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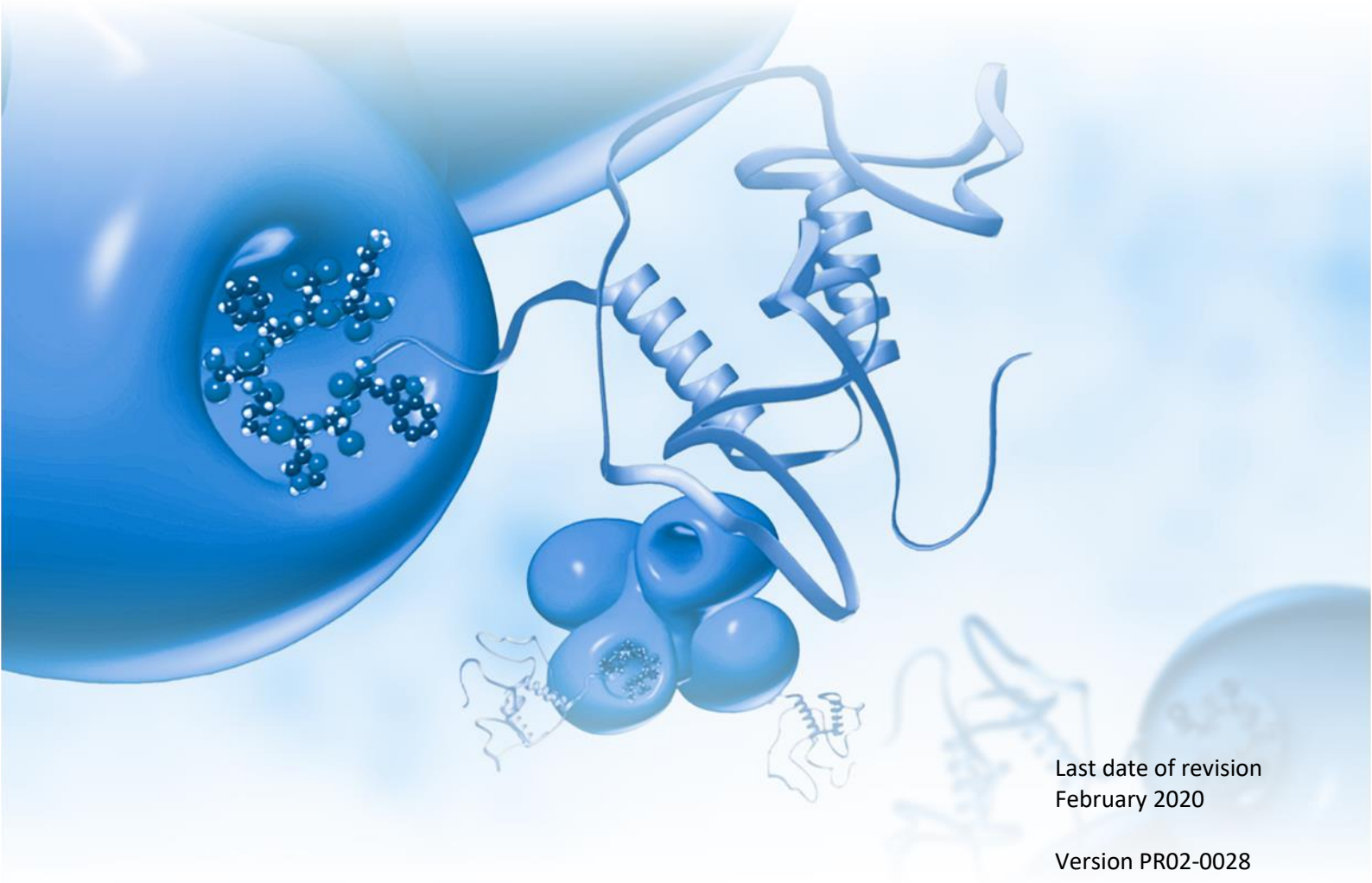
Expression and purification of proteins using Strep-Tactin®

A comprehensive manual



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For Strep-tag® purification from **mammalian cells** a separate manual is available (MEXi – mammalian expression manual).
Manuals are available for download under
www.iba-lifesciences.com/download-area.html.

1 Introduction

1.1 Strep-tag® system overview

The Strep-tag®II is a short peptide (8 amino acids, WSHPQFEK), which binds with high selectivity to Strep-Tactin®, an engineered streptavidin. The binding affinity of Strep-tag®II to Strep-Tactin® (K_d = 1 µM) is nearly 100 times higher than to streptavidin. This technology allows one-step purification of almost any recombinant protein under physiological conditions, thus preserving its bioactivity. The Strep-tag® system can be used to purify functional Strep-tag®II proteins from any expression system including baculovirus, mammalian cells, yeast, and bacteria [1, 2, 3].

Twin-Strep-tag®

Based on the proprietary Strep-tag® technology the **Twin-Strep-tag®** was developed which has a size of 30 amino acids.
The Twin-Strep-tag® combines high specificity and mild conditions with **higher affinity** thereby enabling efficient **purification even in batch** or directly from culture supernatants. Furthermore, it tolerates diverse buffer conditions and additives (high salt, detergents, reducing agents, metal ions and chelating agents) making it a universal tag for varying protein properties, particularly for protein complexes in protein interaction analysis.

After application of the crude extract on a Strep-Tactin® column and a short washing step, gentle elution of purified recombinant protein is performed by addition of low concentrations of desthiobiotin (recommended 2.5 mM). The Strep-Tactin®:Strep-tag®II interaction is compatible with a variety of reagents (see Table 1) making the system attractive for purifying metallo- and membrane proteins, large proteins and protein complexes.
Binding capacity depends on the Strep-Tactin® matrices (25 - 100 nmol/ml using standard Strep-Tactin® resins and up to 500 nmol/ml using Strep-Tactin® Superflow® High Capacity) as well as on the fused recombinant protein. Because of its small size, Strep-tag®II generally does not interfere with the bioactivity of the fusion partner. Thus, removal of the tag becomes superfluous. Comprehensive reviews and scientific publications giving an overview of various Strep-tag® applications are listed at www.strep-tag.com.

1.2 Strep-Tactin® protein purification principle

The basis for the development of the Strep-tag® principle was the well known binding of biotin to streptavidin (Fig. 1). To take advantage of this strong interaction in protein purification applications we found it desirable to have a peptide that is capable of binding to the biotin binding pocket of streptavidin when fused to recombinant proteins. This peptide was supposed to serve as purification tag. The finally engineered short sequence consists of only 8 amino acids (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) and was named Strep-tag®II.
To optimize binding properties, also streptavidin has been engineered to obtain Strep-Tactin®. Thus, the optimal binding partners have been found: **The Strep-tag® system is now one of the most widely used affinity chromatography systems**.

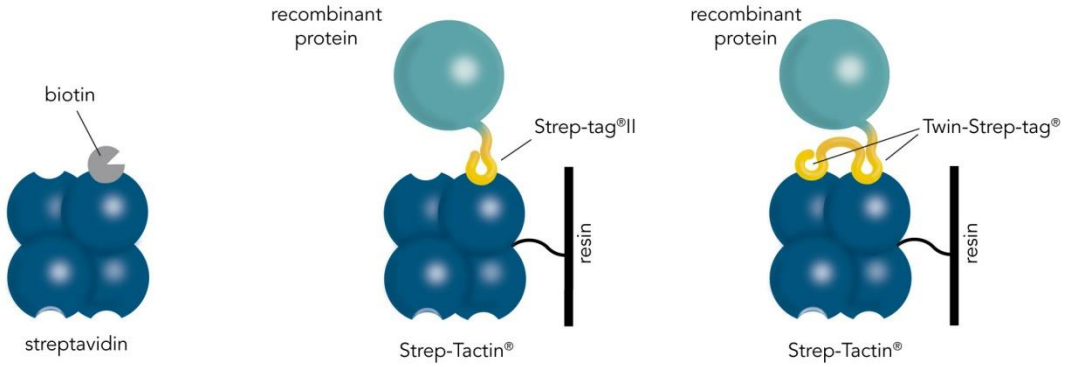


Fig. 1: Schematic view of the Strep-tag®II and Twin-Strep-tag® protein purification principle

Strep-tag®II

The Strep-tag®II is a short peptide tag (8 amino acids, WSHPQFEK) with negligible effect on the recombinant protein due to its chemically balanced amino acid composition (WSHPQFEK). The tag can be placed at the C- or N-terminus. A two amino acid spacer (SerAla) between the protein and the tag is recommended to ensure accessibility of the tag. Generally, it does not interfere with folding or bioactivity, does not react with heavy metal ion buffer impurities, has no ion exchange properties and does not induce protein aggregation. Thus, there is no need for removing the tag.

Twin-Strep-tag

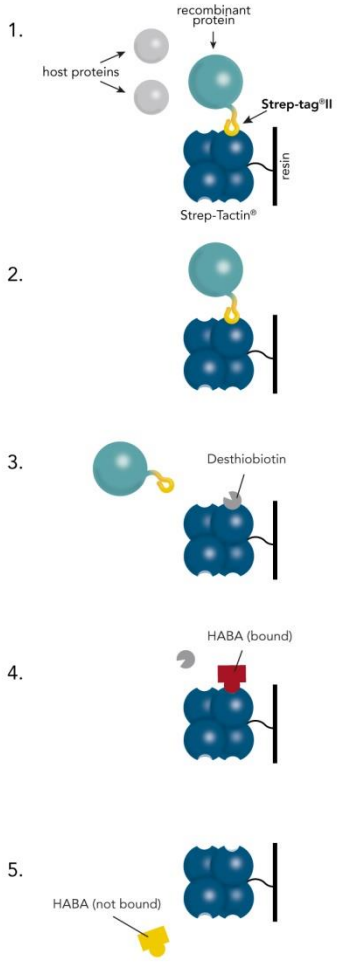
The Twin-Strep-tag® (**WSHPQFEK**-GGGSGGGSGG-SA**WSHPQFEK**) is a tandem version of the Strep-tag®II with an internal linker region. This twin version with the same specificity but higher affinity to Strep-Tactin® enables efficient purification even in batch or directly from culture supernatants.

Strep-Tactin®

Strep-Tactin® is a streptavidin derivative which is one of the most stable proteins known.

1.3 The Strep-Tactin® protein purification cycle

The purification of Strep-tag®II fusion proteins is easy, straightforward and user-friendly. The complete procedure can be performed under nearly physiological conditions, e.g., in PBS. Physiological buffers can also be used in combination with a wide range of additives (see Table 1). For elution 2.5 mM desthiobiotin is added to the buffer (Fig. 2). Desthiobiotin is an inexpensive, reversibly binding and stable analog of biotin - the natural ligand of streptavidin - and serves for the competitive elution of Strep-tag®II proteins.
The system is safe and easy to use since column regeneration and activity status are visualized by a color change on the purification column (see page 31).



Steps 1 + 2: The cell lysate is added to the column. Once the tagged protein has bound specifically to Strep-Tactin® the host proteins are washed away rapidly with small amounts of physiological wash buffer (Buffer W).

Step 3: Then, bound Strep-tag®II protein is gently eluted by wash buffer containing 2.5 mM desthiobiotin (Buffer E) which specifically competes for the biotin binding pocket.
Since the buffer conditions during elution essentially remain unchanged, potentially unspecific binding proteins (without Strep-tag®II) will not be eluted and, thus, will not contaminate the protein of interest. Next to the specific binding of Strep-tag®II to Strep-Tactin®, this is the second specificity conferring step of this purification procedure, yielding extremely high protein purity.

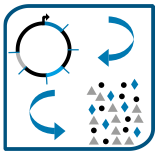
Step 4: To regenerate the column the yellow azo dye HABA (2- [4'-hydroxy-benzeneazo] benzoic acid) is added (Buffer R) in excess to displace desthiobiotin from the binding pocket. Once HABA binds to the binding site, the color turns to red conveniently indicating the regeneration and activity status of the column.

Step 5: HABA can be removed simply by adding wash buffer. Once the red color has disappeared the column can be re-used. Strep-Tactin® resin can be regenerated and re-used 3 to 5 times without loss in performance.

Fig. 2: Strep-Tactin® purification cycle

For Strep-tag® purification from **mammalian cells** a separate manual is available.
Manuals are available for download under:
www.iba-lifesciences.com/download-area.html.

2 Expression



2.1 Expression in *E. coli* with the *tet*-system (pASK-IBA/pASG- IBA vector series)

General considerations

The production of a heterologous protein is often accompanied by an impaired growth of *E. coli* cells. Consequently, regulation of heterologous biosynthesis is generally recommended by the use of a promoter whose activity can be blocked by a repressor. If the foreign protein is cytotoxic, even the production of minute quantities can result in a dramatic selection against the *E. coli* cells which harbor the expression plasmid. In such cases, tight repression of the promoter is required. Synthesis of the gene product is then switched on in a controlled manner simply by adding a chemical inducer. The Strep-tag®II expression vectors pASK-IBA/pASG-IBA that carry the promoter/operator region from the *tet*A resistance gene are the state-of-the-art solution for such an inducible expression system [4,5]. The strength of the *tet*A promoter is comparable with that of the *lac*-UV5 promoter (nearly 25 % activity of the T7 promoter).

It can be fully induced by adding anhydrotetracycline at a concentration that is not antibiotically effective (200 ng/ml). The constitutive expression of the *tet* repressor gene, which is also encoded on the expression plasmids, guarantees the repression of the promoter in the absence of the inducer. In a Western blot, no expression is detectable under these conditions [4,6]. In contrast to the *lac* promoter, which is susceptible to catabolite repression (cAMP-level, metabolic state) and chromosomally encoded repressor molecules, the *tet*A promoter/operator is not coupled to any cellular regulation mechanisms. Therefore, when using the *tet* system, there are basically no restrictions in the choice of culture medium or *E. coli* expression strain. For example, glucose minimal media and even the bacterial strain XL1-Blue, which carries an episomal copy of the tetracycline resistance gene, can be used for expression.

Recommended Buffers/Solutions	Concentration of ingredients	Notes
Ampicillin (pASG-IBA vectors, pASK-IBA vectors except pASK-IBA2C, 3C, 4C, 5C, 6C, 7C)	stock solution 100 mg/ml in H_2O, sterile filtered	Store in aliquots at -20°C
Chloramphenicol (pASK-IBA2C, 3C, 4C, 5C, 6C, 7C)	stock solution 30 mg/ml in ethanol	Recommended for fermentation at high cell densities. Store at -20°C.
Anhydrotetracycline	stock solution 2 mg/ml in Dimethylformamid (DMF)	Store at -20°C
LB medium	10 g/l trypton 5 g/l yeast extract 5 g/l NaCl	

Recommended Buffers/Solutions (continued)	Concentration of ingredients	Notes
Buffer W	100 mM Tris/HCl, pH 8.0 150 mM NaCl 1 mM EDTA	
5x SDS-PAGE sample buffer	0.250 M Tris/HCl, pH 8.0 25 % glycerol 7,5 % SDS 0.25 mg/ml bromophenolblue 12.5 % v/v mercaptoethanol	

Important notes

The *tet* promoter system is independent of the *E. coli* strain. Following strains were successfully tested: JM83, WK6, B, BL21, MG1655, W3110, XL1-Blue, BL21-CodonPlus™

→ We recommend JM83 or W3110 for periplasmic secretion.

Protocol

1. **Preculture: Inoculate 2 ml of LB medium containing 100 µg/ml Ampicillin (pASG/pASK-IBA plasmids except 2C to 7C) or 30 µg/ml Chloramphenicol (pASK-IBA2C to 7C) with a fresh colony harboring the pASG/pASK-IBA expression plasmid and shake overnight (200 rpm) at 37°C.**

   The colony should not be older than 1 week.
   We recommend using overnight colonies.
   Do not inoculate from glycerol stocks.

   The yield of soluble, functional protein can be substantially increased in most cases by lowering the preculture growth temperature to 22°C - 30°C. Take care that cells do not reach the stationary phase for extended periods prior to inoculating the production culture.

2. **Culture for expression: Inoculate 100 ml of LB medium containing 100 µg/ml Ampicillin (or 30 µg/ml chloramphenicol, depending on the plasmid used) with the preculture and shake at 37°C.**

3. **Monitor the optical density at 550 nm (OD_{550}).**
   Cell suspension samples with an OD_{550} over 1.0 should be diluted with LB medium before measuring.

4. **Take a 1 ml sample immediately before induction.**
   This sample is the non-induced control; pellet cells (microfuge, 30 seconds) and resuspend them in 80 µl Buffer W. Add 20 µl 5x SDS-PAGE sample buffer and mix. Store at -20°C until SDS-PAGE analysis.

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Protocol (continued)	Prior to SDS-PAGE the whole sample should be incubated in an ultrasonic bath for 15 minutes to shear the chromosomal DNA into small pieces and should be heated to 70°C for 10 minutes. **5. When OD_{550} equals 0.5-0.6, add 10 µl of anhydrotetracycline stock solution.** The yield of soluble, functional protein may be substantially increased, particularly in case of periplasmic expression, by lowering the growth temperature to 22°C - 30°C. **6. Shake for 3 hours at 200 rpm.** Overnight induction may increase protein yields in some cases. **7. Harvest the cells by centrifugation at 4500 x *g* for 12 minutes (4°C).** **Proceed with chapter 3 (Preparation of cleared lysates) or store cell pellet at -20°C**

2.2 Expression in *E. coli* with the T7-system (pPSG-IBA vector series)

The system uses the T7 promoter and T7 RNA polymerase for high-level transcription of the gene of interest. As the T7 promoter is stronger than the tet promoter, pPR-IBA vectors can be recommended in cases where expression with the tet promoter does not lead to significant yields of the recombinant protein. In other cases, T7 expression may cause insoluble inclusion bodies. In such cases the tet promoter might be a good alternative if the expression of soluble protein is desired.

Expression of the target genes is induced by providing a source of T7 RNA polymerase in the *E. coli* host cell (DE3 lysogen). This is accomplished by using e.g. *E. coli* BL21 (DE3) host which contains a chromosomal copy of the T7 RNA polymerase gene (Novagen, Invitrogen). The T7 RNA polymerase gene is under control of the lacUV5 promoter which can be induced by IPTG [6, 7].

Recommended Buffers/Solutions	Concentration of ingredients	Notes
Ampicillin (pPSG-IBA vectors, pPR-IBA vectors)	stock solution 100 mg/ml in H_2O, sterile filtered	Store in aliquots at -20°C
Buffer W	100 mM Tris/HCl, pH 8.0 150 mM NaCl 1 mM EDTA	
LB medium	10 g/l trypton 5 g/l yeast extract 5 g/l NaCl	
Glucose	20 %, sterile filtered	

Recommended Buffers/Solutions (continued)	Concentration of ingredients	Notes
IPTG	stock solution (1M): 238 mg/ml in H_2O, sterile filtered	Store in aliquots at -20°C
5x SDS-PAGE sample buffer	0.250 M Tris/Cl, pH 8.0 25 % glycerol 7,5 % SDS 0.25 mg/ml bromophenolblue 12.5 % v/v mercaptoethanol	

Protocol

1. **Preculture: Inoculate 2 ml of LB medium containing 100 µg/ml Ampicillin with a fresh colony harboring the pPSG expression plasmid and shake overnight (200 rpm) at 37°C.**

   The colony should not be older than 1 week.
   We recommend using overnight colonies.
   Do not inoculate from glycerol stocks.

   The yield of soluble, functional protein can often be substantially increased by lowering the preculture growth temperature to 22°C - 30°C. Take care that cells do not reach the stationary phase for extended periods prior to inoculating the production culture.

   In case of toxic proteins, the leakiness of the lacUV5 promoter and the resulting expression may lead to cell death or to the selection of non-productive mutants. Add 2 % glucose and/or use pLysS or pLysE cotransformants in such cases [7].

2. **Culture for expression: Inoculate 100 ml of LB medium containing 100 µg/ml ampicillin with the preculture and shake at 37°C.**

3. **Monitor the optical density at 550 nm (OD_{550}).**
   Cell suspension samples with an OD_{550} over 0.3 should be diluted with LB medium before measuring.

4. **Take a 1 ml sample immediately before induction.**
   This sample is the non-induced control; pellet cells (microfuge, 30 seconds) and resuspend them in 80 µl Buffer W. Add 20 µl 5x SDS-PAGE sample buffer and mix. Store at -20°C until SDS-PAGE analysis.
   Prior to SDS-PAGE the whole sample should be incubated in an ultrasonic bath for 15 minutes to shear the chromosomal DNA into small pieces and should be heated to 70°C for 10 minutes.

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Protocol (continued)	**5. When OD_{550} equals 0.5-0.6, add 50 µl of IPTG stock solution (0.5 mM end concentration).** **6. Shake for 3 hours at 200 rpm.** Overnight induction may increase protein yields in some cases. **7. Harvest the cells by centrifugation at 4500 x *g* for 12 minutes (4°C).** **Proceed with chapter 3 (Preparation of cleared lysates) or store cell pellet at -20°C.**

2.3 Expression with other systems

Bacterial expression has the advantage of obtaining the expression product in a short time at low cost. Nevertheless, there are proteins which cannot be expressed in *E. coli*. In such cases, yeast, insect, mammalian or plant cells can be used as alternative expression hosts. IBA´s StarGate Cloning system provides a variety of expression vectors for these hosts (yeast, mammalian and insect cells (pYSG-, pESG-, pCSG- and pLSG-IBA vectors)) for a multitude of affinity tags (Strep-tag®II, Twin-Strep-tag®, 6xHistidine-tag, FLAG-tag and GST-tag). See www.iba-lifesciences.com/stargate_vectors.html.
In addition, IBA's pEXPR-IBA series of classic mammalian expression vectors use the CMV promoter and are available with N-terminal Strep-tag®II. pEXPR-IBA vectors are compatible in many cases with corresponding pASK-IBA vectors so that the same PCR fragment can be cloned in parallel into both vectors for direct comparison of both expression systems.
See www.iba-lifesciences.com/classic-cloning-vectors.html.

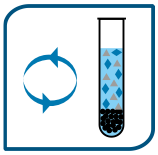
2.4 Precautions to prevent Strep-Tactin® inactivation through biotin

Free biotin binds nearly irreversibly to Strep-Tactin®. Thereby, it prevents binding of the Strep-tag®II fusion protein and, moreover, inactivates Strep-Tactin® resins. Therefore, it has to be removed or masked prior to affinity chromatography. The best and simplest precaution is to add stochiometric amounts of avidin for irreversible masking prior to chromatography. Other solutions are removal via dialysis, ammonium sulfate precipitation or cross-flow filtration/concentration.
The biotin issue is most relevant when cell culture supernatant containing secreted recombinant protein is directly subjected to Strep-Tactin affinity chromatography, because eukaryotic cultivation media (for mammalian or insect cell expression as well as for yeast) may contain significant amounts of biotin.
The cell internal content of biotinylated proteins and free biotin is rather low and not a threat for significant inactivation of the Strep-Tactin resin (biotin capacity ≥ 350 nmol/ml sedimented resin and cell internal biotin content is in e.g. *E. coli* 7 nmol/l/OD).
An overview on biotin contents of standard cell culture media can be found at www.iba-lifesciences.com/faqs.html**, Troubleshooting - Biotin blocking.**

2.5 Trouble shooting – Expression

Problem	Comments and suggestions
No or low expression	Check the culture condition (e.g. IPTG, anhydrotetracycline, antibiotics) Check vector (sequence, frame) Check whether the protein is found in the insoluble fraction. Reduction of temperature during cultivation may solve this problem (e.g. 16°C, 22°C, 26°C, 30°C). Use another expression system (e.g. T7 promoter instead of *tet* promoter, see page 11). Use eukaryotic cells for expression (yeast, insect or mammalian cells)
Protein is degraded	Use protease deficient *E. coli* strains. If degradation occurs during cell lysis, add protease inhibitor If the protein is small (<10 kDa), consider adding a terminal carrier protein. Lower temperature during induction can reduce the problem. Secretion of the recombinant protein to the periplasmic space can reduce the problem.
Protein is secreted	Remove all signal sequences from the coding region.
Inclusion bodies are formed: Protein is insoluble	Reduce expression level by modifying growth and induction conditions, e.g.: lower culturing temperature (16°C, 22°C, 26°C, 30°C) Use another expression system (e.g. tet promoter instead of T7 promoter, see page 9).

3 Preparation of cleared lysates



3.1 Preparation of cleared lysate after cytoplasmic expression of Strep-tag®II fusion proteins

Recommended Buffers/Solutions	Concentration of ingredients	Notes
Buffer W	100 mM Tris/HCl pH 8 150 mM NaCl 1 mM EDTA	It is recommended to work without EDTA when metallo-proteins have been expressed
5x SDS-PAGE sample buffer	0.250 M Tris·Cl, pH 8.0 25 % glycerol 7,5 % SDS 0.25 mg/ml bromophenolblue 12.5 % v/v mercaptoethanol	

Important notes

Strep-tag®II/Strep-Tactin® binding is compatible with many reagents and detergents (see www.iba-lifesciences.com/faqs.html, Troubleshooting – "Reagents compatible with the Strep-tag®II/Strep-Tactin® interaction"; for a complete list)

Protocol

1. **Chill Buffer W at 4°C.**

2. **Resuspend the pellet of a 100 ml culture in 1 ml Buffer W.**

3. **Take a 10 µl sample for analysis of the total protein content via SDS-PAGE and/or Western blotting.**
   The 10 µl sample should be thoroughly mixed with 90 µl Buffer W and 25 µl of 5x SDS-PAGE sample buffer. Store it at -20°C. The whole sample must be incubated in an ultrasonic bath for 15 minutes to shear the chromosomal DNA into small pieces and should be heated to 70°C for 10 minutes prior to SDS-PAGE.

4. **Sonicate the residual suspension under ice-cooling.**
   Take care that the suspension does not become warm or even hot which may denature proteins or activate proteases. Perform bursts with cooling intervals if possible.
   French pressing is possible as well.
   Lysis should be complete and can be monitored by measuring the optical density at 590 nm [% lysis = $(1 - A_{590}^{sonicate}/A_{590}^{suspension}) \times 100$].

5. **(Optional) If the lysate is very viscous, add RNase A (10 µg/ml) and DNase I (5 µg/ml) and incubate on ice for 10 – 15 min.**

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Protocol

6. **Centrifuge the suspension at 13000 rpm (microfuge) for 15 minutes at 4°C.**

7. **Insoluble cell components are sedimented. If the recombinant protein forms inclusion bodies it will be present in the sediment.**

8. **Carefully transfer the clear supernatant to a clean tube.**
   For analysis of the insoluble part of the expressed protein, dissolve the sediment with 1.25 ml 1x SDS-PAGE sample buffer (= 250 µl 5x SDS-PAGE sample buffer mixed with 1 ml Buffer W).
   Store the supernatant on ice until chromatography or store at -20°C if chromatography cannot be performed the same day.

9. **Proceed to protocols for Strep-tag® protein purification under native conditions (see protocols 4.1, 4.2, 4.3).**

Important notes

Strep-tag®II/Strep-Tactin® affinity purification should not be performed discontinuously in batch mode which would result in significantly reduced protein purity and yield in comparison to column chromatography. Further, prolonged batch incubations increase the risk of proteolytic degradation of the target protein including cleavage of the tag. If batch purification with the Strep-tag® system is attempted, the use of Strep-Tactin®XT Superflow® MagStrep “type3” XT beads (cat.no. 2-4090-002) in combination with the Twin-Strep-tag® is recommended.

3.2 Preparation of cleared lysate after periplasmic expression of Strep-tag®II fusion proteins

Periplasmic proteins are secreted into the periplasmic space located between the outer and inner membrane of *E. coli*. Proper secretion is only possible when the recombinant protein has an N-terminal signal peptide (e.g. OmpA) which is cleaved after translocation by *E. coli* leader peptidase. In order to purify proteins secreted into the periplasmic space using Strep-Tactin® technology [8, 9], the Strep-tag® can be fused to the C- or N-terminus using pASK-IBA2, 2C, 4, 4C, 6, 6C, 12, 14, 44 and pASG-IBA2, 4, 44, 102, 104, 142, 144.

Recommended Buffers/Solutions	Concentration of ingredients	Notes
Buffer P	100 mM Tris/HCl pH 8.0 500 mM sucrose 1 mM EDTA	Used for the release of the periplasmic content. It is recommended to work with 2 mg/ml polymyxin B sulfate instead of 1 mM EDTA when metalloproteins are isolated.

Recommended Buffers/Solutions	Concentration of ingredients	Notes
5x SDS-PAGE sample buffer	0.250 M Tris/HCl, pH 8.0 25 % glycerol 7,5 % SDS 0.25 mg/ml bromophenolblue 12.5 % v/v mercaptoethanol	

Important notes

Strep-tag®/Strep-Tactin® binding is compatible with many reagents and detergents (see www.iba-lifesciences.com/faqs.html, Troubleshooting – "Reagents compatible with the Strep-tag/Strep-Tactin interaction"; for a complete list)

Protocol

1. **Chill Buffer P at 4°C.**

2. **Resuspend the pellet of a 100 ml culture in 1 ml Buffer P.**

3. **Incubate 30 minutes on ice.**
   These conditions will usually sufficiently permeabilize the outer membrane of *E. coli* to release the soluble periplasmic components and leave the spheroplasts intact to ensure low contamination of the protein preparation with cytoplasmic proteins [10]. Harsher treatments, e.g. osmotic shock or use of lysozyme may be used if the periplasmic components are not completely released with the EDTA treatment.

4. **Collect a 10 µl sample for total analysis of the protein content via SDS-PAGE and/or Western blotting.**
   The 10 µl sample should be thoroughly mixed with 90 µl Buffer W and 25 µl 5x SDS-PAGE sample buffer. Store at -20°C. The whole sample must be incubated in an ultrasonic bath for 15 minutes to reduce the chromosomal DNA to small pieces and should be heated to 70°C for 10 minutes prior to SDS-PAGE.

5. **Remove spheroplasts by centrifugation at 13000 rpm (microfuge) for 5 minutes at 4°C.**

6. **Carefully transfer the clear supernatant in a clean tube.**
   To check whether a part of the expressed protein remained in the cells, resuspend the sedimented spheroplasts with 1 ml Buffer P and add 250 µl 5x SDS-PAGE sample buffer and perform SDS-PAGE, optionally followed by Western blotting.
   Store the supernatant on ice until chromatography or store at -20°C if chromatography cannot be performed the same day.

Important note

7. **Proceed to protocols for Strep-tag® protein purification under native conditions (see protocols 4.1 to 4.3).**
   Strep-tag®II/Strep-Tactin® affinity purification should not be performed discontinuously in batch mode which would result in significantly reduced protein purity and yield in comparison to column chromatography. Further, prolonged batch incubations increase the risk of proteolytic degradation of the target protein including cleavage of the tag. If batch purification with the Strep-tag® system is attempted, the use of Strep-Tactin®XT Superflow® MagStrep “type3” XT beads (cat.no. 2-4090-002) in combination with the Twin-Strep-tag® is recommended.

4 Purification of Strep-tag®II and Twin-Strep-tag® fusion proteins

To allow an efficient Strep-tag®II/Strep-Tactin® binding we strongly recommend using column purification instead of batch applications for proteins fused to Strep-tag®II. It is crucial that protein binding takes place on the column. Even a pre-incubation of resin and lysate prior to filling the resin into a column will lead to decreased protein yields. Further, prolonged batch incubations increase the risk of proteolytic degradation of the target protein including cleavage of the tag. If batch purification is attempted, the use of Strep-Tactin® Superflow® High Capacity in combination with the Twin-Strep-tag® is recommended.

4.1 Purification of Strep-tag®II and Twin-Strep-tag® fusion proteins using Strep-Tactin® gravity flow columns

Recommended Buffers/Solutions	Concentration of ingredients	Notes
Buffer W	100 mM Tris/HCl pH 8.0 150 mM NaCl 1 mM EDTA	It is recommended to work without EDTA when metalloproteins have been expressed
Buffer E (elution buffer)	100 mM Tris/HCl, pH 8.0 150 mM NaCl 1 mM EDTA 2.5 mM desthiobiotin	When using Strep-Tactin® Superflow® High Capacity resin it may be advantageous to use 5-10 mM desthiobiotin to get the target protein eluted at higher concentration (especially in combination with Twin-Strep-tag®)
5x SDS-PAGE sample buffer	0.250 M Tris/HCl, pH 8.0 25 % glycerol 7,5 % SDS 0.25 mg/ml bromophenolblue 12.5 % v/v mercaptoethanol	

Important notes

- Strep-Tactin® Sepharose, Superflow® and MacroPrep® as well as Strep-Tactin® Superflow® high capacity can be used for gravity flow purification.
- Binding capacity is protein dependent and normally lies between 50 and 100 nmol (up to 500 nmol in case of Strep-Tactin® Superflow® High Capacity) recombinant protein per ml bed volume (100 nmol correspond to 2 mg of a 20 kDa protein).
- The composition of the lysis, wash and elution buffers can be modified to suit the particular application, e.g. by adding 0.1 % Tween, 5-10 mM β-mercaptoethanol, or 1 mM PMSF, or increasing NaCl or glycerol concentrations.

Continue page 20

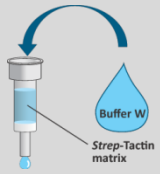
Important notes (continued)

- The pH should not be lower than 7.5, though. For more information see www.iba-lifesciences.com/faqs.html, Troubleshooting – "Reagents compatible with the Strep-tag/Strep-Tactin interaction".
- Generally, it is recommended to perform chromatography at 4°C. Dependent on the individual equipment this is not always possible and chromatography has to be performed at room temperature. If columns are stored at 4°C and transferred to room temperature air bubbles may form since cold storage buffer is able to take up more gas than buffers at ambient temperature. Therefore, it is recommended to equilibrate the columns immediately after exposure to higher temperatures with buffer that is equilibrated at such temperatures.

Column bed volume (CV)	Protein extract volume*	Washing buffer volume	Elution buffer volume
0.2 ml	0.1 - 2 ml	5 x 0.2 ml	6 x 0.1 ml
1 ml	0.5 - 10 ml	5 x 1 ml	6 x 0.5 ml
5 ml	2.5 - 50 ml	5 x 5 ml	6 x 2.5 ml
10 ml	5 - 100 ml	5 x 10 ml	6 x 5 ml

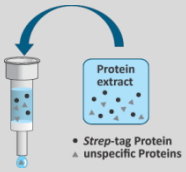
Table 4. Recommended buffer volumes for chromatography on Strep-Tactin® columns
*Adjust protein extract volume according to binding capacity of the column and apply the extract as concentrated as possible in the recommended volume range.

Protocol



1. **Equilibrate the Strep-Tactin® column with 2 CVs (column bed volumes) Buffer W.**
   Remove first top cap from column, then the cap at the outlet of the column. If the caps are removed in reverse order, the column may run dry. Remove storage buffer prior to adding Buffer W for equilibration. The column cannot run dry under gravity flow.
   Use buffer without EDTA for metallo proteins!

2. **Centrifuge cleared lysates (14,000 rpm, 5 minutes, 4°C, microfuge).**
   Insoluble aggregates which may have formed after storage may clog the column and thus have to be removed.

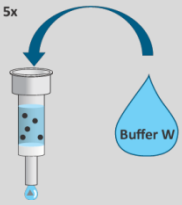


3. **Add supernatant of cleared lysates to the column.**
   The volume of the lysates should be in the range of 0.5 and 10 CVs (see Table 4 this page). Extracts of large volumes with the recombinant protein at low concentration may lead to reduced yields and should be concentrated prior to chromatography.

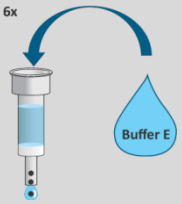
Continue page 21

Protocol (continued)

Concentrated cell extracts are preferred; if quantification is possible, apply cell extract containing 50 to 100 nmol (up to 500 nmol in case of Strep-Tactin® Superflow® high capacity) recombinant Strep-tag®II fusion protein per 1 ml CV.



4. **Wash the column 5 times with 1 CV Buffer W, after the cell extract has completely entered the column.**
Collect the eluate in fractions having a size of 1 CV. Apply 2 µl of the first washing fraction and 20 µl of each subsequent fraction to an analytical SDS-PAGE.



5. **Add 6 times 0.5 CVs Buffer E and collect the eluate in 0.5 CV fractions.**
20 µl samples of each fraction can be used for SDS-PAGE analysis. Most of the purified Strep-tag®II fusion protein usually elutes in the 2nd to 5th fraction.
Desthiobiotin and EDTA can be removed, if necessary, via dialysis or gel chromatography.

4.2 Purification of Strep-tag®II and Twin-Strep-tag fusion proteins on chromatography workstations using Strep-Tactin® cartridges

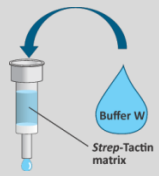
Recommended Buffers/Solutions	Concentration of ingredients	Notes
Buffer W	100 mM Tris/HCl pH 8.0 150 mM NaCl 1 mM EDTA	It is recommended to work without EDTA when metallo-proteins have been expressed
Buffer E (elution buffer)	100 mM Tris/HCl, pH 8.0 150 mM NaCl 1 mM EDTA 2.5 mM desthiobiotin	When using Strep-Tactin® Superflow® High Capacity resin it may be advantageous to use 5-10 mM desthiobiotin to get the target protein eluted at higher concentration especially in combination with Twin-Strep-tag®)
5x SDS-PAGE sample buffer	0.250 M Tris/HCl, pH 8.0 25 % glycerol 7,5 % SDS 0.25 mg/ml bromophenolblue 12.5 % v/v mercaptoethanol	

Important notes

- Cartridges filled with 1 ml or 5 ml Strep-Tactin® Superflow® (High Capacity) or MacroPrep® are designed for use with chromatography workstations with 10-32 fittings (HPLC and Äkta).
- They can, however, also be operated with other workstations, with syringes or with peristaltic pumps by use of appropriate adapter sets (catalogue numbers 2-1021-001 (Luer lock), 2-1022-001(M6), 2-1023-001 (1/4-28), 2-1025-001 (1/16 inch); see www.iba-lifesciences.com)
- Binding capacity is protein dependent and normally lies between 50 and 100 nmol (up to 500 nmol in case of Strep-Tactin® Superflow® High Capacity) recombinant protein per ml bed volume (100 nmol of a 20 kDa protein correspond to 2 mg).
- Cartridges may be connected in series to enlarge capacity. A coupling adapter is needed in this case (Cat. no. 2-1026-001).
- Recommended flow rates: 0.5-1 ml/min for a 1 ml cartridge; 1-3 ml/min for a 5 ml cartridge.
- The composition of lysis, wash and elution buffers can be modified to suit the particular application, e.g., by adding 0.1 % Tween, 5-10 mM β-mercaptoethanol, or 1 mM PMSF, or increasing NaCl or glycerol concentrations. The pH should not be lower than 7.5, though. See www.iba-lifesciences.com/faqs.html, Troubleshooting – "Reagents compatible with the Strep-tag/Strep-Tactin interaction"; for more information.
- Generally, it is recommended to perform chromatography at 4°C. Depending on the individual equipment this is not always possible and chromatography has to be performed at room temperature. If cartridges are stored at 4°C and are transferred to room temperature air bubbles may form due to restricted solubility of air at elevated temperatures. Therefore, it is recommended to equilibrate the cartridges immediately after exposure to higher temperatures with buffer that is equilibrated at such temperatures.
- Cartridges do not generate significant back pressure which makes the use of flow restrictors superfluous. Therefore, IBA recommends not using flow restrictors to avoid inhomogeneity's resulting from buffer changes during chromatography.

Protocol

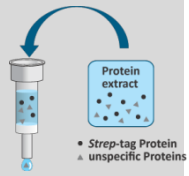
1. **Connect adapters to the cartridge if fittings other than 10-32 are required and connect the cartridge with the chromatography workstation.**



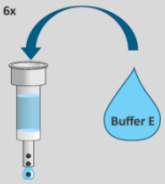
2. **Equilibrate cartridge with 5 CVs (column bed volumes) of Buffer W.**
   The flow rate should be in the range of 0.5-1 ml/min for 1 ml cartridges and 1-3 ml/min for 5 ml cartridges.
   Monitor elution at 280 nm; the baseline should be stable after washing with 5 CVs.

Continue page 23

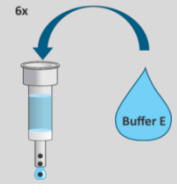
Protocol (continued)



3. Apply lysate to cartridge.
Begin with a flow rate of 1 ml/min. Monitor pressure at this step. If the lysate is very viscous and pressure is increased significantly, reduce viscosity of the extract (please note Table 4 on page 22 in this respect) or reduce flow rate.
Collect the flow-through for SDS-PAGE analysis.



4. Wash with Buffer W until A_{280} is stable.
Usually 5-10 CVs are sufficient to reach the baseline. To get maximal protein yields proceed with step 5 as soon as the baseline is reached. Collect fractions for SDS-PAGE analysis.



5. Elute the protein with Buffer E.
Collect fractions for SDS-PAGE analysis.

4.3 Purification of Strep-tag® fusion proteins using Strep-Tactin® cartridges with syringes

Recommended Buffers/Solutions	Concentration of ingredients	Notes
Buffer W	100 mM Tris/HCl, pH 8.0 150 mM NaCl 1 mM EDTA	It is recommended to work without EDTA when metalloproteins have been expressed
Buffer E (elution buffer)	100 mM Tris/HCl, pH 8.0 150 mM NaCl 1 mM EDTA 2.5 mM desthiobiotin	When using Strep-Tactin® Superflow® High Capacity resin it may be advantageous to use 5-10 mM desthiobiotin to get the target protein eluted at higher concentration
5x SDS-PAGE sample buffer	0.250 M Tris/HCl, pH 8.0 25 % glycerol 7,5 % SDS 0.25 mg/ml bromophenolblue 12.5 % v/v mercaptoethanol	

Important notes

- Use a female luer to 10-32 male adapter for the cartridge inlet and a 1/16 inch to 10-32 male adapter for the outlet (Cat. no. 2-1021-001).
- The composition of lysis, wash and elution buffers can be modified to suit the particular application, e.g., by adding 0.1 % Tween, 5-10 mM β-ME, or 1 mM PMSF, or increasing NaCl or glycerol concentrations. The pH should not be lower than 7.5, though. For more information see www.iba-lifesciences.com/faqs.html, Troubleshooting – "Reagents compatible with the Strep-tag/Strep-Tactin interaction".
- Generally, it is recommended to perform chromatography at 4°C. Depending on the individual equipment this is not always possible and chromatography has to be performed at room temperature:
  If cartridges are stored at 4°C and transferred to room temperature air bubbles may form since cold storage buffer is able to take up more gas than buffers at ambient temperature. Therefore, it is recommended to equilibrate the cartridges immediately after exposure to higher temperatures with buffer that is equilibrated at such temperatures.
- Cartridges may be connected in series to enlarge capacity. A coupling adapter is necessary in this case (Cat. no. 2-1026-001).

Protocol (for running a 1 ml cartridge)

1. **Connect the adapters and fill the inlet with Buffer W.**
2. **Connect a 10 ml syringe filled with Buffer W. Avoid the inclusion of air bubbles.**
3. **Inject 5 ml Buffer W with a flow rate of 1 drop/sec to equilibrate the cartridge.**
4. **Centrifuge the cleared lysate (14,000 rpm, 5 minutes, 4°C, microfuge) to remove aggregates that may have formed during storage.**
   Insoluble aggregates which may clog the cartridge shall be removed.
5. **Fill a syringe with the appropriate amount of the cleared lysate.**
6. **Remove the 10 ml syringe used for equilibration.**
7. **Fill the inlet with Buffer W.**
8. **Apply the cleared lysate with a flow rate of 0.3 to 0.5 drops/sec.**
9. **Remove the syringe, fill the inlet with Buffer W, fill a 10 ml syringe with Buffer W and connect the syringe with the cartridge.**

Continue page 25

Protocol (for running a 1 ml cartridge), continued

10. **Wash with 100 drops Buffer W (corresponding to approx. 5 ml) at a flow rate of 0.3 to 0.5 drops/sec. Collect the eluate in fractions of 20 drops and apply 2 µl of the first fraction and 20 µl of each subsequent fraction to an analytical SDS-PAGE (fraction W1 to W5).**

11. **Remove the syringe and fill the inlet with Buffer E.**

12. **Fill a 5 ml syringe with 4 ml Buffer E and connect it to the cartridge.**

13. **Elute the recombinant Strep-tag®II fusion protein with 60 drops Buffer E (corresponding to approx. 3 ml) at a flow rate of 0.3 to 0.5 drops/sec. Collect the eluate in fractions of 10 drops and apply 20 µl of each fraction to an analytical SDS-PAGE (fraction E1 to E6). Purified protein should be present in fractions E2-E5.**

4.4 Trouble shooting – Strep-Tactin® purification

4.4.1 "No or weak binding to Strep-Tactin® column"

pH is not correct.	The pH should between pH 7.0 and pH 8.5
Strep-tag®II is not present.	Use protease deficient *E. coli* expression strains. Add protease inhibitors during cell lysis.
Strep-tag®II is not accessible.	Fuse Strep-tag®II with the other protein terminus; use other linker.
Strep-tag®II has been degraded.	Make sure that the Strep-tag®II is not associated with a portion of the protein that is processed. *Avoid purification in discontinuous batch mode*. Prolonged batch incubations increase the risk of proteolytic degradation of the target protein including cleavage of the tag.
Strep-tag®II is partially accessible.	Reduce washing volume to 3 CVs.
Strep-Tactin® column is inactive.	Check the binding capacity of the column with HABA. Upon addition of HABA onto the column, HABA should switch its color from yellow to orange. To avoid inactivation of the column due to biotin/biotinylated proteins, add avidin (or Biotin Blocking Buffer) to the cell lysate, if biotin containing extracts are intended to be purified. The total biotin content of the soluble part of the total *E. coli* cell lysate is about 1 nmol per liter culture (OD_{550} = 1.0). Add 2-3 nmol of avidin monomer per nmol of biotin.
Batch purification is carried out	To allow an efficient Strep-tag®II/Strep-Tactin® binding we strongly recommend using column purification instead of batch applications. It is crucial that protein binding takes place on the column. Even a pre-incubation of resin and lysate prior to filling the resin into a column will lead to decreased protein yields. If batch purification with the Strep-tag® system is intended, the use of Strep-Tactin®XT Superflow® or MagStrep "type3" XT beads in combination with the Twin-Strep-tag® is recommended.
Flow rate is too fast	Reduced flow rates may increase yields depending on the given recombinant protein.

4.4.2 "Contaminating proteins"

Note: The soluble part of the *E. coli* total cell extract contains no proteins beyond the nearly irreversibly binding biotin carboxyl carrier protein (BCCP) which binds significantly to the Strep-Tactin® column. Therefore, contaminating proteins interact, specifically or non-specifically, with the recombinant protein itself and are, thus, co-purified.

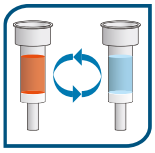
Contaminants are short forms of the tagged protein.	Use protease deficient *E. coli* expression strains. Add protease inhibitors after cell lysis. Fuse the Strep-tag®II with the other protein terminus. Check for the presence of internal translation initiation starts (only in case of C- terminal Strep-tag®II) or premature termination sites (only in case of N-terminal Strep-tag®II). Add 6x*Histidine*-tag to the other terminus and use both tags for purification which will lead to full length protein preparations.
Contaminants are covalently linked to the recombinant protein via disulfide bonds.	Add reducing agents to all buffers for cell lysis and chromatography.
Contaminants are non-covalently linked to the recombinant protein:	Increase ionic strength in all buffers for cell lysis and chromatography (up to 1 M NaCl) or add mild detergents (0,1 % Triton X-100, 0,1 % Tween, 0.1 % CHAPS, etc.).

4.4.3 "Air bubbles in the column"

When the column is taken from the cold storage room to the bench, the different temperatures can cause small air bubbles in the column. The reason is that the cold storage buffer is able to take up more gas than buffers at ambient temperature.

To prevent development of bubbles in the column bed.	Keep on working in the cold room (also recommended for proteins), use degassed buffers or wash the column immediately with buffers at ambient temperature once the column is removed from the cold.

5 Storage and regeneration of Strep-Tactin® resin



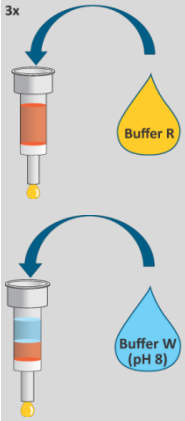
Recommended Buffers/Solutions	Concentration of ingredients	Notes
Buffer R (regeneration buffer)	100 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 1 mM HABA (hydroxy-azophenyl-benzoic acid), pH 8.0	It is recommended to work without EDTA when metallo-proteins have been expressed
Buffer W	100 mM Tris/HCl, pH 8.0 150 mM NaCl 1 mM EDTA	

Important notes

- Strep-Tactin® matrices should be refrigerated at temperatures between 4 and 8°C for longer storage.
- We recommend a maximum of 5 runs per column.
- Resin tolerates washing with 8 M guanidine. Such procedures should not last longer than 30 minutes and the resin should be equilibrated with Buffer W immediately afterwards.
- If HABA cannot be efficiently removed from Strep-Tactin® Superflow® High Capacity by using Buffer W, we recommend using Buffer W at pH 10.5 (or alternatively 100 mM Tris base) for efficient removal of HABA from Strep-Tactin® Superflow® High Capacity.

5.1 Regeneration of gravity flow columns filled with Strep-Tactin® resin

Protocol

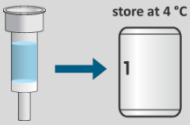


1. **Wash the column 3 times with 5 CVs Buffer R.**
   The **color change from yellow to red** indicates the regeneration process and the intensity of the red color is an indicator of the column activity status.

2. **Regeneration is complete when the red color on the bottom of the column has the same intensity as on top of the column. If this is not the case use more Buffer R.**

3. **Overlay with 2 ml Buffer W or R for storage.**

Continue page 29

Protocol (continued) 	**4. Store the column at 4-8°C. Remove Buffer R by washing with 2 times 4 CVs of Buffer W prior to the next purification run.** Exception: In case of Strep-Tactin® Superflow® High Capacity, use 4 CV Buffer W at pH 10.5 for HABA removal. Immediately afterwards, exchange the column buffer to Buffer W pH 8.0 as long term exposure to pH 10.5 may be detrimental to the resin.

5.2 Regeneration of cartridges with Strep-Tactin® resin

Recommended Buffers/Solutions	Concentration of ingredients	Notes
Buffer R (regeneration buffer)	100 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 1 mM HABA (hydroxy-azophenyl-benzoic acid), pH 8.0	It is recommended to work without EDTA when metallo-proteins have been expressed
Buffer W	100 mM Tris/HCl, pH 8.0 150 mM NaCl 1 mM EDTA	

Protocol	**1. Fill the cartridge inlet with Buffer R.** **2. Fill a 20 ml injection with Buffer R.** **3. Wash with 15 CVs Buffer R at a flow rate of 1 drop/sec.** **4. Regeneration is complete when the red color on the bottom of the column has the same intensity as on top of the column. If this is not the case use more Buffer R.** **5. Store the cartridge at 4-8 °C. Remove Buffer R by washing with 2 times 4 CVs of Buffer W prior to the next purification run.** Exception: In case of Strep-Tactin® Superflow® High Capacity, use 4 CV Buffer W at pH 10.5 for HABA removal. Immediately afterwards, exchange the column buffer to Buffer W pH 8.0 as long term exposure to pH 10.5 may be detrimental to the resin.

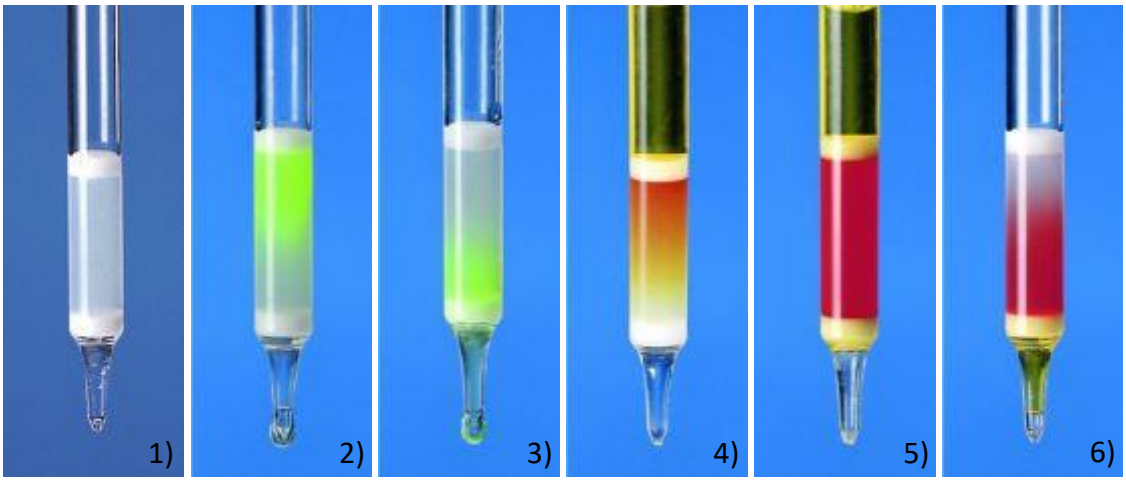


Figure 3: Purification of an overexpressed GFP-Stre*p*-tag®II fusion protein on Strep-Tactin® resin from *E.* coli crude cell extract.

The single steps of the Strep-Tactin® protein purification cycle are shown in Figure 3 (pictures left to right):
1) new or regenerated Strep-Tactin® Sepharose® column;
2) specific binding of GFP-Strep-tag®II fusion protein to Strep-Tactin® Sepharose column while unspecific proteins are rapidly washed away with small amounts of physiological buffer;
3) Strep-tag®II fusion protein is eluted due to addition of the specific competitor "desthiobiotin";
4) to 6) column regeneration: desthiobiotin is displaced by the yellow solution HABA, which turns red once complexed with Strep-Tactin®. In the process the red color shift depends on the respective Strep-Tactin® resin (see also Figure 4). HABA is then removed by washing buffer and the column can be re-used.

Figure 4: Strep-Tactin® columns regenerated with HABA: Strep-Tactin®, Sepharose, Superflow® and MacroPrep® (left to right).
Since MacroPrep® is not as transparent as Sepharose or Superflow®, the color shift with HABA to red appears different.

6 References

For up-to-date references see www.iba-lifesciences.com

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