

Expression and purification of proteins using Strep-Tactin[®] XT and Twin-Strep-tag[®]

A comprehensive manual



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For Twin-Strep-tag[®] purification from **mammalian cells** a separate manual is available.

Manuals are available for download under:

www.iba-lifesciences.com/download-area.html.



1 Introduction



1.1 Strep-Tactin[®]XT:Twin-Strep-tag[®] system overview

The newest generation of the Strep-tag[®] system is a further development of the commonly used Strep-Tactin[®]:Strep-tag[®]II technology. The Strep-tag[®]II is a short peptide, which binds with high selectivity to Strep-Tactin[®], an engineered streptavidin. This technology allows one-step purification of recombinant proteins under physiological conditions, thus preserving their 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].

Based on the proprietary Strep-tag[®] technology the **Twin-Strep-tag[®]** was developed which is a sequential arrangement of two Strep-tag[®]II sequences. This tag enables the same mild and rapid purification as Strep-tag[®]II but, due to its avidity effect, has an increased affinity for Strep-Tactin[®]. Like Strep-tag[®]II, the Twin-Strep-tag[®] 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.

IBA's 3rd generation Strep-tag[®] system is based on the novel **Strep-Tactin[®]XT Superflow[®]** resin in combination with the aforementioned Twin-Strep-tag[®]. Strep-Tactin[®]XT has a **binding affinity in low pM ranges** for the Twin-Strep-tag[®] still maintaining the binding reversibility, the mild recovery of immobilized proteins and the high purity (> 95 %). Furthermore, Strep-Tactin[®]XT now enables new applications in the field of high throughput screening, batch purification, purification using denaturing conditions and protein interaction studies, making the system superior to all other available affinity tag purification systems.



1.2 The Strep-Tactin[®]XT:Twin-Strep-tag[®] protein purification principle

The basis for the development of the Strep-tag[®] system was the well-known binding of biotin to streptavidin (Fig. 1). To take advantage of this strong interaction in protein purification applications we put effort in finding 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 eight amino acids (sequence: WSHPQFEK) and was named Strep-tag[®]II. To optimize binding properties, also streptavidin has been engineered to obtain Strep-Tactin[®]. Constant research led to further developments and finally resulted in the 3rd generation of the Strep-tag[®] system: **Strep-Tactin[®]XT and Twin-Strep-tag[®]**.

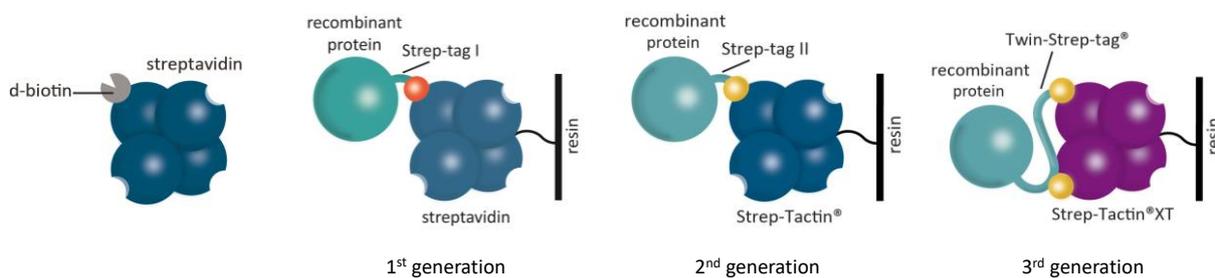


Fig. 1: Schematic view of the Strep-tag[®] core technology

Twin-Strep-tag[®]

The Strep-tag[®]II is a short peptide tag with negligible effect on the recombinant protein due to its chemically balanced amino acid composition (8 amino acids, WSHPQFEK) which can be fused to the protein as either N- or C-terminal tag. A two amino acid spacer (SerAla) between the protein and the tag promotes the accessibility of the tag. The further improved Twin-Strep-tag[®] is a sequential arrangement of two Strep-tag[®]II sequences with an internal linker region (total size of 28 aa). This tag enables the same mild and rapid purification as Strep-tag[®]II but, in addition, has an increased affinity for Strep-Tactin[®] which allows efficient purification even in batch or directly from cell culture supernatants.

The Strep-tag[®] system allows the usage of physiological buffers, like PBS, in combination with a wide range of additives. Generally, the tag does not interfere with folding or bioactivity of the target protein, 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 removal of the tag after purification.

Strep-Tactin[®]XT

Strep-Tactin[®]XT is the further development of Strep-Tactin[®]. The interaction between Strep-Tactin[®]XT and Twin-Strep-tag[®] has a binding affinity in the low pM range making the system superior to all other available purification systems. The near covalent binding of the interaction partners ensures higher protein yields compared to Strep-Tactin[®] and makes the system suitable for purifications under native as well as under denaturing conditions. The



interaction is stable up to 6 M urea thus enabling long-lasting resins which can be re-used several times. Moreover, the neutral pI of Strep-Tactin[®]XT minimizes non-specific protein or nucleic acid binding.

1.3 The Strep-Tactin[®]XT protein purification cycle

The purification of Twin-Strep-tag[®] fusion proteins is easy, straightforward and user-friendly. The complete procedure can be performed under physiological conditions, e.g. in PBS buffer. Physiological buffers can optionally be used in combination with a wide range of additives. For elution, simply biotin is added to the buffer (Fig. 2). Biotin is the natural ligand of streptavidin - and therefore serves for the mild elution of Twin-Strep-tag[®] proteins. Column regeneration is easily achieved by applying NaOH.

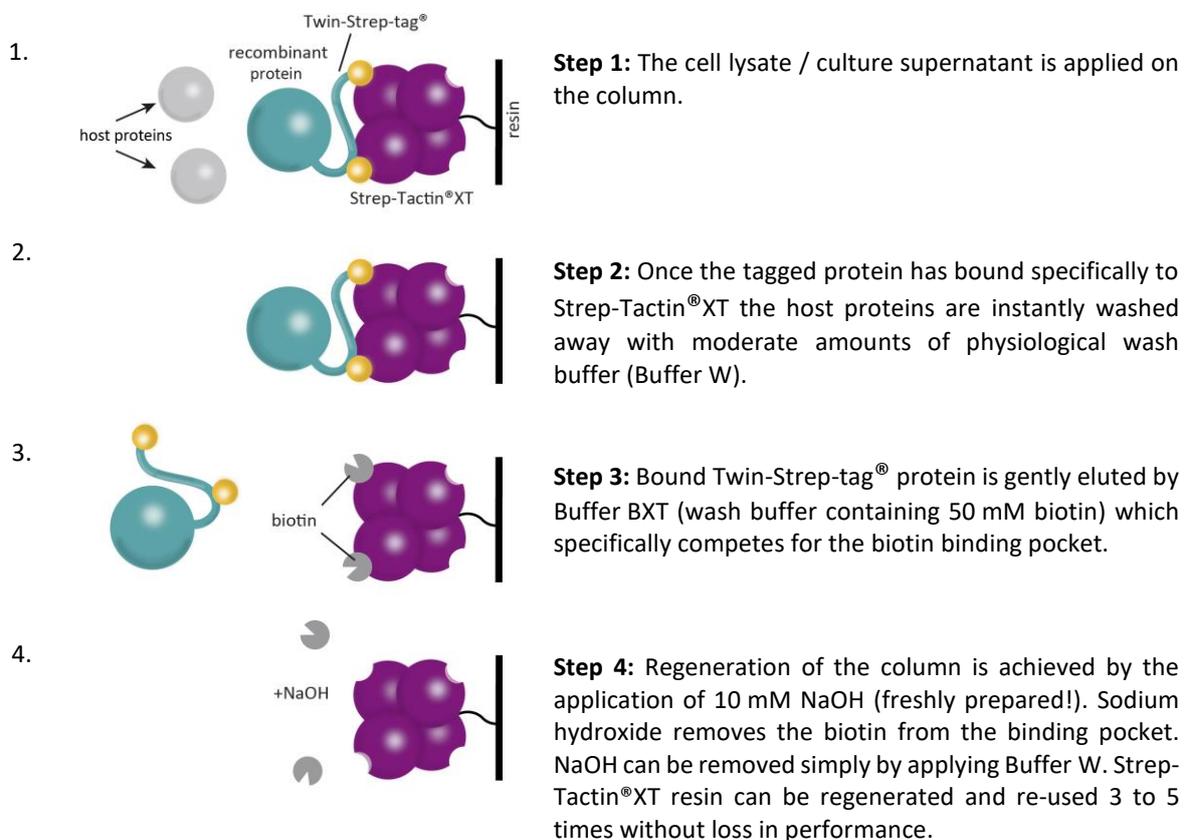
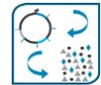
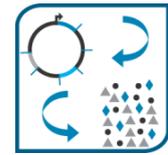


Fig. 2: Strep-Tactin[®]XT purification cycle



2 Expression in *E. coli*



Even minute quantities of recombinant protein caused by expression of a leaky promoter can influence *E. coli* cell growth. Moreover, it can result in a dramatic selection against *E. coli* cells harboring the expression plasmid in case the recombinant protein is cytotoxic. Consequently, regulation of heterologous protein biosynthesis is generally recommended by the use of a promoter whose activity can be blocked by a repressor, and subsequent synthesis of the gene product is switched on in a controlled manner by adding a chemical inducer.

IBA offers *E. coli* expression vectors using two different repression and induction systems: vectors either harboring the tet promoter (see 2.1) or the T7 promoter (see 2.2).

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

The Twin-Strep-tag[®] and Strep-tag[®]II expression vectors pASK-IBA/pASG-IBA carry the promoter/operator region from the *tetA* resistance gene and are the state-of-the-art solution for such an inducible expression system [4, 5]. The strength of the *tetA* promoter is comparable with that of the *lac*-UV5 promoter.

The protein expression can be induced by adding anhydrotetracycline at a concentration that is antibiotically not 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 dependent on chromosomally encoded repressor molecules, the *tetA* promoter/operator is not coupled to any cellular regulation mechanisms. Thus, 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.

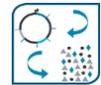
IBA offers a special developed Mammalian expression system (MEXi) which consists of HEK-293 cells adapted to suspension growth in chemical defined media with a very low biotin concentration. Corresponding manuals are available for download under www.iba-lifesciences.com/download-area.html.



Recommended Buffers/Solutions	Concentration of ingredients	Notes
Ampicillin (pASG-IBA vectors, pASK-IBA vectors <u>except</u> : pASK-IBA2C, 3C, 4C, 5C, 6C, 7C)	stock solution 100 mg/ml in H ₂ O, 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	
Buffer W	100 mM Tris/HCl, pH 8.0 150 mM NaCl 1 mM EDTA	
5x SDS-PAGE sample buffer	250 mM 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 harbouring the 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.

In most cases, the yield of soluble, functional protein can be significantly increased by lowering the growth temperature of the preculture to 22°C - 30°C. The cells should not reach the stationary phase for extended periods prior to inoculation.

- 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 (OD₅₅₀).**

Cell suspension samples with an OD₅₅₀ higher than 1.0 should be diluted with LB medium before measuring.

- 4. When OD₅₅₀ 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.

Take a 1 ml sample immediately before induction.

This sample represents 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.

- 5. Shake for 3 hours at 200 rpm.**

Overnight expression may increase protein yields in some cases.

- 6. Harvest the cells by centrifugation at 4500 x g for 12 minutes (4 °C).**

Proceed to 3 (Preparation of cleared lysates) or store cell pellet at -20 °C



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

The system uses the T7 promoter and T7 RNA polymerase for high-level transcription of the gene of interest. Since the T7 promoter is stronger than the tet promoter, pPSG-IBA vectors can be recommended in cases where expression with the tet promoter does not lead to sufficient yields of the recombinant protein.

Expression of the target gene 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. BL21(DE3) *E. coli* 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)	stock solution 100 mg/ml in H ₂ O, sterile filtered	Store in aliquots at - 20 °C
LB medium	10 g/l trypton 5 g/l yeast extract 5 g/l NaCl	
IPTG (Isopropyl β-D-1- thiogalactopyranoside)	stock solution (1 M): 238 mg/ml in H ₂ O, sterile filtered	Store in aliquots at - 20 °C
Glucose	20 %, sterile filtered	
Buffer W	100 mM Tris/HCl, pH 8.0 150 mM NaCl 1 mM EDTA	
5x SDS-PAGE sample buffer	250 mM 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 harbouring the pPSG-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 often be significantly increased by lowering the growth temperature of the preculture to 22°C – 30°C. The cells should not reach the stationary phase for extended periods prior to inoculation.

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 co-transformants in such cases in order to prevent basal expression [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 (OD₅₅₀).**

Cell suspension samples with an OD₅₅₀ higher than 1.0 should be diluted with LB medium before measuring.

- 4. When OD₅₅₀ equals 0.5 – 0.6, add 50 µl of IPTG stock solution (0.5 mM end concentration).**

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.

Take a 1 ml sample immediately before induction.

This sample represents 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.

- 5. Shake for 3 hours at 200 rpm.**

Overnight expression may increase protein yields in some cases.

- 6. Harvest the cells by centrifugation at 4500 x g for 12 minutes (4 °C).**

Proceed to 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®, Twin-Strep-tag®, 6xHistidine-tag, FLAG-tag and GST-tag). See http://www.iba-lifesciences.com/stargate_vectors.html.

2.4 Precautions to prevent Strep-Tactin®XT blocking through biotin

Free biotin binds to Strep-Tactin®XT. Thereby it reduces the binding capacity of the resin for the protein of interest. Therefore, biotin has to be removed or masked prior to affinity chromatography. The best and simplest precaution is to add IBA's Biolock (Biotin blocking solution) or stoichiometric 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®XT resins, since eukaryotic cultivation media (for mammalian or insect cell expression as well as for yeast) may contain significant amounts of biotin.

The MEXi system, however, provides mammalian cell culture media with a minimum biotin concentration for effective Strep-Tactin®XT:Twin-Strep-tag® purification. The amount of biotinylated proteins or free biotin in all extracts is rather low and not a threat for significant reduction of binding capacity of the Strep-Tactin®XT. Cell internal biotin content in e.g. *E. coli* is about 7 nmol/l/OD.

An overview on biotin contents of standard cell culture media can be found at www.iba-lifesciences.com/technical-support.html, **Troubleshooting - Biotin contaminations**.

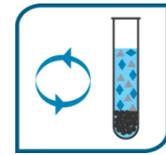
2.5 Troubleshooting – Expression



Problem	Comments and suggestions
No or low expression	<ul style="list-style-type: none"> • 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 <i>tet</i> promoter, see page 11). • Use eukaryotic cells for expression (yeast, insect or mammalian cells).
Protein is degraded	<ul style="list-style-type: none"> • Use protease deficient <i>E. coli</i> 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 expression can reduce the problem. • Secretion of the recombinant protein to the periplasmic space can reduce the problem.
Protein is secreted	<ul style="list-style-type: none"> • Remove all signal sequences from the coding region.
Inclusion bodies are formed: Protein is insoluble	<ul style="list-style-type: none"> • 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. <i>tet</i> promoter instead of T7 promoter, see page 8).



3 Preparation of cleared lysates



3.1 Preparation of cleared lysate after cytoplasmic expression of Twin-Strep-tag® 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	250 mM Tris·HCl, pH 8.0 25 % glycerol 7.5 % SDS 0.25 mg/ml bromophenolblue 12.5 % v/v mercaptoethanol	

Protocol	
	<ol style="list-style-type: none"> 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 10µl 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. 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. 6. Centrifuge the suspension at >15,000 x g (microfuge) for 15 minutes at 4 °C. 7. Insoluble cell components are pelleted. If the recombinant protein forms inclusion bodies it will be present in the pellet.

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8. **Carefully transfer the cleared supernatant to a clean tube.**
Store the supernatant on ice until further usage or store at -20°C if chromatography cannot be performed on the same day.
For analysis of the insoluble part of the expressed protein, dissolve the pellet with 1.25 ml 1x SDS-PAGE sample buffer (= 250 µl 5x SDS-PAGE sample buffer mixed with 1 ml Buffer W).
9. **Proceed to protocols for Twin-Strep-tag® protein purification under native conditions (see protocols 4.1, 4.2, 4.3).**

3.2 Preparation of cleared lysate after periplasmic expression of Twin-Strep-tag® 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, the Twin-Strep-tag® can be fused either to the C- or N-terminus using e.g. pASG-IBA102, 104 and 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.
5x SDS-PAGE sample buffer	250 mM Tris/HCl, pH 8.0 25 % glycerol 7.5 % SDS 0.25 mg/ml bromophenolblue 12.5 % v/v mercaptoethanol	

**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 shear 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 15,000 x g (microfuge) for 5 minutes at 4°C.**
- 6. Carefully transfer the cleared supernatant to a clean tube.**

Store the supernatant on ice until further usage or store at -20°C if chromatography cannot be performed on the same day.

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

**Important note**



4 Purification of Twin-Strep-tag® fusion proteins



To allow an efficient Strep-Tactin®XT:Twin-Strep-tag® binding we 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 can lead to decreased protein yields. Further, prolonged batch incubations increase the risk of proteolytic degradation of the target protein including cleavage of the tag.

For larger volumes we recommend the use of WET FRED – the applicator for Strep-Tactin® 1 ml, 5 ml and 10 ml gravity flow columns. WET FRED enables convenient application of large cell culture supernatant volumes to a gravity flow column in a simple way (Cat.no: 2-0910-001). For small volumes and high throughput applications, we recommend the use of MagStrep type 3 XT Beads (Cat.no: 2-4090-002).

For further information please see <https://www.iba-lifesciences.com/download-area.html> under section IBA Manuals.

4.1 Purification of Twin-Strep-tag® fusion proteins using 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 are purified
Buffer BXT (elution buffer)	100 mM Tris/HCl, pH 8.0 150 mM NaCl 1 mM EDTA 50 mM biotin	

Important notes

- 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.
The pH should not be lower than 7.5, though.
- 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 buffers are 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.



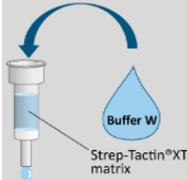
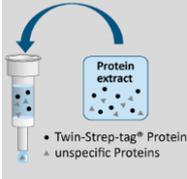
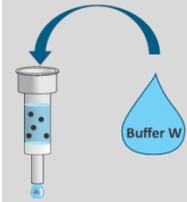
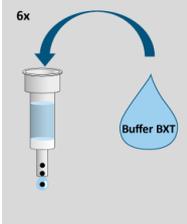
Recommended volumes for working with Strep-Tactin®XT columns

Column bed volume (CV)	Washing buffer volume	Elution buffer volume
0.2 ml	5 x 0.2 ml	6 x 0.1 ml
1 ml	5 x 1 ml	6 x 0.5 ml
5 ml	5 x 5 ml	6 x 2.5 ml
10 ml	5 x 10 ml	6 x 5 ml

Table 1. Recommended buffer volumes for chromatography on Strep-Tactin®XT 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. Remove top cap from column first, then the cap at the outlet of the column.** If the caps are removed in reverse order, air may enter the column bed. Remove storage buffer. The column cannot run dry under gravity flow.
Equilibrate the Strep-Tactin®XT column with 2 CV (column bed volume) Buffer W (100 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA).
- 2. Centrifuge cleared lysates (18,000 x g, 5 minutes, 4°C).**
 Frozen cell extracts have to be centrifuged prior to application in order to remove any aggregates that may have formed.
- 3. Add supernatant of cleared lysate to the column.**
 The volume of the lysate should be in the range of 0.5 and 10 CVs (see Table 1).
- 4. Wash the column with 5 CV Buffer W, after the cell extract has completely entered the column.**
 Collect the wash fractions (1 CV each) and optionally save 2 µl of each subsequent wash fraction for application on an analytical SDS-PAGE.
- 5. Add 6 times 0.5 CVs Buffer BXT and collect the eluate in 0.5 CV fractions.**
 Option: To get high protein concentrations in one fraction add 0.6 CV as elution fraction 1 (E1), then 1.6 CV (E2) and finally 0.8 CV (E3). Main protein content should be in E2. 20 µl samples of each fraction can be used for SDS-PAGE analysis.



4.2 Purification of Twin-Strep-tag® fusion proteins on chromatography workstations using 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 are purified
Buffer BXT (elution buffer)	100 mM Tris/HCl, pH 8.0 150 mM NaCl 1 mM EDTA 50 mM biotin	
5x SDS-PAGE sample buffer	250 mM Tris/HCl, pH 8.0 25 % glycerol 7.5 % SDS 0.25 mg/ml bromophenolblue 12.5 % v/v mercaptoethanol	

Important notes	
	<ul style="list-style-type: none"> • Cartridges filled with 1 ml or 5 ml Strep-Tactin®XT Superflow® 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). • 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 pH should not be lower than 7.5. • 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 buffers are 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. • 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 signal at 280 nm; the baseline should be stable after washing with 5 CVs.
- 3. Centrifuge cleared lysates (18,000 x g, 5 minutes, 4°C).**
Frozen cell extracts have to be centrifuged prior to application in order to remove any aggregates that may have formed.
- 4. 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 1) or reduce flow rate.
Collect the flow-through for SDS-PAGE analysis.
- 5. Wash with Buffer W until A₂₈₀ 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.
- 6. Elute the protein with Buffer BXT.**
Collect fractions for SDS-PAGE analysis.



4.3 Purification of Twin-Strep-tag® fusion proteins using 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 BXT (elution buffer)	100 mM Tris/HCl, pH 8.0 150 mM NaCl 1 mM EDTA 50 mM biotin	
5x SDS-PAGE sample buffer	250 mM 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 pH should not be lower than 7.5.
- 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 buffers are 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.
- 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/s to equilibrate the cartridge.**
4. **Centrifuge the cleared lysate (18,000 x g, 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/s.**
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.**
10. **Wash with 100 drops Buffer W (corresponding to approx. 5 ml) at a flow rate of 0.3 to 0.5 drops/s. 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 W.**
12. **Fill a 5 ml syringe with 4 ml Buffer BXT and connect it to the cartridge.**
13. **Elute the recombinant Twin-Strep-tag[®] fusion protein with 60 drops Buffer BXT (corresponding to approx. 3 ml) at a flow rate of 0.3 to 0.5 drops/s. 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 Troubleshooting – Twin-Strep-tag® purification



4.4.1 “No or weak binding to Strep-Tactin®XT column”

pH is not correct.	<ul style="list-style-type: none"> The pH should be > 7.5
Twin-Strep-tag® is not present.	<ul style="list-style-type: none"> Use protease deficient <i>E. coli</i> expression strains. Add protease inhibitors during cell lysis.
Twin-Strep-tag® is not accessible.	<ul style="list-style-type: none"> Fuse Twin-Strep-tag® to the other protein terminus; use other linker. Reduce washing volume to 3 CVs
Twin-Strep-tag® has been degraded.	<ul style="list-style-type: none"> Check that the Twin-Strep-tag® is not associated with a portion of the protein that is processed. <i>Avoid purification in discontinuous batch mode.</i> Prolonged batch incubations increase the risk of proteolytic degradation of the target protein including cleavage of the tag.
Strep-Tactin®XT column is inactive.	<ul style="list-style-type: none"> Check activity with HABA. Add avidin (Biotin Blocking Buffer) or BioLock solution (Cat.no: 2-0205-050) if biotin containing extracts are intended to be purified. The total biotin content of the soluble part of the total <i>E. coli</i> cell lysate is about 1 nmol per liter culture (OD₅₅₀ = 1.0). Add 2-3 nmol of avidin monomer per nmol of biotin.
Flow rate is too fast	<ul style="list-style-type: none"> Reduced flow rates may increase yields depending on the given recombinant protein.
The column is blocked with Biotin from culture medium.	<ul style="list-style-type: none"> Regenerate the resin with freshly prepared 10 – 20 mM NaOH
The column is not properly regenerated.	<ul style="list-style-type: none"> Prepare fresh 10 – 20 mM NaOH and regenerate again Efficient regeneration can be visualized by addition of HABA. When HABA is added to the column it changes its color from yellow to dark orange/red.

4 Purification of Twin-Strep-tag® fusion proteins

4.4.2 “Contaminating proteins”

Note: The soluble part of the *E. coli* total cell extract contains no proteins beyond the 22 kDa Binding Biotin Carboxyl Carrier Protein (BCCP) which binds significantly to the Strep-Tactin®XT 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.	<ul style="list-style-type: none">• Use protease deficient <i>E. coli</i> expression strains.• Add protease inhibitors after cell lysis.• Fuse the Twin-Strep-tag® with the other protein terminus. Check for the presence of internal translation initiation starts (only in case of C- terminal Twin-Strep-tag®) or premature termination sites (only in case of N-terminal tag).• Add 6xHistidine-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.	<ul style="list-style-type: none">• Add reducing agents to all buffers for cell lysis and chromatography.
Contaminants are non-covalently linked to the recombinant protein.	<ul style="list-style-type: none">• 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.).
Contaminants are biotinylated proteins.	<ul style="list-style-type: none">• Add Avidin or BioLock to supernatant before application onto the column.

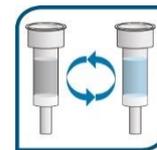
4.4.3 Air bubbles in the column

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 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.
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5 Storage and regeneration of Strep-Tactin®XT resin



Recommended Buffers/Solutions	Concentration of ingredients	Notes
Regeneration buffer	10 mM NaOH	For Strep-Tactin®XT (Always use fresh NaOH!!!)
Buffer W	100 mM Tris/HCl, pH 8.0 150 mM NaCl 1 mM EDTA	

Important notes

- Strep-Tactin®XT matrices should be stored in buffer W at temperatures between 4 and 8°C for longer terms.
- We recommend a maximum of 5 runs per column.
- Resin tolerates washing with 6 M urea. Such procedures should not last longer than 30 minutes and the resin should be equilibrated immediately with Buffer W afterwards.
- Efficient regeneration of Strep-Tactin®XT **high capacity** requires more column volumes of 10mM NaOH than Strep-Tactin®XT (see 5.3)

5.1 Regeneration of Strep-Tactin®XT Superflow gravity flow columns

Protocol

The diagram shows three steps: 1. A column is shown with a blue arrow pointing to a NaOH droplet. 2. The column is shown with a blue arrow pointing to a Buffer W (pH 8) droplet, with '2x' indicating two times. 3. The column is shown with a blue arrow pointing to a storage container labeled 'store at 4 °C' and '1', indicating 2 ml of Buffer W (pH 8) overlaid.

- 1. Wash the column with 4 CV of 10 mM NaOH (fresh!).**
Strep-Tactin®XT Superflow® cannot be regenerated using Buffer R (HABA - hydroxy-azophenyl-benzoic acid, 100 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM HABA). However, after treatment with NaOH, operability can be confirmed by application of Buffer R which induces an orange-shift in case of a successful regeneration.
Note: Regeneration with >4CV 10mM NaOH might be detrimental for the Strep-Tactin®XT on the long run.
- 2. Immediately remove the NaOH by adding two times 4 CV Buffer W (pH 8).**
- 3. Store the column at 4°C overlaid with 2 ml Buffer W (pH 8).**



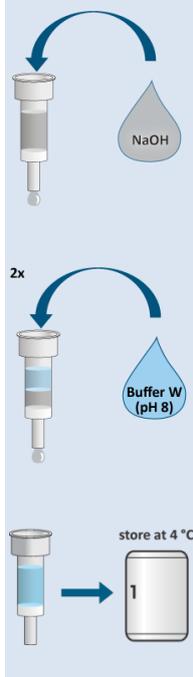
5.2 Regeneration of Strep-Tactin®XT Superflow® cartridges

Protocol

1. Fill the cartridge inlet with 10 mM NaOH (fresh!).
2. Fill a 20 ml syringe with 10 mM NaOH and connect the syringe with the cartridge.
3. Wash with 3 CVs 10 mM NaOH at a flow rate of 1 drop/sec or 1 ml/min.
4. Immediately remove the 10 mM NaOH by washing with 8 CV of Buffer W.
5. Store the cartridge at 4 – 8°C.

5.3 Regeneration of Strep-Tactin®XT Superflow® High Capacity gravity flow columns

Protocol



1. **Wash the column with 15 CV of 10 mM NaOH (fresh!).**
Strep-Tactin®XT Superflow® high capacity resin cannot be regenerated using Buffer R (HABA - hydroxy-azophenyl-benzoic acid, 100 mM Tris/HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM HABA). However, after treatment with NaOH, operability can be confirmed by application of Buffer R which induces an orange-shift in case of a successful regeneration.
Note: Washing with <15 CV 10mM NaOH will not completely regenerate the Strep-Tactin®XT high capacity matrice.
2. **Immediately remove the NaOH by adding two times 4 CV Buffer W (pH 8).**
3. **Store the column at 4°C overlaid with 2 ml Buffer W (pH 8).**

6 References



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

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Please refer to www.iba-lifesciences.com/download-area.html for downloading this manual.

7 Notes



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