# The *Strep*-tag system for one-step purification and high-affinity detection or capturing of proteins

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**The *Strep*-tag II is an eight-residue minimal peptide sequence (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) that exhibits intrinsic affinity toward streptavidin and can be fused to recombinant proteins in various fashions. We describe a protocol that enables quick and mild purification of corresponding *Strep*-tag II fusion proteins—including their complexes with interacting partners—both from bacterial and eukaryotic cell lysates using affinity chromatography on a matrix carrying an engineered streptavidin (*Strep*-Tactin), which can be accomplished within 1 h. A high-affinity monoclonal antibody (*Strep*MAB-Immo) permits stable immobilization of *Strep*-tag II fusion proteins to solid surfaces, for example, for surface plasmon resonance analysis. Selective and sensitive detection on western blots is achieved with *Strep*-Tactin/enzyme conjugates or another monoclonal antibody (*Strep*MAB-Classic). Thus, the *Strep*-tag II, which is short, biologically inert, proteolytically stable and does not interfere with membrane translocation or protein folding, offers a versatile tool both for the rapid isolation of a functional gene product and for its detection or molecular interaction analysis.**



## INTRODUCTION

Efficient procedures for purification, detection and immobilization or separation—possibly in complex with cognate macromolecules—are of key importance in modern protein science. In particular for structural genomics and proteome research, the rapid isolation of a recombinant gene product, preferentially under standardized high-throughput conditions, and the characterization of its biochemical activity or identification of interaction partners are crucial. The *Strep*-tag II (ref. 1) is a small-affinity peptide, which, after fusion with the recombinant protein of interest, offers a practically useful solution to these tasks.

The *Strep*-tag was originally selected from a genetic random library[2] as an eight-amino-acid peptide (WRHPQFGG) that specifically binds to core streptavidin, a proteolytically truncated version of the natural bacterial protein[3]. Owing to its extraordinary affinity for the small group D-biotin, together with its high intrinsic stability and low nonspecific interaction, core streptavidin—as soluble protein, in immobilized form, conjugated with enzymes or even as engineered versions—is in wide use as a powerful reagent for the detection as well as separation of various biotinylated macromolecules or other compounds of interest[4].

As the *Strep*-tag binds reversibly to the same pocket where the natural ligand D-biotin is complexed, it can be applied for the efficient purification of corresponding fusion proteins on affinity columns with immobilized streptavidin[5]. Elution of the bound recombinant protein is effected under mild buffer conditions in a biochemically active state by competition with D-biotin or a suitable derivative with less strong affinity, such as D-desthiobiotin, thus facilitating repeated use of the column.

The *Strep*-tag/streptavidin system was systematically optimized over the years, including X-ray crystallographic analysis of the protein–peptide complex[6], development of the optimized *Strep*-tag II (WSHPQFEK), which permits greater flexibility in the choice of the attachment site[7], and engineering of a variant streptavidin with improved peptide-binding capacity[8], dubbed *Strep*-Tactin. As a result, the *Strep*-tag provides a reliable tool in recombinant protein chemistry, which has proven particularly useful for the parallel isolation and functional analysis of multiple gene products in proteome research[1].

Similarly as with other short-affinity tags[9,10], such as the $His_6$ tag[11,12], the calmodulin-binding peptide[13] or the Flag tag[14], the *Strep*-tag II can be easily fused to a recombinant polypeptide during subcloning of its cDNA or gene, and versatile expression vectors are available for various host organisms (http://www.iba-go.com; http://www.qiagen.com; http://www.merckbiosciences.co.uk/html/NVG/streptagindex.htm), especially for *Escherichia coli*[15,16] but also for eukaryotic cells, including yeast[17–19], insect[17], mammalian[17,20,21] and plant cells[22]. A particular benefit of the *Strep*-tag II is that it does not hamper protein folding or secretion and it usually does not interfere with protein function. Thus, the *Strep*-tag is optimally suited for the preparation and analysis of functional proteins whereas it is less useful for the purification of recombinant proteins under strongly denaturing conditions. The better alternative in such cases is the $His_6$ tag, which allows the isolation of recombinant proteins from solubilized inclusion bodies in the presence of 6 M GdnHCl[23].

However, the *Strep*-tag II is largely resistant to cellular proteases, it can be used in the presence of mild detergents and it is biochemically almost inert. This gives a clear advantage over the $His_6$ tag, the calmodulin-binding peptide and the Flag tag[17], whose function moreover is metal ion dependent. Consequently, for most applications, there is no need to cleave the *Strep*-tag II off the protein of interest; nevertheless, vectors with conventional protease recognition sites are available (http://www.iba-go.com). Notably, intact *Strep*-tag II fusion proteins were successfully employed for protein crystallography in several cases[24,25].

*Strep*-tag II fusion proteins are generally eluted from the *Strep*-Tactin affinity column under physiological buffer conditions using a low concentration of a biotin derivative for competition (**Fig. 1**). This not only allows the isolation of sensitive proteins in a native state, but it is also possible to purify intact protein complexes in a preparative manner, even if just one subunit carries the tag[1]. For example, the cytochrome *c* oxidase, an integral membrane protein,

was isolated after complexation with a cognate recombinant antibody fragment equipped with the *Strep*-tag and the crystal structure of the entire assembly was subsequently determined[26]. Furthermore, the *Strep*-tag II was successfully applied for the identification of protein interaction partners in proteomics research[21,22]. In this context, the use of a tandem arrangement of two *Strep*-tag II sequences appeared to be advantageous for higher purification yields of protein complexes, also allowing the application of elevated detergent concentrations to reduce background.

The readily reversible affinity interaction has more recently been used to develop a method that even permits the isolation of live antigen-specific T cells. To this end, multimers of recombinant MHC/*Strep*-tag II fusion proteins charged with an antigenic peptide are formed in the presence of *Strep*-Tactin and used to stain and separate the cognate T-cell population via cytofluorimetry. Monomerization in the presence of D-biotin leads to rapid dissociation of the biomolecular complex, and the liberated T cells are phenotypically and functionally indistinguishable from untreated cells[27].

Owing to the moderate affinity of the *Strep*-tag II peptide for an individual binding site on *Strep*-Tactin, with a dissociation constant around 1 μM (see ref. 8), the formation of a stable complex between the naturally tetrameric streptavidin (or *Strep*-Tactin) and a *Strep*-tag II fusion protein requires an avidity effect, which involves multivalency as well on the side of the recombinant protein. However, the affinity value for monovalent interaction is sufficient for chromatographic purposes, whereby the strength of interaction is determined by the concentration of available binding sites on the chromatography matrix, and it even constitutes an advantage because the accompanying fast dissociation kinetics ensures sharp elution and high chromatographic resolution (**Fig. 2**). Yet, for many other applications, it is desirable to detect a *Strep*-tag II fusion protein with high sensitivity, for example, on a western blot (**Box 1**) or via histochemical staining, or to stably immobilize it on a solid support (**Box 2**).

For detection applications, several *Strep*-Tactin/enzyme conjugates, such as with calf intestine alkaline phosphatase or horseradish peroxidase (HRP), are commercially available (http://www.iba-go.com). These reagents work nicely for detection of the *Strep*-tag II fusion protein in an immobilized state, for example, on a western blot, as an avidity effect can occur under these conditions. Alternatively, monoclonal antibodies—for example, *Strep*MAB-Classic (http://www.iba-go.com),

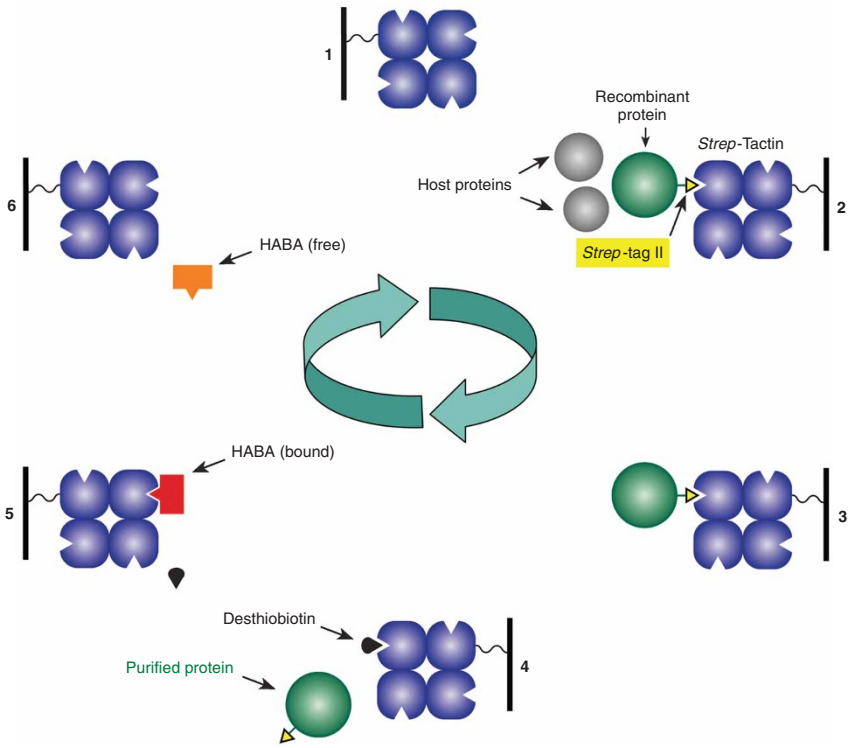


**Figure 1** | Schematic illustration of the *Strep*-tag purification cycle. The cell lysate containing the *Strep*-tag II fusion protein is applied to a column with immobilized *Strep*-Tactin (Steps 1 and 2). After a short washing step with a physiological buffer, host proteins are removed (3) and the purified *Strep*-tag II fusion protein is specifically displaced via competition with a low concentration of D-desthiobiotin (4). D-Desthiobiotin removal is accelerated by application of a HABA solution and indicated via color change from yellow-orange to red (5). Finally, HABA is quickly removed by washing with a small volume of running buffer (6), thus making the column ready for the next purification run (1).

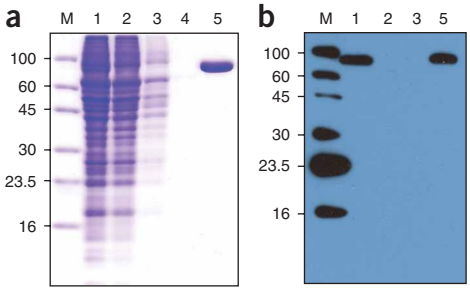


**Figure 2** | Purification of rhtTGase from a mammalian cell lysate. HEK 293 EBNA cells transfected with an expression plasmid encoding rhtTGase fused at its C terminus with the *Strep*-tag II were kindly provided by Dr. Neil Smyth, University of Southampton, UK. The expression plasmid for rhtTGase was a derivative of pCEP4 (Invitrogen), an Epstein–Barr virus-based plasmid that episomally propagates under antibiotic selection and uses the CMV promoter for efficient expression. Cultivation and preparation of the cleared lysate was essentially performed as described before[28]. In the present example, approximately $2 \times 10^9$ cells yielded 2.8 mg purified rhtTGase using a *Strep*-Tactin Superflow column with 5 ml bed volume. (**a**) Analysis of the protein purification using SDS-PAGE stained with Coomassie brilliant blue. Lane 1: cleared lysate (2 μl; 12.5 ml in total) before loading onto the *Strep*-Tactin column. Lane 2: column flow-through (2 μl; 12.5 ml in total). The column was washed five times with 5 ml Buffer W containing 1 mM phenylmethanesulfonyl fluoride (PMSF). Lane 3: pooled washing fractions (2 μl; 25 ml in total). Then, rhtTGase was eluted by the addition of 6 × 2.5 ml Buffer E containing 1 mM PMSF. Lane 4: the first (empty) elution fraction (8 μl; 2.5 ml in total). Lane 5: the pooled elution fractions 2–5 containing the purified protein (8 μl; 10 ml in total). Lane M shows the molecular size standard with masses given in kDa (0.4 μg protein per band). (**b**) Detection of the *Strep*-tag II by *Strep*MAB-Classic HRP conjugate on a western blot according to the protocol given in **Box 1**. SDS-PAGE was first performed as in (**a**), whereby only 2 μl of the pooled elution fractions was applied and the sample of the first elution fraction was omitted.

## BOX 1 | SENSITIVE DETECTION OF A *STREP*-TAG II FUSION PROTEIN ON A WESTERN BLOT BY CHEMILUMINESCENCE

1. After SDS-PAGE, transfer proteins to a nitrocellulose membrane by wet blotting for 16 h at 4 °C using Towbin buffer (25 mM Tris base, 192 mM glycine, 0.05% (w/v) SDS, 20% (v/v) methanol) and a tankblot apparatus operated at a constant current of 0.1 A.
2. Block nonspecific binding sites on the nitrocellulose membrane by incubation with blocking buffer (PBS, 0.1% (v/v) Tween 20, 3% (w/v) BSA) upon agitation at room temperature for 1 h.

■ **PAUSE POINT** Blocking may also be performed for a longer period, for example, overnight, at 4 °C without shaking.

3. Incubate the blot for 1 h in PBS containing 0.2% (w/v) BSA and 0.1% (v/v) Tween 20 containing *Strep*MAB-Classic HRP conjugate at a dilution of 1:32,000.
4. Wash the membrane three times for 5 min each with PBS and 0.1% (v/v) Tween 20 and subsequently once for 5 min with PBS.
5. Detect HRP activity on the nitrocellulose membrane by means of the SuperSignal West Pico chemiluminescence kit according to the instructions of the manufacturer and expose to X-ray film for 1 min.

also available as HRP conjugate, or *Strep*-tag antibody (http://www.qiagen.com)—can be used for sensitive detection of *Strep*-tag II fusion proteins using typical immunochemical methods. Recently, a high-affinity monoclonal antibody—*Strep*MAB-Immo (http://www.iba-go.com)—was developed, which, after immobilization to a solid matrix (e.g., a microtiter plate, paramagnetic beads or a surface plasmon resonance chip), allows the stable capturing of monovalent *Strep*-tag II fusion proteins (**Fig. 3**, **Box 2**).

Taken together, an entire toolbox in conjunction with the *Strep*-tag II is now available, which permits not only the quick purification but also the sensitive detection and tight immobilization of corresponding fusion proteins. Here, we describe a standardized protocol for the isolation of a *Strep*-tag II fusion protein from either a eukaryotic or bacterial cell extract. Recombinant human tissue transglutaminase (rhtTGase), the predominant autoantigen of gluten-sensitive enteropathy[28], serves as a model protein to illustrate the efficiency of one-step protein purification via the *Strep*-tag II from a human embryonic kidney (HEK) cell lysate (**Fig. 2**). In addition, we demonstrate the detection of this protein on a western blot (**Box 1**) as well as its antibody-mediated immobilization on a Biacore chip, thus enabling real-time interaction analysis (**Fig. 3**, **Box 2**).

## MATERIALS

### REAGENTS

- Buffer W (100 mM Tris-HCl pH 8.0, 150 mM NaCl and 1 mM EDTA); EDTA may be omitted from Buffer W in case of metalloproteins
- Buffer E (Buffer W containing 2.5 mM D-desthiobiotin); EDTA may be omitted from Buffer E in case of metalloproteins
- Buffer R (Buffer W containing 1 mM 2-(4-hydroxyphenylazo)benzoic acid (HABA))
- D-Desthiobiotin (IBA) ▲ **CRITICAL** D-Desthiobiotin is synthesized from D-biotin. Commercial preparations may contain residual D-biotin, which inactivates streptavidin as well as the *Strep*-Tactin affinity matrix owing to irreversible complex formation. Ask for a corresponding certificate of analysis.
- HABA (Sigma, #H5126)
- *Strep*-Tactin Superflow, 1 or 5 ml bed volume (IBA)
- Disposable columns (0.5–2 ml bed volume; Pierce, #29920; 3–10 ml bed volume; Bio-Rad, #732-1010)
- PBS (4 mM $KH_2PO_4$, 16 mM $Na_2HPO_4$, 115 mM NaCl, pH 7.4)
- Tween 20 (Sigma, #P7949)
- BSA (Sigma, #A7906)
- *Strep*MAB-Classic HRP conjugate (IBA)
- Rabbit anti-mouse IgG (Biacore, BR-1005-14)
- Nitrocellulose (Protran, Whatman, #BA83 or #BA85)
- SuperSignal West Pico chemiluminescence kit (Pierce, #34077)
- *Strep*MAB-Immo (IBA)
- CM5 sensor chip (carboxydextran surface; research grade; Biacore/GE Healthcare)
- 0.1 M *N*-hydroxysuccinimide (Biacore, #BR-1000-50)
- 0.4 M 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide/HCl, EDC (Pierce, #22980)
- 1 M ethanolamine/HCl (Biacore, #BR-1000-50)
- Buffer B (PBS, 0.005% (v/v) Tween 20)
- Buffer R (10 mM glycine/HCl pH 1.7)

### EQUIPMENT

- Tankblot apparatus (Biometra)
- BIACORE X instrument (Biacore)

### EQUIPMENT SETUP

A disposable column designed for gravity flow chromatography (e.g., Pierce, #29920) may be optimally packed with *Strep*-Tactin resin by performing the steps in **Box 3**.

## PROCEDURE

**1|** Column equilibration: wash the column containing *Strep*-Tactin Superflow matrix with 2 column bed volumes (CV) of Buffer W. Choose a sufficient bed size, for example 1 or 5 ml, to fully capture the recombinant protein present in the amount of cleared lysate to be applied. *Strep*-tag fusion protein capacity is usually in the range of 50–100 nmol $ml^{-1}$ packed resin.

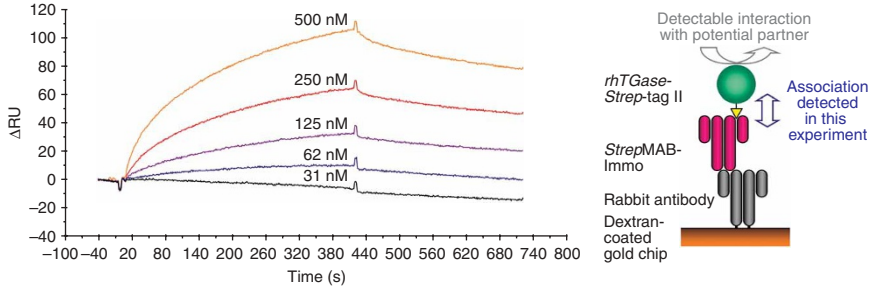


**Figure 3** | Immobilization of rhtTGase carrying the *Strep*-tag II via *Strep*MAB-Immo as detected in a Biacore measurement. *Strep*MAB-Immo was itself captured via a rabbit anti-mouse IgG that had been covalently bound to a CM5 sensor chip. rhtTGase, purified as shown in **Figure 2**, was applied at different concentrations as indicated, and for each measurement cycle the chip was regenerated and freshly charged with *Strep*MAB-Immo according to the protocol described in **Box 2**. Fusion proteins with the *Strep*-tag II, especially when connected via the recommended Ser-Ala linker to their C terminus, are stably immobilized by this procedure, which is useful for real-time association/dissociation studies with interaction partners.

## BOX 2 | ANTIBODY-MEDIATED IMMOBILIZATION OF A *STREP*-TAG II FUSION PROTEIN ON A BIACORE CM5 SENSOR CHIP

▲ CRITICAL STEP It should be noted that for high-affinity recognition by *Strep*MAB-Immo, the eight-residue *Strep*-tag II sequence should be preceded by the N-terminal amino-acid doublet "Ser-Ala," as it is usually encoded on available expression vectors for C-terminal *Strep*-tag II fusion proteins.

1. Set up the BIACORE X instrument at a flow rate of 5 μl $min^{-1}$ using running Buffer B.
2. Activate the chip surface by injection of 1:1 EDC/NHS (see REAGENTS) for 7 min.
3. Immobilize the antibody by injection of 50 μg $ml^{-1}$ *Strep*MAB-Immo, diluted (from a 1 mg $ml^{-1}$ stock solution) into 10 mM Na-acetate pH 4.5, for 7 min.
4. Deactivate residual reactive groups by injecting ethanolamine/HCl for 7 min. Using this procedure, a signal of typically around 15,000 RU (approximately 15,000 pg $mm^{-2}$, corresponding to 0.1 pmol antibody) is generated owing to the immobilization of *Strep*MAB-Immo. The CM5 sensor chip is now ready for capturing by applying a *Strep*-tag II fusion protein in Buffer B.

OPTION: Owing to the high affinity of *Strep*MAB-Immo for *Strep*-tag II fusion proteins, it may not be possible to regenerate the chip without loss of binding activity. In this case, the following protocol for indirect immobilization is recommended.

1. Activate the surface by injection of EDC/NHS (1:1) for 7 min as above.
2. Immobilize rabbit anti-mouse IgG, diluted at a concentration of 50 μg $ml^{-1}$ (from a 1 mg $ml^{-1}$ stock solution), into 10 mM Na-acetate pH 5.0, for 7 min.
3. Block residual reactive groups by injection of ethanolamine/HCl for 7 min. Using this procedure, a signal of typically around 6,000 RU (approximately 6,000 pg $mm^{-2}$, corresponding to 0.04 pmol antibody) is generated owing to the immobilization of the rabbit anti-mouse IgG. The CM5 sensor chip is now ready for reversible capturing of the *Strep*MAB-Immo.
4. Apply 35 μl *Strep*MAB-Immo, diluted to 25 μg $ml^{-1}$ in Running Buffer B, for 7 min.
5. Usually, a signal of typically around 500 RU is generated owing to the immobilization of *Strep*MAB-Immo. The chip is now ready to capture a *Strep*-tag II fusion protein and to subsequently record a sensorgram of the interaction between such a *Strep*-tag II fusion protein and a cognate ligand.
6. Perform a series of sensorgrams at different ligand concentrations using the same chip by regenerating the chip with a pulse of Regeneration Buffer R for 3 min, followed by first charging with *Strep*MAB-Immo again and then immobilizing the *Strep*-tag II fusion protein.

**2|** Column loading: apply the cleared lysate prepared after expression of the recombinant *Strep*-tag II fusion protein in bacterial (cf. **Box 4**) or mammalian cells (**Box 5**) to the affinity column. Use a flow rate of 0.5 CV $min^{-1}$ for all steps as starting condition (in case of 1 ml or smaller bed volume, a higher flow rate of 1 CV $min^{-1}$ may be applied). Work at a temperature that assures stability of the target protein, preferentially 4 °C.

▲ CRITICAL STEP When using gravity flow columns that cannot run dry owing to a built-in stop flow mechanism (e.g., Pierce #29920), the liquid should be completely drained before the next step. This is especially recommended for the cleared lysate, where care should be taken that no residue is left in the column reservoir before application of Buffer W.

## BOX 3 | PACKING A DISPOSABLE COLUMN WITH *STREP*-TACTIN RESIN

1. Equilibrate Buffer W and the wet *Strep*-Tactin resin to ambient temperature.
2. Close the column with the bottom cap, fill it with Buffer W up to the upper reservoir section, float a porous disc on top of the liquid and push it to the column bottom with a suitable glass rod or the blunt end of a Pasteur pipette.
3. Decant Buffer W, fill the column with a desired volume of *Strep*-Tactin slurry (e.g., 2 ml of a 50% suspension), allow any air bubbles that may have formed to ascend by gentle stirring and open the column by removing the bottom cap.
4. Shortly before the resin has fully settled, close the column with the bottom cap again and carefully overlay the resin with Buffer W up to the lower rim of the reservoir section, that is, ca. 2 ml.
5. Let the residual resin settle by gravity.
6. Float another porous disc on top of the liquid and carefully push it on top of the resin.
7. Fill the entire column, including the reservoir, with Buffer W, remove the bottom cap, drain to a residual volume of ca. 1 ml and close the outlet again with the bottom cap.
8. Let the column stand for 24 h. The resin will slightly contract during this period and a small void will form between the upper porous disc and the resin.
9. Readjust the upper porous disc. Any superfluous volume between the upper disc and the gel bed causes a gradient during buffer changes at the different chromatographic steps, which reduces purification efficiency.
10. To use the column for purification, drain any Buffer W and apply the cleared lysate containing the *Strep*-tag II fusion protein. For storage, fill the column with Buffer W up to the lower rim of the reservoir section and close it by applying first the bottom cap and then the top cap. If microbial growth is a problem, 0.02% (w/v) $NaN_3$ may be added as a preservative to Buffer W.

! CAUTION For opening a sealed column, always remove the top cap first, otherwise air will be soaked into the column bed.

## BOX 4 | CYTOPLASMIC EXPRESSION OF A *STREP*-TAG II FUSION PROTEIN IN *ESCHERICHIA COLI* AND PREPARATION OF A CLEARED CELL LYSATE

**Protein expression**

Numerous plasmids for the expression of *Strep*-tag II fusion proteins are available from various companies (IBA, Novagen, Qiagen). Most of them carry the β-lactamase (ampicillin resistance) gene as selectable marker and expression is controlled by the tightly regulated tetracycline promoter/operator[15]. Hence, we describe here expression by means of such a plasmid[1].

1. Preculture: inoculate 2 ml of Luria–Bertani medium (LB)[33] containing 100 μg $ml^{-1}$ ampicillin with a fresh colony harboring the expression plasmid and shake overnight (200 r.p.m.) at 37 °C.

▲ CRITICAL STEP The colony should not be older than 1 week. Do not inoculate from glycerol stocks. The yield of soluble, functional protein may be substantially increased by lowering the growth temperature to between 22 and 30 °C. Take care that cells do not reach the stationary phase for extended periods before inoculation of the main culture, especially when using ampicillin as antibiotic for selection. Ampicillin is rapidly hydrolyzed by the periplasmic β-lactamase and selection pressure is thus abolished. This aspect is even more important if expression plasmids with leaky promoters such as the *lac* promoter are used and the encoded protein has a toxic effect on the host cell.

2. Culture for expression: inoculate 100 ml LB medium containing 100 μg $ml^{-1}$ ampicillin with 1 ml of the preculture and shake at 37 °C or, if appropriate, at a lower temperature.

3. When the $OD_{550}$ reaches 0.5–0.6, add 10 μl of an anhydrotetracycline (aTc) stock solution (2 mg $ml^{-1}$ aTc dissolved in ethanol).

4. Shake for 1 h in the case of expression at 37 °C or for 3 h when using a temperature of 30 °C or lower.

▲ CRITICAL STEP Optimal duration of induction is dependent on the individual recombinant protein and on whether cytoplasmic or periplasmic expression is intended. Thus, the period recommended above can often be optimized and may reach up to 16 h (overnight).

**Preparation of a cleared lysate**

1. Harvest the 100 ml culture by centrifugation (4,500*g*, 15 min, 4 °C) and resuspend cells with 1 ml Buffer W. Add protease inhibitors if necessary.

■ PAUSE POINT As it may be difficult to resuspend partially lysed cells, which usually happens when directly freezing the pellet, it may be advantageous to store the cells in suspension at −20 °C or a lower temperature.

2. Lyse cells via sonication or by means of a French press while chilling.

▲ CRITICAL STEP Take care that the suspension does not get warm, which may denature proteins or activate proteases. Lysis should be as complete as possible, for example by means of repeated passages through the French pressure cell, and may be monitored according to the optical density at 590 nm using the formula % lysis $= (1 - OD_{590}^{sonicate}/OD_{590}^{suspension}) \times 100$.

3. Add DNase (5 μg $ml^{-1}$) or Benzonase to degrade high molecular weight DNA or RNA if the lysate is still viscous and incubate on ice for 15 min.

4. As an alternative to Steps 2 and 3, lyse cells chemically with commercial lysis buffers. For example, B-Per (Pierce #78243) has been shown to be compatible with *Strep*-tag purification in case of *Staphylococcus aureus* GAPDH and chicken ovalbumin and Bug Buster (EMD Biosciences #70584) was used in the context of a recombinant Fab fragment carrying the *Strep*-tag II. IBA-Lyse (IBA #2-1017) is another chemical lysis buffer, which has been specially developed for *Strep*-tag affinity purification.

▲ CRITICAL STEP Such buffers usually contain a mild detergent, DNase, lysozyme and EDTA. Although chemical lysis is more reproducible than sonication, some of these additives may cause problems with protein integrity and subsequent assays.

5. Centrifuge in a benchtop microfuge (13,000 r.p.m., 4 °C, 15 min) to remove cell debris and transfer the resulting clear supernatant into a clean vessel. Take an aliquot (2 μl) for SDS-PAGE analysis and store it at −20 °C or below.

■ PAUSE POINT The supernatant can be stored at −20 °C or below until affinity chromatography. Before affinity chromatography, it should be thoroughly centrifuged again or passed through a 0.4 μm sterile filter to protect the affinity column from aggregates that may have formed.

**3|** Column washing: after the cell extract has completely entered the column, wash with 5 CV Buffer W (in case of a gravity flow column, five batches of 1 CV each should be applied) and collect the eluate in fractions of 1 CV. Apply 2 μl of the first washing fraction and 20 μl of each subsequent fraction to an analytical SDS-PAGE.

**4|** Protein elution: elute the recombinant *Strep*-tag II fusion protein with 3 CV (six times 0.5 CV in case of a gravity flow column) Buffer E and collect the eluate in fractions of 0.5 CV. Analyze 20 μl samples of each fraction by SDS-PAGE. The purified *Strep*-tag II fusion protein usually elutes in the 2nd to 5th fraction. If necessary, remove D-desthiobiotin and EDTA from the purified recombinant *Strep*-tag II fusion protein by dialysis or by using disposable desalting columns.

**5|** Column regeneration: in a first step, remove D-desthiobiotin from the column by washing with 15 CV Buffer R. Buffer R contains HABA, whose red-colored hydrazone isomer gets complexed at the biotin-binding site of *Strep*-Tactin[29]. In this manner, D-desthiobiotin removal is accelerated by preventing rebinding. Full color change of the column bed to red indicates completion of this process. In the final step, remove HABA by washing with 8 CV Buffer W. After destaining, the column is ready for the next purification run.

## BOX 5 | EXPRESSION OF A *STREP*-TAG II FUSION PROTEIN IN EUKARYOTIC CELL CULTURE AND PREPARATION OF A CLEARED CELL LYSATE

**Protein expression**

There exist various strategies for expression of proteins in mammalian cells. While transient expression is fast and may lead to good expression yields—as far as episomal plasmid propagation is possible when using suitable expression plasmids in combination with appropriate host cells and antibiotic selection (see for example, **Fig. 2**)—the generation of a stable expression cell line is recommendable if higher reproducibility from batch to batch is desired.

1. Propagate cells in the appropriate medium. Transfect at least $10^9$ cells for transient protein expression or harvest a similar amount of stably transfected cells if preparation of the recombinant protein in the milligram range is desired.

**Preparation of a cleared lysate**

1. Centrifuge the cells as appropriate and resuspend them in 4 ml Buffer W per $10^9$ cells. In general, addition of protease inhibitors such as Complete protease inhibitor tablets, EDTA-free (Roche #1873580) is recommended.
2. Lyse cells by repeated freeze/thaw cycles: freeze cells in liquid nitrogen and thaw them in a 37 °C water bath; repeat this cycle five times.
3. Shear DNA by passing the lysate four times through an 18-gauge needle. If the lysate is very viscous, add RNase (10 μg $ml^{-1}$) and DNase (5 μg $ml^{-1}$) and incubate on ice for 15 min.

▲ **CRITICAL STEP** Note that various protocols are available for eukaryotic cell lysis. Milder methods can help to keep cell compartments intact, which may be desirable if proteolytic degradation is a problem.

4. Centrifuge lysate at 3,000*g* for 15 min at 4 °C to pellet the cellular debris and transfer the clear supernatant into a clean vessel. Take an aliquot (10 μl) for SDS-PAGE analysis and store it at −20 °C or below. Depending on the individual case, higher *g* values may be necessary to remove the aggregates and fully clear the lysate.

■ **PAUSE POINT** The supernatant can be stored at −20 °C or below until affinity chromatography. After thawing and before affinity chromatography, it should be centrifuged again as above.

● **TIMING**

Preparation of a cleared lysate as applicable (**Boxes 4** and **5**): 60–90 min
Column chromatography, including regeneration: 30–60 min

**? TROUBLESHOOTING**

**Low protein yield**

The volume of the lysate should be between 0.5 and 10 CV, maximally up to 20 CV. Extracts of large volumes with the recombinant protein at low concentration may cause reduced yields, owing to leaching, and should thus be concentrated before chromatography. However, care should be taken that no protein precipitate is formed in the lysate. If quantification is possible, a content between 50 and 100 nmol of the recombinant *Strep*-tag II fusion protein in the cleared lysate per 1 ml *Strep*-Tactin Superflow matrix is optimal.

Check the pH of the cleared lysate and also of Buffer W. Below pH 7, the *Strep*-tag II exhibits reduced affinity for streptavidin or *Strep*-Tactin owing to protonation of its central His residue[30]. In fact, the *Strep*-tag II fusion protein may be eluted at pH 5.0 as an alternative to competition with D-desthiobiotin.

*Strep*-Tactin columns become inactivated by D-biotin. Such inactivation is indicated during the regeneration process with HABA[29] if the upper zone of the column does not turn red. In this case, check whether the cleared lysate is contaminated with free biotin. Cytosolic extracts usually do not contain significant amounts of free biotin, in contrast to synthetic culture media for mammalian cells. For example, the widely used Eagle basal medium contains 1 mg $liter^{-1}$ biotin[31]. Thus, biotin contamination may originate from insufficient removal of media components after cell harvest.

**Biotinylated protein contamination**

Covalently biotinylated host proteins, for example, the biotin carboxyl carrier protein of *E. coli*[32], are present in cytosolic extracts at very low amounts and should not cause a marked reduction of resin capacity. Because biotin binds by several orders of magnitude more tightly to *Strep*-Tactin than the *Strep*-tag II peptide, these proteins will stay bound to the column and not elute together with the *Strep*-tag II fusion protein. However, the situation can change after repeated use of the column. Then small amounts of biotinylated proteins may coelute. Such small amounts are almost not detectable in Coomassie-stained SDS-PAGE but may become visible on western blots. Biotinylated host proteins can, however, be efficiently masked by the addition of avidin to the cleared lysate[1,2]. This may result in a precipitate, which has to be removed by centrifugation before affinity chromatography. As a rule of thumb, apply a twofold molar excess of avidin (calculated for the monomer) with respect to the biotinylated host protein(s). A cleared lysate of *E. coli* derived from a 1 liter culture with $OD_{550} = 1$ contains approximately 1 nmol biotin carboxyl carrier protein. Masking of biotinylated host proteins by avidin may be particularly helpful to reduce background in protein–protein interaction studies[22].

### Low flow rates

When the packed column was stored at 4 °C but chromatography is intended at room temperature (20–25 °C), take care that the column is washed immediately with Buffer W adapted to ambient temperature. Otherwise, air bubbles may form in the column bed and impair flow rates such that the column has to be repacked as described in **Box 3**.

### Impurities resulting from host cell proteins

*Strep*-tag affinity chromatography leads to very high enrichment factors, yielding purities of up to greater than 99% in a single step. Thus, if present, impurities are generally caused by host proteins interacting with the recombinant *Strep*-tag II fusion protein itself. Depending on the nature of these specific or nonspecific interactions, which may be ionic, metal ion-induced, hydrophobic or even covalent, for example, via disulfide bond formation, impurities may be avoided by adding—to the cell lysate and/or to Buffer W—higher concentrations of NaCl, EDTA, mild detergents or reducing agents, respectively (cf. **Table 1**). To test whether an additive has the desired effect and to optimize its concentration, apply increasing concentrations of a corresponding solution to the column directly after the protein lysate application and washing step and collect the eluate for each concentration, using 1 CV per step. SDS-PAGE analysis will reveal whether the impurities can be selectively eluted and which additive concentration will be tolerated by the *Strep*-tag:*Strep*-Tactin interaction for the protein of interest.

**TABLE 1** | Reagents and their concentrations for purification of several *Strep*-tag (II) fusion proteins with concentrations up to the values mentioned.

| Reagent | Concentration |
|---|---|
| **Reducing agents** | |
| DTT | 50 mM |
| β-Mercaptoethanol | 50 mM |
| **Non-ionic detergents** | |
| $C_8E_4$ | max. 0.88% |
| $C_{10}E_5$ | 0.12% |
| $C_{10}E_6$ | 0.03% |
| $C_{12}E_8$ | 0.005% |
| $C_{12}E_9$ (Thesit) | 0.023% |
| Decyl-β-D-maltoside | 0.35% |
| *N*-dodecyl-β-D-maltoside | 0.007% |
| *N*-nonyl-β-D-glucopyranoside | 0.2% |
| *N*-octyl-β-D-glucopyranoside | 2.34% |
| Triton X-100 | 2% |
| Tween 20 | 2% |
| **Ionic detergents** | |
| *N*-lauryl-sarcosine | 2% |
| *N*-octyl-2-hydroxy-ethylsulfoxide | 1.32% |
| SDS, sodium *N*-dodecyl sulfate | 0.1% |
| **Zwitter-ionic detergents** | |
| CHAPS | 0.1% |
| *N*-decyl-*N*,*N*-dimethylamine-*N*-oxide | 0.034% |
| *N*-dodecyl-*N*,*N*-dimethylamine-*N*-oxide | 0.13% |
| **Other additives** | |
| Ammonium sulfate $(NH_4)_2SO_4$ | 2 M |
| $CaCl_2$ | max. 1 M |
| EDTA | 50 mM |
| Ethanol | 10% |
| Guanidine/HCl | max. 1 M |
| Glycerol | max. 25% |
| Imidazole | max. 250 mM |
| $MgCl_2$ | 1 M |
| NaCl | 5 M |
| Urea | max. 1 M |

The label "max." indicates an upper limit and even higher concentrations may be possible. Nevertheless, these values should be considered merely as a recommendation. As binding strength depends on steric accessibility and the protein context of the *Strep*-tag II, compatible working concentrations may vary considerably for individual proteins.

## ANTICIPATED RESULTS

With the protocol described, usually 1–2 mg of a mid-sized protein (ca. 20 kDa) can be obtained with a purity greater than 95% by using a 1 ml bed volume affinity column. Performance of the standard procedure is illustrated in **Figure 2** (and **Box 1**) for the purification of rhtTGase using a 5 ml *Strep*-Tactin gravity flow column. The *Strep*-tag II has been used successfully in numerous examples for the efficient purification of all types of proteins including membrane proteins. As a broad spectrum of buffer additives is possible (**Table 1**), the prerequisites for purifying most proteins under quasi-native conditions can usually be fulfilled. The method is compatible with almost any expression host, probably because the *Strep*-tag II is an artificial sequence, which does not interfere with cellular processes.

The *Strep*-tag affinity chromatography can be easily adjusted in scale without changing its general characteristics. Once optimal loading of the resin with the recombinant *Strep*-tag II fusion protein has been determined at a particular scale—such as the one described in our protocol—up- or downscaling may be simply achieved by multiplying all parameters with the desired factor. If, for example, ten times the amount of recombinant protein has to be purified, just use ten times the amount of cleared lysate and a column having ten times the bed volume of the initial column. Washing the column with 5 CV Buffer W is generally sufficient and protein elution is accomplished by applying 3 CV Buffer E (take fractions of 0.5 CV). The recombinant *Strep*-tag II fusion protein usually elutes in fractions 2–5, with most of the recombinant protein (>80%) present in fractions 3 and 4.

When performing protein purification on a case-by-case basis, the use of gravity flow columns is advantageous as no setup of specialized equipment is necessary. Also, a gravity flow column equipped with appropriate filter discs needs no observation as it cannot run dry and application of surplus washing buffer is impossible. In our laboratories, gravity flow columns with bed volumes between 0.2 and 50 ml are in operation for routine purification up to the 100 mg range. Nevertheless, *Strep*-Tactin affinity matrix packed in pressure-resistant cartridges for purification on HPLC/FPLC workstations is available as well. After the workstation is set up, the *Strep*-tag II fusion protein purification run can be completed in several minutes. Thus, the use of

such workstations can be advantageous for high-throughput purification of *Strep*-tag fusion proteins. Furthermore, for purposes of highly parallel operation, prepacked 96-well affinity columns are commercially available (http://www.iba-go.com).

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