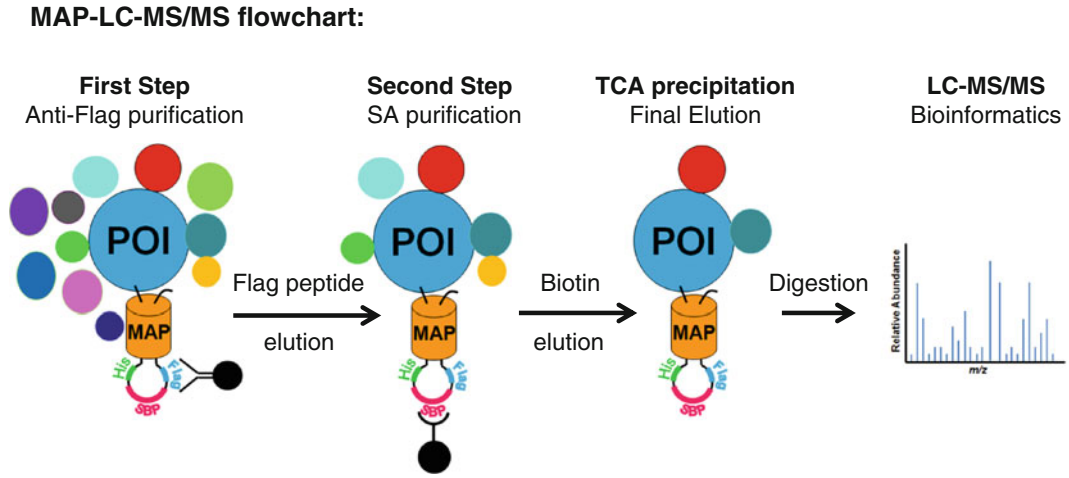



**Fig. 4** Schematic overview of the MAP purification procedure. Cells expressing the mVenus-MAP-tagged POI are lysed and the fusion protein is first purified from total protein extracts using anti-Flag (M2) agarose. After several wash steps, purified proteins are eluted using Flag peptide then purified using streptavidin agarose. Finally, protein complexes are eluted from streptavidin beads with Biotin and precipitated by 20 % TCA for mass spectrometric analysis

### *3.3 MAP Purification Under Native Conditions*

A flowchart of MAP purification is shown in Fig. 4. Briefly, tagged proteins are first purified from cell lysate by anti-Flag immunoprecipitation (IP), followed by streptavidin IP, and finally eluted and TCA-precipitated before being subjected to LC-MS/MS (Fig. 5b).

1. Day 1: U2OS cells stably expressing Cdc14B-MAP are seeded at $2.5 \times 10^6$ cells per 150 mm dish, using a total of ten dishes (*see* **Note 10**).
2. Day 3: Cells are trypsinized and pelleted by centrifugation at $200 \times g$ for 5 min and washed with PBS (gently resuspend cells in PBS).
3. MAP purification: All subsequent steps are performed at 4 °C with precooled buffer and equipment.
   (a) Measure packed cell volume (PCV). Add 5× PCV of lysis buffer A (*see* **Note 11**) containing the protease inhibitor cocktail and phosphatase inhibitor cocktail (*see* **Note 12**). Agitate for 20 min.
   (b) Centrifuge the extract for 10 min at $16{,}000 \times g$ and apply atop anti-FLAG M2-beads (50 µl of beads for every 1 ml of supernatant). Incubate for 3 h (*see* **Note 13**).
   (c) Wash beads with 40 bead volumes of wash buffer in an Eppendorf tube or Micro bio-spin column (*see* **Note 14**) and spin for 15 s at $100 \times g$ and discard the supernatant before proceeding with the elution. Elute the bound

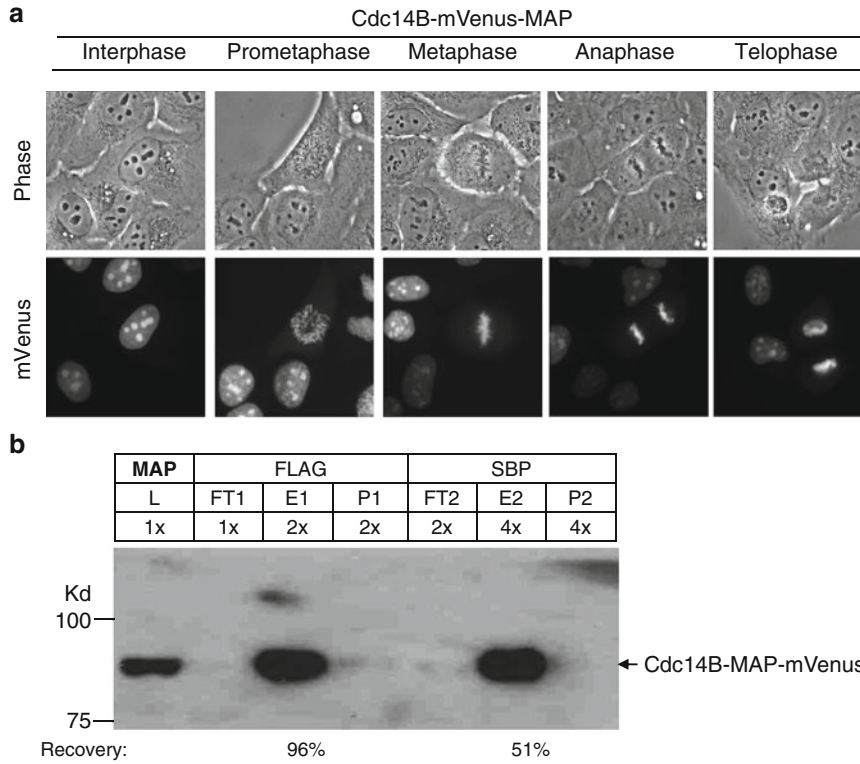



**Fig. 5** Cdc14B-mVenus-MAP localization and purification. (**a**) Cdc14B-MAP localization in cells during different mitotic stages is shown. Cdc14B is in nucleolus and nucleoplasm during interphase, released from nucleoli and redistributed onto condensed chromosomes from prometaphase to anaphase, and then relocated to reformed nucleoli in telophase. (**b**) Western blot analysis of MAP purification yields of Cdc14B(C314S)-mVenus-MAP. U2OS cells stably expressed Cdc14B(C314S)-mVenus-MAP were generated as described in the text and Fig. 3. The purification procedure was monitored by western blot using anti-GFP antibodies. L: Lysate; FT1: flow through after anti-Flag purification; E1: elution from anti-Flag argarose; P1, pellet after Flag peptide elution; FT2: flow through after streptavidin purification; E2: elution from streptavidin agarose; P2, pellet after biotin elution. The *panel* is labeled for the recovery was estimated from measuring band intensity with ImageJ. The concentration of each sample (i.e., 1×, 2×, 4×) relative to the starting lysate is shown

proteins by applying10 volumes of lysis buffer A containing 200 μg/ml Flag peptide and nutating (agitating) for 15 min.

(d) Incubate each 1 ml of eluent with 50 μl of high-capacity streptavidin beads for 3 h. Wash with 40 volumes of wash buffer A and elute the bound proteins with 10 volumes of elution buffer containing 2.5 mM D-Biotin with agitation for 15 min.

(e) Either concentrate the final eluent by Amicon Ultracel-10 Centrifugal Filters (Millipore) for Coomassie blue staining or precipitate purified proteins with 20 % trichloroacetic acid (TCA) (*see* **Note 15**) for eventual identification of interactive partners and modifications by mass spectrometry.

### 3.4 MAP Purification Under Full Denaturing Condition

The details of purification under denaturing conditions have been previously described [8]. Briefly, cells are lysed directly in denaturing lysis buffer and purified using the His-Tag, followed by anti-Flag or SBP purification with streptavidin beads under denaturing conditions.

1. Day 1: Seed U2OS cells stably expressing MAP tagged fusion proteins at $2.5 \times 10^6$ cells per 150 mm dish over ten dishes.
2. Day 3: MAP purification.
   (a) Remove media and directly lyse cells in each dish by applying 0.5 ml lysis buffer B for 10 min.
   (b) Scrape adherent cells from dish (still in lysis buffer B) and move to an Eppendorf tube. Centrifuge for 10 min at $16{,}000 \times g$ to remove cell debris.
   (c) Incubated cell lysate overnight at 4 °C with 50 µl Ni-NTA resin per 1 ml lysate.
   (d) Wash beads with 40 volumes of wash buffer B and elute the bound proteins with 10 volumes of wash buffer B containing 300 mM imidazole plus protease and phosphatase inhibitor cocktails.
   (e) Dilute each 500 µl of eluent to 1 ml and incubate with 50 µl anti-Flag beads or streptavidin beads for 3 h at 4 °C.
   (f) Wash with 40 volumes of wash buffer and elute the bound proteins with 10 volumes of elution buffer B containing 200 µg/ml Flag peptide or 2.5 mM D-Biotin. During elution, agitate sample for 15 min.
   (g) The final elution was precipitated with 20 % TCA (following general TCA ppt procedures) for identification of post-translational modifications by mass spectrometry.

### 3.5 Characterization of Cdc14B Binding Partners by LC-MS/MS

1. TCA pellets, after washing with ice-cold acetone, are resuspended in 20 µl of Tris-buffered 8 M urea, reduced (DTT or other reductant), alkylated (e.g., iodoacetamide), and digested with trypsin overnight. The resulting peptides were then subjected to 2D-LC-MS/MS (MudPIT) analysis on a Thermo LTQ as previously detailed [14]. For a comparison of three directly coupled HPLC MS/MS strategies for identification of proteins from complex mixtures, namely, single-dimension LC-MS/MS, 2-phase MudPIT, and 3-phase MudPIT, please refer to the literatures [14, 15].
2. Convert Thermo RAW files, the output data of the LC-MS/MS runs, to mzML files [16] and search the data with MyriMatch [17] or other database search algorithm against a database containing forward and reverse human proteins (uniref100 2007, 555246 entries; or other relevant protein FASTA database) to identify peptide matches of both the MAP-tagged

bait and co-eluting proteins. Allow for the following modifications: methionine oxidation, cysteine carboxamidomethylation, and serine, threonine, or tyrosine phosphorylation.

3. Filter and assemble peptides into protein with IDPicker [18] [19]. Export spectra counts per protein to Excel and normalized all protein spectra counts to the bait protein—in this case CDC14B (Fig. 6b). Compare the data across samples.
4. Proteins enriched in both biological mitotic (premetaphase) replicates compared to asynchronous cells were imported into Cytoscape v2.8.1 [20] for visualization and grouping (Fig. 6a).

### 3.6 Identification of Cdc14B Phosphorylation Sites by LC-MS/MS

After processing of raw LC-MS/MS data using the bioinformatics tools described above, phosphorylation sites were manually validated according to the following criteria:

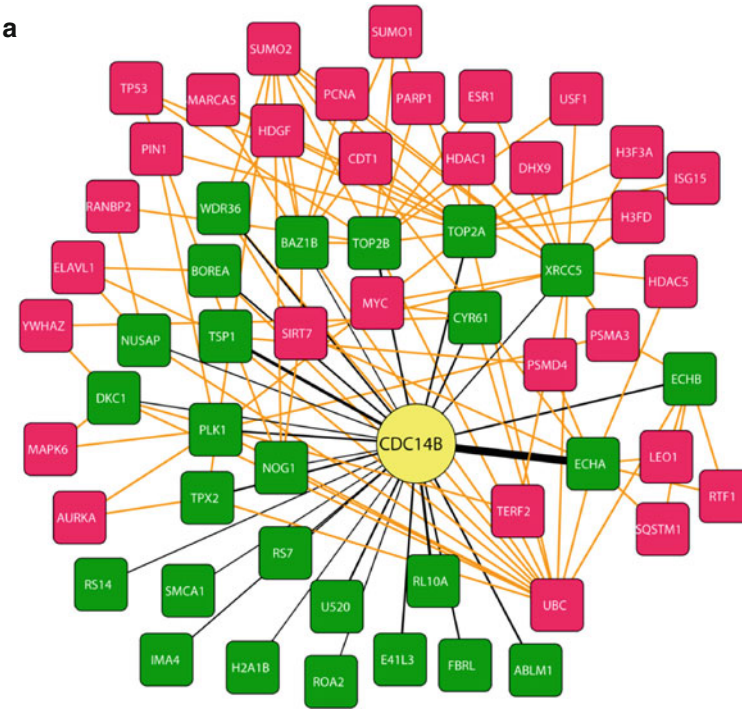
1. Identify peptides with the appropriate mass shift. In the case of phosphorylation a mass addition of +80 Da to peptide is indicative of phosphorylation.
2. Identify $MS^2$ spectra that exhibit a prominent 98 Da ($H_3PO_4$) neutral loss peak.
3. $MS^2$ b and y fragment ion intensities must be greater than 5 signal/noise ratio.
4. $MS^2$ spectra must contain two or more sequential fragments (b and/or y) surrounding the phosphorylation site(s).

Cdc14B phosphosites we identified by this method are listed in Table 1 (*see* **Note 16**).

## 4 Notes

1. The MAP vector contains either the Hygromycin or Puromycin selection marker. Our mVenus-MAP plasmid is designed for fusion of a POI to the N-terminus of the MAP tag, which makes selecting for full length MAP-tagged proteins by fluorescence/FACS much easier. For this reason we suggest cloning POI into the N-terminus of MAP tag unless this orientation is deleterious to the protein's function. However, the mVenus-MAP tag will function when fused to either end of a protein. We typically add linkers (such as GGSGGS) to the C-terminus of our POI to provide extra flexibility and spacing between the POI and the MAP tag.
2. Herculase II DNA Polymerases is especially useful for amplifying difficult and GC-rich targets.
3. Gel purification of MAP vector digested by Kpn I and Not I dramatically decreased the background in the vector only control.

**a**




**b**

| Accession | Gene symbol | Coverage | TSC | Asy | Syn-1 | Syn-2 | Syn/Ayn Ratio | Description |
|---|---|---|---|---|---|---|---|---|
| O60729 | CC14B | 83 | 7353 | 1000.0 | 1000.0 | 1000.0 | **1.0** | Dual specificity protein phosphatase CDC14B |
| Q9Y2J2 | E41L3 | 37 | 280 | 0.5 | 57.0 | 38.8 | **95.7*** | Band 4.1like protein 3 |
| P40939 | ECHA | 36 | 98 | 0.5 | 26.3 | 10.5 | **36.8** | Trifunctional enzyme subunit alpha |
| P11388 | TOP2A | 18 | 119 | 0.5 | 35.1 | 1.6 | **36.7*** | DNA topoisomerase 2alpha |
| P53350 | PLK1 | 25 | 62 | 0.5 | 13.1 | 13.7 | **26.9*** | Serine/threonineprotein kinase PLK1 |
| Q02880 | TOP2B | 6 | 50 | 0.5 | 14.0 | 0.0 | **14.0*** | DNA topoisomerase 2beta |
| P07996 | TSP1 | 20 | 140 | 1.7 | 35.1 | 8.1 | **12.5** | Thrombospondin1 precursor |
| P55084 | ECHB | 27 | 50 | 0.5 | 6.1 | 5.7 | **11.8*** | Trifunctional enzyme subunit beta |
| O75643 | U520 | 14 | 72 | 0.5 | 1.8 | 8.9 | **10.6*** | U5 small nuclear ribonucleoprotein 200 kDa helicase |
| P62906 | RL10A | 53 | 474 | 12.1 | 113.9 | 121.2 | **9.7** | 60S ribosomal protein L10a |
| Q9ULW0 | TPX2 | 28 | 74 | 0.5 | 2.6 | 5.7 | **8.3*** | Targeting protein for Xklp2 |
| P22087 | FBRL | 50 | 210 | 6.9 | 21.9 | 62.2 | **6.1** | rRNA 2'Omethyltransferase fibrillarin |
| Q8NI36 | WDR36 | 23 | 61 | 1.7 | 15.8 | 4.0 | **5.7** | WD repeat protein 36 |
| O00622 | CYR61 | 55 | 86 | 3.5 | 28.9 | 10.5 | **5.7** | Protein CYR61 precursor |
| Q53HL2 | BOREA | 44 | 53 | 1.7 | 9.6 | 8.1 | **5.1** | Borealin |
| O14639 | ABLM1 | 21 | 52 | 0.5 | 0.0 | 4.0 | **4.0*** | Actin-binding LIM protein 1 |
| O00629 | IMA4 | 32 | 67 | 5.2 | 21.9 | 10.5 | **3.1** | Importin alpha4 subunit |
| P62081 | RS7 | 64 | 255 | 17.3 | 35.1 | 69.5 | **3.0** | 40S ribosomal protein S7 |
| P13010 | XRCC5 | 24 | 58 | 5.2 | 18.4 | 12.9 | **3.0** | ATPdependent DNA helicase 2 subunit 2 |
| O60832 | DKC1 | 30 | 83 | 6.9 | 21.0 | 17.0 | **2.7** | H/ACA ribonucleoprotein complex subunit 4 |
| Q9BXS6 | NUSAP | 42 | 96 | 3.5 | 15.8 | 1.6 | **2.5** | Nucleolar and spindle associated protein 1 variant |
| P62263 | RS14 | 43 | 282 | 24.2 | 55.2 | 58.2 | **2.3** | 40S ribosomal protein S14 |
| P22626 | ROA2 | 53 | 287 | 27.7 | 73.6 | 52.5 | **2.3** | Heterogeneous nuclear ribonucleoproteins A2/B1 |
| Q9BZE4 | NOG1 | 40 | 98 | 6.9 | 21.0 | 8.9 | **2.2** | GTP binding protein 4 |
| P28370 | SMCA1 | 18 | 144 | 13.8 | 42.9 | 12.9 | **2.0** | Probable global transcription activator SNF2L1 |
| Q9UIG0 | BAZ1B | 22 | 151 | 13.8 | 42.9 | 12.1 | **2.0** | Bromodomain adjacent to zinc finger domain protein 1B |
| P04908 | H2A1B | 36 | 517 | 41.5 | 96.4 | 67.9 | **2.0** | Histone H2A type 1-B |

**Fig. 6** Mitotic partners of Cdc14B (**a**) Protein interaction map for mitotic Cdc14B partners and selected BioGRID interactions. *Green nodes* represent proteins identified by LC-MS/MS that were consistently enriched in mitotic (premetaphase) Cdc14B(C314S)-MAP purifications by at least twofold (based on normalized spectral counts). *Pink nodes* are physical protein interactors from BioGRID that are shared between at least two Cdc14B interactors (*green nodes*). The edges between the *green nodes* and Cdc14B are *black* and the edge thicknesses indicate the extent of enrichment of that protein in the mitotic Cdc14B purifications. BioGRID interactions are indicated by *orange edges*. This figure was generated in Cytoscape [20]. (**b**) Proteins co-purified with

**Table 1**
**Phosphorylation sites of Cdc14B identified by LC-MS/MS. All sites were manually verified using IDPicker as described in Subheading 3.6**

| | Position | Peptide | Puative Kinases |
|---|---|---|---|
| 1 | S10 | (R)RSpSWAAAPPCSR | Akt, Chk1/2 |
| 2 | S18 | RSSWAAAPPCpSR | |
| 3 | S244 | LESGYHQHpSPETYIQYFK | CDK |
| 4 | S416 | LLSGVDDISINGVENQDQQEPEPpYSDDDEINGVTQGDR | |
| 5 | S417 | LLSGVDDISINGVENQDQQEPEPYpSDDDEINGVTQGDR | Casein Kinase |
| 6 | S440 | RQpSKTNAIPLTVILQSSVQSCK | Aurora Kinase |
| 7 | T442 | RQSKpTNAIPLTVILQSSVQSCK | |
| 8 | S455 | TNAIPLTVILQSpSVQSCK | Polo Kinase |
| 9 | S458 | TNAIPLTVILQSSVQpSCK | |
| 10 | S470 | TSEPNISGpSAGITK | |

4. We picked four clones since there were more than twice as many colonies in the vector plus Cdc14B plate compared to the vector control plate.
5. 250 ng of pMAP-Cdc14B plasmid is good enough to obtain good expression of Cdc14B-MAP in U2OS cells. Normally, less DNA is required for expression of smaller proteins.
6. U2OS cells tolerate Lipofactamine 2000 for incubations overnight; however, some sensitive cells might require replacement of the medium earlier such as 4 or 6 h.
7. Replace medium daily for the first 3 days because Hygromycin selection results massive cell death within 3 days and removal of the dead cells keeps the live cells healthier.
8. It took 10–14 days for single cells to multiply to more than 1,000 cells in 96-well plates. POI-MAP localization can be poorly observed in 96-well plates; however, for better resolution, Lab-Tek coverglass should be used for live imaging of stable cell lines.

**Fig. 6** (continued) Cdc14B(C314S)-MAP that are enriched in mitosis (prometaphase). Accession = Uniprot Accession number, Gene Symbol = representative gene symbol for protein, Coverage = % sequence coverage, TSC = total spectra counts over all purifications, Asy (Asychronous), Syn (Prometaphase) = normalized abundance of protein in each purification (spectral counts of protein/spectral counts for Cdc14B × 1,000), Ratio = average normalized abundance of protein in prometaphase divided by asynchronous abundance, *asterisks* indicate instances where the protein was not identified in the asynchronous purifications and an arbitrary value of 0.5 normalized abundance was assigned

9. The actual sizes are sometime slightly different from the calculated sizes, so it will be important to examine whether there are nonspecific bands on the blot or use an antibody against the POI, which allows comparison of the gel mobility and levels of the POI-MAP fusion to those of the endogenous POI. In our case, we are lacked a good Cdc14B antibody to do so. If the goal is to identify binding partners of the POI, then POI-MAP-tagged proteins that are expressed as close to endogenous levels as possible is ideal.
10. The number of cells used for the purification depends on the expression levels of MAP-tagged protein and the experiment purposes. For our purpose, to identify interactive proteins followed by Mass Spec, we used 4 mm × 150 mm dishes for highly expressed proteins and 10 mm × 150 mm Dishes for POI-MAP fusions with medium to low levels of expression.
11. Lysis buffers could be optimized from very mild to extremely harsh such as denaturing for MAP purification. For Cdc14B-MAP purification under native conditions, we optimized the concentration of detergent NP-40 to 0.2 %, which was the minimum required to release the majority of the protein from the cells. However, MAP purification also works for purification using RIPA buffer and NP-40 up to 1 % if required.
12. Phosphatase inhibitors are essential if the purified protein complexes are to be analyzed for phosphorylation.
13. Shortening the incubation time to 1–2 h could decrease nonspecific background, minimize protein degradation, and maintain more transient interactions; however, it reduced recovery.
14. We washed the beads in either Eppendorf tubes or bio-spin columns. The former normally obtains more binding partners, while the latter results in less background noise. Avoid letting the resin dry out on bio-spin columns (100 g for 15 s is an optimal condition in our purifications), which markedly reduces the yield.
15. We are able to see a yellow pellet from most of our purifications and white flakes after acetone wash.
16. Some phosphosites could not be unambiguously identified (i.e., two potential sites were adjacent as in Y416 and S415 and others). These could not be distinguished based on the spectra, but could be clearly identified as phosphopeptides.

## Acknowledgements

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# Chapter 15

# Bimolecular Affinity Purification: A Variation of TAP with Multiple Applications

**Petro Starokadomskyy and Ezra Burstein**

## Abstract

The identification of true interacting partners of any given bait can be plagued by the nonspecific purification of irrelevant proteins. To avoid this problem, Tandem Affinity Purification (TAP) is a widely used procedure in molecular biology as this reduces the chance of nonspecific proteins being present in the final preparation. In this approach, two different affinity tags are fused to the protein bait. Herein, we review in detail a variation on the TAP procedure that we have previously developed, where the affinity moieties are placed on two different proteins that form a complex in vivo. This variation, which we refer to as Bimolecular Affinity Purification (BAP), is suited for the identification of specific molecular complexes marked by the presence of two known proteins. We have utilized BAP for characterization of molecular complexes and evaluation of proteins interaction. Another application of BAP is the isolation of ubiquitin-like proteins (UBL)-modified fractions of a given protein and characterization of the lysine-acceptor site and structure of UBL-chains.

**Key words** Bimolecular affinity purification (BAP), Mass spectrometry, Tandem affinity purification, Ubiquitin acceptor site, Ubiquitination, Ubiquitin-like proteins (UBL)

## 1 Introduction

Advances in affinity-based protein purification techniques led to simple purification protocols that are accessible to most molecular biology laboratories. A commonly used format to identify novel protein complexes is the Tandem Affinity Purification (TAP) procedure, where a given bait protein is sequentially purified through two affinity resins, coupled with mass spectrometry analysis (MS-analysis) of the purified material [1–3]. However, one disadvantage of the TAP method is the inability to purify specific protein complexes from others that contain the same protein. Thus, the result of TAP is usually an average sum of all interacting partners of the target protein. Meanwhile, the interaction of given protein with specific molecular partners can highly distinguish between several protein complexes and pathways. To overcome this limitation of

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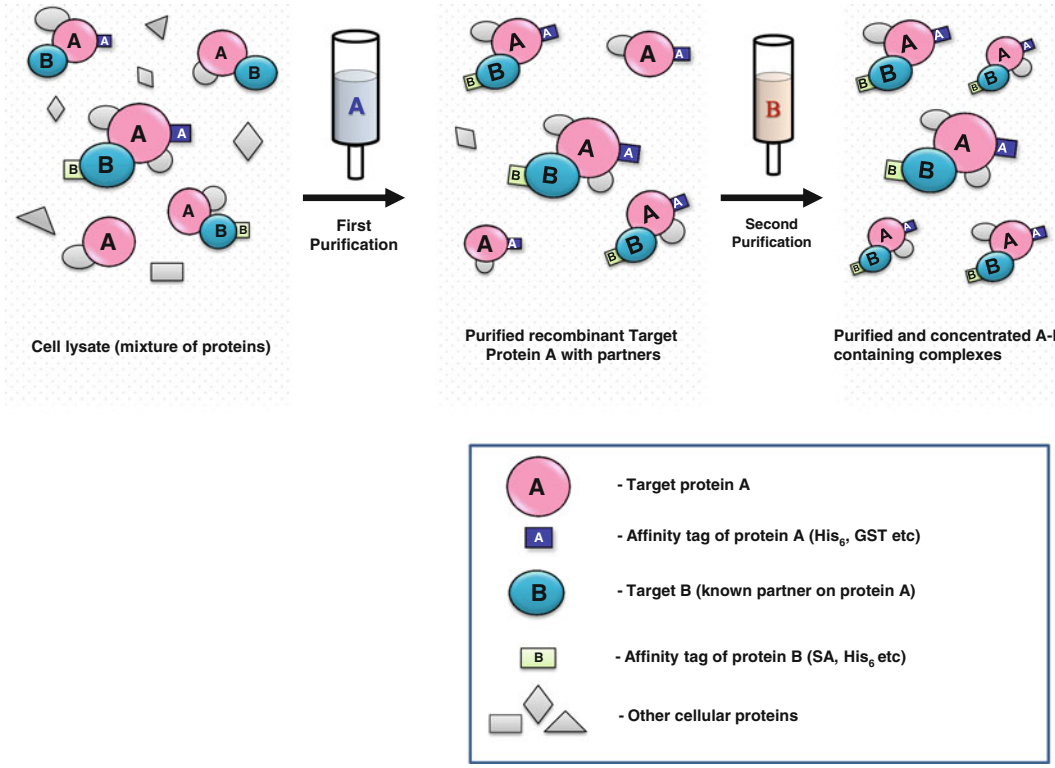


**Fig. 1** Schematic representation of the nBAP purification scheme

TAP, we developed the Bimolecular Affinity Purification (BAP) scheme, which provides the possibility for preparative isolation of a molecular complex characterized by the presence of two baits. In the BAP protocol, the first affinity moiety is fused with a target protein, while the second one is attached to its known partner in a specific pathway (Fig. 1). Using sequential purification through these tags, it is possible to isolate protein complexes of more homogenous composition while ignoring the others. This concept allows the investigation of specific partners and functions of the protein of interest in the selected pathways. The difference in harvested protein complexes that result from the TAP and the BAP protocols are illustrated in Fig. 2, and in fact, the result of BAP can be viewed as a more specific subset of the TAP results.

Moreover, one additional feature of BAP is the possibility of specifically purifying modified forms of the target protein that have been conjugated by ubiquitin-like proteins (UBLs), such as ubiquitin, Nedd8, or SUMO. Covalent modification of protein by UBLs plays essential regulatory functions in a number of pathways. Understanding the nature of the modification and mapping the modified amino acid in the target protein can shed light on the molecular basis of intracellular signal transduction and disease development.

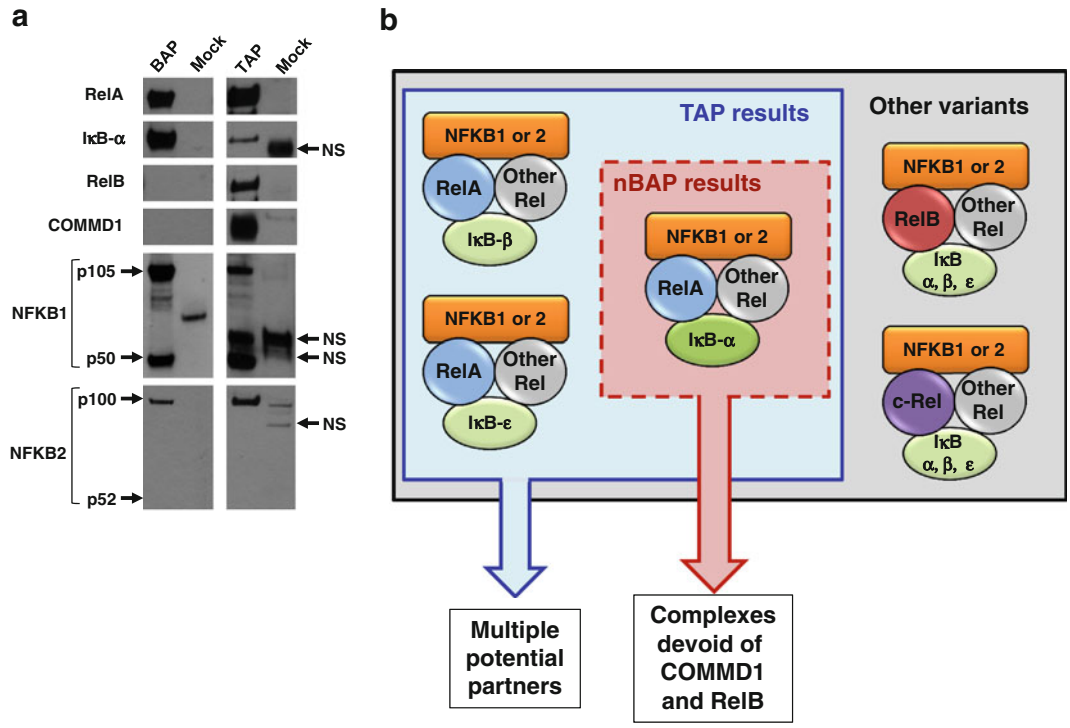


**Fig. 2** (**a**) Comparison of the RelA containing molecular complexes, yielded by TAP and nBAP. The proteins used for this example are GST-RelA and $His_6$-IκB-α for nBAP, and SBP-RelA-HA for TAP. *NS—nonspecific interaction.* (**b**) Schematic representation of the differences between TAP and nBAP. The nBAP method purifies narrower set of protein complexes (*pink box*), which are a subset of the TAP results (*blue box*). Other complexes remain unprecipitated by neither the TAP nor the nBAP procedures (*grey box*)

Using affinity tagged recombinant UBL-proteins, a variant of BAP makes it possible to purify in vivo conjugated target proteins. Subsequent MS-analysis allows for the characterization of a protein's UBL-acceptor sites and the structure of UBL chains if present.

In addition to these unique applications, the BAP procedure is simple and accessible to laboratories that are unable to do more complicated chromatographic separation techniques, but for whom the homogeneity of the sample is an important consideration.

### *1.1 Short Method Review*

We have developed two variations of the BAP procedure. One of them is the native BAP (nBAP), where the purification scheme is carried out under native buffer conditions. Another variant is referred to as denaturing BAP (dBAP), where the purification steps are performed under fully denaturing conditions. Each of them has specific applications and unique advantages and limitations.

The nBAP is used for purification and characterization of molecular complexes, containing two target proteins [4]. This is a reliable way to characterize the cofactors of a protein of interest in

a particular pathway. To achieve this goal, different affinity tags are fused to two proteins that are known to interact in vivo. The first affinity purification pulls down all molecular complexes containing first target protein. The second affinity purification concentrates only the desired complex consisting of the target protein and its partner plus other cofactors associated with them (Fig. 1). The main advantage of nBAP over TAP is the ability to separate specific molecular complexes, avoiding the potential heterogeneity of TAP. As with TAP, a big number of convenient affinity tag can be used (like the GST, $His_6$, Strep-tag, FLAG, HA, etc.). The purified material can be analyzed by SDS-PAGE and subsequent immunoblotting or by LC/MS-MS-analysis. For example, the application of TAP to purify RelA/p65 associated factors leads to the expected co-purification of IκBα, RelB, p105/p50 (NFKB1) and p105 (NFKB2), and COMMD1 (Fig. 2a). However, BAP purification of RelA- IκBα containing complexes demonstrates that these are devoid of RelB, as previously reported, and also devoid of COMMD1 (Fig. 2a). Thus, the BAP purified material is inherently more homogenous in composition (Fig. 2b).

Denaturing BAP (dBAP) is another variation of the method, designed for preparative purification of the UBL-conjugated forms of a target protein. Because of the activity of UBL-specific proteases in native lysis buffers, UBL-modified material is often unstable. To overcome this problem, the cell lysis and purification procedures are performed in 8 M Urea buffer during dBAP. In addition, usage of buffers containing 8 M urea solves the problem of insolubility of many UBL-conjugates. In the dBAP protocol, one tag is fused to the target protein, while another tag is associated with the recombinant UBL molecule. After in vivo UBL conjugation, the protein target acquires two affinity tags, and this forms the basis of the specific tandem purification. The first affinity purification pull down the total fraction of UBL-modified proteins from the lysate, and during the second affinity purification, the desired target protein is further purified out of the first fraction (Fig. 3). However, in specific situation, the sequence of this purification scheme can be reversed as well.

The main advantage of dBAP is the possibility to concentrate the UBL-modified fraction of a specific protein. We have successfully used dBAP for purification of ubiquitinated proteins for mass-spectrometry analysis, and this method has been effectively implemented by others [5]. One of the examples of this procedure is presented in Fig. 4, which depict the specific purification of ubiquitinated IκBα from cells. The main limitation of dBAP is that only two types of affinity tags remain functional under denaturing conditions, namely $His_6$-tag and biotinylation target sequences. Usually we use the C-term TB-tag or the N-terminal BT-tag, consisting of a short peptide sequence from *P. freudenreichii* transcarboxylase,

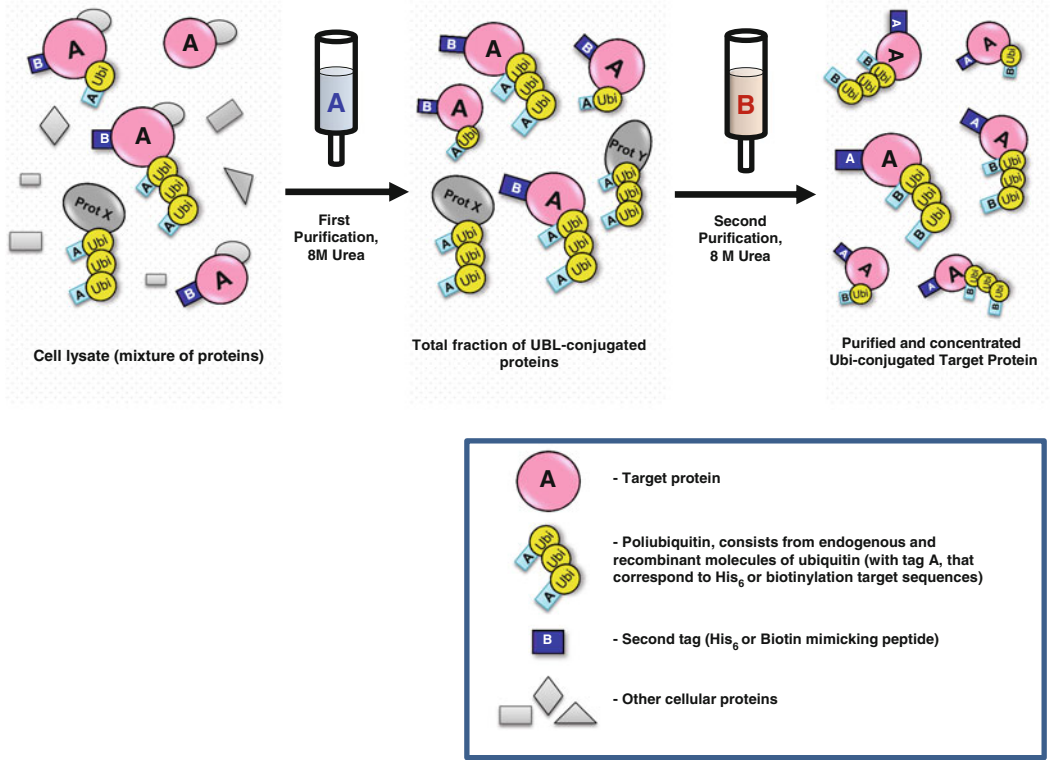


**Fig. 3** Schematic representation of the dBAP purification scheme

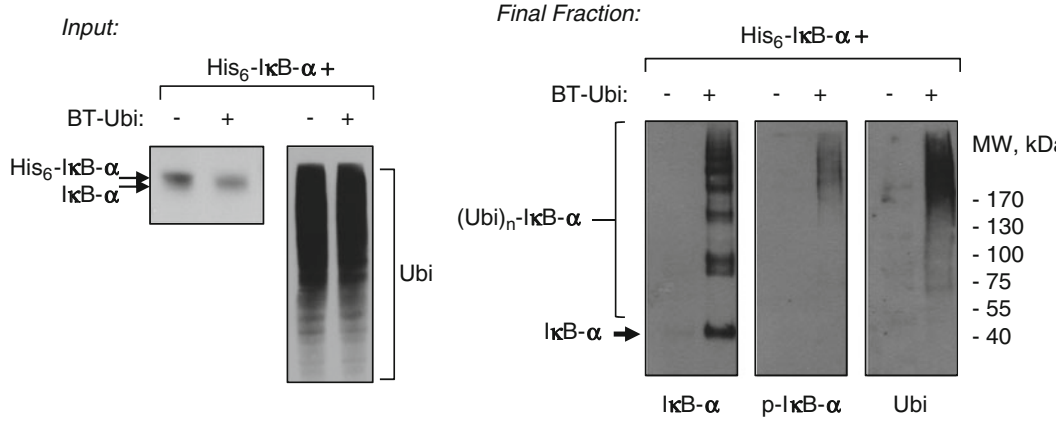


**Fig. 4** Purification of ubiquitinated $His_6$-IκB-α as the example of the applicability of the dBAP-protocol. Cells were transfected to express $His_6$-IκB-α along with BT-Ubi (+) or a vector control (–). Expression levels of $His_6$-IκB-α compared to endogenous IκB-α are shown in the input western blot. Similarly, expression of BT-tagged Ubiquitin did not raise the overall cellular level of ubiquitinated proteins (*left panel*). After TNF stimulation of cells, the dBAP purification was able to recover laddered IκB-α that was also immunoreactive for phospho-IκB-α and ubiquitin, consistent with the isolation of ubiquitinated IκB-α (*right panels*)

that is biotinylated by the endogenous mammalian machinery, preceded or followed by the TEV cleavage site respectively (pEBB-BT (N-term), Addgene #36295, pEBB-TB (C-term variant), Addgene #36099). For proteins exceeding 20 kDa, we exclusive use the C-terminal TB-tag which is more efficiently biotinylated than the N-terminal BT-tag in larger proteins.

The obtained material is usually subjected to LC/MS-MS-analysis, which can identify the sites of UBL-modification and the characteristics of the structure of UBL chains, if present. In the case of ubiquitin the identification of acceptor sites relies on the fact that tryptic digestion of a ubiquitinated protein leaves the carboxyl-terminal diglycine of ubiquitin still attached to the lysine of the target protein. Therefore, a mass shift of 114 Da is generated that can be used to identify the modified peptides that correspond to the acceptor site in the target protein [6]. For the UBLs that can form chains, the dBAP technique also provides similar information about acceptor sites on the UBL itself, thus revealing the branching structure of UBL chains.

The protocol below represents a unified description for the nBAP and the dBAP; both protocols are very similar, excluding the purification part of the procedure (Subheading 3.4). The protocols are focused on two sets of affinity tags: GST-tag with $His_6$-tag for nBAP and $His_6$-tag with BT-tagged ubiquitin [5] for the dBAP procedure. However, the same procedures with minor changes can be applied to other affinity tags following required variations during binding and elution steps [7, 11].

## 2 Materials

### 2.1 Cell Line

We use human embryonic kidney (HEK) 293 cells (available from ATCC): these cells are cultured in DMEM media supplemented with 10 % FBS and 2 mM glutamine. We use the calcium transfection method, but alternative strategies for transfection or viral gene delivering might be needed depending on the choice of cell line to be used.

### 2.2 Common Buffers for nBAP and dBAP

Prepare all solutions using ultrapure water (prepared by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C) and analytical grade reagents. Always handle with gloves to avoid keratin contamination. Prepare and store all reagents at room temperature (unless indicated otherwise). Diligently follow all waste disposal regulations when disposing of waste materials. The following solutions can be made ahead of time.

1. 5 M NaCl.
2. 20 % SDS.
3. 0.5 M EDTA.

4. 1 M $Na_2HPO_4$.
5. 1 M Tris, pH 8.0.
6. 1 M Tris, pH 6.3.
7. 1 M Imidazole (store at 4 °C).
8. 1 M DTT (store at –20 °C).
9. 100 mM PMSF in isopropanol (store at –20 °C).
10. Aprotinin, 1 mg/mL (store at –20 °C).
11. Coomassie staining solution: 0.06 % (w/v) Coomassie Blue G-250 and 10 % (v/v) acetic acid in water.
12. Gel destaining solution: 5 % methanol and 7 % acetic acid in water.
13. Gel fixing solution: 25 % (v/v) isopropanol and 10 % (v/v) acetic acid in water.
14. Gel loading buffer (3×): To make 10 mL stock, add 2.4 mL of 1 M Tris-Cl pH 6.8, 3 mL of 20 % SDS, 3 mL of Glycerol (100 %), 1.6 mL β-mercaptoethanol, 0.006 g of bromophenol blue. Store at 4 °C.
15. Leupeptin, 10 mg/mL (store at –20 °C).
16. PBS (phosphate-buffered saline), 1× without calcium and magnesium (Cellgro 21-040).

#### 2.2.1 Buffers for nBAP

The recipes of buffers provided here are meant for purification of a protein pair tagged with GST and $His_6$. If you intend to use another tag, please change the elution buffer accordingly.

1. *nBAP Triton lysis buffer*: 25 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10 % (v/v) Glycerol, 1 % (v/v) Triton X-100. This buffer can be made ahead of time and stored at RT. Just prior to use, add the following to make "complete" Triton lysis buffer: 1 mM PMSF, 10 mM DTT, 1 mM Sodium orthovanadate, 10 ng/mL Leupeptin, 1 ng/mL Aprotinin. Complete Triton lysis buffer can be stored at –20 °C for 4 weeks.
2. *BAP wash buffer*: 50 mM Tris–HCl (pH 8.0), 100 mM NaCl. This buffer can be made ahead of time and stored at RT. Just prior to use, add the following to make "complete" Triton lysis buffer: 1 mM PMSF, 10 mM DTT, 1 mM Sodium orthovanadate, 10 ng/mL Leupeptin, 1 ng/mL Aprotinin. Complete Triton lysis buffer can be stored at –20 °C for 4 weeks.
3. *Glutathione elution buffer*: 25 mM HEPES (pH 7.0), 100 mM NaCl, 40 mM reduced glutathione. Should be prepared just prior to use.
4. *Ni-NTA equilibration/wash buffer*: 25 mM HEPES (pH 7.0), 100 mM NaCl, 20 mM imidazole.
5. *Ni-NTA elution buffer*: 25 mM HEPES (pH 7.0), 100 mM NaCl, 250 mM imidazole.

#### 2.2.2 Buffers for dBAP

1. 10 M Urea solution (*see* **Note 1**).
2. *dBAP 8 M Urea lysis buffer*: 8 M Urea, 300 mM NaCl, 0.5 % NP-40, 50 mM $Na_2HPO_4$, 50 mM Tris (pH 8.0), 1 mM PMSF, 1 μg/mL Aprotinin, 10 μg/mL Leupeptin. Prepare fresh each time, using the stock solutions listed above including fresh 10 M urea.
3. *Buffer D1* (Nickel Wash Buffers): 8 M Urea, 300 mM NaCl, 0.5 % NP-40, 50 mM $Na_2HPO_4$, 50 mM Tris (pH 8.0). Prepare fresh each time, using the stock solutions listed above including fresh 10 M urea. Two variants of Buffer D1 are as follows:
   (a) *Buffer D1a*: pH 6.3. Adjust pH of Buffer D1 with pure HCl, verify with pH strips or meter.
   (b) *Buffer D1b*: pH 6.3, 10 mM Imidazole. Adjust pH as before; for 50 mL total volume, add 0.5 mL of 1 M Imidazole stock solution to Buffer D1a.
4. *Buffer D2* (Elution Buffer): 8 M Urea, 200 mM NaCl, 2 % SDS, 50 mM $Na_2HPO_4$, 10 mM EDTA, 50 mM Tris (pH 4.3). Adjust pH with pure HCl to pH 4.3, verify with pH strips or meter. Prepare fresh each time, using the stock solutions listed above including fresh 10 M urea.
5. *Buffer D3* (Streptavidin Wash Buffers): 8 M Urea, 200 mM NaCl, 0.2 % SDS, 50 mM Tris (pH 8.0). Prepare fresh each time, using the stock solutions listed above including fresh 10 M urea. Two variants of this buffer are:
   (a) *B*uffer D3a: Buffer D3 with 2 % SDS.
   (b) B*uffer D3b*: Buffer D3 without SDS.
6. *Buffer D4* (Final Wash Buffer): 50 mM Tris (pH 8.0), 0.5 mM EDTA, 1 mM DTT.

### 2.3 Additional Materials

The following materials are grouped according to their use in the various methods listed below. Please read through the entire protocol to evaluate whether the following materials are needed.

#### 2.3.1 Protein Expression and Lysis

1. Supplemented DMEM containing 4 μM biotin.
2. Bradford protein assay to assess protein concentration of cell lysates.
3. MG-132: a proteasome inhibitor that could be useful (*see* Subheading 3.3, **step 1** below).
4. Plasmids to express affinity tagged proteins. For example in this protocol we are using the following: pEBG (N-terminal GST), pEBB-$His_6$, pEBB-BT-ubiquitin (Addgene #36098). Other plasmids from our lab designed to express affinity tagged proteins have been deposited in Addgene (pEBB-SBP-HA (Addgene #36100), pEBB-BT (Addgene #36295), or pEBB-TB (Addgene #36099)).

*2.3.2 Protein Purification*

1. Glutathione agarose beads (Pierce), 50 % slurry.
2. Ni-NTA Agarose (Invitrogen).
3. Streptavidin ImmunoPure Agarose (Pierce).

*2.3.3 Western Blot/ Quality Control*

1. Antibodies to specific target proteins, i.e., the proteins being purified.
2. Anti-polyubiquitin antibody (Stressgen).
3. Streptavidin-HRP (BioLegend).

*2.3.4 LC-MS/MS Identification of Purified Complexes*

1. 2-Chloroacetamide.
2. Acetic acid.
3. Acetonitrile.
4. Ammonium bicarbonate.
5. Methanol.
6. Sequencing grade, modified trypsin (optional, depending on your proteomic core facilities).
7. Trifluoroacetic acid (TFA) (optional, depending on your proteomic core facilities).

### *2.4 Equipment*

1. Benchtop centrifuge with cooling system for 1.5-mL microcentrifuge tubes.
2. Cell scraper, 3-cm blade.
3. Centrifuge.
4. Centrifuge chromatography columns, 22-mL total volume (Pierce, 89898).
5. Conical tubes, 50-mL, able to withstand 15,000 × *g* (Corning, 430828).
6. Inverted microscope.
7. Laminar flow hood and $CO_2$ incubators for cell culture.
8. Microcentrifuge tubes, 1.5-mL.
9. Serological pipets and Pipet-Aid.
10. Tabletop centrifuge with a fixed-angle rotor that can reach 15,000 × *g*.
11. Tissue culture plates, 15-cm
12. Tube rotator.

## 3 Methods

Herein we present a united protocol for nBAP and dBAP. The nBAP is illustrated by GST- and His6-tagged proteins purification as an example; the dBAP protocol describes a purification of a

$His_6$-tagged protein, fused to the BT-tagged ubiquitin. Analogous protocol can be applied with a TB-tagged protein and $His_6$-ubiquitin as previously described [5]. The differences between the protocols will be pointed out throughout the text.

### 3.1 Cloning of Expression Vectors

Use standard molecular cloning techniques to insert the coding sequences of the desired baits in frame with the affinity tags (*see* **Note 2**).

1. For nBAP as described here, one bait should be fused to GST and the second bait is fused to $His_6$-tag.
2. For dBAP the target protein is fused to a $His_6$-tag (pEBB-$His_6$). As second bait we use human ubiquitin, fused to the N-terminal BT-tag (*see* **Note 3**). Alternatively, $His_6$-tagged Ubiquitin can be used and the target protein is fused to the N-term BT using pEBB-BT (Addgene #36295) or C-term TB-tag using pEBB-TB (Addgene #36099).

### 3.2 Protein Expression

Seed $7 \times 10^6$ HEK 293 cells in 15 cm dishes ~18 h prior to transfection (*see* **Note 4**). Transfect cells using a conventional calcium phosphate method [8]. For nBAP we use 5–10 μg of each plasmid per 15 cm dish. For dBAP we use 20–30 μg of the plasmid coding the $His_6$-fusion protein and 3 μg of pEBB-TB-Human Ubiquitin.

Replace media ~8 h post-transfection with fresh media. If you are using a biotinylation tag, supplement with fresh media containing 4 μM Biotin. Allow the cells to grow for about 48 h before the next step.

### 3.3 Preparation of Cell Lysates

1. Two days after transfection, prepare fresh Lysis buffer. As an optional step, cells may be treated prior to lysis in order to promote the accumulation of the ubiquitinated form of the target protein in question. This may consist of proteasomal blockade or ligand stimulation depending on the specific protein being investigated (*see* **Note 5**).
2. Aspirate media from tissue culture plates, gently wash at room temperature with 5 mL of 1× PBS, and lyse cells by applying 1.5 mL/plate of respective Lysis buffer (native or denaturing). Scrape the plates with a cell scraper and collect the lysates and debris in a conical 50 mL tube. Place the plates on ice during this procedure.
3. For dBAP, sonicate samples to complete the lysis and reduce lysate viscosity. For HEK 293 cells, 25 pulses is typically sufficient (Branson sonifier, output control 2.5, 75 % duty cycle). Verify the adequacy of the sonication by pipetting the lysate: if the lysate is still viscous, sonicate again (*see* **Note 6**). Please note, that nBAP does not required sonication.

**Table 1 Quality control analysis**

| Lane | Sample | Rationale |
|---|---|---|
| 1 | Input | To verify expression of the bait proteins |
| 2 | Flow-through of the first column | To assess the ability of the first column to deplete the lysate of target proteins |
| 3 | First column beads before elution | To show binding of target proteins to the first column |
| 4 | First column beads after elution | To show proper elution of target proteins from the first column |
| 5 | First column eluate | To verify presence of the target bait proteins in the eluate |
| 6 | Flow-through of the second column | To show depletion of the target proteins |
| 7 | Second column precipitate | To confirm recovery of target proteins from second column |

4. Remove cell debris by centrifugation of the lysate at 15,000 × *g* for 15 min at 4 °C. Transfer supernatant to a fresh conical tube and determine protein concentration by Bradford assay. Set aside an aliquot (about 100 µL or 1 % of the supernatant) for quality control purposes (Table 1).

### 3.4 Protein Purification

This step is the most dissimilar part of the protocols for nBAP and dBAP. A complete purification procedure for each one of them is presented below.

#### 3.4.1 nBAP Protein Purification

1. Pre-equilibrate glutathione (GSH) resin column: Add 1 mL of 50 % glutathione agarose beads to chromatography column. Wash column with 5 mL of complete Triton lysis buffer (10× volume of beads).
2. Bind first bait to GSH column: Cap bottom of the column, load cell lysate, and then cap top of the column. Rotate column for 1–3 h at 4 °C. Let lysate flow through by gravity (or through centrifugation at 500 × *g* for 5 min). Save an aliquot of the flow-through (100 µL or 1 %) to ensure that the binding to the GSH column resulted in efficient depletion of the first bait from the lysate (*see* Table 1).
3. Wash column four times with 20 mL of complete Triton lysis buffer, rotating for 5 min at 4 °C between each wash. During the last wash, save an aliquot of the beads by removing 200 µL (1 %) of the resuspended beads prior to draining the wash buffer. This will be used to confirm binding of the bait to the column during quality control.

4. Elute the column by adding 2 mL of Glutathione elution buffer and capping the column as before. Rotate the column for 20 min at 4 °C. Drain the column, saving the eluate, and repeat the elution step three additional times. Pool all 4 eluates into a single batch. Save a sample of the GSH eluate (about 80 μL or 1 % of the eluate). After elution is complete, resuspend the beads in 5 mL of *BAP wash buffer* and save a sample of the beads post-elution (50 μL or 1 % of the resuspended beads) to ensure that adequate elution took place.
5. Pre-equilibrate Ni-NTA agarose column. Load column with approximately 25 μL of bead volume for every 100 mg of input lysate, utilized at the beginning of the affinity purification. Apply 5 mL (20× volume of beads) of *BAP wash buffer* and allow draining by gravity flow.
6. Apply GSH eluate to the copped Ni-NTA column and rotate 1–3 h at 4 °C. Drain column by gravity flow and wash Ni-NTA agarose with 10 mL of *BAP wash buffer* five times. After the last wash, resuspend the beads in 1 mL of *BAP wash buffer* and transfer beads to a fresh 1.5-mL microcentrifuge tube. Set aside 10 μL (or 1 %) of the bead suspension for quality control.
7. Store final purification product at 4 °C by precipitating the beads by centrifugation (500 × *g* for 5 min at 4 °C) and adding enough *BAP wash buffer* to keep the beads as 50 % slurry. The stability of the sample at 4 °C depends on the protein complex and the desired application in which this complex will be utilized.

### 3.4.2 dBAP Protein Purification

1. Pre-equilibrate the first column containing Ni-NTA agarose bead slurry with Buffer D1: load column with 30 μL of 50 % slurry for each 100 mg of total protein lysate. Apply five bead volumes of Buffer D1 and allow it to drain by gravity flow.
2. Precipitate total recombinant proteins with Ni-NTA beads: cap bottom of the column, load cell lysate, and then cap top of the column. Rotate loaded column for 1–2 h at RT. At the end of the rotation, save a small amount of resuspended beads (~1–3 %) in a microfuge tube for the quality control purposes (Table 1).
3. Allow column to drain by gravity flow or through centrifugation at 500 × *g* for 5 min. Save flow-through to assess depletion of ubiquitinated proteins for quality control purpose.
4. Wash Ni-NTA agarose with ten bead volumes of Buffers D1 (two times), and subsequently by Buffer D1a and Buffer D1b (each one time, every time rotating for 5 min at RT). At the end of the rotation, allow column to drain by gravity flow or through centrifugation.
5. Elute target protein from Ni-NTA beads by applying ten bead volumes of Buffer D2. Rotate column for 30 min at RT. At the end of the rotation, allow column to drain by gravity flow or through centrifugation.

6. Collect eluate in a fresh conical tube and adjust pH to 8.0 with 10 N NaOH (*see* **Note 7**). Verify with pH strips. Save a small amount of eluate (~1–3 %) and resuspend Ni-NTA beads in Buffer D1 and also save a small amount of resuspended beads for quality control purposes.
7. Pre-equilibrate streptavidin (SA) beads: load column with 50 μL of a 50 % slurry for ~100 mg of total input lysate used for initial affinity purification step with Nickel beads. Apply 5 mL of Buffer D3 and allow it to drain by gravity flow.
8. Add pH-adjusted eluate from **step 6** to the SA beads and rotate from 1 h to overnight at RT (*see* **Note 8**).
9. Drain the column by gravity flow or centrifugation. Save a small aliquot of the flow-through for quality control.
10. Wash SA column with ten bead volumes of Buffers D3 (two times), and subsequently with Buffer D3a and Buffer D3b (each one time, every time rotate for 5 min at RT). At the end of the rotation, allow column to drain by gravity flow or through centrifugation.
11. Add 10 bead volumes of Buffer D4 and rotate for 5 min at RT. At the end of the rotation, allow column to drain by gravity flow or through centrifugation. Repeat this step four times.
12. Store final purification product (ubiquitinated target protein) at 4 °C by precipitating the beads by centrifugation (300 × *g* for 5 min at 4 °C) and adding enough BAP wash buffer to keep the beads as a 50 % slurry.

### 3.5 SDS-PAGE Separation

For dBAP, we routinely separate the recovered material by SDS-PAGE and excise bands that correspond to UBL-modified bait for MS-analysis. For nBAP, gel band excision is also a possibility, but elution and trypsin digestion in solution can provide more fruitful results for MS-analysis.

1. Sample preparation: after the last wash, resuspend beads in 1 mL of last applied buffer (*BAP wash buffer* for nBAP or Buffer D4 for dBAP), gently shake and spin for 1 min at 600 × *g* at RT. Carefully aspirate supernatant, and resuspend beads in 3× gel loading buffer (1 μL of loading buffer per 1 μL of bead volume). Heat sample for 10 min at 80–95 °C for denaturation. Now samples can be stored at –80 °C overnight or for several days before proceeding with SDS-PAGE separation.
2. SDS-PAGE separation: load maximal amount of sample onto a SDS-PAGE gel. Run for desired amount of time for adequate separation.
3. Perform Coomassie blue staining of the gel using a standard protocol (*see* **Note 9**).

### 3.6 Quality Control at the End of the Purification

Quality control (QC) western blot: To ensure that proper precipitation and elution at each step, we recommend a QC western blot using bait-specific antibodies. Equal amounts of the following paired samples should be loaded according to Table 1.

### 3.7 Tandem Mass Spectrometry

Most molecular biology laboratories are unlikely to perform all these steps on their own and will use the services of a protein chemistry core facility. Given below is a general outline for processing samples after separation by SDS-PAGE. If you intend to employ the services of a protein chemistry core facility, consult about their specific requirements.

1. In a laminar flow hood, excise the desired bands from the Coomassie blue stained gel and place them in microfuge tubes (*see* **Note 10**). Many protein chemistry cores will handle the sample at this stage.
2. Destain the gels with 30 % methanol for 3 h.
3. Incubate the gel slices with DTT (10 mM) followed by 2-chloroacetamide (50 mM) at RT for 30 min, to reduce and carbamidomethylate cysteine residues, respectively (*see* **Note 11**).
4. Mince gel slices and dry using a Vacufuge.
5. Add 30 μL of 50 mM ammonium bicarbonate buffer containing 500 ng of sequencing grade, modified trypsin. Incubate sample on ice for 45 min to reswell the gel.
6. Add 40 μL of 50 mM ammonium bicarbonate buffer to the sample and incubate overnight at 37 °C. The next day add 250 ng of trypsin and incubate for 2 h at 37 °C to complete protein digestion.
7. Extract peptides at 30 °C by applying first 150 μL of 60 % acetonitrile–0.1 % TFA (30 min), followed by 150 μL acetonitrile–0.1 % TFA (30 min). Pool both extracts and use a Vacufuge to concentrate the sample to a volume of ~30 μL.
8. Additional sample handling is dependent of the specific mass-spectrometry instrument and application.
9. Data obtained from MS-MS are analyzed against databases of human proteins. During this analysis undesirable contaminants may be evident in the sample (for example, Keratins). Next, in the case of dBAP, a more restricted analysis is recommended using a target protein database containing predicted peptides from tryptic digestion of the target protein, common contaminants, and the UBL in question.
10. When analyzing the MS-MS data for dBAP, potential modifications of amino acids that may lead to changes in mass and/or charge are considered. These modifications may be the result of sample preparation (for example, oxidation of methionine residues or alkylation of cysteines) or may represent in vivo posttranslational modifications. In particular, oxidation of

methionine (+15.9949 Da), carbamidomethylation of cysteines (+57.0214 Da) and ubiquitination of lysines (diglycine, +114.0469 Da) should be considered in the analysis. Various software tools for MS-MS data analysis are available, but manual verification of the MS–MS spectra corresponding to peptides identified as UBL-modified is recommended [5, 10].

## 4 Notes

1. The 10 M Urea solution should be prepared fresh every time. For a 40 mL solution, resuspend 24 g of urea in 20 mL of water, vortex and incubate at 30 °C for 20 min. After urea has been dissolved, adjust the volume to 40 mL. To remove isocyanate impurities incubate solution with 1 % Amberlite ion exchange resin with gentle agitation for 1 h at RT. Use this solution to make all the urea containing buffers described here.
2. Before the experiment it is worthwhile to evaluate the effect of the affinity tag position on the expression of bait proteins. We recommend to transiently transfect the cells with different variants of target protein and analysis the expression by western blotting analysis using antigen-specific antibodies. In case of evaluation of biotinylation tags (TB or BT), we perform parallel immunoblotting with streptavidin-HRP to confirm efficient biotinylation in vivo.
3. Please note that we recommend the use of human and not yeast ubiquitin. Although yeast ubiquitin is nearly identical to human ubiquitin and can be conjugated by the mammalian ubiquitination machinery, the three amino acid differences between these two proteins may complicate the analysis of mass spectrometry data. The coding sequence for human ubiquitin that we use was amplified by PCR from genomic DNA (forward primer 5′-*ataggatccgccaccatgcagattttcgtgaaaacc* and reverse primer 5′-*tctgcggccgcaccaccacgaagtctcaac*). The amplified product was then inserted into pEBB-BT to derive pEBB-BT-human ubiquitin. Please remember that recombinant ubiquitin should be tagged only in its N-terminal tag, as a C-terminal glycine is required for conjugation.
4. For most bait proteins, 5–10 plates should be enough for analysis. Depending on the application, other cell lines might be preferable. Use of different expression vectors or cell lines requires such optimization before large scale purification.
5. In the case of proteasomal blockade, we routinely use a MG-132 at a concentration of 40 μM for 3–5 h. In the case of ligand stimulation, we used standard time points for the ligand. Alternatively, co-expression of the known E3 ligase for the target protein might be useful.

6. Proper sonication is mandatory because of high viscosity of the lysate, produced with 8 M urea buffers.
7. Alkalization of the eluate is necessary in order to achieve binding to the next column. Because of the small volume of the sample, pH testing with paper strips is usually more practical than using a pH meter. We usually drop 1–2 μL of the solution on the paper's strip indicator zone.
8. The samples can be incubated with the second column overnight. In most cases, this step will be reached at the end of the day, and thus, we routinely performed the binding overnight without any apparent detriment to the procedure. Otherwise, this step may be performed in 1 h.
9. During the staining, avoid keratin contamination of the sample. Never touch the gel itself with bare hands; open the gel cassette in the laminar flow hood using gloves. For gel staining, use disposable new plasticware or thoroughly cleaned glassware and maintain the sample covered with cellophane wrap or a lid at all times. A simplified Coomassie staining protocol is provided below.
   (a) Place gel in 50 mL of fixing solution and gentle agitate at room temperature for 10 min. Replace the fixing solution by 50 mL of Coomassie staining solution, gently agitate and place the gel in the microwave for 5–10 s to warm it up to about 40–50 °C (estimated by touch). Agitate the gel on the rocker for 10 min. In the laminar flow hood drain the Coomassie solution and place the gel in destaining solution. Warm up the solution in the microwave for 5–10 s as before. Place the gel on the rocker for 15 min. In the laminar flow hood, replace the destaining solution with new destaining solution. Agitate the gel on the rocker for ~3 h. If needed, the destaining solution can be replaced with ultrapure water and the gel may be left overnight before the next steps.
10. During the procedures avoid touching the gel and always wear gloves. This step is very sensitive to keratin contamination, which can ruin the MS-analysis.
11. When performing the dBAP, a critical technical consideration is to avoid using iodoacetamide as an alkylating agent that prevents disulfide bridges. Iodoacetamide can also lead to the addition of 2-acetamidooacetamide moieties to lysine residues resulting in a modification (114 Da) that is indistinguishable from the diglycine modification [9]. Using 2-chloroacetamide as an alternative agent avoids this potential artifact and is therefore preferred for this application [6, 10].

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# INDEX

## A


Affinity tag
- cellulose-binding module (CBM)....35–44
- chitin binding domain (CBD)....48
- cleavage
  - autoprocessing....59–69
  - intein....41, 44, 48, 49, 53, 54, 56, 57
  - tobacco etch virus (TEV)....115, 123
- 1D4 epitope tag....2, 3, 5–7, 10
- elution....14, 22, 27, 30, 48, 78, 108, 115, 122, 145, 198
- flag tag....2, 4, 138, 145, 148, 151, 196
- glutathione-*S*-transferase (GST) tag....59, 72, 121, 195, 196, 198–202
- green fluorescent protein (GFP) tag....38–41, 43, 121, 151–160, 164, 172, 176, 179
- hemagglutinin (HA)....2, 60–61, 121, 136, 196, 200
- heme-tag....17–32
- hexahistidine-tag (His-tag)....2, 59, 107, 108, 115, 118
- his-MBP tag (Maltose Binding Protein)....72, 81–93, 116
- maltose binding protein (MBP)....72, 81–93, 116
- multifunctional GFP (mfGFP)....152, 156, 158
- Myc....2, 152
- small ubiquitin-related modifier (SUMO)....71–79, 194
- strep-tag....107–108, 116, 117, 158, 196
- streptavidin-binding peptide (SBP)....122, 125–127, 152, 156–158, 176, 200
- synaptobrevin Avi purification-tag (SnAvi)....163–173
- tamavidin....95–105

Agarose
- beads....64, 66, 69, 122, 124, 126, 130, 131, 136, 139, 141, 143–145, 148, 201, 203, 204
- gel....50, 51, 63, 64, 82, 86, 99, 179, 180, 182

Antibiotic resistance marker
- ampicillin....21, 48, 50, 51, 63, 65, 67, 83–87, 93, 100, 110, 112
- blasticidin....139, 141, 153, 155
- chloramphenicol....20, 21, 84, 87, 92
- Hygromycin....139, 140, 153, 155, 159, 178, 179, 182, 183, 187, 189
- kanamycin....77, 93
- Puromycin....178, 182, 187

Antibody
- monoclonal....2–7, 13, 179
- polyclonal....1, 3, 13


## B


Beads
- activated....8, 26
- agarose....64, 66, 69, 122–124, 126, 130–132, 136, 139, 141, 143–145, 148, 184, 201, 203, 204
- anti-FLAG M2....139, 145, 184
- biotinylated....102, 136, 137, 147, 172
- dyna-....98, 102
- glutathione....201, 203, 204
- IgG....122, 123, 126, 131, 132, 145
- magnetic....8
- micro-....96, 98, 102–105
- nickel NTA....205
- protein A....168
- protein G....122, 123, 126, 131, 139, 143, 145, 148
- sepharose....8–10, 13, 18, 20, 26, 31
- Strep-Tactin....154, 156–158
- streptavidin....122–124, 126, 127, 131, 132, 136, 139, 141, 144, 172, 184–186, 205

Biotin
- biotinylated....96, 98, 102, 136, 163, 171
- in vivo biotinylation....135–148, 171

Blotting
- immuno-....101, 156, 157, 207
- western....2–4, 7, 10–13, 108, 114, 117, 124, 125, 127, 130–132, 145, 146, 148, 166, 170, 172, 176, 179, 182, 183, 185, 197, 201, 206, 207


## C


Cells
- bacterial....1, 5, 60, 68, 72, 87, 100, 160, 171
- BL21....24, 63, 65, 68, 77, 110, 112, 170
- competent....49, 51, 55, 63, 65, 82, 83, 86, 92, 110, 177, 181
- DH5α....51, 55, 63, 65, 83, 86, 92, 93, 177
- *Escherichia coli*....71, 82, 83, 85–93, 97
- eukaryotic....60, 72, 122, 125, 156, 164
- *gyrA*$^+$....83, 85, 92
- HEK293T....3–6, 9–10, 12, 153, 155–156, 166, 169, 198, 202
- HeLa....166, 169
- mammalian....1, 5, 153, 155, 164–166, 168, 170, 171, 175–190



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Cells (*cont.*)
mouse embryonic stem cells (mESC) ..... 135–148
sorting (FACS) ..... 176, 179, 182, 183
$T1^R$ ..... 82, 85
T-REx ..... 155, 159
U2OS ..... 182–186, 189
yeast ..... 1, 5, 36, 122, 175, 176
Chromatography
column
chitin ..... 50, 52, 55
glutathione (GSH) ..... 203
HIS ..... 27, 29, 118, 157
Ni-NTA ..... 114, 119, 154
RAC ..... 40, 42
strep-tactin ..... 154, 157, 158
elution ..... 25–26, 29, 35, 40, 42, 77, 78, 108, 115, 116, 118, 119, 157, 158
immobilized metal affinity chromatography (IMAC) ..... 60, 81, 107, 154, 157, 158
Cloning
deoxyribonucleic acid (DNA) ..... 49–51, 55, 61, 63–65, 75, 82, 83, 85, 86, 98–100, 154, 159, 177, 180, 181
plasmid ..... 20, 49, 50, 63, 65, 83, 85, 86, 125, 154–155, 178, 181
polymerase chain reaction (PCR) ..... 5, 48–50, 56, 64, 65, 75, 77, 82, 83, 85, 86, 93, 98, 154, 155, 180–182
recombinant cloning ..... 82–86, 96, 98–100, 154, 155, 159
restriction enzyme ..... 49–51, 64, 154, 155, 177
transfection ..... 182
transformation ..... 51, 55, 86, 93, 99, 181, 183
vector ..... 20, 49–51, 53, 60, 63–65, 67, 75, 77, 82–86, 93, 98, 99, 155, 177, 178, 180–182, 189, 202

## D

Detection and confirmation
co-immunoprecipitation ..... 2, 3
coomassie ..... 3, 20, 42, 69, 97, 157
gel electrophorsis ..... 97, 157
GFP fluorescence ..... 152, 157, 158
in vivo ..... 71, 72, 74, 78, 138, 207
LC-MS ..... 177, 187, 189
mass spectrometry ..... 78, 125, 172
proteomics ..... 136
SDS-PAGE ..... 20, 42, 43, 69, 78, 97, 100–102, 117, 118, 129, 157, 158
western blot ..... 3, 7, 117, 125, 166, 170

## E

Epitope ..... 1–14, 91, 121, 136, 152, 164, 166, 176

## I

Immunoaffinity ..... 3, 5, 8–9, 11, 13, 14
Inducible
expression ..... 24, 59–69, 128, 153, 155–156
promotor ..... 24, 48, 128
Intein ..... 36, 38, 41–44, 48, 49, 53, 54, 56, 57

## L

LC-MS/MS ..... 176, 177, 184, 186–189, 201
Lysis
buffer ..... 25, 38, 41, 64, 66, 68, 76, 77, 123, 125–127, 130, 160, 165, 167, 168, 170, 179, 180, 184–186, 190, 196, 199, 200, 202, 203
cell lysis ..... 38, 41, 52, 56, 68, 75, 76, 79, 165, 167, 168, 196

## M

Mass spectrometry ..... 11, 78, 121, 124, 125, 127, 128, 132, 136, 148, 171, 172, 177, 184–186, 193, 196, 206–207
Membrane
blot ..... 2–4, 7, 10–13, 100, 102, 108, 117, 129, 166, 179, 183
cell ..... 1–6, 9, 10, 12, 13, 110–111, 113, 183
extract ..... 2, 9–10
protein ..... 1–7, 9–14, 18, 26, 31, 97, 100–102, 110–111, 113, 116, 117, 119, 129, 166, 183
PVDF ..... 7, 11, 97, 100, 102

## P

Peptide ..... 3, 5, 6, 10, 11, 14, 17–20, 30, 36, 61, 73, 98, 103, 122, 125–127, 136, 139, 145, 148, 151, 152, 158, 163, 164, 170, 176, 179, 184–187, 196, 198, 206, 207
Plasmid ..... 3, 5, 9, 13, 20, 24, 38, 39, 41, 49, 50, 63, 65, 75, 83, 85, 86, 89, 92, 110, 112, 125, 128, 129, 138–140, 153–155, 166, 170, 178, 181, 187, 189, 200, 202
Polymerase chain reaction (PCR) ..... 5, 48–50, 55, 56, 63–65, 68, 75, 77, 82, 83, 85, 86, 91, 93, 98, 99, 154, 155, 180–182, 207
Protease ..... 2, 6, 9, 59–61, 64, 68, 69, 72, 73, 84–91, 111–116, 118, 119, 122–124, 126, 130, 131, 139, 143, 144, 147, 154, 157, 160, 164–166, 169, 170, 172, 176, 179, 183, 184, 186
Protease inhibitor ..... 6, 9, 64, 68, 111, 113, 114, 123, 124, 130, 139, 143, 144, 154, 160, 165, 169, 179, 183, 184
Protein
bait ..... 121–125, 127–129, 131, 132, 187, 193, 194, 202, 203, 207
complex ..... 2, 36, 135–148, 152, 153, 156, 157, 163, 164, 169, 171, 175, 184, 186, 193–196, 204

elution ..... 10, 11, 14, 22, 27, 43, 44, 48, 3, 54, 62, 64, 76, 79, 87, 90, 108, 118, 122, 124, 127, 129, 132, 148, 154, 156, 158, 170, 186, 199, 204
endogenous ..... 136, 138, 141, 147, 170, 171, 190, 197, 198
expression ..... 1, 3, 11, 13, 17, 19–21, 24, 25, 30, 31, 36–41, 49–52, 61, 65, 67, 68, 72, 73, 75, 77–79, 81–93, 95–105, 112, 113, 117, 138, 151–153, 155, 156, 164, 170, 171, 176, 180, 182, 183, 189, 190, 197, 200, 202, 207
extraction ..... 22, 51, 84, 87, 100, 177
folding ..... 68, 71, 72, 74, 91, 116
fusion ..... 17, 48–52, 60, 61, 64, 67, 69, 71–79, 81, 82, 84–93, 95–105, 110–112, 116, 118, 121, 151–153, 155–158, 160, 164, 165, 76, 183, 184, 186, 202
interaction ..... 2, 95, 107–108, 117, 121–132, 151, 152, 175, 177, 188, 190, 193
localization ..... 2, 7, 12–13, 151, 152, 164, 176, 177, 183
membrane ..... 1–14, 18, 97, 100–102, 110, 111, 113, 116, 117, 119, 129, 166, 183
modification ..... 177
partner ..... 2, 72, 74, 96, 98–101, 103–105, 108, 116, 121–124, 128, 137, 138, 169, 171, 176, 185, 186, 188, 190, 193, 194, 196
production ..... 24, 35, 61, 81, 85, 86, 91, 96, 117
proteomics ..... 136
purification ..... 121–132
  bimolecular affinity purification (BAP) ..... 193–208
  multifunctional affinity purification (MAP) ..... 175–190
  tandem affinity purification (TAP) ..... 121–132, 136, 152, 153, 157, 163, 164, 166–169, 175, 193–208
quantification ..... 5, 13, 20, 23, 26, 29–30, 61, 91, 122, 129, 130
recombinant ..... 35–44, 47, 59, 61, 62, 71, 81, 85, 95–105, 107, 108, 112, 116, 117, 151–160, 170, 195, 196, 204, 207
solubility ..... 24, 31, 59, 61, 62, 68, 69, 71–79, 81, 84, 86–89, 91, 93
stability ..... 35, 59, 61, 204
ubiquitinated ..... 196–198, 202, 204, 205

## R

Restriction
  digestion ..... 51, 64, 65, 67, 68, 99, 181, 206
  enzyme ..... 38, 49–51, 64, 99, 103, 154–155, 177
  site ..... 5, 61, 67, 98, 99, 154–155, 177, 180

## S

SDS-PAGE ..... 7, 10, 11, 20, 22–23, 28–29, 39–43, 52, 53, 56, 57, 62, 63, 65–67, 69, 77–79, 84, 87–89, 93, 97, 100–104, 108, 114, 115, 117, 118, 124, 127–129, 131, 132, 140, 142, 146–148, 157, 158, 166, 169, 172, 179, 183, 196, 205, 206
Solubility
  enhancer ..... 2, 81, 89
  protein ..... 1–14, 24, 31, 59, 71–79, 86, 88–91, 95, 96, 98, 100–103, 105, 108, 118, 119, 128, 130, 160
  tag ..... 1–14, 24, 59, 61, 62, 68, 69, 71–79, 81, 84, 86–91, 93, 95, 96, 108, 129
Supernatant ..... 8, 10, 25, 38–42, 52, 57, 60, 62, 65–67, 69, 77–79, 88, 100, 102, 103, 113, 114, 122, 125–127, 131, 141–145, 148, 156, 158, 160, 167–172, 184, 203, 205

## T

Tag
  activation ..... 6, 8, 9, 22, 26, 31, 43, 48, 53, 59, 60, 68, 73, 108, 110, 117, 119, 163, 171, 196
  affinity ..... 2–5, 13, 14, 17–32, 35–44, 47–57, 59, 60, 72, 74, 78, 81, 95, 96, 107, 108, 112, 115–118, 121–132
  epitope ..... 1–14, 91, 121, 136, 152, 164, 166, 176
  purification ..... 1–14, 17–32, 35–44, 47–57, 59–69, 71, 72, 74, 76–79, 84, 86–91, 95, 96, 107, 108, 112, 115–118, 121–131, 136–138, 140, 151–160, 163–173, 175–178, 182–187, 190, 194–202, 207
  removal ..... 8–12, 14, 18, 19, 25, 26, 28, 29, 31, 36, 38–40, 42, 43, 47, 48, 59, 65–67, 69, 71–74, 79, 88, 90, 93, 102, 108, 115, 118, 123, 125–127, 131, 176, 186
  solubility ..... 1–14, 24, 59, 61, 62, 68, 69, 71–79, 81, 84, 86–91, 93, 95, 96, 108, 129